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Review

Functional and dysfunctional roles of quadruplex DNA in cells

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Abstract

A number of biological roles have been proposed for quadruplex, also referred to as G4 or tetraplex, DNA. The presence of quadruplex DNA may lead to errors in some biological processes and be required in others. Proteins that interact with quadruplex DNA have been identified including those that cause Bloom's and Werner's syndromes. There are small molecules that specifically bind to quadruplex DNA, inhibit telomerase, and are cytotoxic towards tumor cells indicating a role for quadruplex DNA in telomere function. It is now possible to make testable proposals for the possible biological implications of quadruplex DNA in replication, transcription, and recombination as well as possible routes to therapeutic intervention.

Keywords: Quadruplex DNA; G4 DNA; Telomere; Bloom's syndrome; Werner's syndrome; Triplet repeat; Telomerase

1. Introduction

Quite recently proteins have been found that have high affinity for quadruplex, also known as G4 or tetrahelical, DNA and defects in these proteins can lead to errors in replication, transcription, and recombination as well as to increases in the rates of aging and tumor formation [1–9]. Quadruplex DNAs are known to inhibit telomerase [10–13], HIV integrase [14] and thrombin [15,16]. Some of the molecules that specifically bind to quadruplex DNA are cytotoxic to tumor cells presumably by means of telomerase inhibition [10,12,13,17–20].

These and other biological results have kindled interest in quadruplex DNA as therapeutics and as therapeutic targets. The biological information is complemented by the availability of structural information on quadruplex DNAs [21–24] and by progress in understanding the nature of the special interactions between quadruplex DNAs and potassium [23,25,26].

The sections below give overviews of some of the proposed roles of quadruplex DNA in replication, transcription, and recombination and in the structures of triplet repeat and telomere DNAs. The potential of quadruplex DNA as targets for therapeutic intervention and as therapeutics is also presented. As discussed below it appears that the transient formation of quadruplex DNA can lead to errors in transcription, replication, and recombination while quadruplex DNA may play roles in recombination, the formation of the synaptonemal complex, and in the telomere structure of vertebrates. Before beginning the discussion of the biological roles of quadruplex DNAs the diversity in the structural features of quadruplex DNAs needs to be considered.

2. Quadruplex DNA structural types

Several types of quadruplex DNA structure, based on quartets of dG residues, have been determined to high resolution by solution state methods [21–24]. In a chair or edge type structure the dG residues of each quartet alternate *syn–anti–syn–anti* as depicted in Fig. 1. In the crossover or basket type structure the dG residues alternate *syn–syn–anti–anti* within each quartet as shown in Fig. 1 and both intra- and intermolecular basket type structures have been observed. The quadruplex structures formed by four parallel strands [24,27,28] have all residues *anti* as depicted in Fig. 1.

These three types of quadruplex structure have distinctly different shapes and electrostatic potentials [29–31]. The chair type structures have two narrow grooves and two wide grooves. The basket type structures have one narrow, one wide and two medium width grooves.

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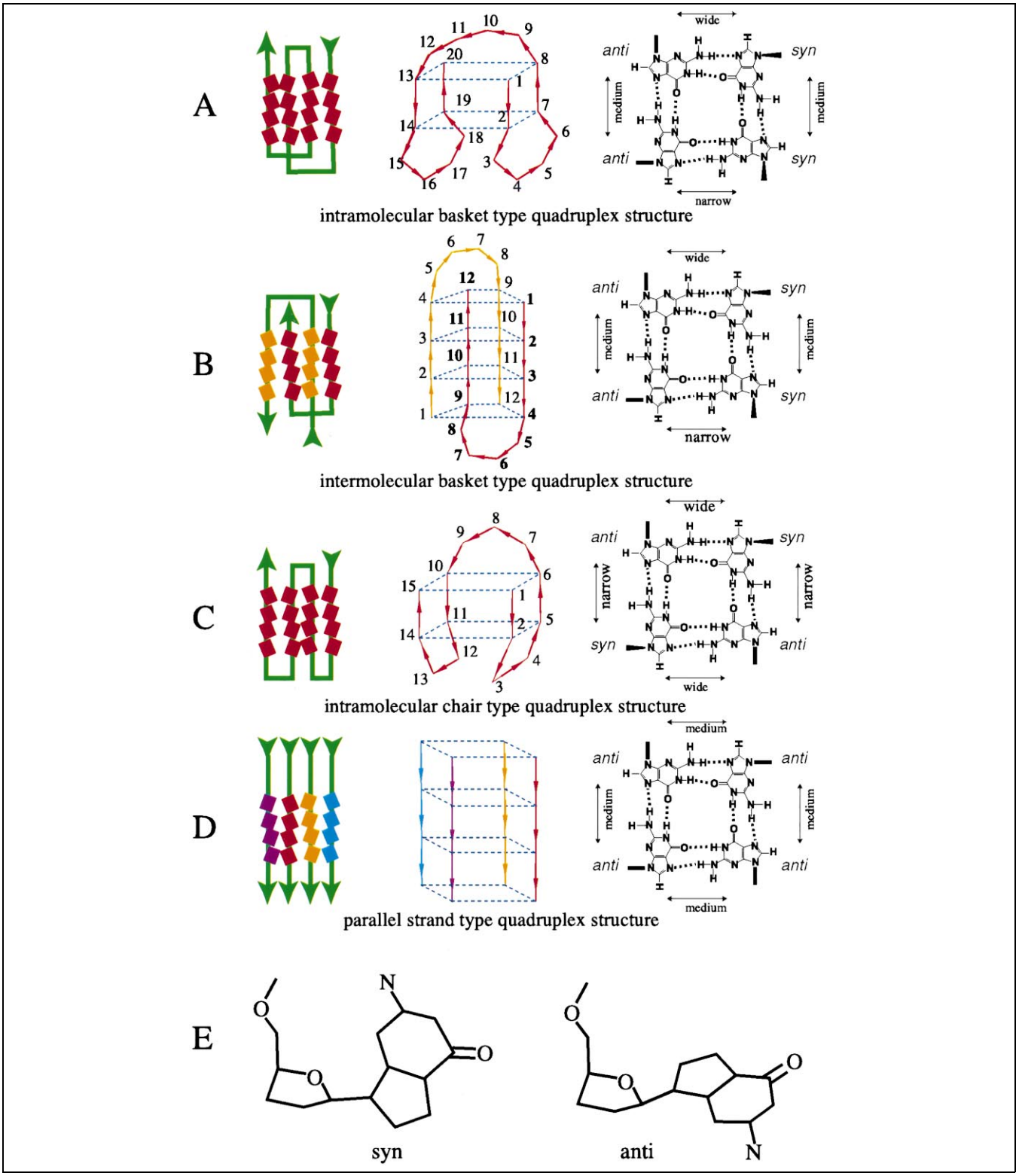

The parallel strand structures have all medium width grooves. The electrostatic potentials of the narrow grooves are quite strong and provide unique binding sites for cations and proteins [29–31]. Chair type structures appear to have a requirement for potassium, or a suitable substitute [23,25].

The terms 'G4', 'tetraplex' and 'quadruplex' have occasionally been used to encompass this entire range of structures and on other occasions to describe a DNA sequence whose quadruplex structural type was not known. Quadruplex DNAs migrate faster in gels than do duplex DNAs containing the same number of nucleotides and gel mobil-

Fig. 1. Four of the possible types of quadruplex structures based on quartets of dG residues are shown. In (A) the quadruplex structure is an intramolecular basket type structure. On the left the structure is schematically depicted with the red boxes indicating the dG residues and the backbone of the DNA shown in green. The structure is depicted in the middle and on the right the width of the grooves and the configuration of the residues in the dG quartets. In (B) the quadruplex structure is an intermolecular basket type structure. On the left the structure is schematically depicted with the red boxes indicating the dG residues of one strand and the orange boxes the dG residues of the other strand. The backbones of the DNAs are shown in green. The structure is depicted in the middle and on the right the width of the grooves and the configuration of the residues in the dG quartets. In (C) the quadruplex structure is an intramolecular chair type structure. On the left the structure is schematically depicted with the red boxes indicating the dG residues and the backbone of the DNA shown in green. The structure is depicted in the middle and on the right the width of the grooves and the configuration of the residues in the dG quartets. In (D) the quadruplex structure is a parallel strand type structure. On the left the structure is schematically depicted with each color of box indicating the dG residues of one of the strands. The backbones of the DNAs are shown in green. The structure is depicted in the middle and on the right the width of the grooves and the configuration of the residues in the dG quartets. In (E) the orientation of the base relative to the sugar is shown for *syn* and *anti* orientations.
←

ity results have often been used as evidence for the presence of quadruplex DNA. There has not been a systematic attempt to correlate the gel mobility with the structural types of quadruplex DNA. The N7 position of dG residues in quartets is involved in Hoogsteen base pairing, as shown in Fig. 1, which protects the site from chemical modification. This feature allows chemical modification experiments to monitor the presence of quadruplex DNA.

It appears that some of the discrepancies that appear in the literature concerning the interactions, formation and other properties of quadruplex DNA arise from the studies being on different structural types of quadruplex DNA. Some DNA sequences can form more than one structure as evidenced by the multiple bands that can be resolved by gel electrophoresis. The mixture of structures can depend on the concentration of the DNA and which cations are present.

Investigations of the interactions and reactions of quadruplex DNA should include results obtained with DNAs of the known quadruplex structure. This can be done since it is now known how to prepare DNAs of each of the quadruplex structure types.

3. Replication and transcription

Bloom's and Werner's syndromes are caused by defects in members of the RecQ family of ATP dependent helicases [3–6,8,32]. Bloom's syndrome is associated with a wide range of symptoms that are linked to a very unstable genome and to high rates of leukemia, solid tumors and many other types of cancers [33,34]. Bloom's syndrome is caused by defects in the BLM gene that encodes a 1417 amino acid protein. Werner's syndrome is associated with an early onset of aging and with genetic instability [33,34]. Increased deletion of genes and the presence of chromosomal defects are associated with Werner's syndrome. Unlike Bloom's syndrome, Werner's syndrome does not appear to give rise to elevated levels of sister chromatid exchange. Werner's syndrome is caused by defects in the WRN gene which encodes a 1432 amino acid protein. The RecQ helicases unwind DNA in the 3′ to 5′ direction and may have important interactions with topoisomerase III [33] and polymerase δ [35].

The yeast protein Sgs1 is also a member of the RecQ family of ATP dependent helicases and defects in Sgs1 can lead to errors in replication, transcription, high levels of recombination and rapid aging [4,6,8]. The premature aging caused by defects in Sgs1 can be compensated for by BLM but not by WRN [36] while both WRN and BLM can suppress the hyperrecombination caused by defects in Sgs1 [37]. Yeast with temperature sensitive Sgs1 have drastically lowered DNA and RNA polymerase activity at the restrictive temperature [8].

The BLM, WRN and Sgs1 RecQ helicases catalyze the ATP dependent unwinding of quadruplex DNA and require the DNA to have a 3′ tail of seven or more residues [3–7]. A function of these RecQ helicases may be the resolution of quadruplex structures that appear during replication and transcription [3–5,9]. A model for this activity, depicted in Fig. 2, predicts that DNA containing dG repeats can cause problems when defects in BLM or WRN are present.

There are a couple of instances in which the presence of quadruplex DNA may be needed to activate transcription. The insulin mini-satellite of the insulin-linked polymorphic region, a 14 bp long tandem repeat of d(ACAGGGGTGTGGGG), is located 363 nucleotides (nt) upstream of the human insulin gene. Mutations that disrupt quadruplex structure formation by this mini-satellite disrupt insulin gene transcription [38]. The presence of potassium activates transcription and also exposes part of the control region DNA to anti-gene silencing by anti-sense triplex formation [39]. This potassium effect supports the notion that quadruplex structures are involved as the chair type quadruplex structures require the presence of potassium [23,25].

3.1. Questions and therapeutic potential

These results indicate that the transient formation of quadruplex DNA can lead to errors in replication and transcription. The errors can appear throughout the genome as there are many regions of DNA that have the potential to form quadruplex DNA. One of the biological

roles of the RecQ helicases may be to resolve such quadruplex structures. Small molecule catalysis of the interconversion between quadruplex and single stranded DNA is a route to therapeutic intervention when the quadruplex resolution activity is missing. Small molecules can catalyze the interconversion between quadruplex structural types [40,41] suggesting that small molecule based resolution of quadruplex DNA may well be possible. Once the DNA is converted to the single stranded form the proteins which normally bind to single strand DNA may inhibit the reformation of quadruplex DNA.

The kinetics, the number of ATP required and thermodynamics of the resolution of quadruplex DNA by the RecQ helicases need to be examined so as to assess whether the activity actually occurs in cells and if so on which of the types of quadruplex DNA. The WRN helicase, for example, can resolve quadruplex DNA formed from repeats of d(CGG), associated with fragile X syndrome, but not those formed by the telomere repeat of vertebrates, d(TTAGGG) [5]. Specific inhibition of the resolution of quadruplex DNA by the RecQ helicases would allow testing of the proposed model and a resolution assay is needed to find inhibitors.

4. Recombination

During meiosis the sister chromatids pair up via the formation of synaptonemal complexes which have shapes that are characteristic for each species. These complexes are formed via single strand cuts in the sister chromatids followed by pairing of the homologous regions and then by ligation of the invading strands of the two duplexes. This leads to formation of a four strand, Holliday junction which is subsequently resolved back into two duplexes by the action of RecA helicase and other proteins. Many of the proteins associated with recombination are also involved in double strand DNA repair [42].

Single stranded DNA is transiently present during recombination which could allow the formation of quadruplex DNA when dG repeats are present [3,4]. The presence of quadruplex DNA would cause errors in recombination. This could lead to the formation of the quadriradial chromosomes, that appear to be trapped in the middle of recombination, similar to the ones that are found in Bloom's syndrome cells [3].

Some of the proteins involved in synapsis are likely to be expressed only during meiosis. About 10 years ago the yeast meiosis specific, 70 kDa protein Hop1, *ho*mology *p*romoter protein, was found [43]. Hop1 is essential for the assembly of the synaptonemal complex [7,43]. Hop1 binds to quadruplex DNA with 0.2 nM affinity and catalyzes the formation of quadruplex DNA from single stranded DNA [7]. These results have been taken to suggest that quadruplex DNA may play a role in synapsis in yeast [7]. Quadruplex DNA could form during recombination due to the transient presence of single stranded DNA by a mechanism similar to that shown in Fig. 2. Quadruplex DNA formed in association with Hop1 may be needed for proper formation of some synaptonemal complexes. It is noted that quadruplex DNA formation is also catalyzed by various polycations [40] and it is not known if Hop1 is acting other than as a non-specific polycation.

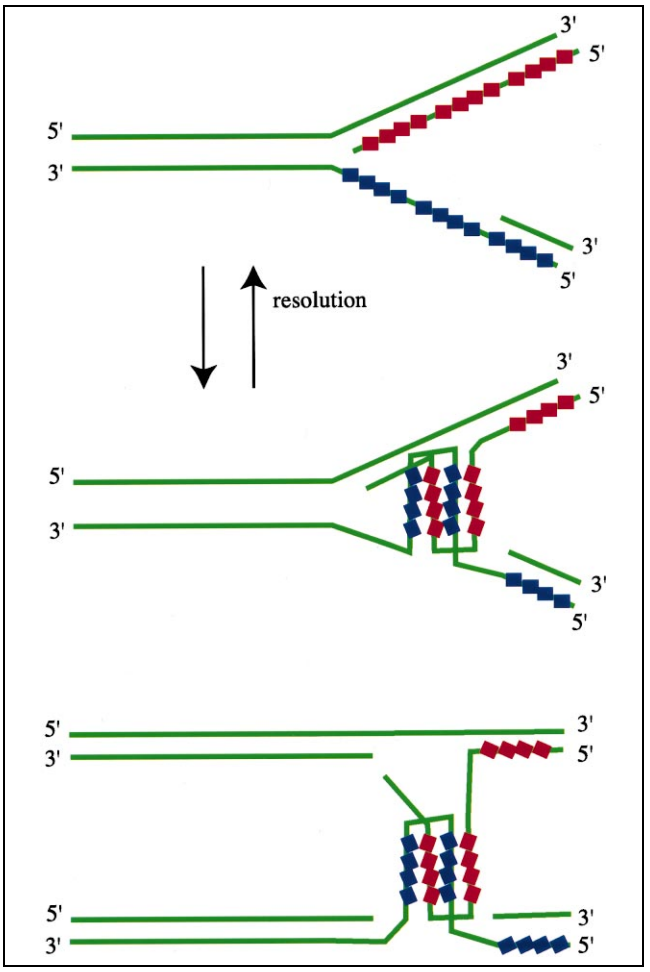

Fig. 2. The process of replication is schematically depicted. The red boxes indicate the positions of dG repeats in the leading strand and the blue boxes the positions of the dG residues in the lagging strand. At the replication fork there is single stranded DNA present which can allow quadruplex DNA to be formed as shown at the top of the figure. The quadruplex DNA could be formed with the strands being parallel or anti-parallel as shown in the middle of the figure. Once formed the quadruplex DNA at the replication fork could act to halt replication. The quadruplex structure could be resolved by the action of enzymes such as the ATP dependent RecQ helicases. The structure shown at the bottom contains a quadruplex DNA bridge between the two strands. Such a structure could be formed from a quadruplex structure at the replication fork. This structure could also be formed in recombination. The single stranded DNA formed in recombination could combine with the dG rich strand of the homologous DNA to form a quadruplex bridge between the two duplexes.

Duplex DNAs that have many consecutive dG–dG pairs have been referred to as 'synapsable'. Two such duplexes

can join via quadruplex DNA structures involving the dG residues on both strands of both duplexes [44]. We are not aware of any situations in which a duplex DNA containing multiple consecutive dG–dG pairs occurs in vivo.

4.1. Questions

The potential role of quadruplex DNA in the synaptonemal complex is primarily based on the results obtained on Hop1 and the model can be tested in several ways. One approach to detecting the presence of quadruplex DNA in synaptonemal complexes is to use one of the porphyrins that become fluorescent only in the presence of quadruplex DNA [40,45]. Another is to determine if one of the quadruplex binding molecules disrupts synaptonemal formation. A third alternative is to compare the reconstitution of the synaptonemal complex using native DNA with that obtained using DNA in which a nucleotide that disrupts quadruplex DNA formation has been incorporated. Nucleotides such as 6-thioguaninosine or 7-deazaguanosine disrupt quadruplex DNA formation [46–48].

5. Triplet repeat DNA

The molecular basis of at least a dozen human, genetic based disorders is the expansion of the number of repeats of a triplet sequence [49,50]. Triplet repeat diseases exhibit non-Mendelian inheritance, as expansion can occur in a single generation, and the genetic characteristic known as anticipation. Anticipation refers to the tendency towards earlier onset and increased severity as the number of repeats increases. The most studied case is fragile X syndrome which is associated with the d(CGG) repeat. The fragile X repeat appears adjacent to the promoter region of the FMR1, fragile X mental retardation-1, gene whose product has an unknown function. Expansion of the number of repeats tends to suppress expression of FMR1 leading to the diseased state. The d(CGG) repeat occurs about 5–50 times in normal individuals, 40–230 times in carriers and 230 to more than 2000 times in diseased individuals [51].

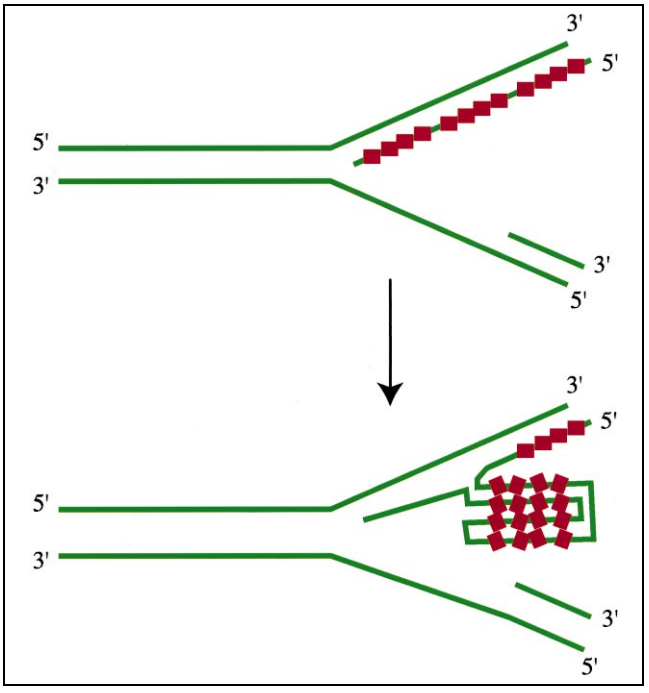

Fig. 3. During replication the formation of a quadruplex structure by the leading strand can lead to the expansion of the DNA. Any stable looped out form of DNA can lead to expansion.

There have been numerous studies of the properties of DNA with the $d(CGG)_n$ repeats and the complementary $d(CCG)_n$ [21,52]. Native gels of repeats of these DNA indicate the presence of multiple forms that have been proposed to have a number of different structural types as reviewed elsewhere [50]. There is considerable evidence that $d(CGG)_n$ repeats can form quadruplex DNA structures under physiological conditions. The formation of quadruplex structures can lead to 'slippage' during replication, as depicted in Fig. 3, which can lead to expansion. Similar quadruplex structures appear to be formed by all $d(NGG)_n$ repeats [53]. WRN helicase can unwind quadruplex structures formed by $d(CGG)_n$ repeats [5].

5.1. Questions and therapeutic potential

The formation of quadruplex DNA by triplet repeats has been proposed to lead to errors in replication [50,51,53]. This model remains to be rigorously tested. A series of yeast, or other, cell lines that have various lengths of d(CGG) repeats adjacent to the promoter of an easy to characterize protein would seem to be a promising line of inquiry. These cells could provide a good in vivo system for testing both the quadruplex model as well as potential therapeutics. The importance of quadruplex DNA in triplet expansion could be checked in such a model system by the incorporation of nucleotides which disrupt quadruplex DNA formation, such as 6-thioguaninosine or 7-deazaguanosine [46–48]. A potential route to therapeutic intervention may be the use of small molecules to promote the resolution of the quadruplex form.

It is not known whether the expansions of all triplet repeats have a common mechanism. There is no compelling reason as to why there should be a common mechanism of triplet expansion. Some of the triplet repeats associated with disease states can not form quadruplex DNA such as the d(AAG) repeat associated with Friederich's ataxia.

6. Telomere DNA and telomerase

One part of the 'telomerase hypothesis' is that telomerase activity is needed for cells to divide indefinitely making telomerase inhibition a prime chemotherapy target. An-

other part of the hypothesis is that the loss of telomerase activity is a source of cellular aging and so turning telomerase on will allow cells to live longer. While therapy based on telomerase inhibition appears promising [17,19,54–59] the benefits of telomerase activation are less clear [60].

Telomerase is used to maintain the length of linear chromosomes. This activity is needed since linear chromosomes shorten each time the DNA is replicated. Tumor cells divide indefinitely and about 85–90% of all tumor cells examined to date have high levels of telomerase activity [61–63] making telomerase a prime target for cancer therapy [64–67]. Telomerase is inhibited by quadruplex DNA [58,68]. The porphyrin T4 that specifically binds to quadruplex DNA [11,45,69] inhibits telomerase and is toxic to tumor cells [10,12] making quadruplex DNA a promising target for therapeutic intervention [20,58,59,70,71]. Additional molecules that bind to quadruplex DNA are also known to inhibit telomerase [58].

While telomerase and HIV reverse transcriptase appear to be closely related the nucleotide analogues effective against HIV reverse transcriptase do not necessarily have activity against telomerase [59]. If the sole function of telomerase was telomere maintenance then telomerase inhibition should not be toxic until several, or more, rounds of replication. It has been shown that some telomerase inhibitors can be toxic more quickly than this model suggests and thus may act via mechanisms other than by inhibiting telomere length maintenance by telomerase.

At one time telomere DNA was considered to be primarily a protective cap to prevent chromosome fusion and chromosome degradation. It is now becoming clear that telomere DNA, and its associated proteins, adopt a unique set of structures and play roles in cell cycle regulation, cellular life span and gene silencing. Human telomere DNA interacts with a large number of proteins including RAP1, Ku, TRF1, TRF2, telomerase and others. Some of these proteins only interact with telomere DNA, like TRF1 and TRF2. Some, like RAP1 and Ku, also interact with other DNA sequences. There is evidence that the double strand break repair proteins may be required for proper telomere maintenance.

Mammalian telomeres may form a loop as depicted in Fig. 4 [72,73]. The proposed invasion of the duplex region by the d(TTAGGG)$_n$ overhang is reminiscent of the junctions present in recombination and replication. The joint of the t-loop may involve the presence of quadruplex structures and one such possibility is depicted in Fig. 4. The structure of the DNA at the point of strand invasion is not known at the present time. There are human nuclear proteins that specifically bind to d(CCCAAT) repeats and these may be involved in stabilizing the complementary strand when the d(TTAGGG)$_n$ strand forms quadruplex structures [74]. The involvement of BLM in meiosis indicates that quadruplex resolution of telomere DNA may be important [37].

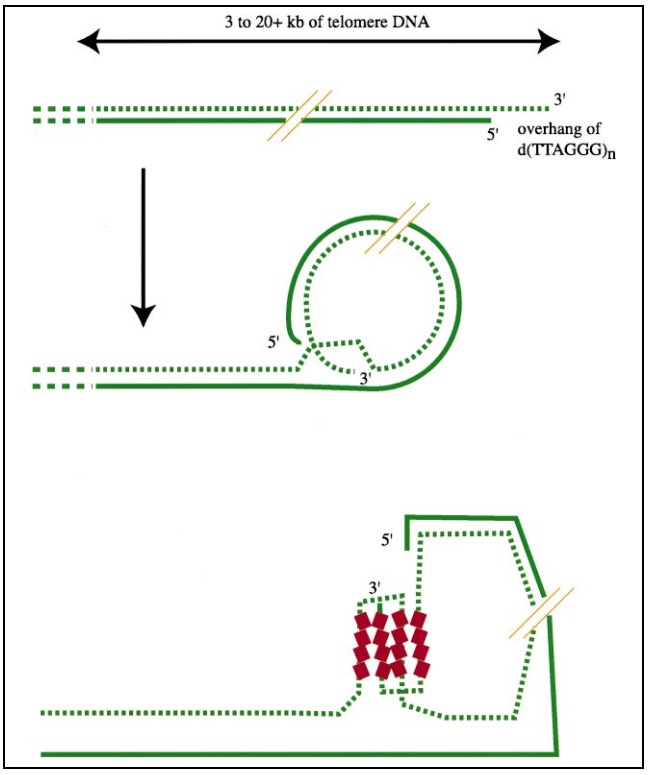

Fig. 4. In mammalian chromosomes the d(TTAGGG)$_n$ repeat may fold back and invade the duplex region to form a t-loop. There is typically 3000–20 000 nt of telomere DNA and the overhang is typically 130–270 nt in length. The size of the t-loop depends on the length of the telomere DNA and if there are not enough repeats of the telomere DNA or if the overhang is too short then the t-loop may not form. A possible structure for the t-loop is shown.

The telomere binding proteins of ciliates bind to the d(GGGGTTTT)$_2$ overhang found in the mini-chromosomes of the macronucleus [72]. Quadruplex DNA is not found in the complexes between the telomere binding protein and d(GGGGTTTT)$_2$ [72]. These results are not directly related to what occurs in mammalian telomeres as the overhang is shorter, the proteins are different and there is no t-loop in the macronucleus of ciliates amongst other differences. The DNA in the ciliate micronucleus appears to form a t-loop like that found in mammalian cells [72].

6.1. Questions and therapeutic potential

The limited data that is currently available indicates that molecules that bind to quadruplex DNA can inhibit telomerase. A working model is that the binding stabilizes the quadruplex form of the DNA and the stabilized complex inhibits telomerase both in vitro and in vivo. Only a small number of molecules that bind to quadruplex DNA have been investigated and relatively little is known about the ones that have been found. Given the unique structural features of each of the types of quadruplex DNA, it should be possible to find drug-like molecules that bind to each type of quadruplex DNA with nanomolar, or

higher, affinity. The availability of such molecules will allow testing of the hypothesis that quadruplex DNA is a good target for chemotherapeutic intervention and which type of quadruplex DNA should be targeted. Some inhibitors that target quadruplex DNA may also inhibit the activity of RecQ helicases, Hop1 and other proteins giving rise to significant side effects.

For a joint, as depicted in Fig. 4, to occur repeats of d(TTAGGG) need to form quadruplex structures. The quadruplex structures formed by $d(TTAGGG)_n$ contain crucial information in deciphering how molecules that bind to quadruplex DNA inhibit telomerase as well as in guiding the design of better inhibitors. Methods for the detection of quadruplex DNA in mixtures are needed to determine the presence of quadruplex DNA in the structures formed by telomere DNA free and in association with proteins.

The quadruplex structures that can be formed by telomere DNA are not well characterized. There is compelling evidence that $d(TTAGGG)_4$ forms a quadruplex structure, at least in the presence of potassium, but the structure is yet to be determined [46,48]. Results obtained by nucleotide substitution on the gel mobility of $d(TTAGGG)_4$ are not entirely consistent [46,48]. The thermal stability of the quadruplex form of $d(TTAGGG)_4$ observed in gel experiments is much higher than that of the sequence used in a nuclear magnetic resonance based structural study.

It is also noted that while quadruplex DNA is an inhibitor of telomerase, so are 7-deazaguanosine [75] and 6-thioguaninosine [76]. Neither of these modified guanines can participate in quadruplex DNA formation [46–48]. Taken together the results suggest that quadruplex DNA is needed at one step since these nucleotide analogs block quadruplex structure formation while quadruplex DNA can inhibit additional steps of telomerase activity. It may be the case that these two steps involve different structural types of quadruplex DNA.

7. Quadruplex DNAs as therapeutics

Directed evolution is based on the mimicry of biological processes for the discovery of potential therapeutic agents [77,78] and can now be carried out in conjunction with high throughput screening. A successful application of a directed evolution methodology has been the discovery of a class of DNA molecules that bind to and inhibit thrombin [15]. An aptamer is an RNA or DNA molecule that binds to a specific molecular target. The sequence d(GGTTGGTGTGGTTGG) was the DNA aptamer with the highest affinity for thrombin in the original screens [15]. This DNA aptamer significantly increases the thrombin catalyzed clotting times of both purified fibrinogen and human plasma and does not compete with known active site inhibitors of thrombin [15]. This DNA adopts an intramolecular chair type quadruplex structure containing dG quartets, as depicted in Fig. 1, and the tertiary structure determines the activity of the aptamer [16,30]. The structural information has been used to guide the development of more effective inhibitors of thrombin [79].

DNAs with sequences consistent with the formation of quadruplex structures have been found to be potent inhibitors of HIV-1 integrase [14]. Potassium appears to primarily effect the structure of the loops of the quadruplex structures formed by those DNAs that inhibit integrase [14].

8. Other interactions of quadruplex DNAs

The nuclear protein LR1 is B-cell type specific and binds to quadruplex DNA with nanomolar affinity [1]. LR1 is thought to play a key role in immunoglobin switch recombination [1]. Nucleolin, an abundant protein in the nucleolus, binds to quadruplex DNA with about 1 nM affinity [1,2]. Nucleolin may be involved in rDNA processing, transcription, replication and recombination [2]. Rat livers express two hnRNP proteins which bind to telomere DNA with nanomolar affinity that can unwind quadruplexes formed from fragile X repeat DNA but not those formed from telomere or immunoglobin repeats [9,80]. A protein with quadruplex DNA resolvase activity has been purified from human placenta [81].

9. Future directions

This survey indicates that quadruplex DNA may be involved in a number of biological systems. However, many of the studies have used DNAs that may form multiple structures making it difficult to ascertain which quadruplex structure type, or types, were involved in the interactions examined. The use of DNAs that form a known, single quadruplex structure will allow the interactions to be better characterized and may also indicate that the binding affinities are stronger than those deduced from results on the DNAs that adopt multiple structures. To further characterize the resolution activity of the RecQ helicases, and other proteins, the catalysis of the formation of quadruplex DNA by Hop1 assays needs to be developed for studying the kinetics and equilibria between single stranded, duplex and quadruplex forms of DNA. Such assays may also allow the development of small molecules that catalyze the resolution of quadruplex DNA as well as inhibitors of the RecQ helicases.

A few molecules have been found which selectively bind to quadruplex DNA. These molecules have offered the opportunity to begin the examination of the effects of targeting quadruplex DNA both in vitro and in vivo. Given the differences between the structural types of quadru-

plex DNA it should be possible to specifically target each of the quadruplex types.

Many systems of interest, like triplet repeat DNAs, appear to form a wide range of structures. Thus, methods are needed which allow the characterization of the structures present and their sensitivity to environmental conditions. This could be accomplished by the combined use of native gels and staining with molecules that specifically bind to quadruplex DNA. More detailed information could be obtained by the use of molecules that specifically stain a single quadruplex structural type.

As molecules that specifically bind to quadruplex DNA have been found, and even more specific and tighter binders are sure to be found, it should be possible to specifically target each type of quadruplex DNA for reaction and possibly subsets of each structural type. Reactive groups can be added to the quadruplex specific binders to specifically cleave quadruplex DNA both in vivo and in vitro to obtain targeted quadruplex specific nuclease activity.

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