Deficiency of RecA-dependent RecFOR and RecBCD pathways causes increased instability of the (GAA·TTC)$_n$ sequence when GAA is the lagging strand template

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ABSTRACT

The most common mutation in Friedreich ataxia is an expanded (GAA·TTC)$_n$ sequence, which is highly unstable in human somatic cells and in the germline. The mechanisms responsible for this genetic instability are poorly understood. We previously showed that cloned (GAA·TTC)$_n$ sequences replicated in Escherichia coli are more unstable when GAA is the lagging strand template, suggesting erroneous lagging strand synthesis as the likely mechanism for the genetic instability. Here we show that the increase in genetic instability when GAA serves as the lagging strand template is seen in RecA-proficient but not RecA-deficient strains. We also found the same orientation-dependent increase in instability in a RecA$^+$ temperature-sensitive E. coli SSB mutant strain (ssb·1). Since stalling of replication is known to occur within the (GAA·TTC)$_n$ sequence when GAA is the lagging strand template, we hypothesized that genetic stability of the (GAA·TTC)$_n$ sequence may require efficient RecA-dependent recombinational restart of stalled replication forks. Consistent with this hypothesis, we noted significantly increased instability when GAA was the lagging strand template in strains that were deficient in components of the RecFOR and RecBCD pathways. Our data implicate defective processing of stalled replication forks as a mechanism for genetic instability of the (GAA·TTC)$_n$ sequence.

INTRODUCTION

Friedreich ataxia is one of over 20 inherited disorders caused by abnormally large expansions of unstable triplet-repeat sequences (1). Whereas all other triplet-repeat expansions involve (CTG–CAG)$_n$ or (CGG–CCG)$_n$ sequences, Friedreich ataxia is so far the only disease caused by the expansion of a (GAA·TTC)$_n$ sequence (2). Disease-causing alleles contain 66–1700 triplets, which interfere with transcription of the FXN gene, resulting in a deficiency of the mitochondrial protein frataxin (3–7). Expanded (GAA·TTC)$_n$ alleles at the human FXN locus display intergenerational and somatic instability. During paternal transmission they often contract by 20–30%, and during maternal transmission they expand or contract by about the same size (8,9). Using small pool PCR to analyze the repeat length in individual somatic cells from multiple human tissues we have shown that expanded (GAA·TTC)$_n$ alleles are somatically unstable in vivo. Indeed, all tissues derived from patients show a significant frequency of large contractions of the (GAA·TTC)$_n$ sequence (10,11).

The mechanisms responsible for generating triplet-repeat instability are not fully understood. Some proposed mechanisms include recombination (12–15), DNA repair (16–18) and epigenetic modification (19). Our previous data using (GAA·TTC)$_n$ sequences, as well as work by others using (CTG–CAG)$_n$ and (CGG–CCG)$_n$ sequences support erroneous replication as a cause of triplet-repeat instability. For instance, instability of (CTG–CAG)$_n$ and (CGG–CCG)$_n$ sequences depend on the orientation of the repeat tract relative to the origin of replication in bacteria and yeast (20–25). Instability of the (CTG–CAG)$_n$ and (GAA·TTC)$_n$ sequences in transiently transfected mammalian cells was shown to require DNA replication, with the orientation and distance from the origin of replication acting as potent modifiers (26,27).

The (CTG–CAG)$_n$ and (GAA·TTC)$_n$ repeats are more unstable when CTG and GAA, respectively, serve as the lagging strand template during replication in Escherichia coli and yeast. It is thought that this instability is mediated...
via secondary DNA structures, which may be promoted by the discontinuous nature of lagging strand synthesis allowing single-stranded regions of DNA in the template strand (28). The contraction bias observed for (CTG–CTG)ₙ sequences in both bacterial and yeast systems when (CTG)ₙ is the lagging strand template is thought to be due to the relative thermodynamic stability of the hairpin formed by single-stranded (CTG)ₙ versus (CAG)ₙ sequences (20,29). The reason for the increased frequency of mutation of the (GAA–TTC)ₙ sequence when GAA is the lagging strand template is less clear. Some data suggest that (GAA)ₙ and (TTC)ₙ single-stranded sequences may also form hairpin structures (30). UV-melting experiments demonstrated that the single-stranded (GAA)ₙ sequence, but not the complementary (TTC)ₙ sequence, can form hairpin-like secondary structures (31). (GAA–TTC)ₙ sequences also form unique structures not observed with the (CNG–CNG)ₙ repeat sequences such as triplexes (32–35) and sticky DNA (7,36,37). The role of these structures in mediating (GAA–TTC)ₙ instability is not known, but triplexes in particular are believed to be responsible for the stalling of replication that occurs in (GAA–TTC)ₙ sequences (34,38–40). Additionally, replication stalling occurs predominantly when GAA is the template for lagging strand synthesis (40), which could be the underlying basis for the increased instability and predilection for contractions observed in this orientation. RecA plays a critical role in restarting stalled replication forks in *E. coli* mainly via its activity of strand invasion in homologous recombination (41–46). Two main pathways exist for the restart of replication forks, both of which require RecA and SSB; the RecFOR pathway is essential for restart of replication following UV damage and for post-replication repair of unfilled gaps, and the RecBCD pathway is needed to resolve regressed forks and for the repair of double strand breaks. Here we show that the orientation-dependent instability of the (GAA-TTC)ₙ sequence, i.e. enhanced instability when GAA is the lagging strand template, is caused by the deficiency of either the RecFOR or RecBCD pathways. Our data, therefore, indicate that fidelity during replication of the (GAA-TTC)ₙ sequence requires efficient restart of stalled replication forks in the GAA orientation.

**MATERIALS AND METHODS**

**(GAA-TTC)ₙ** repeats with minimal flanking intron 1 sequences were cloned in the Pst I/Xba I sites of pUC19 using PCR products of the *FXN* gene from human subjects as previously described (25). The following recombinant plasmids, with the repeat tract cloned in both orientations with respect to the mMB1 origin of replication, were confirmed by sequencing and selected for further analysis (sequences in the TTC and GAA orientations were identical and only the sequence of the ‘GAA’ orientation is shown here): GAA-21 [(GAA)₁₇ (A)(GAA)₃₅], GAA-41 [(GAA)₁₇(A)(GAA)₃₅, and GAA-79 [(GAA)₇₉] (Figure 1). Deletion of the Plac promoter in pUC19, to produce the pDEL-GAA-79 construct (Figure 3A), was accomplished by first introducing an Apa I site at the −35 position using the QuickChange II XL site-directed mutagenesis kit (Stratagene), followed by removal of the fragment between Apa I and Hind III, thus deleting both the −10 and −35 sites. The lack of transcription was confirmed via loss of ability to produce blue colonies on X-gal containing plates. The (GAA)₇₉ insert from the original pUC19 construct was subcloned in the Pst I/Xba I sites to produce pDEL-GAA-79 and confirmed by sequencing. Another plasmid (pINS-GAA-79) (Figure 3A) was created to alter the distance between the origin of replication and the GAA triplet-repeat by inserting a 1525 bp sequence from intron 1 of the human *FXN* gene. The following primers were used to amplify the spacer sequence from human genomic DNA:

**Spacer-F:** 5’-GTCCTGGCTTCCGCTCATCTGTC

**Spacer-R:** 5’-GATGCGACATGTCCAATCTTGTC

The primers contained the Bsp QI and Afl III sites, respectively (underlined), which were then used to insert the spacer sequence into the corresponding sites in pUC19 (i.e. between the origin of replication and the multiple cloning site). The (GAA)₇₉ insert from the original pUC19 construct was subcloned in the Pst I/Xba I sites to produce pINS-GAA-79 and confirmed by sequencing.

**Transformation of bacterial strains**

All wild-type and mutant *E. coli* strains used in this study, their genotypes and sources are listed in Table 1 (47–51). Transformation was performed by making cells competent using 10 mM MgSO₄ and 50 mM CaCl₂ and plating on LB plates containing 100 μg/ml ampicillin (supplemented with 10 μg/ml thymine for CL1 and its derivatives). Sequencing was used to verify the (GAA-TTC)ₙ repeat length.
Analysis of (GAA−TTC)n repeat instability

Repeat instability was measured exactly as we have previously described (25). Briefly, colonies containing full-length repeat tracts were grown in 5 ml LB cultures containing 100 μg/ml ampicillin (and 10 μg/ml thymine for CL1 and its derivatives). Quadraplicate cultures were grown for each strain. All strains were grown at 37°C, except RM121 and C600 cultures were grown at either 25°C (permissive temperature for RM121) or 37°C (non-permissive temperature for RM121). Cultures were grown until mid-log phase (OD600 of 1) at which point glycerol stocks were made from each culture. Previous reports have indicated that transformation of triplet repeat-containing plasmids per se may increase the instability of the repeat tract (52); therefore, mutation analysis was carried out by plating colonies from glycerol stocks. PCR of individual plasmids indicated that transformation of triplet repeat-containing plasmids was performed to assess (GAA−TTC)n instability when GAA is the lagging strand template.

RESULTS

Deficiency of RecA causes increased (GAA−TTC)n instability when GAA is the lagging strand template

To investigate the effect of RecA status on (GAA−TTC)n instability, GAA-79 and TTC-79, constructs containing a (GAA−TTC)n repeat sequence in pUC19 in both orientations relative to the pMB1 origin of replication (Figure 1), were propagated in three RecA-deficient (DH5α, Top10, HB101) and three RecA-proficient strains (C600, KA796, KH1370) (Table 1). Colony PCR was used to visualize products of individual replication events in order to detect repeat instability. GAA-79 was significantly more unstable than TTC-79 in all three orientations (Figure 2A). In contrast, all three RecA + strains did not show a difference in the level of instability between GAA-79 and TTC-79 (Figure 2A). These data suggest that deficiency of RecA causes an orientation-dependent instability of the (GAA−TTC)n sequence. However, the differing genetic backgrounds of the various strains (Table 1) resulted in widely varying absolute levels of instability. Also, it was not possible to determine if the orientation-dependence stemmed from increased instability in the GAA orientation, decreased instability in the TTC orientation, or both. Therefore, to further investigate the role of RecA status and to control for the confounding effects of varying genetic backgrounds, GAA-79 and TTC-79 plasmids were propagated in a set of isogenic strains, M152 (recA) and MM28 (wild-type) (Table 1). Again, GAA-79 was significantly more unstable than TTC-79 in the recA strain (Figure 2B and C), indicating that the orientation-dependent instability is specifically due to the deficiency of RecA. Furthermore, since GAA-79 was
significantly more unstable than TTC-79 in the recA strain, and there was no difference in the instability of TTC-79 between the two strains (P = 0.163; Figure 2C), it indicates that the orientation-dependent instability in the absence of RecA is due to an increase in instability when GAA is the lagging strand template.

Collisions between the replicative and transcriptional polymerases, or the transition from Pol I to Pol III polymerases during plasmid replication, could potentially be the cause for the enhanced instability of the repeat in the recA mutant strains. We, therefore, investigated if the increase in instability in recA strains in the GAA orientation was also seen in the absence of transcription through the repeat tract, or by significantly increasing the distance between the origin of replication and the (GAA-TTC)$_n$ sequence. (A) The (GAA-TTC)$_n$ sequence was additionally subcloned in the GAA orientation into the pDEL (with a deletion of the Plac promoter in pUC19) and pINS (with a 1.5 kb spacer from intron 1 of the human FXN gene inserted in pUC19 between the origin of replication and the repeat tract) vectors (see Materials and Methods section for details). (B) Instability of the (GAA-TTC)$_n$ sequence was significantly enhanced in the M152 (RecA-deficient) versus MM28 (RecA-proficient) strain. Error bars depict +/− 2SEM; **P < 0.01; ***P < 0.001.

Figure 2. RecA deficiency causes orientation-dependent (GAA-TTC)$_n$ instability due to increased instability when GAA is the lagging strand template. (A) Repeat instability was significantly enhanced for GAA-79 (white bars) versus TTC-79 (black bars) propagated in three RecA-deficient strains (DH5α, HB101, Top10), but no such orientation-dependence was seen in the three RecA-proficient strains (C600, KA796, KH1370). (B) Representative gels showing enhanced repeat instability for GAA-79 versus TTC-79 propagated in M152 (RecA-deficient) but not in the isogenic strain MM28 (RecA-proficient). Arrowheads indicate the position of the full-length (GAA-TTC)$_{79}$ repeat. (C) Instability was significantly enhanced for GAA-79 versus TTC-79 in M152 (RecA-deficient) but not in MM28 (RecA-proficient). Error bars depict +/− 2SEM; **P < 0.01; ***P < 0.001; n.s. = not significant.

Reduced SSB activity causes an increase in (GAA-TTC)$_n$ instability when GAA is the lagging strand template

Since SSB is known to play an important role in RecA activity, we also examined the effect of reduced SSB
activity on (GAA-TTC)_n instability. Constructs containing various lengths of the (GAA-TTC)_n sequence, cloned in the GAA orientation relative to the origin of replication (GAA-21, GAA-41 and GAA-79) (Figure 1), were propagated in the wild-type C600 strain and its derivative, RM121, which contains a temperature-sensitive SSB mutation (ssb-1) (47) (Table 1). Instability was measured by culturing both strains at the non-permissive temperature (37°C), at which there is greatly reduced SSB activity in the RM121 strain (53). Instability of the (GAA-TTC)_n sequence was found to be length-dependent in the ssb-1 strain at the non-permissive temperature, and the repeat tracts were significantly more unstable in the ssb-1 strain than in the parental wild-type strain (P < 0.001 for GAA-41 and P < 0.01 for GAA-41 and GAA-79, respectively) (Figure 4A and B). Instability of GAA-41 was significantly higher at 37°C in ssb-1 compared with 25°C. Increase in the temperature per se did not affect instability since C600 showed the same level of instability at both temperatures. The increased instability in the ssb-1 mutant versus wild-type even at the permissive temperature is likely due to leaky expression of the mutant phenotype caused by a partial deficiency of SSB. Error bars depict +/- 2SEM; **P < 0.01; n.s. = not significant.

Therefore, further detailed characterization of the effect of reduced SSB activity was performed using the GAA-41 construct. Propagation of the GAA-41 construct in ssb-1 and the wild-type control at 25 and 37°C, showed a significantly higher level of instability in the ssb-1 strain at the non-permissive temperature (Figure 4C). Although GAA-41 was significantly more unstable in ssb-1 than in the wild-type at both 25°C and 37°C (P = 0.007 and P < 0.001, respectively), no difference in instability was observed in the wild-type strain grown at 25°C and 37°C (P = 0.95). This demonstrates that the specific reduction in SSB activity at the non-permissive temperature is responsible for the increased instability of GAA-41 in ssb-1, and the latter is not simply due to increase in temperature.

Since the ssb-1 mutant is known to be defective in RecA induction at the non-permissive temperature (both C600 and RM121 are RecA-), we investigated whether reduced SSB activity would also show increased instability when GAA is the lagging strand template. GAA-41 and TTC-41 were propagated in ssb-1 and wild-type strains at both 25°C and 37°C, which revealed orientation-dependent instability specifically in ssb-1 cultures grown at the
non-permissive temperature (Figure 5). The observation of similar levels of instability with GAA-41 and TTC-41 in the wild-type strain, and in ssb-1 when grown at the permissive temperature, is consistent with their RecA+ status. However, the observation of increased instability of GAA-41 versus TTC-41 in ssb-1 at the non-permissive temperature indicates that it is the reduction in SSB activity that is responsible for the orientation-dependent instability. Propagation of GAA-41 at the non-permissive temperature resulted in significantly increased instability compared to the permissive temperature (Figure 4B), but no such difference was seen for TTC-41 (P = 0.706) (Figure 5). This indicates that reduced SSB activity has a significant effect on (GAA-TTC)n instability only when GAA serves as the lagging strand template. Therefore, the orientation-dependent instability observed in the ssb-1 strain at the non-permissive temperature is due to an increase in instability when GAA is the lagging strand template. This is further supported by the fact that no orientation-dependent instability was observed at either 25°C or 37°C in C600 (P = 0.461 and P = 0.187) (Figure 5), which has normal activities of both SSB and RecA. Furthermore, even though there was increased instability in the ssb-1 strain at the permissive temperature (compare RM121 versus C600 at 25°C) (Figure 5), no orientation-dependent instability was noted. This suggests that the slight reduction in SSB activity at 25°C is not sufficient to induce the orientation-dependent instability.

As in the case of all recA strains, the mutations encountered with the GAA-41 construct in the ssb-1 strain included mainly contractions. Despite the increase in the overall frequency of contractions, there was no difference in the magnitude of contractions of GAA-41 versus TTC-41 constructs (median magnitude of contractions was 18 and 19 triplets, respectively; P = 0.43), or with the GAA-41 construct grown at the non-permissive versus permissive temperatures (median magnitude of contractions was 18 and 20 triplets, respectively; P = 0.49) in the ssb-1 strain.

Deficiency of RecFOR and RecBCD pathways increases (GAA-TTC)n instability when GAA is the lagging strand template

Stalling of replication occurs in the (GAA-TTC)n tract specifically when GAA is the lagging strand template (40). Since deficiency of RecA and SSB, proteins that play an important role in the recovery of stalled replication forks, showed increased instability in the GAA orientation, we examined the role of other proteins involved in replication restart. GAA-79 and TTC-79 plasmids were propagated in E. coli strains mutant for recBC (CL3), recD (CL4), ruvAB (CL575), recF (CL579), recO (CL554), and recJ (CL10), and their parental wild-type strain, CL1 (Table 1). The same level of instability was noted in the GAA and TTC orientations in the wild-type CL1 strain (P = 0.39 and P = 0.86 in Figure 6A and B, respectively), which has normal RecA and SSB activities. In the recBC, ruvAB, recF and recO mutants, instability was clearly orientation-dependent, with the (GAA-TTC)79 repeat tract showing significantly more instability when GAA was the lagging strand template (P < 0.01 in each strain; Figure 6A and B). These data indicate that both the RecBCD and RecFOR pathways are required for maintaining stability of the (GAA-TTC)n sequence when GAA is the lagging strand template. Orientation-dependent instability was not seen in the recJ (P = 0.24) and recD (P = 0.42) mutants (Figure 6A and B), indicating that the absence of these proteins is not sufficient to mediate the orientation-dependent instability of the (GAA-TTC)n sequence. In all the mutant strains that showed orientation-dependent instability, invariably the orientation-dependence was due to an increase in the mutation frequency when GAA was the lagging strand template compared with the corresponding wild-type strain (P < 0.05 in each case).

Absence of the SOS response does not increase (GAA-TTC)n instability when GAA is the lagging strand template

The observation of increased instability when GAA serves as the lagging strand template in the absence of RecA, SSB and RecFOR, suggested that the absence of one or more proteins induced in the SOS response may be involved in mediating the orientation-dependent instability. We therefore propagated GAA-79 and TTC-79 in the lexA1 (Ind−) mutant (CL103), which is unable to turn on the SOS response because it has a non-cleavable LexA, and CL43, its isogenic normal counterpart (Figure 7) (Table 1). There was a slightly higher instability with GAA-79 versus TTC-79 in the lexA1 (Ind−) mutant (P < 0.05). However, comparison of the instability in the mutant versus wild-type strains showed that this observation is unlikely to be biologically significant, since there
was no difference in instability between the two strains in either the GAA or TTC orientations ($P = 0.14$ and $P = 0.98$, respectively) (Figure 7). These data therefore indicate that the effect of increased instability in the GAA orientation seen in the RecA, SSB and RecFOR mutants is unlikely to be due to deficient induction of the SOS response.

**DISCUSSION**

Friedreich ataxia is one of a relatively large group of diseases caused by the expansion of a triplet-repeat sequence, but it is unique in that it is a recessive disease and that it is so far the only disease caused by expansion of a (GAA–TTC)$_n$ sequence. Moreover, as opposed to the expansion bias of the (CNG–CNG)$_n$ repeat in human somatic cells in vivo (54–57), the expanded (GAA–TTC)$_n$ sequence in Friedreich ataxia patients shows a strong contraction bias in all tissues (10,11). Even in the dorsal root ganglia of Friedreich ataxia patients, where the repeat tract undergoes further large expansions, there is a significant number of contractions (11). Understanding the mechanism(s) that cause contraction of the (GAA–TTC)$_n$ sequence is a prerequisite to eventually being able to reverse the mutation or slow its progressive expansion in specific somatic cells of patients. The contraction bias observed when (GAA–TTC)$_n$ repeat-containing plasmids are propagated in our *E. coli* model is therefore a useful system to study the mechanism(s) involved in the contraction process.

The *E. coli* RecA protein is essential for the restoration of stalled or arrested replication forks (41–46). It plays multiple roles that vary based on the type of lesion encountered. In concert with other proteins it plays a key role in the recent widely recognized mechanisms of recombinational repair for restarting stalled forks. In addition, the presence of widespread DNA damage results in the RecA-dependent upregulation of ~40 different genes, as part of the LexA-mediated SOS response that together contribute to survival and/or genome stability. Replication of the (GAA–TTC)$_n$ sequence with GAA as the lagging strand template has been shown to stall the replication machinery in both prokaryotic and eukaryotic systems (4,25,40). How this stalling is overcome is unclear. However, our previous data (25), and those of others (4,40), had indicated that replication in the GAA orientation is associated with frequent deletions of the (GAA–TTC)$_n$ sequence. While there are likely to be multiple factors responsible for destabilizing the (GAA–TTC)$_n$ sequence, as is evident from the wide variation of repeat instability seen in different strains,
we focused our efforts on characterizing the mechanism(s) of the well-defined phenotype of orientation-dependent instability. Here we have demonstrated that enhanced instability when GAA serves as the lagging strand template is caused by deficiency of RecA, SSB and other key players in the RecFOR and RecBCD pathways for the recovery of stalled replication forks. These data support the role of efficient RecA-dependent restart of stalled replication forks in maintaining stability of the (GAA\textsubscript{n}/C\textsubscript{1}TTC\textsubscript{n}) sequence. Moreover, we also found that the effect of increased instability in the GAA orientation caused by the deficiency of RecA, SSB and RecFOR is unlikely to be mediated via proteins induced in the SOS response. Furthermore, our data also indicate that the increased instability in the GAA orientation in the absence of RecA is unlikely to be due to interactions between the replicative and transcriptional polymerases (since similar instability was observed in the absence or presence of transcription), or due to the transition between Pol I and Pol III occurring within the repeat tract (since similar instability was observed after greatly increasing the distance between the origin of replication and the repeat tract).

Hypothetically visualizing replication through the (GAA\textsubscript{n}/C\textsubscript{1}TTC\textsubscript{n}) sequence in the GAA orientation as a 'lesion' on the lagging strand (Figure 8A), we propose three non-mutually exclusive mechanisms by which repeat stability would be maintained (Figure 8B–D). A lagging strand lesion may be bypassed by dissociation of the lagging strand polymerase thereby creating a daughter-strand gap. Gap repair by the RecFOR pathway would ensue, where the RecFOR complex loads RecA on the gapped DNA, thus effecting DNA strand exchange for homologous recombinational repair. (C) Stalling may result in fork regression with formation of a Holliday junction. The Holliday junction may be resolved without cleavage via exonucleolytic digestion by the helicase-nuclease combination of RuvAB and RecBCD, so that a fork structure is restored. (D) Stalled forks may break, and repair of such a break with restart of replication may be achieved by joint molecule formation between the intact and broken sister arms via the combined action of RecBCD, RuvAB and RecA.

![Figure 8](image-url)
maintaining repeat stability. The helicase–endonuclease complex RuvABC is involved in both the RecFOR and RecBCD pathways thus explaining the orientation-dependent instability in the ruvAB mutant.

Deficiency of RecJ and RecD exonucleases, which preferentially function in the RecFOR and RecBCD pathways, respectively, did not show any orientation-dependent instability. This is consistent with the redundancy afforded by the overlapping functions of these and other similar exonucleases (60). More importantly, RecJ is required for intermolecular plasmd recombination, and recJ mutants show a 4000-fold decrease in such recombination events (61). The (GAA-TTC)$_n$ sequence has been shown to undergo frequent intramolecular and intermolecular recombination in *E. coli* (15). Therefore, the observation that the recJ mutant does not increase instability when GAA is the lagging strand template suggests that intermolecular plasmid recombination is unlikely to be the underlying mechanism of this orientation-dependent increase in instability.

The role of SSB in mediating orientation-dependent instability of the (GAA-TTC)$_n$ sequence is likely more varied. *E. coli* ssb is known to play a role in a variety of DNA metabolic pathways, primarily by preventing single-stranded DNA from forming secondary structures. It promotes primosome assembly, DnaB helicase activity, fidelity and processivity of DNA polymerases, and efficient mismatch repair (62,63). Furthermore, SSB can prevent replication pausing at secondary DNA structures by helping the polymerase overcome the structural barrier (38). Given the 50-fold reduced binding affinity of SSB for poly(A/G) versus poly(T/C) sequences (64,65), and the ability of single-stranded (GAA)$_n$ sequences to adopt stable secondary structures (31), it is conceivable that these factors may also contribute to the development of deletions when GAA is the lagging strand template. Additionally, SSB plays an important role in the RecFOR and RecBCD pathways, and modulates the activity of RecA in homologous recombination and replication restart. SSB and RecA both bind cooperatively to single-stranded DNA and are therefore often in competition for the same substrate. RecA must be able to displace SSB from regions of single-stranded DNA present at stalled replication forks, single-stranded gaps, or double-stranded breaks in order for RecA-coated presynaptic filaments to form, which is an early rate-limiting step in homologous recombination. However, RecA initially requires SSB on these single-stranded DNA regions in order to prevent secondary structure formation so that RecA can bind in a cooperative fashion to form presynaptic filaments. There is also some evidence that SSB enhances the rate of subsequent joint formation and strand exchange events during RecA-mediated recombination (62,63). The significant functional interaction between SSB and RecA, and the fact that deficiency of either protein results in an increase in instability when GAA serves as the template for lagging strand synthesis, suggest that the two proteins may function together in a way that stabilizes (GAA-TTC)$_n$ repeat tracts *in vivo*. It is therefore not surprising, given the myriad roles of SSB, that the ssb-1 mutant displayed a very high level of repeat instability, necessitating the use of a shorter (GAA-TTC)$_n$ repeat sequence in our experiments. The ssb-1 mutant also showed increased frequency of deletions of the (CTG–CAG)$_n$ repeat sequence (48).

Some of our findings contrast with the observations others have made regarding the mechanism of instability of the (CTG–CAG)$_n$ sequence. Whereas RecA plays a role in stabilizing the (GAA-TTC)$_n$ sequence, the (CTG–CAG)$_n$ repeat sequence was paradoxically more stable in a recA mutant (14). However, enhanced instability of the (CTG–CAG)$_n$ sequence was noted in the recA mutant background when a double strand break was introduced within the repeat tract, and this instability was orientation-dependent in recA and recBC mutants (66). Moreover, the (CTG–CAG)$_n$ repeat was stabilized in recO and ruvAB mutants, and destabilized in a recJ mutant (67). These data indicate that the mechanisms underlying the instability of the two triplet-repeat sequences are likely different, probably reflecting the differences in their physical properties. It is noteworthy that there are significant differences in the type of instability that these two triplet-repeat sequences display in human tissues and in various model systems.

In summary, there are clearly multiple mechanisms that mediate instability of (GAA-TTC)$_n$ sequences in *E. coli* given that there is substantial instability in the TTC orientation and that the absolute level of instability is different in various cell lines. However, our results indicate that deficiency of the RecA-dependent RecFOR and RecBCD pathways causes enhanced instability when GAA is the lagging strand template. These observations support the hypothesis that proficient RecA-dependent restart of stalled replication forks is required to maintain the integrity of the (GAA-TTC)$_n$ sequence. Moreover, the differences in the mechanisms underlying the instability of the (GAA-TTC)$_n$ versus (CTG–CAG)$_n$ sequence indicate that the mechanisms are sequence and/or structure dependent.

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