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Interaction of the Dye Ethidium Bromide with DNA Containing Guanine Repeats†

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Received November 18, 1991; Revised Manuscript Received January 7, 1992

ABSTRACT: DNA containing one or more copies of the motifs repeated in telomere sequences has unusual conformational properties. The isolated sequence from the protozoan *Oxytricha*, dT_4G_4 has the potential to form tetramers in the presence of sodium or potassium ions. We report here that these tetramers bind ethidium tightly, with an interaction that fulfills several criteria for an intercalative mechanism in the G sequence. By contrast, the 4-fold tandem repeat of this subunit, $d(T_4G_4)_4$, does not interact specifically with ethidium in the presence of Na^+. This difference might have a simple structural basis: the tetramer of dT_4G_4 forms a stack of four G-quartets in the presence of Na^+ or K^+, whereas the constraint imposed by the T4 "tethers" in the repeat $d(T_4G_4)_4$ allows only two layers to form in the presence of Na^+. In the presence of sufficient K^+, the latter can partially form a four-layer G-quartet structure, which interacts with ethidium. This idea is supported by analysis of a "relaxed" sequence, $dT_4G_4(T_7G_4)_3$, which allows formation of four G-quartets and binds ethidium in the presence of Na^+ as well as K^+. Ethidium (and intercalators generally) should thus be able to retard or inhibit the action of telomerase in the presence of K^+.

The ends of eukaryotic chromosomes contain specialized structures called telomeres (Blackburn & Szostak, 1984; Zakian, 1989; Blackburn, 1991) which protect the natural termini as opposed to internal breaks from degradation or fusion processes. Telomeres contain DNA with variable lengths of simple tandemly repeated sequences (Zakian, 1989), in which clusters of G form in one strand, together with enzymes that can synthesize the G-rich repeat (Blackburn, 1991), as well as accessory proteins. DNA bearing telomeric repeat sequences shows unusual concentration-dependent conformational behavior (Lipps, 1980; Oka & Thomas, 1987), which has focused attention on the in vitro properties of oligonucleotide models containing single or tandemly repeated clusters of G's (Henderson et al., 1988; Sen & Gilbert, 1988, 1990; Williamson et al., 1989; Sundquist & Klug, 1989). At low concentration and in the absence of Na^+ or K^+, the octanucleotide dT_4G_4 migrates as a monomer on electrophoresis in nondenaturing gels (Williamson et al., 1989). In the presence of Na^+, dT_4G_4 has a mobility equivalent to a tetramer and forms a ladder of five tetrameric bands in the assay described by Sen and Gilbert (1990), when annealed with a marker strand such as $dT_{10}G_4$ (data not shown). On the other hand, the tandemly repeated sequence $(dT_4G_4)_4$ exhibits an anomalously high mobility in the presence of Na^+ or K^+ in native PAGE compared with either $d(T_4G_4)_2$ or dT_4G_4 (Williamson et al., 1989). This has been attributed to formation of a compact folded structure thought to contain two layers of four hydrogen-bonded guanine bases (Williamson et al., 1989). Formation of K^+-stabilized G-quartet structure inhibits the action of the telomerase from this organism (Zahler et al., 1991). The interaction of these two molecules with ethidium bromide, a dye that can bind to duplex DNA by intercalating between adjacent base pairs (Bresloff & Crothers, 1975), is described here.

Materials and Methods

Synthesis and Purification of Oligonucleotides. Oligonucleotides used in this study were synthesized on an ABI 391 PCR MATE DNA synthesizer and deprotected by routine phosphoramidite procedures (Caruthers, 1982). Strands were purified by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, following the manufacturer's recommended elution protocol. Oligonucleotides were labeled at their 5′ termini using T4 polynucleotide kinase (Boehringer); the labeled strands were purified by polyacrylamide gel electrophoresis.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded using an AVIV Model 60DS CD spectropolarimeter equipped with a programmable, thermoelectrically controlled cell holder. CD melting curves were recorded at 265 nm. Ellipticities at 5 °C were normalized to 1.0. Samples were heated at a rate of 0.5 °C/min. DNA solutions were prepared in a 10 mM phosphate buffer containing 0.1 mM EDTA and 200 mM NaCl or KCl, adjusted to pH 7. The DNA strands of dT_4G_4 were evaporated to dryness at 50 °C over 10 h and then dissolved in 10 mM phosphate buffer (pH 7) and 0.1 mM EDTA. The DNA samples were incubated at 4 °C for 48 h. The DNA solution of $d(T_4G_4)_4$ was prepared in a 10 mM phosphate buffer (pH 7) containing 0.1 mM EDTA and 200 mM NaCl or KCl. The DNA samples were heated to 95 °C for 2 min, cooled slowly, and incubated at room temperature for 24 h.

Gel Electrophoresis. Native gels: The DNA strands in 10 μL of 10 mM Tris·HCl (pH 7), 0.1 mM EDTA (TE buffer), and 200 mM NaCl were evaporated to dryness at 50 °C over 10 h. The samples were dissolved in 10 μL of TE buffer, incubated at 4 °C for 48 h, and then run on 20% native gels

† This work was supported by Grants CA-24101 (N.R.K.) and GM-42223 (L.A.M.) from the National Institutes of Health and by the generous support from the W. M. Keck Foundation.
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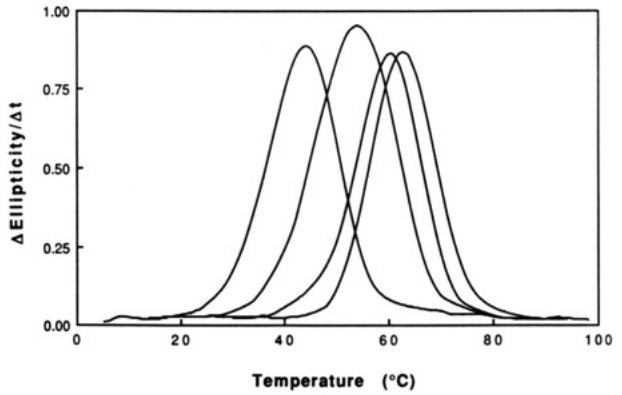

FIGURE 1: Differential CD melting profiles for dT_4G_4 at total strand concentrations of 100 μM in the presence of 200 mM NaCl from left to right without and with ethidium bromide of 25, 50, and 100 μM. Samples were preequilibrated at 5 °C for 1 h. The vertical scale has been changed in sign.

at 4 °C for 20 h at 100 V (ca. 8 V/cm). The electrophoresis plates were jacketed and cooled with circulating water to provide a running temperature of 4 ± 1 °C in the gel throughout the electrophoresis. The buffer system contained 40 mM Tris·HCl, 20 mM acetic acid, and 1 mM EDTA (pH 8.1), with or without 1 μg/mL ethidium bromide. Denaturing gels: The samples were taken up in formamide loading buffer, heated briefly to 90 °C, cooled, and then run on a denaturing polyacrylamide gel (20%) for 3 h at 2000 V (ca. 50 V/cm) and 40 °C.

MPE·Fe(II) Cleavage Reaction. Our procedure followed that of van Dyke and Dervan (1983) and Guo et al. (1989). DNA samples (10 μL, 100 μM) were exposed to 10 μM Fe(II) and 10 μM MPE in TE buffer, without or with indicated salts for 15 min at 4 °C, followed by addition of 4 mM DTT for 45 min. The reaction was stopped by extraction with 1-butanol and precipitation with ethanol.

Fluorescence Measurements. The measurements were carried out on an MPF-2A spectrofluorometer equipped with temperature regulation. Fluorescence emission spectra were recorded at 10 °C with excitation at 510 nm and emission at 590 nm.

Calorimetric Measurements. Experiments were carried out using the Omega titration calorimeter (Wiseman et al., 1989) from Microcal Inc. Each DNA sample was titrated with a concentrated solution of EB in a 100-μL stirring syringe rotating at 400 rpm. The stoichiometry, molar binding enthalpies, and binding affinity were determined by using a nonlinear fit of the experimental data (integral heat vs total concentration of added ligand) with the assumption of one type of binding site.

DMS Protection Assay. Methylation protection experiments were performed as described by Maxam and Gilbert (1980). The DNA strands were heated to 90 °C for 4 min, cooled slowly, and then incubated at 4 °C for 24 h. A total of 1 μL of a fresh 1:100 dilution of DMS in water was added to the 10 μL of DNA samples (25 μM) in TE buffer without or with indicated salts for 30 min at 4 °C. The reactions were terminated by two sequential rapid ethanol precipitations and then lyophilized. The DNAs were cleaved at the methylation sites by treatment with 100 μL of 1 M piperidine at 90 °C for 30 min and lyophilized extensively.

Results and Discussion

The Tetramer of dT_4G_4 Is Stabilized by the Intercalating Drug Ethidium Bromide (EB). One criterion for interaction of a drug with ordered DNA is an increase in the T_m (midpoint

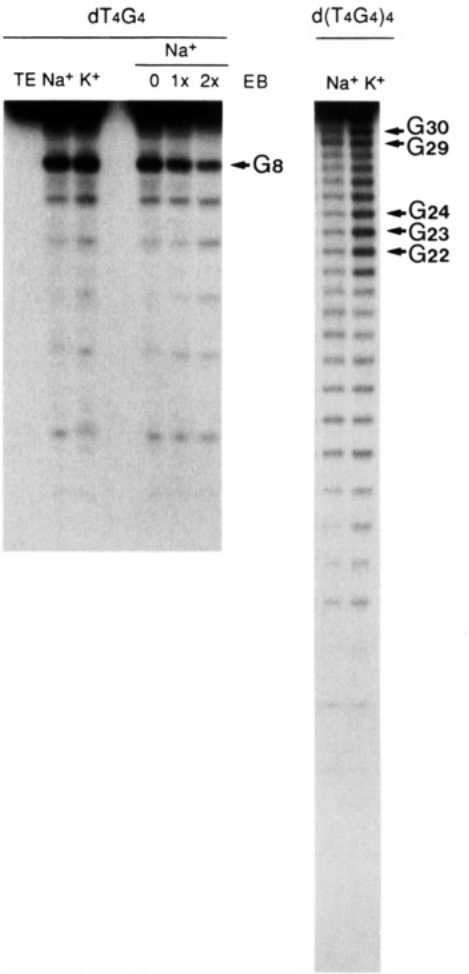

FIGURE 2: MPE·Fe(II) cleavage pattern of dT_4G_4 and $d(T_4G_4)_4$. The cleavage reactions were carried out in TE buffer or in TE with added 200 mM NaCl or KCl, without or with ethidium (EB) indicated.

thermal denaturation temperature) of the DNA in the presence of the drug (Crothers, 1971). Figure 1 shows the derivative plots of the melting curves for ethidium–DNA complexes with varying ratios of ligand to tetramer in the presence of Na^+. The thermal stability of the tetramer in both Na^+ (Figure 1) and K^+ (data not shown) is enhanced significantly on binding ethidium: at a ratio of 1 ethidium per tetramer, for example, an increase of 10 °C is observed. Association of EB with the tetrameric form of dT_4G_4 should shift the equilibrium between the single strands and the tetramer to favor tetramer in the presence of dye. This can be directly detected by polyacrylamide gel electrophoresis (PAGE) at low temperature in nondenaturing conditions (data not shown). As is discussed below, the G-quartet structure formed by the tandem repeat $d(T_4G_4)_4$ does not interact selectively with EB in Na^+ by this criterion.

MPE·Fe(II) Cleavage in the Presence of Na^+ or K^+ Suggests That the G Region in dT_4G_4 Is a Site of Drug Binding. To locate the sites of interaction in dT_4G_4, we used Dervan's chemical probe MPE·Fe(II), which consists of an ethidium-like ring system linked to a reactive EDTA·Fe(II) group that can initiate DNA strand cleavage in the vicinity of the binding site(s) (Hertzberg & Dervan, 1982). Cleavage of tetrameric dT_4G_4 and a single-strand control was compared. As shown in Figure 2, MPE·Fe(II) cleaves the tetramer of dT_4G_4 and the single strand (lane TE). Strong reactivity at G8 in the tetramer is detected, which can be eliminated by adding nonreactive ethidium to the DNA samples (Figure 2). Thus argues that ethidium selectively interacts in the vicinity of G8

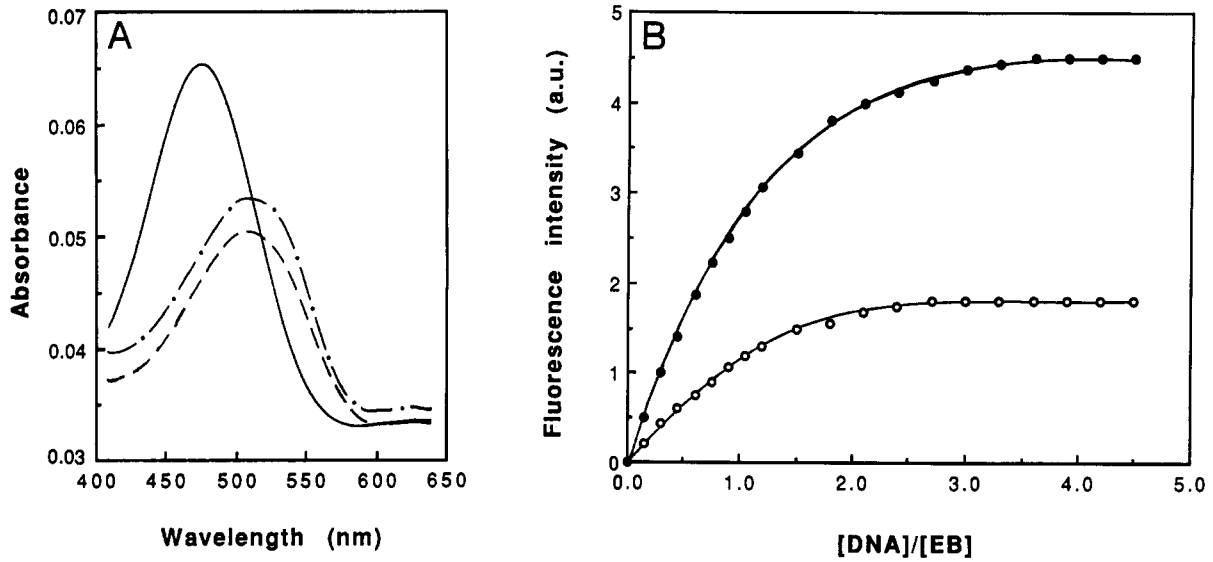

FIGURE 3: (A) Absorption spectra of 10 μM ethidium bromide (EB) with or without 50 μM total strand concentration of DNA added in 10 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, and 200 mM NaCl [free EB (—), EB added with G4 DNA product of dT_4G_4 (-·-), and a ds control (--)]. (B) Fluorescence intensity of EB at 590 nm, with excitation at 510 nm, upon addition of the ds DNA (filled circles) and tetramer (open circles) in 10 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, and 200 mM NaCl. The total concentration of EB is 10 μM.

in the tetramer, possibly at the end of the G region or between G7 and G8. No indication for a strong interaction of MPE·Fe(II) can be seen in the tandem repeat, $d(T_4G_4)_4$, in the presence of Na^+, however (Figure 2).

Ethidium Binds to the G4 Structure of dT_4G_4 in an Intercalative Mode. Aside from association with the end of the tetramer, ethidium could intercalate into the dT_4G_4 complex or bind in the grooves of this structure. Intercalative binding of EB into duplex (LePecq & Paoletti, 1967) or triple-stranded (Scaria & Shafer, 1991; Mergny et al., 1991) DNA is associated with a strong enhancement in fluorescence yield of the dye and a characteristic red shift in its emission band. Figure 3 compares these parameters for binding of EB to dT_4G_4 and to a duplex control. The increase in intensity and shift in wavelength observed are consistent with intercalative binding, although this experiment still does not fully eliminate the possibility of a tight-end binding process in which the phenanthridine ring covers one or more G's at the ends of the helix. To investigate this latter possibility, we synthesized the sequence dT_4G_4T, which forms a similar tetraplex to dT_4G_4 in the presence of Na^+ or K^+. The interaction of MPE·Fe(II) with this complex is identical to that in dT_4G_4 (data not shown). The presence of an additional T residue at the 3′ end of the oligomer does not weaken the interaction observed in the G sequence, favoring the idea that the interaction with the four helix is intercalative.

Ethidium Binds to G4 DNA as Strongly as to Corresponding ds DNA. Both the stoichiometry of binding and the affinity can be assessed directly by monitoring the heat of binding using a titration microcalorimeter (Wiseman et al., 1989). Under unsaturated conditions, the heats obtained for each injection are independent of the total concentration of added ligand. After correction for the heats of dilution of the dye, molar binding enthalpies (ΔH) are calculated by averaging the first four to five injections. The resulting calorimetric binding isotherm is shown in Figure 4. A nonlinear fit of this binding isotherm provides an estimate of the binding constant (K), molar binding enthalpy (ΔH), and stoichiometry of the complex. These values are consistent with association of one EB per tetramer of dT_4G_4, with an affinity comparable to that of normal intercalation by EB in short DNA duplexes, as summarized in Table I.

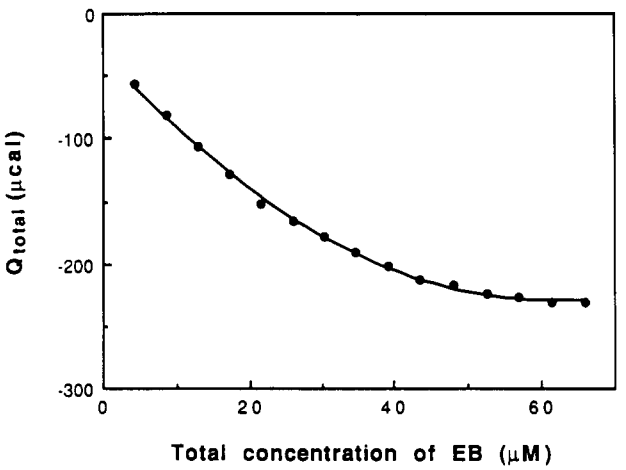

FIGURE 4: Calorimetric titration of dT_4G_4 with ethidium. The measurements were done in 10 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA and 200 mM NaCl at 13.7 °C. The total concentration of dT_4G_4 in single strands in the cell is 0.14 mM, and the ethidium concentration is 1.198 mM. Solid circles indicate experimental heat values; the line corresponds to a three-parameter fit to a binding process with $n = 0.95$, affinity $K = 1.54 \times 10^5$, and $\Delta H° = -5.9$ kcal/mol. The standard deviation of the fit is 1.2%.

Table I: Equilibrium Binding and Thermodynamic Parameters[a] for EB Binding to dT_4G_4 and ds DNA control $d(T_4G_4)/d(C_4A_4)$[b]

	n_b[c]	K (M^{-1})	$\Delta G°$[d] (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
ds DNA	2.7 ± 0.1	5.2×10^4	−6.8 ± 0.3	−12.0 ± 0.6	−5.2 ± 0.7
G4 DNA	1.0 ± 0.1	1.5×10^5	−7.3 ± 0.4	−5.9 ± 0.3	+1.4 ± 0.5

[a] All measurements were done in 10 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, and 200 mM NaCl, at 15.8 °C for ds DNA and 13.7 °C for G4 DNA. [b] Total concentration of single strands = 8.2×10^{-5} M for ds and = 1.4×10^{-4} M for G4 DNA. [c] Number of binding site(s) per duplex or per tetramer DNA molecule. [d] Calculated at 25 °C assuming $\Delta C_p = 0$.

Why Does Ethidium Selectively Interact with $(dT_4G_4)_4$ but Not with $d(T_4G_4)_4$ in the Presence of Na^+? By contrast, the structure and response to ethidium of the tandem repeat $d(T_4G_4)_4$ differ strikingly. One obvious possibility is that the reason is structural, because the G-quartet in the tandem repeat has a distinctly different CD spectrum that is sensitive

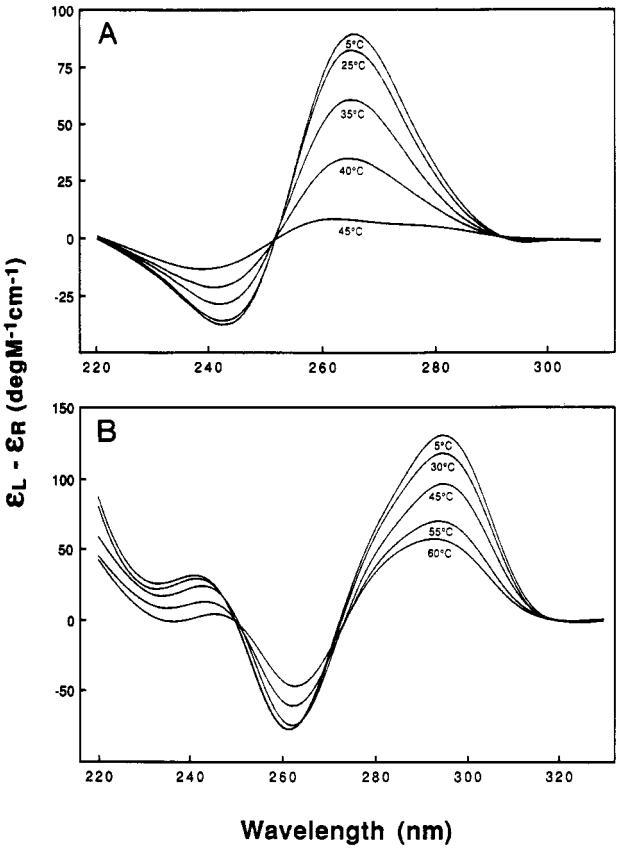

FIGURE 5: Temperature-dependent CD spectra of dT_4G_4 (A) and $d(T_4G_4)_4$ (B) in 10 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, and 200 mM NaCl. The total concentration of dT_4G_4 or $d(T_4G_4)_4$ is 100 and 25 μM, respectively. Samples were preequilibrated at 5 °C for 1 h. Each spectrum corresponds to an average of three scans from which the buffer background was subtracted.

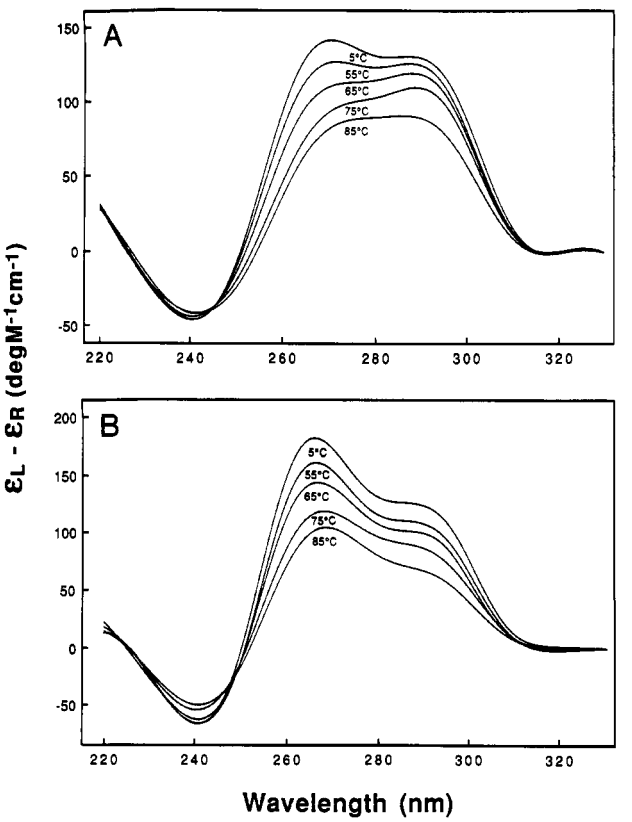

FIGURE 6: Temperature-dependent CD spectra of $d(T_4G_4)_4$ without (A) or with (B) EB in the presence of K^+. The 25 μM $d(T_4G_4)_4$ in 10 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, and 200 mM KCl was incubated without or with 25 μM EB at 5 °C for 1h.

to metal ions (Hardin et al., 1991). Figure 5 contrasts the CD spectra of $(dT_4G_4)_4$ in Na^+ as a function of temperature with that of the tetramer of dT_4G_4. Instead of the nearly conservative spectrum centered at 265 nm seen in the latter, $d(T_4G_4)_4$ has a nonconservative spectrum with a strong positive band at 295 nm (Mergny et al., 1991). Several arguments suggest that, in the presence of Na^+, the G region in the repeat contains two rather than four H-bonded G-quartet layers (Williamson et al., 1989). Apart from the evidence presented by these workers, the CD spectrum of $(dT_4G_4)_4$ resembles that of tetramers formed from oligonucleotides containing only two adjacent G's and thus two layers per tetramer (Jin et al., 1990). Consistent with this idea, the enthalpy of formation of the structure in $d(T_4G_4)_4$ measured in 200 mM Na^+ by differential scanning calorimetry is −52.0 kcal/mol of strand, roughly one-half that in the dT_4G_4 tetramer (−93.4 kcal/mol of tetramer) or the tetramers containing a pair of two G-quartet layers (−110 kcal/mol) (Jin et al., 1990). The CD spectrum of the sequence $dT_4G_4(T_7G_4)_3$ in Na^+ resembles that of the dT_4G_4 tetramer and not that of $d(T_4G_4)_4$ (data not shown). The $dT_4G_4(T_7G_4)_3$ molecule binds EB as well as the dT_4G_4 tetramer. The DMS protection experiment (Sen & Gilbert, 1988) shows that four adjacent G residues are protected from methylation in the presumed four-layer structure in dT_4G_4-$(T_7G_4)_3$, in contrast to the patterns reported in $d(T_4G_4)_4$ (Williamson et al., 1989). Thus we believe that increasing the length of the T sequence allows formation of a four-layer G-quartet structure in the presence of Na^+. The CD spectrum of $d(T_4G_4)_4$ responds differentially to metal ions (Mergny et al., 1991), in contrast to that of dT_4G_4. K^+ appears to allow the repeat to form a partial four-layer structure, as suggested by the CD spectra in Figure 6A. This hypothesis predicts that EB should interact with the K^+ form of the tandem repeat, which has a CD spectrum that can be interpreted as a mixture of both two- and four-layer G-quartet structures. This is confirmed by CD measurements of spectral changes in $d(T_4G_4)_4$ in the presence of EB (Figure 6B). According to this hypothesis, EB should interact with the sequence $dT_4G_4(T_7G_4)_3$ in the presence of Na^+ or K^+, which is again confirmed. These observations are consistent with the idea that EB interacts preferentially with four-layer G-quartet structures but not with the two-layer version.

Conclusions. In the presence of Na^+, the structure of the tetrameric complex of dT_4G_4 differs fundamentally from that of the tandem repeat $d(T_4G_4)_4$, consistent with the idea that, in the presence of Na^+ or K^+, the former forms a four-layer G-quartet structure, while in the presence of Na^+, the repeat has a fold-back structure in which only two layers can form (Williamson et al., 1989). The dye EB associates tightly with the former structure, by a mechanism consistent with some degree of insertion of the phenanthridine ring between the 5′ distal G bases; the dye shows no strong binding to the repeat structure in the presence of Na^+. The presence of K^+ appears to allow partial formation of four-layer G4 structures that now interact with EB. Ethidium thus provides a first example of agents other than metal ions that have the potential to differentially affect telomerase activity, based on the observation that formation of G-quartet structures with K^+ inhibits the telomerase from *Oxytricha* (Zahler et al., 1991). The effect would be different in a single overhang of dT_4G_4 than in tandem repeats; the latter require K^+ in order to interact with ethidium. Blackburn has proposed that telomere synthesis might be a target for selective drug action by agents that interact with the telomerase itself or with its substrate(s) (Blackburn, 1991). Stabilizing structures that inhibit action

of telomerase offer one possible mechanism for interfering with telomere synthesis.

ACKNOWLEDGMENTS

We thank Bing Mao and Dr. Nick Geacintov for their help with fluorescence measurements.

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Structure and Stability of Sodium and Potassium Complexes of dT_4G_4 and dT_4G_4T†

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Received November 26, 1991; Revised Manuscript Received January 7, 1992

ABSTRACT: The ends of eukaryotic chromosomes contain specialized structures that include DNA with multiple tandem repeats of simple sequences containing clusters of G on one strand, together with proteins which synthesize and bind to these sequences. The unit repeat in the protozoan *Oxytricha* with the cluster dT_4G_4 can form structures containing tetrads of guanine residues, referred to G4 DNA, in the presence of metal ions such as Na^+ or K^+. We show here that, in the presence of Na^+, dT_4G_4 forms a tetramer with parallel strands by means of a UV cross-linking assay. In the presence of K^+, two further interactions are observed: at low temperature, higher order complexes are formed, provided the 3′ end of the strand is G; a single 3′T inhibits this association in dT_4G_4T. At high temperature, these complexes dissociate, leading to a tetramer with a different ordered structure that melts only at very high temperatures. These results suggest that the cohesive properties of DNA containing G clusters might depend on associative interactions driven by a free 3′G terminus in the presence of K^+, as well as by connecting antiparallel G hairpins as has been postulated.

One role of telomeres is to stabilize the ends of chromosomes against breakage (Blackburn & Szostak, 1984; Zakian, 1989; Blackburn, 1991) by inhibiting degradation of DNA or fusion processes. DNA containing the sequences repeated in telomeres can show unusual cohesive behavior in solution (Lipps, 1980; Oka & Thomas, 1987), suggesting that short overhanging G clusters might be capable of connecting duplexes, thereby maintaining integrity of chromosome ends (Blackburn, 1991). This has been confirmed by studies of short oligomeric model sequences containing G clusters (Henderson et al., 1987, 1990; Sen & Gilbert, 1988, 1990, 1992; Sundquist & Klug, 1989; Williamson et al., 1989; Hardin et al., 1991). Oligonucleotides corresponding to two copies of dT_2G_4 form an intramolecular, double-stranded hairpin loop structure under low ionic strength. Upon addition of Na^+ or K^+ ions, the two hairpins dimerize to assume a compacted, antiparallel fold-

† This work was supported by Grant CA-24101 from the National Institutes of Health and by the generous support from the W. M. Keck Foundation.
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0006-2960/92/0431-2455$03.00/0