

Expansions, contractions, and fragility of the spinocerebellar ataxia type 10 pentanucleotide repeat in yeast

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Edited* by Thomas D. Petes, Duke University Medical Center, Durham, NC, and approved January 4, 2011 (received for review June 30, 2010)

Spinocerebellar ataxia 10 (SCA10) is an autosomal dominant disease caused by large-scale expansions of the (ATTCT)_n repeat within an intron of the human *ATXN10* gene. In contrast to other expandable repeats, this pentanucleotide repeat does not form stable intra- or interstranded DNA structures, being a DNA unwinding element instead. We analyzed the instability of the (ATTCT)_n repeat in a yeast experimental system, where its expansions led to inactivation of the *URA3* reporter gene. The inactivation was due to a dramatic decrease in the mRNA levels owing to premature transcription termination and RNA polyadenylation at the repeat. The rates of expansions strongly increased with the repeat's length, mimicking genetic anticipation in human pedigrees. A first round of genetic analysis showed that a functional *TOF1* gene precludes, whereas a functional *RAD5* gene promotes, expansions of the (ATTCT)_n repeat. We hypothesize that repeat expansions could occur upon fortuitous template switching during DNA replication. The rate of repeat contractions was elevated in the *Tof1* knockout strain, but it was not affected by the *RAD5* gene. Supporting the notion of replication irregularities, we found that (ATTCT)_n repeats also cause length-dependent chromosomal fragility in yeast. Repeat-mediated fragility was also affected by the *Tof1* and *Rad5* proteins, being reduced in their absence.

DNA repeats | genome instability | DNA repair

Expansion of simple DNA repeats is a cause of more than 30 hereditary diseases in humans (1). Just one repeat within a particular gene undergoes expansions in each case, suggesting that expansion events occur *in cis*, in contrast with *trans*-acting mutations of DNA metabolism destabilizing different repeats (2–4). Many of the repeat expansion diseases are characterized by genetic anticipation (i.e., an increased severity and early onset of the disease as the repeat progressively expands during intergenerational transmissions) (5). The exact mechanisms of repeat expansions in humans are unknown, although data from model systems implicate DNA replication, repair, and recombination as contributors to repeat expansions (6).

Trinucleotide, tetra-, penta-, and dodecanucleotide repeats can expand, leading to disease (1). It was generally believed that unusual secondary structures formed by expandable repeats are central for the expansion process (7). Recently, however, it has become clear that this is not always the case. A pentanucleotide repeat, (ATTCT)_n, large-scale expansions of which cause spinocerebellar ataxia type 10 (SCA10) (8), does not form any unusual structures (9) but is a DNA unwinding element (DUE). Because a DUE is involved in replication initiation *in vivo*, it was proposed that (ATTCT)_n repeats expansions could result from multiple reinitiation of DNA replication within this sequence (9, 10). Another example of an expandable repeat that is not expected to form alternative DNA structures is a sequence, (TGGAA)_n, responsible for SCA31 (11). We were interested, therefore, in whether the same mechanisms govern expansions of the structure-prone and structure-proof repeats.

Here, we concentrated on the mechanisms of expansions of the SCA10 (ATTCT)_n repeat. This repeat is positioned in the ninth intron of the human *ataxin-10* (*ATXN10*) gene (8). Normal individuals have 10–22 copies, whereas affected individuals may have up to 4,500 copies of this repeat. SCA10 is an autosomal dominant disease that is prevalent in Mexico and Brazil. The two populations differ both symptomatically and genetically when it comes to the disease (12). In addition to ataxia, Mexican families are afflicted with epilepsy, but Brazilian families are not (13, 14). At a DNA level, expanded (ATTCT)_n repeats without interruptions are typical for the Mexican population, whereas multiple interruptions throughout the expanded repetitive run are characteristic for the Brazilian population. The SCA10 pathogenesis is not well understood. It was suggested that haploinsufficiency of the *ATXN10* gene, RNA gain of function (15), or chromatin change (12, 16) could contribute to the disease.

The mechanisms responsible for the expansions of the (ATTCT)_n repeats remain unclear. They were never detected in any experimental system, making the genetic analysis of the process impossible. Thus, from a biomedical point of view it was paramount to develop a genetically tractable experimental system to study (ATTCT)_n repeat expansions. Here we achieved this goal using our recently developed strategy to monitor large-scale changes in repeat lengths in yeast (17). Specifically, non-interrupted ATTCT repeats, ranging from 46 to 81 copies, were integrated into an artificial intron of the *URA3* gene, which rendered the gene functional. Expansions of the repeat beyond ≈85 copies blocked the reporter gene's expression, leading to the selectable genotype, 5-fluoroorotic acid resistance. The rate of repeat expansions seemed to increase with the repeat's length, mimicking human pedigrees. We identified several proteins that affected repeat expansions. The *Tof1* protein, a component of the replication fork stabilizing complex (18, 19), seemed to prevent (ATTCT)_n repeat expansions, whereas the *Rad5* protein, responsible for template switching during postreplication repair (20), was necessary for repeat expansions. At the same time, the *Rad52* protein, a master component of homologous recombination in yeast (21), does not seem to play a significant role in (ATTCT)_n repeat expansions. We also found that (ATTCT)_n repeats stimulated chromosomal fragility in a length-dependant

Author contributions: A.A.S., T.A., C.H.F., and S.M.M. designed research; N.C., A.A.S., L.I.S., R.H.T., L.S., and R.M. performed research; P.S.S. and T.A. contributed new reagents/analytic tools; N.C., A.A.S., L.I.S., T.A., C.H.F., and S.M.M. analyzed data; and N.C., C.H.F., and S.M.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009409108/-DCSupplemental.

manner and that fragility was also affected by the Tof1 and Rad5 proteins, being reduced in their absence.

We conclude that repeat expansions occur during DNA replication and/or postreplicative repair. Remarkably, our genetic data are qualitatively similar to that obtained for a different repeat, (GAA)_n—a prominent structure-forming repeat. We conclude, therefore, that the repetitive nature of these sequences might be the key factor that predisposes them to expansions during DNA replication and propose a model for this process. This said, comparison of expansion and contraction rates for different repeats confirms that the structure-forming potential of a sequence dramatically contributes to its instability.

Results

Yeast System to Study Expansions of (ATTCT)_n Repeats. A DNA fragment from a patient with SCA10 containing more than 500 (ATTCT)_n repeats was initially cloned into the pcDNA3.1/Hygro vector as previously described (15). After we transformed the resultant plasmid into *Escherichia coli*, the repeat had contracted down to ≈220 units. Sequencing of the contracted repeat revealed multiple interruptions providing only 41 ATTCT repeats as the longest uninterrupted run of repeats (Fig. 1A). We am-

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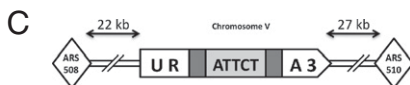
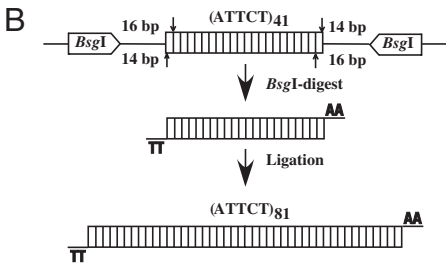


Fig. 1. Yeast system for studying expansions of (ATTCT)_n repeats. (**A**) An (ATTCT)_n repeat from a Brazilian patient with SCA10 contains multiple interruptions (underlined), providing only 41 uninterrupted repeats (bolded). (**B**) Scheme for cloning longer uninterrupted (ATTCT)_n repeats. (**C**) Our experimental cassette, in which various (ATTCT)_n repeats (light gray) are placed within an intron (dark gray) of the artificially split *URA3* gene (white) on chromosome V. This cassette replaced the endogenous *URA3* gene, positioned 22 kb away from *ARS508* on the left and 27 kb away from *ARS510* on the right.

plified this uninterrupted run using PCR with the primers carrying restriction sites for the enzyme *BspI* at their 5' ends. Upon digesting a PCR product, an (ATTCT)₄₁•(AGAAT)₄₁ duplex with AA and TT 3' overhangs was formed (Fig. 1B). These overhangs allow a self-ligation of multiple (ATTCT)₄₁•(AGAAT)₄₁ fragments in a head-to-tail orientation only. Using this approach, we generated an uninterrupted (ATTCT)₈₁ repeat in vitro.

The (ATTCT)₈₁ repeat was then cloned into the intron of the artificially split *URA3* gene (17). The resultant cassettes were excised and integrated into chromosome V of the CH1585 strain, replacing its *ura3-52* allele upon selection for uracil prototrophy (Fig. 1C). PCR analysis of the *URA*⁺ clones revealed properly integrated *URA3* cassettes with 46, 64, and 81 ATTCT repeats, which corresponded to 628-, 718-, and 803-bp-long introns. A yeast strain with 81 ATTCT repeats in the *URA3* gene grew exceptionally slowly on the media lacking uracil. Furthermore, when it was plated on the 5-FOA-containing media, a lawn of small colonies would slowly form as well. We concluded, therefore, that 81 ATTCT repeats inactivated the *URA3* gene strongly enough to make cells partially 5-FOA resistant, which made this repeat useless for further selection. This left us with two repeat lengths suitable for the selection for expansions: (ATTCT)₄₆ and (ATTCT)₆₄.

In a previous study of (GAA)_n repeats in yeast, we found that repeat expansions blocked splicing of the *URA3* gene carrying an intron, when the intron's length exceeded ≈1.1 kb (17). We reasoned that substantial expansions of the (ATTCT)_n repeats within the *URA3* intron should lead to the reporter's inactivation as well, making yeast 5-FOA resistant. Fig. 2 shows PCR analysis of the repeat lengths 5-FOA^R clones originated in the strain carrying 64 (ATTCT)_n repeats in the *URA3* intron. Two types of events are evident: significant repeat expansions and unchanged repeat lengths.

Rates and Scales of (ATTCT)_n Repeat Expansions. To determine the rates of both events leading to drug resistance, 8–12 independent single colonies grown on full media were replated onto the selective, 5-FOA-containing media, as well as on full media for normalization. All 5-FOA^R clones from six to eight selective plates were analyzed by PCR for their repeat length. This gave us the frequencies of expansions of both events for (ATTCT)_n repeats. Their rates were then calculated using the method of mutant accumulation, as previously described (17). The average rate of expansions increased eightfold when the number of repeats increased 1.4-fold (Table 1). The difference in expansion rates between (ATTCT)₄₆ and (ATTCT)₆₄ repeats was highly statistically significant (*P* < 0.0001). The rates of events in which repeat lengths remained unchanged also depended on the repeat's length, but very modestly: a 2.3-fold increase when the number of repeats increased 1.4-fold. Surprisingly, only three of 16 sequenced clones in this group contained mutations in the *URA3* cassette: two had missense mutations in the *URA3* ORF, and one had a point substitution in the *ACT1* intron. The

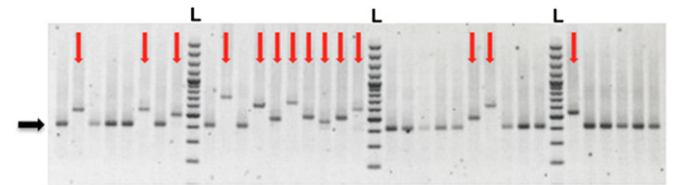


Fig. 2. PCR analysis of 5-FOA-resistant clones. The characteristic results for 5-FOA^R clones originated from (ATTCT)₆₄ repeats. Red vertical arrows point to expanded repeats, whereas the black horizontal arrow marks the position of the original repeat. L, 100 bp-plus ladder (Fermentas).

Table 1. Rates (95% confidence intervals) of expansions and other events leading to 5-FOA resistance

Repeat number	Rate of expansions ($\times 10^{-7}$)	Rate of unchanged repeats ($\times 10^{-7}$)	Rate of mutations ($\times 10^{-7}$)
0	N/A	N/A	0.94 (0.24–2.3)
46	0.44 (0.35–0.79)	3.5 (2.4–4.6)	Not studied
64	3.3 (2.4–4.1)	7.6 (5.6–9.7)	1.4 (1.0–1.8)

remaining 81% of 5-FOA-resistant clones did not have any mutations in the *URA3* cassette (discussed in *SI Results*).

Because the expansion rate for the (ATTCT)₆₄ repeat was sufficiently high ($\approx 4 \times 10^{-7}$ per cell/per generation), we accumulated 56 independent clones to build a distribution for the lengths of expanded repeats (Fig. 3). From this distribution, the range of expansion lengths lies between 81 and 132 repeats (i.e., the expansions are fairly significant). Evidently, the distribution is biased owing to the selection cutoff around 80–85 repeats. This is consistent with previous data that the *URA3* cassette with the (ATTCT)₈₁ repeat makes yeast partially 5-FOA resistant. Importantly, however, the median length of expanded repeats corresponds to ≈ 105 , which is above and beyond the selection cutoff. We believe that an addition of 40 pentanucleotide repeats (200 bp) might reflect an average increment in the expansions of (ATTCT)_n repeats.

Effects of (ATTCT)_n Repeats on Reporter Gene Expression. A priori, repeat-containing clones could become drug resistant if one of the following events occurred: (i) repeats expanded to a point where the intron length exceeded 1.1 kb (22), or (ii) expanded repeats directly block expression of the *URA3* gene at the transcription or posttranscription level. The latter scenario seemed more likely for (ATTCT)_n repeats, because a strain carrying 81 repeats creates an 803-bp-long intron of the *URA3* cassette and is partially 5-FOA resistant.

To study the effects of the (ATTCT)_n repeats on the expression of the *URA3* gene, we first analyzed the levels of its mRNA by RT-PCR. We first conducted reverse transcription reaction with random primers followed by PCR with mRNA-specific primers (Fig. 4A). One can see that even the shortest repeat studied, (ATTCT)₄₆, decreased the amount of *URA3* mRNA by 10-fold compared with the *URA3* cassette with no repeats. As the numbers of repeats increased, there were progressively lesser amounts of *URA3* mRNA (Fig. 4B). With the longest repeat, (ATTCT)₈₁, less than 2% of the amount for the control mRNA was detected. This very low level of expression of the *URA3* gene, carrying 81 (ATTCT)_n repeats, explains the ability of the

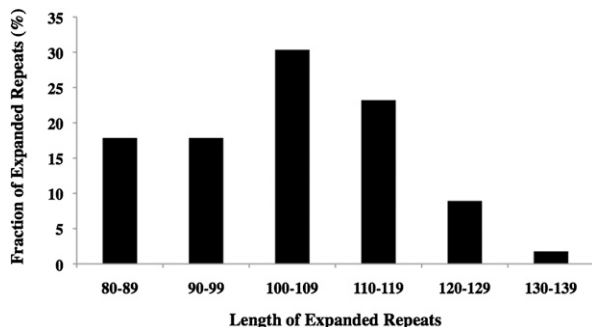


Fig. 3. Length distribution among the expanded repeats originated from the original (ATTCT)₆₄ repeat. The range is from 81 to 132 repeats, the mean expansion corresponds to 104 repeats, and the median expansion length is 105 repeats.

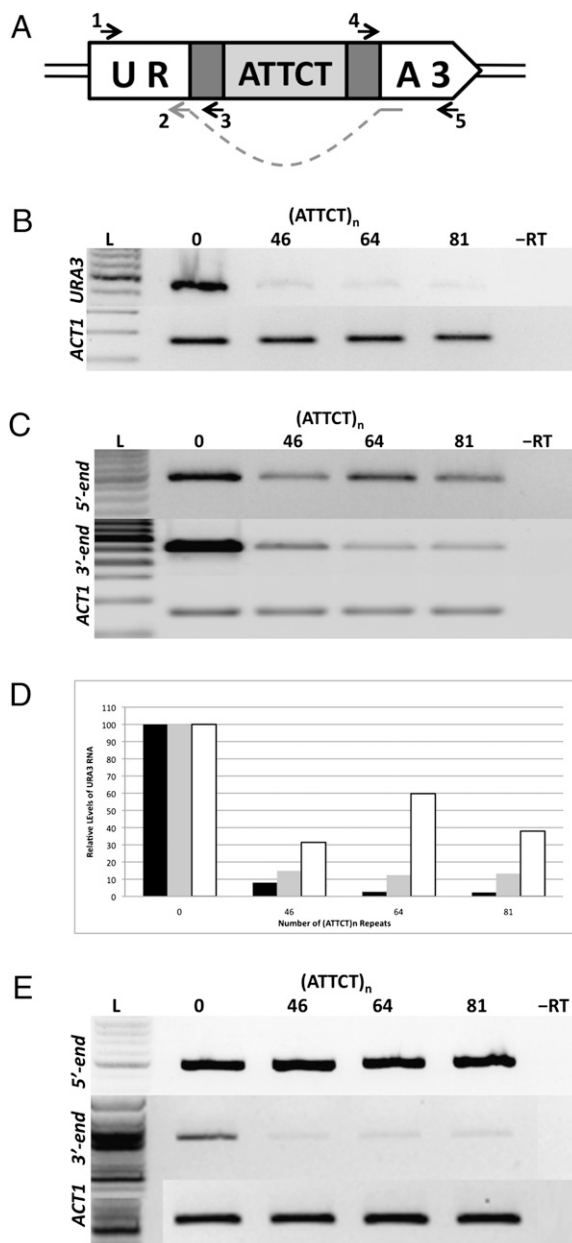


Fig. 4. Effects of (ATTCT)_n repeats on the reporter's gene expression. (A) Schematic representation of the repeat-bearing *URA3* cassette together with various primers used for the RT-PCR. Because primer 2 contains exonic sequences surrounding the intron, primers 1 and 2 were used to specifically amplify *URA3* mRNA. Primers 3 and 4 contain intronic sequences. Consequently, primers 1 and 3 were used to amplify *URA3* pre-mRNA upstream of the repeat (5' end), whereas primers 4 and 5 amplified pre-mRNA downstream of the repeat (3' end). (B) RT-PCR analysis of *URA3* mRNA for clones containing 0, 46, 64, and 81 ATTCT repeats. Actin mRNA was used for the normalization. (C) RT-PCR analysis of *URA3* pre-mRNA upstream and downstream of the repeat tract. (D) Quantitative graph showing relative amounts of *URA3* mRNA and pre-mRNA. Black, gray, and white bars correspond to mRNA, pre-mRNA downstream of the repeat, and pre-mRNA upstream of the repeat, respectively. (E) RT-PCR analysis of the *URA3* RNA upstream and downstream of the repeat tract for the polyadenylated transcripts only.

corresponding yeast strain to slowly grow on both URA⁻ media and 5-FOA⁺ media. Note that a 10-fold decrease in the *URA3* expressions does not make the yeast strain a uracil auxotroph, because orotidine-5'-phosphate decarboxylase is an exceptionally proficient enzyme (23).

Further RT-PCR experiments were performed to examine the levels of the *URA3* pre-mRNA situated 5' and 3' to the $(ATTCT)_n$ repeats. The levels of pre-mRNA downstream of the repeats decreased strongly with an increase in their lengths (Fig. 4C). In contrast, the levels of pre-mRNA upstream of the repeats were only modestly decreased compared with the repeat-free control (Fig. 4C).

Two scenarios could lead to a dramatic difference in pre-mRNA level upstream and downstream of the $(ATTCT)_n$ repeat: (i) transcription could be stalled by the repetitive sequence per se, or a protein bound to it, or (ii) $(AUUCU)_n$ runs in the transcript could set off RNA polyadenylation leading to premature transcription termination. To distinguish between these possibilities, we modified RT-PCR to amplify only polyadenylated transcripts by using oligo(dT) primers for the reverse transcription reaction. Fig. 4E shows that equal amounts of *URA3* pre-mRNA upstream of the repeat are present for all repeat lengths in this setting. This strongly suggests that the repetitive tract signals unruly polyadenylation.

Test of $(ATTCT)_n$ Repeat Fragility. Expanded trinucleotide repeats, as well as some AT-rich minisatellites, are sites of increased chromosomal fragility in human and yeast cells (24). To test whether the expanded SCA10 repeat would be a fragile site, we used a previously designed assay to assess the breakage rate of a yeast artificial chromosome (YAC) containing an expanded $(ATTCT)_n$ repeat. In this assay (Fig. 5A), loss of the right arm of the YAC distal to the repeat tract can be measured by loss of the *URA3* marker and subsequent ability of the cells to grow on 5-FOA media (25). Only breakage events that fail to heal normally and instead result in arm loss are measured, thus the rate of 5-FOA resistance is an underestimation of the true breakage rate, but it can be used to obtain a relative rate compared with a no-tract control, in this case a 386-bp nonrepetitive AT-rich human sequence. When we initially cloned the $(ATTCT)_{81}$ repeat into the YAC, we also obtained a spontaneous expansion in one of the transformants, which was verified to be a pure $(ATTCT)_{132}$ tract by sequencing. The $(ATTCT)_{81}$ tract induced 3.3-fold more chromosome fragility than the control sequence of equal length ($P = 0.003$), and the longer $(ATTCT)_{132}$ tract led to a 10.3-fold increase, a highly significant difference compared with the no-tract control ($P < 0.0001$) (Fig. 5B). Thus, expanded SCA10 repeats induce fragility in a length-dependent manner. The level of fragility observed here for the $(ATTCT)_n$ repeats seems to be comparable to that of a $(CAG)_n$ repeat tested in the same assay (25–27).

Genetic Analysis of Repeat Expansions, Contractions, and Fragility. To gain more insight into the mechanisms of repeat expansions, we analyzed the effects of various mutations affecting DNA replication, recombination, and repair on this process. Specifically, we analyzed three mutations: $\Delta tof1$, $\Delta rad5$, and $\Delta rad52$, because they were useful for our understanding of the $(GAA)_n$ repeat expansions (17).

A *URA3* cassette containing $(ATTCT)_{64}$ repeats was introduced in *Tof1*, *Rad5*, or *Rad52* knockout strains, and the rates of the repeat expansions in those strains were determined. The median rates expansions obtained from at least three independent experiments are presented in Table 2. One can see that in the *tof1* strain, the average rate of expansions was fivefold higher than that in the wild-type strain ($P < 0.0001$), suggesting that *Tof1p* works against repeat expansions. The results in *rad5* strain were strikingly opposite: no expansions at all were observed, indicating that the *Rad5* protein could be the key player promoting repeat expansions. The rate of expansions in *rad52* was threefold less than in the wild-type strain, a difference that was not as dramatic as in the *rad5* strain but still statistically significant.

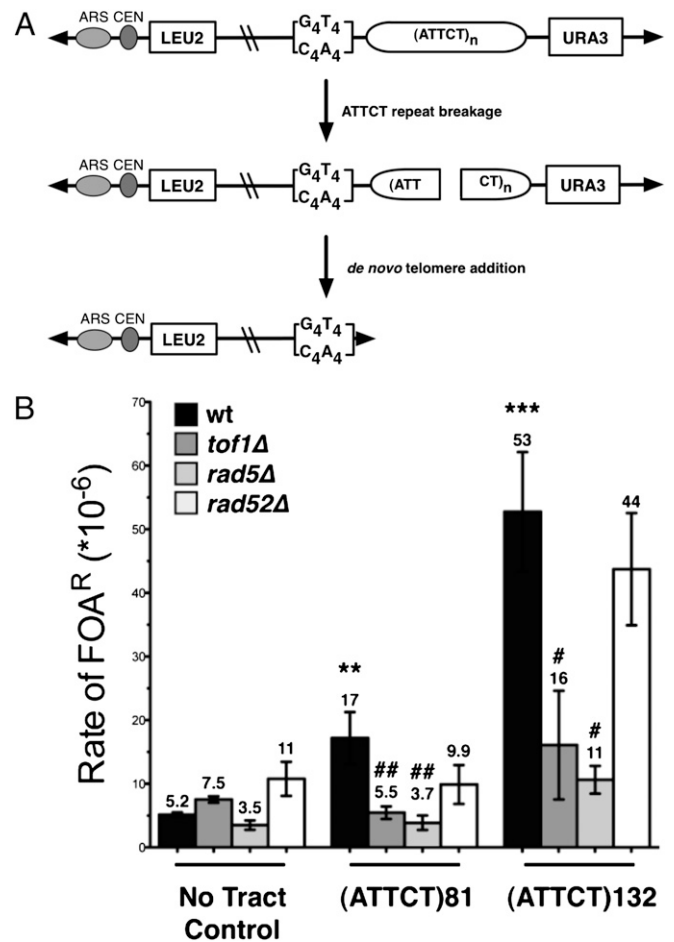


Fig. 5. Effect of $(ATTCT)_{81}$ and $(ATTCT)_{132}$ repeats on chromosome fragility. (A) Diagram of the fragility assay. The $(ATTCT)_n$ repeat tracts were cloned near the end of a YAC, proximal to a *URA3* marker gene. Chromosomes that break at or near the repeat and fail to repair will lose the *URA3* gene and can be rescued by telomere addition onto the G_4T_4 telomere seed sequence. (B) Rate of *Leu⁺FOA^R* cells provides a relative breakage rate compared with a no-tract control. Significance was determined by a pooled variant *t* test: compared with the no tract control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; or compared with the same tract length in the wild-type (wt), # $P < 0.05$, ## $P < 0.01$.

Our system also allowed us to determine the rates of repeat contractions in different genetic backgrounds. To this end, we started from a 5-FOA-resistant clone containing 103 $(ATTCT)_n$ repeats, which was obtained in the course of repeat expansion experiments, and analyzed the rate of accumulation of the *URA⁺* clones, which could only stem from repeat contractions. All *URA⁺* clones obtained in these experiments have shorter than 20 $(ATTCT)_n$ repeats (i.e., they resulted from large-scale

Table 2. Genetic control of expansions and contractions of the $(ATTCT)_n$ repeats

Genetic background	$(ATTCT)_{64}$ repeat rate (95% CI) of expansions ($\times 10^{-7}$)	$(ATTCT)_{103}$ repeat rate (95% CI) of contractions ($\times 10^{-7}$)
WT	3.3 (2.4–4.1)	6.4 (5.0–7.9)
$\Delta tof1$	17.4 (8.5–26.3)	48.0 (35–61)
$\Delta rad5$	$< 5 \times 10^{-8}$	9.5 (4.0–17)
$\Delta rad52$	1.0 (0.55–1.5)	Not studied

CI, confidence interval.

contractions). Table 2 shows that the rate of contractions for the (ATTCT)₁₀₃ repeat was quite low, 6.4×10^{-7} compared with other repeats studied in yeast (Table 3). In the Tof1 knockout strain, the rate of contractions was elevated 7.5-fold compared with the wild-type strain, similarly to what was observed for expansions, suggesting that the Tof1 protein acts to protect against both repeat contractions and expansions. In contrast with the expansion data, the rate of repeat contraction in the Rad5 knockout strain was unchanged. Thus, the Rad5 protein has no bearing on repeat contractions.

We then tested repeat-mediated fragility in *tof1Δ*, *rad5Δ*, and *rad52Δ* backgrounds. Unexpectedly (Fig. 5B), repeat-mediated fragility was significantly decreased (approximately threefold) in the *tof1Δ* strain relative to the wild-type strain for both (ATTCT)_n tracts. In contrast, the absence of the Tof1 protein did not affect the fragility of the no-tract control YAC. Similar to expansions, fragility was highly dependent on the Rad5 protein, being approximately fivefold decreased in the *rad5Δ* background compared with wild-type levels for both repeats (Fig. 5B). Repeat-mediated fragility was slightly depressed in the *rad52Δ* background, but this change was not statistically different compared with wild type.

Discussion

We have shown that expansions of a pentanucleotide repeat (ATTCT)_n responsible for SCA10 in humans can be observed in a yeast experimental system. The propensity of this repeat to expand depended on its length: there was an eightfold increase in the rates of expansions between the repeats differing in lengths just 1.4-fold. This observation mimics what is known about the (ATTCT)_n repeat expansions in human SCA10 pedigrees (12). Admittedly, the (ATTCT)_n repeat can easily expand up to thousands of copies in humans (8) but only to hundreds of copies in our yeast system. This difference is due to the fact that relatively short repeats already cause *URA3* gene inactivation.

As the repeat length within the *URA3* intron increased, the levels of *URA3* mRNA progressively decreased. In fact, even the shortest (ATTCT)₄₆ run studied already caused a 10-fold decrease in the *URA3* mRNA level, whereas (ATTCT)₈₁ repeat decreased it to just 2% of the control level. Furthermore, the amount of *URA3* pre-mRNA downstream of the repeat also decreased dramatically with the repeat's length. In contrast, the amount of pre-mRNA upstream of the repeat was decreased insignificantly compared with the repeat-free control. Furthermore, by analyzing polyadenylated RNA transcripts via RT-PCR with an oligo(dT) primer, we showed the presence of near-identical amounts of pre-mRNA upstream of the repeat independent of its length. We conclude, therefore, that the repeat somehow signals RNA polyadenylation, resulting in the premature transcription termination and accumulation of RNA transcripts truncated at or past the repeat. The mechanisms of triggering polyadenylation by (AUUCU)_n runs in yeast remain elusive. It may have to do with the fact that the polyadenylation signals are not as highly conserved in yeast as in higher eukar-

yotes (28). Notably, to date no decrease of pre-mRNA or processed mRNA for the mutant SCA10 allele has been detected in humans (15).

Genetic analysis of expansions revealed that knocking out the *TOF1* gene leads to a fivefold increase in the rate of expansions of the (ATTCT)₆₄ repeat compared with the wild-type strain. This effect of the Tof1 knockout on the expansions of a pentanucleotide repeat is quantitatively similar to its effect on trinucleotide repeats (17, 29). Thus, functional Tof1 protein precludes expansions of various unstable repeats. Strikingly, the deletion of the *RAD5* gene in the (ATTCT)₆₄ strain led to the complete elimination of repeat expansions. This is far more dramatic than the previous observation of a relatively modest decrease in the rate of (GAA)_n repeat expansions upon *RAD5* inactivation (17).

Tof1 is a fork-stabilizing protein, which in a complex with Csm3 and Mrc1 proteins prevents fork dissociation when it encounters DNA lesions or other stall sites (30). Rad5, in contrast, facilitates template switching, which allows the replication fork to bypass DNA lesions and other impediments (31). Because Rad5 seemed to be vital for the expansion of (ATTCT)_n repeats, we favor a previously proposed model (17) implicating template-switch as the mechanism for expansions (Fig. 6). During the replication of repetitive tracts longer than one Okazaki fragment, the nascent leading strand might occasionally switch from its template to the nascent lagging strand, because the 3' end of the nascent leading strand is complementary to multiple sequences in the nascent lagging strand. Upon reaching the end of an Okazaki fragment, the polymerase would have to switch back to the leading strand template for the replication to continue. After DNA replication is resumed, extra repeats remain in the nascent leading strand. When the *TOF1* gene is inactivated, the replication fork becomes less stable, increasing the likelihood of template switching. Because the Rad5 protein is essential for the template switching, its inactivation should halt template switch altogether, precluding expansions.

Contractions of the (ATTCT)_n repeats are also stimulated by the lack of the Tof1 protein but are independent of the Rad5 protein. This tells us that a destabilization of the replication fork, rather than template switching, is the key for repeat contractions. It is generally believed that contraction could happen when a replicative DNA polymerase skips a looped-out portion of the template strand corresponding to a repeat (Fig. 6).

(ATTCT)_n repeat-mediated fragility is different from both expansions and contractions in that it is decreased upon inactivation of the *TOF1* gene. This suggests that fragility occurs not during the template switching, or template skipping, but in some alternative pathway. Our data suggest that in the wild-

Table 3. Comparison of expansion and contraction frequencies for different repeats in *S. cerevisiae*

Expandable repeat	Frequency of expansions	Frequency of contractions	Reference
(ATTCT) ₆₄	1.1×10^{-6}	NS	This study
(GAA) ₁₀₀	1.0×10^{-5}	NS	17
(CAG) ₇₀₋₇₈	$0.8-1.0 \times 10^{-3}$	3.7×10^{-3}	25, 27, 38
(ATTCT) ₁₀₃	NS	2.3×10^{-6}	This study
(GAA) ₂₁₅	NS	1.2×10^{-2}	This study
(CAG) ₁₅₅	5.0×10^{-2}	1.7×10^{-1}	25

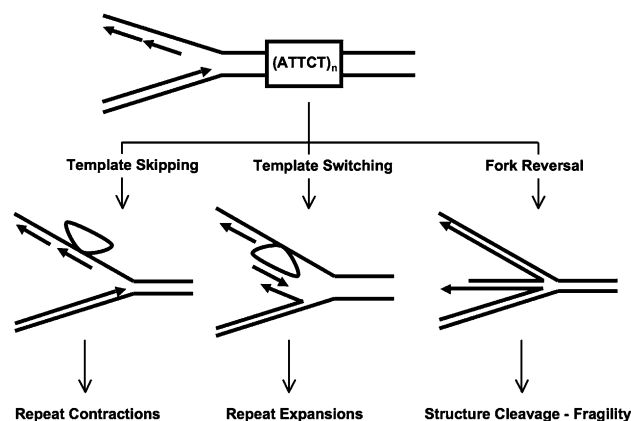


Fig. 6. Template skip (Left), template switch (Center), and fork reversal (Right) models for repeat contractions, expansions, and repeat-mediated fragility, respectively (see text for details).

type strain with an intact Tof1/Csm3/Mrc1 complex, template switching within the (ATTCT)_n repeat is a rare event (Table 1). We suggest that fork reversal caused by either an AT-richness or a slippery nature of this repeat is more common, as was also observed for other expandable repeats (32, 33). In this scenario (Fig. 6), fragility would occur by the cleavage of reversed fork intermediates. Because Rad5 has been shown to catalyze fork reversal in vitro (34), the decrease in fragility in the *rad5Δ* strain would be due to a decrease in the reversed fork substrate. Alternatively, a recombination intermediate formed in the context of the PRR pathway could be a substrate for nuclease cleavage at (ATTCT) repeats, causing fragility.

The (ATTCT)_n repeat differs from other expandable repeats in that it does not form stable secondary structures, such as hairpins, cruciforms, triplexes, and G-quartets (1); instead, it is a DUE (9). The proposed template-switch model does not require a repeat to form stable secondary structures. As such, it is uniquely applicable to direct tandem repeats. Note, however, that although the propensity to form alternative DNA structures is not necessary for a repeat to expand, structure-forming repeats seem to expand at a higher rate. Table 3 illustrates this point by comparing expansion and contraction rates for three repeats differing in their structure-forming ability. Evidently, the (ATTCT)_n repeat has the lowest propensity to expand or contract compared with either a triplex-forming (GAA)_n repeat (17) or hairpin-forming (CAG)_n

repeats (25, 27, 35). We believe, therefore, that the formation of stable secondary structures during replication of a repetitive sequence might additionally increase the likelihood of template switching, resulting in its higher expansion rate.

Materials and Methods

Strains. The *Saccharomyces cerevisiae* strain used for the expansion assay was CH1585 (FY251) (17). The strain used for the fragility assay was BY4705 (36).

Plasmids and Selectable Cassettes. (ATTCT)_n repeats were cloned into the unique *MunI* site in the intron of the pYES2-intron plasmid (17) (more details in *SI Materials and Methods*).

Gene disruption was carried out by direct gene disruption using *kanMX* selectable marker (details in *SI Materials and Methods*). Yeast genomic DNA was isolated as previously described (37).

A protocol for PCR analysis of long (ATTCT)_n repeats is described in detail in *SI Materials and Methods*.

Rates of expansions were determined using the method of mutant accumulation (38) with the modifications described in *SI Materials and Methods*.

Rates of fragility were determined using fluctuation assays as previously described (25) (*SI Materials and Methods*).

RNA isolation and its RT-PCR analysis are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Claire Moore and Mitch McVey for helpful discussions. This work was supported by the National Institutes of Health Grants GM60987 (to S.M.M.), GM063066 (to C.F.), and NS041547 (to T.A.).

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