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Models for chromosomal replication-independent non-B DNA structure-induced genetic instability

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Abstract

Regions of genomic DNA containing repetitive nucleotide sequences can adopt a number of different structures in addition to the canonical B-DNA form: many of these non-B DNA structures are causative factors in genetic instability and human disease. Although chromosomal DNA replication through such repetitive sequences has been considered a major cause of non-B form DNA structure-induced genetic instability, it is also observed in non-proliferative tissues. In this review, we discuss putative mechanisms responsible for the mutagenesis induced by non-B DNA structures in the absence of chromosomal DNA replication.

Keywords

DNA structure; DNA replication; DNA repair; Transcription; Recombination; Genetic instability

INTRODUCTION

DNA has long been considered a passive “victim” of damage and mutagenesis. But studies over the past few decades have also suggested that the DNA itself can be a causative factor for genetic instability leading to expansion of repetitive units, or inducing gross genomic rearrangements (translocations, deletions, insertions, inversions, and duplications) that might cause human disease [1–3]. Many types of non-B DNA structures form at repetitive DNA sequences containing different repeat units [1]. For example, cruciform structures can form at inverted repeats when the duplex is unwound during replication, transcription, or repair, affording the single-stranded repeat sequence the opportunity to base pair with itself in an intramolecular fashion, thereby forming a hairpin or cruciform structure. Cruciform/hairpin structures have been found to induce DNA double strand breaks (DSBs), deletions, and translocations in prokaryotic cells [4,5] and in yeast, mice, and human cells [6–11]. Because purine bases can adopt a *syn* conformation, alternating pyrimidine-purine sequences such as GC or GT repeats can readily adopt left-handed Z-DNA structures with a zigzag arrangement of the DNA backbone [12,13]. Z-DNA-forming CG repeats cause small deletions within the repeats, likely due to slippage events during replication in bacteria [14], and can induce DSBs within or surrounding the repeats in mammalian cells, resulting in large-scale deletions (>100 bp) [15]. Polypurine-polypyrimidine elements with mirror repeat symmetry can adopt intramolecular triplex DNA structures (H-DNA), where unpairing of the duplex in half of the tract allows one of the single strands to pair in parallel with the purine strand in the remaining duplex segment, forming a three-stranded helix with the complement of the third strand remaining unpaired. H-DNA has been detected *in vivo* using

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both triplex antibodies and fluorescently labeled single-stranded DNA oligonucleotides [16,17]. We have shown that the endogenous H-DNA-forming sequence from the human *c-MYC* promoter caused a 20-fold increase in mutation frequency on a mutation reporter gene in mammalian cells [18]. Strand slippage events can occur at tandem repeats, particularly when the DNA within a loop can form intrastrand base pairs, such as in CAG/CTG triplet repeats. These structures can cause contraction or expansion of the tandem repeat units, and have been associated with at least 20 neurological diseases (reviewed in [19–23] and reviews within this issue). Expansion of simple repeats is also related with “fragile sites” in the genome, which are prone to DNA breaks, chromosomal translocations, deletions, and amplifications, and have been implicated in several hereditary disorders. For example, long CTG, CCG, or GAA tracts that are capable of adopting hairpin, Z-DNA [24], H-DNA [25] or sticky DNA [26] structures (*i.e.* two triplex-like-structures formed on GAA repeats), can cause chromosome breakage [1,27–29]. In addition, many non-B DNA structures formed at non-repetitive sequences are mapped in or near chromosome breakage “hotspots” [30–35], suggesting a relationship between non-B DNA structure and DNA breakage and chromosome translocation.

However, the mechanisms involved in expansion, deletion, and particularly DNA DSBs are still not clear, particularly in mammalian cells. Data obtained to date have revealed that the mechanisms involved in DNA structure-induced genetic instability are complex and more than one pathway is likely involved in processing non-B DNA structures. While some pathways are more dominant in certain cases, they may be less important under other conditions, depending on the type of DNA structure, the cis-elements nearby, and the levels of DNA replication, transcription, or the amounts of DNA damage and repair within close proximity to the non-B structures.

Generally, non-B DNA conformations are in higher energy states than B-DNA. To form most types of non-B DNA structures such as hairpins, cruciforms, H-DNA, G4 DNA (*i.e.* stable four-stranded structures formed at guanine-rich DNA sequences such as telomeres [36]), or slipped DNA, Watson-Crick base pairs in the B-form are separated prior to structure conversion, which is an energy-consuming process. Therefore, genomic DNA is likely in the B-form with Watson-Crick base pairing the majority of the time. However, when the DNA is unwrapped from nucleosome cores or the DNA duplex is separated during replication, the single strandedness required for non-B DNA structure conversion is generated, particularly in the lagging strand during DNA synthesis. In addition, many forms of non-B DNA structures appear to interfere with the processivity of DNA polymerases and cause replication fork stalling, which in turn further prolongs the existence of single strandedness and facilitates non-B DNA conformations. For example, H-DNA poses a strong barrier for DNA polymerases such as Taq [37] and polymerase alpha [38] and can pause or arrest DNA replication in mammalian cells [39,40]. 2D electrophoretic analysis of replication intermediates suggested that replication forks stall at hairpin structures formed at inverted repeats in bacteria, yeast, and mammalian cells [41,42]. These findings suggest that chromosomal DNA replication plays important roles in non-B DNA induced genetic instability.

Instability at triplet repeats often occurs in highly proliferative tissues [43,44] or in rapidly dividing cells [45,46], and are relatively stable in differentiated skeletal muscle cells [47–49], supporting a role for replication in triplet repeat stability. CAG or CTG triplets can form hairpins with loops containing a mismatch once in every two C–G pairings. Such a structure may be the result of primer-template slippage in DNA replication and can promote further slippage events [50] giving rise to repeat expansion seen in human disease. In addition, the location and orientation of the triplet repeat relative to the replication origin have dramatic effects on the frequency and types of instability [42,51–56]. Mutations in genes involved in

DNA replication, *e.g.*, FEN-1 [57] or DNA polymerases [58] can have dramatic effects on triplet repeat stability. These data clearly implicate semi-conservative chromosomal DNA replication in non-B DNA-induced genetic instability.

Although the replication slippage model of triplet repeat (or other direct repeats) instability has been considered a major mechanism of repeat instability [59], sequences capable of forming non-B DNA structures can induce instability in non-proliferative tissues as well, suggesting a chromosomal replication-independent process(es) leading to DNA structure-related instability. For example, CAG repeat expansion occurs in the brain [60–62], even though all neuronal cells do not divide, and different regions of brain exhibited different levels of CAG repeat expansion [61,62]. The CAG expansion was even larger in muscle tissue than in lymphocytes from patients with Kennedy's disease and Myotonic dystrophy [48,63]. In cultured cells derived from different tissues of transgenic mice carrying long CAG(162) repeats in the chromosome, CAG repeats exhibited different rates of expansion and no correlation between repeat instability and the cell proliferation rate was found [64]. In addition, we found that H-DNA and Z-DNA structures induced DSBs and large-scale deletions and rearrangements of plasmid DNA in replication-deficient HeLa cell extracts ([15] and our unpublished results). Moreover, Z-DNA and H-DNA induced a higher proportion of large-scale deletions in replication-deficient plasmids than on those recovered from replicating plasmids ([15] and our unpublished results). Obviously, chromosomal DNA replication-independent mechanisms also play important roles in non-B DNA structure-induced mutagenesis, particularly in related neurological disorders. In this review, we summarize different pathways of non-B DNA structure-induced mutagenesis associated with DNA damage and repair.

DNA REPAIR-STIMULATED NON-B DNA STRUCTURE FORMATION

As discussed above, genomic DNA remains in the B-form the majority of the time and needs to be unwrapped from nucleosomes and separated into single strands prior to conversion into non-B DNA structures, such as slippage loops, hairpins/cruciforms, H-DNA, and G4 structures. DNA damage occurs frequently on genomic DNA either spontaneously or induced by endogenous or exogenous damaging agents. DNA repair processes at or near the repetitive sequences could give rise to non-B DNA conformations and induce structure-related genetic instability. This “repair stimulated DNA structure formation” model may not apply to those structures that require torsional stress from negative supercoiling within an intact duplex, such as Z-DNA.

DSBs resulting from many endogenous or exogenous factors occur frequently *in vivo*, and are strong signals for the recruitment of the DNA repair machinery to the breakpoints. Repair of DSBs thousands of base-pairs away from an inverted repeat in the yeast chromosome resulted in 5'-to-3' degradation at the breakpoint and exposure of single-stranded inverted repeat DNA. Folding the ssDNA to form a hairpin structure could bring the two ends of complementary strands at the breakpoint together, resulting in a large hairpin structure. Resolution of the structure and bi-directional replication could result in amplification of large chromosomal regions [65–67]. Alternatively, the single-stranded repair intermediates could initiate a pairing interaction between two inverted repeats from different chromosomes [68]. Both processes could result in the formation of dicentric chromosomes and would lead to gross chromosomal rearrangements, including translocations, truncations, and amplifications.

When DSBs were introduced within or near a CTG repeat tract on plasmids prior to being transfected into mammalian cells, they were predominantly processed into deletions of the repeat and flanking regions. Longer repeats induced a greater proportion of deletions and

expansions in the repeats than did shorter ones [CTG(79) > CTG(47) > CTG(17)], and the deletion areas were larger in those induced by the longer repeats, consistent with the higher inherent capability of longer CTG repeats to form more stable non-B DNA structures [69]. DSBs centrally located within CTG or CGG repeats also increased deletions of the repeating sequence in RecA or RecBC-deficient *E. coli* [70]. A nick or a short 15 nt gap within the CTG repeats did not induce expansions or deletions; however, a longer gap of 30 nt, leaving a 30 nt single-stranded CAG repeat which can form a stable hairpin structure, caused a distinct 30 nt deletion of the entire looped region [70], suggesting that hairpin formation at the repair intermediate is an essential step for repair-stimulated CTG instability [71]. Repair of a DSB within a GAA repeat, which is not able to form an intrastrand hairpin structure, also increased repeat deletions. But unlike DSB repair near CTG or CCG repeats, DSB repair outside the GAA repeat tract did not affect instability [72]. Moreover, repair of DSBs within the repeat resulted in deletion lengths equivalent to the distance of the DSB to the closer end of the repeat tract, suggesting a single-strand annealing mechanism of DSB repair [72].

Alternatively, in bacteria, yeast, or mammalian cells in late S phase where homologous templates are available, the single-stranded repeat sequence derived from the DSB can invade a homologous template, and slippage loops (or hairpin structures if the sequence supports intrastrand base-pairs) can occur during DNA synthesis, resulting in expansions and contractions *via* a synthesis-dependent strand annealing (SDSA) model that allows for crossover [73].

In addition to DSBs, many other types of DNA damage can enhance the instability of repetitive sequences that are capable of forming non-B DNA structures. In human colorectal carcinoma cancer RKO cells, mutations due to DNA strand slippage at repetitive sequences were able to reactivate a frame-shift mutated green fluorescent protein (*GFP*) gene integrated into the chromosome. Treating the cells harboring the tetranucleotide repeat *e.g.*, AAAG(16) repeats within the chromosomally integrated reporter system with N-methyl-N-nitro-N-nitrosoguanidine (MNNG), t-butyl hydrogen peroxide, and UV irradiation (but not γ -irradiation or benzo(a)pyrene diol epoxide), resulted in a significant increase in the number of reactivation mutants compared to cells harboring a reporter gene containing a CA(16) repeat or without a repetitive sequence. In addition, an increase in reactivating frame-shift mutations at the AAAG(16) repeat was observed with increasing doses of UV or MNNG, while control cells did not show any significant changes with increasing doses, suggesting that the (repair of) damage could induce slippage mutations in repeated sequences [74]. However, H₂O₂ treatment on telomerase-immortalized normal human fibroblasts harboring a plasmid containing G(17), A(17), or CA(17) repeats in the *tk-neo* gene, reduced mononucleotide repeat slippage-induced reactivation of the reporter *neo* gene in the (+1) reading frame. No effect was observed when the *neo* gene was in the (−1) reading frame [75]. These observations suggested that the chemical and/or structural nature of different types of repetitive DNA might be important for the fate of the damaged sequence.

Figure 1 depicts our model of “DNA repair-stimulated non-B DNA structure formation” and subsequent genetic instability. This model provides a plausible explanation for non-B DNA structure induced instability in non-dividing cells, particularly for those mutations caused by intrastrand hairpin formation at simple triplet repeats such as CAG and CCG repeats [76]. Clearly, there are still many questions to be answered to better define this model. For example, based on this model it would be predicted that long single-stranded repetitive sequences where stable non-B structures can form would be more likely to be seen in long patch repair pathways such as nucleotide excision repair (NER), mismatch repair (MMR), long-patch base excision repair (BER) and DSB repair, rather than short-patch BER.

However, the monofunctional alkylating agent MNNG, and the oxidative DNA damaging agent t-butyl hydrogen peroxide, induced slippage loops and contraction of AAAG(16) repeats, while γ -irradiation had only a minor effect [74]. Also, oxidative damage that is largely repaired by BER is the predominant type of damage in the brain, where triplet repeat expansion occurs in human disease. And more directly, knocking-out of the base excision repair enzyme, 7,8-dihydro-8-oxoguanine-DNA glycosylase (OGG1), dramatically stabilized CAG repeats in mouse brains, suggesting a critical role for BER in CAG expansion [77]. Thus, the mechanisms involved are more complicated than proposed in the model (Figure 1) and these mechanisms still need to be explored.

DNA STRUCTURE-SPECIFIC CLEAVAGE MODEL IN THE ABSENCE OF DAMAGE

In the “bipartite model” of damage recognition in NER, both DNA distortion and chemical modification are important signals for the damage/distortion recognition proteins (*e.g.*, XPC-RAD23B) to bind DNA lesions [78,79]. Discontinuity and distortion of the DNA double helix induced by lesions are initial signals for recruitment of the DNA damage/distortion recognition proteins to the sites of lesions, as proposed by Yang [80]. XPC-RAD23B can also bind to distorted DNA, such as bubble structures in the absence of a lesion [79]. Further, XPC-RAD23B is not needed for NER if the DNA structure is already sufficiently distorted [81]. During initiation of MMR, MutS heterodimers likely recognize mismatched DNA by the flexible regions of DNA containing both helix distortion and poor base stacking, to distinguish the mismatched DNA as a substrate for MMR processing [82,83]. Recently, by using DNA repair factors fused to the *E. coli* lac-repressor (LacR) which can be recruited to the chromosomally integrated lac operator sequence (*lacO*) in NIH-3T3 cells, Soutoglou and Misteli (2008) found that recruitment of Nbs1, MRE11, MDC1, or ATM to the *lacO* loci activated a DNA damage response in the absence of DNA damage [84].

Formation of non-B DNA structures generally results in discontinuity and distortion of the DNA double helix, either within the structure (*e.g.*, H-DNA and G-DNA) or at the junctions of the non-B and B-forms of DNA (*e.g.*, hairpins/cruciforms and Z-DNA) and may be recognized as “damage” by DNA repair proteins. For example, the *Saccharomyces cerevisiae* Mre11 protein can bind to G4 DNA with much higher affinity than G-rich DNA in the canonical B-form [85]. Given that recruitment of Mre11 or other DNA repair proteins at non-damaged DNA is sufficient to initiate a DNA damage response [84], it is possible that non-canonical DNA structures may also trigger DNA repair and cellular damage responses in the absence of DNA damage *per se*.

DNA structure-induced cellular DNA damage responses

Lahiri et al. (2004) [86] determined the effect of expanded CAG repeats on DNA damage responses in a yeast artificial chromosome system. The study was designed to determine if checkpoint deficiency would allow the replication to progress through the repetitive sequence without removing the deleterious structure and result in a higher breakage frequency. Mutation of the checkpoint factors Mec1, Ddc2, Rad9, Rad17, Rad24, or Rad53 gave rise to increased CAG repeat instability, indicating that (the DNA structure formed at) CAG repeats could activate these checkpoint and repair proteins [86]. Interestingly, Chk1-mediated G2 arrest was not required for CAG repeat-induced processing [86]. Long tracts of CAG(175) repeats on plasmids were also recognized as “damage” in *E. coli*, as evidenced by the induction of an SOS response [87]. However, shorter CAG(25–79) repeats did not appear to induce an SOS response and the SOS system itself did not affect the stability of CAG repeats in *E. coli* [88]. Intron 21 of the human *PKD1* gene contains a 2.5-kb polypurine-polypyrimidine tract that can form an H-DNA structure, and it is thought to

contribute to the high mutation rate of the *PKD1* gene. This polypurine-polypyrimidine tract in plasmids induced an SOS response and delayed host cell growth, resulting in a dramatic decrease in colony-forming capability. This effect of the polypurine-polypyrimidine tract on host cell colony-forming capability was reduced in a *uvrA* deficient strain (~100-fold decrease in colony-forming capability versus 500-fold in isogenic wild-type cells), and was almost diminished in *uvrB* or *uvrC* deficient strain [89]. However, whether or not NER proteins bind directly to polypurine-polypyrimidine tracts is still not known. These data provide evidence for a non-B DNA-structure induced cellular response in the absence of DNA damage.

Structure-induced DNA cleavage and mutagenesis

We speculate that cellular DNA damage responses initiated by non-B DNA structures slow cell cycle progression providing the DNA repair machinery time to remove the “damage”. This process is likely involved in DNA structure-induced cleavage and mutagenesis that has been identified in the absence of DNA replication or exogenous DNA damaging agents (see below). In fact, many types of non-B DNA structures induce mutations that are more complex than simple deletions or insertions within the repeats as proposed in the slippage and misalignment model during replication. For example, in mammalian cells, one half of a palindromic repeat was completely deleted while the other half was maintained (one-sided deletion) [11]. This type of mutation is the result of a breakpoint that occurs at the tip of the hairpin or cruciform structure [11,90,91]. Depending on the lengths of the arms, hairpin structures are processed with different efficiencies in mammalian cells. Short hairpins (≤ 14 nt) are typically cleaved on the complementary un-structured strand [92,93], and repair on non-palindromic loops of the same size or longer hairpins (~26–40 nt) often occurs at the structured strand and results in deletion of the sequence that forms a loop/hairpin structure [93–97]. Although the substrate DNA containing the inverted repeats was replicated in these studies, it is possible that the cleavage occurred independent of DNA replication [93–97]. Our laboratory has found that H-DNA and Z-DNA structures induced DNA single-strand breaks and DSBs in HeLa cell-free extracts in the absence of DNA replication or DNA damage, and caused large-scale deletions and rearrangements which can not be explained by a slippage/misalignment model ([15,18] and our unpublished results). These data suggest a structure-specific cleavage and DNA strand break model of non-B DNA-induced mutagenesis *in vivo*.

The Mre11/Rad50/Nbs1 complex accumulates at DSBs to process the ends of the breakpoints. However, Mre11 also exhibits an incision activity at hairpin/cruciform structures [98]. Although MRN has not been shown to bind to a hairpin/cruciform structure directly, it interacts with BRCA1 which preferentially binds to four-way branched DNA structures (which are similar to cruciforms) [99]. After recruitment of the MRN complex to the cruciform structure, BRCA1 dissociates from the DNA [100], and activates the nuclease activity of the MRN complex. Multiple lines of evidence support an activity of the MRN complex on long (>160 bp) palindromic DNA sequences [9,98,101,102]. In yeast, MRN generates DSBs at or near the inverted repeat at an early stage during pre-meiotic replication, and the Rec12 endonuclease processes hairpin structures to produce DSBs at a later stage, both resulting in meiotic recombination [102]. Spo11, the human ortholog of Rec12 also exhibited nuclease activity at hairpin structures [103,104], and induced DSBs at multiple sites within a CAG(79) repeat, resulting in both expansions and contractions [105]. SbcCD, the bacterial homologs of Rad50 and Mre11, binds to hairpin structures and cleaves at the intact looped strand in the absence of nicks or gaps [106,107]. This breakage can be further extended by ATP-dependent double-stranded DNA exonuclease activity of the SbcCD complex [106]. Deficiency in SbcCD dramatically stabilized a long (571 bp) DNA palindrome, demonstrating the importance of this complex in DNA structure-induced

instability [108]. In addition to its activity on cruciform structures, purified yeast Mre11 binds to G4 DNA and forms a stable complex even in the presence of 0.2–1.0 M NaCl [85]. After binding, the intrinsic endonuclease activity of Mre11 cleaved G4 DNA in a Mn^{2+} -dependent manner, at sites different from the cleavage sites observed at single-stranded G-rich DNA [85], suggesting a structure-, but not sequence-specific endonuclease activity on G4 DNA.

As discussed above, the discontinuity and distortion of the DNA helix induced by non-B DNA structures may serve as a signal to recruit MMR or NER proteins and induce repair processes. Single-base or small loops of several nucleotides are efficiently repaired by the MMR mechanism [109]. Larger loop mismatches (12–247 nt) are repaired by an MSH2-independent mechanism with a bias toward using the non-looped strand as the template during repair synthesis, resulting in loss of the loop [94,95]. Kirkpatrick and Petes (1997) reported that repair of 26-base loops in yeast involved both the MMR protein, Msh2 and the NER protein, Rad1 [110]. A 12-base cruciform was repaired with a 2:1 bias toward loop retention, with the bias shifting from loop retention to loop loss with increasing loop size [93,95,111,112]. The human MSH2-MSH3 complex can recognize and bind to cruciform structures formed at perfect inverted repeats, and the affinity is enhanced when the hairpin contains mismatched (*e.g.* CAG repeats) [113]. Moreover, binding of MSH2-MSH3 to mismatches in the hairpin stem formed at CAG repeats might activate repair mechanisms [114]. The function of the MMR proteins in CAG repeat instability appears to differ among different species. Deficiencies in either *mutS*, *mutL*, or *mutH* greatly reduced large deletion events (greater than 8 repeats) at CAG(180) repeats in *E. coli* [115], and increased the small deletion and expansion events caused by misalignment [116]. In yeast, loops and cruciform structures were repaired less efficiently than in bacterial and mammalian cells [117], and the absence of Msh2 did not significantly affect the frequency of large deletions at a CAG(50) repeat [118] or large expansions at a CAG(25) repeat [52]. However, yeast deficient in *Msh2* did show an increase in the small deletions or additions (of one repeat unit) generated by small loops due to misalignment that are typically repaired by MMR [119]. In mouse models of Huntington's disease or myotonic dystrophy carrying long CNG repeats, MMR appears to be required for repeat instability, as evidenced by reduced repeat instability in the absence of MSH2 [120], MSH3 [121], or PMS2 [122]. Another mouse model carrying >300 CTG repeats from the human DM1 gene showed that absence of MSH2 shifted the mutations toward deletions rather than expansions, although the mutation frequency was not dramatically altered [123]. In cultured human cells, MSH2 and MSH3 are also involved in the transcription-induced deletion resulting from a CAG(95) repeat [124]. Similar effects of MMR proteins on other types of repeats have also been reported. For example, in *E. coli* a GC(15) repeat induced both short (<2 bp) and long (>2 bp) deletion events, which are likely caused by two different mechanisms. Deficiency of either *mutH*, *mutL*, or *mutS* enhanced the frequency of 2 bp deletions, indicating a role for MMR in small loop misalignment, but showed no effect on larger deletion events [125].

The bacterial NER protein, UvrA has been shown to bind to bubble and loop regions in duplex DNA [110,126], and is also involved in CAG triplet repeat deletion on plasmids in *E. coli* [127]. The UvrA protein binds to loops/hairpins of 1–17 CAG repeats on plasmids with a much higher affinity ($K_d \sim 15$ nM) than to the same sequence in linear DNA [127]. In addition, UvrA binding was able to stimulate subsequent efficient excision on structured CTG repeats [127]. Deficiency of the NER genes, *uvrC* or *uvrD* greatly enhanced the instability of a CAG(68) repeat in bacteria, whereas *uvrA* or *uvrB* deficiency stabilized CAG tracts [128]. In contrast, Parniewski et al. (1999) reported that *uvrA* deficiency dramatically increased the instability of CAG repeats, and *uvrB* deficiency stabilized the CAG tract on plasmids in bacteria [129]. The reason for these discrepancies or why NER factors may have different effects on CAG repeats is not clear, and further studies are warranted. In cultured

human cells, knocking-down XPA (as well as MMR proteins, MSH2 or MSH3), but not XPC, significantly reduced the transcription-induced deletion on CAG repeats in a chromosomal context, suggesting that transcription-coupled NER might be involved in CAG repeat instability [124].

In addition to loop or cruciform structures, we found that H-DNA-induced mutagenesis occurs in HeLa cell-free extracts in the absence of replication, suggesting a role for structure-specific cleavage (*e.g.*, DNA repair) activities in inducing mutations in mammalian cells (Wang et al, unpublished results). In MSH2-deficient human Hec59 cells, or XPA-deficient human XP12RO cells, H-DNA-induced mutation frequencies were lower than those found in control cells, implicating MSH2 and XPA in the mutagenic process. However, the plasmids isolated from the MSH2 or XPA-proficient versus deficient cells showed no obvious differences in the amount or location of H-DNA-induced DSBs. These results suggest that while MSH2 and XPA are involved in the structure-induced mutagenesis, MMR or NER pathways are not directly involved in generating the DSBs, consistent with our recent finding that MLH1 deficiency did not affect the H-DNA-induced mutation in human cells (Wang et al, unpublished results). A slightly different result was obtained in *E. coli*. The H-DNA-forming sequence from the human *PKD1* gene induced large deletions in *E. coli*, and MutS or MutL deficiency dramatically reduced the H-DNA-induced mutation frequency, implicating a role for the MMR pathway in H-DNA metabolism in bacteria [130].

Several proteins have been shown to have nuclease activities on non-B DNA structures. For example, DNA topoisomerase II binds to and cleaves hairpins (*e.g.* hairpin structure formed at a negatively supercoiled 52-bp palindromic sequence in the human beta-globin gene [131]), but not cruciforms [132]. It cleaves a *Drosophila melanogaster* DNA topoisomerase II cuts Z-DNA structures on negatively supercoiled minicircles [133]. DNA topoisomerase II cleavage sites near human immunodeficiency virus integration sites in the human genome consist of Z-DNA-forming sequences and other repetitive sequence elements [134], suggesting that non-B DNA structures are substrates for DNA topoisomerase II. The cruciform structure is similar to the recombination intermediate four-way Holliday junction, such that the Holliday junction resolvases might also have activity on cruciforms formed at inverted repeats. H-DNA and G4 DNA structures expose an unstructured single-stranded DNA region, which may also serve as substrates for single-strand specific nucleases. The studies described above clearly support a model of “DNA structure-specific cleavage” by DNA repair proteins, followed by error-generating repair processes at the breakpoints (Figure 2). If the structured DNA itself is recognized as damage in the genome, this could stimulate gratuitous repair on undamaged DNA, which can occur repeatedly regardless of the replication status until the sequence is mutated and non-B DNA formation is no longer favored.

ACCUMULATION OF DNA DAMAGE AT NON-B DNA STRUCTURES

The DNA is constantly being damaged and repaired in living cells. On average, ~19,000 DNA damage incidences occur per cell per day [135], as measured by an alkaline sucrose gradient sedimentation method in human fibroblasts. This number varies among cell type and cellular environment, and the damaged sites are not randomly distributed. The type of DNA sequence motifs to be preferentially attacked depends upon the chemical and/or physical nature of the assaulting agent and the DNA base composition and structure. Some physical characteristics of non-B DNA structures suggest that they may be more susceptible to DNA damage than the canonical B-DNA structure. Although information on the genome-wide distribution of DNA damage and mutation is not available, UV irradiation has been shown to induce a mutation hotspot at a site in the potential hairpin loop of quasi-

palindromic sequences in the *lacI* reporter gene in *E. coli* [136]. UV irradiation also caused significant chromatin fragmentation at repetitive sequences in mammalian cells, particularly at GT repeats (a Z-DNA-forming sequence) and long stretches of homopurine/homopyrimidine regions (potential H-DNA-forming sequences) [137]. It is not clear whether the accumulated mutations were caused by higher levels of UV damage in the non-B DNA-forming sequences, or due to a lower repair efficiency in the structured regions, or both. In fact, non-B DNA plays an important role in both DNA damage formation and repair efficiency and fidelity, as discussed below.

In canonical B-form DNA the base-pairing between strands is protected within the helix. However, single-stranded regions are exposed in non-B DNA structures such as H-DNA, G4 DNA, the tips of a hairpins or looped structures, and in B-Z junctions of Z-DNA, which are less stable than duplex DNA. Supporting this notion, negatively supercoiled DNA (an important molecular force that drives the formation of non-B DNA structures), and single-stranded DNA were more reactive to 4-Nitroquinoline 1-oxide (4-NQO) than relaxed duplex DNA [138], and B-Z junctions in Z-DNA were hyperreactivity to 4-NQO [139], hydroxylamine, and osmium tetroxide [140]. Moreover, in a Z-DNA structure, the guanosine nucleotides are in a *syn* position where the bases are located over the sugar without protection and thus could be more accessible to DNA damaging agents [141]. Diethylpyrocarbonate reacts more strongly with purines in a left-handed Z-DNA structure, and guanines in Z-DNA are more reactive with dimethylsulfate and diethylsulfate [140]. Ionizing radiation preferentially attacks the guanine sites in the Z-DNA structure, while the cytosine sites are more resistant, leading to a characteristic signature damaged pattern of the Z-form that differs from damaged sites seen on B-DNA [142].

In addition, damage occurring to the DNA in a Z conformation is more resistant to processing by DNA repair enzymes. For example, N7-methylguanine, which is typically removed by a DNA glycosylase, was not efficiently repaired when present in Z-DNA, and when the Z-DNA conformation was converted into B-DNA by adding EtBr, the N7-methylguanine became a good substrate for DNA glycosylase [143,144]. O6-methylguanine in alkylated poly(dG-m5dC). poly(dG-m5dC) in the Z conformation was repaired with an efficiency only 10% of that measured on B-DNA [145]. A single nucleotide loop that was otherwise removed efficiently by MMR, was not processed by the repair machinery when present in the stem of a hairpin structure formed at a 33 nt palindromic sequence in *S. cerevisiae* [112]. It not yet clear how or at which step the non-B DNA structure interferes with the repair process, *i.e.* the recognition step, recruitment of subsequent repair factors, enzymatic cleavage, excision, DNA synthesis, and/or ligation.

DNA is well organized and packaged into chromatin *in vivo*, and both DNA damage and repair occur in the context of the chromatin structure. Tertiary chromatin organization in regions of open chromatin is also associated with genetic instability [146–148]. The structures of non-B DNA conformations may interfere with DNA-histone interactions and the non-B DNA regions are less likely to be assembled into nucleosomes. The abnormal positioning or absence of nucleosomes can also change the sensitivity of non-B DNA regions to modification by genotoxic agents. For example, DNA in the Z-form can be refractory to nucleosome formation [149–151], probably because the interaction of the Z-DNA and the positively charged arginines on the histone core are not aligned properly relative to each other [152]. Although the data available are from Z-DNA, it is not hard to imagine that other types of non-B DNA structures may be refractory to nucleosome formation. At an early stage of apoptosis initiated by very low UV doses in rat leukemia cells, the fragmented DNA contained a four-fold excess of repetitive sequences (*e.g.* H-DNA and Z-DNA-forming sequences), suggesting that these non-B DNA-forming

sequences were in an open chromatin structure, rendering them more susceptible to enzymatic cleavage [137].

Based on these observations, we propose a model of “accumulation of DNA damage” at non-B DNA structures (Figure 3), where formation of a non-B DNA structure in the chromosome is favored at an open chromatin region, and this structured region is now refractory to nucleosome assembly and remains open. These modified physical characteristics may increase susceptibility of this particular region to DNA damage and/or enzymatic cleavage or DNA damage, resulting in increased localized genetic instability.

TRANSCRIPTION AND RECOMBINATION-MEDIATED NON-B DNA-INDUCED MUTAGENESIS

DNA transcription opens the chromatin structure, unwinds the DNA helix, exposes single-stranded DNA, and generates negative supercoiling behind the translocating polymerase complex. These processes generate conditions that are conducive to the formation of non-B DNA structures. For example, in permeabilized nuclei from mammalian cells, binding of anti-Z-DNA antibodies to fragments of the *c-MYC* gene near the promoter was observed only when the *c-MYC* gene was actively transcribed [153–155]. For more information on the role of transcription in repeat instability, see the review by Lin et al in this issue. In collaboration with the Hanawalt group, we recently discovered that T7 RNA polymerase transcription was partially blocked within and downstream of the H-DNA-forming homopurine-homopyrimidine mirror repeat sequence from the human *c-MYC* promoter region, and within CG(14) Z-DNA-forming sequences [156,157]. Similarly, G4-forming sequences inhibit transcription by T7 RNA polymerase and mammalian RNA polymerase II [158]. The transcription blockage was dependent on negative supercoiling of the template, and was enhanced in multi-round transcription, implicating the non-B DNA structures in the transcription arrest [156,157]. Detailed discussion regarding this topic can be found in the review by Tornaletti et al. in this issue. These findings suggest the possibility that stalled RNA polymerase machinery might recruit and activate transcription-coupled NER at the non-B DNA region, resulting in genetic instability.

Recombination is another DNA metabolic event that can occur in non-dividing cells and is one of the mechanisms implicated in triplet repeat instability. RecA or RecB deficiency led to decreased CAG repeat contraction of up to 70-fold or 1700-fold, respectively [88]. Z-DNA structures are also correlated with recombination hotspots in eukaryotic cells [159,160]. An ~1,000 bp region in the major histocompatibility complex in mice containing several copies of long GT repeats is a recombination hotspot and accounts for up to 2% of recombination on the entire chromosome [161,162]. H-DNA-forming sequences are also mapped at hotspots of recombination, such as sites of unequal sister chromatid exchange in the *Cγ2a* and *Cγ2b* heavy chain genes in the MPC-11 mouse myeloma cell line [163]. Although structural features of some non-B DNA conformations might facilitate recombination, such as H- and Z-DNA [164], it is also possible that the non-B DNA related recombination events are induced by DNA breakage within or near the non-B DNA structure, which occurs spontaneously (*i.e.*, the “structure-specific cleavage model”) or induced by DNA damaging factors (*i.e.*, “repair-induced non-B DNA structure-related genetic instability”) [102,165,166].

SUMMARY

Non-B DNA structures distinguish themselves from the rest of the genome of B-DNA in several aspects, and may recruit and trigger repair cleavage activity in the absence of DNA damage *per se*. These types of structures occur in more open regions of the chromatin and

are more susceptible to DNA damage. In addition, repair of DNA damage can induce the formation of non-B DNA structures, resulting in mutagenesis. Since DNA damage and repair are active processes in living cells regardless of the replication status, models of DNA repair-associated mutagenesis at non-B DNA structures proposed in this review may explain some observations of non-B DNA-induced genetic instability in non-dividing (or slowly dividing) cells such as the brain and muscle. However, many questions remain to be answered, and different types of non-B DNA structures may have unique processing pathways and should be interpreted separately. The enzymes that recognize and/or process each type of non-B DNA structure may differ and the proteins that have been found to be associated with non-B DNA instability might participate in an unexpected way. For example, MSH2 and XPA proteins are both involved in instability of CAG repeats and H-DNA-forming sequences, but this may be through some as yet un-identified functions, evidenced by the finding that knocking down both proteins did not further reduce repeat contraction, suggesting a new role for these proteins in the same pathway [124]. We have found that MSH2 and XPA are involved in H-DNA metabolism but that the DNA structure may not be processed via canonical MMR or NER mechanisms (Wang et al., unpublished results). Future studies to address these questions could lead to a better understanding of the mechanism(s) of non-B DNA-induced genetic instability and related disease development.

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ABBREVIATIONS

DSBs	double strand breaks
NER	nucleotide excision repair
MMR	mismatch repair
BER	base excision repair
DM1	type 1 myotonic dystrophy

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Figure 1. “Excision-repair stimulated hairpin/slippage structure formation” model. In this model, (1) DNA damage (shown as a star) occurs near (or within) a non-B DNA structure-forming sequence (shown in red), recruiting the DNA repair machinery. (2) Processing of the damage by repair enzymes (indicated as various shapes) exposes a single-stranded DNA region within the non-B DNA-forming sequence. (3) Non-B DNA structures (such as a hairpin as shown in the figure) can form at the newly synthesized strand, which causes expansion of the sequence, or at the template strand leading to deletion of the sequence.

Figure 2. “DNA structure-specific cleavage” model. A non-B DNA structure formed on non-damaged DNA (shown in the figure as a Z-DNA structure) can be mistakenly recognized as a site of damage. The recruited DNA repair machinery generates breaks within or surrounding the non-B DNA structure, followed by error-generating repair of the breaks. This “structure forming-repair” cycle can occur repeatedly as a futile cycle until the sequence is mutated.

Figure 3. “Accumulation of DNA damage in non-B DNA structures” model. Unwrapping of a non-B DNA-forming sequence (highlighted in red) from the histone core during DNA metabolism, *e.g.*, DNA replication, transcription, or repair (Step 1), facilitates the non-B DNA conformation (Step 2, shown in the figure is a Z-DNA structure). The non-B DNA conformation is more susceptible to DNA damage and the damage in the non-B DNA region is more resistant to repair (Step 3), leading to accumulated damage in this region (Step 4).