

Monovalent Cation Induced Structural Transitions in Telomeric DNAs: G-DNA Folding Intermediates[†]

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ABSTRACT: Telomeric DNA consists of G- and C-rich strands that are always polarized such that the G-rich strand extends past the 3' end of the duplex to form a 12–16-base overhang. These overhanging strands can *self*-associate in vitro to form intramolecular structures that have several unusual physical properties and at least one common feature, the presence of non-Watson–Crick G·G base pairs. The term “G-DNA” was coined for this class of structures (Cech, 1988). On the basis of gel electrophoresis, imino proton NMR, and circular dichroism (CD) results, we find that changing the counterions from sodium to potassium (in 20 mM phosphate buffers) specifically induces conformational transitions in the G-rich telomeric DNA from *Tetrahymena*, d(T₂G₄)₄ (TET4), which results in a change from the intramolecular species to an apparent multistranded structure, accompanied by an increase in the melting temperature of the base pairs of >25°, as monitored by loss of the imino proton NMR signals. NMR semiselective spin–lattice relaxation rate measurements and HPLC size-exclusion chromatography studies show that in 20 mM potassium phosphate (pH 7) buffer (KP) TET4 is approximately twice the length of the form obtained in 20 mM sodium phosphate (pH 7) buffer (NaP) and that mixtures of Na⁺ and K⁺ produce mixtures of the two forms whose populations depend on the ratio of the cations. Since K⁺ and NH₄⁺ are known to stabilize a parallel-stranded quadruplex structure of poly[r(I)₄], we infer that the multistranded structure is a quadruplex. Our results indicate that specific differences in ionic interactions can result in a switch in telomeric DNAs between intramolecular hairpin-like or quadruplex-containing species and intermolecular quadruplex structures, all of which involve G·G base pairing interactions. We propose a model in which duplex or hairpin forms of G-DNA are folding intermediates in the formation of either 1-, 2-, or 4-stranded quadruplex structures. In this model monovalent cations stabilize the duplex and quadruplex forms via two distinct mechanisms, counterion condensation and octahedral coordination to the carbonyl groups in stacked planar guanine “quartet” base assemblies. Substituting one of the guanosine residues in each of the repeats of the *Tetrahymena* sequence to give the human telomeric DNA, d(T₂AG₃)₄, results in less effective K⁺-dependent stabilization. Thus, the ion-dependent stabilization is attenuated by altering the sequence. Upon addition of the Watson–Crick (WC) complementary strand, only the Na⁺-stabilized structure dissociates quickly to form a WC double helix. This demonstrates that under some circumstances the K⁺-stabilized G-DNA structure can be kinetically preferred over WC DNA.

Telomeres consist of repetitive contiguous DNA sequences that always occur in a very specific secondary structural motif at the ends of linear chromosomes. The motif consists of a guanosine-rich strand on the 3'-terminus that overhangs the corresponding 5'-terminal complementary C-rich strand by approximately 12–16 bases (Blackburn & Szostak, 1984; Blackburn, 1986; Henderson & Blackburn, 1989). Greider

and Blackburn (1989) have purified an enzymatic ribonucleoprotein complex from *Tetrahymena* (named telomere terminal transferase, “telomerase”) that catalyzes the addition of T₂G₄ units to telomeric ends. This activity is thought to compensate for the loss of telomeric sequences during replication (Blackburn, 1986; Watson, 1972). Similar enzymes have also been isolated from *Euplotes* (Shippen-Lentz & Blackburn, 1989) and HeLa cells (Morin, 1989). If telomeres from *Tetrahymena* or *Oxytricha* are ligated onto yeast DNAs and introduced into yeast cells by transformation, these sequences are recognized and elongated by a putative yeast telomerase (Blackburn, 1986; Pluta et al., 1984). Thus, an intrinsic property of the structures formed by the DNA sequences of telomeres from diverse organisms is recognized by the telomere metabolic machinery.

The structural characteristics of oligonucleotides corresponding to G-rich sequences that are present in the chromosomes of *Tetrahymena* (T₂G₄; the same sequence as in *Glaucoma*), *Dictyostelium* (AG₆GAGAG₆AG₆), *Oxytricha* (T₄G₄), *Trypanosoma* (T₂AG₃; the same as humans), and

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Saccharomyces (TGTGTG₃) were assessed previously (Henderson et al., 1987). These DNAs have the following common characteristics: (i) As the temperature is reduced below ca. 40 °C in NaP¹ buffer, all of the DNAs form discrete species with electrophoretic mobilities that are faster than those of the corresponding single-stranded species. In 20 mM sodium phosphate or 10 mM Tris buffers the electrophoretic pattern obtained with TET4 does not change over an 8000-fold concentration range, indicating the presence of an intramolecular species. These results demonstrated that telomeric DNAs have the unusual property of forming stable intramolecular apparent hairpin- or dumbbell-like or quadruplex-containing species at moderate ionic strength. These species exchange quickly with the single-stranded forms at near-physiological temperatures. (ii) They exhibit hyperchromic shifts in the absorbance spectra at 260 nm. (iii) All of the G-rich telomeric DNAs are recognized as viable substrates for the *Tetrahymena* telomerase (Greider & Blackburn, 1985, 1989). However, the C-rich DNAs, d(T₂G₄)₁, and the perfectly Watson-Crick base-paired DNA d(T₂G₄)₂·d(A₂C₄)₂ were not viable substrates for the enzyme.

Detailed NMR studies were also carried out with TET4 (Henderson et al., 1987). ³¹P NMR thermal denaturation studies demonstrated the presence of one dominant structure with a variety of different types of discrete phosphodiester backbone conformation. ¹H NMR thermal denaturation studies in H₂O buffer showed that the structure contains approximately 22–25 hydrogen bonds that dissociate as the temperature is raised through the 40 °C range. On the basis of the number of hydrogen bonds and the base composition of TET4 and considering the possible types of base pairs, it was concluded that the low-temperature structure contains G-G base pairs (in addition to possible G-T and/or T-T base pairs). Two-dimensional NMR experiments demonstrated that four to six of the guanosine residues are in the unusual synclinal conformation.

On the basis of these results, it was suggested that the physical properties that are inherent in the novel structure that is formed by TET4 dictate in part the sequence specificity in the biological role of telomeres (Henderson et al., 1987). Given the fact that all of these DNAs have the property of being G-rich sequences punctuated by Ts and/or As (but rarely Cs), these studies implicate the ability to form G-G base pairs as a central structural feature of telomeres. These results demonstrate two interesting points: (a) non-Watson-Crick base pairing in DNA may be important within a biological context and (b) conformations other than canonical B- and A- (and possibly Z-) form DNA can occur in specialized physiological structures. The generality of G-rich telomeric structures has now been extended to include [from Forney et al. (1987)] the ciliates, slime molds, fungi, flagellated protozoans, sporozoa, plants (e.g., corn; Richards & Ausubel, 1988), and mammals (e.g., humans; Cooke et al., 1985; Allshire et al., 1988; Roberts, 1988). The term "G-DNA" was coined for this type of structure (Cech, 1988). As discussed in this paper, the term "G-DNA" should be extended to a class definition since the form of "G-DNA" that occurs in K⁺-containing buffer is different from that found in the presence of limiting Na⁺ (Henderson et al., 1987).

Oka and Thomas (1987) initially showed that monovalent cations can specifically affect the interaction between telomere-containing DNAs. They studied DNA fragments that contained *Oxytricha* telomeres at the termini [d(T₄G₄)_n·d(C₄A₄)_m, $n > m$] and found that when incubated at high concentrations the DNAs "cohere" in an apparent interstrand association reaction to form an unusual structure. Unlike WC duplex formation, "coherence" is slow and unpaired 3' overhanging strands are required. Potassium increases the stability of the "cohered" form by ca. 25 °C relative to Na⁺. This is not true for WC base-paired DNAs where there is essentially no difference in duplex stability in the presence of these cations (Krakauer & Sturdevant, 1968). "Cohered" telomeres are relatively poor substrates for nucleases, while the uncohered ("dispersed") forms are not.

Chemical modification/protection/interference, inosine-substitution, and UV cross-linking experiments were used by several groups to determine further details of the structure(s) that is (are) adopted by telomeric G-rich DNAs in vitro (Williamson et al., 1989; Henderson et al., 1990; Sundquist & Klug, 1989; Sen & Gilbert, 1990).

Williamson et al. (1989) studied the effects of monovalent cations on TET4 and *Oxytricha* telomeric DNA d(T₄G₄)₄ (OXY4) using dimethyl sulfate (DMS) modification experiments. They found that the formation of more mobile species on nondenaturing gels depends on ion type. Complexes form in the presence of 50 mM Tris solutions of NaCl or KCl but not in LiCl-containing buffer. On the basis of these experiments and thymidine cross-linking results they concluded that these molecules form intramolecular quadruplex species. They note that at least two conformations can be trapped in their cross-linking reactions.

Henderson et al. (1990) also concluded that there are differential reactivities at the G residues in these structures depending on ion type. Since methylation at GN7 by DMS could be inhibited either due to the presence of syn G residues or because N7 participates in hydrogen bonding with an imino or amino group in the "G-DNA" structure, interpretation of these data in definitive structural terms is difficult. In addition, they found that *Tetrahymena* telomerase can recognize molecules that cannot form quadruplex structures.

Sundquist and Klug (1989) made model telomeric DNA complexes that contained 3'-d(T₂G₄)₂ overhanging DNA. They found that the molecules dimerize in a Na⁺- or K⁺-dependent manner, producing complexes in which all G N7 atoms are inaccessible to DMS modification. These apparent quadruplex-containing complexes are stable in buffers containing >100 mM KCl. The authors suggest that the thymidine residues may act as "spacers" which serve two functions: (i) to reduce steric clash between the quadruplex and adjacent duplex regions and (ii) to allow G-DNA hairpin formation by acting as loops. This is consistent with the affinities of the bases for water since T is the most hydrophilic and G the most hydrophobic of the DNA bases (Saenger, 1984).

Sen and Gilbert (1988) obtained interesting results that may indicate a potential role for nontelomeric G-rich DNAs in meiotic pairing of chromosomes. They studied DNAs derived from the immunoglobulin switching region that constitute the sites for recombination between the variable and constant sequences during the differentiation of B lymphocytes to plasma cells. These DNAs are composed of several G-rich motifs, e.g., G₄T, GAGCT, and (G)₄AGCTG₄. They found that the Watson-Crick base-paired DNAs form structures upon incubation in Tris-HCl buffer at 4 °C in which the C-rich

¹ Abbreviations: CD, circular dichroism; DMS, dimethyl sulfate; DYM, d(AG₆GAGAG₆AG₆); HUM4, d(T₂AG₃)₄; KP, 20 mM potassium phosphate (pH 7) containing 0.1 mM EDTA; NaP, 20 mM sodium phosphate (pH 7) containing 0.1 mM EDTA; OXY4, d(T₄G₄)₄; TET4, d(T₂G₄)₄; TMP, trimethyl phosphate; TSP, sodium 3-(trimethylsilyl)-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane; WC, Watson-Crick.

complementary strands are looped out to form parallel four-stranded DNA complexes ("M" strands) composed of base-paired guanine "quartets". They referred to these complexes as "G4-DNAs". Given the similarity between the sequences of telomeric DNAs and the switch region DNAs, these authors predicted that telomeric DNAs may also form "G4-DNA" *in vivo*, thus acting as an initial point of alignment during meiotic pairing.

Sen and Gilbert (1990) extended these studies to assess the effects of monovalent cations on the structures formed by telomeric DNAs. They found that in the presence of Na⁺ the complexes contain four parallel strands and that formation of the complex is a second-order process. This suggests that the rate-limiting step in quadruplex formation involves dimerization of strands to form an intermediate duplex structure(s). Increasing K⁺ concentrations initially stimulates and then blocks the formation of G4-DNA. As the K⁺ concentration is increased above ca. 50 mM, their results show that intermolecular "fold-back" structures become predominant. In summary, they predict that sequences containing four or more separated runs of guanine residues will produce four-stranded intramolecular "fold-back" structures [see Williamson et al. (1989)], sequences containing two G-tracts will form intermolecular "fold-back" structures, and molecules with single runs of guanines will produce G4-DNA.

In this paper we verify by alternative means the conclusion that specific ionic interactions can induce transitions between intra- and intermolecular duplex and quadruplex forms of "G-DNA" and outline some of the properties of these structures. We then present a model in which monovalent cations differentially control the partitioning of G-rich DNAs between these different structural motifs. Potential genetic implications and several scenarios that could incorporate ion-mediated structural changes in DNA *in vivo* are also discussed.

MATERIALS AND METHODS

Preparation of DNAs. DNA oligonucleotides were synthesized on an Applied Biosystems solid-phase DNA synthesizer using standard phosphoramidite chemistry. The DNA was detached from the silica support, and protecting groups were removed by incubating the silica-bound samples in concentrated ammonium hydroxide for 8 h, removing the supernate, concentrating by lyophilization, and then lyophilizing twice after resuspending in H₂O. The oligonucleotides were purified by either preparative gel electrophoresis or high-pressure liquid chromatography using a Nucleogen DEAE 60-7 column (Machery-Nagel). A gradient from 50 mM to 1.5 M triethylammonium acetate (pH 6.1) in 20% acetonitrile was used. Alternatively, samples were purified by preparative gel electrophoresis on 12% polyacrylamide gels containing 0.6× (54 mM) Tris-borate (pH 7) in 8 M urea (Henderson et al., 1987). DNAs were resuspended into 10 mM Tris-HCl (pH 7) containing 1 mM EDTA (TE) and diluted 1:1 with 100% formamide and applied to the gel. The progress of the electrophoresis was monitored by loading a 0.1% bromophenol blue and xylene cyanol solution in an adjacent well. After electrophoresis, the DNA was located by placing the gel on a fluorescent thin-layer chromatography plate and viewing with a UV lamp. The gel containing the DNA was removed, crushed, and extracted twice with 5–10 mL of TE for ca. 15 h. DNA was then "desalted" and urea removed by C-18 chromatography. The resin was removed from a Sep-Pak (Waters) or NENsorb (New England Nuclear) cartridge and placed in a small column. The DNA was

applied and eluted at ambient pressure according to the manufacturer's instructions.

Samples were prepared for spectroscopy by one of two methods. To obtain minimal Na⁺ conditions, samples were ethanol precipitated, resuspended in TE, applied to a C-18 column (see above), eluted with 1:1 methanol/H₂O, and resuspended directly into 10 mM NaP buffer at the required concentration. Otherwise, samples were ethanol precipitated twice by adding 1/10 volume of 20% potassium acetate (pH 6) and 2 volumes of ethanol. They were then resuspended into 200 μ L of NaP or KP buffer and dialyzed extensively against the same buffer in a BRL 1200MA microdialysis apparatus. Buffer was then added to obtain a final volume of 400 μ L. NMR samples were lyophilized repeatedly from 99.96% D₂O (Aldrich, gold label). Imino proton NMR samples contained 10% D₂O/90% H₂O in the appropriate buffer. Final DNA concentrations were 0.5–2 mM in strands.

HPLC Size-Exclusion Chromatography. Purified TET4 was dialyzed at a concentration of 70–80 A₂₆₀/mL into either NaP, KP, or a 1:1 mixture of the two buffers (10 mM NaP/10 mM KP). These samples were then chromatographed on a Bio-Rad Bio-Sil TSK-125 HPLC size-exclusion column at room temperature in each of the three buffers. In this manner, nine different sample/mobile phase combinations were studied.

Absorbance Thermal Denaturation Studies. Absorbance versus temperature data were obtained on a Gilford Response II apparatus. The temperature was increased from 0 to 90 °C and returned to 0 °C to check for evaporation and hysteresis; absorbance changes due to these factors were less than 1%. The heating and cooling rates were 0.25 °C/min.

Circular Dichroism Spectroscopy. Circular dichroism spectra were collected on a Jasco J-600 spectropolarimeter interfaced to an IBM PC microcomputer. The sample temperature was maintained by placing the sample in a 1-cm path length cylindrical cell surrounded by an external jacket for recirculating water. All CD data were base line corrected for signals due to the cell and buffer.

Samples were prepared for the ion-dependence experiments (Figure 6) as follows: TET4 and d(AG₆GAGAG₆AG₆) from *Dictyostelium* (referred to as "DYM DNA") were dialyzed into 0.1 mM EDTA (pH 7) at ca. 100 μ M DNA concentration without prior "desalting" and then resuspended and preincubated at 60 °C in the following ion solutions: 100 mM lithium, sodium, potassium, or ammonium phosphate (pH 7) and 50 mM magnesium or calcium chloride in 20 mM Tris-HCl (pH 7) containing 0.1 mM EDTA. The oligonucleotides were allowed to cool slowly to 4 °C, and CD spectra were obtained at 10 °C in 1-cm path length cells.

NMR Methods. NMR data were collected on either a General Electric GN-500 or Omega 500 spectrometer. The temperature was controlled by the spectrometer-interfaced computer and heating unit using a dry ice/ethanol bath for cooling and was monitored via a thermocouple implanted in the probe. One-dimensional ¹H spectra were collected in 16K data sets consisting of 128 scans each. Imino proton spectra were obtained according to the "1-1 hard pulse" solvent suppression method (Otting et al., 1987); spectra contained 16K data points consisting of 256 scans each. Partially deuterated sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) was used as the internal ¹H chemical shift reference.

³¹P NMR data were collected by using a broad-band probe tuned to the phosphorus frequency. Spectra were acquired by using a two-stage proton decoupling technique to minimize sample heating and take advantage of the positive ¹H-³¹P

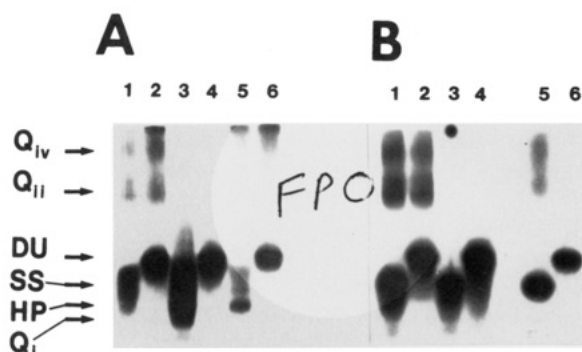


FIGURE 1: Effect of sodium and potassium on the electrophoretic mobility of TET4 and stability of resulting complexes relative to Watson-Crick DNA. Oligonucleotides were dialyzed into 20 mM NaP or KP containing 0.1 mM EDTA, ^{32}P -end-labeled with polynucleotide kinase, and applied to a 12% polyacrylamide gel containing 0.6X Tris-borate (pH 8.3) (=TBE) without further purification. The Na^+ and K^+ concentrations are approximately 40 mM at pH 7 in phosphate buffer. Gels were run at either 7 °C (panel A) or 30 °C (panel B). The stabilities of the complexes formed in the presence of each ion relative to the WC duplexes were probed by adding the WC complementary strand $d(\text{C}_4\text{A}_2)_4$ (=C). Lane 1, TET4 + K^+ ; lane 2, TET4 + K^+ + C; lane 3, TET4 + Na^+ ; lane 4, TET4 + Na^+ + C; lane 5, gel-purified TET4 preincubated at 4 °C in 10 mM Tris-HCl (pH 7) and 1 mM EDTA (=TE) then applied directly to the gel; lane 6, same as lane 5 + C.

nuclear Overhauser effect. Protons were broad band decoupling during the 1-s relaxation delay. Approximately 1200 scans were collected in 8K data sets. Internal trimethyl phosphate (TMP) was used as the ^{31}P chemical shift reference.

Selective spin-lattice relaxation data were acquired by using the standard $(180^\circ_{\text{selective}} - \tau - 90^\circ_{\text{nonselective}} - \text{observe})_n$ pulse sequence and data sets containing 16K data points. Selected thymidine H6 peaks were irradiated by using a decoupler pulse of approximately 2 ms, resulting in ca. 80% inversion (Wemmer et al., 1985; Wolk et al., 1988). Experimentals were actually semiselective in that some neighboring TH6 resonances were also partially irradiated. An 8-s relaxation delay was used to ensure equilibration between scans. Eighteen delay times ranging from 10 μs to 10 s (τ_{∞}) were used; most of the delay times were shorter than 100 ms to facilitate initial slope calculations; 256 scans were acquired per delay time.

RESULTS

Stabilization of Different TET4 Structures by Na^+ and K^+ : Intra- and Intermolecular Forms. TET4 samples were dialyzed into either NaP or KP buffer solutions at a concentration of 200 A_{260} units/mL (ca. millimolar in DNA strands). Samples were 5'-end-labeled with polynucleotide kinase and [^{32}P]ATP, resuspended into TE buffer containing 20 mM NaCl or KCl, and then applied to and electrophoresed on 12% nondenaturing polyacrylamide gels in TBE [89 mM Tris-borate (pH 8.3) containing 1 mM Na_2EDTA]. Electrophoresis patterns obtained at 7 and 30 °C are shown in Figure 1 (panels A and B, respectively). A detailed comparison of lanes 1 and 3 in panel A shows that species with very different electrophoretic mobilities exist when samples are preincubated in the presence of NaP or KP at ca. millimolar DNA concentration and then electrophoresed. Lane 5 shows the pattern obtained when TET4 was stored at 4 °C in TE (containing a minimal 2 mM Na^+), 5'-end-labeled, and then applied to the gel in TE. The patterns in lane 5 (TE preincubation) and lane 3 (NaP preincubation) both consist primarily of species that are *more mobile* than single-stranded DNA. Note that a single form predominates when the sample is preincubated

in TE (panel A, lane 5). In addition to this form (labeled "HP"), the NaP sample also contains a species that is *more mobile (more compact)* than any of the species seen in the lanes corresponding to the KP or TE samples (panel A, lane 3; labeled " Q_i "). In contrast, a distinctly different pattern that demonstrates the presence of *less mobile (larger)* species was obtained when TET4 was preincubated in KP (lane 1; labeled " Q_{ii} " and " Q_{iv} ").

Two related points must be noted and clarified. First, it is evident from the "spreading" of the bands (e.g., in panel A, lane 3) that an exchange process is occurring during the electrophoresis experiment. Second, throughout the text molecular conformations are referred to as "hairpin" (or "duplex") or "quadruplex". These terms are used to refer to two different *classes* of structures. The interpretation presented here is consistent with the experimental data, and the results obtained using different methods are internally consistent. However, the results do not allow assignment of individual specific structures under a given set of conditions. In fact, as noted above, it is clear that mixtures of species, which differ depending upon the solution conditions and method of preparation, are present in most cases.

If one views these results in the context of our previous results (Henderson et al., 1987) and those of Williamson et al. (1989) and Sen and Gilbert (1990), the patterns make sense. We conclude that the conformation in TE is due to a "folded-back" hairpin (HP; Henderson et al., 1987; Sen & Gilbert, 1990). The NaP-preincubated sample is interpreted as containing a mixture of hairpin, the more compact and mobile intramolecular quadruplex (Q_i ; Williamson et al., 1989), and possibly some bimolecular duplex (labeled "DU"; Sen & Gilbert, 1990). Finally, the less mobile species (Q_{ii} and Q_{iv}) are interpreted as being due to bimolecular and tetramolecular quadruplex structures (Sen & Gilbert, 1990).

We were able to gain some insight into the stability of these species by subjecting the samples to either higher temperatures (panel B) or by incubating the sample with the corresponding WC complementary strand (lanes 2, 4, and 6). When the electrophoresis temperature is increased to 30 °C (panel B), the populations of the more mobile species seen in lanes 3 and 5 on the 7 °C gel (panel A; HP and Q_i) decrease and the majority of the sample has the mobility of a single-stranded species (labeled "SS"). A minimal amount of the more mobile species is seen when the sample is preincubated at 30 °C in the presence of KP (lane 1), and the populations of the species with lower mobilities (Q_{ii} and Q_{iv}) do not diminish significantly. Thus, these complexes are more stable than the HP and Q_i species. This is consistent with assignment of the Q_{ii} band to the bimolecular species and the Q_{iv} band to the tetramolecular species. These assignments are consistent with the progressively less compact nature of the complexes and increased stabilities relative to the intramolecular HP and Q_i species. Note that when TET4 is preincubated in TE and electrophoresed at 30 °C, small amount of the Q_{ii} and Q_{iv} species appear, and the Q_i , HP, and DU complexes are gone (panel B, lane 5). The interpretation that Q_{ii} and Q_{iv} are quadruplex species is consistent with results obtained by Sen and Gilbert (1988) upon preincubating G-rich oligonucleotides derived from the immunoglobulin switching region in Tris buffer.

G-DNA Can Be Kinetically Preferred Relative to Watson-Crick Duplex DNA in the K^+ -Induced Form of TET4. In order to gain additional insight into the identity of the different species, we incubated the TET4 sample in NaP and KP with the corresponding WC complementary strand, d-

(C_4A_2)₄ (lanes 2, 4, and 6). The mobility of the Na^+ and Tris samples decreased due to almost quantitative formation of the expected duplex (DU; lanes 4 and 6). In contrast, while the intensity in the HP and Q_i bands is lost and replaced by approximately an equal amount of duplex band, the less mobile species in the KP sample (Q_{ii} , Q_{iv}) do not decrease in intensity at 7 °C (panel A, lane 2). This result demonstrates that the HP and Q_i species are less stable than the WC duplex. In contrast, the Q_{ii} and Q_{iv} species are not converted to the WC duplex. Thus, on the time scale of the experiment and at temperatures below 30 °C the Q_{ii} and Q_{iv} species are kinetically stabilized relative to the WC form. However, given sufficient time and a productive pathway to establish thermodynamic equilibrium, the WC form may predominate. This result makes sense when considered in light of the assigned structures discussed above. On a thermodynamic basis one might expect nearly equal stabilities (Sen & Gilbert, 1988; Aboul-ela et al., 1985). The bimolecular hairpin species would have approximately the same number of hydrogen-bonding interactions as the WC duplex (i.e., 8 G-quartets times 8 stacks, total 64, versus 16 C-G and 8 A-T base pairs, total 64). However, the quadruplex complex would have additional stabilization due to the ion-carbonyl coordination interactions and possibly stronger stacking interactions. The tetramolecular complex should be more stable than the WC duplex (i.e., 8 hydrogen bonds per G-quartet times 16 stacks, total 128) plus stacking interactions and ion-dependent stabilization.

Monovalent Cation-Dependent Structural Differences. (a) **HPLC Size Exclusion.** We chose two alternative methods to determine the effect of ions on the size of solution complexes formed by TET4 DNA, size-exclusion HPLC and semiselective spin-lattice relaxation measurements. The sample was preincubated at a concentration of 200 A_{260} /mL in either NaP, KP, or a 50:50 mixed solution of the two. The HPLC column was pre-equilibrated in either NaP or KP. The faster eluting form or forms were not retained by the column matrix as effectively and are therefore larger than the slower eluting species (Figure S1). When the NaP-preincubated sample was run with NaP elution buffer, almost all of the material elutes as the smaller species. As the KP concentration used during preincubation was increased, the fraction of less compact species increased in both elution buffers. More sample elutes in the enlarged form when KP was used during elution than when NaP was used. When the sample was preincubated in NaP and run with NaP or KP to elute, two small shoulders corresponding to slightly larger species were seen on the right side of the large peak which corresponds to the compact form(s). Note that larger "species" may represent more than one conformation if the peak corresponds to forms that are larger than one that is completely excluded from the column matrix.

(b) **Semiselective Spin-Lattice Relaxation.** The second method involved measuring the semiselective spin-lattice relaxation rates of the TET4 TH6 proton resonances in NaP or KP. The reciprocal of the initial slope of a plot of the extent of relaxation versus the relaxation interval (Figure S2) depends on the detailed spin environment of the proton and the motional characteristics of the molecule and the proton relative to the rest of the molecule (i.e., overall tumbling and internal fluctuations). These characteristics include the rates and motional amplitudes. If the environments and internal motions of the nucleus are similar in different conformations, the reciprocal slope is proportional to the average size of the molecule (Wemmer et al., 1985; Wolk et al., 1988). Since molecular

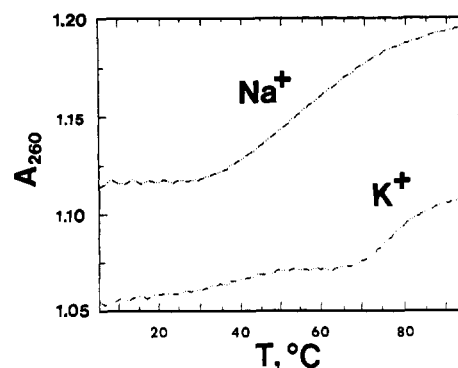


FIGURE 2: Absorbance thermal denaturation of TET4 in 20 mM NaP (pH 8) or KP (pH 8) buffers containing 0.1 mM EDTA monitored at 260 nm. Data were obtained at pH 8 because the complexes are slightly destabilized (relative to pH 7) with a decrease in T_m such that the upper base lines of the "melt" curves are visible in KP buffer. Samples were prepared and dialyzed into the respective buffers as described in Figure 1. The temperature was changed at a rate of 0.5 °C/min. Heating and cooling curves were essentially superimposable, demonstrating reversibility under these conditions.

reorientation about the long axis has a dominant effect on the relaxation rate, given the assumptions described above, the ratio of the reciprocal slopes corresponds to the ratio of the length of the complexes formed under the different conditions. Without making these assumptions, different slopes can unambiguously be interpreted as indicating the presence of different (mixtures of) complexes. The average slope values obtained with TET4 DNA (two determinations) were 4.4 (± 0.5) s^{-1} in NaP and 8.6 (± 1.3) s^{-1} in KP at 9 °C. Control measurements were also made with the hairpin-forming DNA molecule d(CG)₃T₄(CG)₃ in NaP or KP. The same relaxation rates were obtained in these control measurements (within experimental error), showing that different ions do not induce differential relaxation in the absence of significant structural changes. Thus, in the most conservative interpretation and in agreement with NMR results discussed above, different conformational forms of TET4 DNA are present under these conditions. If the assumptions described above are made, it can be concluded that the K^+ -induced conformation is approximately twice the length of the complex that is present in NaP. This interpretation is in agreement with the more qualitative length differences demonstrated by gel electrophoresis and HPLC size exclusion and is consistent with the presence of a tetramolecular quadruplex in KP at ca. millimolar DNA concentration and a hairpin species (bimolecular complex of hairpins, intramolecular quadruplex) in NaP.

The K^+ -Induced Form of TET4 Is Much More Stable Than the Na^+ -Induced Form. (a) **Absorbance Thermal Denaturation.** Absorbance "melt" data obtained with TET4 in NaP or KP at pH 8 are shown in Figure 2. Although a clear upper plateau is not seen, the absorbance approaches a limiting value by 95 °C in each case. Approximate T_m values are 55 °C in NaP and 80 °C in KP. The denaturation temperature range is broader in NaP than in KP, suggesting that more than one structural transition is being monitored in NaP. The onset of the primary transition is about 40 °C lower with the NaP sample than in the presence of KP. In addition, as predicted by the electrophoresis experiments, a minor transition that apparently corresponds to a small population of the hairpin form of TET4 can be seen in the profile for the KP sample in the 40 °C range.

(b) **Base Pairing Interactions Differ in Na^+ and K^+ .** TET4 DNA samples were dialyzed in parallel into either NaP or KP buffer (pH 7) containing 0.1 mM EDTA. Proton NMR

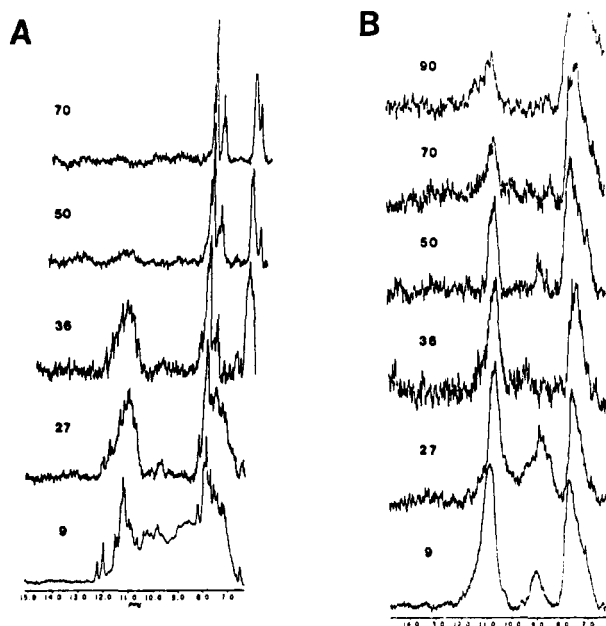


FIGURE 3: Effect of sodium and potassium on thermal denaturation of TET4 DNA as monitored by imino ^1H NMR. Samples were prepared by dialysis into either (A) NaP or (B) KP buffer (see Figure 1), lyophilized, and resuspended into 90% H_2O /10% D_2O . Spectra were obtained by using the 1-l hard pulse solvent suppression technique at the labeled temperatures. Similar T_m s and chemical shifts were obtained with two independently processed samples.

spectra were obtained with these samples at temperatures ranging from 9 to 90 °C (Figure 3). Remarkably, when the sample is dialyzed into KP (Figure 3B), imino proton signals are still present in the 10–12.5 ppm region at 90 °C, 40–50 °C higher than the temperature at which they are lost in NaP (Figure 3A). In addition, the imino proton spectrum obtained in KP is dispersed over a narrower chemical shift range than the spectrum obtained with the NaP sample. These results show that the base pairing interactions or environments are different in the presence of Na^+ and K^+ .

The data obtained with the TET4 sample that was dialyzed into NaP (Figure 3A) are similar to the results shown in our previous study (Henderson et al., 1987). However, while essentially the same peak pattern is present, the resonances are broadened and are less distinct than peaks in the spectrum of the C-18 "desalted"/NaP-resuspended sample used in the previous study. In addition, these resonances appear to be superimposed on a broad peak centered around 11 ppm similar to the pattern seen in KP (Figure 3B). Finally, the denaturation temperature is ca. 10 °C higher when the sample is prepared by the dialysis procedure relative to the value obtained with the C-18 "desalted"/NaP-resuspended sample [ca. 40 °C; see Henderson et al. (1987)].

The reason for the higher melting temperature and broadened imino proton peaks obtained with the NaP-dialyzed sample can be explained by considering the results in the context of a dynamic equilibrium between the structures proposed above. It is likely that KP stabilizes a four-stranded quadruplex since, due to the 4-fold axial symmetry, this structure should have an imino proton NMR spectrum with very similar chemical shifts. In contrast, the less symmetric hairpin would be expected to have a more dispersed NMR spectrum (Henderson et al., 1987). The higher T_m value obtained with the KP-dialyzed sample is also consistent with a quadruplex structures since it should be more stable. In addition, high (NMR-compatible) DNA concentration and K^+ should favor the multistranded quadruplex complex. In

general, NMR peak broadening can be attributed to either an increase in the molecular weight of the complex or exchange of the nucleus between different environments. Both explanations are consistent with the interpretation that the "desalting" procedure produces a preparation that is primarily composed of duplex species. It is possible that additional salt is retained by the DNA during the dialysis procedure relative to the limited amount that was added to the resuspended sample. Thus, exchange could occur more readily under these conditions and the equilibrium(a) would be expected to be driven to the more ion-rich (quadruplex-containing) structure(s). This would account for the apparent superposition of two types of spectra shown in Figure 3A, assuming that the "pure" spectra are due to hairpin [see Henderson et al., (1987)] and quadruplex (Figure 3B) species, respectively. Other NMR data and circular dichroism results (see below) are consistent with this interpretation.

Note that the samples used in the electrophoresis experiments have reduced amounts of the larger quadruplex species Q_{ii} and Q_{iv} . This is to be expected since they were diluted at least several orders of magnitude relative to the NMR conditions prior to electrophoresis and their dissociation should be very concentration-dependent (Sen & Gilbert, 1990).

In order to investigate intermediate states in the proposed monovalent cation-dependent equilibria, we obtained imino proton NMR spectra of TET4 in mixed NaP/KP buffers. Results are shown in Figure 4 for five mixed ion conditions: (A) 20 mM NaP/5 mM KP; (B) 20 mM NaP/10 mM KP; (C) 20 mM NaP/20 mM KP; (D) 10 mM NaP/20 mM KP; (E) 5 mM NaP/20 mM KP. There is a ca. 10 °C increase in denaturation temperature at each step as the KP concentration is increased from 0 to 5 mM then to 10 mM. When equal amounts (20 mM) of NaP and KP are present, the sample does not fully denature below 95 °C. As the KP concentration is increased from 10 to 20 mM, the low-temperatures imino proton spectrum changes from the dispersed pattern seen in NaP alone to the more homogeneous pattern seen in KP alone. However, as the temperature is increased, some fine structure can be seen on the downfield side of this peak envelope. Our interpretation of these results in the context of the proposed equilibrium is that there is an enhanced exchange between different species at elevated temperature and that the state of the equilibria depends on the temperature and on the concentration and type of ionic species.

An additional broad relatively homogeneous resonance becomes increasingly apparent around 9 ppm as the KP concentration is maintained at 20 mM and the NaP concentration is decreased (Figure 4D,E). This is the chemical shift range that might be expected for amino proton resonances (McConnell, 1984). Similar peaks were seen in the ^1H NMR spectra of the quadruplex gel complex formed by 0.5 M 5'-GMP in H_2O (Pinnavaia et al., 1975). It is also possible that these peaks correspond to either "trapped" or hydrogen-bonded thymidine imino protons (Tibanyenda et al., 1984; Henderson et al., 1987). A variety of different semiresolved peaks are seen in this chemical shift range in the presence of NaP (Figure 3A), while a relatively undispersed peak pattern is seen in the presence of KP (Figure 3B). As with the imino protons, all of the guanine amino groups should be in a similar environments in the quadruplex conformation and in relatively distinct environments in the hairpin form. In summary, all of the exchangeable proton data are consistent with the hypothesis that as the temperature decreases, we are monitoring the ion-dependent folding of a single-stranded species into a hairpin

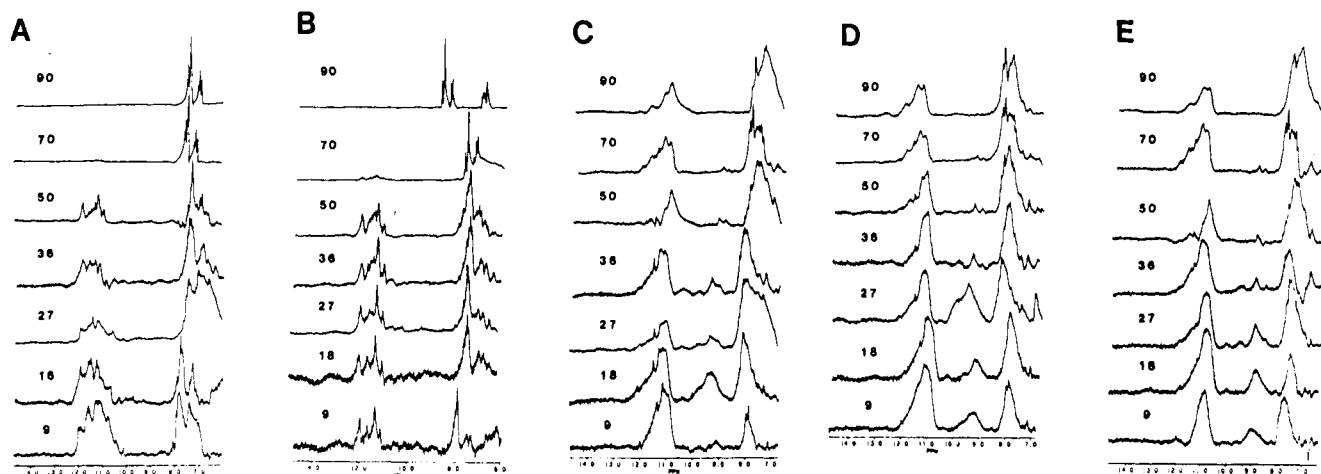


FIGURE 4: Effect of mixtures of sodium and potassium on thermal denaturation of TET4 DNA as monitored by imino ^1H NMR. Samples were prepared as described in Figure 2. Following those experiments, samples were lyophilized, aliquots of the appropriate concentrated buffer were added, and they were resuspended to yield the following conditions (pH 7, 90% $\text{H}_2\text{O}/10\%$ D_2O): (A) 20 mM NaP/5 mM KP; (B) 20 mM NaP/10 mM KP; (C) 20 mM NaP/20 mM KP; (D) 10 mM NaP/20 mM KP; (E) 5 mM NaP/20 mM KP. Spectra were obtained by using the 1-1 hard pulse solvent suppression technique at the labeled temperatures.

that either folds a second time to form an intramolecular quadruplex, condenses with a second hairpin to form a bimolecular complex, or unfolds and condenses with three other strands to form a tetramolecular quadruplex.

(c) ^{31}P and Aromatic Resonances. ^{31}P and ^1H NMR spectra were obtained at low and high temperatures with the NaP- or KP-dialyzed TET4 samples after transfer into D_2O (Figure S3). At 9 °C the Na^+ sample has a relatively dispersed ^{31}P NMR spectrum that collapses at temperatures above 40 °C to a single narrow resonance, corresponding to the random coil conformation. However, when comparing the data obtained after dialysis with results obtained with samples prepared by the C-18 "desalting"/resuspension method (Henderson et al., 1987), we found that like the imino proton resonances the spectra are broadened, apparently due to conformational exchange. In contrast, in KP buffer at 9 °C, the ^{31}P NMR spectrum of TET4 has much less apparent fine structure relative to the spectrum obtained in NaP. At 90 °C the spectrum partially collapses to a narrow peak centered at about 4.1 ppm, corresponding to the random coil conformation; however, a shoulder was also noted on the upfield side of the peak. In light of the imino proton results, we think that this shoulder can be attributed to the residual hydrogen-bonded complex that is still present at 90 °C indicated by the imino proton results (Figure 3B). A comparison of the peak intensities demonstrated directly that the NaP and KP concentrations are approximately the same relative to the DNA concentrations. Thus, the phenomenon demonstrated here is not due to differential ionic strength effects.

The nonexchangeable ^1H NMR spectra of TET4 obtained in the presence of NaP and KP were also very different. At 9 °C in NaP the peaks were dispersed over ca. 1.2 ppm. NOESY results show that almost every peak in the spectrum could be assigned to a specific residue type (Henderson et al., 1987). Again, the peaks in the spectrum of the NaP-dialyzed sample are apparently exchange-broadened relative to the peaks in the spectrum of the C-18 "desalted"/resuspended sample (Henderson et al., 1987). As the temperature was increased to 60 °C, the spectrum of the NaP-dialyzed sample collapsed to that of a random coil conformation, with chemical shifts reflecting only the position within the linear sequence. In contrast, most of the intensity in the spectrum of the KP-dialyzed sample fell within about 0.8 ppm and no distinctly resolved peaks were present. When the temperature was raised

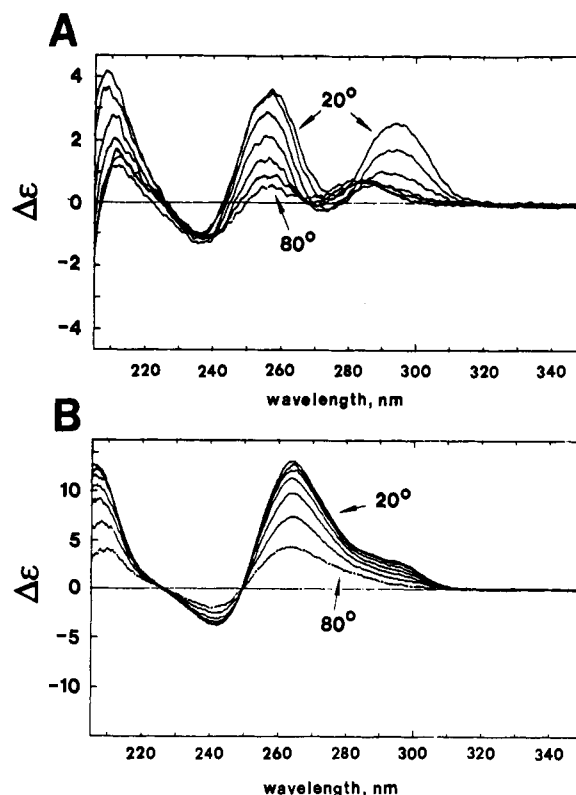


FIGURE 5: Effect of sodium and potassium on thermal denaturation of TET4 DNA as monitored by circular dichroism. (A) Sep-pak pretreated sample in 10 mM NaP; (B) sample was dialyzed into 20 mM KP. Spectra were obtained at 20–80 °C in 10 °C intervals. Spectra corresponding to the temperature extremes are labeled.

to 90 °C, the spectrum did not collapse to that of the random coil, consistent with the imino proton and ^{31}P NMR results. Thus, both the ^{31}P and nonexchangeable ^1H NMR results are consistent with the interpretations presented above regarding a multispecies equilibrium.

(d) *Circular Dichroism.* Figure 5 shows CD spectra for TET4 DNA in NaP and KP obtained as a function of temperature. These spectra demonstrate that the chiral environments of the bases are not the same under these conditions. The bilobed CD spectrum seen in Figure 5A is only obtained when the sample is prepared according to the C-18 "desalting" procedure; however, the ca. 290-nm band persists to a limited

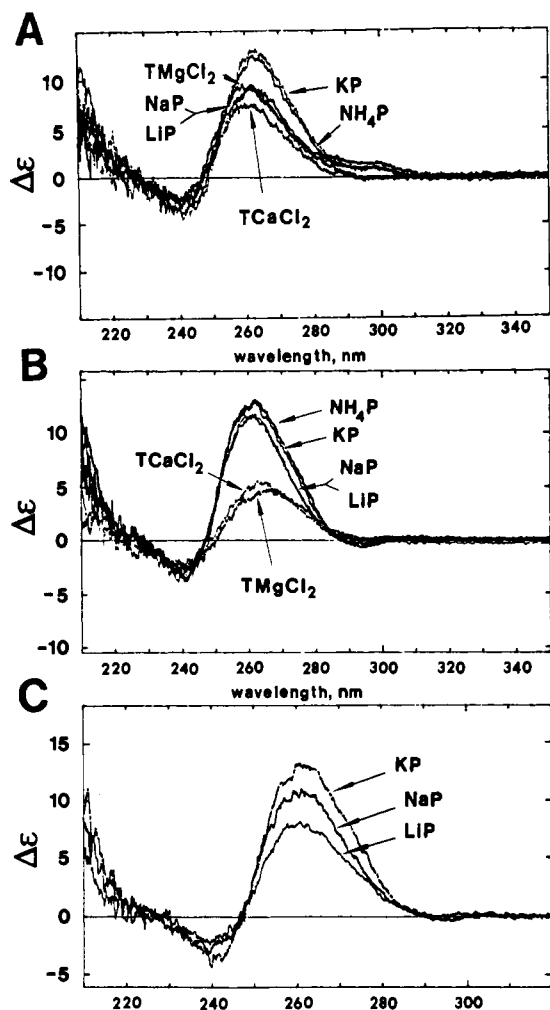


FIGURE 6: Differential effects of mono- and divalent cations on the circular dichroism (CD) spectrum of TET4 and *Dictyostelium* DNAs. (A and B) Samples were dialyzed into 100 mM lithium (LiP), sodium (NaP), potassium (KP), and ammonium (NH_4P) phosphate (pH 7) buffers containing 0.1 mM EDTA, or into 20 mM Tris-HCl (pH 7) and 0.1 mM EDTA containing either 100 mM MgCl_2 (TMgCl_2) or CaCl_2 (TCaCl_2). Samples were heated to 90 °C and allowed to cool slowly to 4 °C. Spectra were measured at 9 °C and were reproducible after storage for >6 months at 4 °C. (A) TET4 DNA; (B) the consensus repeat sequence from *Dictyostelium* (DYM) telomeric DNA, $\text{d}(\text{AG}_6\text{GAGAG}_6\text{AG}_6)$. (C) CD spectra for DYM DNA in 1 M LiP, NaP, and KP (pH 7) buffers at 9 °C.

extent whether the sample is resuspended or dialyzed into NaP (Figure 6). In addition, a TET4 sample that has been dialyzed into KP can be converted to a preparation with the bilobed CD spectrum by the C-18 "desalting" treatment followed by transfer to NaP. Since the bilobed spectrum is recovered after heating to 80 °C and cooling, the conformation that corresponds to this type of spectrum is thermodynamically stable and is not "trapped" due to the "desalting" procedure. Since the presence of the ca. 290-nm band is correlated with the more mobile HP and Q₁ species on nondenaturing gels (Figure 1), the dispersed imino proton NMR spectrum (Figure 3 and 4; Henderson et al., 1987), and less stable structural characteristics (Figures 2–4), we propose that it is due to the chiral environments of the bases in the non-Watson–Crick base-paired hairpin. The CD spectrum shown in Figure 5B with a maximum ellipticity at 264 nm and a smaller shoulder in the 280–305-nm range is then attributed to a quadruplex-enriched preparation.

Additional evidence for the latter assignment comes from separate studies with a model quadruplex complex composed of four strands of $\text{d}(\text{CGCG}_4\text{GCG})$ (C. Bailey, T. Watson, M.

Corregan, and C. C. Hardin, unpublished experiments). This molecule forms a complex that is much more stable in the presence of K^+ or NH_4^+ than in Na^+ . The CD spectrum of this complex is the same in the presence of KP and NaP buffers and is similar to the spectrum obtained with the KP-dialyzed form of TET4 DNA in the 200–280-nm region. In contrast to TET4 DNA, the spectrum of the $[\text{d}(\text{CGCG}_4\text{GCG})_4\text{-ion}]$ complex falls to zero above 280 nm. The molecule cannot form a non-Watson–Crick base-paired hairpin and does not adopt a conformation with a different CD spectrum when "desalted" by C-18 chromatography. Thus, its CD spectrum can be specifically assigned to a quadruplex structure. The molecule $\text{d}(\text{CGCG}_3\text{GCG})$ also forms a quadruplex that is preferentially stabilized by K^+ and has a CD spectrum similar to that of TET4 in KP ($\Delta\epsilon_{\text{max}} = 264$ nm). In contrast to $\text{d}(\text{CGCG}_4\text{GCG})$, however, this molecule can also undergo a reversible transition to a Watson–Crick hairpin (Hardin et al., 1991).

As the temperature is raised, the intensities of both CD bands decrease in the presence of NaP and KP (Figure 5). In the presence of NaP, the intensity of the 290-nm band decreased quickly with increased temperature and is essentially zero by 50–60 °C. This coincides with the denaturation temperature of the hairpin species according to our interpretation of the imino proton NMR experiments (Figure 3A; Henderson et al., 1987). In contrast, in the presence of KP the intensity of this band decreases progressively as the temperature is raised yet does not bottom out by 80 °C. This result is also consistent with the imino proton NMR thermal denaturation experiments (Figure 3B) since it indicates that the molecule has not completely denatured at 80 °C. Similar results were also obtained with the $[\text{d}(\text{CGCG}_4\text{GCG})_4\text{-ion}]$ and $[\text{d}(\text{CGCG}_3\text{GCG})_4\text{-ion}]$ complexes discussed above (Hardin et al., 1991). Thus, we can correlate the decrease in intensity in the 290-nm range with denaturation of a hairpin species and a decrease in intensity at 264 nm with denaturation of a quadruplex species. These results are consistent with the interpretation that at intermediate DNA concentration (ca. 10 μM in the CD experiments) the NaP sample contains predominantly hairpin with some quadruplex, while in KP the sample adopts the quadruplex conformation and is present to a lesser extent as the hairpin.

Note that the putative quadruplex CD bands in Figure 5, panels A and B, have slightly different $\Delta\epsilon_{\text{max}}$ values, ca. 258 and 264 nm, respectively. It is possible that this is due to the presence of different specific quadruplex conformations, i.e., the four-stranded species in KP and the two-stranded species (composed of two hairpins) in NaP. The "tailing" to lower mobility seen in the electrophoresis pattern in Figure 1, panel A, lane 3, may be due to this kinetic exchange process.

Effect of Mono- and Divalent Cations on the CD Spectra of TET4 and the *Dictyostelium* Telomeric DNA. TET4 and the telomeric DNA from *Dictyostelium*, $\text{d}(\text{AG}_6\text{GAGAG}_6\text{AG}_6)$ (referred to as "DYM DNA"), were dialyzed and preincubated in the following ion solutions: 100 mM lithium (LiP), sodium (NaP), potassium (KP), or ammonium (NH_4P) phosphate (pH 7), or 50 mM magnesium (TMgCl_2) or calcium chloride (TCaCl_2) in 20 mM Tris-HCl (at pH 7) containing 0.1 mM EDTA. Spectra are shown in Figure 6, panels A and B, for TET4 and DYM DNAs, respectively. The effects of K^+ and NH_4^+ on the CD spectrum of TET4 DNA are similar and are quite different from those of Na^+ and Li^+ . The CD intensities at 264 nm are more intense in the former cases than in the latter. The intensity of the 264-nm band for the sample in TMgCl_2 is similar to that obtained with LiP and NaP and is

slightly less in the spectrum of the TCaCl_2 sample. The spectra also differ depending on the cation in the 280–305-nm region. Approximately equal intensities are seen with all of the monovalent cations, while the samples containing divalent ions have little CD above 285 nm. The CD intensity in this range is slightly higher in NaP than in the other ion solutions despite the fact that this sample was not "desalted" prior to the dialysis and preincubation procedures. However, note that the intensity of the 290-nm band relative to the 264-nm band is clearly less in 100 mM NaP than in 20 mM NaP.

If we follow the assumption described above that the intensity at ca. 290 nm reflects the presence of the hairpin species, these results indicate that it does not readily form in solutions containing divalent cations. These results are consistent with ion dependences determined with the model quadruplex complex formed by $\text{d}(\text{CGCG}_3\text{GCG})$ (Hardin et al., 1991). These studies showed that divalent cations stabilize the quadruplex better than monovalent cations of equivalent ionic radius. In order to obtain relative stabilities for the quadruplex structure(s) formed by TET4 in different ion solutions, one must make at least two assumptions. First, one must assume that TET4 forms the same type of quadruplex complex in the presence of the different ions at these concentrations. Second, one must assume that changing the ion does not significantly perturb the transition dipoles corresponding to the CD band at 264 nm. When the temperature is raised ca. 60 °C in 20 mM KP (Figure 5b), the CD decreases progressively to yield a spectrum that essentially matches the 10 °C spectrum in 100 mM NaP (Figure 6b). This is reasonable evidence that the first assumption is valid. Thus, if one can make the second assumption, this direct comparison shows that K^+ stabilizes the quadruplex form of G-DNA slightly more effectively than NH_4^+ and that both are considerably more effective than the other ions.

The ionic radii of the hexacoordinated ions are as follows (Dobler, 1981): K^+ , NH_4^+ (1.33 Å); Ca^{2+} (0.99 Å); Na^+ (0.97 Å); Li^+ (0.68 Å); Mg^{2+} (0.66 Å). To reiterate, the CD intensities vary as follows: $\text{K}^+ > \text{NH}_4^+ \gg \text{Na}^+, \text{Li}^+, \text{Mg}^{2+} > \text{Ca}^{2+}$. Thus, with the exceptions of the divalent cations Ca^{2+} and Mg^{2+} , the CD intensities at 264 nm are correlated with the size of the cation. Assuming that the CD intensity at this wavelength is indicative of complex stability, these results indicate that both ionic radius and charge affect the extent of stabilization induced by the ion.

Unlike TET4, DYM DNA has no thymidine residues. However, according to electrophoretic evidence presented in our previous study (Henderson et al., 1987), it can form an apparent intramolecular species in NaP, following the C-18 "desalting" procedure, that is about as homogeneous as the complex formed by TET4. Monovalent cations have similar effects on the CD spectrum of DYM DNA, while divalent ions decrease the CD intensity at 264 nm to almost one-third the value seen with the monovalent cations (Figure 6b). There is a small but clear difference between the spectra obtained in KP or NH_4P and those obtained in LiP or NaP . As with TET4 (Figure 6a), the largest CD intensities at 264 nm are obtained in the presence of K^+ and NH_4^+ . If one assumes that the CD intensity at 264 nm is diagnostic of the degree of stabilization of the quadruplex complex in the different ion solutions, these results show that KP and NH_4P stabilize the DYM DNA complex slightly better than LiP and NaP and that the divalent ions are much less effective. Thus, different telomeric sequences are affected by specific ions to different extents. A better discrimination between the monovalent cations is obtained when they are present at 1 M (Figure 6c).

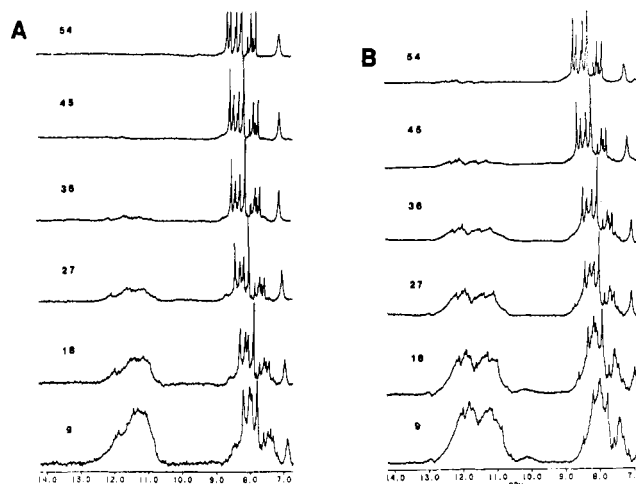


FIGURE 7: Effect of sodium and potassium on thermal denaturation of human telomeric sequence DNA, $\text{d}(\text{T}_2\text{AG}_3)_4$ (HUM4), as monitored by imino ^1H NMR. Samples were prepared by dialysis into either (A) NaP, or (B) KP buffer (see Figure 1), lyophilized and resuspended into 90% H_2O , 10% D_2O . Spectra were obtained as described in Figure 3.

Under these conditions, the change in CD intensity at 264 nm correlates very well with the ionic radius. In contrast, there was little difference in the relative intensities in the CD spectra of TET4 DNA obtained in the presence of 100 mM or 1 M cation.

Imino proton thermal denaturation experiments were also carried with this molecule (results not shown). There is a ca. 10 °C higher denaturation temperature in KP relative to NaP (80 °C versus 70 °C); however, the spectra are both similar to the spectrum of TET4 DNA in KP, indicating that the molecule probably adopts an intermolecular quadruplex conformation in the presence of both cations. This conclusion is consistent with our interpretation of the CD data in light of the results obtained with TET4 DNA since the slight difference in intensity at 264 nm indicates that there is a slight difference in stability but not a radical structural difference. Also, there is no positive CD intensity in the 280–305-nm range, consistent with the interpretation that little if any of the hairpin form of this molecule is present when prepared by dialysis. Sundquist and Klug (1989) noted that adenine can theoretically be accommodated within the quadruplex motif. We show further evidence for this conclusion obtained with the human telomeric sequence $\text{d}(\text{T}_2\text{AG}_3)_4$ below.

K⁺-Induced Structural Stabilization Is Sequence-Dependent. Samples of $\text{d}(\text{T}_2\text{AG}_3)_4$, the human telomeric repeat sequence (referred to as HUM4), were also dialyzed in parallel into either NaP or KP buffer. Results from imino proton NMR thermal denaturation experiments performed with these samples are shown in Figure 7. Different resonance patterns can be seen in NaP and KP; however, unlike the spectra obtained with TET4 DNA, the resonances are less dispersed in the presence of NaP than in KP. Different patterns are also seen in the 8 ppm region at 9 °C; however, the patterns in this region are similar if one compares the 27 °C data in NaP with the 36 °C data in KP. On the basis of these results, the conformational status of HUM4 DNA is different in NaP and KP but is not radically different as seen with TET4 DNA. The disappearance of the imino proton resonances is displaced upward by ca. 10 °C in KP relative to NaP, demonstrating that K^+ has a stabilizing effect relative to Na^+ .

Circular dichroism results obtained in the presence of NaP or KP (Figure 8) concur with this conclusion. Samples were prepared by HPLC and dialyzed into the appropriate buffer;

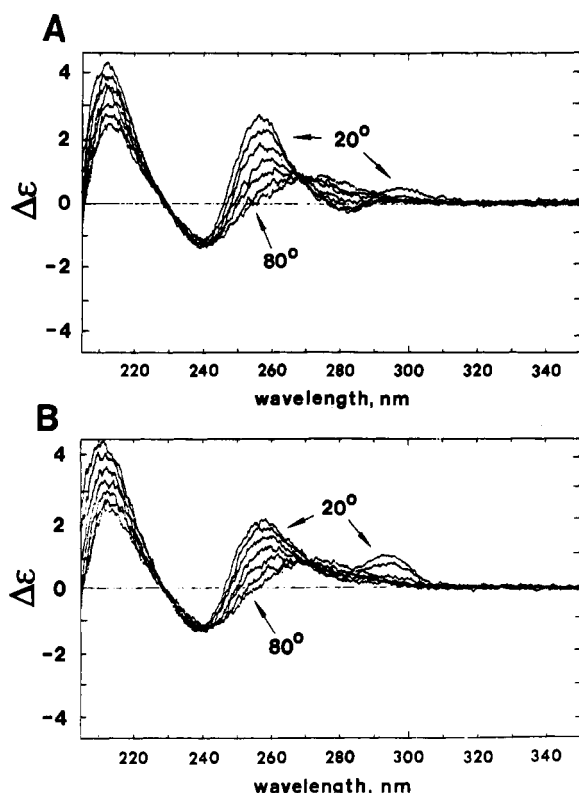


FIGURE 8: Effect of sodium and potassium on thermal denaturation of HUM4 DNA as monitored by circular dichroism. Samples were prepared as described in Figure 1 and then diluted to 0.8 A_{260}/mL . Panels correspond to (A) NaP or (B) KP buffer, respectively.

CD spectra were obtained in 1-cm cells in the 20–80 °C range. It is interesting that in both buffers in the 20–30 °C range the spectra resemble those obtained with TET4 DNA in NaP at 40–50 °C when the sample was prepared by the C-18/“desalting” procedure (Figure 4A). As seen in the NMR experiments, the CD spectra obtained under a given set of conditions in NaP match spectra obtained at a 10 °C higher temperature in KP. Thus, these results are consistent with the imino proton NMR results in showing that K^+ increases the denaturation temperature by about 10 °C relative to Na^+ . A possible interpretation regarding the presence of the CD band at ca. 295 nm based on the results obtained with TET4 DNA is that a limited population of HUM4 DNA can adopt a hairpin conformation and that the equilibrium is less dependent on the ionic conditions than with TET4 DNA. More detailed studies will be required to confirm or discount this possibility. In any case, these results show that substituting an adenine residue for a guanine in the telomeric consensus sequence results in a decrease in the stabilization due to K^+ relative to Na^+ .

DISCUSSION

When samples are “desalted” by C-18 chromatography and then resuspended at high (ca. millimolar) DNA concentration into 20 mM NaP (Henderson et al., 1987), one obtains a well-resolved exchangeable proton spectrum that is dispersed over the 9.4–12.6 ppm range. Since this implies that a number of quite different imino proton environments exist, and because one would expect relatively homogeneous chemical shifts for a quadruplex structure, we think the data are most consistent with formation of a hairpin-like conformation under these conditions. Williamson et al. (1989) proposed that the linear DNA molecule folds into an intramolecular quadruplex structure containing two sets of connected antiparallel strands

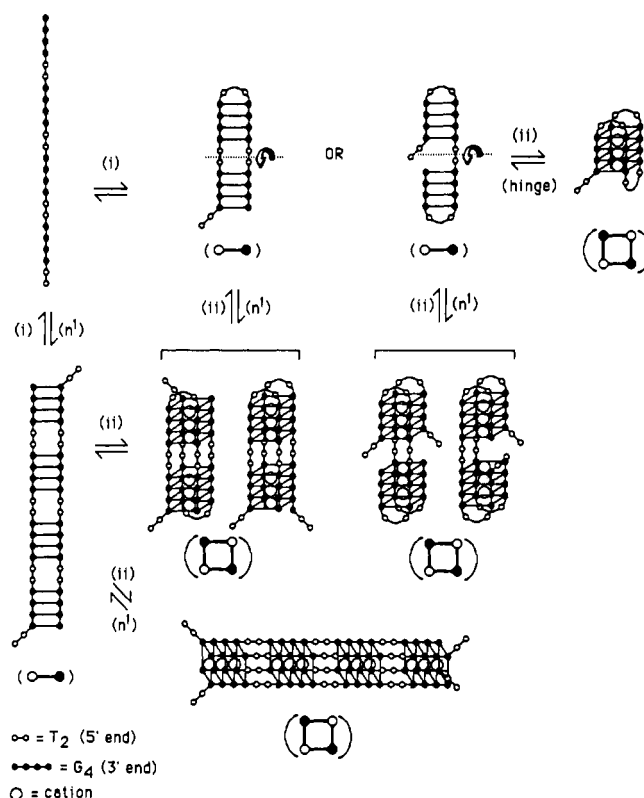


FIGURE 9: Possible folding pathways involved in ion-induced formation of G-quadruplex conformations in TET4 DNA. Individual folding steps involve one of two mechanisms: (i) pairing of individual strands to form non-WC duplex (hairpin) structures driven by counterion condensation and hydrophobic effects or (ii) pairing of non-WC duplexes to form quadruplexes driven by formation of octahedral coordination complexes involving monovalent cations “caged” within two stacked G-quartets. Reactions are zeroth order with respect to DNA concentration in the intramolecular folding steps and first order (n^1) in the intermolecular folding steps. Intramolecular quadruplex formation is identified by the term “hinge”. Symbols: (O) thymidine residues; (●) deoxyguanosine residues. The strand polarities are indicated below the structures. Open circles represent a 5' to 3' strand directionality going into the page, closed circles represent the opposite strand direction. Note that only antiparallel configurations are shown. Analogous parallel-stranded structures may also be possible. Also, note that one of the bimolecular structures formed by the condensation of two hairpins cannot form via the “hinge” mechanism because the loops are at opposite ends.

at low DNA concentration (ca. micromolar) in buffer containing either 50 mM NaCl or 50 mM KCl. In addition, they found that, in the presence of limiting Na^+ (i.e., in 0.5× TBE; 2 mM Na^+), TET4 has the mobility of a 12-mer. In contrast, in the presence of 50 mM Na^+ , the molecule adopts a conformation with the mobility of a 6-mer. Comparing these conclusions with the results shown in Figure 1 demonstrates that the structure adopted by TET4 is very dependent on the method of preparation (i.e., cation type and concentration, DNA concentration). Thus, it is important to keep these factors in mind when comparing results from electrophoresis studies with those obtained in experiments that involve the use of more concentrated samples such as absorbance, circular dichroism, and especially NMR measurements.

G-DNA Structural Equilibrium Scheme. In order to rationalize the results presented in this paper and previous papers (Henderson et al., 1987; Williamson et al., 1989; Sundquist & Klug, 1989; Sen & Gilbert, 1990), we propose the equilibrium scheme shown in Figure 9. The model predicts the type(s) of structures that should occur in a manner consistent with structural and kinetic considerations. In addition, it includes a structural explanation for the specific effect of K^+

that is based on conformations that can conceivably be adopted by $d(T_2G_4)_4$ and reasonable pathways of formation.

The quadruplex structure can be formed in several ways, from one, two, or four strands. Note that two kinetic reaction orders can occur in each of two steps, unimolecular (by intramolecular folding) and bimolecular (by condensation of two strands). The mechanisms for complex formation involve two different modes of ion binding, (i) counterion condensation and (ii) octacoordinate cryptand (host-guest) complex formation. Examples for the cases of one, two, and four strands are shown in Figure 9. Step i in these mechanisms can be driven by two factors. First, since guanosine is by far the most hydrophobic of the bases, stacking and base pairing will exclude water and thus favor the complex in aqueous solution. Second, when the anionic phosphodiester backbones approach each other in the paired complex, the linear charge density increases substantially. This is offset by increasing the ionic strength since cations will "condense" around the structure until thermodynamic equilibrium is achieved, resulting in electrostatic neutralization. Thus, Na^+ and K^+ are essentially equally effective in driving step i. Differences in ionic hydration may also be a limiting factor in the relative abilities of the ions to exchange between solvated and complexed pools (Dobler, 1981; W. S. Ross and C. C. Hardin, unpublished calculations).

Step ii will be preferentially driven by K^+ for the following reason. When a "G-quartet" hydrogen-bonding complex forms, four carbonyl oxygen atoms are juxtaposed in a square-planar geometry (Zimmerman et al., 1975). As stated by Sundquist and Klug (1989), when two G-quartets are on adjacent planes separated by the pitch of WC DNA (≈ 3.4 Å), the geometry is such that the carbonyl oxygen- K^+ distance is 2.8 Å, in good agreement with the value observed in a number of polyether- K^+ cryptand complexes (2.78 Å). In contrast, the value for typical Na^+ chelation complexes is 2.4 Å, significantly smaller than the cavity formed by adjacent G-quartets. Ammonium has a van der Waals radius almost equal to that of K^+ , thus explaining why NH_4^+ stabilizes complex formation (Figure 6; Howard & Miles, 1982a,b).

Uni- or bimolecular reactions can also occur in step ii. In essence the inter- or intramolecular duplex structures are intermediates in the formation of the different types of quadruplex structures. Note that the observed reaction order may reflect the strand stoichiometry of the rate-limiting step in complex formation if the transition-state free energy for that step dominates (Sen & Gilbert, 1990). While we do not present evidence that demonstrates a specific strand polarity for the four-stranded complex, the studies of Sen and Gilbert (1988, 1990) show that the strands probably have the same polarity. Due to the constraints placed on the bimolecular hairpin and intramolecular quadruplex conformations, antiparallel strands must be present. It is possible that isomeric species with different strand polarities can form.

On the basis of results in this paper and previous studies, we can list several factors that can influence the status of the equilibrium shown in Figure 9. These factors include the following: (i) different ions, (ii) DNA and ion concentrations, (iii) length of the G-rich strand, (iv) WC complementary DNA, and (v) sequence differences. The major conclusion is that the predominant physiological monovalent cations, Na^+ and K^+ , have very different affinities for and stabilizing effects on structures that are formed by telomeric DNAs. The availability of ions in physiological situations is usually regarded to be saturating relative to the concentrations investigated in these studies (≤ 40 mM); however, this may not be

true. Cations may be effectively sequestered due to competition between different anionic species in the intracellular (or organellar) milieu (Ling, 1984). Therefore, non-G-quartet-containing structures (i.e., non-WC base-paired hairpins) may be present depending on the solution conditions, temperature, and effective DNA concentration.

Ammonium stabilizes the quadruplex structures formed by TET4 and DYM DNAs to an extent similar to K^+ , while Na^+ , Li^+ , Mg^{2+} , and Ca^{2+} are less effective. The stabilizing effect of the monovalent cations can be correlated with the ionic radii. However, charge also has an influence in the cases shown here since the divalent cations apparently stabilize the complexes less effectively than the monovalent cations despite the fact that Mg^{2+} and Ca^{2+} have radii similar to Li^+ and Na^+ , respectively.

The quadruplex complex formed by TET4 DNA is much more stable in KP than in NaP. Consequently, the WC complement $d(A_2C_4)_4$ does not interact nearly as effectively in KP to form the WC base-paired duplex. The number of contiguous guanine residues in the telomeric repeat can affect the degree of differential stabilization due to the monovalent cations. By changing the sequence from $d(T_2G_4)_4$ to $d-(T_2AG_3)_4$, the difference in denaturation temperature in going from KP to NaP decreases from ca. 45 to ca. 10 °C. In the latter case one would predict that the WC complement should be able to compete more effectively with the quadruplex complex to form the WC duplex in the presence of K^+ .

How relevant are these effects in vivo? Potassium is the predominant monovalent cation in eukaryotic cells while Na^+ is present at less than half the K^+ concentration. While the exact concentrations vary with cell type, typical basal ranges are ca. 30 mM in Na^+ and ca. 110 mM in K^+ (Boynton et al., 1982; Ling, 1984; Lau et al., 1988). However, it has been pointed out that these levels are unlikely to be the free ion concentrations and that they (especially K^+) are subject to change depending on the cell type and physiological status (Ling, 1984). In fact, during the normal cell cycle the K^+ concentration increases from ca. 90 to 130 mM [Lau et al. (1988) and references therein]. This range can be much wider in bacterial cells [e.g., the K^+ concentration in *Escherichia coli* cells can range between 300 and 600 mM; in some halophilic bacteria the K^+ concentration can be as high as 7 M; see Schein (1990)]. On the basis of these facts, our results, and previous proposals (Ling, 1984; Sen & Gilbert, 1988; Williamson et al., 1989; Sundquist & Klug, 1989), we have developed a hypothesis in which ion fluctuations (especially K^+) play a role in the control of nucleic acid dependent physiological functions via their effect on the structure of G-rich sequences.

If one considers the equilibrium scheme shown in Figure 9 in a potential biological context, the complexes may represent different associations that could be adopted by four-repeat overhanging telomeric G-rich strands attached to native chromosomes. While the length of the overhang is thought to be on the order of 12–16 bases (Klobutcher et al., 1981; Pluta et al., 1982; Henderson & Blackburn, 1989), it is possible that more single-stranded DNA forms due to fraying events (or telomerase activity) during part of the cell cycle (i.e., during macronuclear development) or that under some conditions the adjacent WC base-paired duplex can be displaced to form the proposed structures. On the basis of previous results (Henderson et al., 1987) and other studies (Oka & Thomas, 1987; Williamson et al., 1989; Sundquist & Klug, 1989; Sen & Gilbert, 1990), we reiterate the proposal that the properties that are inherent in telomeric sequences impart the charac-

teristics that are important for telomere function. In this regard, the structures shown in Figure 9 may each be functionally relevant and the properties that are demonstrated in this paper may be involved in the control of these functions. As outlined above, the structures can be classified according to the number of strands involved in the complex. In a functional context this suggests that formation of the monomolecular complex, which would be favored by low K^+ and DNA concentrations, helps to protect the chromatids from exonuclease recognition and degradation (Oka & Thomas, 1987) or may be involved in recognition by telomere-specific binding proteins (Gottschling & Zakian, 1986; Price & Cech, 1989; P. Qiu and E. Henderson, unpublished experiments). The net effect would be intact and faithful propagation of the genomic information. Extending this scheme to the bimolecular species, as the K^+ concentration increases during the cell cycle, the bimolecular species, which contains six coordinated cations, would be favored over the monomolecular species with three coordinated cations. On the basis of previous proposals (Sen & Gilbert, 1988; Sundquist & Klug, 1989), this could act as an initial point of alignment for chromosomal pairing in preparation for homologous recombination events. Finally, as the K^+ concentration increases late in the cell cycle, the equilibrium shifts to the tetramolecular species, which could correspond to the process of meiotic (or mitotic) association (Sen & Gilbert, 1988). Following this event the daughter cells form and the next cycle begins.

Sen and Gilbert (1988) have demonstrated the generality of this ion-dependent phenomenon using DNAs derived from the switching region of immunoglobulin genes. The underlying common factor is the occurrence of repeating tracts of three or more contiguous guanine residues. It is interesting and possibly functionally significant that such tracts are present in several important sequence contexts in eukaryotic genomes including telomeres (Blackburn & Szostak, 1984; Blackburn, 1986), immunoglobulin switching regions [Sen and Gilbert (1988) and references therein], and transcriptional promoters (Cooney et al., 1988; Nussinov et al., 1988; Gottschling et al., 1990; Mueller & Wold, 1989; Caddle et al., 1990) and near intron-exon splice sites (Nussinov, 1989). On the basis of these occurrences, it is possible that the ion-dependent structural rearrangements that are demonstrated and partially characterized in this paper may be functionally relevant in a general way.

ADDED IN PROOF

Raghuraman and Cech (1990) recently tested the relative effects of Na^+ and K^+ on the stability of the *Oxytricha* telomeric DNA $d(T_4G_4)_4$ and recognition by a specific binding protein. They estimated ΔG values (at 37 °C) of -2.2 kcal/mol in 50 mM Na^+ and -4.7 kcal/mol in 50 mM K^+ . Estimated half-lives for unfolding (at 37 °C) were 4 h in Na^+ and 18 h in K^+ . They also found that the completely folded DNA was not recognized by the telomere-binding protein. These results suggest that K^+ -induced quadruplex formation may function as a "limiting" mechanism in vivo to prevent "runaway" addition of telomeric DNA to the chromosomal terminus [e.g., Greider and Blackburn (1987)].

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SUPPLEMENTARY MATERIAL AVAILABLE

Size-exclusion chromatography (Figure S1), semiselective inversion-recovery (Figure S2), and ^{31}P and nonexchangeable 1H NMR thermal denaturation (Figure S3) results for TET4 DNA in NaP and KP (4 pages). Ordering information is given on any current masthead page.

Registry No. $d(T_2G_4)_4$, 66976-17-8; $d(T_2AG_3)_3$, 117490-04-7; K, 7440-09-7; Na, 7440-23-5; guanine, 73-40-5.

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Intramolecular Triplex Formation of the Purine·Purine·Pyrimidine Type[†]

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ABSTRACT: Six octadecamers with hairpin motifs have been synthesized and investigated for possible intramolecular triplex formation. Electrophoretic, hypochromic, and CD evidence suggest that d(CCCCTTTGGGGTTTGGGG) and d(GGGGTTTGGGGTTTCCCC) can form G·G·C intramolecular triplexes via double hairpin formation in neutral solutions, presumably with the terminal G tract folding back along the groove of the hairpin duplex. In contrast, d(GGGGTTTCCCCTTTGGGG) and the three corresponding 18-mers containing one G and two C tracts each forms a single hairpin duplex with a dangling single strand. The design of the sequences has led to the conclusion that the two G tracts are antiparallel to each other in such a triplex. Magnesium chloride titrations indicate that Mg^{2+} is not essential for such an intramolecular triplex formation. The main advantage of our constructs when compared to the intermolecular triplex formation is that the shorter triplex stem can be formed in a much lower DNA concentration. The merit of G·G·C triplex, in contrast to that of C⁺·G·C, lies in the fact that acidic condition is not required in its formation and will, thus, greatly expand our repertoire in the triplex strategy for the recognition and cleavage of duplex DNA. Spectral binding studies with actinomycin D (ACTD) and chromomycin A₃ (CHR) as well as fluorescence lifetime measurements with ethidium bromide (EB) suggest that although hairpin duplexes bind these drugs quite well, the intramolecular triplexes bind poorly. Interestingly, the binding densities for the strong-binding hairpins obtained from Scatchard plots are about one ACTD molecule per oligomeric strand, whereas more than two drug molecules are found in the case of CHR, in agreement with the recent NMR studies indicating that CHR binds to DNA in the form of a dimer.

Homopurine-homopyrimidine sequences have been mapped to several sites in the regulatory regions of eukaryotic genes that are found to be hypersensitive to single-strand-specific nucleases such as S1 (Larsen & Weintraub, 1982; Nickol & Felsenfeld, 1983; Elgin, 1984). These sequences have been suspected to exhibit unusual DNA structures, as they are known to undergo a transition in plasmids to an underwound state under conditions of moderately acidic pH and negative supercoiling (Wells et al., 1988, and references cited therein). Recent studies on these systems (Htun & Dahlberg, 1988;

Johnston, 1988) appear to support a model consisting of a triple-stranded (pyr·pur·pyr) plus a single-stranded structure called H-DNA (Lee et al., 1984; Lyamichev et al., 1986).

Triplex formation has also been exploited as a strategy for DNA recognition and site-specific double-helical cleavage (Mosher & Dervan, 1987; Strobel et al., 1988; DeDoan et al., 1987; Praseuth et al., 1988; Povsic & Dervan, 1989; Maher et al., 1989; Francois et al., 1989; Horne & Dervan, 1990; Perrouault et al., 1990) and has generated considerable excitement in recent years. Such a strategy utilizes, for example, homopyrimidine oligodeoxyribonucleotides with EDTA·Fe attached to a single position to form pyr·pur·pyr triplexes and cleave the duplex DNA at those recognition sites. Studies on

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