Divalent transition metal cations counteract potassium-induced quadruplex assembly of oligo(dG) sequences

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ABSTRACT

Nucleic acids containing tracts of contiguous guanines tend to self-associate into four-stranded (quadruplex) structures, based on reciprocal non-Watson–Crick (G*G·G·G) hydrogen bonds. The quadruplex structure is induced/stabilized by monovalent cations, particularly potassium. Using circular dichroism, we have determined that the induction/stabilization of quadruplex structure by K\textsuperscript{+} is specifically counteracted by low concentrations of Mn\textsuperscript{2+} (4–10 mM), Co\textsuperscript{2+} (0.3–2 mM) or Ni\textsuperscript{2+} (0.3–0.8 mM). G-Tract-containing single strands are also capable of sequence-specific non-Watson–Crick interaction with d(G-C)-tract-containing (target) sequences within double-stranded DNA. The assembly of these G*G·C-based triple helical structures is supported by magnesium, but is potently inhibited by potassium due to sequestration of the G-tract single strand into quadruplex structure. We have used DNase I protection assays to demonstrate that strand into quadruplex structure. We have used DNase I protection assays to demonstrate that competition between quadruplex self-association and triplex assembly is altered in the presence of Mn\textsuperscript{2+}, Co\textsuperscript{2+} or Ni\textsuperscript{2+}. By specifically counteracting the induction/stabilization of quadruplex structure by potassium, these divalent transition metal cations allow triplex formation in the presence of K\textsuperscript{+} and shift the position of equilibrium so that a very high proportion of triplex target sites are bound. Thus, variation of the cation environment can differentially promote the assembly of multistranded nucleic acid structural alternatives.

INTRODUCTION

Nucleic acid sequences containing tracts of contiguous guanines tend to self-associate into a four-stranded structure (quadruplex; 1–9). Within the quadruplex, the guanine residues of the four strands are arranged in a square planar array with reciprocal non-Watson–Crick HN-1*O-6 and H2N-2*N-7 hydrogen bonds (a total of eight hydrogen bonds per G-quartet). The G*G·G·G quadruplex structure is inordinately ($T_m$ typically >90\textdegree C) and differentially (K\textsuperscript{+} >> Na\textsuperscript{+}) stabilized by monovalent cations that are positioned within the quadruplex helical core, between adjacent G-quartets, coordinated to carbonyl oxygens. The relationship of cationic radius to the size of the quadruplex helical core is responsible for the differential influence of electrostatically nearly equivalent alkali metal cations on quadruplex structure.

Single-stranded guanine-rich nucleic acids can also bind double-stranded DNA, forming a triple helical structure (10–17). The target for spontaneous triplex formation must generally contain an asymmetrical distribution of purines on one strand and pyrimidines on the other (a pur·pyr region), with major groove dimensions capable of accommodating a third strand (18–19). The purine*purine-pyrimidine (pur*pur-pyr) triple helical structure is based predominately on antiparallel G*G·C interactions, with hydrogen bonds between HN-1 of the third strand and N-7 of the target and between H2N-2 of the third strand and O-6 of the target (20–21). Formation of the purine*purine-pyrimidine triplex requires the presence of divalent magnesium counterions (22–25), though the precise mode of phosphate coordination has not been defined.

Sequences potentially capable of quadruplex and/or triplex formation have been found in critical regulatory regions of the genome and evidence supporting a natural functional or possible therapeutic significance of multistranded conformations in vivo continues to accumulate. We have chosen to utilize the divalent transition metal cations, which exhibit a higher affinity for phosphate relative to the alkali and alkaline earth metals (26–31), to further probe the nature of the G*G-based multiple-stranded structures. Here, the differential influence of the cation environment on competing quadruplex and triplex assemblies and equilibria are presented.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

The oligonucleotides were prepared by automated phosphoramidite synthesis, eluted through reverse phase chromatography and analyzed by polyacrylamide electrophoresis as described (32). Stock solutions were lyophilized from methanol/H2O and stored in distilled, deionized water. The sequences utilized were as follows:

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G-tract oligonucleotides  dist-14a, 5′-CGGGGGGGGGGCGGC-3′,
prox-F, 5′-CGGGGGGGGGGGGACG-3′;
mixed sequence controls 18mer, 5′-CGAGACATGGCAGGGGAC-3′,
10mer, 5′-CGAGCAGTCC-3′.

All major conclusions were confirmed using both G-tract oligonucleotide sequences.

**Circular dichroism assay for quadruplex assembly**

CD spectra (210–340 nm) of oligodeoxyribonucleotides (4.8 µM strand concentration) were obtained with a JASCO 500A spectropolarimeter (sensitivity 2–5 m°/cm). Oligonucleotides were allowed to equilibrate in 20 mM Tris–HCl, pH 7.2, 10 mM total divalent cation concentration (see figure legends), and initial CD spectra were obtained. UV absorbance spectra were obtained concomitantly. Upon addition of KCl (final concentration 5–90 mM), a series of CD spectra were obtained at timed intervals. The change in molar ellipticity at θmax (259–261 nm), expressed either in absolute terms or as a proportion of the total change over a particular time period, was taken as a measure of the increase in relative proportion of oligonucleotide molecules involved in quadruplex structure.

**DNase I protection assay for triplex assembly**

The human dihydrofolate reductase (dhfr) promoter sequence (−112 to +56) containing the two purine-pyrimidine asymmetrical regions (targets for triplex formation) was 3′-32P-end-labeled on either the purine-rich or pyrimidine-rich strand and isolated by native polyacrylamide gel electrophoresis as described (33). Co-incubation of the dhfr promoter fragment (200 000 c.p.m./reaction, ∼40 nM) with one of the G-tract oligonucleotides (32–40 µM) was carried out in 20 mM Tris–HCl, pH 7.2, 10 mM MgCl2 for 45 min at room temperature (22), resulting in >90% intermolecular triplex formation (32). Alkali metal cations (supplied as the chloride salt) were variably included as stated in the text and figure legends. The Mg2+ and transition metal ion composition were also varied as stated, but the total divalent cation concentration was maintained at 10 mM. Unless otherwise stated, the oligonucleotide was the final component added and was thus exposed simultaneously to the monovalent and divalent cations and the double helical target.

Following incubation, samples were subjected to limited digestion with DNase I (45 s on ice, 2–20 U/ml, determined empirically and dependent upon the divalent cation composition) and the products analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. Autoradiographic data were quantitated by laser densitometric analysis. The intensities of bands (within the linear range) within the target sequence were used as a measure of triplex formation. Reference bands above and below the target which were unaffected by triplex formation were utilized to control for degree of digestion and sample recovery. All densitometric parameters (e.g. area scanned, peak width) were standardized and results derived from quadruple integrations.

**RESULTS**

**Induction/stabilization of quadruplex structure by K+**

The oligodeoxyribonucleotides used for these studies each contain two tracts of contiguous guanines. As such, these sequences resemble the telomeric repeats which have served as the prototype for the study of G4G4G4G quadruplexes (4–8,34); the length of the G-tracts is equal to or greater than that found in the telomeric repeats and the isolation of G-tracts from each other or from the terminus of the strand is limited to only one base. The oligonucleotide sequences correspond with inverse orientation to a G-tract pattern found in the human dihydrofolate reductase promoter (see below).

The CD spectra of either of these oligonucleotides in deionized, distilled water or in 20 mM Tris–HCl, pH 7.2, 10 mM MgCl2 (Fig. 1A, solid line = 0 min) exhibits the characteristic pattern of the parallel intermolecular quadruplex (35–37) with a large positive ellipticity at ∼260 nm and a smaller negative peak at ∼240 nm. Thus, even in the absence of monovalent cations, these oligonucleotides exhibit a certain degree of native quadruplex character. Hardin et al. (35) have shown that the relative height of the positive CD peak at 260 nm can be correlated with the prevalence of quadruplex structure. Over time, the ellipticity of dist-14a or prox-F varies ≤10%. However, upon addition of 30 mM KCl, a marked, progressive increase in the magnitude of the CD is observed (discernable within 2 min; Fig 1A, broken lines). Note that the wavelength of maximal ellipticity shifts slightly from 259 to 261 nm upon addition of K+ and that an isosbestic point is present at 248–249 nm, indicating that the transition involves only two species. Thus, exposure of the oligonucleotide to potassium causes a rapid induction and/or stabilization of quadruplex structure.

A series of assays was performed to verify the nature of the abruptly increased ellipticity upon exposure of the G-tract oligonucleotide to K+. If the changes in the CD spectral pattern represented enhanced intermolecular association of oligonucleotide molecules, then a dependence on oligonucleotide concentration should be observed. Furthermore, the role of the potassium cation in quadruplex assembly would suggest that the rate of increase in ellipticity should also be influenced by K+ concentration. Each of these parameters was varied independently and the expected positive correlations between either K+ or oligonucleotide concentration and the initial rate of quadruplex assembly were confirmed (Fig. 1B and C).

Upon exposure of the oligonucleotide to sodium, the increase in molar ellipticity at 260 nm was much slower and lesser in degree than that induced by K+ (data not shown). Furthermore, a shoulder of positive ellipticity at −294 nm developed, a feature which has been correlated with the existence of an alternative quadruplex structure, produced in the presence of Na+ by folding and bimolecular association of G-tract sequences, such that strands of the quadruplex alternate orientation and half of the G residues are in the syn conformation (4,34). The CD spectra of a mixture of the mononucleotides which comprise the G-tract oligonucleotides was of considerably lower magnitude, did not resemble the quadruplex signature pattern and did not change significantly upon incubation with K+. Control mixed sequence oligonucleotides exhibited CD patterns clearly distinct from those of the G-tract oligonucleotides, with no significant changes observed even up to 24 h following addition of K+ (data not shown).

**Effect of the divalent cation composition on quadruplex assembly**

For the next set of experiments, Mg2+ was eliminated or else partially replaced by a divalent transition metal cation while keeping the total divalent cation concentration constant at 10 mM. An essentially identical alteration of the CD spectrum and rate of assembly...
Figure 1. Induction/stabilization of quadruplex assembly by K⁺. (A) The G-tract-containing oligonucleotide prox-F (4.8 µM strand concentration) was allowed to equilibrate in 20 mM Tris–HCl, pH 7.2, 10 mM MgCl₂ and the initial CD spectrum was recorded as detailed in Materials and Methods. Immediately following the addition of 30 mM KCl, a series of CD spectra were obtained. The tracings for 0, 2, 5, 10, 30 and 60 min are shown. Values are plotted as molar ellipticity. (B and C) A series of experiments similar to that described in (A) was performed in which either K⁺ concentration or oligonucleotide concentration was varied. The increase in molar ellipticity at 260 nm at 2 min following addition of K⁺ (relative to that observed upon equilibration for 1 h) is plotted versus K⁺ concentration or oligonucleotide concentration on a logarithmic scale.

increase in molar ellipticity at 260 nm was obtained with ≥ 30 mM K⁺ in the presence or absence of Mg²⁺ (data not shown). However, in the presence of 3–4 mM Mn²⁺, 2 mM Co²⁺ or 0.5 mM Ni²⁺ the rapid increase in molar ellipticity otherwise observed upon addition of K⁺ was severely blunted (by 50–>90%; Fig. 2A and B). Neither Mn²⁺, Co²⁺ nor Ni²⁺ had any immediate appreciable influence on the native CD pattern of either of the G-tract oligonucleotides. Thus, without otherwise altering the secondary structure of the oligonucleotide, these divalent transition metal cations effectively counteract the induction/stabilization of quadruplex structure by potassium.

When the G-tract oligonucleotide is allowed to equilibrate for 24 h in the presence of 30 mM K⁺, the magnitude of the positive CD peak at 260 nm increases by 55–60% from its initial value. However, with the inclusion of Mn²⁺ or Co²⁺ the maximal molar ellipticity is considerably lower, i.e. a smaller proportion of the population of oligonucleotide molecules is involved in quadruplex structure in the

Figure 2. Divalent transition metal cations counteract the induction/stabilization of quadruplex assembly by K⁺. (A) A series of CD spectra of prox-F was obtained following addition of K⁺ as described for Figure 1A, except that 10 mM MgCl₂ was replaced by 8 mM MgCl₂ plus 2 mM CoCl₂. The progressive increase in magnitude of the positive ellipticity at 259–261 nm and of the negative peak at 240 nm seen in Figure 1A is largely abrogated in the presence of Co²⁺. (B) The proportionate initial (2 min) increase in molar ellipticity at 260 nm following addition of 30 mM K⁺ is indicated for various cationic compositions. (C) The proportionate increase in molar ellipticity at 260 nm at equilibrium (24 h) following addition of 30 mM K⁺ is indicated for various cationic compositions.
Inhibition of intermolecular purine*purine-pyrimidine trplex assembly by K+

The human dhfr core promoter sequence (38) contains two closely spaced and very similar regions of purine-pyrimidine (pur-pyr) asymmetry, which consist of (on the purine-rich strand) tracts of contiguous guanines with interspersed individual A or C residues. These two pur-pyr regions represent potential targets for assembly of intermolecular triple helical structures and the G-tract patterns of the oligonucleotides dist-14a and prox-F correlate in an antiparallel orientation with the G-tract patterns of the distal and proximal pur-pyr regions. We have in fact demonstrated specific binding of these single-stranded oligonucleotides to their respective double-stranded target sequences on a restriction fragment of the dhfr promoter in a simple buffer consisting only of 20 mM Tris–HCl, pH 7.2, 10 mM MgCl₂ (32,39). With an excess of oligonucleotide, intermolecular trplex assembly proceeds rapidly at ambient temperature such that essentially 100% of the specific target site is occupied within 10 min. However, we observe a potent concentration-dependent negative influence of potassium cations on formation of the triple helical structure. For the experiment shown in Figure 3A, concentrations of KCl from 0 to 60 mM were included from the onset of an incubation of the oligonucleotide prox-F with the dhfr promoter fragment under conditions otherwise conducive to trplex formation (20 mM Tris–HCl, pH 7.2, 10 mM MgCl₂, room temperature, 45 min, oligonucleotide excess). Without KCl, prox-F produces a high degree of DNase protection over its intended target within the proximal pur-pyr region, as well as a minor footprint resulting from mismatched binding to the homologous distal pur-pyr sequence (lane 1). An increase in KCl concentration is accompanied by a decrease in the proportion of dhfr promoter molecules to which the oligonucleotide third strand is bound (lanes 2–5). A considerable loss of occupation of the proximal (specific) target site by prox-F is observed with ≥30 mM KCl. The mismatched binding of prox-F to the distal target is almost completely inhibited with only 5 mM KCl.

This negative influence of K⁺ is dependent upon order of addition (Fig. 3B). If K⁺ is added prior to or simultaneously with the oligonucleotide, trplex formation is blocked (lane 3). However, if the trplex is pre-assembled in the absence of potassium, the subsequent addition of K⁺ does not appreciably accelerate trplex dissociation (lane 4). Thus K⁺ apparently inhibits the initial binding of the purine-rich oligonucleotide to the pur-pyr double-stranded target, but does not destabilize this triple helical structure if preformed in its absence. Trplex formation is also inhibited by Na⁺, but a 3-fold higher Na⁺ concentration is required for the degree of inhibition of trplex formation exhibited by K⁺ (lane 5 and data not shown). No alterations of the DNase I digestion pattern of the dhfr promoter are detected with any manipulation of the concentrations of MgCl₂, KCl or NaCl alone.

Substantial increases in Mg²⁺ concentration only modestly counterbalance the negative effect of K⁺ on trplex formation (data not shown). According to polyelectrolyte theory and in practice, the divalent alkaline earth cation has a much greater affinity for non-specific electrostatic interaction with DNA than the presence of K⁺ (Fig. 2C). Thus it appears that the transition metal cations shift the equilibrium between the single-stranded form and the quadruplex toward the single-stranded form, by hindering quadruplex association and/or facilitating quadruplex dissociation.

Figure 3. The assembly of an intermolecular pur*pur-pyr trplex at the dhfr promoter is inhibited by K⁺. (A) The concentration of KCl was varied in an incubation of the oligonucleotide prox-F (32 µM) with the labeled 204 bp restriction fragment of the human dhfr promoter. Trplex formation was assayed by DNase I protection as described in Materials and Methods. A Maxam–Gilbert G+A sequencing reaction is included on the left (purine-rich strand labeled). The position of the specific (proximal) target sequence for prox-F is indicated by the solid bracket. (B) K⁺ inhibits trplex formation without accelerating dissociation. The conditions for incubation of the labeled dhfr promoter fragment with the G-tract-containing oligonucleotide prox-F were varied. Lane 1, negative control (oligonucleotide withheld); lane 2, positive control (monovalent cation withheld); lane 3, 30 mM KCl initially included; lane 4, initial 45 min incubation without KCl, followed by 45 min incubation with 30 mM KCl; lane 5, 30 mM NaCl initially included. A Maxam–Gilbert G+A sequencing reaction is included on the left (pyrimidine-rich strand labeled). The position of the specific target sequence is indicated by the broken bracket. (C) Titrations of trplex formation at the dhfr promoter were carried out by independently (i) limiting oligonucleotide concentration or (ii) increasing K⁺ concentration. The proportion of specific target sites bound was determined by quantitative DNase I protection and laser densitometry as described in Materials and Methods. Results of the two titrations were correlated to provide a measure of the effective decrease in single-stranded oligonucleotide concentration caused by K⁺-induced quadruplex assembly.
does the monovalent alkali metal cation (40–42). Therefore, non-specific electrostatic competition between monovalent potassium and divalent magnesium could not reasonably account for the inhibition of triplex formation by K⁺. Rather, the ability of the monovalent cation to apparently compete so effectively against the divalent cation is best understood in terms of the two separate, competing reactions (triplex assembly with Mg²⁺ versus quadruplex assembly with K⁺).

Simultaneous incubation of the single-stranded oligonucleotide, the double-stranded target, Mg²⁺ and K⁺ results in kinetically competing triplex and quadruplex assemblies. Quadruplex self-association of the G-tract oligonucleotide, fostered by potassium, decreases its availability for binding to the double-stranded target sequence. The diminishing pool of free (available) oligonucleotide also accounts for the apparent increase in stringency of triplex formation seen with low concentrations of K⁺; the homologous (mismatched) binding site would not effectively compete with the higher affinity specific target for a limited third strand population. Differential effects of the monovalent alkali metal cations on nucleic acid structure are very unusual; the correlation between the markedly greater degree of induction of quadruplex assembly by K⁺ relative to Na⁺ and the considerably higher efficacy of K⁺ relative to Na⁺ for inhibition of intermolecular triple helical assembly at the dhfr promoter strongly suggest a common mechanism.

Independent titrations of triplex formation with (i) limiting oligonucleotide concentration in the absence of K⁺ and (ii) increasing K⁺ concentration in the presence of a constant excess of oligonucleotide have been correlated to quantitate the relationship between K⁺ concentration and the decline in apparent availability of the single-stranded form of the oligonucleotide (Fig. 3C). The result is an approximate inverse relationship between K⁺ concentration and effective oligonucleotide concentration (exponent = –1.16, r² = 0.990). This result also correlates well with the data of Figure 1, where the initial rate of increase in molar ellipticity (induction/stabilization of quadruplex) was shown to be a function of K⁺ concentration.

Cations which counteract the K⁺-associated induction/stabilization of quadruplex structure foster triplex assembly in the presence of K⁺

With simultaneous exposure of the oligonucleotide to K⁺ as well as Mg²⁺ and the specific double helical target, an initial kinetic

Figure 4. Transition metal cations promote intermolecular purine-purine-pyrimidine association in the presence of K⁺. The labeled dhfr promoter fragment was incubated with a G-tract-containing oligonucleotide under conditions otherwise conducive to triplex formation (20 mM Tris–HCl, pH 7.2, room temperature, 45 min, oligonucleotide excess), except that 30 mM KCl was also included. The total divalent cation concentration was held constant at 10 mM, while the proportion represented by Mg²⁺ and either Mn²⁺, Co²⁺ or Ni²⁺ was varied. (A) Representative DNase I protection assay with oligonucleotide prox-F and variable Mn²⁺ content. Mn²⁺ alone does not alter the pattern of DNase I sensitivity of the dhfr promoter (data not shown). (B) The degree to which triplex assembly in the presence of 30 mM K⁺ is enhanced (relative to 10 mM Mg²⁺ alone) by the various Mg²⁺:non-Mg²⁺ divalent cation compositions is quantitated. Mn²⁺, solid triangles; Co²⁺, open squares; Ni²⁺, closed circles. (C) Effect of Mn²⁺ and Co²⁺ on the titration of triplex assembly against increasing K⁺ content. The absolute proportion of specific target sites bound by the oligonucleotide third strand are plotted versus the K⁺ concentration on a logarithmic scale. Data are included for 10 mM Mg²⁺ alone (solid squares; see Fig. 3A), 5 mM Mg²⁺/15 mM Mn²⁺ (open triangles) and 8 mM Mg²⁺/2 mM Co²⁺ (open circles).
‘race’, a competition between assembly of the triplex (binding of the G-tract oligonucleotide to its double-stranded target sequence) and assembly of the quadruplex (self-association of the G-tract oligonucleotide) is observed. Once assembled, both the triplex and the K⁺-associated quadruplex are very stable structures (4,21,24–25,35,43; CD thermal denaturation data not shown). Thus an apparent equilibrium is rapidly established (in <45 min). In the absence of K⁺ and with an excess of oligonucleotide over target, triplex formation may be driven to near completion, however, in the presence of K⁺, the quadruplex is favored to the exclusion of the triplex. Thus factors differentially affecting the progress of these divergent, competing intermolecular assemblies will determine the resulting relative proportion of triplex or quadruplex products.

A series of experiments was performed in which triplex formation in the presence of K⁺ was measured while Mg²⁺ was replaced to a variable degree by a divalent transition metal cation (total divalent cation concentration held constant at 10 mM). The oligonucleotide was added last to the incubation and thus simultaneously exposed to the monovalent and divalent cations as well as its double helical target. Assay of the binding of oligonucleotide prox-F to the dhfr promoter fragment in the presence of 30 mM K⁺ and variable Mg²⁺:Mn²⁺ composition is shown in Figure 4A. With increasing Mn²⁺ content (2–10 mM), the DNase I footprint of prox-F at its target (which disappeared in Fig. 3A) reappears and becomes increasingly more pronounced, indicative of triplex formation on a considerable proportion of the dhfr promoter molecules. Concentrations as low as 300 μM Ni²⁺ or Co²⁺ significantly enhanced triplex formation in the presence of K⁺ (Fig. 4B). These alterations of the cationic environment effectively counteract the inhibition of intermolecular pur·pur·pyr triplex formation by K⁺, permitting the oligonucleotide third strand to bind to its specific target on 75–90% of dhfr promoter molecules otherwise unbound. These results correlate well with the ability of these divalent transition metal cations to counteract the induction/stabilization of quadruplex structure by K⁺. The biphasic nature of each of these titrations is apparently indicative of an additional mode of cation coordination which is detrimental to triplex formation and is reminiscent of the potential for destabilization of duplex DNA by transition metal cations (44–46).

These results are influenced by the choice of experimental protocol (oligonucleotide added last). If the oligonucleotide is pre-incubated with the effective divalent cation composition prior to exposure to the monovalent cation and double helical target, K⁺-resistant triple helical assembly is enhanced. Conversely, if the transition metal cation is initially withheld from the incubation, its immediate effectiveness in fostering the triple helical association (in competition with K⁺-induced self-association of the G-tract single strand which has already taken place) is drastically reduced (see below).

The titration of triplex assembly versus increasing K⁺ concentration (as in Fig. 3A) is also altered by the transition metal cations (Fig. 4C). In the presence of either 5 mM Mn²⁺ or 2 mM Co²⁺, ~80, 75 and 60% of specific target sites were occupied by the third strand despite 60, 90 or 120 mM K⁺. This represents a minimum 4- to 5-fold decrease in sensitivity of triplex formation to K⁺ relative to that observed with 10 mM Mg²⁺ alone and relatively efficient triple helical association at near physiological K⁺ concentration.

The equilibria between triplex and quadruplex structures are altered by changes in the cationic environment

In order to investigate the influence of the cationic environment on the establishment of a true equilibrium between quadruplex and triplex species, three series of samples were used, differing only in divalent cation composition (10 mM Mg²⁺, 10 mM Mn²⁺ or 8 mM Mg²⁺/2 mM Co²⁺; Fig. 5). In half of the samples of each series, the oligonucleotide was exposed to 30 mM K⁺ prior to the divalent cations and the target sequence. For the other half, the oligonucleotide was exposed to the divalent cations and the target sequence first, after which the K⁺ was added. Thus, in one case, opportunity is presented for initial quadruplex assembly, while in the other case, the triplex is formed first.

Initially, DNase I digestion of the dhfr target sequence reveals a footprint of the triplex structure only in those samples in which K⁺ was initially withheld (the second lane of each pair). Identical samples were incubated at ambient temperature for 4 (middle panel) to 48 h (lower panel). Since the final composition of the reactants was the same, the achievement of true equilibrium would be indicated when the DNase I protection pattern of the two samples of each pair (triplex assembled first and quadruplex assembled first) become identical. By 48 h the trend is evident. The footprint initially present in the Mg²⁺ alone sample is lost over time, indicating that dissociated third strands are being sufficiently sequestered in the presence of K⁺ by quadruplex association to cause the dhfr target sequence to become unoccupied. On the other hand, samples with Co²⁺ or Mn²⁺ in which the quadruplex was pre-assembled gradually developed a footprint at the dhfr target sequence because sufficient oligonucleotide molecules were free from the quadruplex and were available for triplex formation. By 48 h (bottom panel), equilibrium between the competing quadruplex and triplex assemblies was clearly established. The quadruplex is favored to the exclusion of the triplex in 10 mM Mg²⁺ alone, while nearly 100% triplex formation is allowed in the presence of either of the transition metal cations. Identical results were obtained with samples incubated for as long as 7 days (not shown).

DISCUSSION

The K⁺-associated induction/stabilization of quadruplex structure is specifically counteracted by divalent transition metal cations

The G-tract oligonucleotides exhibit a certain degree of native quadruplex character in aqueous solution, however, a significant proportion of molecules remain in the free single-stranded form. Upon exposure to monovalent alkali metal cations, particularly K⁺, an increasing proportion of the oligonucleotide population is recruited into quadruplex structure. The mechanism by which a net increase in quadruplex structure is mediated by K⁺ potentially involves (i) the facilitation of de novo association of oligonucleotide single strands and/or (ii) additional stabilization (decreased rate of dissociation) of the existing quadruplex population. At 1.3 Å, the cationic radius of K⁺ is apparently optimally suited for crosslinking of carbonyl oxygen atoms (6). The divalent transition metal cations Mn²⁺, Co²⁺ and Ni²⁺ potently counteract the effect of K⁺ on the G-tract oligonucleotides: the rate at which oligonucleotide is
and the K⁺-induced shift in equilibrium toward the quadruplex is sequestered into quadruplex structure by K⁺ is slowed considerably (i) the transition metal may chelate or coordinate directly to two mechanisms (which are not necessarily mutually exclusive): the monovalent cation. This could conceptually involve either of these divalent cations in opposing the mechanism of action of lessened. These results are consistent with a direct involvement (B) 10 mM Mg²⁺ ; (C) 10 mM Mn²⁺ ; (D) 8 mM Mg²⁺ /2 mM Co²⁺ . For half of KCl and 10 mM total divalent cation. Three series of samples were prepared: (A) 10 mM Mg²⁺ ; (B) 10 mM Mg²⁺ ; (C) 10 mM Mn²⁺ ; (D) 8 mM Mg²⁺ /2 mM Co²⁺ . For half of the samples (the first of each pair of lanes), the oligonucleotide was exposed to K⁺ for 45 min before the dhfr target and divalent cations were added. For the other half of the samples (the second of each pair of lanes), the oligonucleotide was exposed to the dhfr target in the presence of the divalent cation(s) for 45 min before K⁺ was added. The samples were then allowed to continue incubation at ambient temperature for varying periods of time (indicated to the left of each panel) prior to DNase I digestion. Within each series, the final composition of the samples was the same, thus the establishment of equilibrium is indicated when the degree of DNase I protection in the first and second lanes of each pair become identical. The samples shown in (A) are negative (no oligonucleotide) versus reverse Hoogsteen (triplex) hydrogen bonding; (ii) strand composition [(single strand × 4) versus (single strand plus double-stranded target)]; (iii) geometrical configuration, which certainly results in disparate modes/sites of counterion coordination. The data presented here have demonstrated that the cationic environment influences competition between quadruplex and triplex assemblies (Fig. 6). The same divalent cationic compositions which counteract the induction/stabilization of quadruplex structure by K⁺ also abrogate the inhibition of triplex formation by K⁺. Furthermore, the equilibrium between the competing triplex and quadruplex associations is significantly altered by the cationic environment, such that the occupation of a high proportion of triplex target sites in the presence of K⁺ becomes possible.

**Biological relevance**

Naturally occurring G-tract-containing sequences in the eukaryotic genome have frequently been implicated in important molecular biological functions, such as regulation of gene expression, replication, recombination and chromosomal condensation (50–53). It has been proposed that the potential of these sequences for adoption of non-Watson–Crick structures, such as the quadruplex or the triplex, may be functionally relevant in vivo [e.g. human γ-globin 5'-flanking region, associated with hereditary persistence of fetal hemoglobin (54); Chinese hamster ovary origin of replication (55); human c-myc promoter (56); d(CGG)ₙ repeats, associated with the fragile X syndrome (57); human insulin gene-linked polymorphic region (58); telomeric repeats, associated with integrity of chromosomal termini, chromosomal pairing at meiosis, cellular senescence and immortality of transformed cells (59–61)]. Naturally occurring triplex binding proteins have been identified (62); eukaryotic chromatin is heterogeneously stained by anti-triplex antibodies (63–64); intramolecular triplex structures have been detected in E.coli. (65–66). Miller and colleagues have putatively identified at least two naturally occurring untranslated transcripts which may function through intramolecular triplex formation with genomic target sequences (D.M.Miller, unpublished data). Naturally occurring polypeptides which bind quadruplex structures (67–69), promote quadruplex formation (37,70) or cleave DNA specifically near quadruplex structures (71) have nucleophilic atoms (i.e. N-7 and O-6) of the guanine bases of single-stranded oligonucleotide molecules (27,30–31,47–49) and prevent formation of the Hoogsteen hydrogen bonds essential for quadruplex assembly; (ii) the transition metal may compete directly with potassium for occupation of the quadruplex helical core, possibly for coordination to O-6 atoms, and exert a less stabilizing or even destabilizing effect on the existing quadruplex structure. At 0.6–0.8 Å, the radii of Mn²⁺, Co²⁺ and Ni²⁺ are much smaller than K⁺ and therefore a poorer ‘fit’ within the quadruplex helical core. Further experimentation will be required to define more precisely the nature of these transition metal–nucleic acid interactions.

**Differential influence of the cationic environment on competing non-Watson–Crick intermolecular assemblies**

Both the parallel quadruplex and the antiparallel purine-pyrimidine triple helix are based on non-Watson–Crick guanine* guanine interactions and a G-tract-containing sequence may be simultaneously capable of involvement in either structure. These two non-canonical nucleic acid structures are fundamentally distinct in several ways, including: (i) Hoogsteen (quadruplex) versus reverse Hoogsteen (triplex) hydrogen bonding; (ii) strand composition [(single strand × 4) versus (single strand plus double-stranded target)]; (iii) geometrical configuration, which certainly results in disparate modes/sites of counterion coordination. The data presented here have demonstrated that the cationic environment influences competition between quadruplex and triplex assemblies (Fig. 6). The same divalent cationic compositions which counteract the induction/stabilization of quadruplex structure by K⁺ also abrogate the inhibition of triplex formation by K⁺. Furthermore, the equilibrium between the competing triplex and quadruplex associations is significantly altered by the cationic environment, such that the occupation of a high proportion of triplex target sites in the presence of K⁺ becomes possible.
Figure 6. Scheme illustrating the competitive equilibria between quadruplex assembly and triplex assembly and the influences which monovalent and divalent cations may exert on these equilibria.

been reported. Quadruplex formation has been implicated in the life-cycle of the HIV virus (72–73) and may be involved in developmental regulation (74). Thus several lines of evidence point to the existence and functional significance of quadruplex and triplex nucleic acid structures in vivo. An understanding of the factors which influence the assembly of such multi-stranded nucleic acid structures is crucial to discerning the associated mechanisms of molecular biological regulation.

The data presented here have implicated the existence of a distinct mode of cation coordination through which nucleic acid reactivity is significantly altered, making possible the differential promotion of one non-Watson–Crick G*G interaction over another. The potential for quadruplex or triplex formation in vivo would undoubtedly be influenced by resident cationic moieties or putative accessory proteins. Although total cellular concentrations of Mn²⁺, Co²⁺ and Ni²⁺ are very low (submicromolar), it is conceivable that compartmentalization, synergistic interactions (75) or presentation by accessory molecules could facilitate their interaction with nucleic acids under appropriate conditions. Alternatively, the effects of the transition metal cations in vitro might well be duplicated naturally within the cell (via high positive charge, not coordination to base) by oligovalent polyamines or a specialized basic polypeptide domain (76–78).

Possible applications

The results presented here are also relevant for proposed oligonucleotide-based therapeutic approaches, involving the exogenous pharmacological administration of nucleic acid molecules (some of which would contain G-tracts) intended for antisense, triplex or aptamer strategy (25,79–85). The ability of such an oligonucleotide effector molecule to reach and bind its intended target will be influenced by the specific cationic environments to which it is exposed within the cell. The inhibition of intermolecular pur*pur-pyr triplex assembly by moderate concentrations of potassium appears to represent a considerable obstacle to the natural or practical therapeutic utility of this interaction within a physiological (i.e. ~140 mM K⁺) environment (86–88), however, an effective cationic species may mitigate this obstacle in vivo.

The divalent transition cations which we have utilized in this work are labile metal centers, with relatively short-lived interactions with nucleic acids. This laboratory is currently investigating the feasibility of site-specific incorporation of inert (covalent) metal centers into synthetic oligonucleotide structures. The data presented here have certainly suggested that such an approach might allow the permanent addition of physical/chemical attributes favorable for triplex formation. In related approaches, the incorporation of positive charges in the backbone of synthetic oligonucleotides and the use of a 6-thio-substituted guanine derivative have been investigated as a means to disfavor quadruplex self-association (89–91). Positively charged ligands have been shown to specifically stabilize triplex structures (92–93). Interestingly, the interactions of the various cationic environments on other modified oligonucleotide structures proposed for therapeutic use (methylphosphonate, phosphorothioate, 2′-O-methyl, etc.) largely remain to be determined.

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