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Commentary

DNA secondary structure: A common and causative factor for expansion in human disease

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The discovery of unstable transmission has changed the face of genetics because it provides an alternative to the single-gene/single-trait pattern of Mendelian inheritance. More than 10 hereditary diseases are caused by instability at simple trinucleotides. Expansion causes disease when a particular base sequence is repeated beyond the normal range, interfering with the expression or properties of a gene product (1–2). As the length of the repeat grows, so also do the size of the successive expansions and the likelihood of another unstable event. This accounts for clinical anticipation in which the severity increases and the age of onset decreases in successive generations. In Huntington's disease, for example, instability and pathogenesis are not observed at 28 repeats, occur frequently at 38 repeats, and are almost certain above 60 repeats. Although different genes are affected and different features of pathogenesis are evident, there is a common pattern of unstable transmission among the trinucleotide repeat diseases, suggesting common elements to the mechanism.

Data from human genetic studies, from structural analysis, and from model organisms are consistent with the notion that instability initiates from improper DNA secondary structure during replication and/or repair (3–5). However, unambiguous proof of secondary structure *in vivo* has been extraordinarily difficult because it requires a solution to the controversial problem of whether duplex DNA can adopt single-stranded structures at any time during the cell cycle in the presence of a complementary partner strand. In issue 4 of the *Proceedings*, however, Moore *et al.* (6) may well provide one of the first demonstrations that secondary structure not only forms at repeats *in vivo* but also that structure formation can elude the cellular machinery designed to detect and to repair single-stranded loops. Thus, the notion that secondary structure mediates repeat expansion in double-stranded DNA may finally be more fact than hypothesis. The authors touch on several issues at the very heart of the mechanism for expansion.

DNA Defects Rather than Proteins Drive Instability. Several lines of evidence point to the DNA itself as both common and causative among expansion diseases. One of the first indications came directly from human genetics. In a small number of Huntington's families, patients were identified in which both alleles were of the premutation length. All new mutations in Huntington's disease arise from premutation or carrier alleles between 29 and 35 repeats (7). Thus, both premutation alleles should have roughly the same likelihood of expanding, and they may be followed through several generations. If proteins caused expansion, then both alleles should tend to expand to the same extent in these families, because both alleles are exposed to the same proteins or to similar genetic backgrounds. On the other hand, if expansion were driven by properties of the DNA, then the frequency of instability might be very different between the two alleles. In one family particularly prone to expansion, the two alleles of 33 repeats and 29 repeats were dramatically different with respect to instability. One allele expanded in almost every transmission, while the other was largely stable (7). The difference in frequency could not be explained by protein composition, because in many transmissions, both alleles were carried in the same individual. Similarly, the differences are unlikely to be explained by chromatin structure (8, 9), because 4 repeats (or 12 base pairs) will have little influence on the formation or phasing of even one nucleosome.

Additionally, the pattern of instability in patient populations with expansion disease is dramatically different from instability in diseases known to occur by defective or absent repair proteins. Instability at simple repeats occurs in nonpolyopsis colon cancer, in which mutations in mismatch repair enzymes are inherited (10). However, genomic fingerprinting (direct comparison of a panel of repetitive loci) in Huntington's Disease (HD), Friedreich's ataxia (FRDA), and colon cancer patient populations reveals that instability occurs only at the CAG (in the HD locus) or the GAA (in the FRDA locus) in the respective patient groups (11, 12). This mutation pattern is dramatically different from that observed in nonpolyopsis colon cancer patients, in which instability occurs at most of the microsatellites tested, and in which the size of the mutation tends to be smaller (10–13). Because repair proteins act on DNA at any site, genomewide instability is the expected fingerprint if faulty protein function drives instability (10–14). For example, genomewide instability can also result from the absence of repair proteins such as RAD27/FEN-1 in yeast (14). Thus, single-site mutation in trinucleotide diseases does not reflect a general fault of the protein machinery but reflects the inability of this machinery to function normally at the repeat site.

DNA Secondary Structure as a Causative Factor in Expansion. What property of DNA might limit instability to a particular site in patients? The ability of longer repeat regions to form stable secondary structures might provide site-specific instability if structure inhibited the action of replication or repair proteins at the repetitive site. Moore *et al.* (6) provide evidence that such a mechanism is plausible *in vivo*. In these experiments, haploid yeast strains were constructed in which the His gene contains or lacks an insert containing 10 triplet repeats. Because repeat lengths are short, no instability occurs during the experiments but the effect of structure on escape from repair can be measured. Haploid yeast strains were mated to generate diploid strains containing both types of His genes and forced into recombination at meiosis under low-temperature conditions. In some cases, heteroduplex molecules will form containing a triplet repeat loop if the recombination product differs by an insertion. These loops can form secondary structures or not depending on the insertion sequence. Once loops are formed, they can be maintained or repaired (excised). Failure to repair the loop results in a non-Mendelian segregation of the triplet sequence at the first mitotic division. In other words, the ratio of mutant (insert) to wild-type (no insert) alleles will be higher in the daughter cells during mitosis. The authors find that, indeed, repeats forming stable structures are not repaired. From these observations, three important conclusions can be drawn.

First, only triplet repeats that form secondary structure have non-Mendelian postmeiotic segregation. Because control triplet

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sequences provide evidence that base pairing, rather than sequence, allows escape from repair, secondary structure indeed forms at expandable triplets *in vivo*. This finding culminates several years of investigation since secondary structure formation was reported *in vitro*. At that time, it was recognized that repeat sequences migrated on polyacrylamide gels in a manner consistent with a non-B-form duplex and consistent with hairpins (15). More definitive NMR studies confirmed that both CAG or CTG (16) and CGG (17) formed hairpins with identical overall structure comprising a repeat unit of two GC pairs and a mismatched pair. Despite the number of mismatched pairs, repeat-containing hairpins formed stable structures under physiological conditions. Until 1996, all diseases resulting from trinucleotide instability were associated with unstable CNG repeats (where N is A, C, T, or G) and suggested that a common mechanism for expansion may involve DNA hairpins. However, the mutation underlying FRDA quickly dispelled this notion. Instability in FRDA (a recessive neurodegenerative disorder) was found to occur at GAA repeats in the first intron of the human frataxin gene (18). At these sites, the GAA repeats form a YRY triple helix containing non-Watson–Crick pairs (12). Similar to hairpins, the triplex structures mediate intergenerational instability in 96% of transmissions. Several other structures have been reported including i-motif (19), e-motif (20) and quadruplex DNA (21) which are present at unstable, more complex minisatellites such as telomeres. Thus, multiple structures are likely to play a role in genomic instability.

Important support for this model has been provided in model organisms. Passage of plasmids containing structure-capable triplets in bacteria (12, 22) or yeast (23) render these plasmids susceptible to instability (largely deletions) in a length-dependent fashion as observed in human disease.

Repair Proteins Are Defeated by DNA Secondary Structure at Repeats. Although the repeats used in the Moore *et al.* study are too short to cause instability, the authors establish a second important principle: secondary structure allows a loop to escape repair. This means that loops of DNA (at least those produced during meiosis) can be stably carried without excision to the first mitotic division, where they can serve as templates for new synthesis. These studies suggest that stable base pairing prevents recognition by repair enzymes of bases or junctions requiring repair. However, the identity and number of repair proteins that could be defeated by secondary structure are hotly debated. There is an emerging consensus that multiple mechanisms must operate to generate the full spectrum of trinucleotide instability in human disease.

Loops of less than four unpaired bases are efficiently corrected by methyl-directed mismatch repair (MMR) (24). Consequently, MMR proteins may influence expansion in early stages of disease, when repeat lengths are close to the threshold for instability. In stable alleles, repeat stretches contain many interruptions (1–3). Small loops (or secondary structure) may allow mispairing of bases that are corrected by MMR enzymes, leading to loss of base interruption. Additionally, early stages of disease can be accompanied by an equal incidence of small insertion or deletion events. It is well known that triplet repeats are inherently poor substrates for polymerase (25). Polymerase pausing and/or blocks have been demonstrated at both CGG *in vivo* (26) and CTG (27) and GAA (12) *in vitro*. Difficulty in traversing a repeat stretch may allow the first opportunity for a slip. Polymerase slippage occurs at repeats because directly repeated sequences can provide multiple sites for pairing of a complementary strand—if the duplex becomes unpaired. However, slippage to a matching repeat tends to be small. This is because slippage by more than a few repeating units becomes energetically unfavorable, because many more bonds must be broken in the template than are reformed at a loop. Indeed, defects in MMR significantly enhance instability within trinucleotide repeat loci in human colon tumors (11), and the size of the instabilities are typically within ± 4 repeats. Secondary structure does not prevent binding of MMR proteins (28). However, the efficiency of MMR as tested in model organisms has not generated a consistent picture. MMR defects are known to enhance instability at mononucleotide runs (29) and increase instability up to 700-fold at dinucleotides (30). However, instability of integrated stretches of CTG repeats of 40 and 70 in yeast are not affected by deletion of MLH1 (31). In bacteria, mutations in MMR proteins enhance the stability of triplet repeat-containing plasmids (32). These varying results may reflect a requirement for different MMR proteins at different loop structures. Whatever the source of the differences, it is clear that defeat of MMR by secondary structure cannot account for the full spectrum of mutation observed in human disease. As the repeats grow, the expansion rate increases from 3- to 175-fold over the contraction rate and the size of the change increases (3). These effects are inconsistent with the MMR defects observed in any simple system.

Loop formation also is associated with several mechanisms thought to generate larger expansions. Recent studies in yeast indicate that absence of flap endonuclease activity (RAD27 in *Saccharomyces cerevesiae*; FEN-1 in humans) can destabilize simple tandem repeat loci (14, 31). The $5' \rightarrow 3'$ flap endonuclease is a structure-specific nuclease required for Okazaki fragment processing (33). Lagging strand processing is necessary because polymerase will, occasionally, "read through" the end of a preexisting Okazaki fragment, causing strand displacement at the $5'$ end. FEN-1 forms a ring that slides along the single-strand flap until it binds the junction and cleaves the flap (33). If the flap is not removed, ligation of the displaced strand yields a loop of extra DNA that can potentially lead to an expansion. Indeed, the loss of FEN-1 results in a tendency for duplications rather than deletions in yeast (14, 31). Mutation patterns in human expansion disease are inconsistent with the notion that FEN-1 is lost or defective in disease. However, it has been suggested that secondary-structure formation in the flap might prevent FEN-1 processing in a site-specific manner (34). Several studies indicate that secondary structure forms more readily on lagging strand and that lagging strand is more susceptible to mutation (35, 36). Demonstration of FEN-1 involvement might support an "orientation-centric" model of instability that depends on lagging-strand synthesis.

Repeats can increase from 30 to 1,000 bases in a single transmission event once the repeat is sufficiently long (1–3). Aberrant Okasaki processing at repeats by FEN-1 might facilitate large expansions because the displaced strand has the potential to invade a homologous chromosome or a sister chromatid. Thus, the donor single strand could prime synthesis on a recipient, generating expansion by gene conversion. Alternatively, expansions may be generated by recombination repair at or near the site of an unfilled gap or double strand breaks. At long arrays, polymerase may fail to fully traverse the repeats, resulting in stalling of the replication fork and/or incomplete replication and chromosome breakage. In addition to observed fragile site in CGG repeats in human disease, recent studies have shown that chromosome breakage can occur at CTG repeats at or above 130 in yeast (31). During recombination in yeast, repair of double strand breaks induces CTG instability that depends on both Rad52p and Rad1p (31). These data support a mechanism by which CTG tract instability can occur during double strand break repair through a single-strand annealing mechanism (31, 37). It has not yet been demonstrated whether such a recombination-based mechanism depends on secondary structure at the repeats. However, it is clear that double strand breaks can be a source of amplification of repeats once a break has occurred. As with MMR, recombination during double strand break repair cannot account for the full mutation spectrum observed in human disease. Because double strand break at CAG/CTG repeats are observed only above 130, all of Huntington's disease instability must occur by another mechanism.

Orientation and Developmental Issues of Expansion in Human Disease. Discrimination among these mechanisms awaits

resolution of two additional issues that are touched on in the experiments of Moore *et al.* (6). Loop escape in their system occurs during meiosis. Can loops formed at mitosis also escape? It is still uncertain whether instability is a mitotic or a meiotic event. If instability depended on both homologous chromosomes, then events in a haploid cell could be ruled out. However, recent data from FRDA, the only recessive trinucleotide repeat disorder, reveals that instability does not depend on the number of long alleles and that there is no change in repeat number of normal alleles after unstable transmission (12). These data indicate that the process of instability is likely to be intra-allelic. An intra-allelic event could occur in either a germ cell or somatic cell, and instability occurs in both (39–42). Recent data also indicate that, in both germ cells and somatic tissue of human and mouse, instability increases with age. Instability in a germ cell of an aging animal indicates either that transcription-coupled repair may contribute to instability or that heteroduplex loops made during replicative phase in germ cells take time to repair. The latter mechanism is possible, according to the data of Moore *et al*.

The third and final conclusion of Moore *et al.* is that hairpins form equally well *in vivo* on either strand of a CAG/CTG repeat (because they escape repair to the same extent). This important finding may shed light on the orientation dependence of instability. In both bacteria and yeast, it is well established that orientation with respect to the origin of replication is a factor in unstable transmission. CTG in the lagging strand is more unstable and tends to delete, whereas no change or infrequent expansion occurs if CAG repeats are in the lagging strand (35, 36). These data have suggested that the degree and the outcome (deletion or insertion) will be influenced by which direction polymerase is travelling when the repeat is copied. While the orientation dependence is clear, the cause is not. It has been suggested that the greater hairpin stability of CTG repeats may be responsible for this difference (22). However, the thermodynamic stabilities of CAG and CTG hairpins *in vitro* are nearly identical under physiological salt concentrations (16, 43). Additionally, recent kinetic studies indicate that CAG and CTG hairpins will reform duplex molecules at equal rates under pseudo-first-order conditions, suggesting that the kinetic lifetime of both hairpins *in vitro* are similar (43).

The lack of differential effects imposed by hairpins after they form points to a model in which orientation has a differential influence on the rate of formation of CTG relative to CAG hairpins (6, 43). If the "orientation-centric" model of instability is correct and instability is limited to the lagging strand, then it is possible that differential protein–protein interactions (such as binding of single-stranded binding proteins) impose as-yet-unknown constraints on the formation of CAG relative to CTG hairpins. However, in the absence of definitive data, the orientation dependence of instability may be explained equally well as "sequence-centric" in that a CAG or CTG hairpin has a different effect in the leading strand than in the lagging strand but instability can occur on either.

The importance of the orientation cannot be overestimated. Human studies as well as the variability in transgenic mice models (1–3) have led to the suggestion that an undefined chromosomal component or position is critical for instability. Sensitivity of a repeat to a preferred origin of replication may be one chromosomal component of instability. If expansion and contraction reflect the direction of replication, then random firing may be the protective mechanism the keeps repeats in check in normal individuals. If this were the case, nonrandom copying or origin selection could be the source of the problem in expansion disease. Recent results with FRDA patients reveal that for repeating GAA/CTT, however, the YRY folding is preferred at GAAs (12). Unlike hairpins, then, folding of the nucleotide chain in the frataxin allele is directional. Surprisingly, in FRDA patients, males tend toward deletions, while females tend toward expansion (12, 44). A "direction" for instability can occur only if both the structure and the origin are fixed. Because hairpin formation is equally likely on both strands, directional copying or preferred origin use may be masked at CNG repeats. If orientation dependence reflects different differential origin use, then it remains entirely possible that repeat expansion itself may influence the firing of nearby origins during disease progression. The acquired tendency to "expand" may reflect the tendency to be increasingly replicated in only one direction.

Although substantial progress has been made in understanding this fascinating mutational mechanism, new information continues to raise important questions. While a thorough understanding of the mechanism has not been achieved, the search is well worth the effort. A mechanistic solution to the problem of instability is likely to expose the very nature of heritable traits and evolution.

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