Subtelomeric Repeat Amplification Is Associated With Growth at Elevated Temperature in yku70 Mutants of Saccharomyces cerevisiae

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ABSTRACT

Inactivation of the Saccharomyces cerevisiae gene YKU70 (HDF1), which encodes one subunit of the Ku heterodimer, confers a DNA double-strand break repair defect, shortening of and structural alterations in the telomeres, and a severe growth defect at 37°C. To elucidate the basis of the temperature sensitivity, we analyzed subclones derived from rare yku70 mutant cells that formed a colony when plated at elevated temperature. In all these temperature-resistant subclones, but not in cell populations shifted to 37°C, we observed substantial amplification and redistribution of subtelomeric Y′ element DNA. Amplification of Y′ elements and adjacent telomeric sequences has been described as an alternative pathway for chromosome end stabilization that is used by post-senescence survivors of mutants deficient for the telomerase pathway. Our data suggest that the combination of Ku deficiency and elevated temperature induces a potentially lethal alteration of telomere structure or function. Both in yku70 mutants and in wild type, incubation at 37°C results in a slight reduction of the mean length of terminal restriction fragments, but not in a significant loss of telomeric (CnA/TGn)n sequences. We propose that the absence of Ku, which is known to bind to telomeres, affects the telomeric chromatin so that its chromosome end-defining function is lost at 37°C.

Telomeres are specialized structures at the ends of linear chromosomes. In Saccharomyces cerevisiae, telomeric DNA consists of a simple repeat tract, commonly abbreviated as (CnA/TGn)n, which in wild-type cells is ~300-bp long (for review see Zakian 1996). The exact length of individual telomeric repeat tracts varies, reflecting a dynamic state that is kept approximately stable by a balance between lengthening and shortening reactions. Telomeres shorten after each round of replication because of the inability of DNA polymerase to start de novo synthesis; after removal of the most distal telomeric RNA primer a gap is left at the 5′ end of the newly synthesized strand. This shortening, as well as shortening potentially arising from nucleolytic degradation, is counteracted by the ribonucleo-enzyme telomerase, which elongates the 3′ ends of telomeric DNA by adding TGn repeats (for reviews see Greider 1996; Zakian 1996). The S. cerevisiae genes TEL1 and EST2 encode the RNA and the protein component of the ribonucleoprotein telomerase, respectively (Singer and Gottschling 1994; Counter et al. 1997; Linder et al. 1997a). As expected, inactivation of one or both genes results in a progressive decline of the length of the terminal telomeric repeat tract with each round of replication, as was seen by shortening of terminal restriction fragments (TRFs). This telomere shortening is accompanied by a considerable drop in viability after growth for 50–100 generations (Singer and Gottschling 1994; Lendvay et al. 1996). The same characteristic phenotypes have been observed in strains mutated for the genes EST1, EST3, or EST4 (CDC13; Lundblad and Szostak 1989; Lundblad and Blackburn 1993; Lendvay et al. 1996; Nugent et al. 1996). Although not required for telomerase activity in vitro (Linder et al. 1997b), the products of these genes are thought to be involved in proper telomerase function in vivo, e.g., by loading the telomerase onto the chromosomal ends (for review see Nugent and Lundblad 1998). The loss of viability that is characteristic for tel1 and est mutants has been termed senescence (Lundblad and Szostak 1989). In late cultures of senescent strains so-called post-senescence survivors arise, which employ a RAD52-dependent, telomerase-independent mechanism of chromosome end stabilization that is manifested by global amplification of subtelomeric Y′ elements and telomeric repeat tracts (Lundblad and Blackburn 1993; Lendvay et al. 1996). This alternative chromosome end stabilization may either proceed by recombination of internal (CnA/TGn)n sequences onto the chromosome ends, as was initially proposed (Lundblad and Blackburn 1993), or by a break-induced replication mechanism. According to the break-induced replication model, chromosome ends, which, after telomere attrition, appear as sites of chromosome breaks, invade homologous targets (i.e., telomeric or subtelomeric regions of other chromosome ends), and replicate all the sequences to the end of the “donor” chromosome (Bosco and Haber 1998; Le et al. 1999).

Shortening of telomeric repeat tracts may result not
only from telomerase deficiency, but also from a disturbance of proper telomere length regulation. For example, \textit{tel1} and \textit{tel2} mutants do not respond to factors that influence telomere length in wild-type cells, suggesting that these mutants are impaired in telomere length regulation (Runge and Zakian 1996). In these mutants, which do not exhibit senescence, reduction of the mean TRF length by \( \sim 200 \) bp becomes fully manifest only after \( \sim 100 \) generations of growth; thereafter a new steady-state level of telomere size is established (Lustig and Pets es 1986; Runge and Zakian 1996).

Reduction of the mean TRF length has also been observed in \textit{yku70} and \textit{yku80} mutants (Boulton and Jackson 1996b; Porter et al. 1996). \textit{YKU70} (HDF1) and \textit{YKU80} (HDF2) encode proteins that exhibit significant homology to the mammalian Ku70 and Ku80 proteins, respectively (Feldmann and Winnacker 1993; Feldmann et al. 1996; Milne et al. 1996). Like their mammalian counterparts, Yku70p and Yku80p form a heterodimeric complex that binds with high affinity to double-stranded DNA ends (Milne et al. 1996). By a variety of different assays it has been shown that the yeast Ku-like proteins are required for the repair of DNA double-strand breaks (DSBs) by a nonhomologous end-joining pathway (Boulton and Jackson 1996a; Milne et al. 1996; Tsukamoto et al. 1996; Friedl et al. 1998). In addition to the repair deficiency, \textit{yku70} and \textit{yku80} mutants display a considerable growth defect at elevated temperature. On solid media at 37\(^\circ\)C, the plating efficiency is very low in both mutants and the cells appear to arrest in a large-budded state (Feldmann and Winnacker 1993; Boulton and Jackson 1996b; Feldmann et al. 1996; Siede et al. 1996; Barnes and Rio 1997).

Several recent investigations addressed the role of the yeast Ku-like proteins in telomere metabolism. It has been shown that inactivation of \textit{YKU70} or \textit{YKU80} leads to an alteration of the structure of the chromosomal termini, in that the G-rich strand forms a long single-stranded tail during all phases of the cell cycle (Polotnianka et al. 1998; Zakian et al. 1998). In wild-type cells, this tail is detectable in late S-phase only and is thought to arise by specific 5'-3' exonuclease activity (Wel linger et al. 1993, 1996). Individual, artificially elongated telomeres shrink to the strain-specific average length more rapidly in the absence than in the presence of Ku, leading to the proposal that Ku protects telomeres against nucleolytic degradation and recombination events (Polotnianka et al. 1998). Lack of functional Ku-like proteins has also been shown to result in a deficiency in transcriptional repression of genes located close to the telomeres [telomere position effect (TPE)] but not in derepression of the silent mating type loci (Boulton and Jackson 1998; Evans et al. 1998; Lar oche et al. 1998; Nugent et al. 1998). Ku interacts with the silencing protein Sir4p, which may explain its role in TPE (Tsukamoto et al. 1997). Fluorescence in situ hybridization has shown that the clustering of telomeres and their localization near the nuclear periphery are also affected by inactivation of Ku (Laroche et al. 1998). Although the TRF lengths are comparable in Ku- and \textit{tel1} mutants, the latter are normal with respect to rapid shortening of elongated telomeres (Li and Lust ig 1996), appearance of G-rich tails (Gravel et al. 1998), and telomere clustering and localization (Laroche et al. 1998), and they are affected only slightly with respect to telomere position effect (Boulton and Jackson 1998), demonstrating that these phenotypes in \textit{dfl1} or \textit{yku80} mutants are not simply a consequence of short telomeres. Rather, the data suggest a distinctive role for the Ku proteins at the telomeres. Indeed, in vivo crosslinking demonstrated that Yku80p binds to telomeric DNA (Gravel et al. 1998).

The present report aims at further characterizing the role of \textit{Yku70p} in telomere metabolism. We show that, even after prolonged subcultivation, inactivation of \textit{YKU70} does not affect cell viability at 30\(^\circ\)C, although a new steady-state TRF length is established that is \( \sim 200 \) bp shorter than in wild-type cells. In spite of a severe growth defect at 37\(^\circ\)C, some \textit{yku70} mutant cells are able to escape the temperature-induced lethality and form colonies at 37\(^\circ\)C. We observed considerable, RAD52-dependent amplification of subtelomeric \( Y^+ \) DNA in these temperature-resistant subclones of \textit{yku70} mutant strains, and we propose that the absence of the Ku-like proteins at the telomeres together with some temperature-dependent effect initiates a potentially lethal process that can be bypassed by a telomerase-independent, alternative mechanism of chromosome end stabilization. No indications for an increased loss of telomeric \( (C_{13}A/ T_{13})_n \) DNA in \textit{yku70} mutants compared to wild-type or \textit{tel1} mutants were found after a shift to elevated temperature. We propose that telomeres in Ku-deficient cells lose their telomere-defining chromatin structure at elevated temperature and appear as sites of chromosomal breaks, thus leading to cellular response reactions like recombinative events and G2 arrest.

**MATERIALS AND METHODS**

**Yeast strains:** The strains used in this study are described in Table 1. The diploid \textit{YKU70/ yku70A::URA3} strain WS9132, which was obtained by mating strain SX 46A \textit{yku70A} with strain WS8105-1C (Siede et al. 1996), exhibits the same telomere length as does a cross of wild-type strains SX 46A and WS8105-1C (A. A. Friedl, unpublished data). In the construct used for one-step gene disruption (Rothstein 1983) of the \textit{YKU70} gene, the \textit{URA3} gene replaced a HindIII fragment of the \textit{YKU70} open reading frame (ORF), thus disrupting the ORF at amino acid position 300 (Siede et al. 1996). Strain WS9132 was sporulated using standard methods. Two complete tetrads (spore clones 1a-d and 2a-d) were further investigated. Some of the results obtained with the spore clones were confirmed by experiments performed in strains of different genetic background carrying the same \textit{yku70A::URA3} allele or a \textit{yku70A::TRP1} allele disrupting the ORF at the same position. These strains were derived from MK166 (Liefshitz et al. 1995).
or W303, both kindly donated by M. Kupiec. Because of the formal possibility that these yku70 disruption mutations allow expression of a truncated Yku70 protein, some results were also confirmed in a strain with a genetic background similar to that of the spore clones (i.e., SX46A) carrying a complete deletion of the YKU70 open reading frame. This was obtained by PCR-based gene deletion (Baudin et al. 1993) with a PCR product representing the URA3 gene flanked by sequences homologous to sequences located 5' of the start codon and 3' of the stop codon of the genomic YKU70 gene. For construction of tel1 mutants, the 5' flanking region and the first 1770 bp of the TEL1 ORF were amplified by PCR from strain SX46A. After cloning, an 883-bp EcoRI-AfIII fragment encompassing the first 657 bp of the ORF, the start codon, and the promoter region was released and substituted by an EcoRI-Cfr10I fragment encompassing the TRP1 gene. The resulting tel1Δ::TRP1 construct was released from the vector and transformed into strain SX46A to construct strain SX46A tel1Δ. Construction of strains SX46A yku70Δ rad52Δ and WS8105-1C yku70Δ rad52Δ has been described (Siede et al. 1996).

Subcultivation and determination of growth characteristics: The dissected spore products of strain WS9132 were allowed to grow on solid YPD medium at 30° for 3 days, and then the colonies were completely transferred to 20 ml of liquid YPD medium. After overnight growth at 30°, the cell titer was determined to allow an estimation of the number of generations passed since spore germination. A total of 5 x 10⁶ cells were used to inoculate fresh medium, and aliquots representing 100 and 1000 cells were plated on solid medium and incubated at 30° (4 days) and 37° (up to 14 days) for a determination of the plating efficiency. The remainder of the suspension was concentrated by centrifugation, resuspended in liquid complete medium containing 10% glycerol, and frozen at –20°.

Subcultivation steps were repeated six times to monitor the cells at intervals of ~15 generations between generation 30 and generation 120. Strain SX46A tel1Δ was subcultivated in a similar manner, except that transformants arising after one-step gene disruption on selective solid medium were first cultivated for one round in selective liquid medium before they were further subcultivated in YPD medium. Successful disruption of the TEL1 gene was later verified by Southern analysis. To isolate single cell subclones, colonies arising after plating at 30° and 37° were transferred to 10 ml liquid complete medium and grown at 30° for 2 days. Two milliliters of the suspensions was used to generate frozen stocks, and the remaining suspension was used to prepare DNA for genomic Southern analysis.

Fluctuation analyses were performed by transferring 6-12 complete single colonies (obtained after growth at 30° for 3 days) to 1 ml water and plating onto YPD plates after appropriate dilution. Plates were incubated at 30° or 37° for 4 days. To determine the median frequency of cells forming a colony at 37° per cell growing at 30°, the data of two to three experiments were pooled. The rates of formation of temperature-resistant cells per cell generation were estimated for each experiment individually, using the m estimator, which accounts for situations where only a proportion of the cells of a colony is plated under selective conditions (Jones et al. 1994), and the mean of the values obtained in the different experiments is indicated.

Determination of telomere length and Y′ amplification in genomic Southern blots: Genomic DNA was isolated using a method based on cell wall disintegration by vortexing with glass beads and phenol extraction (Hoffman and Winston 1987), digested overnight with XhoI using standard procedures (Sambriski et al. 1989), and separated on 0.8% agarose gels. After electrophoresis, the gels were blotted by alkaline capillary transfer onto nylon membranes (Qiagen, Inc., Chatsworth, CA). For a determination of telomere length, the blots were preincubated in blocking buffer (5 x SSC, 1% N-lauroyl sarcosine, 1 x Denhardt’s solution) for 1 hr at 34°, and were hybridized for 3 hr at 34° in fresh blocking buffer containing oligonucleotide probes that were end-labeled with 32P by polynucleotide kinase. To probe for the terminal telomeric tract, oligonucleotides (CA)n (C10nA) (CCC ACA CAC ACC CAC AC) or (G11nT) (GTG TGG GTG TGG TGT GGG) were used. After washing for 15 min at room temperature in 2 x SSC, 0.1% SDS, the blots were autoradiographed. The autoradiographs were scanned with a GT 9000 scanner (Epson, Torrance, CA) and evaluated using the RFLPscan software (Scanalytics, Billerica, MA) to determine the peak of the signal intensity distribution of the major telomeric band.

For an estimation of the amount of Y′ elements, the blots were stripped and rehybridized first with a Y′ probe and then with a probe for CEN4, which can be used as internal control for the amount of DNA loaded (Conrad et al. 1990). The Y′ probe, a gel-purified PvuII-KpnI fragment, was isolated from plasmid pCE-T27, which was kindly provided by W. Oertel.
was repeatedly subcultivated in liquid medium at 30°C to determine the plating efficiency at 30°C and samples were taken after each subcultivation step. Length reductions were also observed in spore clones of a mutant allele (data not shown). Each spore clone was grown for further information). We compare the signal locating at 1150 bp), and by::URA3,::URA3 detection and quantification of telomere repeats. The ends of chromosomes bearing Y' elements appear as broad band at the bottom of the gel (arrow), and the ends of chromosomes lacking Y' elements are visible as additional bands (asterisks). Bands of higher molecular weight represent additional Y'-lacking chromosome ends and fragments with internally located CA repeats. Lanes 1–4, DNA isolated from spore 2c after 30 (5), 45 (6), 60 (7), 75 (8), 90 (9), 105 (10), and 120 (11) generations. Lanes 12, DNA isolated from strain SX46A yku70Δ after subcultivation for >200 generations. Size markers on the right side indicate 4, 3, 2, 1.6, and 1 kb (from top to bottom).

Figure 1.—TRF length in spores 2a (YKU70) and 2c (yku70Δ::URA3) in dependence of the number of generations passed since germination. Xhol-digested DNA was probed with a (CA)10 oligonucleotide recognizing the sequence of the terminal telomeric repeats. The ends of chromosomes bearing Y' elements were detected, and the new telomere length was found.

A new steady-state level of telomere length is established in yku70 mutant spore clones. To analyze the phenotype of yku70 mutants in dependence of the number of generations passed since inactivation of the gene, a YKU70//yku70Δ::URA3 heterozygous strain (WS9132) was sporulated, and the products of two complete tetrads (spores 1a-d and 2a-d) were characterized further. Southern hybridization experiments revealed that spore clones 1a, 1d, 2a, and 2b carry the YKU70 wild-type gene, while spore clones 1b, 1c, 2c, and 2d carry the yku70 mutant allele (data not shown). Each spore clone was repeatedly subcultivated in liquid medium at 30°C and samples were taken after each subcultivation step to determine the plating efficiency at 30°C and 37°C (see below) and the telomere length. To analyze the telomere length, genomic DNA was digested with XhoI, which cuts at a conserved site in the subtelomeric Y' elements (Chan and Tye 1983), resolved by gel electrophoresis, transferred to nylon membranes, and hybridized with (CA)10 or (G1-3T)n. Using this method, the terminal telomeric fragments of chromosome ends bearing Y' elements migrate in a single broad band, while subtelomeric C13A/TG13 arrays and chromosome ends lacking the Y' elements result in additional bands (Walmsley et al. 1985).

Figure 1 shows that the length of the fragments in the main telomeric band of spore clone 2a (YKU70) remains constant between 30 and 120 generations after spore germination (the earliest and latest timepoints investigated), with the maximum of the hybridization signal located at ~1330 bp. In spore clone 2c (yku70), the length of the fragments in the main telomeric band is reduced by ~180 bp after 30 generations (maximum signal at 1150 bp), and by ~210 bp after 45 generations (maximum signal at 1120 bp). The minor bands originating from chromosome ends lacking Y' sequences are also shortened by ~200 bp in the yku70 mutant spore. Between generations 45 and 120, no further alterations were detected, and the new telomere length was found to be the same as in a sample of strain SX46A yku70Δ grown for >200 generations (Figure 1, lane 12). Similar length reductions were also observed in spore clones 1b, 1c, and 2d, and in a variety of yku70 mutant strains of different genetic background (data not shown; see materials and methods for further information).
conclude that telomeres do not shorten progressively in yku70 mutant cells, but rather that a new equilibrium length is established after growth for ~30-45 generations.

**Inactivation of YKU70 does not affect viability at 30°, but impairs colony formation at 37°:** To determine the growth characteristics in dependence of the replicative age, samples taken after the individual subcultivation steps were plated on solid medium and incubated at 30° or 37°. In all eight spore clones, the plating efficiency at 30° was similar (close to 100%), and it remained constant between 30 and 120 generations of growth after sporulation. Furthermore, the colony size as well as the cellular yield after each subcultivation step were comparable for all spores (data not shown). Similarly, even after extensive subcultivation (i.e., for >200 generations), we have never detected any indication for growth defects at 30° in any of our various yku70 mutant strains (including a strain lacking the entire YKU70 open reading frame).

When plated at 37°, in the wild-type spore clones 1a, 1d, 2a, and 2b the plating efficiency at 37° was as high as at 30° and remained constant until generation 120. In contrast, already at generation 30 after germination, the plating efficiency at 37° was very low in the yku70 mutant spore clones 1b, 1c, 2c, and 2d, and it remained low (fluctuating between <0.1 and 1%) until generation 120 (Table 2). Many of the temperature-resistant colonies became visible only after incubation for >8 days. Appearance of temperature-resistant subclones was also observed in yku70 mutant strains of different genetic background (A. A. Friedl, unpublished results). Generally, colonies emerging from temperature-resistant yku70 mutant cells were variable in size and often irregular in shape. When single cells derived from temperature-resistant subclones were replated at 37°, the plating efficiency generally was low (<30%), suggesting that the ability to grow at 37° is not stably transmitted.

**Y' elements are amplified in temperature-resistant subclones of yku70 mutant spores:** To elucidate the mechanism by which the yku70 mutant cells obtain an ability to grow at the restrictive temperature, a total of 42 colonies of spores 1b, 2c, and 2d that arose during the subcultivation experiment after plating at 37° were picked and expanded in liquid medium at 30°. DNA isolation, XhoI digest, and hybridization with a (CA)10 oligonucleotide revealed that the mean TRF length in these subclones is comparable to that observed in control samples grown at 30° (Figure 2). In all samples derived from temperature-resistant subclones, however, we observed that, at comparable amounts of total DNA loaded, a band of ~7-kb length hybridized substantially stronger than in the control samples. In temperature-resistant subclones of spores 1b and 2c also a second band of ~5.5 kb gave strongly enhanced hybridization.

### Table 2

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<tr>
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<td>2c</td>
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<tr>
<td>2d</td>
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*Generations passed between spore germination and plating at 37°.*

Figure 2.—Y' element amplification in temperature-resistant survivors isolated from spore 1b (yku70::URA3). DNA from control clones of spore 1b grown at 30° (lanes 1 and 9) and from temperature-resistant survivor clones of spore 1b that were obtained in the subcultivation experiment (lanes 2-8) was digested with XhoI. Clone identification numbers of temperature-resistant clones are 320 (lane 2), 322 (3), 326 (4), 324 (5), 325 (6), 323 (7), and 319 (8). (A) Probing with a labeled (CA)10 oligonucleotide shows that the length of the major TRF class (arrow) is similar in controls and temperature-resistant subclones. Horizontal bars on the left edge indicate 6, 4, and 2 kb (from top to bottom). (B) Rehybridization with a Y' probe reveals that the strong bands of ~5.5 and 7 kb represent the two size classes of Y' elements, Y' long and Y' short. The 5.5-kb band in the control samples reproduces badly, but is clearly visible on the original autoradiographs. Additional bands of higher molecular weight represent fragments containing the most centromere-proximal Y' elements and adjacent sequences. (C) Rehybridization with a CEN4 probe labels an internal fragment that is used as a standard for the amount of DNA loaded.
signals (see Figure 2A for examples derived from spore 1b).

This phenotype was strongly reminiscent of that observed in postsenescence survivors of senescent mutants, where fragments containing unit Y' elements and short stretches of internal telomeric sequence were found to be strongly amplified (Lundblad and Blackburn 1993; Lendvay et al. 1996). Indeed, rehybridization of the Southern blots with a Y' probe showed that the strongly hybridizing fragments in the temperature-resistant subclones represent Y' elements (Figure 2B). Furthermore, we found that derivatives of spores 1b and 2c contain both size classes of Y' elements (6.7 and 5.2 kb), whereas in derivatives from spore 2d only the long Y' elements appear to be present (data not shown).

To allow an estimation of the extent of Y' amplification, in a subset of the temperature-resistant clones, the signal intensities of both Y' bands were quantified after hybridization with the Y' probe and were normalized against an internal standard that was obtained by subsequent rehybridization with a probe for CEN4. Using this assay, we find that the amount of Y' DNA varies up to 2-fold between individual single cell clones of wild-type spore 2b (data not shown). This level of variation, which is seen in both size classes of Y' elements, probably reflects the normal recombinative activity at Y' elements (Louis and Haber 1990a) and/or the technical limitations of the quantification procedure. In contrast, when estimating the amount of Y' DNA in 17 temperature-resistant subclones of spore 1b (Table 3) and 8 subclones of spore 2d (data not shown), we observed a clear increase compared to control samples that were derived from subclones grown at 30°C and run on the same gels. Table 3 shows that amplification factors differ between individual temperature-resistant clones, ranging from ~5- to >100-fold, and that the relative degree of amplification of the two classes of Y' elements within given clones is variable.

When nine subclones derived from yku70 mutant spore 1b grown on solid medium at 30°C were analyzed in a similar manner, the normalized signal intensities of the ~7-kb band varied less than twofold between the clones. In eight of these clones, also the signal intensities of the ~5.5-kb band varied less than twofold. In the remaining clone, the intensity of the ~5.5-kb band was found to be 3.5 times stronger than the median intensity of the other samples (data not shown). These results suggest that Y' amplification is rare and comparatively low in yku70 mutant cells when grown at 30°C.

### Table 3

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<th>Clone ID (generation)</th>
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^a^ Generations passed between spore germination and plating at 37°C.

^b^ Ratio between normalized Y' hybridization signal intensities in temperature-resistant clones and 30°C control samples on the same gel. Normalization was performed with respect to the CEN4 signal after rehybridization.

Some chromosome ends acquire exceedingly high numbers of Y' elements: To investigate whether, in the course of Y' amplification, a comparable number of repeat units is gained by all chromosome ends or whether some individual ends acquire a disproportionately high number of repeats, we separated the chromosomal DNA of the 42 temperature-resistant yku70 mutant subclones by PFGE and analyzed the resulting banding pattern. The limited sensitivity of this method precludes a detection of small alterations in chromosome length, especially if all chromosome species have gained a similar and comparatively low number of new Y' elements. If, however, individual chromosome ends have acquired an exceedingly high number of new repeats, a clear shift in the localization of the respective bands should occur, which can be detected easily upon visual inspection of the gels. Indeed, we detected in 27/42 samples a clearly visible shift of at least one chromosomal band; in 5 of these samples three or more bands were found to be shifted (Figure 3A). The fuzziness of the altered bands (see below) precluded a detailed analysis of the karyotypic alterations in samples carrying more than two shifted bands, but in those samples that were amenable to further analysis, the altered bands were always found to be shifted towards higher molecular weight, with apparent increases in chromosome size of up to ~100 kb. Southern hybridization using a probe for the Y' elements appeared to give particularly high signals with some of the lengthened chromosomal bands (Figure 3, B and C), strongly suggesting that amplification of Y' elements is a major factor contributing to the increase in chromosome size. In some cases, the location of a shifted band was not immediately visible in the altered banding pattern. For example, in clone 319, the band corresponding to chromosome I...
Temperature Resistance in yku70 Mutants

**Figure 3.** Karyotypic alterations in temperature-resistant subclones of spore 1b (yku70Δ::URA3). A shows the negative image of a PFGE gel after staining with ethidium bromide. Lane 1, control sample of spore 1b grown at 30°C. Lanes 2-6, samples taken from temperature-resistant subclones 319 (lane 2), 322 (3), 324 (4), 321 (5), and 334 (6). Note that the amounts of DNA loaded on lanes 1 and 2 are similar; the other lanes contain more DNA. Arrows indicate examples for band shifts in temperature-resistant subclones. Asterisks indicate lost chromosomal bands in subclones 319 (band of chromosome I absent) and 334 (band of chromosome VI absent). B shows the results obtained after hybridizing a blot of the gel shown in A with a probe for Y′ elements and overnight exposure (only lanes 1 and 2 are shown). Indicated are the positions of chromosomes VI and XI, which have gained Y′ elements in the temperature-resistant subclones. C shows the same blot after exposure for 5 hr. Note that shifted bands often give rise to a particularly strong signal (arrows).

(estimated size 260 kb) seems to be lacking (Figure 3A, lane 2), but hybridization with a chromosome-specific probe showed that chromosome I in this sample has a new size of ~300 kb, thus leading to comigration with chromosome VI (data not shown).

Mostly, the bands of the lengthened chromosomes were more fuzzy than normal chromosomal bands, thus suggesting heterogeneous chromosome lengths within the cell population. In addition, we observed in many clones a high degree of heterogeneous smear over the whole length of the gel lane (see, e.g., clone 334 in lane 6 in Figure 3A). This kind of smear does not arise normally when applying our methods of DNA isolation (see, e.g., the control lane 1 in Figure 3A), and it appeared to hybridize strongly with a Y′ probe (Figure 3C, lane 6). Furthermore, Southern hybridization in a sample of clone 334 with a probe for chromosome VI (whose 300-kb band is absent in the karyotype) gave a broad band ranging from ~300 to 600 kb (data not shown), suggesting that the smear is mainly caused by heterogeneous increase in chromosome lengths and not by DNA degradation.

Like most strains of *S. cerevisiae* (Louis and Haber 1990b), spore clone 1b (grown at 30°C) does not carry Y′ elements on all chromosomal termini; rather, the Y′ element is absent from chromosomes VI and XI, as is shown by Southern hybridization (Figure 3B). However, in temperature-resistant subclones of spore 1b, these chromosomes hybridize with probes for Y′ (Figure 3, B and C), hinting at a redistribution of Y′ elements to new chromosomal sites. Since bands indicative for chromosome ends lacking Y′ elements were still visible when DNA of temperature-resistant subclones was digested with XhoI (Figure 2), however, it appears that within a population derived from a temperature-resistant colony not all chromosome ends carry Y′ elements.

When we karyotyped subclones of yku70 mutant spores 1b, 1c, 2c, and 2d that were obtained in the subcultivation experiment after plating at 30°C, we found fuzzy lengthening of one chromosome in 1 out of 56 subclones analyzed; the karyotypes of the other subclones were normal. Assuming that chromosome elongation is at least in part due to Y′ amplification, this shows again that the amplification process occurs rarely in cells grown at 30°C.

**Amplification of Y′ elements in yku70 mutants requires RAD52:** Amplification of subtelomeric repeats in post senescence survivors of senescent yeast mutants depends on RAD52 (Lundblad and Blackburn 1993; Lendvay et al. 1996). To estimate the rate of formation of temperature-resistant cells in yku70 rad52 as compared to yku70 RAD52 strains, we performed fluctuation analyses with the yku70 mutant spore clones 1c and 2d and two yku70 rad52 double mutant strains that were derived from strains SX46A and WS8105-1C, respectively. All strains had been propagated for >150 generations at 30°C prior to the fluctuation experiments. The frequency of temperature-resistant clones per cell in the colony varied from <0.2 × 10⁻³ to 21 × 10⁻³ (median frequency 6.5 × 10⁻³) in spore 1c cells and from <1.2 × 10⁻³ to 360 × 10⁻³ (median frequency 3.9 × 10⁻³) in spore 2d cells. This high range of frequencies suggests that at least a part of the temperature-resistant cells did arise by spontaneous mutations as the colony grew. The rates of formation of temperature-resistant clones in spores 1c and 2d were estimated as 4.7 × 10⁻⁴ and 34 × 10⁻⁴ per cell division, respectively. In the two yku70 rad52 double mutant strains, the median frequencies of temperature-resistant cells were 1.5 × 10⁻⁶ (range <0.3 × 10⁻⁶ to 10 × 10⁻⁶) for strain SX46A yku70Δ rad52Δ and 1.0 × 10⁻⁶ (range <0.6 × 10⁻⁶ to 26 × 10⁻⁶) for strain WS8105-1C yku70Δ rad52Δ. The rates of formation of temperature cells per division were found to be three orders of magnitude lower than in the yku70 single mutants (3.6 × 10⁻² and 7.3 × 10⁻² per cell division for strains SX46A yku70Δ rad52Δ and WS8105-1C yku70Δ rad52Δ, respectively). These data show that the ability to form temperature-resistant cells in yku70 mutants depends largely on a functional RAD52 gene.

We investigated the structure of telomeric and telomere-associated DNA in seven independent temperature-resistant subclones derived from strains SX46A yku70Δ rad52Δ and WS8105-1C yku70Δ rad52Δ and did
not find indications for increased amounts of Y' DNA in these subclones after hybridizing with probes for Y' and CEN4 (Figure 4, B and C). Hybridization with the (G13T) oligonucleotide, however, revealed that in these subclones the main telomeric fragments are on average 50 bp longer than in the parental strains (Figure 4A). When cells derived from these temperature-resistant yku70 rad52 clones were replated at 37\(^\circ\), the plating efficiency was found to be high (close to 100%), indicating that the temperature-resistant phenotype in these clones is stable. Most probably, temperature resistance in the yku70 rad52 double mutant strains is conferred by extragenic suppressor mutations that increase telomere elongation. To test whether this telomere alteration is simply a consequence of the already reduced telomere length in Ku-deficient mutants, we included a tel1 mutant strain in our studies. A fresh tel1 disruption mutant (strain background SX46A) was serially subcultivated to allow analysis of its phenotype at regular intervals between generations 45 and 120. In line with data reported by others (Lustig and Petes 1986; Runge and Zakian 1996; Boulton and Jackson 1998), we found that the tel1 mutants acquire a new equilibrium TRF length of \(~1080\) bp after \(~100\) generations of growth, and that the plating efficiency is similar at 30\(^\circ\) and 37\(^\circ\) (data not shown). We analyzed 20 subclones obtained after colony formation at 37\(^\circ\), which were derived from cells that had undergone 120 generations since inactivation of TEL1. Similar to what was seen in wild-type cells, we observed that the terminal telomeric fragments are slightly shorter in these clones than in clones exposed to a maximal temperature of 30\(^\circ\), but we did not find any indications for Y' amplification (data not shown).

Our data suggest that temperature-induced lethality in yku70 mutants is related to some alteration in telomere length, structure, or functionality at elevated temperature, which can be circumvented by amplification of subtelomeric DNA or processes leading to telomere elongation.

Wild-type and tel1 mutant clones grown at 37\(^\circ\) do not show Y' amplification: When we analyzed the phenotype of temperature-resistant yku70 mutant clones, we included wild-type clones as controls. A total of 56 colonies derived from YKU70 spores were picked from plates exposed to 37\(^\circ\) and expanded in liquid medium at 30\(^\circ\) before DNA isolation. To our surprise, we observed telomere shortening in these clones, i.e., as compared to samples that were exposed to a maximal temperature of 30\(^\circ\); the length of the terminal telomeric fragments giving maximum hybridization signal was reduced by 30–80 bp (Figure 5). This slight reduction in size, which is reproducibly detectable, also occurred in strains of different genetic background (data not shown), suggesting thermodilability of some aspect of telomere metabolism. However, no indication for an amplification of Y' elements was found in these YKU70 subclones, nor did we detect any chromosomal alteration in PFGE gels (data not shown).
Figure 6.—Quantitation of telomere DNA by determination of the hybridization signal intensity after shifting liquid cultures to 37°C. Early logarithmic cultures of wild-type SX 46A (lanes 1 and 2) and SX 46A yku70Δ (lanes 3 and 4) pregrown at 30°C were split and grown for 24 hr at 30°C (lanes 1 and 3) or 37°C (lanes 2 and 4), respectively, before genomic DNA was isolated, digested with XhoI, separated, and probed with (C13T)n, (A)n, (G13T)n, (B), and CEN4 (C). The arrow indicates the major telomeric band; horizontal bars indicate 6, 3, 2, and 1 kb (from top to bottom).

**Discussion**

In this work, we aimed at further characterizing the role of Yku70p in telomere metabolism. We show that, after inactivation of the YKU70 function, shortening of the terminal telomeric repeat tract proceeds rather rapidly and that a new steady-state telomere length is established, which is ~200 bp shorter than in congenic YKU70 strains. Several recent investigations show that the absence of a functional Ku protein leads to a variety of substantial alterations in telomere structure and function (see Introduction). One might expect that these alterations had some negative influence on viability or growth rate. However, a detailed analysis of these parameters over 120 generations after sporulation revealed that yku70 mutant spores and their YKU70 siblings did not differ in these regards when grown at 30°C. In addition, we never found indications for reduced growth at 30°C in any of our various yku70 mutant strains. Hence, it appears that the telomeres in Ku-deficient cells are still sufficiently stable and functional to allow normal growth at 30°C. It should be noted, however, that in one instance a negative impact of the inactivation of YKU70...
TABLE 4
Hybridization signal intensity in the major telomeric band after temperature shift to 37°

<table>
<thead>
<tr>
<th>Strain (temp.)</th>
<th>((G_{13}T)/(CEN4))</th>
<th>((C_{1}A)/(CEN4))</th>
<th>((G_{13}T)/(C_{1}A))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (30°)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>WT (37°)</td>
<td>1.08</td>
<td>1.01</td>
<td>0.88 ± 0.18</td>
</tr>
<tr>
<td>yku70 (30°)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>yku70 (37°)</td>
<td>1.16 ± 0.09</td>
<td>1.10 ± 0.27</td>
<td>0.97 ± 0.21</td>
</tr>
<tr>
<td>yku70 rad52 (30°)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>yku70 rad52 (37°)</td>
<td>1.09 ± 0.18</td>
<td>1.21 ± 0.33</td>
<td>0.91 ± 0.10</td>
</tr>
</tbody>
</table>

WT, wild type.

Hybridization signal ratios were normalized with respect to the 30° sample.

Ratio between the signal intensity of the major telomeric band after hybridization with \((G_{13}T)\) and the signal intensity of the CEN4 fragment. Indicated are mean and SD from two blots (one experiment), except for WT (one blot).

Ratio between the signal intensity of the major telomeric band after hybridization with \((C_{1}A)\) and the signal intensity of the CEN4 fragment. Indicated are mean and SD from two blots (one experiment), except for WT (one blot).

Ratio of the signal intensity in the major telomeric band after hybridization with \((G_{13}T)\) and \((C_{1}A)\), respectively. Indicated are mean and SD from five blots (three independent experiments).

on growth at 30° was described (Barnes and Rio 1997), which may be explained by differing genetic backgrounds.

Ku-deficient mutants display a severe growth defect at 37° (Feldmann and Winnacker 1993; Boult on and Jackson 1996b; Feldmann et al. 1996; Siede et al. 1996; Barnes and Rio 1997), but we observed in yku70 mutant strains of various genetic backgrounds that a small proportion of cells is able to escape temperature-induced lethality and to form colonies at this temperature. In this study, we analyzed 42 subclones derived from colonies that appeared after plating yku70 mutant spore products at 37°. As compared to control samples grown at 30°, all of the temperature-resistant subclones exhibited a strong increase in the number of XhoI-fragments containing unit Y′ elements. A similar phenomenon has so far been observed only in postsenescence survivors derived from strains mutated for TLC1, EST1, EST2, EST3, or EST4/CDC13 (Lundblad and Blackburn 1993; Lendvay et al. 1996; Nugent et al. 1996). For the sake of simplicity we refer to this phenomenon as Y′ amplification, but it should be borne in mind that current models predict a simultaneous amplification and redistribution of internal and/or distal telomeric sequences (Lundblad and Blackburn 1993; Bosco and Haber 1998; Le et al. 1999).

Postsenescence survivors and temperature-resistant yku70 clones share similarity with regard to the instability of the phenotype after replating, the RAD52-dependence of the amplification process, the Y′ element amplification factors, and the transfer of Y′ elements onto chromosome ends previously lacking these elements. However, bands indicative for chromosome ends lacking Y′ elements were readily detectable in our samples of temperature-resistant yku70 clones after hybridization of XhoI-digested DNA with a telomeric probe, while these bands were not seen in samples of est1 survivors (Lundblad and Blackburn 1993). While the reason for this difference remains to be elucidated, it may hint at a differential behavior of Y′-containing ends versus Y′-lacking ends in Ku-deficient cells.

In this and previous (Friedl et al. 1998) work we observed that Y′ amplification or fuzzy chromosomal banding occurs, with a low frequency, also in yku70 mutant clones grown at 30°, while these events have never been detected in wild-type cells so far. The high variability in the frequency of temperature-resistant yku70 cells seen in the fluctuation experiments suggests that events leading to temperature resistance can occur spontaneously during colony growth at the permissive temperature, although we cannot exclude at the moment that additional events (which may be responsible for late-appearing colonies) are induced by the selective treatment. Whether selected for or induced by incubation at 37°, we propose that the Y′ amplification process enables growth at the elevated temperature in yku70 cells. This assumption is based on the observation that all temperature-resistant yku70 single mutant subclones investigated so far exhibited amplification of subtelomeric DNA, whereas this amplification is not evident in liquid cultures of yku70 cells that were shifted to and held at 37° for 24 hr (after which most cells are dead). Hence, Y′ amplification correlates with temperature resistance and does not occur unspecifically in all cells at elevated temperature.

How can Y′ amplification confer viability to Ku-deficient cells growing at elevated temperature? Given that Y′ amplification is not the only mechanism enabling growth at elevated temperatures in Ku-deficient cells (see below), we consider it unlikely that the viability-conferring effect is due to Y′-specific features, such as ARS consensus sequences (Chan and Tye 1980) or the
helicase-like protein encoded by part of the naturally occurring Y′ elements (Yamada et al. 1998). We rather assume that the rescuing effect of the mechanism that manifests itself as Y′ amplification is conferred by recombinative amplification and redistribution of telomeric sequences, similar to what has been proposed to explain the suppression of senescence in yeast mutants deficient in the telomerase pathway (McEachern and Blackburn 1996).

What kind of alteration occurs at telomeres of KU-deficient cells upon incubation at elevated temperature? In mutants deficient for the telomerase pathway, loss of viability is thought to occur once a critical telomere size limit is reached after replication-associated shortening. It seemed possible that a critical telomere size limit could be reached in Ku-deficient cells incubated at 37°C because of a temperature-dependent further reduction of the telomere length. Indeed, in yku70 mutant cells, the average TRF length, as determined by the location of the maximum hybridization signal, is decreased after incubation at the elevated temperature. The extent of the size reduction, however, is comparable to that seen in wild-type and tel1 mutant cells. Since the average TRF length after the temperature shift in the tel1 mutant is slightly shorter than in the yku70 mutant, it appears that the reduced average telomere length per se cannot be responsible for temperature-induced lethality.

Although we cannot exclude that our methods to determine telomere length and amount of telomeric DNA are not sensitive enough to allow detection of small but biologically significant differences between yku70 mutants and tel1 mutants, we do not think that the absence of Ku leads to a more drastic temperature-induced shortening or loss of telomeric sequences than that occurring in Ku-proficient cells (Figure 6). Rather, we think that in the absence of Ku the normal temperature-induced telomere shortening is no longer tolerated by the cell or that some other, not length-related, alteration of the telomere conformation occurs. Ku is a telomere-binding protein, and it may, directly or indirectly, constitute an important factor for the establishment of telomeric chromatin. Telomeres exhibit a special type of chromatin organization in which the DNA is not bound by nucleosomes, but rather by so-called telosomal proteins, including Rap1p (for review see Zakian 1996). This specific chromatin organization is known to be responsible for the TPE; it may also have a role in differentiating telomeric ends from DNA ends at sites of DSBs. In the absence of Ku, formation of proper telomeric chromatin seems to be impaired, as inferred from the reduction of TPE seen in Ku-deficient cells even at permissive temperature. Still, the telomere-defining function of telomeric chromatin appears to suffice when Ku-deficient cells grow at 30°C, since their viability is not reduced and indications for alternative chromosome end stabilizations are rarely found. We propose that the combination of elevated temperature and absence of Ku destabilizes/destroys the telomeric chromatin in a way that its telomere-defining activity is lost. This may be a consequence of the further reduction of telomere length that may further destabilize Ku-lacking telomeric chromatin because of a reduction of the number of other telomere-associated proteins. Alternatively, some protein or its interaction with DNA or other proteins may be less stable at 37°C, and Ku may have a role in ensuring sufficient stability of the telomeric chromatin under these conditions.

In this scenario, the Y′ amplification mechanism could rescue the cells by compensating for the temperature-induced telomere shortening and maintaining a length similar to that seen at 30°C (Figure 2), thus ensuring the necessary number of protein binding sites. In a similar way telomere elongation, as seen in the very rare temperature-resistant yku70 rad52 clones, could facilitate proper chromatin formation. The observation that the severity of the TPE deficiency in Ku− mutants increases with temperature (Evans et al. 1998; Nugent et al. 1998) also supports our assumption that telomeric chromatin is thermostable in the absence of Ku. Overexpression of EST1, EST2, or TLCl in Ku−deficient mutants not only suppresses temperature sensitivity (Nugent et al. 1998) but also alleviates the TPE defect at elevated temperature (Evans et al. 1998). So far, the telomere lengths resulting from overexpression of individual components of the telomerase pathway have not been investigated; recent data, however, are consistent with an assumed role for these proteins in formation of telomeric chromatin that is independent of increased telomerase activity (Evans et al. 1998) but also alleviates the TPE defect at elevated temperature (Evans et al. 1998). Finally, we found that temperature resistance of yku70 mutant cells is increased when they are incubated in media containing 1 m sorbitol (B. Feller-Hoff, unpublished results). Sorbitol is known to stabilize proteins and protein-protein interactions in vitro (e.g., Wimmer et al. 1997); it has been proposed that sorbitol can also stabilize cellular proteins in cells grown in its presence (Heat h et al. 1995). By whatever mechanism caused, a temperature-induced instability of the telomeric chromatin may cause the loss of the privileged status of the chromosome ends, thus making them appear as sites of chromosomal breaks. Alternatively, a loss of defined telomere structure may affect proper chromosome segregation in mitosis. Additional investigations will be required to clarify this point.

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LITERATURE CITED
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Boulton, S. J., 1050 B. Fellerhoff, F. Eckardt-Schupp and A. A. Friedl


Le, S., K. J. Moore, J. E. Haber and C. W. Greider, 1995 RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics 135: 143–152.


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