Did Quadruplex DNA Play a Role in the Evolution of the Eukaryotic Linear Chromosome?

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Abstract: The current evidence on prokaryotic linear chromosomes, the eukaryotes that do not use telomerase and quadruplex DNA has been considered. This has lead to the suggestion that quadruplex DNA may have played a role in the evolution of the protection linear chromosomes rather than in overcoming the end replication problem.

Keywords: DNA, telomere, quadruplex, tetraplex, evolution, linear chromosome.

Linear chromosomes present several challenges to organisms [1,2]. The end replication problem occurs due to the leading and lagging strand replication of DNA which leaves the lagging strand at least one primer short after each round of replication [3]. The other main problems are that the ends of linear chromosomes will recombine with the ends of other chromosomes and that the linear ends are substrates for the DNA repair systems [4-6]. While the end replication problem may be better known the protection of the ends of linear chromosomes is equally important.

The telomere repeat sequences of most eukaryotes contain runs of dG residues as listed in table 1. Most, if not all, of these sequences can form quadruplex, also referred to as tetraplex or G4, structures [7]. This has lead to proposals for the roles of quadruplex structures in the telomeres of eukaryotes [7-13]. Since telomeres have recently become prime targets for chemotherapeutic intervention [8-13] it is of interest to see if there is any useful information that can be obtained by considering what roles quadruplex DNA may have played in the evolutionary origin of telomeres. The evolutionary considerations can be analyzed in terms of the experimental results that are available on the quadruplex structures formed by eukaryotic telomere DNAs.

The evolutionary step from prokayotes to eukaryotes involved a number of fundamental changes in cell structure and function. These changes include the introduction of meiosis, the nucleus, the linear chromosome, mitochondria and chloroplasts, mRNA capping, sexual fusion, organelles, phagocytosis and telomeres. The information from genome sequencing is now being used in an attempt to determine when and how these changes occurred. The limited information that is currently available indicates that there were lateral, or horizontal, transfers of genetic information between the prokaryotic and eukaroytic lines after their original divergence [14]. One of these transfers may have occurred before the introduction of mitochnondria.

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There exists at least one eukaryotic line, *Giardia*, which does not have mitochondria [14]. *Giardia* eukaryotes have nuclei, carry out meiosis and have linear chromosomes. The results of the comparison of the genomes of *Giardia* with that of other eukaryotes and with prokaryotes has been taken as evidence for the evolution of the nuclear aspects of eukaryotic cells before the symbiosis with mitochondria and chloroplasts occurred [14].

Until recently it was thought that linear chromosomes occurred solely in eukaryotes but there are a number of prokaryotes that have linear chromosomes [14-16]. Two types of prokaryotic linear chromosomes have been found. There are organisms, such as the spirochete *Borrelia* which causes Lyme disease, that have linear chromosomes that end in closed, covalent hairpin loops [17,18]. These organisms may have novel chromosome replication mechanisms. The linear chromosomes that are closed by covalent hairpin loops do not seem to be precursors of those found in eukaryotes.

The linear chromosomes of other prokaryotes including *Streptomyces* and *Agrobacterium tumefacens* have proteins that are covalently bound at both ends as depicted in Fig. (**1**) [14-16]. These proteins allow solution to the main problems of linear chromosomes. A covalently bound protein can protect the chromosome from recombination and DNA repair systems. The covalently bound protein can also act as a primer for replication. One of the terminal proteins of *Streptomyces* has a reverse transcriptase like domain [19].

Retroelement Directed Elongation of Linear Chromosomes

Linear chromosomes can be elongated by the action of a retroelement, genetic information that exists both as RNA and/or DNA, as is the case with telomerase. Telomerase uses a captive RNA as a template for the synthesis of DNA and the telomere RNA arises from transcription. Telomerase may have evolved from retrotransposons [20,21].

Drosophila has a non-standard telomeric sequence [22-31]. Investigation of the *Drosophila* telomeres has shown that the elongation of DNA uses retroelements [23-31]. The 3' end of the HeT-A retroelement used in Drospophilia are

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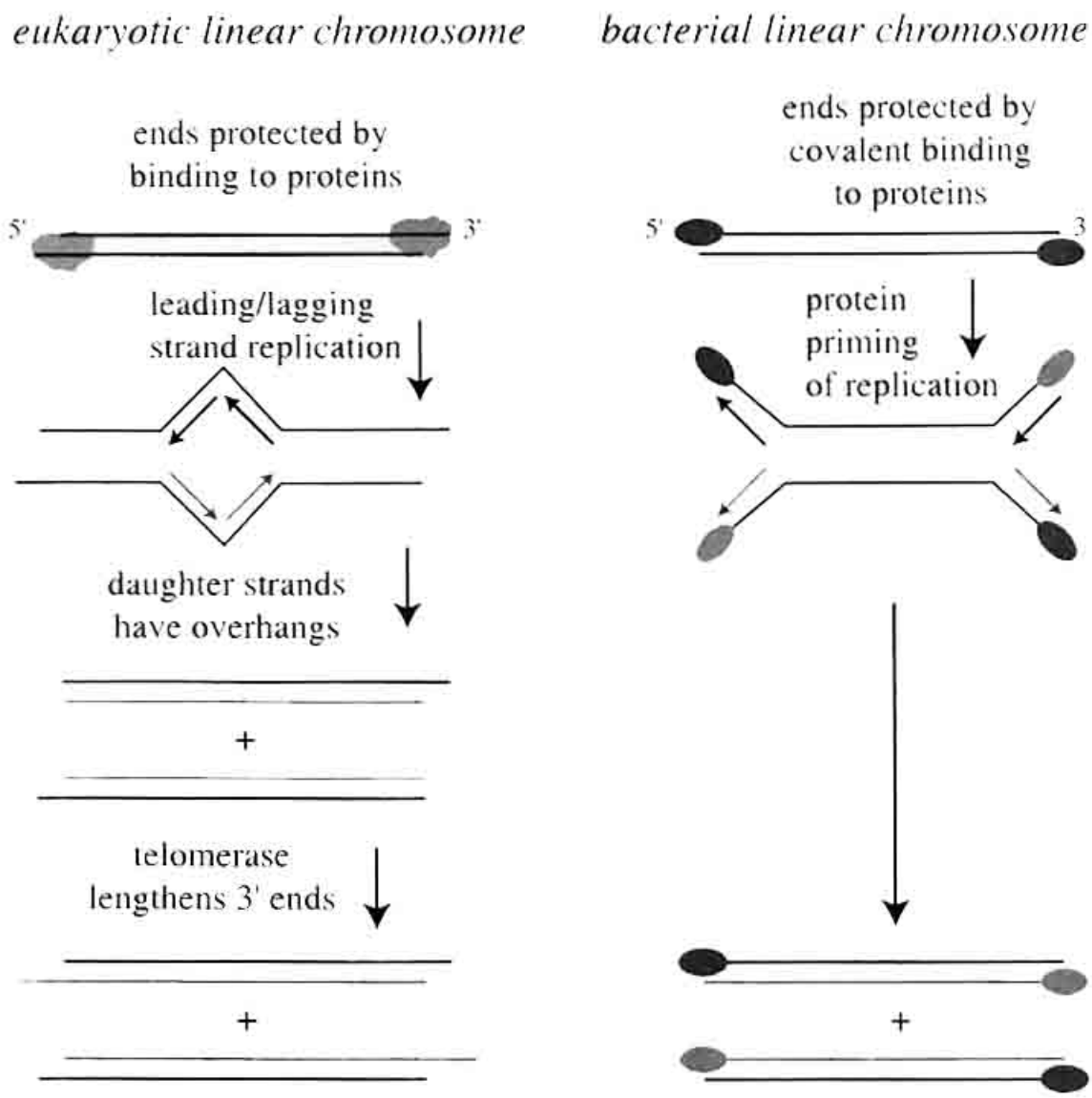

Fig. (1). A schematic depiction of the replication of prokaryotic linear chromosomes that are protected by binding to proteins is shown on the right. On the left is shown a schematic depiction of the replication of eukaryotic linear chromosomes.

long appears to be capable of forming quadruplex structures [32]. A duplex region can be formed between a retroelement that is complementary to the overhang in the leading strand that is the result of incomplete synthesis of the lagging strand as depicted in Fig. (**2**). The retroelement can then act as a template for elongation of the leading strand that in turn can be used as a template for lengthening the lagging strand. This process can be repeated indefinitely to elongate the linear chromosome. The retroelement could also be integrated into the telomere region other than at the end. The retroelement can be formed by the transcription of a gene that may have a locus that is distant from the telomere. Virtually all retroelements use tRNA or an RNA with significant secondary structure to initiate reverse transcription.

The evolution of telomerase from a retroelement appears to be a quite plausible model [20,29,33]. The original retroelement would form tighter and tighter complexes with the reverse transcriptase until the RNA became captive. The original RNA may also have had some reverse transcriptase activity as this is well within the range of activities that have been observed for ribozymes. It seems that this route would offer protection of the RNA and that there could be evolutionary pressure for the repeat sequence to become short. It is not clear to us why there would be selective pressure for the repeat unit to adopt a quadruplex structure. The very limited evidence that is available, in particular that from *Drosphila* and the linear chromosomes of prokaryotes, indicates that the end replication problem can be solved without quadruplex structures.

Stabilization of Linear Chromosomes

There are a number of possible ways to stabilize linear chromosomes, some of which are depicted in Fig. (**3**). The earliest linear chromosomes may have been stabilized by

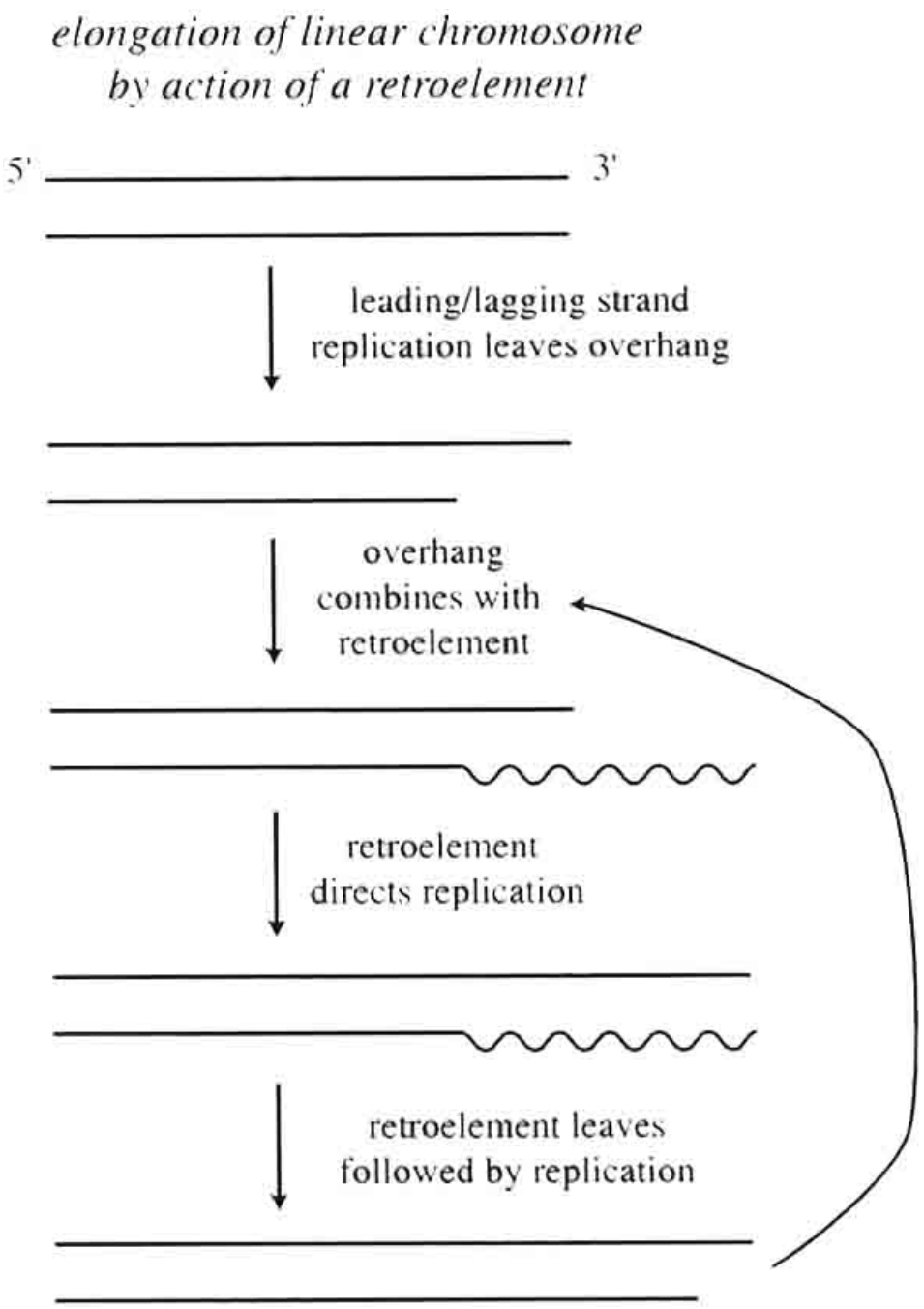

Fig. (2). The elongation of a linear chromosome can proceed through the action of a retroelement as depicted.

binding to a protein as is the case for some of the prokaryotic linear chromosomes and as may be the case for the macronuclear chromosomes of *Oxytrichia nova [34,35]*. The ends of the linear chromosome need to be stable to DNA repair systems and to recombination. To be stable to DNA repair the ends of the chromosome need to be "hidden" and to be stable to recombination by non-homologous end joining.

The overhang of a linear chromosome can form a RNA-DNA hybrid duplex with the retroelement as depicted in Fig. (**3**). This structure could offer temporary capping. However, such a hybrid could be sensitive to RNase H of which multiple forms are present in all organisms. Such a hybrid structure may offer very short term capping to linear chromosomes as RNA-DNA hybrids are apparently not recognized by DNA repair systems. The RNA could, however, provide an important role in the assembly of the telomere structure. Quadruplex DNAs are kinetically very stable [7,36] and telomere sequences can form a range of conformers as discussed below. Thus, the overhang could form structures that are kinetically stable but inappropriate for the assembly of the telomere structure. The RNA could act as a resolvase by unwinding quadruplex structures. This

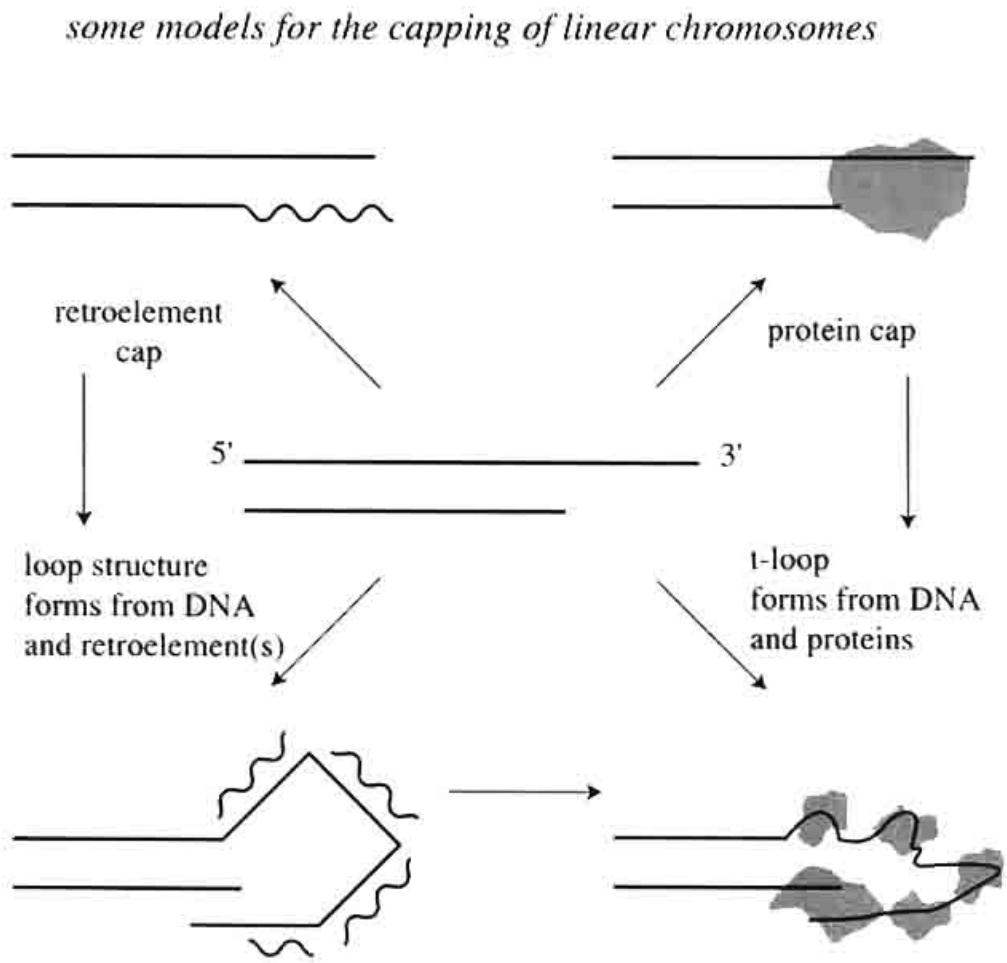

Fig. (3). There are many models for the protection of the ends of linear chromosomes of which four are shown. In the bottom two cases the overhang invades the duplex region to form a "t-loop".

activity may be carried out by RecQ helicases in modern eukaryotes [37-43].

The DNA overhang could form a non-duplex structure with the retroelement used for elongation, or other, RNA as depicted in Fig. (**3**). This structure could form a stable loop arrangement with the lagging strand as shown. The DNA-RNA hybrid structure could contain duplex, triplex, quadruplex or other structural elements which confer resistance to RNase H activity as well as DNA repair and recombination. The formation of the full loop would mean that there is no "end" to the chromosome that could be recognized as a double strand break by DNA repair and recombination systems.

The capping of the linear chromosomes by a non-duplex DNA structure that forms a loop with the lagging strand is depicted in Fig. (**3**). In this type of arrangement the overhang DNA forms a structure that is resistant to DNA repair as well as looping back such that there is no end to the chromosome. Some of the possible non-duplex structure that could be formed include triplex, pseudo-knot and quadruplex structures. The formation of this structure may be aided by the action of one, or more retroelements.

Quadruplex structures are compact which could allow efficient packaging of the ends of linear chromosomes without a system as elaborate as the one used for nucleosomes. Thus, chromosomes that were capped with variable numbers of quadruplexes could form the same basic structure. Another advantage is that quadruplex structures can be formed by a short repeat sequence. The length of the repeat needed for stable triplex structures is rather long probably on the order of a few dozen nucleotides such as $d(A_{12}T_{12}T_{12})_n$. Similarly, a pseudo-knot structure would require a rather long repeat sequence.

While quadruplex structures may be resistant to DNA repair systems they are not particularly resistant to nucleases. Thus, if an early linear chromosome was capped by quadruplex structures then the loop regions of the quadruplex structures would need to be protected. Recent results indicate that quadruplex structures can contain regions that consist of base triples or quadruplexes that do not include dG residues as discussed below. The sequences used for the early linear chromosomes may have utilized these features.

There does not appear to be any structure that can be formed by DNA or DNA-RNA hybrids that could offer long term, stable capping of a linear chromosome. The structures

would be too sensitive to nucleases though quadruplex structures or DNA-RNA hybrids could offer protection from recombination and DNA repair. The formation of a complex with a protein could protect the loop regions from nucleases and offer stable capping.

The macronucleus of *Oxytrichia nova* contains thousands of gene sized linear chromosomes which are apparently capped by a simple mechanism. These chromosomes contain overhangs of $d(G_4T_4G_4)$. The chromosomes bind to OnTEBP, *Oxytrichia nova* telomere end binding protein [34,35]. The original crystal structure indicated that $d(G_4T_4G_4)$ bound to OnTEBP in a single stranded form and this was frequently taken as evidence, though not by the authors of the original structure, as evidence against the involvement of quadruplex structures in telomeres. A subsequent structure determination, by the same group as carried out the original, showed that the quadruplex form of $d(G_4T_4G_4)$ also binds to OnTEBP [34]. This new result indicates that the telomeres of the macronulceus of *Oxytrichia nova* may interact with both single stranded and quadruplex DNA [34]. Curiously, there have been no good studies of the binding of $d(G_4T_4G_4)$ to OnTEBP. A competition experiment between a DNA that can not form quadruplex DNA, for example replacing one of the dG residues with s6G or 7-deazaG which block quadruplex structure formation [7], with $d(G_4T_4G_4)$ could allow determination of which structural forms binds most strongly. The current results on OnTEBP are inconclusive as to the role of quadruplex DNA in the stabilization of the linear chromosomes of *Oxytrichia nova* but the results do not rule out a role for quadruplex DNA.

Very little is known about the interactions of the proteins that interact with the telomere DNA of most eukaryotes. Many proteins are known to bind to the telomeres including RAP1, TRF1 and TRF2 [44,45]. There have not yet been any studies to determine which forms of DNA these proteins preferentially bind to.

There is some evidence that the linear chromosomes of many eukaryotes form a t-loop [46] in which the overhang invades the duplex region of the telomere as shown in Fig. (**3**). It has been proposed that when the overhang becomes less than a critical length then the t-loop can not form. Quadruplex structures may be involved in the stabilization of the t-loop and one model for this is shown in Fig. (**3**). However, the roles of proteins in the formation and stabilization of the t-loops are not yet known. It is also not known whether the formation of telomere structure is a spontaneous process or whether it requires helpers such as the chaperones which assist protein folding.

It may well the case that the formation of telomere structure requires the activity of proteins such as the RecQ helicases [37,40,47]. Defects in the RecQ helicases cause Bloom's and Werner's syndromes both of which are characterized by the formation of many types of tumors [37,38,41-43,48,49]. These ATP dependent RecQ helicases have been shown to be able to preferentially unwind quadruplex structures [37,38,41-43,48,49]. RecQ helicases apparently co-localize with telomeres during the the S phase of the cell cycle [50]. The RecQ helicases may be important in the resolution of improper quadruplex structures that are formed at telomeres.

Structures of Telomere DNAs

Quadruplex DNAs are compact structures with high charge density as indicated by their mobility in gel electrophoresis. The compact nature of the structures can allow efficient packaging of the DNA. The narrow grooves of basket and chair type structures, which are depicted in Fig. (**4**), have strong electrostatic potentials that can provide strongly binding sites for divalent and trivalent cations and could allow discrimination from other forms of DNA.

The structures of a number of quadruplex DNAs have been determined by solution state NMR methods [7] and some of these are depicted in Fig. (**4**). Almost all of the reliable structural information about quadruplex DNAs has been obtained from NMR data. There is only quality one crystal structure of a dG based quadruplex DNA, a parallel strand complex, that has been published [51]. The range of known quadruplex structures has continued to expand. While it was originally thought that the quartets could only be based on dG residues there are now a number of known structures that contain other residues [52-57].

The samples that have been characterized by NMR are those that adopt a single, stable conformer in solution and have a modest molecular weight. NMR methods can offer high resolution information about the structures and interactions of these molecules. For example, the binding of cations to quadruplex DNAs has been studied by NMR methods. NMR methods can not be applied to many of the samples of biological interest simply because the molecular weights are too large. The overhang in human chromosomes contains thousands of nucleotides which is simply beyond the technique.

The NMR results have shown that many of the sequences in Table 1 can form quadruplex structures. There is not yet a full set of rules for predicting the type of quadruplex structure from sequence [58]. Complementary evidence has been obtained by other methods with the most prominent being gel electrophoresis. The examination of the gel mobility of DNAs as a function of DNA concentration, the cations present and by nucleotide substitution has offered considerable evidence about which sequences form quadruplex structures under which conditions [59-61]. These results have been, for the most part, consistent with those obtained by NMR [7]. One of the advantages of the gel approach is that it can allow the investigation of sequences which adopt multiple conformers under a wide range of conditions. These studies have shown that the sequences in Table 1 can adopt quadruplex structures.

The structures adopted by the telomere sequences can depend on subtle changes. For example, the quadruplex structures formed by $d(TTAGGG)_4$ and $d(GGGTTA)_4$ appear to be quite different. One of these apparently forms a monomeric quadruplex structure and the other as a dimer as the results in Fig. (**5**) show. The structures formed by longer repeats of the vertebrate telomere repeat are not known.

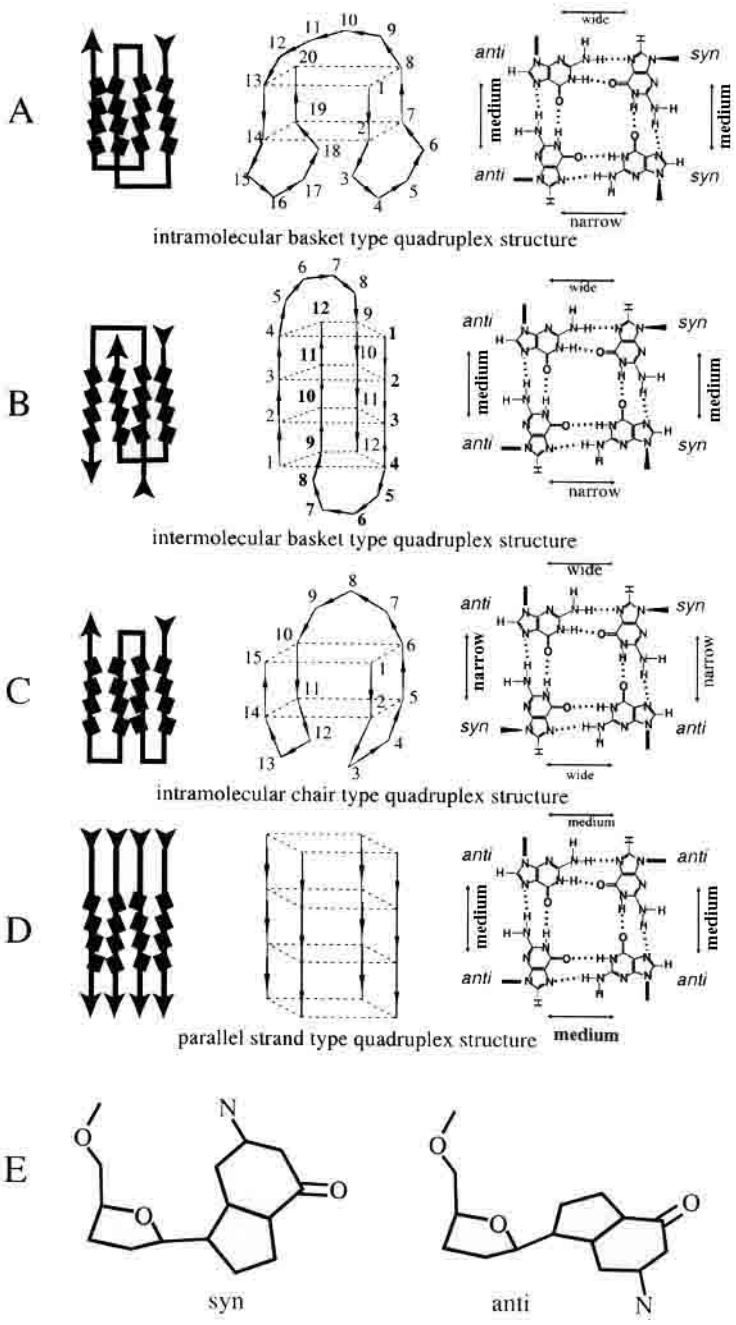

Fig. (4). Four of the possible types of quadruplex structures based on quartets of dG residues are shown.
In part A the quadruplex structure is an intramolecular basket type structure. On the left the structure is schematically depicted with the red boxes indicating the dG residues and the backbone of 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 part 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 part 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 part 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 part E the orientation of the base relative to the sugar is shown for syn and anti orientations.

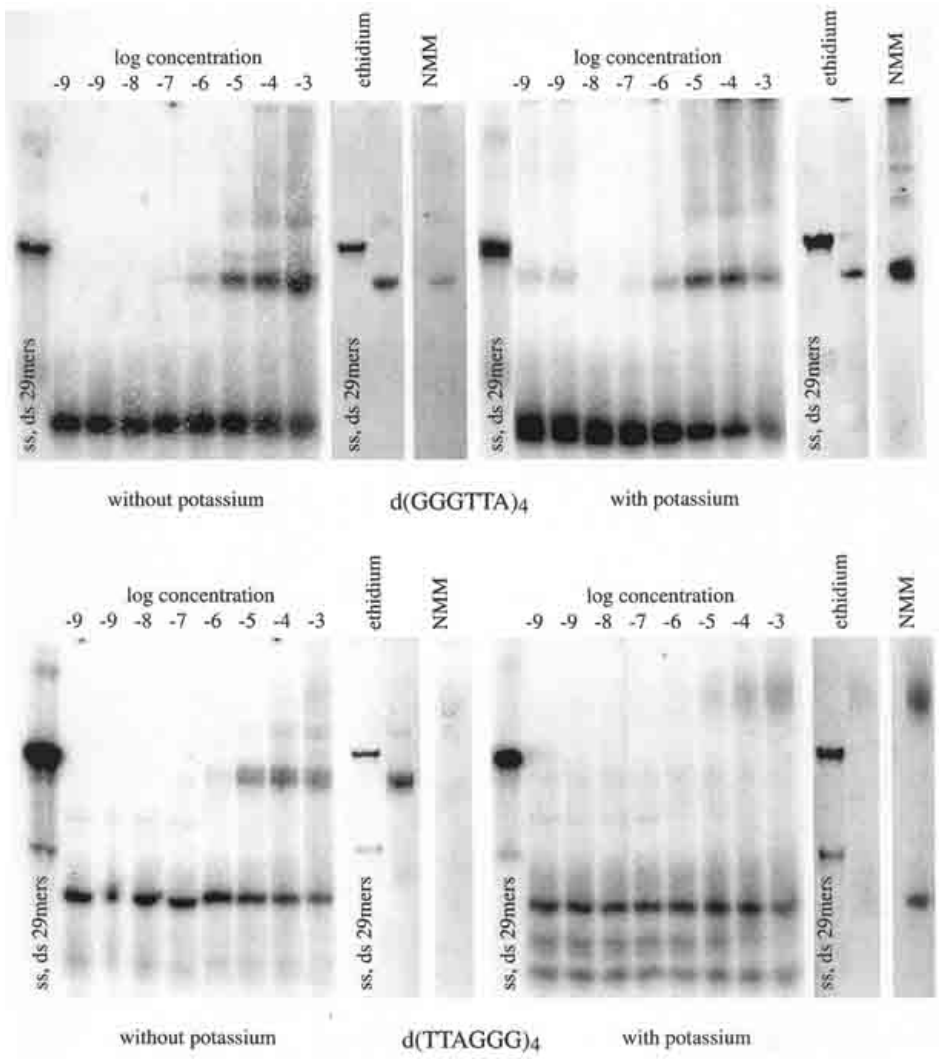

Fig. (5). The gels for d(GGGTTA)$_4$ are shown on the top. On the far left is the image obtained by phosphoimager detection of the ^{32}P in the DNAs. The amount of ^{32}P 5'-end labeled DNA is the same in all of the lanes. There is also a lane that contained ss and ds29mers. The gels marked "ethidium" and "NMM" are the images obtained by detecting the fluorescence of the gel after it had been stained with the indicated dyes. The three gels on the left were run in the absence of potassium and those on the right were run in the presence of 10 mM potassium.

The gels for d(TTAGGG)$_4$ are shown on the bottom. On the far left is the image obtained by phosphoimager detection of the ^{32}P in the DNAs. The amount of ^{32}P 5'-end labeled DNA is the same in all of the lanes. There is also a lane that contained the ss and ds29mers. The gels marked "ethidium" and "NMM" are the images obtained by detecting the fluorescence of the gel after it had been stained with the indicated dyes. The three gels on the left were run in the absence of potassium and those on the right were run in the presence of 10 mM potassium.

These results indicate that d(TTAGGG)$_4$ forms an *intramolecular* quadruplex in the presence of potassium while d(GGGTTA)$_4$ forms an *intermolecular* quadruplex structure that is most likely a dimer. The quadruplex structures formed by d(TTAGGG)$_4$ and d(GGGTTA)$_4$ have a strong potassium dependence indicating that both of these form chair type structures. Neither d(TTAGGG)$_4$ nor d(GGGTTA)$_4$ forms significant quadruplex structure in the absence of potassium under the conditions used.

The positions of the bands of the ss and ds29mers observed by ^{32}P detection and by fluorescence are different since the phosphoimager detects the position of the DNAs containing the 5'-end label while the DNAs used in the fluorescence experiments do not have the extra phosphate. The additional negative charge causes the 5'-end labeled DNAs to have higher gel mobility than the DNAs without the end label.

All of the gel experiments were carried out using 1.5 mm, 20% polyacrylamide gels made with an acrylamide to bisacrylamide ratio of 19:1. The gel buffer contained 89 mM Tris, 89 mM boric acid, 2 mM EDTA, 140 mM NaCl at pH 8.0. For the potassium experiments 10 mM KCl was added to this buffer. The DNA samples for the gel experiments were in a buffer containing 140 mM NaCl, 20 mM Tris at pH 7.0. 10 mM KCl was added to the samples for the potassium gels. The DNA samples were annealed by heating to 80 ^{o}C followed by cooling to room temperature over 4 h. Glycerol was added to 8.3%, by volume, before the DNAs were loaded on the gels. For the ^{32}P detection experiments a constant amount of labeled DNA was added to a variable amount of cold DNA to obtain samples over the concentration range of 10^{-9} to 10^{-3} M.

Table. 1.

organism	Telomere repeat sequence
mammals & trypanosome	d(GGGTTA)
plants	d(GGGTTTA)
tetrahymena	d(GGGTT)
saccharomyces	d(GGGTGTGT)
oxytrichia & euplotes	d(GGGGTTTT)
dictyostelium	d(GGGGAGA)

The results indicate that small changes in sequence can alter the type of quadruplex structure formed. Even small alterations in the sequences in the loop regions can have profound effects on the structure formed. This indicates that the free energy differences between the various types of structures are not large. The gels of many DNAs containing repeats of telomeric sequences contain multiple bands which also indicates that the free energy differences are small. Quadruplex DNAs tend to be kinetically very stable allowing the separation of conformers that have small free energy differences. These results suggest that the binding to proteins could readily shift the equilibrium to a single quadruplex structural type with the preferred structural type being determined by the protein-DNA interactions rather than just the preference of the DNA alone.

Catalytic DNAs, known as deoxyribozymes, have been found by *in vitro* methods. Many of these appear to involve quadruplex structural features including a deoxyribozyme which catalyzes a 5'-5' capping addition of ATP [62]. While there are no known cases of deoxyribozyme activity in cells these results indicate that quadruplexes can form the platform for activities not typically associated with DNA.

Summary. The limited evidence that exists about the evolution of linear chromosomes suggests that quadruplex DNA may have played an important role in capping the ends. The compact nature of quadruplex structures and the fact that they can be formed by short repeat sequences are features that could have been exploited to stabilize the ends of linear chromosomes. There is no obvious reason why other sequences could not have been exploited to solve the end replication problem as has been done by *Drosophila*.

This tentative model suggests that molecules that target quadruplex DNA may be having their effect by disrupting the capping of linear chromosomes. This may be more important than the inhibition of telomerase. This would explain how quadruplex binding molecules have an earlier onset of cytoxicity than predicted on the basis of telomerase inhibition alone [9-11,63]. The disruption of telomere structure may be repairable by slowly dividing but not by rapidly dividing tumor cells or due to loss or reduction in activity of the system that is responsible for checking telomere structure integrity. To test this prediction more needs to be known about the quadruplex structures formed by telomere DNA and the effects of telomere binders on these structures.

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