Cation and sequence effects on stability of intermolecular pyrimidine-purine-purine triplex

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ABSTRACT

A differential effect is found of various bivalent cations (Ba^{2+} , Ca^{2+} , Mg^{2+} , Cd^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} and Hg^{2+}) on stability of intermolecular Py-Pu-Pu triplex with different sequence of base triads. Ca^{2+} , Mg^{2+} , Cd^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} do stabilize the $\text{d}(\text{C})_n \text{d}(\text{G})_n \text{d}(\text{G})_n$ triplex whereas Ba^{2+} and Hg^{2+} do not. Ba^{2+} , Ca^{2+} , Mg^{2+} and Hg^{2+} destabilize the $\text{d}(\text{TC})_n \text{d}(\text{GA})_n \text{d}(\text{AG})_n$ triplex whereas Cd^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} stabilize it. The complexes we observe are rather stable because they do not dissociate during time of gel electrophoresis in the co-migration experiments. Chemical probing experiments with dimethyl sulfate as a probe indicate that an arbitrary homopurine-homopyrimidine sequence forms triplex with corresponding purine oligonucleotide in the presence of Mn^{2+} or Zn^{2+} , but not Mg^{2+} . In the complex the purine oligonucleotide has antiparallel orientation with respect to the purine strand of the duplex. Specifically, we have shown the formation of the Py-Pu-Pu triplex in a fragment of human papilloma virus HPV-16 in the presence of Mn^{2+} .

INTRODUCTION

Two major classes of DNA triplex have been characterized: pyrimidine-purine-pyrimidine (Py-Pu-Py) and pyrimidine-purine-purine (Py-Pu-Pu). Both types of triplexes may be formed in homopurine-homopyrimidine regions of normal Watson–Crick duplex, which accepts into its major groove either pyrimidine (in case of the Py-Pu-Py triplex) or purine (in case of the Py-Pu-Pu triplex, see Fig. 1) strand.

Both types of triplexes may be formed intermolecularly, when corresponding oligonucleotide binds to the homopurine-homopyrimidine sequence (1–9), or intramolecularly, when H-DNA is formed (10–19).

Quite different ambient conditions favor these two types of triplexes. Whereas the Py-Pu-Py triplex is stabilized by lowering pH, due to protonated triad CG^+C^+ , which enters this type of triplex, the Py-Pu-Pu triplex is usually stabilized by bivalent cations.

However, this bivalent-cation stabilization is by no means a simple electrostatic phenomenon. Earlier we have shown that the

intermolecular $\text{d}(\text{TC})_n \text{d}(\text{GA})_n \text{d}(\text{AG})_n$ triplex is stabilized by Zn^{2+} ions but not by Mg^{2+} ions (6,7). By contrast, the $\text{d}(\text{C})_n \text{d}(\text{G})_n \text{d}(\text{G})_n$ triplex is stabilized by Mg^{2+} ions.

Here we present a systematic study of the effect of various bivalent cations on the formation of Py-Pu-Pu triplex at neutral pH to determine general conditions stabilizing this type of triplexes.

We do not consider in the present paper the protonated Py-Pu-Pu triplex, which has been thoroughly studied by us in a recent paper (20).

MATERIALS AND METHODS

Plasmids

The pTC33.4 plasmid carrying two $\text{d}(\text{TC})_{16} \text{d}(\text{GA})_{16}$ inserts was prepared as described (4). The pG31, pTC33, pA56 and pAA32 plasmids were taken from the Moscow laboratory library of plasmids. The inserts were incorporated into the polylinker of the pUC19 plasmid. The sequences of inserts, plus a flanking polylinker, between boundary EcoRI and HindIII sites, are listed. Only strands carrying the homopyrimidine tract of the inserts are shown: pTC33.4: 5'-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGATCC(TC)₁₆GAATTGATCC(TC)₁₆GAATTGGGTACCGAGCTCGAATT-3'

pG31: 5'-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCA(C)₃₁TGCAGGCATGCA-3'

pA56: 5'-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCA(T)₅₆GCAGGCATGCA-3'

pTC33: 5'-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCC(TC)₁₆GAATT-3'

pAA32: 5'-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCTTCCCTCTTTCCCCTATACCCCTTTCTCCC-TTGAATT-3'

The pFR16 plasmid was obtained by insertion of the 132-bp-long PvuII-PvuII fragment of human papilloma virus HPV-16 (positions 554–685 (21)) into the SmaI site of the polylinker of the pUC19 plasmid.

The plasmids pG21 and pG22 were obtained as a result of spontaneous deletion of the pPG37 plasmid, which has been kindly provided by Dr. I. Panyutin. The pG21 and pG22 plasmids carry the inserts $\text{dG}_{21} \text{dC}_{21}$ and $\text{dG}_{22} \text{dC}_{22}$ correspondingly, incorporated into the PstI site of the pUC18 plasmid.

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Figure 1. Isomorphous CG*G and TA*A base-triads corresponding to antiparallel polarity of the third purine strand with respect to the purine strand of the duplex. All nucleotides have the *anti* conformation.

UV photofootprinting

The pTC33.4 plasmid was cut with the EcoRI and HindIII restriction enzymes and the 3'-ends of the EcoRI site were labelled with $\alpha^{32}\text{P}$ dTTP and by Klenow fragment of DNA polymerase I. The fragment carrying the inserts was isolated by electrophoresis in 6% polyacrylamide gel, eluted from the gel slice into 100mM NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA, concentrated by ethanol precipitation, and redissolved in 5mM Tris-HCl (pH 8.0), 0.5mM EDTA. About 0.1 pmols of labeled fragments were incubated with 3 μM of oligonucleotide d(AG)₅ in 20 μl of appropriate buffer (see figure legends) for 3 hours at room temperature.

The samples were irradiated by two germicidal lamps (15W, 254nm) for 5 min. with an intensity 60 J sec⁻¹ m⁻². Irradiated DNA was twice ethanol precipitated.

To reveal the [6-4] photoproducts, DNA was dissolved in 100 μl of 1M piperidine. Reaction with piperidine was carried out at 90°C for 30 min. The products of the DNA scission were resolved in an 8% polyacrylamide denaturing gel.

Co-migration assay

To avoid formation of the H* form, the plasmids were linearized prior to the co-migration experiments. The pTC33 and pA56 plasmids were cut by the HindIII restriction enzyme, the pG31 plasmid was cut by the EcoRI restriction enzyme. 0.4 μg of linearized plasmid DNA was incubated with 4-fold molar excess of corresponding oligonucleotide (see legends to figures) at room temperature for 2 hours. Gel electrophoresis was performed in 1.5% agarose gel for 3.5 hours. Radioautography and staining with ethidium bromide were performed as described in (2).

Probing with dimethyl sulfate (DMS)

To prepare DNA fragments for modification by DMS the pTC33.4, pAA32 and pFR16 plasmids were cut with HindIII restriction enzyme, 3'-end labeled with Klenow fragment of DNA

polymerase I, digested, with restriction enzymes, EcoRI, for the pTC33.4 plasmid, or BglI, for the pAA32 and pFR16 plasmids. Smaller labeled fragments of each plasmid were used for further experiments. In case of the pG21(pG22) plasmids a slightly modified scheme was used: first the plasmid was cut with the EcoRI restriction enzyme, 3'-end labeled and then it was cut with HindIII.

DMS footprinting of $dC_n dG_r dG_m$ and $d(CT)_n d(AG)_r d(GA)_m$. The prepared fragments were dissolved in 20 μl of the buffer: 50mM MOPS, pH6.9, 50mM NaCl and 5mM MeCl₂ (or 2mM EDTA, instead of MeCl₂, in control experiments). All changes of the above standard conditions are indicated in legends to figures. Then 1 μl of the oligonucleotide solution was added (concentrations of stock solution of oligonucleotides were 1.0 A₂₆₀units/ml for d(AG)₅, 5.0 A₂₆₀units/ml for dG₁₀). The mixture was incubated for 2 hours at room temperature. Then 1 μl of 3% DMS was added and the reaction was performed for 15min at 20°C. After double precipitation in ethanol, the samples were treated in 1.0M mercaptoethanol, 1mM EDTA and the products of cleavage were run in 8% polyacrylamide denaturing gel.

DMS footprinting of irregular triplexes. In case of the pAA32 plasmid, 40 μl of solution containing the fragment and the dGAG-AAAGGGG oligonucleotide (3 μl of 5.0 A₂₆₀units/ml stock solution) in the above buffer were incubated for 3 hours. Then 2 μl of 5% DMS was added and incubated at 10°C for 2min. The reaction was terminated with 10 μl DMS-STOP solution (1.5M NaOAc, pH5.2, 1.0M mercaptoethanol, 250 $\mu\text{g}/\text{ml}$ of tRNA). After double precipitation in ethanol, the samples were treated with 100 μl of 10% piperidine at 90°C for 30 min and the products of cleavage were run in 10% polyacrylamide denaturing gel. The 3'-ends of the fragment are at the bottom.

In case of the pFR16 plasmid, 40 μl of solution containing the fragment and the dAGGAGGAGGAGA oligonucleotide (5 μl of 1.0 A₂₆₀units/ml stock solution) were incubated for 3 hours at room temperature. Then 2 μl of 5% DMS was added and incubated for 3 min. The subsequent treatment was the same as in case of the pAA32 plasmid.

RESULTS

Effect of bivalent cations on formation of regular triplexes $d(TC)_n d(GA)_n d(AG)_m$ and $d(C)_n d(G)_n d(G)_m$

We used three assays to test the triplex formation between linear plasmid DNA carrying the homopurine-homopyrimidine insert ($d(TC)_n d(GA)_n$ or $d(C)_n d(G)_n$) and corresponding oligonucleotide. The DMS footprinting is grounded on the protection effect of triplex in the N7 position of guanine of plasmid DNA against alkylation by dimethyl sulfate (12). The photofootprinting assay is grounded on inability of adjacent pyrimidines in the duplex to form the [6-4] photoproducts in the triplex zone (4,6,7). And, finally, the co-migration assay is applicable to very strong, practically irreversible complexes between plasmid DNA and oligonucleotide. If such a strong complex is formed, a labeled oligonucleotide forms in gel, after electrophoresis, the bands coinciding with the bands formed by stained unlabeled plasmid DNA (2).

Fig.2 shows the results of DMS footprinting experiments for d(AG)₅ and the pTC33.4 plasmid carrying two d(CT)₁₆ d(AG)₁₆

Figure 2. Probing with dimethyl sulfate (DMS) of the complex formed between the $d(AG)_5$ oligonucleotide and a fragment of the pTC33.4 plasmid, carrying two $d(CT)_{16}d(AG)_{16}$ inserts, in the presence of different bivalent cations.

inserts, in the presence of different bivalent cation. At neutral pH without bivalent cations (lane 18) or in the presence of ions of alkaline-earth metals (lanes 2,10), the intermolecular Py-Pu-Pu triplex does not form. By contrast, a stable complex is formed in the presence of ions of transition metals, Cd^{2+} (lane 4), Co^{2+} (lane 6), Mn^{2+} (lane 12), Zn^{2+} (lane 16) and Ni^{2+} (lane 14), but not Hg^{2+} (lane 8).

Fig.3 shows that the photofootprinting assay leads to the same conclusion. In the presence of ions of alkali-earth metals (Ba^{2+} , Ca^{2+} and Mg^{2+}) the [6-4] photoproduct forms (lanes 2-4) while in the presence of ions of transition metals, Cd^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} or Ni^{2+} , (lanes 5-9) one can see a sharp decrease of the yield of the photoproduct (see also refs. 7, 22).

Fig.4 shows that the $d(C)_n d(G)_n d(G)_m$ triplex is not so selective with respect to the nature of cation. The stable complex for this sequence is formed in the presence of all bivalent cations tested with the exception of Ba^{2+} (lane 1) and Hg^{2+} (lane 9). Note that both ends of the insert exhibit a strong effect of hyperreactivity with respect to DMS.

Fig.5 shows that in some cases the Py-Pu-Pu triplex is so stable that can be seen even in the co-migration assay. The right panels in Fig.5 present gels stained with ethidium and the bands show the positions of linearized plasmid DNA. The left panels present the radioautograph of the same gel and the bands indicate the position of labeled oligonucleotide.

Fig.5A demonstrates that only labeled oligonucleotide dG_{10} preincubated with the pG31 plasmid, carrying the $dG_{31} dC_{31}$ insert, in the presence of Ca^{2+} moves with plasmid DNA in the buffer containing Ca^{2+} . Under our conditions (neutral pH)

Figure 3. Detection of the triplex formation between the $d(AG)_5$ oligonucleotide and a fragment of the pTC33.4 plasmid, carrying two $d(CT)_{16}d(AG)_{16}$ inserts, by photofootprinting. The [6-4] photoproducts were measured. Irradiation was conducted at room temperature in 10mM Tris at pH7.5, 50mM NaCl, 1mM EDTA (lane1); 15mM Tris at pH7.8, 7mM $BaCl_2$ (lane 2); 15mM Tris at pH7.7, 7mM NaCl, 7mM $CaCl_2$ (lane 3); 15mM Tris at pH7.7, 7mM NaCl, 7mM $MgSO_4$ (lane 4); 15mM Tris at pH7.1, 7mM NaCl, 7mM $Cd(NO_3)_2$ (lane5); 15mM Tris at pH7.1, 7mM NaCl, 7mM $CoSO_4$ (lane 6); 15mM Tris at pH7.0, 7mM NaCl, 7mM Mn Acetate (lane 7); 20mM Tris at pH7.0, 50mM NaCl, 10mM $ZnSO_4$ (lane 8); 15mM Tris at pH7.0, 7mM $NiCl_2$ (lane 9).

pyrimidine oligonucleotides do not form triplex with corresponding homopurine-homopyrimidine sequences (lanes 2, 4). Complex between $d(CT)_{16} d(AG)_{16}$ and $d(AG)_5$ oligonucleotide does not form (lane 3) in the presence of Ca^{2+} . This finding agrees with the data in Fig.3. Although formation of triplexes $dT_n dA_n dT_n$ and $dT_n dA_n dA_n$ was reported under similar ambient conditions (23,24), our assay does not permit their observation due to, probably, their low stability. Similar data on binding of only dG_{10} with $dG_{31} dC_{31}$ and the lack of binding of all other oligonucleotides were observed in the presence of Mg^{2+} ions (data not shown).

Fig.5B shows formation of stable triplexes $d(CT)_n d(AG)_n d(AG)_m$ and $d(C)_n d(G)_n d(G)_m$ in the presence of Mn^{2+} ions (lanes 1, 3). Similar data were obtained for Zn^{2+} , Cd^{2+} and Co^{2+} (data not shown).

Note that some metal ions significantly precipitated on the electrode. Therefore the ion concentration we indicate in figure legends corresponds to the concentration in the buffer, prior to electrophoresis.

Figure 4. Probing with dimethyl sulfate (DMS) of the triplex formation between the dG₁₀ oligonucleotide and a fragment of the pG21 plasmid carrying the dG₂₁dC₂₁ insert (a) and pG22 plasmid carrying the dG₂₂dC₂₂ insert (b). All conditions are clear from the headers with the exception of lane 1 in panel b, which corresponds to the labeled fragment subjected to piperidine treatment without the DMS modification.

Triplex formation between purine oligonucleotide and mirror-repeated homopurine-homopyrimidine duplex

The fact that a number of cations (Cd²⁺, Co²⁺, Mn²⁺, Zn²⁺ and Ni²⁺) stabilize both, d(CT)_n d(AG)_n d(AG)_m and d(C)_n d(G)_n d(G)_m, triplexes implied that in the presence of these metal ions the Py-Pu-Pu triplex may be stable not only for regular sequences but for any homopurine-homopyrimidine sequence. To check this possibility we took the pAA32 plasmid carrying an irregular (almost) homopurine-homopyrimidine mirror repeat, AAGGGAGAAAGGGGTATAGGGGAAAGAGGGAA, and synthesized the corresponding purine oligonucleotide dGAG-AAGGGGG.

Fig. 6 shows the data on DMS footprinting for this system. In the presence of Mg²⁺ ions, the modification pattern is homogeneous along the whole mirror repeat, just as in the control lanes without bivalent cations. In the presence of Mn²⁺ ions one can see clear-cut asymmetry in the modification pattern for two halves of the mirror repeat. Specifically, guanines in the 5'-half of the purine strand of the repeat are modified as heavily as in control lanes without oligonucleotides (see the odd lanes) indicating that this sequence does not form triplex with the oligonucleotide. By contrast, all guanines in the 3'-half of the purine strand of the mirror repeat, which are expected to be covered due to triplex formation, are virtually fully protected against modification unambiguously indicating that this region forms triplex in the presence of Mn²⁺ ions (see lane 4). Note that in lane 4 the 3'-flanking guanine, immediately adjacent to the triplex zone, is hypermodified by DMS. Thus, one can conclude that in the presence of Mn²⁺ ions purine

Figure 5. Experiments on co-migration of labeled oligonucleotides dG₁₀ (lane 1), dC₁₀ (lane 2), d(AG)₅ (lane 3), d(CT)₅ (lane 4), dA₁₀ (lane 5) and dT₁₀ (lane 6) with the plasmids pG31 (lanes 1,2), pTC33 (lanes 3,4) and pA56 (lanes 5,6). **A**, Preincubation was performed in 15mM Tris Ac pH7.7, 15mM NaCl, 5mM CaCl₂; gel electrophoresis was conducted in 20mM Tris Ac pH7.6, 50mM NaAc, 10mM CaCl₂. **B**, Preincubation was performed in 20mM Tris Ac pH7.1, 50mM NaCl, 5mM MnAc; gel electrophoresis was conducted in 20mM Tris Ac pH7.1, 50mM NaAc, 10mM MnAc.

oligonucleotides form triplex in which the third strand is antiparallel with respect to the purine strand of the duplex.

Similar, though less clear-cut, effects are observed in the presence of Zn²⁺ ions (lane 6). Although the protection effect in this case is not so evident, the clear-cut hyperreactivity of the 3'-flanking guanine leaves no doubts that the antiparallel triplex is formed in this case as well. Thus, the data in lane 6 of Fig. 6 confirm our earlier claim that in the presence of Zn²⁺ ions the antiparallel Py-Pu-Pu triplex is formed in an arbitrary homopurine-homopyrimidine sequence (7).

Formation of Py-Pu-Pu triplex between dAGGAGGAGGAG-A oligonucleotide and homopurine-homopyrimidine region, AGAGGAGGAGGA, of human papilloma virus HPV-16

Just as in other cases when the CG*G and TA*A triads should alternate in the triplex, triplex between the HPV-16 homopurine-homopyrimidine region AGGAGGAGGAGA and the dAGG-AGGAGGAGA oligonucleotide is not formed in the presence of Mg²⁺ ions (data not shown).

Again in full agreement with the above data, the presence of Mn²⁺ ions led to formation of the triplex. For this system we have varied both, the Mn²⁺ concentration and the sodium ions concentration (see Fig. 7).

Lanes 2–4 show that 1mM of Mn²⁺ ions is not sufficient to stabilize the triplex. In 3mM of Mn²⁺, the triplex is only partially formed. The stable triplex formation requires high concentrations of Mn²⁺ ions and lane 4 demonstrates that 10mM is quite sufficient concentration, as can be seen also in Fig. 6.

Figure 6. Probing with dimethyl sulfate (DMS) of the triplex formation between a homopurine-homopyrimidine mirror repeat and the dGAGAAAGGGG oligonucleotide in the presence of different bivalent cations. The observed complex is schematically shown. The bracket indicates the binding site for the oligonucleotide in case of parallel polarity of the third strand with respect to the purine strand in the duplex. Preincubation and modification were conducted at 10°C in 25mM Tris pH7.0, 25mM NaCl, 10mM MgSO₄ (lanes 1,2); 25mM Tris pH7.0, 25mM NaCl, 10mM MnAc (lanes 3,4); 25mM Tris pH6.5, 50mM NaCl, 10mM ZnSO₄ (lanes 5,6).

Lanes 6–8 in Fig.7 show that, in the presence of 10mM Mn^{2+} , increasing of concentration of sodium up to 200mM does not virtually affect the triplex stability.

Note that the data in Fig.7 definitely confirm the antiparallel orientation of the third strand in the Py-Pu-Pu triplex with respect to the purine strand of the duplex. Indeed, if the parallel orientation were also possible we would not observe the complete protection of the 5'- and/or 3'-guanine of the insert. Lanes 4 and 6 vividly demonstrate that in the stable triplex these guanines are completely protected against DMS modification.

DISCUSSION

Using a number of experimental techniques we have thoroughly studied the effects of various bivalent cations and of DNA sequence on stability of the pyrimidine-purine-purine triplex. The method often used to detect triplexes consists in chemical or photochemical cleavage of the duplex by an active group tagged to oligonucleotide (1,8,9). It makes possible to detect even very

Figure 7. Probing with dimethyl sulfate (DMS) of the triplex formation between a fragment of human papillome virus (HPV-16) and the corresponding oligonucleotide, dAGGAGGAGGAGA, at different ionic conditions: 30mM Tris pH7, 1mM MnAc, 50mM NaCl (lanes 1,2); 30mM Tris pH7, 3mM MnAc, 50mM NaCl (lane3); 30mM Tris pH7, 10mM MnAc, 50mM NaCl (lanes 4–6); 30mM Tris pH7, 10mM MnAc, 100mM NaCl (lane 7); 30mM Tris pH7, 10mM MnAc, 200mM NaCl (lane 8).

weak complexes. By contrast, all methods we used detect only really strong complexes for which overwhelming majority of binding sites in the sample are stably occupied by oligonucleotide. As a result, we observe either protection of the binding site from chemical/photochemical reaction or co-migration of the labeled oligonucleotide with DNA carrying the binding site.

Note that originally the co-migration assay was used to study pyrimidine-purine-pyrimidine triplexes, which can be tremendously stabilized by lowering pH (2). The co-migration assay may be applied only to virtually irreversible complexes, for which the dissociation time comparable with the time of electrophoresis, i.e. several hours.

Experiments presented above lead to two major conclusions. First, bivalent cations are as strong stabilizers of the Py-Pu-Pu triplex as protons in case of the Py-Pu-Py triplex. Fig.5 most vividly demonstrates this fact showing that in the presence of Ca^{2+} ions the dG₁₀ oligonucleotide co-migrates with DNA carrying the dG₃₁dC₃₁ insert while in the presence of Mn^{2+} ions the d(AG)₇ oligonucleotide co-migrates with pTC33 plasmid

carrying the $d(TC)_{16}d(GA)_{16}$ insert as well as the co-migration of dG_{10} with $dG_{31}dC_{31}$. Secondly, conditions exist under which the Py-Pu-Pu triplex is formed by irregular sequences of adenines and guanines. Studying such triplexes we have shown, beyond any doubt, that only triplex in which two purine strands are antiparallel with respect to each other is stable (see Figs.6,7).

Our data on polarity of the Py-Pu-Pu triplex agree with the results obtained by the method of cleavage of the duplex by chemical or photochemical groups tagged to the oligonucleotide (8,9).

Although X-ray crystallographic data about the Py-Pu-Pu triplex are lacking, the available NMR data strongly support the base-pairing scheme in Fig.1 and indicate that all nucleotides are in the *anti* conformation (25).

In contrast to the nature of the stabilizing effect of protons on the Py-Pu-Py triplex, which is directly evident from structure of the isomorphous TA*T and CG*C⁺ base triads, the nature of the stabilizing effect of bivalent cations on the TA*A and CG*G base triads is by no means obvious. Our experiments provide with empiric data, which indicate that the picture is rather complicated.

Ba²⁺ and Hg²⁺ ions do not stabilize the Py-Pu-Pu triplex at all. One may speculate that these cations are too bulky to be incorporated into the triplex. Mg²⁺ and Ca²⁺ ions stabilize only Py-Pu-Pu triplex consisting exclusively of the CG*G triad and do not stabilize triplexes consisting of both, CG*G and TA*A, triads (see Figs.2–7). Thus, ions of alkali-earth metals are poor stabilizers of the Py-Pu-Pu triplex. By contrast, transition metal ions (Cd²⁺, Co²⁺, Mn²⁺, Zn²⁺ and Ni²⁺) stabilize the Py-Pu-Pu triplex for all sequences studied by us. Thus, we conclude that the presence in sufficient amount (in concentration about 5mM or more) of one of the transition metal ions, Cd²⁺, Co²⁺, Mn²⁺, Zn²⁺ and Ni²⁺, is a sufficient condition for the Py-Pu-Pu triplex to form.

The wider range of conditions for the $d(C)_n d(G)_n d(G)_m$ triplex to exist is by no means surprising because the regular sequences are well known to have less stringent structural limitations. The $d(C)_n d(G)_n d(G)_m$ triplex may exist in two structural forms one of which is consistent with the incorporation of TA*A triads while the other one, which is formed in the presence of Mg²⁺ and Ca²⁺ cations, is inconsistent with such incorporation. The difference in structure may entail the difference in preferential binding sites for Mg²⁺ and Ca²⁺ ions on one side and the transition metal ions on the other. The former most probably bind with phosphates while the latter with the bases.

We can not exclude that the stable Py-Pu-Pu triplex carrying TA*A triads could form in the presence of Mg²⁺ ions if the TA*A triads were rarely incorporated within long stretches of CG*G triads. We believe, however, that in these triplexes, which were observed by some authors (9,26), the TA*A triads behave as 'mismatches' and significantly destabilize the triplex because we have systematically failed to observe stable triplexes in the presence of magnesium ions for sequences with significant alternation of guanines and adenines. By contrast, in the presence of transition metal ions, e.g. the Mn²⁺ ion (see Figs 2,3,5,7), sequences with significant alternation of adenines and guanines (such as ...AGAG... or ...AGGAGG...) form very stable triplexes at room temperature even when they are rather short consisting of only 10–12 base triads.

Note that, as was first demonstrated by Beal and Dervan (8), adenines in the Py-Pu-Pu triplex may be replaced with thymines.

As a result the AT*T triads are formed, which could be even more stable than the TA*A triads. Our data presented elsewhere (27) indicate that such triplexes exhibit the same specificity with respect to bivalent cations as the 'ordinary' Py-Pu-Pu triplexes studied in the present paper.

The data in Figs. 4 and 6 on hyperreactivity of the flanking guanines with respect to dimethyl sulfate are worth mentioning. They most probably indicate that some structural peculiarity occurs at the Py-Pu-Pu triplex-duplex junction. In case of an irregular sequence (Fig. 6) we actually observed the effect only for the guanine adjacent to the triplex from the 3'-end. In case of homogeneous sequence (Fig. 4) we observed the effect at both ends and for guanines within the insert. The apparent difference in the results is due to the fact that for the irregular sequence there was no guanine adjacent to the 5'-end of the region while for the regular sequences, which were 21 and 22 bp-long, two oligonucleotide molecules consisting of 10 nucleotide each always left uncovered guanines at one or both ends of the insert. The uncovered guanines were never observed in the middle of the insert because such a gap between the two oligonucleotide molecules would result in two additional duplex-triplex junctions. The triplex-duplex junctions are known to be highly unfavorable in case of the Py-Pu-Py triplex (28) and it is natural to expect them to be unfavorable for the Py-Pu-Pu triplex as well. Note that our data on hyperreactivity of flanking nucleotides in case of the Py-Pu-Pu triplex correlate very well with the data recently reported by the Wells group (29) for case of the Py-Pu-Py triplex.

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