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Investigation of the formation and intracellular stability of purine·(purine/pyrimidine) triplexes

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ABSTRACT

In a previous work we showed that a short triple helix-forming oligonucleotide (TFO) targeted to the murine *c-pim-1* proto-oncogene promoter gives a very stable triple helix under physiological conditions *in vitro*. Moreover, this triplex was stable inside cells when preformed *in vitro*. However, we failed to detect triplex formation for this sequence inside cells in DMS footprinting studies. In the present work, in order to determine whether our previous *in vivo* results are limited to this particular short triplex or can be generalized to other purine·(purine/pyrimidine) triplexes, we have tested three other DNA targets already described in the literature. All these purine·(purine/pyrimidine) triplexes are specific and stable at high temperature *in vitro*. *In vivo* studies have shown that the preformed triplexes are stable inside cells for at least 3 days. This clearly demonstrates that intracellular conditions are favourable for the existence of purine·(purine/pyrimidine) triplexes. The triplexes can also be formed in nuclei. However, for all the sequences tested, we were unable to detect any triple helix formation *in vivo* in intact cells by DMS footprinting. Our results show that neither (i) chromatinization of the DNA target, (ii) intracellular K^+ concentration nor (iii) cytoplasmic versus nuclear separation of the TFO and DNA target are responsible for the intracellular arrest of triplex formation. We suggest the existence of a cellular mechanism, based on a compartmentalization of TFOs and/or TFO trapping, which separates oligonucleotides from the DNA target. Further work is needed to find oligonucleotide derivatives and means for their delivery to overcome the problem of triplex formation inside cells.

INTRODUCTION

Triplex forming oligonucleotides (TFO) represent a new approach to artificially regulate gene expression by interacting directly at the level of DNA. This concept is based on the hypothesis that hybridization of the oligonucleotide on a genomic DNA target in a sequence-specific manner will modulate transcription of the targeted gene. Two motifs of DNA triple helices have been described; in the pyrimidine motif, an oligonucleotide composed of thymidines and cytosines binds in the major groove of a duplex DNA in parallel orientation to runs of purine acceptors through Hoogsteen base pairing; in the purine motif, an oligonucleotide composed of guanosines and adenosines binds in the major groove of the duplex DNA in an antiparallel orientation to the purine acceptor strand via reverse Hoogsteen base pairing; in the same motif, oligonucleotides composed of guanosines and thymidines may adopt a parallel or antiparallel orientation depending upon the sequence. (reviewed in 1,2).

The concept of the antigene strategy has been clearly verified *in vitro* for purine and pyrimidine motifs: oligodeoxyribonucleotides bound to duplex DNA are able to inhibit *in vitro* transcription by altering DNA–protein interactions (3,4) or by blocking transcription elongation (5,6).

While the concept of oligonucleotide-directed triple helix formation and stability is well documented *in vitro*, little is known about its *in vivo* validity: in the pyrimidine motif, the requirement for cytosine protonation limits triplex formation and stability under physiological conditions; however, methylation of cytosines and addition of an intercalating agent to the third stand has allowed transcription inhibition of reporter genes in intact cells (7); in the purine motif, it has been shown that TFOs are able to inhibit a viral (8,9) or cellular targeted gene (10–12) in a sequence-specific manner, suggesting that these oligonucleotides acted via triplex formation. Recently, Wang *et al.* have demonstrated targeted mutagenesis with intercalator-conjugated (13) or non-modified (14) oligonucleotides which were designed to form triplexes. These results suggest the possibility of triple helix formation inside cells which induces mutagenesis via recognition of the triplex by the transcription repair machinery.

However, a growing number of publications describe non-specific or 'sequence-specific' effects of oligonucleotides on cellular processes via mechanisms unrelated to binding of the intended target macromolecule (15), especially in the case of guanine-rich oligonucleotides. Consequently, it appears to us that a direct demonstration of triple helix formation inside cells is still needed to reach conclusions about the numerous effects already described in the literature.

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In previous studies we have shown that a short guanine-rich oligonucleotide targeted to the murine *c-pim-1* proto-oncogene promoter gives a very stable triple helix under physiological conditions *in vitro* (16), which can even stabilize the duplex DNA in melting studies (17). Moreover, this triplex is stable inside cells when preformed *in vitro* and can be visualised by *in vivo* DMS footprinting. However, we failed to detect triplex formation for this sequence inside cells in DMS footprinting studies (18).

In the present work, in order to determine whether our previous *in vivo* results are limited to this particular short triplex or can be generalized to other purine·(purine/pyrimidine) triplexes, we have tested three other DNA targets already described in the literature: (i) the 'Friend' sequence, a nearly perfect 45mer polypurine tract localized in the *gag* gene of Friend murine leukemia virus (F-MuLV) (TFOs targeted to this sequence block *in vitro* transcription elongation) (6); (ii) the 'supFG1' sequence, which has been successfully used for *in vivo* targeted mutagenesis by TFOs (13,14); (iii) the 'Vpx' sequence, localized in the *vpx* gene of Simian immunodeficiency virus (SIV), which has allowed us to obtain the formation of a very stable triplex at physiological K^+ concentrations (19).

All these purine·(purine/pyrimidine) triplexes are specific and stable at high temperature *in vitro*. These triplexes are stable after electroporation into cells when they are preformed *in vitro*. Thus, it appears that the intracellular conditions are favourable for the existence of purine·(purine/pyrimidine) triplexes. The triplexes can also be formed in nuclei. However, for all the sequences tested we were unable to detect any triple helix formation *in vivo* in intact cells by DMS footprinting. In order to explain our observations we suggest the existence of a cellular mechanism which blocks quantitative formation of purine·(purine/pyrimidine) triplexes in live cells. The nature of this block is discussed.

MATERIALS AND METHODS

Plasmids

Plasmid pWang1 containing the triple helix-forming sequence published by Wang *et al.* (13) was constructed by inserting the oligonucleotides 5′-CTAGAGGGGGAGGGGGTGGTGGGGG-GGGAAGG-3′ and 5′-GATCCTTCCCCCCCCACCACCCCC-TCCCCCT-3′ into the *Xba*I and *Bam*HI sites of the vector pBluescript II.

Plasmid pVpx1 containing the polypurine stretch of the SIV *vpx* gene was constructed by inserting the oligonucleotides 5′-CTAGACCTGGAGGGGGAGGAGGAGGAGGTCCG-3′ and 5′-GATCCGGACCTCCTCCTCCTCCCCCTCCAGGT-3′ into the *Xba*I and *Bam*HI sites of the vector pBluescript II.

Plasmid pFriend1 was made by digestion of pBluescript SK by *Hin*dIII and *Eco*RI and insertion of a 2 kb (*Hin*dIII–*Eco*RI) fragment from p57Friend, a plasmid containing the complete F-MuLV genome (a kind gift of Dr Marc Sitbon). This plasmid contains a 26 base polypurine/polypyrimidine sequence (positions 930–956 in the F-MuLV genome).

Colony detection by the triple helix hybridization test

The ligation product was used to transfect *Escherichia coli* XL1 blue (Stratagene). Colonies carrying the desired plasmids were detected by an *in situ* triple helix hybridization test: the colonies were transferred to a nitrocellulose membrane and fixed by heating the membrane for 1 h at 65°C. Aliquots of 1000 pmol of the third strand oligonucleotide were labelled with T4 polynucleotide kinase at a specific activity of 100 Ci/µmol and hybridized at 42°C for 2 h with the membrane in 5 ml hybridization solution [20 mM Tris–acetate, pH 7.4, 10 mM $MgCl_2$, 0.1% SDS, 50 µg/ml tRNA (Sigma) and 10 µg/ml albumin (Boehringer)]. After three washing steps at 55°C for 10 min with washing buffer (hybridization buffer without tRNA and albumin), the positive colonies were visualised after 4 h of autoradiography. To avoid false positives, the membrane was then washed in the same buffer without $MgCl_2$ and autoradiographied: positive colonies formed spots in the first case and not in the second. All plasmids were purified on a Qiagen column.

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5'-TCCTTCCCCCCCCACCACCCCCTCCCCCTC-3' supFG1 target
3'-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-5'

5'-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-3' wang30



5'-CCTCCTCCCCTCCTTCTTCCCCCTTC-3'     Friend target
3'-GGAGGAGGGGAGGAAGAAGGGGGAAG-5'

5'-GGAGGAGGGGAGGAAGAAGGGGGAAG-3'     Fri26

5'-AGGAGGGGAGGAAGAAGGGGGAA-3'        Fri23



5'-CCTCCTCCTCCTCCCCCTCC-3'           Vpx target
3'-GGAGGAGGAGGAGGGGGAGG-5'

5'-GGAGGAGGAGGAGGGGGAGG-3'           Vpx20

5'-GGAGGAGGAGGAGGGGGAGG-3'           Zip2
3'-CCTCCTCCTCCT-5'
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Figure 1. Sequences of the TFOs and duplex targets used in the study.

Oligonucleotides

Oligodeoxynucleotides were synthesized using the Applied Biosystems 391A DNA synthesizer and purified by electrophoresis in 20% polyacrylamide denaturing gels. Oligonucleotides bearing an amino group on the 3′-end were purchased from Genset (Paris, France). The different oligonucleotides used are summarized in Figure 1.

Cell culture

Three different cell lines were used in this work: a cat fibroblast cell line (G355-5) kindly provided by Dr Thierry Heidmann (Villejuif, France) and two murine cell lines (NIH 3T3 and Dunni). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 µg/ml streptomycin and 100 U/ml penicillin, routinely passaged every 3 days.

Electroporation

The cells were trypsinized and washed with 10 ml DMEM supplemented with 10% FCS. After centrifugation, the pellets were resuspended in RPMI medium containing 10 mM $MgCl_2$ or in phosphate-buffered saline (PBS) in order to obtain 10^8 cells/ml.

Samples of 150 µl cells were mixed with DNA solution in water (the quantity of DNA is specified below), the volume was adjusted to 200 µl and, after 10 min incubation on ice, electroporated with a single pulse (120 V, 960 µF) with a BTX electroporator system in a 2 mm electroporation cuvette (Eurogentec). Immediately after the pulse, the clump of dead cells was removed and the remaining cells were washed three times with 12 ml RPMI medium containing 3 mM EDTA. The cells were then resuspended in DMEM containing 10% FCS and incubated for 6–72 h before treatment (the time is specified in the figure legends).

The efficiency of transfection was checked under the same conditions with 5 µg superhelical plasmid pCMVβGal (Clontech). After 2 days, β-galactosidase activity was determined by a standard X-gal colouration assay (20). In another set of experiments, we checked that linearization of the plasmid did not significantly change the transfection efficiency.

DMS footprinting *in vitro* and *in vivo*

Preparation of the DNA fragment. To prepare a DNA fragment for modification by DMS, 30 µg pWang1, pFriend1 or pVPX1 were cut with *Cla*I restriction enzyme, 3′-labelled by the Klenow fragment of DNA polymerase I (Eurogentec) in the presence of 50 pM [α-^{32}P]dCTP (3000 Ci/mmol) and digested with *Xho*I restriction enzyme. The largest fragments were then purified with a Fisher Gelrec kit (OSI). These labelled fragments (the size of the fragments was 3 kb for pWang1 and pVPX1 and 5 kb for pFriend1) were used for *in vitro* and *in vivo* footprinting experiments.

Purine oligonucleotide-directed triple helix formation. For the *in vitro* assay, the prepared fragment (~0.5 pM) was dissolved in 20 µl 50 mM MOPS, pH 7.2, 50 mM sodium acetate and 10 mM magnesiun acetate. One hundred picomoles of the oligonucleotide designed to form a triplex were then added. The mixture was incubated for 1 h at 37°C.

For the *in vivo* assay with the preformed triplex, 2000 pmol oligonucleotide were added to 1–1.5 pmol radiolabelled fragment in 20 µl buffer solution containing 10 mM magnesiun acetate, 50 mM sodium acetate and 30 mM MOPS, pH 7.5. After 1 h incubation at 37°C the DNA was used to electroporate cells.

For *in vivo* triplex formation, co-electroporation of 5000 pmol TFO with 1–1.5 pmol plasmid was performed in PBS without divalent cations.

Probing with DMS in vitro. This procedure was performed as previously described (16). Two microlitres of 5% DMS were added to the samples and the reaction was performed for 2 min at 24°C. The reaction was stopped by addition of 5 µl solution containing 50% mercaptoethanol and 0.1 M sodium acetate. After double precipitation in ethanol the samples were treated with 50 µl 10% piperidine at 95°C for 20 min and the cleavage products separated in 6% polyacrylamide denaturing gels.

Probing with DMS in vivo. At the times after electroporation specified in the figure legends, cells were rinsed five times with a solution containing 0.9% NaCl and 2 mM EDTA. Five millilitres of 0.5% DMS were then added in a buffered solution containing 0.9% NaCl, 10 mM magnesiun acetate and 50 mM MOPS, pH 7.5, and the reaction performed for 4 min at room temperature. The reaction was stopped by brief washing with a 0.9% NaCl solution followed by washing with the same solution containing 1% β-mercaptoethanol. Cells were then lysed in 5 ml lysis solution (20 mM EDTA, 0.5% SDS, 50 mM Tris–HCl, pH 7.5) to extract the DNA fragment. Cellular DNA was precipitated by centrifugation at 30 000 *g* for 30 min after 20 min incubation of the cell lysate with 2.5 ml 3 M sodium acetate, pH 5.0, on ice. The DNA fragment was precipitated by addition of an equal volume of isopropanol to the supernatant. The DNA fragment was then dissolved in 200 µl water and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). A second DNA precipitation was performed with 2.5 vol. ethanol at –20°C for 2 h. The DNA was collected by centrifugation for 10 min, washed once with 75% ethanol and allowed to dry for 5 min at room temperature. The samples were treated with 50 µl 10% piperidine at 95°C for 20 min and the cleavage products separated in a 6% polyacrylamide denaturing gel.

The level of guanine protection in DMS footprinting experiments was estimated using a Molecular Dynamics Storm phosphorimager. For each type of oligonucleotide (*i*) this value (x_i) was calculated according to the equation:

$$x_i = [1 - (A_t/A_c)/(C_t/C_c)] \times 100\%$$

where A_c indicates the sum of the intensity of the bands in the marked region located near but outside the site of protection and A_t is the sum of the intensity of the bands in the region inside the triplex forming region (see for example Fig. 3). The ratio C_t/C_c, corresponding to the ratio from the same region for the control oligonucleotide, is used to eliminate the influence of variation in total radioactivity from well to well. The regions taken for the quantification are indicated in the figures.

Nuclei preparation

Nuclei were prepared according to Balboa *et al.* (21) with some modifications. Cells were trypsinized, washed with 10 ml DMEM containing 10% serum and resuspended in 12 ml cold lysis buffer [0.3 M sucrose, 50 mM MOPS, pH 7.5, 0.5% Triton X-100 (Sigma), 10 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF)]. After a 5 min incubation on ice, nuclei were centrifuged for 5 min at 4000 r.p.m. through a sucrose cushion (50 mM MOPS, 10 mM $MgCl_2$, 1.2 M sucrose, 1 mM PMSF). The pellet was then washed twice with sucrose buffer (50 mM MOPS, 10 mM $MgCl_2$, 1 mM PMSF, 0.3 M sucrose). The purity and integrity of nuclei were checked by microscopy.

Triplex formation in isolated nuclei. To check the accessibility of electroporated DNA for triplex formation, nuclei were prepared 24 h after electroporation and resuspended in either sucrose or in a potassium buffer (150 mM KCl instead of 0.3 M sucrose) at a concentration of 5×10^7 nuclei/ml. Two microlitres of oligonucleotide solution were then added to 50 µl resuspended nuclei at 37°C (final oligonucleotide concentrations are specified in the figures). After a 1 h incubation at 37°C, 5 µl 5% DMS were added and the reaction was performed for 3 min at 24°C. The reaction was stopped by addition of 5 µl solution containing 50% β-mercaptoethanol, 0.1 M sodium acetate. The nuclei were then lysed in 5 ml 20 mM EDTA, 0.5% SDS, 50 mM Tris–HCl, pH 7.5. DNA extraction was performed as described for *in vivo* footprinting.

Competition experiments with nuclei. Frozen nuclei (stored at –70°C in 50% sucrose buffer, 50% glycerol) were used in these experiments. Prior to incubation with oligonucleotides, nuclei were washed twice with sucrose buffer and then resuspended in sucrose or potassium buffer at a concentration of 5×10^8 nuclei/ml.

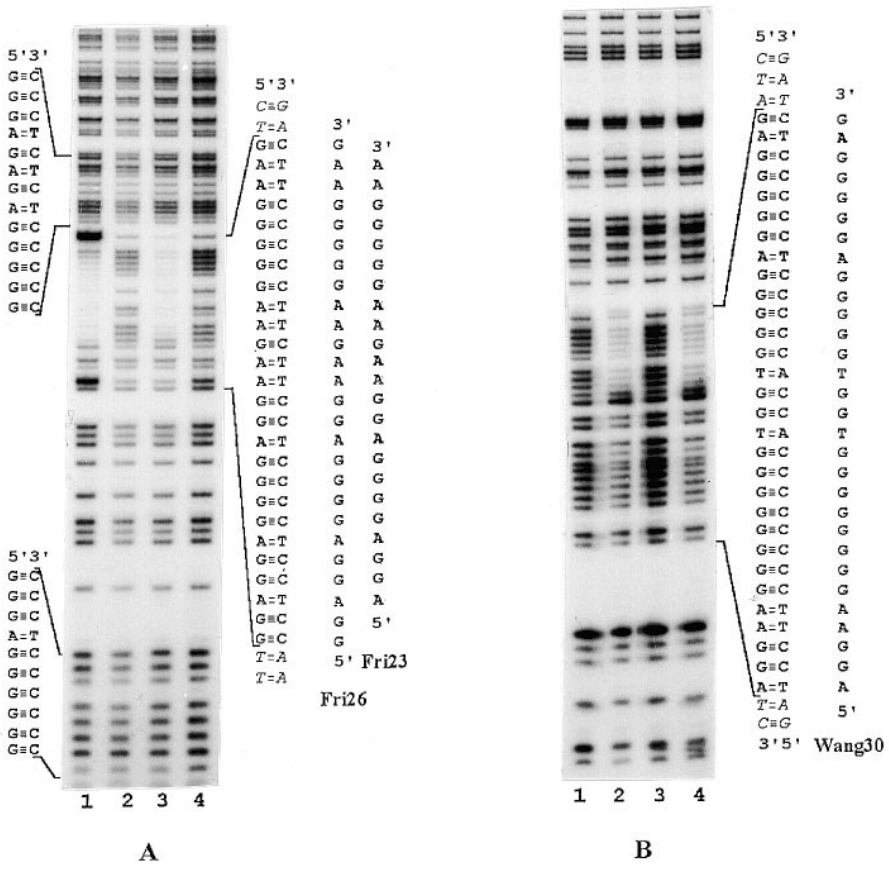

Figure 2. Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of *in vitro* DMS footprinting experiments. (**A**) 'Friend' sequence with various oligonucleotides: 1, Friend23; 2, control Friend23 (same composition as Friend23 but with opposite orientation); 3, Friend26; 4, no oligonucleotide. (**B**) 'SupFG1' sequence: 1, no oligonucleotide; 2, Wang30; 3, control Wang30 (same composition as Wang30 but with opposite orientation); 4, Wang30-amino.

Two microlitres of an oligonucleotide solution (final oligonucleotide concentrations are specified in the figure legends) were added to 50 µl resuspended nuclei or to 50 µl of the same buffer and, after 10 min incubation, ~0.5 pmol radiolabelled plasmid pVPX1 were added. After a 1 h incubation at 37°C the DMS treatment was performed as described above.

RESULTS AND DISCUSSION

Purine·(purine/pyrimidine) triplex formation *in vitro*

DMS footprinting. Before studying triplex formation *in vivo*, we checked that triplex formation with all selected sequences (listed in Fig. 1) could be detected by DMS footprinting. Protection of guanines in the targeted sequence using a DMS footprint experiment has already been demonstrated for TFOs targeted against the *vpx* gene of SIV (19). As shown in Figure 2A, the TFO Friend26 also protects guanines in the targetted sequence, indicating that triplex formation occurs under the experimental conditions. In accordance with the data of Rando *et al.* (6), the TFO binds in an antiparallel orientation. This interaction is very specific: there is no protection on two other polypurine/polypyrimidine sequences, 3′-GGGGGAGAGAGG-5′ and 3′-GGGGGGAG-GG-5′, localized respectively just upstream and downstream of the target (these sequences are indicated in Fig. 2A). The control oligonucleotide with the same composition but with the opposite orientation was unable to protect the target from modification by DMS treatment. The shorter TFO Friend23 was still able to form a stable triplex: in this case the two guanines which flank the target were more sensitive to DMS modification when the triplex was formed, suggesting a change of plasmid DNA conformation at the duplex/triplex junction.

As shown in Figure 2B, triplex formation between the Wang30 TFO and its target could also be detected by DMS footprinting. The protection was not uniform along the sequence: in particular, one guanine in the middle of the target was almost unprotected, almost certainly due to local destabilization induced by the mismatched A·T×T triplet located downstream 3′ of this non-protected guanine. The presence of an amino group 3′ of the TFO did not change the DMS footprint profile.

For all sequences studied, the DMS footprints showed that the level of guanine protection was not identical along the targets. It was more pronounced for the G stretches at the 5′-end of the purine strand of the targets. In our opinion, this observation reflects the preferential initiation of triplex formation starting from the 3′-ends of the TFOs (19).

Co-migration assay. The specificity of the triplexes was confirmed by a co-migration assay: the TFOs Friend26 and Friend23 co-migrated with the targetted plasmid, whereas there was no co-migration of the same TFOs with pBluescript SKII or the control oligonucleotide (same composition as Friend23 but with

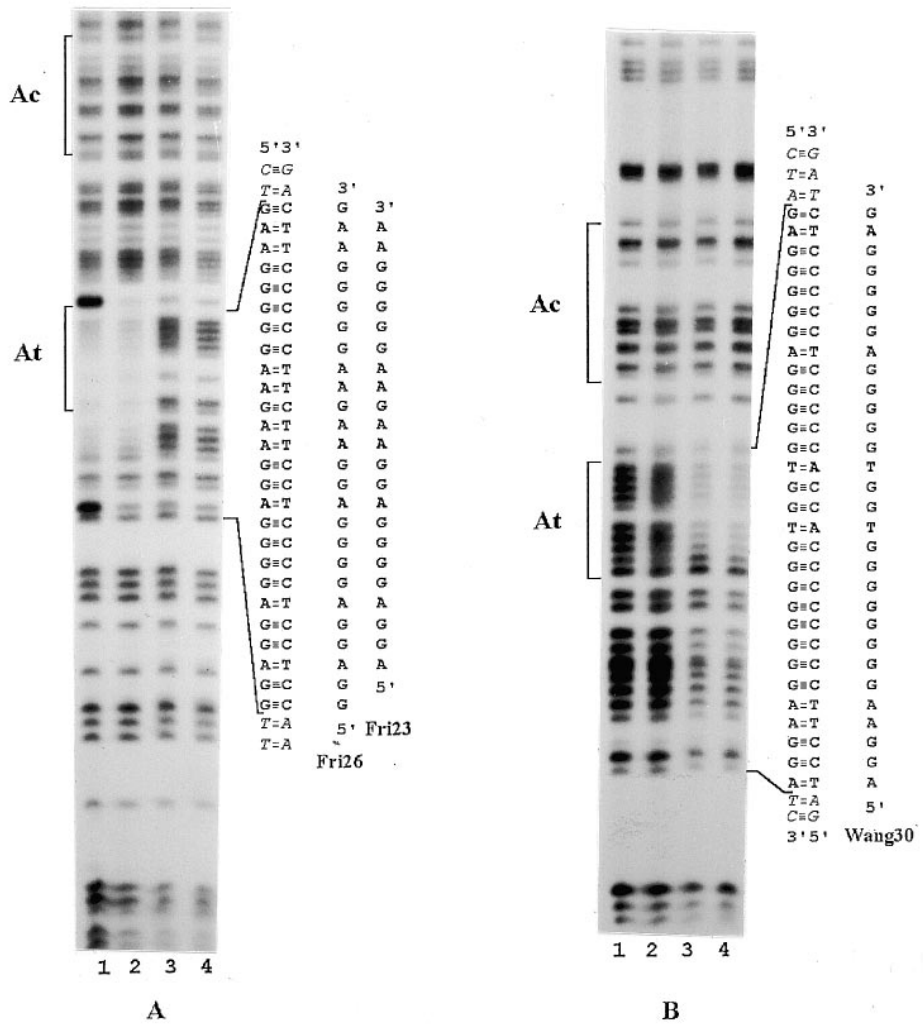

Figure 3. Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of *in vivo* DMS footprinting experiments in Dunni cells 24 h after electroporation. (**A**) 'Friend' target: 1, *in vitro* preformed triplex with Friend23; 2, *in vitro* preformed triplex with Friend26; 3, co-electroporation with Friend26 followed by addition of 10 µM TFO in culture medium; 4, electroporated plasmid without oligonucleotide. (**B**) 'SupFG1' target: 1, electroporated plasmid without oligonucleotide; 2, co-electroporated plasmid with Wang30-amino followed by addition of 10 µM TFO in culture medium; 3, *in vitro* preformed triplex with Wang30 TFO; 4, *in vitro* preformed triplex with Wang30-amino TFO. A_c and A_t indicate the regions used for quantification of the level of guanine protection (see Materials and Methods).

opposite orientation) with plasmid pFriend1. All triplexes were still stable when the co-migration assay was performed at 55°C (data not shown). Similar results were obtained for the 'Vpx' and 'SupFG1' sequences.

The high thermal stability and specificity of triplex formation for purine TFOs have permitted us to use an *in situ* triple helix hybridization test (see Materials and Methods) to detect colonies bearing the desirable plasmids. This high stability may be due to the high percentage of guanines (>70%) in the TFOs used in these three models. This is in agreement with the data of Malkov and Kamenetsky (22), who suggested that a high percentage of guanines and the presence of stretches of guanines are necessary to obtain stable triplexes.

Dependence of triplex formation on the concentrations of the cations K^+ and Mg^{2+}. The level of protection with non-modified TFOs was strongly dependent on the K^+ concentration: in DMS footprint experiments, with ≤30% triplex formation at 10 µM TFO concentration for all tested sequences in the presence of 150 mM K^+. However, for all sequences tested, the triplexes preformed in buffers with low monovalent cation concentrations were stable for several days in the presence of physiological K^+ concentrations. In accordance with our previous data on the *vpx* target, we suggest that the presence of K^+ does not substantially decrease the stability of the triplexes, but rather decreases the kinetics of their formation. This decrease in kinetics may be explained by the formation of certain structures which compete for triplex formation, for example tetraplexes (23,24) or parallel duplexes (25). Fortunately, the negative effect of K^+ on triplex formation can be overcome by using zipper structures, which are obtained by hybridization of a shorter oligonucleotide complementary to the TFO (see Fig. 1). This partial duplex, which we call a zipper oligonucleotide, displays kinetics of triplex formation that are practically independent of the monovalent cation concentration (19).

All the triplexes we studied were absolutely dependent on Mg^{2+} (we did not use other divalent cations). In our experiments the triplexes were stable at all concentration of Mg^{2+} we tested,

ranging from 0.5 to 10 mM. DMS footprinting experiments also show that 0.5 mM Mg^{2+} permits triplex formation. These data indicate that, most probably, the concentration of Mg^{2+} sufficient to support triplex structure is at the same time sufficient for triplex formation. No triplex formation occurred in the absence of divalent cation. Moreover, preformed triplexes were completely dissociated in <1 min after addition of 5 mM EDTA (data not shown). This is in complete agreement with our previous results (16–18) and those of others (22,26) showing that divalent cations are crucial for G-rich triplexes.

Purine·(purine/pyrimidine) triplexes *in vivo*

The strict dependence of the triplexes upon divalent cations allowed us to develop a method to measure triplex formation and stability inside cells (18). All triplexes located outside the cell surface or in dead cells can be disrupted by the addition of EDTA. In contrast to EDTA, DMS can quickly penetrate into live cells. As a result, only triplexes localized inside cells can be visualized by DMS footprinting after treatment of the cells with EDTA.

Purine·(purine/pyrimidine) triplexes are stable inside the cells. Triplexes preformed *in vitro* were electroporated into Dunni cells in order to study triplex stability inside cells. DMS footprinting was performed according to Materials and Methods at various times after electroporation.

Guanine protection inside cells was observed 24 h after electroporation for all three targets (Figs 3A and B and 4). No protection of the targeted guanines was detected without TFOs, which demonstrates that protection of the targetted sequences is indeed due to triplex formation and does not reflect binding of specific proteins to the targeted sequences. The level of guanine protection was between 55 and 75% for each of the three targets in *in vivo* experiments, as compared with 90% in *in vitro* ones (when triplex formation for both types of experiments was performed under the same conditions). This is in agreement with our previous work on the *c-pim* proto-oncogene target, where the corresponding values were 60% inside the cells and 90% *in vitro* (18). We suggest that this difference can be at least partially explained by DNA bound to the external surface of the cell membrane. This external triplex, which is accessible to dissociation by EDTA treatment, could decrease the level of guanine protection in *in vivo* experiments. To check this possibility, we washed the cells with or without EDTA. The difference in protection of guanines of the targetted DNA represents the percentage of external DNA which is accessible to EDTA treatment. The value of external DNA estimated in this experiment was ~15%. The persistency of external DNA after electroporation was also demonstrated in a recent study of Musso *et al.* (27).

In accordance with our previous results with TFOs binding the *c-pim* promoter DNA, the level of G protection did not change over a 3 day period for all the triplexes studied (see Fig. 3). These results are in agreement with recent data of Musso *et al.* (27), who showed that triplexes containing psoralen-conjugated TFOs persist inside cells for at least 3 days. At the same time inhibition of luciferase activity by a non-covalent preformed triplex in their experiments decreased from 40% after 24 h to 10% after 72 h incubation. The difference according to time of this non-covalent triplex persistency when compared with our data could be explained by the different location of the target sequences. In the plasmid we have used it is located outside the promoter and

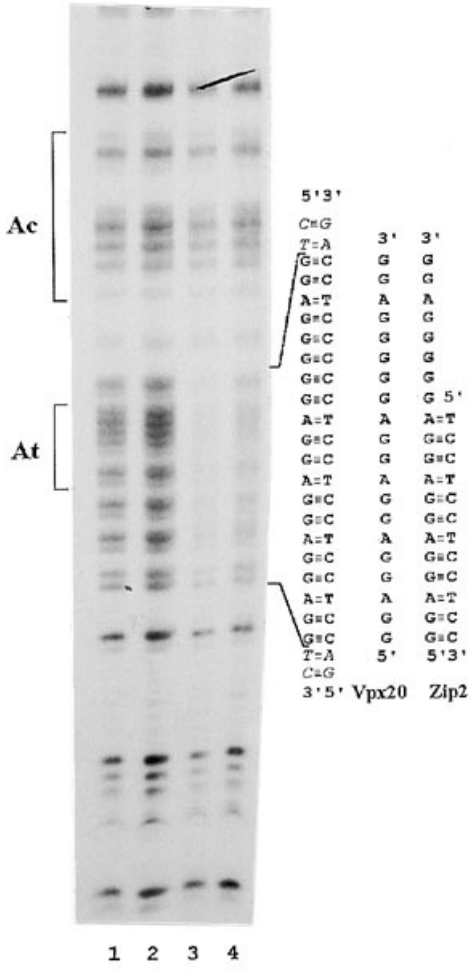

Figure 4. Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of *in vivo* DMS footprinting experiments in Dunni cells on the 'Vpx' target: 1, electroporated with control Vpx20 (same composition as Vpx20 but with opposite orientation) 24 h after electroporation; 2, co-electroporated with Zip2 TFO and 24 h later again electroporated with Zip2 TFO (DMS treatment was performed 6 h later); 3, *in vitro* preformed triplex with Zip2 TFO 24 h after electroporation; 4, *in vitro* preformed triplex with Zip2 72 h after electroporation. A_c and A_t indicate the regions used for quantification of the level of guanine protection (see Materials and Methods).

transcribed region; in the case of Musso *et al.* the triplex could be unwound by the transcription process. These results clearly demonstrate that intracellular conditions and, particularly, free intracellular Mg^{2+} concentration are favourable for the existence of purine·(purine/pyrimidine) triplexes. This high intracellular stability makes non-modified guanine-rich TFOs good candidates for lasting gene regulation inside cells.

Triplex formation in living cultured cells. Two different routes of TFO delivery were used in these experiments.

(i) Co-electroporation of the DNA targets and TFOs. The targeted plasmid and the TFOs were electroporated using PBS without divalent cations to make sure that we were studying triplex formation inside the cells and not the behaviour of the preformed triple helix. Under these conditions, triplex formation can occur only after the plasmid and TFOs have entered the living cells, since divalent cations are only present intracellularly. As

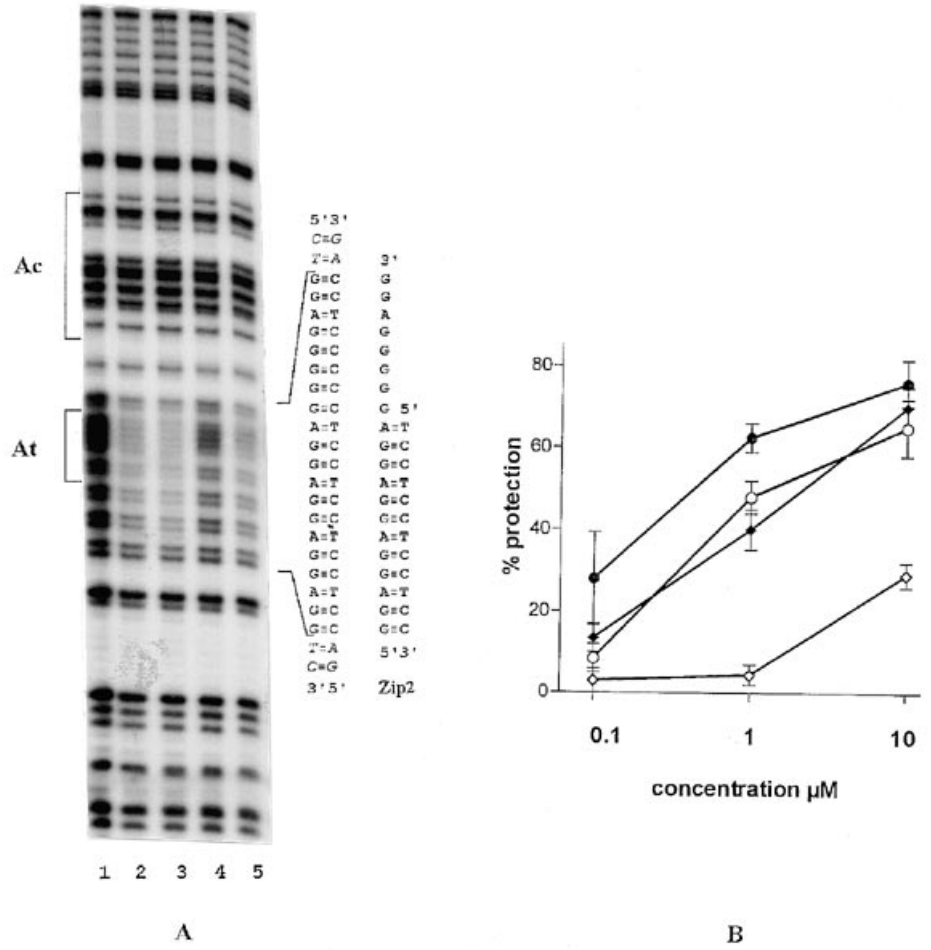

Figure 5. (**A**) Autoradiogram of a 6% polyacrylamide sequencing gel showing triplex formation on the intranuclear plasmid. The nuclei were prepared 24 h after electroporation of the pVPX1 plasmid and, after 1 h incubation with different concentrations of Zip2 oligonucleotides, DMS footprinting was performed: 1, control electroporated DNA; 2, 1 µM TFO in sucrose buffer; 3, 10 µM TFO in sucrose buffer; 4, 1 µM TFO in KCl buffer; 5, 10 µM TFO in KCl buffer. (**B**) Quantitative estimation of guanine protection in the sequence GGGGGAGG of the intranuclear Vpx target. Data were obtained by phosphorimager quantification of the DMS footprinting experiment, partially presented in (A): ●, Zip2 TFO in sucrose buffer; ◆, Vpx20 TFO in sucrose buffer; ○, Zip2 TFO in KCl buffer; ◇ Vpx20 TFO in KCl buffer. A_c and A_t indicate the regions used for quantification of the level of guanine protection (see Materials and Methods).

shown in Figures 3 and 4, no triplex formation was detected by DMS footprinting in Dunni cells, regardless of the TFO tested. We then co-electroporated pVpx1 with Vpx20 in two other fibroblast cell lines, G355-5 and NIH 3T3, in order to check whether the nature of the cell influences triplex formation. We did not detect triplex formation in either of them.

(ii) Passive addition of the third strand. Using pVpx1 as a target, the plasmid and the TFO were co-electroporated and, after washing the cells to remove external plasmid, the cells were incubated with 5 µM 3′-amino-protected VPX20 oligonucleotide for 3 days. The medium was changed daily and a new aliquot of TFO added. After 1, 2 or 3 days incubation the cells were treated with DMS. Once again we did not detect any triplex formation, suggesting that neither co-electroporation nor passive incubation are efficient in permitting triplex formation detectable by DMS footprinting inside cells.

Since we were unable to detect any triplex formation inside cells, we took advantage of this phenomenon to again estimate the external DNA, which is supposedly accessible to triplex formation with TFOs. After electroporation of the plasmid, cells were washed two to five times with DMEM and then incubated with 5 µM VPX20 in the same medium containing 10 mM magnesium. This theoretically allows triplex formation on external DNA. Two hours later, the medium was removed and the cells treated with DMS in the presence of magnesium to avoid triplex dissociation of external DNA. In these experiments the value of external DNA bound to the cells and accessible for triplex formation ranged from 7 to 15%, depending on the number of washing steps. This low protection was indeed due to external DNA, because a washing step with EDTA or with proteinase K and DNase completely suppressed guanine protection. This experiment clearly shows the importance of 'active' washing steps to dissociate external triplexes in studies devoted to intracellular triplex formation.

Investigation of the intracellular mechanisms preventing triplex formation

Surprisingly, no triplex formation was detected with any of the sequences tested, even a sequence known to be efficient for *in vivo* applications (SupFG1). There are many possible reasons for the failure of triplex detection inside cells. We chose to examine the four reasons most often discussed in the literature: (i) formation

of oligonucleotide species refractory to triplex formation in the presence of physiological K^+ concentrations; (ii) chromatinization of the targeted plasmid; (iii) separation of the TFOs and the targeted plasmid into different cellular compartments; (iv) trapping of TFOs by nuclear proteins.

Since all the sequences used in our *in vitro* and *in vivo* experiments showed virtually the same properties, for technical reasons only the Vpx sequence was used to evaluate these hypotheses.

Potassium-dependent oligonucleotide structure. It is well known that guanine-rich oligonucleotides form structures refractory to triplex formation in the presence of physiological potassium concentrations (23–25). We evaluated this hypothesis using a zipper TFO which is able to give 60% guanine protection *in vitro* in the presence of 150 mM K^+ at 1 μM. Moreover, the kinetics of triplex formation with the zipper TFO are relatively fast: 25% triplex is formed after 5 min at an oligonucleotide concentration of 0.7 μM (19).

In the co-electroporation experiments the zipper TFO was used at a concentration of 25 μM. We performed an experiment with labelled TFO to estimate the 'average' intracellular concentration of the TFO after electroporation. To this end 5000 pmol radioactively labelled TFOs were used to electroporate the cells. After 6 and 24 h we found the cellular concentration to be 8 μM. These data are in a good agreement with those of Bazile *et al.* (28), who compared the oligonucleotide concentration in the cell after electroporation and in the medium when electroporation takes place: the ratio of the former over the latter was 25%. However, even when using a zipper TFO in co-electroporation or passive addition experiments, no triplex formation was detected inside cells (see Fig. 4). This suggests that the intracellular K^+ concentration is not the major reason for the absence of intracellular triplex formation.

Inhibition by chromatinization. Another possible reason for the absence of intracellular triplex formation is the nucleosomal structure of the plasmid DNA (29–31), which can prevent triplex formation (32). To determine whether the DNA accessibility of the targeted plasmid is responsible for the lack of triplex formation inside the cells, we measured triplex formation with an intranuclear plasmid. The Vpx1 plasmid was electroporated into Dunni cells and, after 12 or 24 h, the nuclei were extracted by gentle lysis and resuspended in sucrose or KCl buffer. As shown in Figure 5, the guanines in the target DNA were protected from DMS when the TFO Zip2 was added to the nucleus. The levels of protection were respectively 75% in sucrose buffer and 65% in KCl buffer at a 10 μM Zip2 TFO concentration. Under the same conditions oligonucleotide Vpx20 gave corresponding values of 70 and 30%. To our knowledge, this is the first direct demonstration that non-chemically modified oligonucleotides are able to form triplexes in nuclei.

Since the plasmid DNA in the nuclei prepared 24 h after electroporation was fully accessible for triplex formation, we believe that the DNA structure of the plasmid in intact cells should also allow triplex formation.

Cytoplasmic versus nuclear separation of the plasmid and TFO. The precise location of the plasmid DNA and oligonucleotides delivered to the cells by different methods is not known. However, some data concerning the distribution between cytoplasm and nucleus are available. The majority of the plasmid DNA is apparently in the cytoplasm immediately after electroporation. The distribution of plasmid DNA after microinjection into the cytoplasm of post-mitotic cells suggests that a small portion of DNA quickly penetrates post-mitotic nuclei, the remainder being trapped by cytoskeletal proteins (33,34). It appears that the major portion of plasmid DNA requires cells division to enter nuclei (M.Monsigny, personal communication). Taking into account that 12–24 h after cell transfection nearly all DNA is in a chromatin-like structure (29–31), these data suggest that after 12 h the majority of electroporated DNA is inside nuclei. On the other hand, after microinjection or electroporation the vast majority of the oligonucleotide enters the nucleus in minutes (35,36; L.Mir, personal communication). Our results with radioactively labelled TFOs also show that after 5 h >70% of the oligonucleotide is in the nuclear fraction. This could explain the absence of triplex formation when oligonucleotides and plasmid DNA are co-electroporated: the oligonucleotides reach the nucleus almost immediately, whereas the plasmid is not present in the nucleus until 12–24 h. During this time, the major part of the oligonucleotide could be degraded or sequestered in a nuclear compartment (35,37), precluding formation of the triplex. To deliver the plasmid and the TFO to the nucleus at the same time, we performed a double electroporation. TFO Vpx20 or Zip2 was electroporated 24 h after electroporation of the plasmid. Once again, no triplex formation was observed in this experiment (Fig. 4).

Finally, oligonucleotides were also delivered as described by Wang *et al.* (13,14). The cells were trypsinized and seeded on plates in the presence of oligonucleotide either unmodified or modified with a 3′ amino group. Again, DMS footprinting showed no triplex formation. In the case of the amino-protected TFO, this absence of triplex formation was not due to degradation: in agreement with published data (38), we observed a full-length oligonucleotide after 6 h incubation in either culture medium or a cell lysate. This result may be explained by localization of the oligonucleotide: passively added oligonucleotides penetrate cells by endocytosis and mainly localize in cytoplasmic vesicles such as endosomes and lysosomes (39). As a result, only a small proportion of oligonucleotide can escape from cytoplasmic vesicles and interact with plasmid DNA.

Trapping of TFOs by nuclear proteins. In order to estimate the influence of all the nuclear components on triplex formation, we compared the levels of DMS protection obtained *in vitro* in either the presence or absence of nuclei. To this end, oligonucleotides were mixed for 10 min with the nuclei and the targeted plasmid was then added. As shown in Figure 6, the level of protection was weaker in the presence of nuclei when the concentration of the oligonucleotide was <1 μM. This inhibitory effect was undetectable at a 10 μM oligonucleotide concentration. The inhibitory effect appears to be 'nucleus'-specific: the presence of 10 mg/ml BSA did not change the level of protection at any oligonucleotide concentration tested. These results suggest that some nuclear components (probably proteins) may trap TFOs, decreasing the efficiency of triplex formation. Oligonucleotide binding sites in nuclei have already being described (37). The effect of inhibition of triplex formation in live cells could be more pronounced than in fractionated nuclei since: (i) a part of the nuclear proteins can be lost during nuclei preparation; (ii) in live cells energy-dependent

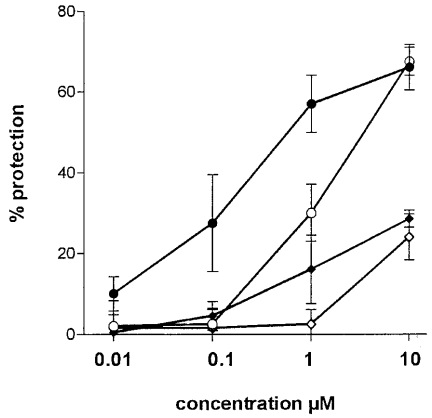

Figure 6. Quantitative estimation of the influence of nuclear components on guanine protection in the sequence GGGGGAGG of the VPX target. Data were obtained by phosphorimager quantification of the DMS footprinting experiments with nuclei as competitor in 150 mM KCl buffer: ●, Zip2 TFO without nuclei; ◆, Vpx20 TFO without nuclei; ○, Zip2 TFO with nuclei; ◇, Vpx20 TFO with nuclei.

mechanisms participate in nuclear oligonucleotide trafficking (35,36).

CONCLUSION

The main goal of this study was to investigate the intracellular formation and stability of purine·(purine/pyrimidine) triplexes. By studying different guanine-rich triplexes, in accordance with published data, we have shown that all studied TFOs form triplexes with their targets *in vitro*. High specificity of formation, high stability at physiological K^+ concentrations and the possibility of constructing TFOs with relatively fast kinetics of triplex formation (zipper TFOs) make these molecules attractive for *in vivo* applications.

In vivo studies have shown that preformed triplexes are stable, clearly demonstrating that intracellular conditions are favourable for the existence of purine·(purine/pyrimidine) triplexes. However, we did not detect any triplex formation with either co-electroporation or passive addition of the TFOs. Our results show that neither (i) chromatinization of the DNA target, (ii) intracellular K^+ concentration nor (iii) cytoplasmic versus nuclear separation of the TFO and DNA target are responsible for the intracellular arrest of triplex formation. Since the average cellular concentration of the TFO (8 µM) in the co-electroporation experiments was insufficient to detect triplex formation, we suggest the existence of a cellular mechanism, based on a compartmentalization process and/or TFO trapping, which would separate oligonucleotides from the DNA target.

Our data apparently contradict those of Wang *et al.* on targeted mutagenesis by TFOs (13,14). The differences in the results might be related to different cell lines used (Dunni cells versus COS 1 cells) or the lower sensitivity of the DMS footprinting method (≤10%) compared with the genetic test of Wang *et al.*, which allows detection of a level of mutation as low as 0.01%. It is also possible that mutagenesis is a rapid phenomenon which takes place with triplex disruption. Nevertheless, the fact that *in vitro* preformed triplex can be detected in cells several days after electroporation is in disagreement with this idea.

Recently, Guieysse *et al.* (40) detected 20% triplex formation inside cells using electroporated plasmid DNA after passive addition of a pyrimidine TFO conjugated to psoralen. It is possible that the route used by pyrimidine oligonucleotides (or oligonucleotides conjugated to an intercalating agent) differs from that of purine oligonucleotides. This could make the former more promising compounds for gene-targeted therapy.

It is evident that definitive proof of formation of a triple helix inside a cell will be obtained if two criteria are fulfilled: the cell is still alive after the triple helix is formed and the target is an endogenous gene. Further work is needed to make TFOs efficient gene-targeted compounds. One possibility is the use of compounds which can simultaneously stabilize triple helices (41) and also modify the intracellular routing of these modified TFOs. Of course, a clinical therapeutic agent could be developed based on some previously studied TFOs, but an understanding of the mechanism of action is important to develop specific gene-targeted compounds and all steps of TFO interaction with cells should be clarified. This information will contribute towards the development of modified TFOs which are more efficient gene-targeted drugs.

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