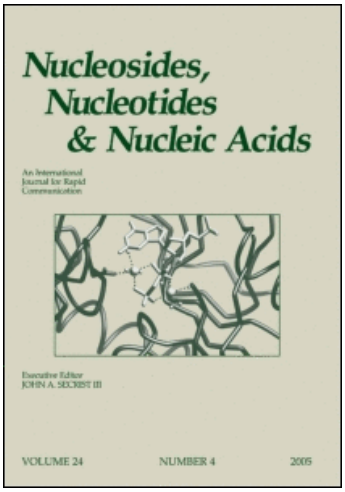

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

G-Rich Triplex-Forming Oligodeoxynucleotides as Transcription Repressors

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To cite this Article Xodo, Luigi E. , Alunni-Fabbroni, Marianna and Manzini, Gtorgio(1997) 'G-Rich Triplex-Forming Oligodeoxynucleotides as Transcription Repressors', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1695 — 1698
To link to this Article: DOI: 10.1080/07328319708006257
URL: http://dx.doi.org/10.1080/07328319708006257

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1695-1698 (1997)

G-RICH TRIPLEX-FORMING OLIGODEOXYNUCLEOTIDES AS TRANSCRIPTION REPRESSORS

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ABSTRACT. The ability of (A,G)- and (G,T)-oligonucleotides to form triple-helices with a critical polypurine-polypyrimidine sequence of the c-Ki-*ras* promoter has been examined, together with their capacity to inhibit the expression of the chloramphenicol acetyl-transferase gene driven by the c-Ki-*ras* promoter in a transfected cellular system.

It is now firmly established that stable triple-stranded DNA structures can be formed when a specific oligonucleotide binds to the minor groove of a sufficiently long polypurine-polypyrimidine (R·Y) DNA duplex[1-3]. Since this association is due to the formation of base-specific hydrogen bonds with Hoogsteen or reverse-Hoogsteen geometry between the purines of the duplex and the bases of the third strand, triplex-forming oligonucleotides (TFOs) have in principle the potential of recognizing, with high selectivity, any genomic R·Y target site of appropriate length. In doing this, they can simply interfere with the biological function of the target sequence or they can locally deliver chemicals to which they have been previously coupled. According to the base content and the types of hydrogen bonds made by the Hoogsteen strand, three classes of TFOs, containing natural bases, have been recognized[4]: (i) (C,T)-oligonucleotides which, under moderately acidic conditions (because of their cytosines requiring protonation), form triple helices stabilized by TA·T and CG·C^+ triads[1,2,5]; (ii) (G,T)-oligonucleotides forming triplexes with non-isomorphous TA·T and CG·G triads, which are stable at physiological conditions but whose stability strongly depends upon the target sequence[6-8]; (iii) (G,A)-oligonucleotides forming triple helices stabilized by non-isomorphous TA·A and CG·G triads[9-13]. It should be noted that most studies reported in the literature regard the first two classes of triplexes[14], although recently the third class has started to draw the attention of various researchers. Contrarily to what had been previously assumed, (A,G) oligonucleotides may have a high triplex-forming ability, provided that they are used under conditions in which they do not strongly self-associate[13,15]. The process of self-association depends on a number of factors, such as the oligonucleotide base content, the

sequence and the presence of certain cations (K^+) in the medium[16,17]. In this communication we report some results of a comparative study concerning the ability of (G,T)- and (A,G)-oligonucleotides to bind specifically to a critical R·Y target located in the murine c-Ki-*ras* promoter. We also investigated their capacity to inhibit *in vivo* the expression of the chloramphenicol acetyltransferase (CAT) gene under the control of this c-Ki-ras promoter in transiently transfected NIH 3T3 cells.

Both the sequences of the critical R·Y c-Ki-*ras* target and the TFOs employed in this study are shown below:

```
5'GCTCCCTCCCTCCCTCCTTCCCTCCCTCCC
3'CGAGGGAGGGAGGGAGGAAGGGAGGGAGGG          c-Ki-ras target

      5'TGGGTGGGTGGTTGGGTGGG                20GT
      5'AGGGAGGGAGGAAGGGAGGG                20AG
5'GGGAGGGAGGGAAGGAGGGAGGGAGGGAGC           30AG
```

The binding between the target duplex and the TFOs has been investigated by polyacrylamide electrophoresis under a variety of experimental conditions[12,13,15]. An interesting aspect of the target-TFO interaction was noticed by performing experiments as a function of temperature. The radiolabeled target was incubated with excess TFOs at 20, 37, 55 and 65°C and the mixtures run in a thermostatted cell. We observed that, while at 20°C all the above TFOs showed a poor capacity to complex the c-Ki-*ras* target, at 37°C the latter was fully converted into triplex by 20GT and 20AG and only partially by 30AG. Interestingly, after incubation at 65°C the triplexes made by 30AG and 20AG were still stable, wheras the triplex made by 20GT was not able to stand such a high temperature and largely dissociated. This is in keeping with UV-absorption melting profiles of the triplexes made by 20GT and 20AG: the first dissociates from its target duplex at T_m=43°C, the second denatures together with the target at T_m=73°C. These data suggest that: (i) (A,G)-oligonucleotides are able to form extraordinary stable triplexes and are therefore interesting molecules to obtain artificial transcription repressors; (ii) at T<37°C, G-rich oligonucleotides tend to adopt unusual structures by self-association, which can hamper their binding to duplex DNA. However, as these structures are easily disrupted with temperature, the capacity of G-rich TFOs to form triplexes increases significantly at T≥37°C[15]. The unusual structures formed by the TFOs employed in this study have been detected by several techniques, including electrophoresis, UV-melting, circular dichroism and hydroxyapatite chromatography[13].

The affinity constants of the above TFOs for the c-Ki-ras target have been determined by electrophoresis and found to be in the order of 10^6-10^7 M^{-1}, at 37°C. As the TFOs show a remarkable affinity for this target, particularly 20AG, a cellular system

Table 1: %Residual CAT activity at various TFO/pKRS-413 ratios

TFO/pKRS-413	20AG	20GT	Control [a]
0	100	100	100
10	80	100	100
25	50	55	90
50	10	30	60

[b] Control is a 25-mer oligonucleotide non-specific for c-Ki-*ras* promoter;

has been used to assess their potential to repress transcription *in vivo*. To this purpose NIH 3T3 cells have been transiently co-transfected with mixtures containing the TFOs and a recombinant plasmid (pKRS-413)[18] carrying the c-Ki-*ras* promoter (and thus the critical R·Y target) located closely upstream from the CAT gene[15]. Transfections have been carried out in the absence and in the presence of increasing amounts of 20GT, 20AG and a non-specific G-rich oligonucleotide used as a control. Cells have been harvested 40 hours after transfection, and the protein extracts were first quantified by the Bradford method and subsequently assayed for CAT activity with [^{14}C] chloramphenicol. Densitometer scannings of the autoradiographies of TLCs have provided the results which are reported in Table 1.

These data show that: i) a moderate inhibition of CAT expression is also induced by the non-specific control oligonucleotide; ii) the effect of 20GT becomes only significative at high TFO/pKRS-413 ratios; iii) of the three TFOs studied 20AG is the most effective transcription repressor, as it is able to reduce the level of CAT activity to less than 10% with respect to the value obtained in the absence of TFOs.

Finally, in order to circumvent the critical step of delivering the TFOs into the cells, we are presently trying to generate triplex-forming oligonucleotides intracellarly by using a DNA construct containing the promoter, capping and terminator sequences of the human small U6 gene[19]. It has already been demonstrated that this plasmid is able to produce a large quantity (μmole/L) of short RNA transcripts which might be directed against any selected genomic site, provided they possess the appropriate sequence[19]. Experiments in this direction are under way in our laboratory.

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