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Functional Analysis of the Yku Complex in Telomere Length Regulation

von

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Erklärung

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Ehrenwörtliche Versicherung

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Kapitel II, III und IV dieser Arbeit entsprechen der Formatvorlage des veröffentlichenden wissenschaftlichen Journals. Alle weiteren Kapitel sind in einheitlicher Weise formatiert. Abbildungen sind für jedes Kapitel separat nummeriert, weisen jedoch in eckigen Klammern die Kapitelbezeichnung auf.

FÜR MEINE ELTERN

„ Es ist nicht das Ziel der Wissenschaft,
der unendlichen Weisheit eine Türe zu
öffnen, sondern eine Grenze zu setzen
dem unendlichen Irrtum.”

Bertold Brecht

Summary

The Ku protein from *Saccharomyces cerevisiae* (Yku) forms, like its human homologue hKu, a heterodimer comprised of a 70kD and an 80 kD subunit. In yeast and mammals, the Ku heterodimer is required for the repair of DNA double strand breaks (DSBs) via nonhomologous end-joining (NHEJ). Interestingly, Ku has been shown to bind to the native chromosome ends. It contributes to the maintenance of wild type telomere length and, moreover, has been implicated in the protection of the telomeres from end-to-end fusions. Telomere-bound Yku delocalizes from telomeric foci in response to DNA damage and accumulates at the sites of a DNA break. This thesis aimed to further characterize the Yku heterodimer and its function at DSBs and the native chromosome ends.

In a genetic screen for mutations that - in combination with a *yku* deletion - lead to cell death, a novel mutation in the yeast telomerase subunit *CDC13/EST4* has been identified earlier in the laboratory. Cdc13p binds to the single stranded DNA overhang at telomeres and is required to recruit the yeast telomerase to chromosome ends. The results presented here suggest that the mutant protein, Cdc13-4p, can still bind to the telomere and does interact with the telomerase subunit Est1p *in vivo*. A model is proposed in which the mutant Cdc13p is altered in its binding to a regulatory protein, thereby modulating telomerase access to the chromosome ends. The lethal effect in *yku* mutants is discussed to result from the loss of additional telomere sequences at the already very short telomeres of *yku* mutants.

In order to fulfill its opposite functions at the 'different' DNA ends, Yku might depend on larger protein networks. Putative Yku interacting proteins have been identified in a two hybrid screen. One interactor, Sir4p, has previously been implicated in NHEJ. The Sir4p domain identified could be shown to interact with the Yku heterodimer via the Yku80p subunit. Experiments that allowed the separation of phenotypes caused by the loss of the Sir4 protein itself and phenotypes induced by a de-repression of silencing in *sir4* mutants revealed no direct involvement of Sir4p in the repair of DSBs. In contrast to Cdc13p, Sir4p acts epistatic with Yku at the telomeres, indicating that the protein-protein interaction detected by two hybrid criteria might take place at telomeres.

Besides defects in DNA repair and telomere protection, mice deficient for Ku have been reported to exhibit phenotypes indicative of premature aging. Loss of *yku70* or overexpression of the Yku heterodimer effects life span in yeast. Experiments presented here rise the possibility that the premature aging is correlated with Ku's function at the telomere.

Chapter I

Introduction

1. The organization of genetic information - linear and circular chromosomes

It is usually assumed that prokaryotic cells have circular chromosomes, whereas eukaryotic nuclear chromosomes are linear. However, there are exceptions to this rule. The existence of linear chromosomes has been detected by pulse-field gel electrophoresis in very distantly related bacteria as *Borrelia burgdorferi* (Ferdows *et al*., 1996) and *Streptomyces lividans* (Lin *et al*., 1993). Furthermore, *Agrobacterium tumefaciens* has been shown to harbor both a circular chromosome of ~ 3 Mbp and a non-homologous linear chromosome of ~ 2,1 Mbp (Allardet-Servent *et al*., 1993; Goodner *et al*., 1999).

Linear chromosomes, however, face a problem during replication, foreshadowed by James Watson (1972), who noted that if conventional DNA polymerases rely on RNA primers placed at the very 5' ends of chromosomes, chromosome ends might not be fully replicated and might shorten during each round of replication (Watson, 1972). Studies in a variety of organisms have revealed different strategies to circumvent this problem. The genome of adenoviruses is a double-stranded linear DNA molecule with inverted terminal repeats about 100 base pairs (bp) in length and a terminal protein covalently linked to the 5' nucleotide of each strand (Challberg *et al*., 1980; Lichy *et al*., 1981). This terminal protein forms a covalent bond with the 5'-OH of dNMP and DNA polymerase uses this base as the first nucleotide to be incorporated into the newly synthesized DNA strand (Salas, 1991). The linear chromosomes of *Streptomyces* also appear to contain terminal inverted repeats and covalently bound terminal proteins (Bao and Cohen, 2001), which might suggest a similar replication mechanisms as it has been detected for adenoviral replication. However, in contrast to adenovirus, replication at *Streptomyces* chromosomes is not initiated at the end but starts at an internal replication origin (Musialowski *et al*., 1994). In *Borrelia burgdorferi* the chromosome ends form a hairpin structure, suggesting that the back-looped DNA strand is used as a primer for replication, one possible mechanism discussed to achieve end replication. A hairpin structure has indeed been reported to be used as a priming site for replication of a viral genome, Vaccinia virus (Baroudy *et al*., 1983; Winters *et al*., 1985).

Simple physical ends of DNA, such as those generated by DNA double-strand breaks (DSBs), are genetically unstable, mutagenic and sometimes oncogenic (reviewed in de Lange, 1995). Interestingly, *Streptomyces* has been shown to have an exceptionally high genetic instability (Leblond and Decaris, 1994; Leblond *et al*., 1990). The linear chromosome shows an increased frequency of deletion that can remove up to 25% of the genome (Leblond *et al*., 1991). Most of these deletions have been detected at the chromosome ends and can result in chromosome circularization (Lezhava *et al*., 1997; Lin *et al*., 1993; Redenbach *et al*., 1993).

Thus, higher eukaryotes have to cope with a variety of problems that arise due to the linear nature of their genome. A special enzyme has evolved to ensure proper replication of chromosome ends and a large protein network is required to protect the chromosomes from degradation and from being mistakenly sensed as a DNA damage. Therefore, the question arises why eukaryotes use linear DNA molecules to pass genetic information to the next generation. The first experimental evidence that shed light on the advantage of having linear chromosomes versus circular chromosomes was obtained from studies in the yeast *Schizosaccharomyces pombe*. Mutations in the telomere binding protein Taz1 were identified in a screen for mutants defective in the ability to impose transcriptional silencing on genes placed near telomeres (Nimmo *et al*., 1998). These *taz1*$^-$ cells failed to form telomere clusters in pre-meiosis, where the six telomeres of the *S. pombe* chromosomes attach to the spindle pole body (Nimmo *et al*., 1998). Clustering of telomeres that is accompanied by oscillating chromosome movements (Chikashige *et al*., 1994) is mandatory for a proper chromosome alignment in *S. pombe*. The defects in *taz1*$^-$ strains led to a reduction in recombination and enhanced chromosome missegregation through meiosis which resulted in a low spore viability (Nimmo *et al*., 1998). Furthermore *tel1 rad3* mutants have been described that have telomeric DNA loss and an increased formation of circular chromosomes (Naito *et al*., 1998). These mutants were not able to form any viable spores (Naito *et al*., 1998), indicating that the linearity of chromosomes is somehow required for the meiotic division in *S. pombe*.

Mitosis is relatively easy in terms of chromosome segregation. Two sister chromatids that have recently replicated and are attached to one another by cohesins, align on the metaphase spindle before being partitioned to either side of a dividing cell. In Meiosis the replication is followed by two nuclear and cellular divisions, Meiosis I and Meiosis II. For the first nuclear division, homologous chromosomes must find each other and pair together in order for a reductional division to occur.

Following chromosome pairing, chromosomes synapse and undergo recombination, which enables both a genetic exchange and a physical linkage of two homologous chromosomes during nuclear division at metaphase I. Recombination and segregation are interdependent since the covalent associations between the two homologues are thought to be required for proper segregation (Figure 1A).

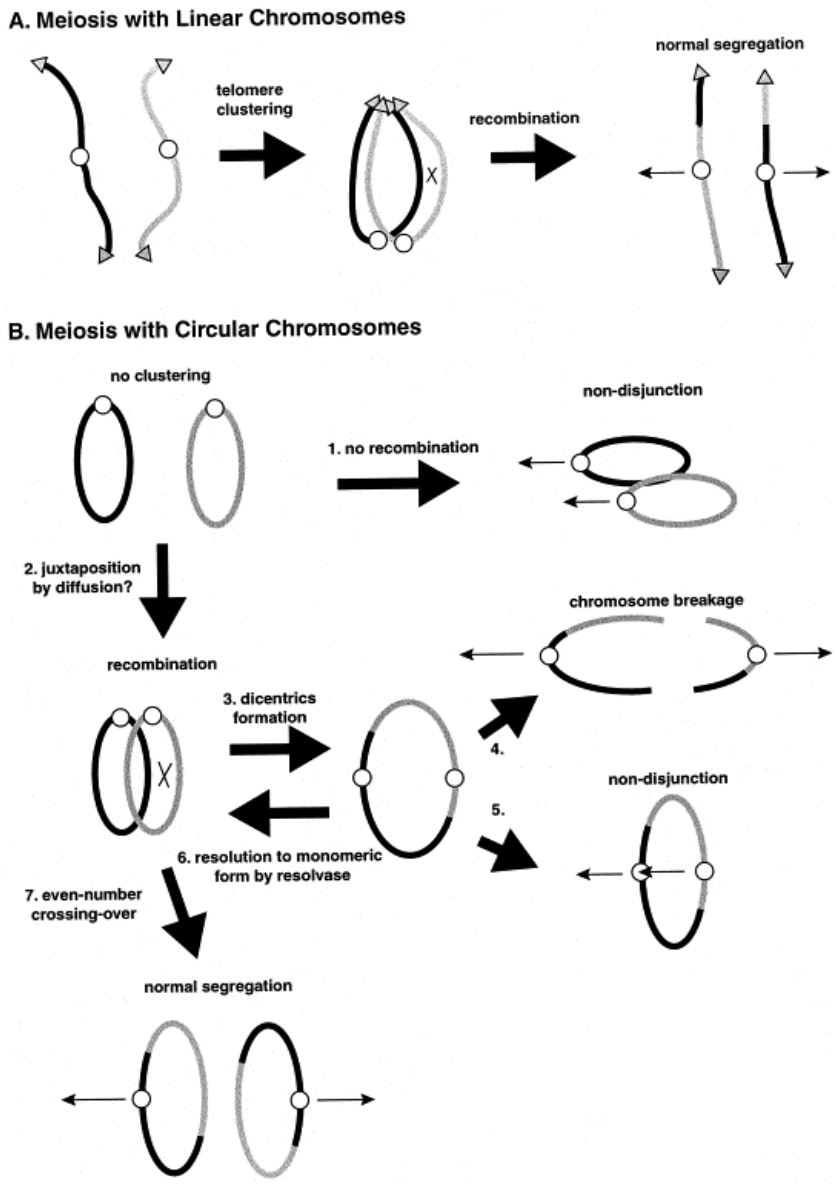


FIG. 1 [I]. Meiosis I of linear chromosomes and possible models for meiosis I of circular chromosomes. Taken from Ishikawa and Naito, 1999.

Some organisms such as *S. pombe* provide evidence that telomeres might play a role in homologue alignment. If pairing of two circular chromosomes lacking telomere sequences cannot be achieved, random segregation of the homologous chromosomes would occur (Figure 1B). If pairing and recombination of circular chromosomes can occur, dicentric chromosomes would be generated which can be resolved by a random break, if the two centromeres are pulled toward opposite poles (Figure 1B). Or the dicentric chromosome might end up in one daughter cell with the other daughter missing the genetic information (Figure 1B). Both models, however, suggest that circular chromosomes induce a strong non-disjunction phenotype during meiosis and would thereby lead to a strongly reduced spore viability and a progeny, if not lethal, that might have increased or reduced gene dosages. Thus linear chromosomes might be essential for a successful meiotic event and might therefore be prerequisites for sexual reproduction and genetic diversity. Strikingly, not all organisms require homologous recombination in order for chromosome pairing and synapsis to occur. In *Drosophila*, no recombination is detectable in male flies or on chromosome IV in females (reviewed in Roeder, 1997). How appropriate segregation of homologues is achieved here is largely unknown.

2. The solution to the end – telomere replication and telomere capping

While linear chromosomes seems to enable sexual reproduction and genetic diversity, they are the origin for at least two major problems: i) how to replicate ends of linear DNA molecules without loss of genetic information and ii) how to prevent chromosome ends to be recognized as DNA double strand breaks that have to be repaired.

2.1. End replication

During DNA replication the two DNA strands of a chromosome are separated and serve as single stranded templates for newly synthesized DNA. A large protein complex containing a DNA polymerase assembles at the replication fork and polymerizes complementary nucleotides in an unidirectional 5’-3’ manner to synthesize the new DNA strands.

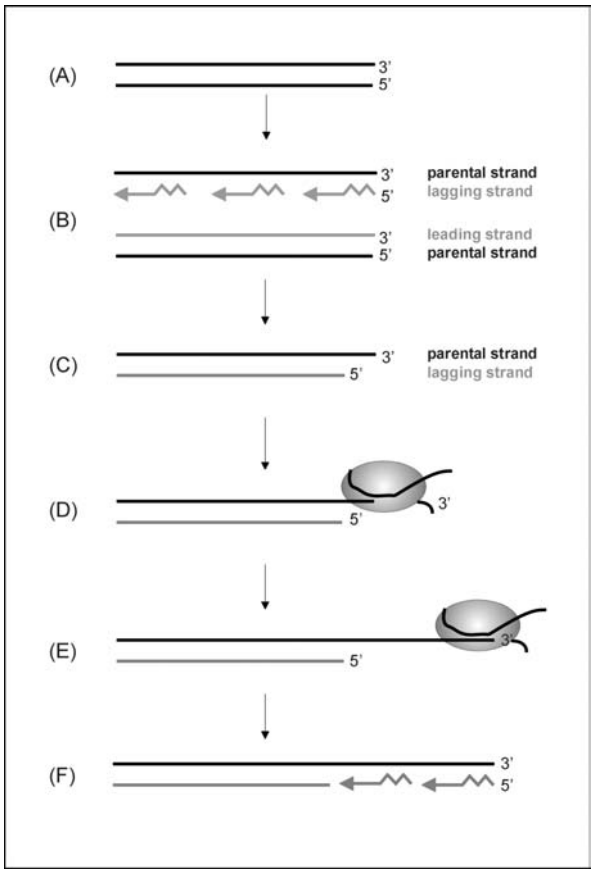


FIG. 2 [I]. Mechanism of telomere elongation. (A) The parental DNA double strand. (B) During replication the leading strand is synthesized as a single DNA strand, whereas the lagging strand is synthesized in short DNA fragments, the Okazaki fragments, using a RNA primer for replication initiation. (C) After RNA primers are removed, a 3' overhang is present at the parental strand due an unreplicated region at the 5' end of the lagging strand. (D) and (E) Telomerase reverse transcriptase uses its RNA subunit to anneal to the GT-rich parental strand and to elongates the strand in the 5' to 3' direction. (F) The CA-rich strand can be filled in by the lagging-strand replication mechanism.

While one daughter strand, the leading strand is continuously generated as the replication fork proceeds, the lagging strand is made discontinuously as short DNA fragments, called Okazaki fragments, using RNA primers to initiate DNA synthesis (Figure 2B). The RNA primers are later removed, the gaps between the Okazaki fragments are filled in and the DNA fragments are joined by a DNA ligase. However, at the very 5' end of the newly synthesized DNA strand a gap remains caused by the removal of the RNA primer (Figure 2C). This short DNA strand would be used as a template in the next round of replication leading to a shortened chromosome end. Different mechanisms have evolved to avoid such DNA loss. In most eukaryotes a telomere specific reverse transcriptase, telomerase, has been identified that is important to maintain linear chromosome ends in a constant length range.

Telomerase recognizes the G-rich parental strand via its RNA subunit. Telomerase RNA forms base pairs with the G-rich single stranded overhang (Figure 2D) and a translocation step allows several rounds of extension of the single stranded DNA (ss DNA) (Figure 2E). Replication of the chromosome is then completed by DNA polymerase using the extended strand as a template for lagging strand elongation (Figure 2F). This replication mechanism leads to a simple, repetitive DNA sequence at the ends of eukaryotic linear chromosomes. The sequence and the length of these repeats varies greatly between different organisms (see Table I), indicating that the RNA template is not identical among species and that different repeat lengths might be required to ensure end protection. However, a 3' single stranded overhang comprised of a few to several repeats of the telomerase synthesized strand has been shown to be present at telomeric DNA in ciliates, yeast and humans (Hemann and Greider, 1999).

TABLE I [I]. Telomere repeat sequences and repeat length in various eukaryotes.

Organism	**Sequence**	**Length of telomeric sequence**	**References**
Tetrahymena thermophila	TTGGGG	300 – 400 bp	(Blackburn and Gall, 1978)
Oxytricha	TTGGGG	3 – 20 kb	(Klobutcher *et al*., 1981; Murti and Prescott, 1999)
Trypanosoma brucei	TTAGGG	10 – 20 kb	(Munoz-Jordan *et al*., 2001)
Saccharomyces cerevisiae	$(TG)_{1\text{-}6}TG_{2\text{-}3}$	300 ± 75 bp	(McEachern and Hicks, 1993; Shampay *et al*., 1984)
Schizosaccharomyces pombe	$TTAC(AG)_{2\text{-}5}$	200 – 300 bp	Sugawara and Szostak, 1986
Caenorhabditis elegans	TTAGGC	2 – 4 kb	(Cangiano and La Volpe, 1993)
Bombyx mori	TTAGG	6 – 8 kb	(Okazaki *et al*., 1993)
Arabidopsis thaliana	TTTAGGG	2 – 4 kb	(Richards and Ausubel, 1988)
Mus ssp.	TTAGGG	10 – 60 kb	(Kipling and Cooke, 1990; Starling *et al*., 1990; Zijlmans *et al*., 1997)
Homo sapiens	TTAGGC	10 – 15 kb	(Moyzis *et al*., 1988)

2.2. Telomerase – the enzyme that ensures complete end replication

The telomere-specific DNA polymerase, telomerase, was identified in 1985 by Greider and Blackburn in *Tetrahymena* using biochemical assays designed to test for an activity that incorporates radioactively labeled dGTP in chromosome ends (Greider and Blackburn, 1985). This incorporation could be abolished by treatment of *Tetrahymena* extracts with RNAse, suggesting that the enzyme activity is dependent on an RNA subunit as proposed for reverse transcriptases (Greider and Blackburn, 1987). The activity was purified and a ~ 160 bp RNA co-purified with the enzymatic activity (Greider and Blackburn, 1989). Single nucleotide exchanges in the telomerase RNA gene were generated and the re-introduction of these mutated RNA genes into *Tetrahymena* resulted, dependent on the mutation, in both shortened or elongated telomeres or the addition of an altered telomere sequence, providing evidence that this RNA is indeed used as a template for telomere replication (Yu *et al*., 1990).

Over the past 12 years telomerase RNA has been isolated from a variety of different organisms (see Chen *et al*., 2000). The telomerase RNAs from different species all contain a domain longer than one full telomere repeat, which is predicted to serve as the template for telomere addition (Greider, 1996). The template region of human telomerase RNA (hTR) encompasses 11 nucleotides 5'-CUAACCCUAAC- 3' complementary to the human telomere sequence (TTAGGG)n (Feng *et al*., 1995). However, this template sequence is not highly conserved among species, which explains the diversity of telomere repeats observed for different organisms (see Table I). Furthermore, telomerase RNAs have diverged greatly not only in sequence but also in length, varying from 159 nucleotides in *Tetrahymena* (Greider and Blackburn, 1989), 450 nucleotides in mammals (Blasco *et al*., 1995; Feng *et al*., 1995) to up to 1.3 kb in *Saccharomyves cerevisiae* (Singer and Gottschling, 1994). Strikingly, recent data predict that the secondary structure of this RNA has been conserved during evolution (Chen *et al*., 2000; Lingner *et al*., 1994; Romero and Blackburn, 1991). A pseudoknot structure has been proposed for ciliate and vertebrate telomerase RNA containing a single stranded template region (Bhattacharyya and Blackburn, 1994; Chen *et al*., 2000). Several other conserved domains, i.e. helices or stem-loops, are present that might play a role in function or stability of the telomerase RNA or might be required for the interaction with the protein subunit of telomerase (Sperger and Cech, 2001).

The catalytic protein component, telomerase reverse transcriptase (TERT), was first identified in *Euplotes aediculatus* (Lingner *et al*., 1997b). The protein that was purified with telomerase activity contains a protein motif essential for reverse transcriptases. The *Euplotes* protein showed homology to a yeast protein, Est2p. Mutations in *EST2* had been previously identified as leading to replicative senescence (Lingner *et al*., 1997a). Homology searches allowed the cloning of *S. pombe* and human TERT (Nakamura *et al*., 1997), indicating that the catalytic protein subunits of telomerase enzymes are phylogenetically conserved reverse transcriptases. Together the two telomerase components, TR and TERT, are necessary and sufficient to produce telomerase activity *in vitro* (Autexier *et al*., 1996; Bachand and Autexier, 1999; Bachand *et al*., 2000).

In the yeast *Saccharomyces cerevisiae* the *TLC1* gene, encoding the telomerase RNA, was initially identified in a screen for genes that, when expressed in high amounts, would suppress telomeric silencing (Singer and Gottschling, 1994). The *TLC1* RNA is ~ 1.3 kb and therefore significantly longer than telomerase RNAs from many other organisms (Lingner *et al*., 1994). It has an irregular 5'-CACCACACCCACACAC-3' template sequence consistent with the irregular sequence of yeast telomeres (see Table I). A genetic screen that was designed to detect mutants that have a defect in telomerase activity, led to the discovery of mutations in four genes as well as in *TLC1* (Lendvay *et al*., 1996). These mutations lead to ever shorter telomeres until senescence and the genes where therefore designated as *EST1*, *EST2*, *EST3* and *EST4* (Lendvay *et al*., 1996). Because *tlc1*Δ and *est*Δ mutants have an identical phenotype and double mutants have no enhanced phenotype, the products encoded by these genes are thought to act in the same pathway of telomerase mediated replication (Counter *et al*., 1997; Singer and Gottschling, 1994; Zakian, 1996).

However, as in higher eukaryotes, the yeast catalytic subunit Est2p and *TLC*1 alone can promote telomerase activity *in vitro* (Lingner *et al*., 1997a) whereas Est1p, Est3p and Est4p/Cdc13p are essential for telomerase activity *in vivo* but dispensable *in vitro*. Est1p and Est4p/Cdc13p, have the properties of single-strand telomere DNA-binding proteins (Virta-Pearlman *et al*., 1996; Wang *et al*., 2000), but genetic analysis argues for different roles of these two proteins *in vivo*. Est1p has been proposed to function in directing telomerase to the chromosomal terminus (Zhou *et al*., 2000). In contrast, the Cdc13 protein has been proposed to play a dual role while bound to the telomere: it protects the end of the chromosome (Garvik *et al*., 1995), and regulates telomerase by mediating, either directly or indirectly through

Est1p, access of this enzyme to the terminus (Evans and Lundblad, 1999; Grandin *et al*., 2000). *EST3* encodes a novel 20-kDa protein, which besides its requirement for telomerase activity *in vivo* has not been further characterized. Thus, in the yeast *S. cerevisiae* more than a catalytic subunit and its RNA are required for telomerase activity *in vivo*.

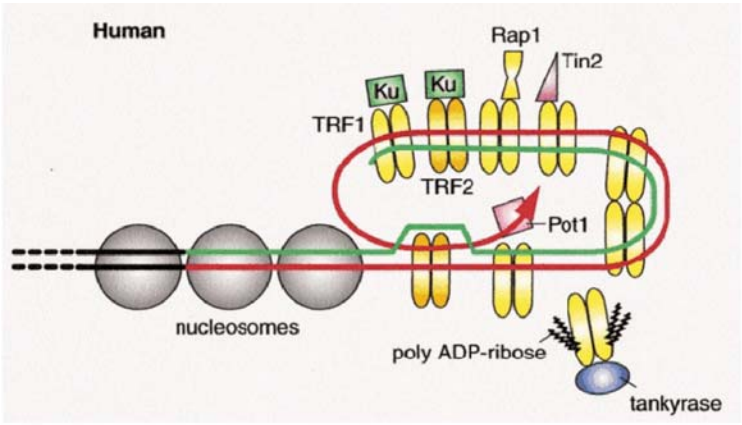
Interestingly the purification of *Euplotes aediculatus* telomerase yielded two proteins, p123, the telomerase reverse transcriptase, and p43, an uncharacterized protein (Lingner and Cech, 1996), indicating that additional proteins might contribute to telomerase function as described for *S. cerevisiae*. Recently, the first mutation in a *C. elegans* checkpoint gene was identified, which leads to progressive telomere shortening and chromosome end-to-end fusions (Ahmed and Hodgkin, 2000). This telomerase negative phenotype was caused by a mutation in the mrt-2 gene, the *C. elegans* homologue of the *S. pombe rad1*$^+$ and the *S. cerevisiae RAD17* checkpoint genes (Ahmed and Hodgkin, 2000). This finding raises the possibility that not yet identified proteins required for *in vivo* telomerase activity might also be present in higher eukaryotes.

Little is known about how telomerase activity is regulated, especially how chromosome replication and telomere elongation are coupled. However, recent studies from yeast and humans suggest that telomerase does not act as a monomer but is present as a multimer *in vivo* (Beattie *et al*., 2001; Prescott and Blackburn, 1997; Wenz *et al*., 2001). It has been suggested that dimerization or multimerization serves as a control mechanism for telomerase activity (Beattie *et al*., 2001).

2.3. Telomere structure and telomere associated proteins

Telomeres, the ends of linear chromosomes are physically indistinguishable from DNA double strand breaks. The genetic information of the yeast *Saccharomyces cerevisiae* is packaged into 16 chromosomes bearing 32 telomeres in the haploid cell. A single DSB leads to death in cells incapable of repairing these breaks (Resnick and Martin, 1976). Thus, sensing the natural end as a break would be deleterious for the cell and therefore telomeres must have properties that preserve these ends from being detected as DSBs.

In mammalian cells double stranded telomeric DNA is bound by two Myb domain proteins, TRF1 and TRF2 (Broccoli *et al*., 1997; Chong *et al*., 1995). Overexpression of TRF1 or TRF2 has been shown to trigger telomere shortening (Smogorzewska *et al*., 2000; van Steensel and de Lange, 1997) indicating that these proteins negatively regulate telomere length and might inhibit telomerase function when bound to telomeres. In mammals, the duplex hexameric repeat TTAGGG runs 5'-3' towards the chromosome end and terminates in a 75-300 nucleotide long single stranded (ss) overhang (Makarov *et al*., 1997; McElligott and Wellinger, 1997; Wright *et al*., 1999). Overexpression of TRF2 lacking a basic domain leads to loss of this ss-overhang at the telomere (van Steensel *et al*., 1998) and results in chromosome fusions and cell cycle arrest or apoptosis (Karlseder *et al*., 1999; van Steensel *et al*., 1998) without a detectable reduction of telomere length (van Steensel *et al*., 1998), indicating that TRF2 is not only involved in negative telomere length regulation but is also essential for telomere capping. Recently, electron microscopy of telomeric DNA purified from human and mouse cells led to the discovery of large loops at chromosome ends *in vivo*, so-called t-loops (telomere loops). TRF2 is capable of promoting and stabilizing the formation of a t-loop structure, whereby the single-stranded G-rich extension is buried into more proximal double stranded regions (Griffith *et al*., 1999; Stansel *et al*., 2001) (Figure 3). The TRF2 mutant, truncated for the basic domain might not be able to facilitate loop formation thereby opening up the chromosome end to fusion events.



taken from Blackburn (2001) *Cell*, 106, 661-73,
with permission of Elizabeth Blackburn.

FIG. 3 [I]. Examples of interactions among the components of the structural DNA-protein complexes comprising the telomeres in humans. Red line: G-rich telomeric repeat strand synthesized by telomerase; green line, complementary C-rich strand of the telomeric repeats; heavy black lines, subtelomeric DNA. The 3' terminal single-stranded telomeric DNA in human telomeres might be in alternative forms: either bound by Pot1 protein or engaged in T-loop formation.

T-loops, have also been detected in *Oxytrichia* (Murti and Prescott, 1999) and *Trypanosomes* (Munoz-Jordan *et al*., 2001), two organisms with a telomere repeat length of ~ 10 kb (see Table 1). In organisms with very short telomere repeat tracts such as *Saccharomyces cerevisiae,* the presence of these usually very large t-loops seems to be unlikely. However, a variety of observations suggest that a back-folding of the telomeric sequence occurs in yeast. *S. cerevisiae* telomeres repress transcription of adjacent genes, a phenomenon called telomere position effect (TPE) (Gottschling *et al*., 1990). Very recently de Bruin and colleagues (2001) have shown that a reporter gene bearing an enhancer positioned 1-2 kilobases downstream of the gene is activated if it is linked to the telomere. The effect is specific for telomeric regions and cannot be induced at internal chromosomal loci (de Bruin *et al*., 2001). This result strongly supports the suggestion that even in yeast the telomere folds back into subtelomeric regions. The finding that Rap1p, a protein that binds sequence-specific to telomeres (Conrad *et al*., 1990), can also be co-immunoprecipitated with subtelomeric chromatin (Strahl-Bolsinger *et al*., 1997) allows the prediction that a ~ 3 kb end structure exists in *Saccharomyces cerevisiae* (Strahl-Bolsinger *et al*., 1997).

2.4. Telomere length regulation

Within a cell population, telomere length is kept within a narrow size due to a balance between elongation and shortening, thereby preserving telomere structure. In mammals additional factors such as tankyrase (Smith *et al*., 1998), Ku (Hsu *et al*., 1999), the Mre11-Rad50-NBS1 complex (Zhu *et al*., 2000), Tin2 (Kim *et al*., 1999) and hRap1 (Li *et al*., 2000) associate indirectly with telomeric DNA via TRF1 or TRF2 (see Figure 3). Furthermore a single stranded binding protein, Pot1, has recently been described to bind to the terminal telomere end (Baumann and Cech, 2001).

A very complex picture of proteins that influence telomere length regulation can be drawn in the yeast *Saccharomyces cerevisiae* (see Figure 4). Several proteins have been shown to be responsible for the regulation of telomerase function at yeast telomeres. A mutation in Cdc13p/Est4p, a protein that binds ss telomeric DNA (Lin and Zakian, 1996) and is essential for *in vivo* telomerase activity (Lendvay *et al*., 1996), causes an increase in telomere length (Grandin *et al*., 1997).

When shifted to the restrictive temperature, this Cdc13-1p mutant accumulates unusually long telomeric single-stranded extensions of the 3' G-rich strand (Garvik *et al*., 1995; Polotnianka *et al*., 1998). A suppressor of this single-stranded phenotype, Stn1p, interacts with Cdc13p by two hybrid criteria (Grandin *et al*., 1997). A third protein, Ten1p, has recently been shown to be involved in this complex (Grandin *et al*., 2001). A temperature-sensitive mutation in Stn1p or Ten1p leads to telomere elongation and the accumulation of ss DNA (Grandin *et al*., 2001; Grandin *et al*., 1997) as described for the *cdc13-1* mutation. Thus, Stn1p and Ten1p are thought to negatively regulate telomerase access to the telomere by their association with Cdc13p. In a two hybrid approach Pol1p and Est1p have been identified to interact with Cdc13p (Qi and Zakian, 2000). Mutations in the catalytic subunit of DNA polymerase α, *POL1*, that disrupt this interaction results in longer telomeres (Qi and Zakian, 2000). The requirement for Est1p for *in vivo* telomerase activity can be overcome by the expression of a Cdc13p-Est2p fusion protein (Evans and Lundblad, 1999), suggesting that Est1p mediates an interaction between Cdc13p and the catalytic telomerase subunit Est2p *in vivo*. A variety of Cdc13p mutations have been studied to analyze certain protein domains. Strikingly, mutations in Cdc13p have been described to lead to telomere elongation while others lead to telomere shortening (Grandin *et al*., 2000; Meier *et al*., 2001). These observations provide strong evidence that Cdc13p may play a key role in regulating the access of telomerase by its interaction with additional proteins.

Yeast telomeric DNA is assembled into a nonnucleosomal chromatin structure, the telosome (Wright *et al*., 1992). The major protein at the telomere, Rap1p, binds sequence-specific to telomere repeats (Berman *et al*., 1986; Conrad *et al*., 1990; Wright *et al*., 1992). Rap1p was originally identified as a transcriptional regulator that can play a role in either repression or activation of transcription (Kurtz and Shore, 1991; Shore and Nasmyth, 1987). The consensus sequence for Rap1p (Buchman *et al*., 1988) is found approximately every 35 bp in telomeric DNA (Wang and Zakian, 1990) and multiple Rap1 proteins bind at telomeres (Gilson et al, 1993). When a telomeric repeat sequence was inserted in the opposite orientation adjacent to telomere repeats, the length of the distal telomere repeat was significantly reduced, indicating that the misoriented repeats have been counted as a part of the telomere (Marcand *et al*., 1997).

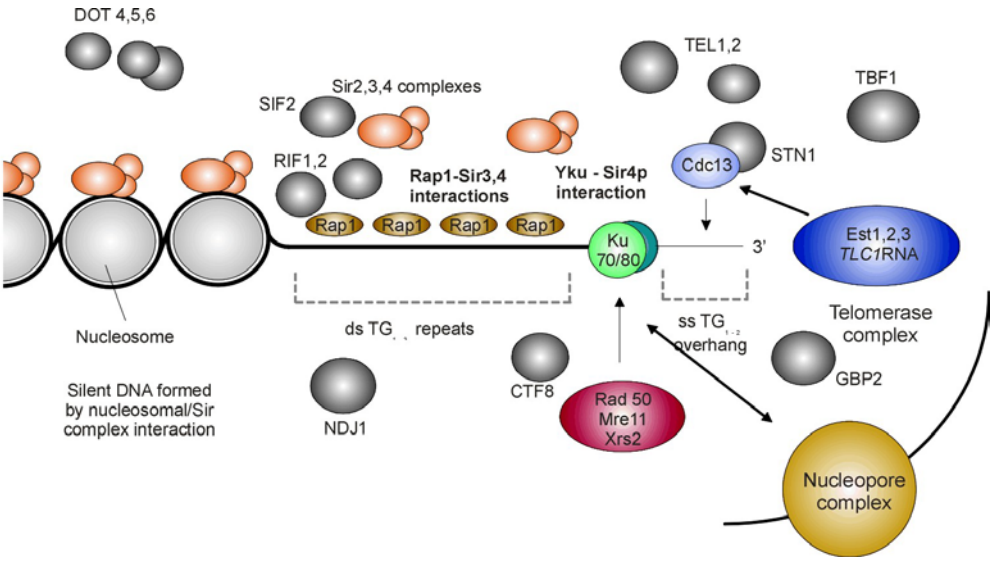


FIG. 4 [I]. A large protein network is required to maintain wild-type telomere length in *S. cerevisiae*.

Yeast strains carrying a C-terminally truncated Rap1p have elongated telomere repeat tracts (Hardy *et al*., 1992). This C-terminal mutation has been described to abolish the interaction of Rap1p with the Sir3 protein (Roy and Runge, 1999). Sir proteins interact with Rap1p at different sites in the genome such as the mating type loci and the telomeres (Gotta *et al*., 1996). The Sir complex is essential for silencing in yeast and *sir2, sir3* or *sir4* mutants are no longer able to repress the mating type loci *HML* and *HMR* (Ivy *et al*., 1986)or telomere adjacent genes (Aparicio *et al*., 1991). Furthermore, *sir4* mutants show a slight but stable reduction in telomere length (Palladino *et al*., 1993).

Interestingly, certain components of the DNA damage response pathways, originally identified by their involvement in the repair of DNA double strand breaks (DSBs) are found at telomeres and are required for wild-type telomere length and function. The Ku heterodimer, essential for the repair of DNA damage by nonhomologous end-joining, localizes to telomeres in budding yeast (Martin *et al*., 1999) and humans (Hsu *et al*., 1999). Yeast Ku is required for the normal localization of telomeres at the nuclear periphery and has been shown to interact with proteins of the nuclear matrix (Galy *et al*., 2000). A disruption of either the *YKU70* or the *YKU80* gene leads to a growth defect at elevated temperatures (Feldmann *et al*., 1996; Feldmann and Winnacker, 1993) and to enhanced sensitivity to the DNA damaging agents bleomycin and methyl methanesulfonate (MMS) (Feldmann *et al*., 1996; Mages *et al*., 1996). Ku is furthermore required for the maintenance of wild-type telomere structure and length.

Yeast strains deficient for *YKU* exhibit stable but shortened telomeres (Boulton and Jackson, 1998; Porter *et al*., 1996) and a G-rich single stranded overhang is detectable in *yku* mutants over the entire cell cycle that in yeast is usually restricted to S-phase (Gravel *et al*., 1998). In cells deficient for Yku, subtelomeric silencing is severely compromised (Galy *et al*., 2000; Gravel *et al*., 1998; Laroche *et al*., 1998; Mishra and Shore, 1999; Nugent *et al*., 1998; Pryde and Louis, 1999). Lack of Ku function leads to telomere-telomere fusions in fission yeast and mammals (Baumann and Cech, 2000; Hsu *et al*., 2000; Samper *et al*., 2000).

Another complex that is required for the repair of DNA damage and also needed for telomere length regulation is the Rad50p, Mre11 and Xrs2p (yeast)/Nbs1(human) complex, which is involved in the repair of DNA lesions. The localization of the Rad50/Mre11/Nbs1 complex at the telomeres has been investigated in mammalian cells (Lombard and Guarente, 2000; Zhu *et al*., 2000) and a deletion of the corresponding yeast genes leads to telomere shortening (Boulton and Jackson, 1998; Nugent *et al*., 1998). Mutations in *TEL1* and *TEL2*, the first genes identified to contribute to telomere length regulation, were isolated in a screen for temperature-sensitive mutants with short telomeres (Lustig and Petes, 1986). Mutations in these genes result in progressive telomere shortening for ~ 150 generations and then telomeres reach a stable level (Lustig and Petes, 1986). Double mutants of a Mre11p-Rad50p-Xrs2p (MRX) component with a *tel1* mutation cause a decrease in telomere length similar to that caused by any of the single mutations, suggesting that Tel1p and the MRX complex act in the same pathway for telomere length regulation (Ritchie and Petes, 2000).

Strikingly, checkpoint genes have been identified that contribute to telomere length regulation. Mutations in, i.e. *DDC1* and *RAD53* of *S. cerevisiae* (Longhese *et al*., 2000), *rad1*$^+$ and *rad3*$^+$ of *S. pombe* (Dahlen *et al*., 1998) as well as mutations in human ATM (Vaziri *et al*., 1999) have been shown to induce telomere shortening and telomere instability. Furthermore *tel1 mec1* double mutants, defective in two ATM homologues of *S. cerevisiae* show replicative senescence (Ritchie *et al*., 1999). The first evidence how a checkpoint gene might influence telomere length regulation was provided by the finding that the telomere shortening of *S. cerevisiae rad53* mutants could be restored by increased dNTP pools, indicating that limiting dNTPs may cause a defect in DNA replication at the telomere (Longhese *et al*., 2000).

However, *ddc1* mutants exhibit short telomeres independent from the intracellular dNTP level, suggesting that the *DDC1* checkpoint gene affects telomere ends by a different mechanism from that defective in *rad53* mutants (Longhese *et al*., 2000). Thus checkpoint related genes, which ensure the proper order and timing of cell-cycle events, also play an important role in maintaining telomere length.

3. Telomere dysfunction triggers the DNA damage checkpoint

The *RAD9* checkpoint in *Saccharomyces cerevisiae* (see Figure 5) serves to arrest the cell cycle in G2 phase when a DNA damage is present or when DNA replication is incomplete (Weinert and Hartwell, 1988; Weinert and Hartwell, 1993). The ends of linear chromosomes are capped and folded into a special structure and a large network of proteins is required to maintain telomere integrity. Analysis of the *cdc13-1* mutation, provided evidence that a defect at telomeres can trigger a *RAD9* dependent cell cycle arrest. *cdc13-1 rad9* double mutants have a higher restrictive temperature than *cdc13-1* single mutants, although the proportion of viable cells in *cdc13-1 rad9* colonies is drastically reduced at higher temperature. This finding suggested that the *RAD9* checkpoint might become activated to arrest the cell cycle at a level of DNA damage that is still sublethal (Weinert and Hartwell, 1993). When *cdc13-1* cells where grown at a semipermissive temperature an increase in recombination events could be detected at telomeric regions but not at the centromere (Garvik *et al*., 1995). Furthermore, an accumulation of single stranded DNA was detectable at elevated temperatures at the telomeres in *cdc13-1* mutants and the ss region in *cdc13-1 rad9* mutants might be as large as 17 kb (Garvik *et al*., 1995), suggesting that the terminal growth defect in *cdc13-1 rad9* might be caused by the loss of essential DNA sequence. The loss of Stn1p, a Cdc13p interacting protein has also been shown to induce ss DNA formation at the telomeres and mutants show an activated *RAD9* checkpoint (Grandin *et al*., 1997). Thus, single stranded DNA might be one lesion that activates the DNA damage checkpoint (Garvik *et al*., 1995; Lydall and Weinert, 1995). Recently, Teo and Jackson (2001) have provided evidence that the DNA damage checkpoint also becomes activated in *yku* mutants when shifted to elevated temperatures.

Yku mutants are temperature sensitive for growth and the temperature sensitive phenotype is proposed to be accompanied by additional telomere shortening (Fellerhoff *et al*., 2000). Rad53p phosphorylation, however, could only be detected in yeast cells grown at elevated temperatures whereas as single-stranded telomeric overhang is already detectable at the permissive temperature (Gravel *et al*., 1998; Teo and Jackson, 2001). Overexpression of telomerase has been shown to suppress the temperature sensitivity of *yku* mutants (Nugent *et al*., 1998) and can suppress the checkpoint activation, however, ss DNA is still present at telomeres (Teo and Jackson, 2001). Thus, telomerase might cap the telomere in *yku* mutants thereby preventing it from being recognized as a DNA break.

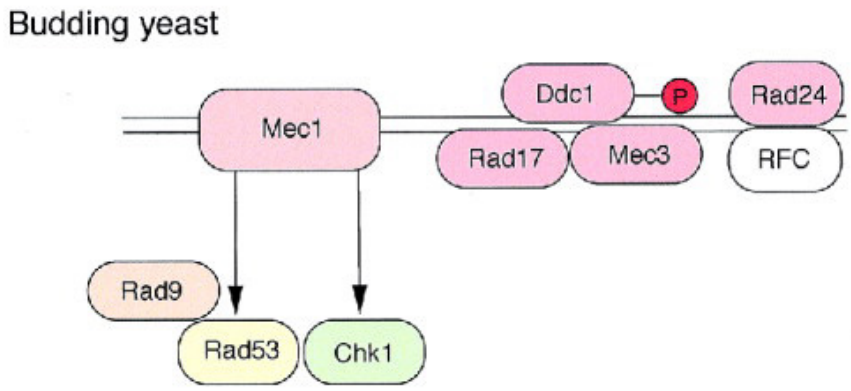


FIG. 5 [I]. Complex formation of the yeast DNA damage checkpoint proteins. Budding yeast Rad17, Mec3 and Ddc1 form a complex regardless of the checkpoint signal. Ddc1 is phosphorylated in a Mec1 dependent manner, which then transmits the checkpoint signal to Rad53 and Chk1. Rad9 is essential for the phosphorylation of Rad53.

In mammalian cells there is some evidence that one critically short telomere may be recognized as a DNA damage and as a consequence induces a p53/p21^{WAF} and p16 dependent cell-cycle arrest to cause senescence (Burkhart *et al*., 1999; Chin *et al*., 1999; Saretzki *et al*., 1999). A dominant negative TRF2 gene has been shown to result in the degradation of the single stranded overhang (van Steensel *et al*., 1998) at human telomeres, which might interfere with t-loop formation. The truncated TRF2 leads to the formation of dicentric chromosomes and eventually to senescence (van Steensel *et al*., 1998) or apoptosis mediated by p53 (Karlseder *et al*., 1999). Thus increasing evidence arises that telomeres indeed are recognized by the DNA damage checkpoint and that a variety of changes at the telomere might contribute to the detection by the cellular repair machinery or the apoptotic pathway.

4. Telomeres, aging and cancer

Early experiments by Hayflick and Moorehead have revealed that normal human fibroblasts show a limited dividing capacity and enter a non-diving state after a defined number of passages (Hayflick and Moorehead, 1961). Only a very small portion of cells can escape this cell cycle arrest, termed crisis, and become immortal, thus diving indefinitely. This effect observed by Hayflick and Moorehead, is restricted to somatic cells, whereas germline cells and tumors are immortal. Telomerase is active in germline cells and in ~ 90% of all tumors. In contrast, most human somatic cells lack significant telomerase activity (Broccoli *et al*., 1995; Counter *et al*., 1994; Kim *et al*., 1994; Shay *et al*., 1993; Vaziri *et al*., 1993) and telomeres shorten every round of DNA replication (Allsopp *et al*., 1992). Somatic cells, that escape crisis stabilize telomere length and activate telomerase (Counter *et al*., 1994) comparable to germline cells. Thus, telomerase is required for indefinite growth of immortal cells in culture. Ectopic expression of hTERT in human fibroblasts restores telomerase activity, stabilizes telomere length and induces indefinite growth (Bodnar *et al*., 1998; Vaziri and Benchimol, 1998), providing strong evidence that the activation of telomerase is sufficient to induce immortalization.

In addition to the limited division potential of somatic cells, Hayflick perceived that fibroblast cultures derived from embryos divided more often than those derived from adults (Hayflick, 1965). This observation, namely that the replicative capacity of a cell correlated with donor age, led to the proposal that the cellular dividing capacity reflects the organismal aging process. However, *in vivo* a variety of somatic cells are quiescent and proliferate seldom if at all. Human fibroblasts taken from elderly individuals undergo an additional 20-50 population doublings in culture (Dice, 1993), representing 40-70% of their replication capacity as defined by Hayflick. Thus, most telomerase-negative cells might never reach their Hayflick limit *in vivo*. A mouse strain deleted for the RNA component of telomerase showed no phenotype during the first 2-6 generations and age associated phenotypes, such as gray hair and wrinkled skin did not occur earlier than in control mice (Lee *et al*., 1998). Only in later mouse generations did the loss of telomerase lead to early-onset of hair graying, hair loss (Rudolph *et al*., 1999) and germline mortality, thereby inducing sterility (Lee *et al*., 1998).These findings argue against a model in which the life span of an individual animal that is born with wild-type telomere length is determined by the activity of telomerase.

The possibility that enhanced telomere shortening might play a role in human aging is supported by the analysis of telomeres from patients with premature aging syndromes. Telomere length of fibroblasts taken from patients with Hutchinson-Gilford progeria, a premature aging syndrome, was reduced as compared to age-matched control cells (Allsopp *et al*., 1992). Furthermore Werner's and Ataxia telangiectasia patients, who experience both premature aging exhibit accelerated telomere shortening (Kruk *et al*., 1995; Smilenov *et al*., 1997). Moreover, overexpression of telomerase could restore wild-type telomere length in Werner's syndrome cells and compensated for the early onset of replicative senescence (Wyllie *et al*., 2000).

Tumors are highly proliferative cells that have escaped growth control and keep diving indefinitely. Maintaining telomere stability is required for long term proliferation of tumors (Shay and Wright, 1996, Wright and Shay, 2001) and telomerase activity has been detected in approx. 90% of all tumors. However, another mechanism to stabilize telomere length, initially identified in telomerase negative yeast cells, has also been detected in tumor cells. This so called alternative lengthening of telomeres (ALT) mechanism is independent of telomerase and relies on homologous recombination (Bryan *et al*., 1995; Dunham *et al*., 2000). Thus, cancer cells seem to have circumvented the progressive telomere loss that is present in normal somatic tissues and is limiting for the cellular replicative potential. Reconstitution of telomerase has been shown to antagonize senescence however it is not sufficient to induce tumor formation (Hooijberg *et al*., 2000). Furthermore, transfection of embryo fibroblasts by a human *ras* oncogene does not convert them into tumor cells unless a second oncogene such as a viral or cellular *myc* gene is introduced together with the *ras* gene (Land *et al*., 1983). Recent studies have implicated c-myc in the transcription activation of hTERT (Greenberg *et al*., 1999; Wang *et al*., 1998; Wu *et al*., 1999).

Tumor formation has been shown to be a multistep process in which telomere integrity plays an important role. Since telomerase is required for the proliferation of most tumors it is an attractive target for anti-cancer drug therapy. Interestingly, the inhibition of telomerase in tumor cancer cell lines by a hammerhead ribozyme can induce an immediate apoptotic signal without prior telomere shortening (Ludwig *et al*., 2001), suggesting that telomerase is not only required for telomere length but also involved in the capping of telomeres (Blackburn, 2000) in the tumors studied.

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Chapter II

HDF2, the Second Subunit of the Ku Homologue from *Saccharomyces cerevisiae*

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Summary

The high affinity DNA binding factor (HDF) protein of *Saccharomyces cerevisiae* is composed of two subunits and specifically binds ends of double-stranded DNA. The 70-kDa subunit, HDF1, shows significant homology with the 70-kDa subunit of the human Ku protein. Like the Ku protein, HDF1 has been shown to be involved in recombination and double stranded DNA break repair. We have purified and cloned HDF2, the second subunit of the HDF protein. The amino acid sequence of HDF2 shows a 45.6% homology with the 80-kDa subunit of the Ku protein. HDF1 by itself does not bind DNA, while HDF2 protein on its own seems to displays DNA binding activity. Targeted disruption of the *HDF2* gene causes a temperature-sensitive phenotype for growth comparable to the phenotype of *hdf1*$^-$ strains. The human Ku protein cannot complement this temperature-sensitive phenotype. *hdf2*$^-$ strains are sensitive to bleomycin and methyl methanesulfonate but this sensitivity is reduced in comparison with *hdf1*$^-$ strains.

INTRODUCTION

As a safeguard against the occurrence of DNA damage prokaryotic and eukaryotic cells have developed at least three different DNA repair mechanisms. In *Saccharomyces cerevisiae* DNA double strand breaks are repaired mainly by the activities of the RAD52 epistasis group (1, 2). An evolutionary conservation of DNA double strand break pathways is suggested by the existence of eukaryotic homologues of *S. cerevisiae* DNA repair genes in this epistasis group (3-7).

Recently, an activity distinct from the RAD52 group, the DNA-activated protein kinase and its regulatory subunit, the Ku heterodimer, was identified as a component involved in the repair of DNA double strand breaks and recombinational events in higher eukaryotes (8-15). The human Ku protein is a heterodimer composed of 70- and 80-kDa subunits (16). The *Drosophila* homologue of the human Ku protein, IRBP (17), has been shown to be involved in repair of DNA double strand breaks, too, indicating a conservation of repair functions in *Drosophila* and mammals (18).

The notion that Ku protein may participate in recombination, replication, or DNA repair events (19, 20) is suggested by the observation that this protein binds to the ends of double-stranded DNA, nicks, and hairpins (19-26). Several lines of evidence appear to corroborate this view. Ku p80 is not detectable in x-ray-sensitive *xrs* hamster cell lines known to be defective in normal V(D)J recombination processes (12, 13). Both mutant phenotypes in these hamster cells can be complemented by the human *XRCC*5 gene encoding Ku p80 (11). Cells derived from mice with severe combined immunodeficiency (*SCID*) have been shown to be sensitive for ionizing radiation and defective in V(D)J recombination. For theses *SCID* cells the catalytic subunit of the DNA-dependent protein kinase is a strong candidate for the afflicted gene (14, 15). A number of DNA-binding proteins, including human p53, have been identified as targets of the DNA-dependent protein kinase (27, 28). The DNA-dependent protein kinase and its regulatory subunit, Ku, may, therefore, play a key role in the signaling pathway of DNA damage (29).

It was shown recently that the Ku heterodimer and human RAD51 coelute with the largest subunit of RNA polymerase II (30). Moreover, DNA polymerase ε, which is involved in DNA repair synthesis (31) and also interacts with the Ku protein (32), is a component of this RNA polymerase II complex (30).

A DNA-dependent protein kinase has not yet been described in *S. cerevisiae*. However, a yeast homologue of the human Ku heterodimer, a high affinity DNA binding factor (HDF), has been identified (33). HDF is a heterodimeric protein binding to the ends of double-stranded DNA. The gene of the 70-kDa subunit, *HDF1*, has been cloned, and the predicted amino acid sequences share significant homology with the 70-kDa subunit of the human Ku protein (33). *hdf1* mutant yeast strains are sensitive for the radiomimetic drug bleomycin (34), an agent causing DNA double strand breaks (35-37). The disruption of the *HDF1* gene also affects mating-type switching and spontaneous mitotic recombination (34). *hdf1 rad52* double mutant strains show an increased sensitivity toward ionizing radiation (38). HDF1 has been shown also to be involved in illegitimate recombination (39). Another phenotype, which may not be related to the DNA repair activities of HDF, is the formation of substantially shorter telomeres in *hdf1* mutant strains and a synthetic interaction of the *hdf1* mutation with *tel*1 mutation, resulting in strains that grow slowly and have very short telomeres (40).

Here we show the cloning of *HDF2*, the second subunit of the HDF heterodimer. HDF2 displays significant homology to human Ku p80, comparable to the homology of HDF1 with Ku p70. While HDF1 does not bind DNA by itself, the HDF2 protein on its own displays DNA binding activity. Disruption of the *HDF2* gene causes a temperature-sensitive phenotype for growth. This temperature sensitivity cannot be complemented by expression of the human Ku protein. *hdf2* mutant strains are also sensitive toward bleomycin and methyl methane sulfonate but in comparison with *hdf1* mutant strain this sensitivity is reduced.

MATERIALS AND METHODS

Yeast strains, Media, Growth Conditions, and Transformation-Strains used in this study are shown in Table I. Only relevant genotypes are listed. The *hdf2*-disrupted strains W303h2a and WaLh2a were generated by one-step gene disruption of the wild-type *HDF2* gene in W303-1A and W303aL. Cells were grown at 30 or 37 °C in YPD liquid medium/plates containing 2% glucose, 1% yeast extract, 2% Bacto-peptone or selective

medium/plates complemented with the appropriate nutrients (SD medium is 2% glucose, 0.67% nitrogen base without amino acids, plus nutrients) (41). Yeast transformation was performed by the lithium acetate method (42).

Purification of the HDF Protein-Protein extracts for HDF purification were prepared from the protease-deficient strain ABYS 60. Purification was performed as described previously (33).

Gel Retardation Assay-A 39-bp long synthetic double-stranded oligonucleotide, designated PGK1,2 (33), was used for gel retardation assays. Protein extracts were incubated with DNA for 5-10 min at room temperature in a buffer containing 150-250 mM ammonium sulfate, 50mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. DNA-protein complexes were analyzed by gel retardation on 10 or 12% polyacrylamide gels in 1 x TBE (0.09 M Tris borate, 0.002 M EDTA). Gels were prerun at 80 V for 30 min and then at 120 V for 6-12 h.

Cloning of the HDF2 Gene-Partial amino acid sequences of purified HDF2 protein were determined and used to identify an unknown open reading frame by a BLAST search. Oligonucleotides derived from the data base sequence information obtained were used for screening an EMBL3A genomic yeast library. A 2.4-kilobase *Sal*I/*Sac*I fragment was subcloned into pGEM4Z (Promega). To verify the sequence the fragment was sequenced from both ends as described previously (33).

Disruption of the HDF2 Gene-A *Sal*I/*Sac*I fragment spanning 175 bp upstream of the ATG codon, the open reading frame of the *HDF2* gene, and 387 bp downstream of the TAG stop codon were cloned into plasmid pGEM4Z. The plasmid was cleaved with *Sfu*I, which cuts once inside the coding region of the *HDF2* gene 300 bp downstream of the ATG codon. 216 bp were removed by Bal31 endonuclease digestion and replaced by a functional kan resistance gene (43)**.** The resulting plasmid, pHDF2kan1, was digested with *Bam*HI/*Sal*I and the DNA was used to transform the haploid yeast strains W303-1A and W303aL for G418 resistance for one-step gene disruption (44). Gene disruption was verified by Southern blot analysis and loss of HDF DNA binding activity in gel retardation assays.

Plasmid Complementation-To complement the *hdf2* deletion in the yeast strain W303h2 a 2.450-bp *Sal*I/*Sac*I fragment was cloned into the multiple cloning site of plasmid pRS313 (45). The fragment contains 175 bp upstream of the *HDF2* start codon, the entire *HDF2* coding region, and 387 bp downstream of the stop codon. The resulting plasmid, pRS313HDF2, containing the *HIS3* selection marker was transformed into the *hdf2* deletion strain W303h2. Positive clones were selected by plating to His^- SD plates. His^+ colonies were tested for temperature sensitivity and HDF DNA binding activity in gel retardation assays.

Expression of Human Ku p70 and Ku p80 in Yeast Cells-For expression of human Ku p80 protein the cDNA was cloned into the plasmid pRS316 (45) under control of the *GAL1-10* promotor. For expression of the Ku p70 protein the cDNA was cloned into the plasmid pRS313 under control of the ADH promotor. The resulting plasmids pRS316Galp80 containing the *URA3* selection marker and pAHp70 containing the *HIS3* selection marker were transformed into different yeast strains. Positive clones were selected by plating to Ura^-, His^-, or Ura^-/His^- SD plates.

Drop Titer Test-A single colony from a YPD or SD plate grown for 3 days was suspended in 500 µl of YPD medium. This cell suspension was diluted from 10^{-1} to 10^{-5}. 10 µl of each dilution were dropped onto a YPD or SD plate. The plates were incubated at 30 or 37 °C for 3-6 days.

If strains transformed with a galactose-inducible promotor were used, a single colony was resuspended in 10-20 ml YP-Gal liquid medium (1% yeast extract, 2% Bacto-peptone, 2% galactose) and incubated with at 30°C for 4 h. Cells were collected and resuspended in 500 µl YP-Gal. This cell suspension was diluted as described above and spread on YP-Gal or SD-Gal plates.

Bleomycin and Methyl Methanesulfonate (MMS) Treatment-Cultures were grown in YPD medium at 30°C over night and diluted to an A_{600} of 0.2-0.3. Cells were grown to mid log phase (A_{600} = 2-3). Individual samples were diluted in water and different cell concentrations were spread immediately in duplicates on YPD plates in the presence or absence of varying concentrations of bleomycin. Plates were incubated for 5-7 days at 30°C. The data from three experiments are given.

Assays for MMS sensitivity were performed in triplicate and analogous to bleomycin treatment. The data of four experiments are given.

For complementation assays cells were grown in selection medium. Samples were prepared as described above and spread on appropriate SD-plates. The data of two experiments are given.

TABLE I [II]

Relevant genotypes of used strains

Strain	Relevant genotype	Source
W303-1A	*Mat**a**, ade2-1, his3-11, leu2-3,112, ura3-1, trp1*	Ref. 34
W303aL	*Mat**a**, hdf1::LEU2, ade2-1, his3-11, ura3-1, trp1*	Ref. 33
W303h2	*Mat**a**, hdf2::KAN, ade2-1, his3-11, ura3-1,leu2-3,112, trp1*	This study
WaLh2	*Mat**a**, hdf1::LEU2, hdf2::KAN, ade2-1, his3-11, ura3-1, trp1*	This study
ABYS60	*Mat**a**, ade-, pra1-1, prb1-1, prc1-1, cpf1-3*	Ref. 33

RESULTS

Cloning of the HDF2 Gene-HDF exists as a stable heterodimer. The purified protein displays two bands in SDS-gel electrophoresis of 70 (HDF1) and about 85 kDa (HDF2) (data not shown). To clone the *HDF2* gene we purified the protein to homogeneity. The purification procedure included four column chromatography steps, phenyl-Sepharose, DEAE-cellulose, phosphocellulose, as well as DNA affinity chromatography on a column-bound oligonucleotide (33).

Starting with crude extract prepared from 500 g of wet yeast cells we obtained about 20 µg of a highly purified protein preparation. The amino acid sequence of four HDF2 peptides were obtained by microsequencing of proteolytic cleavage products. These sequences were used for comparison with sequences in the protein data base. All four peptides matched to one sequence of an unknown protein. The sequence of this unknown protein was identified in connection with the yeast genome project (accession no. SC9718_5). The open reading frame contained 1.890 bp coding for 629 amino acids. The molecular mass predicted from this DNA sequence was 71.25 kDa. This is not in agreement with the molecular mass of about 85 kDa determined by SDS-polyacrylamide gel electrophoreses but may be due to unknown posttranslational modification or the result of an artifact of SDS-gel electrophoresis.

Comparison of the amino acid sequence of the SC9718_5 open reading frame with sequences in the protein data base revealed a significant homology with the p80 subunit of the human Ku autoantigen of 45.6%. This is comparable with the homology of HDF1 with the p70 subunit of the Ku protein of about 46.5%. The amino acid sequence lacks a leucine zipper region shown to be present in the Ku p80 sequence. The SC9718_5 open reading frame sequence also lacks any other known protein domains. An EMBL3A genomic yeast library was screened using oligonucleotides derived from the data base sequence information. Four positive clones were isolated and a 2.450-bp *Sal*I/*Sac*I fragment was subcloned. The sequence was verified by sequencing from the 3´- and 5´-end.

Disruption of the HDF2 Gene-The *HDF2* gene was disrupted by employing the one-step disruption procedure of Rothstein (44) as described under "Materials and Methods". As shown in Fig. 1, *lane 4*, crude extract of the HDF2-deficient strain did not show any HDF-specific DNA binding activity. As a positive control the *hdf2*-deficient strain W303h2 was transformed with yeast expression plasmid pRS313 containing a 2.450-bp long DNA insert with the entire open reading frame as well as upstream and downstream regions of the *HDF2* gene. Crude extract from the transformed strain W303h2 displayed DNA binding activity in gel retardation assays which was indistinguishable from that observed with control strains (Fig. 1, *lanes 1* and 5).

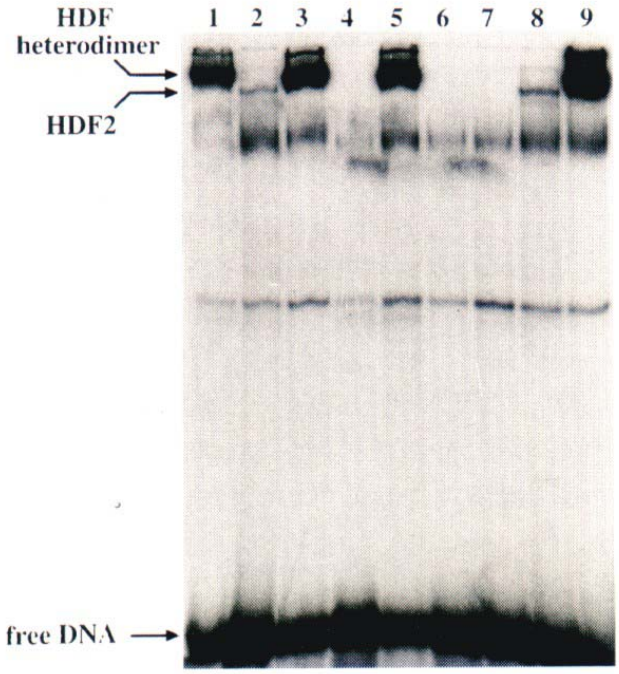

FIG. 1 [II]. HDF2 can bind to DNA on its own. *Lane 1*, wild-type W303-1A; *lane 2*, *hdf1*$^-$ W303aL; *lane 3*, *hdf1*$^-$ W303aL-pRS316HDF1; *lane 4*, *hdf2*$^-$ W303h2; *lane 5*, W303h2-pRS313HDF2; *lane 6*, *hdf1*$^-$ *hdf2*$^-$ WaLh2; *lane 7*, WaLh2-pRS316HDF1; *lane 8*, WaLh2-pRS313HDF2; *lane 9*, WaLh2-pRS316HDF1/ pRS313HDF2.

HDF2 Can Bind to DNA on Its Own-Comparison of the DNA-protein complexes detectable in the crude extracts of *hdf1*- and *hdf2*-deficient strains showed an additional band formed by the crude extract of *hdf1*-deficient strain (Fig. 1, *lane 2*).These results suggest that HDF2 possesses a DNA binding activity of its own. This DNA binding activity of HDF2 was much weaker than the DNA binding activity of the HDF heterodimer in crude extract of wild type cells. (Fig. 1, *lanes 1* and *2*). To verify that the observed DNA-protein complex was formed by the HDF2 protein the *hdf1 hdf2* double mutant strain WaLh2 was used. In crude extracts of the double mutant strain the HDF2-DNA complex was not detectable (Fig. 1, *lane 6*). This complex was also absent if this strain was transformed with the HDF1 expression plasmid, pRS316HDF1 (33) (Fig. 1, *lane 7*). The complex reappeared in crude extracts of the double mutant strain transformed by the HDF2 expression plasmid, pRS313HDF2 (Fig. 1, *lane 8*). Transformation of the double mutant strain with both the HDF1 and HDF2 expression plasmid led to HDF heterodimer DNA binding activity indistinguishable from that observed in the wild type (Fig.1, *lanes 1* and *9*).

HDF2$^-$ Strains are Temperature-sensitive for Growth-Since the HDF1-deficient strain W303aL showed a temperature-sensitive phenotype for growth (33), this growth phenotype was also studied in the *hdf2*-deficient strain W303h2. When haploid wild-type and *hdf2*-deficient strains were kept at 30 °C for 3 days on YPD plates, suspended in liquid medium, and spot-plated onto YPD plates at different dilutions, the wild-type strain grew normally at 37 °C, whereas the *hdf2*-deficient strain did not grow at this temperature. When kept at the permissive temperature (30 °C) both wild-type and *hdf2*-deficient strain grew normally (data not shown). This phenotype of the *hdf2*-deficient strain is similar to that observed for the *hdf1*-deficient strain. Growth of *hdf2*-deficient cells in liquid medium at 37°C for 10-12 h resulted in the development of enlarged single budded cells (data not shown, but see Feldmann and Winnacker (33)). This is in agreement with the phenotype of *hdf1$^-$* cells. Growth phenotypes of the *hdf1 hdf2* double mutant strains were identical with the phenotype of the single mutant strains.

Human Ku Protein Cannot Complement Temperature Sensitivity of HDF-deficient Cells-HDF is the homologue of the human Ku protein. The proteins share biochemical properties and structural homology. We therefore tested the ability of the human Ku subunits to complement the temperature-sensitive phenotypes caused by HDF2 and HDF1 deficiency.

The *hdf2$^-$* strain W303h2 was transformed with a yeast expression plasmid containing the Ku p80 cDNA under the control of a *GAL1-10* promotor. The resulting strain W303h2-pRS316Galp80 was tested for the ability to grow at 30 and 37 °C on Ura$^-$ SD plates containing 2% galactose. As shown in Fig. 2*A*, the *hdf2$^-$* strain expressing the human Ku p80 could not grow at 37 °C. When kept at the permissive temperature (30 °C) this strain grew normally. This result suggested that Ku p80 cannot complement the temperature sensitivity of *hdf2$^-$* strains. We also tested whether Ku p70 could complement the *hdf1$^-$* phenotype. The HDF1-deficient strain W303aL transformed with a yeast expression plasmid containing the cDNA of Ku p70 under control of an ADH promotor could not grow at 37 °C (data not shown), indicating that Ku p70 cannot complement HDF1 deficiency. Finally we tested the *hdf2* mutant strain W303h2 transformed with both plasmids, pRS316Galp80 and pAHp70, for growth at 37 °C. As shown in Fig. 2*B* expression of both subunits of the Ku heterodimer cannot complement temperature sensitivity of the HDF-deficient strain.

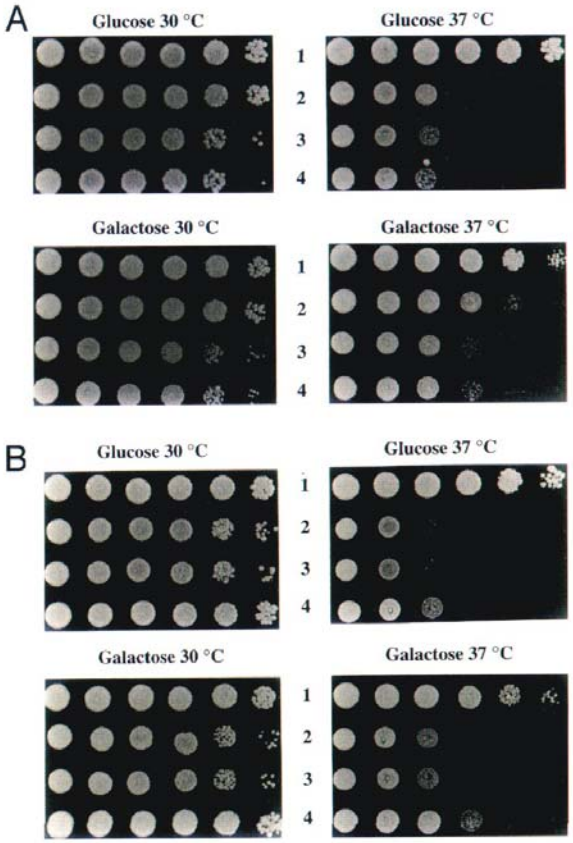

FIG. 2 [II]. *A*, **human Ku p80 cannot complement hdf2 deficiency.** *Lane 1*, wild-type W303-1A-pRS316; *lane 2*, *hdf2*⁻ W303h2-pRS316; *lane 3*, W303h2-pRS316Galp80 clone 1; *lane 4*, W303h2-pRS316Galp80 clone 2. *B,* **human Ku heterodimer cannot complement hdf2 deficiency.** *Lane 1*, wild-type W303-1A-pRS316; *lane 2*, W303h2-pRS316Galp80/pAHp70 clone 1, *lane 3*, W303h2-pRS316Galp80/pAHp70 clone 2; *lane 4*, W303h2-pRS316/pAH.

To verify that the Ku subunits were expressed in yeast cells, we tested DNA binding activity in crude extracts of the transformed strains using gel retardation assays. We could not detect an HDF1/Ku p80 corresponding DNA protein complex with crude extracts of the strain W303h2-pRS316Galp80 (Fig. 3, *lanes 3* and *4*). Extracts from strain W303aL-pAHp70 displayed no HDF2/Ku p70 corresponding DNA binding activity (data not shown). In crude extract of the *hdf2* mutant strain transformed with the Ku p70 and Ku p80 expression plasmids, a new DNA protein complex corresponding to the Ku p70/p80 heterodimer was detectable (Fig. 3, *lanes 5* and *6*).

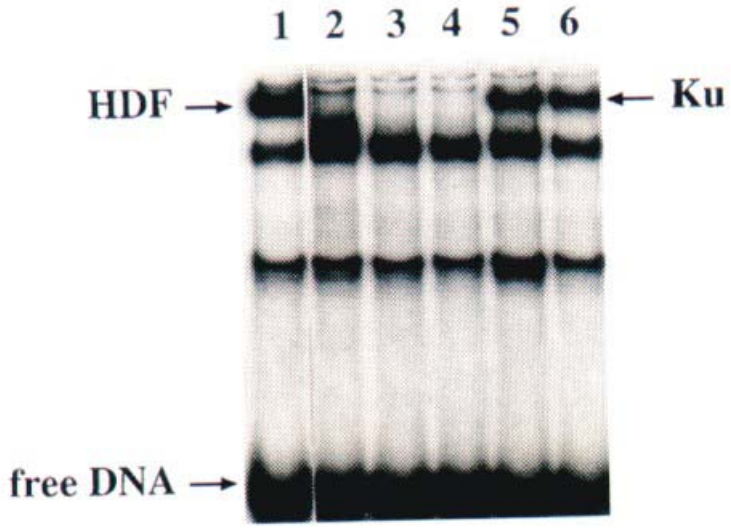

FIG. 3 [II]. Human Ku heterodimer expressed in yeast cells can bind to DNA. *Lane 1*, wild-type W303-1A-pRS316; *lane 2*, *hdf2*$^-$ W303h2-pRS316; *lane 3*, W303h2-pRS316Galp80 clone 1; *lane 4*, W303h2-pRS316Galp80 clone 2; *lane 5*, W303h2-pRS316Galp80/pAHp70 clone 1; *lane 6*, W303h2-pRS316Galp80/ pAHp70 clone 2.

hdf2 Mutants Are Sensitive to Bleomycin and MMS-Bleomycin is known to cause the introduction of double strand breaks into DNA molecules. We have shown previously, that the *hdf1* mutant strain W303aL is strongly sensitive to bleomycin (34). Therefore, we studied the level of sensitivity for bleomycin of *hdf2* and *hdf1 hdf2* double mutant strains. Survival assays were carried out on solid medium in the presence or absence of varying concentrations of bleomycin. A marked reduction by 1.2 orders of magnitude in the survival rates of the haploid *hdf2* mutant strain was observed at a bleomycin concentration of 4 µg/ml. This decrease is not as prominent as the decrease of survival rates in *hdf1* or *hdf1 hdf2* double mutant strains, which showed a reduction by 1.8-2.0 orders of magnitude at the same bleomycin concentration (Fig. 4). Transformation of a *hdf2* mutant strain with a yeast single copy plasmid carrying a functional copy of the *HDF2* gene restored the response to bleomycin to wild-type levels (data not shown).

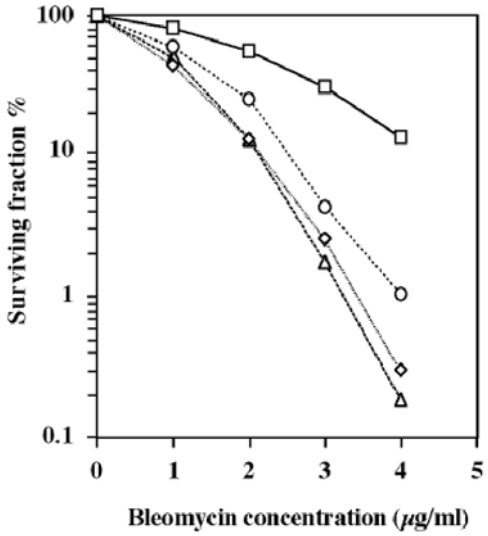

FIG. 4 [II]. ***hdf*1-and *hdf*2-deficient strains show different sensitivities for bleomycin.** ❑ wild-type W303-1A, ◆ *hdf*1 mutant W303aL, ○ *hdf*2 mutant W303h2, and Δ *hdf*1 *hdf*2 double mutant WaLh2 were plated on solid YPD media containing bleomycin in concentrations of 1-4 μg/ml. Colonies were counted after 5 days of incubation at 30 °C.

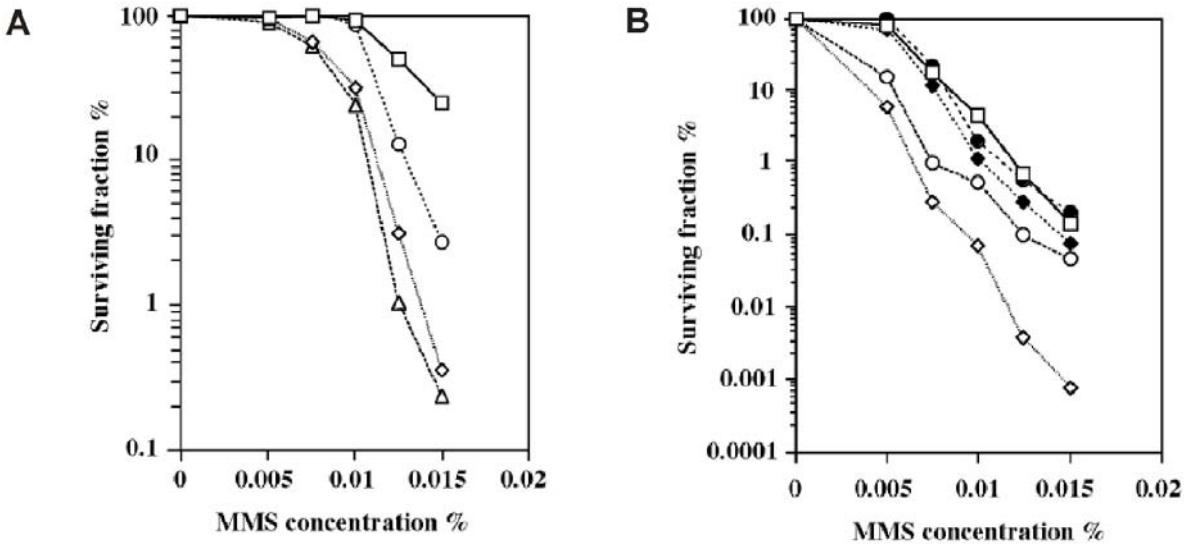

FIG. 5 [II]. *A*, *hdf1*- and *hdf2*-deficient strains are sensitive to MMS. □ wild-type W303-1A, ◇ *hdf1* mutant W303aL, ○ *hdf2* mutant W303h2, and Δ *hdf1 hdf2* double mutant WaLh2 were plated on solid YPD media containing 0.005-0.015% MMS. Colonies were counted after 5 days incubation at 30 °C. *B*, Expression of functional *HDF1* or *HDF2* genes complement *hdf1* or *hdf2* deficiency, respectively. □ wild-type strain W303-1A-pRS316, ◇ hdf1 mutant W303aL-pRS316, ◆ W303aL-pRS316HDF1, ○ hdf2 mutant W303h2-pRS313, and ● W303h2-pRS313HDF2 were plated on solid SD medium containing 0.005-0.015% MMS. Colonies were counted after 5 days incubation at 30 °C.

Another agent known to induce strand breaks in DNA is MMS. Accordingly, *hdf1* and *hdf2* mutant strains and the double mutant strains were also tested for MMS sensitivity.

We observed a significant sensitivity of all three strains to MMS compared with the wild-type strain (Fig. 5*A*). The decrease in survival rate of the *hdf2* mutant strain in response to MMS treatment was not as pronounced as the decrease of the survival rates of the *hdf1* and *hdf1 hdf2* double mutant strains. Survival rates of the *hdf2* mutant strain were reduced by 1 order of magnitude while the decrease in survival rates of the *hdf1* mutant strain and the double mutant strains was by 2-2.2 orders of magnitude. Sensitivity of the *hdf1* and *hdf2* mutant strains to MMS could be restored to wild-type level by expression of a functional copy of the *HDF1* or *HDF2* gene, respectively, from a yeast single copy plasmid (Fig. 5*B*).

DISCUSSION

We have cloned *HDF2* from *S. cerevisiae*, the gene encoding the second subunit of the HDF heterodimer, which is the homologue of the human Ku protein. HDF2 displays a homology of 45.6% with Ku p80. The molecular mass predicted from the sequence of the *HDF2* gene is 71.25 kDa. This is substantially smaller than the mass of the Ku p80 subunit of 82.5 kDa. The significance, if any, of the observed differences in the molecular masses of the two proteins remains unresolved as homology comparisons do not provide any indications for the presence of regional or local decreases in the homology of the two proteins.

No DNA binding activity is detectable for HDF1 on its own. The analysis of extracts of *hdf1*-deficient cells reveals the presence of a weak DNA binding activity which is not detectable in extracts of *hdf2*-deficient cells. This DNA binding activity is also absent in cells of a double mutant strain but it can be restored by expression of a *HDF2* gene from a single copy plasmid. These experiments indicate that HDF2 protein possesses a weak DNA binding activity of its own. Since the DNA binding activity of HDF2 alone is weak compared with the DNA binding activity of the heterodimer it may well be that HDF2 is the DNA binding subunit of the HDF heterodimer, while HDF1 is increasing the affinity of the heterodimer to DNA.

hdf2 mutant strains are temperature-sensitive for growth. Cells grown at 37 °C display the same phenotype as observed for *hdf1* mutant cells, arresting as enlarged single-budded cells. *hdf1 hdf2* double mutant strains display no additional growth defects. These results indicate that this phenotype depends on the missing HDF heterodimer activity.

The temperature-sensitive phenotype for growth caused by HDF deficiency cannot be complemented by the expression of either the single subunits or the heterodimeric human Ku protein. A HDF/Ku corresponding DNA binding activity is not detectable in *hdf1*- or *hdf2*-deficient strains transformed with plasmids expressing human Ku p70 or Ku p80, respectively. These results indicate that HDF1 and Ku p80 or HDF2 and Ku p70 cannot form functional heterodimers. Functional expression of the Ku heterodimer can be shown by detection of the DNA binding activity of the Ku protein in crude yeast extract. We suggest therefore that loss of HDF DNA binding is not responsible for temperature sensitivity but loss of protein-protein interactions. It appears that human Ku cannot take over the function of HDF in protein-protein complexes. One candidate for protein interaction with the HDF heterodimer is the catalytic subunit of a postulated DNA-dependent protein kinase. Until now it was not possible to show the existence of a DNA-dependent protein kinase in yeast. But the functional and structural homology of HDF and Ku heterodimers leads to the assumption that a DNA-dependent protein kinase activity exists in *S. cerevisiae*, as well.

We have shown recently that *hdf1* mutant strains are sensitive for the radiomimetic agent bleomycin and, in addition, show a reduced rate of mating-type switching and mitotic recombination (34). These experiments indicate that the HDF heterodimer is involved in DNA repair and recombination events. In this communication we show that *hdf2*-deficient strains are also sensitive to bleomycin and that *hdf1*- or *hdf2*- deficient strains are sensitive to MMS, an agent inducing DNA breaks. Surprisingly *hdf1* mutant strains are about 10 times more sensitive toward both agents than *hdf2* mutant strains. Deletion of both subunits in *hdf1 hdf2* double mutant strains only slightly increases sensitivities. This observation indicates that HDF1 is the critical component of HDF heterodimers functioning in DNA repair. In conjunction with the data reported for the DNA binding activity of the HDF2 subunit, these results suggest a mechanistic model of the function of the HDF heterodimer.

It may be that HDF2 is the DNA-binding component of the HDF heterodimer, while HDF1 is stabilizing the protein-DNA complex and is the active component in forming protein-protein complexes. In this case the HDF1 subunit alone could function in DNA repair but in a reduced manner. It may be that binding of the HDF heterodimer to DNA localizes the protein toward the position it is needed, and in *hdf2*-deficient cells the HDF1 subunit reaches this position only with a strongly reduced affinity.

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Chapter III

New Function of *CDC13* in Positive Telomere Length Regulation

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ABSTRACT

Two roles for the *Saccharomyces cerevisiae* Cdc13 protein at the telomere have previously been characterized: It recruits telomerase to the telomere and protects chromosome ends from degradation. In a synthetic lethality screen with *YKU70*, the 70-kDa subunit of the telomere-associated Yku heterodimer, we identified a new mutation in *CDC13, cdc13-4,* that points toward an additional regulatory function of *CDC13*. Although *CDC13* is an essential telomerase component in vivo, no replicative senescence can be observed in *cdc13-4* cells. Telomeres of *cdc13-4* mutants shorten for about 150 generations until they reach a stable level. Thus, in *cdc13-4* mutants, telomerase seems to be inhibited at normal telomere length but fully active at short telomeres. Furthermore, chromosome end structure remains protected in *cdc13-4* mutants. Progressive telomere shortening to a steady-state level has also been described for mutants of the positive telomere length regulator *TEL1*. Strikingly, *cdc13-4*/*tel1*Δ double mutants display shorter telomeres than either single mutant after 125 generations and a significant amplification of Y´ elements after 225 generations. Therefore *CDC13*, *TEL1*, and the Yku heterodimer seem to represent distinct pathways in telomere length maintenance. Whereas several *CDC13* mutants have been reported to display elongated telomeres indicating that Cdc13p functions in negative telomere length control, we report a new mutation leading to shortened and eventually stable telomeres. Therefore we discuss a key role of *CDC13* not only in telomerase recruitment, but also in regulating telomerase access, which might be modulated by protein-protein interactions acting as inhibitors or activators of telomerase activity.

INTRODUCTION

Ends of linear eukaryotic chromosomes form a special structure, the telomere. The telomeric DNA-protein complexes are essential for chromosome stability (49). They protect chromosomes from degradation, end-to-end fusion (54) and ensure their complete replication (41). In most eukaryotes, telomeric DNA contains a simple, repetitive sequence with the strand running toward the end of the chromosome being rich in G residues. For some organisms the configuration of the chromosome ends has been defined exactly. In hypotrichous ciliates the double-stranded region is followed by a 12- to 16-nucleotide-long single-stranded (ss) 3´-overhang (22, 24), whereas mouse and human chromosomes contain ss termini of 45 to 200 nucleotides (36, 39, 60). In the yeast *Saccharomyces cerevisiae* the telomere repeats consist of 300 ±75 bp of $C_{1-3}A/TG_{1-3}$ DNA. Detectable ss extensions of the G-rich strand are generated at telomeres specifically during S phase in a telomerase-independent process (11, 57, 58). A specialized enzyme, telomerase, performs synthesis of telomeric DNA by extending the 3´ end of the G-rich strand of the telomere. Telomerase activity in *S. cerevisiae* depends on at least four protein subunits (encoded by *EST1*, *EST2*, *EST3*, and *CDC13*/*EST4*) (28, 34) and the RNA component (encoded by *TLC1*) (52). All subunits are essential for telomerase function in vivo, although only the catalytic subunit *EST2* and the RNA template *TLC1* are necessary for in vitro activity (6, 8, 30). Deletion of most individual components of the telomerase complex leads to inactivation of telomerase and thereby to a decrease in telomere length and to replicative senescence (28, 34).

However, deletion of *CDC13/EST4* leads to immediate cell cycle arrest and cell death (56). This phenotype is triggered by the accumulation of telomeric single-stranded DNA (ssDNA) that activates a *RAD9*-dependent G_2 arrest (16). Therefore Cdc13p was proposed to provide protection of the telomere from nucleolytic degradation by DNA end binding. This role is consistent with the finding that Cdc13p binds ss telomeric DNA in vitro (29, 40) and binds exclusively to telomeric, but not to internal, $C_{1-3}A/TG_{1-3}$-repeat sequences (5). Very recently the DNA binding domain of Cdc13p has been mapped to amino acids 557 to 694.

Heterologous expression in *Escherichia coli* of a small, *CDC13*-derived polypeptide containing this region results in a protein that binds, like the full-length Cdc13p, with high affinity to ss telomeric DNA (23). A single amino acid missense mutation within this region of Cdc13p causes thermolabile DNA binding, and consistent with the presumption that Cdc13p DNA binding is essential to protect chromosome ends, this mutant is temperature sensitive for growth (23).

Besides its role in chromosome end protection, Cdc13p is involved in recruiting telomerase to telomeric DNA. *cdc13-2*^{est} mutant cells exhibit a senescence phenotype but can be rescued by expression of a Cdc13-2^{est}-Est1 fusion protein (12). These data suggest that Cdc13p is essential for loading telomerase to the telomere and that this process is mediated via interaction with Est1p. Interaction of Cdc13p and Est1p has been shown by two-hybrid criteria. Additionally, hemagglutinin (HA)-tagged Cdc13p can be copurified with a glutathione S-transferase (GST)-Est1 fusion protein from yeast extracts, if both proteins were overexpressed (45). Furthermore, Cdc13p seems to be involved in the accurate regulation of telomerase recruitment, as several *CDC13* mutations, not yet mapped at the genomic level, confer either elongated telomeres (41, 18) or shortened telomeres (18).

In *S. cerevisiae* the steady-state level of telomeric GT repeat tract length seems to result from a balance between telomere elongation and telomere shortening (37). Many proteins involved in telomere length maintenance have been identified already. A major factor involved in negative telomere length regulation is the Rap1 protein, which binds with high affinity to specific sequences within the telomeric GT repeat tracts (7). Unregulated telomere elongation is prevented mainly by Rap1p and its interacting partners Rif1p and Rif2p (21, 31, 59). It has been proposed that a negative feedback mechanism determines the exact number of Rap1p molecules bound to telomeric DNA and regulates telomerase activity (37, 38). Recently, a model has been suggested in which a special folded structure prevents telomere elongation (46). In this model, the formation of the folded structure of the chromosome end depends on the length of the GT repeat tract and on the number of bound Rap1p. At least two pathways are involved in positive telomere length control in *S. cerevisiae*. One pathway involves Tel1p and the Mre11-Rad50-Xrs2 complex, and disruption of any of these genes results in stable shortened telomeres (47).

A second pathway affecting positive telomere length regulation involves the Yku heterodimer, which is also an essential component of the nonhomologous end-joining pathway (2-4, 44). As shown by in vivo cross-linking experiments, Ykup binds directly to telomeric DNA (19). Yku mutant strains display short but stable telomeres, and the ss telomeric overhang of the G-rich strand, usually restricted to S phase in wild-type cells, is present in Yku$^-$ cells throughout the entire cell cycle (19).

Using a genetic approach we identified a new mutation in *CDC13*, designated *cdc13-4*, that is lethal in combination with a deletion of either subunit of the Yku heterodimer. The telomeres of *cdc13-4* mutants shorten continuously for about 150 generations before eventually reaching a stable level comparable to telomere length seen in Yku$^-$ mutants. *cdc13-4* causes no senescence phenotype and a *cdc13-4*/*rad52* double mutant is viable for at least several hundred generations. A *cdc13-4*/*tel1*Δ double mutant displays enhanced telomere shortening compared to either single mutant and Y′ element amplification after 225 generations of growth. Coimmunoprecipitations reveal that HA_3-Cdc13-4p still associates with GST-Est1p when both proteins are overexpressed. In addition, in a *cdc13*Δ strain a Cdc13-4-Est1 fusion protein does not induce telomere elongation to the same extent as a wild-type Cdc13-Est1 fusion. The terminal chromosome configuration of *cdc13-4* mutants seems, besides the telomere shortening, unchanged, since no ss G-rich overhang can be detected by native in-gel hybridization. Our data indicate that Cdc13p functions in telomere length regulation independent of its roles in chromosome end protection and telomerase recruitment.

MATERIALS AND METHODS

***S. cerevisiae* strains, media, growth conditions, and transformation.** The strains used in this study are listed in Table 1. Cells were grown at 30°C using yeast extract-peptone-dextrose (YPD), yeast extract-peptone-galactose, or selective media as described elsewhere (14). Screening for synthetic lethal mutations was performed on YPD plates (9). For counterselection plates, 5-fluorootic acid (5-FOA) (bts) was added to selective media at a concentration of 1 mg/ml as described previously (9). To examine telomere length and the senescence phenotypes of strains over many generations, colonies derived from freshly germinated spores were streaked on YPD plates. After 48 h incubation at 30°C, single colonies were restreaked on fresh YPD plates. This procedure was repeated up to nine times.

Single colonies from different generations were then used for overnight inoculation and treated for DNA preparation. Yeast transformation was performed by the lithium acetate method (50).

Plasmids. The plasmid pCH-YKU70 used for the synthetic lethality screen was constructed as follows: a *Xho*I/*Eco*RI fragment containing a functional *YKU70* gene was isolated from the plasmid pRS316-YKU70 (15) and blunted with Klenow enzyme. This fragment was then cloned into pCH1122 (26) linearized with *Sma*I. Expression of Yku70p from pCH-YKU70 was verified by complementation of the temperature sensitive phenotype of a *yku70*-deficient strain and by reconstitution of Yku heterodimer DNA binding activity in a gel retardation assay (15). The *CDC13* expression plasmid pRS314-CDC13 was generated as follows: a 4.7 kb *Apa*I fragment containing 712 bp 5′ of the start codon, the entire open reading frame (ORF) of *CDC13*, and 1,200 bp 3′ of the stop codon was isolated from the library plasmid GP2a. This fragment was ligated to pRS314 (51) linearized with *Kpn*I/*Sac*I and blunted with Klenow enzyme. To generate the plasmid pRS314-cdc13-4 expressing the mutated *CDC13* allele, a 900-bp DNA fragment was amplified by PCR from genomic DNA of mutant LDM29 by using the primers CDC13-ATG (5′-ACG TGT CGA CCC GGG ATG GAT ACC TAG AAG AGC CTG AG-3′) and CDC13-900 (5′-GAA ATA TTT CCC GGT AGA GGA GG-3′). The PCR product was subcloned into pZErO-2 (Invitrogen) and sequenced. A *Xho*I/*Nsi*I fragment carrying the *cdc13-4* point mutation was then excised from pZ-cdc13-4 and ligated to the vector pRS314-CDC13 digested with *Xho*I/*Nsi*I. To generate several *CDC13* disruption constructs, pRS314-CDC13 was digested with *Xho*I/*Aat*II, thereby deleting the entire ORF of *CDC13* except 57 bp at the 5′ end. This fragment was replaced by a marker cassette of either KanMX4 or *URA3*, resulting in plasmids p-cdc13Δ::KanMX4 and p-cdc13 Δ::URA3, respectively. The plasmid pRS-cdc13-4-KanMX4 was generated for genomic integration of the *cdc13-4* allele by linearizing pRS314-cdc13-4 with *Aat*II, blunting it with Klenow enzyme, and inserting the KanMX4 marker cassette.

To generate *CDC13-EST1* and *cdc13-4-EST1* fusion constructs, the *EST1* gene was amplified from genomic DNA of the strain W303a using primers Est1*Sac*I (forward: 5'-GAG CTC ATG GAT AAT GAA GAA GTT AAC G-3') and Est1*Sal*I*Sma*I (reverse: 5'-GTC GAC CCC GGG TCA AGT AGG AGT ATC TGG CAC-3'). A C-terminal fragment of *CDC13* was amplified using primers Cdc13-P3 (5′-CTG GTG CCA GGC GTC AAT TGC-3′) and Cdc13-P4Sma (5′-ATC CCG GGC GAG GTG GGA ACG GCT CCG-3′) and cloned into plasmid pZErO-2. The *EST1* fragment was digested using *Sma*I/*Hpa*I and ligated into pZ-CDC13-P3P4Sma linearized with *Sma*I. The correct orientation of the construct was verified by restriction analysis. The plasmid was then cut with *Sac*II/*Pst*I, and the DNA fragment containing C-terminal-*CDC13-EST1* was isolated. pRS314-CDC13 was digested with *Sac*II/*Not*I and a 3.1-kb fragment containing the N-terminal part of *CDC13* and the *CDC13* promoter was isolated. Both fragments were then ligated to pRS314 *Not*I/*Pst*I, resulting in pRS314-CDC13-EST1. To delete the *Hin*dIII vector site, pRS314-CDC13-EST1 was cut with *Pst*I/*Apa*I, treated with T4-Polymerase, and religated. The religated vector was cut with *Not*I/*Kpn*I, and the *CDC13-EST1* fragment was isolated and ligated to pRS316 *Not*I/*Kpn*I to obtain pRS316-CDC13-EST1. The plasmid pRS316-cdc13-4-EST1 was generated by restriction of pRS316-CDC13-EST1 with *Hin*dIII and replacing the resulting internal *CDC13* *Hin*dIII fragment by the corresponding *cdc13-4* *Hin*dIII fragment.

The correct orientation of the *cdc13-4 Hin*dIII fragment was checked by restriction analysis, and sequencing confirmed the single base pair exchange in pRS316-cdc13-4-EST1.

Gene disruption. The *yku70*-deficient strain KαL7 was generated by disruption of the *YKU70* gene in K2348*α* as described previously (15). Gene disruption was verified by Southern blot analysis. To disrupt the *CDC13* gene, plasmids pRS314-cdc13Δ::URA3 and pRS-cdc13Δ::KanMX4 were digested with *Apa*LI and *Kpn*I, and the resulting linear disruption construct was used to transform several diploid strains to Ura^+ or G418 resistance (Table 1). Disruption of the *CDC13* gene was verified by Southern blot analysis. The yeast strain BMY13 carrying a genomic integrated *cdc13-4* allele was generated by transforming LDY50 using the *Apa*I/*Kpn*I fragment excised from pRS-cdc13-4-KanMX4. The transformed cells were plated on synthetic-dextrose minimal plates lacking uracil and containing 200 mg of G418/liter. Colonies arising from these plates were screened by PCR for correct integration of the marker gene. To verify the integration of the *cdc13-4* point mutation, a PCR fragment spanning the corresponding part of the *CDC13* gene was amplified and sequenced. BMY14 (W303aα *cdc13::URA3/cdc13-4::kanMX4*, *rad52*Δ*::His3MX6/RAD52*) was generated by replacement of the *RAD52* ORF in BMY13 by PCR-based gene disruption (55). Sporulation of BMY13 and BMY14 resulted in haploid spores carrying the *cdc13-4* point mutation (BMY17) and double mutant *cdc13-4/rad52*Δ (BMY18), respectively. Strain BMY56 was generated by crossing BMY17 with W303a. This strain was propagated for several generations and then used to introduce either a *tel1* or a *est2* deletion. *TEL1* was deleted by PCR-based replacement of the entire ORF with a His3MX marker (BMY57), and the *EST2* gene was replaced by the *TRP1* selection marker (BMY58). Transformants arising after incubation on selective media were screened by PCR for integration of the disruption constructs. Both heterozygous strains were then sporulated, and tetratype tetrads BMY59 and BMY60 were used for growth studies and analysis of telomere length phenotypes. To analyze expression of Cdc13-Est1 fusion proteins in a *cdc13*Δ strain, BMY62 was transformed with pRS-CDC13-EST1 or pRS316-cdc13-4-EST1 and sporulated on plates lacking uracil. BMY64 and BMY65 were isolated after tetrad dissection of BMY62+pRS-CDC13-EST1 and BMY62+pRS-cdc13-4-EST1, respectively.

Induced expression of HA_3-*CDC13*, HA_3-*cdc13-4*, and GST-*EST1*. For induced overexpression of HA_3-tagged *CDC13* and *cdc13-4,* the *GAL1* promoter together with the HA_3 tag was introduced in front of the genomic copy of *CDC13* in W303aα or *cdc13-4* in BMY56. Integration of *GAL1*-HA_3 was performed by PCR-based methods as described previously (32) using the HIS3MX6 marker for selection. Correct integration of the HIS3MX-*GAL1*-HA_3 construct in the resulting strains HFY80 (HA_3-*CDC13*) and HFY84 (HA_3-*cdc13-4*) was verified by analytic PCR and sequencing of the PCR product. Expression of HA_3-Cdc13p and HA_3-Cdc13-4p was analyzed by Western blotting using monoclonal anti-HA antibody 9F10 (Roche). The same PCR-based strategy was used to generate strains expressing GST::Est1 fusion protein under control of the *GAL1* promoter. The *TRP1-GAL1*-GST construct was introduced in W303aα, HFY80, and HFY84 resulting in the strains HFY81 (GST::EST1/EST1), HFY82 (HA_3-*CDC13*/*CDC13*, GST::*EST1*/*EST1*) and HFY86 (HA_3*cdc13-4*/*CDC13*, GST::*EST1*/*EST1*). Correct integration was verified by analytic PCR and sequencing of the PCR product.

Expression of GST::Est1p was analyzed by Western blotting using monoclonal anti-GST antibody (Sigma). Strains were grown on yeast extract-peptone media containing 2% galactose for induced expression of HA_3-*CDC13*, HA_3-*cdc13-4*, and GST::*EST1*. Strains HFY81, HFY82, and HFY86 were sporulated to generate haploid strains expressing the tagged Cdc13 and/or Est1 proteins. Tetrad analysis was performed on yeast extract-peptone plates containing galactose to allow expression of HA_3-Cdc13p, HA_3-Cdc13-4, and GST::Est1p. Spores expressing the tagged proteins were identified by marker analysis, and the resulting strains HFY81-8A (GST::*EST1*), HFY82-6B (HA_3-*CDC13*), HFY82-4C (HA_3-*CDC13*, GST::*EST1*), HFY86-3A (HA_3-*cdc13-4*, GST::*EST1*), and HYF86-9D (HA_3-*cdc13-4*, GST::*EST1*) were verified by Western blotting.

Immunoprecipitation. Coimmunopreciptation experiments to analyze the interaction of GST-Est1p-HA_3-Cdc13p and GST-Est1p-HA_3-Cdc13-4p were performed using strains HFY82-4C, HFY86-3A, HFY86-9D, and, as controls, HFY81-8A and HFY82-6B. Crude extracts were prepared as follows: yeast strains were grown overnight in YPGal, diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown to an OD_{600} of 0.8 to 1.2 in yeast extract-peptone-galactose. Cells were lysed in 20 mM Tris (pH 8.0)-200 mM NaCl-1 mM EDTA-1 mM DTT-0.01% NP-40-10% gycerol with one protease inhibitor cocktail tablet per 5 ml (complete, Mini, EDTA-free; Roche) in a bead beater. After centrifugation the soluble protein fraction was diluted 1:1 with lysis buffer containing 1% NP-40 and 0.2% Triton X-100. Crude extract (1,000 μg) was incubated with monoclonal anti-GST antibody, clone GST-2 (Sigma), for 1 h at 4°C, and then G-sepharose (Pharmacia) was added. After incubation for 1 h at 4°C G-Sepharose beads were collected by centrifugation and washed twice with lysis buffer containing 0.5% NP-40-0.1% Triton X-100, twice with lysis buffer containing 1% NP-40, 0.1% Triton X-100, and twice with lysis buffer containing 450 mM NaCl. The beads were treated with 1,000 U DNase I/ml in lysis buffer containing 1 mM $MgCl_2$ and then washed twice with lysis buffer containing 450 mM NaCl and 350 mM potassium acetate. After 15 μl of Laemmli buffer was added, beads were heated 3 min at 95°C and the supernatant was loaded onto an 8% sodium dodecyl sulfate gel. Proteins were visualized by enhanced chemiluminescence Western blotting using anti-HA antibody 9F10 (Roche) and anti-GST antibody clone GST-2 (Sigma).

Synthetic lethality screen. Stationary phase cells of KαL7 carrying the plasmid pCH-YKU70 were mutagenized with 3% ethyl methane sulfonate (EMS) for 90 min resulting in 15.6% survival. After EMS treatment, cells were plated on YPD plates containing 4% glucose to facilitate development of the red pigment. Uniformly red colonies were colony purified three times. Those which remained stably red under nonselective conditions were tested for sensitivity to 5-FOA. To test whether 5-FOA sensitive cells were dependent on *YKU70* expression rather than other components of the plasmid pCH-YKU70, the mutants were transformed with a second plasmid, pRS314-YKU70, expressing Yku70p and containing *TRP1* for selection. As a control, mutants were transformed with pRS314. Mutants carrying pRS314-YKU70 or pRS314 were retested for their ability to form red-white sectors and their growth on 5-FOA. Out of 20,520 mutagenized cells, five mutants were clearly dependent on *YKU70* expression. These mutants were stably red on YPD and sensitive to 5-FOA if transformed with the pRS314 vector control, but displayed red-white sectoring colonies and growth on 5-FOA after transformation with pRS314-YKU70.

Complementation of *YKU70* dependence. The mutant LDM29 was transformed using a single-copy genomic yeast library (ATCC 77164) and plated on Trp^- media. Out of 12,500 primary transformants, 15 plasmids were isolated leading to red-white sectoring colonies even after retransformation. In addition, these 15 plasmids enabled LDM29 cells to grow on 5-FOA-containing media, indicating that those cells were independent of *YKU70* expression. Restriction analysis revealed the isolation of three different genomic fragments capable of complementing the dependence on *YKU70*. To identify the isolated fragments, the 5´ and 3´ ends of the fragments were sequenced using vector-specific primers.

Identification of the *cdc13-4* mutation. The genomic mutation in LDM29 was mapped by gap repair (42). Plasmid pRS314-CDC13 was digested using different combinations of restriction enzymes. The resulting linear plasmids were transformed into LDM29. Generation of a functional *CDC13* gene by gap repair results in cells independent of *YKU70* expression, therefore displaying a red-white sectoring phenotype. Only cells transformed with a pRS314-CDC13 with a *Xho*I/*Nsi*I fragment spanning bp +57 - +830 of the *CDC13* coding sequence deleted did not display red-white sectoring colonies and were sensitive to 5-FOA, indicating that a plasmid carrying the mutated allele of *CDC13* was generated. To identify the mutation, a fragment corresponding to the mutated region in *CDC13* was amplified by PCR from genomic DNA of LDM29 and was sequenced.

Yeast DNA extraction and analysis of telomeric DNA. Genomic DNA was isolated from 5- to 7-ml overnight cultures using the nucleon MiY DNA extraction kit (Amersham Life Science). For analysis of telomere length, genomic DNA was digested overnight using *Xho*I and separated on an 1% agarose gel in 1x Tris-acetate-EDTA buffer. DNA was transferred to nylon membranes (HybondN^+) by vacuum blotting using 0.4 N NaOH. Detection of telomeric DNA fragments was performed as described elsewhere (2). Nondenaturing in-gel hybridization was performed as described previously (11).

TABLE 1. Yeast strains used in this study[a]

Strain	Genotype(s)	Reference
K2348α	*matα ade2-1 ade3 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 Gal*$^+$ *psi*$^+$	(17)
KαL7	K2348α *yku70::LEU2*	This study
CEN.PK2aα	*mat***a**/α *ura3-52/ura3-52 his3-Δ1/his3- Δ1 leu2-3,112/leu2-3,112 trp1-289/trp1-289 MAL2-8*^{c}*/MAL2-8*^{c} *SUC2/SUC2*	(1)
LDY05	CEN.PK2aα *yku70::URA3/yku70::LEU2*	This study
LDY06	CEN.PK2aα *yku80::kanMX4/yku80::kanMX4*	This study
LDY54	LDY05 *cdc13::kanMX4/CDC13*	This study
LDY55	LDY06 *cdc13::URA3/CDC13*	This study
W303aα	*mat***a**/α *leu2-3,112/leu2-3,112 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100 rad5-535/rad5-535*	(15)
WaUαL	W303aα *yku70::URA3/yku70::LEU2*	(15)
LDY50	W303aα *cdc13::URA3/CDC13*	This study
LDY46	W303aUαL *cdc13::kanMX4 /CDC13*	This study
LDY53	W303 *cdc13::URA3* + pRS314-cdc13-4	This study
BMY13	W303aα *cdc13::URA3/cdc13-4::kanMX4*	This study
BMY14	W303aα *cdc13::URA3/cdc13-4::kanMX4, rad52Δ::His3MX6/RAD52*	This study
BMY17	W303a *cdc13-4::kanMX4*	This study
BMY18	W303 *cdc13-4::kanMX4 rad52Δ::His3MX6*	This study
BMY56	W303aα *cdc13-4::kanMX4/CDC13*	This study
BMY57	W303aα *cdc13-4::kanMX4/CDC13 tel1Δ ::His3MX6/TEL1*	This study
BMY58	W303aα *cdc13-4::kanMX4/CDC13 est2Δ ::TRP1/EST2*	This study
BMY59-6A	W303a wt spore derived from BMY58	This study
BMY59-6B	W303α *cdc13-4::kanMX4* derived from BMY58	This study
BMY59-6C	W303a *est2Δ ::TRP1* derived from BMY58	This study
BMY59-6D	W303α *cdc13-4::kanMX4 est2Δ ::TRP1* derived from BMY58	This study
BMY60-11F	W303α *tel1Δ ::His3MX6* spore derived from BMY57	This study
BMY60-11G	W303a *cdc13-4::kanMX4* derived from BMY57	This study
BMY60-11H	W303α *cdc13-4::kanMX4 tel1Δ ::His3MX6* derived from BMY57	This study
BMY60-11I	W303a wt derived from BMY57	This study
BMY62	W303aα *CDC13*/*cdc13Δ::kanMX4*	This study
BMY64	W303 hap *cdc13Δ::kanMX4* + pRS316-CDC13-EST1	This study
BMY65	W303 hap *cdc13Δ::kanMX4* + pRS316-cdc13-4-EST1	This study
HFY80	W303aα *HIS3MX6-GAL1-HA$_3$::CDC13/CDC13*	This study
HFY81	W303aα *TRP1-GAL1-GST::EST1/EST1*	This study
HFY81-8A	W303 hap *TRP1-GAL1-GST::EST1* derived from HFY81	This study
HFY82	W303aα *HIS3MX6-GAL1-HA$_3$::CDC13/CDC13 TRP1-GAL1-GST::EST1/EST1* derived from HFY81	This study
HFY82-6B	W303 hap *HIS3MX6-GAL1-HA$_3$::CDC13* derived from HFY82	This study
HFY82-4C	W303 hap *HIS3MX6-GAL1-HA$_3$::CDC13 TRP1-GAL1-GST::EST1*	This study
HFY84	W303aα *HIS3MX6-GAL1-HA$_3$::cdc13-4/CDC13* derived from BMY56	This study
HFY86	W303aα *HIS3MX6-GAL1-HA$_3$::cdc13-4/CDC13 TRP1-GAL1-GST::EST1/EST1* derived from HFY84	This study
HFY86-3A	W303 hap *HIS3MX6-GAL1-HA$_3$::cdc13-4 TRP1-GAL1-GST::EST1* derived from HFY86	This study
HFY86-9D	W303 hap *HIS3MX6-GAL1-HA$_3$::cdc13-4 TRP1-GAL1-GST::EST1* derived from HFY86	This study

[a] wt, wild-type; hap, haploid.

RESULTS

Isolation of the *cdc13-4* mutant. Yku$^-$ mutant cells are temperature sensitive for growth (14, 15). To investigate the essential role of the Yku heterodimer at 37°C, we performed a synthetic lethality screen to isolate mutants in which *YKU70* would be essential for viability. Therefore we disrupted the *YKU70* gene in K2348α and tested the resulting mutant KαL7 for phenotypes specific for Yku$^-$ mutants. KαL7 is temperature sensitive for growth at 37°C, deficient in nonhomologous end joining, slightly sensitive to methyl methanesulfonate, and displays shortened telomeres (data not shown). The *YKU70* gene cloned into plasmid pCH1122 (pCH-YKU70) complemented the phenotypes of KαL7, indicating a functional expression of *YKU70* from the plasmid. KαL7-pCH-YKU70 colonies grown on YPD displayed a red-white sectoring phenotype, showing that pCH-YKU70 was not essential for growth at 30°C under nonselective conditions.

After EMS mutagenesis of KαL7-pCH-YKU70 we isolated five stably red mutants, which clearly required *YKU70* expression for viability (see Material and Methods). To identify the mutated gene causing the requirement for *YKU70* expression we transformed one mutant, LDM29, with a single-copy yeast library and screened for sectoring colonies indicating that pCH-YKU70 was no longer essential for viability. Plasmids isolated from 15 sectoring colonies revealed three independent clones, two of them carrying a DNA fragment containing the full-length *YKU70* gene. The third plasmid, GP2a, contained a fragment of chromosome IV from YDL57269 to YDL68607. This fragment encoded five ORFs among them YDL220c coding for *CDC13*/*EST4*.

Cdc13p, like the Yku heterodimer, has been shown to be an important factor for telomere maintenance. Therefore we subcloned the *CDC13* gene from plasmid GP2a into the single-copy vector pRS314 (51). After transformation with the resulting plasmid pRS314-CDC13, LDM29 displayed a clear sectoring phenotype indicating that a mutation in *CDC13* caused dependence on Yku70p expression (data not shown). Using the gap repair method (42) we identified a 773-bp fragment near the 5′ end of the *CDC13* gene carrying the mutation.

Sequencing of this fragment revealed the presence of a single point mutation (at position 703, changing a cytosine to a thymine) thereby leading to the amino acid exchange proline 235 to serine (P235S). Since this mutation differs from the *CDC13* mutants already described in the literature, we designated it *cdc13-4*.

Synthetic lethality of *cdc13-4* with Yku. We isolated the *cdc13-4* mutation in a synthetic lethality screen with *YKU70*. To verify the synthetic lethal phenotype we reintroduced the *cdc13-4* mutation in the homozygous *yku70* strain WaLαU. Therefore we disrupted the *CDC13* gene in WaUαL and transformed the resulting strain LDY46, heterozygous for *CDC13*, with pRS-cdc13-4. As expected we obtained only two colony-forming spores after sporulation and tetrad dissection of LDY46-pRS-cdc13-4 (data not shown). None of the viable spores was resistant to G418 (the KanMX marker gene was used for *CDC13* disruption), indicating that all viable spores contain the wild-type allele of *CDC13*. The nonviable spores were examined by microscopy. We found many of these spores germinated but arrested at a two-cell stage. In a very few cases we observed microcolonies containing up to 20 cells, which lysed after 2 to 3 days of incubation at 30°C. To show that these phenotypes were not due to synthetic effects caused by the *RAD5* mutation in the W303 background (13), we repeated the experiment in a CEN.PK2 strain. In this case we examined the synthetic lethality of *cdc13-4* in a *yku70*- and a *yku80*-deficient CEN.PK2 strain, LDY54 and LDY55, respectively.

Again we found only two colony-forming spores for most of the dissected tetrads. In some cases one or two microcolonies arose (Fig. 1). Cells from these microcolonies were not viable after restreaking on YPD plates (data not shown). Our data show that *cdc13-4* is synthetic lethal with either *yku70* or *yku80* deletion. Therefore we suggest that a *cdc13-4* mutant is dependent on a functional Yku heterodimer.

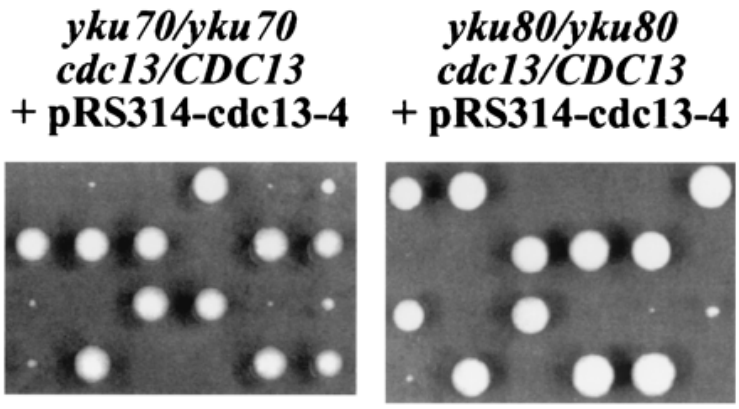


FIG. 1 [III]. Synthetic lethality of *cdc13-4* with the Yku heterodimer. Diploid strains CEN.PK2aα *yku70/yku70 cdc13/CDC13* and CEN.PK2aα *yku80/yku80 cdc13/CDC13* were transformed with plasmid pRS314-cdc13-4. Transformants were sporulated and tetrads were dissected using a Singer SMS Micromanipulator. Individual spores of each tetrad were placed down the columns on the YPD plates and incubated at 30°C for 3 to 4 days.

Telomeres of *cdc13-4* mutants shorten to a steady-state level. To investigate the phenotype of a *cdc13-4* single mutant we generated a haploid strain expressing the mutated *CDC13* gene (LDY53). One allele of *CDC13* was deleted in W303aα and the resulting heterozygous strain LDY50 was transformed using pRS314-cdc13-4. After sporulation and tetrad dissection, some tetrads were able to form three viable colonies (data not shown). Since disruption of *CDC13* is lethal, the tetrads resulting in three colony-forming spores should contain one spore carrying a disrupted *cdc13* allele and the plasmid expressing *cdc13-4*. All three tetrads tested formed two colonies unable to grow on uracil- or tryptophan-lacking media and exhibited wild-type fragment size in a Southern blot. One colony was prototrophic for uracil and tryptophan and displayed a disrupted genomic *CDC13* allele and Southern blot signals corresponding to the plasmid pRS314-cdc13-4 (data not shown). This colony corresponds to the *cdc13-4* mutant LDY53.

One important role of Cdc13p in telomere maintenance is loading telomerase to its ss template at chromosome ends. In *cdc13-2*^{est} mutants the loading function is abolished, presumably by inhibition of the Cdc13p-Est1p interaction, thereby resulting in progressive telomere shortening and senescence (40). To investigate the effect of the *cdc13-4* mutation on telomere stability we performed long-term growth experiments using strain LDY53. No growth reduction was observed for the *cdc13-4* mutant for more than 250 generations, suggesting that this mutation causes no senescence phenotype (data not shown).

To better understand the effect of the *cdc13-4* mutation, we examined telomere length in this mutant after various generations (Fig. 2). *cdc13-4* cells displayed a significant shortening of the telomeric GT repeat tracts after approximately 50 generations (Fig. 2, lane 1). After 150 generations (Fig. 2, lane 3), telomeric GT repeat tracts were almost as short as those observed for *yku70*-deficient strains (Fig. 2, lane 9). However, no further telomere shortening was observed after an additional 100 generations (Fig. 2, lane 5) and after several hundred generations (data not shown). Introduction of the *cdc13-4* mutation in a CEN.PK2 genetic background resulted in a comparable telomere length phenotype (data not shown).

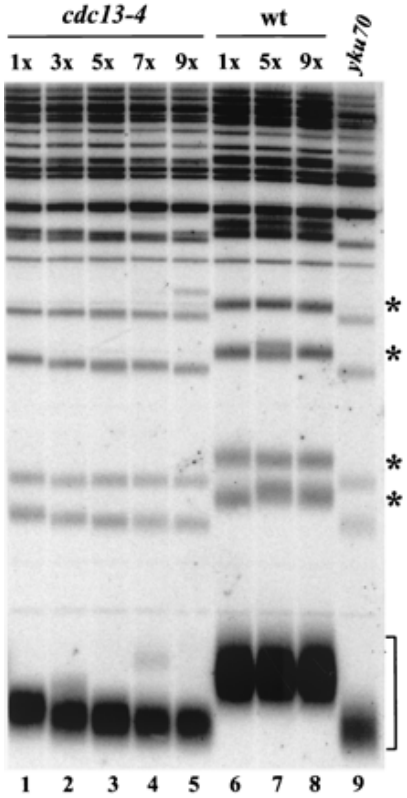


FIG. 2 [III]. Long-term analysis of the telomere length of a *cdc13-4* mutant. Southern blot of genomic yeast DNA, probed with a telomere-specific poly$(GT)_{20}$ oligonucleotide, is shown. The bracket indicates the telomeric GT repeat band derived from Y′ element-containing chromosomes. Asterisks indicate terminal fragments derived from non-Y′ element-containing chromosomes. W303 wild-type (wt) and W303 *cdc13::URA3* + pRS314-cdc13-4 strains from one tetrad were propagated on YPD for 250 generations. Therefore colonies derived from freshly germinated spores were streaked on YPD plates. After 48 h of incubation at 30°C, single colonies were restreaked on fresh YPD plates. Cells were estimated to have undergone 20 to 25 divisions per streakout. Numbering at the top of the lanes (1x, 3x, etc) indicates the number of times of restreaking. Single colonies from different generations were then used for overnight inoculation and treated for DNA preparation. Genomic DNA was prepared as described in Materials and Methods. Lane 1, *cdc13-4*, 50 generations; lane 2, *cdc13-4,* 100 generations; lane 3, *cdc13-4*, 150 generations; lane 4, *cdc13-4,* 200 generations; lane 5, *cdc13-4,* 250 generations; lane 6, W303 wt, 50 generations; lane 7, W303 wt, 150 generations; lane 8, W303 wt, 250 generations; and lane 9, W303a *yku70*.

***cdc13-4* mutants display no senescence phenotype.** Telomerase deficiency results in replicative senescence. Telomeres shorten gradually with increasing generations, eventually leading to cell death (28, 34). However, a few survivors can arise in a senescent yeast culture. These survivors stabilize their telomeres by homologous recombination, adding Y′ elements or GT repeats to the shortened chromosome ends (33). This process is detectable by an increase in intensity of the Y′ element signals in a Southern blot. Deletion of *RAD52* completely abolishes homologous recombination, and therefore no survivors appear in an *est2/rad52*-negative strain (27). To verify the observation that *cdc13-4* mutant cells display shortened telomeres but no senescence phenotype, we generated the diploid strain BMY58, heterozygous for *est2*Δ and *cdc13-4* mutation. After sporulation we compared growth of an *est2*Δ spore and a *cdc13-4* mutant spore (Fig. 3).

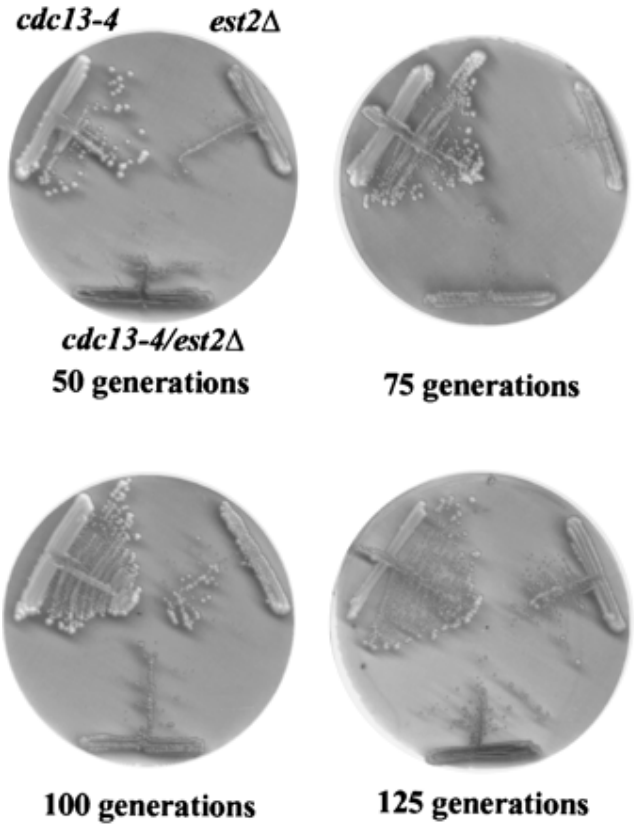


FIG. 3 [III]. Viability of *cdc13-4, est2*Δ and *cdc13-4 est2*Δ strains. After sporulation, *cdc13-4, est2*Δ, and *cdc13-4/est2*Δ mutant cells from a single tetrad were successively streaked on YPD plates to test senescence.

Whereas *est2*-negative cells displayed a significant growth reduction after 50 generations and survivor formation occurred after 75 generations, *cdc13-4* mutant cells grew normally over the entire time frame tested (Fig. 3). We analyzed telomere repeat sequences in BMY59-6C (*est2*Δ) and BMY59-6B (*cdc13-4*) cells by Southern blotting after growth for 25, 50, 75, 100, and 125 generations (Fig. 4).

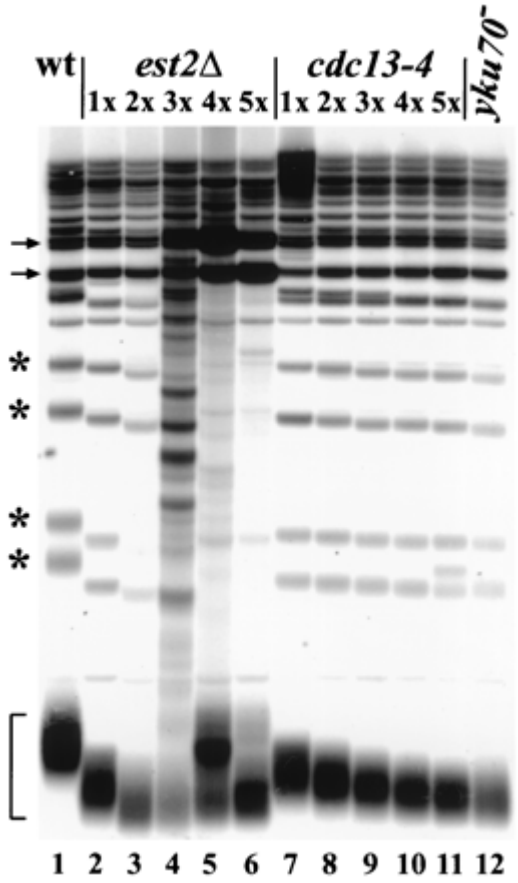


FIG. 4 [III]. Survivor formation in *cdc13-4* and *est2*Δ mutants. Southern blot of *Xho*I-digested genomic yeast DNA probed with a poly(GT)$_{20}$ oligonucleotide specific for telomeric repeats is shown. The bracket indicates the telomeric GT repeat band derived from Y′ element-containing chromosomes. Asterisks indicate terminal fragments derived from non-Y′ element-containing chromosomes. The arrows indicate restriction fragments corresponding to the subtelomeric Y′ elements. After tetrad dissection, spores W303 *cdc13-4* and W303 *est2*Δ from one tetrad were grown for 150 generations as described in Materials and Methods. Lane 1, W303a wild-type (wt); lane 2, *est2*Δ, 25 generations; lane 3, *est2*Δ, 50 generations; lane 4, *est2*Δ, 75 generations; lane 5, *est2*Δ-100 generations; lane 6, *est2*Δ 125 generations; lane 7, *cdc13-4*-25 generations; lane 8, *cdc13-4*, 50 generations; lane 9, *cdc13-4*, 75 generations; lane 10, *cdc13-4*, 100 generations; lane 11, *cdc13-4*, 125 generations.

As expected, telomeric GT repeat tracts shortened dramatically in an *est2*Δ mutant within 50 generations (Fig. 4, lanes 2 and 3). Survivor formation became obvious by the appearance of randomly sized telomeric fragments after 75 generations and the significant amplification of Y´ elements after 100 and 125 generations (Fig. 4, lanes 4 to 6). In contrast, the rate of GT repeat shortening was clearly reduced in a *cdc13-4* mutant (Fig. 4, lanes 7 to 11) compared to *est2*Δ cells and telomeres did not reach the critical length where Y´ element amplification starts in *est2*Δ strains. We observed no increase in Y´ element signals in BMY59-6B cells after growth for 125 generations (Fig. 4, lane 11) or 250 generations (compare Fig. 2, lane 5). Furthermore, a *cdc13-4*/*rad52* double mutant, BMY18, displayed no growth reduction after several hundred generations (data not shown). Telomeres were as short as observed for the single *cdc13-4* mutant and stayed stable at this short level (data not shown).

To investigate whether the rate of telomere shortening is increased in a *cdc13-4*/*est2* double mutant, we compared the growth behavior of an *est2*Δ spore and a *cdc13-4*/*est2*Δ spore from a tetrad of strain BMY58. The double mutant displayed significant growth reduction after 50 generations and survivor formation after 75 generations comparable to the *est2*Δ single mutant (Fig. 3). In addition, telomere shortening was not accelerated and Y´ element amplification occurred in both strains after 75 generations (data not shown).

Cdc13p and Tel1p function in different pathways of telomere length maintenance. The synthetic lethality of the *cdc13-4* mutation with a *yku70* or *yku80* deletion indicates that Cdc13p and the Yku heterodimer have independent but in some way overlapping functions at the telomere. Along with the Yku heterodimer and Cdc13p, a pathway comprised of Tel1p and the Mre11p-Xrs2p-Rad50p complex is involved in telomere length maintenance (47). To investigate if *CDC13* is epistatic to *TEL1* we generated the diploid strain BMY57, heterozygous for *tel1*Δ and *cdc13-4*. Telomeres of BMY57 cells were shorter than the diploid wild-type (Fig. 5, compare lanes 1 and 2), indicating that reduced protein levels in the heterozygous strain already influence telomere length maintenance. For further analysis we used all four spores derived from a tetratype tetrad.

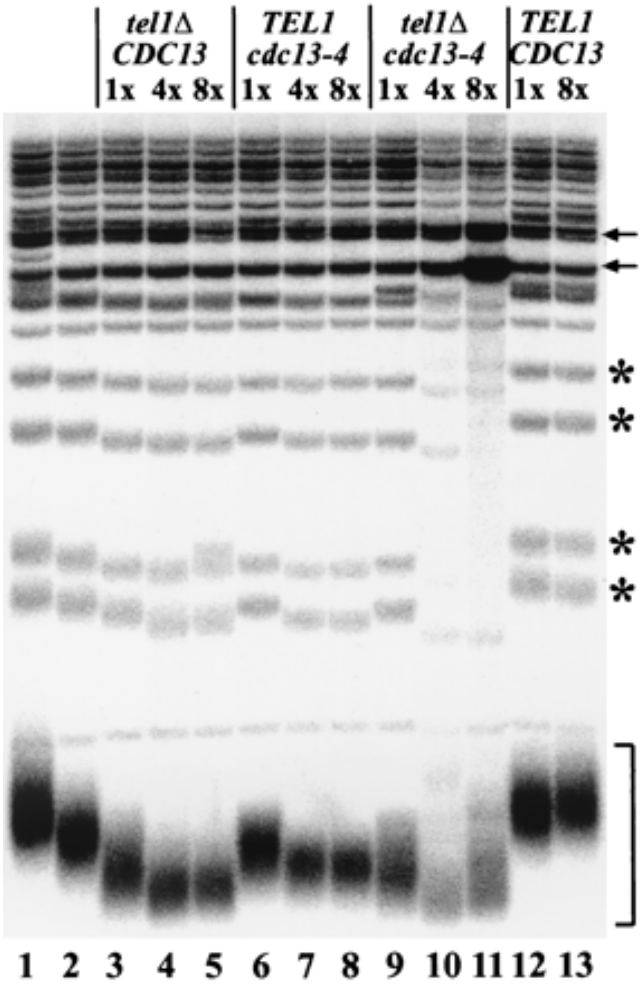


FIG. 5 [III]. Telomere length of *cdc13-4*, *tel1*Δ, and *cdc13-4/tel1*Δ mutants. Southern blot of genomic yeast DNA, probed with a telomere specific poly(GT)$_{20}$ oligonucleotide, is shown. Spores from a tetratype tetrad of BMY57 were propagated for 225 generations as described in Materials and Methods. The bracket indicates the telomeric GT repeat band derived from Y′ element-containing chromosomes. Asterisks indicate terminal fragments derived from non-Y′ element-containing chromosomes. The arrows indicate restriction fragments corresponding to the subtelomeric Y′ elements. Lane1, W303 aα wild-type; lane 2, W303 aα *cdc13-4*/CDC13 *tel1*Δ/TEL1 (BMY57); lane 3, W303α *tel1*Δ, 50 generations; lane 4, W303α *tel1*Δ, 125 generations; lane 5, W303α *tel1*Δ, 225 generations; lane 6, W303a *cdc13-4*, 50 generations; lane 7, W303a *cdc13-4*, 125 generations; lane 8, W303a *cdc13-4*, 225 generations; lane 9, W303α *tel1*Δ*/cdc13-4*, 50 generations; lane 10, W303α *tel1*Δ*/cdc13-4*, 125 generations; lane 11, W303α *tel1*Δ*/cdc13-4*, 225 generations; lane 12, W303a wild-type, 50 generations; lane 13, W303a wild-type, 225 generations.

As shown in Fig. 5, the rate of GT repeat loss was accelerated in *tel1*Δ cells (Fig. 5, lane 3) compared to that in *cdc13-4* mutant cells (Fig. 5, lane 6). At the steady-state level, telomeres of *tel1*Δ cells were significantly shorter than those of *cdc13-4* mutant cells (Fig. 5, lanes 4, 5, 7, and 8). The rate of telomere shortening in the *cdc13-4*/*tel1*Δ double mutant strain (Fig. 5,

lane 9) seemed to be not accelerated compared to that in *tel1*Δ(Fig. 5, lane 3), but the telomeres of the double mutant were shorter than the telomeres of either single mutant after 125 generations (Fig. 5, lanes 4, 7, and 10). After 225 generations we observed a dramatic increase in Y′ element signals in the *cdc13-4*/*tel1*Δ double mutant (Fig. 5, lane 11), indicating that telomeres were stabilized by Y′ element amplification. Although the growth of *cdc13-4*/*tel1*Δ mutants seemed to be reduced after 100 generations, cells did not cease growth completely and no fast-growing survivors occurred. Instead, colonies of the double mutant formed during a further 100 generations of growth were significantly smaller than either single mutant or wild-type (data not shown).

Cdc13-4p is not altered in its binding to Est1p. Expression of a Cdc13-Est1 fusion protein complements a *cdc13* or *est1* deletion and, moreover, results in a dramatic increase in telomere length (12). These data suggest that the telomere-bound Cdc13p recruits telomerase via interaction with Est1p to the ssDNA overhang at chromosome ends. To examine if a reduced association with Est1 causes the telomere shortening phenotype of a *cdc13-4* mutant, we analyzed the effect of expressing a Cdc13-4-Est1 fusion on telomere length. Therefore a Cdc13-Est1 or Cdc13-4-Est1 fusion protein was expressed under the control of the CDC13 promoter from a single-copy plasmid in wild-type, *cdc13-4* and *cdc13*Δ cells.

Expression of either fusion protein resulted in significant telomere elongation in wild-type and *cdc13-4* strains (Fig. 6A). We observed no differences in telomere elongation between mutant Cdc13-4-Est1p- and wild-type Cdc13-Est1p-expressing cells (Fig. 6A, lanes 2, 3, 5, and 6), indicating that both proteins bind with comparable affinities to chromosome ends. The effects of the fusion proteins on telomere length were not as pronounced as expected, and although the expression of the Cdc13-Est1 fusion protein in *cdc13-4* cells leads to GT tract elongation, the telomeres of these cells did not reach wild-type level. These results point toward a competition between the fusion protein and cellularly expressed Cdc13p alleles. When the influence of the fusion proteins on telomere maintenance was examined in a *cdc13*Δ strain, dramatically elongated telomeric GT repeat tracts were observed after 100 generations. However, the Cdc13-4-Est1 fusion protein (Fig. 6B, lane 5) did not induce telomere elongation to the same extend as a Cdc13-Est1 fusion (Fig. 6B, lane 4).

These data suggest that the Cdc13-4-Est1 fusion is capable of binding the telomeric ends and provokes deregulated telomere elongation. Hence, since the Cdc13-4-Est1 fusion does not lead to telomere elongation as observed for Cdc13-Est1p, the establishment of a permanent interaction between Cdc13-4p and Est1p, thereby tethering telomerase to the telomere, seems not sufficient to complement the *cdc13-4* mutation.

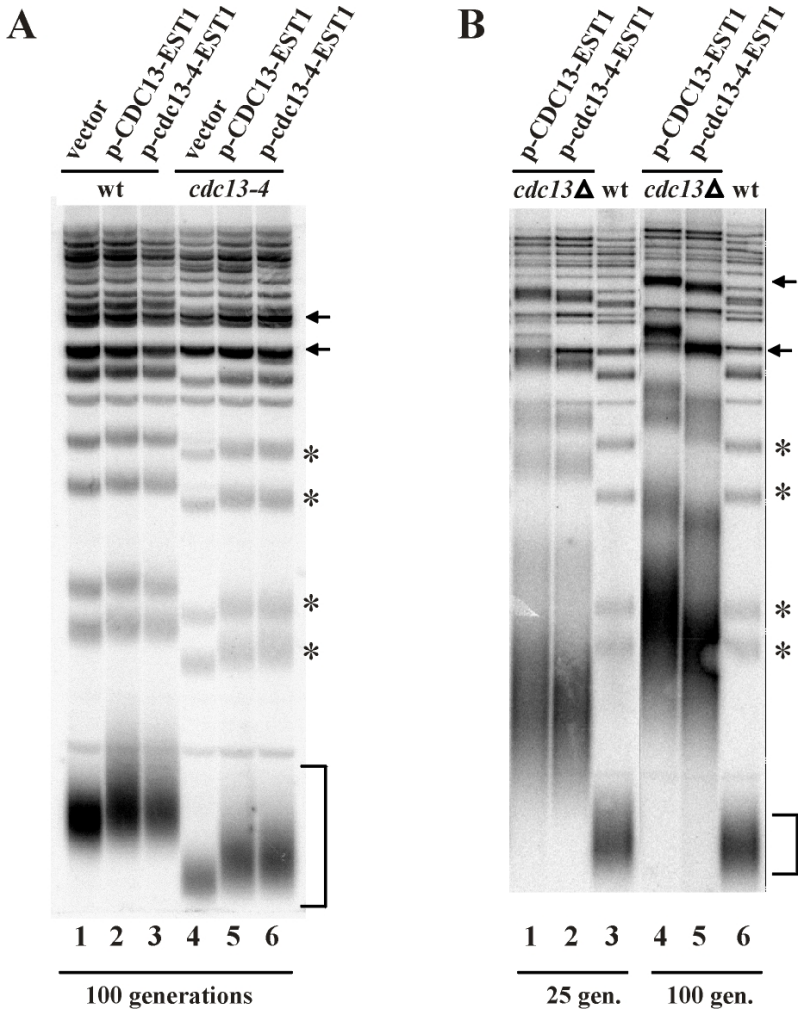


FIG. 6 [III]. Influence of Cdc13-Est1 and Cdc13-4-Est1 fusion proteins on telomere length. (A) Southern blot of W303a wild-type (wt) and W303a *cdc13-4* strains transformed with plasmids pRS316, p-CDC13-EST1 and p-cdc13-4-EST1 probed with a poly$(GT)_{20}$ oligonucleotide. Transformants were cultured for 100 generations on selective media prior to DNA preparation. Lane 1, W303a wt + pRS316 control; lane 2, W303a wt + p-CDC13-EST1; lane 3, W303a wt + p-cdc13-4-EST1; lane 4, W303a *cdc13-4* + pRS316 control; lane 5, W303a *cdc13-4* + p-CDC13-EST1 and lane 6, W303a *cdc13-4* + p-cdc13-4-EST1. **(B)** Southern blot of W303a *cdc13*Δ strains transformed with plasmids p-CDC13-EST1 and p-cdc13-4-EST1 probed with a poly$(GT)_{20}$ oligonucleotide. Transformants were cultured for 25 or 100 generations prior to DNA preparation.

To verify that the Cdc13p-Est1p interaction is not altered in a *cdc13-4* mutant, we performed coimmunoprecipitation experiments. Recently it was reported (45) that a Cdc13-Est1 interaction can be detected biochemically if both protein are overexpressed. Therefore, we generated strains expressing the chromosomal copy of *CDC13* or *cdc13-4* as an N-terminal HA_3-tagged protein and Est1p as a GST fusion protein under control of the inducible, strong *GAL1* promoter. Anti-GST monoclonal antibodies were used to precipitate GST-Est1 fusion proteins, and precipitates were analyzed by Western blotting for HA_3-Cdc13p or HA_3-Cdc13-4p.

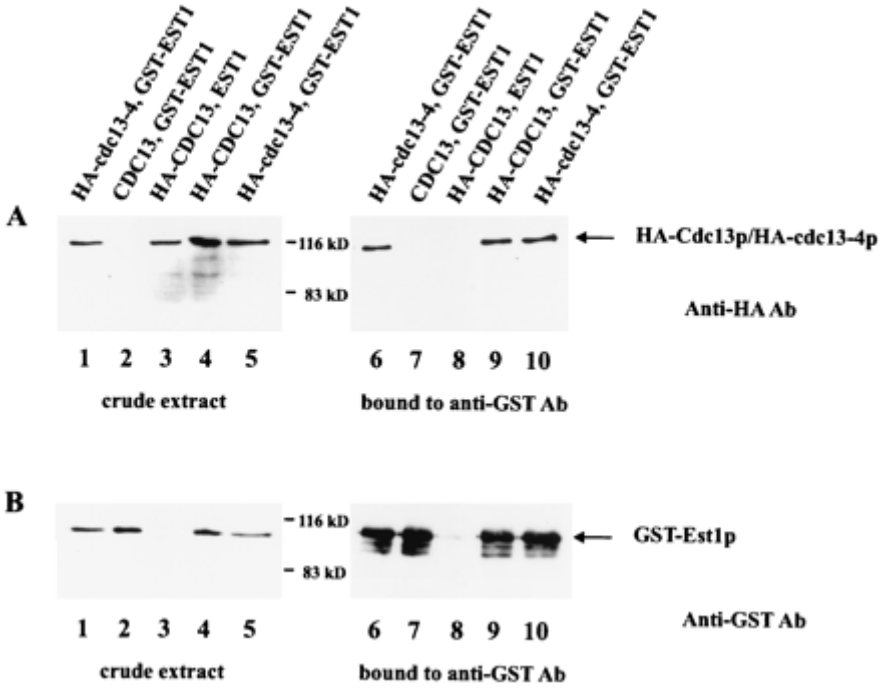


FIG. 7 [III]. Coimmunoprecipitation of HA-*cdc13-4* with GST-Est1. Ten-microgram samples of crude extracts were loaded to compare the protein amounts of different mutants used for coimmunoprecipitation experiments (lanes 1 to 5). Coimmunoprecipitation of Cdc13p and Est1p was performed as described in Materials and Methods (lanes 6 to 10). Crude extract (1,000 μg) was incubated with 5 μg of anti-GST antibody, and G-Sepharose beads were used to isolate antibody and bound proteins. After intensive washing, G-Sepharose beads were heated to 95°C in Laemmli buffer and the supernatant was loaded onto a 8% sodium dodecyl sulfate gel. (A) Lane 1, HFY86-3A (HA-cdc13-4p, GST-Est1p); lane 2, HFY81-8A (Cdc13p, GST-Est1p); lane 3, HFY82-6B (HA-Cdc13p, Est1p); lane 4, HFY82-4C (HA-Cdc13p, GST-Est1p); lane 5, HFY86-9D (HA-cdc13-4p, GST-Est1p); lane 6, HFY86-3A (HA-cdc13-4, GST-EST1); lane 7, HFY81-8A (Cdc13p, GST-Est1p); lane 8, HFY82-6B (HA-Cdc13p, Est1p); lane 9, HFY82-4C (HA-Cdc13p, GST-Est1p); lane 10, HFY86-9D (HA-cdc13-4p, GST-Est1p). HA-Cdc13p and HA-Cdc13-4p were detected by anti-HA antibody. (B) The same blot as in panel A probed with an anti-GST antibody. Note that lanes 6 to 10 were exposed a significantly shorter time to detect the GST-Est1p signals than lanes 1 to 5 and blots probed with anti-HA antibody in panel A. Ab, antibody.

Although only a small portion of the GST-Est1 fusion protein interacts with Cdc13p, no differences in the amount of coimmunoprecipitated HA_3-Cdc13p (Fig. 7, lane 9) or HA_3-Cdc13-4p (Fig. 7, lanes 6 and 10) protein were detectable. We did not observe cross-reaction of HA_3-Cdc13p with the anti-GST antibody (Fig. 7, lane 8), and no signal was detectable when GST-Est1p was immunoprecipitated from extracts containing wild-type Cdc13 without the HA tag (Fig. 7, lane 7). From these data we conclude that the Cdc13-4 mutant protein is not altered in its ability to interact with Est1p.

***cdc13-4* mutation seems not to affect DNA binding.** Very recently, different mutant alleles of *CDC13* that cause stably shortened telomeres comparable to the *cdc13-4* mutation have been described. These mutant Cdc13 proteins seem to display significantly reduced binding activity to telomeric DNA (18). Although expression of Cdc13-Est1p in *cdc13-4* cells indicates that Cdc13-4p and Cdc13p compete for telomere binding, we wanted to determine if the DNA binding activity of Cdc13-4p is reduced compared to that of wild-type Cdc13p. Assuming that overexpression of Cdc13-4p should complement a reduced DNA binding activity, we analyzed telomere length in the diploid strain HFY82 expressing one wild-type copy of *CDC13* and one copy of HA_3-*cdc13-4* under control of the inducible *GAL1* promoter.

After growth on glucose-containing media, the telomere length of HFY82 cells was comparable to that of wild-type (Fig. 8, lanes 1 and 4), indicating that one wild-type copy of *CDC13* was sufficient for telomere stability. Strikingly, after growth under inducing conditions on galactose for approximately 50 generations, telomeres were significantly shorter than those of the wild-type (Fig. 8, lane 5). Telomere shortening is already obvious in the heterozygous strain BMY56, where Cdc13p and Cdc13-4p were expressed from the native *CDC13* promoter (Fig. 8, lane 3), even though GT repeat tract loss was not as pronounced as seen in a haploid *cdc13-4* mutant (Fig. 8, lane 2). In addition, cooverexpression of HA_3-Cdc13-4p and GST-Est1p could not restore wild-type telomere length, but it did induce telomere shortening (Fig. 8, lane 7). Therefore, Cdc13-4p is at least in part dominant on Cdc13p and might compete with Cdc13p for telomere binding. These data indicate that neither DNA binding activity nor interaction with Est1 is reduced in a Cdc13-4 mutant protein.

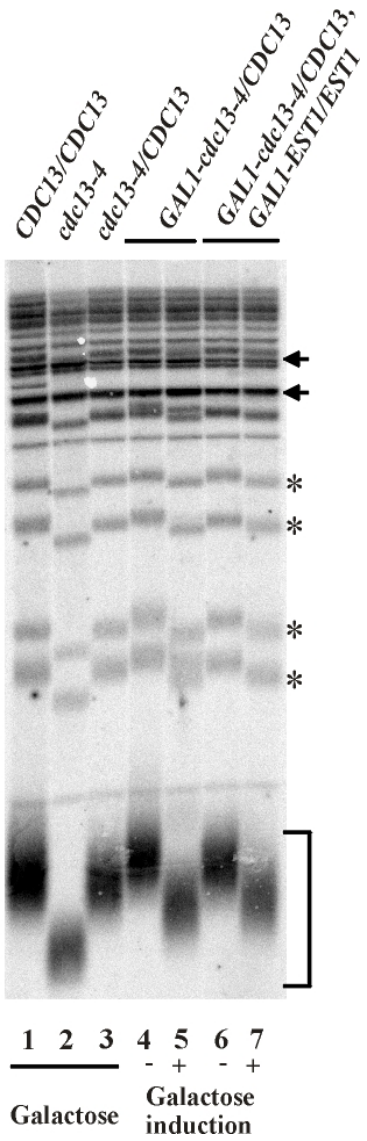


FIG. 8 [III]. Overexpression of *cdc13-4* in a heterozygous mutant strain. Diploid strains W303aα, BMY56 (*cdc13-4*/CDC13), and BMY17 (*cdc13-4*) were grown on galactose-containing media, whereas HFY84 (*GAL1-cdc13-4*/CDC13) and HFY86 (*GAL1-cdc13-4*/CDC13 *GAL1*-EST1/EST1) were simultaneously grown under inducing and noninducing conditions. Telomere length was investigated after 50 generations. Lane 1, W303 aα; lane 2, BMY17 (*cdc13-4*); lane 3, BMY56 (*cdc13-4*/CDC13); lane 4, HFY84 (*GAL1-cdc13-4*/CDC13) grown on glucose; lane 5, HFY84 (*GAL1-cdc13-4*/CDC13) grown on galactose; lane 6, HFY86 (*GAL1-cdc13-4*/CDC13 *GAL1*-EST1/EST1) grown on glucose; lane 7, HFY86 (*GAL1-cdc13-4*/CDC13 *GAL1*-EST1/EST1) grown on galactose. Brackets indicate terminal GT repeats, asterisks non-Y′ elements, and the arrows represent Y′ elements bands.

It has been proposed that Cdc13p protects chromosome ends from degradation by binding to the single-stranded 3′ GT overhang. At the restrictive temperature, *cdc13-1*^{ts} cells exhibit an increased amount of ssDNA in telomeric and subtelomeric regions (16).

To investigate whether a *cdc13-4* mutant displays an accumulation of ssDNA at the telomeres, we performed nondenaturing in-gel hybridization using a synthetic oligonucleotide specific for telomeric GT repeats. As a control we used *yku80* mutant cells that have been shown to contain a long ss overhang of the G-rich strand throughout the cell cycle (19).

In contrast to *yku80* mutants, the ssDNA signal of the *cdc13-4* mutant remained at a wild-type level after growth for 40 (Fig. 9, lanes 2 and 4) and 260 (Fig. 9, lanes 3 and 5) generations. Therefore, chromosome ends still seem to be protected from nucleolytic degradation by the Cdc13-4 mutant protein.

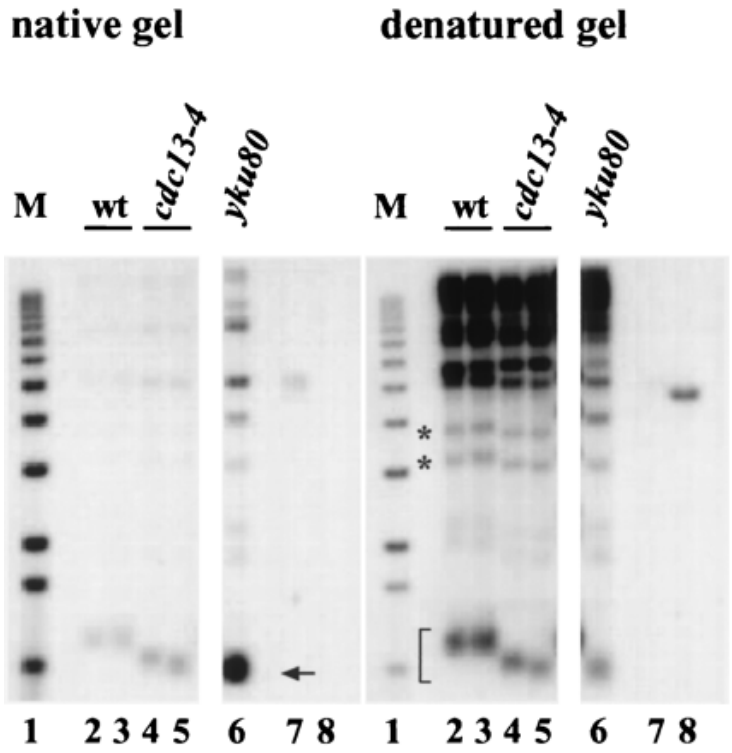


FIG. 9 [III]. Telomeric end structure in *cdc13-4* mutant cells. W303 wild-type (wt) and W303 *cdc13-4* spores derived from one tetrad were streaked on YPD for 250 generations and used for genomic DNA preparation as described under Materials and Methods. **(Left panel)** After *Xho*I digestion, genomic DNA was separated by gel electrophoresis and analyzed by nondenaturing in-gel hybridization using a 22-mer $C_{1\text{-}3}A$ oligonucleotide as a probe. The arrow indicates terminal restriction fragments derived from Y′ element-containing chromosomes. The strong signal in lane 6 corresponds to the elongated ss DNA overhang in *yku80*-deficient cells. Lane 1, 1-kbp ladder DNA; lane 2, W303 wt, 40 generations; lane 3, W303 wt, 260 generations; lane 4, W303 *cdc13-4*, 40 generations; lane 5, W303 *cdc13-4*, 260 generations, lane 6, *yku80* mutant; lane 7, control ssDNA; lane 8, control double-stranded DNA. **(Right panel)** The same gel as in the left panel, after denaturation of the DNA in the gel and rehybridization to the same probe. The bracket indicates the telomeric GT repeat band derived from Y′ element-containing chromosomes. Asterisks indicate terminal fragments derived from non-Y′ element-containing chromosomes.

DISCUSSION

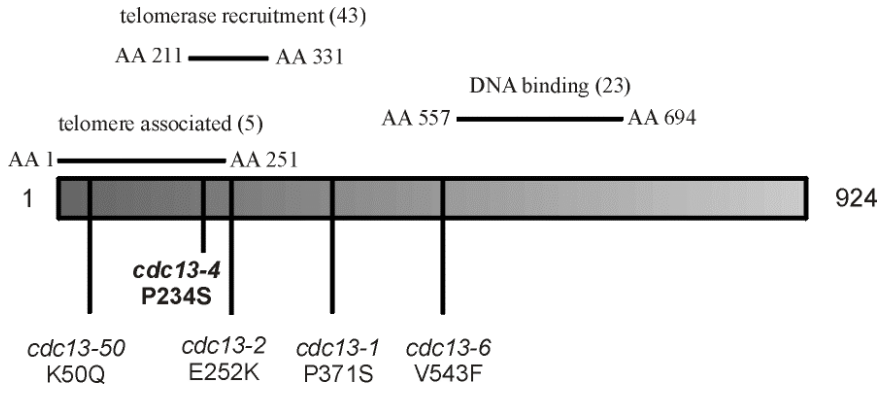
S. cerevisiae CDC13 is an essential gene involved in chromosome end replication and protection. The *cdc13-4* allele, which we isolated in a synthetic lethality screen with *YKU70,* causes a dramatic shortening of GT repeats at the telomeres but the strain remains viable. Telomere shortening proceeds slowly over approximately 150 generations; however, telomere length is stabilized at a short level after 200 generations (Fig. 2). This telomere phenotype is distinct from the senescence phenotype of a *cdc13-2*^{est} allele, which leads to progressive telomere shortening and eventually cell death (40). In a senescent yeast culture a few cells occasionally escape from cell death. These survivors stabilize their telomeres by either adding tandem copies of the subtelomeric Y´ elements or $C_{1-3}A/TG_{1-3}$ repeats in a *RAD52*-dependent recombination process (53). *cdc13-4* mutants do not display Y´ element amplification in a Southern blot as observed in survivors of telomerase-negative yeast strains (Fig. 2 and 4). In addition, a *cdc13-4*/*rad52* double mutant strain is viable for more than 250 generations while maintaining short telomeres (data not shown). Therefore, *cdc13-4* mutants do not show characteristics of a senescent mutant and telomeres do not reach the critical length level which triggers telomere stabilization by homologous recombination. Compared to an *est2*Δ mutant, the rate of telomere shortening in a *cdc13-4* mutant is clearly reduced (Fig. 4), indicating that telomerase activity is altered but not abolished. The stabilization of telomere length at a shorter level shows that telomerase is fully active at the new equilibrium length.

Mutations in *TEL1* and *TEL2* have been reported to cause a progressive telomere shortening phenotype comparable to *cdc13-4*. Telomeres in *tel1-1* and *tel2-1* mutants shorten to a stable level within 150 generations (35, 48), and a *tel1-1*/*tel2-1* double mutant has no telomeres shorter than those of *tel1-1* cells. This suggests that Tel1p and Tel2p function in the same pathway of telomere maintenance (25, 35). In contrast, a *cdc13-4*/*tel1*Δ double mutant displays slightly shorter GT repeat tracts after 125 generations compared to those of a *tel1*Δ or a *cdc13-4* single mutant. In addition, Y´ elements are amplified in the double mutant after 225 generations (Fig. 5), indicating that telomeres have shortened to a critical level. These data point toward a function of *CDC13* in telomere maintenance independent of the *TEL1* pathway.

The *cdc13-4* mutation is synthetically lethal with *yku70* or *yku80* (Fig. 1). This might be explained by a reduced telomere capping ability of the Cdc13-4 protein, which becomes essential at the elongated ssDNA overhang in Yku$^-$ mutants (19). This would then lead to the degradation of chromosome ends and cell cycle arrest. However, our data do not support such a model. Formation of microcolonies from double mutant spores (Fig. 1) makes it more likely that accelerated senescence is the reason for the synthetic lethality. Telomeres in Yku$^-$ mutants are shortened severely, and any further GT repeat tract shortening by the *cdc13-4* mutation would result in reaching a lethal level within a few generations.

Cdc13p has been proposed to control the susceptibility of chromosome ends to the specific degradation of the telomeric $C_{1\text{-}3}A$ strand at the end of S phase (57), and therefore a reduced DNA binding activity of Cdc13-4p could possibly cause a progressive telomere shortening as seen in *cdc13-4* cells. The *cdc13-4* mutation at position 235 is not located in the DNA binding domain of Cdc13p (23) (Fig. 10), although this does not exclude a conformational change in the Cdc13-4 mutant protein resulting in reduced DNA binding activity. Cdc13p protects chromosome ends from degradation and thereby prevents the generation of telomeric ssDNA (16, 40). Therefore we would expect at least a slight increase in ssDNA at the telomeres in *cdc13-4* cells, if Cdc13-4p is reduced in DNA binding. However, native in-gel hybridization experiments revealed no increase in ssDNA formation in *cdc13-4* mutants (Fig. 9).

The expression of a Cdc13-Est1 or Cdc13-Est2 fusion protein in *cdc13*Δ strains has been shown to complement for telomerase deficiency and additionally results in strongly elongated telomeres. Therefore, the expression of a mutant Cdc13-4-Est1 fusion protein should exhibit telomere elongation comparable to that of a wild-type Cdc13-Est1 fusion protein, if DNA binding of Cdc13-4p is not reduced. In fact, telomere elongation was detected in strains expressing the mutant Cdc13-4-Est1 or the wild-type Cdc13-Est1 fusion protein (Fig. 6). Furthermore, GT repeat tract length in *cdc13-4* cells expressing the wild-type Cdc13-Est1 fusion protein, although significantly elongated, did not reach wild-type level after 100 generations (Fig. 6B), suggesting that endogenous Cdc13-4p can compete with the Cdc13-Est1 fusion protein for telomere binding thereby partially preventing telomere elongation.



	mutation	phenotype	reference
cdc13-1	P371S	temperature-sensitive	(40)
cdc13-2	E252K	progressive telomere shortening, senescence	(40)
cdc13-3	n.d.	telomere elongation	(41)
cdc13-6	V543F	temperature-sensitive	(23)
cdc13-50	K50Q	telomere elongation	(45)
cdc13-69	n.d.	stably shortened telomeres	(18)
cdc13-109	n.d.	telomere elongation	(18)
cdc13-4	P234S	stably shortened telomeres	this study

FIG. 10 [III]. Functional domains and selected mutations of *CDC13* mutants. AA, amino acids; n.d. not determined.

Additional evidence that DNA binding is unchanged is provided by the finding that heterozygous *CDC13/cdc13-4* diploid yeast strains show reduced telomere length (Fig. 8), indicating an at least partially dominant phenotype of the *cdc13-4* mutation. Telomere shortening in the heterozygous *CDC13/cdc13-4* diploid strains is not caused by a reduced amount of functional Cdc13p since a *CDC13/GAL1*-HA_3-*cdc13-4* strain exhibit wild-type telomere length (Fig. 8) on glucose where expression of HA_3-*cdc13-4* by the *GAL1* promoter is repressed. The dominant phenotype of Cdc13-4p is even more pronounced if overexpression of HA_3-*cdc13-4* is induced in the heterozygous diploid (Fig. 8). This again indicates that Cdc13-4p competes with wild-type Cdc13p for telomere binding. Therefore, we present evidence that the mutant Cdc13-4 protein is capable of chromosome end binding with an affinity comparable to that of the wild-type Cdc13p. An alternative explanation for the partially dominant phenotype of the *cdc13-4* mutation would be a competition of wild type

Cdc13p and mutant Cdc13-4p for a protein important for telomere elongation. Further experiments have to be performed to address this question.

The recruitment of telomerase to chromosome ends seems to take place via the interaction of Cdc13p and Est1p. Therefore, an attenuated interaction of Cdc13-4p and Est1p could cause telomere shortening to a stable level. The telomerase recruitment site of Cdc13p was recently mapped to amino acids 211 to 331 (43). The *cdc13-4* mutation (P235S) is located near the border of this domain; thus, the interaction of the mutant Cdc13-4 protein and Est1p might be reduced. Nevertheless, we found no reduced interaction of Cdc13-4p-Est1p in coimmunoprecipitation experiments (Fig. 7). In addition, overexpression of Cdc13-4p or cooverexpression of Cdc13-4p and Est1p induces telomere shortening in a heterozygous diploid strain (Fig. 8) and did not complement the *cdc13-4* mutation as we would suggest for a weakened interaction.

Significantly elongated telomeres, most likely the result of unregulated access of the active telomerase complex to the telomere, are detectable in yeast strain expressing a Cdc13-Est1 fusion protein (12). Although the Cdc13-4-Est1 fusion protein causes a dramatic telomere elongation in a *cdc13*Δ strain, the effect is not as pronounced as that observed for a wild-type Cdc13-Est1 fusion. Therefore, establishing a permanent interaction of Cdc13-4p and Est1p is not sufficient to complement the *cdc13-4* mutation to wild-type level, indicating that a function independent of interaction with Est1p is affected in Cdc13-4p. The DNA binding domain of Cdc13p has been mapped to an internal part of the protein (23); nevertheless, the N-terminal 251 amino acids of Cdc13p associate *in vivo* with the telomere (5), indicating tight interaction with telomere bound proteins. This N-terminal domain partially overlaps the telomerase recruiting domain of Cdc13p (43) (Fig. 10), but seems not to be sufficient for Cdc13p-Est1p interaction. Thus, the *cdc13-4* mutation might influence interaction with other proteins at the telomere, thereby preventing appropriate activation of telomerase activity.

In *S. cerevisiae,* telomere length seems to be maintained by the balance of two antagonistic processes - telomere elongation and telomere shortening. Many proteins are necessary to maintain normal telomere length. Deletion of one Yku subunit (3, 44) or inactivation of a member of the *TEL1* pathway, comprised of Tel1p, Mre11p, Xrs2p, and Rad50p, leads to telomere shortening to a stable level (2, 20).

The additional telomere shortening seen in *yku70/tel1* or *yku70/rad50* double mutants indicates that the Yku heterodimer has a *TEL1*-independent role in telomere maintenance (47). In *cdc13-4* mutant cells, telomerase seems to be inactive at normal telomere length, indicating that Cdc13p is involved in positive telomere length regulation by activating telomerase at short GT repeat levels. The further telomere shortening seen in *cdc13-4*/*tel1*Δ double mutants and the synthetic lethality of *cdc13-4* with a Yku subunit deletion lead to the conclusion that at least three independent pathways are involved in positive telomere length regulation and that Ccd13p is an essential part of one of these pathways.

However, the addition of telomeric GT repeats to telomeric ends depends not only on telomerase but also on DNA polymerases Pol α, Pol δ, and DNA primase, most likely by a coordinated regulation of C- and G-strand synthesis (10). Recently, it has been shown that Cdc13p interacts with Pol1p, the catalytic subunit of DNA polymerase α. Single point mutations in either *CDC13* or *POL1* that weaken the interaction of Cdc13p with Pol1p result in telomerase-dependent telomere lengthening (45). Therefore Cdc13p also seems to play an important role in negative telomere length control, presumably by coordinating telomeric C- and G-strand synthesis.

Until now three different functions of Cdc13p in telomere maintenance have been defined by *CDC13* mutations (Fig. 10): i) protection of chromosome ends from nucleolytic degradation (abolished in a *cdc13-1*^{ts} mutant at the restrictive temperature), ii) loading of telomerase onto the ssDNA overhang at the telomere (prevented in *cdc13-2*^{est} cells), and iii) regulation of telomere length. The role of Cdc13p in telomere length control seems to be multifaceted, since mutating *CDC13* can cause either telomere lengthening, seen in *cdc13-50* mutants (45) and different mutant *CDC13* alleles (18), or telomere shortening to a new steady-state level, seen in newly identified *CDC13* mutants (18) and the *cdc13-4* mutant reported here. Our data present evidence that Cdc13p plays a key role not only in recruiting telomerase but also in modulating its access to the telomere, which might be influenced by additional regulatory proteins.

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Chapter IV

Co-expression of a and α Mating Type Induces Resistance to DNA Damage in Haploid *Saccharomyces cerevisiae yku* Mutants

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submitted.

ABSTRACT

The Sir2-4 complex of *Saccharomyces cerevisiae* is required for telomere maintenance and silencing at telomeres and at *HMLα* and *HMR*a. The Yku heterodimer influences telomere length regulation and is essential for DNA repair via nonhomologous end-joining. Recently, *sir4Δ* mutants have been described to display sensitivity to MMS and bleomycin, indicating a role of Sir4p in DNA repair. To further investigate Sir4p function, we analyzed *sir4Δ* and *yku/sir4Δ* double mutants for their capacity to repair DNA damage.

***Sir4Δ* mutants display hardly any sensitivity to bleomycin or MMS, suggesting that Sir4p is not required for DNA repair processes. Surprisingly, *yku/sir4Δ* mutants are significantly more resistant to bleomycin than *yku* mutants. Deletion of *HMLα* in *yku/sir4Δ* mutants reconstitutes bleomycin sensitivity, indicating that the simultaneous expression of *HML*α and *HMR*a causes resistance. Accordingly, episomal expression of *HMLα* in haploid Mata *yku70* mutants leads to resistance to bleomycin comparable to *yku70/sir4Δ* mutants.**

Telomeres of *yku/sir4Δ* mutants are slightly elongated as compared to *yku* mutants and exhibit Y´-element amplification. Deletion of *HMLα* in Mata *yku70/sir4Δ* strain suppresses Y´-element amplification and telomeres become as short as in *yku* mutants, while episomal expression of *HMLα* results in slightly amplified Y´-elements in *yku70* single mutants.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae* two mechanistically related types of silencing have been described: the stable repression of the inactive mating type loci *HML*α and *HMR***a** and the repression of telomere adjacent genes, known as telomere positioning effect (TPE) (LUSTIG 1998). Four *SIR* genes (*SIR1-4*) have been identified as essential components for transcriptional repression of *HML*α and *HMR***a** (RINE AND HERSKOWITZ 1987), however *SIR1* is dispensable for telomeric silencing while *SIR2-4* are prerequisite for TPE (APARICIO *et al*. 1991). Two-hybrid analysis revealed that Sir3p and Sir4p associate with the sequence specific DNA-binding protein Rap1 (MORETTI *et al*. 1994), which is supposed to tether the Sir complex to HM silencers and the telomeric regions. Furthermore, the Sir2-4 complex has been shown to co-localize with Rap1p in distinct staining foci at the nuclear periphery, which coincides with telomeric repeat sequences (PALLADINO *et al*. 1993). Telomeric repeats are shortened in *sir3* and *sir4* mutant strains, and the mitotic stability of chromosome V is reduced (PALLADINO *et al*. 1993), indicating that the Sir complex is not only required for telomeric silencing but also important for chromosome integrity.

A function in DNA repair has been proposed for the Sir proteins based on the finding that *sir4* mutant cells display significantly reduced end-joining efficiency (TSUKAMOTO *et al*. 1997). In addition, Sir4p has been shown to interact with Yku70p, the 70 kDa subunit of the Yku heterodimer, by two hybrid criteria (TSUKAMOTO *et al*. 1997). The Yku heterodimer of *S. cerevisiae* is an essential component of the nonhomologous end-joining (NHEJ) DNA repair pathway (BOULTON AND JACKSON 1996a; BOULTON AND JACKSON 1996b; MILNE *et al*. 1996). *Yku* mutants are deficient in plasmid end-joining and exhibit sensitivity to the DNA damaging agents bleomycin and methyl methanesulfonate (MMS) (FELDMANN *et al*. 1996; MAGES *et al*. 1996; MILNE *et al*. 1996). Furthermore, the Yku heterodimer localizes to the telomere (GRAVEL *et al*. 1998) and is involved in maintaining wild-type telomere length (BOULTON AND JACKSON 1996a; PORTER *et al*. 1996). Recently, it has been shown that Yku80p, Sir4p, Sir3p, and Rap1p are released from telomeric foci in response to bleomycin, MMS, or HO-endonuclease induced DNA damage (MARTIN *et al*. 1999; MCAINSH *et al*. 1999; MILLS *et al*. 1999).

The delocalization of the Sir proteins from telomeres in response to DNA damage as well as the reduced end-joining capacity of *sir* mutants (TSUKAMOTO *et al*. 1997) argues in favor of a direct role of the Sir complex in DNA repair. However, recent studies reveal that a deletion of the de-repressed *HML*α locus in Mat**a** *sir* mutants suppresses the defect in plasmid end-joining (ASTROM *et al*. 1999; LEE *et al*. 1999) and that the Sir proteins have only minors effect on DNA repair (LEE *et al*. 1999).

Here we analyzed the role of the Sir4 protein in the repair of chemical induced DNA damage. In our studies *sir4Δ* and *sir4Δ/hmlΔ* mutants exhibit hardly any sensitivity to bleomycin or MMS, suggesting that Sir4p is not required for the repair of chemical induced DNA double strand breaks (DSBs). Strikingly, *yku/sir4Δ* double mutants are more resistant to bleomycin and MMS than *yku* single mutants. Similar results were observed for *sir3Δ* mutants; single mutants displayed no sensitivity to bleomycin and MMS and the *yku/sir3Δ* double mutant strain exhibited increased resistance to chemical induced DNA damage. In this study we present evidence that the pseudo-diploid state resulting from the loss of silencing at *HML*α and *HMR***a** causes resistance to DNA damage in haploid *yku* mutants. Accordingly, episomal expression of *HML*α in a Mat**a** *yku70* mutant results in significantly enhanced resistance to bleomycin or MMS.

Telomere repeat tracts of *yku/sir4Δ* mutants are slightly longer than telomeres of *yku* single mutants and in addition, Y´-elements are significantly amplified. This seems at least partially be caused by co-expression of both mating type information's in *yku/sir4Δ* strains, since deletion of *HML*α in a Mat**a** *yku70/sir4Δ* strain prevent Y´-element amplification and elongation of the terminal telomere GT repeats.

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used in this study are listed in Table 1. The *yku70* (BMY8) and *yku80* (SPY25) deficient strains were generated as described previously (FELDMANN *et al*. 1996; FELDMANN AND WINNACKER 1993). The *sir3*Δ (BMY44), *sir4*Δ (BMY39), and *hml*Δ (BMY49) strains were constructed using PCR-derived HisMX or kanMX6 modules flanked by short terminal sequences (80 bp) homologous to the ends of the corresponding open reading frame (WACH *et al*. 1994). To generate strains *sir4*Δ/*hml*Δ (BMY51), *yku70/sir4Δ* (BMY40), and *yku80/sir4Δ* (BMY41) the *sir4*Δ deletion construct described above was transformed

into BMY49, BMY8, and SPY25, respectively. The *hml*Δ PCR product was integrated into BMY8 (*yku70*) to generate BMY50 (*yku70/hml*Δ). Transformation of BMY50 (*yku70/hml*Δ) with the *sir4*Δ construct resulted in BMY54 (*yku70/sir4*Δ/*hml*Δ). To generate strains *yku70/sir3Δ* (BMY45) and *yku80/sir3Δ* (BMY46) the *sir3*Δ deletion construct described above was transformed into BMY8 and SPY25, respectively. The correct targeting of the deletion constructs was confirmed by analytical PCR (WACH *et al*. 1994) and sequencing of the PCR products. The plasmid pRS314-C.a.URA3 used for plasmid rejoining experiments was generated as follows: The *Candida albicans URA3* gene was isolated from plasmid Clp10 (MURAD *et al*. 2000) by digestion with *Spe*I, treatment with T4 polymerase followed by *Not*I digestion. The 1.3 kb DNA fragment containing the C.a. *URA3* gene was ligated to the plasmid pRS314 (SIKORSKI AND HIETER 1989) digested with *Sal*I, treated with T4 polymerase and cut with *Not*I. Plasmid pSH1127 used for vector based expression of *HML*α was generously provided by James Haber and is described elsewhere (SUGAWARA *et al*. 1995). Plasmid YCp50 was used as a vector control (ROSE *et al*. 1987).

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source or Reference
HKY 579-10A	MAT**a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 RAD5*	(FAN *et al*. 1996)
BMY8	HKY579-10A *yku70::LEU2*	This study
SPY25	HKY579-10A *yku80::kanMX6*	This study
BMY39	HKY579-10A *sir4Δ::HisMX*	This study
BMY40	HKY579-10A *yku70::LEU2 sir4Δ::HisMX*	This study
BMY41	HKY579-10A *yku80::kanMX6 sir4Δ::HisMX*	This study
BMY44	HKY579-10A *sir3Δ::HisMX*	This study
BMY45	HKY579-10A *yku70::LEU2 sir3Δ::HisMX*	This study
BMY46	HKY579-10A *yku80::kanMX6 sir3Δ::HisMX*	This study
BMY49	HKY579-10A *hmlΔ::kanMX6*	This study
BMY50	HKY579-10A *yku70::LEU2 hmlΔ::kanMX6*	This study
BMY51	HKY579-10A *hmlΔ::kanMX6 sir4Δ::HisMX*	This study
BMY54	HKY579-10A *yku70::LEU2 hmlΔ::kanMX6 sir4Δ::HisMX*	This study

Drop titer test: A single yeast colony grown for 3-4 days on solid media was suspended in 500 µl of dH_2O. This cell suspension was diluted five times by 10-fold serial dilutions. 10 µl aliquots of each dilution were dropped on YPD and YPD plates containing increasing concentrations of bleomycin or MMS. The plates were incubated at 30°C for 3-6 days.

Quantification of bleomycin sensitivity: Cultures were grown in YPD or the appropriate SD medium at 30°C over night and diluted to an OD_{600} of 0.2 - 0.3. Cells were grown to mid log phase (OD_{600} = 1-1.5). Individual samples were diluted in water and different cell concentrations were plated in duplicates on solid YPD and solid YPD containing increasing amounts of bleomycin. Plates were incubated for 4-6 days at 30°C. Data from three independent experiments are given.

Yeast DNA extraction and analysis of telomeric DNA: Genomic DNA was isolated from 5-7 ml over night cultures using nucleon™ MiY DNA extraction kit (Amersham Life Science). For analysis of telomere length genomic DNA was digested over night using *Xho*I and separated on a 1% agarose gel in 1 x TAE buffer. DNA was transferred to nylon membranes (HybondN$^+$) by vacuum blotting using 0.4 N NaOH. Telomeric DNA fragments were detected as described elsewhere (BOULTON AND JACKSON 1998).

End-joining assay: Plasmid pRS314-C.a.URA3 was digested with *Eco*RI to completion, separated on a 0.8% agarose gel and purified using QIAquick gel extraction kit (Qiagen). End-joining assays were performed as described elsewhere (BOULTON AND JACKSON 1996b). Cells were plated onto SD medium lacking Uracil for selection of accurately repaired plasmids. The average from three independent experiments is given.

RESULTS

The Yku heterodimer, comprised of Yku70p and Yku80p, is an essential component for the repair of DNA double-strand breaks by NHEJ. Yeast cells deleted for either Yku subunit are impaired in rejoining of linear plasmids bearing overhanging complementary ends (BOULTON AND JACKSON 1996a; BOULTON AND JACKSON 1996b). In addition, *yku* mutant yeast cells exhibit sensitivity to the DNA damaging agents bleomycin and MMS (FELDMANN *et al*. 1996; MAGES *et al*. 1996). The Yku70 subunit has been shown to interact with Sir4p, a protein of the yeast silencing complex, by two hybrid criteria and *sir4* strains have been reported to display a reduced end-joining efficiency (TSUKAMOTO *et al*. 1997). However, recent observation indicate that the dramatic decrease in plasmid re-joining efficiency in *sir4* mutants is caused by a secondary effect namely the de-repression of the silent mating type loci (ASTROM *et al*. 1999; LEE *et al*. 1999). To further analyze the function of Sir4p in the repair of chemical induced DNA damage, we examined *sir4*Δ and *yku/sir4Δ* mutant strains for their sensitivity to bleomycin and MMS by drop titer tests.

The *sir4Δ* mutants tested exhibited no sensitivity to bleomycin as compared to wild-type (Figure 1, lanes 1 and 2). Furthermore we did not observe an increase in sensitivity to MMS. Strikingly, the deletion of *SIR4* in *yku70* or *yku80* mutants resulted in decreased sensitivity to bleomycin and MMS as compared to *yku70* and *yku80* single mutants (Figure 1, lanes 4-7) - the *yku70/sir4Δ* and *yku80/sir4Δ* double mutant strains were nearly as resistant to bleomycin and MMS as wild-type cells.

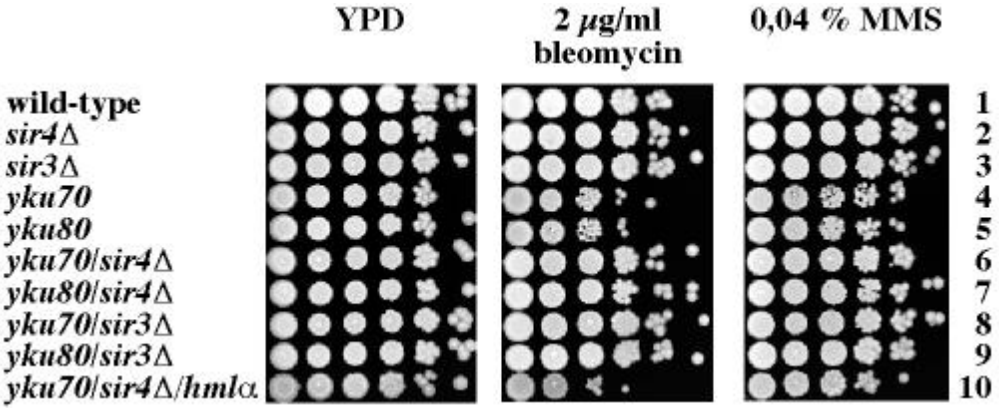

FIG. 1 [IV].- Drop titer assay examining bleomycin and MMS sensitivity. Serial 10-fold dilutions of various strains were grown on YPD plates containing the indicated amount of bleomycin or MMS. All strains were derived from the parental strain HKY 579-10A by gene disruption. The following strains were used: Lane 1: HKY 579-10A (wild-type), lane 2: BMY39 (*sir4Δ*), lane 3: BMY44 (*sir3Δ*), lane 4: BMY8 (*yku70*), lane 5: SPY25 (*yku80*), lane 6: BMY40 (*yku70/sir4Δ*), lane 7: BMY41 (*yku80/sir4Δ*), lane 8: BMY45 (*yku70/sir3Δ*), lane 9: BMY46 (*yku80/sir3Δ*) lane 10: BMY54 (*yku70/sir4Δ/hmlα*).

Similar results were obtained when we analyzed *sir3Δ*, *yku70/sir3Δ*, and *yku80/sir3Δ* strains: While *sir3Δ* single mutant cells displayed no sensitivity to MMS or bleomycin (Figure 1, lane 3), the *yku70/sir3Δ* and *yku80/sir3Δ* double mutants were significantly less sensitive compared to the *yku* single mutants (Figure 1, lanes 8 and 9). These data indicate that the Sir4 protein is not essential for the repair of bleomycin or MMS induced DNA damage. A deletion of either *SIR4* or *SIR3* rather seems to be capable of complementing the repair deficiency in *yku* mutant cells. To verify that these results were not caused by a growth advantage of *yku/sir* double mutants we performed quantitative assays, where we measured the percentage of survival of the *sir4Δ* single and double mutant strains grown on solid media containing one or two µg/ml bleomycin (Figure 2).

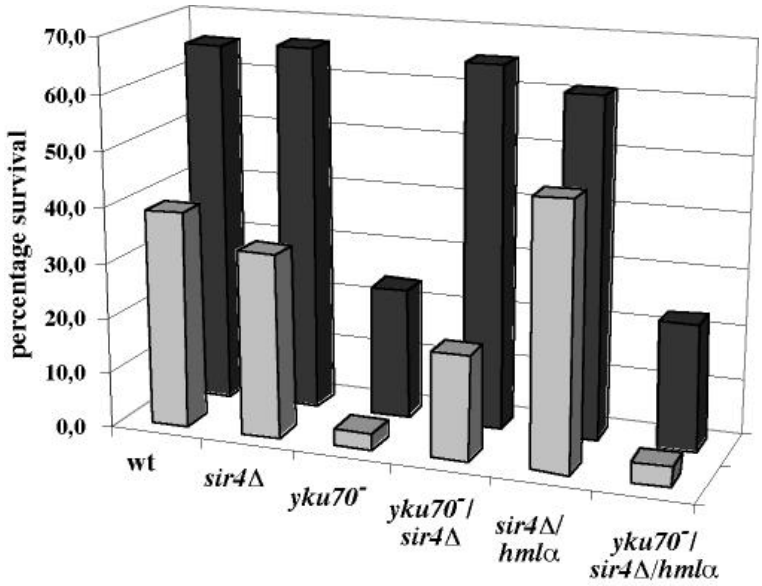

FIG. 2 [IV]. - Survival of *sir4Δ* single and double mutant strains grown on bleomycin. Various dilutions of cells grown to mid log phase in liquid YPD were plated in duplicate on solid YPD and YPD containing one or two µg/ml bleomycin (see Materials and Methods). The row of dark bars indicate the percentage survival for the given strain on 1 µg/ml bleomycin, whereas the light bars indicate the percentage survival on 2 µg/ml bleomycin. The following strains were used: HKY 579-10A (wild-type), BMY39 (*sir4Δ*), BMY8 (*yku70*), BMY40 (*yku70/sir4Δ*), BMY51 (*sir4Δ/hmlα*), BMY54 (*yku70/sir4Δ/hmlα*); The average from three independent experiments is given.

Sir4Δ cells were as resistant as wild-type cells to one µg/ml bleomycin and only a very slight decrease in survival rates could be observed for *sir4Δ* cells grown on two µg/ml bleomycin (Figure 2). In contrast, *yku70* mutant cells displayed significant reduction of survival when grown on one or two µg/ml bleomycin compared to the wild-type. As already observed by drop titer tests, the resistance of *yku70/sir4Δ* double mutants to bleomycin was dramatically increased. No growth reduction was measurable for *yku70/sir4Δ* cells as compared to wild-type on one µg/ml bleomycin and survival rates were greatly enhanced compared to *yku70* single mutant cells on plates containing two µg/ml bleomycin (Figure 2).

These results are in contrast to recently published data (MARTIN *et al*. 1999) where *sir4* mutants have been shown to lead to MMS sensitivity and a hypersensitive phenotype was observed for *yku/sir4* double mutants compared to either single mutant. However, we obtained similar results for *sir4Δ* mutants generated in a W303 *rad5-585* background (data not shown).

In *sir* mutants silencing of the generally repressed mating type cassettes *HML*α and *HMR***a** is abolished (RINE AND HERSKOWITZ 1987), and the resulting co-expression of both mating types induces a reduction in plasmid end-joining efficiency (ASTROM *et al*. 1999; LEE *et al*. 1999). This pseudo-diploid state of *sir* mutants might not only lead to reduced NHEJ but also to an activation of the homologous recombination pathway. Since we observed bleomycin and MMS resistance analyzing a deletion of two different *SIR* genes in a *yku* mutant background, we investigated the influence of the pseudo-diploid state on the resistance to DNA damage in *sir4Δ* and *yku70*/*sir4Δ* mutants. Hence, we deleted the *HML*α locus in Mat**a** *sir4Δ* and *yku70*/*sir4Δ* mutants. End-joining experiments verified that the *SIR4* deletion represses NHEJ efficiency in our genetic background by de-repressing the silent mating type loci. Error-free plasmid end-joining was reduced to 2% of the wild-type level in *sir4Δ* mutants, but was restored to ~ 88% in a *sir4Δ*/*hmlΔ* mutant showing that Sir4p is dispensable for NHEJ as described previously (LEE *et al*. 1999) (Figure 3). However, the repression of the *HLM*α and *HMR***a** loci by the Sir complex is essential to maintain the plasmid end-joining capacity of haploid yeast cells.

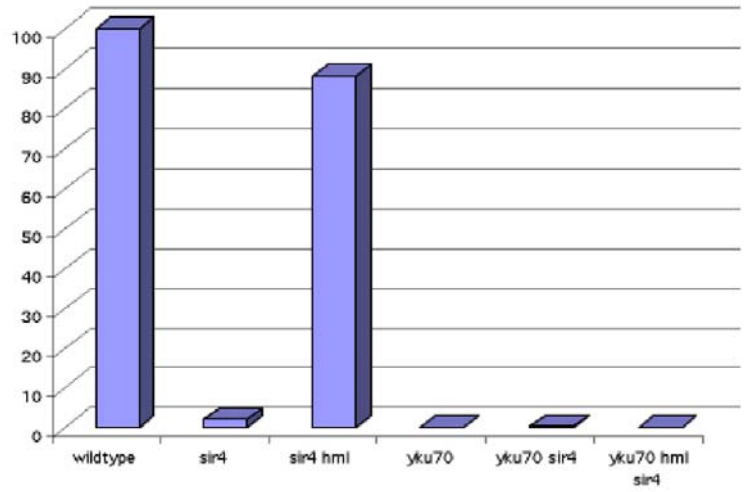

FIG. 3 [IV]. –End-joining efficiency of *sir4Δ* mutant strains. The plasmid pRS314-C.a.URA3 was digested with *Eco*RI, cutting within the coding region of the *Candida albicans URA3* gene. Various strains were transformed with equal amounts of the linearized plasmid or supercoiled plasmid as a control. Cells were plated on uracil lacking SD plates. Only cells able to accurately re-join the linear plasmid were able to form colonies on SD-Ura$^-$ media. The following strains were used: HKY 579-10A (wild-type), BMY39 (*sir4Δ*), BMY51 (*sir4Δ/hmlαΔ*), BMY8 (*yku70*), BMY40 (*yku70/sir4Δ*), BMY54 (*yku70/sir4Δ/hmlα*); The average from three independent experiments is given.

Analyzing chemical induced DNA damage, we found resistance of Mat**a** *sir4Δ* cells deleted for *HMLα* to bleomycin and MMS as seen for wild-type and sir4*Δ* cells (Figure 2 and data not shown). However, when *HMLα* was deleted in a Mat**a** *yku70*/*sir4*Δ mutant, this strain became sensitive to bleomycin and MMS comparable to a *yku70* mutant (Figure 1, lane 10 and Figure 2). These data indicate that the simultaneous expression of **a** and α mating types is responsible for the resistance of a *yku70*/*sir4Δ* double mutant strain to DNA damaging agents. Furthermore we can conclude that the resistance of *sir4* mutants to MMS and bleomycin, we and others (BENNETT *et al*. 2001) have observed is not due to an enhanced resistance, which might be caused by the pseudo-diploid stage of the cell as it can been seen in the *yku/sir* mutant.

To determine whether the resistance to bleomycin only occurs in a *yku70*/*sir4Δ* double mutant or can also be detected in pseudo-diploid *yku70* single mutants, we expressed *HMLα* from a CEN/ARS plasmid in haploid Mat**a** wild-type and *yku70* strains and quantified bleomycin sensitivity (Figure 4).

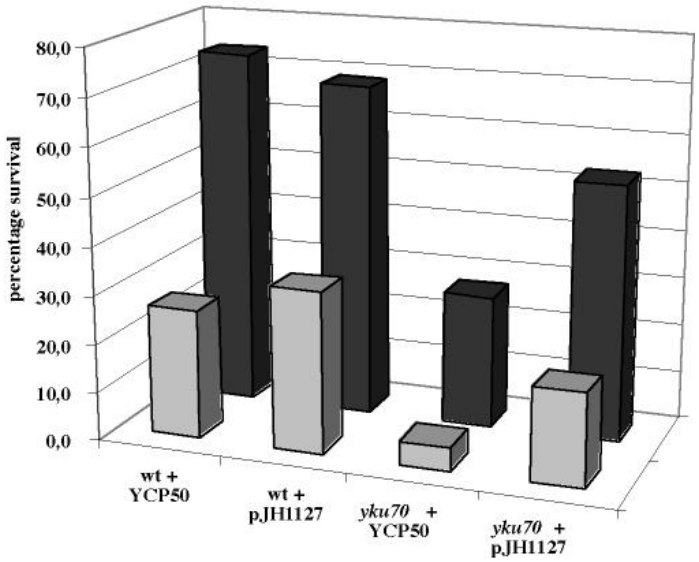

FIG. 4 [IV]. -Bleomycin sensitivity of haploid Mata wild-type and *yku70* mutants in comparison to pseudo-diploid wild-type and *yku70* mutant cells. Strains HKY 579-10A (wild-type) and BMY8 (*yku70*) were transformed with the *HMLα* expression plasmid pJH1127 or plasmid YCp50 as a control. Various dilutions of cells were plated in duplicate on solid YPD and YPD containing one or two µg/ml bleomycin (see Materials and Methods). The row of dark bars indicate the percentage survival for the given strain on 1 µg/ml bleomycin, whereas the light bars indicate the percentage survival on 2 µg/ml bleomycin. The average from three independent experiments is given.

HMLα expression did not alter the resistance of wild-type Mat**a** cells. However, Mat**a** *yku70* mutants gained significant resistance to bleomycin, when expressing *HMLα* from the plasmid (Figure 4), providing additional evidence that the resistance of a Mat**a** *yku70*/*sir4Δ* mutant to bleomycin is primarily induced by the de-repression of the silent mating type cassettes and does not require the loss of the Sir4 protein.

The Yku heterodimer and the Sir4 protein have been shown to contribute to telomere length maintenance (BOULTON AND JACKSON 1996a; PALLADINO *et al*. 1993; PORTER *et al*. 1996). To investigate the impact of a deletion of both activities at the telomere, we analyzed telomere length in *sir4Δ* and *yku/sir4Δ* strains. GT-repeats of *sir4Δ* mutants were only slightly shortened as compared to those of wild-type cells (Figure 5A, lanes 1 and 2), whereas *yku70* and *yku80* mutants exhibited a significant telomere shortening (Figure 5A, lanes 3 and 5). Strikingly, telomeres of *yku70/sir4Δ* and *yku80/sir4Δ* mutants displayed no further GT-repeat tract shortening but telomeres were slightly elongated as compared to *yku70* or *yku80* mutants (Figure 5A, lanes 3-6). In addition, we observed an enhanced Y'-element signal, indicating that recombination events occur at telomeres in these double mutants.

Since simultaneous expression of *HMLα* and *HMR***a** significantly decreased the bleomycin sensitivity in *yku/sir4Δ* mutants, we also investigated the influence of a *HMLα* deletion on telomere length. As expected, we observed no changes in telomere length when *HMLα* was deleted in a Mat**a** wild-type (Figure 5B, lanes 1 and 2) or *yku70* mutant strain (Figure 5B, lanes 5 and 6) in which *HML* and *HMR* remain repressed (GRAVEL *et al*. 1998). Furthermore, telomere length was not altered in a *sir4Δ/hml* mutant as compared to a *sir4Δ* mutant strain (Figure 5B, lanes 3 and 4). The deletion of *HMLα* in *yku70/sir4Δ* mutants had no detectable influence on cell viability at 30ºC (Figure 1, lane 10). However, telomeric GT-repeats were as short as those of *yku70* single mutants and no Y'-element amplification was detectable (Figure 5B, lane 8).

From these data we suggest that homologous recombination takes place to a higher extent at the telomeres of a *yku70/sir4Δ* strain. Activation of homologous recombination seems not to be induced by an additive telomere shortening that could be caused by the simultaneous loss of Ykup and Sir4p at the telomere, but is most likely due to the synchronic expression of both mating types. Telomeres of *yku/sir4Δ/hmlα* cells displayed no additional shortening of telomere length, indicating that the Yku heterodimer and the Sir4 protein are epistatic for telomere maintenance.

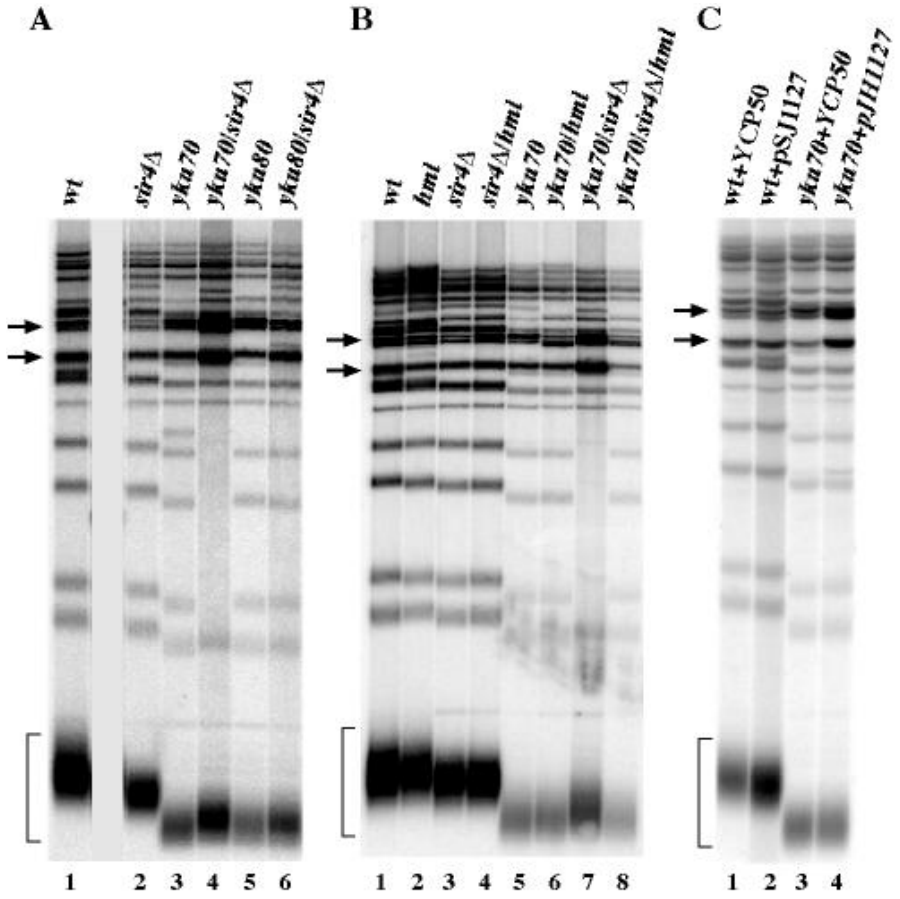

FIG. 5 [IV]. - Telomere length analysis of *sir4Δ, yku* single and *sir4Δ /yku* double mutant strains. Southern blot of *Xho*I digested genomic yeast DNA probed with a poly(GT)$_{20}$ oligonucleotide specific for telomeric repeats. The brackets indicate the telomeric GT-repeat band derived from Y´-element containing chromosomes. The arrows indicate restriction fragments corresponding to the subtelomeric Y´-elements. Genomic DNA was prepared as described in 'Materials and Methods'. (A) Lane 1: HKY 579-10A (wild-type), lane 2: BMY39 (*sir4Δ*), lane 3: BMY8 (*yku70*), lane 4: BMY40 (*yku70/sir4Δ*), lane 5: SPY25 (*yku80*), lane 6: BMY41 (*yku80/sir4Δ*). (B) Lane 1: HKY 579-10A (wild-type), lane 2: BMY49 (*hmlαΔ*), lane 3: BMY39 (*sir4Δ*), lane 4: BMY51 (*sir4Δ/hmlαΔ*), lane 5: BMY8 (*yku70*), lane 6: BMY50 (*yku70/hmlαΔ*), lane 7: BMY40 (*yku70/sir4Δ*), lane 8: BMY54 (*yku70/sir4Δ/hmlα*); (C) HKY 579-10A (wild-type) and BMY8 (*yku70*) cells were transformed with the *HMLα* expression plasmid pJH1127 and YCp50 as a control. Cells were grown at least 100 generations before preparation of genomic DNA. Lane 1: HKY 579-10A + YCp50, lane 2: HKY 579-10A + pJH1127, lane 3: HKY8 + YCp50, lane 4: BMY8 + pJH1127.

To further investigate the influence of pseudo-diploidy on telomere end structure we analyzed telomere length in Mat**a** *yku70* single mutants episomally expressing *HMLα* (Figure 5C). We detected no changes in GT-repeat tract length in wild-type or *yku70* mutant cells expressing *HMLα* or a control plasmid (Figure 5C, lanes1-4). Nevertheless, Y´-element signals were slightly increased in *yku70* mutants transformed with the *HMLα* expression plasmid (Figure 5C, lane 4). From these data we conclude that although the pseudo-diploid state seems to be the reason for elevated recombination at the telomeres in *yku70* mutants, the Sir4p at least partially protects chromosomes ends from recombination.

DISCUSSION

The Sir2-4 proteins of *Saccharomyces cerevisiae* have been described to be involved in the Yku heterodimer dependent nonhomologous end-joining pathway (TSUKAMOTO *et al*. 1997). Further investigations have provided evidence that the dramatic reduction of end-joining efficiency in *sir2-4* mutants is caused by a secondary effect, namely the co-expression of both *HMLα* and *HMR***a** resulting from the loss of silencing at the usually repressed mating type loci (LEE *et al*. 1999). However, several observations point toward a role of Sir proteins in double strand break repair. *sir3, sir3* and *sir4* mutants have been reported to be sensitive to the DNA damaging agent MMS and disruption of *sir4* has been shown to enhance sensitivity in a *yku70* mutant background (MARTIN *et al*. 1999). Moreover, Sir4p and Sir3p delocalize from telomeres (MARTIN *et al*. 1999; MCAINSH *et al*. 1999) and accumulate at sites of a DNA double stranded break (MARTIN *et al*. 1999; MILLS *et al*. 1999). The finding that a *yku70/sir4* double mutant displayed hypersensitivity to MMS and bleomycin compared to either single mutant and the observation that the Sir proteins are recruited to a DNA break with different kinetics than Yku (MARTIN *et al*. 1999), gave evidence for a Ykup independent role of Sir4p in DNA repair. We have investigated the interrelation between Sir4p and the Yku heterodimer on DNA repair and telomere length regulation. Therefore, we analyzed *sir4Δ* and *sir3Δ* mutant strains as well as *yku/sir4Δ* and *yku/sir3Δ* double mutant strains for their sensitivity to chemical induced DNA damage.

In contrast to recent data, *sir4Δ* and *sir3Δ* cells displayed hardly any sensitivity to the DNA damaging agents MMS and bleomycin. We observed no reduced survival of *sir4Δ* cells in drop titer tests (Figure 1) as well as in quantitative assays (Figure 2). Resistance of the *sir4Δ* and *sir3Δ* cells to MMS and bleomycin was not due to an active NHEJ pathway: Accurate recircularisation of a linear plasmid bearing cohesive ends was dramatically reduced in the *sir4Δ* strain (Figure 3), showing that the NHEJ pathway is inhibited. A deletion of *HMLα* in the Mat**a** *sir4Δ* strain restored end-joining capacity nearly to wild-type level (Figure 3) as described previously (LEE *et al*. 1999). This wild-type behavior of *sir4Δ* and *sir3Δ* strains to chemical induced DNA damage shows that the components of the Sir2-4 complex do not significantly influence DNA repair processes and indicates that solely preventing end-joining by co-expression of both mating types is not sufficient to cause sensitivity to chemical induced DNA damage. Strikingly, the *yku70/sir4Δ* and *yku80/sir4Δ* double mutant strains generated in W303-1A *rad5-535* (data not shown) or HKY579-10A *RAD5* (Figure 1) displayed no increased sensitivity to DNA damage. Moreover, both double mutant strains were significantly more resistant to MMS and bleomycin as compared to *yku70* and *yku80* single mutants (Figure 1 and Figure 2). Similar results were obtained when we deleted *SIR3* in *yku* deficient strains: *yku70/sir3Δ* and *yku80/sir3Δ* double mutants again were nearly as resistant as wild-type cells (Figure 1). Therefore, the independent deletion of two components of the yeast silencing complex significantly increased the repair capacity of *yku* deficient cells.

To distinguish between phenotypes that are induced either directly by the absence of the Sir4 protein or indirectly by the de-repression of the mating type loci, we deleted the *HMLα* locus in the haploid Mat**a** strains. When we analyzed a Mat**a** *yku70/sir4Δ/hmlΔ* strain this mutant exhibited MMS and bleomycin sensitivity comparable to a *yku70* single mutant (Figure 1 and Figure 2). From these results we suggest that the reduced sensitivity of *yku70/sir4Δ* mutants is caused by the pseudo-diploid state of theses cells and that Sir4p plays no detectable role in the repair of the DNA damage induced by MMS or bleomycin. These data are corroborated by the finding that an increased resistance can be induced in Mat**a** *yku70* single mutants by the expression of *HMLα* from a plasmid (Figure 4) thereby leading to a pseudo-diploid state in a *yku* mutant proficient for Sir4p.

The decrease in sensitivity to DNA damage seen in *yku70/sir4Δ* mutants and in Mat**a** *yku70* + *HMLα* mutants argues in favour of another repair pathway that becomes activated when both mating types are expressed in a haploid cell. The reduction in NHEJ observed in *sir4Δ* mutants might accompany the activation of this pathway. If this DNA repair mechanism that is induced by co-expression of *HMLα* and *HMR**a*** can compensate for the sensitivity in *yku70*, this effect might also be present in the *sir4Δ* mutant, leading to a wild-type sensitivity. However, Mat**a** *sir4Δ/hmlΔ* mutants exhibit a comparable resistance to MMS and bleomycin (Figure 2) as *sir4Δ* mutant and wild type cells, indicating that the absence of Sir4p *per se* does not induce MMS or bleomycin sensitivity. Therefore, our data provide no evidence for a detectable involvement of Sir4p in the repair of a DNA damage. However, our data do not exclude the possibility that Sir proteins that are released from telomeric sites help to modify the DNA double strand break to ensure a more rapid repair process.

In addition to the contribution of Sir4p to DNA repair processes we have addressed the impact of Sir4p on telomere length maintenance in *yku* deletion strains. *sir4Δ* single mutants exhibited only a slight and stable reduction in telomere length (Figure 5) as described before (PALLADINO *et al*. 1993). Strikingly, *yku70/sir4Δ* and *yku80/sir4Δ* double mutant strains exhibited no enhanced telomere shortening as compared to *yku70* or *yku80* mutants, but a contrary phenotype was observed: both *yku70/sir4Δ* and *yku80/sir4Δ* mutants displayed slightly elongated terminal telomere repeats and an enhanced Y'-element signal. Y'-element amplification has been shown to occur in colonies of telomerase negative strains that arise with a low frequency from a senescent culture (LUNDBLAD AND BLACKBURN 1993). This formation of survivors has been shown to depend on the homologous recombination pathway since a deletion of *RAD52* in telomerase negative strains inhibits survivor formation (LE *et al*. 1999). A comparable effect has been observed for *yku70* mutants where a few cells do survive a shift to the restrictive temperature of 37°C in a *RAD52* dependent manner (FELLERHOFF *et al*. 2000). These temperature-resistant strains also exhibit enhanced Y'-element signals (FELLERHOFF *et al*. 2000). Thus, as in telomerase mutants, the loss of telomere integrity in *yku70* mutants at 37°C induces recombinative events that lead to the stabilization of the chromosome ends (FELLERHOFF *et al*. 2000). However, no reduced survival is observed for *yku70/sir4Δ* and *yku80/sir4Δ* mutants, which would be indicative for a grave loss of telomere repeats.

Since the results we obtained by analyzing *yku/sir* double mutants for chemical induced DNA damage pointed toward an activation of a repair pathway in these double mutants, we tested the effect of the mating type co-expression caused by the *SIR4* deletion on telomeres.

When we deleted *SIR4* in a Mat**a** *yku70/hmlΔ* mutant we could not detect an increase in the Y'-element signal and the terminal telomere repeats were as short as in *yku70* and *yku70/hmlΔ* mutant strains (Figure 5). Moreover, we were able to induce a slight Y'-element amplification in a Mat**a** *yku* strain by the expression of *HMLα* from a plasmid (Figure 5). These results provide evidence that the Y'-element amplification detectable in *yku70/sir4Δ* strains is indeed caused by the co-expression of both mating types and is not a primary effect of the *SIR4* deletion. Furthermore, from the fact that *yku70*/*sir4Δ*/*hmlΔ* mutants have short telomeres comparable to those in *yku70* mutants (Figure 5), we conclude that Sir4p and Yku70p act in the same pathway of telomere length regulation.

Interestingly, the recombination events at the telomeres of *yku70*/*sir4Δ* mutants seem to occur although telomeres are not shortened to a critical level since we do not observe a reduced viability in the *yku70*/*sir4Δ*/*hmlΔ* strain. However, no recombinational effect can be detected in *sir4Δ* mutants or in Mat**a** wild-type strains expressing *HMLα* from the plasmid, both fulfilling the prerequisite of mating type co-expression. Thus, the recombination events at telomeres are not a general feature of the pseudo-diploid state, but seems to dependent on telomere length or structure. One reason that telomeres in *yku* mutants are targeted by recombination events might be the fact that an unusual end structure is present in these mutants throughout the cell-cycle namely a single-stranded overhang that is restricted to S-phase in wild-type strains (GRAVEL *et al*. 1998). This overhang might be sensed by the via pseudo-diploidy induced repair machinery, which then leads to Y'-element amplification. However, several findings support the possibility that only the length of the telomeres in pseudo-diploid *yku* mutants induce Y'-element amplification. *yku* mutants exhibit synthetic accelerated inviability in combination with an *est2* deletion, indicating that not much sequence loss can be tolerated in *yku70* mutants (NUGENT *et al*. 1998). Furthermore, recently published data have shown that short telomeres in the yeast *K. lactis* are highly recombinogenic. *Ter1* mutant cells with stable shortened telomeres display greatly enhanced subtelomeric recombination rates, whereas recombination at internal locations remains unaffected (MCEACHERN AND IYER 2001).

Based on these data it has been suggested that stable shortened telomeres have lost partially their capping function and might be therefore recognized occasionally by the recombination repair pathway (MCEACHERN AND IYER 2001). The appearance of large abnormal cells, comparable to senescent *ter1* cells, in some mutants with stable shortened telomeres rise further evidence that short telomeres might be partially uncapped (MCEACHERN AND IYER 2001). A detectable percentage of large abnormal cells can also be observed in *yku70* or *yku80* mutants (unpubl. observations B. MEIER AND H. M. FELDMANN). The mammalian Ku86 protein already has been implicated in telomere capping based on the finding that deletion of Ku86 in mouse cells leads to enhanced telomere fusion (HSU *et al*. 2000; SAMPER *et al*. 2000). Therefore, we suggest that the short telomeres in *yku* mutants are uncapped to an greater extent and together with an activated recombinational repair pathway in pseudo-diploid cells expressing both mating types this might result in an increase in recombinational events at the telomeres.

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Chapter V

Identification of Yku Interacting Proteins in a Two Hybrid Screen

Introduction

The ends of linear chromosomes of eukaryotic cells, the telomeres, are bound by large protein complexes. These proteins complexes ensure complete replication and protect the chromosome ends from exonucleolytic degradation, recombination and other potential errors, which could lead to a loss of DNA or to DNA rearrangements. Besides telomere specific proteins, however, several proteins implicated in the repair of double strand DNA breaks (DSBs) i.e. the Mre11p, Rad50p, Xrs2p complex and the Ku heterodimer, have been shown to localize to telomeric sites in yeast (Martin *et al*., 1999) and mammals (Hsu *et al*., 1999; Lombard and Guarente, 2000; Zhu *et al*., 2000). Mre11p, Rad50, Xrs2 and Ku are not only bound to telomeres but moreover contribute to the establishment of wild-type telomere length and structure (Boulton and Jackson, 1998; Nugent *et al*., 1998; Polotnianka *et al*., 1998; Porter *et al*., 1996).

Mouse fibroblast deficient for the 80 kD subunit of the Ku heterodimer accumulate a significant percentage of telomere fusions, indicating that Ku is important for the protection of chromosome ends in mammalian cells (Hsu *et al*., 2000; Samper *et al*., 2000). In the yeast *Saccharomyces cerevisiae* a deletion of either subunit of the Yku heterodimer leads to short but stable telomeres (Porter *et al*., 1996) with unusual long G-rich single strand extensions (Gravel *et al*., 1998). Furthermore *yku* mutants are impaired for growth at elevated temperatures presumably due to further telomere shortening (Boulton and Jackson, 1998; Fellerhoff *et al*., 2000). Thus, the Ku protein, which was initially identified as an essential factor required for the repair of double strand breaks lacking homologous regions also facilitates the protection of a special DNA end, the telomere, from its recognition as a double strand break. Recent results provide evidence, that in *Saccharomyces cerevisiae* Yku is released from telomeric sites in response to DNA damage and accumulates at the DNA break (Martin *et al*., 1999). However, the mechanism how Yku fulfils both its protection and repair function is poorly understood. To get insights how Yku might act at different sites, we focused on the identification of proteins that interact with the Yku heterodimer.

The yeast two hybrid system allows the detection of a protein-protein interaction by transcription activation of certain reporter genes. One important step to understand how transcription factors initiate transcription came from the observation that the yeast transcription factor Gal4 consist of two separable domains, a DNA binding domain and a transcription activation domain (Brent and Ptashne, 1985; Hope and Struhl, 1986; Keegan *et al*., 1986). Further studies revealed that the DNA binding domain needs not to be covalently linked to the activation domain to induce transcription activation (Ma and Ptashne, 1988). Moreover, a Gal4 activation domain was able to confer transcription activation when fused to the DNA binding domain of the bacterial repressor LexA, thereby activating genes that contain upstream LexA binding sites (Brent and Ptashne, 1985). These findings provided the basis for the establishment of a system that allows to study the interaction of two proteins, one fused to a DNA binding domain (BD) and one fused to a transcription activation domain (AD) (Fields and Song, 1989; Zervos *et al*., 1993) by a transcriptional read out. If protein-protein interaction occurs, the AD and the BD are brought in close proximity to each other, thereby leading to the assembly of an active transcription factor. The transcription of specific reporter genes is then used as a read out for the interaction of the two proteins (Fields and Song, 1989; Zervos *et al*., 1993). This system allows the study of protein interactions from any organism in the yeast *Saccharomyces cerevisiae*.

In the Interaction Trap System (Zervos *et al*., 1993) the bait vector, pEG202, carries a LexA binding domain (BD) followed by a polylinker sequences that allows the in frame cloning of a gene of interest. An *ADH1* promoter confers constitutive expression of a LexA-BD fusion protein from this plasmid. The prey vector, pJG4-5, contains a nuclear localization signal, a B42 transcription activation domain (AD), and a HA-tag followed by a polylinker for in frame cloning of a DNA library or a gene that should be tested for interaction with the bait. A *GAL1* promoter allows the expression of an AD-fusion protein from pJG4-5 on galactose containing plates but is inhibited if the yeast cells are grown on plates containing glucose as a carbon source. Two reporter genes, that contain upstream LexA binding sites, are used as a read out for protein interactions: the *LEU2* gene that is integrated into the genome of the yeast strain EGY48 and an episomally encoded *lacZ* gene. Thus, cells containing an activation domain-tagged protein that interacts with the LexA-fusion protein, form colonies on leucine deficient galactose plates and accumulate a blue colour on galactose plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-Gal) (Figure 1).

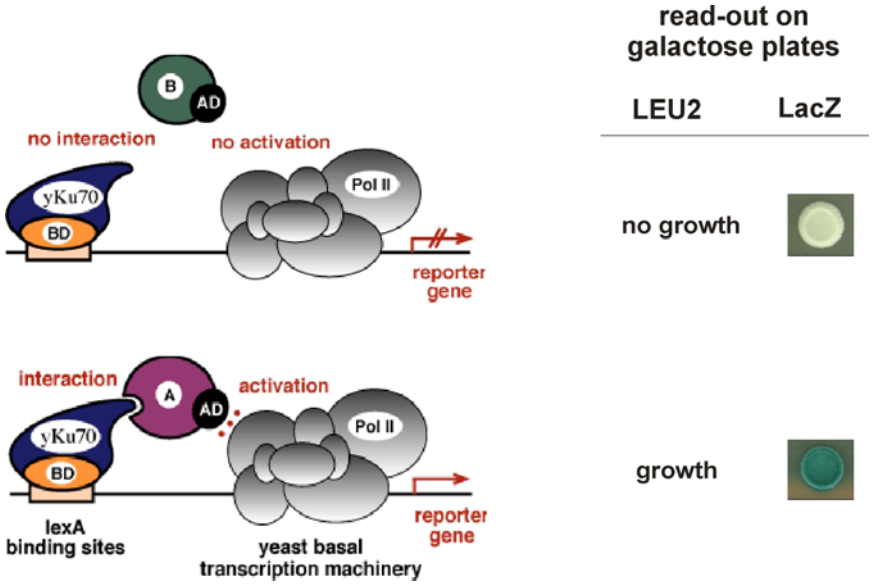

FIG. 1 [V]. Interaction Trap Screening for Yku70p interacting proteins. The Yku70 full-length protein fused to a LexA binding domain (BD) is used as a bait to screen for Yku70p interacting proteins. **(A)** If Yku70-BD is coexpressed with an unrelated protein fused to a transcription activation domain (AD) no transcription activation of the two reporter genes *LEU2* and *lacZ* can occur. Therefore yeast cells do not grow on SC-ura-his-trp-leu Gal and stay white on SC-ura-his-trp x-Gal Gal. **(B)** If a Yku70p interacting protein fused to the AD and LexA-Yku70p are present in the same cell, protein-protein interaction can be detected by the expression of the two different reporter genes. The expression of the *LEU2* gene mediates growth on SC-ura-his-trp-leu Gal media and the expression of the *lacZ* gene leads to the accumulation of a blue pigment in yeast colonies grown on X-Gal plates.

Materials and methods

S. cerevisiae strains, media, growth conditions and transformation

Yeast strains used in this study are listed in Table I. Yeast strains were grown at 30°C using YPD or selective media as described elsewhere (Feldmann *et al*., 1996). Yeast transformation was performed by the lithium acetate method (Schiestl and Gietz, 1989) and the high efficiency transformation of the yeast genomic library was done as described in http://www.umanitoba.ca/faculties/medicine/biochem/gietz/2HS.html.

Plasmid constructions

Two hybrid vectors pEG202, pJG4-5 and pSH18-34 were generously provided by Dr. R. Brent and colleagues. The *YKU70* gene was isolated from pGEM4ZHDF1 (Driller *et al*., 2000) with *Bam*HI and *Sal*I and was ligated to pEG202 cut with *Bam*HI/ *Xho*I, leading to pEG-YKU70. pEG-YKU70 was then cut *Eco*RI and *Sal*I, the Yku70 fragment was purified on an agarose gel and ligated to pJG4-5 linearized with *Eco*RI/*Xho*I to generate pJG-YKU70. The *YKU80* bait and prey plasmids were generated as described previously (Walter, 1997). The N-terminal *SIR4* fragment, AA 1- 397 was PCR amplified from pJGSir4 –40 bp to AA 397 using primers Sir4Nter for 5'-ATC GGA ATT CAT GCC AAA TGA CAA TAA GAC ACC C-3' and Sir4Nter rev 5'-ATC CCT CGA GTG TTT TCT TGG CCT TCA TAT TCA AC-3'. The PCR product was subcloned into pZERO^{TM}-2

(Invitrogen, San Diego USA) and re-isolated after digestion with *Eco*RI/ *Xho*I. This fragment was cloned in pJG4-5 *Eco*RI/ *Xho*I to generate pJG-SIR4 AA1-397. The C-terminal *SIR4* domain described in Tsukamoto *et al.* (1997) was amplified from genomic yeast DNA with primers Sir4CterAA1205 5'-ATC CGA ATT CGA TCG TCG AGT GAA ACA ACT CG-3' and Sir4Cter rev 5'-ATC CCT CGA GGT CAA TAC GGT TTT ATC TCC TTA TTC AC-3'. After cloning into pZERO™-2 the fragment was re-isolated by *Eco*RI/ *Xho*I digestion and ligated to pJG4-5 *Eco*RI/ *Xho*I. A ~ 2200 bp N-terminal *SIR4* fragment was PCR amplified with primers Sir4Nter for and Sir4P2 rev 5'-CCA ATA CAG GAT CAA ACC ATT TGC-3' and a ~ 1900 bp C- terminal fragment with primers Sir4P3 for 5'-CCA GAA AAT AAG ACT GAT AAG G-3' and Sir4P4 rev 5'-GCG GCC GCT CAA TAC GGT TTT ATC TCC-3' to generate pJG-SIR4 AA1-1358. PCR products were ligated to pZERO™-2. The N-terminal fragment was re-isolated by *Eco*RI/ *Bgl*II, the C-terminal part by *Bgl*II/ *Not*I digestion. Both fragments were then ligated to pEG202 cut with *Eco*RI/ *Not*I. pEG-SIR4 AA1-1358 was re-cut with *Eco*RI and *Xho*I, the entire *SIR4* reading frame was purified from an agarose gel and ligated to pJG4-5 digested with *Eco*RI/ *Xho*I. Plasmids were sequenced to ensure in-frame cloning.

Gene disruption

The *yku70* and *yku80* deficient strains were generated as described previously (Feldmann *et al*., 1996; Feldmann and Winnacker, 1993). The *sir2*Δ (BMY61), *sir3*Δ (BMY48), *sir4*Δ (BMY39) strains were constructed using a PCR-derived HisMX or kanMX6 module flanked by short terminal sequences (80bp) homologous to the ends of the corresponding reading frame (Wach *et al*., 1994). To introduce the *rap1-17* mutation into CEN.PK2, a genomic fragment of *rap1-17* was amplified from strain AJL278-4d (Kyrion *et al*., 1992) with primers rap1-17 for: 5'-TGC CGA AGA GCA TGC AGC AC-3' and rap1-17 rev: 5'-CCC TTA GGT ACA CTC CTA CG -3'. The PCR fragment was cloned into pBluescript (Stratagene) and the kanMX4 marker was inserted into the unique *Hin*dIII restriction site of the genomic fragment. The plasmid pBSrap1-17Kan was digested using *Pvu*II/ *Stu*I and the resulting rap1-17::kanMX fragment was used to transform CEN.PK2. The correct integration of deletion constructs was confirmed by analytical PCR (Wach *et al*., 1994) and sequencing of the PCR products.

Sensitivity assay

Yeast colonies were picked, resuspended in dH_2O and diluted five times by 10-fold serial dilutions. Aliquots (6 µl) of each dilution were spotted in duplicate on YPD plates and on YPD plates containing increasing amounts of methyl methanesulfonate (MMS). MMS plates were incubated at 30°C and YPD plates were incubated at 30°C or 37°C for 3-4 days.

Yeast DNA extraction and analysis of telomeric DNA

Genomic DNA was isolated from 5-7 ml overnight cultures using nucleon™ MiY DNA extraction kit (Amersham Life Science). For analysis of telomere length genomic DNA was digested overnight using *Xho*I and was separated on an 0,8% agarose gel in 1 x TBE buffer. DNA was transferred to nylon membranes (HybondN$^+$) by vacuum blotting using 0.4 N NaOH. Detection of telomeric DNA fragments was performed as described previously (Boulton and Jackson, 1998).

Two hybrid screening

Four independent overnight cultures of EGY48 carrying plasmids pEG-Yku70p and pSH18-34 were transformed (Agatep *et al*., 1998) with 2 µg of a yeast genomic library cloned into the prey plasmid pJG4-5 (Watt *et al*., 1995). Cells were then plated at low density onto 16 SC-ura-his-trp plates (24 x 24 cm) and were incubated for 3 days at 30°C. The amount of primary transformants was calculated by counting the colonies in a representative 2,4x2,4 cm square from each plate. Colonies were scraped off the plates, washed twice with dH_2O and were finally resuspended in one pellet volume of storage buffer (65% glycerol; 0.1 M $MgSO_4$, 25 mM Tris pH 7.4). To induce gene expression from the library plasmid 100 µl of yeast cell suspension were added to 10 ml YPGal and incubated on a shaker at 30°C for 5 h. 2 ml and 200 µl culture where plated onto SC-ura-his-trp-leu Gal to select for the expression of the *LEU2* marker gene or onto SC-ura-his-trp-leu Glc control plates. The plates were incubated at 30°C for 3 days. To enhance the stringency of the screening procedure 300 clones, that grew on SC-ura-his-trp-leu Gal, were re-tested for their galactose dependent transcription activation of both marker genes *LEU2* and *lacZ*. Therefore, colonies from SC-ura-his-trp-leu Gal plates were resuspendend in dH_2O and spotted onto SC-ura-his-trp-leu Gal, SC-ura-his-trp x-Gal Gal and the control plates SC-ura-his-trp-leu Glc and SC-ura-his-trp x-Gal, respectively. After 3 days 77 clones, ¼ of the tested colonies, exhibited no growth on SC-ura-his-trp-leu Glc but grew on SC-ura-his-trp-leu Gal plates and gave rise to blue colonies specifically on SC-ura-his-trp x-Gal Gal plates. From these 77 clones the prey plasmid, that allowed selective gene expression on galactose containing plates was isolated.

Plasmid DNA was extracted from yeast as described (Adams et al., 1997) and was transformed in *E. coli* KC8 (Zervos *et al*., 1993), which allowed the selection of the prey-plasmid on tryptophane-lacking media. Plasmid preparations (QIAGEN Plasmid Mini Kit) from *E. coli* KC8 were then retransformed into *E. coli* XL1blue (Stratagene) to avoid DNAse mediated degradation. Plasmids isolated from XL1blue were retransformed into yeast strain EGY48 carrying pEG-Yku70 and pSH18-34 and transformants grown on SC-ura-his-trp were again tested for their ability to activate reporter gene transcription. In addition, the plasmid DNA was digested with *Eco*RI as well as *Hin*dIII and was separated on a 0.8% agarose gel. To identify the 5'-ends of the isolated genomic yeast DNA fragments, plasmids were sequenced using the vector specific primer pJG-ATG: 5'-TTG CTG AGT GGA GAT GCC TCC-3'.

Blast search

To search for homologues of or for sequence motives in the putative Yku70p interacting proteins protein blast searches were performed at http://www.ncbi.nlm.nih.gov/BLAST/. 'Standard protein-protein BLAST', 'PSI- and PHI-BLAST' and 'Search for short nearly exact matches' were used to search for local and overall homologies to the database.

Table I [V]. Yeast strains used in this study

Strain	Genotype	Reference
W303-1A	Mat a *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 rad5-535*	(Fan *et al*., 1996)
W303aL	W303-1A *yku70::LEU2*	(Feldmann and Winnacker, 1993)
EGY48	Mat a *trp1 ura3 his3 LEU2::pLexAop6-LEU2*	(Zervos *et al*., 1993)
CEN.PK2-1C	Mat a *ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8^c SUC2*	(Bojunga *et al*., 1998)
CEN.PK2 aL	CEN.PK2-1C *yku70::LEU2*	AG Feldmann, unpublished
CEN.PK2 ah2	CEN.PK2-1C *yku80::kanMX4*	This study
BMY48	CEN.PK2-1C *sir3Δ::kanMX6*	This study
BMY61	CEN.PK2-1C *sir2Δ::kanMX6*	This study
AJL278-4d	Mat a *rap1-17 ade2-1 ura3-1 HIS3 leu2-3,112 trp1*	(Kyrion *et al*., 1992)
HFY87	CEN.PK2-1C *rap1-17::kanMX4*	This study

Results

The Yku70 fusion protein expressed from pEG202 complements yku70 mutant phenotypes

The YKU70 gene was cloned in frame into plasmids pEG202 and pJG4-5 (see Materials and Methods). To test whether the Yku70 fusion protein is expressed functionally, we transformed pEG-Yku70 and pJG-Yku70 into W303a *yku70::LEU2* (aL) (Feldmann and Winnacker, 1993) as well as pEG202 and pJG4-5 as vector controls. Cells were plated on SC-his and SC-trp media to select for the pEG202 and pJG4-5 growth marker, respectively. After 3 days of growth at 30°C, transformants were resuspended in dH_2O and serial dilutions were used to test the strains for their sensitivity to the DNA damaging agent MMS and for growth at elevated temperatures.

The sensitivity of the W303-1A *yku70* mutant to methyl methanesulfonate was already detectable on YPD plates containing 0,01% MMS (Figure 2A, lane 2). *Yku70* mutants transformed with control plasmid pEG202 or pJG4-5 (Figure 2A, lanes 3 and 5) were as sensitive to MMS as the *yku70* mutant. The introduction of either pEG-Yku70 or pJG-Yku70 in *yku70* strains, however, led to resistance to MMS (Figure 2A, lanes 4 and 6) comparable to wild-type.

Similar results were obtained when we analyzed the temperature-sensitivity of these strains. *Yku70* mutants transformed with pEG202 and pJG4-5 did not grow at 37°C (Figure 2B, lanes 2, 3 and 5) whereas *yku70* mutant strains, which contained a pEG-Yku70 or pJG-Yku70 plasmid expressing the LexA-Yku70p or AD-Yku70p fusion, respectively, exhibited growth at 37°C, which was almost comparable to wild-type (Figure 2B, compare lanes 4 and 6 with lane1).

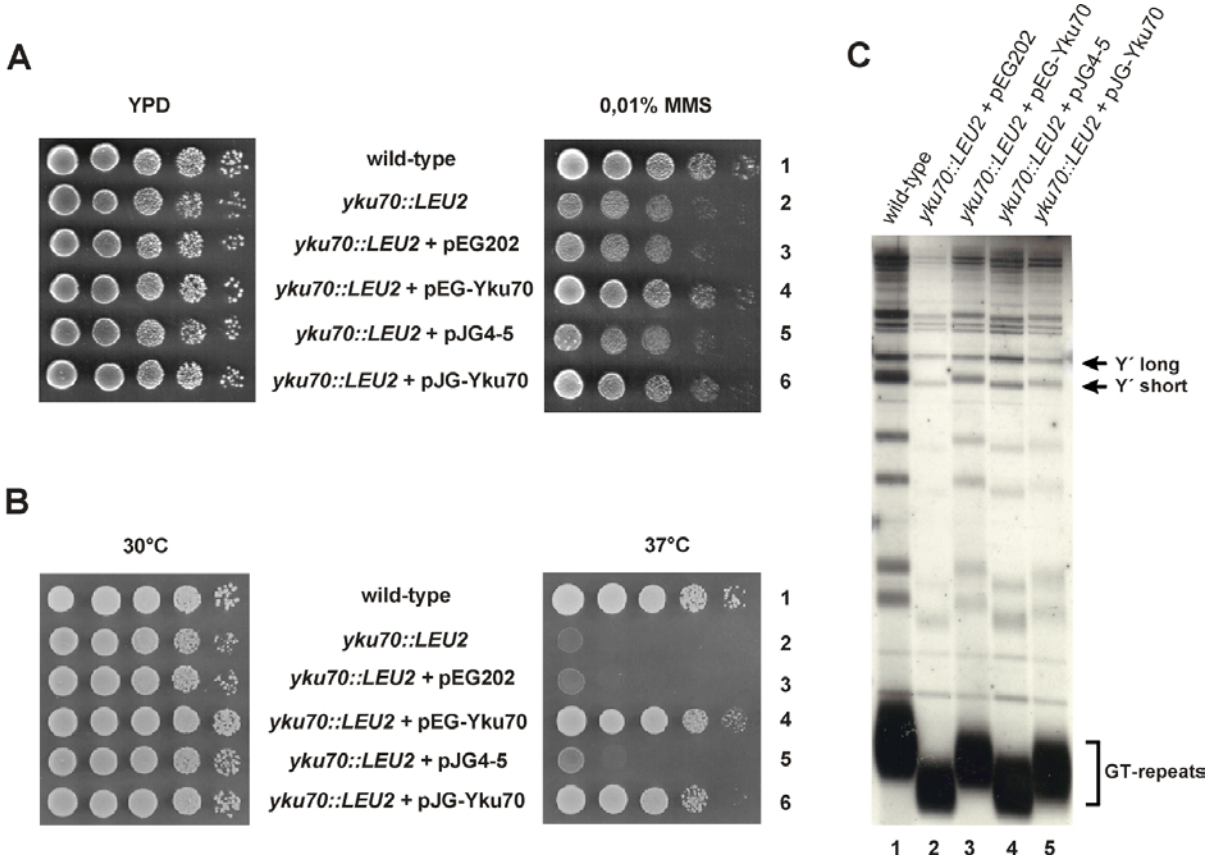

FIG. 2 [V]. The LexA-Yku70 fusion protein complements *yku70* mutant phenotypes. (A) 6 µl of 1:10 serial dilutions of W303-1A wild-type, *yku70* mutant and *yku70* mutant strains carrying various two hybrid vectors were spotted onto YPGal plates and YPGal plates containing 0,01% MMS. **(B)** 6 µl of 1:10 serial dilutions of the W303-1A strains described in (A) were spotted in duplicate onto YPGal plates. Cells were incubated for 3 days at 30°C and 37°C. **(C)** Southern Blot of yeast genomic DNA probed with a poly $(GT)_{20}$ oligonucleotide. Genomic DNA from W303-1A wild-type and *yku70* mutant strains carrying control, LexA-Yku70 and AD-Yku70 expression plasmids was prepared as described in Materials and Methods. Arrows indicate subtelomeric Y' element signals, brackets indicate terminal telomeric GT repeats.

Furthermore we examined the telomere length in yeast strains expressing the Yku70 fusion proteins. In strains W303-1A *yku70::LEU2* + pEG202 and W303-1A *yku70::LEU2* + pJG4-5 we observed a *yku70* corresponding telomere shortening (Figure 2C, lanes 2 and 4). The introduction of either bait or prey vector carrying a LexA- or AD-Yku70 fusion protein, respectively, confers significant telomere elongation (Figure 2C, lanes 3 and 5). Strikingly, telomeres in these strains did not reach wild-type telomere length even though growth at elevated temperatures and MMS sensitivity was rescued to wild-type levels.

Thus, the N-terminal fusion of Yku70p to either the LexA binding domain in pEG202 or the acidic transcription activation domain in pJG4-5 was able to complement the *yku70* mutant sensitivity to the DNA damaging agent MMS, the growth defect at 37ºC and could partially rescue the telomere shortening caused by a deletion of the endogenous Yku70p. From these data we conclude, that the N-terminally fused Yku70p enters the yeast nucleus and can interact with endogenous Yku80p to fulfil its cellular function.

The LexA-Yku70 fusion protein does not induce transcription of the LEU2 and lacZ reporter genes

Since the bait protein cloned into pEG202 is expressed as a LexA binding domain fusion this fusion protein itself may activate transcription of the reporter genes i.e. by an intrinsic acidic domain. To verify that the Yku70p fusion does not activate transcription independent from an interaction with a second protein fused to the B42 activation domain, we transformed EGY48 carrying pSH18-34 with either pSH17-4 (referred to as pGal4AD), pRFHM-1 (referred to as pBicoid AA 2-160), pEG202 or pEG-Yku70. Cells were plated on SC-his-ura and were incubated at 30°C for 3 days. From each transformation 5 independent transformants were resuspended in dH_2O and spotted onto SC-his-ura-leu Glc, SC-his-ura-leu Gal, SC-his-ura x-Gal Glc and SC-his-ura x-Gal Gal plates.

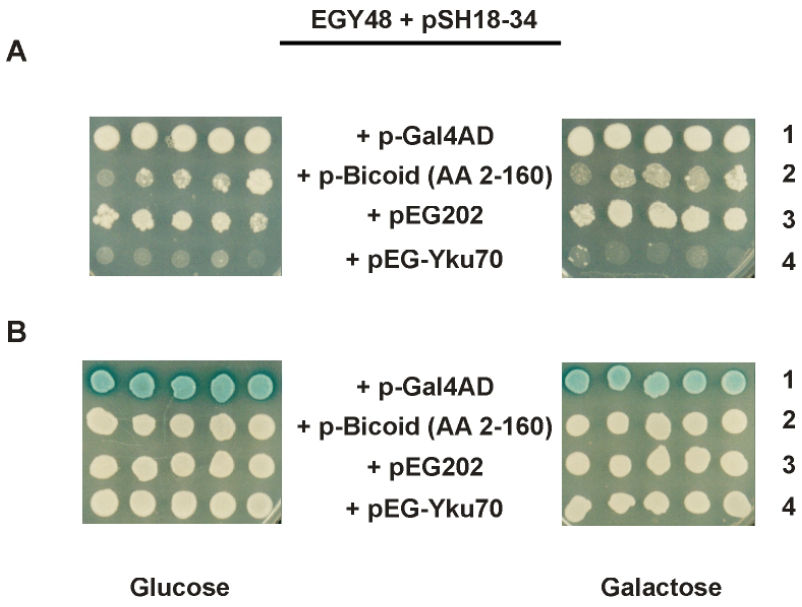

FIG. 3 [V]. The LexA-Yku70 bait protein does not activate reporter gene transcription. Yeast strain EGY48 carrying the *lacZ* reporter plasmid pSH18-34 was transformed with pGal4AD, pBicoid (AA 2-160), pEG202 or pEG-Yku70. Cells were plated on SC-ura-his and were incubated at 30°C for 3 days. (**A**) 5 independent colonies from each transformation were resuspended in dH_2O and spotted onto SC-ura-his-leu Glc and SC-ura-his-leu Gal to assay transcription activation of the genomically integrated *LEU2* reporter gene and were (**B**) spotted onto SC-ura-his x-Gal Glc and SC-ura-his x-Gal Gal to test for an activation of the episomally encoded l*acZ* gene.

The plasmid pGal4AD carries the Gal4 activation domain cloned into the pEG202 backbone and is used as a positive control, leading to growth on SC-ura-his-leu glucose and SC-ura-his-leu galactose plates (Figure 3A, lane 1) and to the accumulation of a blue colour in colonies grown on SC-ura-his x-Gal/Glc and SC-ura-his x-Gal/Gal plates (Figure 3B, lane 1). In contrast EGY48 transformed with pBicoid (AA 2-160), encoding the homeodomain of the Drosophila protein bicoid fused to the LexA binding domain and serving as a negative control for transcription activation, showed strongly impaired growth on leucine lacking media and did not result in the formation of blue colonies (Figure 3A and B, lane 2). The plasmid pEG202, that expressed the LexA DNA binding domain, led to a weak but significant induction of the sensitive growth reporter *LEU2* resulting in growth on Leu^- plates (Figure 3A, lane 3), but no induction of the *lacZ* reporter gene was visible in all five transformants tested (Figure 3B, lane 3). However, the LexA-Yku70p fusion protein overexpressed under the *ADH1* promoter from plasmid pEG-Yku70 exhibited a complete deficiency for growth on leucine lacking media (Figure 3A, lane 4) and did not induce β-galactosidase expression on SC-ura-his x-Gal/Glc as well as on SC-ura-his x-Gal/Gal plates (Figure 3B, lane 4) on which interaction studies are performed.

Furthermore, analysis of reporter gene activation in EGY48 containing not only pSH18-34, pEG-Yku70 but also the prey plasmid pJG4-5 on galactose containing media revealed that LexA-Yku70 did not activate transcription when the B42 activation domain was co-expressed (data not shown). Therefore, the LexA-Yku70p fusion protein did not induce transcription of the reporter genes, when bound to the LexA operator sequences and did not interact with the transcription activation domain expressed from pJG4-5. Thus, the pEG-Yku70 construct fulfils the preconditions to perform a two hybrid screen for Yku70p interacting proteins.

Proteins that interact with Yku70p by two hybrid criteria

To identify Yku70p interacting proteins four independent overnight cultures of EGY48 containing pEG-Yku70p and pSH18-34 were each transformed with 2 µg of the pJG4-5 derived yeast genomic library (Watt *et al*., 1995). Cells were plated on 16 SC-ura-his-trp plates (24 x 24 cm) to select for the three plasmids. Counting the colonies that arose on these plates after 3 days of growth at 30°C revealed approx. 790.000 primary transformants. The colonies were scraped off the plates and resuspended in one pellet volume storage buffer. To induce expression of the library plasmids, 100µl of this solution were used to induce protein expression from the prey plasmid in galactose containing media and two dilution steps were plated onto SC-ura-his-trp-leu Gal media. 300 colonies, able to grow on SC-ura-his-trp-leu Gal, were re-tested for galactose dependent activation of both reporter genes: *LEU2* and *lacZ*. 77 out of these 300 clones exhibited inducible growth on SC-ura-his-trp-leu Gal and accumulated a detectable blue colour on SC-ura-his-trp x-Gal/ Gal. Isolation of the library plasmid from the 77 colonies (see Material and Methods) and sequencing of the fusion part with pEG202/ATG, a primer ~ 40 bp upstream of the *Eco*RI cloning site, revealed various putative Yku70p interacting proteins (listed in Table II).

Table II [V]. Proteins that interact with Yku70p in the LexA two hybrid system. The column 'size of *Eco*RI fragments' allows to calculate the size of the genomic DNA fragment, that was ligated into the isolated pJG4-5 plasmid. Due to the cloning procedure of the genomic library the DNA insert contains flanking *Eco*RI restriction sites but can also contain internal restriction sites. The 'start' determines the first amino acid (AA) from the genomic fragment that is fused to the transcription activation domain. Sequencing was performed using a primer that matches in close proximity to the fusion junction. The isolation frequency of the specific DNA fragment is reflected in 'clones'.

interacting protein	size of *Eco*RI fragments (bp)	start	clones	localization	function
Protein-degradation					
RPN8 (YOR261c)	~ 1400	AA 229	14	nucleus; endoplasmic reticulum	essential gene, non-ATPase subunit of the 26S proteasome-complex (Finley *et al*., 1998)
Nuclear envelope					
NUP84	~ 950	AA 542	7	nuclear pore	nuclear pore component; part of complex with Nup120p, Nup85p, Sec13p, and a Sec13p homologue (Siniossoglou *et al*., 1996) • similar to mammalian Nup107p (Siniossoglou *et al*., 1996) • localizes symmetrically at both sides of nuclear pore (Rout *et al*., 2000)
Telomere/ Silencing					
SIR4	~ 1300	- 40 bp -AA 397	1	nucleus	silencing information regulator, required for silencing at HMR, HML (Ivy *et al*., 1986) and at the telomeres (Aparicio *et al*., 1991)
HAT1 (YPL001W)	~ 900	AA 103	1	component of two HAT complexes, one nuclear one cytoplasmic	histone-acetyltransferase (Parthun *et al*., 1996) • involved in telomere silencing, but not in HMR silencing • affects telomere silencing through histone H4 Lys12 and any of five N-terminal Lys of histone H3 (Kelly *et al*., 2000)
Chromatin structure					
MCM6 (YGL201c)	750 and 300 bp	AA 69	2	nucleus	essential gene, mini chromosome maintenance, protein involved in DNA-replication (Chen *et al*., 1992) • interacts with Dna43p, Est1p, Krr1p and Spt2p in a two-hybrid assay (Uetz and Hughes, 2000)

TOF1 (YNL273w)	2200	AA 549	1	n.d.	topoisomerase I interacting factor • null mutant is not sensitive to UV or MMS (Park and Sternglanz, 1999) • interacts with nucleoporin Nup100p (Allen *et al*., 2001)
YNG2/NBN1 (YHR090c)	~ 950	AA 35	1	nucleus	NuA4 histone acetyltransferase complex component • has over 50% identity to human candidate tumor suppressor p33-ING1 over the C-terminal domain • null mutant is temperature-sensitive, sensitive to UV, but not to gamma irradiation or alkylating agents • null mutant displays a significant decrease in acetylation of histone H4 residues K-5, K-8, and K-12 (Loewith *et al*., 2000)
Proteins of unknown function					
YDR014W/ YD8119.19	~ 1300 and 500	AA 9	5	n.d.	• 40% overall homology to *S. pombe* SPAC11E3.03 (blast search) • domain with 70% homology to integrase-recombinase protein from *Ureaplasma urealyticum* (blast search)
YPR148c	2x 1400 1x 1600	2x AA 292 1x AA 242	3	n.d.	short homology domain to high mobility group proteins (blast search)
YLR440c	3 fragments < 400	AA 49	2	n.d.	essential gene, serine-threonine kinase domain (blast search)
YKR077w	~ 1300	AA 79	1	n.d.	unknown
YPR097W	~ 1750	AA 503	1	n.d.	unknown
YLR052w	~ 1000 and 300	AA 136	2	n.d.	unknown
YDR124W	~ 2700	AA 135	1	n.d.	unknown
YDR520C/ D9719.25	~ 1000	AA 532	1	n.d.	protein with similarity to proteins with Zn-finger domains (blast search)
Transcription/ drug resistance					
SWI6	~ 900	AA 644	8	nucleus	transcription factor (Partridge *et al*., 1997) • mutants have a 60% reduction in RAD51 and RAD54 transcripts (Leem *et al*., 1998) • suppressor of defective silencing (Laman *et al*., 1995)

MDS3 (YGL197w)	~ 900	AA 1048	2	n.d.	putative transcription factor, • negative regulator of early meiotic genes (Benni and Neigeborn, 1997)
PDR3 (YBL005w)	~ 600 and 450	AA 643	1	nucleus	zinc-finger transcription factor • mutants exhibit multidrug resistance (Delaveau *et al*., 1994) • binds to an inverted palindrome (CCGCGG) (Hellauer *et al*., 1996)
Fun30 (YAL019)	~ 950	n.d.	1	nucleus	protein of Snf2p family (Clark *et al*., 1992) • null mutant has increased resistance to UV (Barton and Kaback, 1994) • overproduction from the *GAL1* promoter causes chromosome instability (Ouspenski *et al*., 1999)
Spindlepole					
ASE1 (YOR058c)	~ 1700	AA 654	1	nucleus	microtubule-associated protein (Pellman *et al*., 1995) • loss of function destabilizes telophase spindles • null mutant is temperature-sensitive for growth (Juang *et al*., 1997)
Vacuole					
VPS41 (YDR080w)	~ 1100, 850 and 300	AA 623	1	secretoric/ endocytotic vesicles	"Vacuolar Protein Sorting", protein of the class C Vps protein complex (Pep3p, Pep5p, Vps16p, Vps33p, Vam6p, Vps41p) (Sato *et al*., 2000) • null mutant has abnormal vacuolar morphology • functions in post-Golgi protein processing (Radisky *et al*., 1997)
PEP3 (YLR148w)	~ 1500 and 400	AA 462	1	lysosome/ vacuole; peripheral membrane	protein of the class C Vps protein complex (see VPS41) • null mutant contains no vacuole (Srivastava *et al*., 2000)
Mitochondria, cytoplasm					
DNM1 (YLL001w)	~ 900	AA 518	1	mitochondrial	dynamin-related mitochondrial protein • required for mitochondrial division (Sesaki and Jensen, 1999) • regulates mitochondrial fission (Bleazard *et al*., 1999)

APG1 (YGL180w)	~ 250	AA 773	1	cytoplasmic	serine/threonine protein kinase, mutants defective in vacuolar protein degradation during nitrogen starvation (Matsuura *et al*., 1997) • activation is required for induction of autophagy after nutrient limitation • activation is required for cytoplasm-to-vacuole targeting pathway (Kamada *et al*., 2000)
GPR1 (YDL035c)	~ 1100	AA 821	2	plasma membrane	G protein-coupled receptor containing seven transmembrane domains • involved in the pathway of pseudohyphal differentiation in response to nutrient starvation • coupled to Gpa2p (Xue *et al*., 1998)
transport					
GEA1 (YJR031c)	~ 800	AA 1284	4	n.d.	component of a complex guanine nucleotide exchange activity for the ADP-ribosylation factor ARF • essential function in transport from ER to Golgi in *vivo* (Peyroche *et al*., 1996)

Interestingly, two proteins, the Sir4 protein and a protein of unknown function encoded by the open reading frame YKR077w, were also isolated in a screen using Yku80p as a bait by Kai Walter (Walter K., 1997).

A Sir4p-Yku70p interaction identified by two hybrid criteria is mediated by Yku80p

In the two independent screenings with either Yku70p or Yku80p as a bait, a N-terminal domain of Sir4p encompassing -40 bp to AA 397 was identified by sequencing to interact with Yku. This *SIR4* fragment was isolated once in the screening for Yku70p interacting proteins, and 29 times when using Yku80p as a bait.

Screening for putative interactors of a yeast protein by a two hybrid approach opens up the opportunity that endogenous factors expressed by the yeast strain, in which the screening is performed, can contribute to a protein-protein interaction. Therefore, indirect protein-protein interactions, bridged by another cellular protein can be detected in this system (Moretti *et al*., 1994). To investigate whether the Sir4p domain interacts with both Yku subunits or whether interaction is mediated specifically by one subunit of the heterodimer, we performed two hybrid analysis in *yku* mutant strains.

Therefore, pSH18-34 carrying the *lacZ* reporter gene, pJG-Sir4(1-397) or full-length pJG-Sir4(1-1358) and pEG-Yku70 or pEG-Yku80 were transformed into yeast strains CEN.PK2a (wild-type), CEN.PK2aL (*yku70*) or CEN.PK2ah2 (*yku80*). Protein-protein interactions were measured by the formation of blue yeast colonies on X-Gal/galactose plates.

Co-expression of the Sir4 N-terminal domain Sir4(1-397) with Yku80p on galactose plates led to the accumulation of the blue pigment in wild-type cells (Figure 4A, right panel, lanes 1 and 2). Blue colonies were also detectable when either *yku70* (Figure 4A, right panel, lanes 3 and 4) or *yku80* (Figure 4A, right panel, lanes 5 and 6) deletion strains were used to test this interaction. Comparable results were obtained using the Sir4p full-length construct pJG-Sir4-1-1358. However, the intensity of the blue colour was more pronounced as compared to pJG-Sir4(1-397) indicating that a stronger interaction can be observed between Yku80p and full-length Sir4p as compared to Sir4Np.

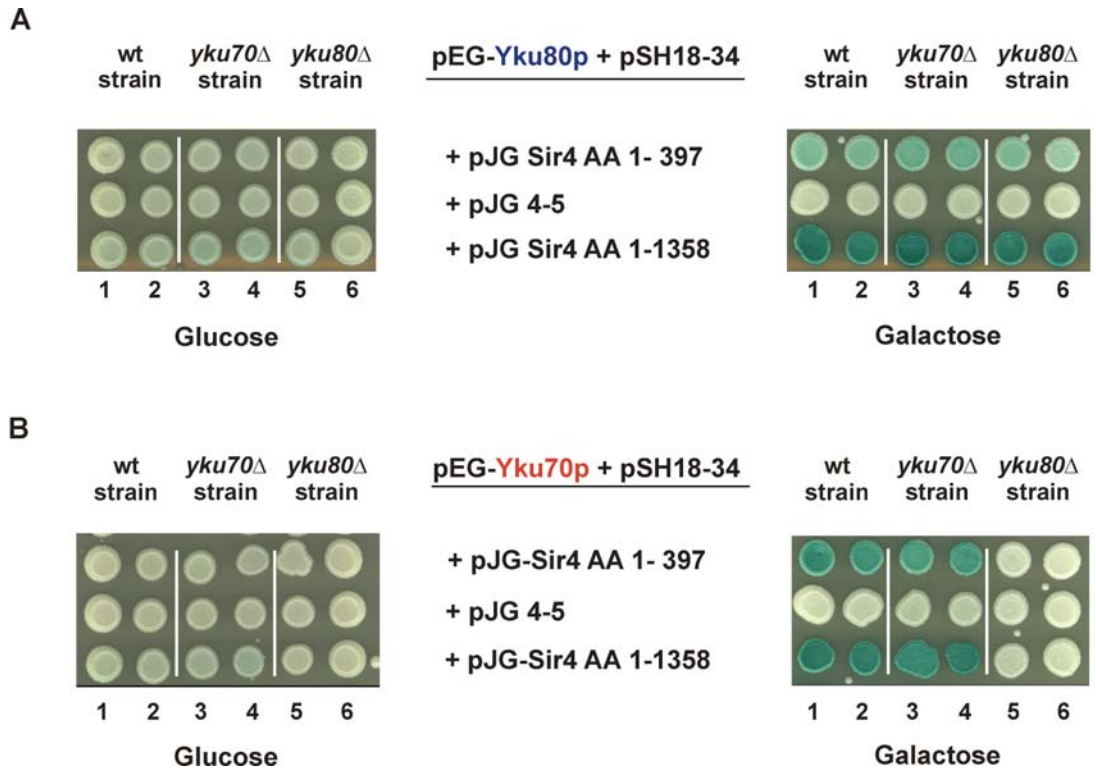

FIG. 4 [V]. The Yku80p subunit interacts with Sir4p in the absence of Yku70p whereas the Yku70p Sir4p interaction requires the presence of Yku80p. From each transformation of pEG-Yku80 with pJG Sir4 AA 1-397, pJG4-5 and pJG Sir4 AA 1-1358 and pEG-Yku80 with pJG Sir4 AA 1-397, pJG4-5 and pJG Sir4 AA 1-1358 6 independent transformants were resuspended in dH_2O. 5 µl of the cell suspension were spotted onto SC-ura-his-trp-leu x-Gal Glc and SC-ura-his-trp-leu x-Gal Gal plates to test for inducible expression of β-Galactosidase detectable by the formation of blue colonies on galactose containing plates.

Whether this reduction is due to a reduced stability of the N-terminal fragment or indicates that the Sir4 AA1-397 is sufficient but does not contain the entire Yku80p interaction domain has to be addressed. However, in both cases the presence of the cellular Yku70p is not necessary to allow interaction between Sir4p and Yku80p. Therefore Sir4p can interact with the Yku80p subunit independently from Yku70p and does not require the presence of a pre-assembled Yku heterodimer.

In contrast, when Sir4(1-397) and Sir4 full-length Sir4(1-1358) were analyzed for their interaction with Yku70p on galactose plates, blue colonies arose in wild-type and the *yku70* mutant (Figure 4B, right panel lanes 1 –4) but not in the *yku80* mutant strain (Figure 4B, right panel, lanes 5 and 6). Thus, the interaction between Sir4p and Yku70p occurs only in the presence of endogenous Yku80p. From these data we suggest that the Sir4p-Yku70p interaction, identified in our two-hybrid screen is bridged by Yku80p.

Sir4p interacts directly with Yku80p

A complex of four proteins, Sir1-4, is required to establish and maintain silencing at the mating type loci *HML* and *HMR* (Rine and Herskowitz, 1987) whereas Sir2p, Sir3p and Sir4p are sufficient to mediate silencing of telomere adjacent genes (Aparicio *et al*., 1991). None of the Sir proteins has been shown to display DNA binding properties. However, interaction of Sir3p with the DNA-binding protein Rap1 (Moretti *et al*., 1994) targets a Sir2-4 sub-complex to telomeric sites (Gotta *et al*., 1997; Maillet *et al*., 1996). A mutant Rap1 protein, *rap1-17*, that creates a stop codon at amino acid 663 (Kyrion *et al*., 1992) thereby lacking its Sir3p interaction domain (AA 679-827) (Moretti *et al*., 1994) results in the loss of telomeric silencing (Kyrion *et al*., 1993). Tethering a LexA-Sir3p fusion protein to telomere adjacent sites can restore telomeric silencing in a *rap1-17* mutant (Lustig *et al*., 1996), indicating that indeed the binding of the Sir-complex to the telomere is abolished in this mutant.

To investigate the requirement of additional components of the *SIR* complex for the interaction of Yku80p with Sir4p, we analyzed the Yku-Sir4p interaction in *sir2*Δ, *sir3*Δ and *rap1-17* mutant backgrounds. The reporter plasmid pSH18-34, pJG-Sir4(1-1358) and either pEG-Yku80 or pEG-Yku70 were transformed into CEN.PK2a *sir2*Δ, CEN.PK2a *sir3*Δ and CEN.PK2a *rap1-17* strains.

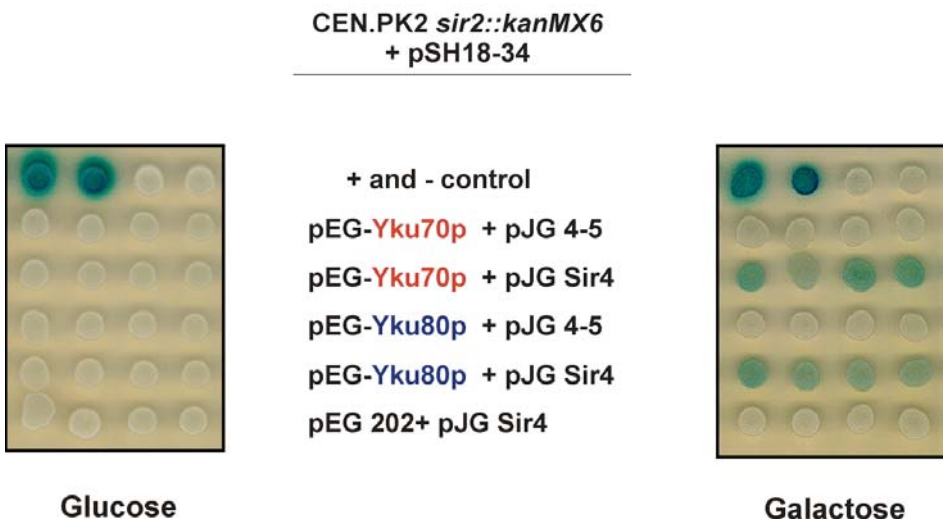

FIG. 5 [V]. The Yku-Sir4 interaction can be detected in the absence of endogenous Sir2p. Four independent transformants from each transformation reaction were resuspended in dH_2O. 5 µl of the cell suspension were spotted onto SC-ura-his-trp-leu x-Gal Glc and SC-ura-his-trp-leu x-Gal Gal plates to test for inducible expression of β-Galactosidase detectable by the formation of blue colonies on galactose containing plates. Cells were grown at 30°C for 3 days.

When full-length Sir4p was expressed together with either Yku80p or Yku70p in a *sir2*Δ strain on galactose containing plates, the *lacZ* reporter gene was expressed and resulted in the formation of blue colonies (Figure 5, right panel), indicating that the Yku-Sir4p interaction takes place in the absence of Sir2p. Identical results were obtained in *sir3*Δ and *rap1-17* mutant strains (data not shown). From these results we suggest that the protein-protein interaction observed between Sir4p and Yku80p is not bridged by other components of the SIR complex or by the Rap1 protein, that recruits the *SIR* complex to the chromosome ends. Thus, Sir4p seems to directly interact with the 80 kD subunit of the Yku heterodimer, mediated by the N-terminal domain of Sir4p**.**

Discussion

The Yku heterodimer, like its human homologue, is required for two important cellular processes, that require large protein complexes. It is i) an essential component of the repair of DNA breaks by non-homologous end-joining (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Milne *et al*., 1996)) and is ii) involved in maintaining the chromosome end structure (Boulton and Jackson, 1996a; Gravel *et al*., 1998; Porter *et al*., 1996).

In a two hybrid screening performed with the 70 kD subunit of the Yku heterodimer, we revealed a variety of putative Yku70p interacting proteins. From 300 transformants that exhibited galactose induced growth on SC-ura-his-trp-leu Gal plates, 77 also showed induced expression of the *lacZ* gene, a reporter gene that is less sensitive than the Lexop-LEU2 reporter (Estojak *et al*., 1995) and confers stringent selection. After re-testing of the isolated library plasmids in yeast and sequencing of the plasmid encoded DNA fragments, we identified 26 putative Yku70p interacting proteins, among them 11 proteins which have been described to localize to the yeast nucleus (Table II).

Strikingly no interaction between Yku70p and Yku80p, the second subunit of the functional Yku heterodimer, was observed in the screening for putative Yku70p interacting proteins. Furthermore, when we directly tested full-length Yku80p for its interaction with Yku70p in either bait or prey position no heterodimer formation was detectable (data not shown). Based on the observation that the LexA-Yku70p did complement *yku70* mutant phenotypes (Figure 2), we suggest that the Yku70p fusion is proficient in its interaction with endogenous untagged Yku80p. Therefore, the Yku80p subunit carrying a N-terminal fusion in the two hybrid context might interfere with heterodimer formation. This hypothesis is supported by the finding that the N-terminally fused LexA-Yku80p fails to complement *yku80* mutant phenotypes (K. Walter, 1997). Nevertheless, a C-terminal fragment of Yku70p could be isolated to interact with LexA-Yku80p as a bait (Walter, 1997). Thus, the interaction between the two subunits of the Yku heterodimer, that have been shown to co-purify (Feldmann *et al*., 1996), cannot be shown in the Interaction Trap system most likely due to sterical hindrance of the two fusion domains.

In a two hybrid screen for protein-protein interactions the occurrence of so called false positive interactors has been reported extensively. Among the most common false positives that have been found with various baits in screenings of independent laboratories are heat shock proteins, ribosomal proteins, mitochondrial proteins, proteasome subunits and Zinc finger proteins (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html). Since the two hybrid system is based on transcription activation all proteins that are expressed from the prey vector pJG4-5 are directed to the nucleus by a nuclear localization signal. Thus, proteins which *in vivo* localize to different cell compartments meet in the nucleus in the two hybrid context.

In our screening for Yku70p interacting proteins VPS41, PEP3, DNM1 and APG1 are found among the putative candidates. However, these proteins localize to the secretory vesicles (Rehling *et al*., 1999), the vacuole (Preston *et al*., 1991), the mitochondria (Otsuga *et al*., 1998) and the cytoplasm (Straub *et al*., 1997) respectively, and are therefore unlikely to interact with Yku70p *in vivo*. The RPN8 protein, a subunit of the 26s proteasome was identified 14 times independently in this screening. Proteasome subunits are known as a group of false positives that arise in two hybrid screenings, however, the observed interaction might indicate that Yku70p is degraded by the 26s proteasome. Nothing is known about Yku protein turnover during the cell cycle. However, Yku70p that is not assembled with Yku80p in the Yku heterodimer seems to be destabilized and preferentially degraded (H.M. Feldmann, pers. communication, Driller *et al*., 2000).

Among the putative interactors we identified a large number of proteins of unknown function, i.e. YDR014w, that was isolated 5 times in the screen and shows homology to *Schizosaccharomyces pombe* SPAC11E3.03, YPR148c, that was isolated 3 times with two different start sites and YLR440c, an essential gene containing a serine-threonine kinase domain. To test whether these proteins contribute to the cellular function of Yku70p, single mutants and double mutants strains carrying an additional *yku70* deletion need to be analyzed for DNA repair and telomere phenotypes. Some proteins were found to interact with Yku70p, which provide phenotypic evidence for a possible involvement in Yku mediated pathways. The MCM6 gene is a highly conserved gene, that is essential for the initiation of DNA synthesis at replication origins. The MCM complex is loaded onto chromatin and together with the origin recognition complex (ORC) assembles the pre-replication complex, that is crucial for limiting origin replication to only once per cell-cycle (for review see Lei and Tye, 2001; Takisawa *et al*., 2000). The MCM6 protein has been found to interact with Est1p, a regulatory subunit of yeast telomerase by two hybrid criteria (Uetz and Hughes, 2000) and in this study as a putative interactor with Yku70p. Therefore it might be interesting to investigate the MCM6-Yku70p interaction and its contribution to telomere function by genetic and biochemical assays. Another interesting Yku70p interacting candidate identified in this screen links the Yku protein to the nuclear pore. In *Saccharomyces cerevisiae* it has been possible to reveal the localization of telomeres to the nuclear periphery using Rap1p antibodies (Klein *et al*., 1992; Palladino *et al*., 1993b).

Recently, Galy and colleagues identified two proteins that are located at the interface between the nuclear envelope and the nuclear interior to co-immunoprecipitate with the Yku heterodimer (Galy *et al*., 2000). Mutations in *MLP1* and *MLP2* lead to a significant mislocalization of telomeres detected by fluorescence *in situ* hybridisation (Galy *et al*., 2000). We have isolated a C-terminal fragment of the nucleopore protein Nup84p to interact with Yku70p. Disruption of the gene encoding Nup84p causes a temperature-sensitive phenotype, which is associated with defects in nuclear membrane and nuclear pore complex organization as well as poly(A)$^+$ RNA export (Siniossoglou *et al*., 1996). Preliminary data suggest that *nup84* mutants exhibit slightly elongated telomeres (data not shown). However, since a defect in RNA export can induce a variety of different pleiotropic effects the generation of a point mutant that disrupts Yku70p interaction would be an important tool to study the relevance of this interaction.

In this study we have analyzed the Sir4 protein, which we isolated in both screenings with either Yku70p or Yku80p as a bait, in more detail. The Sir proteins Sir1p, Sir2p, Sir3p and Sir4p have been shown to be essential for the silencing of the mating type cassettes *HML* and *HMR* (Ivy *et al*., 1986). Moreover, loss of the *SIR4* gene leads to shortened telomeres (Palladino *et al*., 1993a) and to the de-repression of telomere adjacent genes within 6-8 kb of telomeres (Wyrick *et al*., 1999). *Sir4* mutants also exhibit a reduction in chromosome stability (Palladino *et al*., 1993a), indicating that a repressive chromatin structure at the telomere seems to be required to preserve chromosome integrity (Gartenberg, 2000). As the Yku heterodimer, Sir4p has been shown to be released from telomeric sites in response to DNA damage (Martin *et al*., 1999). In our screening for Yku interacting proteins, we identified a N-terminal fragment of Sir4p - encompassing AA 1-397 - to interact with Yku70p (Table II) and as a major interactor with Yku80p (Walter, 1997) in the two hybrid assay. Previously, a C-terminal domain of Sir4p has been described to interact with the Yku70 subunit by two hybrid criteria (Tsukamoto *et al*., 1997). This C-terminal domain (AA 1205-1358) overlaps with the Sir4p region required for dimerisation (Chien *et al*., 1991), for interaction with Sir3p and for the most likely indirect interaction with Rap1p (Moretti *et al*., 1994). We have not been able to reproduce the interaction of the Sir4p C-terminal region with Yku70p or Yku80p in the LexA based system (data not shown). However, the interaction between Yku80p and Sir4p full-length protein (AA 1-1358) leads to a stronger activation of the *lacZ* gene as compared to the interacting N-terminal domain (Figure 4).

This effect could i) be caused by a stability difference between the Sir4 (1-397) fusion and the Sir4 (1-1358) fusion or ii) the Sir4 (1-397) domain could be sufficient to mediate interaction but does not contain the entire interaction domain. Therefore our data do not exclude the presence of a second interaction domain as described by Tsukamoto and colleagues. A more detailed investigation of the Yku-interaction domain in the Sir4 N-terminus isolated here indicates, that AA 1-287 are sufficient to facilitate the interaction with Yku80p (H.M. Feldmann, pers. communication).

Our two hybrid results revealed an interaction of the Sir4p N-terminal domain with both the Yku70p and the Yku80p subunit. However, when we performed two hybrid studies in *yku* mutant strains, no interaction between Sir4p and Yku70p could be observed in a *yku80* mutant (Figure 4B). These results were obtained using either the N-terminal domain or Sir4p full-length. In contrast the Sir4(AA 1-397)-Yku80p as well as the Sir4(AA 1-1358)-Yku80p interactions did not require endogenous Yku70p (Figure 4A). From these data we conclude that the Yku70-Sir4p interaction identified in this approach is mediated by endogenous Yku80p. Strikingly, the isolation frequency of Sir4p in the two independent two hybrid experiments nicely reflects this observation. Furthermore, these results suggest that no direct interaction occurs between full-length Sir4p and Yku70p and that the interaction reported previously (Tsukamoto *et al*., 1996) is most likely indirect.

We have further addressed the requirement of additional proteins involved in Sir4p function at the telomere. Neither a deletion of *sir2*, *sir3* nor a mutation in *RAP1* that abolishes Rap1p binding of the *SIR* complex abrogates Sir4p-Yku interaction (Figure 5 and data not shown). Therefore, the Yku-Sir4p interaction is not bridged by other components of the *SIR* complex or by Rap1p that localizes the Sir complex to the telomeres. In an attempt to biochemically evaluate the interaction between Sir4p and Yku80p detected by two hybrid criteria, we have performed co-immunoprecipitation experiments. However, we have not been able to detect the Sir4p protein in crude extracts using a variety of different approaches (data not shown). Thus the Sir4 protein seems to be very low abundant and its expression might be tightly regulated.

The Yku heterodimer and the Sir4 protein localize to telomeres in *Saccharomyces cerevisiae* (Gotta *et al*., 1996; Martin *et al*., 1999). Furthermore, both proteins have been shown to delocalize from telomeric foci in response to DNA damage and accumulate at sites of a DNA break (Martin *et al*., 1999). Thus, Sir4p might interact with the Yku heterodimer at both the telomere and at the site of a DNA double strand break. Our genetic analysis (see Chapter IV) did not provide evidence for a direct function of Sir4p in DNA repair, however, this does not exclude the possibility of an interaction between Sir4p and Yku at a DNA break. In collaboration with the laboratory of S.P. Jackson we are now in the process of characterizing Yku80p mutants, which influence Yku functions at the telomere (R. Roy and S.P. Jackson, pers. communication). These mutants exhibit a reduced interaction with full-length Sir4p when overexpressed as a LexA fusion in the two hybrid system measured by quantitative β-galactosidase assays (data not shown). These mutants might allow the generation of second site mutations in Sir4p that would restore Sir4p-Yku70p interaction, thereby providing strong evidence for a direct interaction between the Yku80p subunit of the Yku heterodimer and the Sir4p, a component of the silencing information regulator complex in *Saccharomyces cerevisiae*. Furthermore the isolation of a *yku80* point mutant that is abolished in its interaction with Sir4p would allow us to address the importance of the interaction between the Yku heterodimer and Sir4p at the telomere or at a double strand break.

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Chapter VI

Life Span Analysis of *yku70* Mutants and Strains Overexpressing the Yku Heterodimer

Introduction

Linear chromosomes face a problem every time they are duplicated prior to cell division. DNA polymerase, the enzyme replicating the DNA, relies on RNA primers and therefore cannot duplicate the last nucleotides of the lagging strand (Watson, 1972). A special reverse transcriptase, telomerase, fulfils this function by copying repetitive sequences from its RNA template to the DNA ends (Greider and Blackburn, 1985).

Telomerase is absent in most human somatic tissues but is active in germ cells, which produce egg and sperm, to ensure that every generation starts with the same length of telomeres. Interestingly, B and T cells have been shown to activate telomerase during immune response, which requires rapid divisions (Buchkovich and Greider, 1996; Weng *et al*., 1997a; Weng *et al*., 1997b). Cancer cells can divide indefinitely and approximately 90% of all tumors have detectable telomerase activity (Kim *et al*., 1994). Most somatic cells, however, undergo a limited number of cell divisions that is accompanied by a gradual loss of telomere DNA sequences (Harley *et al*., 1990; Harley and Villeponteau, 1995; Prowse and Greider, 1995). Interestingly, a knock out of the telomerase RNA template in mice did not lead to premature aging in the first few homozygous generations but showed wild-type characteristics for phenotypes associated with aging, such as gray hair and wrinkled skin as they grew older (Lee *et al*., 1998). Thus, the life span of an individual animal that is born with wild-type telomere length is not determined by the activity of telomerase in mice. Nevertheless, in later mouse generations the loss of telomerase did lead to early-onset of hair graying, hair loss (Rudolph *et al*., 1999) and germline mortality, thereby inducing sterility (Lee *et al*., 1998).

The possibility, however, that enhanced telomere shortening might play a role in human aging, is supported by Werner's and Ataxia telangiectasia patients, who experience both premature aging and accelerated telomere shortening (Kruk *et al*., 1995; Smilenov *et al*., 1997). This correlation between telomere length and aging is further supported by the finding that overexpression of telomerase leads to a restoration of wild-type telomere length and compensates for the early onset of replicative senescence in Werner's syndrome cells (Wyllie *et al*., 2000).

Recent data revealed an interaction between WRNp, the protein mutated in Werner's syndrome patients, and the Ku heterodimer. The DNA binding protein Ku70/86 is the regulatory subunit of the catalytic active DNA-PK_{cs} and this protein complex is essential for DNA double-strand break repair by nonhomologous end-joining (Chen *et al*., 1996; Finnie *et al*., 1995; Jeggo *et al*., 1994). Furthermore, the Ku heterodimer is required for the protection of the chromosome ends, it binds to telomeres (Hsu *et al*., 1999) and various cell types lacking Ku86 have been reported to show an increased rate of chromosome end-to-end fusion events (Bailey *et al*., 1999; Hsu *et al*., 2000). Mice deficient for either Ku70 or Ku86 are significantly smaller than their control littermates and fibroblasts derived from Ku80-/- embryos showed a reduced proliferative potential (Gu *et al*., 1997; Nussenzweig *et al*., 1996). Moreover, *ku86*-mutant mice have been reported to show an early onset of age-specific alteration, suggesting that the Ku heterodimer influences the senescence process (Vogel *et al*., 1999). An interaction of WRNp and the Ku heterodimer has been determined by co-immunoprecipitation (Cooper *et al*., 2000). In addition, WRNp is phosphorylated by DNA-PK *in vitro* and requires DNA-PK for *in vivo* phosphorylation (Yannone *et al*., 2001). Biochemical data provide evidence that WRN exonuclease activity can be stimulated by Ku (Cooper *et al*., 2000) whereas DNA-PKcs inhibits WRN helicase activity (Yannome, 2001).

In *Saccharomyces cerevisiae*, the entire yeast culture is immortal and yeast cells have active telomerase ensuring that telomere length stays stable in every generation. However, a individual yeast cell has a restricted dividing capacity. The number of cell divisions that mother cells undergo is relatively fixed and has been defined as their replicative life span (Mortimer and Johnston, 1959). The mean and the maximum life span for a given strain are characteristic for that strain but can vary widely from one strain to another (Kennedy *et al*., 1995; Muller *et al*., 1980). Many gene products have been identified that affect life span in *S. cerevisiae*, among them proteins involved in DNA repair, telomere length maintenance or transcriptional silencing (reviewed in Jazwinski, 1999).

A deletion of the *SGS1* gene, the yeast WRN homologue, has been shown to induce a reduction in replicative life span (Sinclair *et al*., 1997). *sgs1* mutants do not exhibit telomere shortening (Watt *et al*., 1996). However, the WRN homologue is required for chromosome stability (Watt *et al*., 1996) and telomere elongation by the addition of long tracts of telomere repeats, and thus for the generation of type II survivors in the absence of the catalytic subunit

of telomerase, Est2p (Huang *et al*., 2001). The *SIR2-4* complex, shown to localize to telomeres (Laroche *et al*., 1998) and to be required for telomere length maintenance and silencing (Palladino *et al*., 1993), has also been implicated in replicative life span (Kennedy *et al*., 1995). Yeast strains deficient for *SIR2, SIR3* or *SIR4* activity exhibit a life span reduction of about 20% as compared to their isogenic wild-type whereas a special mutation in *SIR4* leading to a C-terminally truncated protein has been shown to increase life span (Kennedy *et al*., 1995). The yeast Ku heterodimer (Yku) interacts with Sir4p in two hybrid assays (Tsukamoto *et al*., 1997 and Chapter V). Yku is, like human Ku, required for the repair of double-stranded DNA breaks by nonhomologous end-joining (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Milne *et al*., 1996) and *yku* mutants exhibit stable but shortened telomeres (Boulton and Jackson, 1998; Porter *et al*., 1996). Both the Yku heterodimer and proteins of the *SIR* complex have been shown to delocalize from telomeric sites and accumulate at a DNA break (Martin *et al*., 1999; McAinsh *et al*., 1999; Mills *et al*., 1999).

These findings link the Ku heterodimer to proteins that influence life span in yeast and humans and might point toward an involvement of Ku this process. Therefore, we wanted to address the role of Yku in replicative life span of *Saccharomyces cerevisiae*.

Materials and methods

S. cerevisiae strains, media, growth conditions and transformation

Yeast strains used in this study are listed in Table I. Cells were grown at 30°C using YPD or selective media as described elsewhere (Feldmann *et al*., 1996). Yeast transformation was performed by the lithium acetate method (Schiestl and Gietz, 1989).

Plasmid constructions

Plasmid pFA6a-KanMX (Wach *et al*., 1994) was digested with *Bgl*II, treated with Klenow-enzyme and dephosphorylated. The 1,3 kb *ADH1* promoter was isolated from pADH1001 (Lang and Looman, 1995) by *Hind*III-digestion, was blunted with Klenow-enzyme and ligated to pFA6a-kanMX4 *Bgl*II/Klenow leading to pFA6a-kanMX4-ADH1. The orientation of the *ADH1* promoter was verified by an *Eco*RV digestion. Plasmid pGEM4Z (Promega Corporation, Madison) was digested with *Hin*cII, dephoshorylated and ligated to a blunt ended *URA3* cassette. This plasmid, pGEM4Z-URA3*Hin*cII, was linearized with *Hind*III and dephosphorylated.

A 1,3 kb *ADH1* promoter fragment was isolated from an agarose gel after pADH1001 digestion with *Hind*III. This DNA fragment was ligated to pGEM4ZUra3 *Hin*dIII/dephos. to generate pGEM4ZUra3-P_{ADH1}. The correct orientation of the *ADH1* promoter was verified by restriction analysis.

Gene disruption and genomic integration of overexpression constructs

The *yku70* deficient strains were generated as described previously (Feldmann and Winnacker, 1993). The genomic integration of the *ADH1* promoter in front of the *YKU70* or *YKU80* gene was performed by the PCR based method (Wach *et al*., 1994). The P_{ADH1}-YKU70 integration construct was amplified from pGEM4ZUra3-P_{ADH1} using primers ADH1-YKU70for 5'-GAT TTG TTA AGT GAC TCT AAG CCT GAT TTT AAA ACG GGG AAG TGC AAG ATG GAA ACG C-3' and ADH1-YKU70rev 5'-CTC CAC TAT TGC CAA ATG CAT TAG TGA CTG AGC GCA TCC TTG ATG TAT GCT TGG TAT AGC-3'. The resulting PCR product was used as a template to generate a DNA fragment carrying 80 bp homology to the target sequence with primers ADH1-HDF1LFHfor: 5'-GAC ATT CTC TGT ATT ACT GTT CTA GTT TTC AAC AGT AAA GCT ATG ATT TGT TAA GTG ACT CTA AGC C- 3' and ADH1-HDF1LFHrev: 5'-CAA ACT TCC TAT AAC CTG TTT CAT CCA CTT GAT CGT TAA GTT CTC CAC TAT TGC CAA ATG C-3'. The P_{ADH1}-YKU80 integration construct was generated using plasmid pFA6a-kanMX4-ADH1 as described for P_{ADH1}-YKU70 with primers ADH1-HDF2for: 5'-CGA GAG TGC AGG ACA TAT GCA CAA ATA ATA TAT CTC ACA CCG CCG CAT AGG CCA CTA GTG G-3' and ADH1-HDF2rev: 5'-GTG AAA CAT CCA CGA TGA AAG TTG TTG ACT CAC TTG ACA TGG TCG ACT CTA GAG GAT CC-3' as well as ADH1-HDF2LFHfor: 5'-GAA TAA AAA AAA AGG GCA TCA TCA AGA GAA GAA AAC CTA ATT AAC GAG AGT GCA GGA CAT ATG CAC-3' and ADH1-HDF2LFHrev: 5'-GCC ATA GAT TTG GAA ACA TTA TTA TTT TTC ATC ATT GAT GGT GAA ACA TCC ACG ATG AAA G- 3'. The P_{ADH1}-YKU70 PCR product was transformed into W303-1A leading to BMY9 and the P_{ADH1}-YKU80 PCR product was transformed into W303-1A and BMY9 to generate BMY10 and BMY11, respectively. The correct *ADH1* integration was tested by the Yku70p and Yku80p overexpression in Western blots. Sequences of primers that are underlined represent the homology region to the chosen plasmid.

Yeast DNA extraction and analysis of telomeric DNA

Genomic DNA was isolated from 5- to 7-ml overnight cultures using the nucleon MiY DNA extraction kit (Amersham Life Science). For analysis of telomere length, genomic DNA was digested overnight using *Xho*I or *Pst*I and was separated on an 1% agarose gel in 1x Tris-acetate-EDTA buffer. DNA was transferred to nylon membranes (HybondN^+) by vacuum blotting using 0.4 N NaOH. Detection of telomeric DNA fragments was performed as described elsewhere (Boulton and Jackson, 1996a).

EMSA

Electrophoretic mobility shift assays were performed as described previously (Feldmann *et al*., 1996).

Sensitivity assays

Yeast colonies were picked, resuspended in dH_2O and diluted five times by 10-fold serial dilutions. Aliquots (6 µl) of each dilution were spotted in duplicate on YPD plates and on YPD plates containing various amounts of bleomycin or methyl methanesulfonate (MMS). YPD plates were incubated at 30°C or 37°C and all other plates were incubated at 30°C for 3-4 days.

Western blot

40 µg crude extract were incubated with Laemmli loading buffer for 2 min at 95°C, separated on a 12% SDS gel and transferred to a PVDF membrane (NEN™ Life Science Products) by western blotting. The membrane was blocked with 1x TBST (10 mM Tris-HCl, pH 8,0;150 mM NaCl; 0.05% Tween 20), 1.5% milk powder and was then incubated with the anti-Yku70 antibody HDF-5F2 (Driller *et al*., 2000) or a rat antibody raised against the N-terminus of Yku80p Ku80-6D4-1-1. A mouse monoclonal anti-actin antibody MAB1501 (CHEMICON International, Inc.) diluted 1:5000 in 1x TBST, 1.5% milk was used as a control for protein loading. The membrane was washed three times with 1x TBST and incubated with peroxidase conjugated goat anti-rat IgG and IgM (Dianova) as a secondary antibody diluted 1:5000 in 1x TBST, 1.5% milk. The membrane was washed twice with 1x TBST and the Western Blot Chemiluminescence Reagent Plus (NEN™ Life Science Products) was used for protein detection.

Life span analysis

Yeast cells were taken from freshly restreaked colonies after one day of growth, resuspended in dH_2O and plated at low density on YPD plates. After incubation at 30°C for 4-5 h, virgin cells were isolated as described previously (Kennedy *et al*., 1995) using a Singer MSM Micromanipulator. These starting cells were counted of age zero and the life spans were determined by counting and removing all subsequent daughters that they generated. During the hours of manipulation, the plates were incubated at 28°C; during the night they were incubated at 4°C, which does not influence life span (Muller *et al*., 1980). For each strain usually more than 50 individual virgin cells were examined. To characterize the life span of a strain we determined the mean (average) life span, the age at which 50% of the cells still divide. Virgin cells that did not undergo a single cell division were not included in the calculations (Kennedy *et al*., 1995).

Table I [VI]. Yeast strains used in this study

Strain	Genotype	Reference
W303-1A	Mat**a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 rad5-535*	(Fan *et al*., 1996)
W303aL	W303-1A *yku70::LEU2*	(Feldmann and Winnacker, 1993)
W303aα	Mat**a**/Matα *leu2-3,112/leu2-3,112 ura3-1/ura3-1 his3-11,15/ his3-11,15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100 rad5-535/rad5-535*	Thomas und Rothstein, 1989
W303aUαL	W303aα *yku70::LEU2/yku70::URA3*	(Feldmann and Winnacker, 1993)
CEN.PK2-1C	Mat**a** *ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8^c SUC2*	(Bojunga *et al*., 1998)
CEN.PK2 aL	CEN.PK2-1C *yku70::LEU2*	AG Feldmann, unpublished
JC482a	Mat**a** *ura3-52 leu2 his4-539*	(Pichova *et al*., 1997)
BMY7	JC482**a** *yku70::LEU2*	This study
HKY579-10A	Mat**a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 RAD5*	(Fan *et al*., 1996)
BMY8	HKY579-10A *yku70::LEU2*	This study
BMY9	W303-1A P_{ADH1}-*YKU70::URA3*	This study
BMY10	W303-1A P_{ADH1}-*YKU80:: kanMX4*	This study
BMY11	W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80:: kanMX*4	This study

Results

A yku70 deletion leads to life span shortening in various genetic backgrounds of Saccharomyces cerevisiae

The Yku heterodimer is an essential component of the nonhomologous DNA repair pathway (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Milne *et al*., 1996) and is required for the maintenance of wild-type telomere length and structure (Gravel *et al*., 1998; Porter *et al*., 1996). To investigate the influence of the Yku heterodimer on replicative life span, *yku70* deletion strains were generated for three different strain backgrounds W303-1A, JC482a and

CEN.PK2-1C (Table 1). Furthermore HKY579-10A, a W303 derivative reverted in its *rad5-535* mutation (Fan *et al*., 1996) was included into the analysis.

In wild-type W303-1A, life span determination led to a maximum replicative life span of 38 generations and a mean life span of approximately 24 generations (Figure 1A) as observed previously (Kaeberlein *et al*., 1999). The introduction of a *yku70* disruption in W303-1A decreased the mean life span to 18,5 generations, an average reduction of 24 % (Figure 1A). For HKY579-10A, a W303-1A $RAD5^{+}$ strain, a maximum life span of 40 generations and a mean life span of 26,3 generations was observed for the wild-type (Figure 1B) and the isogenic *yku70* disruption showed a reduction of average life span of about 26 % (Figure 1B and Table 2). Thus a comparable decrease in life span was observed for HKY579-10A *yku70::LEU2* (Figure 1B) and W303-1A (Figure 1A), indicating that the reduced life span observed for W303 *yku70::LEU2* is independent from the *rad5-535* background mutation.

When CEN.PK2, a yeast strain that has not been used for life span analysis before, was tested, the wild-type strain exhibited a maximum of only 25 and a mean life span of 15,6 generations (Figure 1C). A *yku70* deletion in this strain background did induce only a very slight reduction of one generation in mean and maximum life span whereas the median seems to be identical for wild-type and mutant strain. Due to the short overall life span of this strain, the reduction in mean life span is 8% in this genetic background, but the significance is difficult to evaluate based on the overall short replicative life span.

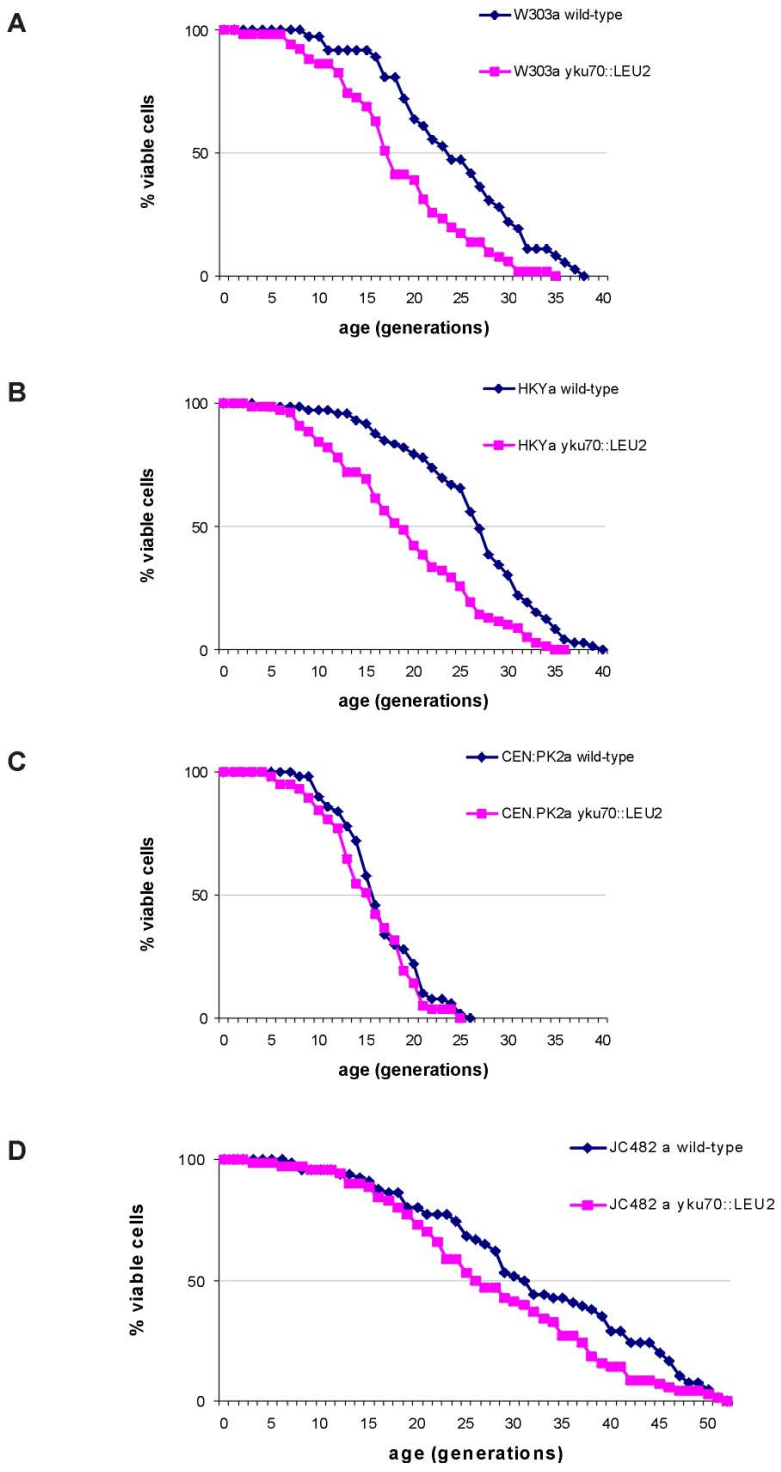

FIG. 1 [VI]. Effects of a *YKU70* deletion on replicative life span. Survival curves are shown for four different *S. cerevisiae* strains carrying the *yku70* disruption. The samples sizes for yeast strains were as follows: **(A)** W303 wild-type (wt), 36 cells; W303 *yku70::LEU2*, 51 cells. **(B)** HKY579-10A, 73 cells; HKY579-10A *yku70::LEU2,* 78 cells. **(C)** CEN.PK2a wild-type (wt), 50 cells; CEN.PK2a *yku70::LEU2*, 70 cells. **(D)** JC482a wild-type (wt), 67 cells; JC482a *yku70::LEU2*, 65 cells.

A reduced life span was also observed in JC482a *yku70::LEU2* when compared to the isogenic wild-type (Figure 1D). The JC482a wild-type mean life span of 30,2 generations was reduced to 27,5 generations in JC482a *yku70::LEU2*. Although the maximum life span in this genetic background was about 54 generations, the effect of the *yku70* disruption was only 9%, which is less severe than in the W303 background but comparable to the data observed in the CEN.PK2a background (see Table 2). In this strain, however, the curves of wild-type and *yku70* mutants are clearly distinguishable and the median life span is reduced (Figure 1D). Taken together, our data reveal that the introduction of a *yku70* mutation leads to a significant decrease in lifespan in at least two different genetic backgrounds (see Table 2).

Table II [VI]. Mean and maximum life span obtained for wild-type and *yku70* mutants in four different genetic backgrounds. Standard deviations were less than 15% of the mean.

Strain	Mean	Maximum	% Reduction in mean life span compared to wild-type
W303-1A	24,3	38	
W303-1A *yku70::LEU2*	18,5	35	23,9 %
HKY579-10A	26,3	40	
HKY579-10A *yku70::LEU2*	19,4	37	26,2 %
JC482a	30,2	54	
JC482a *yku70::LEU2*	27,5	53	8,9 %
CEN.PK2-1C	15,6	25	
CEN.PK2-1C *yku70::LEU2*	14,4	24	7,8 %

The reduction in life span caused by the yku70 disruption is detectable in haploid and diploid cells

The loss of the Sir4 protein, a Yku interacting protein (Tsukamoto *et al*., 1997 and Chapter V), has been reported to reduce the mean life span by 20% as compared to an isogenic haploid W303 RAD5^{+} wild-type (Kaeberlein *et al*., 1999). The Sir4 protein is, in association with Sir2p, Sir3p and Rap1p, required for silencing at the mating type loci *HML* and *HMR* (Rine and Herskowitz, 1987) and at the telomeres (Aparicio *et al*., 1991).

Sir4p is not only essential for silencing but is also involved in telomere length regulation (Palladino *et al*., 1993) and is, as Yku, released from telomeric sites in response to DNA damage (Martin *et al*., 1999). Strikingly, the *sir4* deletion that leads to life span reduction in haploid cells does not alter the replicative capacity of diploid cells (Kaeberlein *et al*., 1999). Furthermore a deletion of the *HML*α mating type locus in a *sir4*Δ strain led to a replicative life span comparable to wild-type (Kaeberlein *et al*., 1999), indicating that the mating-type heterozygosity induced by loss of silencing in *sir4*Δ mutants causes life span reduction. Cells lacking either subunit of the Yku heterodimer show reduced silencing in subtelomeric regions, however, no effect on silencing at *HML*α and *HMR*a could be observed (Gravel *et al*., 1998; Laroche *et al*., 1998; Nugent *et al*., 1998 and Chapter IV). Therefore, the life span reduction observed for *yku70* mutants should also be detectable in homozygous diploid mutant strains. Indeed, we observed a life span reduction of 37% in a diploid W303aα *yku70/yku70* strain.

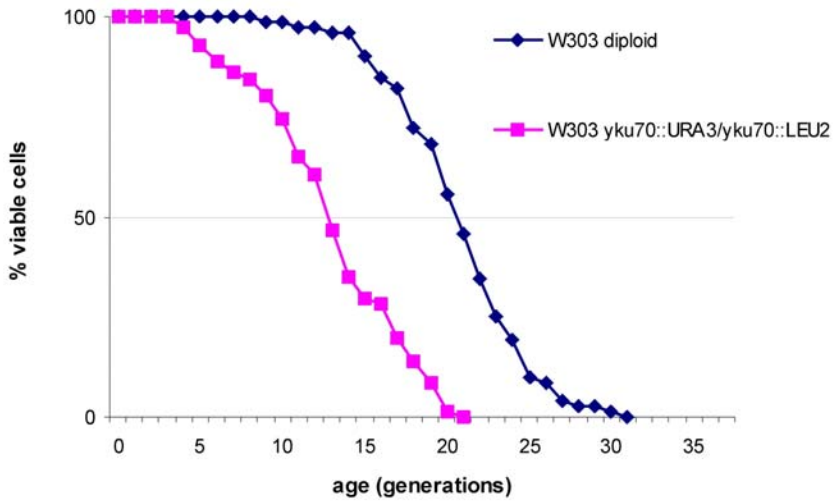

FIG. 2 [VI]. Homozygous diploid W303aα *yku70/yku70* mutants exhibit a shorter life span than the isogenic wild-type strain. Survival curves are shown for W303aα wild-type, 72 cells, and W303aα *yku70::LEU2/yku70::URA3*, 71 cells.

Table III [VI]. Mean and maximum life span obtained for diploid W303 wild-type and *yku70/yku70* mutants.

Strain	Mean	Maximum	% Reduction in mean life span compared to wild-type
W303aα wild-type	20,9	31	
W303 *yku70::LEU2/yku70 ::URA3*	13,1	21	37,2 %

The life span of the diploid W303 wild-type was ~ 15 % shorter than that of the isogenic haploid W303a strain consistent with results reported previously (Kaeberlein *et al*., 1999). Homozygous diploid *yku70/yku70* mutants showed a mean life span of 13,1 generations compared to 20,9 generation in wild-type (Figure 2). The differences in mean and maximum life span between *yku70* mutant and wild-type was more pronounced in the diploid than in the corresponding haploid strain (compare Figure 1 and 2). However, these data provide evidence that the reduced life span of *yku70* mutants is not related to the life span phenotypes reported for *sir4*Δ mutant strains.

The yku70 deletion induces different phenotypes in different strain backgrounds

Whereas proteins involved in nucleotide excision and transcription coupled repair have no significant effects on yeast life span (Park *et al*., 1999), several proteins involved in the repair of double-stranded DNA breaks (DSBs) by homologous recombination have been reported to enhance premature aging in *Saccharomyces cerevisiae* (Park *et al*., 1999). Three types of DSB repair operate in *S. cerevisiae*: homologous recombination, Yku dependent end-joining and error-prone repair (see Boulton and Jackson, 1996b). The life span shortening of *yku70* mutants might indicate that the loss of the nonhomologous end-joining (NHEJ) pathway reduces yeast life span. However, Kaeberlein *et al*., (1997) demonstrated that a W303a strain deleted for Lig4p, the essential ligase for the end-joining mechanism (Schar *et al*., 1997; Teo and Jackson, 1997; Wilson *et al*., 1997), shows a life span comparable to wild-type. Therefore a loss of the NHEJ repair pathway *per se* seems not to influence replicative life span. To investigate what causes shortened life span in *yku70* mutants, we phenotypically characterized all four *yku70* deletion strains.

Yeast strains deleted for either subunit of the Yku heterodimer have been described to exhibit strongly reduced re-ligation of a linearized plasmid lacking homologous regions (Boulton and Jackson, 1996a; Milne *et al*., 1996), which reflects the defect in nonhomologous end-joining. All *yku70* deletion strains tested exhibited a reduction in the plasmid repair assay (data not shown). Whereas the W303-1A, HKY579-10A and CEN.PK2 strains showed a comparable strong reduction in end-joining caused by the introduction of the *yku70* deletion, the transformation efficiency of JC482a wild-type and *yku70* mutant was extremely low (data not shown) probably caused by an enhanced temperature sensitivity of this yeast strain (see Figure 3). Therefore, reduction in end-joining efficiency could not be quantified for the JC482a *yku70::LEU2* mutant. In addition, the sensitivity to the DNA damaging agents bleomycin and methyl methanesulfonate (MMS) has been used to assay a reduced DNA break repair capacity in *yku* mutants strains (Feldmann *et al*., 1996; Mages *et al*., 1996; Milne *et al*., 1996). Surprisingly, although all strains exhibited reduced plasmid religation, only W303-1A and HKY579-10A displayed a significant MMS (Figure 3) and bleomycin (data not shown) sensitivity.

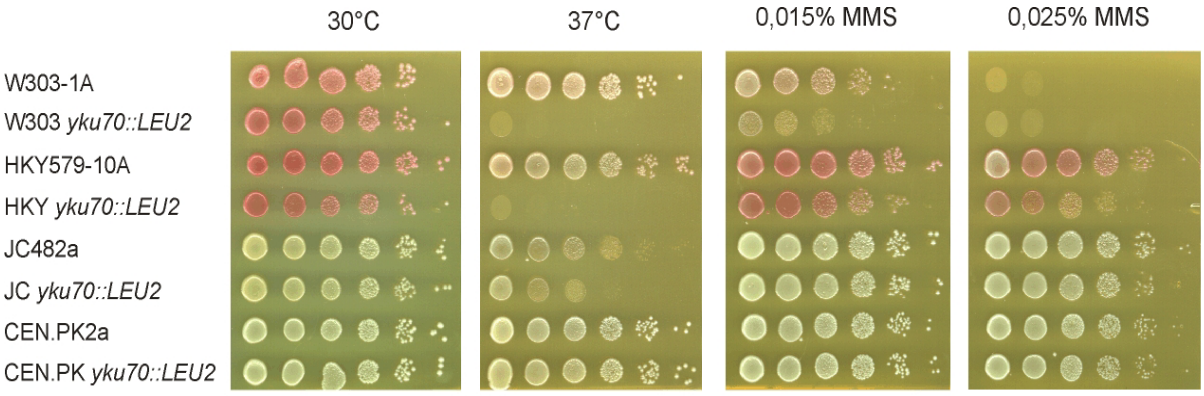

FIG. 3 [VI]. Sensitivity differences of a *yku70* disruption in different genetic backgrounds to the DNA damaging agent MMS and to elevated temperatures. Freshly grown yeast colonies were resuspended in dH_2O and 1:10 serial dilutions were spotted in duplicate onto YPD plates and onto YPD plates containing increasing amounts of methyl methanesulfonate (MMS). MMS plates as well as a YPD control plate were incubated at 30°C for 3 days. The second YPD plate was incubated at 37°C for 3 days to analyze the sensitivity of the various strains to elevated temperatures.

Besides its role in DNA repair Yku also contributes to the maintenance of wild-type telomere length. Accordingly, strains deleted for *YKU70* or *YKU80* show short telomere GT repeat tracts compared to those of wild-type when genomic DNA is analyzed in a Southern blot probed with a telomere specific probe (Boulton and Jackson, 1996a; Porter *et al*., 1996). Therefore we analyzed telomere length in all four genetic backgrounds.

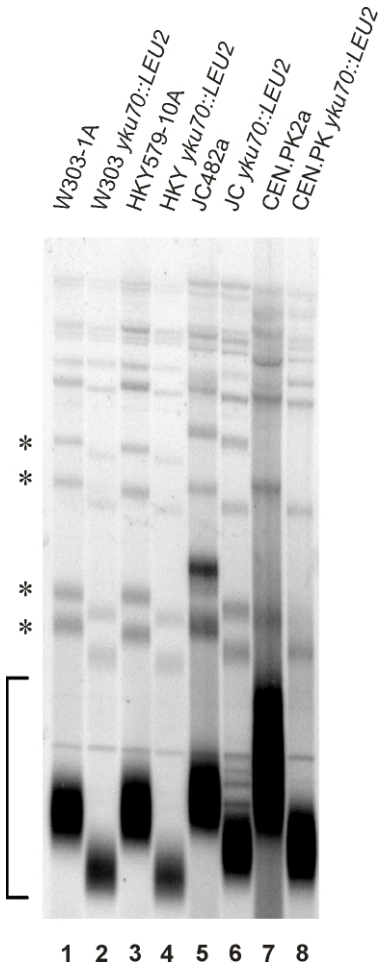

FIG. 4 [VI]. A broad telomere length variability is observed in various genetic backgrounds and the corresponding *yku70* mutant strains. Southern blot of genomic yeast DNA, probed with a telomere-specific poly$(GT)_{20}$ oligonucleotide, is shown. Genomic DNA was isolated as described in Materials and Methods, digested overnight with *Xho*I and run on a 0.8% agarose gel. The bracket indicates the telomeric GT repeat band derived from Y´ element containing chromosomes. Asterisks indicate terminal fragments derived from non-Y´ element containing chromosomes. Lane 1, W303-1A wild-type (wt); lane 2, W303-1A *yku70::LEU2*; lane 3, HKY579-10A wt; lane 4, HKY579-10A *yku70::LEU2*; lane 5, JC482a wt; lane 6, JC482a *yku70::LEU2*; lane 7, CEN.PK2-1C wt; and lane 8, CEN.PK2-1C *yku70::LEU2*.

Interestingly, wild-type telomeric GT repeat tracts varied in length in different genetic backgrounds (Figure 4, compare lane 1 and lane 7) and the telomere shortening reached different levels in *yku70* mutants (Figure 4, lanes 2, 4, 6 and 8). We observed very short telomeres in the strains W303aL (Figure 4, lane 2) and HKY579-10A *yku70* (Figure 4, lane 4), whereas the *yku70* disruption in JC482a and CEN.PK2 resulted in longer terminal restriction fragments and a significantly enhanced size distribution between individual chromosome ends as deduced by the broader smear of GT repeat tract signals (Figure 4, lanes 6 and 8). *Yku70* mutants have been shown to exhibit a growth defect at 37ºC (Feldmann and Winnacker, 1993). This temperature sensitivity can be overcome by a substantial amplification and redistribution of subtelomeric Y'elements (Fellerhoff *et al*., 2000) linking the temperature-sensitive phenotype to the telomere function of the Yku heterodimer. When wild-type and *yku70* mutants were tested for temperature sensitivity, W303aL (W303-1A *yku70::LEU2*) and BMY8 (HKY579-10A *yku70::LEU2*) showed a significant growth reduction at elevated temperatures, whereas only a slight and no difference in temperature sensitivity was observed in the JC482a and CEN.PK2-1C background, respectively (Figure 3).

For life span experiments exponentially growing cells were plated at low density on non-selective media. After 6 h at 30°C small colonies were grown from individual cells. Microscopic analysis of these microcolonies revealed a significant portion of cells that were enlarged in W303-1A *yku70* (data not shown) and HKY579-10A *yku70* (Figure 5) mutants compared to those of the corresponding wild-type. However, these changes were not detectable in the JC482a (Figure 5) and CEN.PK2-1C background (data not shown).

Thus, a *yku70* deletion introduced into different genetic backgrounds leads to impaired plasmid religation and telomere shortening, indicating that these phenotypes are general characteristics of *yku70* mutants. Strikingly, several other phenotypes described for *yku* mutants are restricted to some of the characterized strains. CEN.PK2 *yku70::LEU2* and JC482a *yku70::LEU2,* which exhibited a significant reduction in end-joining showed no sensitivity to MMS, bleomycin or elevated temperatures and displayed no morphological changes in the yeast culture (see Figures 3, 4, 5 and Table 4). Thus, these phenotypes seem to correlate with the W303 background strains W303-1A *yku70::LEU2* and HKY579-10A *yku70::LEU2*, yeast strains also exhibiting very short terminal repeats at the telomere.

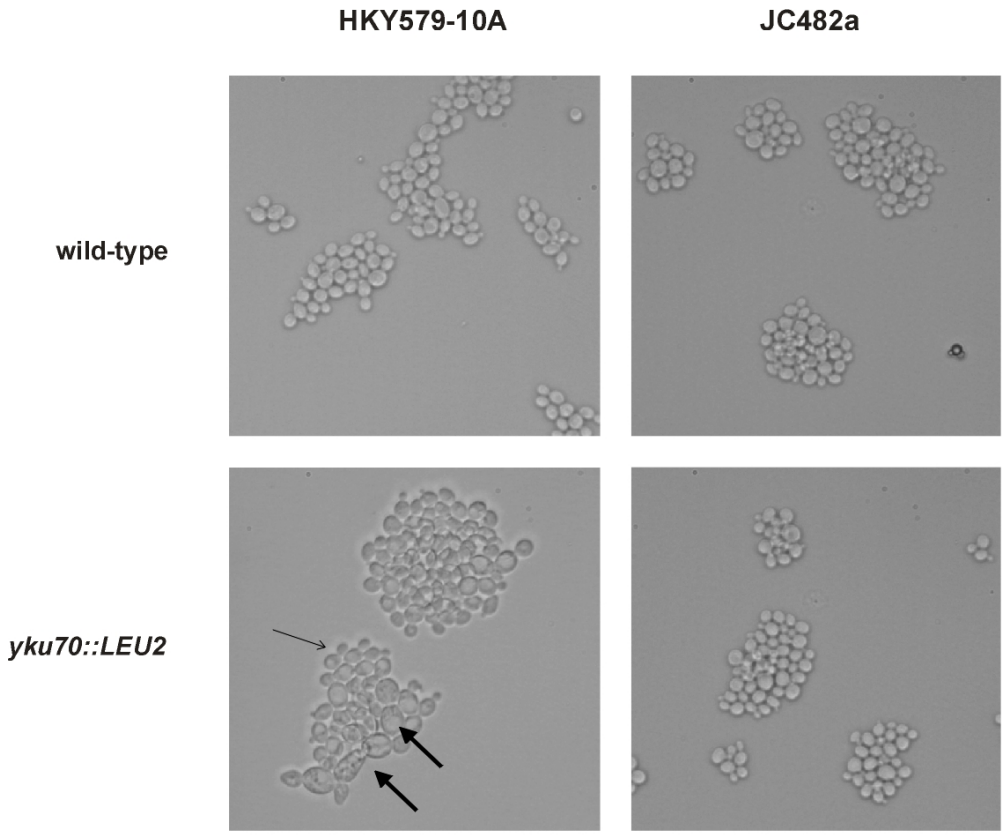

FIG. 5 [VI]. A significant percentage of enlarged cells are detectable in HKY579-10A *yku70* but not in JC482a *yku70* mutant strains. Yeast colonies were streaked onto fresh YPD media and incubated at 30°C for 24-48 h. Cells were then suspended in dH_2O to a low density and were spotted onto a microscope slide covered with a thin layer of solid YPD. After 6-10 hours of growth at 30°C, colonies were analyzed using a Leica DMLS microscope with a 40x/0,65 objective connected to the digital camera Leica DC100. The light arrow indicates cells of wild-type size, the bold arrows indicate significantly enlarged cells in one micro-colony.

Table IV [VI]. A *yku70* deletion shows phenotypic variations in different strain backgrounds.

	W303-1A *yku70::LEU2*	HKY579-10A *yku70::LEU2*	JC482a *yku70::LEU2*	CEN.PK2-1C *yku70::LEU2*
end-joining	impaired	impaired	impaired	impaired
bleomycin	sensitive	sensitive	wild-type	wild-type
methyl methanesulfonate	sensitive	sensitive	wild-type	wild-type
telomere length	very short	very short	short	short
elevated temperature	sensitive	sensitive	slightly sensitive	wild-type
morphological abnormalities	large budded cells	large budded cells	-	-
rad5-535 mutation	+	-	-	-

Overexpression of Yku70p and Yku80p from the ADH1 promoter leads to a 20-fold increase in heterodimer formation

We also investigated the effect of an overexpression of the Yku heterodimer on replicative life span. Therefore we generated haploid W303 strains carrying a genomic integration of an *ADH1* promoter in front of either *YKU70*, *YKU80* or both genes, designated as BMY9, BMY10 and BMY11, respectively (see Materials and Methods). The enhanced expression of both Yku subunits was then analyzed using anti-Yku70 and anti-Yku80 antibodies. An anti-actin antibody was used as a loading control. The affinity of the anti-Yku80 antibody Ku80-6D4 did not allow a detection of wild-type amounts of the Yku80 protein, however, a clear Yku80p corresponding signal was observed in protein extracts from BMY10 (W303-1A P_{ADH1}-*YKU80::kanMX4*) and BMY11 (W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4*) (Figure 6A, upper panel, lanes 3 and 4). The signal intensity of Yku80p was slightly enhanced in BMY11 when compared to BMY10, which might reflect a stabilization of the Yku80p when Yku70 is co-overexpressed. Analysis of the Yku70p expression levels revealed an approximately 50-fold stronger signal in BMY9 (W303-1A P_{ADH1}-*YKU70::URA3*) and BMY11 (W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4*) (Figure 6A, lanes 6 and 8) when compared to wild-type and BMY10 (W303-1A P_{ADH1}-*YKU80::kanMX4*). In both Yku70p overexpression extracts a strong degradation of the Yku70 protein was visible that was not significantly altered when the Yku80 subunit was co-overexpressed (Figure 6A, upper panel lane 8).

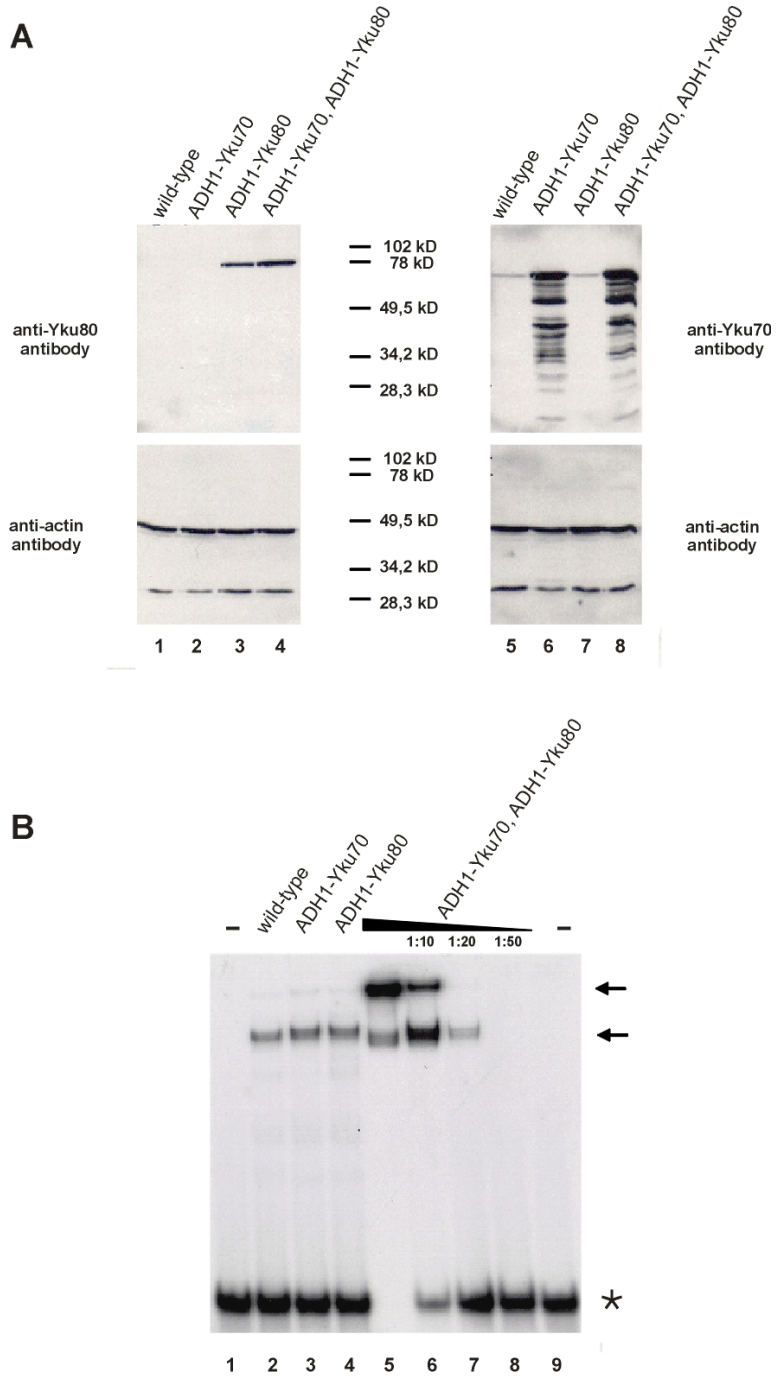

FIG. 6 [VI]. Overexpression of Yku70p and Yku80p from the *ADH1* promoter. **(A)** 40 µg crude extracts prepared from W303-1A, BMY9, BMY10 and BMY11 were used to evaluate the expression levels of Yku70p and Yku80p by Western blot. Yku70p was detected using the rat monoclonal antibody HDF-5F2; Yku80p was detected using the rat monoclonal antibody Ku80-6D4 (upper panel). Actin levels were analyzed to ensure that identical amounts of protein extracts were loaded onto the gel (lower panel). **(B)** DNA end-binding assay with crude extracts from yeast cells overexpressing one or both subunits of the Yku heterodimer. A 39 bp ds DNA oligonucleotide, PGK 1,2, was labeled with Klenow polymerase and used as a probe for DNA end-binding activity. 25 fmol oligonucleotide were incubated with 30 µg crude extract of W303-1A (lane 2), BMY9 (lane 3) BMY10 (lane 4) and BMY11 (lane 5). Additionally, serial 1:10, 1:20 and 1:50 dilutions were analyzed for BMY11, the strain overexpressing Yku70p and Yku80p (lanes 6, 7 and 8). Arrows indicate the two protein-DNA complexes, asterisks indicate the unbound oligonucleotide.

The Yku heterodimer was initially identified by its affinity to bind to double stranded DNA ends (Feldmann and Winnacker, 1993). To determine the binding activity of one or both overexpressed Yku subunits, 30 µg protein crude extracts from BMY9 (W303-1A *P_{ADH1}-YKU70::URA3*), BMY10 (W303-1A *P_{ADH1}-YKU80::kanMX4*) and BMY11 (W303-1A *P_{ADH1}-YKU70::URA3 P_{ADH1}-YKU80::kanMX4*) were tested for binding to the radioactive labeled PGK1,2 oligonucleotide (Feldmann and Winnacker, 1993).

In gel retardation experiments the overexpression of a single subunit of the Yku heterodimers (Figure 6B, lanes 3 and 4) showed no significant increase in DNA binding of crude extracts as compared to wild-type (Figure 6B, lane 2). However, the same amount of crude extracts from cells overexpressing both subunits in the genomic context led to a strongly enhanced DNA binding and no unbound PGK1,2 oligonucleotide was detectable (Figure 6B, lane 5). Moreover, an additional slow migrating DNA-protein complex became prominent that has been described using high protein amounts (Feldmann and Winnacker, 1993) and is caused by the binding of Yku molecules to both ends of the oligonucleotide (H.M. Feldmann, personal communication). This high molecular band disappeared upon progressive dilutions of the crude extract and at a 1:20 dilution the binding of the overexpressed Yku-heterodimer (Figure 6B, lane 7) was comparable to that of the wild-type extract (Figure 6B, lane 2). Thus, crude extracts from strains overexpressing both Yku subunits exhibited at least a 20-fold increase in DNA binding in an electrophoretic mobility shift assay (EMSA).

Whereas protein levels of Yku70p were about 50-fold increased under the control of the *ADH1* promoter, the titration experiments indicate that only a simultaneous overexpression of both subunits increases the amount of a functional heterodimer. The genomically integrated *ADH1* constructs described in this study thereby led to the formation of a functional heterodimer that results in an at least 20-fold increase in Yku DNA binding activity.

Overexpression of the Yku heterodimer induces a slight reduction in telomere length

Since BMY11, carrying genomically integrated overexpression constructs for both Yku subunits, exhibited a strong increase in DNA binding in an EMSA, we tested this strain for Yku related phenotypes. Overexpression of the heterodimer did not induce sensitivity to elevated temperatures, bleomycin or MMS (see Table 5).

Furthermore this strain was morphologically indistinguishable from wild-type cultures (data not shown). However, when BMY11 was analyzed for telomere length, we revealed a slight reduction in length of the terminal GT repeat tract (Figure 7, lane 4) as compared to W303 wild-type (Figure 7, lane 1) and the strains overexpressing a single subunit of the Yku heterodimer (Figure 7, lanes 2 and 3). Thus, a strong overexpression of the Yku heterodimer, as in BMY11, does effect telomere length.

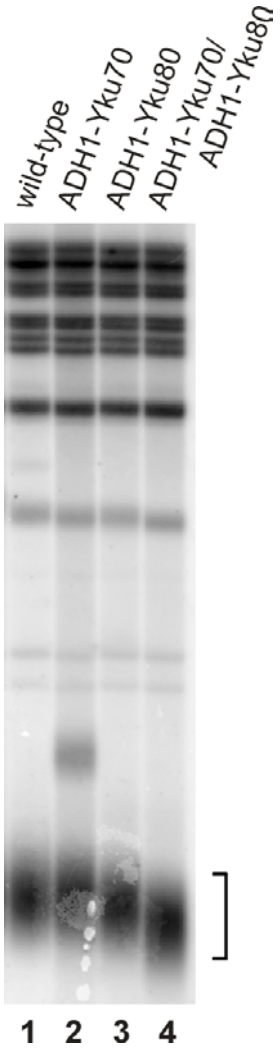

FIG. 7 [VI]. Telomere length of W303a overexpressing one or both Yku subunits. Southern blot of genomic yeast DNA, probed with a telomere-specific poly(GT)$_{20}$ oligonucleotide, is shown. Genomic DNA was digested overnight with *Pst*I and run on a 1% agarose gel. Lane 1, W303a wild-type; lane 2, BMY9 (W303-1A P_{ADH1}-*YKU70::URA3*); lane 3, BMY10 (W303-1A P_{ADH1}-*YKU80::kanMX4*); and lane 4, BMY11 (W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4*). The bracket indicates the telomeric GT repeat band derived from Y´ element containing chromosomes.

Table V [VI]. Phenotypic comparison of W303-1A wild-type, *yku70* mutant and a W303a strain overexpressing the Yku heterodimer.

	W303-1A	W303-1A *yku70::LEU2*	W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80:: kanMX*
end-joining	wild-type	impaired	n.d.
bleomycin	wild-type	sensitive	n.d.
methyl methanesulfonate	wild-type	sensitive	wild-type
telomere length	wild-type	short	slightly shortened
elevated temperature	wild-type	sensitive	wild-type
morphology	wild-type	enlarged cells	wild-type

n.d. = not determined

Overexpression of the Yku heterodimer induces life span shortening comparable to a yku70 deletion

We then investigated the influence of the Yku overexpression on replicative life span. BMY11 (W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4*) showed a significantly reduced life span when compared to the isogenic wild-type (Figure 6). The overexpression of both Yku subunits under the *ADH1* promoter resulted in life span shortening of 26%, from 27 generations in wild-type to 20 generations in BMY11 (Table 6). Thus the overexpression of the Yku heterodimer does not lead to MMS, bleomycin or temperature sensitivity, however the Yku overexpressing strain has slightly shortened telomeres and shows a life span comparable to the life span observed for isogenic *yku70* mutants.

Table VI [VI]. Mean and maximum life span obtained for W303-1A wild-type and BMY11.

Strain	**Mean**	**Maximum**	**% Reduction in mean life span compared to wild-type**
W303-1A	26,7	38	
BMY 11 (W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4)*	21	35	21,4 %

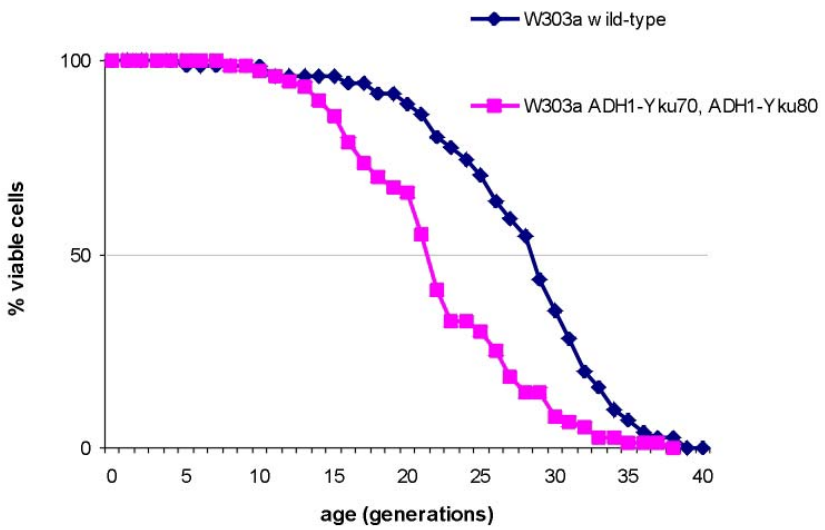

FIG. 8 [VI]. Overexpression of the Yku heterodimer leads to shortened life span in W303-1A. Survival curves are shown for W303-1A wild-type, 71 cells; and W303-1A P_{ADH1}-*YKU70::URA3* P_{ADH1}-*YKU80::kanMX4*, 67 cells.

Discussion

We have investigated the role of the Yku heterodimer on replicative life span in *Saccharomyces cerevisiae*. A deletion of *YKU70* reduced the mean life span by 6 and 7 generations in W303a *rad5-535* (W303-1A) and W303a RAD5$^+$ (HKY579-10A), respectively, whereas a JC482a *yku70* mutant displayed a mean life span that was approximately 3 generations shorter than that of the isogenic wild-type (see Figure 1A, B, D and Table 2). A fourth strain tested, CEN.PK2, showed only a slight reduction of the replicative capacity in the *yku70* mutant. Hence, the analysis of a larger cohort of cells might be necessary, although it will be difficult to evaluate the significance of small differences in generation time due to the very short mean (15 generations) and overall life span (25 generations) of this yeast strain. The comparison of the two genetic backgrounds that exhibit a traceable shortening in life span revealed that the *yku70* deletion reduced life span to a significantly smaller extend in the long living JC482a strain (9%) than in the W303 background strains (24% and 26%) (see Table 2). However, previous experiments performed by Pichova *et al.*, (1997) have shown that the deletion of *ras2* increases life span in the

JC482a background, which has a maximum life span of over 50 generation, to a lower extend than in a KT308, a strain with a maximum life span of about 30 generations. Therefore, the severity of an alteration in life span caused by a specific mutation does not correlate with the overall dividing capacity of the given yeast strain but is influenced by the genetic background.

In addition to the shortened life span in the different genetic backgrounds we obtained a shortened life span in a homozygous diploid *yku70/yku70* mutant strain (Figure 2), indicating that the effect is not restricted to the haploid state as described for *sir4*Δ strains (Kaeberlein *et al*., 1999). Therefore, our data provide evidence that the loss of Yku70p function interferes with wild-type life span in *Saccharomyces cerevisiae*.

To get insights how the *yku70* deletion might influence replicative life span, we determined the phenotypes of all four *yku70* mutant strains used in this study in more detail. Strikingly, several phenotypes that have been described for *yku* mutant strains are not detectable in all strain backgrounds. W303-1A *yku70* and HKY579-10A *yku70* mutants exhibited sensitivity to bleomycin, MMS and elevated temperatures (Figure 3) as described previously (Feldmann *et al*., 1996; Feldmann and Winnacker, 1993; Mages *et al*., 1996). Moreover, we observed an increased portion of enlarged cells in colonies that has been grown from singled cells (Figure 5). In contrast, no enhanced sensitivity to DNA damaging agents could be detected for JC482a and CEN.PK2 (Figure 3) and both strains had no visible morphological abnormalities compared to wild-type (Figure 5). Furthermore CEN.PK2 *yku70* did not exhibit any growth reduction at 37°C as compared to wild-type and only a very mild, if any, effect was detectable in JC482a *yku70*, a background that is *per se* very sensitive to elevated temperatures. The analysis of telomere length in the four mutant backgrounds showed that a broad variability in telomere length exists in different wild-type strains. A deletion of *YKU70* in the two W303 background strains led to shorter terminal restriction fragments than in CEN.PK2 or JC482a. However, all four yeast strains exhibited shortened telomeres and were deficient for nonhomologous end-joining as indicated by a reduced efficiency in plasmid re-ligation (data not shown). Based on these analysis W303-1A *yku70*, HKY579-10A *yku70* and JC482a *yku70*, which induce a life span reduction, shared only two phenotypes namely a defect in NHEJ and reduced telomere length.

Interestingly, the defect in NHEJ was not always accompanied with enhanced sensitivity of *yku70* mutants to bleomycin and MMS. This enhanced sensitivity rather correlated with the appearance of enlarged cells in the culture, temperature sensitivity and very short telomeres.

Several DNA repair proteins involved in homologous recombination have been reported to induce premature aging and early onset of reduced silencing at the mating type loci, whereas single-strand annealing factors and genes involved in nucleotide excision repair and transcription coupled repair do not influence life span (Park *et al*., 1999). Yeast strains deleted for *rad52* show a 70% reduction in mean life span as compared to wild-type. Since a *rad52* mutation abolishes the repair of DNA lesions by homologous recombination (Borts *et al*., 1986), these mutants are likely to be dying prematurely due to unrepaired DSBs (Park *et al*., 1999). Hence, the loss of the non-homologous DNA repair pathway in *yku70* mutants might also result in a reduction in replicative life span. However, a deletion of *LIG4*, the essential ligase for NHEJ, introduced into a haploid W303a strain does not influence replicative life span (Kaeberlein *et al*., 1999), indicating that a defect in NHEJ does not induce premature aging.

Sgs1 mutants, deleted for the yeast WRNp homologue, exhibit a slow growth phenotype (Watt *et al*., 1995) and show a 40% reduced life span (Sinclair *et al*., 1997). They have been reported to stochastically stop dividing as large budded cells due to a G2/M cell cycle arrest (McVey *et al*., 2001). In the *yku* mutant colonies, however, the morphological phenotype is different: the occurrence of enlarged, sometimes mis-shaped cells in *yku* mutant colonies might more likely be a visible effect of an increased amount of old cells in the culture as described in Pichova *et al.* (1997). This effect is detectible only in the W303 background and not in JC482a, which might be correlated to the strong life span reduction. To determine whether these colonies indeed reflect late replicating cells, they could be analyzed for their mating ability, one hallmark phenotype in yeast aging (reviewed in Jazwinski, 1999). In contrast, if cells arrest more often thereby inducing reduced life span one must predict that this phenotype is not caused by a reduced DNA repair efficiency since it can not be detected in *lig4* mutants.

Changes in telomere length and telomere silencing have been described to influence replicative life span in *Saccharomyces cerevisiae* (Kennedy *et al*., 1995; Kennedy *et al*., 1997). Thus the loss of Yku70p function at the telomere might evoke shortened life span. This hypothesis is supported by the finding that *lig4* mutants have been described to have wild-type telomeres (Teo and Jackson, 1997) indicating that Lig4p does not share Yku function at the telomere. Besides telomere shortening, Yku mutants have been described to exhibit reduced telomere silencing (Gravel *et al*., 1998; Laroche *et al*., 1998; Nugent *et al*., 1998) and a special structure namely a ss-overhang at the chromosome ends that persists throughout the cell-cycle (Gravel *et al*., 1998). In our phenotypic analysis we have not investigated telomeric silencing. However, silencing at telomeres as well as at the mating type loci *HML* and *HMR* is fully dependent on the presence of a functional *SIR* complex. A disruption of the *SIR4* gene results in the expression of telomere adjacent genes and *HML* and *HMR*. Loss of either *SIR4* or *SIR3* results in a 20% reduction in mean lifespan (Kaeberlein *et al*., 1999), however, this is caused by the simultaneous expression of the usually silent mating type loci and can not be detected in diploids strains as shown for *yku70* mutants (Figure 2). Therefore it seems unlikely that a loss of telomeric silencing causes life span reduction in *yku70* mutants.

Interestingly, BMY11, a W303 strain overexpressing Yku70p and Yku80p under control of a strong *ADH1* promoter, shows a reduction in life span comparable to that seen in isogenic *yku70* mutants (compare Figure 8 and Figure 1A). A strong overexpression of a protein can induce a variety of pleiotropic effects and might therefore lead to a significant decrease in cell viability. The Yku overexpression, however, did not lead to detectable growth defects and the microscopic analysis revealed no morphological differences between the wild-type and the Yku overexpression strain. In addition, no sensitivity to MMS, bleomycin or elevated temperatures could be observed. Thus, the overexpression did not induce these *yku70* mutant associated phenotypes. Strikingly, when we analyzed the overexpression strains for telomere length, we could detect a slight but significant decrease in telomere length as compared to wild-type or strains overexpressing only one subunit of the Yku heterodimer (Figure 7). It is therefore tempting to speculate that the reduced telomere length induces premature aging in *yku* mutants. However, the telomere shortening in strains overexpressing the Yku heterodimer is very remote.

A comparable slight telomere shortening is observed in *sir4* mutants even if only one MAT locus is expressed (see Chapter IV). Thus telomere shortening *per se* does not induce a life span reduction. Thus, the end structure of the telomere in *yku* mutants might be more likely influencing life span than the telomere length. The overall structure of the telomere is difficult to characterize, however, it would be interesting to determine whether a ss-overhang can be detected for the BMY11 strain overexpressing both Yku subunits.

Preliminary data indicate that overexpression of Est2p under an *ADH1* promoter can compensate for telomere shortening in *yku* mutants (H. M. Feldmann, pers. communication). The life span analysis of a *yku* mutant overexpressing Est2p might therefore provide further evidence that a defect at the chromosome ends leads to a shortened life span in *yku70* strains.

Furthermore, the analysis of *yku* mutants which are only defective in telomere length regulation or DNA repair would not only allow to address the cause of MMS and bleomycin sensitivity but would moreover provide a useful tool to ascertain that the loss of *yku* telomere function correlates with reduced life span. The observation that Yku is not directly bound to subtelomeric regions but is recruited by the *SIR* complex (Martin *et al*., 1999) allows to predict that such separation of function mutants can be found.

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ABBREVIATIONS

aa	amino acid(s)
AD	transcription activation domain
bp	base pair(s)
BD	DNA binding domain
BLAST	basic local alignment search tool
bp	base pair(s)
cm	centimeter(s)
dH_2O	distilled water
DNA	desoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ds	double stranded
DSB	DNA double strand break
dsDNA	double stranded desoxyribonucleic acid
E. coli	*Escherichia coli*
EMSA	electrophoretic mobility shift assay
FIG	figure
G418	geneticin
Gal	galactose
Glc	glucose
HIS3	Imidazoleglycerolphosphate dehydratase gene
kb	kilo base pairs
kD	kilo Dalton
lacZ	β-galactosidase gene
LEU2	3-Isopropylmalate dehydrogenase gene
M	molar
Mbp	mega base pairs
MMS	methyl methanesulfonate
µg	microgram
µl	micro liter
NHEJ	non homologous end-joining
PCR	polymerase chain reaction
RNA	ribonucleic acid
SC	synthetic complete medium
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
ss	single-stranded
ssDNA	single-stranded desoxy ribonucleic acid
TRP1	Phosphoribosylanthranilate isomerase gene
TPE	telomere position effect
URA3	Orotidine-5'-phosphate decarboxylase gene
WRN	Werner syndrome gene
wt	wild-type
x-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast extract peptone dextrose medium