

# Cell-Type-Specific Regulation of the Human Tumor Necrosis Factor Alpha Gene in B Cells and T Cells by NFATp and ATF-2/JUN

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**The human tumor necrosis factor alpha (TNF- $\alpha$ ) gene is one of the earliest genes transcribed after the stimulation of a B cell through its antigen receptor or via the CD40 pathway. In both cases, induction of TNF- $\alpha$  gene transcription can be blocked by the immunosuppressants cyclosporin A and FK506, which suggested a role for the NFAT family of proteins in the regulation of the gene in B cells. Furthermore, in T cells, two molecules of NFATp bind to the TNF- $\alpha$  promoter element  $\kappa$ 3 in association with ATF-2 and Jun proteins bound to an immediately adjacent cyclic AMP response element (CRE) site. Here, using the murine B-cell lymphoma cell line A20, we show that the TNF- $\alpha$  gene is regulated in a cell-type-specific manner. In A20 B cells, the TNF- $\alpha$  gene is not regulated by NFATp bound to the  $\kappa$ 3 element. Instead, ATF-2 and Jun proteins bind to the composite  $\kappa$ 3/CRE site and NFATp binds to a newly identified second NFAT site centered at -76 nucleotides relative to the TNF- $\alpha$  transcription start site. This new site plays a critical role in the calcium-mediated, cyclosporin A-sensitive induction of TNF- $\alpha$  in both A20 B cells and Ar-5 T cells. Consistent with these results, quantitative DNase footprinting of the TNF- $\alpha$  promoter using increasing amounts of recombinant NFATp demonstrated that the -76 site binds to NFATp with a higher affinity than the  $\kappa$ 3 site. Two other previously unrecognized NFATp-binding sites in the proximal TNF- $\alpha$  promoter were also identified by this analysis. Thus, through the differential use of the same promoter element, the composite  $\kappa$ 3/CRE site, the TNF- $\alpha$  gene is regulated in a cell-type-specific manner in response to the same extracellular signal.**

The human tumor necrosis factor alpha (TNF- $\alpha$ ) gene is expressed in multiple cell types and can be induced by a variety of stimuli in a highly specific and temporal manner (reviewed in reference 5). Studies of TNF- $\alpha$  gene regulation have served as a model system for the analysis of mechanisms involved in complex patterns of cell-type- and inducer-type-specific gene transcription (4, 6–8, 14, 29). Several *cis*-acting elements and protein factors involved in the gene's expression have previously been identified. The  $\kappa$ 3 site, for example, located at -97 to -88 nucleotides (nt) relative to the TNF- $\alpha$  gene transcription start site (6), binds two molecules of NFATp (8), and ATF-2 and Jun proteins bind to a cyclic AMP response element (CRE) immediately upstream of  $\kappa$ 3 (8, 29). Consensus sequences for AP-1, AP-2, and SP-1 sites have also been identified (4, 8, 29).

Previous studies of TNF- $\alpha$  gene regulation in the murine T-cell clone Ar-5 stimulated through the T-cell receptor or by calcium ionophore demonstrated that the  $\kappa$ 3 site is both necessary and sufficient to confer cyclosporin A (CsA)-sensitive inducibility on a minimal TNF- $\alpha$  promoter (8). Furthermore, ATF-2–Jun heterodimers bind to the adjacent CRE site and functionally cooperate with NFATp to mediate the CsA-sensitive induction of the gene in activated T cells (29).

TNF- $\alpha$  plays a critical role in B-cell biology and functions as an autocrine growth factor for highly purified human splenic B cells activated via surface immunoglobulin (Ig) or CD40 (1). The NFAT family of proteins have also been implicated in the

regulation of TNF- $\alpha$  in B cells (1, 7, 10). First, the rapid induction of TNF- $\alpha$  gene expression in highly purified human splenic B cells induced by antibodies to the immunoglobulin receptor (anti-Ig) or to CD40 is blocked by pretreatment of the cells with CsA and FK506 and does not require de novo protein synthesis (1, 7). Second, calcineurin phosphatase activity is required for TNF- $\alpha$  induction by anti-Ig, since a panel of calcineurin inhibitors blocks transcription of the gene in human B cells (10). Finally, a panel of calcineurin inhibitors significantly blocked TNF- $\alpha$ -mediated B-cell proliferation induced by either anti-Ig or anti-CD40 (1).

NFATp is a member of a growing family of transcription factors, which includes NFATc, NFAT3, and NFAT4 (11, 16, 19, 22). Outside of their DNA-binding domains, which are distantly related to Rel domains of NF- $\kappa$ B proteins, the NFAT proteins show minimal sequence similarity and have different patterns of tissue expression (11). Originally identified in T cells (hence the name NFAT [nuclear factor of activated T cells]), NFAT proteins typically cooperatively bind with Fos and Jun family members to pyrimidine-rich binding motifs in several cytokine promoters (19, 22, 25, 26; reviewed in references 13 and 23). Other studies have detected the presence of NFAT family members in extracts prepared from a variety of murine and human B-cell lines (30–32). In one study, multimers of the NFAT-binding site from the murine interleukin-2 (IL-2) gene cloned upstream of a minimal *c-fos* gene promoter were shown to be induced by anti-Ig in murine Bal-17 B cells (31). However, the role of NFATp in the regulation of a naturally expressed gene in B cells has not yet been demonstrated.

In this study, we have examined the role of NFATp in the transcriptional activation of the TNF- $\alpha$  gene in B cells. Con-

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trary to our expectations, we found that a novel NFAT-binding site centered at  $-76$  nt relative to the TNF- $\alpha$  mRNA cap site is critical for transcriptional activation of the gene. Thus, in contrast to our findings for Ar-5 T cells, in A20 B cells, the TNF- $\alpha$  NFATp-binding site  $\kappa 3$  is not sufficient or required for induction of the TNF- $\alpha$  gene. Quantitative DNase I footprinting analysis revealed that the newly identified site is a higher-affinity NFATp-binding site than is  $\kappa 3$  and, in contrast to  $\kappa 3$ , plays an important functional role in the CsA-sensitive induction of the gene in A20 B cells.

Furthermore, this analysis revealed a total of three previously unrecognized NFAT-binding sites in addition to  $\kappa 3$ . Similar to our findings in Ar-5 T cells, the CRE immediately upstream of  $\kappa 3$  associates with ATF-2 and Jun proteins and is required for induction of the gene in A20 B cells. Thus, cell-type-specific regulation of the TNF- $\alpha$  gene is achieved through the differential interaction of NFATp with the composite  $\kappa 3$ /CRE site in A20 B cells and Ar-5 T cells.

#### MATERIALS AND METHODS

**Cell culture, activation, and transfection.** The murine lymphoma line A20 was grown in Dulbecco modified Eagle medium supplemented with 12% fetal bovine serum, 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol, 60 U of penicillin per ml, and 60  $\mu$ g of streptomycin per ml. For nuclear extract preparation and RNA analysis, cells were stimulated for 30 min. Where indicated, cells were treated with 1  $\mu$ M cyclosporin A (CsA) (Sandoz) for 10 min before the addition of phorbol myristate acetate (PMA; 200 nM) and/or ionomycin (1  $\mu$ M). Transfections were performed by using DEAE-dextran as previously described (8). Twenty-four hours after transfection, cells were activated with PMA (200 nM) plus ionomycin (1  $\mu$ M) (P+I) and harvested approximately 18 h later. Where indicated, cells were treated for 10 min with 1  $\mu$ M CsA before the addition of PMA or ionomycin. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (14). Quantification of the conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms was obtained by using a Betagen (Waltham, Mass.) Betascope.

**RNA analysis.** RNA was prepared from A20 cells as previously described (6).  $^{32}$ P-labeled RNA probes were prepared as previously described from SP6  $\gamma$ -actin and a murine TNF- $\alpha$  probe, and RNase protection assays were performed as previously described (6).

**Plasmids.** The  $-200$  TNF- $\alpha$  CAT, 5'M, MM, 3'M, 3'FL, SP-1, AP-1, AP-2, C1, C2, C3, and ( $\kappa 3$ ) $^6$ -61 TNF- $\alpha$ /CAT constructs have all previously been described (29). To construct the TNF- $\alpha$  promoter mutants extending to the TATA box, site-directed mutagenesis was performed as previously described (31), using a Mutagen M13 in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). Dideoxy sequencing was used to confirm all of the mutations.

**Preparation of Nuclear Extracts and EMSAs.** Nuclear extracts were prepared as previously described (18), and electrophoretic mobility shift assays (EMSA) were performed with approximately 5  $\mu$ g of protein in a total volume of 15  $\mu$ l at room temperature. Unbound oligonucleotide and protein-oligonucleotide complexes were separated by electrophoresis at 4°C on Tris-borate-EDTA-4% acrylamide gels as described earlier (8).

Antibody competition assays were performed as previously described (31), using a rabbit polyclonal anti-ATF-2 antibody (a gift from Tom Maniatis [3]) or a rabbit polyclonal anti-NFATp antibody specific for NFATp (anti-67.1; a gift from Anjana Rao [23]). Antibodies to Jun family proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

The synthetic oligonucleotides used in the gel shift assays were  $\kappa 3(S)$  (5'-GATCCGAGCTCATGGGTTTCTCCACA-3'),  $\kappa 3(L)$  (5'-GATCCTTCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAA-3'), C3 (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), C2 (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), C1 (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), C1 (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), 5' (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), MM (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), 3' (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), 3'FL (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), -117 (5'-GATCACCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAA-3'), -113 (5'-GATCCTTCTCCGTCAGAGCTCATGGGTTTCTCCACCAAGGAA-3'), and NFAT (5'-GATCCTGTATGAACAAATTTCTCTTTGGGC-3').

**Assembly of the transcription enhancer complexes and DNase I footprinting analysis.** Bacterially expressed and purified ATF-2/Jun, p50, p65, and NFATp were prepared as described previously (12, 28). For DNase I footprinting experiments, TNF- $\alpha$  wild-type (WT) or mutated promoter fragments as indicated (20,000 cpm) spanning the sequences from  $-200$  to  $+87$  were end labeled at the noncoding strand and incubated with the indicated proteins in 20  $\mu$ l of DB buffer

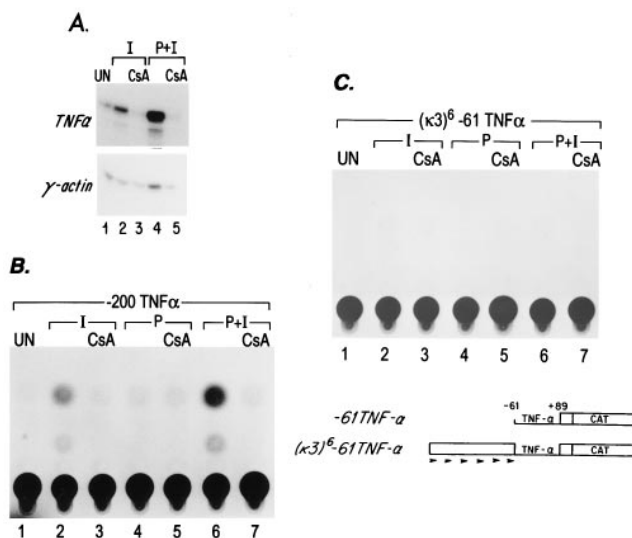


FIG. 1. Induction of TNF- $\alpha$  mRNA in A20 B cells. (A) Autoradiogram of an RNase protection assay mapping TNF- $\alpha$  and  $\gamma$ -actin mRNAs. A20 cells were stimulated with ionomycin (I) or P+I for 30 min in the presence or absence of CsA as described in Materials and Methods. The  $\gamma$ -actin probe was made to have a specific activity one-fifth of the specific activity of the mouse TNF- $\alpha$  probe. (B) Autoradiogram of a CAT assay of extracts prepared from A20 cells transfected with the  $-200$  TNF- $\alpha$  CAT reporter gene. (C) Autoradiogram of a CAT assay of extracts prepared from A20 cells transfected with a construct containing six copies of the  $\kappa 3$  site fused to the  $-61$  TNF- $\alpha$  CAT reporter gene. In panels B and C, 24 h after transfection, the cells were mock induced or induced with ionomycin (I), PMA (P), or P+I in the presence or absence of CsA as described in Materials and Methods.

and digested with DNase I as previously described (28). The DNA was ethanol precipitated, dried, resuspended in 3  $\mu$ l of formamide dye, and loaded onto a 6% sequencing gel as previously described (12).

#### RESULTS

**The TNF- $\alpha$  gene is inducible and CsA sensitive in the murine lymphoma B-cell line A20.** Given that PMA and ionomycin together are capable of triggering the activation of the B lymphocyte cell cycle (15), we stimulated A20 cells with ionomycin or with P+I to recapitulate B-cell activation. Stimulation was performed in the presence or absence of CsA, and the levels of TNF- $\alpha$  mRNA were determined by quantitative RNase protection analysis. A20 cells constitutively express a low level of TNF- $\alpha$  mRNA (Fig. 1A, lane 1) and express higher TNF- $\alpha$  mRNA levels within 30 min of stimulation with ionomycin (lane 2). Thus, in B cells, as in T cells, the TNF- $\alpha$  gene can be induced by calcium flux alone and does not require protein kinase C stimulation. However, the addition of PMA, a known activator of protein kinase C, superinduced TNF- $\alpha$  mRNA levels (lane 4), and induction by ionomycin (lane 3) or by P+I (lane 5) was blocked by pretreatment of the cells with CsA for 10 min prior to stimulation.

To identify the TNF- $\alpha$  promoter sequences required for the transcriptional activation of the TNF- $\alpha$  gene during CsA-sensitive B-cell stimulation, we transfected A20 cells with a TNF- $\alpha$  CAT reporter construct containing 200 nt 5' of the TNF- $\alpha$  mRNA cap site ( $-200$  TNF- $\alpha$  CAT). Increased CAT activity was detected when the cells were stimulated with ionomycin and with P+I (Fig. 1B, lanes 2 and 6, respectively), and this induction was blocked when the cells were pretreated with CsA (lanes 3 and 7). Thus, the pattern of expression of the transfected human  $-200$  TNF- $\alpha$  promoter CAT construct parallels the expression of the endogenous murine A20 TNF- $\alpha$  gene

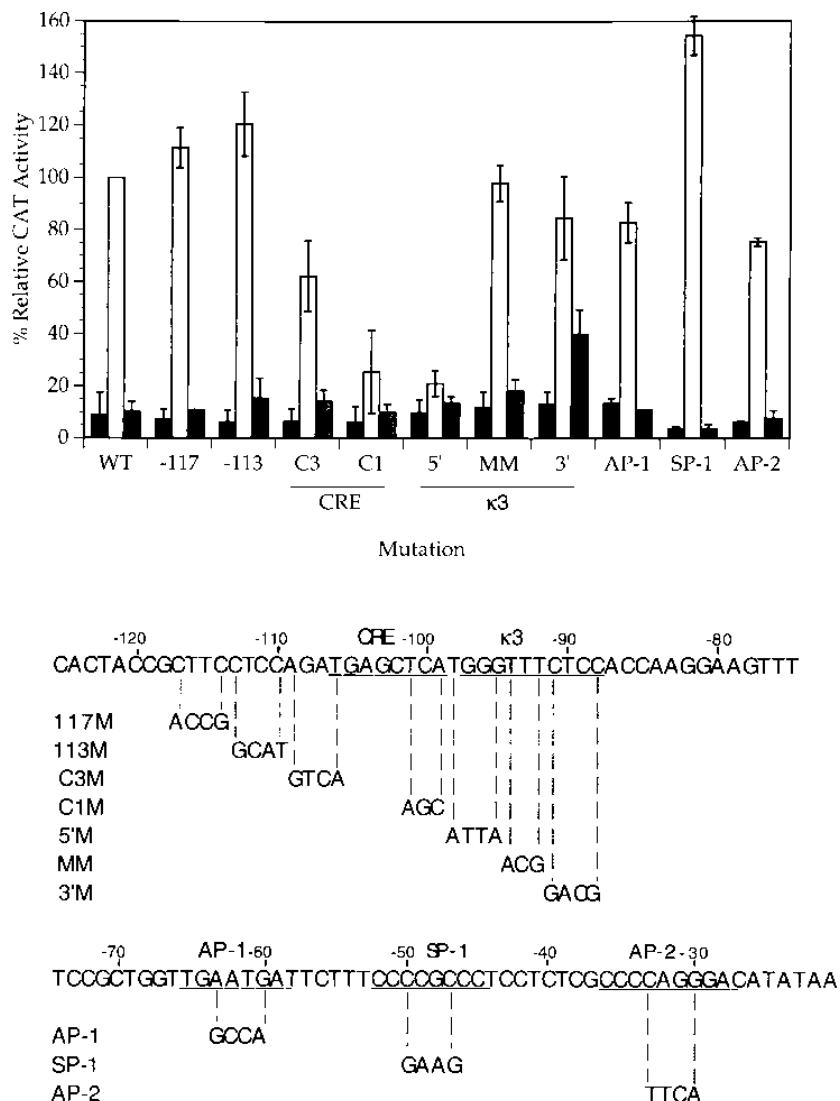


FIG. 2. Relative activities of human TNF- $\alpha$  CAT fusion constructs containing mutations in the CRE,  $\kappa$ 3, AP-1, AP-2, and SP-1 sites. A20 cells were transfected with the WT ( $-200$  TNF- $\alpha$  promoter) CAT and mutated TNF- $\alpha$  promoter CAT plasmids displayed at the bottom. Twenty-four hours later, the cells were mock-stimulated (black bars) or induced with P+I in the presence (gray bars) or absence (white bars) of CsA. CAT assays were performed, and the percent conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms was quantified with a Betagen Betascope. Result for each experiment were normalized to the  $-200$  WT-induced level (100%) and then averaged and plotted. Standard deviations are represented by the error bars. The results of three independent experiments are shown. The patterns of expression of the various constructs were consistent between experiments. The average values for fold induction for the displayed constructs are as follows: WT, 11.23;  $-117$ , 15.7;  $-113$ , 19.4; C3, 9.7; C1, 4.0; 5', 2.1; MM, 8.3; 3', 6.5; AP-1, 6.2; SP-1, 40.6; and AP-2, 11.9. The CRE,  $\kappa$ 3, AP-1, AP-2, and SP-1 sites are underlined.

(Fig. 1B). Similar results were obtained when the  $-200$  TNF- $\alpha$  CAT construct was transfected into the human Burkitt's lymphoma B-cell line Raji (data not shown).

The human TNF- $\alpha$  gene promoter contains three NF- $\kappa$ B consensus sequences, called  $\kappa$ 1 ( $-587$  to  $-576$  nt),  $\kappa$ 2 ( $-210$  to  $-202$  nt), and  $\kappa$ 3 ( $-98$  to  $-87$  nt) (8). Consistent with studies in other cell types (6, 8, 9) and with other inducers, deletion of  $\kappa$ 1 and  $\kappa$ 2 did not affect inducibility of the gene, and 200 nt upstream of the TNF- $\alpha$  transcription start site are sufficient for maximal inducibility of the gene in A20 cells (Fig. 1B and data not shown).

The  $\kappa$ 3 site, but not the  $\kappa$ 1 or  $\kappa$ 2 site, is included in the  $-200$  TNF- $\alpha$  CAT reporter construct. The  $\kappa$ 3 site, in contrast to the  $\kappa$ 1 and  $\kappa$ 2 sites, has been implicated in the regulation of the gene in a variety of cell types (6, 8, 29). In the T-cell clone Ar-5, the  $\kappa$ 3 site binds two molecules of NFATp and an immediately

adjacent CRE binds ATF-2 and Jun proteins and mediates the CsA-sensitive inducible regulation of the TNF- $\alpha$  gene (8, 29). We were therefore surprised to find that the same construct, ( $\kappa$ 3) $^6$ -61 TNF- $\alpha$ , which carries six copies of the  $\kappa$ 3 site fused to a truncated  $-61$  TNF- $\alpha$  promoter and is highly inducible in Ar-5 T cells, was not inducible in A20 B cells (Fig. 1C) although the intact TNF- $\alpha$  promoter was inducible (Fig. 1B). Thus, we were interested in the role that the  $\kappa$ 3 site played within the intact TNF- $\alpha$  promoter in the CsA-sensitive regulation of the gene in A20 B cells.

**The  $\kappa$ 3 and CRE sites are required for inducible TNF- $\alpha$  gene expression in B cells.** To characterize the role of the  $\kappa$ 3 and abutting CRE sites in the inducible regulation of the TNF- $\alpha$  gene in A20 cells, we tested the effects of site-directed mutations introduced into these sites on TNF- $\alpha$  promoter activity in A20 cells. A mutation introduced into the 5' aspect of

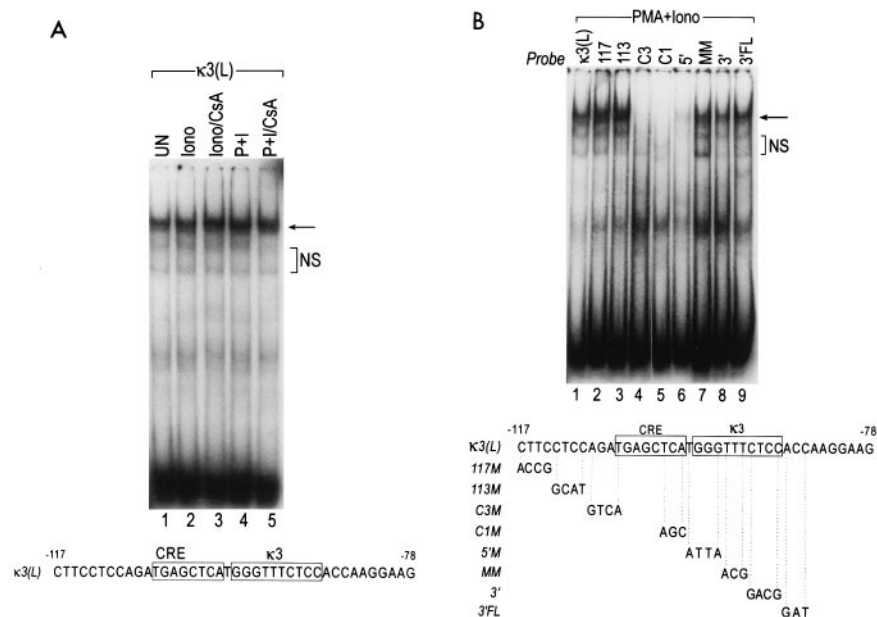


FIG. 3. Binding of nuclear factors to the  $\kappa 3(L)$  probe. (A) Nuclear extracts were prepared from unstimulated (UN) cells or cells stimulated with ionomycin (Iono) or P+I for 30 min in the presence or absence of CsA as described in Materials and Methods. The  $\kappa 3(L)$  probe contains -117 to -78 nt relative to the mRNA cap site and is diagrammed at the bottom of panel B. Nonspecific (NS) binding complexes are bracketed. (B) Binding of factors to WT and mutated  $\kappa 3(L)$  oligonucleotides. Nuclear extracts were prepared from A20 cells stimulated with P+I for 30 min, and an EMSA was performed with the probes displayed at the bottom. Nonspecific (NS) binding complexes are bracketed.

the  $\kappa 3$  site within the context of the -200 TNF- $\alpha$  promoter, called 5'M, resulted in a dramatic reduction of CAT activity (Fig. 2). In contrast, mutation of the 3' aspect of the  $\kappa 3$  site, called 3'M, did not significantly affect induction of the gene. These results were different from those obtained for T cells, in which both of these mutations eliminated ionomycin induction of the gene (8). Moreover, the 3' mutation completely eliminates NFATp binding to  $\kappa 3$  (17). Thus, the fact that the 3'M mutation does not affect the inducible expression of the TNF- $\alpha$  gene in A20 cells suggested that NFATp is not involved in the activation of TNF- $\alpha$  via the  $\kappa 3$  site in A20 cells.

To examine the role of the CRE in the activation of the TNF- $\alpha$  gene, we tested the activities of constructs carrying mutations in the CRE site in A20 cells. The C1 mutation, which mutates the 3' aspect of the CRE dramatically, decreased induction of the gene (Fig. 2). Also, a mutation of the 5' aspect of the CRE, which mutates only the first T of the CRE, called C3, also resulted in a decrease of approximately 50% of CAT activity (Fig. 2). The specificities of these effects were demonstrated by the fact that mutations introduced into the AP-1, AP-2, and SP-1 consensus sequences located downstream of the  $\kappa 3$  element had no detrimental effect on gene induction (Fig. 2). We note that the same pattern of expression for all of the mutant constructs displayed in Fig. 2 was obtained when ionomycin alone was used as the inducer (data not shown). Thus, these results are consistent with a critical role of the 5' aspect of  $\kappa 3$  and the CRE in the induction of the TNF- $\alpha$  gene in A20 cells.

**The composite  $\kappa 3$ /CRE site binds a constitutive complex.** To test the binding activities of the  $\kappa 3$ /CRE site in A20 cells, we used an oligonucleotide probe, called  $\kappa 3(L)$ , matching the sequences from -117 to -78 nt and performed an EMSA using nuclear extracts from A20 cells that had been either mock stimulated or stimulated with ionomycin or P+I in the presence or absence of CsA. Interestingly, in A20 nuclear extracts the  $\kappa 3(L)$  probe bound a major constitutive complex, which is

neither inducible nor CsA sensitive (Fig. 3A). This pattern is in dramatic contrast to the binding pattern of the  $\kappa 3(L)$  probe in Ar-5 T cell nuclear extracts, in which the same probe gives rise to two inducible NFATp complexes and a constitutive complex made up of ATF-2 and Jun proteins (8, 23, 29). Thus, the  $\kappa 3(L)$  oligonucleotide displays different binding patterns in nuclear extracts derived from A20 B cells and those derived from Ar-5 T cells.

**Mutations that abrogate TNF- $\alpha$  gene induction also interfere with  $\kappa 3(L)$  binding.** To determine whether the same mutations which decrease gene expression in vivo also alter binding patterns of nuclear proteins in vitro, we performed gel shift assays with probes carrying those mutations used in the transfection assays. The CRE mutations C1 and C3, which abrogated induction of the gene, disrupted binding to the uppermost complex, indicating that this is the CRE-binding complex (Fig. 3B, lanes 4 and 5). Although the binding of the CRE-binding complex is completely abrogated by the C3 mutation, it lowers inducibility of the TNF- $\alpha$  promoter by approximately 50%. Thus, the C3 mutation's effect in the transfection assay, although not completely concordant, is correlated with the disruption of binding to this complex. We note that the binding assay is done with an isolated promoter element, the CRE/ $\kappa 3$  site, and the induction of the gene is dependent on multiple elements in the transfection assay.

The oligonucleotide carrying the 5' mutation in the  $\kappa 3$  site, 5'M, which abrogated gene induction in the transfection assay, also displayed markedly decreased binding to the CRE-binding complex (Fig. 3B, lane 6). The specificity of binding of this complex to the CRE was demonstrated by the inability of probes bearing mutations outside of the CRE (-117, -113, MM, 3'M, and 3'FL) to affect the formation of the CRE-binding complex; the binding patterns of these mutant probes were similar to the binding pattern of the wild-type  $\kappa 3(L)$  probe (Fig. 3B, lanes 1 to 3 and 7 to 9). Thus, the CRE and the 5' aspect but not the 3' aspect of the  $\kappa 3$  site are required for



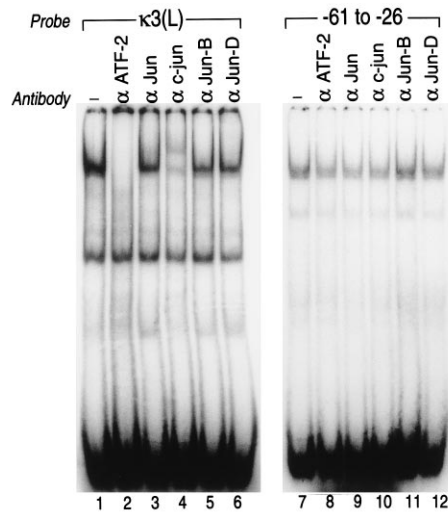


FIG. 4. Reactivities of antibodies to ATF-2 and Jun family members with the  $\kappa 3(L)$ -binding complex. Extracts from A20 cells stimulated with P+I for 30 min were assayed for binding to the  $\kappa 3(L)$  or to the  $-61$  to  $-26$  nt probe in the presence or absence in the indicated antibodies. The  $-61$  to  $-27$  nt oligonucleotide probe, which binds a constitutive factor in A20 cells, was used as a control for the specificity of the ATF-2 and Jun antibodies in the supershift assay.

both the formation of the  $\kappa 3(L)$ -binding complex in vitro and the inducible expression of the gene in vivo in A20 cells.

**ATF-2 and Jun proteins bind to the  $\kappa 3(L)$  oligonucleotide in A20 B cells.** Previously we have shown that ATF-2 and Jun proteins derived from T-cell nuclear extracts specifically associate with the CRE included in the  $\kappa 3(L)$  oligonucleotide (29). Given the importance of the CRE both in TNF- $\alpha$  gene regulation in A20 B cells and in the formation of the major  $\kappa 3(L)$ -binding complex, we were interested in determining whether ATF-2 and Jun proteins present in A20 nuclear extracts also assembled on  $\kappa 3(L)$ . Figure 4 shows that antibodies to ATF-2 (lane 2), Jun family members (lane 3), and c-Jun (lane 4), reacted with the CRE-binding complex in A20 nuclear extracts. The specificity of this reaction was verified by the use of a probe matching the sequences from  $-61$  to  $-26$  nt relative to the TNF- $\alpha$  mRNA start site; we found that none of the antibodies reacted with the complexes bound to this probe (Fig. 4, lanes 7 to 12). The fact that the ATF-2 and Jun antibodies reacted with the entire CRE/ $\kappa 3$  binding complex suggested that ATF-2 and c-Jun are the major components of the A20 CRE/ $\kappa 3$ -binding complex. Again, these results are in contrast to results from T cells, in which a discrete CRE-binding complex and two NFATp-binding complexes are detectable (29).

**NFATp and NF- $\kappa B$  proteins do not bind to the  $\kappa 3(L)$  oligonucleotide in A20 B cells.** We have previously shown that the  $\kappa 3$  element is recognized by NFATp in T cells (23, 29). Moreover, a mutation that disrupts binding of NFATp to  $\kappa 3$  in T cells also abrogates induction of the gene in these cells (8, 17). However, as we showed in Fig. 2 and 3, the same mutation, 3'M, has no effect on the induction of the gene or upon the binding of the  $\kappa 3(L)$ -binding complex in A20 cells. Therefore, these results suggested either that NFATp is not present in A20 cells or that NFATp is present but not required for the regulation of TNF- $\alpha$  via the  $\kappa 3$  site in A20 cells. To determine whether NFATp was present in A20 cells, we used a probe matching an NFAT site from the murine IL-2 promoter and antibodies to NFATp in a gel shift analysis using A20 nuclear extracts. The NFAT probe formed a complex in stimulated A20 nuclear extracts that was recognized by an antibody to

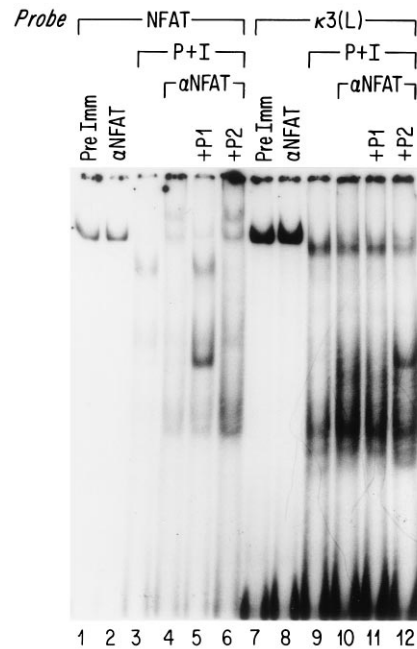


FIG. 5. Reactivities of an NFATp antibody with nuclear  $\kappa 3(L)$ -binding complexes from A20 cells. Nuclear extracts were prepared from A20 cells that were stimulated for 30 min with P+I and were assayed for binding  $\kappa 3(L)$  or NFAT oligonucleotides in the presence of an NFATp antibody ( $\alpha$ NFATp) and the cognate peptide to which the antibody was raised (P1) or an irrelevant peptide (P2), as indicated. PreImm, preimmune serum.

NFATp (Fig. 5, lanes 3 and 4). Addition of the cognate peptide (P1) to which the NFATp antibody was raised reversed the supershift (lane 5), whereas addition of a noncognate peptide (P2) did not (lane 6). However, the antibody to NFATp failed to supershift the complex bound to the  $\kappa 3(L)$  probe (lanes 9 to 12), indicating that although NFATp is present in A20 cells, it does not appear to bind to the  $\kappa 3$  site in these cells.

Given that the  $\kappa 3$  site matches the NF- $\kappa B$  consensus sequence (6), we investigated the possibility that NF- $\kappa B$  proteins present in A20 cells bind to the  $\kappa 3$  site. As a control, we used the NF- $\kappa B$  site taken from the murine  $\kappa$  light-chain enhancer as a probe and compared its binding activity in A20 nuclear extracts with the binding activity of the  $\kappa 3(L)$  probe. Although antibodies to p50, p65, and c-Rel recognized the complexes bound to the  $\kappa B$  control probe, none of the antibodies reacted with the proteins bound to the  $\kappa 3(L)$  probe (data not shown). In addition, we tested antibodies to RelB and p52, which also did not react with the  $\kappa 3$ -binding factors (data not shown). Thus, NF- $\kappa B$  proteins or NFATp do not bind to the TNF- $\alpha$   $\kappa 3$  site in A20 cells.

**Transcriptional activation of synthetic promoters by  $\kappa 3(L)$  is cell type specific.** In the T-cell clone Ar-5, six copies of the  $\kappa 3$  site cloned upstream of a truncated  $-61$  TNF- $\alpha$  gene promoter confers inducibility by T-cell receptor ligands or by calcium ionophore (8). The  $\kappa 3$  element included in the ( $\kappa 3$ )<sup>6</sup>-61 TNF- $\alpha$  CAT construct extends from  $-105$  to  $-85$  nt relative to the TNF- $\alpha$  transcription start site. This oligonucleotide binds two molecules of NFATp but does not contain an intact CRE site and hence does not bind ATF-2 and Jun proteins (29). Although the ( $\kappa 3$ )<sup>6</sup>  $-61$  TNF- $\alpha$  CAT reporter construct is highly inducible in Ar-5 T cells, this construct was not inducible by ionomycin or by P+I in A20 cells (Fig. 1C).

In contrast, the  $\kappa 3(L)$  oligonucleotide, which extends from

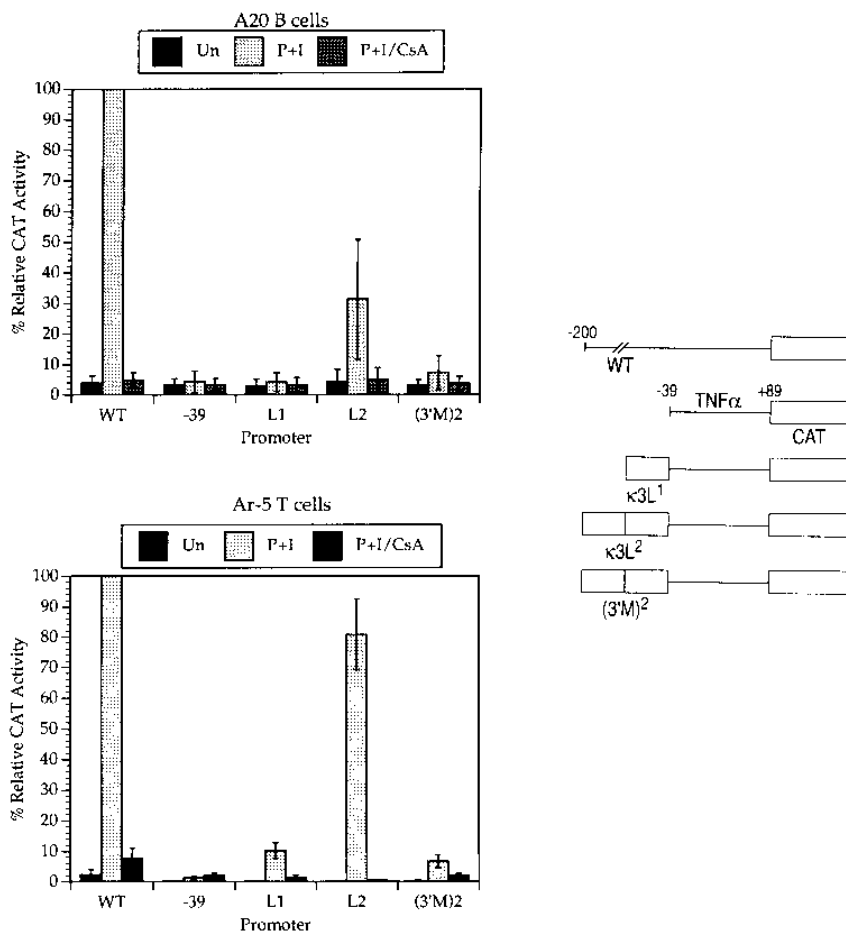


FIG. 6. Activation of synthetic TNF- $\alpha$  promoters containing  $\kappa 3(L)$  multimers in A20 B cells and Ar-5 T cells. A20 B cells or Ar-5 T cells were transfected with the  $-200$  TNF- $\alpha$  promoter CAT construct (WT) or with the truncated  $-39$  TNF- $\alpha$  CAT construct containing either one or two copies of the  $\kappa 3(L)$  site or two copies of the  $\kappa 3(L)$  site containing the 3' mutation. Twenty-four hours later, the cells were mock stimulated (Un) or stimulated with P+I in the presence or absence of CsA. CAT assays were performed, and the percent conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms was quantified with a Betagen Betascope. The results of three independent experiments are shown. In both A20 and Ar-5 cells, the patterns of expression of the constructs were consistent between experiments. Standard deviations are represented by the error bars. Within each experiment, the results were normalized to the  $-200$  WT-induced level (100%) and then averaged and plotted.

$-117$  to  $-78$  nt relative to the TNF- $\alpha$  cap site, includes the intact CRE and binds to ATF-2 and Jun proteins as well as two molecules of NFATp in Ar-5 T cells (29). We cloned one and two copies of this oligonucleotide upstream of a truncated TNF- $\alpha$  promoter containing only  $-39$  nt relative to the transcription start site. In T cells, just one copy of  $\kappa 3(L)^1$  is inducible and two copies confer dramatic inducibility upon the linked promoter, almost reaching the levels achieved with the intact  $-200$  TNF- $\alpha$  CAT (WT) (Fig. 6 and reference 29). Interestingly, in A20 cells, the  $\kappa 3(L)^1$  construct was not inducible in A20 cells (Fig. 6), and two copies of  $\kappa 3(L)^2$  confer only modest ionomycin inducibility upon the linked truncated  $-39$  TNF- $\alpha$  promoter, which is approximately 15% of the levels achieved with the  $-200$  TNF- $\alpha$  CAT construct. Thus, in A20 cells, an intact CRE and multiple copies of  $\kappa 3$  are required to act as a weak inducible enhancer element.

To test whether this induction requires NFATp, we cloned two copies of the  $\kappa 3(L)$  oligonucleotide bearing a mutation in the 3' aspect of  $\kappa 3$ , which is required for NFATp binding (17), upstream of the truncated  $-39$  TNF- $\alpha$  promoter ( $(3'M)^2$ ). As shown in Fig. 6, this mutation abrogates the ability of the oligonucleotide to act as an inducible enhancer element in both cell types. As we demonstrate later, NFATp binding ac-

tivity is lower in A20 B cells than in Ar-5 T cells (see Fig. 9). Thus, in Ar-5 T cells, it is likely that NFATp is recruited to the  $\kappa 3$  site within the context of the intact promoter because of its relatively high abundance in this cell type. This speculation is supported by the finding that a single copy of  $\kappa 3(L)$  is sufficient to confer inducibility. In A20 B cells, at least two copies of  $\kappa 3(L)$  are required for the minimal inducibility of the synthetic promoter. These results are consistent with the lower abundance of NFATp, which is apparently not sufficient for its functional association with the  $\kappa 3$  site within the context of the intact promoter in A20 cells.

#### The TNF- $\alpha$ promoter contains four NFATp-binding sites.

To determine whether there were other, as of yet unrecognized NFATp-binding sites in the TNF- $\alpha$  promoter accounting for the CsA-sensitive inducibility of the  $-200$  TNF- $\alpha$  CAT construct, we performed a quantitative DNase I footprinting analysis using recombinant, purified NFATp and a TNF- $\alpha$  promoter fragment spanning from  $-200$  to  $+87$  nt relative to the cap site as a probe. Interestingly, we identified three additional NFATp-binding sites. One region spanning from  $-104$  to  $-67$  nt, which includes the  $\kappa 3$  element as well as another pyrimidine-rich region centered at  $-76$  nt, was protected from DNase I cleavage. In addition, there are two other regions

located upstream of the CRE site that are protected from DNase I cleavage centered around  $-117$  and  $-149$  (Fig. 7A, lanes 3 to 8). These two footprints span from  $-121$  to  $-107$  nt relative to the transcriptional start site (NFAT  $-117$ ) and from  $-165$  to  $-144$  nt (NFAT  $-149$ ) (Fig. 7A).

**The novel  $-76$  NFATp-binding element is required for TNF- $\alpha$  gene induction in Ar-5 T cells and A20 B cells.** We introduced mutations into the NFAT site centered at  $-76$  nt to determine the function of this newly discovered NFAT-binding site in A20 B cells as well as Ar-5 T cells. Constructs bearing mutations in the core of the site, 76M and 72M (bottom of Fig. 8), were tested in Ar-5 T cells and A20 B cells. In both cell types, the promoter's inducibility was severely affected by these mutations (Fig. 8). As expected, mutations in the sequences flanking the core of the site (80M, 68M, and 59M) also slightly decreased inducibility of the gene.

Deletion of the sequences between  $-200$  and  $-142$  nt relative to the TNF- $\alpha$  transcription start site, which deletes the most upstream NFAT site, decreases absolute levels of CAT activity but had little effect on the promoter's inducibility in A20 cells or Ar-5 T cells (data not shown and reference 29). As demonstrated in Fig. 2, a mutation in the core of the  $-117$  site has no effect on the inducibility of the TNF- $\alpha$  gene in A20 cells (Fig. 2), although this same mutation decreases gene induction in T cells to approximately 50% of wild-type levels (31). Thus, only the NFAT site that is centered at  $-76$ , and not the NFAT site centered at  $-149$  or  $-117$ , is required for induction of the TNF- $\alpha$  gene in A20 B cells. We note that the same pattern of expression for all of the mutant constructs displayed in Fig. 8 was obtained when ionomycin alone was used as the inducer in A20 cells (data not shown).

To test whether NFATp present in A20 and Ar-5 nuclear extracts was capable of binding to the  $-76$  NFAT site, we constructed an oligonucleotide probe spanning the region from  $-85$  to  $-59$  nt and performed an EMSA using nuclear extracts from both cell types. The  $-85$  to  $-59$  nt probe formed a single complex in uninduced Ar-5 and A20 nuclear extracts (Fig. 9A, lanes 1 and 7, respectively), which was inducible by ionomycin (lanes 2 and 8) and was CsA sensitive (lanes 3 and 9). To determine whether the mutation carried in the 76M construct that decreased reporter gene activity in the transfection assays would also have an effect on the binding of this protein complex in vitro, we incorporated the 76M mutation into the  $-85$  to  $-59$  nt oligonucleotide probe (bottom of Fig. 9A). This mutant probe was incapable of forming any complex in uninduced, ionomycin-induced, and CsA-pretreated nuclear extracts prepared from Ar-5 (lanes 4 to 6) or A20 (lanes 10 to 12) cells. Thus, the same mutation that decreased CAT activity in both cell types in vivo also abrogated binding in vitro.

The identity of this complex was determined by using an antibody to NFATp. As shown in Fig. 9B, the antibody to NFATp recognizes the complex in both B- and T-cell nuclear extracts. Thus, the complex binding to the  $-85$  to  $-59$  nt probe in both cell types corresponds to NFATp or an antigenically related protein. Interestingly, with the same amount of nuclear extract, NFATp binding is stronger in Ar-5 T cells than in A20 B cells (compare lanes 1 to 3 with lanes 7 to 9 in Fig. 8A and lanes 2 to 4 in Fig. 9B), consistent with a higher concentration of NFATp in Ar-5 cells than in A20 B cells. Taken together, these results demonstrate a critical role for NFATp binding to the  $-76$  NFAT site in the induction of the TNF- $\alpha$  gene in both A20 B cells and Ar-5 T cells.

Our EMSA experiments using nuclear extracts suggested that the  $-76$  site binds NFATp with a higher affinity than the  $\kappa 3$  site. To confirm this observation, we examined our quantitative DNase I footprinting experiment shown in Fig. 7A. The

nucleotides centered around  $-76$  (NFAT  $-76$ ) and spanning from  $-84$  to  $-67$  nt were protected from DNase I cleavage at the lowest concentrations of NFATp (Fig. 6A, lanes 3 to 8), whereas the footprint centered around the  $\kappa 3$  site (from  $-104$  to  $-84$  nt) appeared at intermediate concentrations of recombinant protein (lanes 5 to 8). Thus, consistent with the EMSA data, which demonstrate that in A20 nuclear extracts the  $-76$  site binds to NFATp (Fig. 9B) whereas the  $\kappa 3$  site does not (Fig. 5), the NFAT-76 site had a higher binding affinity for recombinant NFATp than the  $\kappa 3$  site.

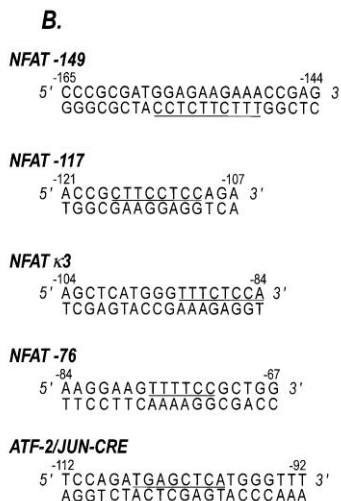
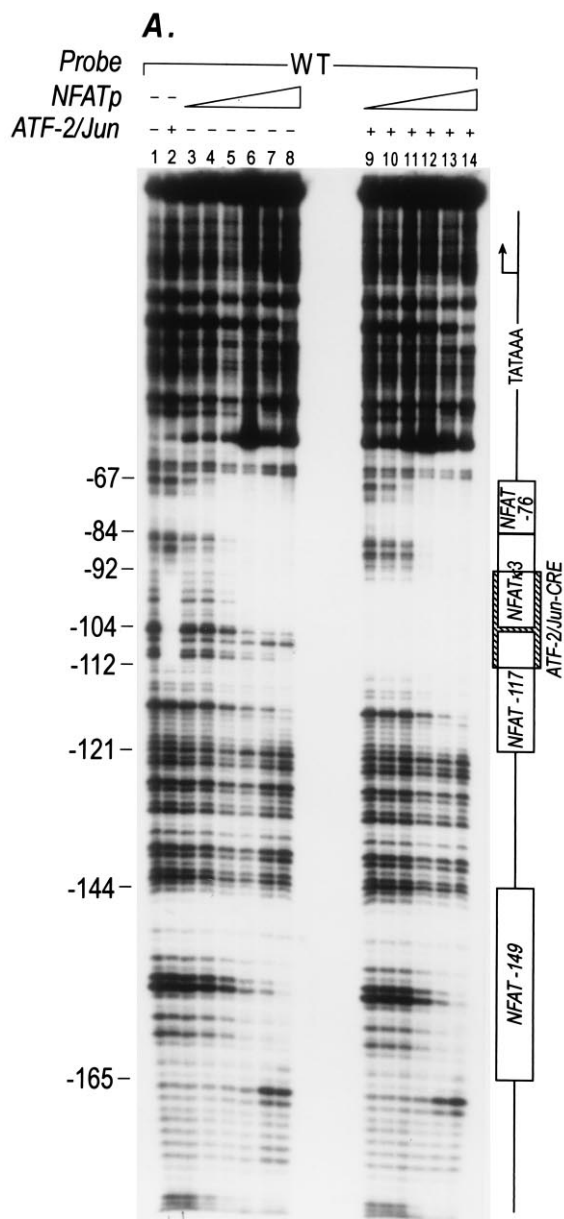
**ATF-2-Jun and NFATp do not bind cooperatively.** We have previously shown that NFATp and ATF-2-Jun do not cooperatively bind to the  $\kappa 3(L)$  site in an EMSA (29). To examine whether the multiple other NFATp-binding sites in the TNF- $\alpha$  promoter could bind ATF-2-Jun and NFATp cooperatively, we performed a quantitative DNase I footprinting analysis using increasing amounts of NFATp in the absence or presence of a fixed amount of ATF-2-Jun. Binding of recombinant ATF-2 and Jun proteins to the TNF- $\alpha$  promoter resulted in the protection from DNase I cleavage of a region centered around the CRE site spanning from  $-112$  to  $-92$  nt (Fig. 7A, lane 2). Furthermore, the affinity of NFATp for any of the TNF- $\alpha$  NFAT-binding sites is not changed in the presence of ATF-2 and Jun (compare lanes 2 to 8 to 9 to 14 in Fig. 6A). Thus, ATF-2-Jun and NFATp do not cooperatively bind on the TNF- $\alpha$  promoter, consistent with our previous EMSA analyses (29).

**NFATp does not bind cooperatively to the TNF- $\alpha$  NFAT sites.** To examine whether NFATp binds cooperatively to the multiple NFAT sites in the TNF- $\alpha$  promoter, we performed a quantitative DNase I analysis on different DNA templates bearing mutations in the  $-76$  ( $-76M$ ) and  $\kappa 3$  ( $5'M$  and  $3'M$ ) sites and compared their abilities to bind NFATp to a WT DNA template. These templates were identical to those promoter fragments (WT, 76M,  $5'$ , and  $3'$ ) used in the transfection assays (Figs 2 and 8). As demonstrated in Fig. 10A, the  $-76$  mutation abrogates binding of recombinant NFATp to the  $-76$  site without disturbing NFATp binding to the  $\kappa 3$  site (compare lanes 2 to 6 with lanes 16 to 20). Thus, there is no cooperative binding between the  $\kappa 3$  and the  $-76$  NFAT sites. As expected, consistent with the EMSA analysis, the  $-76$  mutation abolishes the binding of NFATp to the  $-76$  site; however, the affinity of NFATp for all of the other sites is not affected (lanes 16 to 20). As a negative control, we demonstrated that ATF-2-Jun binds to the  $-76M$  probe with similar affinity as it does to the WT probe (compare lanes 11 to 14 with lanes 25 to 28).

To delineate further any potential cooperative interactions between the TNF- $\alpha$  NFAT-binding sites, we used DNA templates bearing mutations in the  $5'$  or  $3'$  subsite of  $\kappa 3$  in quantitative DNase I analyses. Increasing amounts of recombinant NFATp are able to bind to the  $5'M$  probe with almost the same affinity as to the WT promoter (Fig. 10A, lanes 30 to 34). In contrast, the  $3'M$  probe is unable to bind NFATp at the  $\kappa 3$  site (lanes 39 to 44), consistent with a previous EMSA analysis.

Given that the  $\kappa 3$  site resembles an NF- $\kappa B$  consensus sequence, we tested the ability of p50-p65 heterodimers to bind to the WT DNA template in the quantitative DNase hypersensitivity assay. Recombinant NF- $\kappa B$  proteins do not recognize any of the sequences in the TNF- $\alpha$  promoter shown to be functionally important in the regulation of the gene (Fig. 10A, lanes 7 to 10). These results support previous functional analyses (6, 9) demonstrating that NF- $\kappa B$  proteins do not play a significant role in the regulation of the human TNF- $\alpha$  gene (6, 9, 27).

Binding of NFATp to the sites centered at  $-76$ ,  $-117$ , and



–149 is not affected by either the 5'M or the 3'M  $\kappa$ 3 mutation (compare lanes 1 to 6 to lanes 29 to 34 and 39 to 44 in Fig. 10A). Interestingly, the 5' mutation, which is immediately downstream of the CRE, severely compromises ATF-2–Jun binding (compare lanes 11 to 14 with lanes 35 to 38). These results are consistent with our EMSA data showing that an oligonucleotide probe bearing the 5' mutation was severely compromised in its ability to form the major  $\kappa$ 3(L) CRE-binding complex in A20 nuclear extracts (Fig. 3). However, although the 5'M probe binds ATF-2/Jun with lower affinity than does the WT probe, both NFATp and ATF-2 can still occupy their binding sites simultaneously on the 5' mutant probe (compare lanes 1 to 11 with lanes 12 to 17 in Fig. 10B). Thus, the fact that the 5'  $\kappa$ 3 mutation decreases TNF- $\alpha$  gene expression is not due to interference with NFATp binding but rather is due to a decrease in ATF-2–Jun binding.

**DISCUSSION**

The TNF- $\alpha$  gene provides a unique opportunity to study how complex patterns of gene expression are established. In this report, we show that a single TNF- $\alpha$  regulatory element, the  $\kappa$ 3 site, is differentially used in two cell types in response to the same extracellular signal. Stimulation of both B cells and T cells with calcium ionophore results in the activation of TNF- $\alpha$  gene transcription, and the treatment of either cell type with CsA or FK506 prior to cellular activation blocks gene expression. In this report, we have shown that depending on the particular cellular milieu in which the TNF- $\alpha$  promoter functions, the composite  $\kappa$ 3/CRE site differentially associates with NFATp and with ATF-2 and Jun proteins after cellular stimulation.

The  $\kappa$ 3 site does not bind NFATp in A20 B cells, whereas it does bind two molecules of NFATp in Ar-5 T cells. The CRE is recognized by an ATF-2–Jun heterodimer in both cell types. In A20 B cells, the activation of the TNF- $\alpha$  promoter requires a previously unrecognized NFAT-binding site centered at –76 nt. The –76 NFAT site also plays a critical role in the induction of the gene in Ar-5 T cells. Furthermore, we have identified two other previously unrecognized NFAT-binding sites. The site centered at –117 appears to play a minor role in the induction of the gene in Ar-5 T cells but does not appear to be involved in the induction of the gene in A20 B cells. The most upstream site centered at –149 appears to play a minimal role in both cell types (Fig. 11). Thus, functional binding of NFATp to sites in the TNF- $\alpha$  promoter is cell type specific.

Our finding that in A20 B cells NFATp binding to the  $\kappa$ 3 element is not required in the context of the intact TNF- $\alpha$  promoter but is required for the activation of the synthetic homopolymeric promoter is intriguing. Our footprinting data obtained in assays using recombinant purified proteins demonstrate that the  $\kappa$ 3 site binds NFATp with lower affinity than the newly identified –76 NFAT site. Moreover, we have shown

FIG. 7. There are multiple NFATp-binding sites in the TNF- $\alpha$  promoter, and there is no evidence of cooperative binding between NFATp and ATF-2–Jun. (A) Quantitative DNase I hypersensitivity assay using a TNF- $\alpha$  promoter fragment spanning the sequences from –200 to +87 (WT). The DNA template was end labeled (20,000 cpm) at the noncoding strand and incubated with the indicated proteins. The concentration of ATF-2–Jun added was 300 ng, and NFATp was added in increasing concentrations at 1, 3, 10, 30, 100, and 300 ng, as indicated by the wedges. (B) TNF- $\alpha$  promoter sequences forming footprints with the recombinant NFATp or ATF-2–Jun proteins in the DNase I footprinting analysis displayed panel A. The pyrimidine-rich core sequences of the NFAT-binding sites are underlined, as is the CRE motif within the ATF-2–Jun footprint.



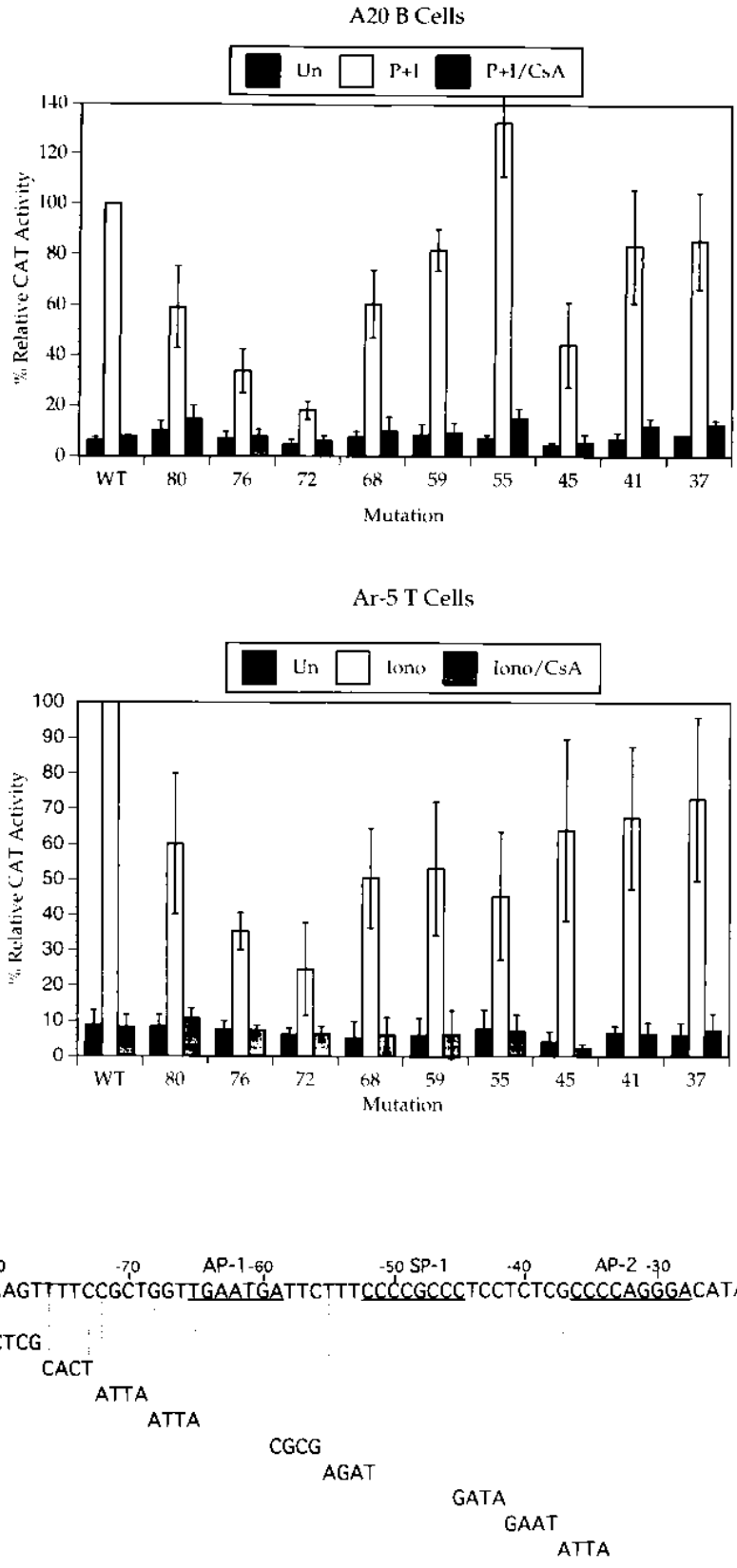


FIG. 8. Further characterization of sequences contributing to TNF- $\alpha$  promoter activity in A20 B cells and Ar-5 T cells: identification of a novel NFATp-binding site. A20 B cells or Ar-5 T cells were transfected with the WT (-200 TNF- $\alpha$  promoter) CAT construct and mutated TNF- $\alpha$  promoter CAT plasmids displayed at the bottom and 24 h later were mock stimulated (Un) or induced with P+I in the presence or absence of CsA. CAT assays were performed, and the percent conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms was quantified with a Betagen Betascope. Within each experiment, the results were normalized to the -199 WT-induced level (100%) and then averaged and plotted. Standard deviations are represented by the error bars. The results of three independent experiments are shown. The average values for fold induction for the various constructs in the A20 cells are as follows: WT, 15.9; -80, 5.7; -76, 4.8; -72, 3.9; -68, 8.0; -59, 9.7; -55, 18.8; -45, 10.1; -41, 12.1; and -37, 10.5. The average values for fold induction for the various constructs in the Ar-5 T cells are as follows: WT, 11.3; -80, 7.0; -76, 4.7; -72, 4; -68, 9.5; -59, 8.9; -55, 5.9; -45, 16; -41, 10.3; and -37, 12.3.

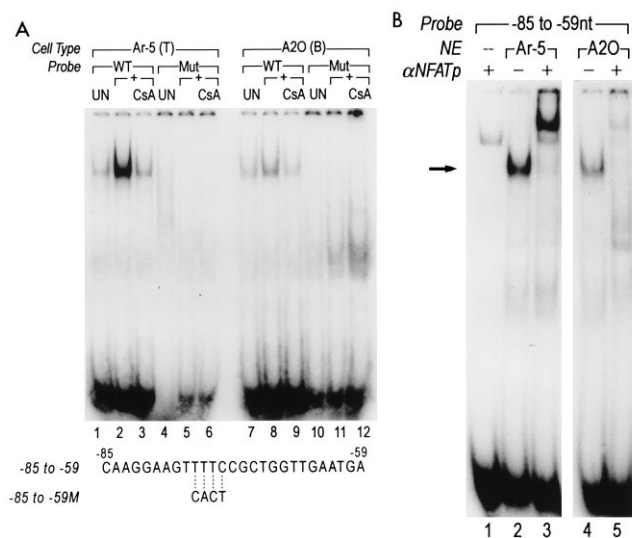


FIG. 9. Binding of complexes in Ar-5 T-cell and A20 B-cell nuclear extracts to the  $-85$  to  $-59$  oligonucleotide probe. (A) Binding of factors to WT and mutated (Mut)  $-85$  to  $-59$  oligonucleotides in nuclear extracts prepared from A20 and Ar-5 cells. An inducible, CsA-sensitive complex binds to the  $-85$  to  $-59$  nt probe (WT) in nuclear extracts derived from either Ar-5 cells stimulated with ionomycin (+) or A20 cells stimulated with P+I (+). A mutation in the core NFAT-binding motif of the  $-85$  to  $-59$  probe (Mut) abrogates binding in both cell types. Ar-5 T cells were stimulated with ionomycin for 30 min, and A20 B cells were stimulated with P+I for 30 min in the presence or absence of CsA. The amounts of mutant probe in lanes 4 to 6 and 10 to 12 are less than the amounts of WT probe in lanes 1 to 3 and 7 to 9. However, even on a much darker exposure, no binding complexes in either cell type that associate with the mutant probe are observed. (B) Reactivity of an NFATp antibody ( $\alpha$ NFATp) with the  $-85$  to  $-59$  nt probe in nuclear extracts prepared from Ar-5 cells or A20 cells. Ar-5 cells were stimulated with ionomycin for 30 min, and A20 cells were stimulated with P+I for 30 min. The EMSA was performed in the presence or absence of the anti-NFATp antibody as described in Materials and Methods.

that NFATp-binding activity is more abundant in Ar-5 T cells than in A20 B cells. Finally, a single copy of the composite  $\kappa 3$ (L) element (which contains only a single copy of the NFAT-binding site), similar to the single copy in the intact promoter, is not inducible in A20 B cells but is induced approximately 10-fold in Ar-5 T cells. Only when the composite site is oligomerized can it confer inducibility on a synthetic promoter in A20 cells. Taken together, these results are consistent with the possibility that a threshold level of NFATp is required for DNA binding to certain sites.

Our footprinting analysis showed that the  $-76$  site bound NFATp at the lowest concentrations. Thus, although the NFATp concentration in both cell types is sufficient for binding to the  $-76$  site, the concentration of NFATp required for binding to the  $\kappa 3$  site is apparently not reached in A20 B cells as it is in Ar-5 T cells. These observations are consistent with the idea that the lower abundance of NFATp in A20 B cells combined with the  $\kappa 3$  element's lower affinity for NFAT accounts for the cell-type-specific association of NFATp with  $\kappa 3$  in the two cell types. Therefore, in Ar-5 T cells, the CRE/ $\kappa 3$  site functions as a composite promoter element binding factors from different transcription factor families, exemplified by NFATp and ATF-2-Jun, whereas in A20 B cells the site functions as a simple site and binds only to one class of proteins, represented by ATF-2-Jun. Thus, the B-cell line A20 serves as a prototype of cells in which there are relatively lower levels of NFAT proteins and in which the TNF- $\alpha$  gene is induced. Our results are consistent with the notion that the TNF- $\alpha$  gene is regulated in a specific manner in cells that is concordant with

their levels of NFAT proteins. In future studies, it will be important to determine whether the results obtained for A20 cells can be generalized to the regulation of the TNF- $\alpha$  gene in other B cells or whether our observations reflect a cell-type-specific phenomenon that may occur in cell types from a variety of tissues and lineages.

NFATp is a member of the NFAT family of transcription factors, which includes NFATp, NFATc, NFAT-3, and NFAT-4 (11, 16, 19, 22). There is evidence that both NFATp and NFATc are involved in the transcriptional regulation of the IL-2 gene in T cells (19, 22), and the binding specificities of these two proteins appear to be identical (11, 26). We note that NFATc is also present in both A20 B cells and Ar-5 T cells (data not shown). Antibodies to NFATc specifically recognize the  $\kappa 3$  NFAT complexes in Ar-5 T-cell nuclear extracts but do not recognize the  $\kappa 3$ -binding complex in A20 nuclear extracts. Moreover, as expected, the NFATc antibodies recognize the complexes binding to the  $-76$  site in both cell types (data not shown). These results lend further support to a cell-type-specific model of  $\kappa 3$ -NFAT binding and are consistent with the notion that the higher concentrations of NFATp and NFATc in Ar-5 cells achieve a certain threshold level required for binding to the  $\kappa 3$  site.

Of interest, NFATp is capable of binding to all four of the TNF- $\alpha$  NFAT-binding sites without a bZIP protein partner. This is in contrast to findings for other cytokine promoters such as IL-2, granulocyte-macrophage colony-stimulating factor, and IL-4, in which at least some of the NFAT-binding sites do require pairing with an AP-1-like partner and in which cooperative binding interactions between the two groups of proteins can be demonstrated (reviewed in reference 21). In the IL-2 promoter, for example, four of the five NFAT-binding sites bind NFAT in association with AP-1 (26).

Thus, it is interesting that the TNF- $\alpha$  CRE site in particular is capable of binding to a variety of recombinant AP-1 family proteins, including Fos, Jun, and ATF-2. However, the  $\kappa 3$  site does not require a bZIP protein partner to bind to NFATp. Instead, the site appears to act as a junction point (as originally defined by Miner and Yamamoto in 1992 [20]) for the interaction of two different transcription factor families, AP-1 and NFAT proteins. This view is supported by the footprinting analysis, which shows that the sequences spanning from  $-121$  to  $-67$  form a large footprint with NFATp and ATF-2-Jun interdigitating at the  $\kappa 3$ /CRE site (Fig. 11).

Furthermore, the  $\kappa 3$ /CRE composite site is capable of producing novel regulatory consequences in certain cell types, perhaps through protein-protein interactions between ATF-2-Jun and NFATp. This hypothesis is supported by the fact that just one copy of the intact CRE/ $\kappa 3$  site is a potent calcium-inducible promoter element when linked to a truncated promoter in T cells, whereas multiple copies of the  $\kappa 3$  site alone are required for inducible activity (8, 29). Our quantitative DNase analysis demonstrated a lack of cooperative binding between ATF-2-Jun and NFATp, as did previous EMSA analyses (29). Perhaps the functional synergy of the CRE and  $\kappa 3$  in T cells is a consequence of the interaction of the proteins that bind to the sites with some other factor or constituent of the transcription apparatus and not with each other. Such interactions have been previously suggested in other cases of eukaryotic transcriptional activators that do not cooperatively bind but do cooperatively activate transcription (2, 15).

In contrast to the regulation of other genes in which NFAT proteins play a role, TNF- $\alpha$  transcription in B or T cells does not require de novo protein synthesis and calcium flux alone is sufficient for the gene's rapid induction (7, 8) (Fig. 1). We have shown here that all four of the TNF- $\alpha$  NFAT-binding sites are

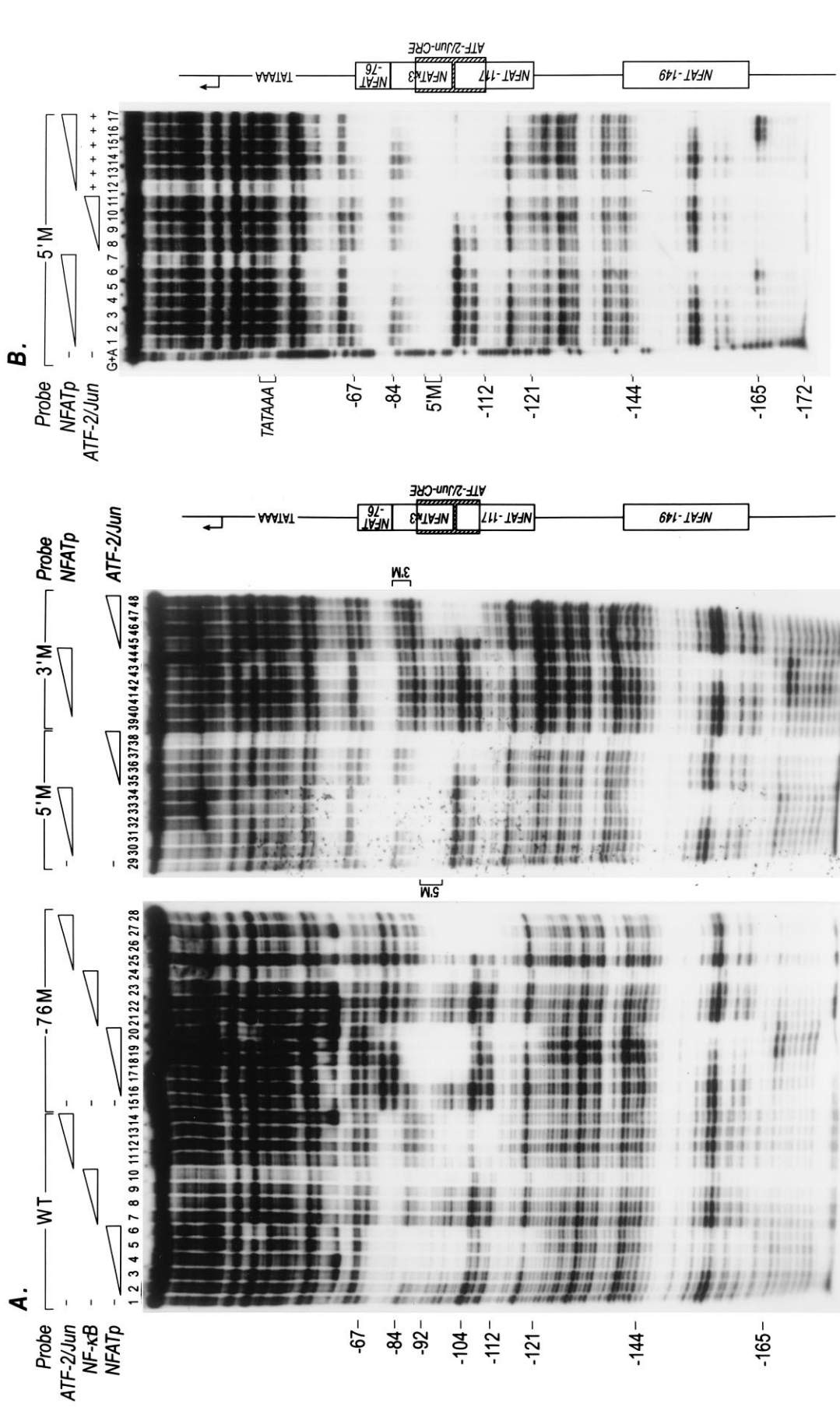


FIG. 10. Effects of TNF- $\alpha$  promoter mutations on NFATp and ATF-2-Jun binding in vitro. NF- $\kappa$ B proteins do not bind to the TNF- $\alpha$  promoter. (A) Quantitative DNase I footprinting assay using WT and mutated TNF- $\alpha$  promoter fragments as templates. Sequences spanning from -200 to +87 (WT) or sequences carrying the 76M, 5M, or 3M mutation were end labeled (20,000 cpm) at the noncoding strand and incubated with the indicated proteins. ATF-2-Jun was added in increasing amounts (3.3, 20, 100, and 500 ng) in lanes 7 to 10, 21 to 24, 35 to 38, and 45 to 48, as indicated by the wedges. NFATp was added in increasing amounts (2, 8, 33, 125, and 500 ng) to lanes 2 to 6, 16 to 20, 30 to 34, and 40 to 44. NF- $\kappa$ B (the p50-p65 heterodimer) was added in increasing amounts (10, 50, and 250 ng and 1  $\mu$ g) to lanes 7 to 10 and lanes 21 to 24. We note that these same concentrations protected the beta interferon NF- $\kappa$ B site, PRDII, from DNase I digestion (data not shown and reference 28). (B) Quantitative DNase I footprinting assay using the mutated (5M) TNF- $\alpha$  promoter fragment as a template. The probe was end labeled (20,000 cpm) at the noncoding strand and incubated with the indicated proteins. ATF-2-Jun was added in increasing amounts (3.3, 20, 100, and 500 ng) in lanes 8 to 11 and added at 500 ng in lanes 12 to 17. NFATp was added in increasing amounts (0.5, 1.67, 5, 16.7, 50, or 166 ng) to lanes 1 to 7 and 12 to 17.

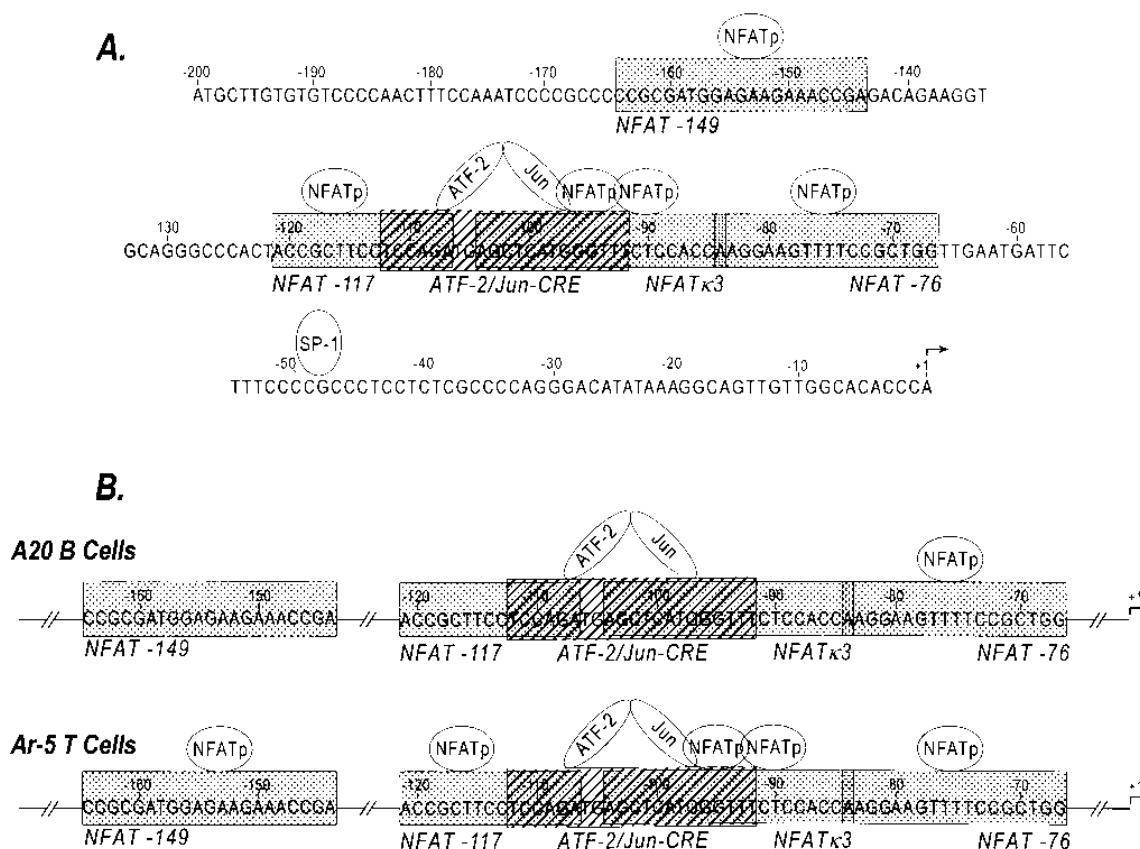


FIG. 11. Model of the TNF- $\alpha$  promoter. (A) Summary of the results of the footprinting analysis with recombinant NFATp and ATF-2-Jun proteins. NFATp associates with four different regions contained within 200 nt upstream of the TNF- $\alpha$  transcription start site. ATF-2 and Jun proteins bind to a CRE consensus sequence. In other studies, we have demonstrated that SP-1 binds to an SP-1 consensus sequence located at -52 to -45 nt (10a). The start site of transcription is at +1. (B) Summary of the cell-type-specific association of NFATp with the TNF- $\alpha$  promoter in Ar-5 T cells and A20 B cells.

capable of binding NFATp in the absence of AP-1. Thus, the regulation of TNF- $\alpha$  can be distinguished from, for example, the pattern of expression of IL-2 in T cells, which occurs with slower kinetics (24). IL-2 gene transcription requires the co-operative binding of Fos and Jun proteins with NFAT proteins, de novo protein synthesis, and protein kinase C activity in addition to calcium flux (24).

The analysis presented here suggests that the binding affinity of a promoter for a particular factor may be sufficient for binding in certain cell types and too low for effective binding in another. In this manner, novel patterns of gene expression can result from the cell-type-specific interaction of a promoter with a particular set of transcription factors. Thus, a promoter may accomplish a level of flexibility in its response to a particular stimulus, depending on the cell type in which it functions. It is interesting to speculate that a gene such as the TNF- $\alpha$  gene, which is expressed in multiple cell types in response to a variety of inducers, may have evolved this level of flexibility to accomplish novel patterns gene regulation.

Our previous studies demonstrated that TNF- $\alpha$  is an auto-crine and paracrine B-cell growth factor that functions much the way IL-2 does in T cells (1). Thus, the understanding of TNF- $\alpha$  gene regulation in B cells could potentially lead to novel therapeutic manipulations that interrupt the signaling cascade resulting in TNF- $\alpha$  gene transcription and subsequent protein production in conditions characterized by immunopathologic proliferation of B cells. A detailed understanding of

TNF- $\alpha$  gene transcription in B cells also offers potential novel insights regarding the role of the shared cytokine transcription factor NFATp in immediate-early gene transcription in B cells.

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