The integral divalent cation within the intermolecular purine*purine·pyrimidine structure: a variable determinant of the potential for and characteristics of the triple helical association

Scott W. Blume1,2, Jacob Lebowitz3, Wolfgang Zacharias1,2, Vincenzo Guarcello1,2, Charles A. Mayfield1,2, Scot W. Ebibinghaus1,2, Paula Bates1,2, David E. Jones Jr1,2, John Trent1,2, Nadarajah Vigneswaran1,2 and Donald M. Miller1,2,*

1Comprehensive Cancer Center and 2Department of Medicine and 3Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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ABSTRACT

In vitro assembly of an intermolecular purine*purine·pyrimidine triple helix requires the presence of a divalent cation. The relationships between cation coordination and triplex assembly were investigated, and we have obtained new evidence for at least three functionally distinct potential modes of divalent cation coordination. (i) The positive influence of the divalent cation on the affinity of the third strand for its specific target correlates with affinity of the cation for coordination to phosphate. (ii) Once assembled, the integrity of the triple helical structure remains dependent upon its divalent cation component. A mode of heterocyclic coordination/chelation is favorable to triplex formation by decreasing the relative tendency for efflux of integral cations from within the triple helical structure. (iii) There is also a detrimental mode of base coordination through which a divalent cation may actively antagonize triplex assembly, even in the presence of other supportive divalent cations. These results demonstrate the considerable impact of the cationic component, and suggest ways in which the triple helical association might be positively or negatively modulated.

INTRODUCTION

Under certain constraints of sequence composition and environmental conditions, a segment of double-stranded DNA may be bound specifically by a third nucleic acid strand lying within the major groove (1–4). Triplex formation is analogous to Watson–Crick hybridization in its dependence upon (i) base-specific hydrogen bonding (5–7) and (ii) counterions to neutralize the charges of the deoxyribose–phosphate backbones in order to overcome the electrostatic repulsion of nucleic acid strands (8–11). Viable targets for spontaneous triplex formation are characterized by a predominance of purines on one strand and pyrimidines on the other. This purine-pyrimidine (pur·pyr) asymmetry is associated with major groove dimensions capable of accommodating a third strand (12–13), and facilitates alignment for the formation of multiple specific non-Watson–Crick hydrogen bonds with bases of the third strand. Assembly of the purine*purine·pyrimidine class of triple helix, based predominantly on antiparallel G*G·C alignments (where * represents the non-Watson–Crick interaction), generally requires the presence of magnesium(II) cations (14–17). This precise mode of coordination of the divalent cation within the triple helical structure has not yet been determined.

This laboratory has investigated the assembly of intermolecular purine*purine·pyrimidine triple helical structures at the human dihydrofolate reductase (dhfr) core promoter (18–19). This sequence contains two closely spaced and very similar regions of purine-pyrimidine asymmetry. Synthetic oligonucleotides were designed to bind in antiparallel orientation specifically to either of these target sequences, producing triplex structures dominated by G*G·C alignments, but tolerating individual A*A·T as well as C*C·G (hydrogen bonding not necessarily inferred) alignments. Here we have employed quantitative DNase I protection titrations to investigate the relationship of the required divalent cation to the intermolecular pur*pur·pyr triplex. The cationic component was found to be continuously necessary for maintenance of triple helical integrity and to exert a considerable yet variable influence on the capacity for triplex association, third strand affinity, stringency of triple helical alignment and destabilization of the triple helical structure secondary to cation efflux. These findings may have important implications for understanding triplex formation as it might occur naturally within the cell and may be also relevant to triplex-based therapeutic antigene strategies.

*To whom correspondence should be addressed at: University of Alabama at Birmingham, Comprehensive Cancer Center, 1824 6th Avenue South, Room 520, Birmingham, AL 35294, USA. Tel: +1 205 934 1977; Fax: +1 205 975 6911: Email: donald.miller@ccc.uab.edu
MATERIALS AND METHODS

Human dhfr promoter fragment

The human dhfr promoter sequence (−112 to +56) containing the two purine-pyrimidine biased regions (−58 to −40 and −25 to −5) was obtained as described (19). This restriction fragment was excised and 3′-32P-end-labeled on either the purine-rich or pyrimidine-rich strand, to optimize electrophoretic/autoradiographic visualization of the proximal or distal pur-pyr region respectively. Following isolation on a non-denaturing polyacrylamide gel, the labeled double-stranded dhfr promoter fragment was precipitated from a 1 M ammonium acetate solution, dried, then resuspended and stored in distilled, deionized water.

Oligodeoxyribonucleotides

The oligonucleotides utilized for intermolecular triplex formation were prepared as described (19). The sequences of the oligonucleotides, along with the human dhfr promoter sequence to which each is specifically targeted (plus 10 bp flanking sequences on either side) are as follows: dist-14a, 5′-CGGGGCGGGGGGGCGTGGCCTGCGTGCGC-3′ (target sequence underlined); distal target, 5′-...CTGGCTGCGCCGGGGCGGGCGGTCTGGCTGCGG...3′; prox-F, 3′-CGAGGGGGGGCGGGG-5′ (target sequence underlined); prox-G, 3′-TGGGGACGAGGGGGGCGGGG-5′ (target sequence italic); proximal target, 5′-...CCTGACAAAAAGGGGAGCGAGGGGGGGCCGGCGCCAATT...3′.

For the purposes of this manuscript, triple helical ‘alignment’ refers to the colinear antiparallel positioning of residues of the third strand along the major groove surface of the native double helix such that exclusively G*G·C, A*A·T and C*C·G triplets would result (19).

Triplex formation

The standard reaction contains the labeled dhfr promoter fragment (75–200 × 10^3 c.p.m./sample, ∼40 nM), an oligonucleotide (32–40 µM), Tris–HCl, pH 7.2 (20 mM) and a divalent metal chloride (10 mM). When carried out for 45 min at room temperature, this incubation allows the system to approach equilibrium, which in the presence of Mg^{2+} results in triplex formation on a very high (>90%) proportion of specific target sites.

DNase I protection assay

Following incubation to allow for triplex formation, samples were placed on ice, then subjected to limited digestion with DNase I (2–40 U/ml for 45 s). The DNase I activity required was determined empirically under the different reaction conditions (particularly cationic composition) to achieve comparable degrees of cleavage and produce an even distribution of bands representing large and small digestion products of the dhfr promoter fragment. Limited digestion products were separated on an 8% polyacrylamide, 8 M urea sequencing gel.

Laser densitometric analysis and evaluation

The autoradiographic results of DNase I protection assays were analyzed by laser densitometry. Scanning, peak area integration and controlled evaluation of numerical data were performed as described in detail (20). The relative intensities of (experimental) bands within the target, corrected for regional intensity of the digest as determined by vicinal reference bands, were compared from lane to lane as a measure of proportionate occupation of the target site on the population of dhfr promoter molecules by the oligonucleotide third strand.

RESULTS

Variable support for intermolecular pur*pur-pyr assembly

In the experiment shown in Figure 1, a series of divalent alkaline earth and transition metal cations were tested for the ability to support formation of an intermolecular purine-pyrimidine triple helix at the proximal target sequence of the human dhfr promoter. For this assay, standard conditions for triplex formation were employed (20 mM Tris–HCl, pH 7.2, room temperature, 45 min), except that the 10 mM MgCl_2 was substituted for by the same concentration of one of the other metal(II) chlorides. For the first of each pair of lanes, the oligonucleotide was withheld from the incubation, thus controlling for the effects of the substituted metal cation alone on DNase I sensitivity of the dhfr promoter fragment. For the second lane of each pair, a single-stranded purine-rich oligonucleotide designed for triple helical alignment with the proximal pur-pyr region of the dhfr promoter was included.
In the presence of Mg$^{2+}$, the oligonucleotide prox-G produces a clear, characteristic footprint (19) over its intended target within the proximal pur-pyr region (lane 2). Definitive endonuclease protection was also observed when Co$^{2+}$ (lane 4) or Mn$^{2+}$ (lane 8) replaced Mg$^{2+}$. The essentially complete disappearance of bands over an area approximating the specific target sequence indicates that nearly 100% of the population of double helical dhfr promoter molecules have been bound by the oligonucleotide third strand at this site when either Mg$^{2+}$, Co$^{2+}$ or Mn$^{2+}$ was present. Furthermore, no significant change in the position and extent of the sequence protected from DNase digestion by the oligonucleotide was seen with change in the identity of the supporting divalent metal cation, consistent with a comparable mode of triplex formation in each case. In contrast, no clear indication of triplex formation was observed in the presence of Ni$^{2+}$ (lane 6). Additional assays have indicated that Ca$^{2+}$ is also supportive of intermolecular triplex formation at the proximal pur-pyr region, while Cd$^{2+}$ is not (data not shown).

Next, these divalent cations were tested for their ability to support triplex formation at the distal pur-pyr region of the dhfr promoter (Fig. 2). Highly efficient binding of the oligonucleotide dist-14a to this specific target sequence was observed with the divalent magnesium (lane 2), cobalt (lane 4) and manganese (lane 8) cations; however, Ni$^{2+}$ (lane 6) and Cd$^{2+}$ (lane 10) were apparently either inadequate for support or inhibited formation of the triple helical structure. Again, the features of DNase protection of the native double helix induced by the binding of the oligonucleotide third strand were essentially unchanged among each of the supportive divalent cations, consistent with facilitation of a qualitatively similar mode of triple helical association.

**Stringency of triple helical alignment**

The distal and proximal purine-pyrimidine regions of the human dhfr core promoter are very similar sequences, differing only by the insertion of two individual A residues. In the presence of Mg$^{2+}$, in addition to its proximal (specific) target, prox-G binds weakly to the homologous distal pur-pyr region producing a minor degree of DNase protection (a light or partial footprint; Fig. 1, lane 2) representing a relatively unstable, terminally misaligned triple helical structure (also 7,17,19,21–23). Dist-14a binds only to its specific (distal) target under these conditions (Fig. 2, lane 2). Thus a sensitive internal control for stringency of triple helical alignment is inherent in the dhfr system.

Examination of the homologous (mismatched) target sites in Figures 1 and 2 revealed an enhanced degree of DNase protection by prox-G over the distal pur-pyr region when either Co$^{2+}$ or Mn$^{2+}$ was substituted for Mg$^{2+}$. In addition, in the presence of Mn$^{2+}$, a substantial footprint produced by the unfavored association of dist-14a with the proximal pur-pyr region appeared. These results suggest that the stringency of triple helical alignment may be somewhat decreased in the presence of Co$^{2+}$ and Mn$^{2+}$. Utilization of divalent calcium in the assembly of pur-pur-pyr structures appears to actually increase the specificity of third strand binding, allowing less mismatched oligonucleotide binding than even Mg$^{2+}$ (data not shown).

**Influence of the cation on affinity of the third strand for its target**

A series of experiments was carried out to further evaluate the support for intermolecular pur-pur-pyr triple helical assembly by Mg$^{2+}$, Co$^{2+}$ and Mn$^{2+}$. In the first of these, the effect of a limiting concentration of oligonucleotide on triplex formation was titrated in the presence of a constant and sufficient concentration of each of the divalent cations. Under otherwise standard conditions, allowing 45 min for each reaction to approach equilibrium, the relative degree of facilitation of the affinity of the third strand for its double-stranded target by each cation could be ascertained (Fig. 3A). The titrations were qualitatively similar; however, manganese clearly exerted a more positive influence on triplex formation. Approximate values for $K_{assoc}$ are listed in Table 1.

![Figure 2. Variable support by divalent cations for intermolecular triplex formation at the distal pur-pyr region of the human dhfr promoter.](image)

**Table 1.** Approximate $K_{assoc}$ for triplex formation at 10 mM divalent cation concentration

<table>
<thead>
<tr>
<th>Divalent Cation</th>
<th>$K_{assoc}$ ± SD (per M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>5.8 ± 0.5 × 10$^6$</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>3.5 ± 1.0 × 10$^7$</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>3.7 ± 2.0 × 10$^6$</td>
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</tbody>
</table>

*The $K_{assoc}$ calculated for magnesium is consistent with that reported by other laboratories quantitating intermolecular pur-pur-pyr triplex formation by footprinting (15,22).
Figure 3. Titration of triple helix assembly with limiting oligonucleotide (third strand) concentration or limiting divalent cation concentration. (A) The dhfr promoter fragment (∼40 nM) was incubated with variable concentrations of the oligonucleotide prox-F in the presence of one of the supportive divalent cations (10 mM). (B) The dhfr promoter fragment was incubated with the oligonucleotide dist-14a (32 µM) in the presence of variable concentrations of divalent metal chlorides. For both (A) and (B), the samples were analyzed by DNase protection assay as described in Materials and Methods and in the legends to Figures 1 and 2 and the proportions of specific target sites bound by the oligonucleotide third strand were quantitated by laser densitometric analysis. •, Mg²⁺; ○, Co²⁺; *, Mn²⁺.

Cation potency

Next, the dependence of triplex formation on the concentration of the metal cations themselves was investigated. A gradient of Mg²⁺, Co²⁺ or Mn²⁺ was used to titrate the binding of an oligonucleotide (in constant excess) to its specific target on the human dhfr promoter (Fig. 3B). A similar decline in triplex formation accompanied the decrease in concentration of each of the divalent metal cations; however, cobalt consistently exhibited a greater potency, retaining the ability to support triplex formation at lower cation concentrations. Together, these results suggest that these individual divalent cations facilitate the intermolecular pur·pyr·pur association by a similar, though not entirely equivalent, mode of action.

Inhibition of triplex formation by divalent cations

Next, the supportive effect of Mg²⁺, Co²⁺ or Mn²⁺ was titrated against the non-supportive tendency of Ni²⁺ (Fig. 4). The total divalent cation concentration was held constant at 10 mM, while the proportionate composition of the two divalent species was varied through the series 10:0, 8:2, 5:5, 2:8 and 0:10. Binding of prox-F or dist-14a to a high proportion of dhfr promoter molecules in the presence of 10 mM MgCl₂, CoCl₂ or MnCl₂ is indicated by the high degree of DNase protection of the appropriate target (lanes 1, 6 and 11). In contrast, these footprints were lost when 10 mM NiCl₂ was utilized (lanes 5, 10 and 15). If Ni²⁺ were an extremely potent inhibitor of triplex formation, then nearly complete loss of the footprint might be expected in the second lane of each panel (8 mM Mg²⁺, Co²⁺ or Mn²⁺, 2 mM Ni²⁺). If a low concentration of one of the other divalent cations could serve to supplement the action of Ni²⁺ and facilitate triplex formation, then a clear footprint might be expected in the fourth lane of each panel (2 mM Mg²⁺, Co²⁺ or Mn²⁺, 8 mM Ni²⁺). If Ni²⁺ were passively non-supportive (inert toward triplex formation), titrations similar to those of Figure 3B (gradient of supportive cation concentration alone) would be expected. In fact, none of these was the case, as the degree of DNase protection produced by oligonucleotide binding to the specific pur·pyr target was incrementally altered throughout the titration, with half-maximal triplex formation seen at approximately an even ratio of supportive to non-supportive cation. Thus it appears that nickel(II) is an active antagonist of intermolecular pur·pyr·pur triplex formation with roughly equivalent potency as the magnesium(II), cobalt(II) or manganese(II) cations, which promote triplex formation.

Triple helical stability and cation efflux

The experimental protocol chosen to measure triple helical stability relies on dilution of component nucleic acid species to
minimize further triple helical association and thereby allow the rate of third strand dissociation from existing triple helical structures to be assayed. Initially, the oligonucleotide in modest excess (2-4 µM) was preincubated with the dhfr promoter fragment under standard conditions to allow for accumulation of triple helical structures on a high proportion of the target DNA molecules. At that point, the reaction was diluted sufficiently to decrease the concentration of the oligonucleotide to the extent (0.04–0.08 µM; Fig. 3A) that de novo triplex formation or re-association would occur slowly or not at all. The diluent was a complete 1× buffer containing Tris–HCl and the same concentration (10 mM) of the divalent metal chloride used in the initial binding reaction. By maintaining these conditions, pre-formed triplexes were not disturbed biochemically by dilution and loss of triple helical structure as a function of time could be followed.

In Figure 5, the stability of a triple helix formed in the presence of 10 mM Mg²⁺ is measured. The absence of DNase protection in the first lane (negative control), in which the oligonucleotide was withheld from the initial preincubation, but added subsequent to dilution, is indicative of the lack of post-dilution triplex formation. The footprint over the specific target in the second lane (positive control) confirms that the oligonucleotide bound a very high proportion of the dhfr promoter molecules during the preincubation period and that these triple helical structures were initially intact following dilution. The incremental decline in DNase protection over time in lanes 3–5 indicates that the <prox-F*proximal pur·pyr> triple helix formed in the presence of Mg²⁺ dissociates relatively slowly at ambient temperature. Once formed, are these triple helical structures free of dependency on supportive divalent cations? Does a population of cations remain stably associated with the three-stranded structure? Are some divalent cations continually required as an integral part of the triple helical structure, yet are susceptible to loss to the environment? For lanes 6–8 of Figure 5, the diluent contained no MgCl₂, effectively decreasing the divalent cation concentration from 10 to 0.2 mM (well below the level otherwise needed to support de novo triplex formation; Fig. 3B). For the triplex assembled in the presence of magnesium, dilution without maintenance of the environmental supportive divalent cation pool resulted in an almost instantaneous destabilization, with >70% loss of DNase protection within 1 min. Supplementation of the diluent with 20 mM NaCl or KCl did not avert this rapid destabilization of triple helical structure (data not shown). Thus, the destabilization of triple helical structure is not solely a consequence of the decrease in total environmental ionic strength, but results instead from specific loss of integral divalent cations to the environment.

This experiment was repeated, substituting MnCl₂ for MgCl₂, and the results of both experiments are presented graphically in Figure 6. With maintenance of the environmental supportive divalent cation pool, the triple helix assembled in the presence of either Mn²⁺ (curve A) or Mg²⁺ (curve B) dissociated slowly with a half-life >>15 min. Upon effective removal of the supportive divalent cation pool, the rate of triple helical dissociation was greatly accelerated. Note, however, that the divalent cation concentration of the triplex formed in the presence of Mn²⁺ (curve C; t½ ~2–4 min) was considerably less dramatic than the nearly instantaneous disintegration of the triplex assembled in the presence of Mg²⁺ (curve D). These results were confirmed using a 2 mM initial divalent cation concentration (which upon dilution became only 0.04 mM) and targeting instead the distal pur-pyr region of the dhfr promoter. Again, a significant difference in the susceptibility to cation efflux of the triple helical structure assembled in the presence of Mg²⁺ (majority lost within 1 min) and Mn²⁺ (t½ ~4 min) was observed. The triple helix assembled in the presence of Co²⁺, like that of Mn²⁺, was also associated with a delayed cation efflux/triplex destabilization relative to Mg²⁺ (data not shown).

**DISCUSSION**

We have obtained evidence for at least three distinct modes of cation coordination which critically affect several parameters relating to intermolecular pur*pur-pyr triplex assembly (Table 2).

**Support of intermolecular pur*pur-pyr triplex formation**

Assembly and stabilization of the intermolecular pur*pur-pyr triple helix is sufficiently promoted by divalent magnesium cations. Since association of the alkaline earth metals with DNA is essentially limited to electrostatic interactions with the anionic oxygen atoms of the phosphodiester backbone (24–25), it must
be this activity as a counterion for phosphate charge by which Mg$^{2+}$ enables the triple helical structure to form. The specific mode of phosphate coordination which supports the pur·pur·pyr structure remains to be determined. (An alkaline earth metal cation may potentially coordinate with one or both of the free oxygen atoms of the phosphate, oscillate between inner and outer sphere binding, migrate from one phosphate to another or coordinate to two phosphates simultaneously; 26–27.)

A subset of other divalent metal cations (Ca$^{2+}$, Mn$^{2+}$ or Co$^{2+}$) also support intermolecular triplex formation at either of the pur·pyr target sequences of the $dhfr$ promoter. It is apparently the capability of electrostatic binding and phosphate charge neutralization shared with Mg$^{2+}$ by which Ca$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ foster a qualitatively similar association of the third strand with its target.

Dependence of triplex formation on oligonucleotide (third strand) concentration

Titrations of oligonucleotide concentration indicate that the degree to which each of the supportive divalent cations facilitates the affinity of the third strand for its double helical target correlates roughly with affinity of the cation for phosphate. Mn$^{2+}$, with a greater affinity for DNA phosphate than Mg$^{2+}$, Ca$^{2+}$ or Co$^{2+}$ (24,28–31), most efficiently utilizes a limiting third strand concentration to promote triplex formation (also 32).

Stringency of triple helical alignment

Relative to Mg$^{2+}$, a modestly relaxed stringency of triple helical alignment is observed with Co$^{2+}$ and particularly Mn$^{2+}$, with a greater allowance for third strand binding to a homologous (but non-identical) target. The decreased triple helical stringency observed is limited to tolerance of unbound oligonucleotide termini (also 17,21,33); no change in position of specifically aligned structures and no clear evidence of actual mispairing is seen. The mismatched triple helical structures exhibit a lower binding affinity and lower stability (data not shown) relative to the specifically aligned triplex.

This tendency towards enhanced facilitation of triplex formation at the expense of specificity also correlates with measured potentials of the divalent metal cations for electrostatic binding to phosphate: Ca$^{2+} <$ Mg$^{2+} <$ Co$^{2+} <$ Mn$^{2+}$ (24,28). Augmented neutralization of anionic phosphate charge may compensate for a lessening of affinity due to misaligned residues (26,34).

Dependence of triplex formation on metal(II) concentration (cation potency)

The titrations of triplex formation with limiting cation concentration are consistent with the notion that a certain finite number of ‘spots’ must be filled by the supportive divalent cation in order for triple helix assembly to occur. Co$^{2+}$ and to a lesser extent Mn$^{2+}$ retain the ability to effectively support triplex formation at considerably lower metal(II) concentrations than Mg$^{2+}$. In addition to binding phosphate, the transition metal cations Co$^{2+}$ and to a lesser extent Mn$^{2+}$ are capable of coordinating to nucleophilic atoms of the nucleotide bases (28,31,35). The increased potency of Co$^{2+}$ and Mn$^{2+}$ for support of triplex formation would appear to be a function of an augmented attraction of these cations for one or both of the nucleic acid molecules, possibly through base binding or phosphate–base chelation.

Efflux of supportive divalent cations from the composite triple helical structure

The consistently accelerated rate of third strand dissociation accompanying dilution of the ambient cation pool allows us to conclude that: (i) the divalent metal cations required for assembly continue to be necessary for maintenance of the triple helical association; (ii) at least a subpopulation of these integral supportive cations are susceptible to loss via diffusion to the environment. Magnesium facilitates a relatively stable triple helical association, yet triple helical integrity is almost instantaneously compromised if the ambient cation pool is not maintained, an effect apparently accounted for by rapid efflux of essential but labile electrostatically
bound magnesium cations from the triple helical structure. A substantial slowing of this cation diffusion/triplex destabilization is seen when either Mn²⁺ or Co²⁺ is utilized as the supportive divalent cation. This would apparently invoke a novel (relative to Mg²⁺) and longer lived metal–nucleic acid interaction, such as an intrastand chelate, with simultaneous coordination of an individual divalent cation to a phosphate and a heterocyclic atom (25–28,36,37), or perhaps an intrastand chelate, involving for instance guanine N-7 or O-6 (as observed for Mn²⁺ and Co²⁺ in tRNA; 38) of the G-rich oligonucleotide, which would provide a temporary crosslink of the third strand to the underlying duplex.

**Antagonism of triplex formation**

Ni²⁺ and Cd²⁺ exhibit an electrostatic affinity for DNA phosphate greater than that of Co²⁺ (24,28), yet are essentially non-supportive and in fact actively antagonize the intermolecular purine–purine–pyrimidine interaction. This suggests the existence of an additional, detrimental mode of base coordination which may prohibit or destabilize the triple helical association through interference with intermolecular hydrogen bonding, disruption of base stacking and/or disturbance of the base triplet geometry required for compatibility with triple helical structure. These divalent transition metals Ni²⁺ and Cd²⁺ are associated with a greater propensity to bind to the nucleotide bases than Mn²⁺ or Co²⁺ (24,26) and are more likely to coordinate by the stronger, more stable inner sphere mode (27,28).

Evidence that cobalt displays both beneficial and detrimental activities toward triplex formation can also be ascertained from the data. The relative ratios of K_assoc for triplex formation at 0.5 mM Mg²⁺, Mn²⁺ or Co²⁺ are 1.0:3.1:11.9, respectively (Fig. 3B), whereas at 10 mM divalent cation concentration these ratios become 1.0:6.0:6.4 (Fig. 3A and Table 1). Thus at low concentrations, cobalt is superior even to manganese for support of triplex formation; however, this advantage is counterbalanced at higher cobalt concentrations.

We have previously observed this biphasic response across the transition series, with regard to the ability of these divalent cations to counteract the K⁺-associated induction/stabilization of quadruplex self-association of G-rich oligonucleotides and thereby facilitate triplex assembly in the presence of K⁺ (20). Without inducing discernible alterations in the circular dichroism spectra of the oligonucleotides, these cations potently suppress the rapid increase in molar ellipticity at 259–261 nm which is otherwise observed upon addition of potassium. In this manner, such alterations of the cationic environment allow triplex formation to effectively compete against quadruplex formation (which otherwise, in the presence of K⁺, sequesters the intended third strand population). This triplex-favorable property (K⁺/quadruplex resistance), like cation potency and relative resistance to cation efflux, initially increases across the transition metal series; however, with higher concentrations or further progression across the series, a distinctly detrimental (to triplex formation) mode of cation coordination becomes evident and potassium-resistant triplex formation is lost.

**Potential biological relevance**

The sequences of the human dhfr promoter which we have studied are representative of a pattern (G-rich purine tracts with isolated pyrimidine interruptions) which is very prevalent in the human genome and which comprise viable targets for the formation of stable, specific triple helical structures in vitro (17–20). The particular composition of these sequences (such as the prevalence of A*T*A*T triplets or alignment at pyrimidine interruptions) may have important ramifications for cation coordination and triplex assembly (39–41).

These studies demonstrate ways in which the potential for triplex formation and the properties of the triple helical structure might be modulated. It has been suggested that triplex formation might be utilized naturally as a means of physiological regulation of molecular biological processes within the cell (42–50). Although free intracellular concentrations of the transition metal cations are considerably lower than those utilized in these experiments, it is conceivable that some of the triplex-modulatory effects we have observed in vitro with naked transition metal cations could be accomplished naturally in vivo by a specialized accessory polypeptide domain, perhaps through presentation of a coordinated metal cation (51) or through a particular arrangement of basic (cationic) amino acid residues (52). As precedents for these concepts, consider that formation of the triple helical intermediate involved in homologous recombination requires an accessory polypeptide factor(s) (53,54) and that possible physiological roles of cations as allosteric effectors of other types of nucleic acid–nucleic acid interactions have been proposed (55,56). It has also been proposed that administration of an exogenous oligonucleotide to induce intermolecular triplex formation site specifically on genomic DNA might be used as a means of therapeutically modulating the expression of specific genes (17–56). The data presented herein support the concept that rational modification of oligonucleotide structure, to include the permanent, site-specific incorporation of a cationic moiety, might be utilized to potentiate the therapeutic efficacy of the sequence-specific triple helical interaction (also 63–67). Such investigations are currently underway in this laboratory.

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