DNA Polymerase III Proofreading Mutants Enhance the Expansion and Deletion of Triplet Repeat Sequences in Escherichia coli*

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The influence of mutations in the 3′ to 5′ exonucleolytic proofreading ε-subunit of Escherichia coli DNA polymerase III on the genetic instabilities of the CGG-CCG and the CTG-CAG repeats that cause human hereditary neurological diseases was investigated. The dnaQ49ts and the mutD5 mutations destabilize the CGG-CCG repeats. The distributions of the deletion products indicate that slipped structures containing a small number of repeats in the loop mediate the deletion process. The CTG-CAG repeats were destabilized by the dnaQ49ts mutation by a process mediated by long hairpin loop structures. The mutD5 mutator strain stabilized the (CTG-CAG)_{175} tract, which contained two interruptions. Since the mutD5 mutator strain has a saturated mismatch repair system, the stabilization is probably an indirect effect of the nonfunctional mismatch repair system in these strains. Shorter uninterrupted tracts expand readily in the mutD5 strain, presumably due to the greater stability of long CTG-CAG tracts (>100 repeats) in this strain. When parallel studies were conducted in minimal medium, where the mutD5 strain is defective in exonucleolytic proofreading but has a functional MMR system, both CTG-CAG and CGG-CCG repeats were destabilized, showing that the proofreading activity is essential for maintaining the integrity of TRS tracts. Thus, we conclude that the expansion and deletion of triplet repeats are enhanced by mutations that reduce the fidelity of replication.

The expansion of triplet repeat sequences (TRS)\(^1\) such as CGG-CCG, CTG-CAG, and GAA/TTG is the causative mutation in a number of human hereditary neurodegenerative diseases including fragile-X syndrome, Huntington’s disease, myotonic dystrophy, and Friedreich’s ataxia (1). The genetic instabilities\(^2\) of these sequences were attributed to their propensities to adopt unusual DNA structures during the replication, transcription, and repair processes (1–3).

Experiments with genetically tractable Escherichia coli and Saccharomyces cerevisiae revealed several biochemical and genetic factors that influence the stabilities of TRS (3–5). The orientation of the repeats relative to the unidirectional ColE1 origin (6–8) and the single-stranded f1 origin (9) of replication are important factors. Other determinants including genetic background (3), host cell growth phase (10), transcription (11), methyl-directed mismatch repair (MMR) (12–14), expression of single stranded DNA binding protein (15), nucleotide excision repair (16), and the presence or absence of the Okazaki fragment flap processing endonuclease (17–20) also affect the TRS stability.

Short single-stranded oligonucleotides composed of TRS can adopt secondary structures like hairpin loops and tetraplexes in vitro (21–23). These conformations were shown to arrest DNA synthesis in vitro (24–26) and in vivo (27). The formation of secondary structures by the TRS on the newly synthesized lagging strand and the template of the lagging strand mediates the expansions and deletions, respectively, of these tracts in E. coli (6). Recently, we showed that expansions and deletions of the CGG-CCG, CTG-CAG, and GAA/TTG repeats can also be mediated by hairpin formation during continuous leading strand synthesis in vivo (9). Repair-resistant hairpin structures were proposed to be formed in vivo by TRS in yeast (28).

The fidelity of DNA replication in E. coli is \(10^{-10}\) errors/replotted base (29, 30). This high fidelity of base incorporation is achieved in three steps, namely base selection by the DNA polymerase, the 3′ to 5′ exonucleolytic proofreading of the inserted bases, and postreplicative MMR. The contribution of proofreading to the overall fidelity is \(10^{-3}\) (30). The proofreading function in E. coli is performed by the 3′ to 5′ exonucleolytic ε-subunit of DNA polymerase III, which is encoded by the dnaQ gene. Several alleles of the dnaQ gene including dnaQ49ts and mutD5 have been identified and studied (30–37). Strains containing the dnaQ49ts mutation are strong mutators. The dnaQ49ts is a temperature-sensitive mutation that affects the physical interaction between the ε-subunit and the polymerizing α-subunit of DNA polymerase III, thereby destabilizing the replication fork (31, 36, 37). In addition, dnaQ49ts strains have a defective exonucleolytic proofreading activity (38). On the other hand, mutD5 impairs the exonuclease activity of the ε-subunit, also rendering it a strong mutator (32, 34). Previous studies (39, 40) showed that the dnaQ49ts and the mutD5 mutations enhance deletions between tandemly repeated DNA sequences in vivo in E. coli. Therefore, these mutants were used to analyze the effect of DNA polymerase III proofreading on TRS instabilities in vivo.

Herein, we show that the instabilities of the long CGG-CCG and the CTG-CAG tracts are significantly enhanced by mutations that inactivate the proofreading system in E. coli. Substantial deletions of the TRS tracts are found on temperature inactivation of the proofreading exonuclease in the dnaQ49ts
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strains. Frequent expansions of the CTG-CAG repeats are observed in strains containing the mutD5 mutation. Thus, the functional proofreading exonuclease in E. coli probably removes slipped structures in the TRS tracts during replication, thereby significantly reducing the frequency of deletions and expansions.

EXPERIMENTAL PROCEDURES

Plasmids Containing TRS—Fig. 1 lists the TRS-containing plasmids used in the study. pRW3320 (7) and pRW4006 (16) contain CGG-CCG repeats in orientation I in pUC18NovI. CTG-CCG repeat-containing plasmids were prepared as follows. Tracts containing 17, 98, and 175 CTG-CCG repeats were isolated by digesting pRW3244, pRW3246, and pRW3248 (6, 10), respectively, with NovI (New England Biolabs, Inc.). The inserts were ligated into the polylinker of the gene targeting vector pG3100 (41) that had been digested with NovI. Clones containing the CTG-CCG repeats in orientations I and II were obtained (Fig. 1) and characterized by restriction mapping and sequencing.

Strains Used—The bacterial strains used in this study were E. coli CD4 (Hfr, metD88, proA3, Δlacz l-Y6, tex-76, λ- relA, malA368 (λ), metB1) (33), KA796 (ara, thi, ΔproE) (33), NR9807 (as CD4 but dnaQ49, zae502::Tn10), and NR9458 (as KA796 but mutD5, zaf13::Tn10) (35), which were kind gifts of Dr. Rool M. Schaaper (NIEHS, National Institutes of Health, Research Triangle Park, NC).

Propagation of Triplet Repeat-containing Plasmids in E. coli—Plasmids containing undeleted TRS tracts were prepared as described previously (10) and transformed into the appropriate E. coli strain, which had been rendered competent. The transformation mixture was inoculated into 10-ml LB tubes containing 100 μg/ml ampicillin at a density of 10^3 transformed cells/ml. The cultures were grown at 25 °C, 30 °C, and 37 °C as appropriate with shaking at 250 rpm. At late log phase (OD₆⁶₀ ~ 1.0 units), an aliquot of the culture was inoculated into a fresh tube containing 10 ml of LB (with ampicillin as before) at a final dilution of 10⁻². Due to the variable rates of cell growth, the time periods required to achieve late log phase varied between the temperatures. The original culture was harvested, and the plasmid DNA was isolated by the standard alkaline lysis method (42). Thus, the cells were propagated in log phase by repeated recultivation (shown in Figs. 2–5 as Number of recultivations).

Growth of Triplet Repeat-containing Plasmids in E. coli in Minimal Medium—Minimal medium (MM) containing 1× Vogel-Bonner salts (43) was prepared by dissolving 0.2 g of MgSO₄·7H₂O, 1.83 g of citric acid (anhydrous), 10 g of K₂HPO₄ (anhydrous) and 3.5 g Na₂HPO₄·4H₂O in 1 liter of double distilled H₂O. After sterilization, the medium was supplemented with 1% dextrose, 10 μg/ml thiamine, and 100 μg/ml proline. pRW3320 and pRW3320 (Fig. 1) that contained undeleted TRS tracts were transformed into E. coli KA796 and NR9458 competent cells and inoculated into 10 ml of MM containing 100 μg/ml ampicillin at a density of 10⁶ transformed cells/ml. The cultures were propagated in MM by repeated recultivation and harvested as described above.

Polycrylamide Gel Analysis of Triplet Repeat Instabilities—The instabilities of the triplet repeat tracts were determined by measuring the relative amount of full-length triplet repeat insert in the plasmids after growth in the respective E. coli strains at the appropriate temperatures. The plasmids were digested with NotI to excise the triplet repeat containing insert, labeled with [α-³²P]dATP by end-filling with the Klenow fragment of E. coli DNA polymerase I (U.S. Biochemical Corp.). The labeled DNA was separated on 5% polyacrylamide gels. The gels were dried and exposed to a phosphorescence-sensitive screen, which was scanned and quantitated using a Molecular Dynamics PhosphorImager as described previously (9).

RESULTS

Temperature Inactivation of the dnaQ Gene Product Destabilizes the (CGG-CCG)₉₃ Tract—CGG-CCG and CTG-CCG repeats expand and delete due to the formation of secondary structures (hairpin loops) on the leading (9) and lagging strands (6, 7, 44) during DNA replication in E. coli. Postreplication repair processes like MMR and nucleotide excision repair did not affect the CGG-CCG repeat instabilities but strongly influenced the CTG-CCG repeats (12–14, 16, 45). Repetitive DNA sequences including tandem repeats, homopolymeric tracts and dinucleotide repeats are destabilized to different degrees by proofreading defects in E. coli (39, 40, 45, 46) and S. cerevisiae (47–49). In vitro primer extension and single-stranded gap filling assays showed that proofreading-deficient DNA polymerases generated fewer expansions and deletions than proofreading-proficient enzymes (50, 51). However, no information exists regarding the in vivo involvement of 3‘ to 5’ exonucleolytic proofreading on the instabilities of triplet repeat tracts.

The in vivo recultivation assay (10) was previously used to determine the influence of various cis- and trans-acting factors on TRS instabilities (6, 9, 11, 12, 15, 16). Therefore, this assay was used to investigate the role of DNA polymerase III proofreading on the instabilities of the CGG-CCG repeats. The dnaQ₄₉ is a temperature-sensitive mutation in the exonuclease proofreading subunit of DNA polymerase III that results in a decrease in polymerase proofreading with an increase in temperature (31, 36, 37). The mutagenicity of the dnaQ₄₉ strain is low at 25 °C, intermediate at 30 °C, and high at 37 °C due to temperature inactivation of the proofreading apparatus. At the nonpermissive temperatures, the dnaQ₄₉ results in the destabilization of the physical interactions between the exonuclease subunit and the polymerizing α-subunit of DNA polymerase III (36, 52, 53). Therefore, recultivation assays of CGG-CCG repeat-containing plasmids were carried out in the wild type and mutant strains at 25, 30, and 37 °C.

pRW3320 (Fig. 1), which contains (CGG-CCG)₉₃ cloned into the polylinker of pUC18NovI in orientation I was propagated by repeated recultivation in log phase in E. coli strains NR9807 (dnaQ₄₉) and CD4 (wild type) at 25, 30, and 37 °C as described under “Experimental Procedures.” After each recultivation, the cultures were harvested, and the plasmid DNAs were isolated and digested with NotI to excise the triplet repeat tract. The digested DNA was labeled and electrophoresed through 5% polyacrylamide gels. Fig. 2A shows the gel analyses of the CGG-CCG repeat tracts excised from pRW3320 grown in NR9807 (dnaQ₄₉) and CD4 (wild type) at the three temperatures. Quantitation of the deletion products (Fig. 2B) revealed a substantial temperature-dependent destabilization of the CGG-CCG repeat tract in the dnaQ₄₉ strain. Whereas at 25 °C the full-length TRS tract constituted 75, 45, and 22% after the first, second, and third recultivations, respectively, less than 5% of the full-length tract remained after three recultivations at 30 and 37 °C. In contrast, the CGG-CCG repeats maintained in the wild type strain showed similar extents of instabilities at all the three temperatures, and ~50% of the full-length tract remained even after three recultivations. Even at 25 °C, the CGG-CCG repeat tracts are more unstable in the dnaQ₄₉ than in the wild type strain. This is consistent with the observations of Fijalkowska et al. (53) that the dnaQ₄₉ is a strong mutator at 25 °C.

These experiments clearly show that the stability of the (CGG-CCG)₉₃ tract is inversely proportional to the temperature in the dnaQ₄₉ strain. Since the instabilities of the repeat tract in the wild type strain are similar at 25, 30, and 37 °C, the possibility of a direct effect of temperature on the instabilities can be conclusively ruled out. Thus, the destabilization of the (CGG-CCG)₉₃ tract in pRW3320 in E. coli NR9807 (dnaQ₄₉) is due to the impairment of DNA polymerase III proofreading apparatus at the elevated temperatures.

The CGG-CCG Repeats Are Unstable in a Length-dependent Manner in the dnaQ₄₉ Strain—The instability of the CGG-CCG repeat tract depends on its length in fragile-X syndrome patients (54). Length-dependent instabilities of plasmid-borne CGG-CCG repeat tracts were also shown previously in E. coli (7, 9). Therefore, we investigated the effect of proofreading deficiency on short CGG-CCG repeat tracts. The plasmid...
pRW4006, which contains a (CGG-CCG)$_{32}$ tract in the pUC19NotI polynucleotide, was propagated in the dnaQ49 strain and wild type strains at 25, 30, and 37 °C. Polyacrylamide gel analysis of TRS-containing restriction fragments showed that the (CGG-CCG)$_{32}$ tract was completely stable (>95% full-length) even after three recultivations in both strains at all three temperatures (data not shown). Thus, the deleterious effect of the impaired proofreading exonuclease on CGG-CCG repeats depends on the length of the repeat tract. This observation is in agreement with the well-established paradigm of the length-dependent biological properties of TRS (3).

Slippage Mediates the Instabilities of the CGG-CCG Repeats—We proposed (9) that the TRS instabilities in the filamentous phagemid replication system were mediated by hairpin loops of discrete sizes based on the analysis of deletion and expansion products. In order to determine the intermediate steps of the deletion process in the absence of proofreading, the lengths and relative distributions of the deletion products of the CGG-CCG repeat tracts grown in the dnaQ49 strain were analyzed. The gels from the recultivation assay of pRW3320 in NR9807 (dnaQ49) at 25, 30, and 37 °C shown in Fig. 2A were quantitatively traced to reveal peaks corresponding to deletion products of specific sizes. As the temperature increased, a greater number of discrete deletion products were found. The length of each of the deletion products from cells grown at 30 °C was determined and plotted against the distance migrated (Fig. 2C). Fifty-five different deletion products of lengths ranging from 78 to 8 repeats were identified. The products were distributed almost throughout the entire range of possible deletion products. A cluster of deletion products of sizes ranging between 55 and 8 repeats was found whose members were separated by a single triplet repeat. The multiplicity of deletion products observed suggests that the CGG-CCG repeat instabilities are mediated by slipped structures that contain one or a small number of repeats, in agreement with prior findings (14).

**Length-dependent Instabilities of the CGG-CCG Repeats Are Enhanced by the mutD5 Mutation**—The mutD5 is a mutation in the dnaQ gene that impairs the exonucleolytic activity of the ε-subunit of DNA polymerase III, rendering it a strong mutator (32, 34, 55, 56). The mutD5 is dominant to the dnaQ strain (wild type) allele (52). We investigated the effect of the mutD5 mutator on the stability of the CGG-CCG repeats. pRW3320 (Fig. 1) was propagated for three recultivations in E. coli strains NR9458 (mutD5) and KA796 (wild type) at 37 °C. The plasmids were isolated after each recultivation, and the triplet repeat tracts were excised by NotI restriction digestion, labeled, and analyzed on polyacrylamide gels (Fig. 3A). The extents of the instabilities were quantitated and plotted against the number of recultivations (Fig. 3B). The CGG-CCG repeats are clearly more unstable in the mutD5 than in the wild type strain. Whereas after three recultivations, ∼80% of the full-length TRS remained in the wild type strain, only 40% of the (CGG-CCG)$_{32}$ tract was left in the mutD5 strain. Also, pRW4006 containing a (CGG-CCG)$_{32}$ tract was propagated and found to be completely stable in the wild type and mutD5 strains (data not shown). After three recultivations, >95% of the full-length TRS remained intact in both strains (Fig. 3B).

Thus, the impairment of the ε-subunit of DNA polymerase III results in the destabilization of CGG-CCG repeat tracts in a length-dependent manner.

**Long CTG-CAG Repeat Tracts Are Destabilized by the Temperature Inactivation of the dnaQ Gene**—The instabilities of the CTG-CAG repeats have been shown to be influenced by repair systems (i.e. MMR and nucleotide excision repair) as well as their orientations relative to the ColE1 origin and the single-stranded phage Φ origin (6, 9, 12–14, 16). We studied the behavior of tracts containing (CTG-CAG)$_{25}$ in orientations I and II in strains containing a defective exonuclease proofreading system. pRW3506 and pRW3505 (Fig. 1) were constructed by subcloning a NotI restriction fragment containing (CTG-CAG)$_{25}$ from pRW3248 into the 12-kilobase pair gene-targeting vector pGS100 in orientation I and II, respectively. In addition, tracts containing (CTG-CAG)$_{25}$ and (CTG-CAG)$_{17}$ were cloned also into pGS100 in both orientations (Fig. 1).
Recultivation assays in E. coli HB101 and SURE cells of pGS100-based plasmids containing long TRS tracts revealed that the triplet repeats were significantly more stable in these constructs than in pUC19-based plasmids, presumably due to the size of the plasmid and the distance from the origin of replication (~1.2 kilobase pairs). Therefore, these plasmids were used to study the instabilities of the CTG-CAG repeat tracts in proofreading-deficient E. coli strains.

pRW3505 and pRW3506 were transformed into E. coli strain NR9807 (dnaQ49) and maintained in log phase at 25, 30, and 37 °C for three successive recultivations. The plasmids were isolated after each recultivation, and the TRS instabilities were analyzed by the gel analyses of the labeled NotI restriction fragments (Fig. 4A). Also, recultivation assays were performed with pRW3505 and pRW3506 in the isogenic wild type strain CD4 at 25, 30, and 37 °C, and the TRS instabilities were analyzed. Quantitative analyses of these gels showed that the (CTG-CAG)$_{175}$ tract in orientation II in pRW3505 is more stable in the wild type strain than in the dnaQ49 strain (Fig. 4B). Due to the inherently high instability of the (CTG-CAG)$_{175}$ tract in orientation II, the destabilization by temperature observed in the dnaQ49 strain was marginal. However, in the wild type strain there was a clear destabilizing effect of temperature on the (CTG-CAG)$_{175}$ tract (Fig. 4B). In orientation I, the (CTG-CAG)$_{175}$ tract in pRW3506 was not significantly destabilized by temperature in the wild type strain (Fig. 4C). In contrast, the stability of the tract in the dnaQ49 strain was substantially reduced by increasing the temperature from 25 to 30 °C. Further increase to 37 °C did not have a significant effect on the instabilities.

These data show that increase in temperature has a deleterious effect on the stability of the (CTG-CAG)$_{175}$ Repeat tract. The effect of temperature is more pronounced in the dnaQ49 strain than in the wild type strain. Thus, the temperature-dependent

in orientation I was propagated in log phase in NR9807 (dnaQ49) and in CD4 (wild type) at 25, 30, and 37 °C. Labeled NotI restriction fragments containing the triplet repeat tracts were separated on 5% polyacrylamide gels, which were dried and exposed to x-ray film and to a PhosphorImager screen for quantitation.

Lanes 1–3 contain DNA isolated from cultures after 1–3 recultivations, respectively. The arrow indicates the band that contains the full-length triplet repeat. The deletion products migrate in the region encompassed by the full-length TRS. The extents of the instabilities of the (CGG-CCG)$_n$ tracts after the third recultivation of growth in the dnaQ49 strain at 25 °C shown in Fig. 2A was

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Plotted on the x axis against the migration distance (in pixels) on the y axis.

A K. R. Iyer and R. D. Wells, unpublished observations.
instability of the replication complex at 30 and 37 °C in the mutant strain is responsible for the higher instability of CTG-CAG repeats. The instability of the CTG-CAG repeats in the wild type strain is exacerbated by temperature preferentially in orientation II. Our data are consistent with previous studies (10, 20), which showed that at 37 °C, CTG-CAG tracts are studied also in the E. coli strains NR9845 (mutD5) and KA796 (wild type). Fig. 5A shows the gel analyses of labeled restriction fragments containing TRS tracts from pRW3505 and pRW3506 grown in the mutD5 and the wild type strains at 37 °C. As revealed by the quantitation (Fig. 5B), the (CTG-CAG)₁₇₅ Tracts in orientations I and II are stabilized by the mutD5 mutator. The stabilization of the tract is greater in orientation I in which ~60% of the full-length tract was left in the mutD5 strain after three recultivations. In contrast, only ~5% of the full-length tract was intact after three recultivations in the wild type strain. In orientation II, the tracts were substantially more unstable in both strains. As little as 10% of the full-length tract remained intact after the first recultivation in the wild type strain. In contrast, ~45% of the (CTG-CAG)₁₇₅ tract was undetected at the same stage of growth in the mutD5 strain. However, due to the inherently greater instability of TRS tracts in orientation II (6), the tracts were almost completely deleted by the second recultivation (~10% undetected).

In order to test the influence of TRS lengths on their instabilities in the mutD5 and the wild type strains, we propagated pRW3501 and pRW3502 (Fig. 1) in the wild type and mutD5 strains. The (CTG-CAG)₁₇₅ tract in both plasmids was found to be completely stable (>95% intact full-length) in the wild type and the mutD5 strains (data not shown).

Thus, the CTG-CAG repeats are stabilized in a length-dependent manner by the mutD5 mutator. Previous studies showed that the mutD5 mutator strain suffers from the saturation of the MMR system and its consequent inactivation (32, 34). Also, a functional MMR system was shown to destabilize long CTG-CAG tracts in vivo (12). Our observation that the mutD5 mutator has a stabilizing effect on the (CTG-CAG)₁₇₅ tracts is consistent with these studies.

(CTG-CAG)₆₀ Readily Expands in the mutD5 Strain—Previous (12–14) and ongoing studies⁴ show that whereas long (CTG-CAG)₁₇₅ tracts containing two interruptions are destabilized by a functional MMR system, uninterrupted intermediate length tracts containing 64 repeats are stabilized. The differential effect of mismatch repair on the (CTG-CAG)₁₇₅ and the (CTG-CAG)₆₀ repeat tracts may be due to their different lengths. Alternately, the presence or absence of interruptions may account for the differences in the instabilities. Therefore, we investigated the effect of the mutD5 mutator on the stability

⁴ P. Parniewski, A. Jaworski, R. D. Wells, and R. P. Bowater, manuscript in preparation.
Fig. 4. Instabilities of (CTG\textsubscript{z}CAG)\textsubscript{175} in NR9807 (dnaQ\textsuperscript{49}) and CD4 (wild type) strains. A, plasmids pRW3505 and pRW3506, which contain the (CTG\textsubscript{z}CAG)\textsubscript{175} tract in orientation II and I, respectively, were propagated in the dnaQ\textsuperscript{49} strain at 25, 30, and 37 °C and analyzed as described in the legend to Fig. 2. All (CTG-CAG)\textsubscript{n}-containing NotI restriction fragments also carry 129 base pairs of nonrepetitive flanking sequence.

B, the instabilities of the (CTG-CAG)\textsubscript{175} tract from pRW3505 cultivated in the dnaQ\textsuperscript{49} strain at 25 (○), 30 (■), and 37 °C (▲) and in the isogenic wild type strain at 25 (○), 30 (■), and 37 °C (▲) were quantitated as the legend to Fig. 2B from three independent experiments. The data treatment is also as in the legend to Fig. 2B. C shows a similar analysis of the instabilities of the (CTG-CAG)\textsubscript{175} tract from pRW3506 grown in the dnaQ\textsuperscript{49} strain at 25 °C (○), 30 °C (■), and 37 °C (▲) and the wild type strain at 25 °C (○), 30 °C (■), and 37 °C (▲). The reasons for the apparent difference in the shape of the curves for pRW3506 between 30 and 37 °C are not clear but may be due to the signal to noise ratios on the gels.
of an uninterrupted intermediate length (CTG-CAG)$_{98}$ tract. pRW3503 and pRW3504 contain (CTG-CAG)$_{98}$ cloned into the pGS100 polylinker in orientations II and I, respectively (Fig. 1). E. coli NR9845 (mutD5) was transformed with pRW3503 or pRW3504 and grown at 37 °C in log phase for three recultivations. The plasmids were isolated, and the triplet repeat insert was excised with NotI, labeled, and separated on polyacrylamide gels (Fig. 6). Several expansion products of the (CTG-CAG)$_{98}$ tract were identified (Table I), some of which are indicated in Fig. 6 by the white arrows. The expansion products were observed in orientation I as well as orientation II. In contrast, gel analysis of the triplet repeat inserts from pRW3503 and pRW3504 grown in E. coli KA796 (wild type) at 37 °C for three recultivations revealed very few expansion products (Table I). Whereas the two expansion products from the wild type strain each constituted <5% of the total TRS, each of the individual expansion products from the mutD5 strain represented >10% of the total amount of triplet repeat inserts. Quantitation of the relative amounts of the full-length

**Table I**

| Host strain (which contain 98 repeats) | Plasmids | Lengths of the expansion products | Increase in repeat length |
|---------------------------------------|----------|----------------------------------|--------------------------|
| NR9458 (mutD5)                        | pRW3503  | 135                               | 37                       |
|                                       | pRW3504  | 129                               | 34                       |
|                                       | pRW3504  | 125                               | 27                       |
|                                       | pRW3503  | 119                               | 21                       |
|                                       | pRW3504  | 114                               | 16                       |
|                                       | pRW3503  | 110                               | 12                       |

**Fig. 6. Expansions and deletions of (CTG-CAG)$_{98}$ in the mutD5 and the wild type strains.** pRW3503 and pRW3504, which contain (CTG-CAG)$_{98}$ in orientation II and orientation I, respectively, were propagated for three recultivations in the mutD5 strain at 37 °C. After each recultivation, the plasmids were analyzed as described in the Fig. 2 legend. The white arrows indicate expansion products of the (CTG-CAG)$_{98}$ tracts. The lengths of the expansion products observed in three independent experiments are listed in Table I.
Thus, our data show that the (CTG\textsubscript{CGG})\textsubscript{98} tract preferentially expands in the \textit{mutD5} strain. However, the extents of the instabilities of this tract are similar in the wild type and the \textit{mutD5} strains.

**Impaired Exonucleolytic Activity Destabilizes TRS Tracts in the \textit{mutD5} Mutator in Minimal Medium**—We postulate that the increased stability of the (CTG\textsubscript{CGG})\textsubscript{175} tract in the \textit{mutD5} strain relative to its isogenic wild type strain in LB medium is due to the indirect effect of the saturated MMR system (see “Discussion”). In contrast, since the instabilities of the (CTG\textsubscript{CGG})\textsubscript{z} repeats are unaffected by MMR (12), the destabilization of these tracts in the \textit{mutD5} strain in LB is probably due to a direct effect of inactive exonucleolytic proofreading. Since the \textit{mutD5} mutator has two defects, inactive exonucleolytic proofreading and saturated MMR, it was not possible to directly determine the role of proofreading in TRS instabilities. Therefore, it was necessary to perform additional investigations under conditions where MMR is functional but the proofreading activity is impaired.

It has been known for over 2 decades that \textit{mutD5} strains have a 10–100-fold higher mutator activity in rich media than in minimal media (57, 58). More recent studies have shown that this increase is due to the preferential saturation (and hence inactivation) of the MMR system in rich medium (32, 56, 59, 60). Interestingly, when the \textit{mutD5} strains were cultivated in MM, they possessed a proficient MMR system, which resulted in a lower mutation rate (60). However, the strains still suffered from a defect in the 3’ to 5’ exonucleolytic proofreading activity.

We predicted that the (CTG\textsubscript{CTG})\textsubscript{175} tract would not be stabilized by the \textit{mutD5} mutation under conditions where the MMR system is active, such as in MM. On the other hand, the CGG-CGG repeats would be expected to behave similarly in LB and in MM. Thus, the CGG-CGG tracts were likely to be destabilized in MM in the \textit{mutD5} strain relative to the wild type strain. Also, any differences observed between the instabilities of the (CTG\textsubscript{CTG})\textsubscript{175} tract in the \textit{mutD5} and in the wild type strains in MM would have to be attributed to the impaired 3’ to 5’ exonucleolytic activity.

To test these predictions, pRW3530 and pRW3506 (Fig. 1) were transformed into the KA796 (wild type) and NR9548 (\textit{mutD5}) strains and grown in MM. The plasmids were recovered after successive recultivations and digested with NotI to excise the TRS tracts. The restriction fragments were labeled and electrophoresed through polyacrylamide gels, and the TRS instabilities were analyzed quantitatively. Table II lists the percentages of full-length TRS tracts that remained after growth in the wild type and \textit{mutD5} strains in MM. The (CGG-CGG)\textsubscript{91} tract was substantially more unstable in the \textit{mutD5} strain than in the wild type strain over three recultivations in MM. Also, the (CTG\textsubscript{CTG})\textsubscript{175} tract was destabilized by the \textit{mutD5} mutator relative to the isogenic wild type strain in MM.

Thus, the growth of the wild type and \textit{mutD5} cells in minimal medium created conditions where both strains had a functional MMR system, but the \textit{mutD5} cells alone had an impaired exonucleolytic proofreading function. Under these conditions, we observed that the \textit{mutD5} mutation had a deleterious effect on the stability of the CGG-CGG and the CTG-CAG repeats. Therefore, our data clearly implicate the proofreading exonucleolytic activity as the culpable factor in the genetic instabilities of TRS.

**DISCUSSION**

The dnaQ49\textsuperscript{ts} Mutation Increases TRS Instabilities—Our data show that the instabilities of the CGG-CGG and the CTG-CAG repeats are influenced by mutations that impair the 3’ to 5’ exonucleolytic proofreading \(\varepsilon\)-subunit of DNA polymerase III. The temperature-sensitive dnaQ49\textsuperscript{ts} mutation disrupts the ability of the \(\varepsilon\)-subunit to physically interact with the polymerizing \(\alpha\)-subunit of DNA polymerase III at the nonpermissive temperatures, resulting in an unstable replication complex (36, 52, 53).

The (CGG-CGG)\textsubscript{91} tract is unstable at the higher temperatures (30 and 37 °C) in the dnaQ49\textsuperscript{ts} strain. However, temperature has no effect on the stability of the tract in the wild type strain. Thus, we conclude that the destabilizing effect of temperature on the CGG-CGG repeats is due to the inactivation of the 3’ to 5’ exonucleolytic \(\varepsilon\)-subunit of DNA polymerase III at the elevated temperatures. An increase in the temperature also destabilized the CTG-CAG repeats to a greater extent in the dnaQ49\textsuperscript{ts} than in the wild type strain. Hence, we propose a model (Fig. 7) for the involvement of the proofreading apparatus in TRS instabilities. This hypothesis is based in part on previous suggestions that TRS can adopt transient secondary structures, such as hairpins (21–23, 28) and slipped structures (61, 62), which can retard the progression of the replication fork (24–27). The model is also consistent with previous proposals (39, 40) for the role of the \textit{E. coli} dnaQ gene product in preventing deletions between tandemly repeated sequences.

Thus, in the dnaQ49\textsuperscript{ts} strain, the replication fork stalls when it encounters the secondary structures in the CGG-CGG and the CTG-CAG repeat tracts. Since the replication fork in the dnaQ49\textsuperscript{ts} strain is unstable at the nonpermissive temperatures, DNA synthesis through a region containing secondary structures would be highly error-prone. The bypass of these structures by the DNA synthetic machinery may occur by a template-primer misalignment mechanism. Upon completion of DNA synthesis, the TRS tract is substantially deleted. In contrast, in the wild type strain, the replication fork may stall at the slipped structures, but the stable replication complex is
From a saturation of the MMR system, the stabilization of the repeat tracts in the MMR system (12). Since the loops may be stabilized by the formation of hairpins, which can be synthesized (19), incorporation of bases by the 3′DNA polymerase III replication complex efficiently proofreads any mis-replication fork (20). The sizes and the stabilities of the hairpins. In contrast, when the proofreading machinery in maintaining the integrity of the TRS tract remains undeleted (open triangles (d)). Next, the loops are destabilized and repaired efficiently, and the fork progresses (e). Finally, DNA synthesis is completed, and the TRS tract remains undeleted (f).

The (CTG-CAG)\textsubscript{175} tract was stabilized by MMR mutations, a recent study (13) showed that for the much shorter and uninterrupted (CTG-CAG)\textsubscript{64} tracts, MMR mutations were deleterious. Thus, the length and the purity of the TRS tract may determine the effect of the MMR system on its stability. Analogous to the findings with the MMR mutants, we observe that an uninterrupted (CTG-CAG)\textsubscript{98} tract is not stabilized by the mutD5 mutator. Thus, the differential effects of the mutD5 mutator on the (CTG-CAG)\textsubscript{175} and the (CTG-CAG)\textsubscript{98} repeats may be due to the different lengths of these tracts, and recent studies\textsuperscript{4} support this notion.

It was suggested previously (14) that in a cell with a functional MMR system, the formation of slipped structures in a TRS tract with interruptions may result in mismatches that recruit the MMR proteins. Secondary structure formation during MMR can consequently result in TRS instability. This process is avoided in cells deficient in MMR, thereby stabilizing the repeat tracts. The (CTG-CAG)\textsubscript{175} tract used in this study contains two G to A interruptions at repeats 28 and 69. Therefore, the possibility that the stabilization of the tract by the mutD5 mutator occurs due to the presence of interruptions cannot be ruled out.

Interestingly, the CGG-CCG repeats are destabilized by the mutD5 mutator. Jaworski et al. (12) showed previously that the CGG-CCG repeats were unaffected by mutations in the MMR system. Therefore, unlike the CTG-CAG repeats, the destabilization of the CGG-CCG repeats by the mutD5 mutator is probably due to the inactivation of the proofreading activity.

**Defective Exonucleolytic Proofreading Causes CTG-CAG and CGG-CCG Repeat Instabilities in the mutD5 Strain with a Functional MMR System.**—As discussed above, the competing influences of MMR and exonucleolytic proofreading in the mutD5 strain did not allow an unequivocal resolution of the role of the proofreading activity per se in the TRS instabilities. To dissect these factors, it was necessary to analyze the expansion and deletion of TRS tracts under conditions where the MMR system was functional, but exonucleolytic proofreading was impaired. The medium-dependent phenotypic variation of mismatch repair proficiency in mutD5 cells has been well characterized (55, 57, 58). There is a substantial body of literature (32, 56, 59, 60) that reveals that in rich medium, mutD5 strains have a defective 3′ to 5′ exonucleolytic proofreading system as well as a saturated and hence inactive MMR system. Interestingly, these studies also showed that the MMR system in the mutD5 cells is active but the proofreading activity is still impaired when grown in MM.

The (CGG-CCG)\textsubscript{81} and (CTG-CAG)\textsubscript{175} tracts were substantially more unstable in the mutD5 mutator strain than in the isogenic wild type strain in MM. Since the MMR system is functional under these conditions, we conclude that the TRS instabilities are due to the inactivation of 3′ to 5′ exonucleolytic proofreading. Also, these results are consistent with our proposal that the stabilization of the CTG-CAG tracts by the mutD5 mutator strain in LB is due to the indirect effect of the saturated MMR system.

Thus, whereas MMR affects only the CTG-CAG repeats, exonucleolytic proofreading appears to be essential for maintaining the integrity of CTG-CAG as well as CGG-CCG repeat tracts. The reasons for this difference are unclear, but it may be speculated that the differences in the propensities of these sequences to form secondary structures (21–23) could influence this behavior.

**Different Loop Lengths Mediate CGG-CCG and CTG-CAG Instabilities.**—Analyses of the deletion products of the (CGG-CCG)\textsubscript{81} tract revealed 55 individual products ranging...
from 78 to 8 repeats in length. The majority of these products were separated by just one triplet repeat. The intermediates of the deletion process may be loops containing one or a small number of triplet repeats possibly by the mechanism described previously (14). DNA synthetic bypass of the small loops can result in the sequential formation of deletion products of lengths that cover almost the entire spectrum of possible products. In contrast, the CTG-CAG repeats show specific deletion and expansion products of discrete lengths. This pattern is likely to reflect the preferential formation of hairpin intermediates of specific lengths (at least five repeats).

Thus, we hypothesize that the instabilities of the TRS tracts occur due to the formation of transient but stable slipped structures that contain a small number of repeats in the loop. These structures may block the progression of the replication fork. In the case of the CGG-CCG repeats, the stalled replication complex eventually bypassed the slipped structures, resulting in a large family of deletion products. For the CTG-CAG repeats, the stalling of the replication machinery provides an opportunity for the consolidation of the slipped structures by base pairing between the CTG repeats in the loop. This results in the formation of long hairpins, which are bypassed eventually by the polymerase, giving rise to discrete deletion products.

Expansion of CTG-CAG Repeats in Mutator Strains—A number of expansion products were observed when (CTG-CAG)$_{175}$ was propagated in the mutD5 mutator strain. The expansion products varied in length from 108 to 139 repeats. Furthermore, the expansions were orientation-independent, since both orientations of the CTG-CAG tract yielded these products. In contrast, very few expansions were observed in the wild type strain. Since the balance between the expansion and deletion processes determines the formation of longer and shorter products, the observation of longer products indicates a shift in the balance in favor of expansion. We speculate that the lack of interruptions and the length of the (CTG-CAG)$_{175}$ tract may promote expansions in both wild type and mutD5 strains. However, an expanded product may be more likely to be maintained stably in the mutD5 strain than in the wild type strain because of the greater stability of long CTG-CAG tracts in the mutator strain. Alternatively, the expanded products may arise due to the inability of the mutant epsilon subunit to correct hairpin loops on the nascent strand.

Okazaki Fragment Size May Influence TRS Instabilities—Previous studies showed that temperature strongly affects the instabilities of the CTG-CAG repeats (10, 20). We have also observed an effect of temperature on the instabilities of the CTG-CAG repeats. The (CTG-CAG)$_{175}$ tract is destabilized in the wild type CD4 strain when the temperature is increased from 25 °C to 30 and 37 °C. This effect is more pronounced when the repeats are in the less stable orientation II than in orientation I. The CTG-CAG repeats delete in orientation II because of the formation of hairpin structures on the lagging strand of DNA replication.

Based on in vitro studies of the T4 replication fork, Selick et al. (63) proposed that the sizes of the Okazaki fragments were proportional to the rate of the replication fork progression. Subsequently, Mariappan and colleagues (64, 65) determined that the slower progression of E. coli replication forks in vitro resulted in Okazaki fragments that were substantially shorter (300 nucleotides) than the normal length (1000–2000 nucleotides). We speculate that the progression of the replication fork is much slower at 25 °C than at 30 or 37 °C. Therefore, the Okazaki fragments may be smaller at the lower temperatures. As a consequence, the extent of single strandedness of the lagging strand may also be low. Hence, there may be fewer opportunities for the template to fold back into hairpin loops at 25 °C, where the TRS tracts may be more stable. A prediction of this model is that triplet repeats should be substantially less deleted in mutant strains of E. coli that synthesize shorter Okazaki fragments. Whereas expansion products of TRS tracts propagated in E. coli are observed less frequently than deletion products, these tracts predominantly expand in humans. This behavior may be explained by the shorter Okazaki fragments in eukaryotes (~250–300 nucleotides) relative to prokaryotic cells (66).

Our data clearly show that the impairment of the proofreading system in E. coli enhances the genetic instabilities of triplet repeats. The observation that these tracts expand frequently in mutator strains may be of special relevance to human hereditary diseases. Compromised repair systems in humans cause a variety of diseases associated with unstable microsatellite sequences (1, 67). Furthermore, it has been suggested that proofreading defects may also account for the etiology of these diseases (68). Therefore, it is possible that triplet repeat expansion disorders (1) occur due to direct or indirect effects of disruptions in human polymerase proofreading systems.

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