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Telomere instability caused by subtelomeric Y′ amplification and rearrangements in *Saccharomyces cerevisiae* (*ku70 tel1* and *ku70 rad50*) double mutants

Miguel J Ruiz-Gómez[†]

Radiobiological Institute, University of Munich. Schillerstr. 42, 80336 Munich, Germany

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Telomeres solve the end-replication problem. Previous results suggested a relation between Yku70/80 and proteins Tel1 and Rad50 in telomere stabilization. Inactivation of any of these genes lead to a shortening of telomeres, while in *ku70 tel1* or *ku70 rad50* double mutants a drastic amplification of Y' elements was found. The biological significance of this observation is not clear. To further characterize Y′ amplification 25 strains and isolates of *S. cerevisiae* were analyzed. As expected, amplification was seen in *yku70 tel1* and *yku70 rad50* double mutants, but not in other strains. The extent of Y′ amplification was also tested to determine if excessive numbers of Y′ repeats appear. A variation in chromosome lengths within the population of cells has been found. Hybridisation study indicated that chromosomes only increase in length in these double mutants, but never get shorter. A high degree of variability was observed in single cell clones, in spite of their close relationship, indicating that alterations in subtelomeric regions are not stable but occur continuously in these mutants. Therefore, these genes are essential to chromosome stability

Keywords: Chromosome stability, Telomere, Y′ amplification, Yeast

Telomeres are specific nucleo-protein structures at the ends of linear chromosomes that help to solve the end-replication problem. Telomere length is regulated by a complex interplay of several factors, including telomerase, telomere-binding proteins, DNA replication machinery and recombination[1]. In addition, they provide a sort of cap that protects the chromosome ends from degradation, recombinative processes and end-to-end fusions, thus ensuring chromosomal stability[2]. Although the capping function is lost when telomeres erode, capping appears not to be a direct function of telomere length. Recent results rather suggest that capping has been promoted by telomere-associated proteins. If these are absent because of mutational inactivation or if they cannot longer bind to telomere because of significant telomere shortening, end-to-end fusions or recombination of telomere-associated DNA occur. Ku is a conserved DNA end-binding protein that plays various roles including protection of chromosome end at telomeres and promotion of DNA repair[3]. Inactivation of Ku70/Ku80 complex in mammalian cells resulted in end-to-end fusions, leading to chromosomal instability, independent of length of telomeric tract[4-7].

Yeast ku has an essential role at the telomere; in particular, ku deficiency produces telomere shortening, loss of telomere clustering and silencing. In the yeast *S. cerevisiae*, inactivation of Ku does not affect cellular proliferation at normal growth temperature (30°C), but at elevated temperature (37°C) only few cells are able to form a colony[8]. Generation of temperature-resistant survivor clones depends on recombination factor Rad52, and all survivors exhibit amplification of subtelomeric repetitive Y′ elements. This phenotype reminisces of that observed in clones surviving cellular senescence after inactivation of telomerase. These data suggest that absence of Ku together with a temperature-induced instability of another factor lead to a loss of capping function. In some Ku-deficient clones, slight Y′ amplification was also seen at 30°C, demonstrating that absence of capping provided by Ku, although not affecting viability, may in some situations lead to hyper-recombination between Y′ elements. Other authors have reported that Ku inhibits hyper-recombination within the telomeric tract itself[9].

[†]Present address:
Departamento de Radiología y Medicina Física, Facultad de Medicina, Universidad de Malaga. Bulevar Louis Pasteur 32, 29071 Malaga, Spain
Telephone: +34-95-2131578
Fax: +34-95-2131630
E-mail: mjrg@uma.es

Cells bearing *rad50Δ* mutation exhibited a progressive attrition of distal telomeric tract as they divided, displayed slow growth and a shortening of non-Y′-containing elements[10]. Mutations in genes encoding yeast ku proteins as well as *RAD50* were shown to affect telomere length maintenance which suggests that more than one recombination pathway may play a role in telomere metabolism[6]. The Rad50 complex is required for both homologous recombination (HR) and non-homologous end joining (NHEJ). Tomita *et al.*[11] reported that Rad50 complex can process double-strand breaks (DSB) ends and telomere ends in the presence of ku heterodimer. In addition, mutations in *tel1* and *tel2* confer shortened telomeres on cells, indicating a defect in telomere length regulation[12]. The *TEL1* gene encodes a very large (322 kD) protein that shares homology with a family of lipid/protein kinases involved in DNA repair and/or telomere replication[13]. In cells lacking *TEL1*, rate of elongation of a telomere that has been shortened progressively declines upon return to equilibrium length. In addition, lack of this gene does not affect telomerase activity, but structure of the end-complex is modified to impair telomerase accessibility[14]. Therefore, *TEL1* seems to be required mainly for association of telomerase to short telomeres in yeast. Moreover, it may also regulate telomere length by enhancing the processivity of telomerase at short telomeres[15].

The aim of this work is to investigate the importance of recombinative Y′ amplification for continuing growth in *ku70 tel1* and *ku70 rad50* double mutants. Since loss of capping function may promote mechanisms that allow for telomerase-independent telomere stabilization, results obtained in the model organism *S. cerevisiae* may also have an impact on tumour biology.

Materials and Methods

Yeast strains and culture—From the laboratory's strain collection, a variety of strains and isolates of *S. cerevisiae* (25) were thawed, streaked and phenotypically characterised (Table 1). *MATα* strains are derived from WS8105-1C (*MATα ade2 arg4-17*

Table 1—Yeast strains

Strain nº	Strain	Genotype
1	WS8105-1C (wild type)	*MATα ade2 arg4-17 trp1-289 ura3-52*
2	WS8105-1C *yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 yku70::URA3*
3	WS8105-1C *yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 yku70::URA3*
4	WS8105-1C *rad52Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad52::TRP1*
5	WS8105-1C *rad54Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad54::kanMX*
6	WS8105-1C *rad54Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad54::kanMX yku70::URA3*
7	WS8105-1C *rad54Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad54::kanMX yku70::URA3*
8	WS8105-1C *rad54Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad54::kanMX yku70::URA3*
9	SX46A *tel1Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1*
10	SX46A *tel1Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1*
11	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
12	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
13	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
14	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
15	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
16	SX46A *tel1Δ yku70Δ*	*MATa ade2 trp1-289 ura3-52 his3-532 tel1::TRP1 yku70::URA3*
17	WS8105-1C *rad50Δ rad52Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG rad52::URA3*
18	WS8105-1C *rad50Δ rad52Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG rad52::URA3*
19	WS8105-1C *rad50Δ rad52Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG rad52::URA3*
20	WS8105-1C *rad50Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG yku70::TRP1*
21	WS8105-1C *rad50Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG yku70::TRP1*
22	WS8105-1C *rad50Δ yku70Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG yku70::TRP1*
23	WS8105-1C *rad50Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG*
24	WS8105-1C *rad50Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG*
25	WS8105-1C *rad50Δ*	*MATα ade2 arg4-17 trp1-289 ura3-52 rad50::hisG*

trp1-289 ura3-52) and *MATa* strains from SX46A (*MATa ade2 trp1-289 ura3-52 his3-532*). The tested strains were: wild type, *yku70::URA3, rad52::TRP1, rad54::kanMX, tel1::TRP1 and rad50::hisG* single mutants, as well as *yku70::URA3 rad52::TRP1, yku70::URA3 rad54::kanMX, yku70::URA3 tel1::TRP1, yku70::TRP1 rad50::hisG* double mutants. Deletion/disruption of these genes have been described previously[16,17]. To identify strain identities, auxotrophic markers were tested by replica plating on various selection media. Mating type status was tested by mating with strains with defined mating type. Growth characteristics were determined by analysing colony size and growth in liquid culture. Yeast cells were grown in Yeast extract-Peptone-Dextrose (YPD) broth (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) with or without 2% Bacto-agar (all reagents from Difco, Becton Dickinson and Co. Sparks, MD, USA).

Preparation of genomic yeast DNA and electrophoretic separation in pulsed-field gels—Preparation of genomic DNA from each strain by agarose plug method and pulsed field gel electrophoresis (PFGE) using CHEF DRII system (Bio-Rad, Richmond, CA) were performed as described previously[18]. After electrophoresis, gels were stained with ethidium bromide and recorded by a Polaroid camera.

Determination of chromosomal lengths—Length of chromosomal molecules of strains used were determined by comparing their electrophoretic mobility in CHEF gels, according to sizes described previously by Friedl *et al.*[17]. The following lengths were determined: 260 kb (I), 300 kb (VI), 360 kb (III), 450 kb (IX), 590 kb (V, VIII), 690 kb (XI), 790 kb (X), 830 kb (XIV), 855 kb (II), 1000 kb (XIII, XVI), and 1135 kb (VII, XV). For chromosomes IV and XII, lengths of 1600 and 2200 kb were assumed.

Southern hybridization with chromosome-specific probes—For the identification of altered chromosomes, southern hybridizations were performed using centromere- or gene-specific probes. Chromosomal DNA separated by PFGE was denatured in 0.25 N HCl (15 min) and 0.4 N NaOH (15 min), and transferred onto nylon membrane (Qiagen, Chatsworth, CA) by capillary transfer using the same buffer (0.4 N NaOH). Prehybridization and hybridization were carried out according to membrane manufacturer's directions. Fragments were labelled with Dig-labeled DNA probes and detected immunologically on a membrane with Anti-Dig-alkaline phosphatase and chemiluminescent detection (Roche-Kit) (Roche Diagnostics, Mannheim, Germany).

DNA digestion—To determine Y′ amplification, genomic DNA was digested overnight at 37°C with *XhoI* using standard procedures[19], and separated on 0.8% agarose gel.

γ Irradiation—Cells were grown in YPD medium to stationary growth phase and resuspended in water. Appropriate dilutions of samples were plated on YPD plates and then irradiated in a ^{60}Co source at a dose rate of 7.21Gy/min (Atomic Energy of Canada, Ltd., Kanata, Ontario, Canada). After irradiation, the plates were incubated 6 days at 30°C and colonies scored.

Results and Discussion

To determine whether Y′ elements amplification is present at 30°C, various single and double mutant strains were investigated. Genomic DNA was prepared using an agarose plug method to avoid shearing of the DNA. Chromosomal molecules were then separated by pulsed-field gel electrophoresis (PFGE) (Fig. 1). A clear banding pattern indicative of stable chromosome lengths was observed in wild type and the following single mutant strains: *yku70::URA3, rad52::TRP1, rad54::kanMX, tel1::TRP1, rad50::hisG.* In addition, the *rad54::kanMX yku70::URA3* double mutant strain exhibited stable chromosome lengths. Fuzzy chromosomal bands suggesting heterogeneous alterations in chromosome lengths were observed in at least some clones of *tel1::TRP1 yku70::URA3* and of *rad50::hisG yku70::URA3* double mutants. In one clone of *rad50::hisG rad52::URA3* double mutants (strain 17) an elongation of chromosome IV is evident, but not a general heterogeneity of chromosome lengths.

To determine whether Y′ amplification has occurred in any of these strains/isolates, DNA was isolated by phenol extraction, digested with the restriction enzyme *Xho*I and separated by gel electrophoresis. Gels were stained with ethidium bromide and recorded by photography. Y′ amplification is indicated by the increase in intensity of distinct bands with fragment lengths of 5.8 or 6.7 kb in these stained gels (Fig. 2). Strains 15 (*tel1::TRP1 yku70::URA3),* 16 (*tel1::TRP1 yku70::URA3)*, and 20 (*rad50::hisG yku70::TRP1)*, which show very fuzzy bands in electrophoretic karyotyping, also exhibit a strong increase in the Y′ *Xho*I fragments.

To test for the type of alteration in individual chromosome species, pulsed-field gels were blotted onto nylon membranes and hybridised with chromosome-specific DNA probes for chromosomes I and V. As expected, chromosomal elongation was seen in some *yku70::URA3 tel1::TRP1* and *yku70::TRP1 rad50::hisG* double mutant isolates, but not in any of the other strains (Table 2).

To investigate whether the subtelomeric region in *yku70::URA3 tel1::TRP1* and *yku70::TRP1 rad50::hisG* double mutants is subjected to continuously ongoing alteration process, single cell

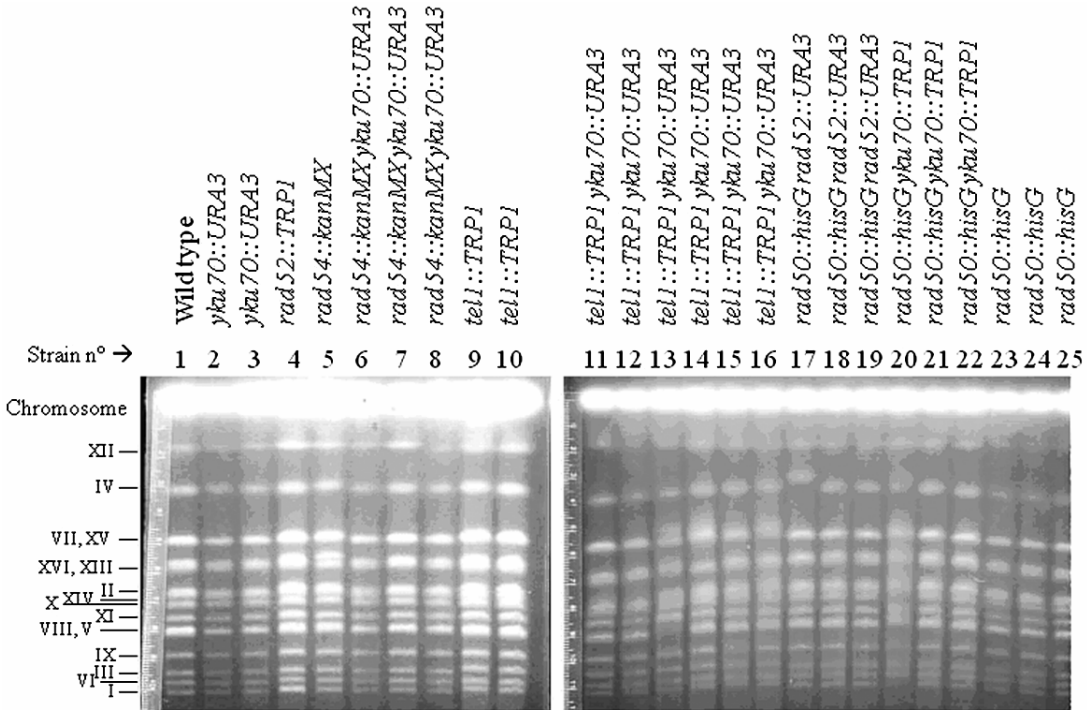

Fig. 1—Pulsed Field Gel Electrophoresis (PFGE) of the strain collection. The chromosomal pattern of different single and double mutant strains was obtained. The WS8105-1C (wild type) strain (line 1) was used as control.

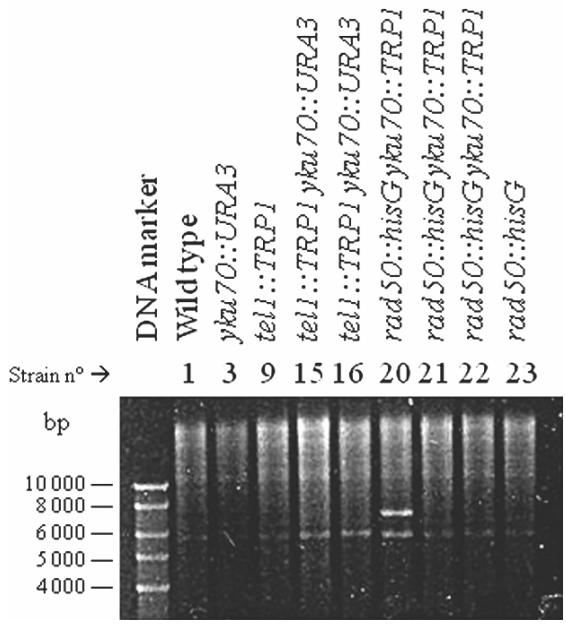

Fig. 2—DNA digestion with *XhoI*. [DNA marker: 1kb DNA ladder Promega, Germany].

Table 2—Chromosomal pattern of chromosomes I and V obtained by Southern blot

Strain	Genotype	Chromosomal pattern
11	*tel1::TRP1 yku70::URA3*	Normal
12	*tel1::TRP1 yku70::URA3*	Normal
13	*tel1::TRP1 yku70::URA3*	Chr. I normal; Chr. V bigger
14	*tel1::TRP1 yku70::URA3*	Normal
15	*tel1::TRP1 yku70::URA3*	Normal
16	*tel1::TRP1 yku70::URA3*	Chr. I normal; Chr. V fuzzy band
17	*rad50::hisG rad52::URA3*	Normal
18	*rad50::hisG rad52::URA3*	Normal
19	*rad50::hisG rad52::URA3*	Normal
20	*rad50::hisG yku70::TRP1*	Chr. I and V, fuzzy bands
21	*rad50::hisG yku70::TRP1*	Normal
22	*rad50::hisG yku70::TRP1*	Normal
23	*rad50::hisG*	Normal
24	*rad50::hisG*	Normal
25	*rad50::hisG*	Normal

Chr.: Chromosome

clones were generated and investigated by streaking cells from one colony and comparison of each 5 colonies thus obtained. In the two isolates of *yku70::URA3 tel1::TRP1* analysed and in one out of three *yku70::TRP1 rad50::hisG* double mutant isolates analysed, a high variability of chromosome lengths, in part accompanied by fuzzy bands were observed (Fig. 3). The results of the hybridisation study indicated that chromosomes increase in length in the *yku70::URA3 tel1::TRP1* and the *yku70::TRP1 rad50::hisG* double mutants. A high degree of variability between the colonies was observed in spite of their close relationship, indicating that alterations in subtelomeric regions are not stable but occur continuously in *yku70::URA3 tel1::TRP1* and the *yku70::TRP1 rad50::hisG* double mutants (Tables 3 and 4).

To investigate whether Y′ amplification is necessary to ensure viability in these double mutants, it was planned to inactivate the *RAD52* gene in these mutants. Colonies grown after transformation were investigated to verify the correct gene disruption by testing the sensitivity to gamma-irradiation at 0, 200 and 500 Gy. None of the candidates obtained in two independent transformation experiments to disrupt the *RAD52* gene exhibited the hyper-sensitivity typically shown by *rad52* mutants (Fig. 4). Analytical PCR showed that correct disruption of *RAD52* gene has not occurred.

The regulation of telomeric length in *S. cerevisiae* is thought to be a complex process. Mutations in different genes affect the correct balance between elongation and shortening.

Linear chromosomes in *Saccharomyces* could be maintained either by telomerase or by recombination-driven amplification of subtelomeric Y′ DNA[20]. Another mechanism that can maintain the ends of yeast chromosomes is the telomere-telomere recombination[21].

Rad50 protein is bound to human telomeres and it has been proposed that Rad50 is involved in the establishment of the t-loop structure[22]. Cells carrying the *rad50Δ* mutation display shorter telomeres and cell senescence. Kironmai and Muniyappa[10] reported

Table 4—Chromosomal pattern of chromosome XIV obtained by Southern blot

Strain	Genotype	Chromosomal pattern
15	*tel1::TRP1 yku70::URA3*	Chr. XIV in clone 5 (right line) is bigger
16	*tel1::TRP1 yku70::URA3*	Bigger in clone 1 and fuzzy in clone 4
20	*rad50::hisG yku70::TRP1*	Not enough DNA
21	*rad50::hisG yku70::TRP1*	No alterations
22	*rad50::hisG yku70::TRP1*	No alterations

Chr.: Chromosome

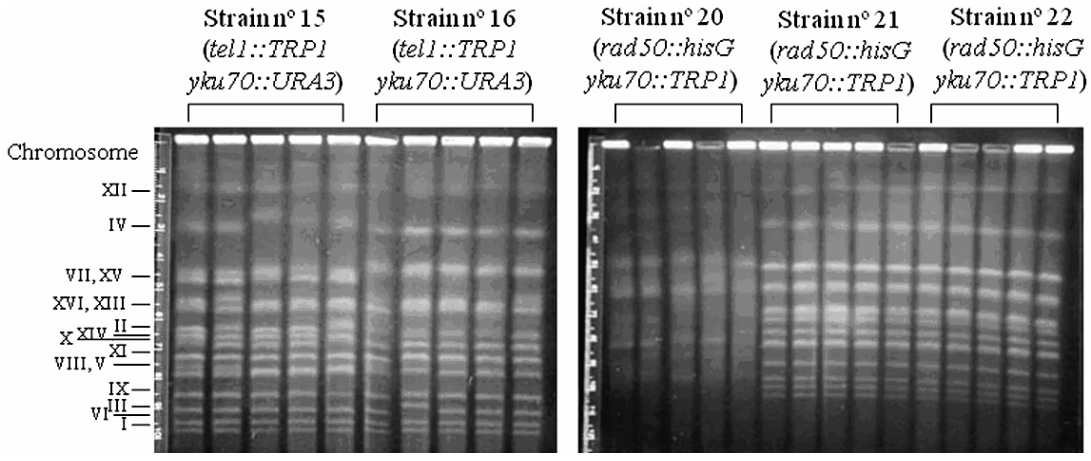

Fig. 3—Pulsed Field Gel Electrophoresis (PFGE) of 5 clones isolated from *yku70::URA3 tel1::TRP1* and *yku70::TRP1 rad50::hisG* strains possibly subjected to continuously alteration process, to study the extent of Y′ amplification.

Table 3—Chromosomal pattern from Pulsed Field Gel Electrophoresis

Strain	Genotype	Chromosomal pattern between clones in the same strain	Result
15	*tel1::TRP1 yku70::URA3*	Lots of alterations in the intensity, location and fuzzy bands	Unstable
16	*tel1::TRP1 yku70::URA3*	Some alterations in the location of bands between clones	Unstable
20	*rad50::hisG yku70::TRP1*	Lots of alterations in distribution, intensity and fuzzy of bands	Unstable
21	*rad50::hisG yku70::TRP1*	Some little not significant alterations	Stable
22	*rad50::hisG yku70::TRP1*	No alterations	Stable

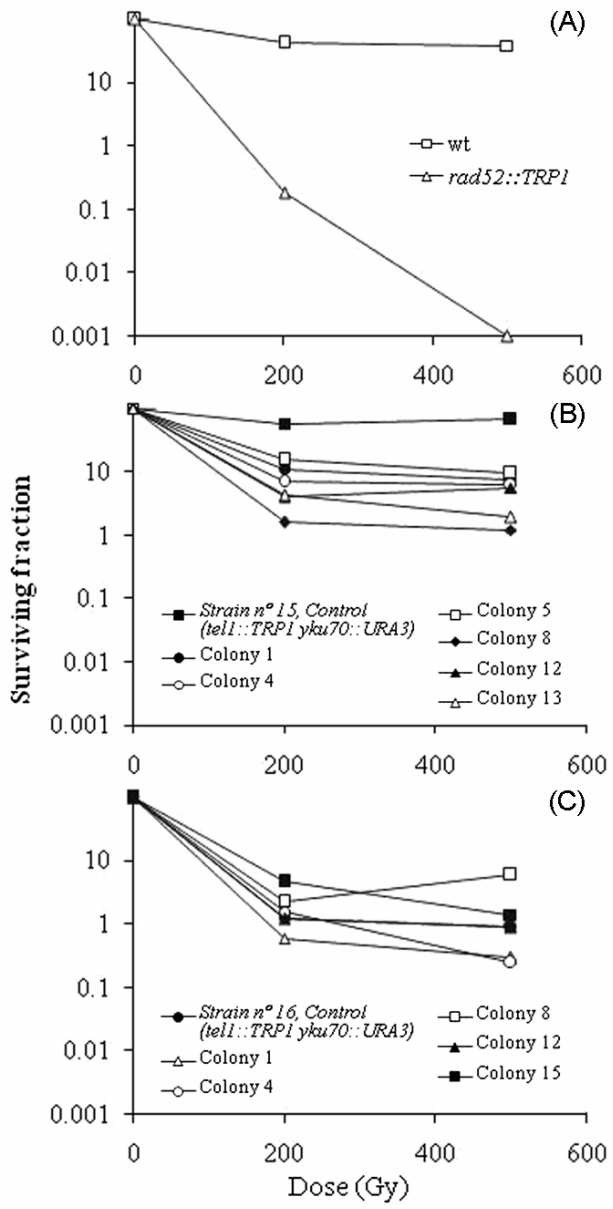

Fig. 4—Exposure to gamma rays of different clones inactivated for *RAD52* gene. [A: Control wild type strain (wt) and control *rad52* strain, B: Control *yku70::URA3 tel1::TRP1* (strain 15) and 6 colonies *rad52* inactivated, C: Control *yku70::URA3 tel1::TRP1* (strain 16) and 5 colonies *rad52* inactivated]. None of the strains assayed exhibited the hypersensitivity typically shown by *rad52* mutants.

that the simplest model to explain the shorting telomeres in *rad50Δ* cells could be due to an indirect effect of the failure of these mutants to repair DNA damage, to process damaged DNA molecules properly or to prepare telomeric DNA as substrates for repair machinery. Our findings are consistent with the results published by these authors who reported the idea that one of the functions of the Rad50 protein is to stabilize the telomeric and telomere associated DNA in dividing cells. *RAD50* encodes a DNA-binding protein, participates in DNA-end joining pathway, confers mitotic cells resistance to clastogenic agents and interfaces a structure that connects sister chromatids[23,24].

Single mutants in *rad50* gene show initial telomere shortening followed by stabilization. In this way, Le *et al*.[6] reported that mutations in *RAD50*, *XRS2* and *MRE11* have similar genetic effects on recombination, indicating that these proteins interact to form a complex that appears to provide or regulate an exonuclease function. The data published by these authors suggest that at least two independent recombination pathways are involved in telomere length maintenance. Moreover, Craven and Petes[25] concluded that all yeast telomeres cannot be regarded as identical substrates for the enzymes involved in telomere maintenance. The data reported by them demonstrate the complications of epistasis analysis as applied to telomere length regulation.

Mutation in either ku subunit enhanced the instability of elongated telomeres to degradation and recombination[9]. A chromosome end is not recognized as DNA damage and is thus protected form the action of repair enzymes. Several repair proteins, including ku, are involved in both DNA DSB repair and telomere maintenance[11]. In this way, Tomita *et al*.[11] suggest that the Rad50 complex is required for the processing of DSB and telomere ends in the presence of ku heterodimer. In contrast, they reported that ku inhibits processing of telomere ends by alternative nucleases in cells lacking Rad50 protein. In addition, Berthiau *et al*.[14] observed that the absence of *TEL1* induces an alteration in the structure of telomeric chromatin modifying the organization of the telomeres.

Preliminary results had suggested an involvement of the ku70/80 heterodimer and of the proteins Tel1 and Rad50 in telomere stabilization. Inactivation of any of these genes, while causing a stable reduction of telomere length, did not affect the growth characteristics of the respective single mutants. There were, however, indications for occasional telomere instability: some cells exhibited amplification of subtelomeric Y′ elements. Drastic amplification of Y′ elements were observed in *ku70 tel1* or *ku70 rad50* double mutants, suggesting partially redundant functions of these proteins in telomere capping. The biological significance of the Y′ amplification

observed in these mutants is not clear. From studies in telomerase-deficient mutants it is known that Y′ amplification constitutes one of two telomerase-independent pathways for telomere stabilization, the use of which allows to circumvent cellular senescence following telomere attrition. It is possible that *ku70 tel1* or *ku70 rad50* double mutants similarly depend on Y′ amplification to ensure survival. Alternatively, the amplification may simply reflect derepressed recombination activities in telomere-associated regions as caused by loss of capping function, without having relevance for cell survival.

Not all mutants (single or double) studied that show mutations in genes related to telomere metabolism leads to Y′ amplification. This amplification has been only seen in *yku70::URA3 tel1::TRP1* and *yku70::TRP1 rad50::hisG*. These mutants showed differences in the chromosome lengths within the population and reflected Y′ amplification only. Alterations in subtelomeric regions are not stable in this strain but occur continuously in these mutants. The genes disrupted in these strains are essential to the stability of the chromosome.

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