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Haploinsufficiency of Yeast FEN1 Causes Instability of Expanded CAG/CTG Tracts in a Length-Dependent Manner

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Abstract

Trinucleotide repeat diseases, such as Huntington's disease, are caused by the expansion of trinucleotide repeats above a threshold of about 35 repeats. Once expanded, the repeats are unstable and tend to expand further both in somatic cells and during transmission, resulting in a more severe disease phenotype. Flap endonuclease 1 (Fen1), has an endonuclease activity specific for 5' flap structures and is involved in Okazaki fragment processing and base excision repair. Fen1 also plays an important role in preventing instability of CAG/CTG trinucleotide repeat sequences, as the expansion frequency of CAG/CTG repeats is increased in FEN1 mutants *in vitro* and in yeast cells defective for the yeast homolog, *RAD27*. Here we have tested whether one copy of yeast FEN1 is enough to maintain CAG/CTG tract stability in diploid yeast cells. We found that CAG/CTG repeats are stable in *RAD27* +/− cells if the tract is 70 repeats long and exhibit a slightly increased expansion frequency if the tract is 85 or 130 repeats long. However for CAG-155 tracts, the repeat expansion frequency in *RAD27* +/− cells is significantly higher than in *RAD27* +/+ cells. This data indicates that cells containing longer CAG/CTG repeats need more Fen1 protein to maintain tract stability and that maintenance of long CAG/CTG repeats is particularly sensitive to Fen1 levels. Our results may explain the relatively small effects seen in the Huntington's disease (HD) FEN1 +/− heterozygous mice and myotonic dystrophy type 1 (DM1) FEN1 +/− heterozygous mice, and suggest that inefficient flap processing by Fen1 could play a role in the continued expansions seen in humans with trinucleotide repeat expansion diseases.

Keywords

RAD27; Trinucleotide repeat; Huntington's disease; Myotonic dystrophy; DNA replication; DNA repair

1. Introduction

Thirteen inherited genetic diseases have been found to be caused by CAG/CTG trinucleotide repeat expansions, including Huntington's disease (HD), myotonic dystrophy type 1 (DM1), and several spinocerebellar ataxias (SCAs) (Cleary and Pearson, 2003;Pearson et al., 2005). Thenormal gene loci typically have 6 to 34 copies of the CAG/CTG repeats. When the copies

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exceed a threshold of about 35 repeats they become unstable and are prone to further expansions both somatically and in the following generations (Siianova and Mirkin, 2001;Cleary and Pearson, 2003;Pearson et al., 2005). The disease-causing repeat size ranges from 36 to 121 in Huntington's disease, and 80 to over 1000 in myotonic dystrophy (Siianova and Mirkin, 2001;Cleary and Pearson, 2003;Pearson et al., 2005).

The mechanisms of trinucleotide repeat expansion have been studied in different organisms, including *E. coli*, yeast, mice and mammalian cells (Siianova and Mirkin, 2001;Lenzmeier and Freudenreich, 2003). One of the models to explain the expansions of trinucleotide repeats is that expansions occur due to incorrect Okazaki fragment processing (Gordenin et al., 1997;Freudenreich et al., 1998;Schweitzer and Livingston, 1998;Lenzmeier and Freudenreich, 2003). Fen1, the human homolog of yeast Rad27, is a structure-specific endo/exonuclease that cleaves the 5' flap created by strand displacement during Okazaki fragment maturation (Rossi et al., 2006). *In vitro,* Fen1 prefers to cleave a double flap substrate containing a 5' flap with a one nucleotide 3' overhang (Rossi et al., 2006). Secondary structures formed by trinucleotide repeats on the 5' flap can inhibit Fen1 cleavage, leading to ligation of the unprocessed flap and repeat sequence expansion (Spiro et al., 1999;Henricksen et al., 2000). The inhibition is length dependent: the longer the repeats are, the more inefficient the flap processing (Henricksen et al., 2000).

Fen1 is an evolutionarily conserved nuclease and has been isolated and characterized from many organisms, from archaebacteria to humans (Greene et al., 1999). Human Fen1 protein shares 61% identity and 79% similarity with *Saccharomyces cerevisiae* Rad27 protein (Shen et al., 1998). Recent work has shown that expression of human Fen1 can correct a number of mutant phenotypes in *rad27* deletion (*rad27*Δ) yeast cells, including sensitivity to DNA-damaging agents, high rates of recombination and mutation, and synthetic lethality with double-strand-break repair mutants (Hansen et al., 2000).

In *S. cerevisiae*, the expansion and the contraction of CAG/CTG repeats is dramatically increased in a *rad27*Δ strain compared to wild-type (Freudenreich et al., 1998;Schweitzer and Livingston, 1998;Spiro et al., 1999), consistent with the idea that defective flap processing can lead to sequence expansions *in vivo*. CTG repeat expansions are also observed when human or yeast FEN1 mutants defective in nuclease activity are used in an *in vitro* assay for flap processing (Liu and Bambara, 2003;Liu et al., 2004). In mammals, the loss of FEN1 is embryonic lethal. In order to investigate the role of flap processing in repeat instability in mammals, Spiro and McMurray used mice heterozygous for FEN1 and harboring an expanded CAG-120 repeat within the human HD gene (Spiro and McMurray, 2003). Expansion of CAG repeats was increased in the progeny of the FEN1 heterozygous male mice, showing a shift from about equal expansions and contractions to five-fold more expansions than contractions. However no differences in somatic cell repeat instability were observed compared to wild-type mice (Spiro and McMurray, 2003). Recently another group observed that a $(CAG/CTG)_n$ ($104 \leq n \geq 110$) repeat at the DM1 locus did not exhibit increased instability either somatically or intergenerationally in FEN1 +/− heterozygous mice (van den Broek et al., 2006). These results raise the question of whether one copy of FEN1 was sufficient to prevent most repeat expansions in the HD and DM1 mouse models. We wished to address this question in order to gain further insight into whether defective flap processing by Fen1 may play a role in expansion of trinucleotide repeats in humans.

2. Materials and methods

2.1 Yeast strains and yeast mating

All the yeast haploid strains containing CAG-70 or 155 repeat tracts, cloned on a yeast artificial chromosome (YAC), were the VPS105 background (*MATα ade2 can1 leu2-3,112* Δ*trp1*

ade3 Δ*ura3 lys2-801* amber). All the yeast haploid strains containing CAG-85 and 130 repeat tracts, cloned on yeast chromosome II, were the YPH500 background (*MATα ura3-52*, *lys2-801* amber, *ade2-101* ochre, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*). In order to create diploid yeast cells, a *MATα* strain containing a chromosome harboring an expanded CAG repeat tract (CAG-70, 85, 130 or 155) was mated with an isogenic *MATa* strain at 30°C for over night. Cells were plated for single colonies onto either YEPD for *RAD27* +/+ or selective plates for *RAD27* +/− and *rad27* −/−. Single isolated colonies were checked for diploid status in two ways: (1) a mating type test that can distinguish diploid and haploid (*MATa* or *MATα*) cells (Sprague, 1991) and (2) By PCR for *RAD27* +/− and *rad27* −/− strains, which have different markers at each *RAD27* locus resulting in different size PCR products which can be distinguished by gel electrophoresis.

2.2 CAG stability assay

To test CAG repeat expansion and contraction frequencies, 10 independent colonies with correct tract length (CAG-70, 85, 130 or 155) were chosen as starting colonies from either *RAD27* + haploid cells, *RAD27* +/+, *rad27* −/−, or heterozygous *RAD27* +/− diploid cells, and grown in liquid media for 16 hours at 30°C. These 10 individual cultures were mixed together and plated for single colonies using proper dilutions. Mixing 10 individual cultures was done to make sure we sampled multiple independent events and to avoid “jackpot” effects of a single early expansion or contraction event which could make up a significant fraction of a culture and bias the observed frequency. About 50 daughter colonies were then tested for repeat contractions or expansions by colony PCR. The assay was repeated a minimum of three times for each tract length in each strain background (Table S1). For CAG-70 and 155 which are on the YAC, repeat sequences were amplified by primers CTG rev2 (CCCAGGCCTCCAGTTTGC) and T720 (TAATACGACTCACTATAGGG). For CAG-85 and 130 on chromosome II, repeat sequences were amplified by primers CTG rev (GTGGAGGATGGAACACGG) and T720. Reactions were run as described previously (Callahan et al., 2003).

In order to check if loss of heterozygosity occurs in *RAD27* +/− cells during the course of the experiment, the presence of both *RAD27* and *rad27*Δ alleles were assessed independently by PCR for three sets of putative *RAD27* +/− daughter colonies from the stability assay. All 152 daughter colonies tested retained both alleles, indicating that loss of heterozygosity was not occurring or was very rare.

The Fisher's exact test was used to analyze whether the total number of contractions or expansions in *RAD27* +/−, *rad27* −/− or *RAD27* + strains was significantly different from that in the *RAD27* +/+ strain. Data from each repeated experiment were combined together for the statistical analysis using a 2 × 2 contingency table. Analysis using a one-tailed non-parametric Mann-Whitney test was also done, and values reported as significant by the Fisher's exact test were also significant using this method. The means +/− standard error that are plotted in Fig. 1 are averaged across at least three repetitions of each experiment (Table S1).

2.3 Western blot analysis

Yeast protein was purified as described (Knop et al., 1999) with the following changes: 5ml cells (OD 2-3) were collected and resuspended in 1ml ice-cold water. The cell suspension was mixed with 150μl 1.85M NaOH and placed on ice for 10 min. 150μl 55% TCA was added and the mixture was incubated for 10 min on ice. Cells were pelleted for 5 min at 14000 rpm at 4° C. The supernatant was removed and the cell pellet resuspended in 150 μl 2X SDS-sample buffer (200mM DTT, 4% SDS, 100mM Tris-HCl pH 6.8, 20% glycerol and 30 μl/ml bromphenol blue). In order to neutralize the remaining traces of the TCA, 3-5 μl of 1.5M Tris-HCl, pH 8.8 was added. Proteins were separated by 10% SDS-polyacrylamide gel

electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Amersham Biosciences). Rad27 protein was detected by anti-Rad27 antibody (Santa Cruz Biotechnology, Inc.). Actin was detected by anti-actin (Chemicon International). Signals were detected with an ECL detection reagent (Millipore) according to the manufacturer. Blots were stripped by washing with 1x TBST (10mM Tris-HCl pH 7.4; 150mM NaCl; 0.1% Tween20) and incubating for 30 min at 50°C with freshly made strip-buffer (100mM 2-mercaptoethanol; 2% SDS; 62.5mM Tris-HCl, pH 6.7). After stripping, blots were washed twice for 10 min in TBS-T.

3. Results

3.1 The effect of RAD27 haploinsufficiency on CAG repeat expansions and contractions is tract-length dependent

In yeast, cells can grow with either haploid or diploid chromosome content. In order to create heterozygous *RAD27* +/− diploid yeast cells, a *rad27*Δ *MAT*α strain containing a chromosome harboring an expanded CAG repeat tract (CAG-70, 85, 130 or 155) was mated with an isogenic wild-type *MATa* strain. For the cells containing CAG-85 or 130, the repeats were cloned on yeast chromosome II (Freudenreich et al., 1998), and for CAG-70 and 155, the repeats were cloned on a yeast artificial chromosome (YAC) (Callahan et al., 2003). In all strains, the repeat tract is oriented such that the CAG sequence is on the lagging strand template and the CTG sequence on the nascent lagging strand (i.e. the more expansion-prone orientation, referred to as orientation I in Kang et al., 1995 and Freudenreich et al., 1997). Based on available data on the location of replication origins (Nenguke et al., 2003) and also the expansion bias seen at the human disease loci, this orientation is likely to be equivalent to that found at the HD locus. The heterozygous yeast is similar to the HD and DM1 mouse models used (Spiro and McMurray, 2003;van den Broek et al., 2006), since each diploid yeast cell contains two copies of each yeast chromosome, one copy of the yeast FEN1 gene, and one expanded CAG repeat tract. To test CAG repeat expansion and contraction frequencies, stability assays were performed on *RAD27* +/+, *rad27* −/−, or heterozygous *RAD27*+/− diploid cells containing repeat tracts of different lengths (CAG-70, 85,130 or 155). The stability assay was repeated at least three times for each strain for a total of at least 148 colonies tested per strain (Table S1). For the *RAD27* +/− cells, it was verified that loss of heterozygosity did not occur in the course of the experiment (0/152 colonies checked; see Methods 2.2).

For CAG-70 heterozygous *RAD27* +/− diploids, the frequency of repeat expansion is similar to that of *RAD27* +/+ cells (Fig. 1A; Table 1). The frequency of repeat expansion increased slightly as the repeat length increased to CAG-85 or 130 (~2 to 4-fold), although the increase was not enough to be statistically significant. However, when the repeat tract length reached CAG-155, the *RAD27* +/− heterozygote exhibited a significantly greater frequency of expansions than the *RAD27* +/+ wild-type diploid control ($P<0.006$). The increase in CAG-155 expansions in the *RAD27* +/− heterozygote was not as great as the expansion frequency observed in the absence of Rad27 protein, but rather showed an intermediate phenotype (Fig. 1A; Table 1). These results indicate that one copy of *RAD27* in a diploid cell is sufficient to maintain the shorter CAG-70 repeat tracts to the same degree as in a wild-type cell, but that expansion of longer CAG/CTG repeat tracts is sensitive to *RAD27* dose.

A similar pattern was observed for the frequency of repeat contractions (Fig. 1B; Table 1). CAG-70 and CAG-85 *RAD27* +/− heterozygotes exhibited a low level of contractions that was similar to the *RAD27* +/+ strains. For CAG-130 and 155, a significant increase of contraction frequency in the *RAD27* +/− heterozygotes compared to *RAD27* +/+ wild-type diploids was observed (Fig. 1B; Table 1).

3.2 The frequency of contraction is cell type dependent for CAG-155

To determine whether CAG/CTG repeat tract stability is affected by the haploid or diploid status of the cells, CAG repeat instability was compared in wild-type diploid cells and wild-type haploid cells. For both cell types only one expanded CAG repeat tract was present. For CAG-70, 85 and 130, no significant difference in CAG repeat instability between wild-type haploid (*RAD27* +) and wild-type diploid (*RAD27* +/+) strains was observed (Fig. 1). Similarly for CAG-155, the wild-type diploid expansion frequency (1.3%) is approximately equal to the wild-type haploid frequency (0.5%). Interestingly, CAG-155 contractions were more frequent in wild-type diploid cells compared to wild-type haploids (33.3% vs 16.8%) (Fig. 1B; Table 1). The CAG-155 tract is carried on a YAC which is present in only one copy in both the haploid and diploid cells used. Therefore the increase cannot be explained by increased recombination between homologs.

3.3 Yeast Fen1 levels are not upregulated in RAD27 +/− heterozygote cells

The phenotypic difference in repeat instability between the *RAD27* +/+ diploid cells and *RAD27* +/− diploid cells strongly suggests that Rad27 protein is not upregulated in the heterozygote. In order to directly determine Rad27 protein levels, protein was extracted from each cell type and detected using an antibody to yeast Rad27 protein (Fig. 2A). Quantification compared to a control protein (actin) confirmed that Rad27 protein levels in the heterozygote were half that detected in *RAD27* +/+ diploids (Fig. 2B), indicating that Rad27p is not upregulated in *RAD27* +/− cells.

4. Discussion

Our data indicate that maintenance of long CAG/CTG repeats is particularly sensitive to Fen1 levels. Comparing the *RAD27* +/− diploids with *RAD27* +/+ diploids, both contain one expanded CAG repeat tract in the genome and two copies of each natural yeast chromosome, but in the *RAD27* +/− cells the total amount of Rad27p is half the level found in *RAD27* +/+ cells. This situation is not a problem under normal circumstances. However, 5' flaps containing a CTG repeat (as in our case) pose a special challenge since they can form a stable hairpin structure that inhibits Fen1 cleavage (Spiro et al., 1999;Henricksen et al., 2000). In this case, flap equilibration can usually eventually result in correct processing by Fen1 (Liu et al., 2004), followed by DNA ligation and no repeat expansion. However, when Fen1 concentration is limiting, the unprocessed flaps could equilibrate into a variety of intermediate structures, some of which will be ligated by DNA ligase to cause a sequence expansion. This model is supported by *in vitro* data that shows that there is a competition between Fen1 and DNA ligase at the flap, with increasing amounts of ligase resulting in ligation of the unprocessed flaps to produce expansions (Henricksen et al., 2002). Similarly, limiting amount of Fen1 could also favor flap ligation to produce expansions.

In the *RAD27* +/− heterozygote cells we studied, the same amount of Rad27 protein is present in cells containing the shorter or longer repeat tracts, yet only the longer repeat tracts were prone to expansions and contractions. One explanation is that there are more Okazaki fragments with CTG repeats on the 5' flap in cells containing the long repeat tracts. In eukaryotes, Okazaki fragments are 135-145 nucleotides long (Cleary and Pearson, 2005). Assuming that the average length of Okazaki fragments is ~ 140 nucleotides, in cells containing shorter repeats (CAG-70 and 85) there will be no more than 2 Okazaki fragments containing CTG repeats on a 5' flap during one replication cycle. However, in CAG-130 or 155 cells, 3 or 4 Okazaki fragments will have CTG repeats located on 5' flaps waiting for Rad27p cleavage, increasing the probability that ligation will result before flap cleavage to cause a sequence expansion. Equilibrating flaps could also allow hairpin formation on the template strand opposite the unligated nick, which combined with extension of the 3' end of the next Okazaki fragment over

the template hairpin, could lead to a contraction. Another explanation is that the longer repeat is more likely to incur DNA lesions that require Rad27 for repair. It has been shown that *rad27*Δ cells show an increased fragility of CAG repeats compared to wild type cells which is exacerbated as tract length increases (Callahan et al., 2003). Faulty repair could lead to either expansions or contractions.

A novel finding from this study was that wild-type diploid cells have a significantly higher contraction frequency than haploid cells, but again only for the long CAG-155 tract. Because of the experimental setup, both cell types had only one copy of the chromosome with the expanded CAG repeat, therefore the difference between haploids and diploids could not be due to recombinational repair between CAG repeats on homologs. It has been shown that non-homologous end-joining is much less efficient in diploid cells than in haploid cells (Astrom et al., 1999;Lee et al., 1999). Possibly, the repair choice for CAG repeats could shift towards a more contraction-prone repair pathway, such as single-strand annealing, in diploid cells. The chance of utilizing such a pathway could depend on the level of lesions in the cell, or could also be influenced by a specific structure or lesion formed more frequently at CAG-155 tracts. One candidate for this structure is a stalled replication fork (Freudenreich and Lahiri, 2004).

The sensitivity to repeat length we observed could explain the results seen in the FEN1 +/− mice with 120 CAG repeats at the HD locus (Spiro and McMurray, 2003). Full levels of Fen1 are apparently required for proper maintenance of 120 repeats in the developing male germ cells, indicating that significant flap processing is occurring in this cell type during either premeiotic germ cell divisions or postmeiotic gap repair (Kovtun and McMurray, 2001;Spiro and McMurray, 2003). However, in the somatic cells studied that are not undergoing rapid division or differentiation, one copy of FEN1 appears sufficient to maintain a CAG-120 tract without expansions (Spiro and McMurray, 2003). One copy was also sufficient to maintain a CTG-110 repeat at the DM1 locus (van den Broek et al., 2006). Our results suggest that a FEN1 +/− HD or DM1 mouse model with longer repeats might show a more dramatic phenotype. Similarly, a FEN1 heterozygote individual with a trinucleotide repeat in the long-normal range might accumulate a trinucleotide repeat expansion in a germ cell, even though general genome stability is not affected.

In humans, CAG/CTG expansions can occur in both dividing pre-meiotic germ cells and nondividing postmeiotic germ cells (Yoon et al., 2003) as well as in both proliferating and non-proliferating somatic cells (Sinden, 2001). Our data indicate that cells with larger expanded repeat tracts that are actively undergoing either replication or repair may be the most sensitive to Fen1 protein levels. The myotonic dystrophy locus, where CTG tracts larger than 150 repeats are often observed, exhibits instability in proliferating cell types that are presumably undergoing Fen1-dependent flap processing during replication (Fortune et al., 2000;Pearson et al., 2005). The SCA2 and SCA7 loci also expand to 200-300 repeats (Siianova and Mirkin, 2001). The majority of inherited Huntington's alleles are less than 150 repeats, however there is evidence that somatic expansions in affected human brain cells are common, with a significant percentage of cells containing CAG tracts greater than 150 repeats, and that these increases precede pathogenesis (Kennedy et al., 2003). These non-dividing neural cells could be prone to repeat expansion events during Fen1-dependent repair. Since brain cells are known to have reduced repair capacity, it is possible that the normal expression level of Fen1 in the affected cells is not sufficient to prevent expansions in a long CAG repeat tract.

In summary, we have shown that the balance between Fen1 levels and repeat maintenance is critical, implying that any condition that upsets this balance (more flaps because of DNA damage or longer repeat lengths; less Fen1 because of cell type, developmental window, or a mutated allele) could lead to repeat expansions. Overall, our data imply that inefficient flap

processing by Fen1 is a mechanism that may be operating to generate CAG/CTG expansions in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FEN1, Flap endonuclease 1; Rad, Radiation sensitive; HD, Huntington's disease; DM1, Myotonic dystrophy type 1; SDS, Sodium dodecyl sulfate; PAGE, Polyacrylamide gel electrophoresis; MAT, Mating type locus; YAC, Yeast artificial chromosome; SCA, Spinocerebellar ataxia.

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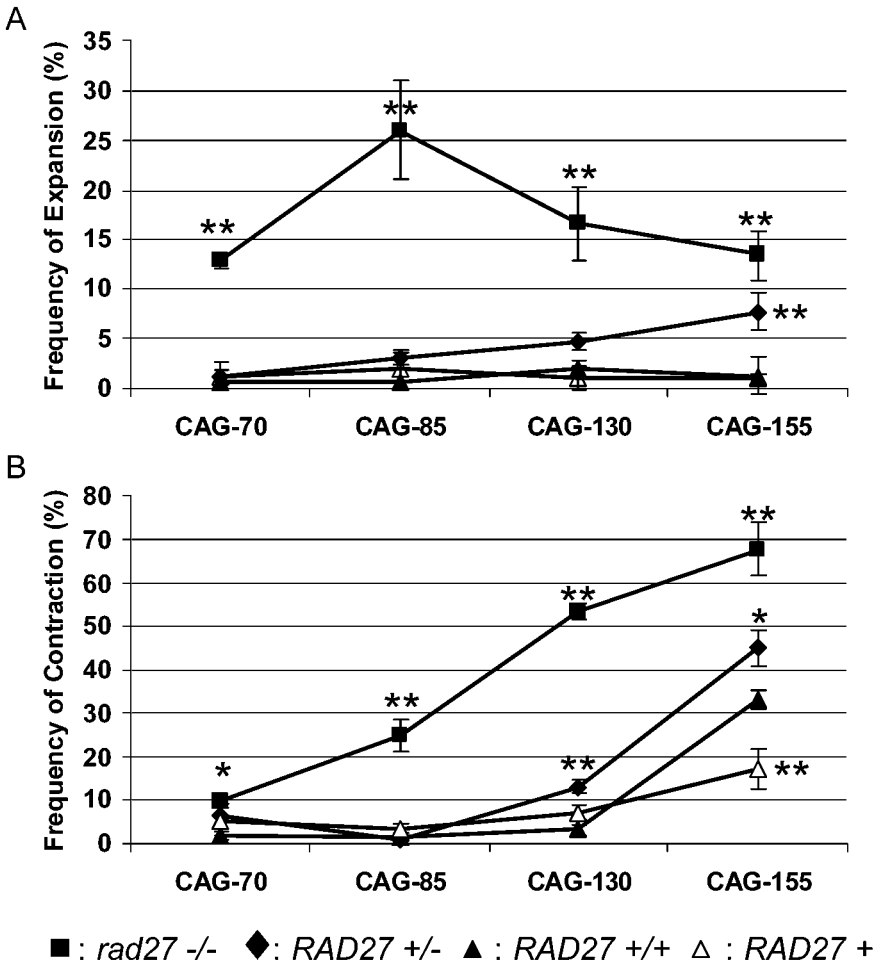

Figure 1.

Frequency of CAG repeat expansion (A) and contraction (B) in *RAD27* + (Δ), *RAD27* +/+ (▲), *rad27* −/− (■) and heterozygous *RAD27* +/− (◆) strains. 148 to 312 colonies were evaluated for each tract length of each strain in at least three independent experiments. We note that the frequency of expansions for the *rad27* −/− CAG-130 and 155 strains in (A) could be an underestimate due to inefficient PCR amplification of tracts longer than about 180 repeats in this strain background. **, P<0.01; *, P<0.05 compared to *RAD27* +/+ value, by Fisher's Exact Test. Standard error is shown.

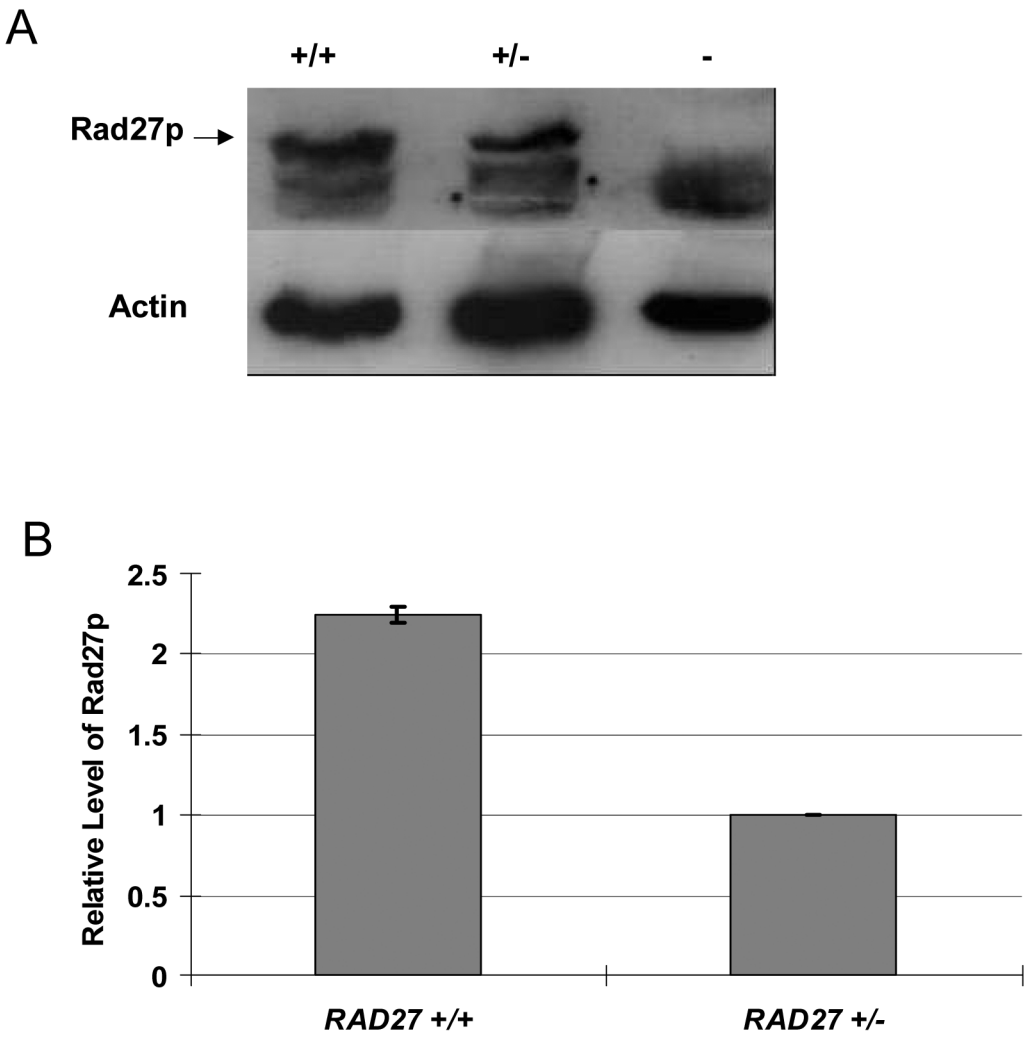

Figure 2.

Detection of Rad27 protein levels by Western blot. (A) Protein extracts were separated on an SDS polyacrylamide gel, blotted, and probed with anti-Rad27 antibody. The same blot was stripped and re-probed with anti-actin antibody. Results for *RAD27* +/+, *RAD27* +/− heterozygotes and *rad27* − strains are shown. (B) The relative level of Rad27p was first normalized by the amount of Actin in the same blot by comparing the band density. Quantification of band density was performed by Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). The normalized Rad27p level in *RAD27* +/+ cells was compared to that in the *RAD27* +/− cells to get the relative level of Rad27p. The experiment was done twice. Standard error is shown.

Table 1

Frequency of Expansions and Contractions

	Expansion (%)				Contraction (%)			
	CAG-70	**CAG-85**	**CAG-130**	**CAG-155**	**CAG-70**	**CAG-85**	**CAG-130**	**CAG-155**
rad27 –/–	12.8**	25.6**	16.6**	13.4**	9.6*	24.4**	53.4**	67.8**
RAD27 +/–	1.3	2.6	4.7	7.7**	6.4	1.3	12.8**	45.2*
RAD27 +/+	0.6	0.6	1.9	1.3	1.9	1.6	3.2	33.3
RAD27 +	1.3	1.9	1.3	0.5	5.1	3.2	7.1	16.8**

** P<0.01

* P<0.05 compared to *RAD27* +/+ value, by Fisher's Exact Test.