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Constitutive and Inducible In Vivo Protein–DNA Interactions at the Tumor Necrosis Factor-α Promoter in Primary Human T Lymphocytes

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Tumor necrosis factor-α (TNF-α) is a key cytokine of lymphocytes with major regulatory functions in immunomodulation, chronic inflammation, and septic shock. However, only limited information on TNF promoter regulation in vivo in primary lymphocytes is available. To determine and compare protein–DNA interactions at the native TNF locus in primary lymphocytes, we analyzed the human TNF-α promoter by ligation-mediated polymerase chain reaction (LM-PCR) techniques. Accordingly, primary CD4+ T lymphocytes from peripheral blood were cultured in the presence of various stimuli and analyzed by LM-PCR. Inducible in vivo protein–DNA interactions at the TNF promoter were detected between −120 and −70 bp of the human TNF promoter relative to the transcriptional start site. This area includes binding sites for transcription factors such as ETS-1, NFAT, ATF-2/c-*jun*, SP-1/Egr-1, and NF-κB. In contrast, no protein–DNA interactions were observed at various binding sites with reported regulatory function in tumor cell lines such as the k2 element, the NFAT site at −160, the AP1 site at −50, and the SP1 site at −65. Additional mutagenesis and transfection studies demonstrated that NF-κB and CREB/ AP-1 are important regulators of inducible TNF promoter activity in primary human T lymphocytes. These results provide novel insights into the complex regulation of TNF gene transcription in primary T lymphocytes in vivo by constitutive and inducible protein–DNA interactions that appear to be at least partially different compared to previously characterized tumor cell lines.

TNF Promoter In vivo footprinting AFT-2/c-*jun* SP1/Egr

TUMOR necrosis factor (TNF) is a pleiotropic cytokine that plays an important role in chronic inflammation (5), immunomodulation (31), cachexia (39), and septic shock (18,38). It is produced by a wide variety of cells of the immune system including mononuclear cells, macrophages, T and B lymphocytes, NK cells, and mast cells (4,34–36). TNF may form monomeric transmembrane or homotrimeric secreted molecules that bind to two distinct cell surface receptors, designated p55 and p75 TNF-R (13,21,30,33). These receptors are aggregated by TNF into clusters of two or three molecules of either the 55 or 75 kDa TNF-R whose activation mediates biologic activities of TNF (13,21,30,33). TNF is a key mediator of both acquired and natural immunity and appears to contribute to the initiation and progression of many immune-mediated disorders such as rheumatoid arthritis (17), multiple sclerosis (16), Crohn's disease (23), and diabetes (3). These data on the functional importance of TNF in the immune system are underlined by the finding that transgenic mice overexpressing TNF develop severe destructive arthritis (28). In ad-

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dition, it was recently demonstrated that mice in which the TNF gene has been inactivated by homologous recombination exhibit multiple defects of immune cell function (26).

Expression of the TNF gene in cells of the immune system is tightly regulated at the transcriptional, posttranscriptional, and translational level (6, 14,15). For instance, several groups have used in vitro binding assays to identify binding sites for various transcription factors (e.g., ETS-1, NF-κB, NFAT, ATF-2/c-*jun*, and C/EBP) in the TNF promoter that appear to regulate TNF gene expression at the trancriptional level in cell lines (11,12,20,27,40,41). Additional reporter gene studies revealed that the 200 bp immediately upstream of the TATA box are sufficient to confer maximal inducibility and cyclosporin (CsA) sensitivity of the TNF promoter (11). Subsequent studies identified two elements within this region, denoted CRE and k3, that appear to play a key role in regulating inducible TNF promoter activity. Studies in the Ar-5 T cell line demonstrated that while the CRE element is bound by a constitutive complex of ATF-2 and c-*jun*, the adjacent k3 site is recognized by two molecules of the CsA-sensitive transcription factor NFATp (40). Because site-directed mutagenesis of the CRE and k3 sites caused strikingly reduced TNF promoter activity in transient transfection assays (11,40), these data suggested an important role for ATF-2/c-*jun* and NFAT in the regulation of TNF gene transcription in lymphocytes. Furthermore, other transcription factors such as AP-1, SP-1, Egr-1, C/EBP, AP-2, ETS-1, and NF-κB have been proposed to regulate TNF promoter activity (10,11,19,40). However, only limited data are available with regard to their role in TNF promoter regulation in vivo.

In the present study, we analyzed protein–DNA interactions at the human TNF promoter in primary T lymphocytes in vivo by genomic footprinting. The data suggest that TNF gene transcription in vivo is regulated by the coordinate binding of a set of transcription factors including ATF-2/c-*jun*, *junB*/c-*fos*, Egr-1, and NF-κB to the TNF promoter. We propose a model where TNF gene transcription in primary T lymphocytes is regulated in vivo at the level of constitutive and inducible protein–DNA interactions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The Jurkat T cell line was obtained from ATCC (Rockville, MD). Cells were cultured in RPMI-1640 supplemented with 10% FCS (PAA, Linz, Austria), 5% NCTC 135 medium (Gibco BRL, Gaithersburg, MD), 20 mM HEPES buffer (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 1000 U/ ml penicillin/ streptomycin (Biochrom, Berlin, Germany).

Isolation and Culture of Primary Human CD4+ T Lymphocytes

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using Ficoll-Hypaque gradients. PBMC were then further purified to isolate CD4+ T cells using immunomagnetic beads specific for CD4 (obtained from Dynal, Oslo, Norway). The beads were finally removed from the cells by treatment with Detachabead (Dynal). Freshly isolated cells were counted and subjected to FACS analysis. Only cell populations with purity greater than 95% were used in the experiments described below. The cells were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) in humidified atmosphere with 5% CO_2 at 37°C.

Primary T cells were stimulated with 50 ng/ml PMA (Sigma, St. Louis, MO), 1 μg/ml ionomycin (calcium salt; Sigma), anti-human CD3 antibody (Cat. No 30110D, Pharmingen, San Diego, CA) or anti-human CD28 antibody (Cat. No. 33740D, Pharmingen) as specified in the Results section. Antibodies were coated at a concentration of 1 μg/ml in coating buffer (100 mM $NaHCO_3$, pH 8.2) overnight to tissue culture plates prior to stimulation.

Dimethylsulfate (DMS)-Piperidine Treatment of DNA for In Vivo Footprinting

DMS (0.1%, Sigma) was added directly to the culture medium. DNA extraction was performed by an overnight incubation in cell lysis buffer (1 mM Tris-HCl, pH 7.5, 400 mM NaCl, 2 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) at 37°C. The strand scission reaction was performed by resuspending the DNA in 1 M piperidine (Sigma) and subsequent incubation for 30 min at 90°C. The DNA was finally resuspended in water and diluted at a concentration of 1 mg/ml. For control reactions naked genomic DNA was treated with DMS in vitro. The DNA was incubated for 30 s with 0.1% DMS at room temperature. In vitro methylated control DNA was subsequently treated with piperidine as described above.

Ligation-Mediated Polymerase Chain Reaction

In vivo footprinting by ligation-mediated polymerase chain reaction (LM-PCR) was carried out essentially as described (1,9,22). In brief, primer annealing was performed with 0.5 pmol primer 1 for 1 μg genomic DMS-treated and piperidine-cleaved DNA. For primer extension Sequenase 1.0 (United States Bio-

chemical, Cleveland, OH) was used. Linker ligation was performed overnight at 15°C. Exponential PCR amplification was done with primer 2 and the linker primer for 15–22 cycles (94°C for 1 min, Tm+ 1°C for 2 min, 76°C for 3 min). Finally, the ^{32}P-labeled third primer (10^6 cpm) was added together with 2 units of Taq DNA polymerase and 2 µl of dNTPs (5 mM each) and a final PCR cycle was performed, followed by phenol/chloroform extraction of the samples, ethanol precipitation, and analysis on a 6% denaturing urea/polyacrylamide gel.

Primer sequences for LM-PCR were as follows:

Set A	1. 5′-TATCTTTTTCCTGCATCCTG-3′
	2. 5′-CTGGAAGTTAGAAGGAAACAGACC-3′
	3. 5′-GGAAACAGACCACAGACCTGGTCCCC-3′
Set B	1. 5′-GTCTGCAGTTGCTTCTCTCC-3′
	2. 5′-TCTTAGCTGGTCCTCTGCTGTCC-3′
	3. 5′-TGGTCCTCTGCTGTCCTTGCTGAGGG-3′
Linker top strand	5′-GCGGTGACCCGGGAGATCTGAATTC-3′
Linker bottom strand	5′-GAATTCAGATC-3′

The in vivo footprinting ladders were verified by comparison to sequencing ladders of cloned TNF promoter DNA. Therefore, the TNF promoter was amplified by PCR from Jurkat genomic DNA using two gene-specific primer sequences (upstream primer 5′-GAAAAGTCAGGGTCTGGAGGGGC-3′, downstream primer 5′-AGAACCTGCCTGGCAGCTTGT CAGG-3′) derived from previously published sequence data (37). The resulting 1.2-kb TNF promoter fragment ranging from −1173 to +25 relative to the TSS was cloned into the pCRII-vector (Invitrogen, Leek, Netherlands) by TA cloning.

Isolation of Nuclear Proteins From Primary Lymphocytes

Extraction of nuclear proteins was carried out by the method of Schreiber et al. (35). Protein concentrations were measured with a protein assay kit (BIO-RAD, Munich, Germany).

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides for EMSA were synthesized, annealed, gel purified, and end-labeled with [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Radiolabeled DNA probe (25,000 cpm) was added to the binding reaction that also contained 1 µg synthetic DNA duplex of poly(dI-dC) (Pharmacia, Piscataway, NJ), 3 µg nuclear proteins, and binding buffer [25 mM HEPES (pH 7.5), 150 mM KCl, 5 mM DTT, 10% glycerol]. For supershift assays 2 µg specific rabbit anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was used. For competition analysis an excess of unlabeled oligonucleotides containing consensus binding sites for transcription factors was added to the binding reaction. Complex formation was allowed to proceed for 30 min at room temperature. Finally, the complexes were separated from unbound DNA by native polyacrylamide gel electrophoresis on 5% gels. The gels were exposed to Kodak MS films on intensifying screens at −80°C.

The sequences of oligonucleotides for EMSA were as follows:

GC-box	5′-CCAAATCCCCGCCCCCGCGATGG-3′; and 5′-CCATCGCGGGGGCGGGGATTTGG-3′
CRE	5′-CCAGATGAGCTCATGGGTT-3′; and 5′-AACCCATGAGCTCATCTGG-3′
Egr-1	5′-GGATCCAGCGGGGGCGAGCGGGGGCGA-3′; and 5′-CCTAGGTCGCCCCCGCTCGCCCCCGCT-3′
NF-kB1	5′-GATCGAGGGGACTTTCCCTAGC-3′; and 5′-CTAGATCCCCTGAAAGGGATCG-3′
NF-kB2	5′-CTA GTG ATG AGT CAG CCG GAT C-3′; and 3′-GAT CAC TAC TCA GTC GGC CTA G-5′
CREB	5′-GATTGGCTGACGTCAGAGAGCT-3′; and 5′-AGCTCTCTGACGTCAGCCAATC-3′
AP-1	5′-CTAGTGATGAGTCAGCCGGATC-3′; and 5′-GATCCGGCTGACTCATCACTAG-3′
SP-1	5′-GATCGATCGGGGCGGGGCGATC-3′; and 5′-GATCGCCCCGCCCCGATCGATC-3′
NFAT	5′-CTGTATCAAACAAATTTTCCTCTTTGGGC-3′; and 5′-GCCCAAAGAGGAAAATTTGTTTGATACAG-3′

ELISA for TNF-α

To measure TNF protein production, 10^6 primary CD4+ T lymphocytes/well were seeded out in 1 ml culture medium in triplicate in 48-well tissue culture plates and incubated at 37°C in humidified 5% CO_2 atmosphere in the presence or absence of different stimuli as indicated above. After 48 h cell-free culture supernatants were removed and assayed for TNF concentration by ELISA (24).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed with the Quikchange™ Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Cat. No. 200518) according to the manufacturer's instructions. Primer sequences were as follows:

Egr-1 mut:	5′-TCC CCG CCC CAT CGA TGG AGA AGA AAC C-3′ and 5′-GGT TTC TTC TCC ATC GAT GGG GCG GGG A-3′
k3-mut:	5′-GGT GGA GAA ATA CAT GAG CTC ATC TGG-3′ and 5′-CCA GAT GAG CTC ATG TAT TTC TCC ACC-3′
k3-mut2:	5′-GCT CAT GGG TTT CTT AAC CAA GGA AGT-3′ and 5′-ACT TCC TTG GTT AAG AAA CCC ATG AGC-3′

CRE-mut: 5′-AAC CCA TGA GCT ATT CTG GAG GAA G-3′ and 5′-CTT CCT CCA GAA TAG CTC ATG GGT T-3′

DEAE Transfection of Primary CD4+ T Lymphocytes and Reporter Gene Analysis

The TNF promoter DNA was excised with *Eco*RI from the pCRII vector (see above), treated with Mung Bean nuclease (Amersham) to create blunt ends, and cloned into the *Sma*I site of the promoterless pXP1 luciferase reporter gene vector (25) yielding the pXP1-TNF-Luc construct. The pXP1-TNF-Luc vector (10 μg) was transfected into 10^7 primary CD4+ T cells using the DEAE transfection method. After 24 h the cells were stimulated as described above. The stimulation was allowed to proceed for 18 h before the cells were harvested, washed in PBS, and lysed in cell lysis buffer (Promega). Luciferase activity was measured as light emission over a period of 10 s after addition of luciferase assay buffer (Promega) with a luminometer (Lumat, Berthold). Luciferase activity was finally normalized to the β-galactosidase activity of the lysate.

Statistical Analysis

Data from transfection experiments were analyzed by the Wilcoxon test using the program Statworks for MacIntosh.

RESULTS

The TNF Promoter Is Inducible in Primary T Lymphocytes

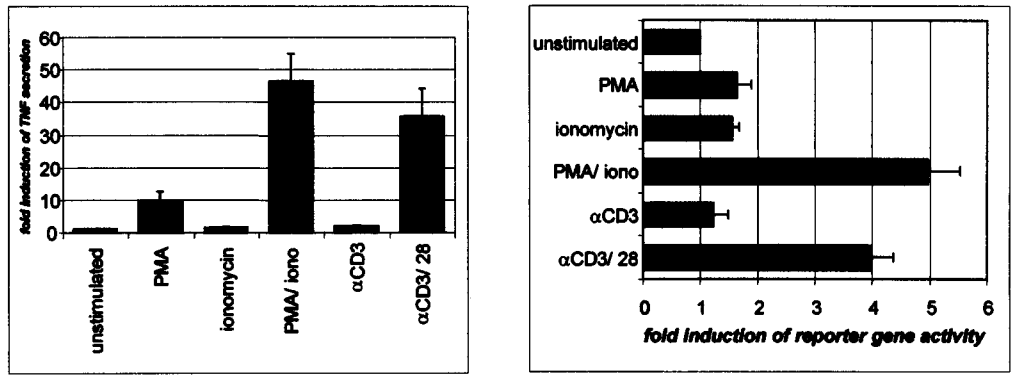
Previous studies have demonstrated that TNF is produced by human T cell lines upon a variety of exogenous stimuli. In order to verify the effects of chemical and cell-specific reagents on TNF induction in freshly isolated human T cells, we performed an initial series of studies using enzyme linked immunosorbant assays (ELISA) and transient transfection experiments. In primary CD4+ T cells, stimulation with PMA and ionomycin caused high production of TNF protein while neither PMA nor ionomycin alone was able to induce similar amounts of secreted protein (Fig. 1A). Although anti-CD3 antibodies have been reported to induce TNF promoter activity in transient transfection experiments of various T cell lines (40), such stimulation did not induce significant TNF protein production by primary CD4+ T cells under our culture conditions. However, costimulation of T cells with antibodies to the CD28 surface molecule induced high TNF secretion comparable to the PMA/ionomycin stimulation pathway.

To verify if induction of TNF protein in primary T lymphocytes by the above stimulation methods could be due to increased TNF promoter activity, we next performed transient transfection experiments in primary CD4+ T cells. Accordingly, we cloned the human TNF promoter upstream of a luciferase reporter gene and transfected the resulting construct, designated pXP1-TNF-Luc, in primary CD4+ T lymphocytes. Stimulation of T cells with PMA and ionomycin augmented reporter gene activity about fivefold compared to unstimulated T cells (Fig. 1B). A similar increase of reporter gene activity was found using antibodies to CD3 and CD28. In contrast, stimulation of primary T cells with PMA, ionomycin, or antibodies to CD3 alone failed to induce high reporter gene activity (Fig. 1B). These data suggest that high TNF protein production after stimulation of primary T lymphocytes with PMA and ionomycin is at least partially due to increased TNF promoter activity. Furthermore, maximum induction of TNF promoter activity in primary T lymphocytes requires costimulation via CD28 or synergistic activation of both the calcium- and protein kinase C-dependent pathways.

In Vivo Footprinting of the Human TNF Promoter in Primary T Lymphocytes Demonstrates Constitutive and Inducible Protein–DNA Interactions

Various studies on transcriptional regulation of the TNF promoter have used in vitro assays to determine potential binding sites for *trans*-acting regulatory factors. However, it is clear that gene regulation in vivo is complicated by many additional factors such as the nucleosomal context. Recently, methods have been described to analyze protein–DNA interactions in intact cells in vivo. We therefore explored in subsequent studies whether binding sites of the TNF promoter with reported regulatory function were occupied in primary lymphocytes in vivo by using LM-PCR.

Accordingly, we isolated in vivo methylated DNA from unstimulated and stimulated primary CD4+ T lymphocytes and subjected the DNA to the LM-PCR procedure using primer sets for both the coding and noncoding strands (see Materials and Methods). Altered DMS reactivities at various sites with previously described regulatory function were observed upon stimulation with αCD3/αCD28 and PMA/ionomycin. For instance, both stimulation conditions resulted in a complete protection of the canonical CRE sequence (5′-gagctc-3′) at position −105 and the adjacent k3 site at position −95 of the TNF promoter (Fig. 2A, B). Because previous studies employing site-di-



A B

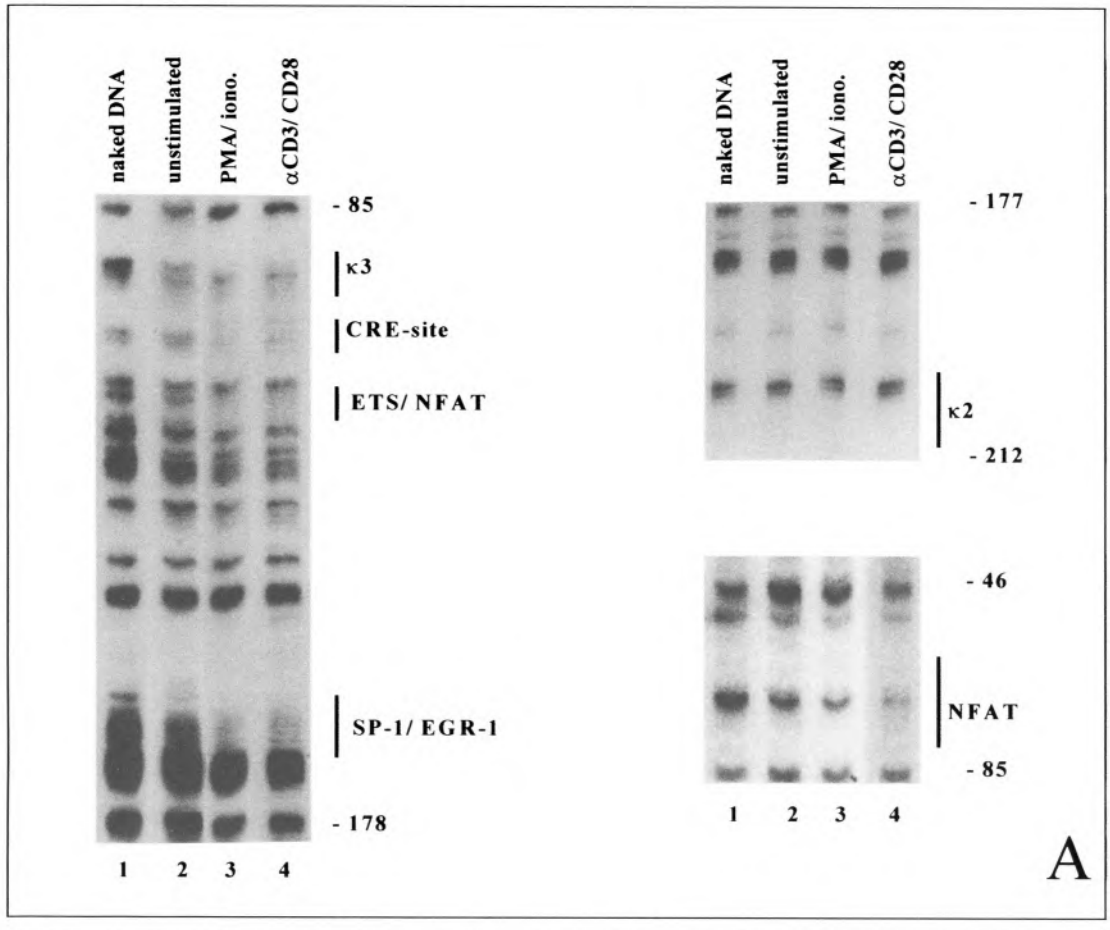
FIG. 1. TNF is inducible in freshly isolated human T lymphocytes. (A) TNF protein production by stimulated and unstimulated primary T lymphocytes. Primary T lymphocytes were stimulated with PMA, ionomycin (iono), or antibodies to CD3/CD28, as specified in Materials and Methods. Cell-free supernatant was collected after 48 h and cytokine concentration was determined by ELISA. Data were normalized to the values from unstimulated control samples and represent mean values ± SD of three independent experiments. (B) Activity of TNF promoter constructs in primary CD4+ T cells. Primary CD4+ T lymphocytes were transfected with the pXP1-TNF-Luc vector containing the human TNF promoter upstream of a luciferase reporter gene (see Materials and Methods). Cells were stimulated with indicated stimuli 24 h after transfection and luciferase activity was assessed after an additional 18 h. Data represent mean values ± SD of three independent experiments and are expressed as fold induction of each stimulation condition compared to the unstimulated control.

rected mutagenesis suggested a key role for these sites in inducible TNF gene expression (40), our data further support a pivotal role for the CRE and the k3 element in vivo. In addition, an inducible footprint over a GC-rich sequence at position −165 was found in stimulated T lymphocytes (Fig. 2) that has previously been demonstrated to bind SP1 and Egr-1 based on in vitro assays (19). Furthermore, other previously identified binding sites were found to be protected from methylation in vivo: in stimulated primary T cells the recognition sites for ETS-1/NFAT (at position −115 upstream of the transcriptional start site) and NFAT (−76) were found to be protected. In contrast, no footprints were observed at the k2 element (Fig. 2A), which has been reported to be a low-affinity binding site for NF-κB (11). Furthermore, the NFAT site at −160 and the AP1 (at −50) and SP1 (at −65) binding sites showed no altered DMS reactivity in primary T lymphocytes under our cell culture conditions (Fig. 2B). In summary, the in vivo footprinting data demonstrated that the TNF promoter in vivo in primary T lymphocytes is regulated at the level of constitutive and inducible protein–DNA interactions. A detailed summary of all observed protein–DNA interactions is shown in Fig. 3.

Constitutive and Inducible Protein Complexes Bind to the CRE/k3 Site and the GC-Box of the TNF Promoter in Primary T Lymphocytes

To further analyze inducible protein–DNA interactions in primary T cells, we next performed EMSA with oligonucleotides encompassing the in vivo protected binding sites. In an initial series of studies, we focused on the CRE site that has been reported to be a target site for CREB/AP-1 with essential importance for inducible and CsA-sensitive TNF promoter activity (40). An oligonucleotide encompassing the CRE site was labeled and incubated with nuclear extracts of unstimulated and stimulated primary T lymphocytes. Two strongly inducible complexes (see Fig. 4) using primary T cell extracts were found. Competition experiments with consensus binding sites for transcription factors revealed that these two complexes were related to CREB and AP-1 as competition with the respective binding sites abolished the appearance of these complexes (Fig. 4). Furthermore, addition of antibodies against CREB/AP-1 family members affected the appearance of both complexes (Fig. 4): while antibodies to ATF-2 and c-*fos*/c-*jun* abrogated the upper complex, antibodies both against c-*fos*/c-*jun* and *junB* reduced intensity of the lower complex. Taken together with the genomic footprinting data, these findings suggested that inducible CREB/AP-1 family members such as ATF-2/c-*jun* and c-*fos*/*junB* bind to the TNF promoter in primary T lymphocytes in vivo.

In further studies, we analyzed the capacity of an oligonucleotide encompassing the in vivo protected GC-rich region at −165 to bind to nuclear proteins. Using extracts from primary T lymphocytes two specific complexes were observed by EMSA analysis (Fig. 5). Both complexes were present in extracts of unstimulated cells and were induced upon stimulation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unstimulated
PMA/ iono.
αCD3/ CD28
- 85
κ3
CRE-site
ETS/ NFAT
SP-1/ EGR-1
- 178
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NFAT
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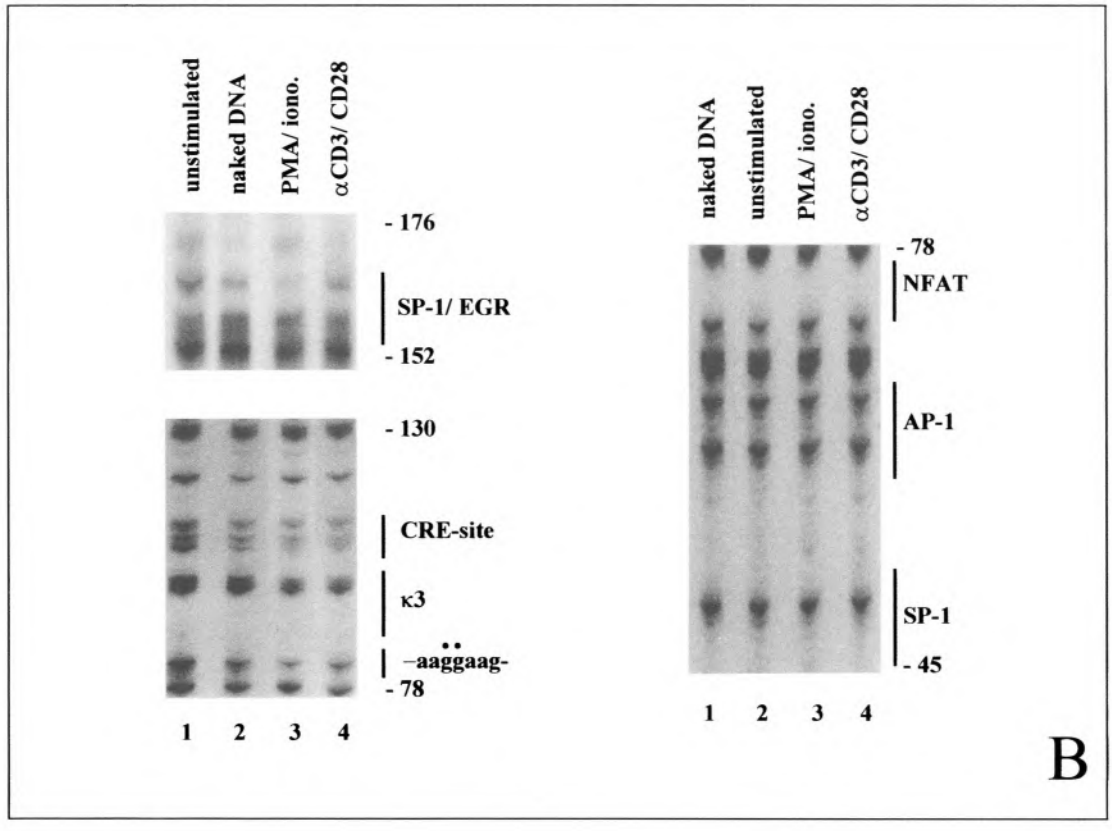
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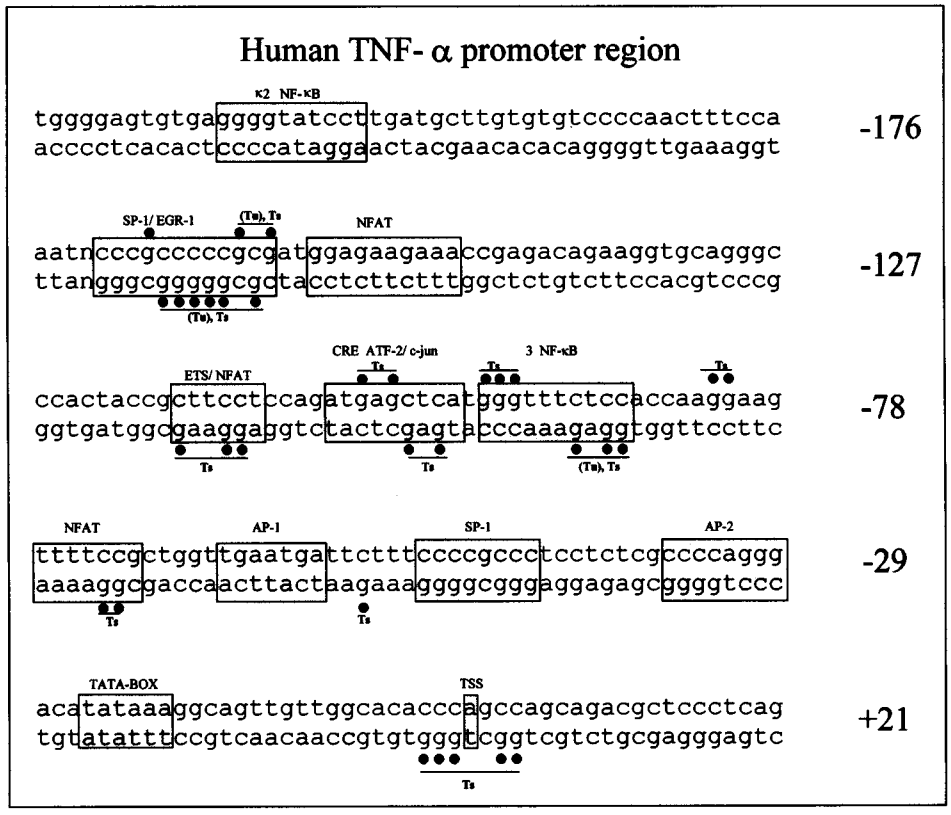

FIG. 3. Summary of in vivo protein–DNA interactions in the 5′ flanking region of the TNF gene in unstimulated (Tu) and PMA plus ionomycin-stimulated (Ts) CD4+ T lymphocytes. Circles indicate protected bases. The location of previously characterized binding sites for transcription factors in the TNF promoter region is indicated.

with PMA/ionomycin and anti-CD3/CD28. Competition and supershift experiments revealed that the lower complex contained SP-1, while the upper inducible complex contained Egr-1. Because the induction of the Egr-1-containing complex in EMSA correlated with the presence of the in vivo protection at the GC-box in the LM-PCR analysis (Fig. 2A), these data suggested that Egr-1 binds to the GC-element of the TNF promoter in vivo in primary T lymphocytes.

Finally, we focused on the k3 site in primary T lymphocytes. Using primary cell extracts we found two specific complexes that bind to this site (Fig. 6). Both complexes were inducible upon stimulation with PMA/ionomycin or anti-CD3/CD28 and could be cross-competed with two different NF-κB consensus binding sites. Addition of specific antibodies to p50 led to reduced intensity of both complexes, whereas addition of antibodies to p65 caused a reduced intensity of the lower complex only. Taken together, these results suggested that NF-κB family members such as p50 and p65 bind to the k3 site of the TNF promoter in vivo in primary T lymphocytes.

Site-Directed Mutagenesis Reveals Importance of the CRE and k3 Sites for Inducible TNF Promoter Activation in Primary T Lymphocytes

To determine the functional importance of the CRE, Egr-1, and k3 sites for inducible TNF promoter activity, we performed transient transfection assays in primary CD4+ T cells. Accordingly, we constructed mutagenized reporter gene plasmids containing mutations of these sites of the TNF promoter that abrogated the capacity of these sites to compete specific

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FIG. 2. In vivo protein–DNA interactions at the TNF promoter in primary T lymphocytes. CD4+ T lymphocytes were stimulated for 4 h prior to DMS treatment. After isolation of methylated DNA the DNA was subjected to the LM-PCR method and analyzed by denaturing polyacrylamide electrophoresis. Footprinting ladders on the coding (A) and noncoding (B) strands were compared to DMS reactivity of naked DNA that was treated with DMS in vitro. The location of previously described binding sites of transcription factors is indicated.

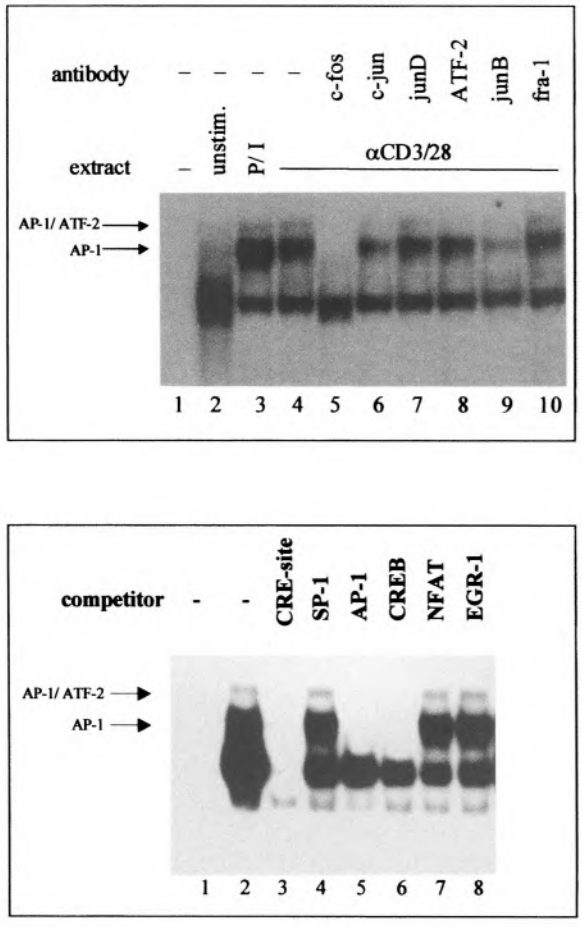

FIG. 4. Upper panel: Activation-induced changes of nuclear proteins binding to the CRE site of the human TNF promoter in primary CD4+ T lymphocytes. Nuclear proteins were extracted from primary cells 1 h after activation with indicated stimuli. Two inducible specific complexes were seen using extracts from primary T lymphocytes. For supershift assays antibodies were added to the binding reaction as indicated. Lower panel: Competition assays at the CRE site using nuclear extracts from PMA plus ionomycin-stimulated CD4+ T lymphocytes. Competitor DNA was added to the binding reaction as indicated.

complexes obtained with the wild-type sites. As shown in Fig. 7, mutation of the Egr-1 site caused no significant inhibition of inducible TNF promoter activity in primary T cells. In contrast, transfection of CRE and k3 mutant TNF promoter constructs in primary T cells significantly ($p < 0.01$) reduced reporter gene activity in PMA plus ionomycin-activated cells compared to the wild-type construct. Whereas each mutation in the k3 site led to an about 50% reduction of reporter gene activity, mutation of the CRE site caused an almost 70% loss of inducible promoter activity in primary T lymphocytes. Finally, mutations of both the CRE and k3 sites led to further reduction of reporter gene activity in primary CD4+ T lymphocytes (Fig. 7).

DISCUSSION

Previous studies on the transcriptional regulation of TNF gene expression have identified various regulatory target sites for transcription factors in the TNF promoter including for ATF-2/c-*jun*, AP-1, SP-1, ETS-1, NFATp, and NF-κB (11,19,40). However, only limited information on their contribution to TNF promoter regulation in vivo in primary lymphocytes has been available. In the present study, we analyzed in vivo and in vitro protein–DNA interactions at the human TNF promoter in primary T lymphocytes. We provide evidence that the TNF promoter is regulated in primary lymphocytes at the level of constitutive and inducible protein–DNA interactions. We propose a model where transcription of the TNF gene in primary T lymphocytes is regulated by the coordinate binding of a set of inducible transcription factors to their respective target sites in vivo (Fig. 8). These data provide new insights into the complex regulation of TNF gene transcription in primary T lymphocytes in vivo.

The human TNF promoter was initially characterized by transfection of reporter gene constructs into T, B, and myeloid cell lines representative of different developmental stages and physiological activation states (11,29). Such studies demonstrated that the TNF promoter in lymphocytes can be strongly activated upon a variety of exogenous stimuli. Recent studies of the TNF promoter by several groups have provided additional insights into the functional complexity of this regulatory element (7,20,40). These data suggested that the promoter contains independent positive and negative regulatory elements whose coordinate interactions permit regulated TNF gene expression. However, insights into the protein–DNA interactions in vivo of the TNF promoter in primary cells have been limited. In one approach towards this goal, we used in vivo genomic footprinting techniques in intact T lymphocytes to determine the occupancy of various sequence elements in the TNF promoter in vivo.

Our in vivo footprinting data indicated the presence of various altered DMS reactivities between −120 and −70 bp upstream of the transcriptional start site of the TNF promoter in primary T lymphocytes. This area includes a potential ETS-1 site, the CRE element, and the k3 site, as well as a NFAT site (at −76). Kinetic studies showed that protections at these sites appeared simultaneously, suggesting a mechanism where the binding of one factor to its site favors protein binding at adjacent sites. A similar mechanism of protein–DNA interaction in vivo was suggested recently for the interleukin-2 and IFN-γ promoters in T lymphocytes (2,8). In contrast to the

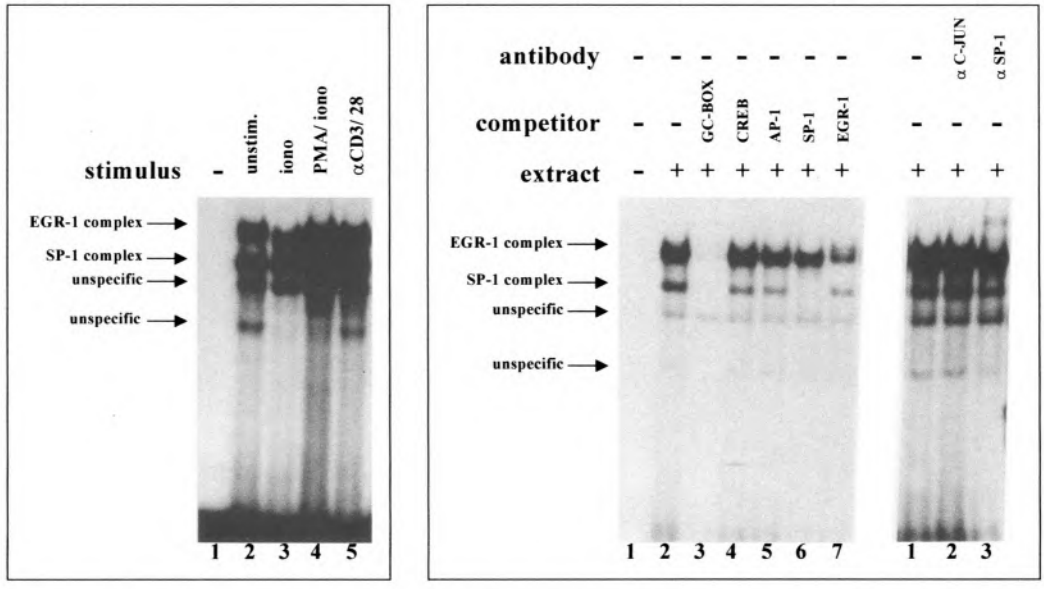

FIG. 5. Left panel: Activation-induced changes of nuclear proteins binding to the GC-box of the human TNF promoter in primary CD4+ T lymphocytes. Two specific complexes were observed in the gel retardation studies as indicated. Right panel: Competition and supershift analyses at the GC-box using nuclear extracts from PMA plus ionomycin-stimulated CD4+ T lymphocytes. Antibodies (2 μg) or competitor DNA were added to the binding reaction as indicated. A supershift of the lower complex was observed after addition of antibodies to SP-1.

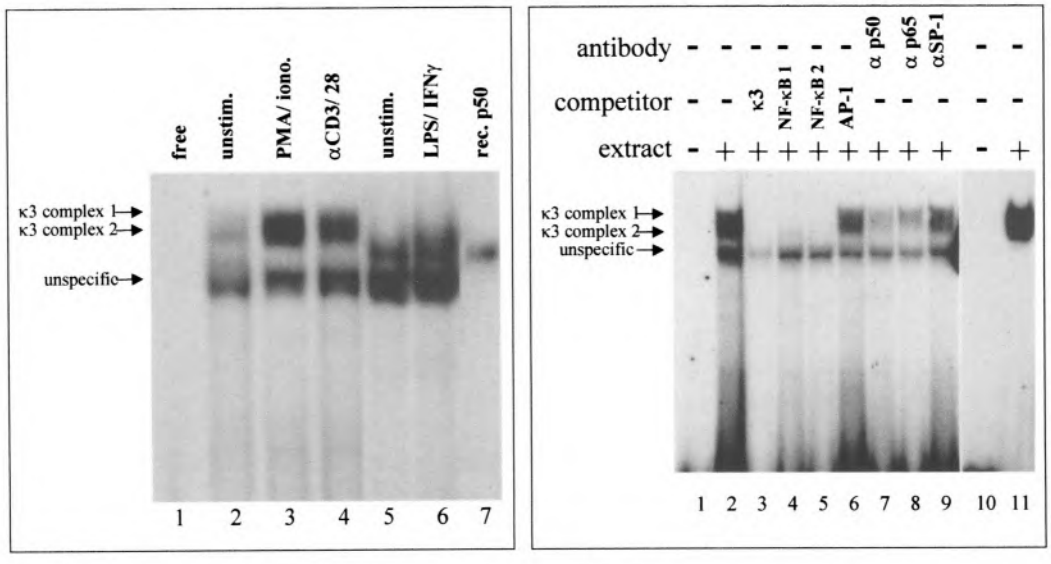

FIG. 6. Left panel: Binding analysis at the k3 site using nuclear extracts from unstimulated and stimulated T lymphocytes (lanes 1–4) and CD14+ monocytes (lanes 5–6). An inducible specific double complex (upper complex: 1; lower complex: 2) was found upon stimulation with PMA/ionomycin or anti-CD3/CD28 in T cells. Two complexes at the k3 site were found in monocytes: an inducible complex (lane 6) was observed that comigrated with complex 2 in T cells. Furthermore, a slower migrating constitutive complex was seen in CD14+ monocytes that comigrated with recombinant p50. Right panel: Competition and supershift assays at the k3 site using nuclear extracts from stimulated T lymphocytes. The specific complexes could be cross-competed with two different NF-κB consensus oligonucleotides and were reduced in intensity after addition of antibodies to the p50 subunit of NF-κB. Antibodies to p65 caused a reduced intensity of the lower complex, suggesting the presence of p50/p65 in complex 2.

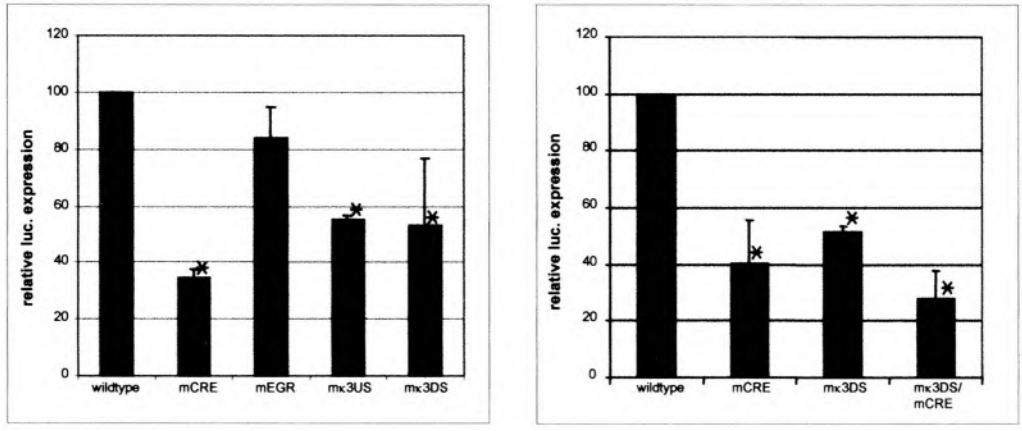

FIG. 7. Transfection analysis of TNF promoter constructs carrying single or double mutations of the CRE/Egr-1 sites and the proximal or distal parts of the k3 site, respectively. Wild-type and mutagenized luciferase reporter gene constructs were transfected into PMA plus ionomycin-stimulated primary human CD4+ T cells. Data represent mean values ± SD of four (left panel) and three (right panel) independent experiments after normalization for transfection efficiency. Luciferase activity is reported as percentage compared to T cells transfected with the wild-type construct (100%).

above sites, no footprints were observed in primary T cells at various binding sites with reported regulatory function in tumor cell lines. These sites included the k2 element, the NFAT site at −160, the AP1 site at −50, and the SP1 site at −65. This finding indicates a lack of protein–DNA interactions at these sites of the TNF promoter under our culture conditions and suggests that protein–DNA interactions at the TNF promoter may strikingly differ between tumor cell lines and primary CD4+ T lymphocytes.

In further studies, gel retardation analysis of the CRE and k3 sites was performed to examine whether these in vivo protected sequences were target sites for rapidly inducible or cell type-specific regulatory proteins in primary T lymphocytes. We found that stimulation of primary T lymphocytes with PMA/ionomycin strongly induced binding of specific ATF-2/c-*jun*- and c-*fos*/*junB*-related protein complexes at the CRE site. Furthermore, mutagenesis of the CRE site strongly reduced inducible TNF promoter activ-

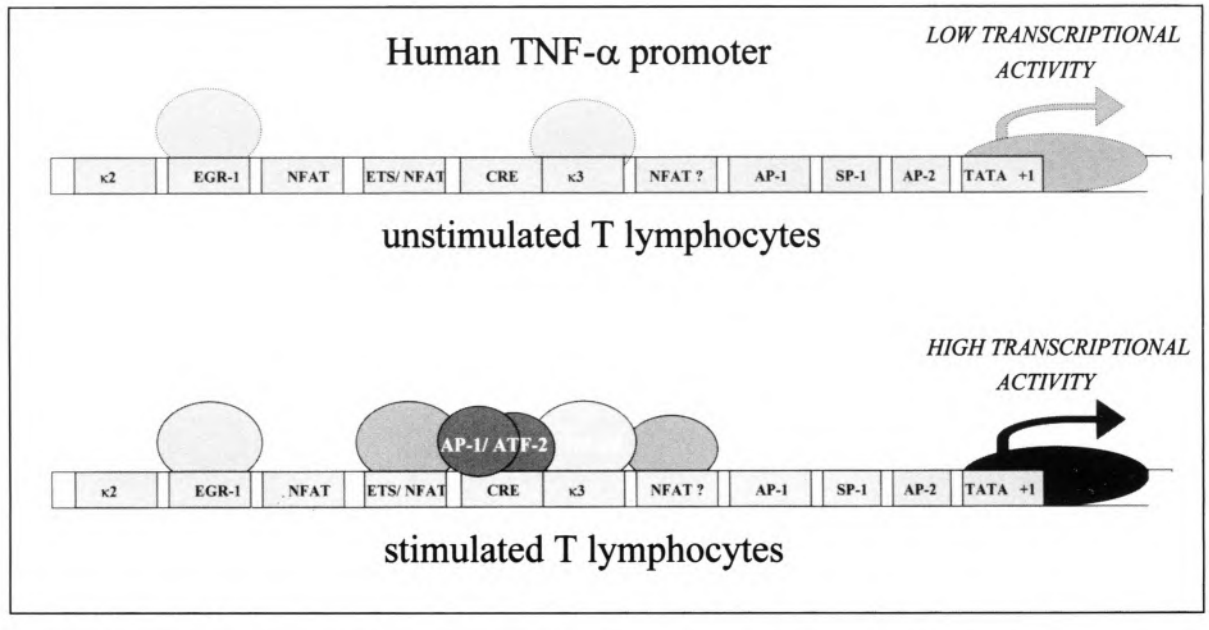

FIG. 8. Schematic model of protein–DNA interactions at the TNF promoter in vivo in unstimulated and PMA plus ionomycin-stimulated primary human CD4+ T lymphocytes. Dotted lines indicate weak protein–DNA interactions in vivo.

ity in primary T lymphocytes. This finding is consistent with previous studies demonstrating that a c-*jun*- and ATF-2-containing complex binds to this site in the Ar-5 T and A-20 B cell lines that regulates inducible and CsA-sensitive TNF promoter activity in lymphocytes (40). Taken together with these data, our results thus suggest that ATF-2/AP-1 complexes are key regulators of TNF gene transcription in primary T lymphocytes in vivo.

In vivo DMS reactivities at the adjacent downstream k3 site were also altered in T lymphocytes, suggesting protein binding to this element. Further studies indicated that NF-κB p50/p65 proteins can bind to this site in activated T lymphocytes. In contrast to these observations in primary T cells, Goldfeld et al. (11) showed that two molecules of NFAT can bind to the k3 site in the Ar5 T cell line. Mutation of this site led to a 50% reduction of inducible promoter activity in primary CD4+ T lymphocytes. These findings suggest that binding of NF-κB p50/p65 complexes to the k3 site regulates inducible TNF promoter activity in primary T cells under our culture conditions. Taken together with the genomic footprinting data, the data presented here thus support a model where binding of NF-κB proteins strongly contributes to inducible TNF promoter activation in primary T lymphocytes in vivo. Because a double CRE/k3 mutant construct yielded reduced reporter gene activity compared to the CRE and k3 single mutant plasmids, these data may also indicate functional cooperativity between the CRE and k3 sites in primary lymphocytes in vivo.

Furthermore, we found an extensive protection in vivo in activated primary lymphocytes that mapped to a GC-box and further studies showed that SP-1 and Egr-1 can bind to this site in primary T lymphocytes. However, site-directed mutagenesis of this site did not cause a significant reduction of inducible TNF promoter activity in primary T lymphocytes. Furthermore, double mutations of the Egr-1/SP-1 site and the k3/CRE site revealed no synergistic effects between these sites on inducible TNF promoter activity in primary T cells (Becker et al., unpublished data). In support of this finding, Kramer et al. (19) showed that mutation of the Egr-1 site does not alter TNF promoter activity in transient transfection assays in T cell lines. Thus, although the Egr-1/Sp-1 site is protected in vivo, it appears that this site plays only a minor, dispensable role for the control of TNF promoter activity in primary T cells. Alternatively, one may speculate that this site plays an important role for TNF promoter activation in the nucleosomal context that is not detectable by transfection studies using cloned promoter fragments.

In summary, the data presented here suggest that TNF promoter activity in primary T lymphocytes in vivo is regulated by the coordinate binding of a set of transcription factors including c-*jun*/ATF-2, c-*fos*/*junB*, NFAT, and NF-κB (Fig. 6). These results provide novel insights into the complex regulation of TNF gene transcription in primary lymphocytes in vivo by constitutive and inducible protein–DNA interactions that appear to be at least partially different compared to previously characterized tumor cell lines.

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