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Keywords: trinucleotide repeat; expansion; checkpoint; DNA damage response; replicational coupling

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Abstract: Trinucleotide repeats frequently expand and contract in humans and model organisms. Protein factors that modulate this process have been found by candidate gene approaches or mutant screens for increased expansion rates. To extend this effort, Saccharomyces cerevisiae mutants with higher CAG•CTG repeat contraction rates were sought using a disruption library. This screen identified Mrc1, the homolog of human Claspin, which mediates the replication and DNA damage checkpoints, and also couples the replicative helicase and polymerase. Genetic analysis showed that Mrc1, along with Tof1 and Csm3, inhibits instability in two distinct ways. Contraction rates of (CAG)20 tracts are elevated by loss of Mrc1, Tof1 or Csm3, but not by defects in most replication checkpoint or DNA damage checkpoint proteins. The three proteins likely inhibit contractions primarily through their coupling activity, which would prevent accumulation of single-strand template DNA prior to the formation of aberrant secondary structure. In contrast, expansion rates of (CTG)13 are elevated in strains defective for Mrc1, Tof1, Csm3, Mec1, Ddc2, Rad24, Ddc1, Mec3, Rad17, Rad9, Rad53 or Chk1, suggesting that the DNA damage checkpoint inhibits expansions after formation of repeat-dependent structures. Together, these results indicate that at least two Mrc1-dependent mechanisms function to reduce CAG*CTG repeat instability.

Razidlo and Lahue Author Agreement

This is to confirm that all authors on the paper have agreed to publishing the manuscript, if accepted.

Dear Sir/Madam,

I am pleased to present this manuscript for consideration at *DNA Repair*. We feel that this work represents important new insights into mechanisms that control triplet repeat instability.

This study used an unbiased mutant screen in yeast and found that disruption of *MRC1* leads to consistently elevated expansion and contraction rates of CAG•CTG repeat tracts. Subsequent analysis showed that Tof1 and Csm3 are also necessary to inhibit both classes of TNR mutations. In the absence of any of these proteins, triplet repeats expand and contract significantly more often than normal. The starting lengths for the expansion and contraction reporters were specifically chosen to monitor length changes near the threshold. These length changes thereby span the range between genetically stable, subthreshold alleles and the longer, unstable tracts that can give rise to further mutation and disease in humans. The demonstration that Mrc1, Tof1 and Csm3 inhibit instability for subthreshold alleles confirms and extends previous studies showing that long, expanded alleles of 85-155 CAG repeats are also prone to fragility and contraction in the absence of the DNA damage checkpoint pathway (Lahiri et al., 2004 *Mol. Cell*), and to fragility, contraction and expansion in the absence of Mrc1 (Freudenreich and Lahiri, 2004 *Cell Cycle*). Our work provides two additional important insights to the role of Mrc1, Tof1 and Csm3. First, *mrc1* and *tof1* mutants have a highly selective mutator phenotype where only structure-forming CAG•CTG tracts are destabilized. Other sequences, including the *CAN1* gene, poly(GT) tracts and unstructured CTA repeats are not affected by *mrc1* or *tof1* mutations. These findings are entirely consistent with the structure forming requirement of pathogenic TNRs as a major part of accepted models of instability (Pearson et al., 2005 *Nat. Rev. Genet.*; Mirkin, 2007 *Nature*). Furthermore this selectivity strongly supports the idea, as described below for expansions, that the DNA damage checkpoint responds to TNR-mediated secondary structure or to subsequently processed forms (Lahiri et al., 2004; Freudenreich and Lahiri, 2004). The second important finding was that expansions and contractions are differentially sensitive to defects in DNA damage checkpoint factors, indicating that at least two mechanisms are at play in preventing triplet repeat instability through Mrc1, Tof1 and Csm3. Together these results significantly extend what is known about Mrc1, Tof1 and Csm3 and their action at TNRs.

Sincerely,
Robert Lahue

*** Disclosure Statement**

Razidlo and Lahue Disclosure Statement

This is to confirm that the authors have no conflict of interest. Furthermore, we confirm that all resources used to support the manuscript are cited, and that the manuscript has not been published elsewhere.

Mrc1, Tof1 and Csm3 inhibit CAG·CTG repeat instability by at least two mechanisms

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Keywords: trinucleotide repeat; expansion; checkpoint; DNA damage response; replicational coupling

Abstract

Trinucleotide repeats frequently expand and contract in humans and model organisms. Protein factors that modulate this process have been found by candidate gene approaches or mutant screens for increased expansion rates. To extend this effort, *Saccharomyces cerevisiae* mutants with higher CAG·CTG repeat contraction rates were sought using a disruption library. This screen identified Mrc1, the homolog of human Claspin, which mediates the replication and DNA damage checkpoints, and also couples the replicative helicase and polymerase. Genetic analysis showed that Mrc1, along with Tof1 and Csm3, inhibits instability in two distinct ways. Contraction rates of $(CAG)_{20}$ tracts are elevated by loss of Mrc1, Tof1 or Csm3, but not by defects in most replication checkpoint or DNA damage checkpoint proteins. The three proteins likely inhibit contractions primarily through their coupling activity, which would prevent accumulation of single-strand template DNA prior to the formation of aberrant secondary structure. In contrast, expansion rates of $(CTG)_{13}$ are elevated in strains defective for Mrc1, Tof1, Csm3, Mec1, Ddc2, Rad24, Ddc1, Mec3, Rad17, Rad9, Rad53 or Chk1, suggesting that the DNA damage checkpoint inhibits expansions after formation of repeat-dependent structures. Together, these results indicate that at least two Mrc1-dependent mechanisms function to reduce CAG•CTG repeat instability.

1. Introduction

Trinucleotide repeats (TNRs) are unstable repetitive DNA elements found in both coding and non-coding regions of numerous human genes. Expansions in specific TNRs cause at least 15 heritable neurodegenerative human diseases, including Huntington's disease and fragile X syndrome [1, 2]. Expansion patterns follow non-Mendelian inheritance patterns in afflicted families [3], indicating that complex and unique molecular mechanisms underlie the propensity of triplet repeats to expand and contract [1, 2]. Disease-causing TNRs almost exclusively have the sequence $(CNG)_n$, and single-stranded DNA containing these repeats readily forms secondary structures *in vitro* that correlate

strongly with the genetic instability of these sequences *in vivo* [4, 5]. Furthermore, DNA polymerases *in vitro* [6] and replication forks in *E. coli* [7] and yeast [8] have difficulty synthesizing G-C rich TNRs. These and other observations led to well supported replication-based models for TNR instability in proliferating cells that are all founded on the premise that aberrant replication of the lagging strand is linked to secondary structure formation in single stranded DNA (ssDNA) [1, 2, 9]. Generation of ssDNA on the nascent strand of the Okazaki fragment may trigger hairpin formation, allowing formation of this crucial structured intermediate that ultimately yields an expansion. Similarly, generation of excess ssDNA on the template strand is thought to permit collapse into a hairpin, and aberrant synthesis past this hairpin would result in contraction on one strand. Thus, for both expansions and contractions, the availability of ssDNA at TNRs is a critical factor determining the likelihood of hairpin formation and subsequent genetic instability.

Expansions and contractions in somatic cells can exhibit differing levels of instability in various tissues [10-13], suggesting that tissue-specific *trans*-acting factors modulate TNR instability. In accordance with this idea, several pathways in yeast modulate TNR mutagenesis, including Okazaki fragment maturation [14-16] and post-replication repair [17, 18]. To identify novel *trans* factors, we performed a blind screen for *S. cerevisiae* mutants that increase rates of TNR contractions. This screen revealed an *mrc1* mutation, suggesting that Mrc1 protein normally prevents contractions in wild-type cells. We focused on Mrc1 because of recent findings implicating it and associated proteins in limiting accumulation of ssDNA, discussed below, and also in prevention of chromosome fragility and instability in yeast with a long, disease-length $(CAG)_{85}$ tract [19, 20].

Mrc1 was initially identified as a mediator of the replication checkpoint [21], which responds to stalled replication forks arising from treatment with hydroxyurea (HU). In the presence of a stalled fork, ssDNA coated with RPA stimulates the recruitment of Mec1/Ddc2 (in yeast) or ATR/ATRIP (in

humans) to the replication fork [22, 23]. Mec1 phosphorylates and activates Mrc1, which recruits and facilitates the activation of the effector kinase Rad53 (Chk2 in humans). Activated Rad53 then phosphorylates a variety of downstream targets, resulting in the inhibition of late origin firing and the upregulation of genes involved in DNA repair [24]. The loss of Rad53, combined with HU treatment, leads to excess ssDNA formation at the replication fork that is detectable by electron microscopy [25]. Mrc1 is also involved in a second checkpoint, the intra-S phase DNA damage response. A number of proteins in the DNA damage response overlap with those in the checkpoint response, including Mrc1, Mec1/Ddc2, Rad53 and others [26]. This overlap may be due to damage sensing through promotion of single-strand gaps. However the DNA damage response also requires additional factors, such as the alternative clamp loader Rad24 and the alternative clamp Rad17/Mec3/Ddc1 (9-1-1 in humans) [26]. Thus phenotypes associated with defects in Rad24, Rad17, Mec3 or Ddc1 distinguish the DNA damage response from the replication checkpoint. In addition to signaling, Mrc1 also has a structural role at the replication fork that is central to normal replisome function [27]. Mrc1 functions with Tof1 and Csm3 to form the replication pausing complex, which maintains fork stability and prevents the uncoupling of helicase and polymerase activities under conditions of replication stress [28, 29]. In cells lacking Mrc1, Tof1 or Csm3, helicase activity occurs without polymerization, and leads to accumulation of excess ssDNA [30]. Thus Mrc1 is involved both in preventing accumulation of ssDNA through its structural role, and response to ssDNA via the replication checkpoint and the DNA damage response [29].

The evidence summarized above shows that genetic instability at TNRs is potentially suppressed by Mrc1 either through replicational coupling to avoid ssDNA and secondary structure formation, or to checkpoint response(s) after structure formation to reduce the likelihood of completing the mutagenic process. Previous work showed that long CAG•CTG tracts, which are disease-causing in humans, can be further destabilized by defects in the DNA damage response [19] or Mrc1 [20]. Our independent

discovery of an *mrc1* mutant that also destabilized shorter CAG•CTG runs, more similar to those seen in normal humans, suggested that checkpoint activities help prevent triplet repeat mutations between genetically stable, subthreshold alleles and the longer, unstable tracts that can give rise to further mutation and disease in humans. Furthermore we found that Mrc1, Tof1 and Csm3 are highly selective in protecting TNRs from instability, and that they use two distinct mechanisms to help avoid CAG•CTG repeat expansions and contractions. Together these results significantly extend what is known about Mrc1, Tof1 and Csm3 and their action at TNRs.

2. Materials and methods

2.1. Saccharomyces cerevisiae strains

Most strains used in this study were derived from BY4741 (*MAT-a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), a derivative of *Saccharomyces cerevisiae* strain S288C (Open Biosystems). Mutants used in this study were created by targeted deletion and confirmed by PCR, and when possible, by phenotypic traits such as UV or hydroxyurea sensitivity. TNR-containing plasmids were digested and integrated into the yeast genome; single integrants were confirmed as described previously [31].

2.2. Plasmids

The pBL94 vector was used to construct all TNR-containing plasmids as described previously [32]. The dinucleotide repeat-containing plasmid, pSH44 [33], was a gift from Tom Petes, Duke University. The *CEN/ARS* based recovery plasmid p*MRC1* and the p$mrc1^{AQ}$ mutant plasmid [34] were gifts from Stephen Elledge, Harvard University.

2.3. Genetic assays and molecular analysis of mutated TNR alleles

Expansion and contraction rates were measured by fluctuation analysis as described previously [31, 32] and as shown in Fig. 1. Mutation rates were calculated by the method of the median [35]. Single-

colony PCR analysis of expansions and contractions was performed as previously described [32, 36], and rates were corrected by multiplying the percent bona fide expansions/contractions by the apparent mutation rates obtained by fluctuation analysis [31]. Dinucleotide mutation rates were measured as described previously [33]. Forward mutation rates for the *CAN1* gene were determined by fluctuation analysis using selection for canavanine resistance. At least two independent clones were tested for all the above assays to ensure reproducibility. Statistical analyses for data shown in Table 2 were performed using the t-test (two-tailed distribution and two-sample equal variance) and P values of less than 0.05 were considered statistically significant. Any outliers were determined using the Q-test. For contraction and expansion experiments in Table 1, mutant strains were directly compared to wild-type in each experiment, and the results are expressed as fold change in rate compared to wild-type. Statistical analyses were performed using the Wilcoxon Mann Whitney test. P values of less than 0.05 were considered statistically significant.

2.4. Genetic screen for modifiers of TNR stability

To identify novel proteins involved in preventing TNR contractions, a random screen of yeast insertion mutants was performed. A plasmid disruption library [37] was used to disrupt random genes in yeast strain BY4741 containing a $(CAG)_{20}$ contraction reporter. A high throughput replica plating method was used to identify mutants with increased rates of instability, based on excess number of papillae when transferred to media lacking uracil. Mutants with increased rates of contractions as compared to wild type were further analyzed by fluctuation analysis. Several mutants showed increased rates of TNR contractions; mutant genes were identified by Vectorette PCR as described in [38].

3. Results

3.1. CAG repeat contractions are inhibited by Mrc1, Tof1 and Csm3

To find *trans*-acting factors that help prevent TNR instability, a yeast gene disruption library was used to screen for mutants with increased rates of TNR contractions. Screens have not previously been focused on mutants affecting contractions; therefore this approach complements previous screens that identified mutants with elevated expansion rates [17, 38]. Also, contractions in wild type yeast show a threshold-like effect, unique to TNRs, near 22 repeats [39]. Therefore a $(CAG)_{20}$-*URA3* reporter was chosen, based on the hypothesis that mutants that elevate contraction rates near the threshold might be highly selective for TNR instability. The strain is initially Ura^-, but contractions removing 5 repeats or more generate an Ura^+ phenotype (Fig. 1A) [32]. This strain was transformed with a library of *LEU2*-marked disruption cassettes [37] and approximately 15,000 Leu^+ isolates were screened for increased rates of CAG repeat contractions. The initial positives were screened twice more with increasing stringency. Using Vectorette PCR (described in [38]) we identified the disrupted *MRC1* gene in a mutant with 3.6-fold increased rates of contractions. These preliminary findings were confirmed when another *mrc1* strain, acquired commercially, displayed a 7.1 fold increase in contractions (Table 1). Furthermore, the contraction rate phenotype could be rescued in both strain backgrounds by add-back of the wild type gene on the low copy p*MRC1* plasmid (Table 1 and data not shown). Strains with *tof1* or *csm3* mutations had elevated contraction rates of 8.6- and 6.5-fold over wild type, similar in magnitude to the *mrc1* strain, and consistent with the functional interdependence of Mrc1, Tof1 and Csm3 [30]. Analysis of the contraction size spectra showed that *mrc1*, *tof1* and *csm3* mutants all yielded –16 to –20 repeat changes, overlapping the range seen in wild type cells [39]. We conclude that the lack of Mrc1, Tof1 or Csm3 leads to a higher rate of contractions, rather than the appearance of a new size class of contractions.

3.2. Mrc1, Tof1 and Csm3 are selective for preventing TNR instability

It is of particular interest to know if Mrc1, Tof1 and Csm3 selectively protect triplet repeats from instability or whether these proteins have a general effect to repress mutation rates throughout the

genome. To assess the mutator selectivity of *mrc1* and *tof1* mutants, three additional spontaneous mutator assays were performed (Table 2). Forward mutations in the *CAN1* gene, resulting in resistance to canavanine, detect many types of inactivating alterations. There was no detectable increase in the rate of canavanine resistance, compared to wild type, in strains lacking either Mrc1 or Tof1 (Table 2). In contrast, a *rad27* control strain lacking flap endonuclease 1 had an elevated rate, consistent with a previous report [40]. The second assay measures frameshift mutations of a dinucleotide repeat tract, $(GT)_{16.5}$, which most often occur as changes of ±1-2 repeats [33] rather than the larger mutations associated with TNRs. The rate of dinucleotide repeat changes was not statistically different from wild type for both *mrc1* and *tof1* (Table 2), whereas the rate in an *msh2* mismatch repair mutant is elevated several hundred fold [17]. In the third assay, we tested whether the TNR phenotype of *mrc1* and *tof1* mutants was dependent on the ability of the TNR to adopt stable secondary structure, which is generally believed to be very important in the mechanism of instability [1, 2]. Expansions were measured for a CTA repeat, which has poor structure forming ability *in vitro* [4] and is genetically stable in yeast as a $(CTA)_{25}$ allele [32]. There was no detectable increase in CTA expansion rates in cells lacking Mrc1 or Tof1 (Table 2), suggesting the phenotype of increased contractions occurs by a mechanism that requires the ability of the TNR to form secondary structures. In summary, these data support the idea that cells lacking Mrc1 or Tof1 (or, by inference, Csm3) are selectively defective in preventing TNR mutagenesis.

3.3. The replication stalling complex, Mrc1/Tof1/Csm3, reduces CAG repeat contraction rates independently of replication checkpoint and DNA damage checkpoint factors

In addition to its replication coupling role, Mrc1 also acts as a mediator of the replication checkpoint and the DNA damage checkpoint. To determine whether CAG repeat contractions are inhibited by either checkpoint, contraction rates were measured in additional mutants. We took advantage of a specific signaling deficient mutant form of Mrc1 called $mrc1^{AQ}$. This mutant has its Mec1 kinase target

phosphorylation sites modified so that it is unable to mediate signaling but is still capable of performing its coupling role [34]. When p*mrc1*^{AQ} was introduced into an *mrc1* knockout strain, the contraction phenotype was nearly normal (Table 1), suggesting that checkpoint signaling is dispensable for the prevention of contractions. Similarly, loss of the upstream kinase Mec1 showed no significant increase in contraction rates (Table 1), although there was a marginally significant increases in contraction rates in the strains lacking the Mec1-associated protein Ddc2 and the downstream kinase Rad53. Complementation by p*mrc1*^{AQ} and the lack of a *mec1* phenotype suggests that, for the most part, the checkpoint signaling function of Mrc1 is dispensable for the prevention of TNR contractions. To test the influence of the replication checkpoint and the DNA damage checkpoint, we investigated $(CAG)_{20}$ contraction rates for strains lacking the RFC-like Rad24 protein, components of the yeast 9-1-1 complex (Rad17, Mec3 and Ddc1), the signaling mediator Rad9 or the effector kinase Chk1. There was no significant contraction phenotype in strains lacking any of these factors (Table 1). Together, the lack of a contraction phenotype in most replication checkpoint or DNA damage checkpoint mutants suggests that the mechanism for preventing contractions may be different for the near-threshold tracts of 20 repeats used here versus the longer, 85 repeat alleles reported previously [19].

Efficient coupling of the replicative helicase and polymerase helps minimize single-stranded template DNA [25, 30]. We considered the possibility that the absence of coupling in *mrc1*, *tof1* or *csm3* strains would lead to more single-stranded template that could fold into a stable secondary structure and lead to a higher rate of contractions. If so, treatment of cells with hydroxyurea to increase ssDNA levels might give an elevated contraction phenotype. Wild type cells were grown continuously in the presence of 50 mM HU and then assayed for $(CAG)_{20}$ contractions. Modestly higher contraction rates were observed in HU treated cells compared to untreated controls (2.0-fold, $P = 0.037$). In contrast, there was no significant increase (<1.4-fold) in contraction rates when *mrc1* cells were treated with HU. Together with the absence of a checkpoint effect on contractions, the HU result supports the

coupling mechanism for preventing contractions. We conclude that the replication coupling activity of Mrc1, Tof1 and Csm3, not their checkpoint activities, is most important for inhibiting contractions of CAG repeats near the apparent threshold length.

3.4. Expansions are inhibited by the DNA damage checkpoints

Are expansions inhibited by Mrc1, Tof1 and Csm3, and, if so, do these proteins act through their coupling activity or in conjunction with checkpoint activation? Expansions were assessed using a $(CTG)_{13}$ repeat reporter (Fig. 1B) because this allele length in our system lies near the apparent threshold for expansions [32]. Similar to the increases seen for contraction rates in cells lacking Mrc1, Tof1 or Csm3 proteins, expansion rates of a $(CTG)_{13}$ repeat tract were elevated 4.0- to 7.4-fold in *mrc1*, *tof1* and *csm3* strains (Table 1). To test a longer repeat tract, the expansion rate of $(CAG)_{25}$ in the *mrc1* mutant was found to be 8-fold increased above wild type (*mrc1* 9.0 (±4.8) x 10^{-7} per cell generation; wild type 1.2 (±1.3) x 10^{-7} per cell generation; $P < 0.05$). These data show that Mrc1 helps prevent expansions of CAG•CTG tracts of 13-25 repeats, in accordance with a previous report for an 85 repeat allele [20].

However, contrary to what is seen for contractions, the checkpoint activity of Mrc1, Tof1 and Csm3 seems to be key for blocking expansions. First, when Mrc1-deficient cells were supplemented with the signaling deficient *mrc1*^{AQ} mutant, the mutant phenotype on $(CTG)_{13}$ was not suppressed (3.7-fold, compared to 4.0-fold for the *mrc1* strain). Second, there was significant increase in expansion rates for both *mec1* (6.0-fold) and *ddc2* (4.9-fold) mutants compared to the *sml1* (1.3-fold) parental strain. Together with the *mrc1*^{AQ} result, this suggests the replication and/or DNA damage checkpoint is an important inhibitor of expansions for CTG tracts near the apparent threshold. Third, increased expansion rates, 3.5- to 6.4-fold above wild type, were seen in strains specifically lacking DNA damage checkpoint components, such as the alternate clamp loader component Rad24, or any member

of the PCNA-like Rad17, Mec3, and Ddc1. Fourth, strains lacking the downstream mediator Rad9 or the effector kinases Chk1 or Rad53 also exhibited expansion rates that were 3.5- to 8.2-fold higher than wild type. All of the mutants tested for expansions gave phenotypes of similar magnitude (3.5- to 8.2-fold), consistent with a DNA damage checkpoint response to help block CTG expansions. The range of expansion sizes in all mutants (+5 to +10 repeats) was similar to wild type [17] suggesting that the higher rates are due to more expansions of the same size rather than appearance of a new size category. Clearly, expansions and contractions are inhibited by different Mrc1-dependent mechanisms for the repeat lengths tested here.

There was a formal possibility that the drug used to select cells with an expansion, 5-fluoroorotic acid (5FOA; Fig. 1B), might trigger a DNA damage response in wild type cells and slow their growth relative to the checkpoint mutants. Thus the higher expansion rates in the mutants might be an artifact of faster growth than wild type cells on 5FOA. This was tested in growth experiments on media containing 5FOA compared to rich media (YPD; yeast extract/peptone/dextrose). To mimic an expanded allele, cells were transformed with the *URA3* reporter in the unexpressed configuration, due to the presence of 33 CAG repeats. Comparison of the growth of the wild type strain and representative mutants (*mrc1*, *mrc1* + p*MRC1*, *sml1*, and *sml1 ddc2*) revealed no consistent differences between the strains. We deem it unlikely that the expansion phenotype observed for checkpoint mutants is due to differences in growth rate on 5FOA media used here.

4. Discussion

This study used an unbiased mutant screen and found that disruption of *MRC1* leads to consistently elevated expansion and contraction rates of CAG•CTG repeat tracts. Subsequent analysis showed that Tof1 and Csm3 are also necessary to inhibit both classes of TNR mutations. In the absence of any of these proteins, triplet repeats expand and contract significantly more often than normal. The starting

lengths for the expansion and contraction reporters were specifically chosen to monitor length changes near the threshold. These length changes thereby span the range between genetically stable, subthreshold alleles and the longer, unstable tracts that can give rise to further mutation and disease in humans. The demonstration that Mrc1, Tof1 and Csm3 inhibit instability for subthreshold alleles confirms and extends previous studies showing that long, expanded alleles of 85-155 CAG repeats are also prone to fragility and contraction in the absence of the DNA damage checkpoint pathway [19], and to fragility, contraction and expansion in the absence of Mrc1 [20]. Our work provides two additional important insights to the role of Mrc1, Tof1 and Csm3. First, *mrc1* and *tof1* mutants have a highly selective mutator phenotype where only structure-forming CAG•CTG tracts are destabilized. Other sequences, including the *CAN1* gene, poly(GT) tracts and unstructured CTA repeats are not affected by *mrc1* or *tof1* mutations. These findings are entirely consistent with the structure forming requirement of pathogenic TNRs as a major part of accepted models of instability [1, 2]. Furthermore this selectivity strongly supports the idea, as described below for expansions, that the DNA damage checkpoint responds to TNR-mediated secondary structure or to subsequently processed forms [19, 20]. The second important finding was that expansions and contractions are differentially sensitive to defects in DNA damage checkpoint factors, indicating that at least two mechanisms are at play in preventing triplet repeat instability through Mrc1, Tof1 and Csm3.

Two lines of evidence suggest that checkpoint signaling is not important to prevent contractions of $(CAG)_{20}$ in our system. First, the high rate of contractions in the *mrc1* strain was complemented by a mutant allele, $mrc1^{AQ}$, which is selectively defective in checkpoint responses [34]. Second, contraction rates are also elevated by loss of Tof1 or Csm3, but not by defects in most replication checkpoint or DNA damage checkpoint proteins (Mec1, Rad17, Rad24, Ddc1, Mec3, Rad9 or Chk1). There was a small contraction phenotype in *sml1 ddc2* and *sml1 rad53* strains, although the reasons for this remain unclear. Nonetheless, these data suggest that Mrc1, Tof1 and Csm3 inhibit contractions primarily

through their coupling activity on the replicative helicase and polymerase. In *mrc1*, *tof1* or *csm3* mutants, the helicase becomes uncoupled from the polymerase, leading to accumulation of single-stranded template [25, 30] and spontaneous secondary structure formation at single-stranded CAG or CTG repeats [1, 2, 9]. DNA synthesis at the shortened template would lead to a contraction. The major role of Mrc1, Tof1 and Csm3 in this model is to prevent contractions by minimizing single-strand template DNA. More complex models envisage repeat-mediated breakage, leading to recombinational repair, single-strand annealing or inability to restart a stalled replication fork at the TNR. These break repair activities typically require recombinational proteins such as Rad52 but previous data in our system showed no contraction phenotype in a *rad52* mutant [41]. We favor the simpler model where Mrc1, Tof1 and Csm3 help prevent contractions by limiting the accumulation of single-strand template DNA prior to the formation of aberrant secondary structure.

Our data suggest that expansions of subthreshold CTG tracts are prevented by DNA damage checkpoint response to TNR-mediated secondary structure, such as a hairpin, or to subsequently processed forms. The major difference from contractions is that expansions are inhibited by the DNA damage checkpoint after structure formation. Defects in either checkpoint fail to respond to aberrant secondary structure, which persists and leads to expansions. This model is supported by the mutator specificity of *mrc1* and *tof1* mutants, and by the fact that the $mrc1^{AQ}$ mutant was defective in blocking expansions. Similarly high rates of expansions occurred in mutants defective in both replication and DNA damage checkpoints (*mec1*, *ddc2*, *mrc1*, *tof1*, *csm3* or *rad53*) or in strains selectively deficient in the DNA damage checkpoint (*rad24*, *ddc1*, *mec3*, *rad17*, *rad9* or *chk1*). It is possible that both checkpoints are capable of responding to the damage intermediate, but epistasis experiments could not be performed due to inviability of double mutants. It is unlikely that recombination plays a role in promoting or preventing expansions in our system since no increase in expansion rates was observed in *rad52* or *mrc1 rad52* mutants (data not shown) and because previous studies showed no *rad51* or

rad52 phenotype on expansions [17, 36]. Mec1 dependent signaling through Mrc1 specifically responds to replication stalling at DNA lesions [34]. In the case of triplet repeats, our data suggest that repeat-mediated secondary structure formation adds to or possibly replaces replication stalling as the signal. Alternatively, the aberrant secondary structure could be cleaved or otherwise altered enzymatically to produce the signal. Finally, unreplicated single stranded DNA, resulting from downstream priming, may serve as the signal for checkpoint activation. Although we currently lack the molecular tools to examine what is occurring as the replication fork encounters a triplet repeat *in vivo*, it is likely that the absence of the DNA damage checkpoint creates an opportunity for mutagenic replication or repair leading to an expansion.

The results of this study are largely in agreement with previous reports of DNA damage response to expanded CAG tracts of 85-115 repeats [19, 20]. However, there are some interesting differences. One key difference is that we found an expansion phenotype for mutations in many genes that inactivate the DNA damage checkpoint. In contrast, the earlier work found a significant expansion phenotype only for *mrc1* [19, 20]. This distinction could be due to differences in assay sensitivity; our genetic assay can distinguish changes in expansion rate over several orders of magnitude [32] whereas the bulk PCR-based method has a more limited range. Also, longer tracts show instability in wild type cells at relatively high frequencies in the ~1-3% range, so there is less sensitivity available to distinguish an elevated mutant phenotype. Another difference was that contractions of long CAG tracts are sensitive to the absence of Mec1, Ddc2, Rad17, Rad24 or Rad53 [19] whereas we found no significant change in contraction rates for these mutations (Table 1). Perhaps the fragility associated with long tracts stimulates a checkpoint response that helps avoid contractions, whereas the shorter tracts examined in our study do not break often enough to generate a checkpoint response. In summary, it is now clear from previous work [19, 20] and from this study that the DNA damage checkpoint helps avoid instability of CAG•CTG repeat tracts. We show here that triplet repeat

sequences are selectively protected by Mrc1, Tof1 and Csm3, and that they act in two distinct ways to inhibit contractions through replicational coupling activity and expansions via the DNA damage checkpoint.

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Figure Legend

Figure 1. A genetic assay to monitor trinucleotide repeat (TNR) contractions and expansions in yeast. The regulatory region controlling expression of the reporter gene *URA3* is shown. Important features include the TATA box, the trinucleotide region, an out-of-frame initiator codon (in red), the preferred transcription initiation site "I", and the start of the *URA3* gene with initiator ATG codon in green. Anticipated transcription is shown as the right-angle arrow. For both panels, the top strand (i.e., the sense strand of *URA3*) is the lagging strand template. **(A)** Yeast cells that have undergone a TNR contraction can be selected by their ability to grow in the absence of uracil. The starting strain is Ura^- due to the inserted triplet repeat sequence, $(CNG)_{20+13}$. (This nomenclature refers to 20 repeats of the trinucleotide CNG, where N=any nucleotide, plus 39 bp of randomized, genetically inert sequence [39]. The total DNA length is therefore equivalent to 33 repeats.) Insertion of this many nucleotides between the TATA box and the preferred transcription initiation site places "I" too far from the TATA box, such that transcription is predicted to begin upstream. This incorporates an out-of-frame ATG (red), resulting in translational incompetence (indicated by X) and leading to a non-functional *URA3* product. Cells have a Ura^- phenotype. If a contraction occurs, losing 5 to 20 repeats, initiation will

occur at the proper site "I," leading to expression of *URA3* and attainment of the Ura^+ phenotype. **(B)** Yeast that have undergone an expansion can be identified by growth in the presence of 5FOA. $(CNG)_{13+12}$ refers to 13 repeats of the trinucleotide CNG plus 36 bp of randomized sequence [32]. The total DNA length is equivalent to 25 repeats. Proper initiation at "I" results in functional expression of *URA3*, which confers sensitivity to the drug 5-fluoroorotic acid (5FOA). If the TNR expands, gaining 5 or more repeats, upstream transcription initiation will include the red out-of-frame ATG, resulting in translational incompetence and resistance to 5FOA.

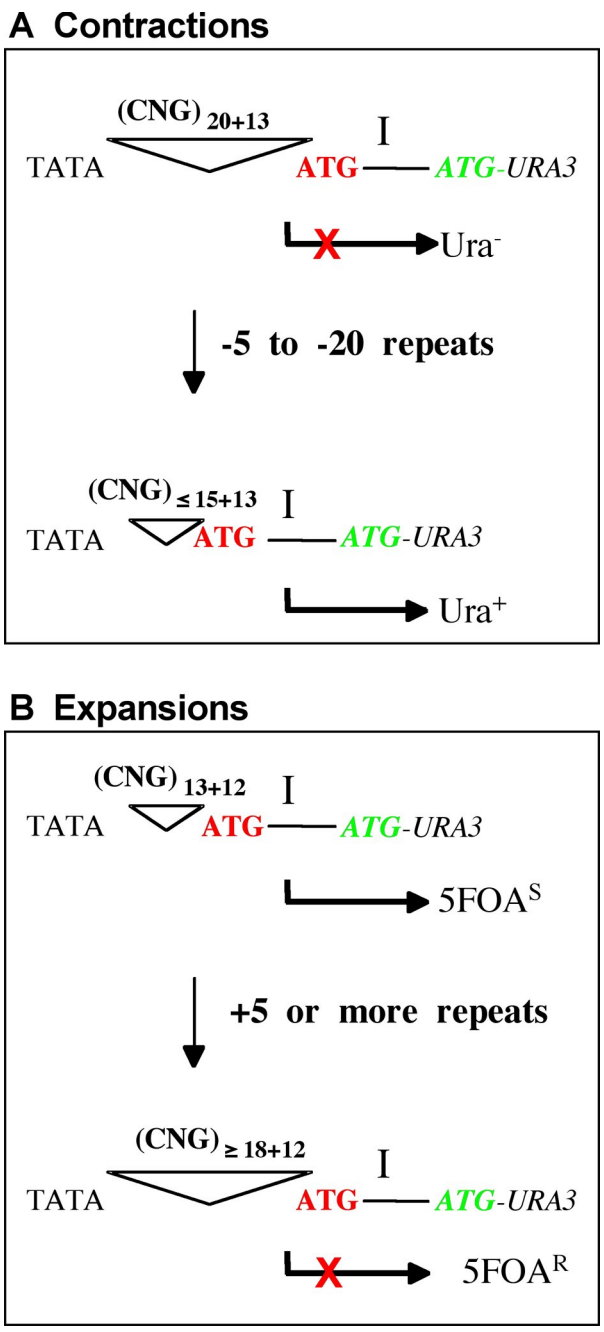
TABLE 1. CAG•CTG Repeat Mutation Rates

	Ratio (fold over wild type)	
Genotype	Contractions of $(CAG)_{20}$	Expansions of $(CTG)_{13}$
mrc1	7.1*	4.0*
mrc1 + p*MRC1*	1.6	1.7
mrc1 + p*mrc1*^{AQ}	2.0	3.7*
tof1	8.6*	7.4*
csm3	6.5*	6.8*
sml1	0.7	1.3
sml1 mec1	1.6	6.0*
sml1 ddc2	3.7*	4.9*
rad17	1.4	3.7*
rad24	1.0	6.4*
ddc1	1.4	4.5*
mec3	0.8	3.5*
rad9	2.2	8.2*
chk1	0.8	7.7*
sml1 rad53	3.7*	3.0*

*Significantly different from wild type ($P < 0.05$). The wild type rate for contractions is 1.4×10^{-7} per cell generation, and for expansions is 1.8×10^{-6} per cell generation.

TABLE 2. Spontaneous Mutation Rates

Mutation rate and genotype	Mean no. of mutations/ cell generation (±SD), 10^{-n}	Ratio (fold over wild type)
CAN1 forward mutations ($x10^{-7}$)		
Wild type	3.0 ± 0.0	1.0
mrc1	1.8 ± 0.9	0.6
tof1	3.0 ± 1.8	1.0
rad27	23 ± 6.6	7.5
Dinucleotide repeat mutations ($x10^{-6}$)		
Wild type	1.7 ± 0.1	1.0
mrc1	3.7 ± 1.4	2.2
tof1	3.6 ± 0.9	2.1
Expansions of $(CTA)_{25}$ ($x10^{-8}$)		
Wild type	< 4.9	1.0
mrc1	< 3.1	0.6
tof1	< 3.1	0.6


A Contractions
(CNG) 20+13
I
TATA
ATG
ATG-URA3
Ura-
-5 to -20 repeats
(CNG) ≤ 15+13
I
TATA
ATG
ATG-URA3
Ura+
B Expansions
(CNG) 13+12
I
TATA
ATG
ATG-URA3
5FOAS
+5 or more repeats
(CNG) ≥ 18+12
I
TATA
ATG
ATG-URA3
5FOAR

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