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Break-induced replication links microsatellite expansion to complex genome rearrangements

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Abstract

The instability of microsatellite DNA repeats is responsible for at least forty neurodegenerative diseases. Recently, Mirkin and colleagues presented a novel mechanism for microsatellite expansions based on break-induced replication (BIR) at sites of microsatellite-induced replication stalling and fork collapse. The BIR model aims to explain single-step, large expansions of CAG/CTG trinucleotide repeats in dividing cells. BIR has been characterized extensively in *S. cerevisiae* as a mechanism to repair broken DNA replication forks (single-ended DSBs) and degraded telomeric DNA. However, the structural footprints of BIR-like DSB repair have been recognized in human genomic instability and tied to the etiology of diverse developmental diseases; thus, the implications of the paper by Kim et al. extend beyond trinucleotide repeat expansion in yeast and microsatellite instability in human neurological disorders. Significantly, insight into BIR-like repair can explain certain pathways of complex genome rearrangements (CGRs) initiated at non-B form microsatellite DNA in human cancers.

Keywords

break-induced replication; chromothripsis; DNA repair; DNA replication; FoSTeS; genome instability; microsatellite instability

Introduction – Microsatellite expansions lead to human disease

Microsatellite DNAs are runs of repetitive sequences in which short motifs, typically 1–6 base pairs in length, are repeated 5–50 times at numerous loci across the genome in wild type cells [1, 2]. These tandem repeats are zones of replication fork stalling and DNA breakage, and are frequently correlated with sites of mutations, copy number variation, replication template switches or structural variant breakpoints [3–8]. Although the repeat tracts responsible for microsatellite expansion-related disorders share the common tendency to form noncanonical DNA structures, they comprise diverse sequences. Thus, CAG/CTG microsatellites are found in the coding regions of polyglutamine (poly-Q) disease-related genes where trinucleotide repeat expansion causes Huntington disease (HTT; Online Mendelian Inheritance in Man (OMIM) 613004), dentatorubral–pallidoluysian atrophy

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(ATN1; OMIM 607462), spinal and bulbar muscular atrophy (AR; OMIM 313700) and several forms of spinocerebellar ataxia. In contrast, Fragile × mental retardation (OMIM 300624) is attributed to expansion of CGG/CCG repeats in the FMR1 gene, Freidrich ataxia (FXN, OMIM 229300) is caused by expansion of GAA/TTC repeats in the frataxin gene, and myotonic dystrophy type 2 (DM2, OMIM 602688) is due to expansions of CCTG/CAGG repeats in the zinc-finger protein-9 gene.

At the Huntington disease (HD) locus the range of CAG/CTG repeats in asymptomatic individuals is between nine and thirty-seven. Complete penetrance of HD was observed for tract sizes equal to or greater than forty-two CAG/CTG repeats, while incomplete penetrance was observed with repeat lengths of thirty-six to forty-one [9]. The phenomenon of ‘genetic anticipation’ is a hallmark of HD and other microsatellite expansion disorders, where earlier onset and increased severity of symptoms are correlated with intergenerational expansions of microsatellite tracts. Because expansions of diverse microsatellites are the causative factors in multiple neurodegenerative disorders, understanding the mechanisms of microsatellite instability is of significant clinical relevance [10–12].

The analysis of microsatellite instability and chromosome breakage has been complicated by the diversity of microsatellite repeats, differences between model systems, variable cellular responses to different microsatellite repeat lengths, and the contribution of chromosome context [13–23], as well as observations that microsatellite-induced replication fork stalling does not unavoidably cause instability [24]. Nevertheless, there is general agreement that microsatellite repeats as a group cause replication stress and chromosome fragility in a length-dependent manner [25, 26]. In human cell culture, expanded CAG/CTG tracts are sites of replicative polymerase stalling [27], hairpin structure formation on leading and lagging strand replication templates in vivo [28, 29], and chromosome DSBs [6, 30]. Neutralization of lagging strand hairpins by transfection of cells with oligonucleotides complementary to either CTG or CAG lagging strand templates simultaneously eliminated both leading and lagging strand hairpin formation and relieved polymerase stalling [27]. These observations suggest that replication is coordinated on leading and lagging templates such that barriers formed on the single stranded lagging template can impede leading strand polymerization and promote leading strand hairpin formation. Hence, CAG/CTG microsatellite hairpin structures are foci of replication fork stalling which generate substrates susceptible to fork collapse and DNA breaks [6, 14, 31, 32].

Multiple replication- and repair-based mechanisms have been proposed to contribute to microsatellite instability including slippage/stuttering of DNA polymerases [33–36], and hairpin formation in nascent strand DNA during replication [28, 37] (Figure 1). Aberrant DNA damage signaling [38], replisome destabilization [14, 39], collisions with transcription machinery [40], R-loop formation [41, 42], POLβ/δ DNA synthesis during base excision repair (BER) of oxidized nucleotides [43–45] and binding of mismatch repair (MMR) proteins [10, 46]) have additionally been proposed to exacerbate microsatellite instability.

Replication- and repair-based mechanisms of microsatellite instability are not mutually exclusive in dividing cells, whereas DNA repair pathways presumably account for expansions in postmitotic tissues [17, 44, 46, 47]. Indeed, the MSH2-MSH3 (MutSβ) MMR

proteins have been shown to contribute to the expansion of long CAG/CTG tracts, irrespective of replication polarity [24, 44, 48–52]. In HeLa cell extracts MutSβ promotes expansion of CAG/CTG repeats by interaction with hairpin structures and recruitment of DNA polymerases β and δ [44]. In humans, MMR deficiency leads to short expansions and contractions of microsatellites (microsatellite instability (MSI, MIN)) in the cancer predisposition disorders Lynch syndrome (OMIM 120435) and Muir-Torre syndrome (OMIM 158320) in dividing cells of the colon and elsewhere [53–55], presumably due to the inability of MMR to resolve nascent and template strand DNA hairpins arising during replication, repair, or transcription.

The break-induced replication fork is unstable and highly mutagenic

The process of break-induced replication (BIR) is a homologous recombination pathway conserved from phage to eukaryotes [56–58] which serves to repair single-ended double-stranded breaks (seDSBs) such as might arise at collapsed or broken replication forks (Figure 2A). BIR has also been implicated in the recombination-dependent alternative lengthening of telomeres (ALT) [59]. In theory, any process that leads to breakage at single stranded DNA (e.g. stalled replication forks or transcription complexes, base/nucleotide excision repair tracts, non-B DNA secondary structures) could lead to BIR once a replisome collides with the end of the broken DNA template.

BIR has been extensively characterized in molecular detail in budding yeast [57, 60–64], where 5′ DNA end resection at a seDSB is followed by RPA binding to the newly exposed 3′ single strand DNA (ssDNA) (Figure 2B) [62]. RPA is subsequently replaced by the RAD51 recombinase (Figure 2C), which directs strand invasion and D-loop formation at a complementary sequence. The complementary sequence is most often acquired from the proximal sister chromatid [62, 63], although annealing to non-allelic homologous or homeologous sequences is possible [65]. Establishment of a replisome containing the canonical CMG (CDC45-MCM-GINS) replicative helicase, PCNA and DNA polymerase at the 3′ ssDNA end [66] leads to extensive break-induced replication from the site of invasion, which can progress hundreds of kilobases [61, 63] but can be limited by resolvase (MUS81/YEN1) cleavage of BIR intermediates or fusion with an oncoming replication fork [67].

The initiation of BIR is slow following a seDSB, requiring several hours [68], and involving multiple template switches [69]. Although the components of the replicative helicase and the three major replicative DNA polymerases (α, δ, ε) are required for BIR, additional observations indicate that the BIR replication fork differs from a typical S-phase replication fork. Thus, BIR requires the nonessential POL32 (human POLD3) subunit of POLδ and POLζ, and the PIF1 helicase for processive DNA synthesis, and certain alleles of the PCNA clamp that can support semiconservative replication are dominant negative for BIR [66].

During BIR, POLδ is the primary polymerase for extension of the RAD51-coated invading strand, as the N-terminal polymerase catalytic domain of POLε is not essential in *S. cerevisiae*, and yeast mutants lacking this region of POLε are not hypersensitive to DSBs [70]. Additionally, yeast POLε is unable to perform displacement synthesis required to

extend the invading strand D-loop in vitro, although a template unwinding function might be provided by the PIF1 helicase in vivo [71]. Nevertheless, based on the pattern of mutations during BIR in cells containing a proofreading-deficient allele, POLε has been implicated in second-strand synthesis [72].

Polymerization from the 3′ end of the invading strand is non-processive during the initial stages of break-induced replication [73], which include repeated cycles of invasion, synthesis, and D-loop dissociation, as well as multiple template switches [69]. Template switches are associated with modification of the PCNA processivity clamp by RAD18-dependent ubiquitination and SIZ1-dependent sumoylation [74, 75], as well as the recruitment of postreplication repair (PRR) translesion polymerases to the replication fork [76, 77]. Indeed, both RAD18 and SIZ1 are essential for efficient BIR [78].

The initial instability of the BIR D-loop suggested that translesion polymerases (Table 1) might contribute to the 1,000-fold greater mutagenicity of BIR vs. semiconservative replication [69, 79, 80]. Moreover, in vitro studies using yeast or human proteins to model homology-dependent DNA synthesis showed that POLη could extend RAD51-mediated D-loops [81–83] and efficiently utilize 3′ ends in the D-loop to synthesize several hundred base pairs of DNA. Unlike POLδ, D-loop extension by POLη did not depend on PCNA. In budding yeast, however, deletion of RAD30 (encoding DNA POLη) or REV3 (encoding the catalytic subunit of DNA POLζ) did not decrease the BIR-dependent repair of a segmented drug marker gene [66]. Nor did these TLS polymerase gene deletions have major effects on the frequency of frame shift mutations at A/T homopolymer tracts during BIR [84]. While these results leave open the possibility of other types of TLS mutations at alternative sequence motifs, the high mutation rate during BIR has been attributed instead to increased dNTP pools, an increased rate of POLδ errors due to decreased proofreading, and decreased efficiency of mismatch repair (MMR). Following leading strand synthesis, replication of the nascent strand displaced from the migrating D-loop is conservative, and serves to fix BIR mutations (Figure 2C) [61].

Break-induced replication can lead to large expansions of CAG/CTG microsatellites

Now, the work by Kim et al. [85] adds the novel observation that break-induced replication is among the mechanisms leading to expansion of CAG/CTG microsatellites in budding yeast. The authors have engineered a system in which large CAG expansions lead to the transcriptional inactivation of a neighboring CAN1 arginine permease gene that can be scored by the conversion to canavanine resistance. In contrast to other potential causes of replication fork stalling and breakage such as R-loop formation or protein binding, the combination of two characteristics of microsatellites promote expansion by BIR; first, the tendency of repetitive sequences to form noncanonical structures that stall replication and cause chromosome fragility [5, 19, 25, 32, 38], and second, the likelihood of stochastic, homology-mediated repeat misalignment between sister chromatid microsatellites during strand invasion.

In the most parsimonius model of Kim et al., nuclease cleavage generates a seDSB at a replication fork stalled by stable hairpin formation (Figure 3A). 5′ excision is followed by RPA binding to the resected seDSB (Figure 3B). RPA is then replaced by RAD51 to form the invading 3′ end nucleoprotein filament and D-loop (Figure 3C). Base pairing of a 3′ CAG repeat to a template 3′ CTG repeat results in microsatellite expansion. Additional expansions may occur through hairpin formation during synthesis of the nascent leading or lagging strands (Figure 3D).

Kim et al. have shown that genetic knockout of proteins involved in homologous recombination (RAD51, RAD52, MUS81/YEN1, MRE11) and break-induced-replication (POL32, PIF1) markedly decreased large CAG/CTG expansions, while knockout of proteins that positively (MSH2, MSH3, MSH6) or negatively (SRS2) affect small scale expansions did not affect large expansions. In the context of the blocking effects of *rad51Δ* and *rad52Δ*, the observation that deletion of the SRS2 helicase did not increase homology-dependent BIR [66, 85, 86] suggests that the SRS2 anti-recombinase activity is targeted to other forms of homologous recombination.

Treatment with HU to slow replication, or with camptothecin to introduce single strand DNA breaks at topoisomerase I cleavage sites, led to an approximate four-fold increase in large CAG expansions, indicating that replication stalling contributes to the BIR process, which can then continue into G2 or M phases [61, 87]. The authors also observed that transcription of the CAG/CTG repeat enhanced BIR, suggesting that RNA synthesis or an altered chromatin structure of the repeat tract may promote breakage. These observations raise the question of whether replisome components present in highly transcribed regions of postmitotic nuclei (e.g. in neurons) could promote BIR [88–90].

Under nonselective growth, large (BIR-related) expansions were observed at ~1×10^{-5} per replication, while small expansions (~1×10^{-2} per replication) and small contractions (~5–17×10^{-2} per replication) were approximately 1000-fold more frequent. In the experiments of Kim et al. [85], the expanded CAG tracts that were sequenced were found not to be mutated aside from changes in length, possibly because of the small number of tracts that were sequenced or the short length of the sequenced DNAs. Significantly, the authors showed that yeast containing an expanded $(CAG/CTG)_{140}$ microsatellite could add large (>20 repeats) expansions, some of which exceeded the initial length of the $(CAG/CTG)_{140}$ tract. Since It is well known that the tendency towards repeat instability increases with tract length, and repeated rounds of template switching occur during the early stages of BIR, it is possible that the largest expansions resulted from iterative rounds of strand invasion [69], as has been observed in synthesis-dependent strand annealing (SDSA) [91] and single-strand annealing (SSA) [92]. Additionally, alternative expansion mechanisms including polymerase stuttering, fork slippage and hairpin formation may also be at work during BIR of the leading or lagging nascent strands [93].

The demonstration by Kim et al. that BIR can lead to expansion of microsatellite DNAs takes on added significance because repeated sequences are prone to forming noncanonical DNA secondary structures (hairpins, slipped strands, triplexes, G-quadruplexes) that are hotspots of replication fork stress and DNA breakage [4, 94–97]. Thus, the same structures

that interfere with semiconservative replication and promote BIR may also lead to stalling or collapse of a BIR replication fork, causing a transition to microhomology-mediated break-induced replication (MMBIR) [77]. Significantly, in contrast to the relatively long homology tracts necessary for RAD51-dependent BIR (>100–200 nucleotides [98]), MMBIR involves multiple template switching events to non-allelic sequences of 0–6 nucleotide homology or homeology and utilizes the error-prone POLζ and REV1 translesion polymerases [77].

Does BIR/MMBIR occur in human cells?

As discussed above, a break in the replication template can initiate BIR [57, 61]. Particularly relevant to breaks at non-B DNA structures, once homology-dependent invasion of a donor chromosome has occurred, microsatellite-induced replisome stress in the donor template, template breaks or replication fork collapse [77, 79] could trigger the transition to MMBIR, especially if levels of RAD51 are limiting [73] (Figure 4A, B). Further rounds of template switching can occur to nonallelic templates containing 0–6 bases of homology or homeology, including snapping back to use the nascent strand as template (Figure 4C, D). Template switching may be promoted by DNA damage or noncanonical structures in the MMBIR template, imperfect primer synthesis by TLS polymerases, the lack of TLS polymerase processivity, D-loop instability, or collapse of the MMBIR fork.

MMBIR has been proposed to be an important mechanism responsible for non-recurrent chromosomal rearrangements (i.e. rearrangements at the same genetic locus that differ in size and sequence between individuals) associated with developmental disorders and cancer [73, 99–101]. In contrast to models in which cancer results from the gradual accumulation of driver gene mutations that successively enhance tumorigenesis [102, 103], a crucial finding was the discovery that tens to hundreds of complex genomic rearrangements comprising at least two breakpoint junctions can be formed during a single catastrophic DNA repair event termed chromothripsis [99, 104]. Thus, in contrast to the fusion of distal DNA sequences after “chromosome shattering”, BIR/MMBIR models posit that breakpoint junctions result from fork stalling and template switching (FoSTeS) [100]. The complex genomic rearrangements characteristic of chromothripsis are observed in 2%–3% of all cancers [104]. The MMBIR mechanism is proposed to be common to chromothripsis, chromoanasynthesis (inherited, constitutive CGRs) [105], and kataegis (localized clusters of single nucleotide somatic hypermutation) [106] in that these chromosome catastrophes arise from local and long range template switching and ordinarily are localized to only one or a few chromosomes.

Epidemiological analyses of diverse tumors across human populations estimate that well over one-third of oncogenic driver mutations arise from replication-based error mechanisms, as opposed to inherited or environmentally induced mutations [103]. DNA sequencing of structural variants from individuals exhibiting similar disease phenotypes has revealed signature features of MMBIR. In contrast to NHEJ, NAHR (nonallelic homologous recombination) or BIR, MMBIR is characterized by junction microhomologies, complex breakpoint junctions (duplications, triplications, inversions) attributed to short-range template switching in *cis* within the same replication fork, and juxtaposition of DNA sequences ordinarily separated by large genomic distances (>10–100 kb) due to FoSTeS in

trans between distinct replication forks [101, 107]. DNA synthesis during CGR repair also exhibits increased single-nucleotide variation and enhanced indel (50–100 bp) mutation rates near breakpoint junctions (~2.1×10^{-4} mutations/bp, ~1.7×10^{-3} events/bp, respectively) [107], consistent with highly error-prone DNA synthesis following template switching.

Recently, Hickson and colleagues have reported BIR-like mitotic DNA synthesis (MiDAS) to occur at common fragile sites (CFS) [87]. Like MMBIR, MiDAS is not RAD51 dependent. Similar to BIR, MiDAS involves generation of a single-ended DSB by the MUS81-EME1 nuclease, RAD52 loading of the noncatalytic POLδ subunit POLD3, and conservative DNA synthesis. Costantino et al. have also reported that RAD51-dependent BIR occurs in replication-stressed (cyclin E overexpressing) human cells [60]. This form of BIR is nominally sensitive to siRNA knockdown of POLD3 (the human ortholog of POL32), or RAD52, or to genetic deletion of RAD52 [108], but not to knockdown of PIF1 [108].

Gu et al. have proposed that Alu-mediated recombination is the result of BIR-like recombination-coupled DNA replication [109]. Human Alu repeats contain fork-stalling homonucleotide (A/T) tracts of up to 100 base pairs, and are present at approximately one million copies per genome [110, 111], making them prime candidates for replication fork stalling, homology-dependent annealing and FoSTeS. While NAHR and NHEJ mechanisms of DNA repair have been associated Alu-mediated recombination leading to more than a dozen human diseases, including Fanconi anemia (OMIM 300514), Alzheimer's disease (OMIM 157140) and Gaucher disease (OMIM 231000), Alu-mediated MMBIR/FoSTeS is specifically implicated in hereditary spastic paraplegia (OMIM 604277) and Waardenberg syndrome type 4 (OMIM 613266) [111].

Microsatellite repeats comprise approximately 3% of the human genome [112]. Taken with the results of Kim et al. [85], examples of microsatellite-dependent fork stalling and DSBs during replication stress enlarge the significance of BIR/MMBIR to include not only neurological and developmental diseases but the formation and progression of tumors. Many new questions are raised by these studies, viz., Does replication stress lead to microsatellite seDSBs at structure-prone repeats other than CAG/CTG tracts [30]? Do concurrent breaks at multiple microsatellites add to the complexity of FoSTeS and "chromosome catastrophes" [113]? What PCNA modifications occur during BIR/MMBIR to allow TLS polymerase recruitment and template switching? How does the distance from a replication origin affect the frequency of BIR, and D-loop stability? What are the effects of ataxia telangiectasia and RAD3-related (ATR) protein binding and downstream kinase signaling on BIR/MMBIR? Can BIR occur in postmitotic cells such as neurons? What is the role of BIR/MMBIR in the response to chemotherapy-induced ssDNA breaks? Thus, the questions raised by BIR in yeast and human cells have far reaching implications for genome stability in normal and pathological conditions.

Outlook

Several themes ought to be considered in the context of the conclusion by Kim et al. [85] that broken replication forks can lead to microsatellite instability by chromosome template switching, namely that microsatellites are prone to replication fork stalling and breakage;

that microsatellites in human cells are hotspots of recombination; and that CGRs show evidence of BIR/MMBIR-driven recombination. One next step will be to construct human model systems that exhibit microsatellite breakage under replication stress to test the effects of non-B structures on fork collapse, quantitate the rate of BIR-derived mutagenesis, and characterize the genomic consequences of BIR/MMBIR initiated at specific loci.

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Abbreviations

(MM)BIR (microhomology-mediated) break-induced replication break-induced replication

(se)DSB (single-ended) double strand break

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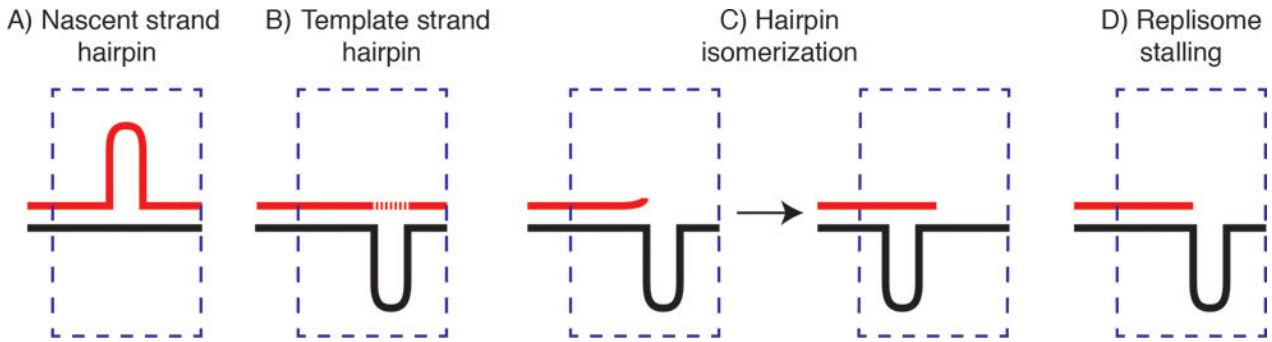

Figure 1.

Hairpin formation during DNA replication or repair. **A)** Polymerase stuttering at microsatellite repeats leads to excess nascent strand microsatellite sequence (expansion). **B)** Terminal transferase-like nontemplated synthesis (dashed line) across a hairpin abasic gap [114–120] or **C**) Template hairpin isomerization following destabilization of a stalled polymerase [121] leads to contraction. **D)** Polymerase stalling leads to replication stress, fork collapse, single-ended DSB (seDSB).

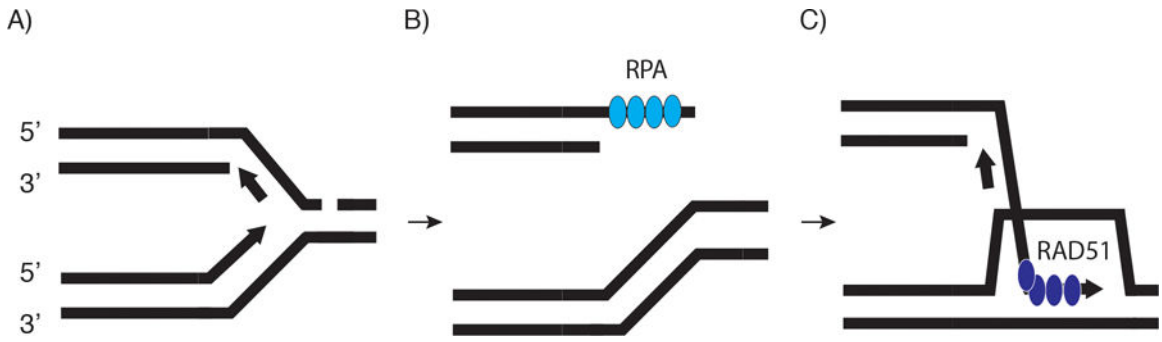

Figure 2.

Model of break-induced replication. **A)** A single-ended DSB leads to BIR. A break in the lagging strand template is shown for simplicity, but other causes of fork collapse or nuclease cleavage (e.g. HO endonuclease [68] and camptothecin inhibition of topoisomerase I [85], have been used to produce seDSB. **B)** Displacement of the lagging strand template allows leading strand ligation to form an intact chromatid. The seDSB is subject to 5′ end resection and RPA binding. **C)** RPA is replaced by RAD51 to form and invading (acceptor) filament. The acceptor DNA released by branch migration of the unstable D-loop is a template for lagging strand conservative DNA synthesis.

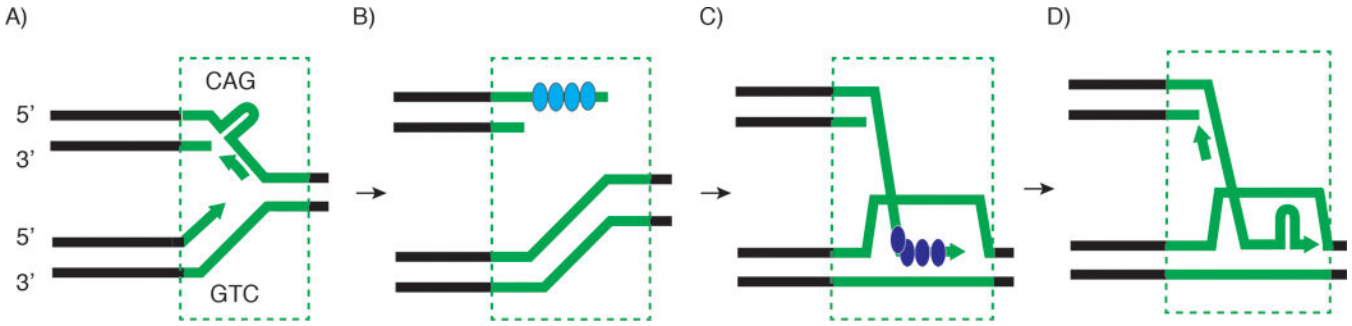

Figure 3.

Model for (CAG/CTG) microsatellite expansion by break-induced replication. **A)** Replication stalling at a (CAG/CTG) repeat tract (green). **B)** DNA cleavage (MUS81-EME2) at the site of stalling leads to a single-ended double strand break, fork collapse (replisome dissociation), resection of the 5′ end of the seDSB and binding of RPA to the extended 3′ ssDNA. **C)** Replacement of RPA by RAD51 and homology-dependent invasion of the sister chromatid repeat forms a displacement loop (D-loop). Misalignment of the acceptor (CAG) and donor (CTG) repeats at the start or middle of the template repeat tract leads to large expansions. **D)** Repeat expansions larger than the initial repeat tract length arise after continued template misalignment, mutation-prone replication fork slippage and hairpin formation across the repeat. The acceptor DNA released from the unstable D-loop is a template for conservative lagging strand replication. Break-induced replication is subsequently terminated by fusion with a leftward moving replication fork and/or resolvase (MUS81, YEN1) cleavage.

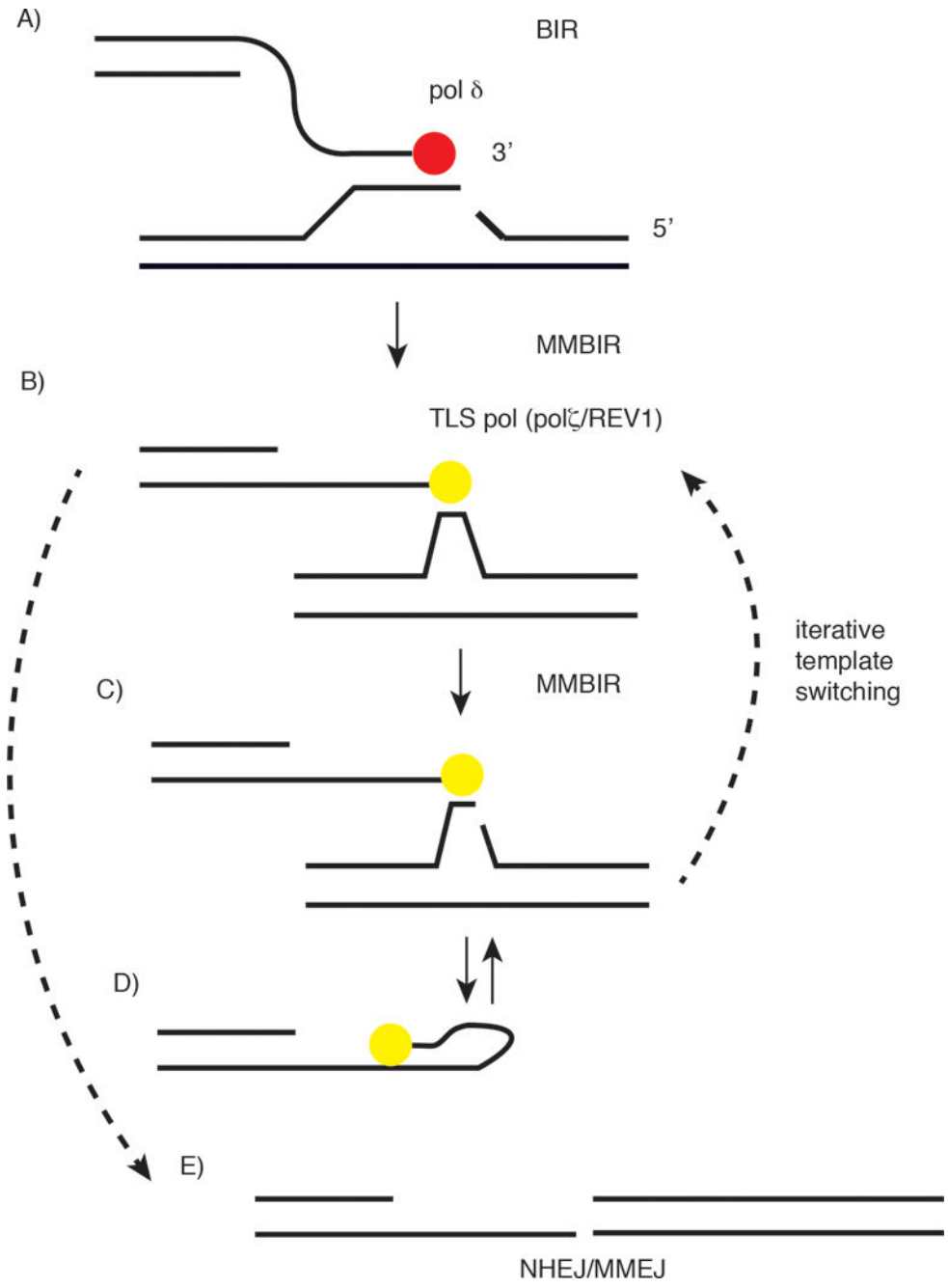

Figure 4.

Template switching and the transition from BIR to MMBIR. **A)** Fork stalling within or beyond microsatellite sequences causes fork collapse/breakage. **B)** TLS polymerases (Polζ/Rev1) enable microhomology-mediated BIR (MMBIR). TLS polymerase synthesis is not processive and fork collapse leads to template switching and microhomology-mediated BIR at a new site. **C)** Successive cycles of fork stalling and template switching (FoSTeS) lead to complex genomic rearrangements (CGRs). **D)** Self-annealing and DNA synthesis at microhomologies in nascent DNA. **E)** DSBs at simultaneously broken microsatellites may

recombine by nonhomologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ).

Table 1

TLS DNA polymerases potentially involved in BIR

DNA polymerase	Human gene (*S. cerevisiae* gene)	Biological activity	References
POL ζ	POLZ (REV3) hREV7 (REV7) POLD3 (POL31) POLD4 (POL32)	Lower fidelity than other B family polymerases; extends mismatches; interacts with REV1, PCNA, POL δ (POL31, POL32); replication from nascent DNA template during MMBIR.	[70, 113–116]
POL η	POLH (RAD30)	Low fidelity polymerase; bypass of pyrimidine dimers; extensive D-loop synthesis; knockdown decreases HR in DT40 cells; interacts with nonubiquitinated and ubiquitinated PCNA	[74, 76, 115, 117–119]
POL κ	POLK	Highly error-prone polymerase (T → G transversions); strong mismatch-extending ability; extends RAD51-dependent D-loops	[76, 117]
REV1	REV1L (REV1)	Deoxycytidyl transferase; inserts CdR across from abasic sites; interacts with multiple TLS polymerases and monoubiquitinated PCNA; promotes MMBIR	[70, 116, 120–122]
POL μ	POLM (N/A)	Template-independent terminal deoxynucleotidyl transferase (TdT) activity, able to accept template distortions	[110, 111]
POL θ	POLQ (N/A)	Template-independent terminal transferase (TdT) activity, implicated in alt-EJ/MMEJ	[106–109]