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The long repetitive polypurine/polypyrimidine sequence $(TTCCC)_{48}$ forms DNA triplex with PU-PU-PY base triplets *in vivo*

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ABSTRACT

Polypurine/polypyrimidine repetitive sequences occur with high frequency in eucaryotic genomes, particularly around transcription units. Since such sequences are known to adopt triple stranded-structures under appropriate conditions *in vitro*, it is of major interest to know if they occur *in vivo*, and thus if they can have some biological importance by inducing structural constraints in the genomic DNA. To this end, we have isolated a $(TTCCC)_{48}$ sequence, present in the promoter of an avian gene, and tested its ability to form PU-PY-PY and PU-PU-PY triple helices *in vitro*, through the oligonucleotide gel shift technique and single strand-specific nuclease footprinting. We have then developped an oligonucleotide protection assay, which can be adapted to *in vivo* investigations. This strategy leads us to conclude that *in vivo* conditions allow preponderant formation of triplex of the PU-PU-PY class.

INTRODUCTION

The first observed triple-stranded structure of nucleic acids was obtained *in vitro* in acidic conditions, and involved PU-PY-PY base triplets (1; 2). A low pH favours such structures since the G-C-C^+ triplet is stabilized by the protonation of a C residue at the N_3 position. The biological meaning of these structures remains hypothetical since the intracellular pH is unlikely to be acidic. However, a methylation of C residues (3), and polyamines (4) were shown to allow formation of PU-PY-PY triplexes at neutral pH. More recently, it has been demonstrated that formation of DNA triplexes can occur spontaneously at neutral pH and in the presence of Mg^{2+} ions. In such conditions, the base triplets observed are G-G-C, and thus PU-PU-PY (5; 6). More generally, whatever their base composition, PU-PY DNA molecules are expected to adopt one of the two triplex structures PU-PU-PY or PU-PY-PY displayed in Figure 1. In the present work, we have studied the relative influence of pH and Mg^{2+} on these alternative structures *in vitro*, using the oligonucleotide gel-shift technique (7) and an enzymatic footprinting assay. We have next developped a new method yielding analogous results, but which, in addition, can be transposed to *in vivo* investigations. As shown in the first part of this article, ionic conditions have a preponderant influence on the formation of the alternative structures PU-PU-PY and PU-PY-PY. This makes impossible the determination of the triplex structure prevailing *in vivo* through techniques that use synthetic buffers, such as single strand specific nuclease digestion, single strand chemical modification, or oligonucleotide hybridization, even if care is taken to preserve the nucleus structure (8). The indirect method presented below avoids the bias introduced by the incubation, or hybridization buffers, by exploiting the presence of single strand-specific exonucleases in eucaryotic cells, which are responsible for the rapid degradation of oligonucleotides after their internalization inside cells. We have checked for the presence of the single stranded half-repeat yielded by the triplex formation, through its ability to protect complementary oligonucleotides from such nuclease digestion.To further confirm the biological relevance of the results, this work was carried out with a natural homo-pyrimidic motif, lying in the promoter sequence of an avian gene.

MATERIALS AND METHODS

DNA and plasmids

For all experiments, we have used the same $(TTCCC)_{48}$ mirror repeat, containing 60% G+C, cloned from the quail genome, together with original surrounding sequences. This repetitive sequence is both extremely long and homogeneous since it contains no modification or disruption. It lies in the promoter region of the oncogene-inducible T64 gene (9) and is inserted between two cis-acting domains responsible for its transcription, as determined by functional deletion analysis (Herault *et al.* In preparation). A 287 bp-long genomic fragment encompassing the $(TTCCC)_{48}$ repeat was subcloned between the XhoI and HindIII sites of a MP12-derived plasmid, such that the 5′ and 3′ ends of the puric strand correspond respectively to the XhoI and HindIII sites. For controls, a sequence of similar length, devoid of mirror repeat, corresponding to the 280 bp-long EcoRV-PvuII segment from the pBSK (Stratagene) polylinker was subcloned in the same plasmid environment. Two 20 mer-oligonucleotides $(5'\text{-CCCTT-}3')_4$, and $(5'\text{-AAGGG-}3')_4$ respectively named PY and PU, were synthesized using the solid phase phosphoramidite method (Applied Biosystem).

The T4 polynucleotide kinase (Boehringer) was used for the 5′-labeling of oligonucleotides.

Gel shift experiments

Plasmids containing the $(TTCCC)_{48}$ sequence were incubated for 15 min at 37°C with ^{32}P-labeled PU and PY oligonucleotides, with or without 10 mM $MgCl_2$ and in various pH conditions. Buffers at pH 5 and 6 were made with 0.1 M Na-Acetate, and at pH 7 and 8 with 0.1 M Tris-Acetate. The mixtures were then loaded on 1% agarose gels and electrophoresed in the same buffers at 20 volts/cm. The gels were dried onto Hybond N membranes (Amersham) and exposed to Hyperfilm MP (Amersham).

Intramolecular footprinting of triplex structures

5μg of native plasmids containing the repetitive sequence were first incubated for 15 min. at 37°C in 1×incubation buffers for S1 (50 mM NaOAc, pH:4.5; 200mM NaCl; 1mM $ZnCl_2$; 1% Glycerol) or P1 (10 mM Tris-Cl^- pH: 7.6; 50 mM NaCl; 10 mM $MgCl_2$; 1mM dithiothreitol) nucleases. The enzymes S1 (1.4 units/μg DNA) or P1 (2 units/μg DNA) were then added and allowed to digest at 37°C for 10 min respectively. Nuclease concentrations and incubation times were chosen to obtain an average of one nick per molecule. After phenol extraction and ethanol precipitation, the plasmids were cleaved with XhoI and then ^{32}P-labeled either at the 5′ end with T4 polynucleotide kinase or at the 3′ end with the Klenow fragment of DNA polymerase I. The labeled DNA was digested with HindIII and the 287 bp-long fragment containing the repetitive sequence was isolated from a native 6% polyacrylamide gel by electroelution. It was resolved in a denaturing gel (Acryl/ Bis. : 80/ 1) containing 8.3 M urea (pH:8.3), 1 M Tris-Borate and 10 mM EDTA (Merck), with a thickness gradient of 0.5 mm-1.5 mm from top to bottom. In these conditions, a linear relationship between migration and fragment length was achieved.

Triplex-mediated oligonucleotide protection *in vitro*

^{32}P-labeled PU or PY oligonucleotides (16000 cpm) were incubated for 5 min. at 37°C with a molar excess of control or $(TTCCC)_{48}$ containing plasmids in 1×incubation buffers for S1 or P1 nucleases, and an excess of these enzymes was added for 20 min at 37°C. Reactions were electrophoresed on denaturing 20% polyacrylamide gels (Acryl/ Bis. : 38/ 2), and the presence of protected oligonucleotides was visualized by autoradiography.

Triplex-mediated oligonucleotide protection *in vivo*

Radiolabeled PU or PY oligonucleotides (10^5 cpm) were incubated, and electroporated with 40 μg of plasmids containing or not the $(TTCCC)_{48}$ sequence in 10^7 Hela cells. Electroporations were performed with the Biorad Gene Pulser apparatus in optimal conditions as tested for Hela cells (960μF and 250v). Cells were then seeded at a density of10^7 cells per 140 mm diameter petri dish and grown in DMEM (Boehringer) supplemented with 10% newborn calf serum. 24 hours after transfection, cells were harvested and lysed with Nonidet P-40. Nuclei were pelleted and lysed in 10 mM Tris-Cl-, pH: 7.8, 10mM NaCl, 2mM MgCl2, 5mM dithiothreitol, 0.5% Na-dodecyl-sulfate, and 50 μg/ml RNase A) for 1 hour at 37°C, and then treated with proteinase K for 1 hour at 37°C. DNA was phenol-extracted independently from nuclei or cytoplasm, and samples containing the same amount of radioactivity (1500 cpm) were electrophoresed through denaturing 20% (w/v) polyacrylamide gels.

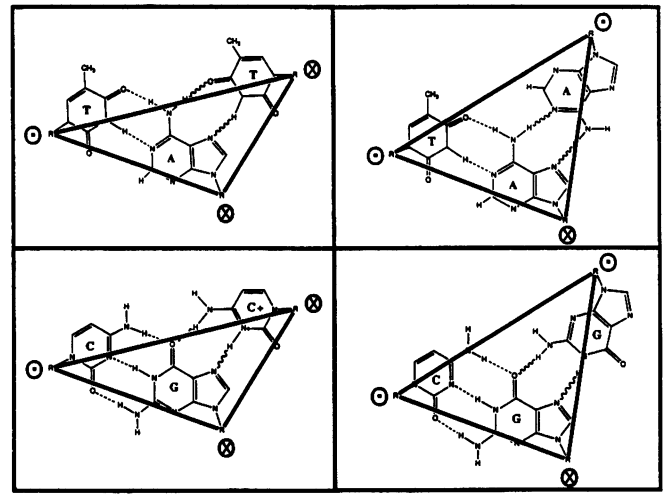

Figure 1. Proposed alternative pairing of PU-PY-PY and PU-PU-PY triplexes. In the major grove of a classical Watson-Crick duplex, a third strand, either pyrimidic (left panel, in absence of Mg^{2+} and at low pH) or puric (right panel, in presence of Mg^{2+}) is hybridized through non-Watson-Crick hydrogen bonds. In both cases, the third strand is antiparallel to the identical Watson strand present in the acceptor duplex DNA. The phosphate backbones of a pyrimidic third strand is close to the puric strand of the duplex DNA, while a puric third strand is located in the middle of the major grove, so that the triplex PU-PU-PY is composed of uniformly spaced strands.

RESULTS

Influence of ionic conditions on the triplex formation of $(TTCCC)_{48}$ *in vitro*

While a low pH induces the formation of PU-PY-PY triple stranded structures, the presence of divalent cations such as Mg^{2+} has been shown to favour the PU-PU-PY triplexes (6). Since the two parameters pH and Mg^{2+} have independently a strong influence on triplex formation, we decided to investigate their relative effects, through the oligonucleotide gel shift technique of Lyamichev *et al.* (7). As shown on fig. 2, the retarded migration of ^{32}P-labeled PU or PY oligonucleotides induced by the plasmids depends on the combination conditions between pH and Mg^{2+}. Extreme conditions (pH: 5.0, no $MgCl_2$) and (pH: 8.0, 10 mM $MgCl_2$) lead respectively to the formation of PU-PY-PY and PU-PU-PY structures. It can be observed that starting from the sole PU-PY-PY structure detected at (pH: 5.0, no $MgCl_2$), the addition of 10 mM $MgCl_2$ or the increase of pH by 2 units have the same effect and result in the formation of an equal proportion of PU-PU-PY structures. The co-existence of PU-PY-PY and PU-PU-PY structures is observed in all intermediate conditions and suggests that low pH and Mg^{2+} have opposite effects in displacing an equilibrium between these alternative structures. In addition, figure 2 shows that supercoiling is not required for triplex formation of this long mirror repeat. Indeed, both supercoiled and relaxed forms of the plasmid present in the preparation can accommodate oligonucleotides. Experiments carried out with linearized plasmids and with the eluted 287 bp-long insert containing $(TTCCC)_{48}$, have confirmed this observation (data not shown).

Determination of the DNA tertiary structure by intramolecular footprinting

Opposite conditions leading to the exclusive formation of PU-PU-PY or PU-PY-PY triplexes perfectly match the composition

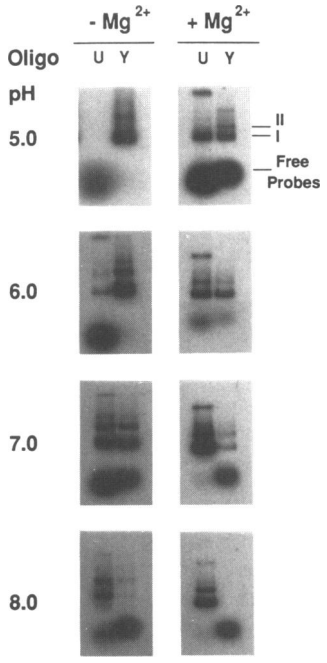

Figure 2. Effects of ionic conditions on triplex formation probed by oligonucleotide gel retardation. 300 ng DNA aliquotes of plasmids containing the $(TTCCC)_{48}$ sequence, were incubated with ^{32}P-radiolabeled PU or PY oligonucleotides, respectively termed U and Y at 4 different pH values, with or without 10 mM $MgCl_2$, and electrophoresed in the same ionic conditions through horizontal 1% agarose gels. Dried gels were autoradiographied. Both supercoiled (I), and relaxed (II) plasmids retain oligonucleotides. Free probes, when present, are visible below the supercoiled forms of plasmids.

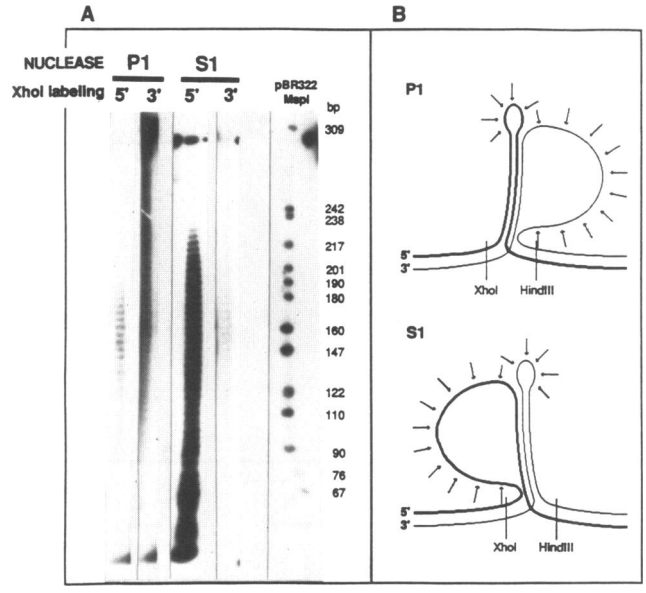

Figure 3. A) P1 and S1 mapping of single stranded regions in triplex-mediated structures. The strictly homogenous $(TTCCC)_{48}$ repeat present in the 287 bp-long XhoI-HindIII fragment show different degradation patterns by P1 and S1 nucleases. 5 μg of intact plasmids DNA containing this insert were first incubated in buffers for P1 (pH:7.6, 10 mM $MgCl_2$) or S1 (pH:4.5, no $MgCl_2$) nucleases, and subsequently incubated in presence of the corresponding enzymes. Enzyme concentrations and incubation times were determined in order to yield an average of one nick per molecule. The XhoI sites were then labeled either at the 5′ or the 3′ end, the XhoI-HindIII fragments were eluted and resolved in denaturing 6% polyacrylamide gel. A constant ratio:fragment length/distance of migration was obtained with a thickness gradient from top to bottom of the gel, and a low content of bisacrylamide. **B)** Proposed structures of the PU-PU-PY and PU-PY-PY conformers in keeping with the above results. Note that in both cases, the single-stranded DNA corresponds to the 5′ half of the repeat. Puric and pyrimidic strands are drawn with thick and thin lines respectively.

of incubation buffers for respectively the P1 and S1 single strand-specific nucleases (fig.1). This provides a tool for mapping the single stranded regions of the $(TTCCC)_{48}$ sequence. Limited digestions with these nucleases were performed to map precisely the S1 or P1 cleavage sites. Fig. 3A shows that the frequence of the nicks are distributed differently in the puric and pyrimidic strands. The puric strand appears globally more sensitive to the S1 nuclease action, while it is only weakly nicked in its central region by the P1 nuclease. An opposite situation is obtained for the pyrimidic strand. These degradation patterns can be interpreted in terms of DNA extrusions schematized on figure 3B. Interestingly, the half repeat remaining single stranded in P1 or S1 buffers, corresponds to the 5′ region of the pyrimidic or puric strands respectively. Thus, the conformers (10) with single stranded 5′-half are highly favoured in our conditions, for PU-PU-PY as well as PU-PY-PY triplexes.

Oligonucleotide degradation prevented by triplexes *in vitro*

The triplex structures deduced from the experiments presented above agree with the existence of single stranded regions covering at least the half-repeat. Such long single stranded regions, are obtained without denaturing treatments and thus can be exploited for hybridization with short complementary oligonucleotides.

In the following experiment, whose principle is summarized on fig.5, the hybridization of PU and PY oligonucleotides to the single stranded regions present in triplexes protects them from cleavage by the single strand-specific nucleases S1 and P1, even when added in excess. As predicted, figure 4 clearly shows that the PU oligonucleotides are protected from P1 degradation while the PY probe is protected from S1 degradation when pre-incubated with plasmids containing the $(TTCCC)_{48}$ repeat. In contrast, the PU and PY oligonucleotides are always degraded respectively by the S1 and P1 nucleases.

Oligonucleotide degradation prevented by triplexes *in vivo*

We have taken advantage of the presence of intracellular exonucleases to reproduce the above *in vitro* experiment in *in vivo* conditions. Hela cells were transfected with radiolabeled oligonucleotides PU or PY and plasmids containing or not the $(TTCCC)_{48}$ repeat. 24 hours later, cellular DNA was extracted and tested for the presence of intact oligonucleotides. As shown in figure 4, the original 20 b-long PU oligonucleotide can be recovered from nuclei, and in a more limited extent from the cytoplasm of cells containing the $(TTCCC)_{48}$ sequence. Radioactivity counting of nuclear extracts and of PU oligonucleotides isolated from the gels has shown that 90% of the total radioactivity remain present in the oligonucleotides after a 24 hours-long stay in cells. In contrast, the same oligonucleotides are predominantly eliminated from cells containing a control plasmid. Interestingly, it must be noted that when associated with the $(TTCCC)_{48}$ sequence, the PY oligonucleotide can also be detected but at levels 10 times lower than the PU counterpart (data not shown), which indicates that a small proportion of PU-PY-PY helices can form in cells.

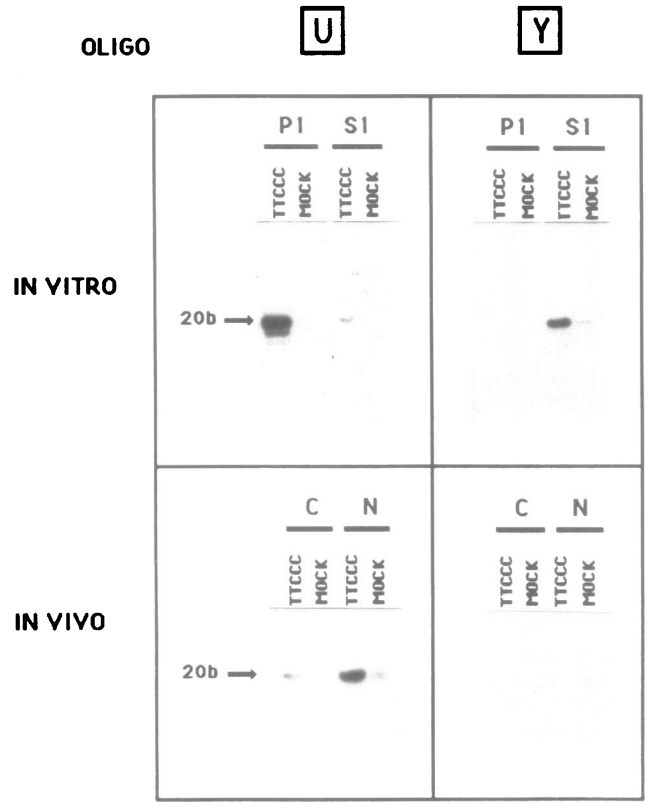

Figure 4. Triplex-mediated protection of oligonucleotides *in vitro* and *in vivo*. *In vitro*: ^{32}P-labeled PU or PY oligonucleotides (respectively noted U and Y) were incubated with 1 mg of plasmid containing or not the $(TTCCC)_{48}$ repetitive sequence, in P1 or S1 incubation buffers. They were then submitted to an excess of the corresponding enzymes. The triplex-dependent protection was assessed by testing the presence of intact oligonucleotides after nucleic acids recovering, denaturation and electrophoresis. The autoradiogram of a the 20% polyacrylamide gel containing these samples shows that PU and PY oligonucleotides can be protected from P1 and S1 degradation respectively, only in presence of the $(TTCCC)_{48}$ sequence. Partially degraded oligonucleotides are visible under the initial 20 mer. *In vivo*: Hela cells were transfected with ^{32}P-labeled PU or PY oligonucleotides and plasmids containing or not the $(TTCCC)_{48}$ sequence. 24 hours later, nucleic acids were extracted independently from nuclear and cytoplasmic fractions, and the presence of intact oligonucleotides was tested by electrophoresis through a denaturing 20% polyacrylamide gel. These results are representative of 3 independent experiments.

DISCUSSION

The biological relevance of non B DNA helices *in vivo* remains an open question. The unusual ability of PU-PY sequences to form triple helices *in vitro* is all the more intriguing since these sequences occur in higher than statistical abundance in eucaryotic genomes (16) and especially in the vicinity of transcription start sites (17, 18, 19, 20, 21). In the present work, we have focused our attention on a typical PU-PY mirror repeat, found in the promoter region of the T64 quail gene homologous to the mammalian cell death marker TRPM2 (Herault *et al.* In preparation). This repeat is very similar to other repetitive sequences close to avian transcription units and highly represented in avian genomes including that of chicken, pheasant and duck (17).

A prerequisite for investigating a possible structural function of such sequences, is to ascertain that the *in vivo* context allows the formation of DNA triplexes. Indeed, many parameters have been shown to influence the formation and the nature of triple

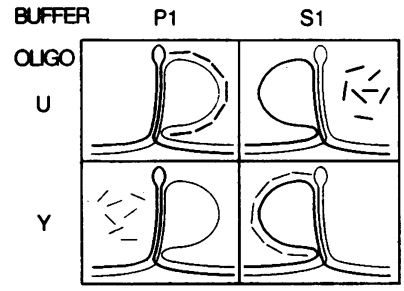

Figure 5. Interpretative scheme of the above *in vitro* results. PU-PU-PY and PU-PY-PY triplexes form in P1 and S1 buffer conditions, yielding respectively single stranded pyrimidic and puric stretches able to hybridize, and thus to protect complementary oligonucleotides. Puric and pyrimidic strands are called U and Y, and drawn with thick and thin lines respectively.

stranded DNAs, as pH, Na^+ (22), divalent cations (23; 24), polyamines (4) or superhelical stress (24). It is then particularly hazardous to predict the actual *in vivo* state of sequences capable to adopt a triple stranded structure *in vitro*. Although we have limited our investigation to only two parameters, namely pH and Mg^{2+} for *in vitro* studies, it appears that the nature of the triplex structure formed is subtletly dependent on the balance between them. It is however important to note that not any couple pH-Mg^{2+} precludes the triplex formation. Moreover, in most conditions, both PU-PU-PY and PU-PY-PY forms co-exist. Although the supposed values of the intracellular pH and Mg^{2+} concentration would rather bring to the formation of PU-PU-PY triplexes, the presence of other interfering factors in the cells cannot be excluded. Hence, we have decided to check the occurence and the exact nature of *in vivo* triplexes. Most methods aimed at identifying triplexes take advantage of the fact that their formation from a PU-PY mirror repeat yields a residual single strand. The gel shift method of Lyamichev is based on the ability of complementary oligonucleotides to hybridize this single strand, present in non-denaturing conditions. It is thus particularly useful to discriminate which of the puric or pyrimidic strands remains single stranded in the terminal structure. An alternative procedure consists in testing the vulnerability of this residual strand to single strand-specific nucleases. An interesting opportunity is provided by the availability of two such nucleases, namely the S1 and P1 nucleases, whose incubation buffers correspond respectively to optimal conditions for formation of PU-PY-PY and PU-PU-PY triplexes, as shown by preceding experiments. Original S1 digestion experiments, carried out with the rat β-globin gene promoter, containing a poly(dG) stretch, suggested the presence of PU-PY-PY triplets. However, the question has soon raised, as to know if the S1-sensitive structure pre-existed before S1 challenge (18). It is now admitted that the S1 degradation patterns do not reflect the pre-existing DNA conformation, but rather the influence of the S1 incubation buffer on the DNA structure. To avoid such bias, we have chosen to maintain the whole cell integrity as long as possible for *in vivo* study.

The basis for this experiment is provided by the instability of natural single stranded oligonucleotides in most eucaryotic cells. Their rapid degradation, which ocurs generally within the first hour following their introduction into cells (11, 12), is a major limitation for their use in strategies based on antisense inhibitors of gene expression, and has led to the development of unnatural oligonucleotides resistant to nucleases (12, 13, 14, 15). We have shown that this instability can be prevented for at least 24 hours,

by the presence of PU-PY mirror repeats containing the oligonucleotide sequence. This result makes clear that triplexes can form *in vivo*. Non-requirement of supercoiling for triplex formation of the long $(TTCCC)_{48}$ sequence is a crucial element for allowing its *in vivo* formation. In spite of the rapid relaxation of plasmids introduced inside cells, the protective effect of the $(TTCCC)_{48}$ sequence for oligonucleotides is not affected. Moreover, the same experiments, done with previously linearized plasmids have given the same results (data not shown). The higher protection extent observed in nuclei can result from a preferential cytoplasmic localization of nucleases, or from a cytoplasmic export of nuclear degradation products. By analogy with the *in vitro* protection experiment, our results suggest that the $(TTCCC)_{48}$ repeat adopts a triple stranded structure *in vivo*, and that this structure involves PU-PU-PY base triplets. Such PU-PU-PY triplexes are in agreement with the high Mg^{2+} concentration present in eucaryotic cells, although the molecular basis of the preferential stabilization of PU-PU-PY triplexes by Mg^{2+} is unknown. It is possible that this Mg^{2+} effect can proceed from a more uniform negative charge density in this type of triplex, resulting from a more central position of the third strand in the major grove of the acceptor duplex DNA (fig. 1). However, the existence of a small proportion of PU-PY-PY triplexes in cells is also likely, as suggested by a faint protection of PY oligonucleotides, obtained in presence of the $(TTCCC)_{48}$ sequence after long time exposures (data not shown). The presence of more original structures, composed of PU-PU-PY and PU-PY-PY triplexes fused together (25), cannot be detected *in vivo* by our approach, because they lack single-stranded stretches.

The set of observations reported above makes the presence of long Pu-Py tracts in eucaryotes more intriguing. Actually, some of them, over 100 base pairs in length, can represent up to 1% of some eucaryotic genomes (26). Their ability to form triplexes *in vivo* may have structural consequences on the chromosomal organization. Moreover, their frequent localization near transcription units, like for the $(TTCCC)_{48}$ element used in this work, suggests that they are not fortuitous but may ensure some, yet unknown, functional regulation of the gene expression. Since PU-PY-PY triplexes are also detected *in vivo*, it can be expected that transitions between the PU-PU-PY and PU-PY-PY forms can have major consequences, particularly in the context of promoter regions by affecting the relative positions of cis-acting elements.

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