Ku Suppresses Formation of Telomeric Circles and Alternative Telomere Lengthening in *Arabidopsis*

Barbara Zellinger,[1] Svetlana Akimcheva,[1] Jasna Puizina,[1,2] Martina Schirato,[1] and Karel Riha[1,*]
[1] Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, 1030 Vienna, Austria
[2] Present address: Department of Biology, University of Split, Teslina 12, Croatia.
*Correspondence: karel.riha@gmi.oeaw.ac.at
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SUMMARY

Telomeres in mammals and plants are protected by the terminal t loop structure, the formation of which parallels the first steps of intrachromatid homologous recombination (HR). Under some circumstances, cells can also utilize an HR-based mechanism (alternative lengthening of telomeres [ALT]) as a back-up pathway for telomere maintenance. We have found that the Ku70/80 heterodimer, a central nonhomologous end-joining DNA repair factor, inhibits engagement of ALT in *Arabidopsis* telomerase-negative cells. To further assess HR activities at telomeres, we have developed a sensitive assay for detecting extrachromosomal telomeric circles (t circles) that may arise from t loop resolution and aberrant HR. We show that Ku70/80 specifically inhibits circle formation at telomeres, but not at centromeric and rDNA repeats. Ku inactivation results in increased formation of t circles that represent $\sim$4% of total telomeric DNA. However, telomeres in *ku* mutants are fully functional, indicating that telomerase efficiently heals ongoing terminal deletions arising from excision of the t circles.

INTRODUCTION

The ends of eukaryotic chromosomes form unique structures that differentiate native chromosome termini from DNA double-strand breaks (DSBs). Telomeres in most organisms consist of G-rich repeats that terminate with a single-stranded 3' G overhang. In mammals and plants, telomeres have been shown to fold into a structure called the t loop (Cesare et al., 2003; Griffith et al., 1999), in which a G overhang invades a duplex region of the same telomere. T loops are thought to protect chromosome termini as they conceal telomere ends and thus prevent activation of the DNA damage response. It is assumed that t loop formation parallels the first steps of homologous recombination (HR). In this regard, a t loop resembles a stabilized homologous recombination intermediate in which a G overhang invades a double-stranded (ds) telomere to generate a displacement loop, but progression into branch migration and Holliday junction resolution is suppressed. Resolution of a t loop results in a circular telomeric molecule and a terminally deleted telomere in mammals (Wang et al., 2004). HR is also responsible for telomere rapid deletion (TRD) that has been described in yeast (Lustig, 2003). Because TRD could be detrimental for chromosome stability, HR at chromosome termini must be precisely regulated. Studies in human and mouse cell lines indicate that the telomere binding proteins TRF2 and Pot1 are important for preventing t loop resolution at telomeres in mammals (Wang et al., 2004; Wu et al., 2006).

Deregulation of telomeric recombination appears to be a prerequisite of the telomerase-independent ALT pathway that is important for telomere maintenance and immortalization of up to 10% of human tumors (Muntoni and Reddel, 2005). Interestingly, one of the hallmarks of ALT is an elevated level of t circles, indicating that ALT cell lines also exhibit t loop resolution and TRD (Cesare and Griffith, 2004; Wang et al., 2004). However, mechanisms that cause telomeres to be permissive of HR and ALT are currently not well understood.

The KU70/KU80 heterodimer is a DNA repair complex that binds to and stabilizes the ends of broken DNA molecules. It also facilitates the recruitment of downstream factors that mediate nonhomologous end joining (Lieber et al., 2003; Riha et al., 2006). Ku is localized to telomeres, and its inactivation leads to various defects in telomere function. In mammals, depletion of Ku results in telomere length deregulation and end-to-end chromosome fusions (d'Adda di Fagagna et al., 2001; Espejel et al., 2002; Myung et al., 2004), whereas in *Saccharomyces cerevisiae*, Ku protects 5′ chromosome ends from exonucleolytic resection and TRD (Gravel et al., 1998; Maringele and Lydall, 2002; Polotnianka et al., 1998) and promotes recruitment of telomerase to telomeres (Fisher et al., 2004; Stellwagen et al., 2003). In previous studies, we have shown that Ku plays an essential role at telomeres in plants. It prevents extensive degradation of the telomeric C-rich strand, but in contrast to yeast, Ku acts as a negative regulator of telomerase in *Arabidopsis* (Riha and Shippen, 2003; Riha et al., 2002). Plants doubly deficient for KU70 and telomerase reverse transcriptase (TERT) exhibit accelerated telomere shortening and an early onset of developmental defects (Riha and Shippen, 2003).

In this study, we show that Ku suppresses formation of t circles in *Arabidopsis* and that Ku deficiency promotes engagement of the ALT mechanism for telomere maintenance. These data indicate that Ku regulates HR activities at *Arabidopsis* telomeres and that excision of t circles may contribute to the accelerated loss of telomeres in *ku70 tert* mutants. Furthermore, we have developed a highly sensitive technique for detecting t circles that facilitates the analysis of HR at telomeres.

RESULTS AND DISCUSSION

Detection of T Circles in *ku70* and *ku80* Mutants

Terminal restriction fragment (TRF) analysis has shown that telomeres in *Arabidopsis ku70* or *ku80* mutants are two to three times longer than in wild-type plants and that they exhibit significant length heterogeneity at individual chromosome ends (Gallego et al., 2003; Riha and Shippen, 2003). Genomic blots revealed that a fraction of TRFs from KU70-deficient plants produced a retarded signal that migrated in wells. This signal diminished after DNA separation by alkaline electrophoresis, indicating that a portion of the telomeric DNA is partially single stranded (ss) and forms tangled structures unable to migrate into the gel (Figure S1 in the Supplemental Data available with this article online). We used neutral-neutral two-dimensional (2D) gel electrophoresis to further analyze the structural properties of telomeric DNA in *ku* mutants. 2D telomere blots of AluI-digested DNA prepared from a *ku70* callus culture revealed arcs corresponding to the migration of open-circular (oc) and super-coiled (sc) double-stranded (ds) circles (Figures 1A and 1B). The circular nature of these molecules was confirmed by their insensitivity to exonuclease V (Figure 1C), which rapidly degrades linear DNA molecules, but not oc or sc circles (Figure S2). The size of the t circles spanned the range of the linear TRFs (Figure 1C), which is reminiscent of the t circles generated in mammalian cells lacking fully functional TRF2 or Pot1 (Wang et al., 2004; Wu et al., 2006).

2D gel analysis of DNA extracted from a *ku70* suspension culture showed an additional arc that ran faster than linear DNA in the second dimension (Figure 1D). This arc contained G-rich as well as C-rich telomeric strands and was exclusively observed in rapidly dividing cell cultures, suggesting that it represents ss or partially ssDNA replication intermediates. Interestingly, ds t circles were also readily detected in the DNA from *ku70* and *ku80* adult plants (Figure 1E), which contain only a very small proportion of actively proliferating cells. This finding suggests that either t circles are relatively stable or that their excision occurs in postmitotic cells.

We next asked whether Ku deficiency promotes formation of circular DNA molecules from other chromosome loci. We degraded the bulk of linear genomic DNA with exonuclease V and resolved the fraction resistant to the treatment by 2D gel electrophoresis. In this way, we detected circular molecules consisting of 5S rDNA and the major *Arabidopsis* 180 bp centromeric repeat. These data indicate that tandem repeats in *Arabidopsis* are prone to excision as extrachromosomal circles (Figure 1F), a phenomenon that has been observed in other organisms, including flies and mammals (Cohen et al., 2003; Kuttler and Mai, 2007). Importantly, although levels of 5S rDNA and centromeric circles were similar in wild-type and KU70-deficient plants, t circles were only detected in *ku70* mutants (Figure 1F). Thus, Ku specifically affects circle formation at telomeres.

No t circles were detected on 2D blots with DNA from wild-type plants or cell cultures (Figure 1E, data not shown). Because telomeres in wild-type plants are significantly shorter than in *ku* mutants, t circles derived from such telomeres may be difficult to resolve by 2D gel electrophoresis. We, therefore, developed a sensitive new technique for detecting t circles (t circle amplification [TCA]). The assay utilizes the highly processive ϕ29 DNA polymerase that has efficient strand displacement activity (Blanco and Salas, 1996). The ϕ29 polymerase can generate ssDNA products in the range of 100 kb from circular template molecules via a rolling circle replication mechanism (Figure 2A) (Lizardi et al., 1998). The presence of t circles in the TCA reaction primed with a telomere-specific oligonucleotide was expected to produce long ss telomeric DNA molecules that could be separated from much shorter extension products synthesized from TRFs. TCA with AluI-digested *ku70* genomic DNA yielded high molecular weight products that could be detected by Southern hybridization with a strand-specific probe (Figure 2B). The specificity of the reaction was confirmed by pretreatment of the DNA with exonuclease V. Although the high molecular weight signal generated by ϕ29 polymerase was resistant to exonuclease V, the signal derived from linear TRFs was completely eliminated (Figure 2B), arguing that TCA specifically detects circular telomeric molecules. TCA products generated in the reactions lacking dCTP (for G strand synthesis; Figure 2C) or dGTP (for C strand synthesis; Figure 2D) indicate that t circles consist almost exclusively of telomeric repeats and do not include subtelomeric sequences (for further discussion, see Figure S3).

Strong TCA signals in samples from *ku70* mutants (Figure 2E) confirmed data obtained by 2D gel electrophoresis and demonstrated that the absence of Ku results in the formation of abundant extrachromosomal t circles. Although no t circles were detected on 2D blots in wild-type plants, weak ϕ29-dependent TCA signals were observed in wild-type samples (Figure 2E and Figure S4). These data illustrate the sensitivity of the TCA technique and indicate that even fully functional telomeres permit t circle excision. This is consistent with a recent finding of sporadic TRD events in Ku-proficient *Arabidopsis* (Watson and Shippen, 2007).