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Potential roles for G-quadruplexes in mitochondria

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Abstract

Some DNA or RNA sequences rich in guanine (G) nucleotides can adopt non-canonical conformations known as G-quadruplexes (G4). In the nuclear genome, G4 motifs have been associated with genome instability and gene expression defects, but they are increasingly recognized to be regulatory structures. Recent studies have revealed that G4 structures can form in the mitochondrial genome (mtDNA) and G4 forming potential sequences are associated with the origin of mtDNA deletions. However, little is known about the regulatory role of G4 structures in mitochondria. In this short review, we will explore the potential for G4 structures to regulate mitochondrial function, based on evidence from the nucleus.

Keywords

G-quadruplexes; mtDNA; mitochondrial gene expression; mitochondrial genome instability; mtDNA deletions; mtDNA depletion; G4 ligand

Introduction

DNA or RNA sequences particularly abundant in adjacent guanines (G) can fold into non-canonical four-stranded nucleic structures known as G-quadruplexes (G4; Fig. 1)[1,2]. G4 structures have been widely studied *in vitro,* and under physiological conditions they display high thermodynamic stability[2,3]. In brief, the G4 core is formed by the assembly of four guanines bound through Hoogsteen-hydrogen bonds into a planar conformation called a G-tetrad[4] (Fig. 1A), and two or more G-tetrads stack together to form a stable quadruplex (Fig. 1B). The source of guanines can be intra- or inter-strand, DNA or RNA, that may be connected by loops of variable length[1]. The relative orientation of the strands can form parallel, antiparallel or mixed orientation G4 structures[5]. G4 stability depends on **π-π** interactions between the stacked G-tetrads and metal cations, particularly K^+ and to a lesser extent Na^+, which fit into the central anionic cavity within or between the G-tetrads to coordinate the carbonyl oxygens of the guanines[3]. Additional details regarding the structural dynamics and stability of G4 structures have been reviewed elsewhere[6,7].

Computational approaches have been used to predict sequences with quadruplex forming potential (QFP)[8–10], usually based on the G_{3+}-$N_{\geq 1}$-G_{3+}-$N_{\geq 1}$-G_{3+}-$N_{\geq 1}$-G_{3+} consensus motif (Fig. 1C). Some of these early tools predicted more than 370,000 QFP sequences in the

human genome[8,11]. Many QFP sequences from plants[12], protozoa[13], bacteria[14], yeast[15] and mammalian cells[16,17] have been confirmed *in vitro* to form G4 structures. Unfortunately, these computational approaches suffer from significant false negative and positive predictions compared with *in vitro* data, and the type of G4 topological fold is also not readily predicted. In a recent study, Bedrat A. et al. developed a more sophisticated algorithm (G4Hunter) that takes into account both the G-richness and G-skewness of sequences, and which they validated using over 200 G-quadruplexes formed *in vitro*[9]. Using G4Hunter in comparative studies, genomes with high guanine and cytosine (GC) content and GC strand asymmetry showed higher incidence of QFP sequences, and the authors estimated that the QFP regions present in the human genome could be from 2 to 10-fold more abundant than previously predicted[9]. Despite these advances, it is important to consider that the rules dictating G4 levels *in vivo*, where G4 stability could be modulated by many factors (including molecular crowding, dehydration, DNA supercoiling, helicase activity, and binding by RNA and proteins), are still only poorly understood.

The mitochondrial DNA (mtDNA) contains hundreds of QFP and is a classic example of a genome with high potential to form G4. Recently, evidence of potential roles for G4s in mtDNA replication[18,19] and their association with human mtDNA deletions[20–22] have been described; however, the contribution of these structures to mitochondrial function may be broader. The purpose of this review is to provide a perspective on potential functions of G4 in the mitochondria that draw from lessons learned in the study of nuclear nucleic acids and to discuss the current data on G4 structures in mtDNA.

A BRIEF REVIEW OF G-QUADRUPLEXES IN THE NUCLEAR GENOME

Evidence for G4 in the nucleus

Before reviewing the evidence for G4s in the nucleus, we emphasize that proving definitively that G4s form and function *in vivo* is challenging. Demonstrating that a G4 ligand (e.g. a small molecule or antibody) binds to a subcellular target is only weak evidence of physiologic G4 formation, because such ligands may also have substantial affinity for non-G4 targets, and also because they may artifactually induce or stabilize G4s to yield levels that do not exist naturally. Similarly, there is no genetic manipulation (e.g. the deletion or overexpression of gene encoding a G4-unwinding helicase) that is known to fully and selectively impact G4 levels. Nonetheless, strong indications of G4 function have been obtained by intersecting independent lines of evidence.

Attempts to directly identify genomic regions that can form G4s have to date primarily used anti-G4 antibodies or the capacity of G4s in a DNA template to block DNA polymerase synthesis[23]. Immunoprecipitation of purified genomic DNA using a single chain antibody fragment (scFv), HF2, revealed enrichment for sequences with QFP[24]. Similarly, enhanced blocking of DNA polymerase under conditions of G4 stabilization (using K^+ or the G4 small molecule ligand PDS) on a purified genomic DNA template occurred preferentially at QFP sequences, which were termed “observed quadruplexes” (OQs)[25]. Because these techniques use isolated DNA, they do not reveal direct evidence that these sequences form G4s in live cells.

However, more recent genome-wide chromatin immunoprecipitation (ChIP-seq) experiments using different scFvs, BG4 or D1, and chromatin isolated from cultured human cells showed a preferential enrichment for QFP sequences[26,27]. Such BG4 peaks occurred at only about one percent of the OQs. This may indicate that only some OQs form G4 *in vivo*, or that BG4 does not bind all G4s. We suspect both are true, and indeed it would be surprising if any antibody (or small molecule ligand) could bind all possible G4 folds, given their structural diversity. Isolation of G4s within chromatin may also be possible using G4 small molecule ligands, although it is not yet clear if any have sufficient affinity and specificity. To test the fidelity of the above approaches, it will be important to determine whether apparent G4 levels in chromatin change as expected under conditions expected to alter G4 stability, for example loss of expression of a G4 helicase.

Telomeres

Early interest in G4 biology focused primarily on telomeres, the structures at the ends of linear chromosomes. Telomeres are critical for chromosome stability, but they tend to shorten with cell division, which can lead ultimately to loss of telomere function, called telomere “uncapping”, and thus cell death or permanent cell cycle arrest. The enzyme telomerase can lengthen telomeres, and most cancers (80–90%) overexpress telomerase to support their replicative immortality[28]. Therefore, to block the perpetual division of cancer cells, one experimental strategy has been to inhibit telomerase activity. Telomeres typically comprise tandem repeats of DNA sequences possessing a run of consecutive guanines which endow them with high QFP. For example, vertebrate telomeres contain thousands of repeats of the sequence 5’-TTAGGG-3’. Indeed, telomere repeats have long been known to form stable G4s *in vitro*[2,29], and several lines of evidence support potential roles for telomeric G4s *in vivo*[30], but the extent to which telomeric G4s play beneficial or deleterious roles under natural conditions remains poorly understood.

Evidence that G4s and G4 small ligands can inhibit telomerase stimulated the development of such molecules as potential anti-cancer drugs[31] and more in depth summary of G4 ligands with anti-cancer activity has been reviewed elsewhere[32]. The predominant mechanism of *in vitro* cancer cell death is associated with telomerase inhibition and/ or telomeres uncapping along with DNA damage and consequent activation of senescence and apoptotic pathways[33]. Several G4 binding ligands have been shown to induce selective cancer cell senescence[34–36]. Additionally, exogenous G4 DNAs (both telomeric and non-telomeric sequence G4s) transfected into cancer cells can induce apoptosis[37], suggesting that the presence of stabilized G4s can induce apoptosis in cancer cells. On the other hand, there is evidence in both *O. nova* and human cells that telomeres in G4 conformations can be good substrates for telomerase[38,39], arguing that stabilization of G4s might enhance cancer cell survival by improving the healing of uncapped telomeres. In summary, to what extent G4 ligands inhibit cancer cells *via* effects on telomerase and telomeres is generally not established. The anti-cancer activity of G4 ligands or stabilized G4 structures might be through effects on mitochondria, on DNA integrity, or on gene expression.

Gene Expression

A large number of studies indicate that G4s are involved in the regulation of transcription. More than 40% of promoter regions of human genes contain sequences that have the potential to fold into G4 structures[40], and QFP is also enriched in the promoters of other species including yeast[41] and bacteria[14]. Functional evidence for regulation of transcription by G4 come from genome-wide analyses of cells treated with G4 small molecule ligands[41,42], expressing anti-G4 scFvs[26], or lacking the activity of DNA helicases with G4-unwinding activity, for example yeast Sgs1 and human Werner's syndrome protein (WRN), Bloom syndrome protein (BLM)[43–45]. These treatments lead to preferential alteration of mRNA levels from genes with high QFP, indicating G4-based mechanisms. Although QFP on the sense strand of such genes could reflect G4-DNA or G4-RNA based effects, QFP in these studies were found upstream of transcription start sites and without strand bias, suggesting at least some role for G4-DNA. Numerous studies have revealed the high propensity of proto-oncogene promoters to form G4 structures[40,46], such as *c-MYC*[47,48], *VEGF*[49], *c-KIT*[50], *KRAS*[51], *hTERT*[52], and *BCL2*[53]. There are G-quadruplexes located upstream of the transcription start site of these genes and at least some can negatively modulate gene expression when treated with small molecule G4 ligands[48,54–57]. G4s are also associated with upregulation of gene expression, and one underlying mechanism may involve their tendency to destabilize nucleosomes, since QFP is associated with reduced histone occupancy at promoters, with DNase I hypersensitive sites, and BG4 ChIP revealed enrichment for nucleosome poor chromatin[25,41,58].

Other mechanisms by which G4s may affect gene expression include regulation of RNA splicing, polyadenylation, stability, targeting, and translation into protein. These have been well reviewed elsewhere[59]. We note that although it has long been assumed that G4-RNA might be more prevalent than G4-DNA, because G4-RNA formation can be unimpeded by competitive base pairing by complementary strand as in duplex DNA, a recent study concluded that in eukaryotes potential G4-RNA is primarily in an unfolded state[60]. More work will be essential to fully understand to what extent transient formation of G4-RNA occurs and plays an important role in RNA biology.

DNA replication and genome stability

Several lines of evidence indicate that G4 structures can impact DNA replication and genome stability[61–63]. For example, ~80 % of apparent replication origins overlap with QFP elements in mouse and human cells[61,64]. Functional dissection of two model replication origins in chicken DT40 cells demonstrated that their QFP elements are required, though not sufficient, for origin firing[65]. At the same time, particularly under conditions of replicative stress, G4 structures may imperil DNA replication and genome stability. Insertion of the human minisatellite CEB1, which has high QFP, into the *S. cerevisiae* genome generates a replication fork stalling and genome instability when the cells lack the Pif1 G4 DNA helicase or are treated to the G4 ligand Phen-DC_3[66,67]. Similarly, Pif1 is important for replication though natural QFP elements in yeast[77]. Furthermore, cultured human cells treated with the G4 ligand PDS develop replication-dependent phosphorylation of H2AX near QFP elements, indicating G4 stabilization might cause damage to replication forks[68]. In cancer cells, a strong association between nuclear genome deletion breakpoints and QFP

sites has been identified, suggesting G4-induced genome instability may contribute to carcinogenesis[69]. Furthermore, cancer cells deficient in homologous recombination (HR) secondary to BRCA1 or BRCA2 deficiency were found to be highly susceptible to PDS induced DNA double strand break generation and toxicity[70]. The HR dependent repair is further corroborated by recent evidence showing cells deficient in BLM helicase show increased sister chromatid exchange events at QFP sequences throughout the human and mouse genome[71]. These findings suggest that HR-dependent mechanisms are important for the repair of G4-related genome damage, and for maintaining genome stability.

MTDNA: A FAVORABLE ENVIRONMENT FOR G4 FORMATION

The mitochondria

Mitochondria have a double membrane structure and play numerous crucial roles in cell biology (Fig. 2). They are signaling organelles, involved in bioenergetics signaling, apoptosis, calcium signaling, and immune signaling. As the hub of cellular metabolism, mitochondria are the home of heme biosynthesis, iron-sulfur cluster biosynthesis, branched-chain amino acid biosynthesis, fatty acid biosynthesis and catabolism, the Kreb's cycle, and high efficiency ATP production through oxidative phosphorylation (OXPHOS). During OXPHOS, electron transport coupled proton (H^+) translocation across the inner membrane into the intermembrane space generates an electrochemical gradient harnessed by Complex V to catalyze the ATP synthesis (Fig. 2A). It is important to note that OXPHOS Complexes I, III, IV, and V are derived from the protein products of mitochondrial and nuclear genomes, and are thus bigenomic in origin.

The mitochondrial DNA

The human mitochondrial genome is a double-stranded circular molecule of 16,569 nucleotides that encodes 13 proteins essential for OXPHOS function, as well as the 22 tRNAs and two rRNAs required for their translation[72] (Fig. 2B). The other OXPHOS proteins are nuclear encoded, cytoplasmically translated, and imported into the mitochondria. Mitochondria contain multiple copies of their genome mtDNA with copy numbers ranging from ~10^2 to ~10^5 copies in somatic cells and to even higher levels (~4×10^6) in oocytes[73]. Somatic cells with a high metabolic demand, such as neurons and muscle cells, require more ATP and have a higher number of mtDNA compared to those with low energy demands, such as fibroblasts.

The mitochondrial genome displays several unique features relative to the nuclear genome. In brief, the mtDNA is packaged into small structures termed nucleoids, with estimates of 1–2 genome copies per nucleoid[74]. Notably, the mtDNA lacks histones and the diverse DNA repair mechanisms of the nucleus, with only base excision repair (BER) activities having been detected[75]. The limited DNA repair pathways available in the mitochondria, coupled with its close proximity to the OXPHOS complexes, which are a major source of reactive oxygen species (ROS), render the mtDNA more vulnerable to damage when compared to nuclear DNA[76]. The primary defense against damage may be the high mtDNA copy number. The constant damage to mtDNA from ROS is thought to contribute to progressive age-related decline in mitochondrial function[77]. Whether aging lowers copy number, oxidative

damage contributes to mtDNA mutations, and mtDNA mutations contribute to aging, are subjects of continued debate[78–80].

Introduction to mtDNA replication

The mammalian mitochondrial genome replicates in a manner distinct from nuclear DNA. Although there is a clear leading (first) and lagging (second) strand, Okazaki fragments are not apparent in mtDNA replication[81]. The lack of Okazaki fragments is due to asymmetric replication of mtDNA, which is the predominant mechanism of DNA synthesis and generates primarily continuous nascent strands. There is significant debate about the specifics of mtDNA replication, so a simplified view of the common features of the mitochondrial genome replication in cultured cells is provided in Fig. 3. The major start site of first strand synthesis is within the control region, at ori-H (O_H; Fig. 3A left). Truncated mtDNA replication molecules, which form a D-loop structure, are common *in vivo*[82], so elongation beyond the D-loop region is thought to indicate productive replication. First strand replication produces a nascent heavy strand, displacing the parental heavy strand. The single-stranded region can be bound by single-stranded DNA binding protein[83] or hybridized with mitochondrial RNA[84]. Although second-strand replication initiation can occur anywhere on the displaced strand, replication primed by hybridized RNA initiates predominantly at ori-L[83] (O_L; Fig. 3A middle). Continued elongation of both strands yields two DNA molecules replicated in a semiconservative fashion (Fig. 3A right).

THE MITOCHONDRIAL VIEWPOINT OF G-QUADRUPLEXES

Mitochondrial G4 forming sequences

The mode of mtDNA replication may contribute directly to G4 structures formation and genome instability, thereby causing disease. The mammalian mitochondrial genome shows significant asymmetry in strand composition with a two-fold enrichment of guanines on one strand (inner strand; Fig. 3B). The reason for this highly conserved strand asymmetry arises from selective pressure to limit cytosine content in the displaced strand, as cytosine can be deaminated to uracil to cause cytosine to thymine transversions[85]. This enrichment of guanines contributes to the higher QFP density of the mitochondrial vs. nuclear genome. In a recent *in silico* study, QFP per kb in the human mtDNA is estimated to be 2.4 to 3.6-fold higher than in the nuclear DNA[9]. During replication and transcription, the DNA strand rich in QFP sequences is temporarily single stranded, suggesting an increased opportunity to form G4 structures. Additionally, the mitochondrial environment would be permissive to G4 formation, as the potassium concentration in the matrix is estimated to be 150 mM[86].

Mitochondrial G4 formation and transcription/replication switching

An intriguing feature of mtDNA is the mechanism of replication initiation at O_H (Fig. 4). Transcription from the light strand promoter (LSP) generates all RNAs that contain heavy strand sequence. Immediately downstream of the LSP are three conserved sequence boxes (CSBs), which are conserved from yeast to human mtDNA[87]. Although the specific transition point reported varies, CSBs are the sites where transcript termination can occur, forming the primer for replication. In *in vitro* transcription assays, multiple groups have shown that mitochondrial RNA polymerase (POLRMT) terminates at CSB II[18,88]. This

termination depends of the formation RNA-DNA G4 hybrid structures[18,8889]. Termination is more efficient when CSB II contains the typical sequence G_6AG_8, rather than the rare variant G_5AG_7, consistent with a higher stability G4 promoting termination. Furthermore, the RNA helicase TEFM, which can be efficiently photo-crosslinked to the G4 forming sequence of CSB II, inhibits termination[19]. Therefore, these *in vitro* studies support a contribution of G4 structure formation and resolution to the switch between mtDNA replication and transcription.

QFP sequences and mtDNA instability

Several groups have queried whether predicted secondary structures in mtDNA might associate with mtDNA deletion breakpoints in human diseases[20–22]. As described above, G4 stabilization can lead to genome instability, and QFP sequences are common sequence elements associated with deletion breakpoints in nuclear genomes of cancer cells, suggesting that G4s could affect the stability of both nuclear and mitochondrial DNA. Among the various sequences that form non-B form secondary structures tested by the different groups[20–22], QFP sequences showed the strongest association with the ends of deletions in the mitochondrial genome. When tested, the QFP sequences form G4 structures *in vitro*[20,21]. Given their apparently negative impact on mtDNA stability, the persistence over evolutionary time of QFP sequences in mitochondrial genomes raises the possibility that their negative effects are more than counterbalanced by beneficial regulatory functions.

A potential role for G4 structures to alter mitochondrial replication, transcription, and translation

Evidence continues to build for G4 structures altering the function of mitochondrial sequences[18,21,90,91]. Alteration of G4 stability at CSB II would be expected to alter the balance between transcription and replication from LSP[18]. This switch likely specifies a gene expression or mtDNA copy number maintenance role to each mitochondrial genome (Fig. 4). More broadly, the enrichment of QFP on the displaced strand, which is the template for second strand synthesis, promotes the speculation that G4 stabilization would interfere with second-strand synthesis to cause mtDNA depletion[20,21] (Fig. 5A). These studies point toward a regulatory role of G4 structures in both DNA replication initiation and elongation. Because QFP are found in both RNA sequences and transcription templates, the formation of G4 structures may also regulate mitochondrial transcription elongation (Fig. 5B), perhaps through hybrid formation as observed in CSB II or template interference. Similarly, RNA G4 may have a regulatory role in translation (Fig. 5C). As was the case in the nucleus, development of tools specific to the mitochondria will be instrumental in testing this hypothesis.

The potential for G4 helicases to regulate mitochondrial function

The Pif1 helicase shows strong G4 resolving activity and is dual localized to both telomeres and mitochondria[92–95]. Pif1 helicases are an evolutionary conserved class of enzymes present in bacteria, yeast and mammals[96]. Depletion of the nuclear Pif1 isoform in human cell lines induces replication fork pausing and DNA deletions[96]. In yeast, mutations in the *PIF1* gene affect mtDNA stability and its susceptibility to oxidative stress[97]. Moreover, it has been observed that the presence of unresolved G4 structures due to Pif1 helicase

deficiency causes DNA instability and DNA breakpoint formation[98,99]. Interestingly, PIF1 ablation in mouse shows no effect on telomere length[100], but causes a modest increase in mtDNA deletion load in skeletal muscle[101].

Other potential G4-interacting proteins have been identified to have a role in mitochondria. Human primary fibroblasts knocked-down for RECQL4, which localizes in part to the mitochondria[102], show an increased susceptibility to mtDNA damage and impaired maximal respiratory function. More recently, an *in vitro* study has identified the mtDNA binding protein TFAM as a G-quadruplex binding protein[103], suggesting that G4 structures might occur in the mitochondrial genome. TFAM has also been suggested to bind recombination intermediates[104], but neither G4 binding nor Holliday-junction binding has been validated in mammalian cells. Like single-stranded DNA, RNA may also be implicated in forming G4 structures. The mitochondrial RNA-binding protein G-rich binding factor1 (GRSF1) shows strong preference for non-coding mitochondrial RNA sequences that are predicted to form G4 structures[105]. The mitochondrial SLIRP RNA helicase that regulates mitochondrial gene expression post-transcriptionally[106] has recently been identified biochemically to bind G4 structures *in vitro*[107]. The formation of G4 in mitochondrial RNA, and a role for GRSF1 and SLIRP have yet to be established. Identifying the key G4 resolving activities may be crucial to understanding the impact of G4 on mitochondrial function.

G4-ligands in mitochondria

Whether G4s play a specific role in transcription/replication switching, or more broadly in replication, transcription, and translation has yet to be established in mammalian mitochondria. Key helicases need to be identified. In addition, the development of mitochondrial G4 reagents may play an important role in probing G4 in mitochondria and understanding their potential implications in respiratory function. Recently, it has been reported that a derivative of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) localizes to mitochondria in cancer cells and induces apoptosis[108]. The mechanisms of action of BMVC on mitochondrial nucleic acids and the impact on respiratory function has not been clarified. Other known G4 ligands, TMPyP4 and DoDC, do not localize preferentially to the mitochondria, but do cause mitochondrial dysfunction concomitant with cytochrome C release and apoptosis[109,110]. Further investigation is necessary to identify and characterize mitochondrial targeted G4 ligands to test their impact on respiratory function.

Future perspectives

Our current knowledge of G4 structures role in the mitochondrial has been limited due the lack of efficient experimental tools. Using the information that have been provided by the studies of G4 structures in the nucleus, we speculate that the mitochondrial G4 formation may affect mitochondrial replication, transcription and translation. To study G4 structures in mitochondrial biology, ligands and antibodies would need to be developed to selectively detect or isolate mitochondrial G4 structures in mtDNA or RNA. As such, scFv antibodies currently expressed in the nucleus might be applied to mitochondrial G4 detection, although they will need to be modified so they can be imported efficiently into the mitochondria. Additionally, scFv molecules may be used to capture and characterize by sequencing the

changes in G4 forming sequences in response to helicase or ligand alterations. For application to mitochondria, known G4 ligands need to be tested, or new molecules need to be generated, that specifically localize to mitochondria and then assessed for function in mitochondrial nucleic acid biology. Furthermore, the identification of the mitochondrial-localized helicases that resolve G4 structures, the development of mitochondrial-specific G4 antibodies, and the identification of mitochondrial-localized G4 ligands may represent a powerful combinatorial approach for investigating G4 function in mitochondrial biology.

In summary, the mitochondrial genome is highly skewed for guanine content and QFP sequences. The high copy number and gene density of mtDNA has the potential to amplify the effects of G4 dysregulation on mitochondrial respiratory function and cell viability. The potential impact of G4 structures on DNA replication and deletion formation is shared between the nuclear and mitochondrial DNAs, as QFP sequences are a common feature near the junctions of deletions in both genomes. To extend our understanding further, however, requires testing if the G4-functions described in the nucleus apply to the mitochondria. Ultimately the mitochondrial G4 reagents development will lead to an improved understanding of the major G4s, how they are resolved, and their normal and pathophysiological functions in the mitochondria.

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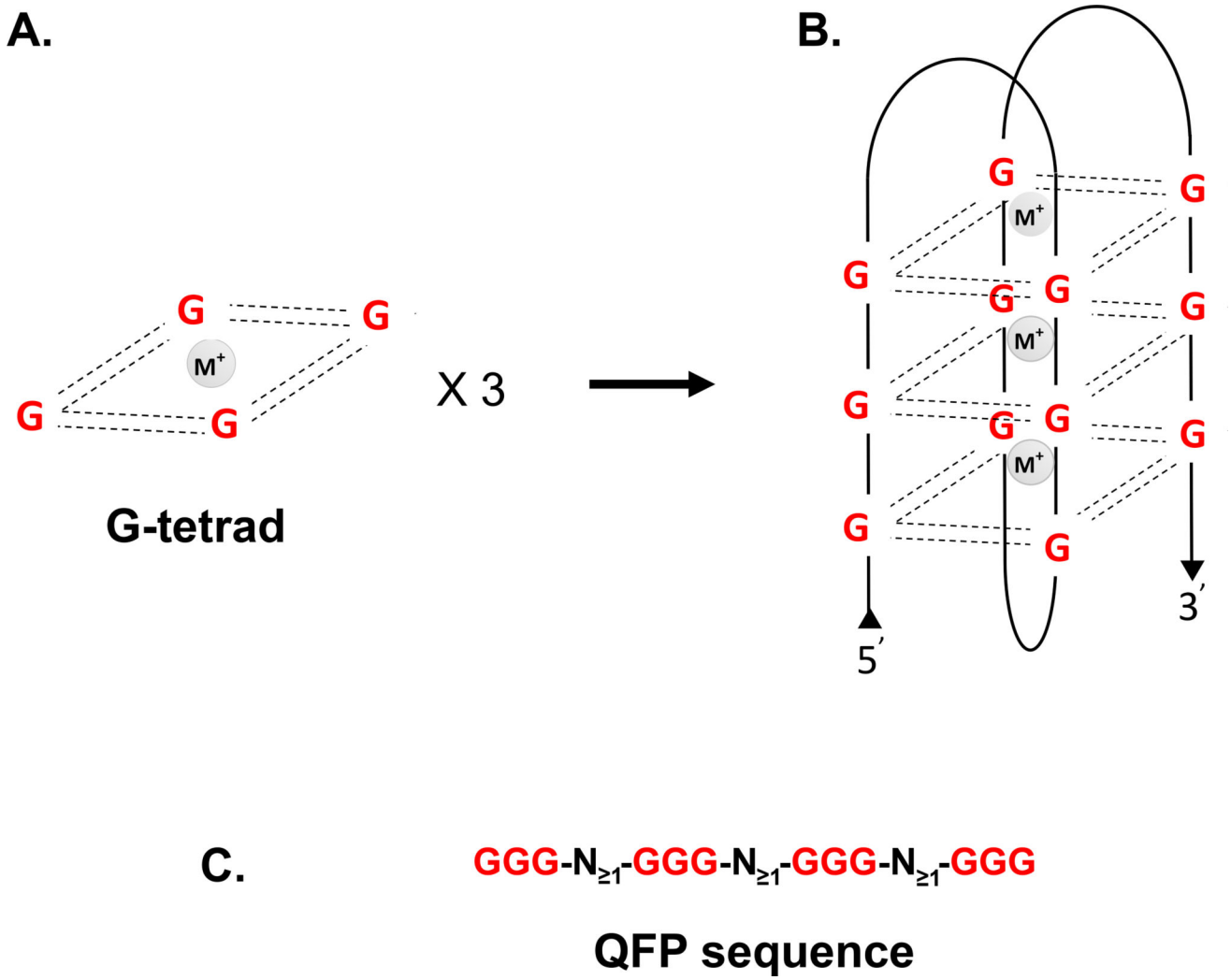

Figure 1. G-quadruplex structure

(A) Four guanines (G) form planar cyclic conformations through eight Hoogsteen-hydrogen bonds (dashed lines) and stabilized by the presence of a central metal cation (M^+). **(B)** G4 structure in antiparallel conformation. Numerous topologies are possible. **(C)** From the requirement of four runs of guanine, QFP sequences can be predicted within the same strand. Traditional prediction requires at least two G per run and one nucleotide to form a loop. Shown is a prediction scheme typically used for identifying three guanine stack G4-forming potential (QFP) sequences. Limits to size of window for search usually range from 30–50 bp.

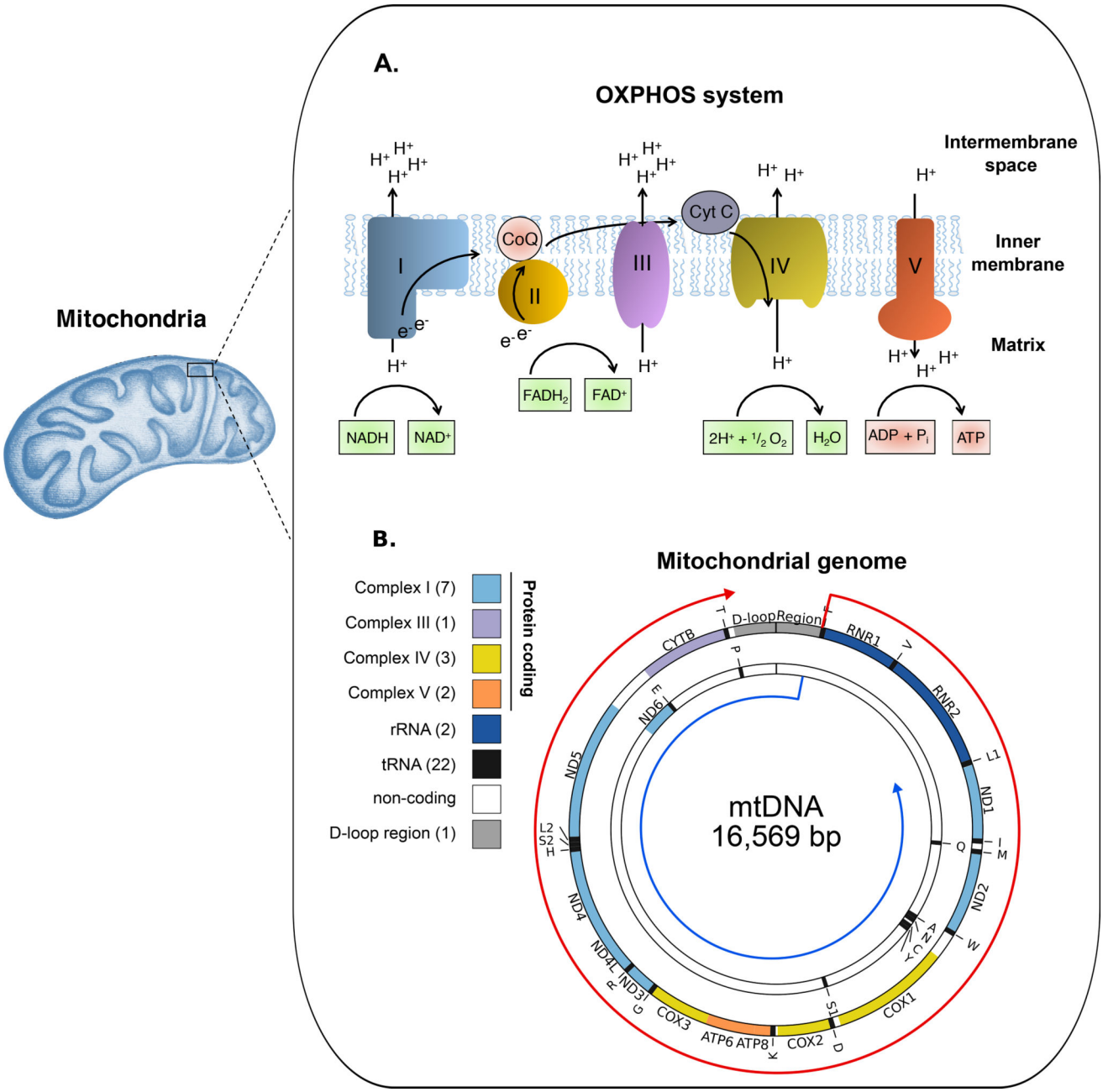

Figure 2. Schematic representation of the oxidative phosphorylation (OXPHOS) system and mitochondrial DNA (mtDNA)

(A) Main components of the mitochondrial OXPHOS system: Complex I (blue), Coenzyme Q (CoQ; pink), Complex II (orange), Complex III (purple), Cytochrome C (CytC; grey), Complex IV (yellow) and Complex V (red). The redox metabolites are shown in green boxes and the final OXPHOS reaction is represented in red boxes. **(B)** The mitochondrial genome is a 16,569 bp circular double-stranded DNA molecule that contains 37 genes encoding for 13 OXPHOS proteins (colors matched panel A) and the tRNAs (black) and rRNAs (dark blue) required for their expression. The number of genes per group are indicated in parenthesis. The genes are encoded on either strand, shown with the two strands separated to

indicate the relevant coding sequences on each polycistronic pre-mRNA transcript (indicated by red and blue arrows).

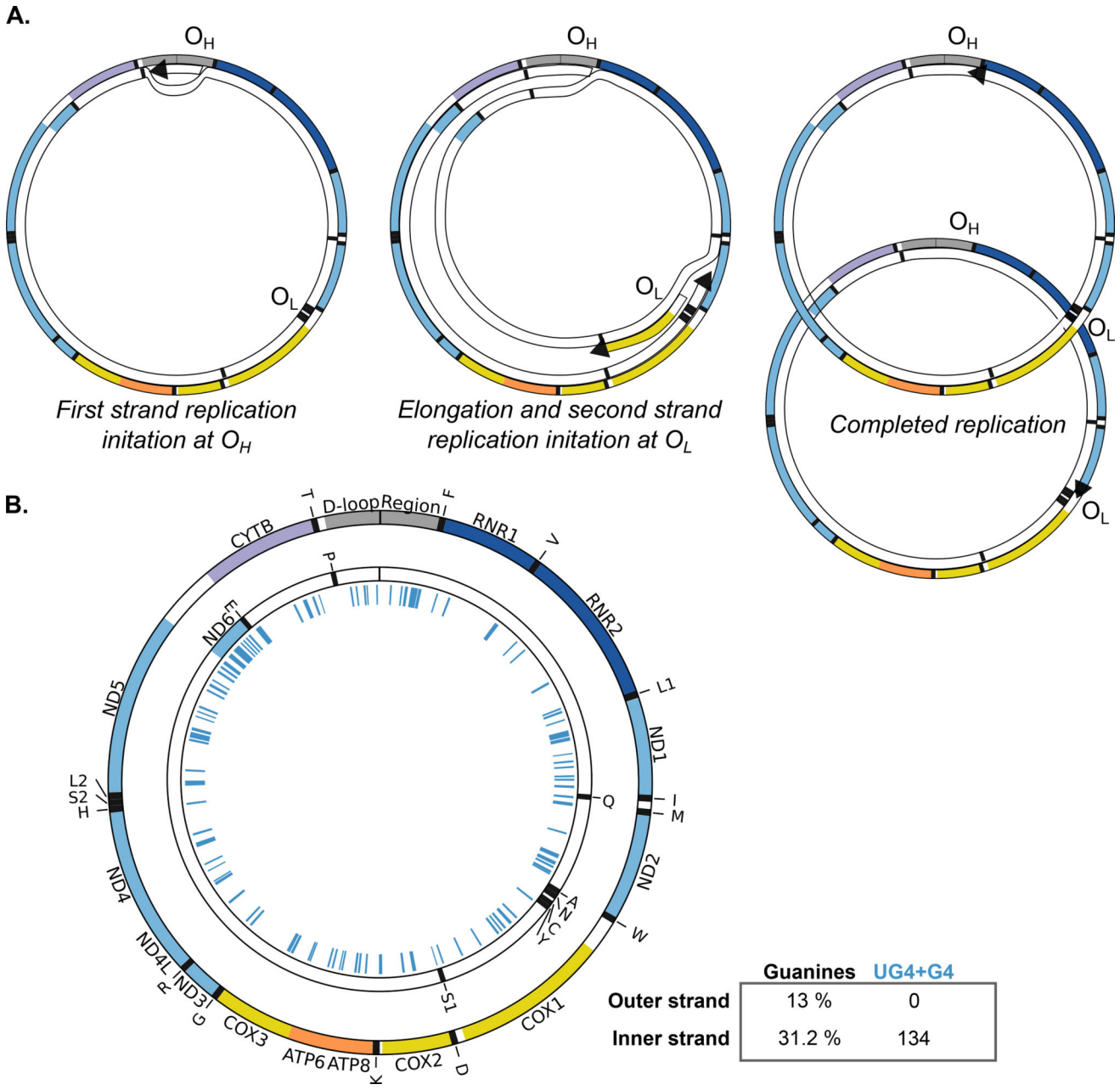

Figure 3. Mitochondrial genome replication and predicted G4 forming potential sequences

(A) Simplified diagram of mtDNA asynchronous replication. Left: first strand replication initiation at ori-H (O_H). DNA starts as duplex, with synthesis of the heavy strand initiated in the heavy strand origin (O_H). Middle: elongation of the first strand and initiation of the second strand replication at ori-L (O_L). Continuous elongation of the first strand displaces the parental heavy strand of mtDNA with nascent DNA. Once replication crosses the primary second strand origin (O_L), replication in the other direction begins. Right: Elongation of both strands continues until two semi-conservative molecules are generated. Note that only the heavy strand is single stranded in this process. **(B)** Representation of the G4-forming sequences in the heavy strand (inner strand; 31.2% guanines) of the human

mtDNA. The figure represents validated unstable G4 (UG4; n=63) and G4 (n=71) out of 209 sequences experimentally tested[9].

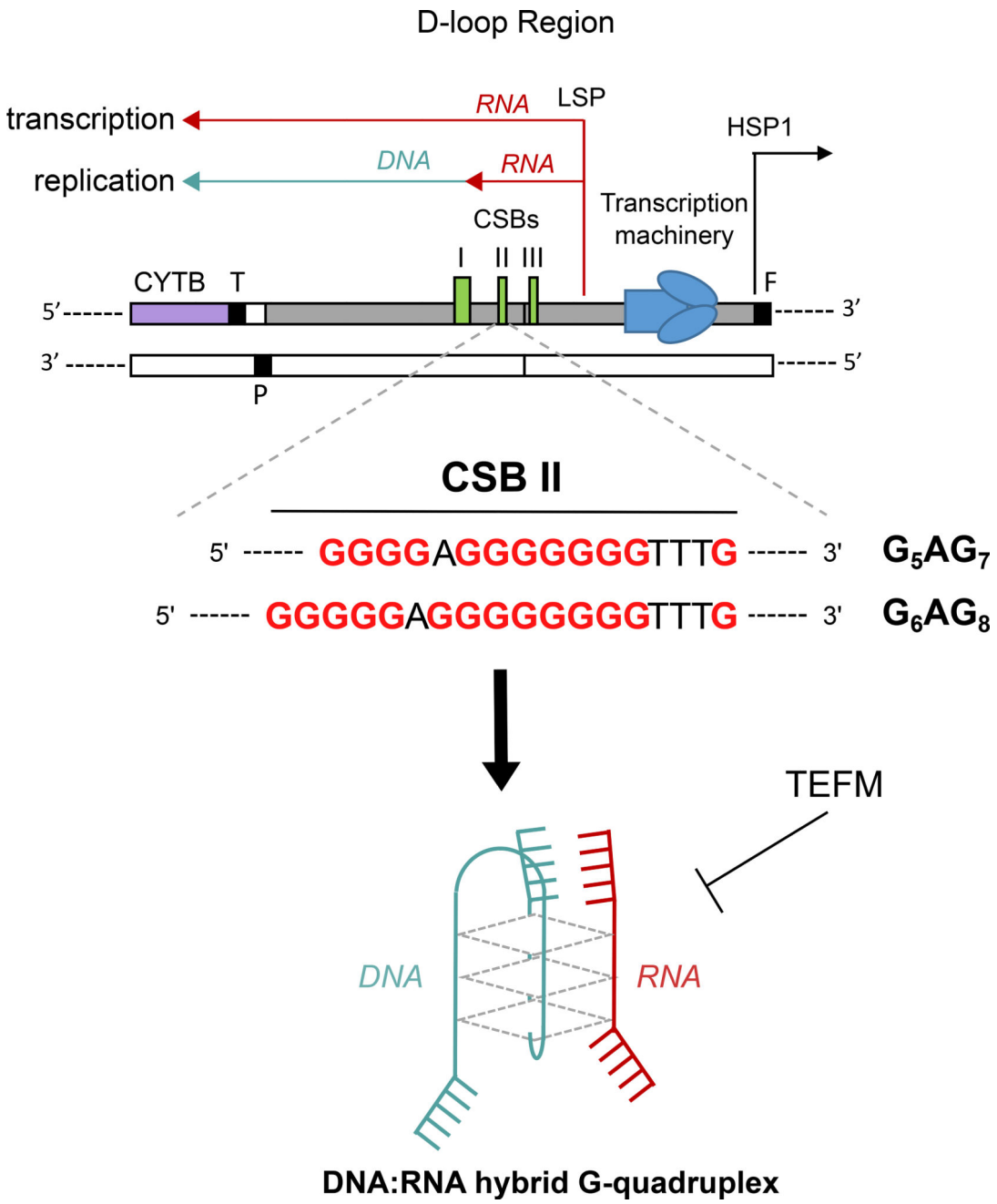

Figure 4. Mitochondrial control region and transcription termination induced by G4 structure

The light strand promoter (LSP; red) transcription can be arrested by a hybrid DNA:RNA G4 structure formed at the conserved block sequence II (CSB II). The arrested RNA is thought to serve as a primer to initiate the leading-strand DNA (green) replication. Two alternative regions at the CSB II could lead to the G4 formation, the G_5AG_7 (rare polymorphism) and the G_6AG_8 (most genomes) sequences. The mitochondrial transcription elongation factor (TFEM) prevents the CSB II transcription termination.

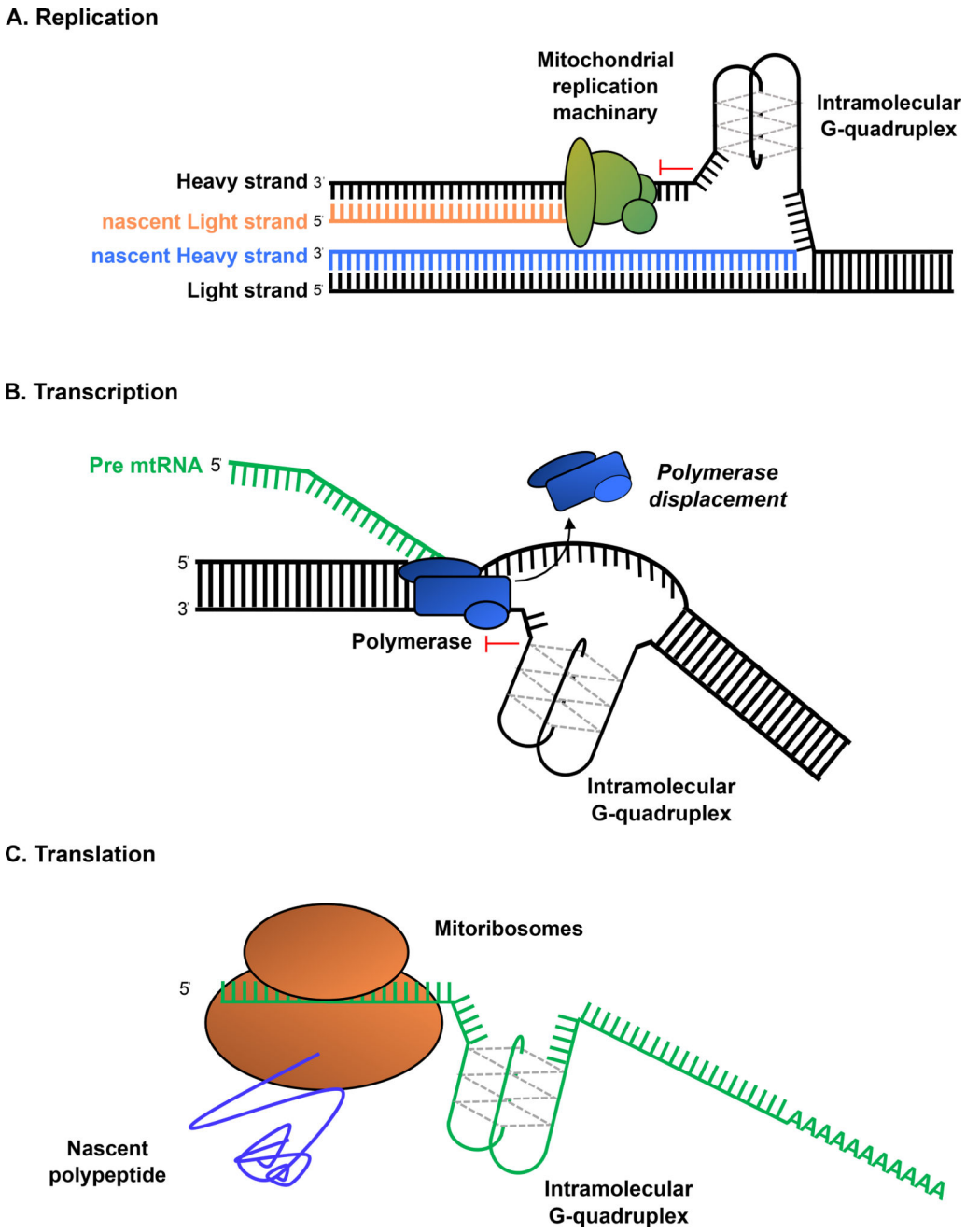

Figure 5. Potential G4 formation during mtDNA replication, transcription and translation

G4 structures can form in single-stranded nucleic acids. **(A)** During mtDNA replication, the displaced G-rich heavy strand is a potential hotspot for the G4 formation, which would arrest the mitochondrial replication machinery (green) during second strand synthesis. **(B)** A model of transcription inhibition by G4 structures. The unresolved G4 structures would occur in the mtDNA template strand preventing the polymerase (blue) activity. **(C)** A model of translation inhibition by G4 structures forming in the mRNA.