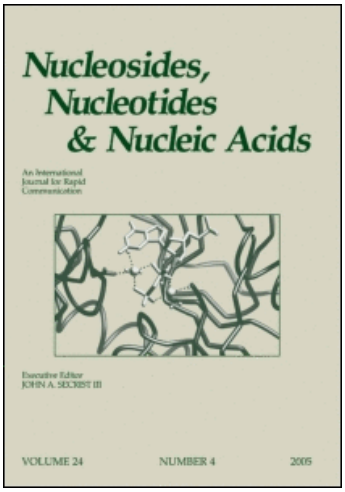

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Characterization of Telomeric Oligonucleotides: Fidelity of Repetitive Nucleotide Sequences

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CHARACTERIZATION OF TELOMERIC OLIGONUCLEOTIDES: FIDELITY OF REPETITIVE NUCLEOTIDE SEQUENCES§

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ABSTRACT: To clarify the reasons for the high fidelity of repetitive telomeric sequences, a series of d(TTXGGG)$_4$ (X: A, G, C, and T) were synthesized and characterized by UV absorption, CD, chemical modification, and resistance to nucleases. d(TTCGGG)$_4$, which is lacking in nature, has similar structural stability and resistance to nucleases, compared with d(TTAGGG)$_4$, which is widely present at the telomeres of many organisms. d(TTGGGG)$_4$ is the most stable as a result of formation of the four G-quartet layers in the presence of potassium ion.

INTRODUCTION

The ends of the linear chromosomes of eukaryotes are termed telomeres and are composed of tandem repeats of short DNA sequences. Most organisms exhibit long telomeres, up to several kilobases, that are variable in length. The telomeric DNA sequences of a variety of organisms are generally rich in guanine and their complementary strands in cytosine. The sequences containing a regular short repeat fit into the consensus d[T$_{1-3}$(T/A)-G$_{3-4}$]$_n$[1, 2, 3]. For example, the telomeric d(TTAGGG) sequence is conserved from the higher eukaryotes such as mammals (human[4]) to the lower eukaryotes such as fungi (*Neurospora*[5])(TABLE 1). In most cases, the 5' side residue of the dG cluster is limited to dT or dA. This strict conservation of dG-rich consensus sequences suggests that the structures formed by these repetitive sequences may play an important role in telomere functions. The telomeres may be required not only for telomere elongation during replication[6] but also for stability of the ends of the chromosome[7].

§This paper is dedicated in memory of Prof. Tsujiaki Hata.

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TABLE 1. Sequences of telomeric DNAs[a]

Telomeric repeat[b]	**Group**/organisms
TTGGGG	**Protozoa**/*Tetrahymena*
TTTTGGGG	**Protozoa**/*Oxytricha*
TAGGG	**Protozoa**/*Giardia*
TTAGGG	**Protozoa**/*Trypanosoma*, **Slime molds**/*Physarum*, *Didymium*, **Fungi**/*Neurospora*, *Podospora*, *Cladosporium*, **Mammals**/ *Homo sapiens*
TTTAGGG	**Plants**/*Chlorella*, *Arabidopsis*
TTTTAGGG	**Algae**/*Chlamydomonas*
TTAGG	**Invertebrates**/*Bombyx*
$T_{1-3}(T/A)G_{2-4}$	**Consensus sequence**

[a]References for the table are from Ref. 1, 2, and 3. [b]Sequence repeat unit shown from 5' to 3' is the 3' end of strand of the chromosome.

In recent years, much attention has been focused on characterization of dG-rich telomeric DNA strands using various types of synthetic oligonucleotides corresponding to dG-rich strands. Previously, we investigated the properties of oligonucleotides containing a dG cluster, d(TmGnTm), which are models of single-stranded parts of telomeric DNA and substitutes for poly(dG)[8]. We have suggested that the parallel four-stranded structure of ribopolymer poly(G) is reproducible within self-associated oligomers containing a dG cluster. Recently, the structures of a parallel-stranded quadruplex formed by the hexamer d(TGGGGT)[9] and an antiparallel-stranded quadruplex formed by the dodecamer d(GGGGTTTTGGGG)[10] were determined using X-ray crystallography. Furthermore, many nuclear magnetic resonance (NMR) studies have been carried out on several types of dG-quartet structures[11,12,13]. Based on these data, it has been shown that oligonucleotides containing two or four repeats of dG clusters readily form antiparallel-stranded dimeric and monomeric quadruplexes, respectively.

In this study, we prepared a series of telomeric 24-mer oligonucleotides made up of four repeats of the d(TTXGGG) sequence. The sequence d(TTGGGG) (X: G) was discovered in the telomere of *Tetrahymena*, and the d(TTAGGG) sequence (X: A) was discovered in various organisms such as protozoa, slime molds, fungi, and mammals. This report describes the observations of the influence of a nucleotide residue adjacent to the 5' side of the d(GGG) cluster on stability of the quadruplex structure and resistance to nuclease.

MATERIALS AND METHOD

Oligonucleotide synthesis and labeling

The oligonucleotides used in this study (shown in Table 2) were synthesized on an Applied Biosystems 391S DNA synthesizer. The oligomers were purified by high performance liquid chromatography on a reverse-phase C18 column at 60°C. The 5' end of the oligomer was labeled with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase (Takara, Kyoto). DNA solution was boiled for 1 min followed by rapid cooling to room temperature just before use.

UV spectroscopy and UV melting curves

UV absorption spectra were recorded on a Shimadzu UV-250 spectrophotometer. For melting temperature measurement, a Hitachi 30 spectrophotometer equipped with a JASCO PTC-343 temperature programmer was used. The temperature was scanned at a heating rate of 1°C/min. CD spectra were recorded on a JASCO J-600 spectropolarimeter. The temperature was controlled using a thermo-jacketed cell and a circulating bath.

Polyacrylamide gel electrophoresis

The formation of the quadruplex was detected by native gel electrophoreses on a 20% polyacrylamide gel (19:1 ratio of acrylamide to bisacrylamide) using 0.4 X TBE buffer containing 90 mM Tris-borate, 2 mM EDTA, and additional salt (20 mM NaCl), or no salt, pH 8.3. DNA bands were observed by UV shadowing and methylene blue staining.

DNA modification and cleavage

G-specific cleavages were performed by a modification of the standard procedure[14]. The oligonucleotide (5 pmol) was dissolved in 50 µl of 0.5 X TE buffer and additional salt (50 mM NaCl or a mixture of 40 mM NaCl and 10 mM KCl) and then annealed after boiling for 2 min. To the DNA solution, 0.1 µl of dimethyl sulfate (DMS) was added and the reaction mixture was kept either at 25°C for 20 min or at 5°C for 40 min. The reaction was stopped by the addition of 1 µl of 20 mM deoxyguanosine. The sample was desalted using a MicroSpin Column S-200HR (Pharmacia) and then dried under vacuum. The residue was dissolved in 1 M piperidine solution and heated at 90°C for 30 min. After evaporation of the piperidine solution under vacuum, the sample was dissolved in 10 µl of 90% formamide and loaded onto a denaturing (7 M urea) 16% polyacrylamide gel.

Cleavage reactions by hydroxyl radicals were performed using a modification of the published procedure[15]. The oligonucleotide (1.5 pmol) was dissolved in 15 µl of 0.5 X TE buffer and additional salt (50 mM NaCl or a mixture of 40 mM NaCl and 10 mM KCl) and then annealed after boiling for 2 min. One µl of 20 mM sodium ascorbate, 0.6% H_2O_2 and a mixture of 0.2 mM iron(II) chloride and 0.4 mM EDTA were added to the DNA

TABLE 2. UV absorption data and melting temperature of telomeric DNAs

Compound[a] d(TT**X**GGG)$_4$	Cation [b]	λ_{max} (nm)	ε[c]	Hypochromicity[d] (%)	Tm[e] (°C)
d(TT**G**GGG)$_4$	Na^+	254	9,400	15	60.8
	Na^++K^+	254	9,600	13	72.5
d(TT**A**GGG)$_4$	Na^+	256	9,100	22	50.0
	Na^++K^+	257	8,900	23	54.5
d(TT**C**GGG)$_4$	Na^+	257	8,600	14	50.2
	Na^++K^+	258	8,700	11	54.7
d(TT**T**GGG)$_4$	Na^+	258	8,700	14	42.5
	Na^++K^+	258	8,900	14	n.d.[f]

[a]The bold-faced capital letter (X) indicates the 5' side nucleotide of the d(GGG) cluster. [b]UV spectra were measured in 0.1 M NaCl or 0.08 M NaCl -0.02 M KCl, buffered with 0.01 M Na cacodylate (pH 7.0). [c]Molar absorption coefficient per base residue at λ_{max}. [d]Absorbance at 260 nm before and after nuclease P1 digestions were compared. [e]Tms at 275 nm were measured. [f]Not determinable. The melting profile of d(TTTGGG)$_4$ was not typical definitive sigmoid curve.

solution and then kept at 25°C for 3 min. The reaction was quenched by the addition of 2 μl of the stop solution (0.1 M thiourea, 20 mM EDTA). The samples treated with piperidine as described above were loaded onto a denaturing (7 M urea) 16% polyacrylamide gel.

Nuclease digestion

The 5'-end labeled oligomers (1 pmol) were dissolved in nuclease P1 digestion buffer (50 mM ammonium acetate, pH 5.0) containing 100 mM NaCl or 80 mM NaCl-20 mM KCl. The solutions (20 μl) were boiled for 1 min and then cooled immediately to reaction temperature (37°C). Nuclease P1 (1 U)(Nippon Gene,Toyama) was added to this solution. The reactions were quenched by the addition of the stop solution (7 M urea, 10 mM EDTA, 0.1% BPB, and 0.1% XC). Similarly the 5'-end labeled oligomers (2.2 pmol) were dissolved in venom phosphodiesterase (VPDase) digestion buffer (10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) containing 100 mM NaCl or 80 mM NaCl-20 mM KCl. The solutions (40 μl) were boiled for 1 min and then cooled immediately to reaction temperature (25°C). VPDase (1 U)(Sigma) was added to this solution. The reactions were quenched by the addition of the stop solution (7 M ureea, 10 mM EDTA, 0.1% BPB, and 0.1% XC). The reaction products were analyzed by 20% PAGE containing 7 M urea and 0.4 x TBE buffer (90 mM Tris-borate, pH 8.3 and 2 mM EDTA), followed by autoradiography.

RESULTS AND DISCUSSION

Native polyacrylamide gel electrophoresis

In the case of *Tetrahymena*, the telomeric DNA sequence is d(TTGGGG) (TABLE 1). The intramolecularly folded G-quartet structure should have four telomeric repeats, $d(TTGGGG)_4$. We synthesized a series of oligonucleotides $d(TTXGGG)_4$, where X is G, A, C, or T (TABLE 2). The mobility of these oligonucleotides in a gel containing 0.4 X TBE buffer at 40°C depended approximately on their length (FIG. 1). The markers are $d(AGTC)_3$ (M12) and $d(AGTC)_6$ (M24), which do not form a higher ordered structure by inter- and intramolecular interaction. When the electrophoresis was performed at 5°C in the presence of 20 mM NaCl, the mobilities of all telomeric DNAs became larger than that of the marker M24. This result indicates that the telomeric oligomers become more compact in the presence of NaCl. There was no band corresponding to complexes formed by intermolecular interaction. Based on these data, it has been suggested that the telomeric oligomers used in this study form monomeric antiparallel-stranded quadruplexes.

UV absorption spectra

For quadruplex formation, Na^+ ion is the most effective among the alkali metal ions[1]. On the other hand, K^+ ion is the most effective for stabilization of the quadruplex[1, 16]. Therefore, UV spectra were measured in NaCl solution or a mixed solution of NaCl and KCl. UV spectral data for the oligomers (approximately 5 μM strand concentration) are shown in TABLE 2. The hypochromicities of the $d(TTAGGG)_4$ at 260 nm under both salt conditions were larger than those of the other telomeric oligomers whose hypochromicities were nearly equal in spite of the salt conditions. The relative stabilities of the telomeric DNAs were assessed by thermal denaturation monitored by measuring hyperchromism at 275 nm. The melting temperatures (Tms) of the telomeric DNAs are shown in TABLE 2. In 0.1 M NaCl, the Tm of $d(TTGGGG)_4$ was the highest and Tm of $d(TTTGGG)_4$ was the lowest among the telomeric DNAs. The difference between them was approximately 20°C. Tms of $d(TTAGGG)_4$ and $d(TTCGGG)_4$ were nearly equal and corresponded to a middle value between the Tms of $d(TTGGGG)_4$ and $d(TTTGGG)_4$. It is known that potassium ion stabilizes the quadruplex structure by chelation with the guanine quartet. The Tms of all telomeric DNAs in 0.08 M NaCl and 0.02 M KCl were higher than the Tms in 0.1 M NaCl. In particularly, the Tm of $d(TTGGGG)_4$ rises approximately 12°C on the addition of KCl. In the case of $d(TTAGGG)_4$ and $d(TTCGGG)_4$, the rises in temperatures (approximately 4.5°C) were not large, compared with the case of $d(TTGGGG)_4$. These results suggest that the stability of the quadruplex structure depends not only on the number of the dG residues in the dG cluster but also the 5' side of the nucleotide residue of the dG cluster influences the stability of the quadruplex.

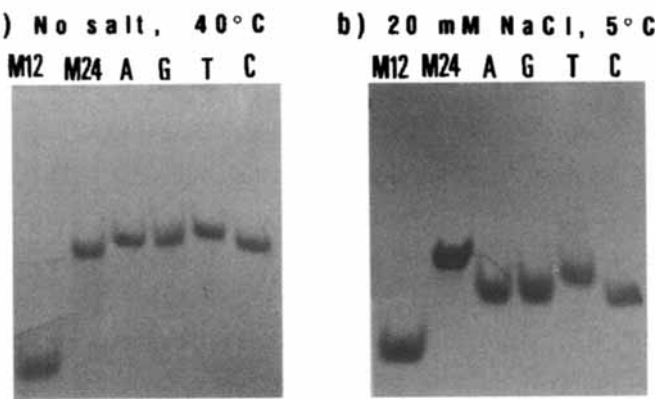

FIG. 1. Formation of monomeric quadruplexes. a) Electrophoresis of oligonucleotides in a native gel (0.5 X TE buffer, 40°C). The marker lanes (M12 and M24) are $d(AGTC)_3$ and $d(AGTC)_6$, respectively. Lanes A, G, T, and C are $d(TTXGGG)_4$ (X: A, G, T, and C, respectively). b) Electrophoresis of oligonucleotides in a native gel (20 mM NaCl and 0.4 X TBE buffer, pH 8.3, 5°C).

Structural characterization using chemical probes

Chemical probes are useful tools for the structural study of several types of DNAs. The most characteristic signature of G-quartet formation is the strong protection from methylation of the guanine N7 by dimethyl sulfate (DMS)[17]. FIG. 1 shows the results of methylation of the telomeric DNAs by DMS. In 0.5 X TE buffer, all of the guanines of telomeric DNAs were sensitive to the DMS methylation. In the presence of both NaCl and KCl, methylation of all the guanines was reduced almost to the background piperidine cleavage level. In the presence of NaCl, G_{15} of $d(TTGGGG)_4$ (lane G), which is the 15th residue from the 5' end of the oligomer, was particularly hypersensitive to the methylation. FIG. 2b shows the degree of methylation of the guanine residues of the telomeric DNAs. These results reveal that the guanine residues at the positions 9 and 15 from the 5' end of $d(TTGGGG)_4$ are not involved in the G-quartet. These guanine residues seem to be in a favorable configuration for the methylation. Furthermore, these guanines seem to be involved in the G-quartet in the presence of KCl.

FIG. 3 shows the hydroxyl radical cleavage pattern on all four telomeric DNAs in the absence and the presence of NaCl and KCl. In the absence of salt, all of the residues of the telomeric DNAs were cleaved to somewhat the same degree. In the presence of salt(s), dT residues are attacked more efficiently by hydroxyl radicals. However, potassium ion does not influence the cleavage pattern. These results suggest that the dT clusters of

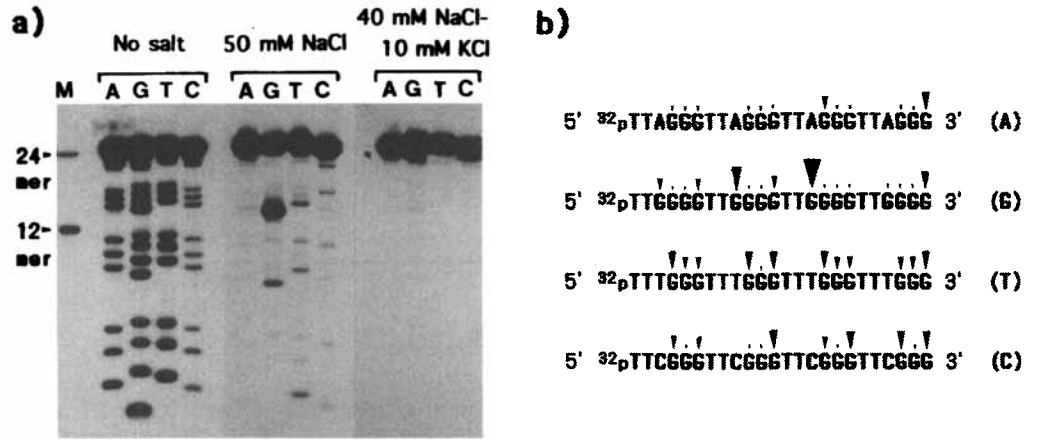

FIG. 2. Methylation protection of the telomeric DNAs. a) The DNA strands 5'-labeled with ^{32}P were methylated with DMS in TE or TE containing 50 mM NaCl or 40 mM NaCl-10 mM KCl and then cleaved with piperidine. The products were analyzed by denaturing PAGE. b) Summary of methylation on the telomeric DNAs in TE containing 50 mM NaCl. The arrowheads indicate the sites of methylation and the size of the arrowheads reflects the relative degree of methylation.

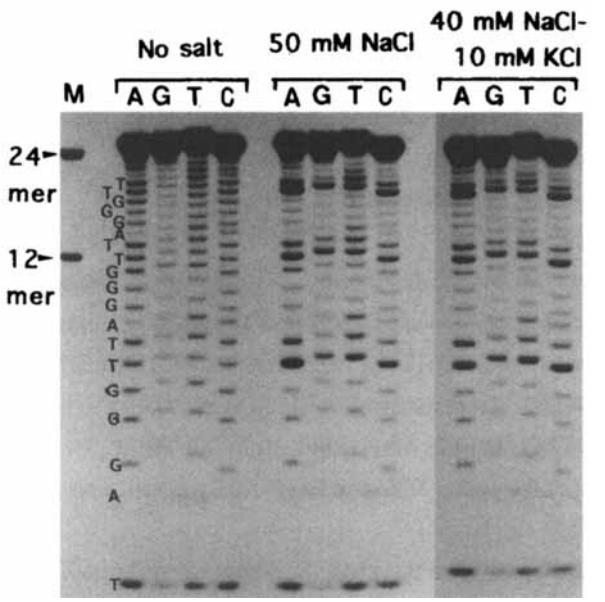

FIG. 3. Hydroxyl radical sensitivity of the telomeric DNAs. The telomeric DNAs were treated with hydroxyl radicals, and the products were analyzed by denaturing PAGE.

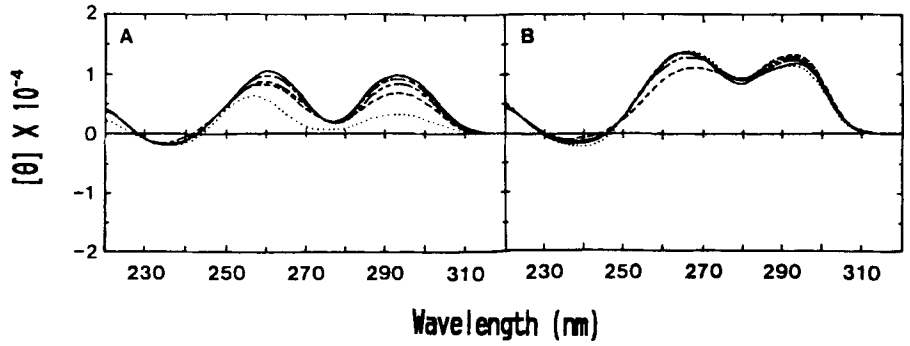

FIG. 4. CD spectra of d(TTGGGG)$_4$. A) Measured in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0). —, 5°C; –·–, 20°C; –··–, 35°C; ----, 50°C; ····, 65°C. B) Measured in 0.08 M NaCl, 0.02 M KCl, and 0.01 M Na cacodylate (pH 7.0). —, 5°C; –·–, 20°C; –··–, 35°C; ----, 50°C; ····, 65°C.

telomeric DNAs form a loop structure and conserve same sensitivity to hydroxyl radicals in spite of the salt condition.

Circular dichroic spectra

Figure 4 (A, B) shows the temperature-dependent CD spectra of d(TTGGGG)$_4$ in the absence and presence of potassium cations, respectively. The CD band around 280 nm was increased by the addition of KCl. The spectra of d(TTGGGG)$_4$ in the presence of KCl were little affected by the rise in temperature. This finding was in agreement with the results of the Tm obtained from the UV-temperature profile. These changes would be attributable to a conformation change in the quadruplex.

Nuclease sensitivity

Dimer forms of *Oxytricha* macronuclear DNA are resistant to both endo- and exonucleases[18]. In this study, we used nuclease P1 (endo-type digestion) and VPDase (3'-5' exo-type digestion) to evaluate the resistance of the telomeric DNAs to nucleases (FIG. 5). In the absence of KCl, the resistance of the telomeric DNAs to both enzymes decreased in the order of the Tms. On the other hand, little d(TTGGGG)$_4$ was digested by both enzymes and the other telomeric DNAs indicate nearly equal sensitivity to the enzymes in the presence of KCl.

From the data obtained in this study, it is suggested that d(TTGGGG)$_4$ forms four layers of the G-quartet and this structure is the most stable among the telomeric DNAs in the presence of KCl. However, in the absence of KCl, d(TTGGGG)$_4$ forms three layers of the G-quartet. The other telomeric DNAs form three layers of the G-quartet in spite of the presence of KCl. The kind of 5' side nucleotide of the dG cluster scarcely affects the

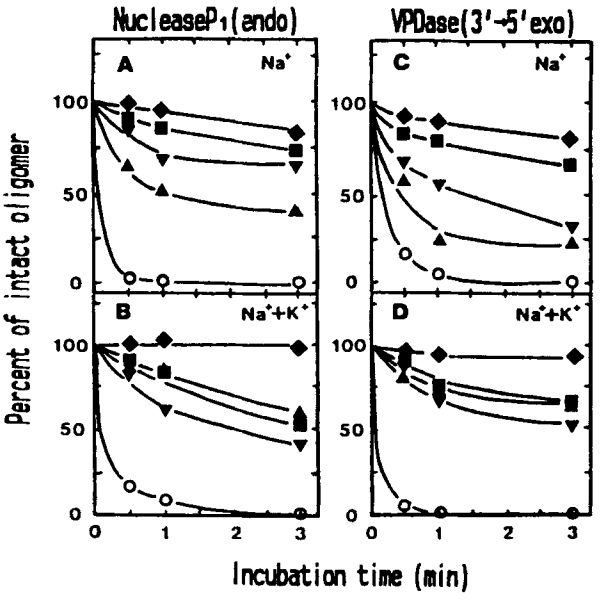

FIG. 5. Time course of degradation of the telomeric DNAs by nuclease P1 and venom phosphodiesterase (VPDase). Nuclease P1 digestion was performed at 37°C in 50 mM ammonium acetate (pH 5.0) containing 100 mM NaCl (A) or 80 mM NaCl-20 mM KCl (B). VPDase digestion was performed at 25°C in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$, containing 100 mM NaCl (C) or 80 mM NaCl-20 mM KCl (D). ◆, d(TTGGGG)$_4$; ■, d(TTAGGG)$_4$; ▼, d(TTCGGG)$_4$; ▲, d(TTTGGG)$_4$; o, d(AGTC)$_6$.

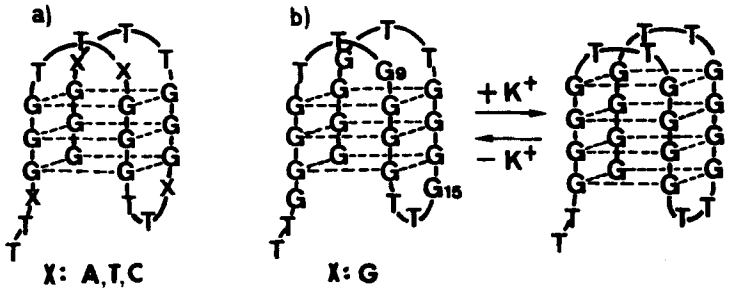

FIG. 6. Schematic diagram showing the predictable folded quadruplex structures of d(TTXGGG)$_4$. a) Edge-looped structure with X (A, T, or C) in the loop. b) Edge-looped structure with G in the loop in the absence of KCl and with G in the tetrad stem in the presence of KCl.

stability of the quadruplex structure in the presence of KCl. We propose a schematic diagram showing the predictable folded quadruple structures of $d(TTXGGG)_4$ (X: A, G, C, or T)(FIG. 6). In the case of $d(TTGGGG)_4$ in the absence of KCl, it is not determined exactly which guanines (G_1 or G_4, G_{21} or G_{24}) in the two parts are involved in the G-quartet. The role of the G-quartet structures in the biology of telomeres has not yet been proven. Many organisms retain high fidelity of their telomeric repetitive sequences, that is, there is a dA residue at the 5' side of the dG cluster. In this study, we could not attribute the fidelity of the 5' side residue of the dG cluster to its specific role in the quadruple structure. Despite the increasing studies on G-quartet structures, many fundamental biological questions remain to be answered.

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