DNA structures, repeat expansions and human hereditary disorders
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Expansions of simple DNA repeats are responsible for more than two dozen hereditary disorders in humans, including fragile X syndrome, myotonic dystrophy, Huntington’s disease, various spinocerebellar ataxias, Friedreich’s ataxia and others. During the past decade, it became clear that unusual structural features of expandable repeats greatly contribute to their instability and could lead to their expansion. Furthermore, DNA replication, repair and recombination are implicated in the formation of repeat expansions, as shown in various experimental systems. The replication model of repeat expansion stipulates that unusual structures of expandable repeats stall replication fork progression, whereas extra repeats are added during replication fork restart. It also explains the bias toward repeat expansion or contraction that was observed in different organisms.

Introduction
The phenomenon of genetic anticipation was first described in 1918 for the human hereditary disorder myotonic dystrophy. This disease appeared to have earlier onset and increased severity as the mutant gene was transmitted from one generation to the next [1]. Genetic anticipation was subsequently detected for other hereditary neurological disorders, including Huntington’s disease, Friedreich’s ataxia and different spinocerebellar ataxias. The penetrance of mutations responsible for these diseases also appeared to increase in successive generations. The latter trend, first described for fragile X syndrome, became known as the Sherman paradox [2]. Neither genetic anticipation nor the Sherman paradox could be explained in terms of classic Mendelian genetics and were often ruled out as a result of ascertainment bias.

The genetic nature of these disorders was finally understood upon cloning and characterizing the fragile X mutation in 1991 [3–5]. This mutation appeared to be caused by the progressive intergenerational expansion of a simple DNA repeat, (CGG)n, located within the 5'-untranslated region (UTR) of the \emph{FMR1} gene. This striking discovery was soon followed by the demonstration of (CAG)n repeat expansions in spinobulbar muscular atrophy [6], (CTG)n repeat expansions in myotonic dystrophy [7–9] and (GAA)n repeat expansions in Friedreich’s ataxia [10], among others. As of today, more than two dozen human hereditary disorders are linked to simple repeat expansions.

In all cases, repeats are stably inherited until their lengths exceed a threshold of approximately 100–200 bp. Beyond this threshold, they start to expand during intergenerational transmission, making up to several thousand copies in the course of a few generations for some diseases. The progressive character of repeat expansions provided the first clue to understanding the hereditary pattern described above [11]. The earlier onset and severity of the disease, and the probability of the repeat’s expansion increase with its length, accounting for genetic anticipation and the Sherman paradox, respectively. During the past decade, it became clear that these genetic phenomena are probably grounded in the unusual structural characteristics of expandable repeats. This review attempts to describe how the formation of unusual DNA structures by expandable repeats during DNA replication and/or repair could lead to their expansion, resulting in disease.

Main characteristics of repeat expansions
Originally, expansions were limited to trinucleotide (CGG)n●(CCG)n [4,5], (CAG)n●(CTG)n and (GAA)n●(TTT)n repeats. It is now clear that tetrameric (CCTG)n●(CAG)n [12], pentameric (AATCT)n●(AGAT)n [13] and even dodecameric (C4GC4GCG)n●(CGCG2CG4)n [14] repeats can also expand, leading to human disease. For each disease, expansions are limited to just one repeat of a given gene. Thus, repeat expansions are not caused by mutations in the \emph{trans}-acting factors involved in DNA replication, repair or recombination, which would induce general microsatellite instability [15]. The primary events leading to repeat expansion occur in \emph{cis}.

The major characteristics of different repeat expansions are fundamentally similar. A repeat starts to expand when its length exceeds a threshold of roughly 100–200 bp. Normal alleles usually have much shorter repetitive runs.
The so-called 'long normal alleles' contain long repeats with several stabilizing interruptions. In expanding alleles, the interruptions at one end of a repeat are usually absent, creating lengthy homogenous repetitive runs [16]. Thus, there exists a link between the repeat's integrity and its propensity to expand. The longer the repeat becomes, the more likely it is that it will expand further. Although expansions occur with mutation frequency for repeats near the threshold length, long repeats expand with near 100% probability. Finally, expansions are large-scale events, such that dozens or even hundreds of repeats could be added during a single transmission step. This indicates that significant DNA synthesis is needed to generate these extra repeats.

**Structures of expandable DNA repeats**

Which properties of the repeats could account for their propensity to expand? The simplest explanation was that the repetitive nature of expandable elements leads to occasional strand slippage during DNA replication [17,18]. An unrepaired 'slip out' in the nascent DNA strand would convert into an expanded repeat after a second round of replication (Figure 1). This hypothesis could not adequately explain several characteristics of repeat expansions, however. First, not every repeat expands. Second, strand slippage usually causes limited repeat length polymorphism, rather than large-scale expansion. Third, the bias toward repeat expansion in humans remains unaccounted for.

By the mid 1990s, it became evident that expandable repeats have unusual structural properties [19]. In a pioneering study [20], soon supported by others [21–24], d(CGG)$_n$, d(CCT)$_n$, d(CTG)$_n$ and d(CAG)$_n$ stretches were shown to fold into hairpin-like structures, comprising both Watson–Crick (WC) and non-WC base pairs (Figure 2a). These hairpins contain different mismatches, contributing to their stability in the following order: 

CGG > CCG ≈ CTG > CAG [20]. Formation of hairpin-like structures by d(GAA)$_n$ and d(TTC)$_n$ repeats was also later proposed [25].

Individual strands of expandable repeats can fold into other unusual DNA conformations as well. For example, single-stranded d(CGG)$_n$ repeats fold into a peculiar tetrahelic structure stabilized by intertwined G-quartets, as shown in Figure 2b [26].

The structure-forming potential of expandable repeats drastically changes the outcome of DNA strand slippage. Denaturing and renaturing repeat-containing duplexes leads to the formation of unusually stable slip-stranded DNAs, in which the 'loop outs' form hairpin-like structures (Figure 2c). These stable hairpins kinetically trap repetitive DNA in the otherwise unfavorable slip-stranded conformation [27].

The (GAA)$_n$•(TTC)$_n$ repeat belongs to the group of so-called homopurine–homopyrimidine mirror repeats,
which form intramolecular triplexes (H-DNA) under the influence of negative supercoiling [28]. Apparently, this particular repeat can form a variety of three-stranded DNA structures (Figure 2d). Individual repetitive runs were shown to adopt either H-γ (pyrimidine/purine/pyrimidine triplex) [29] or H-γ (pyrimidine/purine/purine triplex) [30] conformations under physiological conditions. Two distant directly repeated (GAA)_n(CTT)_n tracts within the same plasmid also form a composite triplex structure, called 'sticky DNA' [30,31]. The fine structure of sticky DNA remains unknown, but it might be reminiscent of the composite triplex structure of the two distant homopurine–homopyrimidine runs, as proposed in [32].

Finally, an AT-rich repeat implicated in spinocerebellar ataxia type 10, (ATTCT)_n(AGAAT)_n, forms an extensively unwound DNA conformation under the influence of superhelical stress [33].

Notably, the formation of unusual DNA structures is less likely for those repeats that are not known to expand [20]. Furthermore, stabilizing interruptions within trinucleotide repeat runs in long normal alleles make the formation of unusual DNA structures more difficult [20,34]. It is generally believed, therefore, that there is a link between a repeat’s ability to form unusual DNA structures and its propensity to expand. It was even speculated that the threshold length for the repeat expansions might simply reflect the stability threshold for the corresponding structures. The latter idea is, however, questionable for two reasons. First, various repeats form unusual DNA structures, such as hairpins, quadruplexes, triplexes and so on, that differ enormously in their stability, yet their expansion threshold is quite similar. Second, very stable secondary structures, capable of obstructing major DNA transactions, are formed by repeats that are much shorter than the expansion threshold [35].

From the biophysical view point, the majority of unusual DNA structures formed by expandable repeats look peculiar. The hairpin-like and slip-stranded structures contain various mismatched base pairs and multiple junctions between substructural elements. Similar defects haunt quadruplex-like structures built from (CGG)_n repeats. Triplexes formed by (GAA)_n repeats are structurally sound, but require significant DNA supercoiling to offset their high nucleation energy. It seems that these structural imperfections, together with the interruptions within repetitive runs, exist to ensure that repeats remain stable. Only in rare instances, when a repetitive run becomes excessively long and homogeneous by losing its interruptions, do expansions begin.

Mechanisms of repeat expansion
The principal event that triggers the formation of unusual DNA structures by expandable repeats in genomic DNA is DNA unwinding or even complete strand separation. The main cellular process involving DNA strand separation is DNA replication. During replication fork progression, a portion of the lagging strand template, called the Okazaki initiation zone (OIZ), remains transiently single stranded to ensure coordinated syntheses of the leading and lagging DNA strands. It was plausible to suggest, therefore, that expandable repeats could fold into unusual secondary structures while within the OIZ of the lagging strand template.

The first support for the lagging strand hypothesis came from a landmark study that found that the stability of (CTG)_n(CAG)_n repeats in bacterial plasmids depended on their orientation relative to the replication origin [36]. When the structure-prone (CTG)_n runs were situated in the lagging strand template, repeats frequently contracted. In the opposite orientation, that is, with (CTG)_n runs in the nascent lagging strand, contractions were less pronounced and expansions became detectable. It was suggested, therefore, that hairpin-like structures formed by the structure-prone repetitive strand in either the lagging strand template or nascent lagging strand caused contractions or expansions, respectively (Figure 3). This hypothesis gained additional support after the expansion frequencies of various repeats in yeast and mammalian cells were also found to depend on their orientation within the replicon [37–39]. Furthermore, mutations in the replication apparatus of bacteria and yeast, particularly those affecting lagging strand synthesis, greatly influenced repeat stability [40].

The model presented in Figure 3 provides a ready explanation for repeat contractions, as the folding of the lagging strand template seemed very feasible. What could trigger slippage of the nascent lagging strand, producing expansions, remained less clear. A valuable hint came from studies of replication fork progression through expandable repeats in bacterial, yeast and mammalian cells [41–43]. It appeared that expandable (CGG)_n(CTG)_n, (CAG)_n(CTG)_n and (GAA)_n(CTT)_n repeats stalled the replication fork in all these systems. The lagging strand around the stall site appeared to be under-replicated [43], suggesting repeat-mediated blockage of lagging strand synthesis. For all repeats, replication stalling was length dependent, becoming evident around the expansion threshold. In most cases, replication blockage depended on repeat orientation relative to replication origin. Finally, both expansions and contractions preferentially occurred in the repeat’s orientation, which was responsible for replication stalling [41].

Together, these results point to an alternative model of repeat instability, based on replication fork stalling and restart. When the leading strand DNA polymerase runs into an expandable repeat, a single-stranded part of the lagging strand template becomes repetitive (Figure 4a)
and can fold into a stable secondary structure (Figure 4b). This becomes particularly likely when the repeat exceeds the threshold length for expansions, making the whole OIZ a smooth repetitive run. Formation of the stable secondary structure in the lagging strand template would stall the lagging strand DNA polymerase and, as leading and lagging strand syntheses are coordinated, slow down the replication fork overall. DNA replication could bypass this obstacle in two different ways. First, lagging strand synthesis could simply resume after skipping an Okazaki fragment, leaving a gap in the nascent lagging DNA around the repeat (Figure 4c). Subsequent repair of this gap would lead to repeat contraction, if the lagging strand polymerase skips the structured portion of its template (Figure 4d). Second, replication stalling can lead to fork reversal. The reversal of a fork, stalled within an expandable repeat, leads to the formation of a four-way junction followed by single-stranded repeat extension at the 3' end of the leading strand (Figure 4e). This single-stranded repetitive tail would easily fold into a secondary structure (Figure 4f). To restart replication, the reversed fork is flipped back and, if the repetitive structure holds, extra repeats will be added to the leading strand (Figure 4g).

This model adds an extra level to our understanding of genetic anticipation, described at the beginning of this review. For repeats comparable to the OIZ in length, expansions should be rare, because both the formation of stable secondary structure on the lagging strand template and subsequent slippage of the leading strand during the restart are low probability events. For repeats longer than the OIZ, the replication fork can stall and restart several times, roughly proportionally to the number of Okazaki fragments in it. These recurrent replication stalls and restarts should greatly increase the instability of long repeats.

Most importantly, this model gives an explanation for the bias toward expansion or contraction that was observed in humans or unicellular model organisms, respectively. It stipulates that the ratio of expansions to contractions depends on the balance between fork reversal and Okazaki fragment skipping upon replication fork stalling. This balance could obviously vary for different organisms or different cells within the same organism. For example, fork reversal could dominate Okazaki fragment skipping in rapidly dividing pre-meiotic cells in mice and humans, in which case expansions are more likely to occur [44]. In contrast, Okazaki fragment skipping might be prevalent in *Escherichia coli*, in which case deletions would be much more frequent than expansions [36].

Proteins involved in DNA replication and repair could also significantly affect the equilibrium between expansion and contraction. A very plausible candidate is the MSH2/MSH3 mismatch repair complex. This complex binds tightly to imperfect hairpins comprising expandable repeats, but its ATPase activity is impaired in the process [45]. As a result, the MSH2/MSH3 dimers are trapped on repeat-containing hairpins, inadvertently stabilizing them. This stabilizing effect might be responsible for the fact that repeat expansions are significantly diminished in mice with *MSH2* or *MSH3* knockouts [46–48].

The replication model might also help understand the observation that expandable repeats stimulate...
Homologous recombination in bacterial [49,50], yeast [51,52] and cultured mammalian cells [53], undergoing expansion and contraction in the process. Reversal of the replication fork stalled within the repetitive run leads to the appearance of a 3'-repetitive extension. 3' overhangs normally serve as platforms for loading the recombination proteins that are responsible for the strand exchange reaction. Loading of these proteins on the repetitive overhang (Figure 4h), followed by strand invasion into a homologous repeat of the sister chromatid, could lead to expansion or contraction, depending on the precise invasion spot.

Unusual structures of expandable repeats could also contribute to their instability in non-dividing cells. Repeat expansions are evident in patients’ post-mitotic tissues, such as brain and skeletal muscle [54,55]. This is commonly explained as the consequence of gap repair of the repeats [48]. Oxygen radicals or other environmental agents could generate nicks and/or small gaps within repetitive tracts (Figure 5a). In the process of gap repair DNA synthesis, the non-template DNA strand is displaced, forming a flap (Figure 5b). This flap would normally be removed by the flap-endonuclease FEN1, which loads onto its 5' end, migrates to its junction with the duplex and cleaves the flap. If the flap contains a repetitive run, however, it can fold into a hairpin-like structure, preventing FEN1 loading [56–58]. Upon completion of repair DNA synthesis, stable slipped-stranded intermediates are formed (Figure 5c) and can be

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**Figure 4**

Model of repeat instability generated during replication fork stalling and restart within the repetitive run [40].

(a) Entrance of the leading strand polymerase into the repetitive run.
(b) Formation of unusual structure by the lagging strand template, stalling the lagging strand polymerase and, ultimately, the replication fork.
(c) Replication continuation by skipping an Okazaki fragment.
(d) Contraction of the repeat as the lagging strand polymerase skips the structure on its template.
(e) Fork reversal generates a ‘chicken foot’ structure with a single-stranded repetitive 3’ extension.
(f) Folding of the repetitive extension into a hairpin-like conformation.
(g) Replication restart upon flipping back the chicken foot, leading to repeat expansions.
(h) Loading of the recombination proteins responsible for the strand exchange reaction onto the 3’-repetitive extension.

A structure-prone strand of the repeat is shown in red, its complementary strand is in green and flanking DNA is in black. Golden ovals represent DNA polymerases, purple lines Okazaki primers and blue circles recombination proteins.
Figure 5

Flap model of repeat expansion in non-dividing cells [58]. (a) Oxidative radicals generate a small gap in the structure-prone strand of a repetitive tract. (b) FEN1 fails to load onto the structured repetitive flap generated during repair DNA synthesis. (c) Stable slipped-stranded DNA intermediates are formed upon completion of DNA synthesis. A structure-prone strand of the repeat is shown in red, its complementary strand is in green and flanking DNA is black.

converted into expanded products via a recently discovered error-prone repair pathway [59].

Conclusions
It is evident that the phenomenon of repeat expansion has broad biological and medical implications. Although the mechanisms of repeat instability vary in their fine detail in different organisms and/or different cell types within the same organism, formation of unusual structures by repetitive DNA seems to be at the heart of all processes. These structures apparently bewilder the machinery for major genetic transactions, primarily DNA replication but also recombination and repair, ultimately leading to repeat instability. Molecular models of repeat expansion, including the replication model discussed here, can satisfactorily explain the majority of the experimental data. Yet none of them has been proven definitively. Thus, further biochemical and genetic studies of repeat expansion are warranted. Of particular interest are the events leading to the conversion of long normal repeats into their expandable versions, which are poorly understood at present.

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References


