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

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ABSTRACT

The most common mutation in Friedreich ataxia is an expanded $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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-deficient but not RecA-proficient 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 $(GAA{\cdot}TTC)_n$ sequence when GAA is the lagging strand template, we hypothesized that genetic stability of the $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ alleles are somatically unstable *in vivo*. Indeed, all tissues derived from patients show a significant frequency of large contractions of the $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(CTG–CAG)_n$, $(CGG–CCG)_n$ and $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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

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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 enhanced frequency of mutation of the $(GAA{\cdot}TTC)_n$ 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). $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ instability is not known, but triplexes in particular are believed to be responsible for the stalling of replication that occurs in $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ sequence requires efficient restart of stalled replication forks in the GAA orientation.

MATERIALS AND METHODS

Plasmid construction

$(GAA{\cdot}TTC)_n$ repeats with minimal flanking intron 1 sequence 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 pMB1 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 $[(GAA)_{17}(A)(GAA)_4]$, GAA-41 $[(GAA)_{37}(A)(GAA)_4]$, and GAA-79 $[(GAA)_{79}]$ (Figure 1). Deletion of the Plac promoter in pUC19, to produce the pDEL-GAA-79 construct

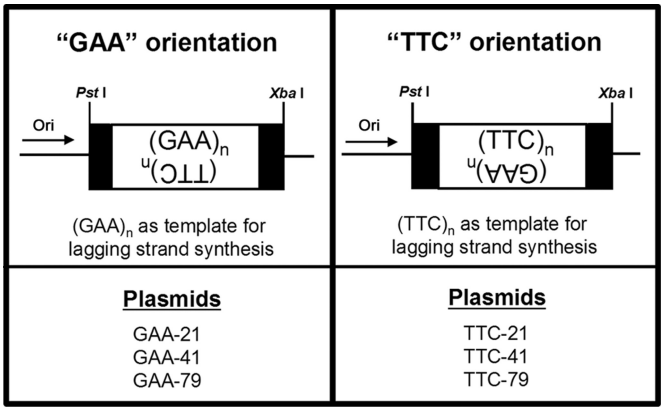

Figure 1. $(GAA{\cdot}TTC)_n$ constructs used to analyze repeat instability. $(GAA{\cdot}TTC)_n$ sequences of the indicated lengths were cloned into the Pst I/Xba I sites of pUC19 in both orientations relative to the unidirectional pMB1 origin of replication. Repeat-containing plasmids are depicted in either the 'GAA' or 'TTC' orientations, based on whether $(GAA)_n$ or $(TTC)_n$ serves as the lagging strand template, respectively. The plasmid constructs contain repeat lengths of $n = 21$, 41 and 79. The black boxes flanking the repeat represent minimal flanking sequence from intron 1 of the *FXN* gene.

(Figure 3A), was accomplished by first introducing an Apa I site at the −35 position using the QuikChange 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′-GCTCCGGCTCTTCCGCTCATCTGTC CATTTTCCTAGAGGG-3′

Spacer-R: 5′-GATGCGACATGTCCAATCATTGCC TACCCCCTG-3′.

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 $MgSO_4$ and 50 mM $CaCl_2$ 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 $(GAA{\cdot}TTC)_n$ repeat length.

Table 1. Strains used in this study

Strain	Genotype	Description
DH5α	*recA1 ϕ80△lacZ△M15△(lacZYA-argF) U169 endA1*	RecA-deficient
	hsdR17(r_k^-,m_k^+) phoA supE44 λ$^-$thi-1 gyrA96 relA1	RecA-deficient
HB101	*recA13 hsdS20 supE44 thi-1 ara-14 galK2 rpsL20 StrR proA lacY1 xyl-5 mtl-1 leuB6*	RecA-deficient
Top10	*recA1 F- mcrA△(mrr-hsdRMS-mcrBC) ϕ80lacZ△M15△lacX74 araD139 △(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*	RecA-proficient
KA796[a]	*thi ara △pro-lac*	
KH1370[b]	*λ$^-$relA1 metD-88 △(cod-lacI)6 tsx-7 srl-8 spoT1 metB*	RecA-proficient
MM28[b]	*galK2(OC) λ$^-$IN(rrnD-rrnE)1 rpsL200(strR)*	Wild-type
M152[b]	*recA3*	Isogenic to MM28 except *recA3*
C600[c]	*supE44 hsdR? thi-1 thr-1 leuB6 lacY1 tonA21*	Wild-type
RM121[c]	*ssb-1(t.s.)*	Isogenic to C600 except *ssb-1* (t.s.)
CL1[d]	*λ$^-$thyA36 deoC2 IN(rrnD-rrnE)1 rph*	Wild-type
CL3[d]	*recB21 recC22 argA81:Tn10*	Isogenic to CL1 except *recB21 recC22 argA81:Tn10*
CL4[d]	*recD1011 argA81:Tn10*	Isogenic to CL1 except *recD1011 argA81:Tn10*
CL10[d]	*recJ284:Tn10*	Isogenic to CL1 except *recJ284:Tn10*
CL554[d]	*recO6218*	Isogenic to CL1 except *recO6218*
CL557[d]	*ruvAB6203*	Isogenic to CL1 except *ruvAB6203*
CL579[d]	*recF6206*	Isogenic to CL1 except *recF6206*
CL43[d]	λ$^-$ rph$^-$1	Wild-type
CL103[d]	*lexa1(Ind−)*	Isogenic to CL43 except *LexA1(Ind−)*

[a]Provided by Dr Roel Schaaper (NIH-NIEHS).
[b]Provided by *E. coli* Genetic Stock Center (Yale University).
[c]Provided by Dr Richard Sinden (Florida Institute of Technology) (47,48).
[d]Provided by Dr Justin Courcelle (Portland State University) (49,50).

Analysis of $(GAA{\cdot}TTC)_n$ 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). Quadruplicate 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 (OD_{600} 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 colonies was performed to assess $(GAA{\cdot}TTC)_n$ repeat instability using primers GS-F and GS-R (25). Relative sizes of PCR products were determined by electrophoresis on 2.5% agarose gels, coupled with direct sequencing of selected products following extraction from gel slices (QIAquick gel extraction kit, Qiagen). Approximately 100 colonies were analyzed from each of the quadruplicate cultures (i.e. from four separate glycerol stocks), for each combination of repeat length, orientation and *E. coli* strain. Instability was calculated as the number of mutation events (change in repeat tract length) observed per successful colony PCR amplification. Orientation-dependent instability was analyzed by comparing the mutation frequencies in the GAA versus TTC orientations (note: the corresponding GAA and TTC constructs were always grown at the same time using identical growth conditions).

RESULTS

Deficiency of RecA causes increased $(GAA{\cdot}TTC)_n$ instability when GAA is the lagging strand template

To investigate the effect of RecA status on $(GAA{\cdot}TTC)_n$ instability, GAA-79 and TTC-79, constructs containing a $(GAA{\cdot}TTC)_{79}$ 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 *recA* mutant strains (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 $(GAA{\cdot}TTC)_{79}$ 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

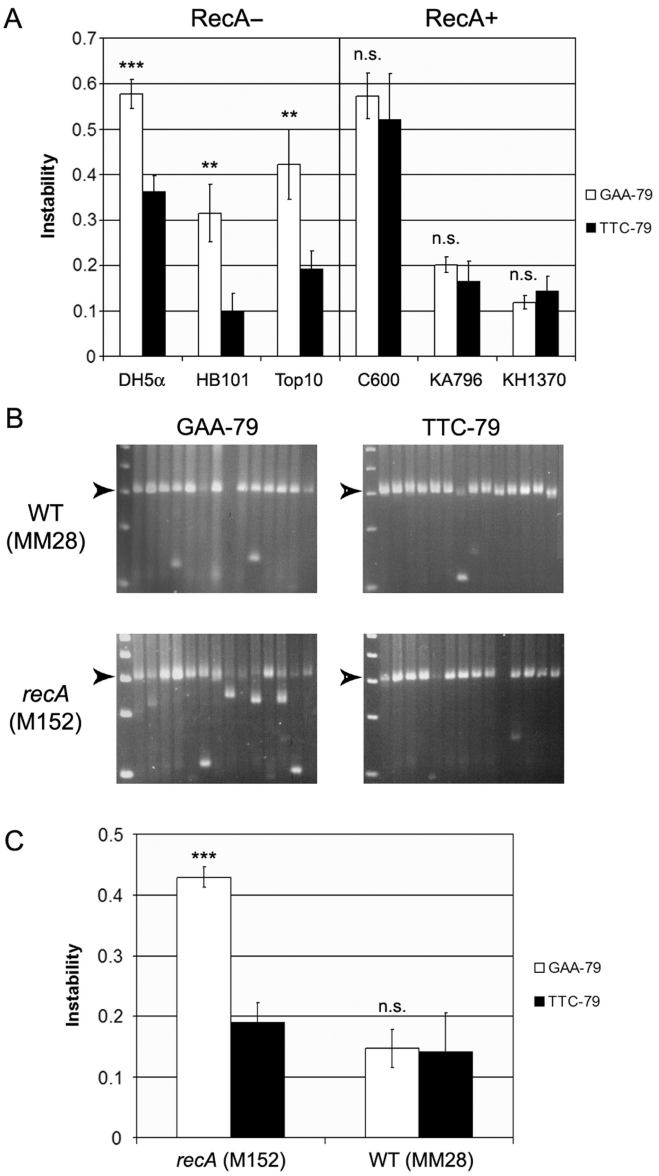

Figure 2. RecA deficiency causes orientation-dependent $(GAA{\cdot}TTC)_n$ instability due to increased instability when GAA is the lagging strand template. **(A)** Repeat instability was significantly enhanced for GAA-79 (white bars) versus TTC-79 (black bars) propagated in three RecA-deficient strains (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 $(GAA{\cdot}TTC)_{79}$ 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.

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

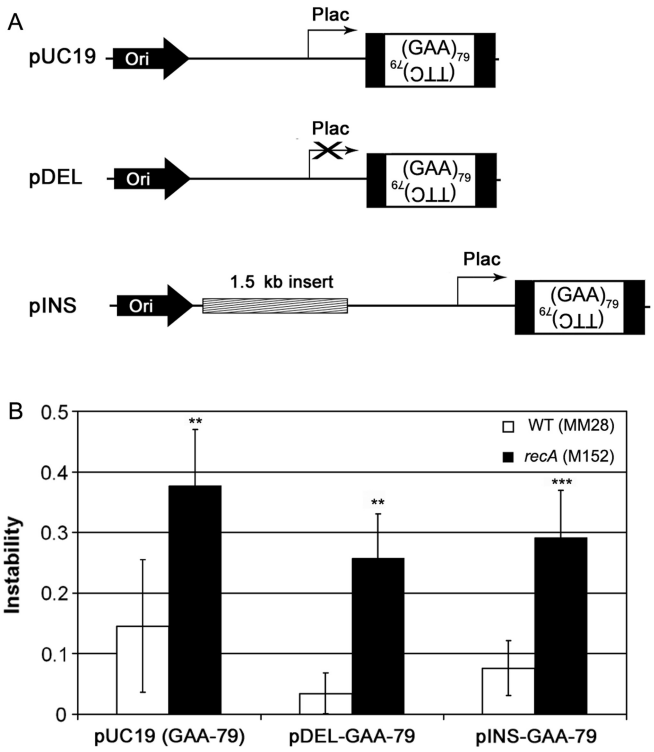

Figure 3. RecA deficiency causes increased instability when GAA is the lagging strand template irrespective of transcription through the repeat tract, or the distance between origin of replication and the $(GAA{\cdot}TTC)_{79}$ sequence. **(A)** The $(GAA{\cdot}TTC)_{79}$ sequence was additionally subcloned 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 $(GAA{\cdot}TTC)_{79}$ sequence was significantly enhanced in the M152 (RecA-deficient) versus MM28 (RecA-proficient) strain. Error bars depict +/− 2SEM; $^{**}P < 0.01$; $^{***}P < 0.001$.

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 repeat tract. pDEL-GAA-79, with the Plac promoter deleted, and pINS-GAA-79, with a 1.5 kb spacer inserted between the origin and the repeat tract, were propagated in M152 and MM28. The *recA* mutant showed a significant increase in the instability of the repeat in both constructs, which was comparable to the GAA-79 sequence within the context of the unmodified pUC19 vector (Figure 3), indicating that neither transcription nor the transition between replicative polymerases was required for the enhancement of repeat instability.

Reduced SSB activity causes an increase in $(GAA{\cdot}TTC)_n$ 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

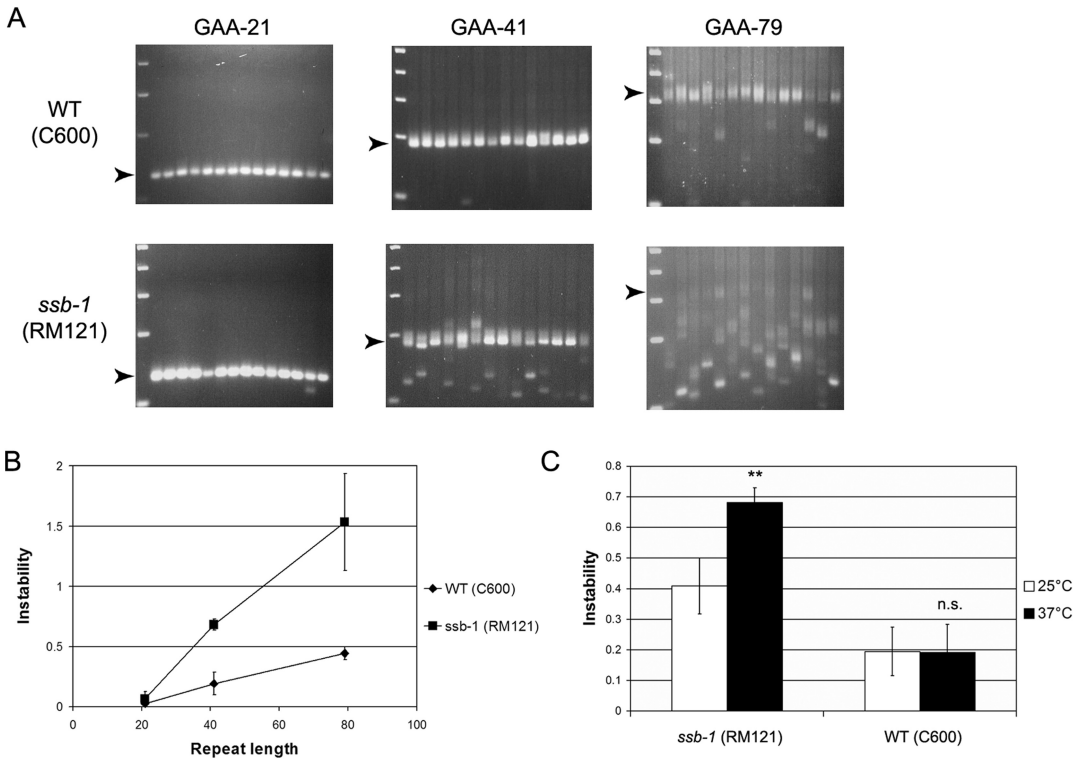

Figure 4. Reduction in SSB activity causes a length-dependent increase in $(GAA{\cdot}TTC)_n$ instability. **(A)** Representative agarose gels showing PCR products generated from colonies containing plasmids with the indicated lengths of $(GAA{\cdot}TTC)_n$ repeat tracts in the GAA orientation in C600 (wild-type (WT) for SSB), and in the isogenic temperature sensitive *ssb-1* mutant, RM121. Arrowheads indicate the position of the respective full-length repeats. **(B)** $(GAA{\cdot}TTC)_n$ instability is length-dependent in both strains, however, the GAA-41 and GAA-79 repeat tracts are significantly more unstable in RM121 ($P<0.001$ and $P<0.01$ for GAA-41 and GAA-79, respectively). Error bars depict +/− 2SEM. **(C)** Repeat instability was determined for GAA-41 propagated in the *ssb-1* mutant and C600 strains at the permissive versus non-permissive temperatures (25°C (white bars) versus 37°C (black bars)). 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.

activity on $(GAA{\cdot}TTC)_n$ instability. Constructs containing various lengths of the $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $P=0.02$ for GAA-79) (Figure 4A and B), indicating that reduction of SSB activity increases $(GAA{\cdot}TTC)_n$ instability. The much higher level of instability of the GAA-79 construct propagated in RM121 at the non-permissive temperature (which made it very difficult to quantify accurately; compare Figures 2B and 4A), indicates that reduction in SSB activity produces a more severe destabilization of the $(GAA{\cdot}TTC)_n$ repeat sequence compared with the deficiency of RecA. 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

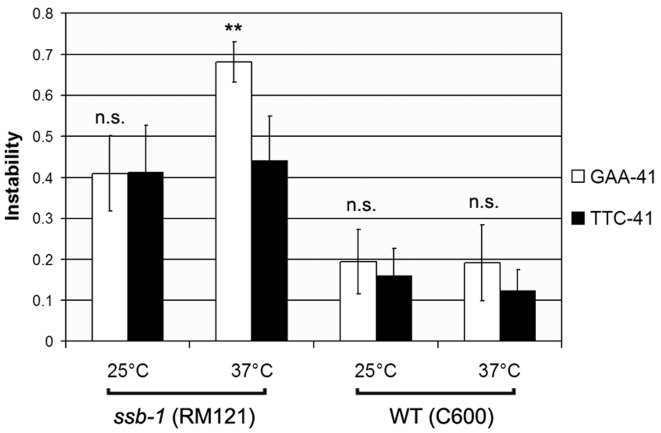

Figure 5. Reduction in SSB activity causes increased instability when GAA is the lagging strand template. Repeat instability was determined for GAA-41 (white bars) versus TTC-41 (black bars) propagated in C600 and *ssb-1* at 25 and 37°C. GAA-41 was significantly more unstable than TTC-41 only in the *ssb-1* mutant strain at the non-permissive temperature. There is increased instability in the *ssb-1* mutant versus wild-type in both the GAA and TTC orientations even at the permissive temperature, which is most likely due to the partial deficiency of SSB at 25°C. However, the orientation-dependent instability manifests only when the *ssb-1* mutant is grown at the non-permissive temperature, and that this is specifically due to the increase in instability in the GAA orientation. Error bars depict +/− 2SEM; $^{**}P < 0.01$; n.s. = not significant.

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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ instability when GAA is the lagging strand template

Stalling of replication occurs in the $(GAA{\cdot}TTC)_n$ 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* (CL557), *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 $(GAA{\cdot}TTC)_{79}$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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

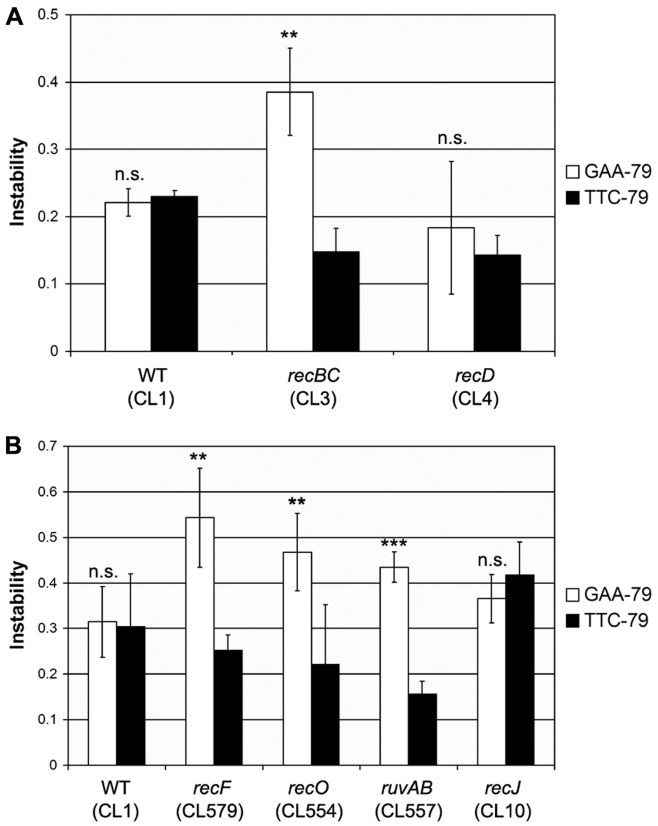

Figure 6. Deficiency of proteins in the RecA-dependent **(A)** RecBCD and **(B)** RecFOR pathways for the restart of stalled replication forks results in enhanced instability when GAA is the lagging strand template. Repeat instability was determined for GAA-79 and TTC-79 in *recBC*, *recD*, *ruvAB*, *recF*, *recO* and *recJ* mutants as well as an isogenic wild-type (WT) strain (CL1). GAA-79 is significantly more unstable than TTC-79 in the *recBC*, *ruvAB*, *recF* and *recO* mutants, but not in the *recD* and *recJ* mutants, or in the wild-type strain. In all mutants that showed orientation-dependent instability, instability in the GAA orientation is greater in the mutant versus the isogenic wild-type ($P < 0.05$ in each case). Error bars depict +/− 2SEM; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = not significant.

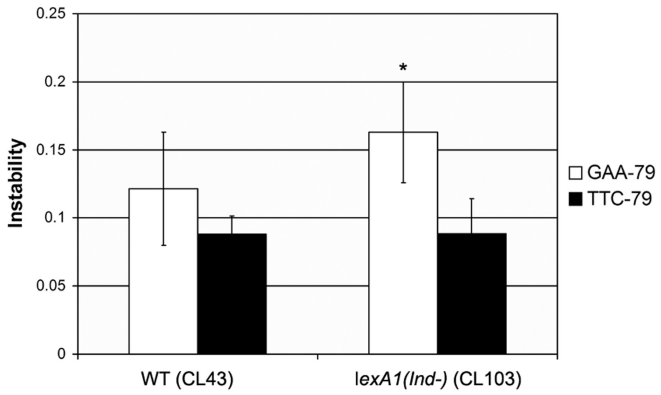

Figure 7. The inability to induce the SOS response is not associated with enhanced instability in the GAA orientation. Repeat instability was determined for GAA-79 and TTC-79 in the *lexA1*(*Ind*−) mutant (CL103) as well as an isogenic wild-type (WT) strain (CL43). GAA-79 is slightly more unstable than TTC-79 in the *lexA1*(*Ind*−) mutant, however, there was no difference in instability in the GAA or TTC orientations when compared with the wild-type strain ($P = 0.14$ and $P = 0.98$, respectively). Error bars depict +/− 2SEM; $^{*}P < 0.05$; n.s. = not significant.

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 $(GAA{\cdot}TTC)_n$ sequence (2). Whereas the other dominant diseases rely on frequent *de novo* expansions of $(CNG–CNG)_n$ repeats, Friedreich ataxia is maintained in the population via a large number of asymptomatic heterozygous carriers of alleles already containing an expanded $(GAA{\cdot}TTC)_n$ sequence. Moreover, as opposed to the expansion bias of the $(CNG–CNG)_n$ repeat in human somatic cells *in vivo* (54–57), the expanded $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ sequence. While there are likely to be multiple factors responsible for destabilizing the $(GAA{\cdot}TTC)_n$ sequence, as is evident from the wide variation of repeat instability seen in different strains,

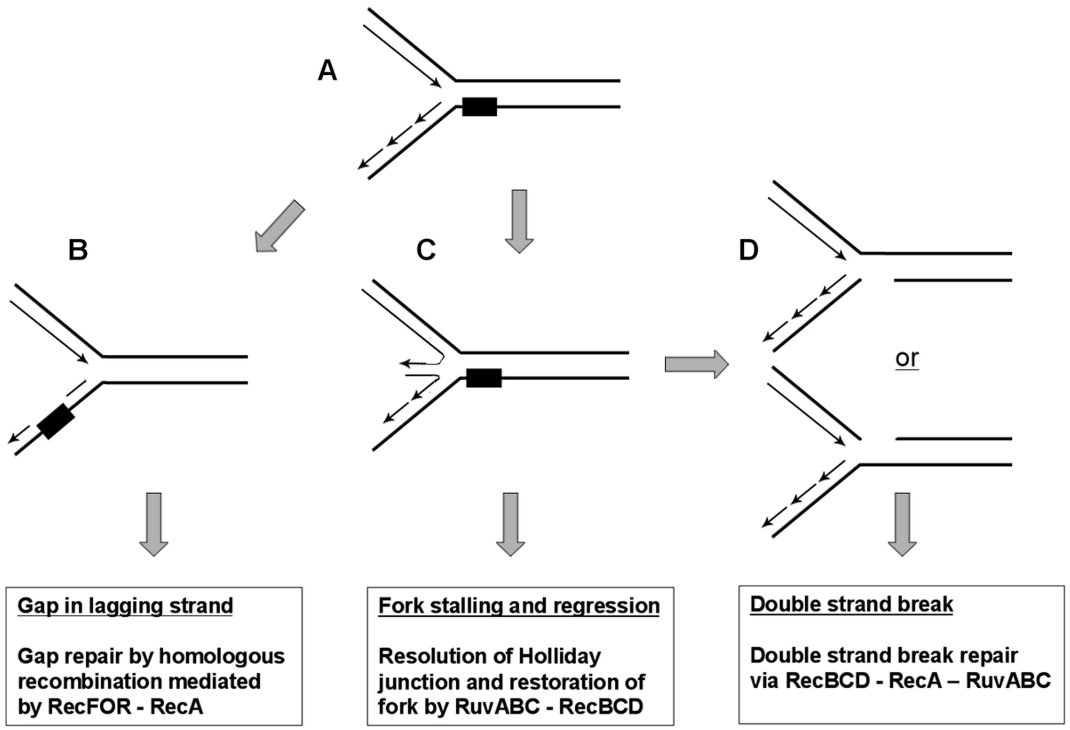

Figure 8. Pathways involved in maintaining stability of the $(GAA{\cdot}TTC)_n$ sequence in *E. coli* (see Discussion section for details). **(A)** Replication of the $(GAA{\cdot}TTC)_n$ sequence in the GAA orientation may be hypothetically visualized as a 'lesion' on the lagging strand. **(B)** A lagging 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.

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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 from the template and restarting replication of the nascent lagging strand on the 3′ side of the lesion thereby creating a daughter-strand gap (Figure 8B). Such a gap would be repaired by the RecFOR pathway, where the RecFOR complex loads RecA on to the gapped DNA that is precoated with SSB, thus effecting DNA strand exchange for homologous recombinational repair (58). Consistent with the importance of this pathway in maintaining repeat stability, both *recF* and *recO* mutants resulted in enhanced instability in the GAA orientation. On the other hand, the stalled replication fork may regress wherein the nascent strands pair to form a Holliday junction (Figure 8C). Holliday junctions may be resolved without the need for cleavage by processing of the free double-stranded DNA end via exonucleolytic digestion by the helicase-nuclease combination of RuvAB and RecBCD, so that a fork structure is restored (46,59). However, stalled forks may break (Figure 8D), either spontaneously or by the Holliday junction resolvase RuvC (45,59). Repair of such a break and restart of replication would be achieved by joint molecule formation between the intact and broken sister arms via the combined action of RecBCD and RecA (45). The enhanced instability in the GAA orientation seen in the *recBC* mutant supports these mechanisms for

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 plasmid recombination, and *recJ* mutants show a 4000-fold decrease in such recombination events (61). The $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ sequence, the $(CTG–CAG)_n$ repeat sequence was paradoxically more stable in a *recA* mutant (14). However, enhanced instability of the $(CTG{\cdot}CAG)_n$ 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 $(GAA{\cdot}TTC)_n$ 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 $(GAA{\cdot}TTC)_n$ sequence. Moreover, the differences in the mechanisms underlying the instability of the $(GAA{\cdot}TTC)_n$ versus $(CTG–CAG)_n$ sequence indicate that the mechanisms are sequence and/or structure dependent.

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