DNA Triplex Formation Selectively Inhibits Granulocyte-Macrophage Colony-stimulating Factor Gene Expression in Human T Cells*

(Received for publication, January 12, 1996, and in revised form, March 8, 1996)

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hemopoietic growth factor that is expressed in activated T cells, fibroblasts, macrophages, and endothelial cells. Although GM-CSF does not appear to be essential for normal hemopoiesis, overexpression of GM-CSF has been implicated in the pathogenesis of some diseases such as myeloid leukemia and chronic inflammation. An NF-κB binding site within the GM-CSF proximal promoter, termed the κB element appears to be important for controlling expression in reporter gene assays in response to a number of stimuli in T cells. We investigated oligonucleotide-directed triplex formation across this regulatory sequence as a potential tool to inhibit GM-CSF gene transcription. A 15-base oligonucleotide, GM3, was targeted to a purine-rich region in the GM-CSF proximal promoter, which overlaps the κB element. Gel mobility shift assays and DNase I footprinting demonstrated that GM3 formed a sequence-specific colinear triplex with its double-stranded DNA target. Triplex formation by GM3 blocked recombinant GM-CSF expression in human T-cell lymphotrophic virus-1 Tax transactivator-induced luciferase activity from a reporter construct driven by the GM-CSF promoter in Jurkat T cells. Finally, GM3 greatly reduced the concentration of endogenous GM-CSF mRNA induced by different stimuli in Jurkat T cells but did not affect interleukin 3 mRNA levels in the same cells. We conclude that the κB element in the GM-CSF promoter plays a central role in the transcriptional activation of the endogenous GM-CSF gene. Colinear triplex formation acts as a selective transcriptional repressor of the GM-CSF gene and may have potential therapeutic application in cases of undesirable overexpression of this protein.

Cytokines are a rapidly growing family of proteins that act as local or systemic intercellular regulatory factors. They are involved in various developmental and differential processes and are important in the immune response to infection and injury. However, cytokines are also implicated in the pathology of many clinical conditions including septic shock, parasitic infections, chronic inflammation, atherosclerosis, and cancer (1).

Granulocyte-macrophage colony-stimulating factor is a cytokine involved in the control of survival, proliferation, and differentiation of hemopoietic progenitor cells as well as in the functional activation of mature myeloid cells (reviewed in Ref. 2). GM-CSF expression occurs in different cell types, including fibroblasts and endothelial cells in response to proinflammatory agents such as tumor necrosis factor and interleukin-1 (IL-1), monocytes in response to endotoxin, and activated T cells (reviewed in Ref. 3).

Despite the clinical application of GM-CSF as an activator of hemopoietic cell production after bone marrow transplantation, evidence has accumulated to suggest that GM-CSF may play a role in the pathogenesis of several disease conditions, in particular myeloid leukemia and chronic inflammation (4–7). GM-CSF was found to be abnormally expressed in atopic patients and in patients with rheumatoid arthritis (4, 5). In terms of leukemia, GM-CSF has been shown to be produced by some leukemic clones in an autocrine fashion in vitro, and the growth of the cells in vitro depends on the presence of GM-CSF (8). Recent in vivo experiments have also shown that GM-CSF increased the blast cell counts in 9 of 12 acute myeloid leukemia patients (9). Taken together these data suggest that it would be of significant therapeutic importance to be able to modulate GM-CSF gene expression.

Synthetic oligonucleotides are attracting increasing interest as tools for specific manipulation of gene expression and have potential therapeutic application. Several strategies that employ short nucleic acid fragments targeted to different intracellular components are currently under investigation with variable success (reviewed in Ref. 10). The “antigene” (11) strategy is based on inhibition of transcription of the selected gene by oligonucleotide-directed intermolecular DNA triple helix formation either through prevention of regulatory proteins binding to the control regions (12) or by acting as transcriptional repressors (13). Triplex formation may have certain advantages over the other oligonucleotide-mediated strategies, as there are fewer target molecules per cell and it is expected to be more selective. In vitro experiments demonstrated that the formation of such sequence-specific structures can inhibit DNA replication (14) or block transcription factors binding to the gene of interest and thereby directly affect transcription initiation (15). Triplex-forming oligonucleotides (TFOs) were also found to repress transcription in intact cells, resulting in highly specific inhibition of the synthesis of promoter reporter gene targets (16–18).

Predominantly polypurine:polypyrimidine (Pur:Pyr) se-

* This work was supported by a grant from the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; TFO, triplex-forming oligonucleotides; bp, base pair(s); PMA, phorbol 12-myristate 13-acetate; HTLV, human T-cell lymphotrophic virus.
quences have been shown to be suitable targets for intermolecular triple helices. Different structures of base triplets have been described, forming two groups of DNA triple helices. Both have the third strand binding in the major groove of the duplex DNA through Hoogsteen (Y*RY motif) or reverse Hoogsteen (R*RY motif) base pairing. The best studied Y*RY type, formed by the addition of a polyuridine third strand, consists of C*G,C and T*AT base triplets with the third strand parallel to the purine-rich strand of the underlying duplex (19, 20). These triple helices are stable only under acidic pH, but that condition can be partly overcome by methylation of the third strand cytosines (22). Stable R*RY type triple helices have been shown to form at physiological pH in the presence of divalent cations, where oligonucleotide interaction with double-stranded DNA involves G*GC, A*AT, or T*AT triplets with an antiparallel orientation of the third strand (21). TFO binding to the DNA duplex target appears to be highly sequence-specific, as triple-forming oligonucleotides have been shown to recognize unique sites in the yeast chromosomc (23).

The human GM-CSF gene contains an enhancer located approximately 3 kilobases from the transcription start site (24) and a proximal promoter spanning at least the first 120 bp from the transcription initiation site (3, 25). Activation of the human GM-CSF promoter in T cells appears to be tightly regulated by transcription factors, the CK-1 element, which binds only specific members of the family such as p65 (Rel-A) and c-Rel (Rel), and the K1 element that binds mainly classical NF-xB p65/p50 (NF-xB1) complexes (26, 27). The K1 element appears to play a central role in the response of the GM-CSF promoter to T cell receptor type signals as well as HTLV-1 Tax activation in T-cells (25, 27).

Here we attempted to modulate transcription from the GM-CSF gene by DNA triple helix formation within the promoter region. A triple-forming oligodeoxyxynucleotide was targeted to a 15-bp purine-rich sequence that overlaps the K1 element. Sequence specificity of the third strand coupling to its duplex target was investigated, as well as the ability of triple formation to inhibit recombinant and nuclear NF-xB proteins binding to the GM-CSF promoter. We also evaluated the effect of the triple-forming oligomer on luciferase activity from GM-CSF promoter-driven reporter constructs and on endogenous GM-CSF mRNA and immunoreactive protein levels in activated Jurkat T cells and show that triple formation can selectively suppress human GM-CSF gene expression.

MATERIALS AND METHODS

Oligonucleotides and Plasmids—Oligodeoxynucleotides were synthesized by the 5'-cyanoethylphosphoramidite method on an Applied Biosystems synthesizer (Applied Biosystems, Foster City, CA) using solid support for nonmodified oligomers or solid support coupled with 3'-amino-modifier (Glen Research, Sterling, VA) for oligomers with a 3'-amino group at the 3' terminus. Deprotected oligonucleotides were purified by preparative 12-20% gel electrophoresis. Concentrations were determined by absorption measurements at 260 nm using molar extinction coefficients. Oligonucleotide sequences are presented in Fig. 1b.

The HTLV-1 x expression plasmid (pCAxTax) was used as a gift from Dr. W. R. Schlossman, National Cancer Institute, San Francisco, CA, and has been previously described (28). The reporter construct pGM1uc contains 620 bp of the GM-CSF promoter (27). The reporter plasmid p3L3uc, containing a 670-bp (550 to +120) polymerase chain reaction fragment of human IL-3 gene promoter made using (C)1-16 genomic clone (24) was provided by Dr. Peter Cockerill (IMVS, Adelaide, S.A., Australia).

Triplex Gel Shift Assays—Double-stranded oligodeoxynucleotides were end-labeled with [32P]-ATP and T4 polynucleotide kinase. TFOs were incubated with double-stranded DNA at indicated concentrations of TFOs (see legend for Fig. 2) in 89 mM Tris-HCl (pH 7.5), 0.5 mM MgCl2, and, sometimes, 0.5 mM CaCl2 after being preincubated at 20°C for 30 min. Incubation was stopped by adding 20 mM EDTA and 0.2% SDS. A + G chemical sequencing of the probe was carried out according to the Maxam-Gilbert method (29). Samples were then electrophoresed through 6% denaturing gels, dried, and visualized using a Molecular Dynamics PhosphorImager.

Protein Binding Assay—Triplex formation was performed as described above using 32P-end-labeled double-stranded fragments of the GM-CSF gene promoter and immunoglobulin x gene enhancer (see Fig. 1). After initial incubation for triplex formation of approximately 0.2 ng of the probe with specific or control single-stranded oligonucleotides as described above recombinant p65 (27) or nuclear extracts from transfected and stimulated Jurkat T cells (prepared according to Ref. 30) were added, and samples were incubated at 37°C and, sometimes, at 0°C. Digestion was stopped by adding 20 mM EDTA and 0.2% SDS. A + G chemical sequencing of the probe was carried out according to the Maxam-Gilbert method (29). Samples were then electrophoresed through 6% denaturing gels, dried, and visualized using a Molecular Dynamics PhosphorImager.

Cell Culture and Transfection Procedure—The Jurkat T cell line was cultured in RPMI medium containing 10% fetal calf serum and supplemented with l-glutamine and penicillin/streptomycin. Cells were transfected by electroporation at 270 V and a capacitance of 960 microfarads using a Bio-Rad gene pulse. 5 × 106 cells in 350 μl of RPMI containing 20% fetal calf serum were used per transfection. Ten μg of HTLV-1 x expression vector and 5 μg of pGM1uc or p3L3uc reporter plasmid was used per transfection. Twenty-four hours post-transfection, TFO GM3 or control oligodeoxynucleotide GMc was added, and samples were incubated for a further 30 min at 20°C. Total RNA was purified using guanidine isothiocyanate 8 h after cell stimulation. The method for quantitating specific mRNA by RNAase protection and the vectors for antisense RNA synthesis have been previously described (31). Ten μg of total RNA was used for each assay with 32P-labeled antisense probe. Specific bands were quantified using a Molecular Dynamics PhosphorImager and normalized against glyceraldehyde-3-phosphate dehydrogenase as internal control.

Assessment of the GM-CSF Protein Levels in the Culture Medium—The concentration of GM-CSF protein in the supernatants of the cell samples, used for RNAase protection assay was quantified by enzyme-linked immunosorbant assay (Quantikine R & O Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. The detection limit for this assay was 1.5 pg/ml.

RESULTS

Oligonucleotide Design and Triplex Formation on the GM-CSF Promoter—It was previously shown that triplex formation by oligonucleotides can inhibit DNA-protein interaction at specific regions by not overlapping and not spanning the entire protein binding site (16). The 15-bp sequence from −80 to −65, which overlaps the x element in the GM-CSF gene proximal promoter (Fig. 1a) was targeted to form an intermoe...
Repression of GM-CSF Transcription by DNA Triple Helix

A

Triplex target sequence

5’-TTCCCCCGGCCTCCCT
3’-AAAGGGCGCGGCCAGGA

G/C

NFAT/ETS

-100

-50

+1

T

B

FIG. 1. The GM-CSF proximal promoter (A) and oligodeoxynucleotides used in this study (B). Map of the GM-CSF gene proximal promoter with previously identified functionally important elements or conserved elements shown by shaded boxes. The 15-bp target site (−80 to −65) for triplex formation is indicated. An arrow indicates the transcription initiation point. B, GM is a 30-mer from −90 to −60 in the GM-CSF gene promoter used as a duplex target in gel shift assays. The underlined sequence indicates the region targeted for triplex formation with a point of “mismatch” shown in small capitals. Igκ is a 27-mer (3931–3957) containing the NF-κB consensus sequence from the immunoglobulin κ gene enhancer used as a control duplex. The GM3 oligonucleotide was designed to bind to the target site as a third strand, and GMc was used as a control oligonucleotide.

The ability of these oligonucleotides to form triplexes was analyzed by gel mobility shift assay and DNase I footprinting. A radiolabeled 30-bp fragment of the GM-CSF promoter from −90 to −60 (GM, Fig. 1b), which encompassed the TFO target and overlapping κB element, was incubated with increasing concentrations of the GM3 oligonucleotide in the presence of Mg2+ and subjected to electrophoresis on native polyacrylamide gel for 18 h at 4 °C. Arrows show the mobility of double-stranded (D) and triple-stranded (T) DNA.

GM-CSF gene promoter. The DNase I footprint occurred at 4 μM concentration of the specific TFO, and full protection was obtained at 16 μM (Fig. 3, lanes 3 and 4), which is consistent with the affinity of GM3 for its target duplex observed in the gel shift assay. The nonspecific oligomer GMc did not allow any protection of the target from DNase I digestion even at a concentration of 20 μM. These data suggest that oligonucleotide GM3 forms a stable and sequence-selective triple helix with its double-stranded target from the GM-CSF gene proximal promoter.

Inhibition of Transcription Factor Binding by Triplex-forming Oligonucleotides—The effect of triplex formation by GM3 on the ability of NF-κB/Rel proteins to bind to the κB element within the 30-bp fragment of the GM-CSF promoter was examined by gel mobility shift assay. The radiolabeled double-stranded oligonucleotide was incubated in the absence or presence of increasing concentration of GM3 followed by incubation with recombinant p65 protein or nuclear extracts from HTLV-1 Tax-expressing Jurkat T cells. The formation of the recombinant p65-DNA complex was inhibited by TFO GM3 in a concentration-dependent manner (Fig. 4a). The GMc un-
Repression of GM-CSF Transcription by DNA Triple Helix

Figure 4. Effect of the triplex formation on recombinant (A) and nuclear (B) NF-κB protein binding to the GM-CSF gene promoter fragment containing the κB element. A, effect of the TFO GM3 on the binding to DNA of purified recombinant truncated p65. Arrows indicate the p65-DNA complex and unbound free probes. Mobility shift assays were carried out on native polyacrylamide gels using 32P-labeled double-stranded DNA fragments from GM-CSF promoter (GM) and immunoglobulin κ gene enhancer (Igκ) containing the κB consensus sequence. Double-stranded probes (−0.2 ng) were preincubated alone or with oligonucleotides at the following concentrations: GM3 at 10 μM (lanes 4 and 10), 3, 1, 0.3, and 0.1 μM (lanes 5–8, respectively), and GMc at 10 μM (lane 9) followed by further incubation with recombinant p65. B, triplex formation by GM3 inhibits nuclear NF-κB protein binding to the DNA. Nuclear extracts were isolated from nonstimulated (N/S) or HTLV-1 Tax expressing Jurkat T cells (tax). An arrow indicates the κB-like DNA-protein complex. Mobility shift assays were performed as described above for the recombinant p65, except that nuclear NF-κB proteins were used. TFO and control oligonucleotides were added to the double-stranded probes at the following concentrations: GM3 at 10 μM (lanes 5), 3, 1, and 0.3 μM (lanes 6–8, respectively), and GMc at 10 μM (lane 9). The figure shows only the region of the gel containing inducible κB-like DNA-protein complex and some constitutive DNA-protein complexes with similar electrophoretic mobility. Identification of specific DNA-NF-κB complexes was performed by competition with 20 ng of unlabelled Igκ in both assays (panel A, lane 2, and panel B, lane 3).

A related oligomer that could not form a triplex did not have any significant effect on p65 interaction with the GM-CSF promoter fragment (Fig. 4a, lane 9). p65 protein binding to the Igκ control probe was not significantly affected by the GM3-specific oligonucleotide, used at the highest concentration (Fig. 4a, lane 10).

Jurkat T cells transfected with the HTLV-1 Tax expression plasmid contain NF-κB/Rel complexes that can bind to the GM-CSF κB element (Fig. 4b) (27). The specific NF-κB complex was determined by competition with an excess of cold Igκ oligomer containing the κB consensus sequence (Fig. 4b, lane 3). Several constitutive DNA-protein complexes were also detected under the conditions used (Fig. 4b). Preincubation of the probe with increasing concentrations of GM3 inhibited the appearance of the κB-DNA specific complex in a concentration-dependent manner (Fig. 4b, lanes 5–8), whereas no effect was detected with control GMc oligomer at the highest concentration (Fig. 4b, lane 9).

These results indicate that triplex formation specifically prevents binding of NF-κB/Rel transcription factors (either recombinant or nuclear proteins) to the κB element of the GM-CSF gene promoter. It is interesting to note that constitutive proteins binding to the GM fragment used as a probe in the gel retardation assay were also blocked by the related TFO but not by the control oligonucleotide (Fig. 4b). This fact could be viewed as additional evidence that the observed inhibition of NF-κB protein binding to the DNA probe was due to triplex formation at the targeted site and not TFO-NF-κB protein interactions.

Inhibition of Transcription from GM-CSF Promoter Reporter Constructs—It was shown previously (17, 36) that short single-stranded oligonucleotides, when applied to the culture medium, can internalize into cells and equilibrate the concentration inside and outside the cell membrane within a few hours. This phenomenon is not universal and varies depending on the cell type used in the experiment possibly as a consequence of different mechanisms of oligonucleotide uptake (reviewed in Ref. 37). When Jurkat T cells were incubated with an intrinsically 32P-labeled oligonucleotide we also found that the intracellular concentration of the oligomer reached approximately 50% of that in the culture medium after 2 h and approximately 80% after 5 h of incubation (data not shown).

To investigate the effect of triplex formation on GM-CSF gene transcription in cell culture, 3′-amino-derivatized (specific and control) oligomers were directly added to the culture medium at the indicated concentrations (Fig. 5). 3′-modification with a primary amino group has been shown to significantly increase the resistance of natural phosphodiester oligo-
oxynucleotides to nuclease degradation (17). Two luciferase reporter constructs were used to analyze the ability of the TFO to inhibit inducible transcriptional activity in J urkat T cells. A 620-bp GM-CSF promoter reporter vector (pGMIuc) containing the triplex target site and a plasmid with 670 bp of the IL-3 gene promoter (pIL3luc), which does not have a sequence capable of forming a DNA triplex with GM3, were transfected into J urkat T cells together with an HTLV-1 tax expression vector. Both IL-3 and GM-CSF promoter activities are induced by HTLV-1 Tax (38). After 24 h, TFO GM3 and control oligomer GMc were added to the culture medium at the concentrations shown in Fig. 5, and cells were cultured for an additional 8 h and then harvested.

The specific triplex-forming oligonucleotide GM3 demonstrated a 65% inhibition of the reporter gene activity from pGMIuc at a concentration of 1 μM, whereas no effect was detected when cells transfected with pGMIuc were treated with GMc at the same concentration of 1 μM (Fig. 5). Also no significant differences in luciferase activity induced by the IL-3 promoter were detected when cells transfected with pIL3luc were exposed to medium alone, GM3, or GMc at the highest concentrations (Fig. 5).

Triplex Formation Inhibits Transcription from the Endogenous GM-CSF Gene—We used RNase protection assays to determine whether the GM3 oligonucleotide, targeted to form a triplex helix within the GM-CSF promoter, would selectively suppress the induction of mRNA from the endogenous GM-CSF gene. The effect on the response of the GM-CSF gene to PMA/Ca2+ ionophore treatment and HTLV-1 Tax expression in combination with PMA/Ca2+ ionophore was measured. J urkat T cells transiently transfected with HTLV-1 tax or untransfected were preincubated for 2 h with triplex-forming oligonucleotide GM3 or control oligomer GMc at the indicated concentrations (Fig. 6). After stimulation for a further 7 h with PMA/Ca2+ ionophore, total RNA was extracted and assayed. GM-CSF mRNA levels in PMA/Ca2+ ionophore-stimulated cells were significantly reduced when cells were treated with GM3 at 2.4 μM concentration compared with samples exposed to the culture medium only or to the control GMc oligomer at the highest concentration. (Fig. 6). GM3 was also effective when the expression of GM-CSF mRNA was synergistically activated by HTLV-1 Tax and PMA/Ca2+ ionophore (26) (Fig. 6). GM3 caused up to 70% reduction of the GM-CSF mRNA level induced by PMA/Ca2+ ionophore and up to 50% decrease after activation with HTLV-1 Tax and PMA/Ca2+ ionophore together (Fig. 6). Concentrations of immunoreactive GM-CSF in the supernatants of cells stimulated with both stimuli and treated with GM3, but not GMc, were also decreased by an average 45% with the highest concentration of GM3 (from an average 560 ± 13 pg/ml (mean ± S.D.) to an average 320 ± 16 pg/ml in two experiments) in accordance with mRNA levels in the cells. In the same cell samples, concentrations of IL-3 mRNA were not affected either by TFO GM3 or by control GMc oligonucleotides regardless of the type of activation used (Fig. 6).

These results lead to the conclusion that a 3′-amino derivatized oligodeoxyribonucleotide, directed to form a triplex helix with a 15-bp target sequence overlapping the NF-κB binding site in the GM-CSF promoter, significantly inhibits transcription from the endogenous gene in human T cells, and this inhibition appears to be oligomer- and gene-specific.

**DISCUSSION**

We show here that a triplex-forming oligonucleotide can repress transcription from a reporter construct containing the human GM-CSF gene promoter and more importantly can reduce expression from the endogenous GM-CSF gene in J urkat T cells. Moreover, we show that GM-CSF protein concentrations in cell supernatants were reduced in parallel with the GM-CSF mRNA levels. Although there are many reports of triplex formation inhibiting transcription from reporter gene constructs, only a few reports describe the inhibition of human endogenous gene expression in cell culture (17, 40–42).

The TFO binds to a sequence overlapping a κB element and blocks in vitro protein binding to this site. This is consistent with the previous mutation analysis of the GM-CSF promoter in our laboratory showing that this κB element is required for the response of the promoter to PMA/Ca2+ ionophore activation (25) and to transactivation by the HTLV-1 Tax protein (27) in reporter gene assays. The data presented here confirm these results for the endogenous gene. Activation of the GM-CSF gene by PMA/Ca2+ ionophore is also mediated via NFAT/AP-1 binding sites in both the promoter and enhancer (25, 43). HTLV-1 Tax, on the other hand, requires the κB element as well as the adjacent CK-1 element for promoter induction but does not activate NFAT/AP-1 sites (27). The fact that the GM3 TFO inhibits the response of the endogenous gene to both PMA/Ca2+ ionophore and Tax/PMA/Ca2+ ionophore costimulation suggests a central role for this κB element in GM-CSF gene transcriptional activation.

2 Himes, S. R., Katsikeros, R., and Shannon, M. F. (1996) J. Virol., in press.
The effect of the TFO GM3 appears to be specific for the GM-CSF gene and has no effect on a reporter construct containing the IL-3 promoter or on mRNA levels from the endogenous IL-3 gene. This implies that the TFO is not binding NF-κB or other transcription factor function in general but is acting selectively through triplex formation on the GM-CSF promoter.

It is also worth noting that the GM3-targeted DNA sequence covers a G/C element (see Fig. 1) that represents a putative Sp1 binding site. The Sp1 protein has been implicated in the transcriptional activation of numerous genes, and the Sp1 site was found to be important in the expression of murine GM-CSF (45). The G/C region of the human gene has a 2-bp difference with the functional site in mouse and appears to be a weak Sp1 binding site. However, blocking of Sp1 binding to the GM-CSF promoter by the TFO may also contribute to the observed inhibitory effect of GM3 on the expression of the gene.

We have also shown that the GM3 oligonucleotide caused a slightly greater decrease in the GM-CSF mRNA levels in J urkat T cells induced by PMA/Ca2+ ionophore than in cells activated by both PMA/Ca2+ ionophore and the HTLV-1 transactivator Tax. Costimulation of GM-CSF expression by these two stimuli has been shown to have a strong synergistic effect leading to high levels of gene transcription. The overexpression of Tax significantly increases binding of NF-κB/Rel transcription factors to the κB element in the GM-CSF promoter compared with that seen with PMA/Ca2+ ionophore, which may make it more difficult for the TFO to compete with proteins for binding to the target site. In addition, NFAT/AP-1 sites and the CK-1 element all contribute to this high level of synergism, and it may be more difficult to block gene activation via a single site. In order to achieve complete inhibition of GM-CSF transcription it may be necessary to use TFOS that block not only the κB element but also NFAT/AP-1 sites in the gene enhancer or promoter since we have shown that all of these elements are required for full activation of the gene (25, 27, 43). It may be also possible to increase the affinity of TFO binding to the target site on the GM-CSF promoter. This may be achieved by using, for instance, oligonucleotides coupled to an intercalating agent (46, 47) or an oligomer that is linker-substituted at the position of the “mismatch” in the duplex target (48).

The observed relatively high KD (10 μM) of the GM3 oligonucleotide binding to its duplex target could be due to the one mismatch in the otherwise homopurine-homopyrimidine sequence of the double-stranded DNA. Pyrimidine interruptions in the purine tract of the targeted DNA sequence have been shown to be significantly unfavorable for triplex formation because of loss of Hoogsteen hydrogen bonding (21) and can increase the value of the relative binding affinity of the third strand by approximately 1 order of magnitude (49). The central location of the interruption in the purine sequence of the duplex, as seen in the target on the GM-CSF promoter, has also been found to cause greater distortion in the stability of the DNA triplex than the same mismatch situated at the end of the targeted site (49, 50). Another factor that may contribute to the relatively low affinity of GM3 for its target DNA is the stretch of eight consecutive guanines in the TFO, which makes this oligonucleotide highly susceptible to self-aggregation through the formation of G-tetrad (51, 52). It also should be noted that some degradation of the triplex may occur during the course of electrophoresis, thus increasing the value of the KD observed.

In a previous number of studies it has been noted that the extracellular concentration of the TFO required for inhibition of target gene transcription in intact cells was in the range of 10–25 μM (17, 18, 40), which was 10–50 times the dissociation constant for triplex formation in vitro. The data presented in this study are not consistent with these observations. A significant decrease of GM-CSF mRNA levels by GM3 was achieved at a concentration of 2.4 μM of the TFO in the culture medium, which was below the KD value observed in the binding gel shift assays. This may be attributed to the intrinsic properties of the GM3 oligonucleotide sequence. But this finding may also be explained by the possible changes in the chromatin structure upon activation of the inducible GM-CSF gene that could make the target duplex in the gene promoter more easily accessible for the exogenous oligonucleotide and thus promote more efficient triplex formation by GM3.

50–70% of cases of acute myeloid leukaemia have been shown to depend on GM-CSF for proliferation both in vitro (8) and in vivo (9) and also for engraftment into SCID mice (53). A specific antagonist of GM-CSF receptor interactions can block the growth of acute myeloid leukaemia cells in vitro (54), as can antibodies against GM-CSF (55) and its receptor (56). It has been suggested, in fact, that paracrine production of GM-CSF is responsible for progression of the disease (57). Furthermore, 20–30% of acute myeloid leukaemia cases constitutively produce and respond to GM-CSF (39, 58, 59), suggesting that it can also act in an autocrine manner. The GM3 TFO may, therefore, have a potential therapeutic role in blocking either the paracrine or autocrine production of GM-CSF in cases of acute myeloid leukaemia. The detection of GM-CSF mRNA in the bronchoalveolar lavage of atopic patients and GM-CSF protein in the synovial fluid of rheumatoid arthritis patients (4, 5) suggests another role for the therapeutic use of TFOS that block GM-CSF transcription.

Acknowledgments—We gratefully acknowledge Dr. Roy Himes for the pGM1uc plasmid, Dr. Peter Cockerill for the pl3luc plasmid, Dr. Steve Gerondakis for the recombinant p65 protein, and Dr. Tim Hercus for performing the enzyme-linked immunosorbent assay. We also thank Professors Angel Lopez and Mathew Vadas for critical review of the manuscript and helpful comments.

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J. Biol. Chem. 1996, 271:14438-14444.
doi: 10.1074/jbc.271.24.14438

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