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Journal:	*Toxicological Sciences*
Manuscript ID	TOXSCI-16-0361.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	18-Aug-2016
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Key Words:	Triplex forming oligonucleotides, supF assay, mutagenesis < Genetic Toxicology

The lack of mutagenic potential of a guanine-rich triplex forming oligonucleotide in physiological conditions

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Running title:

Mutagenesis induced by triple helix formation

Abstract

Triplex forming oligonucleotides (TFOs) bind in the major groove of DNA duplex in a sequence-specific manner imparted by Hoogsteen hydrogen bonds. There have been several reports demonstrating the ability of guanine-rich TFOs to induce targeted mutagenesis on an exogenous plasmid or an endogenous chromosomal locus. In particular, a 30mer guanine-rich triplex forming oligonucleotide, AG30, optimally designed to target the *supFG1* reporter gene was reported to be mutagenic in the absence of DNA reactive agents in cultured cells and *in vivo*. Here, we investigated the mutagenic potential of AG30 using the supFG1 shuttle vector forward mutation assay under physiological conditions. We also assessed the triplex binding potential of AG30 alongside cytotoxic and mutagenic assessment. In a cell free condition, AG30 was able to bind its polypurine target site in the *supFG1* gene in the absence of potassium chloride and also aligned with a 5-fold increase in the mutant frequency when AG30 was pre-incubated with the *supFG1* plasmid in the absence of potassium prior to transfection into COS-7 cells. However, when we analysed triplex formation of AG30 and the *supFG1* target duplex at physiological potassium levels, triplex formation was inhibited due to the formation of competing secondary structures. Subsequent assessment of mutant frequency under physiological conditions, by pre-transfecting COS-7 cells with the *supFG1* plasmid prior to AG30 treatment led to a very small increase (1.4-fold) in the mutant frequency. Transfection of cells with even higher concentrations of AG30 did result in an elevated mutagenic response but this was also seen with a scrambled sequence, and was therefore considered unlikely to be biologically relevant as an associated increase in cytotoxicity was also apparent. Our findings also provide further assurance on the low potential of triplex-mediated mutation as a consequence of unintentional genomic DNA binding by therapeutic antisense oligonucleotides.

Keywords

Triplex forming oligonucleotides, antisense oligonucleotides, supF assay, mutagenesis.

Introduction

Oligonucleotide-based therapeutics offers the potential to modulate gene expression at the transcriptional and translational levels (Mahato *et al.*, 2005). In particular, triplex forming oligonucleotides (TFOs) received much interest as gene targeting reagents because of their potential to bind specific sites on the genomic DNA imparted by Hoogsteen hydrogen bonds (Mukherjee and Vasquez, 2011). Triplexes are formed when the oligonucleotide bind to homopurine:homopyrimidine sequences in duplex DNA in a sequence-specific manner whereby a few mismatches in the triplex binding code can destabilise the triplex helix (Knauert *et al.*, 2005; Semenyuk *et al.*, 2010). There are two main structural motifs for triplexes depending on their sequences. In the purine motif, TFOs (G/A) bind in an antiparallel orientation to the purine strand of the duplex to form A*A:T and G*G:C triplets. In the pyrimidine motif, TFOs (C/T) bind in a parallel orientation to the purine strand of the duplex and occurs at low pH in order for the cytosine residues be protonated at N-3 to form T*A:T and C^{+}*G:C triplets. In general, G-rich duplex target form stable complexes with guanine-rich (G-rich) TFOs whereas adenine-rich (A-rich duplex) targets are favoured by TFOs in the pyrimidine motif (Vekhoff *et al.*, 2008). Because of its sequence-specificity, the triplex technology has been studied as a potential therapeutic strategy to induce site-specific gene mutation, recombination, or transient transcription inhibition (Catapano *et al.*, 2000; Knauert *et al*., 2005; Nagatsugi *et al.*, 2003).

One of the applications of the triplex technology is to modify or inactivate a target genome site for potential therapeutic applications (Majumdar *et al.*, 1998; Vasquez *et al.*, 1999). The work presented in those studies aimed to evaluate the mutagenesis of a TFO that is designed to specifically bind to a triplex target on an extrachromosomal plasmid vector or an endogenous chromosomal locus (Wang *et al*., 1995; Majumdar *et al*., 1998). To stabilise the interaction with the target duplex DNA, TFOs were usually chemically modified by covalent attachment of a DNA cross-linking agent such as psoralen at the end of the TFO. For example, Majumdar and co-workers have demonstrated efficient knockout of the endogenous *Hprt* gene in mammalian cells using a modified pyrimidine TFOs linked to

psoralen following photoactivation (Majumdar *et al*., 1998)..Several publications by Wang and co-workers have studied targeted mutagenesis of TFOs in the target *supF* gene carried by a shuttle vector plasmid transfected into COS monkey cells (Wang *et al*., 1995; Wang *et al*., 1996). Using this gene-reporter system, purine TFOs were shown to induce mutagenesis in the presence (up to 70-fold increase) and absence (up to 13-fold increase) of a psoralen-linker. Whilst triplex-induced mutagenesis by unmodified TFOs offers a potential therapeutic approach, it raises a safety concern for other classes of therapeutic oligonucleotides (e.g. antisense oligonucleotides) where non-specific binding to genomic DNA may cause mutagenicity. The European Medicines Agency (EMA) initially raised concern for potential off-target mutation induced by therapeutic oligonucleotides via a triplex-mediated mechanism

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003149.pdf. However, the potential mutagenic risk from genomic interaction of general antisense oligonucleotides is unlikely as these oligonucleotides tend not to have optimal homology or long stretches of purine/pyrimidine to support triplex formation. This was recently supported by a publication by the Oligonucleotide Safety Working Group (OSWG) on the genotoxicity testing of therapeutic oligonucleotides in the clinic (Berman *et al.,* 2016). However, there are limited studies to confirm this risk in the context of mutagenicity assessment alongside cytotoxicity measures and in relation to human exposure conditions.

Much of the work assessing the mutagenic potential of TFOs has used the supF bioassay. This utilises a polypurine-polypyrimdine target site for triplex formation in the *supF*reporter gene on a shuttle-vector plasmid DNA (Kraemer and Seidman, 1989). The activity of the *supF* gene, which encodes a suppressor tRNA, is determined in an indicator bacterial strain. Two main strategies have been used to evaluate triplex-induced mutagenesis by the supF assay. The first involves establishing the triplex in optimal buffer conditions *ex vivo* prior to cell transfection (Havre and Glazer, 1993). The second is more biologically relevant as it involves transfection of the oligonucleotide into cells already containing the supF shuttle-vector DNA plasmid (Wang *et al*., 1995). Several approaches have been used to introduce

TFOs into cells including electroporation, conjugation to cell penetrating peptides or lipid-based transfection (Lin *et al.*, 2000; Rogers *et al.*, 2004; Vasquez *et al.*, 2001). In some cases, high concentration of oligonucleotide has been used to achieve efficient bioactivity but the potential effect on cytotoxicity was not evaluated (Rogers *et al.*, 2014). It is possible that high concentrations of oligonucleotide adversely affect the transfected cells causing non-specific toxic effects or promoting binding to non-specific intracellular targets (Stein and Cheng, 1993). Accordingly, we have concerns that high TFO concentrations used for transfection in cell systems could induce mutagenic responses that are not physiologically relevant.

Here we sought to evaluate the impact of physiological conditions on the mutagenic potential of G-rich TFOs. In addition, we tested the effect of various TFO concentrations on mutation induction with concomitant analysis of cytotoxicity. As a benchmark, we selected AG30, a 30mer guanine-rich TFO, shown previously to induce targeted mutagenesis in the *supFG1* reporter gene containing a 30-bp polypurine triplex target site, in COS-7 cells (Wang *et al*., 1995). First, we showed that pre-incubation of AG30 and the plasmid in the absence of potassium prior to transfection into COS-7 cells resulted in higher mutagenicity compared to the addition of AG30 to cells already containing the plasmid. These results indicated that intracellular TFO delivery to the target site is a major barrier for triplex formation. Next, we found that pre-heating of AG30 prior to transfection resulted in enhanced mutation suggesting the tendency of AG30 to from secondary structures restricting triplex formation. Finally, we found a correlation between mutation induction and AG30 concentration which was associated with cytotoxicity at high TFO concentrations. Importantly, at high oligonucleotide concentration (1 μM), the scrambled oligonucleotide, Mix30, induced a significant increase in the mutation frequency unrelated to the target site. To our knowledge, this is the first report to show non-specific mutagenicity, unrelated to the intended oligonucleotide, induced at high oligonucleotide concentration in the supF assay. These results indicate that the bioactivity of G-rich TFOs can be constrained by various physiological conditions and that artificial systems used to evaluate TFO-induced mutation

may not be physiologically relevant. We discuss the mutagenic potential of triplex formation in the context of antisense oligonucleotides and the relevance of this data in a therapeutic setting.

Materials and Methods

Reagents

The oligonucleotides used in this study and the target DNA duplex of the *supFG1* gene in plasmid pSupFG1 are listed in Table 1 and were obtained from Integrated DNA Technologies (Coralville, IA, USA). All oligonucleotides contained a propanolamine group at the 3′-end to minimise nuclease degradation. Labelled oligonucleotides contained fluorescein at the 5′-end. AG30thio contained five phosphorothioate linkages at each end. The supFG1 plasmid was a gift from M. Seidman (National Institute of Aging, USA). The *E.coli* strain MBM7070 was a gift from K. Brown (Leicester University, UK).

Mutagenesis assay

Monkey COS-7 cells were obtained from American type culture collection. COS-7 cells were maintained in DMEM media supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (4 mM), 4500 mg/L glucose and sodium pyruvate (1 mM). Cells were cultured at 37 °C in a 5% CO_2 humidified incubator. Cells were seeded in 6-well culture plates at a density of 1.5 x 10^5 cells per well in 2 ml of culture media. On the next day, the cells were transfected with the supFG1 plasmid DNA using Lipofectamine® 3000 according to the manufacturer's instructions (3 µl of P3000 reagent per 1µg of plasmid DNA (~ 3.3 µg of plasmid / 1 x 10^6 cells)). Twenty four-hours later, cells were washed and transfected with the indicated oligonucleotide using Oligofectamine™ according to the manufacturer's instructions. Alternatively, the plasmid pSupFG1 was pre-incubated with the indicted oligonucleotide prior to transfection. For these studies, the plasmid DNA (0.3 pmol) was incubated with 2 µM of the indicated oligonucleotide in triplex binding buffer (10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$) overnight at 37 °C. The excess oligonucleotide was removed by gel exclusion using MicroSpin Sephacryl S400 HR column. For the pre-formed complexes, cells were seeded in 6-well plates at 2 x 10^5 cells per well 24 hr prior to

transfection with complexes containing 1 µg of plasmid DNA were then transfected into COS-7 cells using 3µl of Lipofectamine® 3000 in 2ml culture media. Following 48 h, the cells were then detached with 0.1% v/v trypsin/EDTA in phosphate buffered saline (PBS) and the vector DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The plasmid DNA was treated with *DpnI* for 1 hr at 37 °C to remove any non-replicated plasmid. The restriction enzyme *DpnI* recognises adenine methylation at GATC sites and cleaves the input non-replicated plasmid DNA which carries methylated adenines. The plasmid DNA was then extracted by QIAquick gel extraction kit (Qiagen) and used to transform electrocompetent *Escherichia coli* strain MBM7070 cells as previously described (Wang *et al*., 1996). Electrocompetent cells are cells that have been subjected to serial washes of cold water and suspended in non-ionic buffer at high cell density to provide high transformation efficiency upon electroporation. In brief, 5 µl of the recovered plasmid DNA was incubated with 20 µl of electrocompetent cells (1 x 10^{11} cell/ml) and incubated in ice for 5 min. The mixture was then transferred to 0.1-cm diameter cuvette and electroporated at 25 µF, 250 W, 1800 V using Bio-Rad Gene Pulser. The transformed bacteria were plated into LB-agar plates containing 50 mg/mL ampicillin, 50 mg/mL Bluo-Gal (Fisher Scientific) (300 µg/ml) and 50 mg/mL of Isopropyl β-D-1-thiogalactopyranoside (IPTG). Plasmids with inactivated *supF* gene cannot suppress the amber mutation in the host bacterial β-galactosidase gene, so mutant colonies do not develop a blue colour when metabolising Bluo-Gal in the agar and thus will form white colonies among the blue wild-type colonies. The mutation frequency was calculated by dividing the number of white colonies by the total number of colonies (blue and white).

Triplex binding assays

The ability of the indicated oligonucleotide to form triplexes was measured using gel mobility shift assay with a synthetic 57-mer duplex containing the triplex target sequence. The duplex DNA was prepared by annealing (30 min at 60 °C) complementary strands in 50 mM NaCl and then cooling for 2 h at room temperature. A fixed concentration of the duplex DNA (0.2 µM) was incubated with increasing concentrations of the indicated oligonucleotide for 24 h at

37 °C in a final volume of 20 µl in triplex binding buffer containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$. Alternatively, the complexes were prepared in a buffer containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$,, 140 mM KCl. To study the effect of heat on triplex formation, the duplex DNA (0.2 µM) was incubated with increasing concentrations of the oligonucleotide in triplex binding buffer and heated for 10 min at 80 °C before analysis. The samples were then analysed by gel electrophoresis in a 20% polyacrylamide gel at 70 V at 4 °C in 17.8 mM Tris (pH 7.2), 17.8 mM boric acid and 10 mM $MgCl_2$. The gel was stained with 0.5 µg/mL ethidium bromide and was then visualised on ChemiDoc™ MP System (Bio-Rad).

Circular dichroism

The Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectrophotometer using a 1-mm path length quartz cuvette using a final volume of 200 µl oligonucleotide solution. The CD spectra were recorded between 200 and 320 nm at a scanning speed of 100 nm / min and a bandwidth of 1 nm. The indicated oligonucleotide at a concentration of 5 µM in 10 mM Tris (pH 7.2), 10 mM $MgCl_2$ (in the presence or absence of 140 mM KCl) was heated to 90 °C for 10 min and then cooled to 25 °C before measurement.

Colony formation assay

Forty-eight hours after oligonucleotide transfection (as described above), the cells were seeded at a density of 500 cells per well in a 6-well plate. After 7 days of incubation at 37 °C, the media was removed and the cells were washed with 1 ml of PBS and then 1 ml of 70% ethanol. The colonies were then stained with 0.5% of crystal violet solution for 20 min. The wells were then washed and allowed to dry in normal air at room temperature.

Confocal microscopy

To allow fluorescence visualisation, the pSupFG1 vector was labelled with rhodamine using the Mirus Label IT® nucleic acid labelling Kit at a 0.1:1 (v:w) ratio of the Label IT reagent to DNA as described by the manufacturer. The labelled plasmid was purified by G50 microspin column and suspended in water at 1µg/µl. COS-7 cells were seeded into 6-well culture plates containing sterile non-coated coverslips until 70% confluent. Cells in each well were transfected using Lipofectamine® 3000 (3 µl) with 1 µg of rhodamine-labelled plasmid DNA

pre-incubated with fluorescein-labelled oligonucleotide in triplex binding buffer as described above. For subsequent transfection analysis, the cells were first transfected using Lipofectamine® 3000 with 1 µg of rhodamine-labelled plasmid DNA, followed 24h later by transfection of the fluorescein-labelled oligonucleotide (0.2 µM) using Oligofectamine™. DRAQ5™ was then added to the cells at a final concentration of 5 µM for 5 min at 37 °C to stain the nucleus. The cells were washed 3 times with PBS and then mounted using fluorescent mounting medium (DakoCytomation) on glass microscopic slides. Cells were then visualised with Zeiss LSM-710 (Zeiss, Germany) microscope using a 40 X oil immersion-objective lens.

Results

AG30 binds to the target site with high affinity

Triplex formation between the supFG1 triplex target sequence and AG30 was examined by gel mobility shift assay (Figure 1). AG30 is designed to bind as a third strand in the antiparallel motif and was previously shown to form a triplex with the target sequence in the supFG1 gene with high affinity (Wang *et al*., 1996). AG30thio has the same sequence of AG30 but contains five phosphorothioate backbone linkages at each end to enhance nuclease stability. Fixed concentration of a 57-bp duplex DNA containing the triplex target site was incubated with increasing concentrations of AG30 in the presence of a buffer containing 10 mM $MgCl_2$. In an initial gel mobility shift experiment, almost complete shift from duplex to triplex was observed with AG30 at a concentration of 0.5 µM (Figure 1A). In contrast, no detectable triplex formation was observed using Mix30, an oligonucleotide with a random mix of four bases, up to a concentration of 2 µM. Further gel shift analysis over a range of concentrations (15 nM to 1 µM) showed that AG30 was able to bind to the target site with high affinity (dissociation constant Kd ~ 7 x 10^{-8} M) (Figure 1, B and C) in agreement with previous studies (Wang *et al*.,1995). The binding affinity of AG30thio was similar to that of AG30 suggesting that the presence of 10 phosphorothiote linkages had minimal effect on the binding affinity for the target duplex (Figure 1, B). Overall, these results

demonstrate that AG30 and AG30thio bind with high affinity to the target site under conditions of 10 mM $MgCl_2$ binding buffer.

AG30 self-associates into tetraplex structures

To examine the effect of physiological cytoplasmic potassium concentrations on triplex formation, we repeated the gel mobility shift assay in the presence of 140 mM KCl (Lang *et al*., 2007). Neither AG30 nor AG30thio showed detectable binding to the target site in the presence of potassium (Figure 2, A). This result indicated that triplex formation by AG30 and AG30thio is inhibited by potassium. When the samples were heated to 80 °C for 5 min, AG30 and AG30thio showed enhanced binding affinity compared to the unheated samples. These results show that without heat, AG30 and AG30thio undergo self-association into secondary structures inhibiting triplex formation (Figure 2, B). Circular dichroism (CD) measurements were used to further investigate the secondary structures formed by AG30 and AG30thio. As shown in Figure 2C, the CD spectrum of AG30 and AG30thio showed strong positive ellipticity bands at 210nm and 263 nm and a negative ellipticity band at 240 nm, characteristic of G-quadruplex structures (Kypr *et al.*, 2009). Addition of 140 mM KCl increased the intensity of the bands observed for AG30 and AG30thio suggesting the potassium stabilises G-quadruplex structures.

AG30/pSupFG1 pre-formed complexes induces mutation

To investigate the ability of oligonucleotide to increase the mutation frequency, we used the plasmid-based supF assay. Initially, we pre-incubated the plasmid pSupFG1 and AG30, AG30thio or Mix30 under optimal buffer conditions (in the absence of KCl) to promote triplex formation. The complexes were then subsequently transfected into COS-7 cells using cationic lipids (Lipofectamine 3000®). After incubation for 48 hr at 37 °C, the plasmid DNA was harvested for analysis in the indicator bacteria. As shown in Figure 3, approximately 5-fold increase in the mutant frequency over the background was detected using AG30. In contrast, no increase in the mutant frequency was observed with the negative control, Mix30 (p value > 0.05). Treatment of cells with AG30thio resulted in approximately 3-fold increase in the mutation frequency above background.

Limited mutation was detected when AG30 was transfected into cells containing the pSupFG1 vector

We subsequently examined the ability of AG30 to induce increase in the mutant frequency in cells pre-transfected with pSupFG1. First, we optimised the transfection conditions for oligonucleotides using Oligofectamine® (supplementary Figure S1). COS-7 cells were incubated with various volumes of Oligofectamine® (2, 3, and 4 µl) with 0.2 µM of fluorescein-labelled AG30 (AG30-Fluo) for 4hr and the cellular fluorescence was evaluated by flow cytometry. The fluorescence intensity increased with increasing the volume of Oligofectamine® up to 4 µl. Next, we kept the volume of Oligofectamine® constant (i.e. 4 µl) and evaluated various incubation time on the cellular uptake of AG30-Fluo. As shown in Figure 1S, AG30-Fluo displayed time-dependent increase in the fluorescence intensity but there was no significant difference between 4h and 6 hr. Thus, 4 µl of Oligofectamine® and an incubation time of 4 hr were used for subsequent transfection experiments. When AG30 (0.2 µM) was transfected into cells containing the shuttle vector, 1.4-fold increase in the mutant frequency above the background level was found ($p < 0.05$) (Figure 4 A). This was similar to the increase induced by AG30thio. Conversely, Mix30 failed to induce mutations above background (*p* value > 0.05) (Fig 4, A). Cell viability was analysed by colony formation to assess the toxicity of the oligonucleotide treatment. No significant cytotoxicity was observed with oligonucleotide treatments (Figure 4B, *p* value > 0.05). These data suggest that when AG30 is applied to cells pre-transfected with the target plasmid, the intracellular conditions greatly reduce the ability of TFO to react with the target inside the cell.

Cellular toxicity contributes to non-specific mutagenesis unrelated to the intended target

To investigate the concentration dependence of AG30-induced mutagenesis, COS-7 cells pre-transfected with the pSupFG1 vector were transfected using Oligofectamine® with increasing concentrations of AG30 up to 1 µM (Figure 5A). We used the optimal volume of Oligofectamine® and maintained the ratio of Oligofectamine®/oligonucleotide as selected above. Furthermore, because AG30 form secondary structures that can compete with triplex

formation (Fig. 2), the oligonucleotide solution was heated for 10 min at 55 °C and cooled to room temperature before transfection. As shown in Figure 5 (A), AG30 at a concentration of 0.2 μM induced ~ 3-fold increase in the mutant frequency above background whereas ~ 6-fold increase in the mutant frequency was detected at 1 μM (p value < 0.05). Interestingly, when cells were transfected with Mix30 at a concentration of 1 μM, approximately 5-fold increase in the mutant frequency above background was detected (p value <0.01). Both AG30 and Mix30 displayed a concentration-dependent decrease in cell viability as determined by colony formation assay dropping to 40% cell survival at a concentration of 1 μM (Fig 5, B). These results suggest the increase in the mutant frequency in the *supFG1* gene is dependent on the concentration of oligonucleotide and cytotoxicity could contribute to non-specific mutagenesis unrelated to the intended oligonucleotide.

AG30 co-localises with co-transfected plasmid DNA but not when added to cells pre-transfected with the pSupFG1 vector

The above data suggests that AG30 can bind to its intracellular duplex target in plasmid pSupFG1 and induce increase in the mutant frequency. To further investigate whether AG30 localises with pSupFG1 after transfection into COS-7 cells, the intracellular localisation of fluorescein-labelled AG30 and the rhodamine-labelled shuttle vector DNA was analysed using confocal microscopy. The nucleus was stained with DRAQ5 (blue). Figure 6 A shows representative image of CO7-cells incubated for 4 h at 37 °C with fluorescein-labelled AG30 pre-incubated and co-transfected with rhodamine-labelled pSupFG1 vector. AG30 and the shuttle vector DNA displayed punctated fluorescence that was mainly observed in the cytoplasm of the cells with significant co-localisation detected around the nucleus. Conversely, when AG30 was transfected into cells pre-transfected with the shuttle vector DNA, minimal co-localisation of the fluorescein and the rhodamine signal was observed after 4 h of AG30 transfection (Figure 6 B).

Discussion

In this work, we investigated the potential of G-rich TFOs to induce a mutagenic response in the supF mutation reporter system under non-physiological conditions. We chose a purine

G-rich TFO as they were previously demonstrated to induce mutagenesis in the target *supFG1* gene in the absence of a DNA-reactive agent in cultured cells and *in vivo* (Wang *et al*.,1996; Vasquez *et al*., 2000). For example, Vasquez *et al*. (2000) demonstrated that AG30 can induce about 5-fold increase in the mutation frequency in the targeted *supFG1* gene while no mutagenesis were detected in the control *CII* gene transgenic following administration into transgenic mice. In those studies, the TFOs were designed to specifically bind as a third strand in the antiparallel orientation to the polypurine/polypyrimidine triplex target site (30 bp region) in the *supFG1* reporter gene. Although various chemical modifications to the backbone, sugars or bases have been developed to improve the bioactivity of TFOs, we used chemical modifications representative of oligonucleotide-based therapeutics currently being investigated in the clinic (Kalish *et al*., 2005).

Here we were able to detect a 5-fold increase in mutant frequency above background level when the pSupFG1 plasmid and AG30 were pre-incubated under optimal buffer conditions prior to transfection into COS-7 cells. As expected, a lower increase in mutant frequency (1.4-fold over control) was detected when AG30 (0.2 μM) was transfected into cells already containing the plasmid DNA. This result suggests intracellular triplex targeting by AG30 is less efficient and represents a major factor for AG30-induced mutagenesis. To form intracellular triplex on the shuttle-vector DNA, AG30 needs to enter the cell, escape endo-lysosomal compartments and bind to its target site on the plasmid DNA. Our confocal microscopy studies showed punctate staining of AG30 with minimal co-localisation with pre-transfected plasmid DNA in COS-7 cells. This is consistent with previous data from experiments with AG30 transfected with cationic lipids, suggesting that low efficiency of intracellular delivery of AG30 to the target site is a major barrier for triplex-mediated mutagenesis (Rogers *et al*., 2004). However, it should be noted that co-localisation was assessed 4 hours after transfection while cells incubated with the oligonucleotide were assessed for mutagenesis over two days, it is possible that with additional time more of the oligonucleotide would co-localise with the plasmid DNA. In fact, the mutagenic activity induced by AG30, after Oligofectamine transfection into cells, suggests a fraction of the

oligonucleotide is able to escape the endo/lysosomal compartments and form stable complexes with the plasmid DNA to reach the nucleus where replication and mutation occur. Furthermore, we show the intracellular ionic conditions (in the presence of 140 mM KCl) are inadequate for intracellular triplex formation with AG30. Previous studies show that G-rich oligonucleotides, unless chemically modified, self-associate through G-quartet formation inhibiting triplex formation (Cheng and Van Dyke, 1997; Reshat *et al*., 2012; Rogers *et al*., 2014). Indeed, in the presence of physiological potassium concentration, we could not detect triplex formation using gel mobility analysis even at the highest concentration of AG30 used. The circular dichroism data suggest AG30 adopts a G-quadruplex structure in the presence of potassium ions. Consistent with these results, we previously observed that quadruplex formation by AG30 is stabilised by potassium and that replacing four guanine residues with adenines to interrupt the contiguous run of guanines within AG30 attenuates quadruplex formation (Reshat *et al*., 2012). Similar results have recently been reported where partial substitution of AG30 with 8-aza-7-deaza-guanine can improve its bioactivity (Rogers *et al*., 2014). Therefore, self-assembly of AG30 into secondary structures, which is more likely in the presence of potassium, limits its availability for binding to the target site on the plasmid DNA to form a triplex. Moreover, it has been demonstrated that transfected plasmid DNA can be assembled into a chromatin structure and occupied by DNA binding proteins again limiting accessibility of AG30 to the triplex target site (Criado *et al.*, 1990).

In recent work, Rogers *et al*. showed 4-fold increase in the mutant frequency following transfection with 1 μM of AG30 using a mouse cell line containing the supFG1 shuttle vector DNA (Rogers *et al*., 2014). This prompted us to examine whether the mutant frequency can be increased by elevating the concentration of AG30. Not surprisingly, there was an increase in the mutant frequency with increasing concentration of AG30, reaching 6-fold over control at 1μM. Unexpectedly, the negative control, Mix30, also induced approximately 5-fold increase in the mutant frequency at a concentration of 1 μM. A possible explanation for this response could be an increase in the cytotoxicity at higher concentrations of oligonucleotides. In fact, the significant increase in the mutant frequency using 1 μM of

AG30 or Mix30 correlated with more than 60% reduction in cell viability as determined by the colony formation assay. For AG30, the reduction in cell viability might be mediated via an apoptotic-mechanism as recently reported (Kaushik *et al*., 2013), or via a non-specific toxicity-related mechanism. However, the observation that the negative control Mix30 at 1 μM also induced a significant increase in the mutant frequency above the background favours a non-specific toxicity-based mechanism contributing to the observed mutations. The data does support an association between the mutation induction and cytotoxicity, although the mechanism for mutation induction secondary to cytotoxicity is not clear. It would seem unlikely that the observed increase in mutation frequency would be biologically significant. In fact, these high concentrations, especially for antisense oligonucleotides with phosphorothioate modified backbones, do not reflect the levels achieved following *in vivo* administration (Watanabe *et al.*, 2006). It is possible that the non-specific-mutagenesis observed at high oligonucleotide concentrations could result from plasmids with double-strand breaks following degradation by nucleases in dying cells (Razzaque *et al*., 1984). It is also important to note that Mix30 was not able to form a triplex with the duplex target as determined by mobility gel shift assay which is consistent with the lack of mutagenicity detected at low concentration. Finally, while we found a variation in the frequency of spontaneous mutations (0.03 to 0.1 %) between experiments, the frequencies reported here are consistent with similar experiments with the supFG1 plasmid in a previous reports by Wang *et al*. (1995) and Wang *et al*. (1996) (0.02 to 0.08 %).

In addition, we found the mutagenic activity of AG30 was strongly enhanced when the oligonucleotide solution was heated at 55 °C for a few minutes prior to transfection. For example, at 0.2 μM of AG30, there was ~3-fold increase in the mutant frequency over control when the oligonucleotide solution was heated compared to 1.4-fold increase without heating. This is consistent with the observation that heating increases the binding affinity of AG30 to the target duplex DNA as determined by the gel mobility shift assay. Taken together these results suggest that the increase in the mutant frequency induced by AG30 appears exaggerated under artificial conditions and are unlikely to be clinically relevant.

In previous work, mutagenesis was reported with AG30 directed toward the 30-mer triplex target site in the *supFG1* gene in an exogenous plasmid DNA or a chromosomal context (Wang *et al*., 1996; Vasquez *et al*., 2000). Here, using a plasmid DNA, AG30 was again shown to produce a mutagenic response, albeit at lower frequency than that of Wang *et al*. (1996). It is difficult to directly compare the results as no cytotoxicity measures were taken in the previous studies. Differences in the level of mutation frequencies by G-rich TFOs can be explained by various factors including the oligonucleotide transfection efficiency, the cell cycle, the transcriptional activity, or chemical modifications of oligonucleotides (Rogers *et al*., 2004; Majumdar *et al*., 2003; Faruqi *et al*., 1997). For example, Rogers *et al*. (2004) have demonstrated limited mutagenesis in the *supFG1* gene by AG30 until it was coupled to a cell penetrating peptide. In addition, several reports have shown that the bioactivity of G-rich TFOs can be improved by backbone or base modifications to minimise G-quartet formation (Dagle and Weeks 1996; Faruqi *et al*., 1997). In accordance with the these reports, we show here that the mutagenic response induced by AG30 is influenced by the cell-free conditions and that intracellular environment are promoting alternate secondary structures, thereby restricting the possibility of triplex formation by AG30. In addition the mutagenic potential of AG30 seems to depend on the context of intended application. Luo *et al*. (2000) showed that AG30-indcued intrachromosomal recombination between the tandem thymidine kinase (*TK*) genes flanking a triplex target site was not accompanied with mutation at the target site.

Even though triplex formation based on an exogenous plasmid vector *in vitro* can be achieved with high efficacy under appropriate and controlled buffer conditions, triplex formation on genomic duplex DNA is more difficult to achieve. This is limited by various challenges including cellular components and physiological conditions. For example, triplex formation is restricted to oligopurine/oligopyrimidine target sequences of DNA, typically more than 12 nucleotides in length, which limits the number of potential TFO binding sites in genomic DNA (Wang *et al*., 1996). The binding affinity of the TFO to its target site is also influenced by the TFO length and the level of homology between the TFO and target site. Even a few mismatches result in a remarkable loss in TFO binding affinity emphasising the

stringent specificity of TFO binding but also the low potential of non-specific binding (Knauret *et al*., 2005; Wang *et al*., 1995; Gowers *et al*., 1997). In addition, chromosomal DNA is tightly packed into a chromatin structure restricting the accessibility of chromosomal DNA to TFOs (Brown *et al*., 1996). Using a restriction protection assay to detect triplex-directed psoralen crosslink in genomic DNA, Macris and Glazer (2003) found the accessibility for a chromosomal site to a TFO was substantially increased when transcription was induced at the target site. In addition, the cell cycle could affect the accessibility of genomic target genes whereby cells in the S phase have more access to TFOs than those in the quiescent phase (Majumdar *et al*. 2003). Thus, the transcriptional activity, the cell cycle, and the state of the chromatin structure of the target gene could have a direct effect on chromosomal targeting by TFOs. Efficient TFO delivery into the nucleus is another important factor for triplex formation at chromosomal DNA. Luo *et al*. (2000) described a delivery method based on intranuclear delivery of TFOs by direct injection into mouse cells. In their work, an estimated TFO concentration in the range of 2×10^{-7} to 2×10^{-6} M is required to detect a biological effect such as intramolecular recombination. However, this method might not be suitable for *in vivo* applications and other improved delivery methods should be considered. Finally, the intracellular physiological conditions including the ionic environment (high potassium levels and low magnesium levels) and the neutral pH are not supportive of triplex formation by unmodified TFOs. Triplex formation by pyrimidine motif TFOs requires low pH for the N3 protonation of the cytosines to form Hoogsteen bonds, while physiological levels of potassium can inhibit triplex formation with purine motif TFOs by promoting self-association into secondary structures inhibiting triplex formation (Lee *et al*., 1984; Faruqi *et al*., 1997). Furthermore, the stability of triplexes formed by the purine or pyrimidine motifs rely on the presence of magnesium ions concentration in the range of 5 - 10 μM to minimise charge repulsion between the TFO and the duplex (Blume *et al*., 1999). Those magnesium concentrations do not occur inside the cell (Hartwig, 2001). Taken together, the above strict factors involved in the intracellular triplex formation on a chromosomal site under physiological conditions constrain the *in vivo* TFO activity.

The European Medicines Agency (EMA) raised concern of potential mutagenesis by antisense oligonucleotides via triplex formation at genomic DNA. However, in general, antisense oligonucleotides are typically designed to bind to complementary RNA target and are not expected to bind to genomic DNA. Furthermore, antisense oligonucleotides have more “normal” varied sequences and typically lack the necessary homology and the long tract of purine/pyrimidines limiting the risk of triplex-induced mutagenesis. As described above, multiple cellular and physiological challenges further limit the likelihood of triplex formation by antisense oligonucleotides. In fact, a recent publication by the subcommittee of the OSWG on genotoxicity testing of therapeutic oligonucleotides, issued recommendations on genotoxicity testing of clinical candidate oligonucleotides where triplex formation and resultant mutagenesis are considered highly unlikely (Berman *et al*., 2016). Taken together with our data reported here using a G-rich TFO optimally designed to bind to a triplex target site, further supports the statements in the OSWG report on the low likelihood of off-target triplex-induced mutagenesis by antisense oligonucleotides.

Although the risk associated with non-specific triplex formation with duplex DNA by most oligonucleotides is unlikely to be of clinical relevance, from a risk assessment perspective, several steps need to be addressed to avoid, or at least to lower, such risks. The first step involves sequence analysis of the oligonucleotide for the presence of continuous purine nucleotides. It is unlikely that an antisense oligonucleotide with a long purine stretch especially guanines (>10 nucleotides) would be considered for development (Berman *et al*., 2016). However if it is necessary to consider such a sequence, it will be important to employ a bioinformatics search engine to screen for potential triplex susceptible regions in the human genome sequence with uninterrupted oligopyrimidine-oligopurine tracts (typically 10-30 nucleotide long) as potential off-target triplex forming sequences. If a homology between the oligonucleotide sequence and the corresponding duplex sequence is identified, then the mutagenic consequences could be further investigated. Considerations in the evaluation of triplex-induced mutagenesis should include targets of greatest concern including oncogenes and tumour suppressors where the mutation consequences are amplified by outgrowth of

the individual cell in which the mutation occurred. One approach is to identify the frequency and locations of possible targets for a particular oligonucleotide-based drug. For example, regulatory promoter regions often contain polypurine/polypyrimidine tracts of an appropriate length (Goni *et al.*, 2004). Finally, as mentioned above, triplex formation could be influenced by the accessibility of the target gene in the genomic DNA and the stability of triplexes under physiological ionic conditions (Macris and Glazer, 2003; Lin *et al*., 2000). In our earlier study, a 27mer TFO (TFO27) designed to bind to a purine rich tract in the *HPRT* locus, was unable to induce mutation at the chromosomal targetin human lymphoblastoid TK6 cells even though this TFO was able to bind to its target with high affinity in a cell free system (Reshat *et al*., 2012). Therefore, while the theoretical potential for triplex target sequences could raise a mutagenic concern; it does not guarantee the bioactivity of TFOs as it could vary from one gene target to another depending on their transcription levels and accessibility to TFO as mentioned above (Macris and Glazer 2003).

In summary, using biochemical analysis to measure triplex formation combined with bioanalysis of mutagenesis, this work provides insight into that the mutagenic activity of AG30 under physiological potassium levels and in cells. We further show that the mutant frequency induced by G-rich TFO, AG30, in the supFG1 reporter vector, can be influenced by various conditions (temperature, potassium level, TFO concentration)which do not necessarily represent a clinically relevant event. Our findings further provide assurance on the limited risk of triplex formation and resultant mutation by of antisense oligonucleotides.

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Figure legends

Figure 1. (**A**) Analysis of triplex formation by gel mobility shift assay The duplex DNA (0.2 μM) was incubated with increasing concentrations of AG30 in 10mM Tris (pH 7.2), 10 mM $MgCl_2$ for 24 h at 37 °C and analysed on 20% polyacrylamide gel in 17.8 mM Tris (pH 7.2), 17.8 mM boric acid and 10 mM $MgCl_2$. (**B**) Increasing concentrations of AG30 or AG30thio were incubated with 0.2 μM of the target duplex in 10 mM Tris (pH 7.2), 10 mM $MgCl_2$ and analysed as described above.(**C**) Graphical comparison for the concentration dependence of triplex formation.

Figure 2. Gel mobility shift analysis of the effect of KCl and heating on triplex formation by AG30. (**A**) The duplex DNA (0.2 μM) was incubated with increasing concentrations of the indicated oligonucleotide in 10mM Tris (pH 7.2), 10 mM $MgCl_2$ in the presence or absence of 140 mM KCl and then incubated overnight at 37 °C and analysed on 20% polyacrylamide gel in 17.8 mM Tris (pH 7.2), 17.8 mM boric acid and 10 mM $MgCl_2$. (**B**) Analysis of triplex formation by AG30 and AG30thio as described above but the samples were heated at 80 °C for 10 min prior to incubation at 37°C. (**C**) Circular dischroism spectra of the listed oligonucleotide (5 μM) in 10mM Tris (pH 7.2), 10 mM $MgCl_2$ in the absence or presence of 140 mM KCl.

Figure 3. Targeted mutagenesis of pSupFG1 induced by oligonucleotides. The pSupFG1 vector was pre-incubated with the indicated oligonucleotide in 10mM Tris (pH 7.2), 10 mM $MgCl_2$ and transfected into COS-7 cells using Lipofectamine ®3000. After 2 days, the shuttle vector DNA was isolated from cells and then electroporated into the indictor bacteria. The mutation frequency was calculated by dividing the number of white colonies by the total number of colonies. Experiments were done in triplicate and the standard errors were calculated for the mutation frequencies. The colonies were counted per experiment and the counts from three different experiments were added up to give the total number of colonies counted as shown above each column. Error bars were calculated for the mutation frequency values. *$p < 0.05$ as compared with control.

Figure 4. Mutagenesis of pSupFG1 within COS-7 cells induced by oligonucleotides. (**A**) COS-7 cells were transfected using Lipofectamine® 3000 with the supFG1 vector, the cells were then transfected 24 hr later with the indicated oligonucleotide (0.2 μM final concentration) using Oligofectamine®. After 2 days, the shuttle vector DNA was isolated from cells and then electroporated into the indicator bacteria. The mutant frequency was calculated by dividing the number of white colonies by the total number of colonies. Experiments were done in triplicate and the standard errors were calculated for the mutant frequencies. (**B**) The effect of the above described transfection condition on cell viability was evaluated by colony formation assay. The percentage of colony formation in the control (without oligonucleotide treatment) is considered 100%. Means ± S.D. of three independent experiments are indicated. The colonies were counted per experiment and the counts from three different experiments were added up to give the total number of colonies counted as shown above each column. *$p < 0.05$ as compared with control.

Figure 5. Mutagenesis of pSupFG1 within COS-7 cells. (**A**) COS-7 cells were transfected using Lipofectamine® 3000 with the pSupFG1 vector and followed 24 h later by transfection with the indicated oligonucleotide at the indicated concentration using Oligofectamine. The oligonucleotide was heated at 55 °C for 10 min and cooled to R.T. before transfection. After 2 days, the shuttle vector DNA was isolated from cells and then electroporated into the indictor bacteria. The mutant frequency was calculated by dividing the number of white colonies by the total number of colonies. Experiments were performed in duplicate and the standard errors were calculated for the mutant frequencies. (**B**) The effect of the above described transfection condition on cell viability was evaluated by colony formation assay. The percentage of colony formation in the control (without oligonucleotide treatment) is considered 100%. Means ± S.D. of two independent experiments are indicated. The colonies were counted per experiment and the counts from two different experiments were added up to give the total number of colonies counted as shown above each column. *$p < 0.05$ and ***$p < 0.001$, as compared with control.

Figure 6. Intracellular localisation of TFO/plasmid complexes in COS-7 cell. (**A**) 4 h post transfection of fluorescein-labelled AG30 (green) pre-incubated with rhodamine-labelled plasmid DNA (red) or (**B**) 4 h post transfection of fluorescein-labelled AG30 (green) into COS-7 cells pre-transfected with rhodamine-labelled plasmid DNA. The nucleus was stained with DRAQ5 (blue) and examined by confocal microscopy.

Tables

TABLE 1

Oligonucleotide sequences and the *supFG1* target sequence

Name	Sequence
AG30	5′-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-3′
AG30 thio	5′-A*G*G*A*A*GGGGGGGGTGGTGGGGGAGG*G*G*G*A*G-3′
Mix30	5′-AGTCAGTCAGTCAGTCAGTCAGTCAGTCAG-3′
supFG1 duplex	3′-CAAGCTTAGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-5′ 5′-GTTCGAATCCTTCCCCCCCCACCACCCCCTCCCCCTC-3′

Phosphorothioate linkages are indicated by asterisks

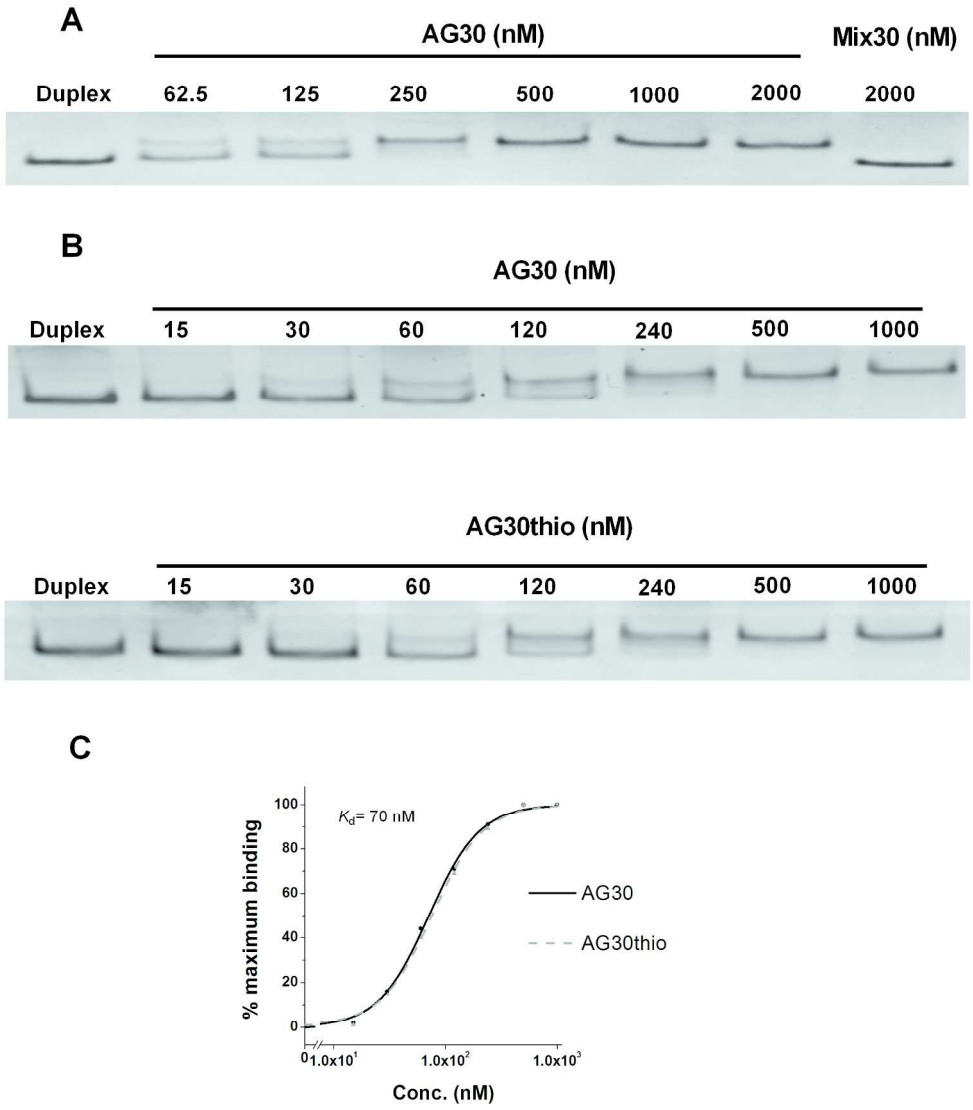

Figure 1. Analysis of triplex formation by gel mobility shift assay

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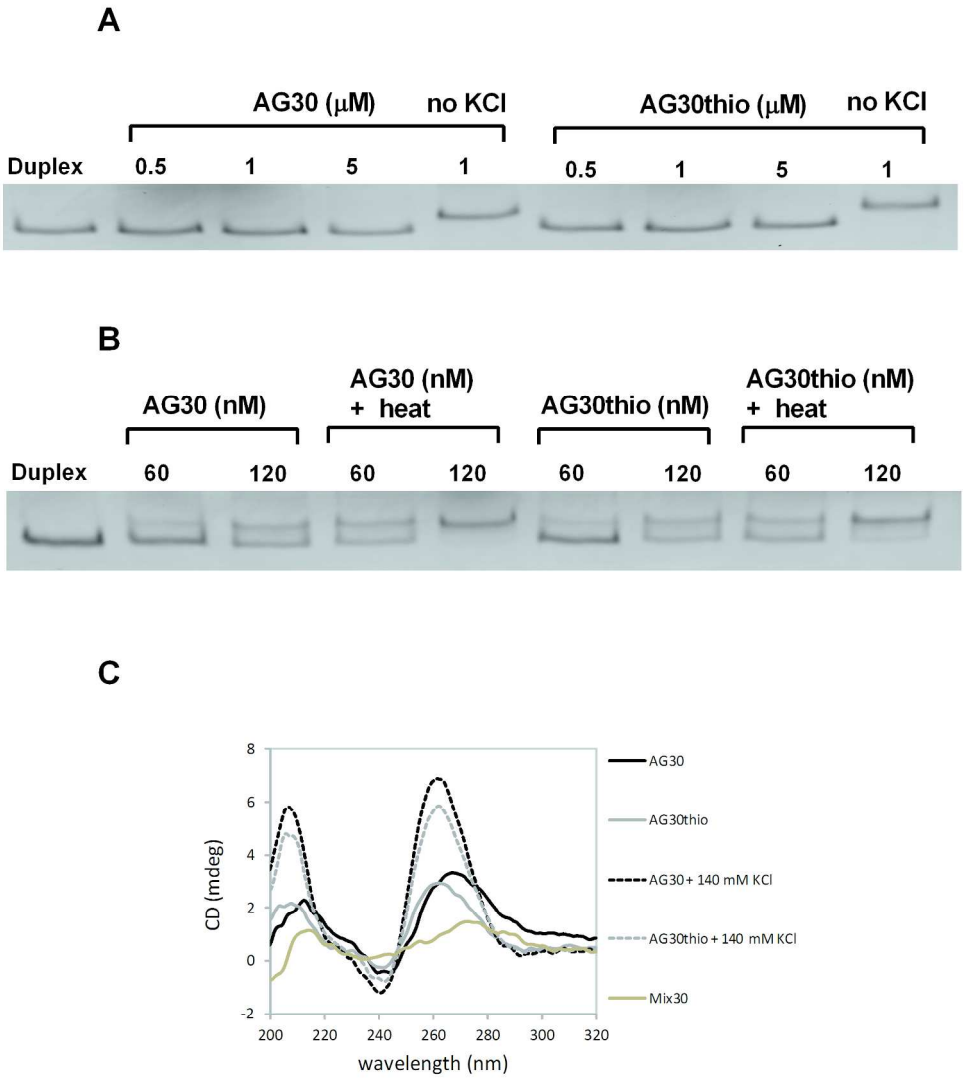

Figure 2. Gel mobility shift analysis of the effect of KCl and heating on triplex formation

217x245mm (300 x 300 DPI)

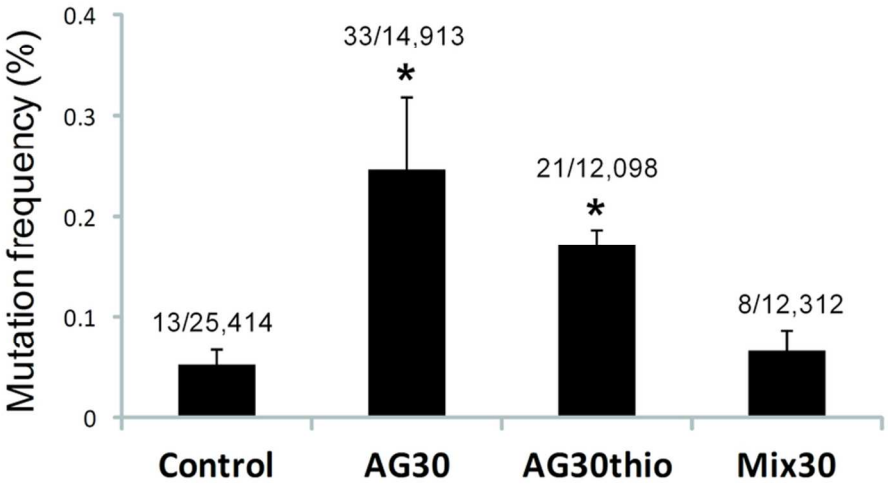

Figure 3. Targeted mutagenesis of pSupFG1 induced by oligonucleotides.

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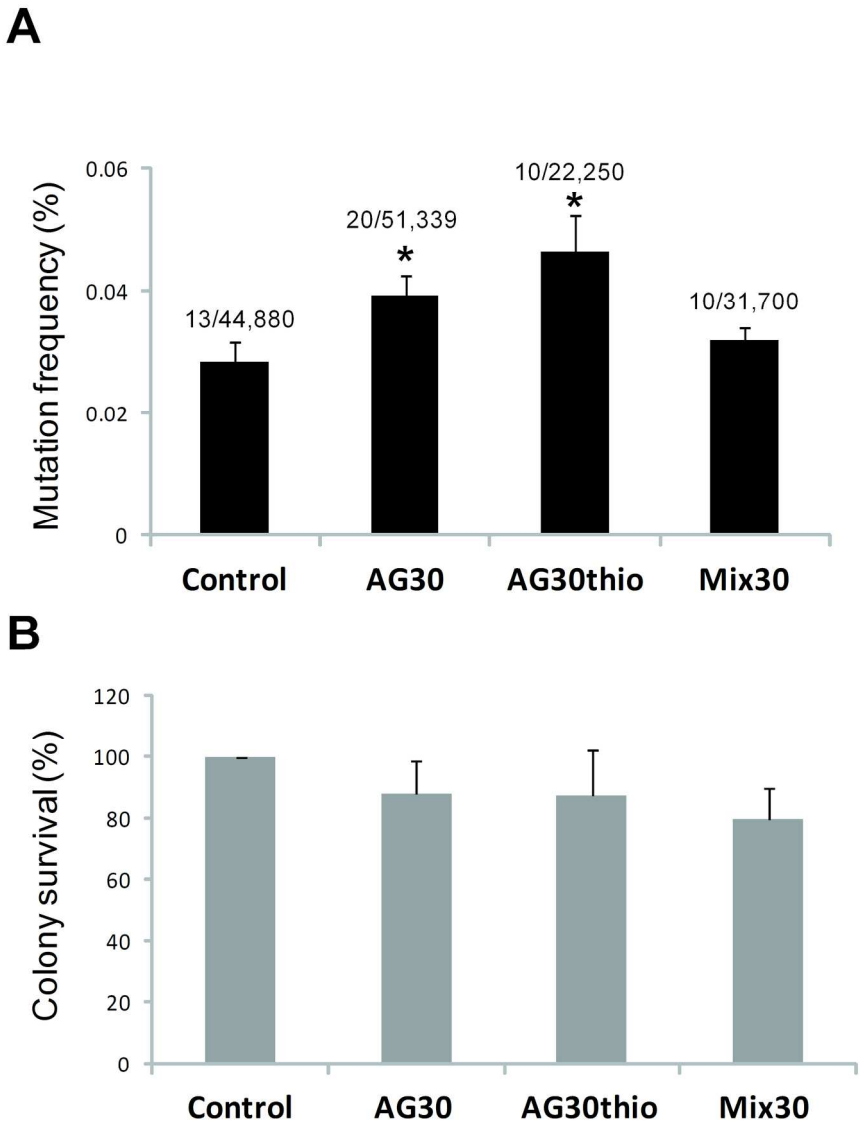

Figure 4. Mutagenesis of pSupFG1 within COS-7 cells induced by oligonucleotides

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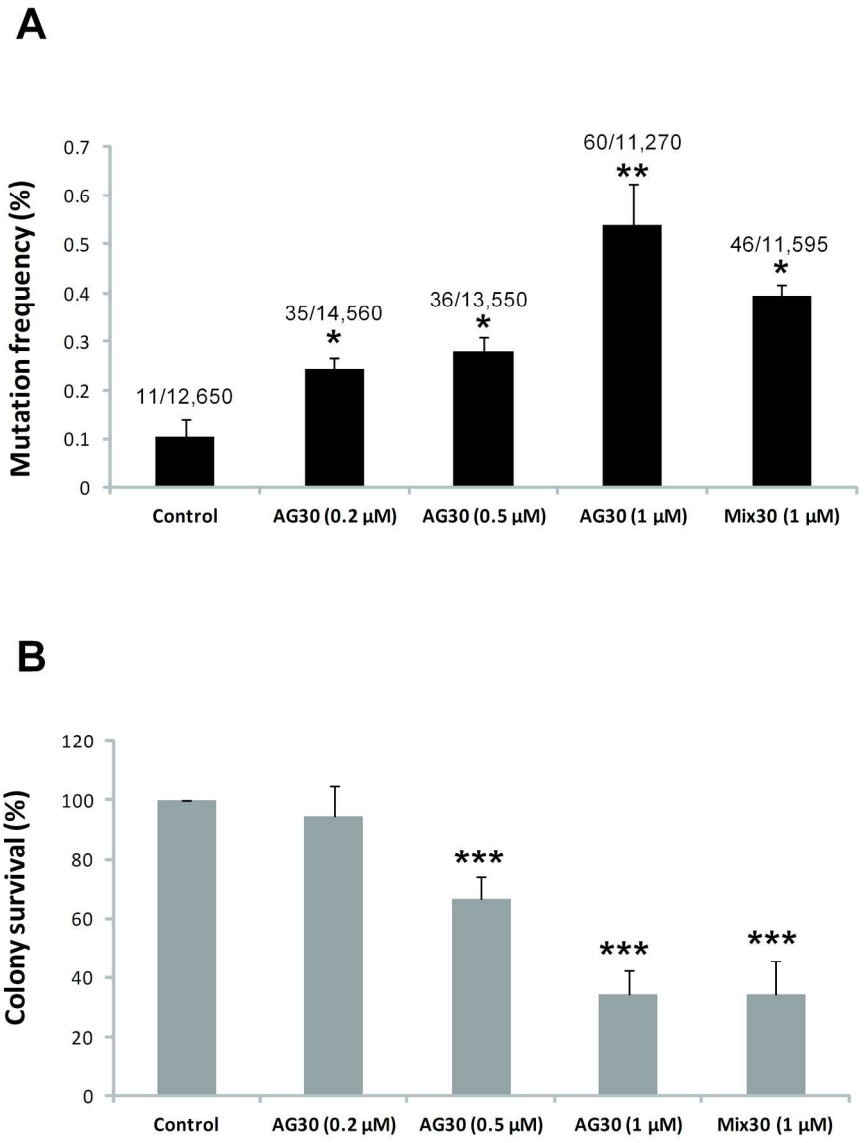

Figure 5. Mutagenesis of pSupFG1 within COS-7 cells using various conc. of AG30.

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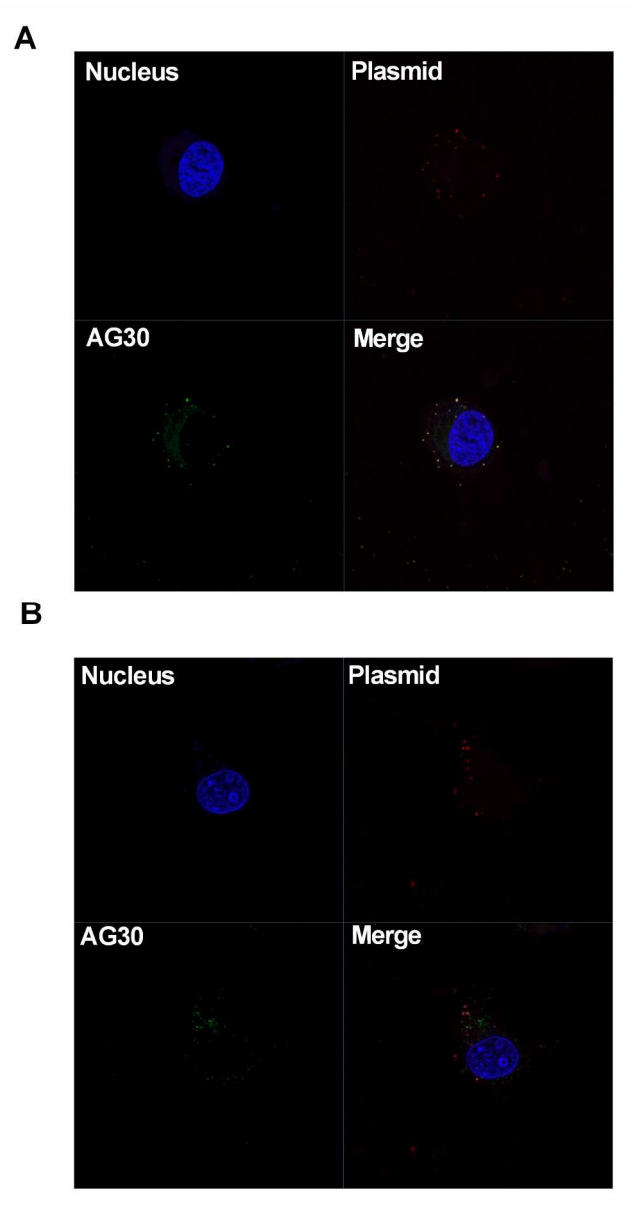
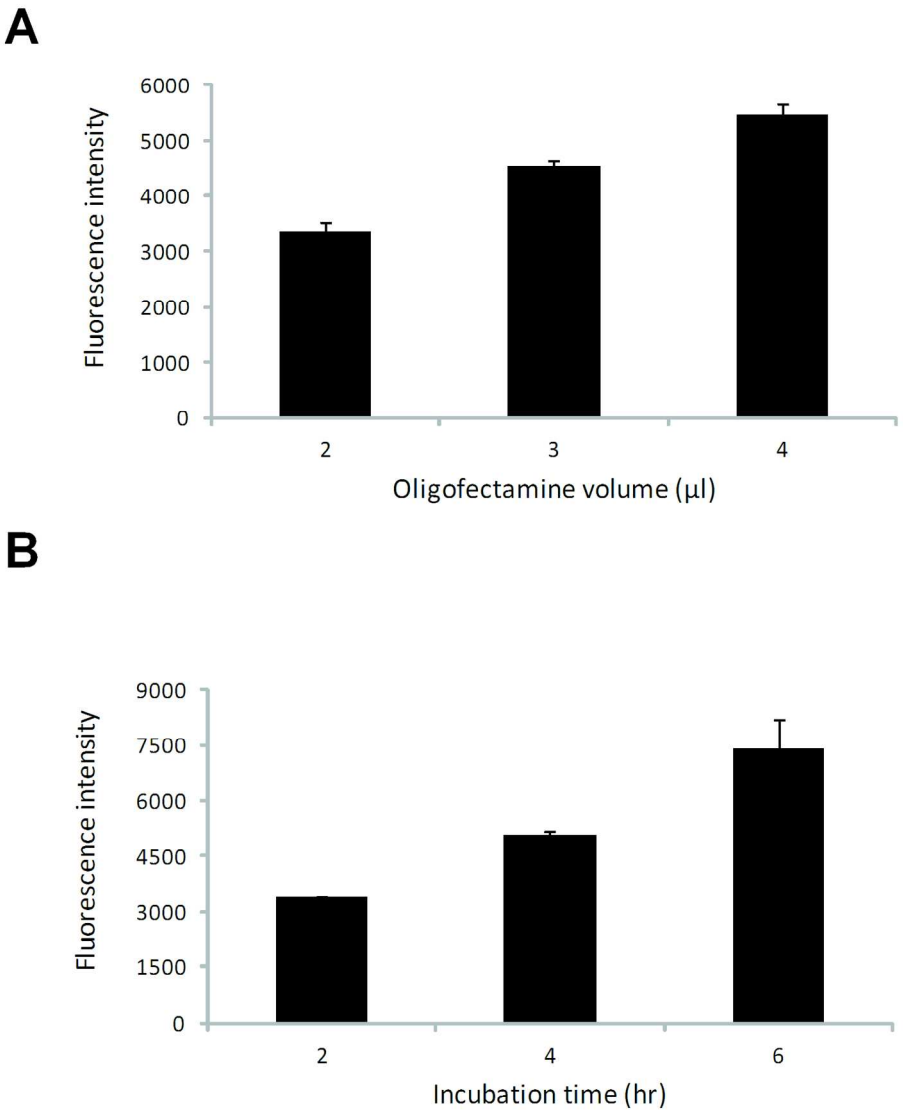


Figure 6. Intracellular localisation of TFO/plasmid complexes in COS-7 cell

140x267mm (300 x 300 DPI)



Supplementary figure

141x174mm (300 x 300 DPI)