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The Effect of DNA Replication Mutations on CAG Tract Stability in Yeast

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ABSTRACT

CAG repeat tracts are unstable in yeast, leading to frequent contractions and infrequent expansions in repeat tract length. To compare CAG repeats to other simple repeats and palindromic sequences, we examined the effect of DNA replication mutations, including alleles of pol α, pol δ, pol ε, and PCNA (proliferating cell nuclear antigen), on tract stability. Among the polymerase mutations, the pol δ mutation (*pol3-14*) destabilizes tracts with either CAG or CTG as the lagging strand template. One pol α mutation, *pol1-1*, destabilizes the orientation with CAG as the lagging strand template, but it has little effect on the CTG orientation. In contrast, the *pol1-17* mutation has no effect on either orientation. Similarly, mutations in the proofreading functions of pol δ and pol ε, as well as a temperature-sensitive pol ε mutation, *pol2-18*, have no effect on tract stability. Three PCNA mutations, *pol30-52*, *pol30-79*, and *pol30-90*, all have drastic effects on tract stability. Of the three, *pol30-52* is unique in yielding small tract changes that are indicative of an impairment in mismatch repair. These results show that while CAG repeats are destabilized by many of the same mutations that destabilize other simple repeats, they also have some behaviors that are suggestive of their potential to form hairpin structures.

REPEATED DNA sequences are inherently unstable because of their ability to misalign during cellular processes that separate the two strands of DNA. Such misalignments can lead to expansions or deletions within the repeated sequence. Different types of repeated sequences, varying in length and sequence of the repeated unit, exhibit unique patterns and degrees of instability. Repetitions of the trinucleotide CAG and its complement, CTG, display instability that was initially recognized because several neurodegenerative diseases in humans are caused by expansions of these repeat tracts (Paulson and Fischbeck 1996).

Studies of CAG tracts placed within yeast chromosomes show that while these repeat tracts share an inherent instability with other simple repeated sequences, such as repetitions of the dinucleotide GT, there are features of CAG tract instability that may be distinct (Kang *et al.* 1995a; Maurer *et al.* 1996; Freudenreich *et al.* 1997; Miret *et al.* 1997). One distinctive feature is that the instability of long CAG tracts is greatly influenced by whether CAG or its complement, CTG, serve as the lagging strand template (Maurer *et al.* 1996; Freudenreich *et al.* 1997). In addition, the majority of changes in CAG tracts are contractions in tract length, whereas dinucleotide repeat tracts undergo expansion and contraction with near equanimity (Strand *et al.* 1993; Wierdl *et al.* 1997). Furthermore, long CAG tracts undergo large changes of many repeat units, while simple mono- or dinucleotide repeat tracts of more moderate length undergo many changes of a single repeat unit (Henderson and Petes 1992; Maurer *et al.* 1996; Freudenreich *et al.* 1997; Greene and Jinks-Robertson 1997; Miret *et al.* 1997; Wierdl *et al.* 1997). These differences between CAG repeat tract instability and the instability of tracts of other simple repetitions could result from experimental differences in the length of the repeat tracts that are examined and their placement within chromosomes and plasmids, but many of the differences are most likely inherent to unique properties of CAG repeats.

The reasons for this unique instability are becoming clearer. Many *in vitro* studies have shown that CAG and CTG oligonucleotides can form stable hairpin structures (Gacy *et al.* 1995; Yu *et al.* 1995; Mariappan *et al.* 1996). The potential for CAG/CTG hairpin formation *in vivo* adds another level of complexity to these repeat tracts by allowing them to act as inverted as well as direct repeats. Hairpin formation could facilitate misalignment throughout the repeat tracts and physically disrupt cellular processes such as DNA replication. Indeed, a careful study using bacteriophage λ indicated that CAG/CTG repeats can form hairpins *in vivo* (Darlow and Leach 1995). Further studies showed that CAG repeat tracts cause polymerase pausing during their replication both *in vitro* and *in vivo* (Kang *et al.* 1995b; Samadashwily *et al.* 1997).

To more fully understand how CAG tracts may be influenced by cellular processes that manipulate DNA, we have been studying CAG tracts embedded in a yeast

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chromosome (Maurer *et al.* 1996). Initial investigations demonstrated that changes in CAG repeat tract length in wild-type, mitotically growing yeast cells are mostly contractions with few expansions (Maurer *et al.* 1996; Freudenreich *et al.* 1997; Miret *et al.* 1997). Also, CAG tract stability is greatly influenced by the direction of DNA replication through the tract (Maurer *et al.* 1996; Freudenreich *et al.* 1997). A further indication of the importance of replication in tract stability came from studies of a *rth1Δ/rad27Δ* mutant lacking the flap endonuclease needed to process the ends of Okazaki fragments. CAG repeat tracts are highly unstable in a *rth1Δ/rad27Δ* mutant, undergoing frequent expansions as well as contractions (Freudenreich *et al.* 1998; Maurer *et al.* 1998; Schweitzer and Livingston 1998). This result suggested a unique mechanism for tract expansion and emphasized the importance of lagging strand replication in the stability of CAG repeat tracts.

In this report, we have examined mutations in different components of the replication machinery in yeast (Newlon 1996) to determine how they contribute to CAG tract instability. The instability of CAG tracts has been investigated in strains bearing mutations in three different DNA polymerases and in proliferating cell nuclear antigen (PCNA). The results reveal the similarities and differences that these repeats exhibit in comparison to other simple repeats and palindromes that have been studied in yeast.

MATERIALS AND METHODS

Yeast strains: All strains are based on the isogenic pair of parental strains SSL204a and SSL204α (Dornfeld and Livingston 1991). Placement of CAG repeat tracts in a clone of *ADE2* and disruption of *ARO2* on chromosome VII in the parental strains have been described previously (Maurer *et al.* 1996). We designate tracts with CAG in the *ADE2* coding strand to be of the C orientation and tracts with CTG in the coding strand to be of the D orientation (Figure 1).

The various replication mutations were all introduced into wild-type SSL204a and α using the two-step replacement method (Scherer and Davis 1979). Cloned copies of mutant alleles were obtained as YIp5 derivatives or eventually subcloned into YIp5. The alleles were generous gifts from the following sources: *pol1-1* (Pizzagalli *et al.* 1988) from G. Lucchini; *pol1-17* (Budd and Campbell 1987), *pol2-18* (Araki *et al.* 1992), and *pol3-14* (Giot *et al.* 1995) from J. Haber; *pol2-4* (Morrison *et al.* 1991) and *pol3-01* (Morrison *et al.* 1993) from A. Sugino; and *pol30-52* (Ayyagari *et al.* 1995), *pol30-79*, and *pol30-90* (Eissenberg *et al.* 1997) from P. Burgers. Potential mutants were screened when possible by identifying phenotypes such as temperature or methyl methanesulfonate (MMS) sensitivity, and were then confirmed by sequencing (primers and sequences available upon request). The strains containing the replication mutations were then mated to strains containing the CAG repeat tracts, and the resulting diploids were sporulated. Spores containing the repeat tract and the replication mutation provided isogenic strains with repeat tracts of the same length. In general, we used two or more spores from the matings that placed repeat tracts in the replication mutants for subsequent studies. In the case of *pol30-52*, a long repeat tract in the D orientation containing 71 repeat units could not be recovered because of

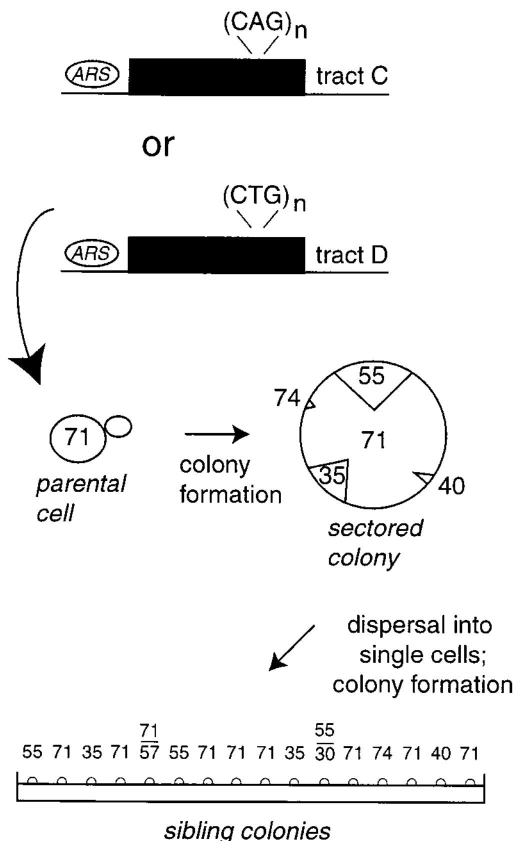

Figure 1.—Colony assay to determine the stability of CAG repeat tracts. Depicted at the top are the two tract orientations used in this study: tract C, which uses CAG as the template for lagging strand synthesis directed by the upstream ARS element, and tract D, which uses CTG as the template for lagging strand synthesis. A cell harboring a tract with 71 repeat units is shown in the diagram. When such a cell divides to form a colony, designated as a parental colony, sectors of cells originating from a cell having undergone a tract length change radiate outward. The numbers in or near the sectors represent the length of the altered tracts in the number of repeat units. Most of these changes are contractions, but tract expansions (shown here as a small sector with a tract length of 74 repeat units) also occur. When the parental colony is dispersed into single cells and plated on an agar dish, sibling colonies arise (represented at the bottom as small mounds on a schematized agar plate). The length of the repeat tracts within the individual sibling colonies is ascertained by PCR (see Figures 2 and 3). Many sibling colonies retain the parental tract length, as indicated by a parental-size PCR product. The products arising from the sectors do not contain the parental-size product, but they contain one of the altered length. For purposes of determining the frequency with which tracts undergo length changes in the parental colonies, the fraction of sibling colonies that lacks the parental-size product is used as a metric because they represent changes that have occurred during parental colony growth. Tracts may be so unstable that changes occur soon after plating of sibling cells; these examples are revealed in the PCR products by the retention of the parental product plus a product of an altered size (see Figures 2 and 3). These are depicted in the diagram by a stack of two numbers. For purposes of quantitation, these are not used because they represent changes that occur after parental colony formation. To make comparisons among frequency values more reliable, we permitted the parental colonies to reach a uniform size of $\sim 5 \times 10^6$ cells.

the severe instability of the repeat tract in this mutant, so shorter repeat tract lengths were used for this part of the study.

Changes in CAG tract length: To examine the effect of DNA replication mutations on CAG repeat tract instability, we grew cells at 30° (unless stated otherwise). This temperature is semi-permissive for the heat-sensitive polymerase mutations used in this study. To ascertain tract length changes, we started by choosing 5 colonies for each strain and tract orientation (Figure 1). These 5 parental colonies were dispersed into single cells and grown on agar to produce a collection of sibling colonies. DNA was purified from 32 sibling colonies that arose from each of the 5 parental colonies.

The purified DNA was used as a template for PCR with primers that flank the repeat tract as described previously (Maurer *et al.* 1996; Schweitzer and Livingston 1997, 1998). Sometimes, the PCR conditions used to amplify the repeat tracts were varied slightly from those described previously in that the buffer used was obtained from Epicentre Technologies (Madison, WI). As described previously, one primer was end labeled with ^{32}P. The PCR products were displayed on a DNA sequencing gel with appropriate standards (Figures 2 and 3). The PCR product from the parental colony was commonly run with the products from the sibling colonies.

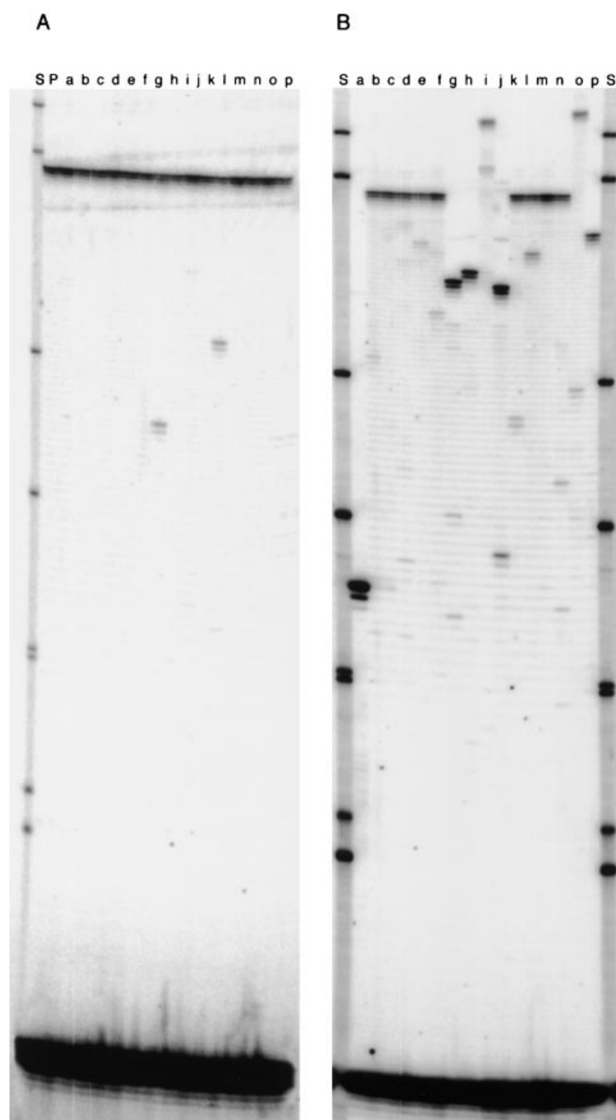

Figure 2.—Analysis of tract length changes in mutant cells. Portions of two gels, labeled A and B, are shown; each is half of a 32-sibling colony analysis. ^{32}P-labeled PCR products copied from template DNA purified from sibling colonies have been run on a sequencing gel. (A) Lanes a–p are from tract C in a *rad52*Δ mutant. (B) Lanes a–p are from tract C in a *pol30-79* mutant. These represent one mutant that does not lead to a significantly greater tract instability than wild-type levels and one mutant that does, respectively. P is the PCR product of the parental colony for the *rad52*Δ mutant (part A). The parental size repeat tract, 78 CAG repeats, was the same for both the *rad52*Δ and *pol30-79* mutants. Size standards of ^{32}P-labeled *Hpa*II fragments of KS+ are run in the lanes marked S. The largest products are those from the repeat tract. Stutter bands that differ by a repeat unit often descend from the band of the largest product. The very dark band across the bottom is the PCR product from the mutant copy of *ADE2* on chromosome XV. It serves as a positive PCR control. As repeat tracts become longer, they become more difficult to polymerize during the PCR. This is why the bands from the smallest repeat tracts and the control template have the greatest intensity. The three patterns of PCR products copied from the DNA of sibling colonies described in materials and methods and in the legend to Figure 1 can be seen. First, some lanes do not contain the band of the parental repeat length (a, g–j, o, and p in B). These are the ones counted in our study. Note that lanes i and o in B are tract expansions. Second, all the lanes (a–p in A) from the *rad52*Δ siblings have the parental band, and lanes b–f and k–n in B from the *pol30-79* mutant do as well. In some of these, the third pattern that represents sectoring after plating of the sibling cells is apparent as well. While lanes g and l in A and l in B have prominent bands smaller than the parental size, they contain the band of parental length. Bands of smaller length are visible in other lanes as well.

Our analysis of tract length changes relied on the phenomenon that as the parental colony grows, some cells undergo tract length changes that result in sectors of cells within the parental colony that have an altered tract length (Figure 1). To ascertain the percentage of cells with altered tract length, we dispersed the parental colony and permitted individual cells to grow. By sampling the sibling colonies, we were able to recognize those that came from sectors with altered repeat tract lengths by the absence of the parental-length tract. In place of the parental-length tracts, siblings arising from cells within the sectors displayed a PCR product of altered length. The length of the altered product could be measured within one or two repeat units, using the size standards that are run in the gel and by the stutter bands that often descend from the longest products (Figures 2 and 3). Small changes of less than five repeats could be measured accurately from the stutter bands (Figure 3). Rather than list all the length changes, we have reported their mean sizes (Table 1). A complete data set is available upon request.

Most parental colonies do not have a large amount of sectoring and, consequently, many of the sibling colonies display the parental-length PCR product. In some cases, the PCR products from sibling colonies not only display the parental-length product, but also a prominent product of altered length. These were not counted in our analyses because they are indicative of sectoring that occurs during growth of the sibling cell colony.

Statistical analysis: To ascertain whether repeat tracts are more unstable in mutant cells than in the wild-type cells, we compared the sets of data from each mutant, *i.e.*, the number

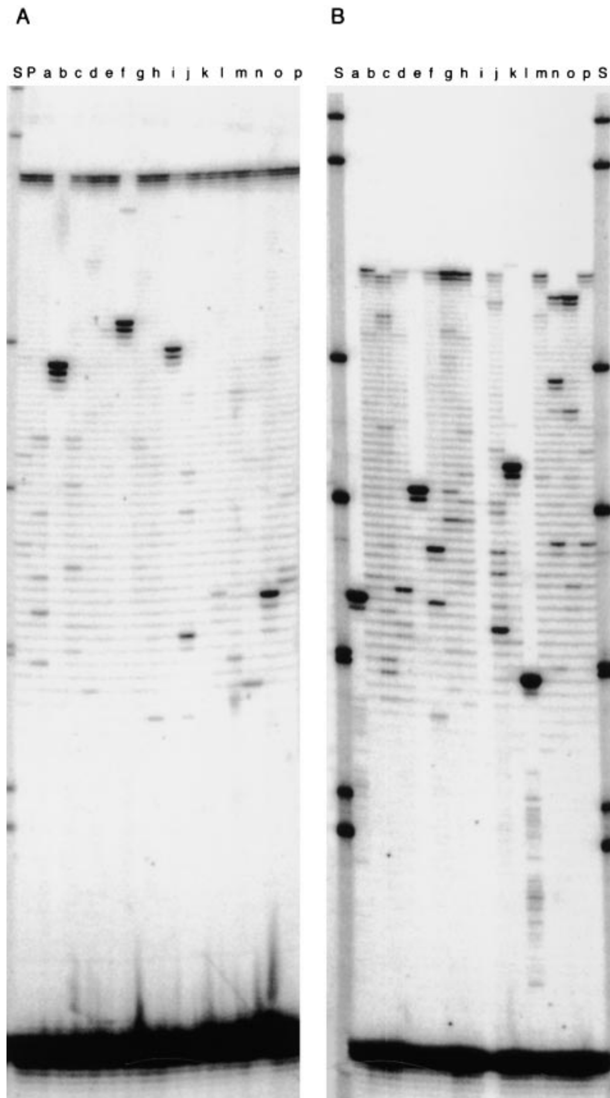

Figure 3.—Analysis of tract length changes in mutant cells. The analyses, as described in the legend to Figure 2, have been carried out for tract D from the *rad52*Δ (lanes a–p in A) and the *pol30-52* (lanes a–p in B) mutants. These represent one mutant that does not lead to significantly greater tract instability than the wild type and one mutant that does, respectively. As described in materials and methods, the parental size of the D tract in the *pol30-52* mutant was 52 repeat units, as opposed to the 71 repeat units used in the *rad52*Δ mutant. Lanes b and f in A and a, c, e, k, l, n, and o in B do not contain the parental size band. Note that the change in lane c in B is a contraction of a single repeat unit. All the remaining lanes (a, c, d, e, and g–p in A and b, d, f, g, h, j, m, and p in B) contain the parental repeat band. Lane i in B is blank; a repeated PCR trial revealed that it contained the parental band.

of changes per sample of 32 sibling colonies from 5 independent parental colonies, to the corresponding set of data from the wild-type cells (Table 1). The statistical test we applied was a randomization test that calculates the probability that, by chance, we found more high values within the set of 5 mutant samples than we did within the set of 5 wild-type samples. First, we calculated the probability that all 5 values for the mutant are larger by chance than the 5 values from the wild type. This probability is 1/252 ($P = 0.004$) {$5/10 \times 4/9 \times 3/8 \times 2/7 \times 1/6$}. Second, we calculated the probability that the set of 5 mutant samples had the 4 largest values plus a specific 1 of the 5 smallest values. This probability is again 1/252 {$4/10 \times 3/9 \times 2/8 \times 1/7 \times 5/6$}. Because there are 5 ways to include the 4 highest values plus any 1 of the 5 lowest values, we multiplied the probability by 5 to obtain the overall probability of 5/252 ($P = 0.02$). Thus, accepting $P < 0.05$ as the limit for statistical significance, we judge the increase in tract length changes in mutants to result from the mutation, rather than by chance, when either all 5 of the mutant values are higher than the 5 wild-type values, or when 4 of the mutant values are the highest 4 among the combined data for mutant and wild type.

RESULTS

Introduction of replication mutations into strains bearing repeat tracts: To create a collection of isogenic strains bearing repeat tracts that differ only in the polymerase and PCNA mutations, we first introduced the polymerase and PCNA mutations into one of our isogenic parental strains (either SSL204a or α, Scherer and Davis 1979; Dornfeld and Livingston 1991). These were then mated to derivatives of SSL204 that we had constructed previously containing either a C repeat tract of 78 repeat units or a D repeat tract of 71 repeat units embedded in a clone of *ADE2* disrupting *aro2* on chromosome VII (Maurer *et al.* 1996; Schweitzer and Livingston 1997, 1998). Haploid meiotic segregants were recovered containing the repeat tract and the replication mutation.

The nature of tract length changes in yeast: We ascertain tract stability by examining whether sibling colonies that arise from the dispersal of cells of a parental colony no longer contain a repeat tract of parental length, but one of an altered length (Figure 1). Tracts of the D orientation are more unstable than tracts in the C orientation, both in wild-type and mutant cells (Table 1). For example, in the data generated for this study of wild-type cells, we found that one tract length change occurred among 160 sibling colonies for tract C, while for tract D, we found 29 tract length changes among 158 siblings (Table 1). This difference arises because the *ADE2* clone in which we have inserted the repeat tract contains an ARS element, tract C using CAG as the lagging strand template, and tract D using CTG as the lagging strand template (Figure 1). With few exceptions, the majority of tract length changes found in wild-type and mutant cells, regardless of tract orientation, are contractions that remove five or more repeat units (Table 1). Tract expansions also occur, but they are rarer. Similarly, changes of three or fewer repeat units are rare, except in mismatch repair mutants (Schweitzer and Livingston 1997) and in the *pol30-52* mutant described in this article.

CAG tract instability in polymerase mutants: CAG tract stability was examined in yeast strains containing mutations in the major replicative polymerases, α, δ,

TABLE 1

Changes in CAG repeat tract length in replication mutants

Strain	Genotype	Tract	No. of repeat units	No. of changes among siblings from five parental colonies	Total no. of changes/ no. of siblings	No. of expansions	No. of contractions	Mean size of expansions ± SD (repeat units)	Mean size of contractions ± SD (repeat units)
CAG101	Wild-type[a]	C	78	0, 0, 0, 0, 1	1/160	1	0	8	—
CAG119	Wild-type[a]	D	71	5, 5, 6, 6, 7	29/158	0	29	—	36 ± 17
CAG158	*pol1-1**	C	78	0, 3, 3, 3, 4	13/159	2	11	6.0 ± 2.8	27 ± 14
CAG159	*pol1-1*	D	71	3, 6, 7, 12, 13	41/159	2	39	2.5 ± 2.1	33 ± 18
CAG160	*pol1-17*	C	78	0, 0, 0, 0, 0	0/160	0	0	—	—
CAG161	*pol1-17*	D	71	4, 6, 6, 7, 7	31/159	1	29	12	35 ± 17
CAG162	*pol3-14**	C	78	0, 2, 3, 5, 6	16/160	3	13	11 ± 5.3	43 ± 17
CAG163	*pol3-14**	D	71	12, 14, 19, 21, 24	90/157	1	89	7	43 ± 16
CAG163	*pol3-14* at 23°	D	71	5, 8, 9, 11, 16	49/157	1	48	8	39 ± 17
CAG163	*pol3-14*	D	30, 31	0, 0, 0, 2, 3	5/159	0	5	—	21 ± 6.5
CAG119	Wild-type[b]	D	30	0, 0, 0, 0, 2	2/157	0	2	—	23
CAG127	*rth1/rad27Δ*[b]	D	30	2, 2, 8, 10, 10	32/159	14	18	6.4 ± 2.6	13 ± 4.4
—[c]	*pol3-14 rad52Δ*	C	78	1, 1, 1, 4, 4	11/157	4	7	7.3 ± 4.9	34 ± 25
—[c]	*pol3-14 rad52Δ**	D	71	17, 20, 20, 23, 26	106/160	0	106	—	38 ± 16
CAG174	*rad52Δ*	C	78	0, 0, 0, 1, 3	4/160	0	4	—	34 ± 10
CAG175	*rad52Δ*	D	71	2, 5, 5, 8, 10	30/160	1	29	7	41 ± 13
CAG172	*pol2-18*	C	78	0, 0, 0, 0, 2	2/160	0	2	—	26 ± 25
CAG173	*pol2-18*	D	71	3, 6, 7, 8, 13	37/160	0	37	—	38 ± 18
CAG123	*pol3-01*	C	78	0, 0, 0, 0, 1	1/158	1	0	1	—
CAG121	*pol3-01*	D	71	3, 4, 6, 7, 7	27/159	0	27	—	45 ± 12
CAG170	*pol2-4*	C	78	0, 0, 0, 0, 2	2/159	0	2	—	49 ± 19
CAG171	*pol2-4*	D	71	2, 5, 6, 6, 7	26/155	0	26	—	39 ± 15
CAG164	*pol30-52**	C	78	1, 5, 8, 16, 20	50/159	11	39	6.5 ± 5.2	26 ± 20
CAG165	*pol30-52*	D	51–55	17, 20, 21, 22, 24	104/158	3	101	4.7 ± 3.8	22 ± 14
CAG166	*pol30-79**	C	78	4, 5, 10, 10, 12	41/160	5	36	18 ± 7.3	39 ± 18
CAG167	*pol30-79**	D	71	23, 24, 28, 28, 28	131/160	0	131	—	41 ± 15
CAG168	*pol30-90**	C	78	1, 2, 2, 3, 4	12/160	5	7	23 ± 17	30 ± 16
CAG169	*pol30-90**	D	71	13, 16, 17, 18, 21	85/158	0	85	—	39 ± 16

All strains were grown at 30° unless otherwise noted.

* Tract instability in these strains was found to be significantly higher ($P < 0.05$) than that in wild-type cells by a randomization test (materials and methods).

[a] The data for two of the five parental colonies were reported in a previous study (Schweitzer and Livingston 1998).

[b] Unpublished data collected for a previous study (Maurer *et al.* 1998).

[c] These strains were not maintained as a stock.

and ε. Polymerase α initiates DNA synthesis during both leading and lagging strand replication (Newlon 1996). While it is needed only once for leading strand synthesis proceeding from each origin, it is required repeatedly during lagging strand synthesis for the initiation of each Okazaki fragment. We used two different temperature-sensitive pol α mutations, *pol1-1* (Pizzagalli *et al.* 1988) and *pol1-17* (Budd and Campbell 1987). *pol1-1* is an N-terminal mutation that appears to disrupt the interaction between pol α and primase (Lucchini *et al.* 1988). The *pol1-17* mutation lies in the C-terminal region and disrupts the polymerization activity of pol α (Budd *et al.* 1989). Although both pol α mutants are phenotypically temperature sensitive, they have disparate effects on CAG tract stability. In the *pol1-1* mutant, tract C, which is oriented with CAG in the lagging strand template, is destabilized. The frequency of changes rose from 1 in 160 sibling colonies in wild-type cells to 13 among 159 sibling colonies in this mutant (Table 1). In the same mutant, for tract D, which is oriented with CTG in the lagging strand template, the difference between the mutant and wild-type values are not significant by the randomization test (see materials and methods). Thus, we conclude that the *pol1-1* mutation destabilizes the C orientation, but has little or no effect on the D orientation. Trials with the *pol1-17* mutant showed that neither tract is destabilized significantly by this mutation (Table 1).

Next, we examined mutations in polymerases δ and ε, two other polymerases that are essential for chromosomal replication in yeast (Budd and Campbell 1993). At least one of these enzymes is thought to extend Okazaki fragments during lagging strand synthesis (Newlon 1996). The pol δ mutation, *pol3-14*, causes yeast cells to arrest as doublets within 5 hr after being shifted to the nonpermissive temperature, 38° (Giot *et al.* 1995). In this mutant, both tracts C and D are destabilized at the semipermissive temperature of 30° (Table 1). For tract C, the frequency of tract length changes was 16 out of 160 sibling colonies in the mutant as compared to the single change out of 160 wild-type sibling colonies. For tract D, the frequency of tract length changes was 90 out of 157 sibling colonies in the mutant as compared to 29 changes out of 158 wild-type sibling colonies.

To further characterize the effect of the *pol3-14* mutation, the stability of tract D was also examined in this mutant at a lower temperature, 23°, and at a shorter repeat tract length. At 23°, the instability of tract D in the *pol3-14* mutant is reduced to near the wild-type frequency, which is consistent with the thermolability of the *pol3-14* gene product. Similarly, the shorter tracts of the D orientation, ∼30 repeat units, are more stable in the *pol3-14* mutant at 30°. For comparison, we have presented the results from wild-type cells and a *rth1*Δ/*rad27*Δ mutant for similar short tracts of the D orientation (Table 1). The results show that the behavior of a short tract of the D orientation in the *pol3-14* mutant is closer to its behavior in wild-type cells than to its behavior in *rth1*Δ/*rad27*Δ mutant cells.

We also tested CAG tract instability in a *pol3-14 rad52*Δ double mutant (Table 1). Previous studies examining the excision of a large hairpin noted that the effect of a temperature-sensitive *pol3* mutation was dependent on a functional *RAD52* gene (see discussion; Gordenin *et al.* 1992). However, the increased instability of CAG repeat tracts in a *pol3-14* mutant does not appear to be dependent on *RAD52* (Table 1).

We tested a mutation in pol ε, *pol2-18*, that causes cessation of cell division and DNA synthesis within 4 hr after cells are shifted to the nonpermissive temperature, 37° (Araki *et al.* 1992). We found that this mutation has no apparent effect of CAG tract instability. In addition, we examined mutations that eliminate the proofreading functions of polymerases δ and ε, *pol3-01* (Morrison *et al.* 1993), and *pol2-4* (Morrison *et al.* 1991). We found that neither proofreading mutation has an appreciable effect on CAG tract stability (Table 1).

CAG tract instability in PCNA mutants: CAG repeat tract stability was investigated in three different PCNA mutants, *pol30-52*, *pol30-79*, and *pol30-90* (Ayyagari *et al.* 1995; Eissenberg *et al.* 1997). PCNA is a homotrimeric protein that encircles DNA like a clamp, interacting with polymerases δ and ε to make them more processive when synthesizing new DNA strands (Newlon 1996). PCNA also interacts with a number of other proteins and is required for several repair processes (Kelman 1997). Both the *pol30-52* and *pol30-79* mutations destabilize tracts C and D to a greater degree than does the *pol30-90* mutation (Table 1). For tract C, the frequencies of tract instability were 50 changes out of 159 sibling colonies, 41 changes out of 160 sibling colonies, and 12 changes out of 160 sibling colonies for the *pol30-52*, *pol30-79*, and *pol30-90* mutants, respectively. The frequencies for the *pol30-52* and *pol30-79* mutants are nearly as large as the values reported previously for the *rth1*Δ/*rad27*Δ mutant using a tract of the same length (Schweitzer and Livingston 1998). In the *pol30-52* mutant, tract D was so unstable that a long repeat tract (71 repeat units) could not be recovered, so shorter tracts (51–55 repeat units) were analyzed instead and yielded a frequency of 104 changes per 158 sibling colonies, 3.5-fold larger than the wild-type value measured at a tract length of 71 repeat units. Similarly, frequencies for tract D in the *pol30-79* and *pol30-90* mutants were 131 changes out of 160 sibling colonies and 85 changes out of 158 sibling colonies, respectively. These frequencies are 3- and 4.5-fold higher than the wild-type frequency, respectively.

We also observed that there are significantly more small changes in repeat tract length in the *pol30-52* mutant than in the *pol30-79* or *pol30-90* mutants. For tract C in the *pol30-52* mutant, 12 changes out of 159 sibling colonies were of 3 repeat units or less, and for tract D in the same mutant, 10 changes out of 158 sibling colonies were of 3 repeat units or less (data not shown).

For tract C, neither the *pol30-79* nor *pol30-90* mutations induced any small changes in repeat tract length. For tract D, there were no changes of 3 repeat units or less in the *pol30-79* mutant, while 1 change out of 158 sibling colonies was 2 repeat units in the *pol30-90* mutant (data not shown). The statistical significance of the difference was calculated using Fisher's exact test by comparing the number of parental colonies in which small changes occurred between the *pol30-52* and *pol30-90* mutants. For the *pol30-52* mutant, 4 of 5 C tract parental colonies yielded siblings with small changes, and 5 out of 5 D tract parental colonies yielded small changes. In contrast, none of the C tract parental colonies from the *pol30-90* mutant yielded siblings with small changes, and only 1 of the D tract parental colonies did so. Using Fisher's exact test, $P = 0.048$ for tract C and $P = 0.0079$ for tract D. In addition, among the remaining data sets for long C and D tracts at 30°, we found only 5 other changes of 3 repeat units or less among 2860 sibling colonies. Thus, the small changes, reminiscent of the changes that occur in mismatch repair mutants (Schweitzer and Livingston 1997), are peculiar to the *pol30-52* allele.

Characterization of CAG tract length changes in polymerase mutants: We also scrutinized the data for any trends in the pattern of tract length changes in the replication mutants. As is the case in wild-type cells, tract C is more stable than tract D in the replication mutants. However, more examples of expansions were found among siblings containing C tracts than those with D tracts (Table 1). Taking the data for the long C and D tracts as a whole (and excluding the values for the small D tracts that include data for the *rth1*Δ/*rad27*Δ mutant), we found 32 expansions out of 1912 sibling colonies for tract C and only 8 out of 1903 sibling colonies for tract D. (We have not placed a statistical significance on these values because they were collected among a variety of mutants that may have significant differences among themselves.) This orientation bias is consistent with what has been found in both *Escherichia coli* (Kang *et al.* 1995a) and wild-type yeast cells (Miret *et al.* 1998).

Measurement of the sizes of tract length contractions in the wild type and mutants yielded broad distributions that averaged between 26 and 45 repeat units. Comparison of the mutant distributions to the wild-type distribution by a *t*-test with two tails revealed that the *pol3-01* distribution for tract D is significantly different, with $P < 0.05$, and that the *pol3-14* distribution for tract D (71 repeat units at 30°) approaches this significance, with $P = 0.051$. Because of their relatively low incidence, no tests were done on the tract expansions.

DISCUSSION

In this study, we tested a variety of replication mutations for an effect on CAG tract stability. Many of these mutations impacted CAG tract stability. In some cases, differences in the relative stabilities of the two orientations or in the patterns of changes were observed among the mutants. As described below, the overall pattern of destabilization by the various mutations places CAG repeats in a unique position among other simple repeats and palindromes that have been studied in yeast.

In applying this mutational analysis to the study of CAG repeats, one of the questions we were testing directly was whether the various mutations might exacerbate the instability of CAG repeat tracts. A few of the mutations we used create an absolute loss of activity, such as the exonuclease mutations of pol δ and pol ε. Other mutations in the DNA polymerase genes are temperature-sensitive, conditional mutations. Studies were carried out at a temperature semipermissive for these mutations. When a conditional mutation grown at the semipermissive temperature effects greater instability, it is an indication that the encoded polymerase plays a role in maintaining the fidelity of the repeat tracts. Failure to observe an effect has an ambiguous meaning. While it could mean that the polymerase plays no role in maintenance of repeat tract stability, it could also mean that the mutation is not severe enough to cause a noticeable change in repeat tract stability.

We note that tracts of the D orientation never undergo the same degree of destabilization in the mutant backgrounds as do tracts of the C orientation (Table 1). This is partially the result of the very high base line value we measure for tract D in wild-type cells that leaves little room for further destabilization. Presumably, this phenomenon occurs because the replication machinery in wild-type cells has little capacity to limit the rate of D tract instability, but it has greater power to limit the rate of C tract stability at the long tract lengths we employ. We also note that the fold differences in frequencies we observe between mutant and wild-type cells are never more than ∼10-fold. Most simple repeats studied in similar mutants, such as homonucleotide and dinucleotide repeats, most often yield a greater fold difference in rates between mutant and wild-type cells (Tran *et al.* 1997; Kokoska *et al.* 1998). This probably reflects the greater stability of the other repeats in wild-type cells.

CAG tract stability in pol α mutants: Temperature-sensitive mutations in pol α, the polymerase that initiates new DNA strands during replication, affected CAG tract stability in an allele-specific manner. The *pol1-1* mutation destabilizes tract C by ∼11-fold, but it does not appreciably destabilize tract D. In contrast, *pol1-17* has no measurable effect on the stability of either orientation. Other studies have examined hairpin excision in pol α mutants. The excision of an 80-bp hairpin was increased in a *pol1-1* mutant (11-fold in rate), but not in another pol α mutant, *cdc17-1* (Ruskin and Fink 1993). In the case of a Tn*5* element (1.5-kb terminal inverted repeats separated by 2.7 kb of unique sequence) embedded in the *LYS2* gene, both *pol1-1* and *pol1-17* increased the rate of excision up to 100-fold in

rate (Gordenin *et al.* 1992). Our data resemble those seen for the excision of the smaller 80-bp hairpin. As described above, the allele specificity could simply reflect the relative stability of the mutant pol α copies at the semipermissive temperature, but it might also correlate with the region in which the respective pol α mutations lie. While the *pol1-1* mutation is in the N-terminal region at amino acid 493, the *pol1-17* and *cdc17-1* mutations are in the C-terminal half of the protein at residues 1004 and 904, respectively (Lucchini *et al.* 1990; Campbell and Newlon 1991).

Although the *pol1-1* mutation affects CAG tract stability, it destabilizes only tracts of the C orientation significantly. This suggests that when CAG is the lagging strand template (tract C), this mutant pol α allows for a significantly greater degree of replication slippage that it does normally. In contrast, when CTG serves as the lagging strand template (tract D), replication slippage occurs readily in wild-type cells and is not limited by the faulty polymerase.

CAG tract stability in pol ε and pol δ mutants: A temperature-sensitive mutation in pol ε, *pol2-18*, does not affect CAG tract stability. Since we only examined one *pol2* temperature-sensitive mutant, we cannot rule out the possibility that other *pol2* mutants would impact CAG tract stability. However, our result is consistent with Tn*5* excision in yeast, which is not increased in two different *pol2* mutants (Gordenin *et al.* 1992).

A temperature-sensitive mutation in pol δ, *pol3-14*, significantly increases the instability of CAG tracts in both orientations. The degree to which the *pol3-14* mutation affects the stability of tract C is ~16-fold over wild-type. The effect on tract D at 30° is approximately a threefold increase. In the case of the 80-bp hairpin mentioned above, a temperature-sensitive mutation in pol δ (*pol3-t*) did not affect the level of hairpin excision (Ruskin and Fink 1993). The *pol3-t* mutation did increase the rate of Tn*5* excision and was dependent on *RAD52* (Gordenin *et al.* 1992). The *RAD52* dependence indicated that the mechanism of Tn*5* excision may involve a recombination-mediated step. We have found that the instability of CAG tracts in a *pol3-14* mutant does not appear to be dependent on *RAD52*, at least for the D orientation. Considering that Tn*5* is not a palindrome, comparison to our studies may not be relevant. In addition, we have used a different allele of *pol3.* Nevertheless, our studies of *rad52*Δ and *pol3-14* suggest that the large number of changes in CAG repeat tract length induced by the *pol3-14* mutation occur by pure slippage during replication.

Shorter repeat tracts of the D orientation (30–31 repeat units) are relatively stable in the *pol3-14* mutant. The stability of such small tracts contrasts with our results with shorter repeat tracts in a *rth1*Δ/*rad27*Δ mutant (Table 1). For long repeat tracts, the *rth1*Δ/*rad27*Δ and *pol3-14* mutations cause similar degrees of destabilization (Schweitzer and Livingston 1998; this study). For short repeat tracts (~30 repeat units), *rth1*Δ/*rad27*Δ induces a high level of instability, whereas *pol3-14* does not (Table 1). This difference suggests that the CAG tract destabilization observed in these two mutants may be induced by different mechanisms since the destabilization caused by *pol3-14* is much more length dependent than that caused by *rth1*Δ/*rad27*Δ. This may not be surprising because Rth1p/Rad27p, the flap endonuclease, acts at the ends of newly synthesized Okazaki fragments, while pol δ must tract along the entire length of the template strand to synthesize new DNA. In addition, a recent study examining micro- and minisatellite instability found that the *pol3-t* and *rad27*Δ mutations had different effects on the instability of these sequences (Kokoska *et al.* 1998). Both mutations destabilized all micro- and minisatellites examined, but *pol3-t* had the largest effect on tracts with longer repeat units (8 and 20 bp), while *rad27*Δ affected tracts with shorter repeat units (mono- and dinucleotides) the most.

The proofreading functions of polymerases δ and ε do not appear to play a role in controlling CAG tract instability. The *pol3-01* and *pol2-4* mutations eliminate the 3′ to 5′ exonuclease functions of pol δ and pol ε, respectively (Morrison *et al.* 1991, 1993), yet they have no effect on the instability of CAG tracts in yeast. This result is in contrast to their effects on homonucleotide runs and GT tracts. Homonucleotide runs are destabilized 40- to 300-fold in rate by *pol3-01* and somewhat less by *pol2-4* (Tran *et al.* 1997). GT tracts are destabilized 5- to 11-fold in rate by *pol3-01*, but not by *pol2-4* (Strand *et al.* 1993). These results suggest that the proofreading activities of pol δ and pol ε may prevent changes in the length of simple mono- or dinucleotide repeat tracts, but that they are unable to control the changes that occur within CAG repeat tracts. One reason for this difference could be that most of the tract length changes in CAG repeat tracts that occur in yeast do so by collapse of the template strand into a hairpin structure (Figure 4). Because the proofreading functions of the polymerases deal mostly with the nucleotides on the newly synthesized strand, they may not contribute to template strand phenomena. This would also indicate that the proofreading exonuclease activities either do not cause pausing when transversing a template of CAG repeats, or that they are not critical to passage of the polymerase through the repeat tract.

CAG tract stability in PCNA mutants: Three *pol30* mutations also destabilize CAG tracts, but they show allelic variation. The three alleles we used have varied phenotypes *in vivo* and *in vitro.* The *pol30-52* mutation is the most severe. It leads to MMS and cold sensitivity, and it has a defect in mismatch repair that is as severe as a deletion of the mismatch repair gene *mlh1* (Ayyagari *et al.* 1995; Umar *et al.* 1996). *In vitro*, the *pol30-52* protein fails to form trimers and is completely defective for DNA synthesis with both polymerases δ and ε (Ayyagari *et al.* 1995). The *pol30-79* and *pol30-90*

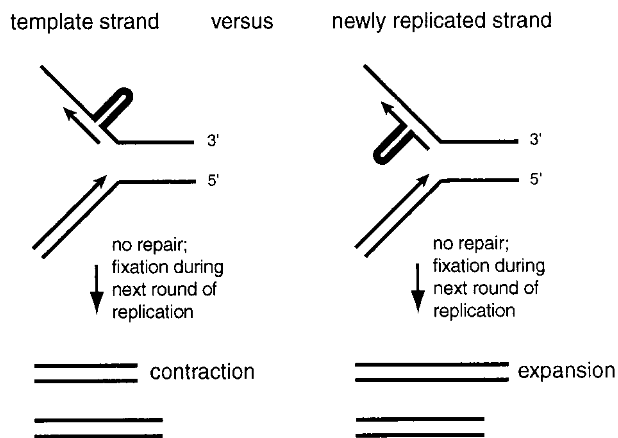

Figure 4.—Changes in CAG tract length can occur during replication. This figure illustrates replication forks opening from left to right through a repeat tract. The solid lines are the template strands, and the arrows represent newly synthesized strands. The top strand in each case is the lagging strand, and the bottom strand is the leading strand. The left portion of the diagram shows that the formation of a hairpin on the template strand within the repeat tract causes the newly synthesized strand to be shorter than the template, leading to a contraction in repeat tract length. The right portion shows that the formation of a hairpin on the newly synthesized Okazaki fragment within the repeat tract ultimately results in an expansion.

mutations are less severe, but they also have distinct phenotypes. The *pol30-79* mutation results in a target-specific mutator phenotype with minimal effects on mismatch repair, whereas *pol30-90* leads to a general mutator phenotype as well as a noticeable defect in mismatch repair (Eissenberg *et al.* 1997). *In vitro*, the *pol30-79* protein is defective in pol-δ-mediated DNA synthesis by 1000-fold, while the *pol30-90* protein is defective in pol-ε-mediated DNA synthesis, also by 1000-fold. In addition, the *pol30-90* protein is defective in its interaction with the flap endonuclease (Rth1p/Rad27p) and in its stimulation of flap endonuclease activity.

Because PCNA is involved in repair as well as replication, the interpretation of CAG tract instability in the *pol30* mutants is not straightforward. However, some of the differences in CAG tract instability among the *pol30* mutants may be correlated with the phenotypes of these mutants. Our previous work showed that small changes of one to three repeat units in tract length were recovered from mismatch repair mutants (Schweitzer and Livingston 1997). Only the *pol30-52* mutation induces a similar class of small changes in CAG repeat tract length, and these are likely to be a consequence of the severe mismatch repair deficiency observed in this mutant (Umar *et al.* 1996; Kokoska *et al.* 1999). The *pol30-90* product is also defective in mismatch repair (Eissenberg *et al.* 1997), but that phenotype was not manifested by an increase in small tract length changes. Also, we note the relatively large number of tract expansions observed in the *pol30* mutants. The highest ratio of tract expansions to tract contractions occurred in the *pol30-90* mutant for tract C (Table 1). This is the mutant PCNA product that has the greatest loss of interaction with the flap endonuclease (Rth1p/Rad27p), and previous studies have shown that *rth1Δ/rad27Δ* mutants create a high ratio of tract expansions to tract contractions in CAG repeat tracts (Freudenreich *et al.* 1998; Schweitzer and Livingston 1998). Finally, the *pol30-79* mutation (defective for *in vitro* DNA synthesis with pol δ) destabilizes CAG tracts more than the *pol30-90* mutation (defective for *in vitro* DNA synthesis with pol ε). With the caveat that the translation of the *in vitro* studies into the *in vivo* studies that we have carried out does not dictate a one-to-one correspondence, our result correlates with the observation that a pol δ mutation (*pol3-14*) affects CAG tract instability while a pol ε (*pol2-18*) mutation does not.

Contractions in replication mutants: Somewhat surprising was the consistency among the size distributions of the tract contractions among the various mutants. If the progress of the replication complex through the repeat tracts is slowed by mutations in the DNA polymerases or in PCNA, this might leave longer stretches of the template strand in a state to collapse into the hairpin structures (Figure 4). In turn, this should be evidenced by a lengthening of tract contractions. We found scant evidence for such lengthening. The distributions of the large contractions were broad, and only two examples that were close to a statistical difference were observed. These were for D tracts in the *pol3-14* and *pol3-01* mutants. Considering that these two mutants represent one mutation that greatly affects tract stability and one that does not, these results do not support the hypothesis that mutations that might slow the progress of the replication complex, as evidenced by greater tract instability, would lead to longer tract contractions. The significance of these results is unclear at this time.

Relationship between CAG repeat tracts and other simple repeats and palindromes: CAG repeat tracts occupy a unique niche among simple repeats and palindromes in yeast. While their stability is sensitive to many of the same mutations, such as the *msh2* and *pol3* mutations, which exacerbate the instability of simple repeats of mono- and dinucleotides (Strand *et al.* 1993; Kokoska *et al.* 1998), they are different because of their insensitivity to the exonuclease mutations of the replicating polymerases (Strand *et al.* 1993; Tran *et al.* 1997). Their insensitivity to the exonuclease mutations of the polymerases more than likely is the result of their apparent palindromic-like nature, as evidenced by the preponderance of tract contractions that arise from the collapse of the template strand into a hairpin (Figure 4). Also, like some palindromes, the extrusion of CAG and CTG tracts is dependent on tract orientation with respect to the direction of replication (Tran *et al.* 1995; Maurer *et al.* 1996; Freudenreich *et al.* 1997). Unlike palindromic sequences, CAG tract contractions are not

dependent on a competent recombinational repair system mediated by *RAD52* (Freudenreich *et al.* 1997; Miret *et al.* 1997). We are interested in knowing in what other ways CAG repeat tracts differ from other simple repeats and from palindromes.

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