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Identification of *RTG2* as a Modifier Gene for CTG•CAG Repeat Instability in *Saccharomyces cerevisiae*

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ABSTRACT

Trinucleotide repeats (TNRs) undergo frequent mutations in families affected by TNR diseases and in model organisms. Much of the instability is conferred *in cis* by the sequence and length of the triplet tract. *Trans*-acting factors also modulate TNR instability risk, on the basis of such evidence as parent-of-origin effects. To help identify *trans*-acting modifiers, a screen was performed to find yeast mutants with altered CTG•CAG repeat mutation frequencies. The *RTG2* gene was identified as one such modifier. In *rtg2* mutants, expansions of CTG•CAG repeats show a modest increase in rate, depending on the starting tract length. Surprisingly, contractions were suppressed in an *rtg2* background. This creates a situation in a model system where expansions outnumber contractions, as in humans. The *rtg2* phenotype was apparently specific for CTG•CAG repeat instability, since no changes in mutation rate were observed for dinucleotide repeats or at the *CAN1* reporter gene. This feature sets *rtg2* mutants apart from most other mutants that affect genetic stability both for TNRs and at other DNA sequences. It was also found that *RTG2* acts independently of its normal partners *RTG1* and *RTG3*, suggesting a novel function of *RTG2* that helps modify CTG•CAG repeat mutation risk.

THE genetic behavior of trinucleotide repeats (TNRs) is unusual. There is a non-Mendelian inheritance pattern of disease-causing TNR expansions in families afflicted with fragile X syndrome, Huntington's disease (HD), or other syndromes (Richards and Sutherland 1994; Paulson and Fischbeck 1996; Cummings and Zoghbi 2000). Not only do the expanded alleles enhance the clinical symptoms, but there is also an increased expansion risk at each generation. Although each disease has its own genetic characteristics, one common theme is that tract length is a very important indicator of the tendency toward mutation. Tracts at or above a crucial threshold length of ~35 repeats (Paulson and Fischbeck 1996) are much more prone to expansion than shorter tracts. Because thresholds designate a sharp boundary between stable and unstable alleles, they are distinct from simple length dependence where the likelihood of mutation increases with tract size. Length dependence occurs for TNRs above the threshold, but is also true for elements that do not have thresholds, such as mono- and dinucleotide sequences in yeast (Tran *et al.* 1997; Wierdl *et al.* 1997). A threshold effect is therefore specific to TNRs, although its molecular nature is not yet known. Another interesting feature of TNR diseases is that human alleles at or above the threshold level exhibit a 3- to 175-fold bias toward expansions rather than contractions, depending on the disease gene and other factors (McMurray 1995). The reasons for the disparity between expansions and contractions are also unknown. A third characteristic of TNR diseases is that unstable triplet repeats have been reported only for the sequences C*N*G (where *N* is any nucleotide) or, in the sole case of Friedreich's ataxia, for the sequence GAA (Campuzano *et al.* 1996). The sequence specificity arises from the tendency of these sequences to adopt unusually strong secondary structures, such as hairpins or triplexes (Gacy *et al.* 1995, 1997; Sakamoto *et al.* 1999). These structures are thought to cause aberrant DNA synthesis during replication, gene conversion, and repair, leading to expansions and contractions. Thus the length and sequence of the TNR are powerful *cis*-elements in determining the likelihood of unstable transmissions.

In addition to the strong dependence on *cis*-elements like tract length and sequence, there is also evidence that *trans*-acting genetic modifiers affect TNR instability. For example, the same allele can show different instability, depending on whether it was inherited paternally or maternally. Generally speaking, the smaller expansions associated with translated CAG repeats are more often transmitted from the father, and very large expansions and contractions associated with untranslated CGG or

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CTG repeats are usually maternal in origin (see Jin and Warren 2000; Kovtun *et al.* 2000; Usdin and Grabczyk 2000; and references therein). Although not all transgenic mice with long TNR alleles show instability, some mouse lines show parental influence on intergenerational instability (Mangiarini *et al.* 1997; Monckton *et al.* 1997; La Spada *et al.* 1998; Sato *et al.* 1999; Shelbourne *et al.* 1999; Wheeler *et al.* 1999; Kovtun *et al.* 2000; Seznec *et al.* 2000). In addition to parent-of-origin effects, there is additional evidence that genetic polymorphisms outside the TNR-containing gene may also play a role in human TNR instability. For example, sperm analysis from men with identical 35 CAG repeat alleles showed a significant difference in instability between the general population and sporadic HD families (Chong *et al.* 1997). Another study on germline mutation spectra at HD suggested that genetic background or other factors lead to variations in instability among individuals with similar allele lengths (Leeflang *et al.* 1999). Taken together, these results indicate that genetic modifiers can act *in trans* to influence TNR instability.

Model systems such as bacteria, yeast, and transgenic mice have been examined for factors that influence TNR instability. These studies utilized candidate gene approaches, focusing on factors that influence DNA metabolism. Examples of candidate genes or pathways that have been characterized include the flap processing activity encoded by the yeast *RAD27* (Freudenreich *et al.* 1998; Schweitzer and Livingston 1998; Spiro *et al.* 1999; White *et al.* 1999), mismatch repair (Jaworski *et al.* 1995; Schweitzer and Livingston 1997; Manley *et al.* 1999; Parniewski *et al.* 2000; Schmidt *et al.* 2000; Kovtun and McMurray 2001), and recombinational repair (Sarkar *et al.* 1998; Richard *et al.* 2000). The candidate gene approach is useful because the role(s) of each gene or pathway can be evaluated in detail. One drawback is that many of the candidate genes examined for effects at TNRs also show destabilization of other DNA sequences. It would be beneficial to identify modifier genes with the highest possible selectivity for TNRs. To our knowledge, no genetic screens have been described in the literature to identify *trans*-acting factors with the desired selectivity.

To assist in the identification of new modifier genes, we took advantage of yeast genetic assays for TNR expansions and contractions that are selective, sensitive, and quantitative (Miret *et al.* 1998; Rolfsmeier *et al.* 2001). An earlier article made the key finding that the rate of expansion of CTG repeats is dramatically increased above an apparent threshold level of ~15 repeats (Rolfsmeier *et al.* 2001). The difference in TNR thresholds in yeast and humans (15 repeats *vs.* ~35 repeats) suggested that cellular proteins play an important role in determining the threshold. If so, mutants that alter TNR instability at tract lengths near the threshold might have a higher selectivity for TNRs than the candidate genes examined so far. We report here that mutations in *RTG2* lead to altered CTG•CAG repeat instability but do not seem to affect other DNA sequences that were tested. An unexpected finding was that the *rtg2* mutation also led to more expansions than contractions, as is more like the case in human families affected by TNR diseases. *RTG2* is therefore a modifier gene with selectivity toward CTG•CAG repeats, which helps control the ratio of expansions to contractions. The known role of *RTG2* is to help to activate certain genes in the tricarboxylic acid cycle and the glyoxylate cycle in response to stress signals in yeast harboring defective mitochondria (Parikh *et al.* 1987; Liao and Butow 1993). No role of *RTG2* in genetic instability has been previously reported or anticipated.

MATERIALS AND METHODS

Strains: The *Saccharomyces cerevisiae* strains included BL035 (*MAT*α Δ*trp1 ura3-52 ade2*Δ *ade8 hom3-10 his3-Kpn1 met4 met13 leu2*Δ), a *leu2*Δ derivative of MW3317-21A (Kramer *et al.* 1989), and an *msh2::LEU2* derivative of BL035 (Miret *et al.* 1998). A *rad27*Δ mutant in the MW 3317-21A background was described earlier (Spiro *et al.* 1999). Strains harboring *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ mutations (Sekito *et al.* 2000), derived from PSY142 (*MAT*α *leu2 lys2 ura3* [ρ^+]), were kindly provided by Dr. Ron Butow (University of Texas Southwestern Medical Center). The yeast strains BL490 (*MAT***a** *leu2*Δ1 *trp*Δ*63 ura3-52 his3-200*) and its *pol32*Δ derivative BL492 were generous gifts from Sergei Mirkin (University of Illinois, Chicago). TNR-containing plasmids were directed to integrate into the yeast chromosome at the *LYS2* locus by *Bsu*36I digestion (Miret *et al.* 1998) via transformation using the lithium acetate protocol (Schiestl and Gietz 1989). Correctly integrated alleles were identified by Southern hybridization.

Plasmids: All triplet-repeat-containing plasmids were constructed using the pBL94 vector as described previously (Rolfsmeier and Lahue 2000). TNR sequences, present as oligonucleotide duplexes, were inserted into the *Sph*I site of pBL94. The primary sequence of all plasmids was confirmed by DNA sequence analysis. Plasmid pRS416-*RTG2* was the generous gift from Dr. Ron Butow. The *RTG2* coding fragment was recovered from pRS416-*RTG2* by restriction digestion with *Kpn*I and *Bam*HI and subcloned into the same restriction sites of pRS314. The low-copy pRS314-*RTG2* plasmid was used in these studies and is referred to as p*RTG2*. The reporter plasmid pSH44, containing 16.5 repeats of the dinucleotide repeat GT (Henderson and Petes 1992), was a generous gift from Dr. Tom Petes (University of North Carolina).

Vectorette PCR: To identify the disrupted genes in our mutagenesis study, genomic DNA flanking the disruption site was PCR amplified using the Vectorette PCR technique (C. Friddle, http://genome-www.stanford.edu/group/botlab). DNA sequencing was used to identify flanking sequence from the disrupted gene, and the gene sequence was identified by comparison with the yeast genome database (http://genome-www.stanford.edu/Saccharomyces).

Genetic assays: To determine the rates of expansion and contraction, we used a quantitative genetic assay that monitors changes in TNR tract lengths by means of changes in cellular phenotype (Miret *et al.* 1998; Rolfsmeier *et al.* 2001). Briefly, TNR tracts are cloned as oligonucleotide cassettes into a reporter construct that includes the *Schizosaccharomyces pombe adh1* promoter fused to a *URA3* reporter gene. The transcription initiation site in this construct becomes dependent on the length of the triplet repeat. A starting tract of 25 repeats

or less allows expression of the *URA3* reporter gene with concomitant sensitivity of the cells to the cytotoxic drug 5-fluoroorotic acid (5-FOA). Expansions of the tract to lengths of 30 or more repeats inactivate the *URA3* reporter and the cells accordingly become resistant to 5-FOA. Expansion rate is proportional to the number of 5-FOA-resistant colonies. For determining contraction rates, the assay is performed in reverse (Rolfsmeier *et al.* 2001), and cell growth is monitored in a selective media lacking uracil. When p*RTG2* was used for complementation studies of the *rtg2::LEU2* allele, the p*RTG2* plasmid was always added to the strain already containing the triplet repeat reporter. The assay for mutation in dinucleotide repeats was performed as described by Petes and colleagues (Henderson and Petes 1992). In this assay, a plasmid harboring a poly(GT) tract was introduced into the strains, and alteration in the reading frame of a reporter gene was monitored. Forward mutation rates for the *CAN1* gene were determined by selection for canavanine resistance. Three to five independent clones were tested for each of the above assays to ensure reproducibility.

To assess the growth behavior of strains carrying *rtg2* mutations, the media used were YNBD (0.67% yeast nitrogen base, 2% glucose, 0.02% glutamate) or YNB acetate (0.67% yeast nitrogen base, 2% potassium acetate and 0.5% yeast extract, 0.02% glutamate, pH 5.5).

Fluctuation analysis: Fluctuation analysis was performed as described previously (Miret *et al.* 1998). The method of median (Lea and Coulson 1948) was used to determine the rates of TNR instability. Briefly, yeast cells harboring a TNR reporter were resuspended in water and appropriate dilutions were plated onto nonselective media (YPD for most strains or YNBD + 0.02% glutamate for *rtg* strains). After growth for 2–3 days (wild type) or 7–10 days (mutants) at 30°, ~10 colonies were resuspended in water and an appropriate dilution was plated on nonselective media for total cell counts. The remaining suspension was plated on selective media containing 1 mg/ml 5-FOA but lacking histidine, to determine expansion rates, or on selective media lacking both histidine and uracil to determine contraction rates. At least three independent clones were tested to generate an average rate with standard deviation.

PCR analysis of independent expansion and contraction events: Single-colony PCR analysis was performed using a published method (Miret *et al.* 1998). Briefly, template DNA was isolated from single yeast colonies by treatment with dithiothreitol and Triton X-100. DNA preparations were subjected to PCR in the presence of 0.125 μCi [α-^{32}P]dCTP and oligonucleotide primers oBL91 (AAACTCGGTTTGACGCCTCCCATG) and oBL157 (AGCAACAGGACTAGGATGAGTAGC) that flank the triplet repeat region. The products of the PCR reaction were resolved on 6% denaturing polyacrylamide gels and their sizes were ascertained to within one to two repeats by comparing them with the reaction products of an M13 DNA sequence ladder. The percentage of *bona fide* expansions or contractions, determined by PCR analysis, was multiplied by the apparent expansion and contraction rates derived from fluctuation analysis. All rates reported here reflect this correction factor.

RESULTS

Identification of an *rtg2* mutant affecting CTG•CAG repeat stability: To identify new cellular proteins that affect TNR instability, we performed a screen for yeast mutants that showed an increased rate of expansion for CTG•CAG tracts near the threshold length. Since the CTG sequences reside in the Okazaki fragment in these expansion experiments, the CTG•CAG tract will be referred to as CTG whenever possible for brevity. [It is important to note the nomenclature for these experiments. For expansions, the cited sequence resides on the lagging daughter strand, since most models for TNR expansions envision hairpin formation in the Okazaki fragment (Richards and Sutherland 1994; Kang *et al.* 1995; Freudenreich *et al.* 1997; Gordenin *et al.* 1997; Schweitzer and Livingston 1997; Miret *et al.* 1998). The lagging daughter strand corresponds to the antisense strand of *URA3*. In contrast, contractions are thought to occur by folding of the TNR in the lagging template strand; therefore we use the convention that the template strand contains the indicated sequence. These assignments are possible because the direction of DNA replication through the integration locus is known (Freudenreich *et al.* 1997).] As shown in previous work (Rolfsmeier *et al.* 2001), CTG expansions in our system exhibit an apparent threshold of ~15 repeats. The response curve of expansion rate *vs.* tract length is very steep in this range, with a 100-fold increase in expansion rates as the tract size is doubled from 10 to 20 CTGs. The steepness of this curve suggested that *trans*-acting mutations might be found with even modest phenotypes on CTG instability. We also reasoned that since thresholds are apparently unique to TNRs, mutations affecting tract instability in this size range might destabilize TNRs more than other DNA sequences. CTG repeats were chosen as a representative TNR because they are unstable in human disease and in many model systems. Furthermore, extensive information is available for CTG tracts in our system (Miret *et al.* 1998; Spiro *et al.* 1999; Rolfsmeier *et al.* 2000, 2001), allowing direct comparison of the behavior of mutant strains with wild-type cells.

The starting strain contained 13 CTG repeats within the *Padh1*-TNR-*URA3* reporter system (Miret *et al.* 1998) described in materials and methods and reported previously (Rolfsmeier *et al.* 2001). In addition to the $(CTG)_{13}$ tract, randomized nucleotides equivalent to 12 additional repeats were included to make a total insert length equal to 25 triplets. Expansions within the $(CTG)_{13}$ region that increase the length by 5 repeats or more inactivate the *URA3* reporter and thereby convey resistance to 5-FOA. In a wild-type background, the rate of expansion for $(CTG)_{13}$ is very low (Rolfsmeier *et al.* 2001). When unmutagenized cells are first grown on nonselective media (YPD) and are then replica plated to 5-FOA-containing media, very few 5-FOA^R papillae are observed. We used frequency of papillation on 5-FOA-containing media as a phenotype to screen 20,000 transformants from a yeast genomic library containing random insertions of the mTn-lacZ/*LEU2* marker (Burns *et al.* 1994). Hereafter these mutants are referred to simply as *LEU2* disruptions. Three rounds of screening were performed to identify mutants that reproducibly displayed more 5-FOA^R papillae than wild type did when

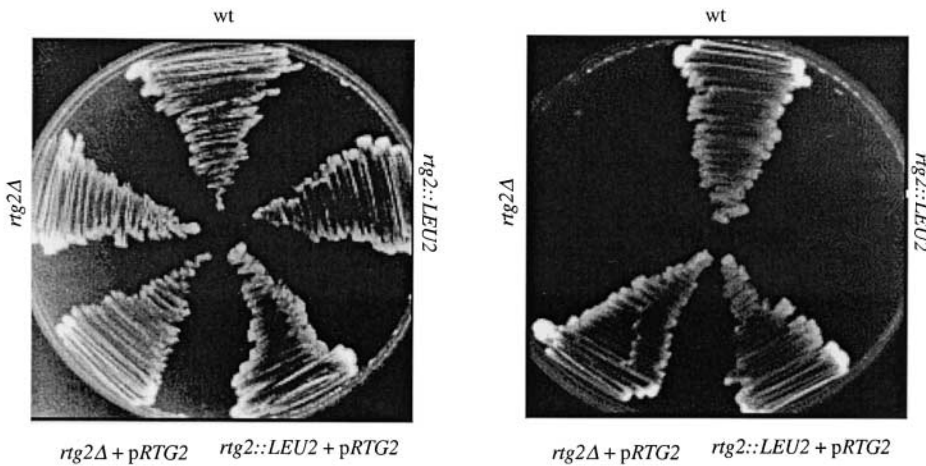

FIGURE 1.—Confirmation of *RTG2* as the mutated gene. Cells with the indicated genotypes were grown for 5 days at 30° on plates containing either YNBD + 0.02% glutamate (left) or YNB acetate + 0.02% glutamate (right). The extended time accommodated the slow-growing *rtg2* mutant strains.

replica plated onto 5-FOA-containing media. The first screening was performed with transformant colonies, whereas rounds 2 and 3 were conducted with 1- to 2-cm^2 patches of cells (to give more total papillae). Next, DNA was isolated from 5-FOA^R papillae and tested by PCR for *bona fide* expansions of the CTG tract (MIRET *et al.* 1998). Several mutants frequently exhibited PCR sizes larger than the starting tract. To identify the disrupted genes, genomic DNA flanking the disruption site was PCR amplified, sequenced, and compared to the yeast genome database. *RTG2* was identified as the disrupted gene in two independent mutant strains.

Confirmation of the *rtg2* mutation: *RTG2* is one of the pivotal genes involved in controlling interorganelle communication between mitochondria, peroxisomes, and the nucleus (LIAO and BUTOW 1993). It encodes a novel cytoplasmic protein possessing an amino-terminal ATP-binding domain with sequence motifs bearing some similarity to hsp70 (BORK *et al.* 1992), and it also shares some sequence similarity with bacterial polyphosphatases and phosphatases that hydrolyze guanosine penta- and tetraphosphate (KOONIN 1994). *RTG2* is also required to maintain a functional metabolic interaction between the TCA and glyoxylate cycles when there is mitochondrial dysfunction. Under such conditions, a decrease in TCA cycle activity is compensated by an increase in the activity of a peroxisomal enzyme via the metabolic interactions between the glyoxylate and TCA cycle. Hence *RTG2* plays an important physiological role for retrograde regulation in linking the two metabolic pathways. It has been demonstrated that *rtg2* deletion mutants are glutamate auxotrophs (LIAO and BUTOW 1993; LIU and BUTOW 1999). Various nucleotides and amino acids are synthesized from glutamate, which is synthesized from α-ketoglutarate, a TCA cycle intermediate. Any change in TCA cycle activity will affect the production of α-ketoglutarate and hence of glutamate. Thus, glutamate level is considered an important signaling component in the retrograde response pathway (LIU and BUTOW 1999; SEKITO *et al.* 2000; EPSTEIN *et al.* 2001).

Since its known role is in interorganelle signaling, not in control of genetic stability, *RTG2* was unexpected in our screen. Several genetic tests were therefore initiated to verify the assignment. *rtg2* mutants are unable to thrive on media containing acetate as the major carbon source, even when supplemented with glutamate (LIAO and BUTOW 1993; JIA *et al.* 1997; LIU *et al.* 2001). We confirmed that our mutant was defective in *RTG2* function by monitoring the growth of the cells on the nonselective media YNBD and the YNB-acetate selective media, both containing 0.02% glutamate (Figure 1). On YNBD, all strains grew well. On acetate-containing media, however, the *rtg2::LEU2* mutation resulted in no growth, similar to an *rtg2*Δ strain (kindly provided by Ron Butow). Growth on acetate-containing media was restored to both *rtg2* mutant strains when a low-copy-number plasmid harboring the wild-type *RTG2* gene was present in the cells. Also, as shown below, the p*RTG2* plasmid reversed the phenotypes of the *rtg2::LEU2* mutation when assayed for two phenotypes on CTG•CAG repeat tracts. Together, the sequencing data, the cellular phenotypes, and the complementation data establish *RTG2* as the mutated gene from our screen.

The effect of *rtg2::LEU2* on CTG expansions: To better understand the effects of *rtg2::LEU2* on expansions, quantitative rate measurements were performed. Several different CTG tract lengths were tested to compare phenotypic effects at tract lengths near the wild-type threshold of ~15 repeats (ROLFSMEIER *et al.* 2001) and above the threshold (25 repeats). We found a subtle but significant effect with the $(CTG)_{13}$ tract. The expansion rate was 2.5-fold higher in the *rtg2::LEU2*-containing strain than in the wild type (Table 1A). The control strain with an *rtg2*Δ allele gave a similar increase in rate of 3-fold. Both mutant values are significantly different from wild type (*P* values of 0.005 and 0.003; see legend to Table 1). The increased expansion rate for *rtg2::LEU2* was reversed to wild-type levels when the p*RTG2* plasmid was present (Table 1A). These results indicate that the *rtg2* mutation conferred a hyperexpansion phenotype

TABLE 1

Mutation rates for *rtg2* mutants

Genotype	Mutations/cell division mean (±SD)	Ratio (-fold over wild type)
	A. Expansions of $(CTG)_{13}$ + $(C,T,G)_{12}$	
RTG2	$1.4 (\pm 0.2) \times 10^{-7}$	1
rtg2::LEU2	$3.5 (\pm 0.2) \times 10^{-7}$	2.5[a]
*rtg2*Δ	$4.2 (\pm 0.2) \times 10^{-7}$	3.0[a]
rtg2::LEU2 + p*RTG2*	$0.9 (\pm 0.4) \times 10^{-7}$	0.6[a]
*rtg1*Δ	$1.2 (\pm 0.2) \times 10^{-7}$	0.9
*rtg3*Δ	$1.5 (\pm 0.1) \times 10^{-7}$	1.1
RTG2 + 0.2% glutamate	$1.5 (\pm 0.1) \times 10^{-7}$	1.1
RTG2 − glutamate	$1.3 (\pm 0.2) \times 10^{-7}$	0.9
	B. Expansions of $(CTG)_{25}$	
RTG2	$1.0 (\pm 0.3) \times 10^{-5}$	1[b]
rtg2::LEU2	$0.9 (\pm 0.1) \times 10^{-5}$	0.9
*rtg2*Δ	$1.2 (\pm 0.4) \times 10^{-5}$	1.2
	C. Contractions of $(CTG)_{25}$ + $(C,T,G)_{8}$	
RTG2	$2.7 (\pm 0.2) \times 10^{-5}$	1[b]
rtg2::LEU2	$1.9 (\pm 0.3) \times 10^{-6}$	0.07
*rtg2*Δ	$1.7 (\pm 0.2) \times 10^{-6}$	0.06
rtg2::LEU2 + p*RTG2*	$3.5 (\pm 0.4) \times 10^{-5}$	1.3
*rtg1*Δ	$3.4 (\pm 0.2) \times 10^{-5}$	1.3
*rtg3*Δ	$2.9 (\pm 0.4) \times 10^{-5}$	1.1
RTG2 + 0.2% glutamate	$3.2 (\pm 0.2) \times 10^{-5}$	1.2
RTG2 − glutamate	$3.2 (\pm 0.1) \times 10^{-5}$	1.2
	D. Mutations of poly(GT)	
RTG2	$3.8 (\pm 0.2) \times 10^{-5}$	1
rtg2::LEU2	$4.3 (\pm 0.2) \times 10^{-5}$	1.1
*msh2*Δ	$1.4 (\pm 0.1) \times 10^{-2}$	370
	E. Mutations at *CAN1*	
RTG2	$3.0 (\pm 0.4) \times 10^{-7}$	1
rtg2::LEU2	$3.9 (\pm 0.4) \times 10^{-7}$	1.3
*rad27*Δ	$1.8 (\pm 0.1) \times 10^{-5}$	60

[a] *P* values from Student's *t*-test, compared to *RTG2*, were 0.005 for *rtg2::LEU2*, 0.003 for *rtg2*Δ, and 0.14 for *rtg2::LEU2* + p*RTG2*.

[b] Expansion rate for $(CTG)_{25}$ (Miret *et al.* 1998) and the contraction rate for $(CTG)_{25}$ + $(C,T,G)_{8}$ (Rolfsmeier *et al.* 2001) were published previously and are included here for comparison.

for $(CTG)_{13}$ tracts. In contrast, strains with 25 CTG repeats showed expansion rates that were indistinguishable among wild type, *rtg2::LEU2*, and *rtg2*Δ (Table 1B). With 15 repeats, we found an intermediate effect. The expansion rate for *rtg2::LEU2* strain was 1.5-fold higher than that for the wild type (data not shown). Thus the CTG repeat hyperexpansion phenotype associated with *rtg2* mutations is lost as the repeat tract is lengthened. Since expansion rates near the threshold are somewhat higher in *rtg2* mutants, these data suggest that *RTG2* plays a modest role in determining the apparent threshold length for CTG expansions.

Two explanations exist for the higher rate of expansions for the $(CTG)_{13}$ tract in the *rtg2::LEU2* mutant. Either there is an increased incidence of the same size expansions as seen in wild-type cells or a new size class of expansions, such as a shift toward larger or smaller alleles, occurs in the mutant. We examined the distribution of expansion sizes in the mutant for the 13 repeats by single-colony PCR (Figure 2). The distribution of expansion sizes for *rtg2::LEU2* ranged from +5 to +10, with a median of 7.5 compared to 8 for the wild-type cells. The two mutational spectra overlap and therefore do not support a different size class of expansions in the mutant. We conclude therefore that the increase in expansion rate associated with the *rtg2* mutation is due to an increased number of events that are of the same size range as in wild type. This analysis also showed that in the *rtg2::LEU2* background, 97% of the expansions were limited to gains equal to or less than the original tract length, consistent with a replicational mechanism of instability (Gordenin *et al.* 1997). One expansion of +29 repeats was observed.

We asked whether *RTG2* dosage was limiting for control

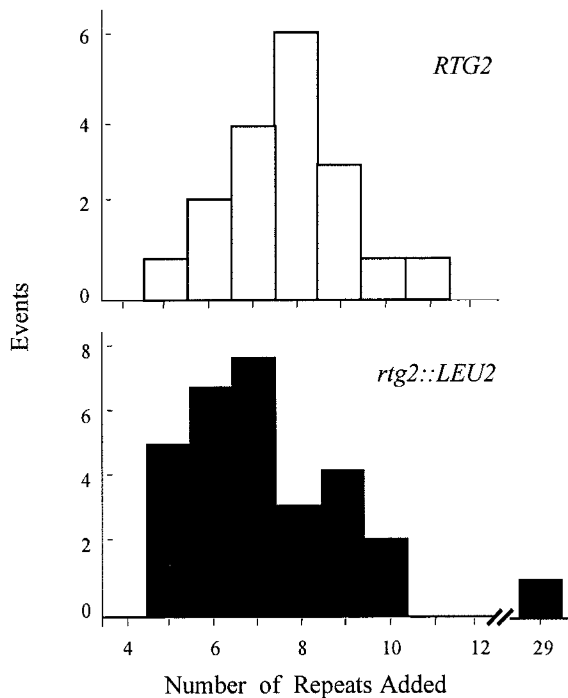

FIGURE 2.—Comparison of the distribution of $(CTG)_{13}$ expansion sizes between wild type and *rtg2::LEU2*. Single-colony PCR analysis was performed on genetically independent 5-FOA^R colonies from fluctuation tests. Expansion sizes were measured using high-resolution denaturing polyacrylamide gels (MIRET *et al.* 1998). Expansion sizes are accurate to within $\pm$1–2 repeats. The *x*-axis denotes the number of repeats added with respect to the starting tract length (*e.g.*, +5 repeats added means a final tract length of 18 repeats). The *y*-axis denotes the observed number of expansion events of that size.

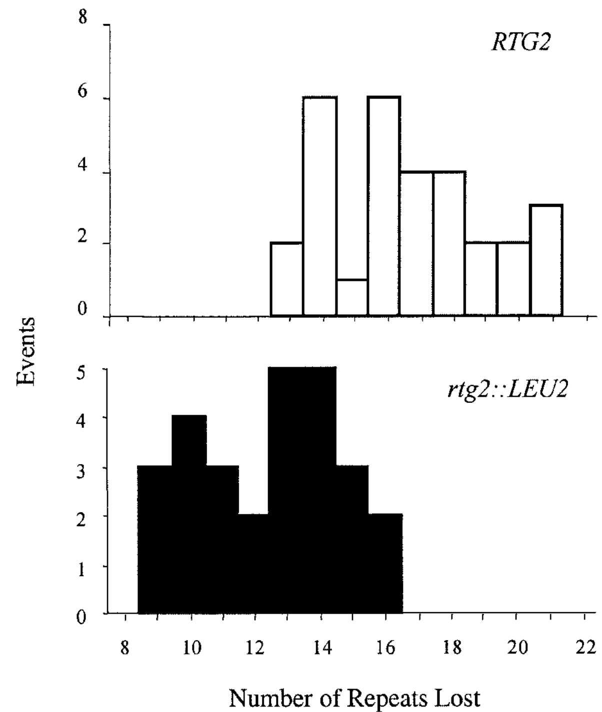

FIGURE 3.—Distribution of contraction sizes from $(CTG)_{25+8}$ in wild-type and *rtg2::LEU2* mutant strains. Single-colony PCR was performed on genetically independent Ura$^+$ colonies from fluctuation tests. The size data were acquired as described in the legend to Figure 2. The *x*-axis here denotes repeats lost.

of CTG repeat expansions. This question was first addressed in heterozygous diploids. When the *rtg2::LEU2* strain was mated to an *RTG2* strain, the resulting heterozygous diploid showed the wild-type phenotype when assayed for expansions of the $(CTG)_{13}$ tract. This indicates that a single copy of *RTG2* is sufficient to control expansions in a diploid cell. We also introduced the low-copy-number *RTG2* plasmid into wild-type haploids and found that the cells retained their wild-type expansion phenotype for $(CTG)_{13}$. This indicates that one to two extra copies of *RTG2* in a wild-type background do not improve the control of CTG expansions.

The effect of *rtg2* on CTG contraction rates: If expansion rates for at least some CTG alleles are increased in *rtg2* mutants, then perhaps contraction rates would be similarly affected. To see if this were true, we examined CTG contraction rates under conditions where the CTG repeat sequences occupy the lagging strand template (see previous comments on nomenclature; ROLFSMEIER *et al.* 2001). A contraction construct was used that contained a $(CTG)_{25}$ repeat tract to which 24 bp of scrambled sequence, equivalent to eight repeats, was added ("25 + 8" configuration). This assay scores for losses of −5 to −25 repeats (ROLFSMEIER *et al.* 2001) and allows a direct comparison with the $(CTG)_{25}$ expansion measurements reported earlier (MIRET *et al.* 1998). Contrary to expectations, we found that the contraction rate was reduced 15-fold for the *rtg2::LEU2* mutant compared to that of the wild type (Table 1C). A similar, 16-fold reduction was seen for the *rtg2*Δ allele. The contraction rate was restored to wild-type levels by introducing the *RTG2* plasmid into the *rtg2::LEU2* background.

To help verify the unexpected finding that *rtg2* mutants suppressed CTG contractions, we examined the contraction sizes in the mutant arising from the "25 + 8" starting tract. Contractions ranged from −9 to −16 repeats in the *rtg2::LEU2* mutant, compared to −13 to −21 in the wild-type background (Figure 3). It therefore appears that the *rtg2* mutation results in the loss of larger expansions and gives rise to a predominance of smaller size contractions. The influence of *RTG2* on expansions and contractions is exciting because it leads to a greater propensity for expansions, at least for one CTG repeat allele. For the $(CTG)_{25}$ tract that can be directly compared by our results, the *rtg2::LEU2* background leads to a preponderance of expansions over contractions of ~5:1, whereas in the wild-type strain the ratio is ~1:3 (ROLFSMEIER *et al.* 2001). Unlike the case in most model systems, these results indicate that expansions are the preferred event in *rtg2* yeast cells.

***rtg2* shows little-to-no phenotype on mutation rates at other DNA sequences:** One goal of this mutant hunt was to find genes that affect CTG•CAG repeat instability substantially more than other DNA sequences. The effects of *rtg2::LEU2* on a poly(GT) dinucleotide repeat were examined because the behavior of this microsatellite is well established. However, dinucleotide repeat mutations occur by a mechanism different from that for TNRs. Dinucleotide repeats most often show variation of plus or minus one to two repeats (Henderson and Petes 1992), and mutation rates are very sensitive to the mismatch repair status of the cell (Strand *et al.* 1993). In contrast, CTG•CAG repeat expansions and contractions are identified in our system when they change length by five triplets or more, and these rates are not affected by mismatch repair deficiencies (Miret *et al.* 1998; Rolfsmeier *et al.* 2000). We measured the mutation rates for a $(GT)_{16.5}$ tract in *rtg2* mutants (Table 1D) by the method of Petes and colleagues (Henderson and Petes 1992). The *rtg2::LEU2* allele did not result in a detectable change of dinucleotide mutation rate, relative to wild type. In contrast, the mutation rate in an *msh2*Δ control strain was elevated >300-fold, similar to published values (Strand *et al.* 1993). We conclude that dinucleotide repeat instability is not significantly changed in an *rtg2* background. Table 1E shows the results of a similar experiment with the *CAN1* gene for determining the mutation rate in nonrepeating sequences. Again, the *rtg2::LEU2* mutant gave a result very close to wild-type levels, whereas a *rad27*Δ control strain was elevated ~60-fold, similar to published values (Tishkoff *et al.* 1997). Like dinucleotide repeats, the results of the forward mutation rate analysis at *CAN1* suggest that the *rtg2* allele does not confer a general mutator phenotype. Rather, the *rtg2* phenotypes on mutation rates appear specific for CTG•CAG repeats.

Another possibility is that the *rtg2* mutation leads to increased instability of triplet repeats in general. To test this idea, we examined expansions of $(CTA)_{25}$. Unlike CTG tracts, CTA repeats show little or no propensity to fold into hairpins (Gacy *et al.* 1995) and they are stable in our yeast assay (Miret *et al.* 1998). Fluctuation analysis revealed that the $(CTA)_{25}$ sequence was equally stable in the *rtg2* mutant and in wild-type cells, with an expansion rate of $\sim 3 \times 10^{-8}$ (Miret *et al.* 1998). The low rate suggests that the *rtg2* phenotype is specific for hairpin-forming triplet repeat sequences only. Our assay may not be suitably sensitive to detect changes in $(CTA)_{25}$ instability at this low level, however.

***rtg1* and *rtg3* mutants do not show effects on CTG•CAG repeat stability:** In response to changes in the mitochondrial state in yeast, an interorganelle signaling pathway known as retrograde regulation is activated (Liao and Butow 1993). The *RTG1*, *RTG2*, and *RTG3* genes are all required in this pathway (Liao and Butow 1993; Jia *et al.* 1997). In cells with dysfunctional mitochondria, *RTG1* and *RTG3* encode transcription factors that bind as a heterodimer to promoters of certain nuclear genes. Although the precise biochemical function of Rtg2p is not known, it helps facilitate the nuclear relocalization of Rtg1p and Rtg3p when the retrograde pathway is activated (Sekito *et al.* 2000). To assess the potential role of *RTG1* and *RTG3* on CTG•CAG repeat instability, we performed quantitative genetic assays for expansions and contractions in *rtg1*Δ and *rtg3*Δ strains. The expansion rates of the $(CTG)_{13}$ repeat in the *rtg1*Δ and *rtg3*Δ mutants were unchanged compared to wild type (Table 1A). Similarly, the contraction rates of $(CTG)_{25+8}$ in the *rtg1*Δ and *rtg3*Δ mutants were comparable to wild type (Table 1C). These results suggest that, in our system, *RTG2* acts independently of *RTG1* and *RTG3*, whereas in the retrograde response all the genes are involved and *RTG2* works in conjunction with *RTG1* and *RTG3* (Sekito *et al.* 2000).

To further establish that the retrograde regulatory pathway is not involved in conferring triplet repeat instability, we tested CTG•CAG repeat expansions and contractions in wild-type cells by inhibiting the retrograde regulation with a high concentration of glutamate. Patches of wild-type cells were grown on YNBD media supplemented with proper nutrients, both in the presence and the absence of 0.2% glutamate (Table 1, A and C). Glutamate at this concentration has been shown to inhibit retrograde regulation by ~99% (R. Butow, personal communication). Both expansion and contraction rates were unchanged by the presence or absence of glutamate (Table 1, A and C). Since the presence of 0.2% glutamate failed to alter the behavior of *RTG2* cells, these results suggest that the retrograde regulation pathway is not involved in instability of CTG•CAG tracts. Furthermore, the retrograde pathway is activated in cells with compromised mitochondrial function (Liao and Butow 1993). Our *rtg2::LEU2* mutant strain is respiratory competent, judged by its ability to grow in the presence of glycerol as the sole carbon source. The results from the above experiments indicate that *RTG2* affects triplet repeat stability via a novel pathway, independent of the retrograde regulatory pathway.

DISCUSSION

Significance: This study revealed *RTG2* as a yeast modifier gene for CTG•CAG repeat instability. The *rtg2::LEU2* allele was found in a screen for mutations that increased expansion frequencies of the $(CTG)_{13}$ reporter. The disrupted allele was identified first by PCR and DNA sequencing. The assignment was subsequently verified by comparisons with a known *rtg2*Δ mutant strain, by growth phenotypes in different media, and by complementation by plasmid-borne *RTG2* for growth defects and for instability phenotypes on CTG•CAG repeats. Clearly the altered behavior of these tracts in the mutant is linked to defects in *RTG2*. Three new phenotypes were found associated with the loss of *RTG2*: (1) modest

increases in rates of repeat expansions but significant decreases in contraction rates; (2) selectivity of the mutation for its effects on CTG•CAG repeat sequences, compared to no detectable change in instability of dinucleotide repeats or at the *CAN1* mutation reporter gene; and (3) the apparent independence of *RTG2* from its normal partners, *RTG1* and *RTG3*. Each of these points is considered in more depth below. The significance of this work includes two major conclusions. First, yeast modifier genes can be identified by mutant screens. Second, mutant modifier genes may provide novel insights into the genetic controls that selectively govern TNR instability. While no human homolog of *RTG2* is known from sequence-similarity analysis of the human genome database, perhaps there is a functional homolog that remains unidentified.

Effects of *rtg2* on expansions and contractions: The *rtg2::LEU2* and *rtg2*Δ mutations resulted in a modest but significant hyperexpansion phenotype for the $(CTG)_{13}$ reporter. The increased expansion rate was traced to more mutations of the same size class as that seen in wild type. Nearly all (97%) of the expansions were within twofold of the original tract length, consistent with a replicational mechanism of instability (Gordenin *et al.* 1997), although a recombinational mechanism (Jankowski *et al.* 2000) has not been ruled out in the *rtg2* background. The hyperexpansion phenotype was reduced when the reporter contained 15 repeats, and no detectable change from wild type was observed when 25 CTG repeats were the target. When plotted as a graph of the log (expansion rate) *vs.* starting tract size (not shown), the data suggest that *rtg2* mutations slightly shift the sigmoidal curve to the left, compared to the wild-type response (Rolfsmeier *et al.* 2001). This is consistent with the idea that the *rtg2* mutation modestly reduces the apparent threshold length for CTG expansions, without affecting the plateau expansion rate at 25 repeats. However, the changes in expansion rate due to *rtg2::LEU2* are too subtle to conclusively indicate an alteration in the threshold for expansions. To provide more compelling evidence for *trans*-control of threshold, additional mutations will be needed in other modifier genes with a stronger influence on expansion rates.

Surprisingly, the *rtg2::LEU2* and *rtg2*Δ mutations also caused a suppression of contractions. The contraction rates were reduced by 15-fold for repeat tracts of 25. Fewer large size contractions were observed in the *rtg2::LEU2* mutant than in wild type. The reduction in contraction rates creates a situation where expansions outnumber contractions by ∼5:1 at CTG repeat lengths of 25. In this model system, expansions are more frequent than contractions, as is more like the case in human families afflicted with TNR diseases. One exciting possibility is that some limiting factor that controls expansion and contraction ratios is altered in *rtg2* mutants. For comparison, *rad27* mutants result in approximately equal numbers of expansions and contractions, primarily due to increases in expansion rates (Schweitzer and Livingston 1998; Spiro *et al.* 1999). Mutations in *rad50* or *mre11* yield recombination-dependent expansions and contractions in nearly equal ratios, but the primary effect of these mutants is to reduce the frequency of contractions (Richard *et al.* 2000). Regarding the opposing effects of *rtg2* on expansion and contraction rates, it remains to be seen whether the two types of alterations occur through different mechanisms or whether the mechanisms are similar but are differentially sensitive to changes caused by the *rtg2* mutation.

Selectivity of the *rtg2* mutation for CTG•CAG repeat tracts: As the existence of a threshold is thought to be unique to TNRs, we hypothesized that mutations affecting the expansion rate of a CTG repeat tract near the yeast threshold length (Rolfsmeier *et al.* 2001) would help identify mutants that destabilize TNRs more than other DNA sequences. The *rtg2::LEU2* allele confers the desired selectivity. There was no detectable difference, compared to wild type, for mutation rates at a poly(GT) tract or for forward mutations inactivating *CAN1*. This feature distinguishes *rtg2* from most other mutations that have been reported to alter the genetic stability of TNRs, but that also affect stability of other sequences. For example, mutation of the flap-processing activity encoded by yeast *RAD27* increases TNR instability (Freudenreich *et al.* 1998; Schweitzer and Livingston 1998; Spiro *et al.* 1999; White *et al.* 1999), but *rad27* strains are also hypermutable at a number of microsatellite repeats (Johnson *et al.* 1995; Kokoska *et al.* 1998) and they result in excess duplications (Tishkoff *et al.* 1997). Mismatch repair defects in bacteria (Jaworski *et al.* 1995; Parniewski *et al.* 2000; Schmidt *et al.* 2000) or eukaryotes (Schweitzer and Livingston 1997; Manley *et al.* 1999; Kovtun and McMurray 2001) have been reported to alter TNR instability, although other studies found no evidence of a role for mismatch repair at perfectly repeating TNRs (Kramer *et al.* 1996; Freudenreich *et al.* 1998; Rolfsmeier *et al.* 2000). In either case, it is clear that loss of mismatch repair function creates instability at many sequences in the genome. Defects in recombinational repair systems in bacteria (Sarkar *et al.* 1998) and yeast (Richard *et al.* 2000) also have been reported to change TNR mutation frequencies, but again these defects have widespread effects throughout the genome. Based on this analysis *RTG2* seems to be the best example so far of a gene that shows a high selectivity for a TNR sequence.

Independence of *RTG2* effects at CTG•CAG repeats from *RTG1* and *RTG3*: The retrograde regulation pathway helps maintain cellular metabolism in yeast with mitochondrial dysfunction (Liao and Butow 1993). The retrograde response involves the products of *RTG1*, *RTG2*, and *RTG3*, plus other genes. Mutation in any one of the three *RTG* genes leads to loss of the retrograde response (Liao and Butow 1993; Jia *et al.* 1997). Since the *rtg2::LEU2* allele was identified in our screen, there

was good reason to examine *rtg1* and *rtg3* mutants for potential effects on CTG•CAG repeat instability. However, we found no evidence that the complete retrograde regulatory pathway was involved. Neither *RTG1* nor *RTG3* was identified in our mutant screen. When *rtg1*Δ and *rtg3*Δ strains were assayed for expansions and contractions, both strains behaved like wild type. Furthermore, in wild-type cells there was no apparent difference in CTG•CAG tract instability whether or not the retrograde response was inhibited by the presence of high concentrations of glutamate in the growth media. Also our *rtg2::LEU2* mutation was found in cells that are respiration competent, whereas signaling of dysfunctional mitochondria is a necessary input signal for activation of retrograde regulation (Liao and Butow 1993). Together these findings indicate that the action of *RTG2* in our system is independent of *RTG1* and *RTG3* and probably independent of the retrograde response. There is a report of the independent function of Rtg2p in the regulation of nitrogen catabolism. Evidence is available that *rtg2*Δ mutations, but not *rtg1*Δ or *rtg3*Δ alleles, affect the ability of cells to take up ureidosuccinate (Pierce *et al.* 2001). Another study concluded, however, that glutamate levels dependent on the *RTG* pathway indirectly control ureidosuccinate uptake (Sekito *et al.* 2002). Thus it is controversial whether or not *RTG2* can act independently of *RTG1* or *RTG3* in functions other than control of CTG•CAG repeat instability.

Possible models for *RTG2* action: Our evidence indicates that *RTG2* is involved in triplet repeat stability via a novel pathway, distinct from the retrograde regulatory response. Although the biochemical function of Rtg2p is not known, it is a cytoplasmic protein involved in the nuclear relocalization of Rtg1p and Rtg3p in response to activation of the retrograde pathway (Sekito *et al.* 2000). Rtg2p does not appear to enter the nucleus, nor is the protein known to interact with DNA. Thus, Rtg2p most likely plays an indirect role in triplet repeat instability. One feasible scenario is that loss of *RTG2* leads to altered expression of some gene or genes that influence CTG•CAG repeat instability. We examined available literature data on Rtg2p for clues to its potential action at CTG•CAG tracts. Microarray analysis provides one means of identifying genes whose expression is altered in an *rtg2* background. Microarray data are available for *RTG2 vs. rtg2* cells (Epstein *et al.* 2001), but only for cells that are ρ^-. Since our cells are ρ^+, the comparison is not a perfect one but it does provide some suggestions. Some of the genes with downregulated expression in an *rtg2* background (but not in *rtg1* or *rtg3* strains) include *HTB2* and *HHF1*, encoding histones H2B and H4, respectively. Two other downregulated genes in the *rtg2* background are *POL32*, which encodes a noncatalytic subunit of DNA polymerase δ, and *POL5*, encoding a putative DNA polymerase of unknown function. Expression of these genes is reduced three- to fivefold in the *rtg2* background. These findings implicate chromatin structure and/or DNA polymerase activity in TNR instability. We tested the idea that downregulation of *POL32* might be the mechanism of action by examining the contraction rate of $(CTG)_{25+8}$ in a *pol32*Δ background. If this idea is correct, the *pol32*Δ mutant would show a reduction in CTG repeat contraction rates similar to *rtg2*, assuming that a *pol32*Δ strain mimics downregulation of the gene. On the contrary, we found that the contraction rate in a *pol32*Δ background [5.2 ($\pm$3.5) $\times 10^{-5}$] is slightly higher than that in wild type [2.0 ($\pm$2.5) $\times 10^{-5}$]. This increased rate suggests that *POL32* is not involved in triplet repeat stability, but that *POL32* helps suppress genomic deletions, as suggested in a recent report (Huang *et al.* 2002). Proteomic analysis is a second possible avenue for understanding *RTG2* action at TNR sequences. Proteomic results with yeast protein complexes (Gavin *et al.* 2002) suggest that Rtg2p is part of a large complex that also includes Hca4p. The activity of this complex is thought to be in protein/RNA transport and its cellular localization is listed as nuclear or unknown (Gavin *et al.* 2002). A recent study reported that Rtg2p also forms complexes with Mks1p, a regulatory factor in several cellular pathways, and this complex may play an important role in the regulation of *RTG*-dependent gene expression (Sekito *et al.* 2002). These reports, in conjunction with our results, underscore the fact that more must be done to fully understand the action of *RTG2* at triplet repeat sequences. Nonetheless, the identification of *RTG2* as a modifier gene and verification of its mutant phenotypes at CTG•CAG repeats provide an important first step.

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