The Impact of Lagging Strand Replication Mutations on the Stability of CAG Repeat Tracts in Yeast

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Manuscript received December 16, 1999
Accepted for publication April 26, 2000

ABSTRACT

We have examined the stability of long tracts of CAG repeats in yeast mutants defective in enzymes suspected to be involved in lagging strand replication. Alleles of DNA ligase (*cdc9-1* and *cdc9-2*) destabilize CAG tracts in the stable tract orientation, *i.e.*, when CAG serves as the lagging strand template. In this orientation nearly two-thirds of the events recorded in the *cdc9-1* mutant were tract expansions. While neither DNA ligase allele significantly increases the frequency of tract-length changes in the unstable orientation, the *cdc9-1* mutant produced a significant number of expansions in tracts of this orientation. A mutation in primase (*pri2-1*) destabilizes tracts in both the stable and the unstable orientations. Mutations in a DNA helicase/deoxyribonuclease (*dna2-1*) or in two RNase H activities (*rnh1*Δ and *rnh35*Δ) do not have a significant effect on CAG repeat tract stability. We interpret our results in terms of the steps of replication that are likely to lead to expansion and to contraction of CAG repeat tracts.

THE instability of repetitive CAG tracts was first recognized in the pedigrees of families with particular hereditary neurological disorders (Paulson and Fischbeck 1996). The disease genes in these families contain long tracts of CAG repeats encoding polyglutamine sequences in the respective proteins. Upon passage from parent to child, the length of the repeat tract often changes. Particularly intriguing are the observations that many of the tract-length changes are biased toward expansion of the CAG tract. The increase in tract length gives rise to the phenomenon of anticipation—the earlier onset and more severe presentation of the pathological conditions—often observed in human pedigrees.

In addition to the studies carried out in humans and on human cells, many studies on CAG repeat tract stability have been performed in mice, yeast, and *Escherichia coli* (Wells and Warren 1998). None of these three model organisms completely recapitulates the expansion-biased instability observed in human pedigrees. CAG repeat tracts in mice are relatively stable in comparison to their instability during human gametogenesis. In both the prokaryote *E. coli* and the simple eukaryote *Saccharomyces cerevisiae*, CAG repeat tracts are very unstable, but most of the tract-length changes are large contractions. Nevertheless, much has been learned about the causes of repeat tract instability from these organisms. In particular an appreciation for the role of replication in repeat tract stability has come from work in *E. coli* and yeast.

Our studies using a CAG repeat tract embedded in a yeast chromosome have focused on using the many mutations in the replication and repair genes to understand the molecular events leading to CAG repeat tract expansion and contraction (Schweitzer and Livingston 1997, 1998, 1999; Maurer *et al.* 1998). Already known is the effect of a mutation in the yeast flap endonuclease gene (*rth1*Δ/*rad27*Δ) that destabilizes CAG tracts and increases the frequency of tract expansions (Freudenreich *et al.* 1998; Schweitzer and Livingston 1998; Spiro *et al.* 1999). The flap endonuclease is an enzyme that appears to be needed to remove primers from Okazaki fragments during lagging strand replication. Consequently, we have examined mutations in other genes involved in lagging strand replication to understand how they contribute to CAG repeat tract stability. We have shown that mutations in DNA polymerase α, DNA polymerase δ, and PCNA (proliferating cell nuclear antigen) all destabilize CAG repeat tracts (Schweitzer and Livingston 1999). In these mutants, as well as in wild-type yeast cells, the predominant changes in tract length that occur are contractions. In this work, we have examined additional mutations in lagging strand replication: DNA ligase, DNA helicase, primase, and RNase H (Bambara *et al.* 1997; Waga and Stillman 1998). While some of the mutations destabilize CAG repeat tracts, one DNA ligase allele yields an increase in the ratio of tract expansions to tract contractions, comparable to the ratio observed in the flap endonuclease mutant.

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MATERIALS AND METHODS

Yeast strains: The parental strains used in this study are SSL204a and α (*MAT***a** or *MAT*α *his3 leu2 ura3 ade2 trp1*;

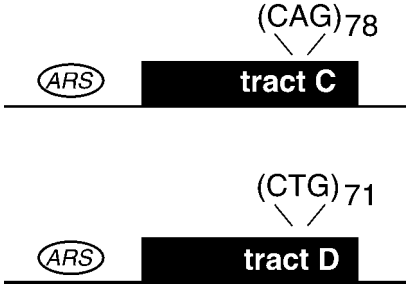

Figure 1.—Stable and unstable orientations of CAG repeat tracts embedded in a yeast chromosome. This figure is a representation of the chromosomal placement of CAG repeat tracts that were made by embedding repeat tracts in the coding region of *ADE2*. *ADE2* clones were used to disrupt the *ARO2* locus on chromosome VII (Maurer *et al.*, 1996). The tracts are oriented so that CAG or CTG are on the top strand of *ADE2* in tracts C and D, respectively. Replication proceeds from the ARS in the *ADE2* clone (Stotz and Linder 1990), making tract C with CAG as the lagging strand template the stable orientation and tract D with CTG as the lagging strand template the unstable orientation.

Dornfeld and Livingston 1991). Replication mutations and CAG repeat tracts have been placed in this background. CAG repeat tracts were previously inserted into the 3′ end of the yeast *ADE2* gene, and the *ADE2* construct was used to disrupt the *ARO2* locus on chromosome VII (Figure 1, Maurer *et al.* 1996). To place the DNA ligase alleles in our strain background with the CAG repeat tracts, we outcrossed strains 244 (*cdc9-1*) and 280 (*cdc9-2*) (Hartwell *et al.* 1973) to SSL204. We then backcrossed spores three times to SSL204 to recover strains that contained the appropriate markers and the repeat tract. The presence of the *cdc9* alleles was scored by temperature-sensitive growth. In the case of *cdc9-1*, sequencing of a portion of the gene ensured that it contained the amino acid substitutions and adjacent polymorphisms of the original *cdc9-1* isolate (Unternahrer and Hinnen 1992). The mutation(s) in *cdc9-2* has not been identified but sequencing of isolates revealed the polymorphisms present in the original background (strain A364A, Hartwell *et al.* 1973). Two or more independent spores containing the *cdc9* alleles were used for each mutant. A similar procedure was used for the DNA helicase/nuclease mutation (*dna2-1*), provided in strain 3X154-9A by Martin Budd and Judith Campbell (California Institute of Technology; Budd *et al.* 1995). The *dna2-1* mutational change has been found to be a P504S substitution (Formosa and Nittis 1999), and this was confirmed in our strain background. The primase mutation (*pri2-1*) was obtained from James Haber (Brandeis University) and substituted into SSL204 by two-step transplacement (Scherer and Davis 1979). DNA from candidates was sequenced to ensure the identity of the G152Q mutation (Francesconi *et al.* 1991). A *URA3* disruption of RNase HI (*rnh1*Δ) was obtained from Anita Hopper (Pennsylvania State University Medical Center) and used for single-step disruption of that gene in SSL204 (Rothstein 1983). To disrupt RNase HII (*rnh35*Δ) we designed PCR primers that produced a product containing 45 bases of *RNH35* on either end and the complete sequence of *URA3*. The PCR product was used for single-step disruption (Manivasakam *et al.* 1995).

Yeast culturing and repeat tract-length determination: Methods used to culture yeast and to determine CAG repeat tract length are given in greater detail in a previous publication (Schweitzer and Livingston 1999). Colonies started from individual cells were grown on YPD agar at 30°, a temperature semipermissive for growth of the temperature-sensitive strains (*cdc9*, *dna2*, and *pri2*). These parental colonies were dispersed in water and individual cells were plated to YPD agar. After growth for 3 days at 25°, whole colonies were collected and DNA was extracted. The DNA from these sibling colonies was used as a template for PCR, and radiolabeled PCR products were displayed on a sequencing gel (Figures 2 and 3). Sibling colonies that no longer possess the repeat tract length of the parent colony have undergone a change in tract length during the 20 generations of growth undergone by the parental colony. For most mutants, we analyzed six sets of 32 sibling colonies. Because the sets of colonies are independent, we use a randomization test to compare the results from sets of mutant cells to sets of wild-type cells (Schweitzer and Livingston 1999). When all six of the mutant values are higher than the six sets of wild-type values, the probability that the mutant and wild-type values are the same is 0.001. For tracts of the C orientation, this probability could be achieved with a sixfold increase in frequency, *i.e.*, if each mutant set had two changes. For tracts of the D orientation, this probability could be achieved with a 1.5-fold increase in frequency, *i.e.*, if each mutant set had eight changes. Another statistical calculation of exact probability, the Fisher's Exact Test (http://www.matforsk.no/ola/fisher.htm), was used to compare the number of sibling sets that contained tract expansions between mutant and wild-type strains.

RESULTS

Studying CAG repeat tracts in yeast: To study CAG repeat tract instability in yeast cells, we embedded repeat tracts in the yeast *ADE2* gene and used this construction to disrupt the *ARO2* gene on chromosome VII (Figure 1). Repeat tracts were cloned with either CAG (tract C) or CTG (tract D) in the *ADE2* coding strand. In yeast and in *E. coli*, tract orientation with respect to the leading and lagging strands of replication influences tract stability (Kang *et al.* 1995; Maurer *et al.* 1996; Freudenreich *et al.* 1997). Tracts are more unstable when CTG serves as the lagging strand template than when CAG serves as the lagging strand template. Because replication initiates at an ARS sequence (replication origin) that lies upstream of *ADE2* (Figure 1), this configuration makes tract D more unstable than tract C. In wild-type yeast cells most changes in tract length are tract contractions.

CAG repeat tract instability is increased in some lagging strand replication mutants: We placed CAG repeat tracts into yeast strains harboring mutations in genes encoding enzymes that are thought to play a role in lagging strand replication (Bambara *et al.* 1997; Waga and Stillman 1998). DNA ligase I is responsible for joining nascent Okazaki fragments. The temperature-sensitive DNA ligase I alleles *cdc9-1* and *cdc9-2* were from the original *cdc* collection of Leland Hartwell (Hartwell *et al.* 1973). *cdc* mutations confer temperature lethality and cell-cycle-specific arrest. The identification of *CDC9* as the gene encoding DNA ligase was made by Johnston and Nasmyth (1978). DNA primase comprises two subunits of the DNA polymerase α complex

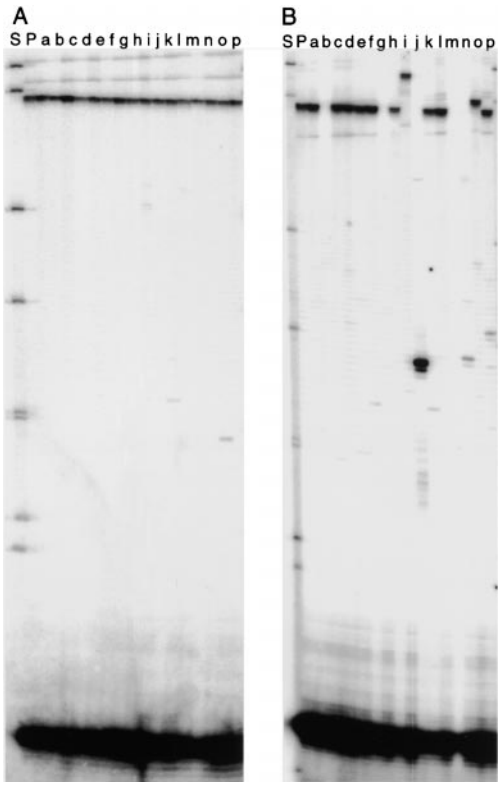

Figure 2.—Analysis of tract-length changes in mutant cells. Portions of two gels, labeled A and B, are shown. Each is half of a 32-sibling colony analysis. ^{32}P-labeled PCR products copied from template DNA purified from sibling colonies have been run in a sequencing gel. (A) Lanes a–p are from tract C in a *rnh1*Δ mutant. (B) Lanes a–p are from tract C in a *cdc9-1* mutant. These represent one mutant that does not yield significantly greater levels of tract instability than wild-type levels and one mutant that does, respectively. "P" is the PCR product of the parental colony, and the parental size repeat tract, 78 CAG repeats, was the same for both the *rnh1*Δ and *cdc9-1* 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 differing 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 through during the PCR. This is why the bands from the smallest repeat tracts and the control template are of greatest intensity. For the *rnh1*Δ mutant (A) the parental band is present in all 16 sibling colonies (a–p) and no changes are recorded. In the case of the *cdc9-1* mutant (B) the parental band is missing in lanes b, g, i, j, m, n, and o. In lanes i and o, a larger band indicative of tract expansions replaces the parental band, and in lane j, a smaller band representing a tract contraction replaces the parental band. The PCR was repeated using the template DNA for lanes b, g, m, and n and each was found to contain the parental size product. Thus, three changes, two tract expansions and one tract contraction, were recorded for this set of 16 sibling colonies.

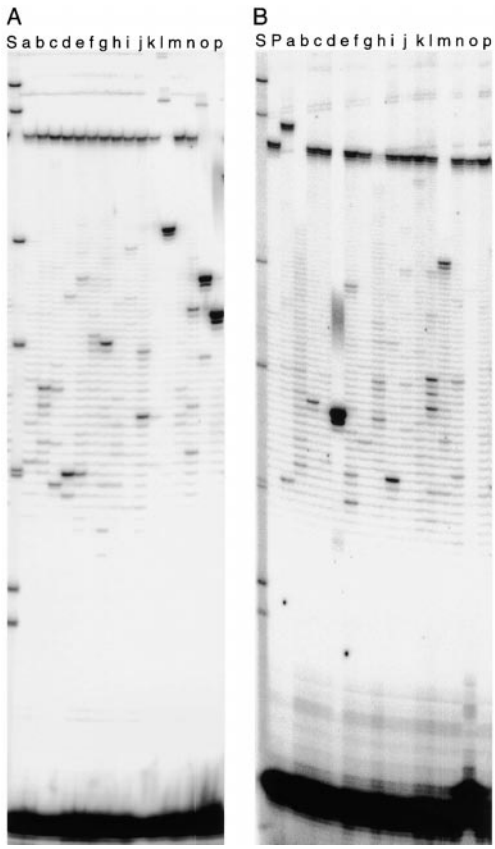

Figure 3.—Analysis of tract-length changes in mutant cells. The analyses, as described in the legend for Figure 2, have been performed for tract D from the *rnh1*Δ (A, lanes a–p) and the *cdc9-1* (B, lanes a–p) mutants. In this case the instability is similar for the two mutants, but a significant number of tract expansions have been found in the *cdc9-1* mutant. For tract D the parental size band of 71 repeat units is the same in both mutants and can be seen in lane P of B. (A) Lanes l, o, and p do not contain the parental band but instead have smaller bands indicative of tract contractions. (B) Lanes a, b, e, and m do not contain the parental size band. The replacement band in lane a is an expansion and the bands in lanes e and m are contractions. Repeated PCR on the template for lane b revealed the parental band. The parental band in lane h is present, but faint. In A and B a few lanes contain a prominent parental band along with smaller bands reinforcing some of the stutter bands. In accord with our experimental regimen, none of these lanes represent a change (Schweitzer and Livingston 1999).

and synthesizes the primers needed for discontinuous synthesis along the lagging strand. The *pri2-1* mutation is a temperature-sensitive mutation in the larger of the two primase subunits. It leads to arrest of DNA synthesis at the elevated temperature of 37° (Francesconi *et al.* 1991). The *dna2-1* mutation is also a temperature-sensitive mutation that lies outside the helicase domain of a protein that has both helicase and nuclease activities (Budd *et al.* 1995; Bae *et al.* 1998; Formosa and Nittis 1999). The conditional lethality of this mutation can be suppressed by high-level expression of the flap endonuclease (Rth1p/Rad27p) with which the helicase interacts (Budd and Campbell 1997). On the basis of this evidence, the enzyme may play a role in unraveling the RNA/DNA primer, allowing excision of the primer by

the flap endonuclease. The RNase H mutations, *rnh1*Δ and *rnh35*Δ, eliminate RNase H activities (Itaya *et al.* 1991; Frank *et al.* 1998). Neither deletion mutation appears to have a phenotype, and a double mutant has been reported to be viable (Frank *et al.* 1999). Whether either enzyme plays a role in the removal of RNA primers from nascent Okazaki fragments is unknown.

To perturb the mutant cells without killing them, we grew all strains at the semipermissive temperature of 30°. We compiled the frequencies of tract-length changes that occurred in these mutants (Figures 2 and 3) and compared them to frequencies recorded in wild-type cells (Table 1). Using a randomization test to calculate significance (Schweitzer and Livingston 1999), we judge that the two DNA ligase mutations (*cdc9-1* and *cdc9-2*) and the DNA primase mutation (*pri2-1*) significantly increase the instability of tract C at the semipermissive temperature of 30° ($P < 0.008$). The increase in the frequencies of tract-length changes for the three mutants are 12-fold, 16-fold, and 10-fold for the *cdc9-1*, *cdc9-2*, and *pri2-1* mutants, respectively. These values are within the range of values we have previously recorded for destabilization of C tracts by mutations in the replicative DNA polymerases (Schweitzer and Livingston 1999). Of the mutations tested in this study, only the *pri2-1* mutation destabilizes tract D to a significant extent ($P = 0.001$). This represents only a 2-fold difference in the frequency of events above the wild-type value. The fold difference is in keeping with the higher baseline value for the instability of D tracts in wild-type cells (Schweitzer and Livingston 1999). The *dna2-1* mutation appears to destabilize tract C but the result is not significant by the test we use. Repeat tracts are no more unstable in either of the two RNase H mutants (*rnh1*Δ or *rnh35*Δ) than they are in wild-type cells.

Tract expansions in mutant strains: We also examined the frequency with which tracts expand or contract in the mutants (Table 1). While the majority of tract-length changes in wild-type and in many replication-defective yeast cells are tract contractions, a large number of tract expansions occur in the DNA ligase mutant *cdc9-1* (Figures 2 and 3). In this mutant nearly two-thirds of the events occurring in the tract of the relatively stable orientation (tract C) are expansions (Table 1). This ratio of expansions to contractions in the DNA ligase mutant is slightly higher than what we previously recorded for the *rth1*Δ/*rad27*Δ mutant, which is deficient for the flap endonuclease that prepares Okazaki fragments for ligation (Table 1; Schweitzer and Livingston 1998). Furthermore, other replication mutants that destabilize CAG repeat tracts yield expansion frequencies that are <40% of the combined total of expansions and contractions. For example, in a previous study (Schweitzer and Livingston 1999), we found that a DNA polymerase δ mutant (*pol3-14*) destabilizes tract C and yields ~20% expansions. In the current study we found that the primase mutant (*pri2-1*) destabilizes tract C and yields ~33% expansions (Table 1). A comparison of the ratios of expansions to contractions between the *cdc9-1* and *pri2-1* mutants by a chi-square test shows that the ratios are significantly different ($P = 0.03$). (The chi-square test could be applied because no two events from any set of sibling colonies were of the same length, signifying the likely independence of all events.) Because the ratio of expansions to contractions has not been determined for tracts of the stable orientation in wild-type cells (Miret *et al.* 1998), we are unable to apply a statistical comparison with the wild type.

While we could not compare the ratio of expansions to contractions for *cdc9-1* with the wild-type ratio for tract C, we were able to make a calculation for tract D. In this case the results in Table 1 show that no expansions were recorded among 32 tract-length changes in samples from wild-type cells. In the DNA ligase mutant *cdc9-1*, eight expansions and 40 contractions were recorded from a similar number of sibling colonies. Using the Fisher's Exact Test, we judged the significance by comparing the number of sets of wild-type sibling colonies that contained an example of a tract expansion (zero sets/six sets) to the number of sets of *cdc9-1* sibling colonies that had at least one example of a tract expansion (five sets/six sets). The difference is significant ($P = 0.008$). Again, we note that the ratio of tract expansions to contractions is comparable to previous results for tract D of comparable lengths in the *rth1*Δ/*rad27*Δ mutant (Maurer *et al.* 1998; Schweitzer and Livingston 1998).

Of the other mutants that we examined, the mutant that yielded the next highest ratio of expansions to contractions was the *cdc9-2* mutant. In this mutant nearly 50% of the events occurring in tract C were expansions and ~10% of the events occurring in tract D were expansions. These results are not significantly different from the wild-type results by the Fisher's Exact Test.

We also measured the sizes of the tract expansions in the *cdc9-1* mutant (Table 2). For tract C the average expansion length is 14 ± 13 repeat units, and for tract D the average expansion length is 7.5 ± 4.7 repeat units. We previously measured the mean sizes of tract expansions in a *rth1*Δ/*rad27*Δ mutant using tracts of the same lengths to be 17 and 8.9 repeat units for the stable and unstable orientations, respectively (Table 1; Schweitzer and Livingston 1998). Thus, in both the *cdc9-1* and *rth1*Δ/*rad27*Δ mutants, expansions are longer in the stable orientation than in the unstable orientation.

Genetic interactions: The similarity in phenotype with respect to CAG tract expansions between the DNA ligase (*cdc9-1*) and flap endonuclease (*rth1*Δ/*rad27*Δ) mutations led us to explore their genetic interaction. We recovered spores from a cross of the two strains that contained both mutations. Unlike either of the parental strains, the double mutant is incapable of forming colonies at 30° and grows poorly at 25°. Examination of a C tract in a double mutant shows that the tract exhibits instability similar to the severe instability found in the

TABLE 1

Changes in CAG repeat tract length in replication mutants

Strain	Genotype	Tract	No. of repeat units	No. of changes among siblings from six 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	2/192	1	1	8	38
CAG119	Wild type[a]	D	71	3,5,5,6,6,7	32/189	0	32	—	37 ± 17
CAG126	*rth1*Δ/*rad27*Δ[a]	C	78	13,20	33/61	14	19	17 ± 10	40 ± 15
CAG128	*rth1*Δ/*rad27*Δ[a]	D	71	20,24	44/62	8	36	8.9 ± 6.5	26 ± 15
CAG213	*cdc9-1*[b]	C	78	1,4,4,5,5,6	25/187	16	9	14 ± 13	38 ± 19
CAG214	*cdc9-1*	D	71	5,6,7,9,9,12	48/190	8	40	7.5 ± 4.7	31 ± 15
CAG229	*cdc9-1* *rth1*Δ/*rad27*Δ	C	78	11,14	25/63	9	16	14 ± 11	25 ± 13
CAG215	*cdc9-2*[b]	C	78	2,3,6,6,7,9	33/190	15	18	16 ± 11	23 ± 17
CAG216	*cdc9-2*	D	71	3,4,6,7,8,11	39/192	4	35	4.2 ± 2.4	31 ± 17
CAG221	*pri2-1*[b]	C	78	2,2,2,3,4,6	19/191	6	13	14 ± 14	32 ± 15
CAG222	*pri2-1*[b]	D	71	9,10,10,12,12,15	68/188	5	63	7.4 ± 5.4	38 ± 16
CAG217	*dna2-1*	C	78	0,0,1,1,2,4	8/191	2	6	9.5 ± 3.5	32 ± 11
CAG218	*dna2-1*	D	71	5,7,8,8,9,13	50/192	0	50	—	33 ± 19
CAG219	*rnh1*Δ	C	78	0,0,0,0,0,1	1/188	0	1	—	27
CAG220	*rnh1*Δ	D	71	4,5,6,8,8,14	45/191	2	43	7.0 ± 5.7	39 ± 12
CAG226	*rnh35*Δ	C	78	0,0,0,0,1,1	2/192	0	2	—	11 ± 7
CAG227	*rnh35*Δ	D	71	2,3,6,6,7,9	33/192	0	33	—	39 ± 17
CAG230	*rnh1*Δ/*rnh35*Δ	C	68	0,0,1,2,2,3	8/184	1	7	9	23 ± 16

[a] The data for five of the six wild-type parental colonies and the *rth1*Δ/*rad27*Δ data were reported in previous studies (Schweitzer and Livingston 1998, 1999).

[b] Tract instability in these strains was found to be significantly higher ($P < 0.008$) than that in wild-type cells by a randomization test (Schweitzer and Livingston 1999).

TABLE 2

Sizes of CAG tract expansions in DNA ligase and flap endonuclease mutants

Mutation	Tract	No. of repeat units	Sizes of tract expansions (repeat units)
*rth1*Δ/*rad27*Δ[a]	C	78	1,9,10,12,12,12,13,13,14,17,17,30,32,40
*rth1*Δ/*rad27*Δ[a]	D	71	1,5,5,7,7,11,13,22
cdc9-1[b]	C	78	1,1,1,3,3,5,8,9,14,16,16,17,19,34,36,38
cdc9-1	D	71	1,3,6,6,7,9,14,14
cdc9-2	C	78	1,3,5,7,8,11,14,15,18,19,24,25,25,28,40
cdc9-2	D	71	1,4,6,6

[a] Values from Schweitzer and Livingston (1998).
[b] Duplicate values derive from different sets of sibling colonies.

*rth1*Δ/*rad27*Δ mutant (Table 1; Schweitzer and Livingston 1998). The apparent decrease in the ratio of expansions to contractions in the double mutant, in comparison to the single mutants, could reflect a disturbance of processive replication leading to tract contractions (see discussion).

We were unable to recover a *cdc9-1 dna2-1* double mutant. In this case we included a wild-type copy of *DNA2* on a *URA3*-CEN plasmid in the sporulation of the cross. Suspected double mutants could not survive on agar containing fluoroorotic acid. We did not attempt to construct the *dna2-1 rth1*Δ/*rad27*Δ double mutant because it has been shown to be inviable (Budd and Campbell 1997).

We also attempted to create a *cdc9-1 rad52*Δ double mutant, but were unsuccessful. In this case we failed to recover a spore from 40 tetrads that was both temperature sensitive (*cdc9-1*) and Leu$^+$ (*rad52*-Δ*HSLEU2*). Our failure is in accord with previous studies that had shown that many other alleles of *cdc9* (including *cdc9-2*) are incompatible with the *rad52-1* mutation (Montelone *et al.* 1981).

We were successful in creating haploid strains that are *rnh1*Δ *rth1*Δ/*rad27*Δ and *rnh35*Δ *rth1*Δ/*rad27*Δ. An examination of repeat tract changes in the two double mutants (two sets of 32 sibling colonies with tract C) showed that the degree of instability and the frequency and sizes of the expansions were no different from that previously recorded in the *rth1*Δ/*rad27*Δ single mutant (data not shown). These results suggest that the absence of either RNase H activity has no gross effect in modifying the phenotype of the *rth1*Δ/*rad27*Δ mutant with respect to CAG repeat tract instability.

We recovered a *rnh1*Δ *rnh35*Δ double mutant with a C tract from a cross of the two single mutants. Examination of tract stability in the double mutant showed more changes than were recorded for either single mutant, but the results do not support a significant difference between the double mutant and the wild type or between the double mutant and either of the single mutants (Table 1).

DISCUSSION

We have compared the stability of long CAG tracts in yeast replication mutants harboring possible defects in lagging strand replication. The comparison shows that the primase mutation *pri2-1* significantly destabilizes tracts when either CAG or CTG serves as the lagging strand template, while the two DNA ligase I mutations, *cdc9-1* and *cdc9-2*, significantly destabilize tracts only when CAG serves as the lagging strand template. Mutations in a DNA helicase gene (*dna2-1*) or in two RNase H genes (*rnh1*Δ and *rnh35*Δ) do not destabilize repeat tracts in either orientation.

Using conditional lethal mutations, such as the primase and DNA ligase I mutations, requires a statement concerning the comparability of results. Because we grew the cells at a semipermissive temperature where cells remain viable, the degree to which the altered enzymes are impaired may vary among the different mutants. This means that the magnitude of the effect of different mutations on CAG repeat stability cannot be used to state that one enzymatic event is more critical than another. In particular, the *DNA2*-encoded helicase/nuclease may play a vital role in replication, but the severity of the *dna2-1* mutation may not render the enzyme sufficiently impaired to destabilize CAG repeat tracts.

In the case of the RNase H mutations that are a complete deletion of the respective genes, we can state that neither of the encoded enzymes has a large effect on tract stability. This was expected from previous studies that show that singly or doubly the two mutations do not confer a profound phenotype (Frank *et al.* 1999).

The destabilization of CAG repeat tracts by the primase mutation (*pri2-1*) is interesting because it is in accord with our previous study on *pol1* mutations of DNA polymerase α (Schweitzer and Livingston 1999). We found that the *pol1-1* mutation destabilizes tract C while the *pol1-17* allele did not. The *pol1-1* mutation is known to alter the interaction of polymerase α with primase, while the *pol1-17* mutation alters the polymerizing action of polymerase α (Lucchini *et al.* 1988; Budd *et al.*

1989). Thus, mutations that affect priming of Okazaki fragments appear to increase CAG repeat tract instability. In the case of both the *pri2-1* mutation, presented in the current study, and the *pol1-1* mutation, presented in our previous study (Schweitzer and Livingston 1999), destabilization of C tracts leads to some tract expansions, and both mutants give rise to a few expansions among events of the D orientation. While the frequency of tract expansions in these mutants is not as great as that observed in the flap endonuclease and DNA ligase mutants, the results suggest that alteration of Okazaki fragment priming might be an additional step in replication that can give rise to CAG repeat expansions.

The temperature-sensitive *dna2-1* mutation did not lead to significant destabilization of CAG tracts. *DNA2* encodes a protein with both helicase and nuclease activities (Budd *et al.* 1995; Bae *et al.* 1998). While the role of this enzyme is not fully established, its physical association with the flap endonuclease suggests that one of its important activities is to unwind primers to provide a substrate for the flap endonuclease (Budd and Campbell 1997). We note that the *dna2-1* helicase mutation must not impair the action of the flap endonuclease significantly, or else we might have observed a phenotype approaching the severe phenotype of a *rth1*Δ/*rad27*Δ mutant. Possibly, our failure to record a significant effect of the *dna2-1* mutation results from a redundant helicase or nuclease activity that has yet to be identified.

Finally, we comment on the DNA ligase mutations *cdc9-1* and *cdc9-2*. One allele, *cdc9-1*, leads to the destabilization of tract C and a preponderance of tract expansions. In the case of tract D, we did not record greater instability in this mutant but did find a significant number of expansion events. The allele *cdc9-2* was similar in its destabilization of C tracts but did not lead to a significant increase in the number of expansions in D tracts. We presume that the variation between the two alleles results from allelic differences drawn out by our employment of a semipermissive growth temperature.

The phenotypes of the *cdc9-1* allele and the *rth1*Δ/*rad27*Δ mutation exhibit a number of similarities but also some differences. The primary similarity is that both increase the incidence of tract expansions. In addition, the mean sizes of the expansions in the two mutants are close in value and show that C tracts yield longer expansions than D tracts. The longer length of C expansions than D expansions has been hypothesized to result from the greater propensity of flaps containing CTG repeats (Okazaki fragments from C tracts) to form hairpin structures than flaps containing CAG repeats (Okazaki fragments from D tracts; Miret *et al.* 1998; Schweitzer and Livingston 1998). The interesting result from this study is that longer expansions in the tracts of the stable orientation occur in both mutants, in the presence (*cdc9-1*) and in the absence (*rth1*Δ/*rad27*Δ) of the flap endonuclease. The similarity suggests that the difference is not proscribed by the flap endonuclease.

Some differences between the DNA ligase allele and the flap endonuclease mutation are also apparent from our studies. In particular, the *rth1*Δ/*rad27*Δ mutation has a much stronger phenotype than the DNA ligase allele. In the *rth1*Δ/*rad27*Δ mutant nearly half of the sibling colonies from a cell with tract C had undergone a tract-length change and nearly two-thirds of the sibling colonies from a cell with tract D had undergone a change (Table 1; Schweitzer and Livingston 1998). This is contrasted to the milder phenotype of the *cdc9-1* mutant where <15% of C tracts and ~25% of D tracts had undergone changes (Table 1). One consequence of the greater instability in the *rth1*Δ/*rad27*Δ mutant is that the frequency of tract contractions significantly increases along with the frequency of tract expansions in this mutant. Another consequence is that the double mutant of *cdc9-1* and *rth1*Δ/*rad27*Δ reflects the phenotype of the *rth1*Δ/*rad27*Δ mutant more than it does that of the *cdc9-1* mutant. That the *cdc9-1* allele should not have as severe a phenotype as the *rth1*Δ/*rad27*Δ mutation is likely a direct consequence of the nature of the two genes and the types of mutations. The DNA ligase allele *cdc9-1* is a conditional allele, and the mutant was challenged at a semipermissive temperature unlikely to completely ablate the enzyme activity. In contrast, the *rth1*Δ/*rad27*Δ mutation results in the loss of activity, and this null mutant has numerous phenotypes indicative of problems in DNA replication and repair (Prakash *et al.* 1993; Reagan *et al.* 1995; Tishkoff *et al.* 1997).

We can speculate upon the pathway by which a failure to ligate Okazaki fragments together leads to tract expansions. One possibility is that the *cdc9-1* mutant DNA ligase impairs the flap endonuclease. At present no evidence exists that the two enzymes, the *CDC9*-encoded DNA ligase and the flap endonuclease, bind to each other, but both are known to interact with PCNA in a similar region (Levin *et al.* 1997; Jonsson *et al.* 1998). Possibly, alteration of this indirect interaction could be strong enough to impair the flap endonuclease, leading to tract expansions. We favor a more direct explanation based on the diminished capacities of the DNA ligase and the flap endonuclease. In the *cdc9-1* mutant the ligase is only partially active because of its mutational alteration, and the activity of the flap endonuclease is susceptible to inhibition by a flap containing CAG repeats (Gordenin *et al.* 1997; Kunkel *et al.* 1997; Spiro *et al.* 1999). We envision that an initial failure to ligate results in the recreation of a flap of DNA at the 5′ end of the penultimate Okazaki fragment (Figure 4). This flap might be created either by the action of a DNA helicase such as Dna2p or by strand displacement synthesis (Masamune and Richardson 1971) through the action of DNA polymerase δ. The flap then folds upon itself creating a loop that inhibits the flap endonu-

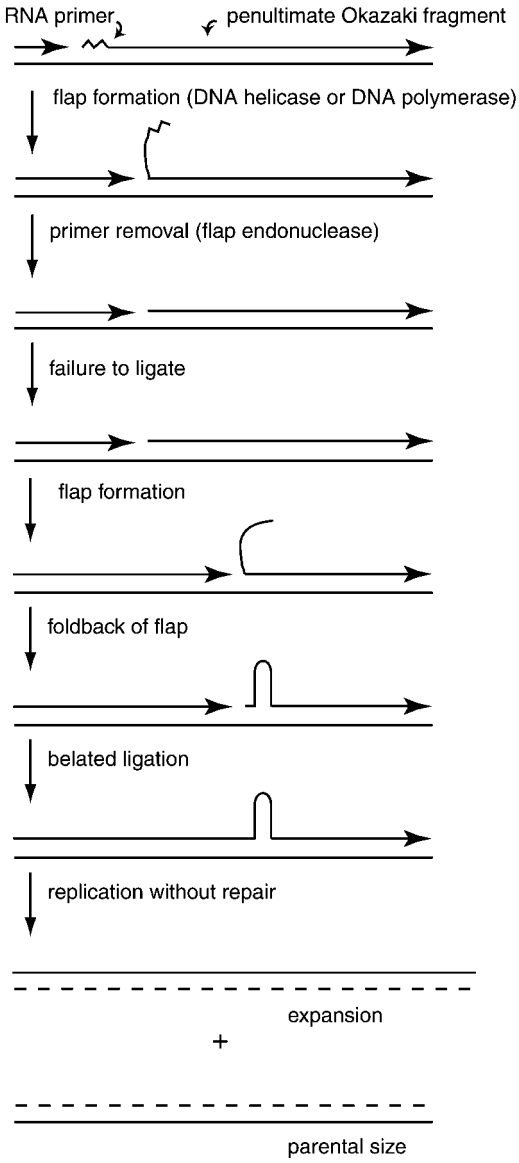

Figure 4.—A potential mechanism for tract expansions in the DNA ligase mutant *cdc9-1*. This diagram shows that if ligation fails after the removal of the flap of nucleotides containing the Okazaki fragment primer, a second round of flap formation ensues. When the flap contains a CAG repeat tract, the tract can fold back into a hairpin and assimilate into the newly made strand. Belated ligation followed by a subsequent round of replication produces a tract expansion.

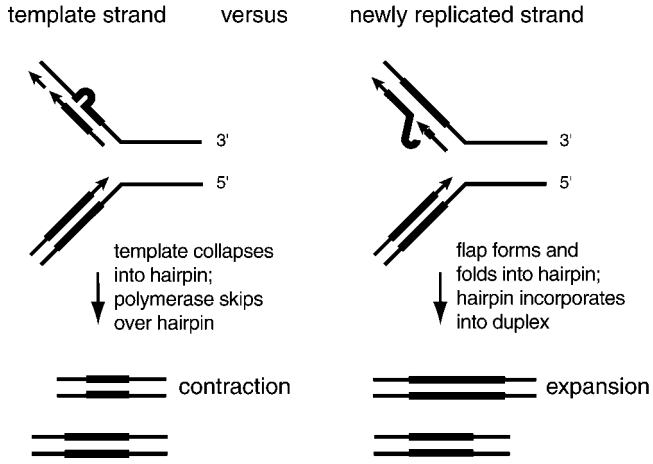

Figure 5.—Contraction and expansion of CAG repeat tracts. The model depicts an opening replication fork where the repeat tracts are represented by the heavier line. Contractions likely occur by collapse of the lagging strand template into a hairpin structure followed by polymerization that skips over the hairpin. When a subsequent round of replication occurs, copying of the newly synthesized strand leads to a shortened repeat tract. Most contractions occur distal to the opening fork in the region of the template that would be expected to remain single stranded for a longer time than template sequences closer to the opening fork (Maurer *et al.* 1998). In contrast, most expansions occur at the end of the tract closer to the opening fork (Maurer *et al.* 1998; Rolfsmeier and Lahue 2000). Flaps of DNA containing CAG repeats that fold back into hairpins resist excision by the flap endonuclease (Spiro *et al.* 1999). If polymerization occurs to complement the template during the formation of the flap, then incorporation of the flap into the duplex creates a loop of extra DNA. The loop on the newly made strand is then fixed as an expansion in the next round of replication.

clease (Spiro *et al.* 1999). Assimilation of the loop into the growing chain and belated ligation would lead to tract expansion.

Our studies on CAG repeat tracts in replication mutants indicate that expansion and contraction of CAG repeat tracts occur by different mechanisms (Figure 5). In wild-type yeast cells most events are contractions of many repeat units. These events likely result from the collapse of the lagging strand template into a hairpin structure. A failure to processively polymerize along the template leaves the template as a single strand of DNA capable of hairpin formation (Maurer *et al.* 1996, 1998; Schweitzer and Livingston 1999). This scenario explains why mutations, such as a DNA polymerase δ mutation and some PCNA mutations, lead to an increase in the frequency of tract contractions (Schweitzer and Livingston 1999). In contrast, tract expansions probably occur by a pathway that involves excess synthesis of DNA along the newly synthesized lagging strand. This conclusion is based on the result that CAG tract expansions can be increased by mutations in the *RTH1*/*RAD27* (Freudenreich *et al.* 1998; Schweitzer and Livingston 1998; Spiro *et al.* 1999) and *CDC9* genes. Mutations in both genes have the potential to lead to a flap of nucleotides at the 5′ end of an Okazaki fragment. When the flap contains CAG repeats, it can fold into a hairpin and be incorporated into the newly synthesized strand as a loop, leading to tract expansions. We have also shown that *rth1*Δ/*rad27*Δ-induced tract expansions are more likely to occur close to the opening fork, while tract contractions in wild-type cells are polarized to the distal end of the opening fork (Maurer *et al.* 1998). This again suggests that the pathways for expansion and contraction are different. In conclusion, we view the

phenotypic similarities between the DNA ligase and flap endonuclease mutations to be the genetic demonstration that perturbations in the final steps of Okazaki fragment maturation are the most likely steps in replication that cause CAG repeat tract expansions.

We appreciate Jill Schweitzer's help and insight. We also thank those who sent us strains and plasmids: Martin Budd, Judith Campbell, James Haber, and Anita Hopper.

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Communicating editor: M. Lichten