Novel functional requirements for non-homologous DNA end joining in *Schizosaccharomyces pombe*

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DNA double strand break (DSB) repair by non-homologous end joining (NHEJ) in mammalian cells requires the Ku70–Ku80 heterodimer, the DNA-PK catalytic subunit DNA-PKcs, as well as DNA ligase IV and Xrcc4. NHEJ of plasmid DSBs in *Saccharomyces cerevisiae* requires Ku, Xrcc4 and DNA ligase IV, as well as Mre11, Rad50, Xrs2 and DNA damage checkpoint proteins. *Saccharomyces cerevisiae* Ku is also required for telomere length maintenance and transcriptional silencing. We have characterized NHEJ in *Schizosaccharomyces pombe* using an extrachromosomal assay and find that, as anticipated, it is Ku70 and DNA ligase IV dependent. Unexpectedly, we find that Rad32, Rad50 (the *S.pombe* homologues of Mre11 and Rad50, respectively) and checkpoint proteins are not required for NHEJ. Furthermore, although *S.pombe* Ku70 is required for maintenance of telomere length, it is dispensable for transcriptional silencing at telomeres and is located throughout the nucleus rather than concentrated at the telomeres. Together, these results provide insight into the mechanism of NHEJ and contrast significantly with recent studies in *S.cerevisiae*.

Keywords: DNA ligase IV/Ku70/Mre11/silencing/
telomere length

Introduction

DNA double strand breaks (DSBs) have the potential to disrupt genomic integrity. If the damage is left unrepaired or is misrepaired, it may lead to chromosomal abnormalities or loss of genetic material, events that can cause cell death or ultimately the onset of cancer. Cells have evolved at least two pathways that can repair DSBs: homologous recombination, which repairs the breaks by retrieving genetic information from a sister chromatid or a homologous chromosome, and non-homologous end joining (NHEJ), a process involving direct sealing of the ends utilizing little or no homology.

NHEJ is the major mechanism for the repair of radiation-induced DSBs in higher organisms. Five proteins involved in the process have been identified in mammalian cells (reviewed in Featherstone and Jackson, 1999); the DNA-PK complex, which comprises the Ku70–Ku80

heterodimer and a catalytic subunit (DNA-PKcs), and a DNA ligase IV–Xrcc4 complex. Cells with mutations in any of the genes corresponding to these factors display radiosensitivity and defective rejoining of DSBs as measured by pulsed-field gel electrophoresis. DNA-PKcs-defective mice grow normally (Gao *et al.*, 1998a), Ku70 and Ku80 null mice are viable but show premature senescence (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997), whereas deletion of the gene for either DNA ligase IV or Xrcc4 results in early embryonic lethality (Gao *et al.*, 1998b). Thus, either NHEJ differs in its requirements for these proteins or they have separate functions in addition to their role in NHEJ. All five proteins are involved in V(D)J recombination, a site-specific recombination event responsible for generating antibody and T-cell receptor diversity in mammalian cells. It has been reported that DNA-PK is also necessary for the maintenance of the telomere structure in mammalian cells (Bailey *et al.*, 1999).

Although NHEJ was first characterized in mammalian cells, *Saccharomyces cerevisiae* has served as a useful model organism to understand NHEJ and to identify additional functions of the proteins. DNA-PKcs has not been identified in the completed sequence of the *S.cerevisiae* genome, but *KU70* and *KU80* homologues (named *YKU70* and *YKU80*, respectively) are present and are essential for NHEJ repair of DSBs in plasmids that have been transformed into cells. In addition, budding yeast *YKU70* and *YKU80* are also required for the maintenance of telomere length, telomere clustering, localization of telomeres to the nuclear periphery and for transcriptional silencing adjacent to telomeres (Boulton and Jackson, 1996a, 1998; Laroche *et al.*, 1998). This demonstrates that in *S.cerevisiae*, Ku, at least, has roles in addition to NHEJ. *Saccharomyces cerevisiae* *LIG4* and *LIF1* (the homologues of DNA ligase IV and Xrcc4, respectively) are also necessary for NHEJ (Teo and Jackson, 1997; Wilson *et al.*, 1997; Herrmann *et al.*, 1998). Several other proteins have also been implicated in NHEJ in *S.cerevisiae*. These include: members of the Mre11–Rad50–Xrs2 complex (Boulton and Jackson, 1998), which are also involved in DSB repair by homologous recombination (reviewed in Petrini, 1999); DNA integrity checkpoint proteins Rad9, Mec1, Mec3, Rad17 and Rad24 (de la Torre-Ruiz and Lowndes, 2000); and three proteins involved in transcriptional silencing Sir2, 3 and 4 (reviewed in Gartenberg, 2000). It has been shown that the *S.cerevisiae* Ku and Sir proteins are located predominantly at telomeric regions in undamaged cells and that they become redistributed throughout the nucleus upon induction of DNA damage. This redistribution is dependent on the Mec1/Rad9 checkpoint proteins (Martin *et al.*, 1999; McAinsh *et al.*, 1999; Mills *et al.*, 1999). The model emerging from these studies in *S.cerevisiae* is that

Ku is stored at the telomeres in a pre-formed complex (possibly containing the Sir proteins among other factors). In the presence of DSBs, the DNA damage checkpoint signals the release of Ku and Sir proteins from these telomeric complexes, Ku then relocates to DSBs and enhances repair by DNA end protection and by attracting other NHEJ proteins such as the DNA ligase IV–Xrcc4 complex. Although the Sir proteins are also recruited to DSBs, their role in the repair process remains to be elucidated.

The fission yeast *Schizosaccharomyces pombe* has also proved to be a good model system for studying many cellular processes. In particular, various aspects of cell cycle regulation, including the DNA damage response in fission yeast, have provided insight into similar pathways in human cells. In this study, we have used *S.pombe* to study NHEJ and the roles of its component proteins. Here we report the characterization of the homologues of Ku70 and DNA ligase IV in *S.pombe* (from now on referred to as Pku70 and Lig4, respectively) and show that they are, as expected, required for NHEJ of plasmid DSBs. However, we find that *S.pombe* cells deleted for either *rad32* or *rad50* (homologues of *MRE11* and *RAD50*, respectively) as well as a range of DNA damage/replication checkpoint mutants are proficient in NHEJ. Thus, the genetic requirements for NHEJ differ between *S.cerevisiae* and *S.pombe*. Further characterization of Pku70 shows that, while it is involved in telomere length maintenance, in marked contrast to *S.cerevisiae*, it is dispensable for telomeric silencing and is not located predominantly at the telomeres.

Results

Identification of the Ku70 and DNA ligase IV homologues in *S.pombe*

To identify factors involved in NHEJ in *S.pombe*, we used the sequences of known NHEJ proteins to search the *S.pombe* database (www.sanger.ac.uk/Projects/S_pombe). Using the TBLASTN program, we identified two open reading frames in cosmids SPCC126 and SPCC1183 with strong homology to Ku70 and DNA ligase IV, respectively. Both are located on chromosome 3. The *pku70* gene contains five introns and encodes a protein of 607 amino acids with a predicted mol. wt of 69.1 kDa. The *lig4* gene encodes a 923 amino acid protein with a predicted mol. wt of 107.3 kDa and contains nine introns. Both proteins show ~42% similarity and 30% identity to their respective human homologues. Phylogenetic trees showing the evolutionary relationship of Ku70 and DNA ligase IV proteins from different organisms are shown in Figure 1A.

Deletion of genes encoding fission yeast Ku70 or DNA ligase IV abolishes NHEJ

To assess the involvement of Pku70 and Lig4 in DSB repair by NHEJ, we adapted the *in vivo* plasmid DSB repair assay (see Materials and methods). Plasmid PS, containing a *LEU2* selectable marker, has two *PvuII* and two *PstI* restriction sites flanking a small fragment. Restriction with either enzyme results in the excision of the fragment and generation of linear molecules with blunt ends, or cohesive ends with 3' overhangs, respectively. Similarly, an *EcoRI* fragment was excised from plasmid PI

for the generation of substrates with 5' overhangs. These substrates were then transformed into *S.pombe* cells. The excision of a fragment allows us to distinguish contaminating uncut plasmids (that retain the fragment) from accurately rejoined ones by restriction digestion. The frequency of NHEJ was determined by measuring the number of *leu*⁺ colonies (capable of growth on plates lacking supplementing leucine) arising from cells transformed with linear DNA divided by the colonies arising following transformation of undigested control plasmid. The region in the vicinity of the DSB does not bear homology to any *S.pombe* genomic sequences and the break can therefore be repaired only by NHEJ. Consistent with previous work (Wilson *et al.*, 1999), we found that *S.pombe* wild-type cells were equally proficient in the repair of blunt- and cohesive-ended DSBs (data not shown). In contrast, *S.cerevisiae* wild-type cells are inefficient at repairing blunt-ended DSBs by NHEJ and no further reduction is observed in *YKU* deletion strains (Boulton and Jackson, 1996b). In agreement with the independence of our NHEJ assay from homologous recombination activities, we found that *rhp54Δ*, a strain defective in homologous recombination (Muris *et al.*, 1996), is proficient in NHEJ (Figure 1B). We generated *pku70* and *lig4* deletion strains and found that the frequency of rejoining of blunt DSBs is decreased ~1000-fold in these cells compared with wild-type cells (isogenic *pku70*⁺ and *lig4*⁺) (Figure 1B). This demonstrates that repair of blunt-ended DSBs is not only efficient in *S.pombe* but is also dependent on Ku and DNA ligase IV. *pku70Δ* and *lig4Δ* strains displayed an ~350-fold reduction in the rejoining of cohesive-ended breaks with 3' or 5' overhangs compared with the wild type (Figure 1C).

The fidelity of NHEJ was analysed by investigating the nature of the junctions. First, the accuracy of repair was determined by examining the ability of plasmids to be redigested with the original restriction enzyme used for the preparation of the substrate. Using this criterion, in wild-type *S.pombe*, it was found that ~40% of blunt DSBs were repaired accurately, whereas rejoining of cohesive DSBs (with either 5' or 3' overhangs) was rarely accurate (Table I). This finding contrasts with the error-free rejoining of cohesive DSBs by NHEJ observed in *S.cerevisiae* wild-type cells, using a similar assay (Boulton and Jackson, 1996b).

Repair in *pku70Δ* and *lig4Δ* strains was always associated with loss of the restriction site (Table I). Forty percent of the small number of plasmids recovered from these strains proved to be undigested contaminants and have been eliminated from the calculations since they can be distinguished from accurately repaired junctions, as they give two bands following restriction digestion. Undigested contaminants were never recovered from strains that showed wild-type levels of NHEJ but were recovered from *pku70Δ* and *lig4Δ*, presumably because they constitute a high proportion of surviving circular plasmids in these strains.

To ascertain the nature of the repair that gave rise to undigestible junctions, some of the *PvuII*-resistant plasmids were sequenced. All cases involved deletions, which were sometimes associated with single base pair insertions. The number of bases lost during repair in wild-type cells was usually small (1–3 bp) (Figure 2), although

Fig. 1. Genetic requirements for NHEJ in *S.pombe*. (A) Phylogenetic trees showing the evolutionary relationship of (I) Ku70 and (II) DNA ligase IV proteins from different organisms. The trees are based on multiple sequence alignments created using the PILEUP program of the GCG software. DDBJ/EMBL/GenBank accession Nos are as follows: HsKu70 (*Homo sapiens*), P12956; GgKu70 (*Gallus gallus*), O93257; XlKu70 (*Xenopus laevis*), BAA76953; SpKu70 (*Schizosaccharomyces pombe*), CAA22471; DmKu70 (*Drosophila melanogaster*), Q23976; ScKu70 (*Saccharomyces cerevisiae*), P32807; CeKu70 (*Caenorhabditis elegans*), CAB55094; DmLigIV (*D.melanogaster*), AAF48298; HsLigIV (*H.sapiens*), NP_002303 with the addition of 67 amino acids at the N-terminus as described in Grawunder *et al.* (1998); SpLigIV (*S.pombe*), CAA21085; ScLigIV (*S.cerevisiae*), Q08387. (B–D) Analysis of NHEJ in *S.pombe* mutants using a plasmid-based assay: (B and D) blunt DSBs, (C) cohesive DSBs. FY0367 is the *pku70* parental strain.

Table I. Accuracy of NHEJ

Strain	Blunt DSBs		DSBs with 5' overhangs		DSBs with 3' overhangs	
	Accurate	Total	Accurate	Total	Accurate	Total
Wild type (501)	22	56	2	21	1	13
<i>pku70Δ</i>	0	2 ^a	0	5 ^a	0	3 ^a
<i>lig4Δ</i>	0	3 ^a	0	2 ^a	ND	ND
<i>rad32Δ</i>	25	42	2	17	0	18
<i>rhp50Δ</i>	15	55	1	20	0	22

^aThe small number of junctions obtained in these cases is due to the severe inability of *pku70Δ* and *lig4Δ* strains to rejoin linear plasmids. ND, not done.

there was one junction with a 47 bp deletion. Only two out of 15 junctions involved utilization of short homologies. Similar data were found for *Pst*I junctions (data not shown). Only a small number of junctions were obtained from *pku70Δ* and *lig4Δ* strains but all involved large deletions (49–81 bp) and the use of 5 bp or greater microhomology (Figure 2). Taken together, these findings show that in *S.pombe*, the plasmid rejoining assay monitors a Ku70- and DNA ligase IV-dependent process and that, in the absence of NHEJ, a microhomology-based mechanism is utilized for repair of DSBs.

NHEJ is not impaired in strains lacking *rad50* or *rad32*

We next extended our analysis of the genetic requirements of NHEJ to include other factors that have been implicated in the process. The Mre11–Rad50–Xrs2 complex has been

shown to be required for efficient NHEJ of linear plasmids in *S.cerevisiae* and, therefore, *S.pombe* strains lacking *rad32* or *rad50* (the homologues of *MRE11* and *RAD50*, respectively) were investigated. Unexpectedly, *rad32Δ* and *rad50Δ* strains were able to repair blunt DSBs proficiently, displaying a range of frequencies similar to those observed for two wild-type strains 501 and FY367 (Figure 1B). Furthermore, NHEJ repair of cohesive-ended DSBs (with either 3' or 5' overhangs) was also normal in *rad32Δ* and *rad50Δ* (Figure 1C). To examine the fidelity of repair, rejoined plasmids were analysed by restriction digestion and sequencing. The accuracy of blunt end rejoining in *rad32Δ* was 60% and in *rad50Δ* 27% (Table I); this is similar to that observed in wild-type cells (40%). Repair of *Pst*I- or *Eco*RI-generated DSBs was less accurate and resembled the low fidelity cohesive end rejoining observed in wild-type cells (Table I). Moreover,

wild type		microhomology
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> CTGGCGTAATAGCGAAGAGGCCCGCAC	
(x2)	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> .TGCGTAATAGCGAAGAGGCCCGCAC	-
(x2)	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .CTGGCGTAATAGCGAAGAGGCCCGCAC	-
(x4)	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .TGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .tTGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> . . .GCGTAATAGCGAAGAGGCCCGCAC	G
(x2)	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u>cGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATCTGGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCGTAATAGCGAAGAGGCCCGCAC	TGGC
	CGCGCGTTGGCCGATTCATTAATG <u>C</u>47bp deletion	-
<i>rad32</i>		microhomology
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> CTGGCGTAATAGCGAAGAGGCCCGCAC	
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> .TGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>C</u> . .CTGGCGTAATAGCGAAGAGGCCCGCAC	-
(x2)	CGCGCGTTGGCCGATTCATTAATG <u>Cg</u> .CTGGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>C</u> . .gTGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTGCGTAATAGCGAAGAGGCCCGCAC	T
<i>rhp50</i>		microhomology
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> CTGGCGTAATAGCGAAGAGGCCCGCAC	
	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .CTGGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .CTGGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>CA</u> .tTGCGTAATAGCGAAGAGGCCCGCAC	-
	CGCGCGTTGGCCGATTCATTAATG <u>C</u> . .TGCGTAATAGCGAAGAGGCCCGCAC	C
	CGCGCGTTGGCCGATTCATTAATG <u>Cg</u> . .TGCGTAATAGCGAAGAGGCCCGCAC	C
	CGCGCGTTGGCCGATTCATTAAT . . .TGCGTAATAGCGAAGAGGCCCGCAC	-
(x2)	CGCGCGTTGGCCGATTCATTAATG . . .GCGTAATAGCGAAGAGGCCCGCAC	TG
(x2)	CGCGCGTTGGCCGATTCATTAATG <u>C</u>GTAATAGCGAAGAGGCCCGCAC	GC
	CGCGCGTTGGCCGATTCATAGCGAAGAGGCCCGCAC	AT
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u>49bp deletion	-
<i>pku70</i>		microhomology
	CGCGCGTTGGCCGATTCATTAATG <u>CAG</u> CTGGCGTAATAGCGAAGAGGCCCGCAC	
	CGC49bp deletionCCCGCAC	CCCGC
	72bp deletionGCCCGCAC	GAAGAG
<i>lig4</i>		
(x2)	GCCCCGAC	GAAGAG
	72bp deletionAGAGGCCCGCAC	AGCGA
	81bp deletion	

Fig. 2. Sequence analysis of inaccurately rejoined *PvuII* junctions. The top sequence in each panel shows the sequence that would result from accurate joining (the *PvuII* recognition sequence is underlined). Lower case italic letters indicate insertions. Numbers in parentheses on the left of a sequence indicate the number of times the sequence was found.

sequence analysis of *PvuII* junctions showed that the extent of deletions and microhomology utilization was generally small and similar to that of wild-type cells (Figure 2). We conclude that Rad32 and Rad50 are not involved in NHEJ of plasmid DSBs in *S.pombe*.

Loss of the DNA damage/replication checkpoint does not affect the frequency of NHEJ

The *S.cerevisiae* DNA damage checkpoint proteins Mec1 and Rad9 have been shown to be necessary for the relocalization of Yku and Sir proteins from telomeres to DSBs in response to DNA damage (Martin *et al.*, 1999; Mills *et al.*, 1999). Furthermore, a modest requirement for Mec3, Rad17 and Rad24 in addition to Mec1 and Rad9 for NHEJ has been demonstrated using a plasmid-based NHEJ assay (de la Torre-Ruiz and Lowndes, 2000). To assess the requirement for proteins involved in the *S.pombe* DNA damage checkpoints in NHEJ, we analysed strains lacking one of the following genes: *rad3*, *rhp9(crb2)*, *cds1 rad17* and *chk1* (the *S.pombe* homologues of *S.cerevisiae* *MEC1*, *RAD9*, *RAD53*, *RAD24* and *CHK1*, respectively). The products of these genes are required for the DNA damage and/or replication checkpoint in the fission yeast (reviewed in Caspari and Carr, 1999). All these strains were proficient in the repair of blunt and cohesive DSBs (Figure 1D and data not shown). *Saccharomyces cerevisiae* cells lacking both *RAD9* and *RAD24* display a similar reduction in the frequency of NHEJ as strains lacking *YKU* alone (de la Torre-Ruiz and Lowndes, 2000). We therefore examined the possibility of a synergistic interaction between the *S.pombe* homologues *rhp9(crb2)* and *rad17* by generating a *rhp9Δrad17Δ* double mutant. In this double mutant, a level of blunt end rejoining similar to that of wild type was observed (Figure 1D). Thus we conclude that NHEJ can operate independently of the DNA damage-sensing activity of the DNA damage/replication checkpoint in *S.pombe*.

NHEJ can take place in the absence of both *rad3* and *tel1*

Several factors involved in NHEJ in *S.cerevisiae* (including Yku70 and Yku80) have also been implicated in the maintenance of telomeric length. It has been proposed that the telomeres of *S.cerevisiae* act as storage sites for NHEJ protein complexes that include the Yku heterodimer and Sir proteins (Martin *et al.*, 1999; Mills *et al.*, 1999). The *S.pombe* Rad3 and Tel1 proteins are members of the family of phosphatidylinositol 3-kinases that includes DNA-PKcs and ATM. *Schizosaccharomyces pombe rad3Δ* and *tell1Δ* strains display telomeric shortening (Dahlen *et al.*, 1998; Naito *et al.*, 1998). Interestingly, when both *rad3* and *tell* genes are deleted, cells initially form irregular shaped colonies reflecting cell death due to telomere loss. However, following prolonged culture, regular shaped derivatives arise, which have lost all telomeric sequences resulting in all chromosomes circularizing due to fusion of chromosome ends (Naito *et al.*, 1998). We found that the Tel1 kinase is dispensable for NHEJ using either blunt ends (Figure 1D) or cohesive ends (data not shown). To assess the requirement for telomeric structures for NHEJ, we performed the plasmid assay in *rad3Δtell1Δ* derivative cells and found that they can repair blunt-ended DSBs proficiently (Figure 1D). Therefore,

neither the activities of these proteins nor the presence of normal chromosome ends are required for NHEJ in *S.pombe*.

The effect of DNA-damaging agents on *pku70Δ* and *lig4Δ*

To assess the role of Pku70 and Lig4 in the repair of chromosomal DNA damage, we examined the sensitivity of *pku70Δ* and *lig4Δ* strains to DNA-damaging agents. Methyl methanesulfonate (MMS) and bleomycin cause methylation damage and DSBs, respectively. We found that *pku70Δ* and *lig4Δ* strains resemble wild type in their sensitivity to 0.005% MMS or 5 µg/ml bleomycin—concentrations that kill most of the *rad32Δ* and *rad3Δ* cells (Figure 3A). Both *pku70Δ* and *lig4Δ* were also resistant to γ-irradiation (Figure 3B). Therefore, it seems that homologous recombination plays the major role in the repair of genomic DSBs in *S.pombe* as is the case in *S.cerevisiae*. Additionally, *pku70Δrhp54Δ* and *lig4Δrhp54Δ* double mutants were no more sensitive to γ-irradiation than the *rhp54Δ* strain (Figure 3B).

Reduced telomere length and linear minichromosome stability in cells lacking *pku70*

In other systems, Ku70/80 and DNA-PKcs have been implicated in regulating telomere length (Boulton and Jackson, 1996a; Bailey *et al.*, 1999). Since a telomere is a naturally occurring chromosome break, it potentially can bind Ku but its activity must be tempered in a normal telomere environment in order to participate in the formation of a chromosomal cap and not to mediate end to end joining. Deletion of the *S.cerevisiae* *YKU* genes results in reduction of telomere length and temperature sensitivity. We found that the average telomere length in *pku70Δ* cells is clearly shorter compared with wild type (Figure 4). Telomere length was also analysed in *rad3Δ* and *rad32Δ* cells (Figure 4) and found to be decreased, at levels similar to those previously published (Dahlen *et al.*, 1998; Wilson *et al.*, 1999). However, as expected, deletion of *lig4* did not affect telomere length (Figure 4). Furthermore, *pku70Δ* and *lig4Δ* strains did not display a temperature-sensitive phenotype and grow as well as wild-type cells at 36°C (Figure 3A, panel II). The absence of Yku in *S.cerevisiae* results in further shortening of telomeres at higher temperatures (Boulton and Jackson, 1998) that may lead to activation of a DNA damage sensor checkpoint and cellular arrest/senescence. The resistance of *pku70Δ* fission yeast strains to increased temperature is consistent with the absence of further telomere shortening under these conditions (data not shown).

To determine if Pku70 has other effects on telomere function, we compared the rate of loss of circular and linear minichromosomes in wild-type and *pku70Δ* cells. The rate of loss of a linear minichromosome (Ch16) was increased 8-fold from 0.017% per cell per generation observed in wild-type cells to 0.14% in *pku70Δ*. However, the rate of loss of a circular minichromosome lacking telomeres was only 2-fold higher (0.52% in wild-type cells and 0.88% in *pku70Δ*). The fact that linear minichromosomes are more unstable than circular minichromosomes in *pku70Δ* cells indicates that Pku70 might normally act at telomeres to protect and stabilize chromosome ends, and is

Fig. 3. *pku70Δ* and *lig4Δ* are resistant to MMS, bleomycin and γ -irradiation and are not temperature sensitive. (A) A 5 μ l aliquot of serial 10-fold dilutions of log-phase cells was spotted onto YEA plates and incubated for 3 days at 30°C (I, III and IV) or 36°C (II). Cells were exposed to 0.005% MMS in III and to 5 μ g/ml bleomycin in IV. (B) Survival of strains subjected to γ -irradiation, determined as described in Materials and methods.

consistent with the reduced telomere length observed in cells lacking Pku70.

Transcriptional silencing of the subtelomeric region is not alleviated in *pku70Δ*

When marker genes are placed close to telomeres in *S.pombe*, their transcription is repressed (Nimmo *et al.*, 1994). Several factors have been identified that contribute to this telomere-mediated transcriptional silencing in *S.pombe*, including Ctr4, Rik1, Swi6, Lot2 and Taz1. Mutation of these factors results in increased expression of telomeric reporter genes (Allshire *et al.*, 1995; Cooper *et al.*, 1997; Nimmo *et al.*, 1998). Since *S.cerevisiae* Yku proteins participate in the formation of silent telomeric chromatin, the involvement of Pku70 in transcriptional silencing of telomeric *ura4* or *his3* marker genes was assessed. Surprisingly, in cells lacking Pku70 function, these telomeric marker genes remained transcriptionally silent and no increase in the frequency of colony growth was observed on plates selecting for telomeric marker expression (Figure 5). Furthermore, we were unable to detect the presence of a *ura4* transcript from the telomeric *ura4* gene in *pku70Δ* cells by quantitative RT-PCR (data not shown). Likewise, telomeric silencing was not alleviated in *rad32Δ*, *rad3Δ* or *lig4Δ* cells but, as shown previously (Nimmo *et al.*, 1998), it was decreased in the

lot2 mutant (Figure 5). If a telomeric pool of Ku and associated proteins is redistributed as a result of DNA damage, then the induction of damage might interfere with telomeric silencing. However, treatment of cells with bleomycin did not alleviate transcriptional silencing at *S.pombe* telomeres in wild-type or mutant cells (data not shown). The finding that Pku70 does not affect transcriptional silencing adjacent to telomeres suggests that it is not a primary constituent of the chromatin complex that mediates this process.

The *S.pombe* Ku70 localization is nuclear but not confined to the telomeres

To gain further insight into Pku70 function, we created a C-terminal fusion of Pku70 with 13 Myc epitopes expressed from its own promoter at the *pku70* locus. The addition of the Myc epitopes did not affect the telomere maintenance or the NHEJ function of Pku70 (data not shown). Following cell fixation, analysis of Pku70 localization by immunostaining revealed a diffuse pattern of Pku70-Myc that generally coincided with the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining (Figure 6A, panels a, c and d). This contrasts with the distinct foci of *S.cerevisiae* Yku80 shown to co-localize with the telomeric Rap1 protein (Martin *et al.*, 1999). The uniform and diffuse localization of Pku70-Myc was confirmed

Fig. 4. Telomere length is decreased in *pku70Δ* cells. Genomic DNA was prepared from the indicated strains and analysed by Southern blotting. A fragment consisting of two 250 bp copies of telomere repeats was used as a telomere probe. The FY1870 strain was used as wild type.

using confocal microscopy (Figure 6B). To assess the localization of Pku70 relative to telomeres, we utilized a fusion of the green fluorescent protein (GFP) with the telomeric protein Taz1. Taz1 is involved in telomere length maintenance (Cooper *et al.*, 1997) and has high affinity for telomeric repeats (Vassetzky *et al.*, 1999; Spink *et al.*, 2000). The Taz1–GFP fusion therefore was utilized to visualize the telomeres. The *taz1-gfp* strain has normal telomere length (data not shown) and, in wild-type cells, Taz1–GFP is seen to concentrate in 1–3 distinct telomeric foci (Figure 6A, panel b). Since *S.pombe* has six telomeres, this finding is consistent with there being some clustering of telomeres in interphase nuclei (Funabiki *et al.*, 1993). The strain used to detect Taz1–GFP at telomeres also contains the *pku70-myc* fusion and allowed us to demonstrate that the diffused Pku70 staining generally does not co-localize with the telomeric Taz1 spots (Figure 6A, panel d). As expected, there is some overlap between the two signals: we found that 53% of Taz1 spots overlap with the diffused Pku70 staining. Treatment of cells with bleomycin or MMS prior to immunofluorescence did not alter the Pku70 staining, which remained diffused throughout the nucleus (data not shown). In addition, the percentage of Taz1 spots that overlap with Pku70 staining remained relatively unchanged after bleomycin or MMS treatment (data not shown). We conclude that Pku70 is not constitutively enriched at the telomeres but is located throughout the nucleus and does not form foci after DNA damage.

Discussion

We have identified homologues of Ku70 and DNA ligase IV in *S.pombe*. Using a plasmid *in vivo* NHEJ assay, we demonstrate that these two proteins are essential for NHEJ. Therefore, the basic NHEJ components and their functions are conserved in *S.pombe*, providing another yeast model in which to investigate this process. Our studies show that NHEJ in *S.pombe* differs in two

Fig. 5. Transcriptional silencing of the subtelomeric region is not alleviated in *pku70Δ* and *lig4Δ*. (A) Serial dilutions of strains containing a telomeric *ura4* marker were spotted onto non-selective (NS) medium, medium containing the drug 5-fluoro-orotic acid (which is toxic to cells expressing uracil) and medium lacking uracil. (B) The expression of a *his3* telomeric marker was assessed on medium lacking histidine as well as NS medium.

important aspects from the process in *S.cerevisiae*: repair of plasmid DSBs in *S.cerevisiae* is accurate and functions only to rejoin cohesive-ended breaks, whereas blunt-ended breaks are rejoined by a Ku-independent single strand annealing mechanism (Boulton and Jackson, 1996b). In *S.pombe*, blunt-ended breaks are rejoined as efficiently as cohesive-ended breaks in a Ku70- and DNA ligase IV-dependent process, and repair of both types of DSBs frequently is associated with small deletions at the junctions. This may reflect greater levels of exonuclease activities in *S.pombe* that can degrade DNA ends. The single-stranded overhangs of cohesive ends may be especially susceptible to such nucleases since cohesive-ended junctions were associated with deletions more frequently than blunt-ended ones. Significantly, ~40% of the blunt ends are rejoined accurately, showing that NHEJ can repair DSBs without utilization of complementarity from overlapping sequences and that repair of blunt-ended breaks is not simply the result of nuclease action on these ends. Similar assays in mammalian cells show only a small dependence on known NHEJ components; however, as reported above with *S.pombe*, blunt ends are rejoined efficiently and the repair of DSBs frequently is associated with deletions (Escarceller *et al.*, 1998; Kabotyanski *et al.*, 1998; K.G.Manolis and P.A.Jeggo, unpublished observations).

Since a DNA-PKcs homologue has not been identified in yeast, we examined strains lacking either or both of the related kinases Rad3 and Tel1. The ability of these strains to carry out NHEJ efficiently showed that they do not act as functional homologues of DNA-PKcs in *S.pombe*. The

Fig. 6. Pku70 is located throughout the nucleus. (A) A logarithmically growing culture of the *taz1-gfp/pku70-myc* strain was used. (a) Cells stained with DAPI; (b) Taz1-GFP fusion visualized at the fluorescein isothiocyanate (FITC) wavelength; (c) Pku70-Myc visualization at the Texas red wavelength using anti-Myc (primary) and Texas red (secondary) antibodies; (d) overlay of the three signals. Bar length is 2 μ m. (B) Confocal intervals of 1 μ m of untreated *pku70-myc* cells. Pku70 was visualized with an FITC-conjugated secondary anti-mouse antibody against the mouse anti-Myc primary antibody used to target Pku70-Myc. Bar length is 1 μ m.

absence of telomeres in the *rad3 Δ tel1 Δ* strain indicates that intact telomeres do not play an intrinsic role in NHEJ, e.g. as storage sites or potential loading points for Ku.

Having established an assay for NHEJ and shown that it is dependent upon known components (Pku70 and Lig4), we next examined the requirement for additional proteins reported to function in NHEJ based on studies in *S.cerevisiae*. Most significantly, we found that loss of Rad32 and Rad50 does not impair either the frequency or accuracy of NHEJ of plasmid DSBs. *Saccharomyces cerevisiae* strains lacking *MRE11* or *RAD50* are as defective in plasmid NHEJ as *YKU* deletion strains. Although it has been reported that the nuclease activity of Mre11 is utilized during NHEJ using an *in vitro* assay (Paull and Gellert, 1998), an *S.cerevisiae* strain bearing an *mre11* mutation that abolishes its nuclease function shows normal NHEJ of plasmid DSBs *in vivo* (Moreau *et al.*, 1999). In addition to these reports, a previous study in *S.pombe* showed a slight reduction (3-fold) in frequency and elevated fidelity of a *rad32 Δ* strain (Wilson *et al.*, 1999). In our study, which involved an extensive examination of junctions formed in *rad32 Δ* as well as *rad50 Δ* , we did not observe any significant difference between these mutants and wild type in either the frequency or fidelity of NHEJ. Furthermore, the 350- to 1000-fold defect of *pku70 Δ* and *lig4 Δ* strains in our study serves as a control to demonstrate a NHEJ-defective phenotype. Taken together, our results suggest that neither of the Mre11 or Rad50 homologues in *S.pombe* are involved enzymatically in the NHEJ pathway and do not contribute to the end resection that results in the deletion formation observed in the wild-type NHEJ junctions. We also observed that a series of mutants defective in the DNA

damage and/or replication cell cycle checkpoint display a normal frequency of NHEJ.

Homologues of Mre11 and Rad50 have been identified in mammalian cells and interact with Nbs1 (a functional homologue of the *S.cerevisiae* Xrs2). Nbs1 is defective in Nijmegen breakage syndrome (NBS), and defects in Mre11 have been observed in ATLD (ataxia telangiectasia-like disorder) patients (Stewart *et al.*, 1999). Although NBS and ATLD cells are radiosensitive, neither display major defects in DNA DSB rejoining using pulsed-field gel electrophoresis, the hallmark of the NHEJ defect in mammalian cells (Kraakman-van der Zwet *et al.*, 1999; Stewart *et al.*, 1999). Furthermore, *MRE11* is not epistatic to *KU* in chicken cells: the sensitivity to γ -irradiation and the extent of chromosomal aberrations are higher in an *MRE11^{-/-}/KU70^{-/-}* cell line compared with cell lines deleted for a single one of the two genes (Yamaguchi-Iwai *et al.*, 1999). In contrast, there is mounting evidence that supports the involvement of Mre11 and its partner proteins in meiotic and mitotic homologous recombination (Petrini, 1999; Yamaguchi-Iwai *et al.*, 1999) and sister chromatid recombination (Bressan *et al.*, 1999). Additionally, cells from patients with ataxia telangiectasia (AT) lack checkpoint responses to DSBs but have a largely normal DSB rejoining profile (Blocher *et al.*, 1991). Also, in chicken cells, *ATM* (the gene mutated in AT) is not epistatic to *KU* but epistatic to the gene for the homologous recombination protein *RAD54* (Morrison *et al.*, 2000). In combination, these results indicate that the genetic requirements for NHEJ in *S.pombe* closely resemble those in vertebrate cells and underline the usefulness of the fission yeast as a model system for the dissection of this process.

We did not observe any sensitivity of *pku70Δ* and *lig4Δ* strains to DNA-damaging agents. Thus homologous recombination is the major pathway for the repair of DSBs in *S.pombe*. *Saccharomyces cerevisiae yku* and *lig4* mutants are also resistant to γ -irradiation, and sensitivity is only observed in *yku/rad52* or *lig4/rad52* double deletions (i.e. in the absence of homologous recombination) and only when cells are in a haploid G₁ phase (in stationary phase cultures) (Boulton and Jackson, 1996b; Herrmann *et al.*, 1998). We carried out our analysis using logarithmically growing cells since stationary cells in *S.pombe* are in a diploid G₂-like phase. *Schizosaccharomyces pombe pku70Δ/rhp54Δ* or *lig4Δ/rhp54Δ* double mutants did not show increased sensitivity to γ -irradiation compared with the *rhp54Δ* strain. We attribute this difference to the fact that *S.pombe* cells have a relatively short G₁ phase. The investigation of the importance of NHEJ for the repair of genomic DSBs in *S.pombe* will require further studies.

An intriguing aspect of our studies is the observation that, although Pku70 is required for telomere length maintenance, there is no alleviation of transcriptional silencing adjacent to telomeres in the absence of Pku70. Thus, telomere length regulation and silencing represent two distinct functions for Ku that differ in their evolutionary conservation. Separation of these two processes is consistent with other findings, which demonstrate that their genetic requirements differ. For example, Mre11 is required for telomere length maintenance but not transcriptional silencing in *S.pombe* and *S.cerevisiae* (Boulton and Jackson, 1998; Wilson *et al.*, 1999; this work) and active NHEJ *per se* is not required for telomere length regulation or telomeric silencing. In *S.cerevisiae*, Yku70 directly associates with silencing proteins such as Sir4 to mediate silencing (Tsukamoto *et al.*, 1997; Boulton and Jackson, 1998), and tethered Yku70 or Yku80 can mediate Sir-dependent silencing (Martin *et al.*, 1999). However, no homologues of Sir3 or Sir4 have been described outside of the budding yeast, and thus the link between Ku and telomeric silencing may not be conserved. It is possible that a distinct class of silencing proteins perform a similar role in other organisms.

Immunostaining showed that the bulk of Pku70 is distributed throughout *S.pombe* nuclei, in contrast to the high concentration of Yku observed in the vicinity of *S.cerevisiae* telomeres. Since the high concentration of Ku at or near the telomeres occurs only in *S.cerevisiae*, this would appear to correlate with a role in transcriptional silencing rather than telomere length maintenance. One possibility would be that these high levels of telomeric Ku are involved in recruiting silencing factors. We surmise that the role of Ku in telomere length maintenance does not require a high concentration of Ku at telomeres. It is likely that a small fraction of Ku is located at or near chromosome ends in *S.pombe*, although we have been unable to demonstrate this directly. The direct role of *S.cerevisiae* Yku in telomeric silencing may thus result in its accumulation at telomeres, making it necessary to regulate the redistribution of *S.cerevisiae* Yku, and potentially other components of the NHEJ machinery, to the sites of DNA damage.

By comparison with *S.pombe*, it seems likely that in *S.cerevisiae* the indirect participation of the DNA damage

checkpoint sensor proteins and Mre11–Rad50–Xrs2 in NHEJ may be to effect relocation of Yku. The independence of *S.pombe* NHEJ for DNA damage checkpoint proteins and Mre11/Rad50 may reflect the fact that Pku is not ‘stored’ at telomeric regions. Significantly, NHEJ of plasmid DSBs in *S.cerevisiae mre11*, *rad50* or checkpoint mutants shows a severe decrease in the frequency of rejoining, but the fidelity of repair is the same as in the parental cells. This suggests that the function of Mre11 and Rad50 in NHEJ in *S.cerevisiae* is distinct from that of Yku70 and Lig4 and would be consistent with a regulatory role, rather than a direct role in the process. Interactions between the Mre11–Rad50–p95 complex and the DNA damage checkpoint have also been demonstrated in mammalian cells (Lim *et al.*, 2000), which is again consistent with a role in the signalling function in response to DSBs. Such interactions may be conserved throughout eukaryotes and remain to be characterized in *S.pombe*; however, there is no evidence that they participate in NHEJ in mammalian cells.

The conservation of the NHEJ machinery between *S.pombe* and mammalian cells suggests that the fission yeast is a good model system for the further characterization of this process. Comparison of the fission yeast NHEJ pathway with that of other eukaryotes will provide increased insight into the functional requirements of DSB repair by NHEJ in the future investigations.

Materials and methods

Genetic manipulations and sensitivity assays

The strains used in this study are listed in Table II. Media and standard genetic techniques were as described previously (Gutz *et al.*, 1974). The *pku70* and *lig4* deletion strains, the C-terminal tagging of *pku70* and the reversion of the *ura4* marker to *Kan^R* or *his3* (in *lig4* or *pku70*, respectively) were created using the PCR-based method (Bahler *et al.*, 1998) and the following primers. For *pku70* knockout: Q484, 5′-TTTCTTTTGTGCGTATTTAAAAAGCCAAGGAAATTGTTTGT-ATAATCTGTTTTTACGCTTAGCTACAAATCCCACTGG-3′; and Q485, 5′-ATATCTTTAAGTTGACTAATCTTAACGCCCTTCATATGAAGTGTACTAATCAAGATCTCCAACACCAATGTTTATA-ACC-3′. For *lig4* knockout: UP76F, 5′-TCGGTTATTTATTTG-AGAAATATTATTGATCATAAACTGATTATAAAACGATTGCTTCAAGCCAAAACCTAAGATCGCCAGGGTTTCCCGATC-ACGAC-3′; and DOWN76R, 5′-ATTAACAAATTTGGGTTCAATTGATATCTATTTGGTTAACAACAACATCATACTCTTTCT-TTTTGTGTTAGCTACAGCGGATAACAATTTACACAGGA-3′. For *pku70-myc* tagging: tag70UPF, 5′-TTCTCAGGGATCGTGA-CCTAGAGTGAGCGGTAAAAAGGCAGATTTATTAGACAATTAACGAACCTATGTCAAAAAATTACGGATCCCCGGGTAT-ATTAA-3′; and tag70DOWNR, 5′-TGTTTGTGCGAAAGTTCA-GAGAAAGACGTAGTCAGGTAATAACATATTACATTCTAC-AATATACACATTATCATTTGCGGCGAATTCGAGCTCGTTT-AAAC-3′. For reversion of *ura4* to *Kan^R*: *ura45*′, 5′-AAGCTTAGC-TACAAAATCCCACTGGCTATATGTATGCATTTGTGTTAAAAA-AAGTTTGTATAGATTATTTAATCTACTCAGCCGGATCCCCGGGTTAATTAA-3′; and *ura43*′, 5′-AAGCTTGTGATATTGAC-GAAACTTTTGTGACATCTAATTTATCTGTTTCCAACACCAA-TGTTTATAACCAAGTTTATCTGTAATTCGAGCTCGTTTAAAC-3′. For reversion of *ura4* to *his3*: P720, CTTCGTCGGCAT-CTCTGCACATGTCGTGTTTCTTACCCTATTGTCCTACCAA-GAACCTCTTCTCTTTCAGGTTTCTGA; and P724, GAGAA-AAGATTGTGGTAATGTTGTAGGAGCATGTTTAATAAATTATCTATAGCAAATTACATGG-ACTGTTGGCTGCTCTTTG. All the genetic alterations were confirmed by PCR. For MMS, bleomycin and temperature sensitivity assays, 5 μ l of 10-fold serial dilutions of log-phase cells were spotted on YEA plates or YEA containing 0.005% MMS or 5 μ g/ml bleomycin, and incubated at the required temperature. For γ -ray survival assays, logarithmically growing cells were irradiated using a ¹³⁷Cs source at a dose rate of 12.5 Gy/min.

Table II. Strains used in this study

Strain	Genotype	Reference
501	<i>ura4.D18 leu1.32 ade6.704 h-</i>	Murray <i>et al.</i> (1992)
972	wild-type <i>h-</i>	
FY0367	<i>ura4.D18 leu1.32 ade6.210 h+</i>	Ekwall <i>et al.</i> (1997)
<i>lig4Δ</i>	<i>ura4.D18 leu1.32 ade6.704 lig4::ura4+ h-</i>	this study
<i>pku70Δ</i> (FY 3506)	<i>ura4.D18 leu1.32 ade6.216 his3.D1 his3-tel pku70::ura4+</i>	this study
<i>pku70-myc</i>	<i>ura4.D18 leu1.32 ade6.704 ku70:myc:Kan^R h-</i>	this study
<i>taz1-gfp</i> (FY 3806)	<i>ura4.D18 leu1.32 ade6.210 his3D1 his3-tel taz1:gfp:ura4+ h⁹⁰</i>	R.Allshire, unpublished
<i>pku70-myc/taz1-gfp</i>	<i>ura4.D18 leu1.32 ade- ku70:myc:Kan^R taz1:gfp:ura4+ h⁹⁰</i>	this study
<i>rhp9Δ</i>	<i>ura4.D18 leu1.32 ade6.704 rhp9::ura4+</i>	Willson <i>et al.</i> (1997)
<i>rad17Δ</i>	<i>ura4.D18 leu1.32 ade6.704 rad17::ura4+</i>	Griffiths <i>et al.</i> (1995)
<i>rhp9Δrad17Δ</i>	<i>ura4.D18 leu1.32 ade6.704 rhp9::ura4+ rad17::ura4+</i>	this study
<i>rad32Δ</i>	<i>ura4.D18 leu1.32 ade6.704 arg3.D4 rad32::ura4+ h+</i>	Tavassoli <i>et al.</i> (1995)
<i>rad50Δ</i>	<i>ura4.D18 ade6.210 smt0 rad50::Kan^R h-</i>	E.Hartsuiker and J.Kohli, unpublished
<i>rad50Δ</i>	<i>ura4.D18 leu1.32 ade6.210 smt0 rad50::Kan^R h-</i>	this study
<i>rad3Δ</i>	<i>ura4.D18 leu1.32 ade6.704 rad3::ura4+ h-</i>	Bentley <i>et al.</i> (1996)
<i>cds1Δ</i>	<i>ura4.D18 leu1.32 ade6.704 cds1::ura4+ h-</i>	Murakami and Okayama (1995)
<i>chk1Δ</i>	<i>ura4.D18 leu1.32 ade6.704 chk1::ura4+ h+</i>	Al-Khodairy <i>et al.</i> (1994)
<i>rhp54Δ</i>	<i>ura4.D18 leu1.32 ade6.704 arg3.D4 rhp54::ura4+ h+</i>	Muris <i>et al.</i> (1996)
<i>tel1Δ</i>	<i>ura4.D18 leu1.32 ade6.704 arg3.D4 tel1::arg3+ h-</i>	N.Bentley, unpublished
<i>rad3Δtel1Δ</i>	<i>ura4.D18 leu1.32 ade6.704 arg3.D4 rad3::ura4+ tel1::arg3+ h-</i>	this study
FY1554	<i>ura4.D18 leu1.32 ade6.210 his3D1 h-</i>	this study
FY1870	<i>ura4.DS/E leu1.32 ade6.210 his3D1 ade6sphOTR his3-tel</i>	this study
FY1872	<i>ura4.DS/E leu1.32 ade6.210 his3D1 ade6sphOTR ura4-tel h⁹⁰</i>	this study
<i>pku70-myc</i> (FY4101)	<i>ura4.DS/E leu1.32 ade6.216 his3D1 ura4-tel pku70/myc::Kan^R h-</i>	this study
<i>lig4Δ</i> (FY4102)	<i>ura4.DS/E leu1.32 ade6.210 his3D1 ura4-tel lig4::Kan^R h-</i>	this study
<i>rad3Δ</i> (FY4109)	<i>ura- leu1.32 ade6.210 his3D1 his3-tel rad3::ura4+ h-</i>	this study
<i>lig4Δ</i> (FY4110)	<i>ura- leu1.32 ade6.216 his3D1 his3-tel lig4::ura4+ h+</i>	this study
<i>pku70Δ</i> (FY4111)	<i>ura4.DS/E leu1.32 ade6.210 his3D1 ura4-tel pku70::his3+ h-</i>	this study
<i>rad50Δ</i> (FY4169)	<i>ura4.DS/E leu1.32 ade6.210 his3D1 ura4-tel rad50::Kan^R h-</i>	this study
<i>rad32Δ</i> (FY4170)	<i>ura- leu1.32 ade6.210 his3D1 his3-tel rad32::ura4+ h-</i>	this study
FY3824	<i>ura4.D18 leu1.32 ade6.210 his3D1 arg3-D4 (Ch16 ade6.216 leu2+) h+</i>	this study
FY757	<i>ura4DS/E leu1.32 ade6.704 (CM3112 Sup3e) h+</i>	Allshire <i>et al.</i> (1995)
<i>pku70Δ</i> (FY4239/4240)	<i>ura4DS/E leu1.32 ade6.210 (Ch16 ade6.216 leu2+) pku70::ura4+</i>	this study
<i>pku70Δ</i> (FY4241/4242)	<i>ura4DS/E leu1.32 ade6.704 (CM3112 Sup3e) pku70::ura4+</i>	this study
<i>pku70Δ/rhp54Δ</i>	<i>ura4.D18 leu1.32 ade6.704 pku70::his3+ rhp54::ura4+</i>	this study
<i>lig4Δ/rhp54Δ</i>	<i>ura4.D18 leu1.32 ade6.704 lig4::Kan^R rhp54::ura4+</i>	this study

Duplicates of irradiated cells and unirradiated controls were plated on YEA medium plates and incubated at 30°C for 3–4 days. Telomere length detection and transcriptional silencing assays were carried out as described previously (Allshire *et al.*, 1995; Nimmo *et al.*, 1998). The minichromosome loss assays were carried out as described previously (Allshire *et al.*, 1995). At least three separate isolates were examined in each case, and the total number of colonies scored for each experimental point were: 5806 for wild type/linear, 3685 for wild type/circular, 20 308 for *pku70Δ*/linear, and 4996 for *pku70Δ*/circular.

NHEJ assay

Plasmid pAL19 was constructed essentially as described for pUR19 (Barbet *et al.*, 1992) except that *LEU2* was used in place of the *ura4* marker. Plasmid PS was obtained by cloning an ~500 bp *Pst*I fragment excised from vector pSTA (Carr *et al.*, 1989) into the unique *Pst*I site of pAL19. Similarly, plasmid PI was created by the insertion of an ~540 bp fragment into the unique *Eco*RI site of pAL19. pAL19 was used as the undigested control and PS and PI for the preparation of the linear substrates. The cohesive-ended substrates for the NHEJ experiments were prepared by excision of the *Pst*I fragment from PS or the *Eco*RI fragment from PI and purification of the remaining linear vector using the Qiagen Gel Extraction Kit. The blunt-ended substrate was prepared by the removal of an ~700 bp *Pvu*II fragment from PS. Logarithmically growing cells were transformed with equal amounts (1 μg) of undigested or linear DNA using the lithium acetate method (Okazaki *et al.*, 1990). Each experiment was carried out in duplicate and the NHEJ frequency was calculated as the percentage of *leu+* colonies arising from cells transformed with linear over those transformed with undigested DNA. This value was normalized to the value obtained from wild-type cells. At

least three experiments were performed for each point, and the average and standard deviation are shown on the bar diagrams. For the analysis of the fidelity of repair, plasmids were extracted from *leu+* colonies as described previously (Caspari, 1997) and electroporated in XL1-Blue electrocompetent cells. The plasmids were then amplified from ampicillin-resistant colonies using the Qiagen miniprep kit and analysed by restriction digestion or sequenced using an ABI 370A automated sequencer.

Immunofluorescence

Immunofluorescence experiments were carried out essentially as described (Ekwall *et al.*, 1995). For drug treatment, logarithmically growing cells were incubated with 0.03% MMS or 40 μg/ml bleomycin or left untreated for 3 h at 30°C with shaking prior to fixation. The mouse monoclonal c-Myc antibody (Santa Cruz Biotechnologies) was used to target Pku70-Myc. For confocal microscopy, cells were prepared as before and observed using a Biorad MRC 600 confocal microscope.

Acknowledgements

We would like to thank all the members of the A.M.C. laboratory and especially T.Caspari for technical advice. We also thank R.Antonelli, S.Macfarlane and G.Hamilton for strain constructions, J.Murray for the *ura4* to *Kan^R* primers, and N.Bone for assistance with confocal microscopy. Work in the laboratory of P.A.J. contributing to this study has been funded by grants from the Leukaemia Research Foundation, the Human Frontiers Science Programme, the Industry-funded UKCCCR Radiation Research Programme and European Union grant F13PCT920007. Telomere research by E.N. and R.A. is funded by The Cancer Research Campaign project grant SP2345/0102.

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Received July 7, 2000; revised October 24, 2000;
accepted November 22, 2000