# Identification and characterization of a novel repressor site in the human tumor necrosis factor α gene

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## ABSTRACT

In human monocytic cell lines, tumor necrosis factor α (TNFα) expression is induced by phorbol myristate acetate (PMA). We have identified positive and negative cis-acting elements in the TNFα promoter by deletion analysis. Here we present the initial characterization of the repressor element. The repressor element was shown to function in either orientation and at various distances upstream from the positive element of the TNFα promoter. The TNFα repressor site (TRS) has been localized to a 25 bp region between base pairs −254 and −230 in the promoter. This region contains a 10 bp sequence with homology to the binding site of the activator protein AP-2. Mutation of the 6 C's of this 10 bp AP-2-like site abolish TRS repressor function. However, this AP-2-like site is not a binding site for AP-2 protein based on gel retardation analysis. In addition, a well-characterized AP-2 binding site placed upstream of the positive element of the TNFα gene did not cause repression. Therefore, this repression is very likely mediated by a novel protein(s) which interacts with the AP-2 consensus site in the TRS.

## INTRODUCTION

Tumor necrosis factor α (TNFα), also known as cachectin, is as an important cytokine produced by macrophages with wide-ranging systemic effects including protection from infections (1), surveillance against tumors (2), stimulation of inflammatory responses (3), mediation of septic shock in chronic infections (4), and cachexia in cancer patients (1). At the cellular level TNFα also stimulates cytokine production from macrophages and lymphocytes (5) and inhibits several metabolic enzymes including lipoprotein lipase in adipocytes (6). The beneficial and deleterious effects elicited by this important cytokine impose a stringent control on its synthesis. Transient increases in TNFα mRNA in response to several inducers have been reported (5,7,8,9). Understanding the regulatory elements involved in TNFα expression will provide a better understanding of its synthesis.

Recently several groups have examined the transcriptional regulation of human and mouse TNFα by viruses (10),
lipopolysaccharide (LPS) (6,10,11,12,13) and phorbol esters (PMA) (9,14,15,16,17,18). Regulation of the mouse and human TNFα gene appears to differ, since different DNA motifs are believed to be involved in induction of the gene by LPS. The NF-κB motif along with its transactivator was shown to be responsible for LPS induction of the TNFα gene in mouse primary macrophages (11,12,13,18). Several copies of the mouse NF-κB motif confer LPS inducibility in mouse cells (13), whereas the NF-κB-like sequences from the human TNFα gene does not mediate the same response in a mouse macrophage cell line (10).

There are conflicting reports about the location of the cis-acting elements involved in PMA inducible regulation of the human TNFα promoter. A PMA responsive element has been reported to be present either between base pairs −285 and −101 (15), or located between base pairs −95 and −36 from the transcription initiation site (14,17). Another report has shown that the TATA box of the TNFα gene is involved in phorbol ester responsiveness of the promoter (16). Furthermore, the molecular mechanisms involved in the down regulation of the TNFα gene are not well defined. Therefore we have undertaken a deletion analysis of the human TNFα promoter to better define the important cis-acting elements which may be involved in the transcriptional regulation of the gene. In this report, we will provide evidence for the presence of both positive and negative regulatory elements in the TNFα promoter and the initial characterization of a novel repressor element.

## MATERIALS AND METHODS

### Cell culture and growth conditions

Human premonocytic cell line U937 was kindly provided by Dr A.Howard, (Merck Research Laboratories, Rahway, NJ). The U937 cells were maintained in RPMI1640 (Gibco/BRL) medium supplemented with 10% fetal calf serum (Hyclone), 100 units of penicillin−streptomycin (Gibco/BRL), and 2 mM glutamine (Gibco/BRL).

### Transient transfection assays

The U937 cells were transfected using lipofectin reagent (Gibco/BRL). In a standard experiment, the cells were transfected

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with 20 $\mu$g of the indicated *TNF$\alpha$* reporter plasmid and 2 $\mu$g of plasmid pCMV.GAL (kindly provided by Dr E.O'Neill, Merck Research Laboratories), which contains the $\beta$-galactosidase coding region expressed from the CMV early promoter (19). Plasmid DNA and lipofectin were incubated in a 60 mm petri dish with $1 \times 10^7$ cells in OptiMEM1 media (Gibco/BRL) for 5 hours at 37°C with an atmosphere of 5% $CO_2$. Following the incubation, an equal volume of RPMI1640 containing 20% fetal calf serum, penicillin, streptomycin, and glutamine was added. The cells were further incubated for 24 hours and then induced for 24 hrs with 2.5 ng/ml of PMA (Sigma). Cell extracts were prepared by 3 freeze and thaw cycles of cells resuspended in 250 mM Tris–HCl [pH 7.5], followed by centrifugation for 5 minutes at 10,000×g at 4°C to remove cell debris. The protein content of cell extracts was determined using the BIO-RAD dye system (Bio-Rad). The cleared supernatant of cell extracts was used to assay for chloramphenicol acetyltransferase (CAT) activity as described by Gorman *et al.* (20). CAT assays were routinely performed for two hours at 37°C, and the reactions were linear throughout this time. Amounts of assayed cell extract were adjusted to achieve acetylation of at least 1–5% of the substrate during the assay. TLC plates were analyzed using either an AMBIS computer controlled radioanalytic imaging system (San Diego, CA) or a phosphoimager system (Molecular Dynamics, Sunnyvalle, CA). The $\beta$-galactosidase activity was assayed in the same supernatants using chlorophenol red-$\beta$-D-galactopyranoside (CPRG) as a substrate (21). Serial dilutions of $\beta$-galactosidase (Boehringer Mannheim) were made to construct a standard curve in each assay. Unit of $\beta$-galactosidase is as defined by Boehringer Mannheim. The $\beta$-galactosidase activity was used to normalize for transfection efficiency among different plasmids in the transfection. Transfection data are expressed as % acetylation of $^{14}$C-chloramphenicol/unit of $\beta$-galactosidase activity.

## Plasmid constructions

The source of *TNF$\alpha$* promoter sequences was plasmid pTNFP1 (kindly provided by Dr M.Tocci, Merck Research Laboratories) which is a pUC19 plasmid with sequences between base pairs −615 and +113 of the *TNF$\alpha$* promoter cloned into the *Sma*I site of the polylinker. The *TNF$\alpha$* sequences were placed in the *Sma*I and *Hind*III restriction sites of the promoterless CAT plasmid pNHCAT (kindly provided by Dr M.Tocci, Merck Research Laboratories). The endpoints of all the deletions were confirmed by sequencing using the dideoxy chain termination procedure outlined in the Sequenase kit from United States Biochemicals.

For constructing 5' promoter deletions, naturally occurring restriction sites were used. Plasmid pAS1 containing sequences between base pairs −615 and +113 of the *TNF$\alpha$* promoter was constructed by digesting plasmid pTNFP1 with *Eco*RI, repairing the cohesive ends with the Klenow fragment of DNA polymerase I, and further digesting the DNA with *Hind*III. The resulting *TNF$\alpha$* promoter was cloned into the *Sma*I and *Hind*III sites of pNHCAT. Similarly, plasmids pAS3, pAS4, pAS5, and pAS2 were constructed using restriction enzyme sites at −493 bp (*Stu*I), −356 bp (*Bxt*XI), −130 bp (*Apa*I), and −107 bp (*Sac*I) respectively. The 3' protruding ends of *Bxt*XI, *Apa*I, and *Sac*I were made blunt by using T7 DNA polymerase according to standard procedures (22). Plasmids pAS6 and pAS7 were generated by using specific primers to amplify *TNF$\alpha$* DNA sequences from pTNFP1. The 3' PCR primer was GGGGGG-

AAGCTTGCATGCCTG for both plasmids. The 5' primer for pAS6 was GGGGGAGGCCTACACACAAATCAGTCA-GTGGCCCAGA, and that for pAS7 was GGGGGAGGCC-TCCCCGCCCCCGCGATGGAGAAGAAACCG. The amplified DNA fragments were then cloned in pNHCAT as described above.

Plasmids depicted in Figs. 3a, 5 and 6b were derived by cloning different DNA inserts into the blunt-ended *Bgl*II site of pAS5. The source of the insert for pCF32 and pCF33 was pCF1, which contains base pairs −280 and −172 region of the *TNF$\alpha$* promoter. Plasmid pCF1 was generated by PCR amplification of *TNF$\alpha$* sequences from pAS1, and digestion with *Eco*RI and *Pst*I, followed by cloning into the *Eco*RI and *Pst*I sites of the vector pBluescript SK+ (Stratagene). Sequences of the 5' and 3' primers were GGGAATTCGGTACCACACACAAATCA-GTCAGTGG and GGCTGCAGTCGACATTTGGAAA-GTTGGGGACAC, respectively. Plasmid pCF1 was digested with *Bam*HI and *Eco*RV, the *Bam*HI site was filled-in with Klenow fragment of DNA polymerase I, and the resulting *TNF$\alpha$* DNA fragment was inserted into the filled-in *Bgl*II site of plasmid pAS5 in either the correct (plasmid pCF32) or the opposite orientation (plasmid pCF33). The source of DNA insert for plasmid pCF40 was a 118 bp fragment of bacteriophage $\Phi$X174 digested with *Hae*III. The 118 bp insert was placed in pAS5 as described above.

Plasmids pCF46, pCF48, and pCF49 were generated by cloning various copies of the 118 bp fragment of $\Phi$X174 into the *Sma*I site of pCF32. Plasmids pCF89 and pCF62 were
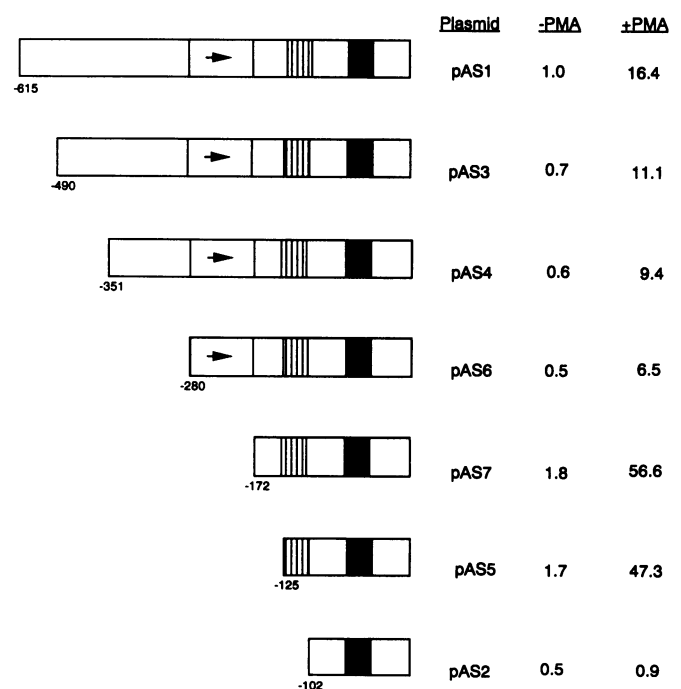


| Plasmid | -PMA | +PMA |
|---|---|---|
| pAS1 | 1.0 | 16.4 |
| pAS3 | 0.7 | 11.1 |
| pAS4 | 0.6 | 9.4 |
| pAS6 | 0.5 | 6.5 |
| pAS7 | 1.8 | 56.6 |
| pAS5 | 1.7 | 47.3 |
| pAS2 | 0.5 | 0.9 |

**Figure 1.** Identification of positive and negative *cis*-acting elements in the human *TNF$\alpha$* promoter. Plasmids pAS1–pAS7 were constructed and U937 cells were transfected as described in Materials and Methods. The results reported are the average of three independent transfection experiments and are normalized to the uninduced value of pAS1. Symbols are: solid box represents TATA element; lined box represents positive activator of *TNF$\alpha$*; box with the arrow represents the repressor element, with the direction of arrow showing the orientation of the element.

generated by cloning an annealed oligonucleotide containing sequences between base pairs −263 and −212 of the *TNFα* promoter into pAS5 as described earlier. Sequences of the complementary oligonucleotides used were:

1) GTGGCCCAGAAGACCCCCCTCGGAATCGGAGCAGGGAGGATGGGGAGTGTGA
2) TCACACTCCCCATCCTCCCTGCTCCGATTCCGAGGGGGGTCTTCTGGGCCAC

The two oligonucleotides were phosphorylated with T4 kinase and annealed using standard procedures (22). The promoter sequences are present in the correct orientation in pCF89 and in the opposite orientation in pCF62. Plasmid pAS80 was constructed as described above with a 25 bp oligonucleotide (AS80) containing the wild-type *TNFα* sequences between base pairs −254 to −230: AAGACCCCCCTCGGAATCGGAGC-
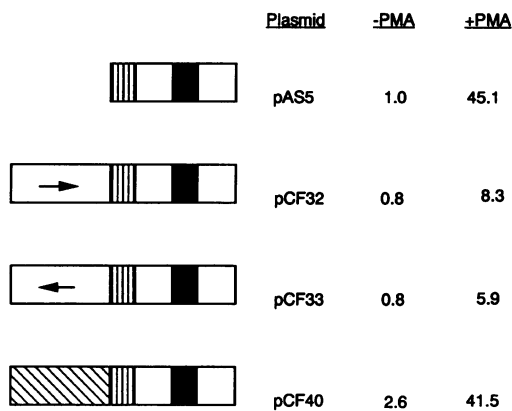


**Figure 2.** The repressor element can function in both orientations. The sequences present between base pairs −280 and −172 were cloned into pAS5 either in the correct (pCF32) or opposite orientation (pCF33) and transfected into U937 cells as described in Materials and Methods. The results reported are the average of 3 transfection experiments, corrected for transfection efficiency using the β-galactosidase activity, and normalized to the uninduced level of pAS5. Symbols are the same as in Fig. 1. Cross-hatched box represents a 118 bp *Hae*III digested DNA fragment from the phage φX174.
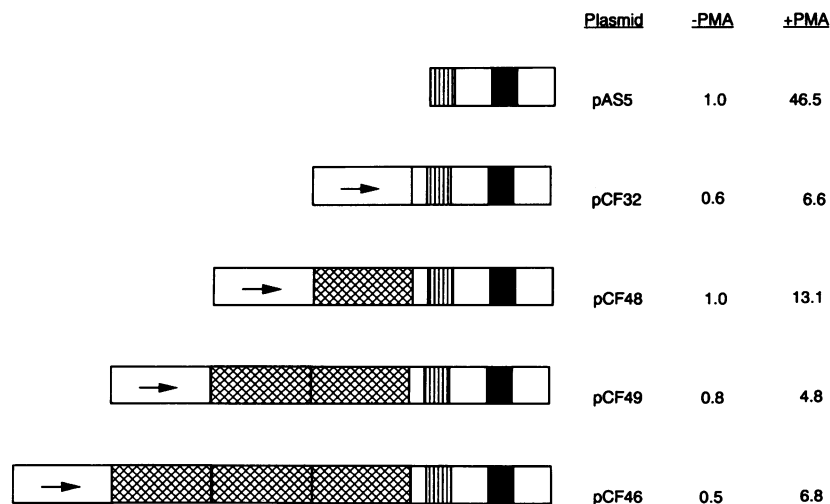
AG. Similarly, pAS81 was constructed with the mutant oligonucleotide AS81: AAGAAATTAGTCGGAATCGGAGC-AG; and pAAP2 with the oligonucleotide AP-2: GATCGAA-CTGACCGCCCGCGGCCCGT as described by Williams *et al.* (22).

## Gel retardation DNA binding assays

Nuclear extracts from the U937 cells were prepared by the procedure of Dignam *et al.* (24), with the following modifications. A cocktail of protease inhibitors was added to buffers A and C. This cocktail included the following inhibitors: 1mM phenylmethylsulfonyl flouride (PMSF), 10 ng/ml N-Tosyl-phenylalanine chloromethyl ketone (TPCK), 10 ng/ml soybean trypsin inhibitor, 0.3 ng/ml benzamidine, 1 ng/ml leupeptin, 1 $\mu$M pepstatin, and 1 ng/ml aprotinin (all purchased from Sigma chemicals). The probe used for gel retardation assays shown in Figs. 4b and 6c contains *TNFα* promoter sequences between base pairs −280 and −172. It was obtained by digesting plasmid pCF1 with *Hind*III and *Bam*HI and filling in with Klenow fragment and [$\alpha^{32}$P]dCTP, followed by isolation of the probe from a 5% polyacrylamide gel. The 26 bp probe used for experiments reported in Fig. 7 was obtained by annealing the two complementary oligonucleotides GATCGAACTGACCGCCCGCGG-CCCGT and ACGGGCCGCGGGCGGTCAGTTCGATC. The 5′ ends were labelled with polynucleotide kinase and [$^{32}$P]$\gamma$ATP. The binding reactions were carried out for 30 minutes on ice in 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes; pH 7.5), 100 mM potassium chloride (KCl), 0.05% nonidet P40, 0.5 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM dithiothreitol, and 5% glycerol. Each 20 $\mu$l binding reaction contained 3.5 $\mu$g of nuclear proteins and 4 $\mu$g of poly (dA−dT) as carrier DNA. The ratio of radioactive probe to competitors was adjusted to either 250 fold molar excess (Fig. 4b) or 500 fold molar excess (Figs. 6c and 7). Purified AP-2 protein used in Fig. 7 was purchased from Promega. The reaction products ere separated on a 5% polyacrylamide gel containing 5% glycerol in 0.5×TBE buffer, followed by gel drying and autoradiography. The double-stranded



**Figure 3.** The repressor element can function at various distances. Plasmids pCF48, pCF49 and pCF46 contain one, two and three copies respectively of the 118 bp *Hae*III fragment of φX174 inserted between the repressor element and the activator element of pCF32. The results reported are the average of 3 transfection experiments, and the data normalized as described in Fig. 2. The symbols used are the same as in Fig. 2.

**Figure 4.** Identification of nuclear factor(s) binding to the *TNFα* repressor element. Nuclear extracts from uninduced U937 cells were prepared as described in Materials and Methods. (A) Sequence of the 108 bp DNA fragment containing the *TNFα* repressor site used as a radioactive probe in the gel retardation assays and sequences of oligonucleotides AS1, AS2, AS3 and AS4 used as competitors. AS1 and AS4 contain short linker sequences (in italics) not found in the 108 bp fragment. (B) Competition analysis of the specific DNA—protein complexes with overlapping oligonucleotides of the *TNFα repressor site*. The binding and electrophoresis conditions were as described in Materials and Methods. Position of the specific complexes are indicated to the left of the panel. The competitors were added at 250 fold molar excess to the radioactive probe. Lanes: 1, no nuclear extract; 2, no competitor; 3, unlabeled probe fragment; 4, oligonucleotide AS1; 5, oligonucleotide AS2, 6, oligonucleotide AS3; 7, oligonucleotide AS4.

competitor oligonucleotides used in gel retardation assays are shown in the figure legends and in Fig. 4a.

## RESULTS

### Identification of positive and negative cis-acting elements in the human TNFα promoter

To identify the regulatory elements in the human *TNFα* promoter, we have constructed a series of plasmids containing 5′ deletions of the promoter fused to the bacterial CAT reporter gene. Deletion plasmids were transiently transfected into a human premonocytic cell line U937, and the expression of CAT was monitored in the presence (2.5 ng/ml) or absence of PMA. As shown in Fig. 1, deletion of sequences between base pairs −615 to −280 did not affect the promoter activity significantly, but deletion of base pairs −280 to −172 gave rise to 9 fold higher PMA-induced CAT expression and approximately 3.5 fold higher basal CAT expression (compare pAS6 and pAS7), indicating the presence of a repressor element in this region. Further deletion of base pairs −172 to −125 did not have a significant effect on promoter activity, but deletion of base pairs −125 to −102 resulted in reduction of promoter activity and PMA inducibility (compare pAS5 and pAS2), suggesting the existence of a positive regulatory element in this region. The negative element identified between base pairs −280 and −172 was subjected to further analysis.

### TNFα repressor element is orientation and distance independent

To determine the functionality of the repressor element identified above, the 108 bp fragment spanning base pairs −280 and −172 was placed in either orientation in front of the positive element (located between base pairs −125 and −102) of the *TNFα* promoter present in plasmid pAS5. As shown in Fig. 2, the 108 bp *TNFα* sequence in the correct orientation (pCF32) was capable of repressing induced gene expression. In addition, the repressor site was also functional when placed in the opposite orientation (see pCF33). This repression is sequence-specific, since a random DNA fragment of approximately the same size did not repress CAT expression to the same extent (compare pCF32 and pCF40).

Furthermore, the repressor function is distance independent because it repressed CAT expression when placed several hundred base pairs upstream from the positive regulatory element (Fig. 3).

### Identification of the protein factor(s) binding to the repressor site

To identify nuclear protein(s) binding to the repressor site, a 108 bp DNA fragment spanning nucleotides −280 to −172 was used as a probe in *in vitro* gel retardation assays (Fig. 4a) with nuclear extracts prepared from uninduced U937 cells. Four complexes were identified that can be specifically competed by an excess of cold probe (lane 2 and 3 , Fig. 4b). The 108 bp probe contains several putative transcription factor binding sites such as AP-1, AP-2, and NF-κB (Fig. 4a). However, oligonucleotides containing well-characterized binding sites for AP-1 (25) and AP-2 (23) failed to compete any of these complexes, while only a partial competition for the complex A was observed with oligonucleotide containing the NF-κB site (26) (data not shown). The potential role of NF-κB in the formation of complex A was not pursued any further.

To localize the region involved in specific complex formation, we used overlapping oligonucleotides of approximately 50 bp in length, spanning the 108 bp element, as competitors in the gel retardation assays (see Fig. 4a). As shown in Fig. 4b, the oligonucleotide AS1 (spanning base pairs −276 and −236) competed efficiently for the binding of complexes B, C, D and to a lesser degree, complex A (compare lane 2 with lane 4). The oligonucleotide AS2 (spanning base pairs −260 and −211) competed efficiently for all four specific complexes (compare lane 2 with lane 5). The oligonucleotide AS3 (spanning base pairs −235 and −186) partially competed for complexes A, C and D, while the oligonucleotide AS4 (spanning base pairs −210 and −172) did not compete for any of the specific complexes (lane 6 and lane 7).

### Localization of the repressor site

To determine if the region in the *TNFα* promoter which can compete efficiently for complex formation can also function as a repressor site *in vivo*, the 52 bp region between base pairs −263
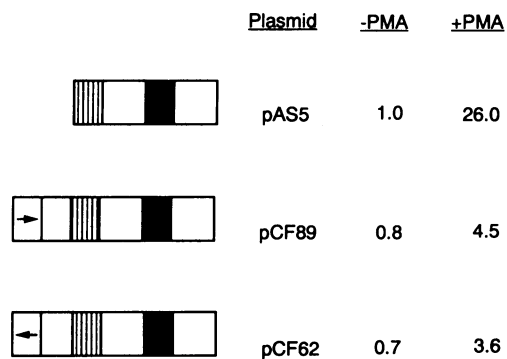
| Plasmid | −PMA | +PMA |
|---|---|---|
| pAS5 | 1.0 | 26.0 |
| pCF89 | 0.8 | 4.5 |
| pCF62 | 0.7 | 3.6 |

**Figure 5.** Localization of the repressor site. Promoter sequences between base pairs −263 and −212 are present either in the correct (pCF89) or the opposite (pCF62) orientation. The results reported are the average of three independent transfections and the data normalized as described in Fig. 2. Symbols are the same as in Fig. 1.
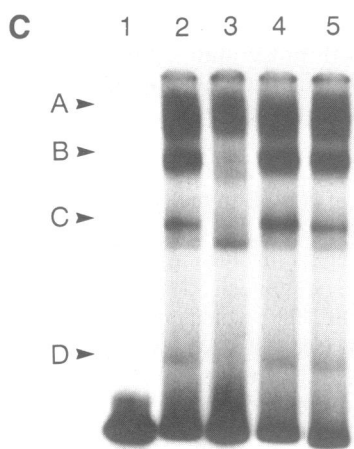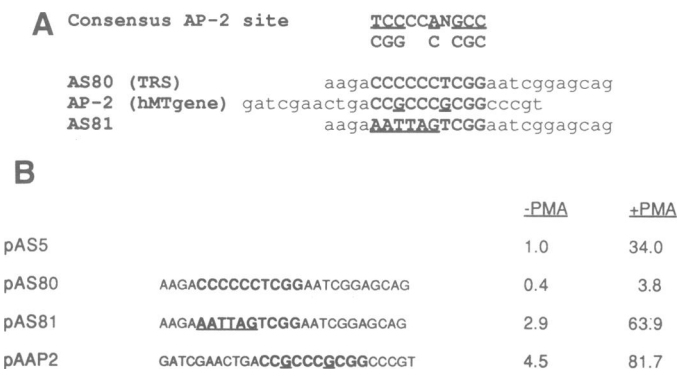
**A** Consensus AP-2 site    TCCCCANGCC
                             CGG C CGC

    AS80 (TRS)          aagaCCCCCCTCGGaatcggagcag
    AP-2 (hMTgene) gatcgaactgaCCGCCCGCGGcccgt
    AS81                aagaAATTAGTCGGaatcggagcag

**B**

| | | −PMA | +PMA |
|---|---|---|---|
| pAS5 | | 1.0 | 34.0 |
| pAS80 | AAGACCCCCCTCGGAATCGGAGCAG | 0.4 | 3.8 |
| pAS81 | AAGAAATTAGTCGGAATCGGAGCAG | 2.9 | 63.9 |
| pAAP2 | GATCGAACTGACCGCCCGCGGCCCGT | 4.5 | 81.7 |

**C**



**Figure 6.** Mutational analysis c the *TNFα* repressor site. (A) Oligonucleotides containing AP-2 like sites. The RS has sequence similarity to a consensus AP-2 binding site (27), and differs om the hMT AP-2 site (23) by 2 bp. The oligonucleotide AS81 contains mutations in the AP-2 like site as shown. (B) Transfection analysis of the plasmids containing different AP-2 like sites. The data presented are averag of three indepedepent transfections and are calculated as described earlier. (C) Competition analysis for the TRS-nuclear factor(s) complexes with oligonucleotides AS80, AS81 and AP2. The probe and binding conditions used are the same as described for Fig. 4, except that the competitors were added at 500 fold molar excess. Lanes: 1, no nuclear extract; 2, no competitor; 3, oligonucleotide AS80; 4, oligonucleotide AS81; 5, oligonucleotide AP-2.
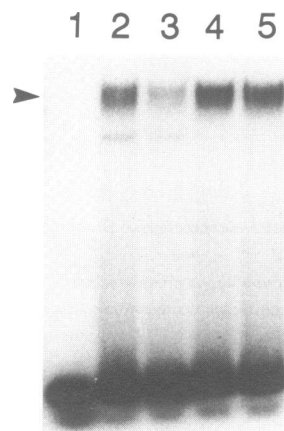


**Figure 7.** The TRS cannot compete for the AP-2 complex formed on the hMT AP-2 site. The probe used was a 26 bp oligonucleotide (AP-2) containing the hMT AP-2 site (23). Binding reactions were carried out with approximately 50 ng (2 μl) of the purified AP-2 protein (Promega) and binding conditions of the manufacturer were used. Lanes: 1, free probe; 2, no competitor; 3, unlabeled probe fragment; 4, oligonucleotide AS80; 5, oligonucleotide TFIID (29): GC-AGAGCATATAAGGTGAGGTAGGA.

and −212 was cloned into pAS5 and transfected into U937 cells. As shown in Fig. 5, this region repressed the *TNFα* promoter (see pCF89) and like the 108 bp promoter region, it also functioned in the opposite orientation (see pCF62). Through the use of gel retardation assays, a 25 nucleotide fragment spanning base pairs −254 and −230 (AS80) was shown to compete for the specific complexes (Fig. 6c). When this sequence was cloned into pAS5 to generate pAS80, and transfected into U937 cells, repression of both basal and PMA-induced expression were observed (see Fig. 6b).

### The *TNFα* repressor site is distinct from the AP-2 site

The 25 bp *TNFα* repressor site (TRS) has 10 bases which share significant homology with the AP-2 consensus binding site (Fig. 6a). Mutation of the 6 C's within the 10 bp AP-2-like site, results in a total loss of repressor activity (pAS81, Fig. 6b), indicating the importance of this region in the repressor function. To further define the role of the AP-2-like site in repressor function, a well-characterized AP-2 site from the human metallothionein gene (23) was cloned into plasmid pAS5, however, the resulting plasmid pAAP2 failed to repress the *TNFα* promoter in U937 cells (Fig. 6b).

Consistent with the transfection data, the gel retardation analysis showed that oligonucleotide AS80 competed efficiently for the formation of complexes B,C, and D, and partially for complex A. Whereas oligonucleotides AS81 and AP-2 were unable to compete for any of these complexes (Fig. 6c). It is worth mentioning that the competition by AS80 was not as efficient as the 108 bp fragment or the 50 bp AS1 and AS2 oligonucleotides (especially for the complex A). When AS80 was used as a probe in gel retardation analysis, multiple specific complexes were also observed (data not shown). However, these complexes were very unstable and they have similar mobility in the gel, therefore we have not been able to resolve unambiguously if there are three or four of these complexes.

To investigate the potential role of AP-2 protein in forming any of these complexes, purified AP2 protein was used in the gel retardation assay with the 108 bp fragment as a probe. No specific complex was detected. However, in a parallel experiment using the hMT AP-2 oligonucleotide as a probe, a specific complex was detected (lane 2, Fig. 7), which cannot be competed by oligonucleotide AS80 or by the non-specific competitor containing the TFIID binding site (lane 4 and 5, Fig. 7). This indicated that the repressor function of the AP-2-like site in the TRS is not mediated through complex formation with the AP-2 protein.

## DISCUSSION

Using promoter deletion analysis in a transient gene expression system, we have investigated the cis-acting elements involved in the regulation of the human TNFα gene by PMA. We report here two regulatory elements identified on the TNFα promoter. A positive element is present in a 23 bp region between base pairs −125 and −102 of the TNFα promoter and a negative element in a 108 bp region between base pairs −280 and −172. Although we report only one positive and one negative regulatory site in the TNFα promoter, we do not exclude other potential regulatory elements in the TNFα promoter which may manifest themselves through a different set of deletion mutants (14−18). The TNFα protein has pleiotropic effects and the transient nature of its induced expression has been well documented (5,7,8,9), therefore it is reasonable for the gene to be tightly regulated by both (and possibly multiple) positive and negative regulatory elements.

We have focused our studies on the repressor element identified between base pairs −280 and −172. Primer extension analysis experiments confirmed the presence of higher levels of correctly-initiated TNF message from cells transfected with plasmid pAS5 than with the wild type plasmid pAS1 (data not shown), suggesting that the repressor element regulates transcription. This element also functions in either orientation and at various distances upstream from the positive element of the TNFα promoter. Based on transient transfection experiments, the repressor element (TRS) was localized to a 25 bp region between base pairs −254 and −230 which contains a 10 bp sequence homologous to the consensus AP-2 site (27), and mutations in this sequence relieve repression. Although the TRS contains a sequence similar to the consensus AP-2 site, it does not bind the AP-2 protein. We have shown that the monocytic nuclear factor(s) binding to this element are distinct from AP-2 based on several lines of evidence: the nuclear factor(s) binding to the repressor element is not competed by an oligonucleotide containing a well known hMT AP-2 binding site; the purified AP-2 protein is unable to bind the repressor element; and in transfection experiments, an oligonucleotide containing the hMT AP-2 binding site failed to repress when placed in the same context as the TRS. We have observed considerable variations in the transfection efficiency of the U937 cells depending on the age of the cells and the lipofectin reagent, which resulted in variation of the absolute magnitude of the PMA-induced response (e.g., pAS5 in Figs. 1, 2, 3, 5 and 6b), but the trend of the responses among different plasmids were always consistent.

There are three AP-2-like sites present in the 108 bp repressor sequence located between base pairs −280 and −172 (Fig. 4a). Using this sequence as a probe in the gel retardation assays, four

specific complexes were identified. The AP-2- like sites may all contribute to the formation of these complexes since the 50 bp oligonucleotides (AS1, AS2 and AS3) containing one or more of these sites all showed some degree of competition. In addition, the AP-2-like sites may interact co-operatively since the 108 bp sequence competed much better than any of the shorter oligonucleotides. Alternatively, the different degree of competition may be due to the different lengths of the competitors, instead of the specific sequence present in the competitors. There are at least two possibilities for the formation of more than one complex on the 108 bp sequence. These include binding of the repressor factor to more than one site, and binding of multiple repressor proteins to the same site. Even though a 25 bp sequence was shown to compete for all of the complexes formed on the 108 bp probe, and when this 25 bp TRS was used as a probe in gel retardation assays, multiple specific complexes were also observed, we cannot rule out any of these possibilities at this time.

The repressive effect of the negative element identified in this study was more noticeable in the PMA-induced expression, however, a small but reproducible reduction in the basal promoter activity was also observed. We have compared nuclear extracts prepared from uninduced and PMA-induced U937 cells in gel retardation assays using both the 108 bp and the 25 bp probes, and observed no difference in complex formation (data not shown). It is possible that PMA induces modification of the TRS-binding protein(s), which affects their ability to mediate the repression, but does not affect their ability to bind to the repressor site. It is also possible that there may be PMA-dependent co-operative binding in the cells with proteins not yet identified from the in vitro gel retardation assays, which is essential for the repressor function in vivo. Lastly, it is possible that the repression mediated by this repressor element may be independent of PMA induction while the higher level expression of the TNFα promoter under such conditions enabled us to observe the repression effect more clearly.

To determine if the repression described in this study is specific for the TNFα promoter, we have placed the repressor elements (both the 108 bp and the 25 bp elements) in front of a heterologous promoter that contains the NFκB binding site from the CMV-IE promoter (19). No obvious repression were observed (data not shown) suggesting that the TRS may only function in the context of the TNF promoter.

Silencers are promoter elements which repress transcription and function independently of orientation and position. As described earlier, the TRS represses the TNFα gene in either orientation and at various positions upstream from the promoter. It is possible that the TRS functions as a silencer in down-regulating the TNFα promoter. Recently, Sato et al. (28) reported a G-rich silencer in the catalase gene, and reviewed several G-rich silencers in other genes. Similar to the TRS described here, the catalase G-rich silencer has a core sequence which closely resembles a known Sp1 activator binding site, but it is not a functional Sp1 site. The TRS may belong to this class of G-rich silencers, which share sequence homology with certain GC-rich activator sites, but function as repressor elements. We have demonstrated that the nuclear factors binding to the repressor element are distinct from the AP-2 protein. If the repression is mediated by trans-acting factor(s), they must be different from AP-2. However, we have no evidence so far to rule out the possibility that TRS may repress the TNFα expression in cis by affecting chromatin structure in the promoter region or decreasing the rate of transcription.

## ACKNOWLEDGEMENTS

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