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RecQ and RecG Helicases Have Distinct Roles in Maintaining the Stability of Polypurine-Polypyrimidine Sequences

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Abstract

DNA triplex structures can block the replication fork and result in double-stranded DNA breaks (DSBs). RecQ and RecG helicases may be important for replication of such sequences as RecQ resolves synthetic triplex DNA structures and RecG mediates replication restart by fork regression. Primer extension on an 88bp triplex-forming polypurine-polypyrimidine (Pu·Py) tract from the *PKD1* gene demonstrated that RecQ, but not RecG, facilitated primer extension by T7 DNA polymerase. A high-throughput, dual plasmid screening system using isogenic bacterial lines deficient in RecG, RecQ, or both, revealed that RecQ deficiency increased mutation to sequence flanking this 88bp tract by eight to ten fold. Although RecG facilitated small deletions in an 88 bp mirror repeat containing sequence, it was absolutely required to maintain a 2.5 kb Pu·Py tract containing multiple mirror repeats. These results support a two-tiered model where RecQ facilitates fork progression through triplex forming tracts and, failing processivity, RecG is critical for replication fork restart.

Keywords

Triplex DNA; DNA secondary structure; RecG helicase; RecQ helicase; Structure-mediated mutation

1. Introduction

Genomic stability is the result of the interaction of intrinsic factors such as the type of sequence or the action of DNA replication and repair proteins, and extrinsic factors such as environmental mutagens. Triplex-forming Pu·Py sequence tracts pause replication *in vitro* [1,2], and such pausing can lead to replication fork collapse and DSBs *in vivo*. The formation of alternative DNA secondary structures can also activate nucleotide excision and SOS pathways [3] resulting in segments of single-stranded DNA (ssDNA). Such ssDNA regions can be converted to DSBs during replication and lead to mutations through mechanisms such as homologous recombination or non-homologous end-joining. Gross rearrangements and deletions as well as microinsertions, microdeletions and point mutations appear to result from alternative structure-mediated mutagenesis [4–9]. Although such mutagenic potential could be hypothesized to reduce the prevalence of such triplex-forming Pu·Py tracts, they are not uncommon and in fact appear to be enriched in the 5' untranslated regions (UTR) of genes [10], and can also have a

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role in the regulation of gene expression. For example, the γ -globin gene contains a triplex-forming sequence that appears to be responsible for developmental gene regulation [11].

Sequences with the capacity to form triplex structures have been implicated in human diseases (reviewed in reference [12]). Intron 21 of the polycystic kidney disease 1 (*PKDI*) gene contains a Pu-Py tract that adopts a triplex DNA conformation as determined by S1 nuclease sensitivity, two dimensional gel electrophoresis [13] and atomic force microscopy [14]. Sequences from this large tract block both prokaryotic and eukaryotic replication machinery *in vitro* [1] and are mutagenic in prokaryotic assays [8]. Such a blocked replication fork could utilize fork reactivation, accomplished in bacteria by a concerted effort of RecG and PriA helicase [15], or lead to fork collapse and a DSB requiring homologous recombination for repair [16]. In humans, the *PKDI* at risk sequence could fail repair, leading to loss of heterozygosity and disease [17,18].

Alternative structures such as intermolecular triplex and G-quartet (G4) DNA are stabilized by the formation of Hoogsteen basepairing. The *Escherichia coli* helicase RecQ has been demonstrated to melt such basepairing using a synthetic G4 DNA construct [19]. In humans, the RecQ family of helicases includes WRN, BLM, RTS, RECQ1, and RECQ5. Both WRN and BLM demonstrate the ability to melt synthetic triplex [20] as well as G4 DNA constructs *in vitro* [21,22]. Deficiencies in WRN, BLM, and RTS result in Werner, Bloom, and Rothmund-Thomson syndromes, respectively. These three syndromes are characterized by genomic instability and increased susceptibility to certain malignancies [23].

RecG helicase plays a critical role in fork regression-based replication restart. Although there is no known human homolog to RecG, both WRN [24] and BLM helicases [24,25] promote fork regression in synthetic constructs of a stalled replication fork.

Whether *E. coli* RecQ or RecG helicases can alleviate intramolecular triplex-induced replication blockade *in vivo* remains unknown. We sought to characterize the effects of these helicases on the intramolecular triplex formed by the *PKDI* mirror repeat tract sequences.

2. Experimental Procedures

2.1. Overexpression and purification of *E. coli* RecQ and RecG

His-tagged RecQ and RecG proteins were purified as previously described [19,26]. The complete *E. coli recQ* cDNA cloned in the pQE31 expression vector (Qiagen) resulting in the plasmid pEG88 was a generous gift from Dr. Charles Radding (Yale University, New Haven, CT). The plasmid pGS772 containing the complete *E. coli recG* gene expressed from an IPTG-inducible promoter was a generous gift from Dr. Robert G. Lloyd (University of Nottingham, Queen's Medical Centre, Nottingham, UK). BL21(DE3) competent cells, containing plasmid pREP4 that expresses the lacI^q repressor, were transformed with pEG88 or pGS772. Cells were grown at 37°C in 8 l of Luria Broth [27] supplemented with ampicillin (100 µg/ml) and kanamycin (25 µg/ml) until the culture reached OD₆₀₀ = 0.6. At this density, IPTG was added to a final concentration of 1 mM in order to induce RecQ or RecG expression. After 4 hours of induction, cells were harvested by centrifugation at 4°C and suspended in 80 ml cell lysis buffer (20 mM potassium phosphate pH 7.4, 20% sucrose, 500 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 10 mM β-mercaptoethanol and 1 mg/ml lysozyme), incubated at 4°C for 20 min, sonicated, and centrifuged for 1 h at 14 000 g. (NH₄)₂SO₄ was slowly added to the supernatant with stirring to reach 45g/dl final concentration. The resultant supernatant was centrifuged for 40 min at 14 000 g and the pellet was resuspended in 15 ml purification buffer (20 mM potassium phosphate pH 7.4, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol) and dialyzed overnight. The sample was applied to a 13 ml SP-Sephadex column, washed with 60 ml purification buffer and fractionated with a 100 ml gradient of 50–

750 mM KCl in purification buffer. Peak fractions, identified by SDS-PAGE, were pooled and dialyzed against 700 ml purification buffer for an hour and then loaded on a 6 ml Ni-NTA agarose column (Qiagen), washed with 20 ml purification buffer containing 20 mM imidazole, then eluted with 80 ml purification buffer containing a linear gradient of 50–100 mM imidazole. Peak fractions were pooled, dialyzed against 1 l storage buffer (20 mM Tris-HCl, 200 mM NaCl, 0.2 mM EDTA, 5 mM β -mercaptoethanol, 20% glycerol) overnight, aliquoted and stored at -80°C until used. Homogeneity of the resulting enzyme preparations was $>95\%$, as determined by SDS-PAGE.

2.2. Primer Extension Reactions

To assess the possible roles of the RecQ and RecG helicases in replication through a *PKDI* mirror repeat Pu-Py tract, primer extension reactions were performed using T7 polymerase. A 50 μl reaction containing 5 U T7 DNA polymerase, 40 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 5 mM DTT, 50 mM NaCl, excess (2 μg) unlabeled template DNA, 300 μM 4dNTP mix, 2 mM ATP, 100 $\mu\text{g/ml}$ BSA and 1 nM purified RecQ or RecG was incubated with 25 pM annealed radiolabeled primer for 20 minutes. This concentration of helicase was chosen because the activity for the duration of the primer extension would not denature the duplex primer-template construct. After incubation, the primer extension reaction products were mixed with formamide as a denaturant and bromophenol blue as a marker, heated to 94°C for 3 minutes, immersed immediately in ice, and resolved on an 8% denaturing polyacrylamide gel. For size determination, a 25 bp molecular weight marker was used. The gel was dried at 80°C for 75 minutes, exposed to a PhosphorScreen (Molecular Dynamics, Sunnyvale, CA, USA) overnight, and imaged using the Storm 860 (Molecular Dynamics) PhosphorImager. Primer extension products were quantified by densitometry using NIH ImageJ 1.37a software.

2.3. Bacterial Strains, Media, and Antibiotics

The *E. coli* strain DH5 α (F^- $\Phi 80\text{lacZ}\Delta\text{M15}$ $\Delta(\text{lacZYA-argF})$ U169 *recA1 endA1 hsdR17* (r_k^- , m_k^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*) was used for all cotransformation experiments and was obtained from American Tissue Culture Collection. TOP10 competent cells (F^- *mcrA* $\Delta(\text{mrr-hsdRMS-mcrBC})$ $\Phi 80\text{lacZ}\Delta\text{M15}$ ΔlacX74 *recA1 araD139* $\Delta(\text{ara-leu})7697$ *galU galK rpsL* (Str^R) *endA1 nupG*) were used for cloning experiments (Invitrogen). To analyze the effects of RecG and RecQ helicases on replication fidelity, isogenic bacterial strains AB1157, DL1104, SWM1003 and N6916 were used. AB1157 (F^- *thr-1 leuB6 thi-1 lacY1 galK2 ara14 xyl5 mtl-1 proA2 his4 argE3 rpsL3* (Str^R) *tsx-33 supE44 kdgK51*) and DL1104 (AB1157 $\Delta\text{recG263::kan}$) were generous gifts of David Leach (University of Edinburgh, Edinburgh, UK). SWM1003 (AB1157 $\Delta\text{recQ::kan}$) and N6916 (AB1157 $\Delta\text{recG263::kan}$ $\Delta\text{recQ::apra}$) were generous gifts of Steven Matson (University of North Carolina, Chapel Hill, NC) and Robert Lloyd (University of Nottingham), respectively. Luria Broth [27] was used to determine resistance to kanamycin. Terrific Broth [27] was used for preparative plasmid amplification and for Western blot lysate cultures. SOC media [27] was used in reversion analysis experiments to enhance transformation efficiency. Carbenicillin (Invitrogen) and kanamycin (Sigma) were diluted to concentrations of 50 $\mu\text{g/ml}$ in liquid and solid media. Tetracycline (Sigma) was diluted to 10 $\mu\text{g/ml}$ in liquid media, and 25 $\mu\text{g/ml}$ in solid media. Chloramphenicol (Sigma) was diluted to 25 $\mu\text{g/ml}$ in both solid and liquid media. Bacterial cells were made competent by the Hanahan RbCl method [28].

2.4. Mutation Screening Systems: Mutation Detection Adjacent to the Sequence

The bacteriophage lambda repressor gene was amplified by PCR from pEA303 ([29], gift from Pieter L. deHaseth, Case Western Reserve University, Cleveland, OH) and inserted into pUC19 at the multiple cloning site. The constitutive β -lactamase promoter was then cloned

immediately upstream of the lambda repressor cassette to create the test plasmid pLR in which lambda repressor expression is constitutively driven by this promoter (Figure 1A).

The complimentary oligonucleotides

TCCCCCTCCTCCCCCTCCTCCCTCCTCCCCCTCCTCCCCCTCCTCCTCCCCCTCC
TCCCTCCTCCCTCCTCCCCCTCCTCCTCCTCCC and
GGGAGGAGGAGGAGGGGGAGGAGGGAGGAGGGAGGAGGGGGAGGAGGAG
GGGGGAGGAGGGGAGGAGGGAGGAGGGGAGGAGGGGGGA with *Eco*RI ends were
synthesized, phosphorylated with T4 kinase (Invitrogen), and annealed by heating to 95°C and
slowly cooling in 10 mM Tris-HCl pH 7.4, 1mM EDTA, and 50 mM NaCl. Duplex fragments
were purified from acrylamide gels and cloned into the *Eco*RI site immediately upstream of
the constitutive promoter in pLR. The tract was confirmed to be inserted in both the forward
(pFLR) and reverse (pRLR) orientations (grey and white arrows in Figure 1A) by sequencing.

The reporter plasmid pKR, containing a pACYC184 backbone, was created by PCR amplification of a cassette containing the overlapping lambda promoter right and lambda repressor operator sequences. The cassette was cloned immediately upstream of a kanamycin resistance gene cassette amplified from pACYC177, such that lambda promoter right drives the expression of kanamycin resistance under the control of lambda repressor (Figure 1B). The new pKR (kanamycin resistance) vector was transformed into DH5 α , and the transformed bacteria were made competent to create the reporter system. All plasmids were prepared in Terrific Broth, and harvested from the cleared lysate by CsCl ultracentrifugation [27].

pLR, pFLR, pRLR, and pUC19 as a negative control were used to transform the DH5 α reporter system. Cotransformants were selected and assayed for kanamycin resistance by inoculation into Luria Broth containing kanamycin ranging in concentration from 0 to 50 μ g/ml and spectrophotometric measurement at 650nm after 15 hours of incubation at 37°C 225rpm.

The purified plasmids pLR, pFLR, or pRLR were also transformed into wild type (AB1157), *ΔrecG* (DL1104), *ΔrecQ* (SWM1003), and *ΔrecQ ΔrecG* (N6916) cell lines and harvested following growth in 10ml Terrific Broth to stationary phase with the Wizard Plus Miniprep kit (Promega) using the EndA⁺ modification. Harvested plasmids were subsequently transformed into the DH5α reporter system. Mutation frequency was scored by the number of revertant colonies counted with an Alpha Innotech imaging system on kanamycin-containing plates per total number of viable bacteria as documented on dilution plates without kanamycin. Each reversion experiment was performed in triplicate. Mutation frequency of plasmids amplified in isogenic cell lines were normalized to that of CsCl-purified plasmid to account for mutations occurring from transformation [30], and the fold increase in mutation was measured as the ratio of normalized mutation frequency of the Pu-Py sequence containing plasmid (pRLR or pFLR) to that of the empty vector (pLR). Student's t-test was used to compare the risk of mutation as a function of the presence or absence of the helicase.

2.5. Mutation Detection Within the Sequence

The Pu-Py tract sequence used above was cloned into the *EcoRI* site of pBR325 as previously described [31,32]. The plasmid pBR325 contains three genes conferring antibiotic resistance to tetracycline, ampicillin, and chloramphenicol [33,34]. The unidirectional ColE1 origin of replication in pBR325 [35–38] makes the assignment of the leading and lagging strands possible. Cloning of the sequence in both orientations into the unique *EcoRI* site in the CAT gene disrupted the gene, rendering bacteria harboring the plasmid containing an intact insert chloramphenicol sensitive (Cm^s). In-frame deletions of the insert restored chloramphenicol resistance (Cm^r). The rate of in-frame deletion was scored as the number of revertant colonies on a chloramphenicol selective plate divided by the total number of viable bacteria as determined by serial dilutions plated onto nonselective media. Six repetitions were performed

for each plasmid in each cell line. Reversion rate was determined by the method of the median frequency, and values greater than two standard deviations from the median were discarded as jackpot mutations [39]. Statistical analysis was performed by Student's t-test on reversion rates between the various RecQ and RecG deficient cell lines harboring plasmid with the same insert orientation, as well as by the Mann-Whitney Rank Sum Test between the two insert orientations within an individual cell line. Revertant colonies were picked and grown under antibiotic selective pressure, plasmid harvested by Promega Miniprep and then retransformed into DH5 α cells. Chloramphenicol-resistant retransformants were analyzed by PCR screening, and then by sequencing.

2.6. Western Blot

Single colonies of pLR, pRLR, pFLR and pUC19 cotransformed into the DH5 α reporter system were selected and grown to stationary phase in 10ml Terrific Broth with carbenicillin. Bacterial lysates were prepared by sonication. After standardization of protein content by bicinchoninic acid (BCA) protein assay (Pierce), 1 μ g or 10 μ g of protein from each lysate were denatured by the Laemmli method [40], and separated by 12% SDS-PAGE. Gels were blotted to PVDF membrane, blocked with 5% nonfat milk in phosphate-buffered saline (PBS), and probed with either a 1:64 000 dilution of rabbit anti-lambda repressor antibody (Invitrogen), or a 1:256 000 dilution of rabbit anti-beta-lactamase antibody (Millipore). After washing with TBST (20 mM Tris, 140 mM NaCl, 0.1% v/v Tween-20, pH 7.5) the membrane was probed with a 1:3000 dilution of horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Biosciences). The membrane was again washed with TBST, and the membrane was developed by ECL Plus Western Blot Detecting kit (Amersham Biosciences) and imaged by autoradiography. Bands corresponding to the proteins of interest were quantified by densitometry using NIH ImageJ 1.37a software.

2.7. Plasmid amplification of a large Pu-Py tract and restriction fragment analysis

The plasmid pCW31 (kind gift from Peter Harris, Mayo Clinic, Rochester, MN) containing the entire 2.5kb Pu-Py tract from intron 21 of the *PKD1* gene in a pBlueScript II KS+ backbone was transformed into wild type (AB1157), Δ recG (DL1104), Δ recQ (SWM1003), and Δ recQ Δ recG (N6916) cell lines and selected by carbenicillin resistance. Single colonies of transformants were grown to stationary phase in 10ml of Terrific Broth. Plasmid was harvested, digested with *EcoRI* or *AluI*, and resolved by agarose gel electrophoresis, stained with ethidium bromide, and imaged.

3. Results

3.1. RecQ Facilitates Fork Progression through Triplex-forming DNA

To assess if helicase activity enhanced polymerase processivity through the mirror repeat sequence, RecQ and RecG helicases were used in primer extension reactions (Figure 2). Regardless of the addition of purified RecG, T7 polymerase shows normal processivity through a control construct, but is prevented from utilizing the triplex forming construct (Figure 2B). However, addition of purified RecQ improved the processivity (Figure 2C) and allowed a ten-fold increase in partial extension products when compared to the reaction with polymerase alone (12.1% vs. 1.3%, Figure 2D). Primer extension reactions with the addition of RecG and RecQ were also performed in the absence of ATP, effectively rendering the helicases dead and demonstrating that true helicase activity was required for primer extension on the triplex-forming template (data not shown).

3.2. Lambda Repressor Inhibits Kanamycin Resistance in Reporter System

To test lambda repressor expression and function, growth of the reporter system bacteria containing purified plasmids pLR, pFLR, pRLR, or pUC19 in the presence of kanamycin was measured turbidometrically. Without passage through repair-deficient cell lines, the repressor gene would not be mutated, and hence the bacteria should exhibit kanamycin sensitivity. As anticipated, reporter clones containing either pLR or pRLR showed a marked sensitivity to kanamycin at concentrations greater than 10 µg/ml, while pUC19 and, unexpectedly, pFLR showed kanamycin resistance up to 50 µg/ml. Recovered plasmid pFLR was free of mutation by sequence analysis and function was completely restored by removing the triplex forming sequence by restriction digestion and re-ligation. Movement of the insert site 50 bp and 500bp upstream to the promoter failed to alter this resistant phenotype, but movement 3' to the lambda repressor gene cassette restored kanamycin sensitivity.

3.3. Production of Lambda Repressor Protein Correlates with Kanamycin Resistance

Western blot analysis was used to assess lambda repressor protein production by reporter system clones harboring purified pLR, pRLR, pFLR, or pUC19 (Figure 3a). Lysates of reporter cells containing either pLR or pRLR demonstrated robust expression of lambda repressor while lysates from cells containing pFLR showed eightfold less lambda repressor expression as determined by densitometry. Lysates from cells containing pUC19 had no detectable lambda repressor present. Beta-lactamase, a constitutively expressed gene present in pUC-based vectors, was expressed at similar levels in pLR, pRLR, pFLR, and pUC19 (Figure 3b), indicating that the decreased expression of lambda repressor in clones harboring pFLR was not due to global suppression of plasmid-encoded protein production.

3.4. Loss of RecQ Significantly Increases Mutations Caused by Triplex-forming DNA

To measure the fidelity of replication in sequence flanking a mirror repeat Pu-Py tract, the novel dual plasmid mutation reporter system was used. This system employs the test plasmid pLR, or its derivative pRLR that contains triplex forming sequence, which is then transformed into DH5α cells harboring the P15 origin-containing reporter construct pKR. A P15-based system was chosen because of its compatibility with the pUC origin [41]. Assays with pFLR were not undertaken because the system failed in this orientation. In this reporter construct, lambda promoter right drives the kanamycin resistance gene such that when both plasmids were present, lambda repressor protein would bind to promoter right and repress the kanamycin resistance gene rendering the bacteria sensitive to the aminoglycoside. Point or frameshift mutations induced by the mirror repeat sequences cloned immediately upstream would interfere with the activity of the promoter, the N-terminal DNA binding [42] or C-terminal cooperativity domains [43,44] of lambda repressor, derepressing the kanamycin resistance gene and allowing for selection of the mutant clones.

The loss of RecQ accentuated the mutation frequency of the triplex forming construct (pRLR) eight to ten fold ($p < 0.03$) when compared to the wild type and RecG-deficient cell lines (Figure 4). Deficiency of both RecQ and RecG, however, completely abolished this increased mutation activity, suggesting that RecG may play a role in the increased mutation rate when RecQ is absent. To understand the mutation spectrum leading to this result, thirty revertants were sequenced. In all but one of the colonies analyzed, kanamycin resistance was the result of an inversion of the Pu-Py tract resulting in the equivalent of pFLR. In the remaining plasmid, a transposable element, IS-1, was inserted into the lambda repressor gene, emphasizing the sensitivity of our system.

3.5. Role of Triplex-forming Sequence and Replication Orientation on Tract Stability

Mutations within the Pu-Py tract itself would be silent in the above dual plasmid reporter system, therefore a mutation detection system was used that reconstituted a selectable marker when an in-frame deletion of the tract occurred. The mirror repeat Pu-Py sequence used previously in the dual plasmid reporter system was cloned in both orientations into the *EcoRI* site of the CAT gene in pBR325, rendering clones harboring this plasmid sensitive to chloramphenicol. Reversion analysis of all the RecQ- and RecG-deficient isogenic bacterial strains with these constructs revealed between a hundred and a thousand fold increased mutation rate ($p < 0.0022$) when the polypyrimidine strand was in the leading versus the lagging strand of replication (Figure 5). The spectrum of mutation varied based on the orientation of the tract (Figure 6). Revertants containing the polypyrimidine strand in the leading strand (pBRR) had a variety of partial in-frame deletions between 41 and 74bp in length that disrupted the mirror repeat symmetry, whereas revertants containing the polypyrimidine strand in the lagging strand (pBRF) were identified as having undergone complete deletion of the tract and one of the flanking *EcoRI* site direct repeats. Comparing reversion rates of pBRR between the various isogenic cell lines in Figure 5, the $\Delta recQ$ (SWM1003) strain was not statistically different from wild type (AB1157) (44.8×10^{-6} vs. 49.7×10^{-6} , $p = 0.85$). However, reversion rate of the $\Delta recG$ strain (DL1104) was significantly decreased from wild type (12.4×10^{-6} , $p = 0.0001$) and reversion rate of the double mutant strain was significantly increased from wild type (131.1×10^{-6} , $p = 0.008$). Likewise the double mutant exhibited a higher reversion rate than the $\Delta recQ$ (SWM1003) strain ($p = 0.016$).

3.6. RecG is Critical for Long Pu-Py Tract Stability

The results from the two systems above support the hypothesis that RecQ may help prevent replication fork blockade at triplex forming sequences and that RecG may facilitate fork restart, albeit with imperfect fidelity, on the 88 bp mirror repeat containing Pu-Py tract. We postulated that due to unresolved stalled replication, strains without functional RecG would exhibit greater instability of the entire *PKDI* 2.5 kb mirror repeat-containing Pu-Py tract. To test this hypothesis, pCW31, which contains this tract, was propagated in isogenic bacteria including the parental, $\Delta recQ$, $\Delta recG$, and double mutant cell lines. After propagation, harvested plasmid was digested with either *EcoRI* to linearize the plasmid or *AluI* to isolate the 2.5 kb tract and electrophoretically resolved on an agarose gel (Figure 7). Compared to stock plasmid, plasmids amplified in parental and $\Delta recQ$ cell lines revealed Pu-Py tract stability with preservation of the tract and no detectable evidence of gross deletion. In contrast, plasmids amplified in the $\Delta recG$ cell line showed a 2.5kb deletion in all plasmids tested, corresponding to the entire *PKDI* Pu-Py tract. Plasmids amplified in the double mutant ($\Delta recG \Delta recQ$) strain revealed an attenuated propensity for deletion, with a mixed population of both unchanged plasmid and plasmid containing the 2.5kb deletion as seen in the $\Delta recG$ cell line.

4. Discussion

The findings of these studies demonstrate the potent mutagenic effects of mirror repeat Pu-Py tracts both within the *PKDI* Pu-Py tract itself as well as the surrounding sequence and the important role of specific helicases in preserving genetic stability at such DNA sequences (Figure 8). The mutagenic potential of such tracts can be keenly dependent on the orientation encountered by the replication fork.

Several members of the RecQ subfamily, specifically bacterial RecQ and mammalian WRN and BLM helicases, melt triplex [20] and G-quartet (G4) DNA [19,22]. We hypothesized that inactivation of RecQ helicases would accentuate mutagenesis surrounding Pu-Py tracts due to the increased replication blockade. We demonstrated an eight to tenfold increased risk of reversion of a triplex-forming plasmid in a RecQ-deficient background. Sequencing of these

mutants show the overwhelming majority (>96%) involve inversion of the Pu-Py tract into the permissive orientation. Such an inversion event has been described in the inversion of the bacterial FimA3 promoter regulating fimbrial protein production [45], acting as a genetic “on-off” switch. The inversion bias appears to be influenced by the degree of negative supercoiling [46] and directed by recombinases such as HbiF, an invertase [47]. This suggests that the inversion event that occurred in our system could be mediated by recombination, and stimulated by stabilized triplex formation in the absence of RecQ. This would be consistent with the hyperrecombination phenotype of RecQ deficiencies in humans, such as Werner and Bloom syndromes [23].

RecG helicase participates in fork regression and replication restart reactions. Both BLM and WRN helicases have a similar fork regression activity [24]. We did not find an increase in mutation frequency in sequence adjacent to triplex-forming Pu-Py tract in a RecG-deficient background, but we did find that loss of RecG helicase appeared to confer an approximately four fold decrease in mutation of the 88 bp tract itself as seen in previous studies [48], suggesting that fork reversal by RecG may be an error-prone repair mechanism on repeated sequences. Loss of both RecG and RecQ caused an approximately three-fold increase in mutation frequency over that of wild type and RecQ-deficient cells of the 88bp tract, suggesting a synergism between both helicases. RecG did appear to be critical to maintaining stability of large Pu-Py tracts as evidenced by deletion of the entire 2.5kb tract in pCW31, but with the additional loss of RecQ, the efficiency of complete deletion was dampened suggesting that RecQ may be participating in the deletion of the large tract by its role as a component of the RecFOR recombination pathway.

Structure-mediated mutagenesis can be influenced by the orientation of the sequence. The expansion of (GAA·TTC)_n triplet repeat sequences within the *FRDA* gene responsible for Friedreich’s ataxia has been ascribed to triplex formation in the nascent strand and pausing of replication machinery, leading to polymerase misalignment. [49]. These triplet repeat sequences may also undergo deletion due to structure formation in the template strand [50], absorbing negative superhelical tension [51]. We found both complete and partial deletions when a Pu-Py tract was inserted into the CAT gene of pBR325. The two to three log order difference between orientations of the tract suggest that mutagenic triplex forms preferentially when the polypyrimidine sequence is in the leading strand, similar to hairpin formation studied by Sinden et al in this system [32]. Furthermore, the nature of mutations as shown in Table 1 suggests that when triplex forms in this unidirectional plasmid in the leading strand, partial deletions occur. Loss of RecQ helicase did not appear to increase reversion rate above that of RecQ⁺ bacteria, but due to the nature of the assay out-of-frame deletions would remain chloramphenicol-sensitive and be undetected.

We detected a decreased expression of lambda repressor in the plasmid containing the Pu-Py tract in one orientation (pFLR), preventing its use in reversion analysis. This orientation-specific effect may be the result of the formation of replication-stalling structures by the tract, biasing the direction of replication of the pUC-based plasmid to preferentially create a “head-on” collision of the replication fork with elongating transcription machinery [52,53]. This hypothesis is supported by the persistence of the effect on lambda repressor expression independent of distance from the promoter/lambda repressor cassette, and restoration of kanamycin sensitivity and thus lambda repressor expression with removal of the tract, as well as placing the sequence downstream of the repressor gene. An alternative explanation of this orientation-specific suppression of lambda repressor expression is the production of non-coding regulatory RNA (reviewed in reference [54]) or the cryptic presence of a clustered regularly interspaced palindromic repeat (CRISPR) sequence [55] from within adjacent pUC backbone. Deletion of the pUC backbone between the Pu-Py tract and the beta-lactamase gene of pFLR did not alter the orientation-specific effect (data not shown), suggesting that this

possibility is unlikely. The expression of other plasmid-encoded proteins such as beta-lactamase (Figure 3), plasmid copy number, methylation, and organism growth rates (data not shown) were also found to be similar between the different plasmids.

In summary, these studies suggest that RecG and RecQ serve distinct roles in maintaining replication fidelity and genomic stability of Pu-Py tracts. Mirror-repeat tracts may require RecQ activity to melt alternative DNA structures such as triplex to prevent fork blockade and sequence-mediated mutagenesis, but a series of these inverted repeats in tandem may provoke replication fork pausing and collapse, necessitating RecG activity to facilitate fork regression to maintain the stability of such large tracts.

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Figure 1. Dual plasmid reporter system

A. Plasmid pLR, which contains the lambda repressor gene downstream of a constitutive promoter and an *EcoRI* restriction site. Plasmid pFLR contains the 88bp overlapping inverted mirror repeats from the *PKD1* Pu-Py tract cloned into the *EcoRI* site upstream of the promoter/lambdarepressor cassette in the forward orientation (white arrow). Plasmid pRLR contains the 88 bp insert in the reverse orientation (grey arrow). B. Reporter plasmid, pKR. The kanamycin resistance gene is under the control of lambda promoter right, containing three lambda repressor operator sites. This is a strong promoter except in the presence of lambda repressor that binds cooperatively to the operator and thereby suppresses transcription.

Figure 2. RecQ, but not RecG, partially alleviates triplex formation and blockade of primer extension reactions

A. Design of radiolabeled primer on the synthetic mirror repeat containing substrate. B. Primer extension using T7 DNA polymerase on a purine-rich template ('R') capable of forming triplex DNA during extension, or a control template ('C') with no triplex-forming sequence with the addition of RecG helicase. C. Same templates as in B, but using RecQ helicase. Note the partial extension bands (bracket) in the primer extension reactions indicating polymerase utilization and read-through of the triplex forming portion of the 'R' construct. D. Densitometric evaluation of primer extension reactions shown in (B) and (C). Addition of RecQ, but not RecG, to the primer extension reaction of the 'R' construct produced a ten-fold increase in partial extension products (12.3% vs. 1.3% of total activity). Primer extension reactions were carried out in the absence of single-stranded binding protein (SSB), decreasing the overall efficiency of all reactions, including those of the control 'C' template (note 55–65% of total activity).

Figure 3. Lambda repressor protein expression is decreased in bacteria with kanamycin resistance, whereas other constitutive protein expression is unchanged

A. Western blot of lambda repressor in lysates from DH5 α clones harboring pLR vs. pFLR vs. pRLR vs. pUC19 as well as the reporter construct pKR. 10 μ g of lysate were run per sample. Strong expression of lambda repressor is seen with pLR and pRLR, but minimal expression with pFLR and absent expression with pUC19 is seen when cotransformed with the reporter construct. B. Western blot of beta-lactamase in lysates from DH5 α clones harboring pLR vs. pFLR vs. pRLR vs. pUC19 as well as the reporter construct. 1 μ g of lysate was run per sample. Strong expression of beta-lactamase is seen with all lysates at approximately equivalent levels.

Figure 4. Deficiency of RecQ, but not RecG, increases the mutation frequency of triplex-forming Pu-Py tracts in surrounding sequence

Risk of reversion was assessed in pRLR compared to pLR in wild type (AB1157), $\Delta recG$ (DL1104), $\Delta recQ$ (SWM1003), and $\Delta recQ \Delta recG$ (N6916), cells. An eight to tenfold increase in mutation frequency is noted of triplex-forming plasmids amplified in the absence of RecQ, but not in the RecQ RecG double mutant. Numbers above the bars depict mean value.

Figure 5. Deficiency of both RecQ and RecG leads to increased mutation within a triplex forming Pu·Py tract

Reversion analysis was assessed in pBRR vs. pBRF in wild type (AB1157), $\Delta recG$ (DL1104), $\Delta recQ$ (SWM1003), and $\Delta recQ \Delta recG$ (N6916) cells. In all cell types, plasmids with the polypyrimidine strand of the Pu·Py tract in the leading strand (pBRR) have a two to three log order increase in mutation rate than those that form triplex in the lagging strand (pBRF). Numbers above the bars depict mean value.

pBRR

32/40 = 80%
 3/40 = 7.5%
 1/40 = 2.5%
 1/40 = 2.5%
 1/40 = 2.5%
 1/40 = 2.5%

GAATTCGGGAGGAGGAGGGGGAGGAGGGAGGAGGGAGGAGGGGGAGGAGGAGGGGGAGGAGGGGAGGAGGGGAGGAGGGGGAGGAGGGGGACGAATTC

pBRF

20/20 = 100%

GAATTCGCCCCCTCCTCCCCTCCTCCCTCCTCCCCTCCTCCCCTCCTCCTCCCCTCCTCCTCCTCCTCCTCCCCTCCTCCTCCTCCTCCTCCGAAATTC

Figure 6. Mutation spectrum of the triplex-forming plasmids pBRF and pBRR, as determined by sequencing of retransformed plasmids from revertant (Cm^r) colonies

The full-length tracts are shown with the overlapping mirror repeat sequences denoted under the tract with apposed arrowheads. Mutations are denoted above the sequence by black bars, with the frequency of identification to the left of each mutation. With pBRR, a number of mutations were isolated that deleted one or both mirror repeat sequences, whereas with pBRF mutations, all mutations isolated involved deletion of the entire tract and one of the flanking *EcoRI* sites.

Figure 7. RecG is required for the stability of large Pu-Py tracts

EcoRI and *AluI* restriction digests were performed on pCW31 amplified in wild type (AB1157), $\Delta recQ$ (DL1104), $\Delta recQ$ (SWM1003), and $\Delta recQ \Delta recG$ (N6916) cells. Compared to lane I (stock plasmid), plasmids in lanes A–H harvested from wild type and $RecQ^-$ cells have stable linear size (*EcoRI* digestion) and size of the 2.5kb *PKD1* Pu-Py tract (*AluI* digestion). However, plasmids harvested from $RecG^-$ cells reveal a large 2.5kb deletion that corresponds to the Pu-Py tract, and plasmids harvested from $RecG^- RecQ^-$ cells have a mixed population of plasmid, both unaltered and mutant.

Figure 8. Model of RecQ and RecG helicases in replicative stress from triplex-forming sequences
 When the replication fork encounters sequence with mirror repeat symmetry such as a polypurine-polypyrimidine tract, alternative structures such as triplex may form, pausing the fork. In the presence of RecQ, the triplex may be melted and replication may proceed. In the absence of RecQ, the stalled fork may be reactivated by the fork regression activity of RecG. Either unresolved triplex formation or the inability to restart a stalled fork may lead to fork collapse, and ultimately a double strand break.