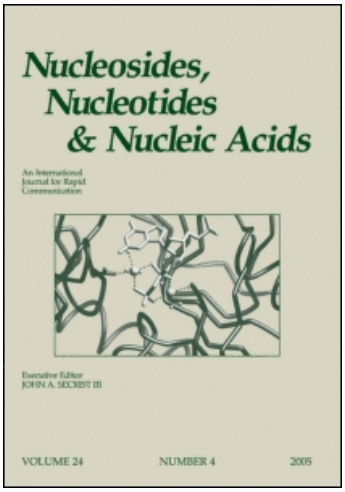

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Structural Polymorphism of Telomeric DNA Regulated by pH and Divalent Cation

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Structural Polymorphism of Telomeric DNA Regulated by pH and Divalent Cation

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ABSTRACT

DNA oligonucleotides can form multi-stranded structures such as a duplex, triplex, and quadruplex, while the double helical structure is generally considered as the canonical structure of DNA oligonucleotides. Guanine-rich or cytosine-rich oligonucleotides, which are observed in telomere, centromere, and other biologically important sequences in vivo, can form four-stranded G-quadruplex and I-motif structures in vitro. In this study, we have investigated the effects of pH and cation on the structures and their stabilities of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$. The CD spectra and thermal melting curves of DNAs at various pHs demonstrated that acidic conditions induced a stable I-motif structure of $d(C_4A_4C_4)$, while the pH value did not affect the G-quadruplex structure and stability of $d(G_4T_4G_4)$. The CD spectra of the 1 : 1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ indicated that the acidic conditions inhibit the duplex formation between $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$. Isothermal titration calorimetry measurements of the duplex formation at various pHs also quantitatively indicated that the acidic

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conditions inhibit the duplex formation. On the other hand, the CD spectra and thermal melting curves of DNAs in the absence and presence of Ca^{2+} indicated that Ca^{2+} induces a parallel G-quadruplex sructure of $d(G_4T_4G_4)$ and then inhibits the duplex formation. These results lead to the conclusion that both the pH and coexisting cation can induce and regulate the structural polymorphisms the oligonucleotides in which they form the G-quadruplex, I-motif, and duplex depending on the conditions. Thus, the results reported here indicate pivotal roles of pH and coexisting cations in biological processes by regulating the conformational switching between the duplex and quadruplexes structures of the guanine-rich or cytosine-rich oligonucleotides in vivo.

Key Words: G-quadruplex; I-motif; Telomere; pH; Divalent cation.

INTRODUCTION

Guanine-rich oligonucleotides form guanine quadruplex (G-quadruplex) structures in the presence of certain cations.[1–3] The G-quadruplex is stabilized by G-quartets, which are formed by the cyclic hydrogen bonds of four guanine bases in a coplanar arrangement (Fig. 1a) . While direct evidence of G-quadruplex formation and function in vivo is still lacking, there is growing interest in the possible roles of the G-quadruplex structure as a target of a drug design and as a general structural motif of telomere DNA, centromere DNA, and other biologically important sequences.[4–8] In addition, functional molecules such as a catalytic porphyrin metallation DNA form a G-quadruplex structure in vitro.[9–11] On the other hand,

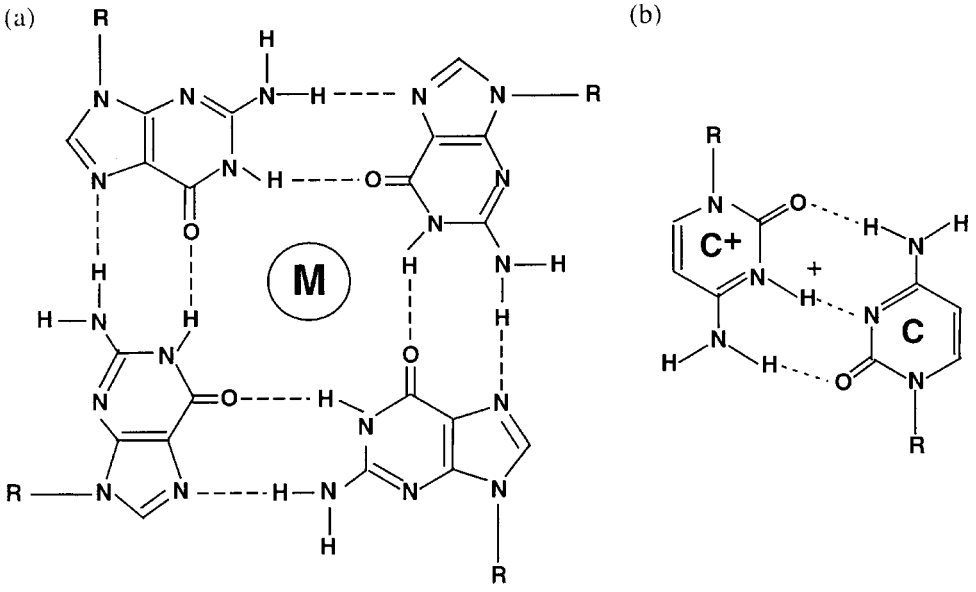

Figure 1. Chemical structures of (a) G-quartet in G-quadruplex and (b) C^+·C base pair in I-motif. R and M indicate ribose and metal ion, respectively.

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cytosine-rich sequences are also interesting because they are the complements of the guanine-rich oligonucleotides and because they are observed in telomeres and centromeres. It was found that cytosine-rich oligonucleotides form a tetrameric four-stranded structure that is the so-called I-motif.[12–15] The I-motif, which is formed by DNA stretches of some cytidines, involves the systematic base intercalation of two parallel duplexes with hemiprotonated $C^+ \cdot C$ base pairs (Fig. 1b) . The I-motif structure forms at acidic or even neutral pHs because the pairing and stacking of the $C^+ \cdot C$ base pairs are critical for the folding topology of the I-motif. NMR and X-ray diffraction studies as well as gel and spectroscopic techniques have shown that both the G-quadruplex and I-motif fold into monomeric, dimeric, and tetrameric structures depending on their sequences, and thus generate a structural polymorphism.[16] Therefore, investigation of the structural polymorphism of the G-quadruplex, I-motif, and duplex with the guanine-rich and cytosine-rich oligonucleotides is of currently significant interest to provide a novel methodology for controlling biological phenomena and functional molecules related to the quadruplexes, while few systematic structural studies of a guanine-rich oligonucleotide with the complementary cytosine-rich oligonucleotide are available. In the present study, we report the effect of pH and divalent cation on the structural formation of the G-quadruplex of $d(G_4T_4G_4)$, the I-motif of $d(C_4A_4C_4)$, and the duplex of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$. The result shows that acidic conditions induce the stable I-motif formation of $d(C_4A_4C_4)$, while it does not significantly affect the structure and stability of the antiparallel G-quadruplex of $d(G_4T_4G_4)$. On the other hand, Ca^{2+} induces a structural transition from the antiparallel to parallel G-quadruplex of $d(G_4T_4G_4)$, while Ca^{2+} does not affect significantly the I-motif of $d(C_4A_4C_4)$. These effects of pH and Ca^{2+} lead to preventing the duplex formation of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ because the stable quadruplex structures of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$ are induced by acidic conditions and Ca^{2+}, respectively. This provides insight into the structural polymorphism of the guanine-rich or cytosine-rich regions, which are often observed through the entire genome, depending on both the pH value and coexisting cation.

MATERIALS AND METHODS

Material Preparations

Oligonucleotides were synthesized on solid supports using standard β-cyanoethyl phosphoramidite methods as previously described.[17,18] The synthesized DNA oligonucleotides containing 5′-end dimethoxytrityl (DMT) groups were removed from the solid support, and then the base blocking groups were removed by treatment with concentrated 25% ammonia at 55°C for 8 h. After drying in a vacuum, the oligonucleotides were passed through a Poly–Pak cartridge (Glen Research Co., Ltd.) with 2% trifluoroacetic acid (TFA) to remove the 5′-end DMT groups. After deblocking operations, the oligonucleotides were desalted through a C–18 Sep-Pak cartridge column (Waters). The oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC) on a TSKgel Oligo DNA RP column (Tosoh) with a linear-gradient of 0–50% $MeOH/H_2O$ containing triethylammonium acetate (TEAA, pH 7.0). The final purity

of the oligonucleotides were confirmed to be >98 % by HPLC. The purified oligonucleotides were desalted again with a C–18 Sep–Pak cartridge before use. The single–strand concentrations of the oligonucleotide were determined by measuring the absorbance at 260 nm at high temperature. The single–strand extinction coefficients were calculated from the mononucleotide and dinucleotide data using the nearest–neighbor approximation.[19]

Circular Dichroism Spectra and Thermodynamic Analysis

All the circular dichroism (CD) titration experiments utilizing a JASCO J-820 spectropolarimeter were measured for a 50 μM total strand concentration of $d(G_4T_4G_4)$ and/or $d(C_4A_4C_4)$ in a 0.1 cm path length cuvette at 5°C, and interfaced to a Dell OptiPlex Gxi computer. The CD spectrum was obtained by taking the average of at least three scans made at 0.1 nm intervals from 200 to 350 nm. Before the CD spectroscopy, the DNA sample was heated to 90°C, gently cooled at a rate of 3°C min^{-1}, and incubated at 5°C for several hours.[20] The temperature of the cell holder was regulated by a JASCO PTC-348 temperature controller and the cuvette-holding chamber was flushed with a constant stream of dry N_2 gas to avoid water condensation on the cuvette exterior. Thermal denaturation profiling of the DNAs were also carried out utilizing the JASCO J-820 spectropolarimeter. CD melting curves were measured for 50 μM total strand concentration of $d(G_4T_4G_4)$ and/or $d(C_4A_4C_4)$ in a 0.1 cm path length cuvette. The CD melting curves were collected as a function of temperature. The melting temperatures of DNAs were calculated from the maximum point of the first derivative of the CD intensity change versus temperature profile, dA/dT.[21]

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on the duplex formation of $d(G_4T_4G_4)/d(C_4A_4C_4)$ using a Microcal VP-ITC isothermal titration calorimeter. The titration of 60 μM $d(G_4T_4G_4)$ on 2.5 μM $d(C_4A_4C_4)$ was carried out in buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM Tris-HCl (pH 8.0), 50 mM MES (pH 6.0), or 50 mM Tris-acetate (pH 4.0) at 20°C. The syringe was rotated at 300 rpm, the time between injections was 600 s, the injection volume was 5 μL, and the injection was started after an initial delay of 60 s. The reference cell contained distilled and deionized water. Before the ITC experiment, the DNA samples were heated to 90°C, gently cooled at the rate of 3°C min^{-1}, and incubated at 20°C for several hours. The data were analyzed with Origin 5.0 (Microcal Software) with an automatically generated baseline to estimate the thermodynamic parameters and the stoichiometry between $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$.

Polyacrylamide Gel Electrophoresis

G-quadruplex, I-motif, and duplex formations at various pHs were investigated using native polyacrylamide gel electrophoresis. DNA solutions with the total

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concentration of 200 μM were prepared in a buffer containing 100 mM NaCl and 10 mM Tris-HCl (pH 8.0), MES (pH 6.0), or Tris-acetate (pH 4.0). DNA samples were heated to 90°C, gently cooled at the rate of 3°C min^{-1}, incubated at room temperature (ca. 20°C) for several hours, and run on 16% non-denaturing polyacrylamide gels at 75 V (ca. 5 V cm^{-1}) for 5 h. The same buffer was used in the polymerization of the acrylamide for the relevant gels. The gel was stained in a 0.01% Stain-All formamide solution (9 : 11 formamide : H_2O ratio).

RESULTS AND DISCUSSION

Effect of pH on Structures of $d(C_4A_4C_4)$, $d(G_4T_4G_4)$, and $d(G_4T_4G_4)/d(C_4A_4C_4)$

Figure 2a shows the CD spectra of 50 μM $d(C_4A_4C_4)$ at various pHs. These measurements were carried out in buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM Tris-acetate (pH 4.0-pH 5.0), 50 mM MES (pH 5.5-pH 7.0), or 50 mM Tris-HCl (pH 8.0) at 5°C. The CD spectra of $d(C_4A_4C_4)$ have positive peaks near 290 and 220 nm and negative peaks near 265 and 210 nm in acidic conditions (pH 4.0 to 6.0), while a shift and decreasing of the positive peak near 290 nm were observed under neutral and alkaline conditions. Previously, it was revealed that $d(5mC_2T_3AC_2)$ forms a two-stranded I-motif.[22] The loops of the dimer are situated on either end of the I-motif core and span the wide grooves. It was also reported that the human insulin minisatellite $d(C_4TGTC_4)$ forms a two-stranded I-motif.[23] These results indicate that a cytosine-rich oligonucleotide including two cytosine stretches can form a two-stranded I-motif. We also reported that the CD spectra of $d(C_3TA_2)_3C_3$, which forms an intramolecular (one-stranded) I-motif structure under acidic conditions, has positive and negative peaks at 288 nm and 256 nm, respectively, and its maximal intensity is shifted with higher pHs.[24] Therefore, the change in the CD spectra in Fig. 2a indicates a structural change in the two-stranded I-motif of $d(C_4A_4C_4)$. The CD intensity at 289.5 nm decreased from 4.77×10^5 to 0.88×10^5 deg cm^2 $dmol^{-1}$, and its maximal position is shifted from 289.5 to 276.5 nm through a pH change from 4.0 to 8.0 (Fig. 2b). The pK_a value of the I-motif formation of $d(C_4A_4C_4)$ was estimated to be 6.4 from the peak position data and 6.2 from the intensity data at 289.5 nm. These results indicate that the formation of the I-motif of $d(C_4A_4C_4)$ was induced by the acidic condition.

It was reported that $d(G_4T_4G_4)$ forms an antiparallel G-quadruplex in the presence of Na^+.[25,26] Figure 2c shows the CD spectra of 50 μM $d(G_4T_4G_4)$ at 5°C and at various pHs. Recent conformational analysis of the G-quadruplex structures revealed that the CD spectra of an antiparallel G-quadruplex structure had a positive peak near 295 and a negative peak near 265 nm, while a parallel G-quadruplex structure had positive and negative peaks near 260 and 240 nm, respectively.[27,28] With this information, the structural type of a G-quadruplex can be determined by a CD measurement. All the CD spectra of $d(G_4T_4G_4)$ at various pHs have positive and negative peaks at 295.5 nm and 265.0 nm. The position of the positive peak and its CD intensity at 295.5 nm are unchanged ($(2.19 \pm 0.18) \times 10^5$ deg cm^2 $dmol^{-1}$) from acidic to alkaline conditions as shown in Fig. 2d. This indicates that the pH

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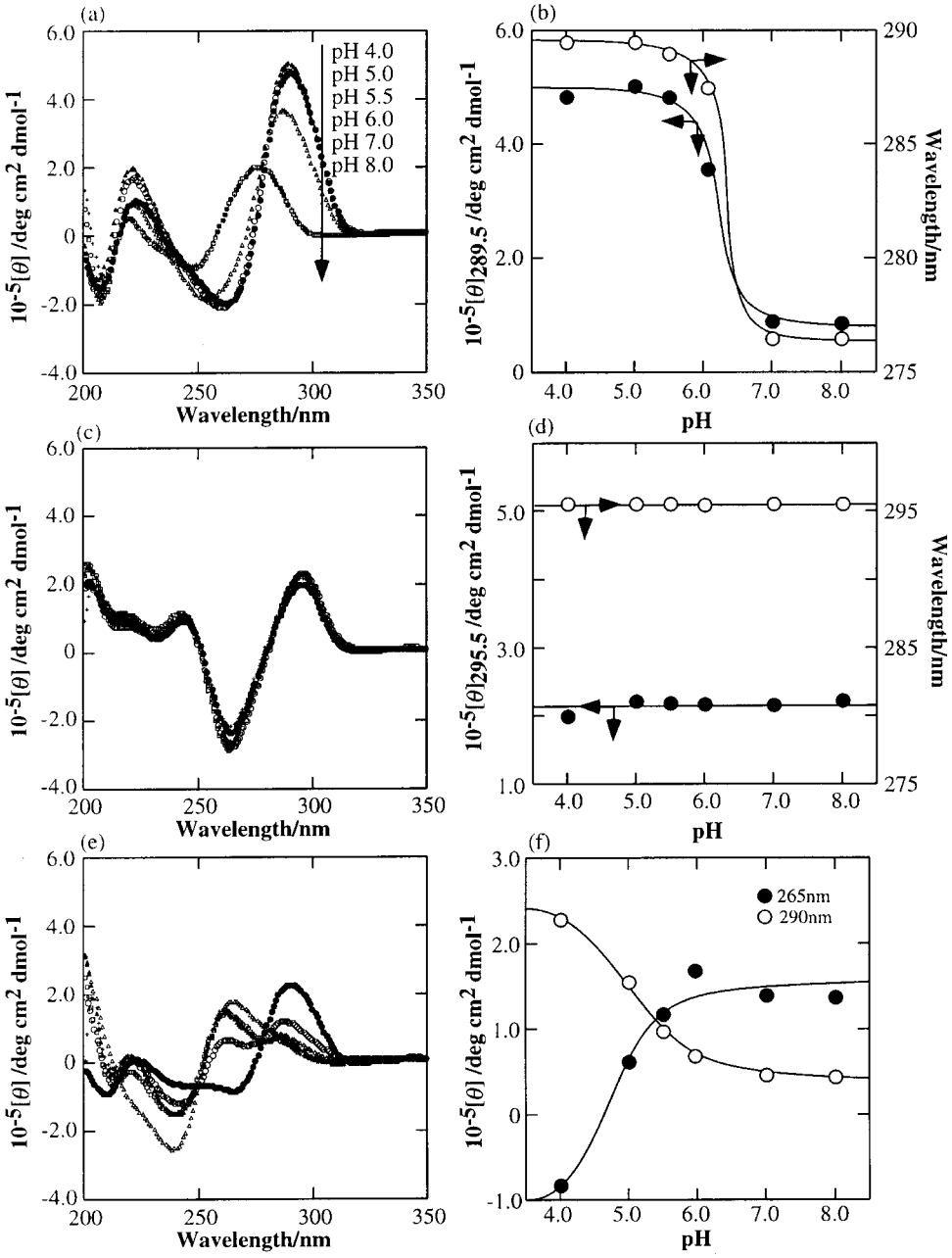

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does not significantly affect the structure of the antiparallel G-quadruplex of $d(G_4T_4G_4)$.

Figure 2e shows the CD spectra of a 1 : 1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ (total concentration is 50 μM) at various pHs. The CD spectra of $d(G_4T_4G_4)/d(C_4A_4C_4)$ have a positive peak at 265 nm and a small shoulder at 285 nm in alkaline and neutral conditions, which is distinct from the spectra of the I-motif of $d(C_4A_4C_4)$ (Fig. 2a) and the G-quadruplex of $d(G_4T_4G_4)$ (Fig. 2c) under the same conditions, indicating that $d(G_4T_4G_4)/d(C_4A_4C_4)$ forms a duplex.[24] The CD intensity of $d(G_4T_4G_4)/d(C_4A_4C_4)$ at 265 nm decreased, and the shoulder at 285 nm shifted to 290 nm and increased through the pH change from 4.0 to 8.0. Figure 2f shows that the CD intensity at 265 nm increased from -0.93 to 1.24×10^5 deg cm^2 dmol^{-1}, while the CD intensity at 290 nm decreased 2.22 to 0.57×10^5 deg cm^2 dmol^{-1} with the pH changes from 4.0 to 8.0. Previously, we reported the native PAGE images of $d(G_3(T_2AG_3)_3/d(C_3TA_2)_3C_3$ with a molar ratio of 2 : 1, 1 : 1, and 1 : 2.[24] These previous results indicated that the guanine-rich and cytosine-rich oligonucleotides form a duplex but not a triplex. These results lead to the conclusion that the 1 : 1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ may form a duplex under neutral and alkaline conditions, although each strand forms a G-quadruplex and I-motif, respectively, under acidic conditions. This demonstrates that pH can generate and regulate the structural polymorphism of telomere DNA that are guanine-rich or cytosine-rich oligonucleotides.

Effect of pH on Stabilities of $d(C_4A_4C_4)$, $d(G_4T_4G_4)$, and $d(G_4T_4G_4)/d(C_4A_4C_4)$

Figure 3a shows the thermal melting curves of 50 μM $d(C_4A_4C_4)$ monitored by the CD intensity at each peak position (Fig. 2b) in the buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM Tris-acetate (pH 4.0-pH 5.0), 50 mM MES (pH 5.5-pH 7.0), or 50 mM Tris-HCl (pH 8.0). The melting temperatures of the 50 μM $d(C_4A_4C_4)$ at pHs 4.0, 5.0, 5.5, and 6.0 are estimated to be 55.2, 50.5, 41.4, and 26.2°C, respectively, although it can not be determined under neutral and

Figure 2. Effect of pH on CD spectra of 50 μM $d(C_4A_4C_4)$, $d(G_4T_4G_4)$, and $d(C_4A_4C_4)/d(G_4T_4G_4)$. All the measurements were carried out in buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM Tris-acetate (pH 4.0-pH 5.0), 50 mM MES (pH 5.5-pH 7.0), or 50 mM Tris-HCl (pH 8.0) at 5°C. (a) CD spectra of $d(C_4A_4C_4)$ at pH 4.0 (black circle), pH 5.0 (white circle), pH 5.5 (black triangle), pH 6.0 (white triangle), pH 7.0 (cross), and pH 8.0 (white square). (b) CD intensity (black circle) at 290 nm and peak position (white circle) of $d(C_4A_4C_4)$ at various pHs. (c) CD spectra of $d(G_4T_4G_4)$ at pH 4.0 (black circle), pH 5.0 (white circle), pH 5.5 (black triangle), pH 6.0 (white triangle), pH 7.0 (cross), and pH 8.0 (white square). (d) CD intensity (black circle) at 295 nm and peak position (white circle) of $d(G_4T_4G_4)$ at various pHs. (e) CD spectra of 1 : 1 mixture of $(C_4A_4C_4)$ and $d(G_4T_4G_4)$ at pH 4.0 (black circle), pH 5.0 (white circle), pH 5.5 (black triangle), pH 6.0 (white triangle), pH 7.0 (cross), and pH 8.0 (white square). (f) CD intensity at 265 nm (black circle) and 290 nm (white circle) of 1 : 1 mixture of $(C_4A_4C_4)$ and $d(G_4T_4G_4)$ at various pHs.

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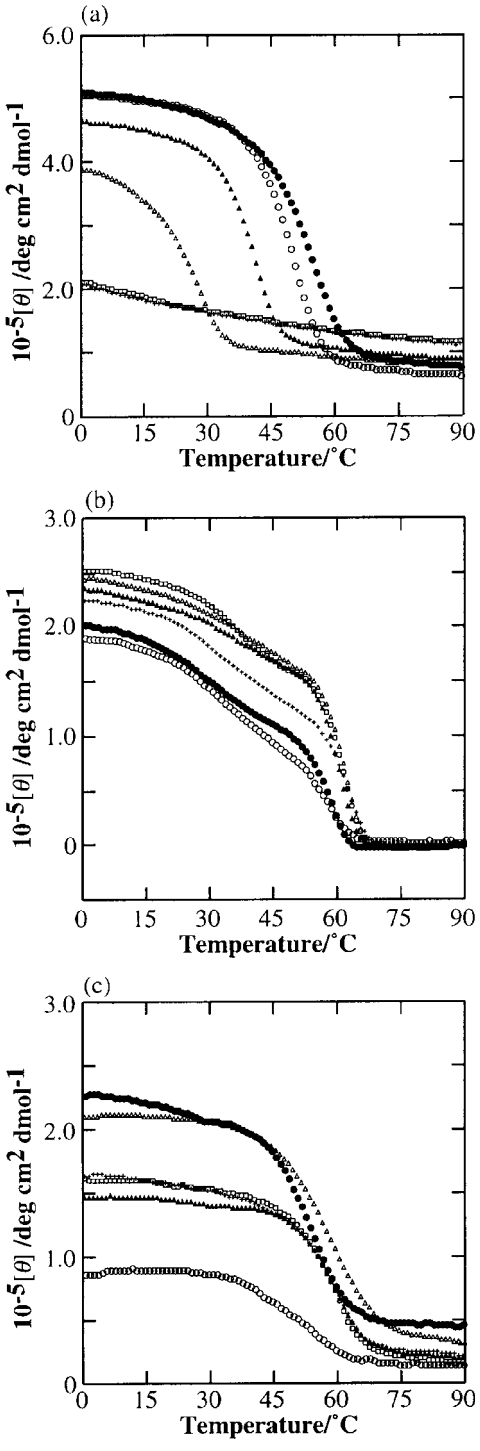

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alkaline conditions (pH 7.0 and 8.0). This confirms that acidic conditions induce the stable I-motif structure as shown in Fig. 2a.

Figure 3b shows the thermal CD melting curves of 50 μM $d(G_4T_4G_4)$ at 295 nm at various pHs. The melting temperature of $d(G_4T_4G_4)$ was estimated to be $59.7 \pm 2.8°C$ at all the pHs. These results show that not only the structure (Fig. 2b) but also the stability of the antiparallel G-quadruplex of $d(G_4T_4G_4)$ are independent of pH. Since the pK_a values of N1 of guanosine and N3 of 2′-deoxythymidine, which are positions that can form hydrogen bonds with other bases, are 9.42 and 9.93, respectively,[29] these results are comparable to the theoretical consideration. The effect of pH on the duplex stability of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ was also investigated. Figure 3c shows the melting profiles of a 1 : 1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ (total strand concentration is 50 μM) at various pHs. The melting temperature of $d(G_4T_4G_4)/d(C_4A_4C_4)$ was estimated to be $54.4 \pm 2.8°C$ in all the conditions, indicating that the pH does not affect the stability of the $d(G_4T_4G_4)/d(C_4A_4C_4)$. The duplex formation of $d(G_4T_4G_4)/d(C_4A_4C_4)$ is independent of the cytosine protonation. Therefore, this independence of the duplex stability versus pH is supported by the theoretical consideration.

Effect of Ca^{2+} on Structure of $d(C_4A_4C_4)$, $d(G_4T_4G_4)$, and $d(G_4T_4G_4)/d(C_4A_4C_4)$

We previously showed that Ca^{2+} induces a structural switch between the antiparallel and parallel G-quaduplexes of $d(G_4T_4G_4)$, and the structures of $d(G_4T_4G_4)$ in the presence of Ca^{2+} include not only a homogeneous four-stranded parallel G-quadruplex but also higher-ordered structures such as a G-wire.[20] Therefore, Ca^{2+} should be one of the promising molecules to regulate the structural polymorphism of the telomere DNAs. Figure 4a shows the CD spectra of 50 μM $d(C_4A_4C_4)$ in a buffer containing 100 mM NaCl, 1 mM Na_2EDTA, 50 mM MES (pH 6.0) in the absence and presence of 100 mM $CaCl_2$ at 5°C. The CD spectra are almost unchanged both in the absence and presence of Ca^{2+}, indicating that Ca^{2+} does not significantly affect the I-motif of $d(C_4A_4C_4)$. On the other hand, Fig. 4b indicates that Ca^{2+} induces a structural transition of the G-quadruplex of $d(G_4T_4G_4)$ from antiparallel to parallel as shown in a previous report.[20]

Figure 4c shows the CD spectra of a 1 : 1 mixture of $d(C_4A_4C_4)/d(G_4T_4G_4)$ in the absence and presence of 100 mM $CaCl_2$ at 5°C. The CD spectrum of $d(C_4A_4C_4)/d(G_4T_4G_4)$ in the absence of Ca^{2+} has a positive peak at 265 nm and a shoulder at 280 nm, while the spectrum in the presence of Ca^{2+} has a positive peak at 280 nm and a shoulder at 265 nm. These CD spectra and the effect of Ca^{2+} on the

Figure 3. CD melting curves of (a) $d(C_4A_4C_4)$ monitored at 289.5 nm (pH 4.0–pH 5.5), 287.5 nm (pH 6.0), or 276.5 nm (pH 7.0 and pH 8.0), (b) $d(G_4T_4G_4)$ monitored at 295.5 nm, and (c) $d(C_4A_4C_4)/d(G_4T_4G_4)$ traced at 265 nm at various pHs. All the measurements were carried out in buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM Tris-acetate (pH 4.0) (black circle), 50 mM Tris-acetate (pH 5.0) (white circle), 50 mM MES (pH 5.5) (black triangle), 50 mM MES (pH 6.0) (white triangle), 50 mM MES (pH 6.0) (cross), or 50 mM Tris-HCl (pH 8.0) (white square) with a 50 μM total strand concentration.

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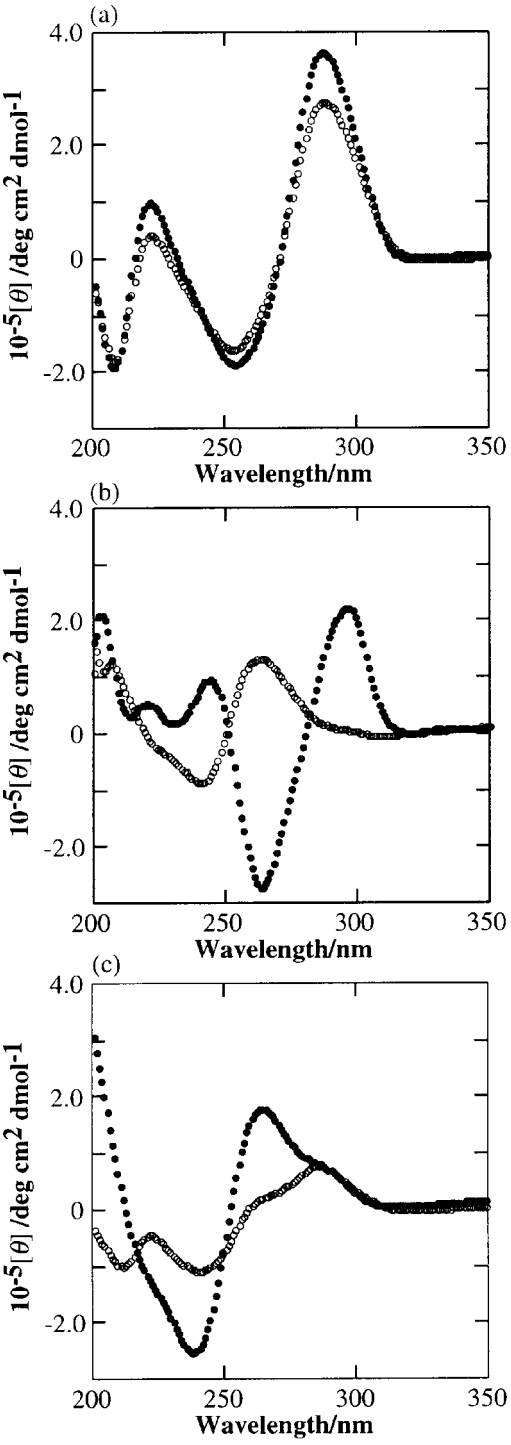

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duplex stability lead to characterization of the $d(C_4A_4C_4)/d(G_4T_4G_4)$ structures in the absence and presence of Ca^{2+} as described below.

Effect of Ca^{2+} on Stabilities of $d(C_4A_4C_4)$, $d(G_4T_4G_4)$, and $d(G_4T_4G_4)/d(C_4A_4C_4)$

Figure 5a shows the thermal CD melting curves of 50 μM $d(C_4A_4C_4)$ at 289.5 nm in the buffer containing 100 mM NaCl, 1 mM Na_2EDTA, 50 mM MES (pH 6.0) in the presence and absence of 100 mM $CaCl_2$. The melting temperature of I-motif of 50 μM $d(C_4A_4C_4)$ decreases from 25.6 to 18.8°C by adding 100 mM Ca^{2+}. The destabilization of the $d(C_4A_4C_4)$ I-motif by Ca^{2+} is smaller than the destabilization by pH change from 4.0 to 8.0 as shown in Fig. 3a. Figure 5b shows thermal CD melting curves of the antiparallel and parallel G-quadruplexes of 50 μM $d(G_4T_4G_4)$ in the presence and absence of 100 mM $CaCl_2$, respectively. The CD melting curve of the antiparallel G-quadruplex was monitored at 295 nm, and the CD melting curve of the parallel G-quadruplex was monitored at 260 nm. The melting temperature of the parallel G-quadruplexes of $d(G_4T_4G_4)$ is slightly higher than that of the antiparallel G-quadruplex (64.8 and 60.4°C, respectively). On the contrary, obvious destabilization of $d(G_4T_4G_4)/d(C_4A_4C_4)$ by Ca^{2+} was observed as shown in Fig. 5c. The CD melting curve of the 1:1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ in the absence of 100 mM $CaCl_2$ was monitored at 260 nm, while the CD melting curve of the mixture in the presence of $CaCl_2$ was monitored at 280 nm. The melting temperature of the structure of $d(G_4T_4G_4)/d(C_4A_4C_4)$ decreased from 57.0 to 18.5°C with the addition of 100 mM $CaCl_2$. These results of the Ca^{2+} effects on $d(C_4A_4C_4)$,$d(G_4T_4G_4)$, and $d(G_4T_4G_4)/d(C_4A_4C_4)$ indicate that Ca^{2+} affects the structure and stability of the G-quadruplex and duplex, and regulates the structural polymorphism of the telomere DNAs.

Duplex Formation of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$

Heat changes caused by the interaction between $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ were investigated by ITC measurements at various pHs. The heats generated by successive injections are plotted versus the molar ratio of $d(G_4T_4G_4)$ to $d(C_4A_4C_4)$ in order to obtain the binding isotherms of the DNA binding at pH 8.0 (Fig. 6a), pH 6.0 (Fig. 6b),and pH 4.0 (Fig. 6c). The data were fitted to a one-site model to estimate the binding constant, K_a, the enthalpy of binding, ΔH°, and the stoichiometry, N, using Microcal software. The binding free energy, ΔG°, was calculated using the following equation:

$$\Delta G^\circ = RT \ln K_a \quad (1)$$

Figure 4. CD spectra of (a) 50 μM $d(C_4A_4C_4)$, (b) $d(G_4T_4G_4)$, and (c) $d(C_4A_4C_4)/d(G_4T_4G_4)$ in the absence (black circle) and presence (white circle) of Ca^{2+}. All the measurements were carried out in a buffer containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM MES (pH 6.0) in the absence and presence of 100 mM $CaCl_2$ at 5°C.

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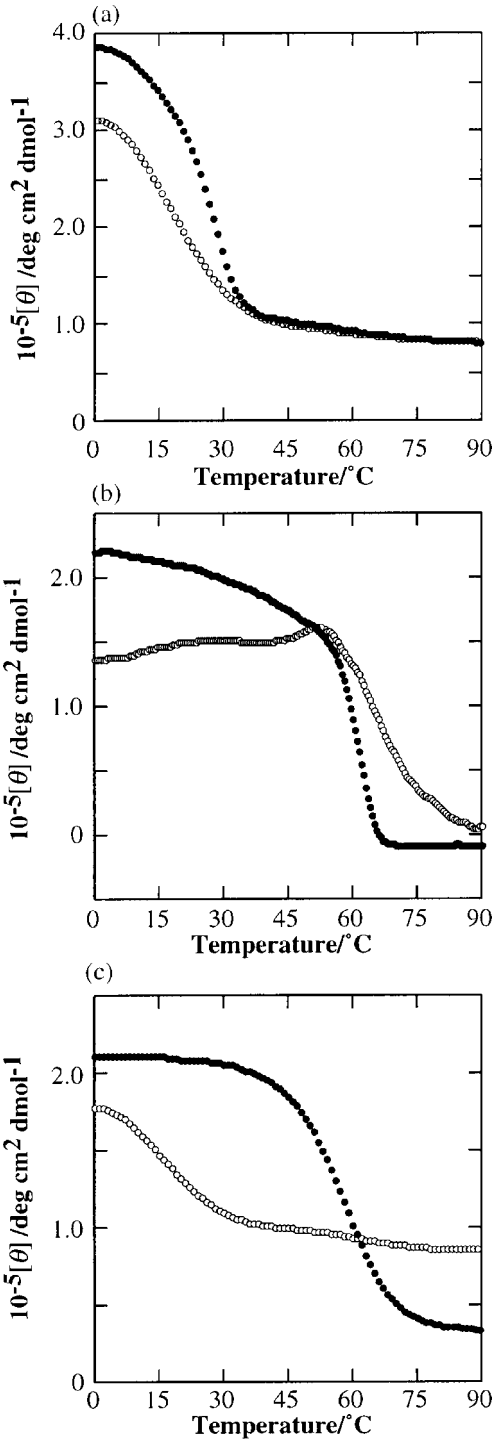

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Table 1. Thermodynamic parameters of the binding between $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$.[a]

pH	N	K_a (10^6 M^{-1})	ΔG°_{20} (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal mol^{-1} K^{-1})
8.0	2.74 ± 0.05	6.09 ± 1.55	-9.10 ± 0.17	-14.4 ± 0.04	-17.9 ± 0.25
6.0	2.65 ± 0.02	5.68 ± 0.64	-9.06 ± 0.07	-14.2 ± 0.02	-17.9 ± 0.14
4.0	–	–	–	–	–

[a]All measurements were performed at 20°C.

where R and T are the gas constant (1.987) and temperature (293.15),respectively. The entropy, ΔS°, was calculated from ΔG° and ΔH° by using the following equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

These parameters obtained here are listed in Table 1. The stoichiometry of $d(G_4T_4G_4)$ binding to $d(C_4A_4C_4)$ at pHs 8.0 and 6.0 were calculated to be 2.74 and 2.65, respectively. These values may indicate that not only the duplex formation of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$, but also other side reactions such as triplex formation and/or G-quadruplex formation of $d(G_4T_4G_4)$ occurred through the titration experiments. Furthermore, the thermodynamic parameters, ΔG°, ΔH°, and ΔS°, were predicted to be –16.1 kcal mol^{-1}, -88.4 kcal mol^{-1}, and -246.1 cal mol^{-1} K^{-1}, respectively, using the nearest-neighbor model, in which the helix-coil transition is in the two-state.[30,31] The differences between the predicted and calculated values also indicate that the binding of $d(G_4T_4G_4)$ to $d(C_4A_4C_4)$ is not in the two-state, although $d(C_3TA_2)_3C_3$ and $d(G_3(T_2AG_3)_3$ form a duplex in the presence of 100 mM NaCl at pH 7.0.[24] On the other hand, ΔG° at 20°C at pH 8.0 and 6.0 were calculated to be -9.10 and -9.06 kcal mol^{-1}, respectively, indicating that the pH does not affect the binding stability between $d(G_4T_4G_4)$ to $d(C_4A_4C_4)$. Other parameters also quantitatively show that the alkaline and neutral conditions have fewer effects on the binding (Table 1). However, small heat changes were observed in the ITC measurement at pH 4.0 and so the parameters cannot be calculated (Fig. 6c). This indicates that $d(G_4T_4G_4)$ cannot bind to $d(C_4A_4C_4)$ under this condition. These ITC results indicate that the I-motif of $d(C_4A_4C_4)$, which is stabilized under acidic conditions, prevents the duplex formation of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$. Although further investigations of the structures of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ complexes are required, the The ITC results confirm that structural polymorphism between the duplex and quadruplex of the telomere DNAs can be regulated by pH.

Figure 5. (a) CD melting curve of 50 μM $d(C_4A_4C_4)$ at 289.5 nm in the absence (black circle) and presence (white circle) of 100 mM Ca^{2+}. (b) CD melting curve of 50 μM $d(G_4T_4G_4)$ at 295 or 265 nm in the absence (black circle) or presence (white circle) of 100 mM Ca^{2+}, respectively. (c) CD melting curve of 50 μM $d(C_4A_4C_4)/d(G_4T_4G_4)$ at 265 or 280 nm in the absence (black circle) or presence (white circle) of 100 mM Ca^{2+}, respectively. All measurements were carried out in buffers containing 100 mM NaCl, 1 mM Na_2EDTA, and 50 mM MES (pH 6.0) in the presence and absence of 100 mM $CaCl_2$.

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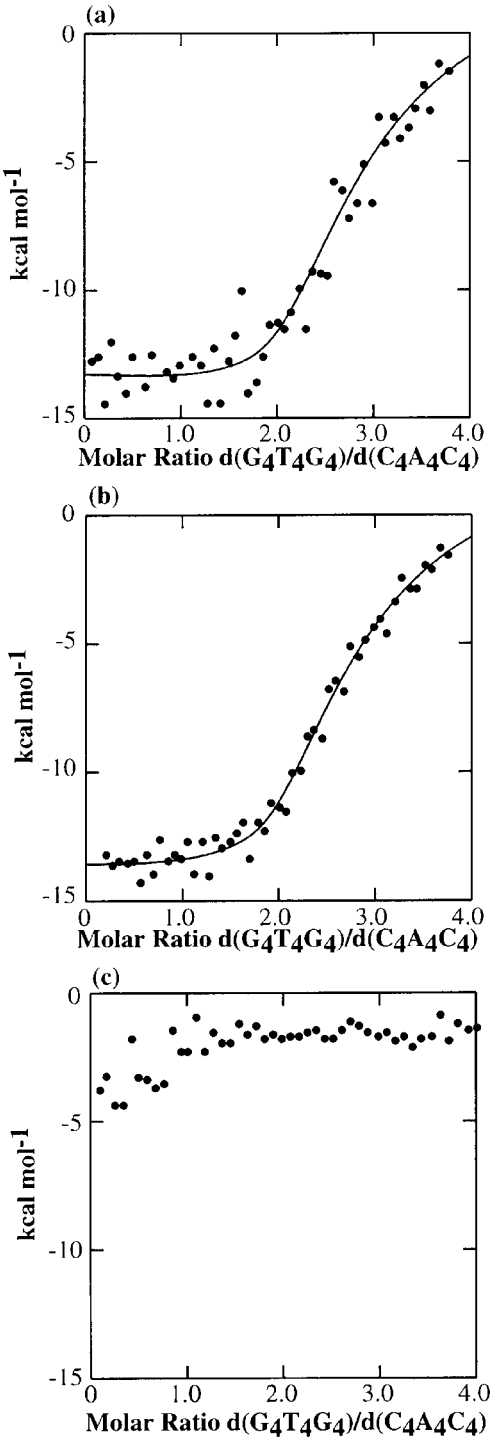

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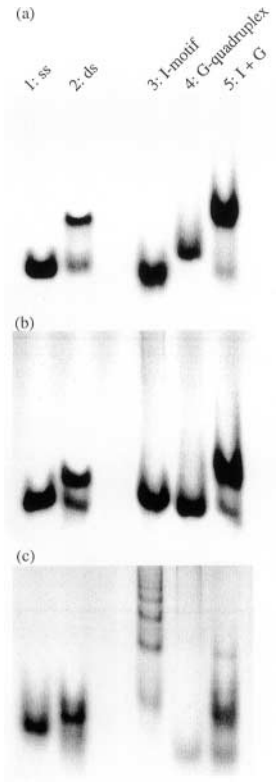

Figure 7. Gel electrophoresis of oligonucleotides in 16% non-denaturing gel containing 100 mM NaCl at (a) pH 8.0, (b) 6.0, and (c) 4.0. Lane 1, d(AATGGATTACAA); lane 2, d(AATGGATTACAA)/d(TTGTAATCCATT); lane 3, $d(C_4A_4C_4)$; lane 4, $d(G_4T_4G_4)$; lane 5, 1 : 1 mixture of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$.

The interaction between $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ were also investigated by non-denaturing gel electrophoresis at various pHs. Figure 7 shows the electrophoretic mobilities of d(AATGGATTACAA) as a 12-mer single-stranded DNA marker (lane 1), d(AATGGATTACAA)/d(TTGTAATCCATT) as a 12-mer double-stranded DNA marker (lane 2), $d(G_4T_4G_4)$ (lane 3), $d(C_4A_4C_4)$ (lane 4), and the 1 : 1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ (lane 5) at pH 8.0 (Fig. 7a), pH 6.0 (Fig. 7b), and pH 4.0 (Fig. 7c). At pH 8.0 and pH 6.0, $d(G_4T_4G_4)$ folds into G-quadruplex, whereas $d(C_4A_4C_4)$ does not fold into I-motif. The migration of the 1:1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ shows two bands in the alkaline and neutral conditions. The migration of main band corresponds to the 12-mer duplex, and that

Figure 6. The integrated heats from the ITC data (black circle) and the best-fit curve (solid line) to a 1 : 1 binding model for the binding between and $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$ in a buffer containing 100 M NaCl, 1 mM Na_2EDTA, and (a) 50 mM Tris-HCl (pH 8.0), (b) 50 mM MES (pH 6.0), or (c) 50 mM Tris-acetate (pH 4.0). All the measurements were carried out with 2.5 μM $d(C_4A_4C_4)$ and 60 μM $d(G_4T_4G_4)$ at 20°C.

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of the other small band corresponds to G-quadruplex and/or I-motif. The small band indicates that a part of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ cannot form the duplex in these conditions. These results support the ITC results that indicate the duplex formation of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ at pH 8.0 and 6.0. On the other hand, banding pattern of DNAs at pH 4.0 is quite different from that of pH 8.0 and 6.0. The migration of $d(C_4A_4C_4)$ at pH 4.0 shows obvious ladder bands, although the migration of $d(G_4T_4G_4)$ is almost same with that at pH 6.0 and pH 8.0. The ladder bands suggest that $d(C_4A_4C_4)$ folds into multi-stranded I-motifs at pH 4.0. The migration of the 1:1 mixture of $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ at pH 4.0 shows three bands, which corresponds to I-motif, duplex, and G-quadruplex. The result of gel electrophoresis also confirms that $d(G_4T_4G_4)$ and $d(C_4A_4C_4)$ cannot the form duplex at pH 4.0 due to the stabilization of the I-motif of $d(C_4A_4C_4)$ in acidic condition.

Mechanism of Structural Polymorphism of the Telomere DNAs

In this study, we investigated the effects of pH and Ca^{2+} on the I-motif of $d(C_4A_4C_4)$, the G-quadruplex of $d(G_4T_4G_4)$, and the duplex of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$. The acidic conditions induces the I-motif structure of $d(C_4A_4C_4)$, and therefore, prevents the duplex formation of $d(C_4A_4C_4)/d(G_4T_4G_4)$. On the other hand, Ca^{2+} induces the parallel G-quadruplex (G-wire) of $d(G_4T_4G_4)$ and destabilizes the duplex of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$. The general mechanism of the structural polymorphism of the telomeric DNAs, which is generated and regulated by pH and Ca^{2+}, is explained by the following considerations of the guanine-rich and cytosine-rich oligonucleotides.

Since the I-motif structure is formed and stabilized by the hemiprotonated $C^+ \cdot C$ base pairs (Fig. 1b), pH is critical to determine stabilities of the I-motif. However, pH does not affect the structure and stability of the G-quadruplex because the G-quadruplex formation does not require the protonation of a base (Fig. 1a). On the other hand, a coexisting cation affects the structure of the G-quadruplex because cation coordinations to O6 of the guanine bases are essential to the structure and stability of the G-quadruplex. However, the I-motif does not have a specific cation binding site and does not require cation coordinations to bases for its stable structure. From these considerations and our results, schematic illustrations of the structural polymorphism of the telomere DNAs including the guanine-rich and cytosine-rich oligonucleotides are shown in Fig. 8. In Fig. 8a, pH regulates the duplex formation by regulation of the I-motif structure of $d(C_4A_4C_4)$. On the other hand, Ca^{2+} regulates the duplex structure and stability of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$ by inducing the parallel-stranded G-wire of $d(G_4T_4G_4)$ as shown in Fig. 8b.

Numerous studies have shown that a number of proteins recognizes the duplex form of the telomere DNAs. Recently, not only the G-quadruplex but also the I-motif recognition proteins have been found such as ST-1 of *Trypanosoma brucei* and qTBP42 of rat hepatocytes.[32,33] These protein bindings are some of the strongest evidence for the biological role of the G-quadruplex and I-motif that are formed by natural oligonucleotides placed in many biologically important regions of the genome. In this study, it was demonstrated that structures of not only $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$ but also $d(C_4A_4C_4)/d(G_4T_4G_4)$ can be regulated by pH, the divalent

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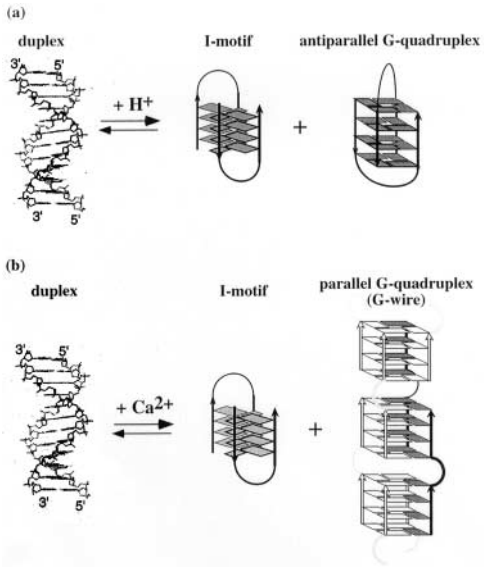

Figure 8. Schematic illustrations of structural polymorphism of $d(C_4A_4C_4)$ and $d(G_4T_4G_4)$ regulated by (a) pH and (b) Ca^{2+}. Squares indicate cytosine and guanine bases. Arrows indicate direction of strands. Thymine bases are omitted for clarity. (a) The structural polymorphism of the telomere DNAs is regulated by pH because the acidic conditions induce the stable I-motif of $d(C_4A_4C_4)$. (b) The structural polymorphism of the telomere DNAs is regulated by Ca^{2+} because Ca^{2+} induces the G-wire of $d(G_4T_4G_4)$.

cation, and their combination. We also found that the intramolecular and intermolecular associations of $d(C_3TA_2)_3C_3$ and $d(G_3(T_2AG_3)_3$ can be regulated by pH and a cation.[24] Thus, the results reported here indicate the important roles of pH and divalent cations in biological processes by regulating the conformational switch between the duplex and quadruplexes structures in vivo.

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