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The Role Of Nonhomologous End-Joining Components in Telomere Metabolism in *Kluyveromyces lactis*

Sidney D. Carter,*[,1] Shilpa Iyer,[†,1,2] Jianing Xu,[†] Michael J. McEachern[†] and Stefan U. Åström*[,3]

*Department of Developmental Biology/Wenner-gren Institute, Stockholm University, SE-106 91 Stockholm, Sweden and [†]Department of Genetics, Fred C. Davison Life Science Complex, University of Georgia, Athens, Georgia 30602

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ABSTRACT

The relationship between telomeres and nonhomologous end-joining (NHEJ) is paradoxical, as NHEJ proteins are part of the telomere cap, which serves to differentiate telomeres from DNA double-strand breaks. We explored these contradictory functions for NHEJ proteins by investigating their role in *Kluyveromyces lactis* telomere metabolism. The *ter1-4LBsr* allele of the *TER1* gene resulted in the introduction of sequence altered telomeric repeats and subsequent telomere–telomere fusions (T–TFs). In this background, Lig4 and Ku80 were necessary for T–TFs to form. Nej1, essential for NHEJ at internal positions, was not. Hence, T–TF formation was mediated by an unusual NHEJ mechanism. *Rad50* and *mre11* strains exhibited stable short telomeres, suggesting that Rad50 and Mre11 were required for telomerase recruitment. Introduction of the *ter1-4LBsr* allele into these strains failed to result in telomere elongation as normally observed with the *ter1-4LBsr* allele. Thus, the role of Rad50 and Mre11 in the formation of T–TFs was unclear. Furthermore, *rad50* and *mre11* mutants had highly increased subtelomeric recombination rates, while *ku80* and *lig4* mutants displayed moderate increases. *Ku80* mutant strains also contained extended single-stranded 3′ telomeric overhangs. We concluded that NHEJ proteins have multiple roles at telomeres, mediating fusions of mutant telomeres and ensuring end protection of normal telomeres.

TELOMERES are specialized protein–DNA complexes at the ends of linear chromosomes. Telomeric DNA in most eukaryotes consists of tandem repeats of short G-rich sequences. In *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, telomeric repeats are bound by repressor activator protein 1 (Rap1) (Conrad *et al.* 1990; Krauskopf and Blackburn 1996), a sequence-specific DNA-binding protein containing two Myb domains. Myb-domain proteins bind to telomeres in other organisms as well, for example, Taz1 in fission yeast (Cooper *et al.* 1997) and Trf1/Trf2 in mammals (Broccoli *et al.* 1997).

Telomeres are synthesized by a specialized enzyme called telomerase (Chan and Blackburn 2004). In both *S. cerevisiae* and *K. lactis*, the catalytic core of this enzyme is composed of a reverse transcriptase, Est2, and an internal RNA component (*TLC1* in *S. cerevisiae* and *TER1* in *K. lactis*) that serves as template during DNA synthesis. Complete loss of telomerase causes shortening of telomeres and leads to replicative senescence due to the inability of the traditional DNA replication machinery to fully synthesize linear DNA ends (Lundblad and Szostak 1989; McEachern and Blackburn 1996).

Natural chromosome termini are protected from fusing with other DNA ends, indicating that telomeres have properties that differentiate them from DNA double-strand breaks (DSBs) elsewhere in the genome. This protective feature of telomeres, first observed by McClintock (1941) and Müller (1938), is often referred to as the telomere-cap function. One result of a compromised telomere cap is the recognition of chromosome termini as DSBs and subsequent attempts by the cell to repair these breaks. One pathway for DSB repair is the homologous recombination (HR) pathway, dependent on the *RAD52* group of genes (Symington 2002). The hallmark of the HR pathway is the use of a sister chromatid or homologous chromosome as a template during a mostly error-free repair process. In contrast, the nonhomologous end-joining (NHEJ) pathway for DSB repair (Daley *et al.* 2005) requires little or no homology and simply fuses two free DNA ends, often generating small deletions and insertions in the process. Normally, these ends are the result of DSBs that arise at internal chromosome positions. The Ku70/Ku80 heterodimer (Milne *et al.* 1996) is required for NHEJ and is thought to protect and align the DNA ends for subsequent end processing and ligation (Feldmann *et al.* 2000). The DNA ligase, Lig4 (Dnl4 or Lig IV), (Schär *et al.* 1997; Teo and Jackson 1997; Wilson *et al.* 1997)

[1]These authors contributed equally to this work.

[2]*Present address:* Department of Neurology, University of Virginia, Charlottesville, VA 22902.

[3]*Corresponding author:* Department of Developmental Biology/Wenner-gren Institute, Stockholm University, Svante Arrheniusv., 16-18 106 91 Stockholm, Sweden. E-mail: stefan.astrom@devbio.su.se

and Lif1 (Lig IV interactingfactor 1) (Herrmann *et al.* 1998) provide the ligase activity during NHEJ. Lif1 is the yeast functional homolog of mammalian Xrcc4 and is required for the stability and full activity of Lig4 (Teo and Jackson 2000). In addition, the MRX complex (Mre11, Rad50, and Xrs2) is also required for efficient NHEJ in *S .cerevisiae* and *K. lactis* (Moore and Haber 1996; Boulton and Jackson 1998; Kegel *et al.* 2006). Mre11 is an endo/exo nuclease, but mutant forms of Mre11 lacking detectable nuclease activity still support NHEJ (Lewis *et al.* 2004). Cell type regulates NHEJ in *S. cerevisiae*, such that haploid *MAT***a** or *MAT*α strains perform NHEJ efficiently, while diploid *MAT***a**/*MAT*α strains perform NHEJ inefficiently (Åström *et al.* 1999; Lee *et al.* 1999). This regulation of NHEJ is the result of transcriptional repression of the *NEJ1* gene in diploid cells. *NEJ1* encodes a protein that interacts with Lif1 and is essential for NHEJ by all standard assays (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Ooi *et al.* 2001; Valencia *et al.* 2001). The *K. lactis NEJ1* ortholog is also essential for NHEJ, but its transcription is not regulated by cell type, and *K. lactis* diploid cells perform NHEJ with an efficiency similar to either haploid cell type (Kegel *et al.* 2006). In humans, the recently identified *XLF/Cernunnos* gene was suggested to be the ortholog of yeast *NEJ1* (Callebaut *et al.* 2006).

It is known that telomeres with a compromised cap can engage in both HR and NHEJ (Lundblad and Blackburn 1993; McEachern and Blackburn 1996). HR repair can result in telomerase-independent telomere elongation or shortening. In contrast, NHEJ of telomeres results in telomere–telomere fusions (T–TFs). This is illustrated in telomerase-deficient fission yeast in which the majority of cells die as a result of gradual telomere attrition. In most surviving cells, chromosomes lacking detectable telomeric sequence circularize as a result of T–TFs, while a smaller fraction of survivors appear to maintain their telomeres by recombination (Nakamura *et al.* 1998). In addition, in *S. cerevisiae* and fission yeast, both telomerase and Tel1, a member of the ATM family of protein kinases, protect telomeres from fusions (Nakamura *et al.* 1998; Chan and Blackburn 2003; Mieczkowski *et al.* 2003).

Other mutations related to telomere function can also compromise the telomere cap. For example, overexpression of a dominant-negative form of the human telomere protein hTrf2 resulted in T–TFs in which telomere sequences were still present (van Steensel *et al.* 1998). Fission yeast Taz1 (Ferreira and Cooper 2001) and *S. cerevisiae* Rap1 (Pardo and Marcand 2005) also protect telomeres from fusing, suggesting that these Myb-domain proteins are part of the DNA–protein complex constituting the cap. In *K. lactis*, T–TFs are observed in strains with specific mutations in the template region of the telomerase RNA. These mutations are predicted to abolish or decrease Rap1 binding to the telomere (McEachern *et al.* 2000). T–TFs containing telomeric repeats appear to arise by a NHEJ-dependent mechanism since their formation requires Lig4 in both yeasts and mammals (Smogorzewska *et al.* 2002; Liti and Louis 2003; Mieczkowski *et al.* 2003; Ferreira and Cooper 2004). The role of individual NHEJ components in generating T–TFs is not clear, however. *S. cerevisiae* Ku has been reported to be both required (Pardo and Marcand 2005) and dispensable (Mieczkowski *et al.* 2003) for the formation of T–TFs. Paradoxically, knockdown alterations of mammalian Ku86 expression promote chromosome fusions involving telomeric sequences (Hsu *et al.* 2000; Samper *et al.* 2000), suggesting that Ku acts to prevent formation of T–TFs. In addition, *S. cerevisiae* Nej1 has been reported to prevent T–TFs, despite being required for promoting NHEJ (Liti and Louis 2003).

In this study, we explored the role of NHEJ proteins in telomere metabolism in *K. lactis*. We found that Ku80 and Lig4 were required for the formation of T–TFs and that subtelomeric gene conversion rates were dramatically increased in strains lacking Mre11 or Rad50. Surprisingly, Nej1 was not required for T–TF formation.

MATERIALS AND METHODS

Yeast strains: The strains used in this study are listed in Table 1. Strains lacking *LIG4* (AKY124), *NEJ1* (SAY572), *KU80* (SAY573), *MRE11* (SAY559), and *RAD50* (SAY557) were generated previously (Kegel *et al.* 2006). The double-mutant strains *sir4 lig4* (SAY703), *sir4 nej1* (SAY701), *sir4 ku80* (AKY116), and *sir4 mre11* (SAY704) were generated by crossing a *sir4* strain (SAY100) with the respective nonhomologous-end-joining mutant strain followed by tetrad analysis. For T–TF assays, a strain containing a duplicated *TER1* locus containing both a wild-type and the mutant *ter1-4L Bsr* allele (generated by plasmid loop-in) was crossed to strains carrying null mutations in *LIG4*, *NEJ1*, *KU80*, *MRE11*, and *RAD50*. Tetrad analysis resulted in the isolation of *TER1::URA3-ter1-4L-Bsr ku80* (SAY600), *TER1::URA3-ter1-4L Bsr mre11* (SAY579), *TER1::URA3-ter1-4L Bsr rad50* (SAY604), *TER1::URA3-ter1-4L Bsr nej1* (SAY562), and *TER1::URA3-ter1-4L Bsr lig4* (SAY563) double-mutant strains. The *TER1::URA3-ter1-4LBsr nej1 lig4* triple-mutant strain (SAY564) was obtained through the same procedure following a cross between 4LBsr and a *nej1 lig4* double-mutant strain (SAY545). Additionally, *TER1::URA3-ter1-4L Bsr* single-mutant strains were isolated from each of the above-mentioned crosses to serve as controls. Each strain was subsequently plated onto 5-FOA medium (Boeke *et al.* 1987) and clones that had lost the wild-type *TER1* gene but retained the mutant *ter1-4L Bsr* allele were identified. The resulting strains were maintained by serial restreaks on rich medium (YEPD) for 48–72 hr at 30°.

Random spore analysis: The *ter1-AccSna* strain used in this experiment is a derivative of the wild-type haploid strain 7B520 (*ura3-1 his2-2 trp1*) (Wray *et al.* 1987). Analysis was performed on the spores generated by mating the *ter1-AccSna* strain with GG1958 (*ade2*). Diploid cells were plated onto sporulation media for 4 days. Cells were then incubated in 200 μl of zymolyase (0.17 mg/ml) in 1 M sorbitol at 37° for 10 min to digest the asci. Subsequently, cells were subjected to heat treatment at 55° for 10 min to kill all vegetative cells. The resulting spores were serially diluted in TE (10 mM Tris and 1 mM EDTA) and plated onto YPD plates.

TABLE 1

Yeast strains used in this study

K. lactis	Genotype	Reference
4LBsr	*MAT*α *his2-2 trp1 ura3-1 TER1::URA3-ter1-4LBsr*	McEachern *et al.* (2000)
CK213-4C	*MAT***a** *leu2 lysA1 metA1 trp1 uraA1*	Chen and Clark-Walker (1994)
AccSna	*MAT***a** *his2-2 trp1 uraA1 ter1-AccSna*	McEachern *et al.* (2000)
AKY116	MAT**a** *lysA1 uraA1 ku80::LEU2 sir4::LEU2*	This study
AKY124	*MAT***a** *leu2 lysA1 trp1 uraA1 lig4::KANMX*	Kegel *et al.* (2006)
SAY100	*MAT*α *ade2 leu2 uraA1 sir4::LEU2*	Åström and Rine (1998)
SAY545	*MAT***a** *leu2 lysA1 metA1 trp1 uraA1 nej1::LEU2 lig4::KANMX*	This study
SAY557	*MAT***a** *leu2 lysA1 metA1 trp1 uraA1 rad50::KANMX*	Kegel *et al.* (2006)
SAY559	*MAT***a** *leu2 lysA1 metA1 trp1 uraA1 mre11::KANMX*	Kegel *et al.* (2006)
SAY561	*MAT*α *his2-2 leu2 uraA1 trp1 TER1::URA3-ter1-4LBsr*	This study
SAY562	*MAT***a** *lysA1* or *LYSA1trp1uraA1 nej1::LEU2 TER1::URA3-ter1-4LBsr*	This study
SAY563	*MAT***a** *lysA1* or *LYSA1 leu2 uraA1 trp1 lig4::KANMX TER1::URA3-ter1-4LBsr*	This study
SAY564	*MAT*? *his2-2 lysA1* or *LYSA1 trp1 uraA1 lig4::KANMX nej1::LEU2 TER1::URA3-ter1-4LBsr*	This study
SAY572	*MAT***a** *leu2 lysA1 metA1 trp1 uraA1 nej1::LEU2*	Kegel *et al.* (2006)
SAY573	CK213-4C *ku80::LEU2*	Kegel *et al.* (2006)
SAY579	*MAT***a** *his2-2 metA1 uraA1 mre11::KANMX TER1::URA3-ter1-4LBsr*	This study
SAY580	*MAT*α *his2-2 uraA1 TER1::URA3-ter1-4LBsr*	This study
SAY600	*MAT*α *uraA1 ku80::LEU2 TER1::URA3-ter1-4LBsr*	This study
SAY604	*MAT***a** *his2-2 leu2 metA1 uraA1 rad50::KANMX TER1::URA3-ter1-4LBsr*	This study
SAY605	*MAT*α *his2-2 TER1::URA3-ter1-4LBsr*	This study
SAY606	*MAT***a** *leu2 lysA1 uraA1 rad50::KANMX TER1::URA3-ter1-4LBsr*	This study
SAY701	*MAT*α *leu2 metA1 uraA1 nej1::LEU2 sir4::LEU2*	This study
SAY703	*MAT*α *ade1 leu2 lysA1 metA1 trp1 uraA1 lig4::KANMX sir4::LEU2*	This study
SAY704	*MAT***a** *leu2 lysA1 uraA1 mre11::KANMX sir4::LEU2*	This study
Kl7B520	*MAT***a** *his2-2 trp1 uraA1*	Wray *et al.* (1987)
GG1958	*MAT*α *ade2*	This laboratory

Hybridizations: DNA blots were hybridized with the previously described wild-type *K. lactis* telomeric probe (Klac1-25) at 50° following a standard protocol (McEachern and Blackburn 1995). In-gel hybridization experiments were performed as previously described (Dionne and Wellinger 1996), using oligonucleotide probes complementary to either the 5′ telomeric strand (G-probe) (Klac1-25) or the 3′ telomeric strand (C-probe). Approximately 3 μg of digested DNA was electrophoresed through a 0.7% agarose gel and analyzed using the conditions described in Underwood *et al.* (2004).

Subtelomere gene conversion assay: The gene conversion assay was performed according to the protocols described previously (McEachern and Iyer 2001). In brief, a native telomere in *rad50*, *mre11*, *ku80*, *lig4*, and wild-type strains was replaced by transformation with an ~2.0-kb *Eco*RI and *Sac*II **s**ub**t**elomeric ***U****RA3* (STU) fragment containing the *URA3* gene from *S. cerevisiae*. The marker was inserted ~120 bp from the junction of the cloned telomere and subtelomere fragments of *K. lactis*. Serially diluted cells of the clones containing a single-copy STU fragment were plated onto synthetic complete (SC) medium, SC + 5-FOA, and YPD. Measurement of the loss of the *URA3* gene was performed by counting colonies grown on 5-FOA and comparing them to the total number of colonies grown on YPD and SC.

Analysis of telomere–telomere fusions: The strains used for analysis of T–TFs were *ter1-4LBsr* strains SAY605 and SAY561 following 25 and 5 serial restreaks, respectively. Genomic DNA was prepared using the MasterPure Yeast DNA purification kit (Epicentre, Madison, WI). One primer (P1 5′-CAGGGCGGGT AACATGAC-3′) contained a sequence unique to *K. lactis* chromosome 2L and corresponded to 268–285 nucleotides upstream from the telomeric repeat sequence start (Nickles and McEachern 2004). The second primer (P2-11 5′ GAAAGAG GAAATCCGTTTCG-3′) contained a sequence common to a subtelomeric region of at least nine chromosome ends other than 2L. Potential annealing sites for this primer ranged between 167 and 254 nucleotides from the telomeric repeat sequence start, depending upon the chromosome end (Nickles and McEachern 2004). PCR reactions (50 μl) contained 0.2 ng genomic DNA, 1× PCR reaction buffer (10 mm Tris–HCl, 1.5 mm MgCl2, 50 mm KCl), dNTP 0.1 mm each, primers 0.5 μm each, and 1 unit Taq DNA polymerase. Reaction conditions were as follows: 95° for 1 min; 35 cycles of 95° for 30 sec, 55° for 30 sec, 72° for 2 min, followed by 72° for 10 min. The products were separated on a 1% agarose gel and visualized by ethidium bromide (EtBr) staining. Portions of the resulting DNA smear were cut from the gel and the DNA products were recovered. Isolated products were then cloned into the Promega (Madison, WI) pGEM T-Easy vector using the suggested protocol and transformed into the *Escherichia coli* strain DH5α cells. Approximately 1 μg of plasmid DNA was digested with *Eco*RI or doubly digested with *Eco*RI and *Bsr*GI. The mixtures were separated on a 2% agarose gel and visualized by ethidium bromide staining to determine fragment sizes. Clones were submitted for DNA sequencing by Macrogen (Seoul, South Korea).

RESULTS

Telomere lengths in strains lacking NHEJ components: To explore if NHEJ proteins were required for maintaining normal telomere length in *K. lactis*, we determined telomere lengths in strains lacking individual NHEJ proteins. The 12 telomeres in *K. lactis* give rise to a

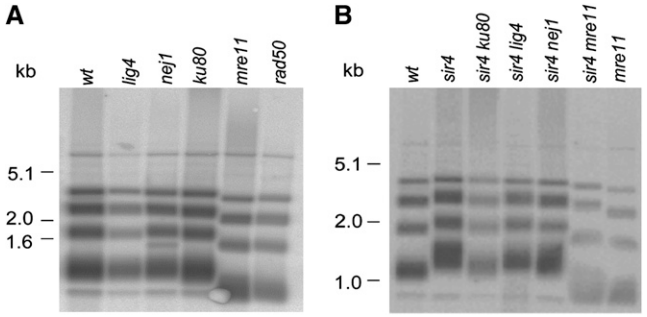


FIGURE 1.—Telomere length in strains compromised for NHEJ. DNA blot of *Eco*RI-digested chromosomal DNA hybridized with a probe specific to *K. lactis* telomeric repeats. (A) Telomere lengths from strains CK213-4C (*wt*, or wild type), AKY124 (*lig4*), SAY572 (*nej1*), SAY573 (*ku80*), SAY559 (*mre11*), and SAY557 (*rad50*). (B) Telomere lengths of strains SAY100 (*sir4*), AKY116 (*sir4 ku80*), SAY703 (*sir4 lig4*), SAY701 (*sir4 nej1*), SAY704 (*sir4 mre11*), and SAY559 (*mre11*). Molecular weight markers are in kilobases.

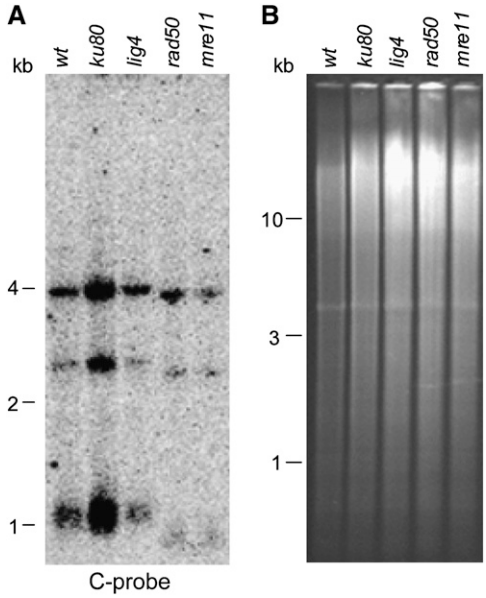


FIGURE 2.—Long 3′ overhangs in *ku80* cells. Nondenaturing in-gel hybridization of genomic DNA digested with *Pst*I from *wt* (wild type), *ku80*, *lig4*, *mre11*, and *rad50* strains. (A) Shows hybridization to a G-strand telomeric probe. (B) Shows the EtBr-stained gel prior to hybridization. Molecular weight markers are in kilobases.

characteristic pattern of four to five bands on DNA blots after digestion of the genomic DNA with *Eco*RI (Figure 1A). Comparing the pattern using DNA from strains with null alleles in NHEJ genes revealed that *lig4*, *ku80*, and *nej1* mutant strains had normal telomere lengths, but telomeres were shorter in *rad50* and *mre11* mutants. The shortened telomeres in strains lacking the MRX complex were consistent with previous results obtained in *S. cerevisiae* (TSUKAMOTO *et al.* 2001). The observation that a *ku80* mutant strain had normal length telomeres was surprising since the corresponding mutation leads to telomere shortening in *S. cerevisiae*.

Next we investigated if we could observe a telomere length defect in the absence of NHEJ proteins in a *sir4* background. It had previously been shown that strains lacking Sir4 in *K. lactis* have longer telomeres than wild-type strains (ÅSTRÖM and RINE 1998). We hypothesized that using a *sir4* mutant background might sensitize the assay. The telomere lengths of a *sir4* single and a *sir4 ku80* double-mutant strain indeed differed. The double mutant had telomeres longer than the wild-type strain, but shorter than the *sir4* single-mutant strain (Figure 1B). In contrast, telomere lengths in the *nej1 sir4* and *lig4 sir4* double-mutant strains were similar to the *sir4* single-mutant strain, confirming that NHEJ function was not required for maintaining normal telomere length (Figure 1B). Telomere length in the *mre11 sir4* double-mutant strain was shorter than that of the wild-type strain, but longer than that of the *mre11* single mutant, indicating that Mre11 and Sir4 affected telomere length homeostasis by different and opposing mechanisms.

Single-stranded 3′ overhangs were formed in the *ku80* mutant: One manifestation of telomere capping loss is the generation of extended single-stranded 3′ telomeric overhangs. We therefore employed nondenaturing in-gel hybridization, a method that detects relative amounts of 3′ overhangs, to analyze *ku80*, *lig4*, *rad50*, and *mre11* mutant strains (DIONNE and WELLINGER 1996). Using a probe complementary to the 3′ strand (C-probe), a strong signal was visible in the *ku80* strain relative to wild type, while only weak signals were detected in the *lig4*, *mre11*, and *rad50* mutant strains (Figure 2A). No signal was observed using a probe complementary to the 5′ telomeric DNA (G-probe) (data not shown). EtBr staining of the gel prior to hybridization confirmed that similar amounts of DNA were loaded in all lanes (Figure 2B). These results showed that extended 3′ overhangs were present in the *ku80* strain and are consistent with Ku80 contributing to telomere end protection. The precise length of these overhangs and whether they were cell cycle dependent remains unclear.

Increased subtelomeric recombination in *rad50*, *mre11*, *lig4*, and *ku80* mutants: Both shorter telomere length and elongated 3′ telomere overhangs have been associated with increased recombination in and near telomeres (MCEACHERN and IYER 2001; UNDERWOOD *et al.* 2004; IYER *et al.* 2005). We therefore hypothesized that *ku80*, *mre11*, and *rad50* mutant strains would exhibit elevated levels of subtelomeric recombination. To test this idea, we performed assays on *ku80*, *mre11*, *rad50*, *lig4*, and wild-type strains to measure the rates of subtelomeric gene conversion as previously described (MCEACHERN and IYER 2001). This assay measures the loss of a *URA3* marker gene, which was artificially positioned close to a telomere in each of the different strains (see MATERIALS AND METHODS). Silencing of the *URA3* gene is not observed using these constructs since 108/108

TABLE 2

Elevated levels of recombination near telomeres in *rad50*, *mre11*, *ku80*, and *lig4* mutant strains

Strains	Gene conversion rates [mutation rate ± SE (*n*)]	Relative rate
Wild type	$2.8 \times 10^{-6} \pm 1.5 \times 10^{-6}$ (13)	1
ku80	$2.3 \times 10^{-5} \pm 9.5 \times 10^{-6}$ (15)	~8
lig4	$3.5 \times 10^{-5} \pm 6.0 \times 10^{-6}$ (10)	~13
rad50	$6.5 \times 10^{-4} \pm 8.8 \times 10^{-5}$ (18)	~230
mre11	$2.8 \times 10^{-3} \pm 1.1 \times 10^{-3}$ (15)	~1000

The gene conversion rates of the mutants are based on measurement of several (three to four) independent transformants of the individual mutant strains, containing the *URA3* gene inserted near one telomere (see MATERIALS AND METHODS). For each strain, the number of samples, *n*, used for the assay is in parentheses. The standard error (SE) was calculated as the standard deviation divided by the square root of *n*.

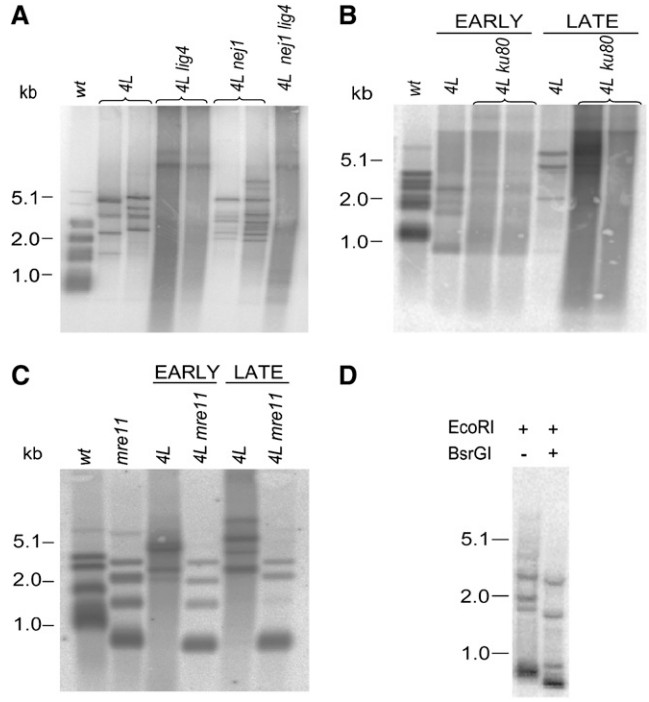


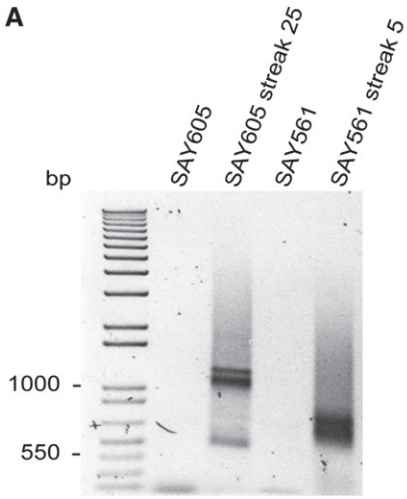
FIGURE 3.—T–TFs in NHEJ mutant strains. (A) *LIG4*, but not *NEJ1*, was required for formation of T–TFs. Shown is a DNA blot using two independent isolates of *ter1-4LBsr* (*ter1-4L*), *ter1-4L lig4*, and *ter1-4L nej1* and one *ter1-4L lig4 nej1* strain. Chromosomal DNA was prepared from the strains following 10 streaks. (B) *KU80* was required for formation of T–TFs. The blot is divided into early and late lanes. Chromosomal DNA from early lanes was isolated immediately following the generation of the mutant strains. Late lanes are of the same mutant strains following 24 serial restreaks. Two independent isolates of *ter1-4L ku80* and one isolate of a *ter1-4L* single mutant are shown. (C) *ter1-4L mre11* double-mutant strains had short, stable telomeres. The blot is divided into early and late lanes as described above. Shown are *ter1-4L*, *mre11*, and *ter1-4L mre11* strains. Wild types for all blots are *TER1* strains recovered from the same tetrads used in the generation of the respective mutant strains. (D) A *ter1-4L rad50* double-mutant strain incorporated mutant repeats. *Eco*RI and *Eco*RI/*Bsr*GI digestions of genomic DNA isolated from a *ter1-4L rad50* mutant strain following 24 serial restreaks. Molecular weight markers are in kilobases.

5-FOA resistant clones derived from cells with the same *URA3*-tagged telomere had all lost the *URA3* gene as judged by DNA blots (NATARAJAN *et al.* 2006). Relative to the wild-type control strain, we observed dramatic increases in *URA3* loss (Table 2). The ~250- to 1000-fold increased gene conversion rates in *rad50* and *mre11* strains indicate that these mutants were particularly compromised in their ability to protect telomeric and subtelomeric regions against recombination. An interesting observation is the modest increase in subtelomeric recombination in the absence of Lig4. This is the first observation that Lig4 might have a role in telomere end protection. We confirmed that the *URA3* gene had been lost from 5-FOA-resistant clones from the *lig4* and *ku80* mutant strains using DNA blots (data not shown). Our results indicate that despite possessing telomeres of normal length, *ku80* and *lig4* mutants have a telomere-capping defect.

Telomere–telomere fusions were produced by an atypical NHEJ mechanism: Mutations in the template region of telomerase RNA, encoded by the *K. lactis* *TER1* gene, lead to the introduction of the corresponding changes in newly synthesized telomeric repeats (MCEACHERN and BLACKBURN 1995; UNDERWOOD *et al.* 2004). Specific mutations in the *TER1* gene, predicted to abolish Rap1 binding to the newly synthesized repeats, can lead to the formation of T–TFs (MCEACHERN *et al.* 2000). We used strains containing a combination of a particular *TER1* allele, termed *ter1-4LBsr* (abbreviated *ter1-4L*), with mutations in NHEJ components and asked if T–TFs were formed.

The *ter1-4L* single-mutant strains used as controls showed the expected pattern of immediate severe telomere elongation and degradation, as indicated by a smeared DNA blot signal. After prolonged passaging, the smeared signal was resolved into a series of sharp, discrete bands (Figure 3B, 4L early *vs.* late). These bands were previously shown to be indicative of T–TFs (MCEACHERN *et al.* 2000). Two independent isolates of *ter1-4L* strains lacking Lig4 or Ku80 showed no signs of T–TFs even after 10 and 25 streaks, respectively (Figure 3, A and B). In contrast, strains lacking Nej1 formed T–TFs at a rate similar to those of a wild-type strain (Figure 3A and data not shown). We concluded that *K. lactis* T–TFs induced by the *ter1-4L* allele appeared to arise by an atypical NHEJ mechanism, a mechanism that required Lig4 and Ku80, but not Nej1.

Combining the *ter1-4L* mutation with *rad50* or *mre11* mutations revealed a surprising result with respect to the initial telomere elongation phenotype. The *ter1-4L* *rad50* and the *ter1-4L mre11* double-mutant strains did not show a loss of telomere length regulation and the



B

Telomere fusion #	Estimated # of mutant repeats -BsrG1 digest	Sequenced repeats (wt / mutant)
1	1	[30bp]
2	1	[25bp]
3	1	[0bp]
4	4	[20bp]
5	19	[280bp]
6	2	[70bp]
7	10	[10bp]

FIGURE 4.—Molecular characterization of T–TFs. (A) Agarose gel electrophoresis of PCR amplifications using genomic DNA from pre- and postfusion strains, as indicated above the lanes, and subtelomeric specific primers. Results were from strains SAY561 and SAY605 following 5 and 25 serial restreaks, respectively. Control lanes result from PCR of genomic DNA isolated from the parental strains used in the generation of each mutant strain prior to the *TER1* loop-out procedure. Size markers indicated on the left. (B) *K. lactis* T–TFs analyzed by restriction digests and DNA sequencing. Seven different T–TFs (first column) were analyzed, one to three originating from strain SAY605 and four to seven from strain SAY561. The second column shows an estimate of the number of mutant repeats present as determined by *Bsr*GI digestion of each cloned fragment followed by agarose gel electrophoresis. The third column is a schematic of the sequencing results. Sequenced wild-type repeats (black arrows) and mutant repeats (blue arrows) are indicated. Red and yellow boxes represent subtelomeric sequences. The numbers within brackets indicate the estimated difference, in base pairs, between the fragment submitted for sequencing and the actual sequence recovered from the analysis.

telomeres remained stably short even after 25 streaks (Figure 3C and data not shown). In addition, we observed no signs of T–TFs in either strain. A useful feature of this particular *TER1* mutant allele is that it introduces a *Bsr*GI restriction site into each newly synthesized telomeric repeat. Digestion with *Bsr*GI showed that in spite of failing to undergo telomere elongation, a few mutant repeats had indeed been incorporated in the *rad50* and *mre11* mutant strains (Figure 3D and data not shown). Hence, these results were consistent with a role for Mre11 and Rad50 in telomerase recruitment, but the contribution of these proteins to the formation of T–TFs remains unclear.

Molecular characterization of telomere–telomere fusions: The fusions between telomeres were further characterized using a PCR-based assay similarly to a previously described strategy in *S. cerevisiae* (MIECZKOWSKI *et al.* 2003; PARDO and MARCAND 2005). Taking advantage of recent sequence data (NICKLES and MCEACHERN 2004), we designed four PCR primers that annealed within the subtelomeric regions of *K. lactis* telomeres. We tested the specificity of these primers using chromosomal DNA from nonfused and postfusion strains as template. One primer pair specifically and consistently amplified DNA from several postfusion strains (Figure 4A), resulting in a smeared signal. This pair corresponded to a primer complementary to a sequence unique to the left end of chromosome 2 (P1) and a primer that was complementary to at least nine subtelomeric regions (P2-11) (see MATERIALS AND METHODS for details). PCR with only primer P2-11 failed to generate a product. This result did not rule out fusions between telomeres not involving telomere 2L, since such fusions, being almost perfect palindromes, probably resist PCR amplification.

The PCR products from two postfusion strains were cloned and amplified in *E. coli*. Digestion with *Bsr*GI

enabled us to make estimations of the number of telomeric repeats containing *Bsr*GI sites. In addition, DNA sequencing made it possible to count most wild-type repeats. The template DNA used originated from independently isolated *ter1-4L* single-mutant strains from late (25) and early (5) streaks. Given the smeary nature of the signal, three or four independent clones were sequenced. In all cases, the anticipated subtelomeric sequences as well as basal telomeric repeats were identified, but the T–TFs could not be sequenced through the fusion point. The three clones originating from the late streak (Figure 4B, telomere fusions 1–3) had very few *Bsr*GI-containing repeats, while the four clones originating from the early streak contained numerous mutant repeats (4–19). In all of the fusions sequenced, the number of wild-type repeats on each end was very limited (0–5).

We assume that the PCR-based assay for T–TFs was much more sensitive than the DNA blots used previously. Consistent with this assumption, we found that T–TFs could be detected earlier using the PCR-based assay, in fact, immediately following the pop-out procedure. Therefore, we investigated if T–TFs could be detected in the NHEJ mutant strains using the PCR-based assay. The results showed evidence of T–TFs in *ter1-4L nej1* mutant strains, but not in *ter1-4L* strains lacking Ku80, Lig4, Mre11, or Rad50 (data not shown). The two assays used for detection of T–TFs generated the same results and were thus confirmatory.

Multiple T–TFs are highly detrimental to *K. lactis* meiosis: The *ter1* template mutants that cause fusions between most or all telomeres have been postulated to select for derivatives that have circularized each of their six individual chromosomes (McEachern *et al.* 2000). Mutant cells with no detectable free telomeres and six telomere fusions displayed only moderately slow mitotic growth. Nevertheless, such cells were expected to incur difficulties while undergoing meiosis. This is because crossovers involving a circular chromosome would dimerize a homologous chromosomal pair and interfere with their disjunction at meiosis I. To test this notion, we used a *ter1-AccSna* mutant for conducting mating and sporulation analysis. This mutant strain, which contains a single base substitution in both the left half and the right half of the Rap1-binding site (Figure 5), was slower to undergo fusions than the *ter1-4L* mutant strain. However, the T–TFs formed in this strain were more stable, presumably because of the smaller average size of the fused telomere sequences.

A *ter1-AccSna* fusion strain showed no defect in the ability to mate with GG1958, a strain with normal telomeres, when compared to an isogenic wild-type control (7B520). However, when diploids generated by crossing wild-type 7B520 with GG1958 and *ter1-AccSna* with GG1958 were sporulated, a dramatic difference in successful tetrad production was observed. Microscopic observation showed that tetrads were abundant among

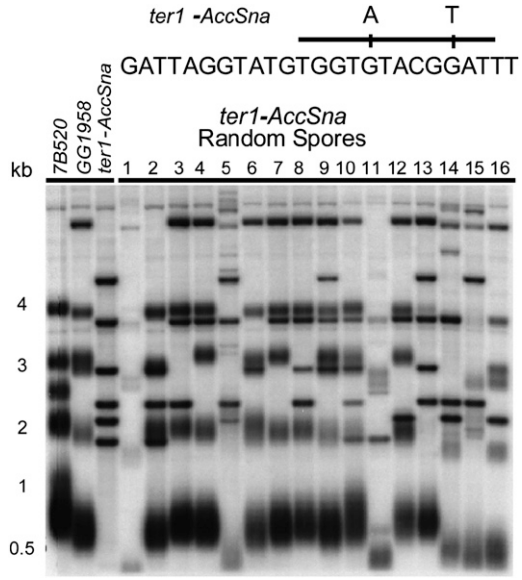


Figure 5.—Multiple T–TFs are detrimental to the *ter1-AccSna* strain undergoing meiosis. Shown is a DNA blot of *Eco*RI-digested genomic DNA from 16 haploid strains (lanes 1–16) derived from a cross between a *ter1-AccSna* strain with stable T–TFs and the wild-type strain GG1958. Lanes marked as 7B520, GG1958, and *ter1-AccSna* represent the parental haploid controls used to set up the crosses. The sharp bands visible in lanes 1–16, which are identical in size to bands present in the *ter1-AccSna* parent, represent T–TFs passed unaltered through meiosis. The genomic DNA was hybridized to a G-strand telomere probe (Klac1-25). Molecular weight markers are in kilobases. Above the blot is a schematic of the *ter1-AccSna* allele, indicating the position of the introduced mutations.

cells of the control strain but seemingly absent from the diploids derived from the telomere fusion strain. To further characterize this meiotic defect, random spore analysis was performed on the sporulating cultures of the same diploid strains. Recovery of viable spores, following heat treatment at 55°, was consistently reduced by ~100-fold for cells derived from the *ter1-AccSna* diploids relative to the control. Those cells that did survive exhibited one or more phenotypes (Ade−, Ura−, His−), which suggested that they were derivatives from the same haploid parental strains that were used to set up the initial cross. We next examined the structure of the telomeres in 16 of these segregants from the *ter1-AccSna* fusion diploids by DNA blots. The results confirmed that each of the clones contained mixtures of fused and unfused telomeres (Figure 5). The unfused telomeres were all either of normal length or shorter than normal, consistent with the presence of either wild-type *TER1* or *ter1-AccSna* (which produces short telomeres prior to fusion formation). In most cases, the fusion bands were identical in size with those of the *ter1-AccSna* haploid parent, consistent with the fusion bands being unaltered by meiosis. In at least two instances (Figure 5, lanes 14 and 15), novel sharp bands were also visible in *ter1-AccSna* haploids.

DISCUSSION

We examined the role of several NHEJ genes in telomere metabolism in *K. lactis*. The impetus behind our work is the seemingly contradictory roles of NHEJ proteins at telomeres *vs.* at other genomic locations. At telomeres, several NHEJ proteins have been implicated as integral components of the protective telomere cap. In this capacity, NHEJ proteins contribute to the prevention of the very same processes that they promote within the genome. We explored several aspects of telomere maintenance, including length homeostasis, the extent of 3′ overhangs, subtelomeric gene conversion rates, and formation of T–TFs. In addition, we examined the ability of strains containing T–TFs to undergo meiosis. Taken together, this study shows that NHEJ proteins have multiple and complex roles in imparting normal telomere metabolism.

With respect to telomere length, the short telomeres of the *mre11* and *rad50* strains were similar to results seen in *S. cerevisiae*, where unusually short but stable telomeres have been seen upon deletion of certain genes in DNA repair pathways, including the MRX complex and the Tel1 kinase (Kironmai and Muniyappa 1997; Boulton and Jackson 1998). Our data argue that the *K. lactis* MRX complex functions in maintenance of telomere length in much the same way that it does at *S. cerevisiae* telomeres. In contrast, both the *ku80* and *sir4* mutant strains showed telomere length phenotypes different from the corresponding *S. cerevisiae* mutant strains. The modest telomere elongation observed in a *K. lactis sir4* mutant differs from the result observed in *S. cerevisiae*, where both *sir3* and *sir4* mutations produce modest telomere shortening (Palladino *et al.* 1993). However, our results are consistent with the Sir4 protein being present at *K. lactis* telomeres and contributing in a minor way to telomere length regulation.

The Ku70/Ku80 heterodimer has been shown to be involved in both telomere length regulation and telomere capping in *S. cerevisiae* (Boulton and Jackson 1998; Gravel *et al.* 1998; Featherstone and Jackson 1999; Grandin *et al.* 2000; Bertuch and Lundblad 2003a,b; Stellwagen *et al.* 2003). Genetic analysis has shown that MRX and Tel1 function in the same pathway of telomere maintenance but in a different pathway than Ku (Ritchie and Petes 2000). Loss of either Ku70 or Ku80 caused a large decrease in telomere length and an increase in the length of 3′ overhangs present at telomeres (Bertuch and Lundblad 2003a) due to degradation of the 5′ strand of telomeric DNA (Gravel *et al.* 1998; Polotnianka *et al.* 1998; Maringele and Lydall 2002). These two roles are genetically separable (Bertuch and Lundblad 2003b). In *S. cerevisiae*, Ku's effect on telomere length appears to result from its ability to bind directly to a particular stem loop region of the telomerase RNA, thereby assisting in telomerase recruitment (Peterson *et al.* 2001; Miyoshi *et al.* 2003; Stellwagen *et al.* 2003). In this study we found that loss of the *K. lactis KU80* gene does not appreciably alter telomere length. A possible explanation for this result is that the binding site for Ku at the stem loop region is absent in the telomerase RNA of *K. lactis* (Tzfati *et al.* 2003). As a consequence, the *K. lactis* Ku protein may not have a major role in recruitment of telomerase to telomeres. However, the observation that a *ku80 sir4* double mutant exhibited slightly shorter telomeres than a *sir4* single mutant is consistent with Ku contributing to telomere length regulation to some degree. Although a Ku deficiency leads to telomere shortening in many organisms studied to date (Fisher and Zakian 2005), there are exceptions to this rule. For example, loss of Ku in Arabidopsis results in considerable telomerase-dependent telomere elongation (Riha and Shippen 2003). An increased length of telomere 3′ overhangs was shared between *S. cerevisiae* and *K. lactis ku80* mutants, indicating that the role of Ku in protecting telomeres from extensive degradation is conserved.

Large increases in subtelomeric gene conversion were previously seen in *K. lactis* telomerase RNA gene (*TER1*) mutants with stably shortened telomeres (McEachern and Iyer 2001). These events may represent break-induced replication (BIR) as subtelomeric loss of *URA3* is accompanied by replacement of tagged telomeric repeats (Natarajan *et al.* 2006). The increased subtelomeric BIR rates seen in *mre11* and *rad50* mutants may therefore be an indirect consequence of shorter telomere length rather than an additional separate defect in telomere capping. A previous report found that *S. cerevisiae mre11* and *rad50* mutants did not display a large increase in the rate of telomeric recombination despite having very short telomeres (DuBois *et al.* 2002). However, this study employed a telomere capture assay that required recombination with a telomeric repeat tract that was only ~80 bp in length. Subsequent work has shown that recombination involving homologous sequences of <100 bp can require *RAD50* (Ira and Haber 2002). We hypothesize that a Rad51 filament formed on a single-stranded 3′ telomeric end could invade another telomere with the invasion extending into subtelomeric sequences. Certain outcomes of this strand invasion complex could result in loss of the *URA3* gene and replacement of the original telomeric repeats with a sequence copied from the donor molecule. As the subtelomeric sequence that is shared between at least 11 of 12 *K. lactis* telomeres extends for well over 1 kb, there would be ample homology available for Rad51-dependent recombination to occur, even if the adjoining telomeric repeat tract was very short. Additionally, Ku deficiency has been reported to trigger increased recombination (Polotnianka *et al.* 1998; Baumann *et al.* 2002; DuBois *et al.* 2002; Miyoshi *et al.* 2003). Our results are consistent with these observations as we show that loss of *ku80* results in a

detectable, although moderate, increase in subtelomeric recombination.

Lig4 is an essential part of the NHEJ pathway along with Ku80, but no role has yet been assigned to Lig4 in telomere metabolism. Therefore, the 13-fold increase in recombination near telomeres in a *K. lactis lig4* mutant observed in this study provides the first suggestion of Lig4 contributing to chromosome end protection at the telomeres. Alternatively, HR and NHEJ compete for repairing DSBs in subtelomeric regions and the absence of Lig4 shuttles more repair events into the HR pathway. In support of this idea, we have observed competition between HR and NHEJ for an ectopic DSB induced ~25 kb from a telomere in *K. lactis* (P. Martinez and S. U. Åström, unpublished observation). It will be worthwhile to conduct further experiments to ascertain if Lig4 plays a role in telomere maintenance in the absence of telomerase.

On rare occasions, chromosome circularization has been observed in the genomes of yeasts and mammals as a result of a compromised telomere cap (Nakamura *et al.* 1998; Iyer *et al.* 2005). Several lines of evidence have pointed out that the genetic requirements for the formation of T–TFs can differ between species. In addition to the concomitant loss of all telomeric sequence, the circularized chromosomes of telomerase-deficient fission yeast have been shown to form independently of Ku80 and Lig IV (Nakamura *et al.* 1998; Fisher and Zakian 2005). In contrast, the T–TFs of *Schizosaccharomyces pombe* mutants lacking Taz1 formed between long telomeres and were dependent on Ku80 and Lig IV (Ferreira and Cooper 2001). In this study, the essential requirement of Ku80 and Lig4 provides the first evidence that fusions in the *ter1-4L* mutant were dependent on the NHEJ pathway. In addition, deleting the *RAD50* and *MRE11* genes, crucial for NHEJ at internal DSBs, led to very short telomeres and lack of any T–TFs. The simplest explanation for this result was that Rad50 and Mre11 were required for both telomere length homeostasis and for the formation of T–TFs. Another possibility could be that a certain minimum number of mutant telomeric repeats were required for T–TF formation and that this threshold was not reached in the *mre11* and *rad50* strains. This explanation was suggested for a T–TF-free *ter1* template mutant that contained two sets of base changes that, when present in cells individually, did cause T–TFs (McEachern *et al.* 2000).

A key finding from this body of work was that strains lacking Nej1, a protein necessary for NHEJ at internal DSBs, formed fusions with similar rates compared to the wild type. One possibility is that the function of Nej1 was replaced by another protein during the formation of T–TFs. It was also possible that the nature of NHEJ at telomere termini differs from NHEJ at internal chromosome positions in such a manner as to render Nej1 function dispensable. Given that the role of Nej1 during NHEJ is unknown, it is difficult to speculate about the nature of such a function. In any event, NHEJ-mediated T–TFs had different molecular requirements than NHEJ at other parts of the genome. In addition, we showed that the fusions formed in a *ter1-4L* strain did in fact contain a variable number of mutant and/or wild-type telomeric repeats. As observed with DNA blots, the size of telomere fusions in the *ter1-4L* mutant strains appeared to vary between individual isolates. This variation could contribute to the observed difference in mutant telomere repeat number between the assayed early and late streaks. However, it is possible that loss of one or several telomeric repeats occurred as a result of complications in cloning repetitive DNA sequences in *E. coli*. It is therefore difficult to draw extensive conclusions from the telomeric sequence data or to speculate about the total number of telomeric repeats present before cloning. Nevertheless, the sequencing data provide a verification of the presence of mutant telomeric repeats, which suggests that a reduced number of Rap1 molecules were bound to the telomeres prior to fusions. This presumably led to uncapping and a possibility for the NHEJ pathway to fuse telomere termini. The PCR-based assay revealed that T–TFs begin to arise immediately, while remaining undetectable on DNA blots. To explain this difference, we favor a model in which T–TFs initially occur both intra- and interchromosomally. In the subsequent streaks, the interchromosomal fusions are selected against due to their disomic nature. Intrachromosomal fusions, which are predicted to be more stable, gradually accumulate. When a majority of cells have formed intrachromosomal fusions, these fusions are possible to detect as discrete bands on DNA blots.

Other studies have shown that *S. pombe* strains containing T–TFs displayed severe defects in their ability to undergo meiosis (Naito *et al.* 1998). This defect was interpreted as either a problem inherent to circular chromomsomes or a consequence of a complete lack of telomeric repeats. Our results showed that *ter1-AccSna* cells containing T–TFs also have severe problems going through meiosis, displaying 100-fold reduced sporulation efficiency. As the T–TFs observed in this study retained telomeric repeats, this result suggests that the circular chromosomes themselves caused the observed meiotic defect. A possible explanation for the rare spores that did result from meiosis is that an even number of crossover events took place between each homologous pair. This would prevent formation of dicentric chromosomes and enable chromosome disjunctions with fused telomeres, thus allowing the cells to proceed through meiosis.

This study provides further evidence for the complex interplay between DSB repair pathways and telomeres. It will be especially interesting to continue exploring the mechanism underlying T–TF formation in *K. lactis*, as the mechanism appears different from NHEJ at internal DSBs.

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LITERATURE CITED

Åström, S. U., and J. Rine, 1998 Theme and variation among silencing proteins in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Genetics **148:** 1021–1029.

Åström, S. U., S. M. Okamura and J. Rine, 1999 Yeast cell-type regulation of DNA repair. Nature **397:** 310.

Baumann, P., E. Podell and T. R. Cech, 2002 Human pot1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing. Mol. Cell. Biol. **22:** 8079–8087.

Bertuch, A. A., and V. Lundblad, 2003a The Ku heterodimer performs separable activities at double-strand breaks and chromosome termini. Mol. Cell. Biol. **23:** 8202–8215.

Bertuch, A. A., and V. Lundblad, 2003b Which end: dissecting Ku's function at telomeres and double-strand breaks. Genes Dev. **17:** 2347–2350.

Boeke, J. D., J. Trueheart, G. Natsoulis and G. R. Fink, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:** 164–175.

Boulton, S. J., and S. P. Jackson, 1998 Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. EMBO J. **17:** 1819–1828.

Broccoli, D., A. Smogorzewska, L. Chong and T. de Lange, 1997 Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat. Genet. **17:** 231–235.

Callebaut, I., L. Malivert, A. Fischer, J. P. Mornon, P. Revy *et al.*, 2006 Cernunnos interacts with the XRCC4 × DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1. J. Biol. Chem. **281:** 13857–13860.

Chan, S. R., and E. H. Blackburn, 2004 Telomeres and telomerase. Philos. Trans. R. Soc. Lond. B Biol. Sci. **359:** 109–121.

Chan, S. W., and E. H. Blackburn, 2003 Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol. Cell **11:** 1379–1387.

Chen, X. J., and G. D. Clark-Walker, 1994 *sir2* mutants of *Kluyveromyces lactis* are hypersensitive to DNA-targeting drugs. Mol. Cell. Biol. **14:** 4501–4508.

Conrad, M. N., J. H. Wright, A. J. Wolf and V. A. Zakian, 1990 RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell **63:** 739–750.

Cooper, J. P., E. R. Nimmo, R. C. Allshire and T. R. Cech, 1997 Regulation of telomere length and function by a Myb-domain protein in fission yeast. Nature **385:** 744–747.

Daley, J. M., P. L. Palmbos, D. Wu and T. E. Wilson, 2005 Nonhomologous end joining in yeast. Annu. Rev. Genet. **39:** 431–451.

Dionne, I., and R. J. Wellinger, 1996 Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc. Natl. Acad. Sci. USA **93:** 13902–13907.

DuBois, M. L., Z. W. Haimberger, M. W. McIntosh and D. E. Gottschling, 2002 A quantitative assay for telomere protection in *Saccharomyces cerevisiae*. Genetics **161:** 995–1013.

Featherstone, C., and S. P. Jackson, 1999 Ku, a DNA repair protein with multiple cellular functions? Mutat. Res. **434:** 3–15.

Feldmann, E., V. Schmiemann, W. Goedecke, S. Reichenberger and P. Pfeiffer, 2000 DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. Nucleic Acids Res. **28:** 2585–2596.

Ferreira, M. G., and J. P. Cooper, 2001 The fission yeast Taz1 protein protects chromosomes from Ku-dependent end-to-end fusions. Mol. Cell **7:** 55–63.

Ferreira, M. G., and J. P. Cooper, 2004 Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. Genes Dev. **18:** 2249–2254.

Fisher, T. S., and V. A. Zakian, 2005 Ku: a multifunctional protein involved in telomere maintenance. DNA Rep. **4:** 1215–1226.

Frank-Vaillant, M., and S. Marcand, 2001 NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev. **15:** 3005–3012.

Grandin, N., C. Damon and M. Charbonneau, 2000 Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol. Cell. Biol. **20:** 8397–8408.

Gravel, S., M. Larrivee, P. Labrecque and R. J. Wellinger, 1998 Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:** 741–744.

Herrmann, G., T. Lindahl and P. Schär, 1998 *Saccharomyces cerevisiae LIF1*: a function involved in DNA double-strand break repair related to mammalian *XRCC4*. EMBO J. **17:** 4188–4198.

Hsu, H. L., D. Gilley, S. A. Galande, M. P. Hande, B. Allen *et al.*, 2000 Ku acts in a unique way at the mammalian telomere to prevent end joining. Genes Dev. **14:** 2807–2812.

Ira, G., and J. E. Haber, 2002 Characterization of *RAD51*-independent break-induced replication that acts preferentially with short homologous sequences. Mol. Cell. Biol. **22:** 6384–6392.

Iyer, S., A. D. Chadha and M. J. McEachern, 2005 A mutation in the *STN1* gene triggers an alternative lengthening of telomere-like runaway recombinational telomere elongation and rapid deletion in yeast. Mol. Cell. Biol. **25:** 8064–8073.

Kegel, A., J. O. Sjöstrand and S. U. Åström, 2001 Nej1p, a cell type-specific regulator of nonhomologous end joining in yeast. Curr. Biol. **11:** 1611–1617.

Kegel, A., P. Martinez, S. D. Carter and S. U. Åström, 2006 Genome wide distribution of illegitimate recombination events in *Kluyveromyces lactis*. Nucleic Acids Res. **34:** 1633–1645.

Kironmai, K. M., and K. Muniyappa, 1997 Alteration of telomeric sequences and senescence caused by mutations in *RAD50* of *Saccharomyces cerevisiae*. Genes Cells **2:** 443–455.

Krauskopf, A., and E. H. Blackburn, 1996 Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats. Nature **383:** 354–357.

Lee, S. E., F. Paques, J. Sylvan and J. E. Haber, 1999 Role of yeast *SIR* genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. Curr. Biol. **9:** 767–770.

Lewis, L. K., F. Storici, S. Van Komen, S. Calero, P. Sung *et al.*, 2004 Role of the nuclease activity of *Saccharomyces cerevisiae* Mre11 in repair of DNA double-strand breaks in mitotic cells. Genetics **166:** 1701–1713.

Liti, G., and E. J. Louis, 2003 *NEJ1* prevents NHEJ-dependent telomere fusions in yeast without telomerase. Mol. Cell **11:** 1373–1378.

Lundblad, V., and E. H. Blackburn, 1993 An alternative pathway for yeast telomere maintenance rescues *est1*- senescence. Cell **73:** 347–360.

Lundblad, V., and J. W. Szostak, 1989 A mutant with a defect in telomere elongation leads to senescence in yeast. Cell **57:** 633–643.

Maringele, L., and D. Lydall, 2002 *EXO1*-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast *yku70*Δ mutants. Genes Dev. **16:** 1919–1933.

McClintock, B., 1941 The stability of broken ends of chromosomes in *Zea mays*. Genetics **26:** 234–282.

McEachern, M. J., and E. H. Blackburn, 1995 Runaway telomere elongation caused by telomerase RNA gene mutations. Nature **376:** 403–409.

McEachern, M. J., and E. H. Blackburn, 1996 Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in *Kluyveromyces lactis* lacking telomerase. Genes Dev. **10:** 1822–1834.

McEachern, M. J., and S. Iyer, 2001 Short telomeres in yeast are highly recombinogenic. Mol. Cell **7:** 695–704.

McEachern, M. J., S. Iyer, T. B. Fulton and E. H. Blackburn, 2000 Telomere fusions caused by mutating the terminal region of telomeric DNA. Proc. Natl. Acad. Sci. USA **97:** 11409–11414.

Mieczkowski, P. A., J. O. Mieczkowska, M. Dominska and T. D. Petes, 2003 Genetic regulation of telomere-telomere fusions in the yeast *Saccharomyces cerevisae*. Proc. Natl. Acad. Sci. USA **100:** 10854–10859.

Milne, G. T., S. Jin, K. B. Shannon and D. T. Weaver, 1996 Mutations in two Ku homologs define a DNA end-joining repair pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 4189–4198.

Miyoshi, T., M. Sadaie, J. Kanoh and F. Ishikawa, 2003 Telomeric DNA ends are essential for the localization of Ku at telomeres in fission yeast. J. Biol. Chem. **278:** 1924–1931.

Moore, J. K., and J. E. Haber, 1996 Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 2164–2173.

Müller, H. J., 1938 The remaking of chromosomes. The Collecting Net **8:** 182–195.

Naito, T., A. Matsuura and F. Ishikawa, 1998 Circular chromosome formation in a fission yeast mutant defective in two *ATM* homologues. Nat. Genet. **20:** 203–206.

Nakamura, T. M., J. P. Cooper and T. R. Cech, 1998 Two modes of survival of fission yeast without telomerase. Science **282:** 493–496.

Natarajan, S., K. Nickles and M. J. McEachern, 2006 Screening for telomeric recombination in wild-type *Kluyveromyces lactis*. FEMS Yeast Res. **6:** 442–448.

Nickles, K., and M. J. McEachern, 2004 Characterization of *Kluyveromyces lactis* subtelomeric sequences including a distal element with strong purine/pyrimidine strand bias. Yeast **21:** 813–830.

Ooi, S. L., D. D. Shoemaker and J. D. Boeke, 2001 A DNA microarray-based genetic screen for nonhomologous end-joining mutants in *Saccharomyces cerevisiae*. Science **294:** 2552–2556.

Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus *et al.*, 1993 SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75:** 543–555.

Pardo, B., and S. Marcand, 2005 Rap1 prevents telomere fusions by nonhomologous end joining. EMBO J. **24:** 3117–3127.

Peterson, S. E., A. E. Stellwagen, S. J. Diede, M. S. Singer, Z. W. Haimberger *et al.*, 2001 The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. Nat. Genet. **27:** 64–67.

Polotnianka, R. M., J. Li and A. J. Lustig, 1998 The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. Curr. Biol. **8:** 831–834.

Riha, K., and D. E. Shippen, 2003 Ku is required for telomeric C-rich strand maintenance but not for end-to-end chromosome fusions in Arabidopsis. Proc. Natl. Acad. Sci. USA **100:** 611–615.

Ritchie, K. B., and T. D. Petes, 2000 The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in a single pathway for telomere maintenance in yeast. Genetics **155:** 475–479.

Samper, E., F. A. Goytisolo, P. Slijepcevic, P. P. van Buul and M. A. Blasco, 2000 Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. EMBO Rep. **1:** 244–252.

Schär, P., G. Herrmann, G. Daly and T. Lindahl, 1997 A newly identified DNA ligase of *Saccharomyces cerevisiae* involved in *RAD52*-independent repair of DNA double-strand breaks. Genes Dev. **11:** 1912–1924.

Smogorzewska, A., J. Karlseder, H. Holtgreve-Grez, A. Jauch and T. de Lange, 2002 DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. Curr Biol **12:** 1635–1644.

Stellwagen, A. E., Z. W. Haimberger, J. R. Veatch and D. E. Gottschling, 2003 Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. Genes Dev. **17:** 2384–2395.

Symington, L. S., 2002 Role of *RAD52* epistasis group genes in homologous recombination and double-strand break repair. Microbiol. Mol. Biol. Rev. **66:** 630–670.

Teo, S. H., and S. P. Jackson, 1997 Identification of *Saccharomyces cerevisiae* DNA ligase IV: involvement in DNA double-strand break repair. EMBO J. **16:** 4788–4795.

Teo, S. H., and S. P. Jackson, 2000 Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks. Curr. Biol. **10:** 165–168.

Tsukamoto, Y., A. K. Taggart and V. A. Zakian, 2001 The role of the Mre11-Rad50-Xrs2 complex in telomerase-mediated lengthening of *Saccharomyces cerevisiae* telomeres. Curr. Biol. **11:** 1328–1335.

Tzfati, Y., Z. Knight, J. Roy and E. H. Blackburn, 2003 A novel pseudoknot element is essential for the action of a yeast telomerase. Genes Dev. **17:** 1779–1788.

Underwood, D. H., C. Carroll and M. J. McEachern, 2004 Genetic dissection of the *Kluyveromyces lactis* telomere and evidence for telomere capping defects in *TER1* mutants with long telomeres. Eukaryot. Cell **3:** 369–384.

Valencia, M., M. Bentele, M. B. Vaze, G. Herrmann, E. Kraus *et al.*, 2001 *NEJ1* controls non-homologous end joining in *Saccharomyces cerevisiae*. Nature **414:** 666–669.

van Steensel, B., A. Smogorzewska and T. de Lange, 1998 TRF2 protects human telomeres from end-to-end fusions. Cell **92:** 401–413.

Wilson, T. E., U. Grawunder and M. R. Lieber, 1997 Yeast DNA ligase IV mediates non-homologous DNA end joining. Nature **388:** 495–498.

Wray, L. V., Jr., M. M. Witte, R. C. Dickson and M. I. Riley, 1987 Characterization of a positive regulatory gene, *LAC9*, that controls induction of the lactose-galactose regulon of *Kluyveromyces lactis*: structural and functional relationships to *GAL4* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:** 1111–1121.

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