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Replication fork regression in repetitive DNAs

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ABSTRACT

Among several different types of repetitive sequences found in the human genome, this study has examined the telomeric repeat, necessary for the protection of chromosome termini, and the disease-associated triplet repeat $(CTG)\cdot(CAG)_n$. Evidence suggests that replication of both types of repeats is problematic and that a contributing factor is the repetitive nature of the DNA itself. Here we have used electron microscopy to investigate DNA structures formed at replication forks on large model DNAs containing these repeat sequences, in an attempt to elucidate the contributory effect that these repetitive DNAs may have on their replication. Visualization of the DNA revealed that there is a high propensity for a paused replication fork to spontaneously regress when moving through repetitive DNAs, and that this results in a four-way chickenfoot intermediate that could present a significant block to replication *in vivo*, possibly leading to unwanted recombination events, amplifications or deletions.

INTRODUCTION

Repetitive DNA sequences found in the human genome consist of repeat units ranging from mono-, di- and tri-nucleotide repeats to long repeating units found in Alu and LINE elements. Overall, repetitive DNA makes up ~30% of the human genome with the Alu and LINE elements constituting the greatest amount (1). The short sequence units which include the triplet and telomeric repeats are of particular interest due to the high number of repeats per unit length of DNA which may bestow unique biological and physical properties.

The triplet repeats which include $(CGG)_n\bullet(CCG)_n$, $(CAG)_n\bullet(CTG)_n$ and $(GAA)_n\bullet(TTC)_n$ have been implicated in numerous human hereditary diseases, a hallmark of which is the appearance of disease pathology when the repeat blocks expand beyond certain tight length thresholds generally exceeding 35 repeats (2). In addition, tetrameric $(CCTG)_n\bullet(CAGG)_n$ (3), pentameric $(AATCT)_n\bullet(AGATT)_n$ (4) and dodecameric $(C_4GC_4GCG)_n\bullet(CGCG_4CG_4)_n$ (5) repeats have been linked to the genetic diseases myotonic dystrophy type 2 (DM2), spinocerebellar ataxia type 10 (SCA10) and progressive myoclonus epilepsy, respectively. The length of disease-related repeats can vary from as little as a few repeats in normal individuals to up to 40 kb in the SCA10 expansions (6). Whereas the exact mechanism of repeat expansion in humans remains unknown, one feature common to all expanded repeats is that they are highly unstable above a threshold of ~100–200 bp (7).

Telomeric repeats which are composed of the hexameric unit TTAGGG in all mammals and many animals (TTTAGGG in plants) are essential for chromosome stability and regulating the replicative lifespan of somatic cells (8). These repeats comprise the DNA component of the telomere (9,10), a nucleoprotein structure which protects the ends of chromosomes and enables cells to distinguish telomeric ends from random double-strand (ds) break ends (11,12).

Telomeric repeats can reach lengths of 15 kb in humans and as much as 150 kb in plants. In the absence of telomerase, a telomere reverse transcriptase, telomeric repeat sequences are gradually lost during cell division, due in part to the 'end replication problem' that results from the inability of the lagging strand to be replicated to the very end of the chromosome (13,14). Large blocks of telomere repeat sequences can also be lost stochastically when the proteins required for end protection functions are disrupted or problems are encountered during DNA replication or repair (8). In the absence of telomerase, certain human cancer cells have been shown to exhibit highly unstable telomeres (ALT phenotype) with rapid increases or decreases in telomere lengths (15–17).

Evidence suggests that the nature of repetitive DNA may itself be a causative factor in mutagenesis (18–21). The relative instability of long blocks of short repeats may also be related to inherent difficulties of the DNA synthesis machinery in replicating through this type of DNA. A large body of evidence shows that there is frequent polymerase pausing in triplet blocks, that both the lagging and leading strands may form hairpins, G-quartets or triplex structures when composed of certain repeats, that the polymerase can slip during synthesis through repeat tracts, and that primer template misalignment can occur as a result of hairpins in the template strands [reviewed in (7,22–24)]. Also,

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telomeric sequences undergoing replication have the potential of forming G-quartets. These impediments to the replication fork could then give rise to repeat expansions or deletions due to reiterative DNA synthesis or replication restart via recombination intermediates. Indeed, recent evidence in *Escherichia coli* suggests that a major mechanism for $(CAG)_n \cdot (CTG)_n$ repeat instability is replication restart, via a Holliday junction 'chickenfoot' intermediate, after DNA polymerase pausing and the resultant collapse of the replication fork (25,26).

Relatively little is known about the replication of mammalian telomeric DNA. Overexpression of the telomeric-binding proteins, TRF1 and TRF2, has been shown to lead to replication fork stalling *in vivo* (27) and *in vitro* human telomeric DNA is replicated much less efficiently than non-telomeric DNA [(27) and N. Fouché, unpublished data]. Further in addition to the normal replicative helicases present at forks, the RecQ helicases WRN and BLM, implicated in premature ageing diseases, have been shown to be important for proper telomere replication and maintenance in human cells (28–30). These helicases have been shown to unwind G-quartets and four-stranded junctions similar to chickenfoot structures (31–36), suggesting that these or other secondary structures unique to telomeric repeats may form and present barriers to replication.

These observations suggest that the polymerase machinery is much more prone to pause or stall during replication of long blocks of short repeats. In the absence of stabilizing proteins, this could lead to fork regression and the generation of four-stranded chickenfoot molecules (as shown in Figure 2B). Resolution of these chickenfoot intermediates could lead to restoration of replication but inappropriate resolution could lead to expansion or contraction of the DNA tracts or possibly the generation of extrachromosomal repeat DNA.

To begin testing this hypothesis, we generated model replication fork templates which mimic a replication fork that has transited a long block of either telomeric repeats or triplet (CTG) repeats. Examination of the structure of this stalled fork by EM provided a means of detecting forks which may have either fully regressed or partially regressed leading to a chicken foot structure. Previously we used this model system with sequences which were not repetitive and we showed that chickenfoot structures could be observed when p53 was present to trap these forms during fork regression (37). In this paper, we show that nearly half of all repeat-containing templates had spontaneously regressed, with a large percentage of molecules forming chickenfoot intermediates. These four-way junction molecules were not observed in the absence of p53 in our previous study of non-repetitive DNA (37). We discuss a model in which repetitive DNA is highly slippery with a high tendency to generate long-lived chickenfoot structures and the implicit implications related to triplet and telomere DNA stability.

MATERIALS AND METHODS

Construction of plasmids

Complementary oligonucleotides (oligos) (i) 5′-TCGAAGAC TTAGGGGCTGAGGTT-3′ and (ii) 5′-CCCTAACCTCAGC CCCTAAGTCT-3′ containing a site for the nicking endonuclease N.BbvC IA (New England Biolabs, Beverley, MA) were annealed and cloned into the XhoI and BbsI sites of the plasmid pRST5 (38), to create pRST5NICK. Dr Y. H. Wang (UMDNJ) generously provided the plasmid $pGEM(CTG)_{130}$, which was constructed by digesting the pSH2 plasmid (39) with restriction endonucleases SacI and HindIII (New England Biolabs) to isolate the CTG fragment, and then cloning this fragment into the same restriction sites in the plasmid pGEM3zf(+) (Promega Corporation, Madison, WI) (Y. H. Wang, unpublished data). Site-directed mutagenesis was performed on the plasmid pGEM(CTG)130, resulting in the creation of two sites for the restriction endonuclease BsmI (New England Biolabs) directly adjacent to, and on either side of, the CTG repeat tract. During this process, some CTG repeats were lost, resulting in a tract length of [CTG]110. The complementary oligos (i) 5′-TCAGCCAGGCCGAAAGAA-AGAAAAGGACAGAGAAAGCC-3′ and (ii) 5′-CTTTCTC-TGTCCTTTTCTTTCTTTCGGCCTGGC-3′ were annealed and cloned into the BsmI and BbvCI sites of this plasmid, downstream of the CTG repeat tract, generating a 221 bp region devoid of As followed by a site for the nicking endonuclease N.BbvC IB (New England Biolabs). During this process, more CTG repeats were lost, generating the plasmid $p(CTG)_{60}$NICK with a final tract length of $[CTG]_{60}$. Plasmid constructs were confirmed by DNA sequencing (The DNA Facility, Office of Biotechnology, Iowa State University).

Construction of replication fork templates

Model replication forks were synthesized by a modification of the method described by Subramanian and Griffith (37). In short, the plasmid pRST5NICK was digested with N.BbvC IA to generate a nick in the G-rich strand at the start of the telomeric tract $[TTAGGG]_{96}$. Similarly, the plasmid $p(CTG)_{60}$NICK was digested with N.BbvC IB to generate a nick in the A-rich strand at the start of the 221 bp region containing the CTG repeat tract. The nicked DNA was incubated with the Klenow fragment (exo^-) of DNA polymerase 1 (New England Biolabs), and 0.5 mM each dTTP, dATP, dGTP (telomeric template) or dGTP, dCTP, dATP (CTG template) to generate a single-strand (ss) tail by strand displacement of the repeat tract. The ss tail was then converted to a ds tail by annealing a 228-fold molar excess of the oligo 5′-CCCTAACCCTAACCCTAACCCTAA-3′ (telomeric template) or a 120-fold molar excess of the oligo 5′-CTGCTGC-TGCTGCTG-3′ (CTG template) to each template for 30 min at 37°C in 100 mM NaCl, then ligating with T4 DNA Ligase (400 U; New England Biolabs) at 16°C overnight in a buffer containing 50 mM NaCl, 10 mM Tris–HCl (pH 7.9) and 1 mM dithiothreitol. The plasmids were then linearized with XmnI (New England Biolabs), allowing detection of the replication fork junction relative to the DNA ends, and measurement of the asymmetrically generated long linear segments of the plasmid, to occur (Figure 1).

Electron microscopy

Replication fork template DNA was diluted to 1 μg/ml in 10 mM HEPES, pH 7.5, 0.1 mM EDTA and prepared for EM visualization as described previously (40). An FEI Tecnai 12 instrument was used at 40 kV and images were photographed using a Gatan US4000SP Ultrascan camera.

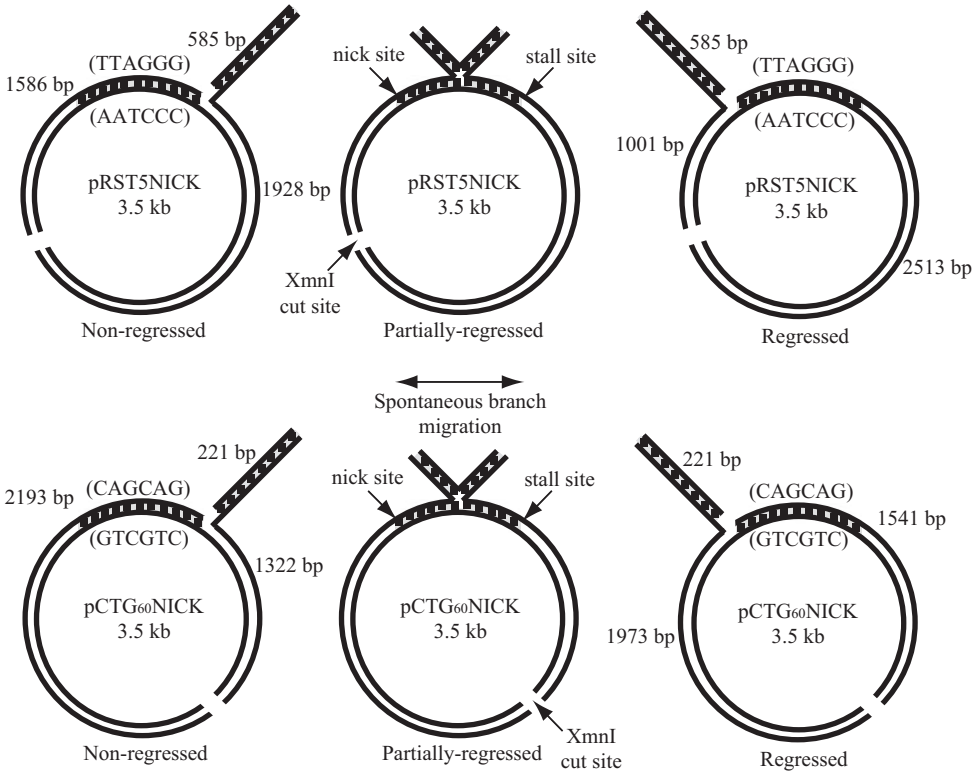

Figure 1. Schematic representation of replication fork templates. Details of the synthesis steps are in Materials and Methods. The telomeric replication fork template was constructed on the plasmid pRST5NICK and the CTG repeat template was made using the $pCTG_{60}$NICK plasmid. The lengths of the long linear segments of the plasmid are indicated in both the non-regressed and fully regressed forms of molecules containing only a single ds tail. Patterned regions indicate repetitive DNA. Positions of the nicking site, the site of replication stalling and the XmnI restriction site used to linearized the plasmid DNA are shown in the center panel of each template diagram.

Molecule lengths (in nm) were measured from Gatan digital images using Gatan Digital Micrograph software and converted to base pair.

The mean and standard deviation of the total DNA length (nm) of all measured molecules were determined, per experiment, and only molecules falling within the range: mean ± SD, were used to determine percent of molecules regressed or non-regressed.

RESULTS

Synthesis of replication fork templates

Synthetic model replication forks were prepared by nicking the repeat-containing plasmids pRST5NICK and $p(CTG)_{60}$-NICK adjacent to the repeat tract and replicating in the absence of one of the four nucleotides. Replication through the repeats stalled at the end of the tract, generating a ss tail that was converted to a ds tail by annealing and ligating complementary oligos along the length of the displaced strand. In both cases, the positions of the nicking sites and sites of replication fork stalling were sufficiently close to (within 2–4 bp of) the repeat tract that there was very little non-repeat DNA present in the ss tail and most of the displaced ss DNA could be converted to ds DNA by ligating complementary oligos. The plasmids were then linearized so that the position of the replication fork junction relative to this restriction site could be determined by measuring the length of each of the longest segments of the replication fork template from the DNA end up to the fork junction (Figure 1).

Visualization of replication fork templates

Visualization of the replication fork molecules containing telomeric or CTG repeat tracts revealed an array of DNA configurations (Figure 2). For both model DNAs the most common species consisted of a linear replication fork template (Figure 2A, C, E and G) containing a single ds tail. Also present were molecules containing two shorter ds tails (shown at higher magnification in Figure 2B, D, F and H). These are typical of chickenfoot intermediates generated by fork regression as seen by EM (37). Six separate preparations of telomeric DNAs and three separate preparations of the CTG triplet repeat template were scored, with >200 molecules counted per experiment. On average, 32 ± 10% of all telomere model DNAs contained a chickenfoot structure within the repeat tract whereas 15 ± 7% of the CTG repeat-containing DNAs contained such structures (Figure 3). The substantial presence of these four-way junctions is highly significant, since these structures were absent in similar preparations of non-repeat-containing replication fork templates synthesized under the same conditions and prepared for EM using the identical protocol (37). Thus, the

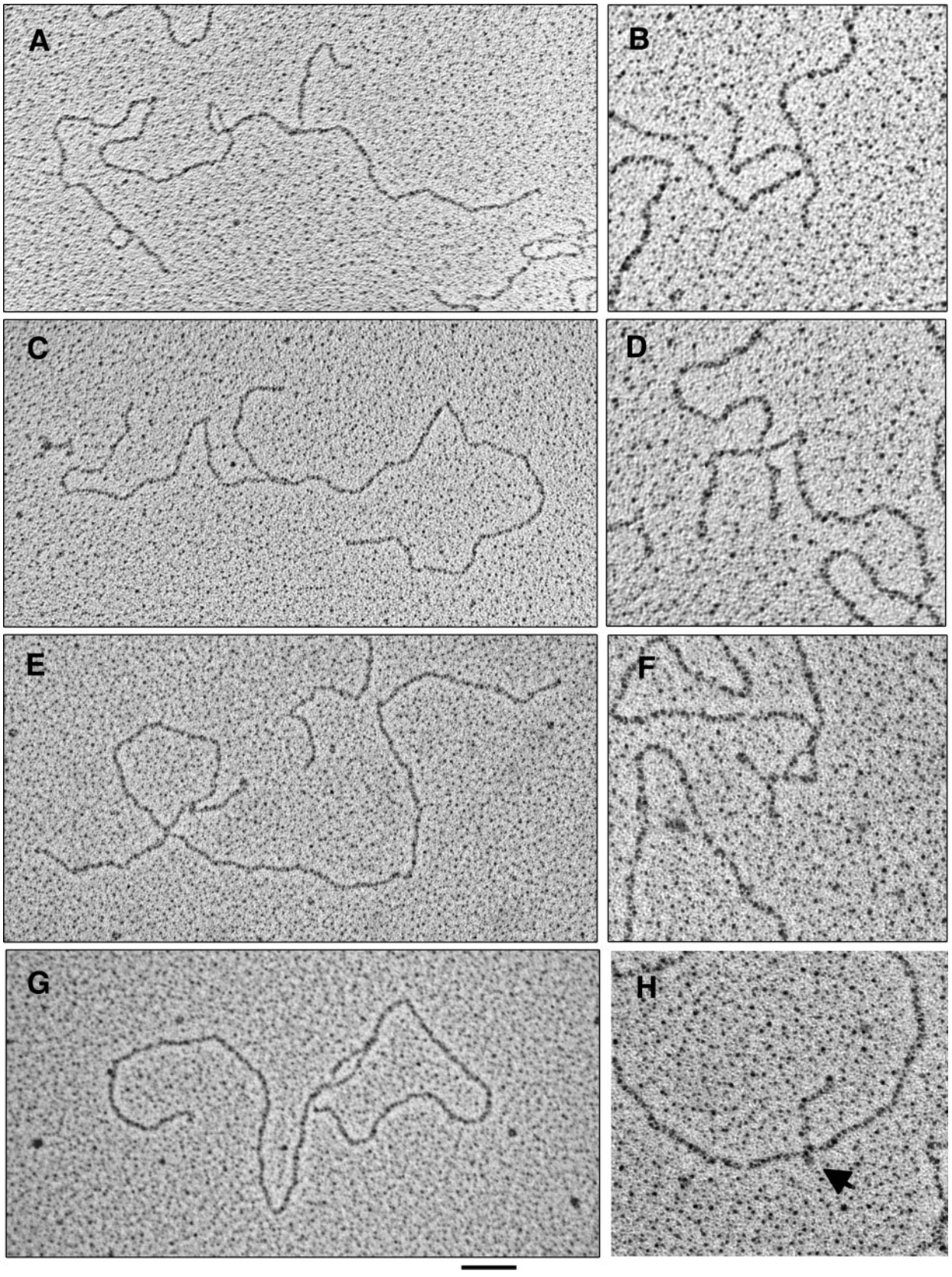

Figure 2. Visualization of DNA configurations by EM. Model replication forks were prepared for EM by mounting on carbon-coated EM grids and rotary shadowcasting with tungsten (Materials and Methods). Examples of linear molecules seen include replication fork templates comprising telomeric (**A**–**D**) or CTG (**E**–**H**) repeats and containing only a single ds tail (A, C, E and G) or two shorter ds tails (B, D, F and H). Bar is equivalent to 150 bp in panels showing full-length molecules.

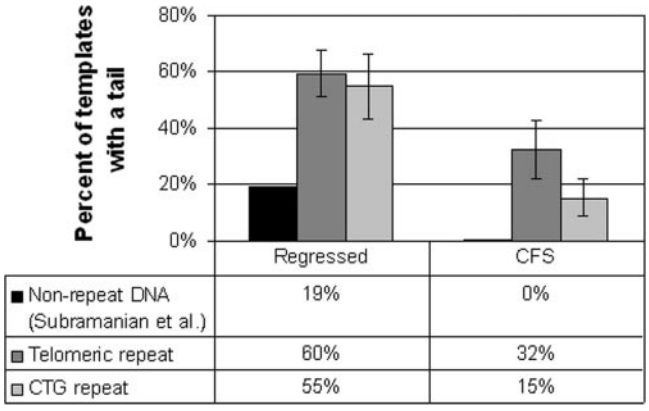

Figure 3. Graph of spontaneous regression of replication forks *in vitro*. All tailed molecules were counted and measured, per experiment, and the average fraction of these molecules that were determined to have regressed was calculated (lane 1). Previously reported values for the regression of non-repeat DNA (37) allowed for comparison to the repeat DNA results. The average fraction of chickenfoot structures visualized by EM was also graphed as a percentage of all tailed molecules seen (lane 2).

appearance of these chickenfoot structures must reflect the result of spontaneous fork migration caused by features unique to repetitive DNA.

Spontaneous replication fork regression in repetitive DNAs

Template DNAs were linearized so that the repeat block measured from 28 to 45% (telomere repeat) or 38 to 44% (CTG repeat) of the total DNA length from the nearest end, providing a means to uniquely determine the position of the replication fork (Figure 1). Molecules containing a single ds tail were photographed, and a minimum of 75 molecules for each template were analyzed to determine the position of the replication fork junction (see Materials and Methods). This was necessary because molecules containing a single ds tail could represent one of the three types of replication fork templates—unregressed; fully regressed; or partially regressed chickenfoot molecules in which one of the ds tails was too short to be visualized by EM (Figure 2H, arrow). This was particularly important in the case of the CTG template, where the repeat tract was relatively short and the likelihood was high that one of the two ds tails of the chickenfoot intermediate would go unobserved.

Telomeric forks were considered to have begun regressing when the longer segment of the plasmid was >1928 bp plus one standard deviation of the mean total DNA length, per experiment. Similarly, the CTG repeat forks were considered to have begun regressing when the shorter segment of the plasmid was >1322 bp plus one standard deviation of the mean total DNA length, per experiment. On the average, 60 ± 8% of telomeric replication forks and 55 ± 12% of CTG repeat forks had regressed to some degree (Figure 3).

Previously, when replication fork molecules which lacked any repetitive sequences at or near the fork and which contained only a single ds tail were examined, only 19% of all molecules were found where measurement of the long linear segments indicated that the replication fork had begun to regress (37). Spontaneous replication fork regression in repetitive DNA was therefore determined to be 41 and 36% higher in telomeric and CTG repeat DNA, respectively, than in non-repeat DNA. These results suggest that there is a high propensity for a paused replication fork to spontaneously regress when moving through repetitive DNA, resulting in a chickenfoot intermediate that would present a significant block to replication, requiring the action of recombination proteins to restart replication.

DISCUSSION

In this study we used EM to visualize stalled replication forks containing long runs of repetitive DNA sequences. We have shown that these forks have a much greater tendency to spontaneously regress, resulting in four-stranded chickenfoot intermediates, than non-repeat-containing DNA. Specifically, 60% of telomeric and 55% of CTG repeat forks had regressed to some degree, in contrast to 19% regression seen in non-repeat DNA. Of particular interest were the greatly increased fractions of chickenfoot molecules seen in the repeat-containing DNAs: 32% of the telomeric templates and 15% of the CTG repeat templates were in four-stranded chickenfoot forms. In contrast, our previous study of non-repeat-containing forms revealed that these four-stranded intermediates were absent unless p53 was present to trap them (37).

The accumulation of chickenfoot structures is intriguing, given that four-way junctions have a higher number of broken base pairs and are likely less energetically favorable than three-way junctions. A possible explanation may be that chickenfoot structures carrying repetitive runs may be stabilized by additional secondary structures or repeat slippages in each of the repeat-containing DNA arms. However, we did not see any T- or Y-shaped protrusions in the slipped DNA arms, arguing that if they are present, they are not large. These DNAs seem inherently more 'slippery' than non-repeat-containing DNA, such that the replication forks are able to more easily transition back and forth between non-regressed and fully regressed states. During replication, repeat-containing DNA could therefore spend a significantly larger fraction of time in the partially regressed state than other DNAs and this could possibly account for the large percentage of chickenfoot structures seen. This model assumes that fork regression would be equal, regardless of the orientation of the repeats. However, since our studies concentrated on just one orientation for both repeats studied, we cannot rule out the possibility of orientation dependence on replication fork regression in these DNAs.

This study has thus revealed a new feature of repetitive DNA that could present a significant barrier to replication. Furthermore, we believe that the four-stranded chickenfoot structures could present a significant problem to the cell, resulting in the recruitment of unwanted recombination factors or leading to deleterious recombination events if repaired. We have shown in our laboratory that p53 will bind to chickenfoot structures with great affinity (37) and it greatly increases the rate of Holliday junction cleavage by resolvase enzymes (41) *in vitro*. Recently, similar results have been shown for the homologous recombination DNA repair protein XRCC3 in complex with Rad51C

(S. Compton, unpublished data). Thus, an abundance of chickenfoot structures in the cell may have significant downstream consequences for cellular signaling and DNA repair.

Although the number of chickenfoot structures present in CTG repeat-containing DNA was approximately half of that seen in the telomeric samples, we believe that this number would have been higher if the repeat tract in the plasmid was longer. From measurements of the long linear segments of the plasmids, it was determined that approximately the same number of molecules had regressed to some degree in the telomeric samples as in the CTG repeat samples. The triplet repeat DNA therefore appears to be as slippery as the telomeric DNA. Thus it seems probable that a large percentage of the chickenfoot molecules in the CTG repeat samples contained one ds tail that was too short to be visualized by EM (Figure 2H, arrow). Also, we found that annealing of the oligos along the ss tail to make the ds tail in these samples was somewhat inefficient, resulting in replication fork templates containing a ds tail shorter than the expected 221 bp. Although the presence of ssDNA regions in the displaced tail may have had the ability to bind back to the template, resulting in replication forks with a loop at the fork junction, no evidence of these structures was seen. More significantly, ssDNA regions resulting from poor oligo annealing did not seem to interfere with the ability of the replication forks to regress to a high degree, and binding back of the displaced ss tail to the template could not account for the chickenfoot structures seen.

Because of the considerable instability of repeat tracts in bacteria, this study was limited to the telomeric repeat TTAGGG and the triplet repeat $(CAG)_n\bullet(CTG)_n$. However, in the future studies with replication fork templates containing sufficiently long stretches of the repeats $(CGG)_n\bullet(CCG)_n$, $(GAA)_n\bullet(TTC)_n$, $(CCTG)_n\bullet(CAGG)_n$, $(AATCT)_n\bullet(AGATT)_n$ and $(C_4GC_4GCG)_n\bullet(CGCG_4CG_4)_n$ will be important to extend and generalize these observations to all of the known disease-related repeats.

Our observations are therefore consistent with repetitive DNA being a poor substrate for replication *in vitro* (22,27,42,43). The existence of stable four-stranded chickenfoot structures may explain the need for additional helicases such as BLM and WRN for efficient replication through telomeres *in vivo* (28–30,44,45). The data also favor a model for expansion of disease-related repeats that involves replication restart via chickenfoot intermediates, particularly in human cells where the repeat blocks can be much longer than those investigated in the bacterial model study (25).

Interestingly, whereas replication of telomeric DNA tends to stall *in vitro* [(27) and N. Fouché, unpublished data] and the G-rich strand may form G-quartets *in vivo* (46), human telomeres are replicated as rapidly as bulk DNA (47–49). Our data therefore also raise the possibility that factors at the telomere are actively involved in recognizing regressed-fork chickenfoot structures and rapidly resolving them in a tightly regulated process to restart replication, without allowing significant changes to the length of the telomere. The recent report that the telomeric-binding protein Taz1 is required for the replication of telomeres in the fission yeast *Schizosaccharomyces pombe* (50) further suggests that factors such as the telomere-binding proteins TRF1 or TRF2 might play such a role in human cells.

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Conflict of interest statement. None declared.

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