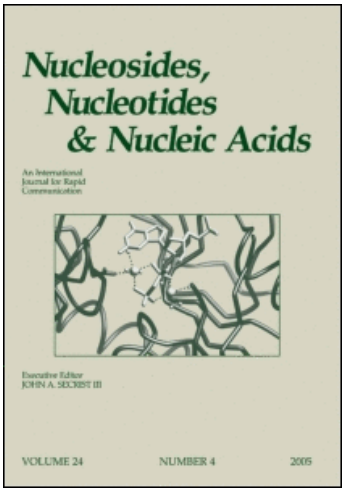


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# DNA Triplex Formation on the Granulocyte-Macrophage Colony-Stimulating Factor Gene Proximal Promoter

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# DNA Triplex Formation on the Granulocyte-Macrophage Colony-Stimulating Factor Gene Proximal Promoter

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**ABSTRACT:** Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hemapoietic cytokine that has been implicated in certain pathological conditions. We have previously demonstrated that an intermolecular DNA triplex formation on a target overlapping the NF-κB binding site on the GM-SCF proximal promoter inhibited gene transcription. A substitution of guanine (G) for thymine (T) in the triplex forming oligonucleotide (TFO) opposite a C:G inversion in the underlying duplex led to a significant increase in the TFO binding affinity and its ability to block NF-κB protein binding to the DNA. However, the substitution did not significantly affect the inhibition of GM-CSF promoter reporter gene activity by the TFO in Jurkat T cells.



Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine produced by a variety of cells including endothelial cells, fibroblasts and activated T cells. GM-CSF stimulates growth and differentiation of different hemopoietic cell lineages and the effector function of mature myeloid cells[1]. Despite *in vivo* use of GM-CSF to stimulate haemopoiesis after bone marrow transplantation, overexpression of this protein has been implicated in numerous pathological conditions, in particular chronic inflammation (asthma[2], rheumatoid arthritis[3]) and leukemia. Certain acute myeloid leukemias (AMLs) and lymphoblastic leukemias have been shown to exhibit disregulated growth in response to autocrine[4] or paracrine[5] GM-CSF. These findings indicate the potential therapeutic importance of specific agents, that will be able to modulate GM-CSF gene expression in cases of undesirable overproduction of this protein. The "antigene" strategy, based on triple helix formation on the target duplex and thus inhibition of the binding of regulatory proteins to the DNA has been successfully

employed to alter gene expression *in vitro* and in living cells[6]. We have shown that a TFO directed to a target overlapping NF-κB and SP1 transcription factor binding sites on the GM-CSF gene proximal promoter represses GM-CSF transcription in activated Jurkat T cells[7]. Recently we have also demonstrated that this TFO significantly and specifically inhibited GM-CSF production and colony growth in primary cells from patients with juvenile myelomonocytic leukemia (JMML)[8]. JMML cells are dependent for their survival and proliferation on GM-CSF produced in an autocrine manner.

The 15-bp duplex DNA stretch targeted for triplex formation on the GM-CSF promoter contains one "mismatch" in an otherwise Pur:Pyr tract. The original TFO used in the experiments described above (termed GM3, Fig. 1 A) contained a G opposite the interrupting C:G pair, as it was shown in a number of studies to be tolerated in this triplex motif[9,10]. However, the observed $K_D$ value of GM3 binding to its duplex target was relatively high (>10μM)[7]. This is consistent with other observations[11,12], that pyrimidine interruptions in the purine strand of the underlying duplex are very unfavourable for triplex formation and one "mismatch" can increase the apparent $K_D$ value of the TFO binding by approximately 1 order of magnitude. Aiming to increase the affinity of the TFO for its target on the GM-CSF promoter, we substituted the G base for a T base opposite the mismatch in targeted duplex. The new TFO was designated GMT3 (Fig.1A). It has been demonstrated that T, being placed opposite a C:G interruption, did not disrupt triplex formation and also showed substantial binding to the C:G base pair[13].



First we compared the relative ability of the GM3 and GMT3 TFOs to form a triplex with the chosen target by gel mobility shift assay (Fig. 1B). A 30 bp double stranded fragment from the GM-CSF gene proximal promoter which contained the triplex target overlapping the NF-κB and SP1 binding sites was radiolabelled and incubated with increasing concentrations of the GM3 or GMT3 oligonucleotides in 10 mM Tris-HCl, pH 7.4, 20mM $MgCl_2$, and 10% sucrose at 20°C for 2 h. followed by electrophoresis through a native polyacrylamide gel. The affinity of GMT3 for the target was at least 10 times higher than that of GM3 (compare lanes 4 and 8, Fig. 1B). The observed apparent $K_D$ for the GMT3 was <0.3 μM as at this concentration of the TFO more than half of the double stranded probe shifted into the triplex conformation (Fig. 1B, lane 5). These results indicate that the T×C:G interaction is significantly more favourable in the context of this triplex motif than the G×C:G interaction.

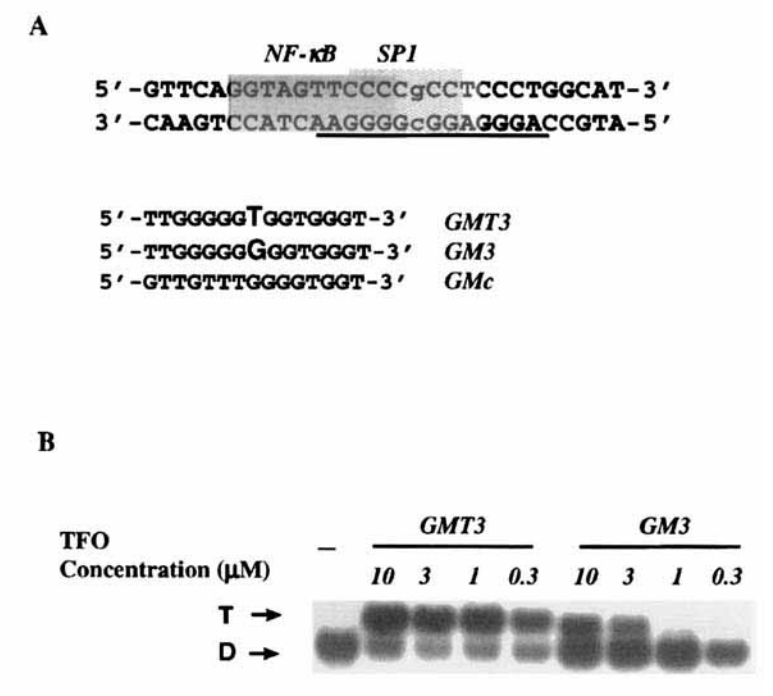


**Figure 1. GM3 and GMT3 bind to their duplex target on the GM-CSF promoter with different affinities.**

A. Oligonucleotide sequences used in this study. The double stranded fragment (90 to -60) from the GM-CSF proximal promoter containes the triplex target (underlined). The NF-κB and SP1 transcription factor binding sites are shown in a shadowed boxes. The C:G inversion in the triplex target is indicated in lower case. Bases in the TFOs GM3 and GMT3 that are placed opposite the "mismatch" in the duplex are shown in big capitals. The GMc oligonucleotide was used as a control.

B. Electro-mobility shift assay of triplex formation. End-labelled duplex (~0.2ng) was preincubated alone or with GM3 or GMT3 at the indicated concentrations and assayed by electrophoresis through a native 12% polyacrylamide gel with 1×TBE running buffer containing 20mM $MgCl_2$. Arrows show the mobility of double-stranded (D) and triple-stranded (T) DNA.

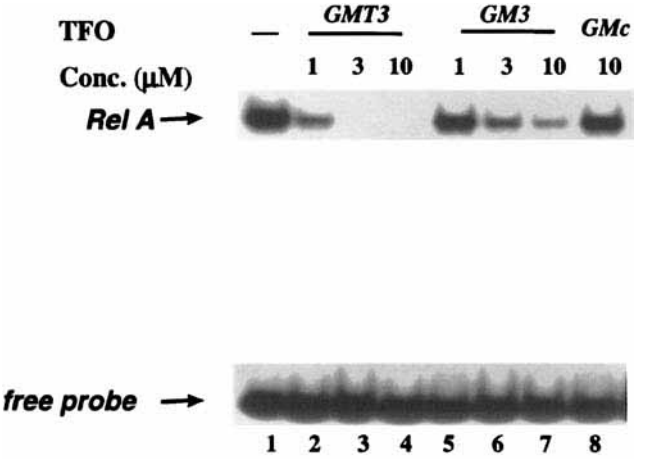


**Figure 2. The effect of triplex formation by the TFOs GM3 and GMT3 on recombinant Rel A binding to the GM-CSF promoter fragment.**

The radiolabelled double-stranded GM-CSF gene promoter fragment (~0.2ng), containing the triplex target site and κB element, was preincubated alone or with GM3, GMT3 or control oligomer GMc at the indicated concentrations. After addition of purified RelA followed by a further 20min incubation, samples were subjected to electrophoresis through a native 5% polyacrylamide gel with 0.5×TBE running buffer. Arrows indicate the Rel A-DNA complex and unbound free probe.



Next we examined whether the GMT3 oligonucleotide was more effective than GM3 in blocking recombinant NF-κB Rel A binding to the κB element within the GM-CSF gene promoter fragment. The radiolabelled double-stranded oligonucleotide was preincubated with increasing concentrations of GM3 or GMT3 followed by addition of the Rel A protein. The resulting complexes were analysed by electrophoresis through a native polyacrylamide gel. In agreement with its increased relative affinity GMT3 inhibited the formation of the Rel A-DNA complex much stronger than GM3 (Fig. 2). GMT3 was able to completely block Rel A binding to the DNA at a concentration of 3 μM (lane 3), whereas GM3 was not 100% effective even at a concentration of 10 μM (lane 7). The control oligonucleotide, GMc, with random sequence of G and T bases (Fig. 1A) was used at the highest concentration to confirm the specificity of the observed

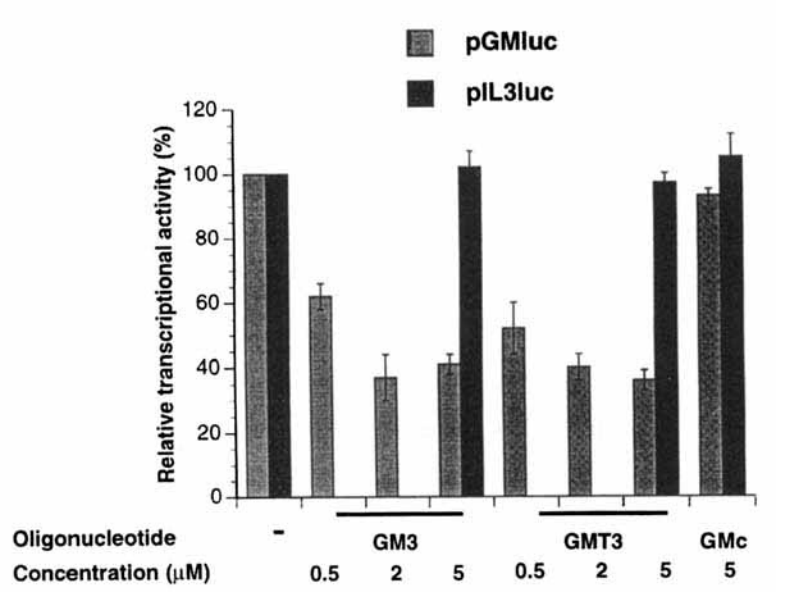


**Figure 3. Effect of TFOs on reporter gene activity in Jurkat T cells.**

Cells were transfected with 5 μg of pGMluc or pIL3luc by electroporation. 3'-amino modified TFOs (specific and control) were added at the indicated concentrations 24 hr post transfection directly to the cell medium and after 2 hr incubation cells were activated with PMA/$Ca^{2+}$ ionophore[16]. Columns show relative levels of luciferase activity for pGMluc and pIL3luc respectively where reporter activity in transfected and stimulated cells treated with medium alone were assigned a value of 100%. Columns and error bars on the graph (mean ± SEM) represent combined data from triplicate measurements in two independent experiments

inhibition. GMc did not have any significant effect on Rel A-DNA complex formation (Fig. 2, lane 8).

To determine whether the increase in binding affinity of the TFO GMT3 for its target site on the GM-CSF promoter will potentiate its inhibitory effect on GM-CSF gene transcription we used Jurkat T cells transiently transfected with two luciferase reporter constructs: the GM-CSF promoter driven pGMluc[14] containing triplex target site and the IL-3 promoter driven pIL3luc[14] as a control. All oligonucleotides used in the experiments on living cells were 3'-aminoderivatised to increase nuclease resistance. The TFOs GM3,

GMT3 and GMc were added at the concentrations indicated in Fig. 3 to the medium of transfected cells 2 hours prior to activation with PMA/$Ca^{2+}$ ionophore. Cells were harvested 8 hours post activation and luciferase activity was assayed. Unexpectedly both GM3 and GMT3 exhibited a similar inhibitory effect of about 60% on GM-CSF reporter gene transcription. The observed effect was specific since treatment of transfected cells with GMc did not decrease luciferase activity from the reporter pGMluc and neither GM3 nor GMT3 affected reporter activity from pIL3luc. The result that the GMT3 oligonucleotide was not a more effective inhibitor of transcription from the GM-CSF promoter reporter gene than GM3 could possibly be due to the limited potential to decrease in transcriptional activity trough the κB regulatory element on the GM-CSF promoter. This hypothesis is supported by our previous finding that mutation of the κB element within the GM-CSF promoter resulted in approximately 70% reduction in luciferase reporter activity in Jurkat T cells stimulated with PMA/$Ca^{2+}$ ionophore[15]. Other factors such as different cellular uptake and intracellular stability and distribution of GMT3 vs. GM3 can also have contributed to the observed result.

In conclusion we showed that a T for G substitution opposite C:G base pair in the TFO targeted to a functionally important site in the GM-CSF gene promoter led to more effective triplex formation, consistently with other reports, and better DNA-protein binding blocking. However in the cell system employed T-containing TFO was not a better inhibitor of the luciferase reporter gene activity, driven by the GM-CSF promoter.



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