

## Calcineurin Mediates Human Tumor Necrosis Factor $\alpha$ Gene Induction in Stimulated T and B Cells

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### Summary

The tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) gene is rapidly transcribed in activated T cells via a calcium-dependent pathway that does not require de novo protein synthesis, but is completely blocked by the immunosuppressive drugs cyclosporin A (CsA) and FK506. Here we show that calcineurin phosphatase activity is both necessary and sufficient for TNF- $\alpha$  gene transcription in T cells, and identify the factor that binds to the  $\kappa 3$  element of the TNF- $\alpha$  gene promoter as the target for calcineurin action. The ability of analogues of CsA and FK506 to block calcineurin phosphatase activity correlates completely with their ability to inhibit induction of TNF- $\alpha$  mRNA, induction of a TNF- $\alpha$  promoter reporter plasmid in transiently transfected T cells, and induction of the  $\kappa 3$  binding factor in an electrophoretic mobility shift assay. Moreover, a cDNA encoding the constitutively active form of calcineurin is sufficient to activate the TNF- $\alpha$  promoter and the  $\kappa 3$  element. TNF- $\alpha$  gene transcription is also highly inducible, CsA-sensitive, and protein synthesis-independent in B cells stimulated through their surface immunoglobulin receptors. Using the panel of CsA and FK506 analogues, we show that calcineurin participates in the induction of TNF- $\alpha$  transcription in activated B cells. These results extend our previous demonstration that the  $\kappa 3$  binding factor is related to NFATp, the preexisting subunit of nuclear factor of activated T cells, and suggest that calcineurin-mediated modification of the  $\kappa 3$  binding factor in T cells is of key importance in the induction of TNF- $\alpha$  transcription.

Stimulation of T cells through the T cell receptor results in several early metabolic events that culminate in the transcriptional induction of a variety of cytokine genes (reviewed in 1 and 2). Among these, the TNF- $\alpha$  gene is unusual because it possesses the characteristics of an immediate early gene. TNF- $\alpha$  mRNA is induced to maximal levels within 15–30 min of exposure to T cell receptor ligands, and this induction does not require de novo protein synthesis (3). Moreover, induction of TNF- $\alpha$  mRNA does not require a combination of calcium ionophore and phorbol ester as observed for most other cytokines (4, 5), but can be mediated by calcium ionophore alone (3). These results suggest that TNF- $\alpha$  gene induction is mediated by transcription factors present in unstimulated T cells, which are posttranscriptionally modified and activated via calcium-dependent signaling pathways that do not require protein kinase C.

The calcium- and calmodulin-dependent phosphatase calcineurin plays a major role in the process of T cell activation (reviewed in 6). Stimulated T cells show increased levels of

calcineurin phosphatase activity (6), presumably as a result of the increase in intracellular free calcium that accompanies T cell activation (reviewed in 1). When bound to specific intracellular receptors (immunophilins), cyclosporin A (CsA) and FK506 inhibit calcineurin phosphatase activity and block the transcription of most (if not all) cytokine genes in activated T cells (7, 8), thus underscoring the importance of calcineurin in the T cell immune response. Analogues of CsA and FK506 that inhibit the phosphatase activity of calcineurin also inhibit the transcription of the cytokine IL-2 (9, 10); likewise, expression of a constitutively active form of calcineurin substitutes for the requirement for calcium in IL-2 gene transcription, permitting transcription in response to phorbol ester alone (11, 12).

Recent work suggests that calcineurin mediates IL-2 gene transcription by directly or indirectly modifying the transcription factor NFATp, which is essential for induction of IL-2 and other cytokine genes (13, 14). NFATp, a 120-kD phosphoprotein, is present in cytosolic extracts of unstimu-

lated T cells (15, 16). Activation of T cells with T cell receptor ligands or with calcium ionophores results in the appearance of NFATp in nuclear extracts; this process is mediated by calcineurin since it is blocked by CsA and FK506 (15, 16). Within the nucleus, NFATp binds as a complex with members of the Fos and Jun (AP-1) family of proteins to two sites in the IL-2 promoter, thereby mediating IL-2 gene transcription (15–19). NFATp or a closely related protein also binds to the  $\kappa 3$  element of the TNF- $\alpha$  promoter, the element that mediates calcium-dependent, CsA-sensitive induction of the gene in T cells (3 and McCaffrey, P.G., A.E. Goldfeld, and A. Rao, manuscript submitted for publication).

The TNF- $\alpha$  gene is distinguished from other cytokine genes expressed in activated T cells in that it is also highly inducible in immunologically stimulated B cells (20–23). TNF- $\alpha$  gene transcription in B cells stimulated through their surface Ig receptors is similar to that in T cells, in that it is also rapidly inducible and protein synthesis-independent (23). Furthermore, as in T cells, the rapid induction of the gene in B cells can also be blocked by the immunosuppressive drugs CsA and FK506 (23), suggesting that the mechanisms governing TNF- $\alpha$  gene induction may be similar in T and B cells.

Here, we show that calcineurin is a major mediator of TNF- $\alpha$  gene transcription in activated T and B cells. These results suggest strongly that the immediate early transcription of the TNF- $\alpha$  gene in antigen stimulated lymphocytes is dependent on a single calcineurin-mediated signal transduction pathway that leads to the posttranslational modification of preexisting transcription factors. This contrasts with the transcription of most other cytokine genes in activated T cells, which require activation of multiple signaling pathways including those dependent on protein kinase C. Thus, the TNF- $\alpha$  promoter represents a simple, direct system for the study of transcriptional events initiated by calcineurin in lymphocytes. Furthermore, the ability of immunosuppressive compounds to block TNF- $\alpha$  gene transcription, indicate that the TNF- $\alpha$  promoter and the  $\kappa 3$  element may serve as novel targets in drug screening assays designed to identify new immunosuppressive agents.

## Materials and Methods

**Cell Culture, Activation, Transfections, and RNA Analysis.** The murine IL-2-dependent T cell clone Ar-5 was grown and activated with monoclonal antibodies or ionomycin as previously described (3). The IgG positive human Burkitt's lymphoma cell line, Akata, was grown and stimulated with anti-IgG as previously described (23). 10 min before activation, cells were treated with CsA (Sandoz Pharmaceuticals Corp., E. Hanover, NJ) at concentrations of  $10^{-6}$  M,  $5 \times 10^{-7}$  M, or  $10^{-7}$  M or with 1 nM FK506 (Fujisawa, Deerfield, IL) or with 1  $\mu$ M of MeBm $_2$ t $^1$ -CsA or with 1  $\mu$ M of MeAla $^6$ -CsA as indicated. 15-O-demethyl-FK520 (referred to as FK520Me throughout this paper), was used at the concentrations of 1  $\mu$ M or 1 nM as indicated. Rapamycin was used to pretreat cells at the concentrations indicated in the figures. MeBm $_2$ t $^1$ -CsA, MeAla $^6$ -CsA, FK520Me, and rapamycin were synthesized as previously described (9). Transfections, activation and CAT assays were performed as described (3). RNA was prepared and RNase protection assays were performed as previously described (3, 23).

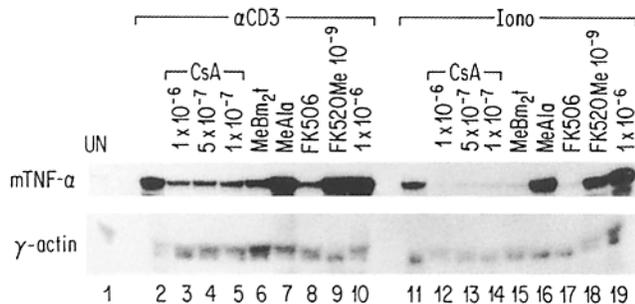
**Preparation of Cellular Extracts and Electrophoretic Mobility Shift Assays (EMSA).** Ar-5 cells were pretreated with CsA (1  $\mu$ M), MeBm $_2$ t (1  $\mu$ M), MeAla (1  $\mu$ M), FK506 (1 nM), FK520Me (1  $\mu$ M), or rapamycin ( $5 \times 10^{-7}$  M) before stimulation with ionomycin (1  $\mu$ M) for 30 min and nuclear extracts were prepared as previously described (3). EMSA using approximately 5  $\mu$ g of nuclear proteins were performed using  $^{32}$ P-labeled  $\kappa 3$  or -61n to -27n oligonucleotide probes as described (3).

**Plasmids.** The -199 TNF- $\alpha$  promoter/CAT gene construct and the ( $\kappa 3$ ) $^{6-61}$  TNF- $\alpha$  CAT construct have been previously described (24). The  $\delta$ CAM and SR2 $\alpha$  plasmids have been previously described (11).

## Results and Discussion

To determine whether calcineurin was an intermediate in the signal transduction pathway that results in the induction of TNF- $\alpha$  gene expression in activated lymphocytes, two approaches were employed. The first approach involved the use of CsA and FK506 analogues (MeBm $_2$ t $^1$ -CsA, MeAla $^6$ -CsA, FK520Me, and rapamycin), which when complexed with cyclophilin A (CyPA) and FKBP-12 respectively, show different binding affinities for calcineurin and differentially inhibit its phosphatase activity (9). The ability of these agents to inhibit cytokine gene transcription is strikingly correlated with their ability to inhibit calcineurin activity in vitro (9, 10). For example, the MeBm $_2$ t $^1$ -CsA/CyPA complex is a potent inhibitor of calcineurin phosphatase activity in vitro and MeBm $_2$ t $^1$ -CsA inhibits IL-2 gene transcription when added to T cells together with an activating stimulus (9). In contrast, neither the MeAla $^6$ -CsA/CyPA and FK520Me/FKBP-12 complexes inhibit calcineurin activity in vitro, and neither inhibits IL-2 gene transcription when T cells are activated (9). Another analogue, rapamycin, binds to FKBP-12 but does not inhibit calcineurin phosphatase activity (reviewed in 10 and 13). Rapamycin inhibits an event in a distinct mitogenic signaling cascade involving a target other than calcineurin and has no effect on the CsA- or FK506-sensitive calcium-dependent pathway involved in IL-2 gene induction (25).

To determine whether induction of TNF- $\alpha$  mRNA in T cells was differentially inhibited by the CsA and FK506 analogues, the murine T cell clone Ar-5 was stimulated with either anti-CD3 or ionomycin in the presence or absence of these compounds and RNA was prepared. As shown in Fig. 1, 1  $\mu$ M MeBm $_2$ t $^1$ -CsA substantially inhibited induction of TNF- $\alpha$  mRNA by anti-CD3 (Fig. 1, lane 6), or ionomycin (Fig. 1, lane 15), as did 0.1 or 1  $\mu$ M CsA (Fig. 1, lanes 3–6, and 12–14). In contrast, an equivalent concentration of the inactive CsA analogue MeAla $^6$ -CsA did not affect induction by anti-CD3 (Fig. 1, lanes 7 and 16). Similarly, although only 1 nM of FK506 greatly inhibited TNF- $\alpha$  mRNA induction in response to either anti-CD3 (Fig. 1, lane 8) or ionomycin (Fig. 1, lane 17), the inactive FK520Me analogue had no effect even at 1,000-fold higher concentration (Fig. 1, lanes 9, 10, 18, and 19). Likewise, rapamycin did not block induction of TNF- $\alpha$  mRNA levels in response to anti-CD3, anti-TCR- $\alpha/\beta$ , or ionomycin (data not shown). These results suggest that calcineurin is a critical intermediate in the signal trans-



**Figure 1.** Inhibition of Anti-CD3 and ionomycin induction of TNF- $\alpha$  gene expression in murine Ar-5 T cells. Cells were stimulated with anti-CD3 ( $\alpha$ CD3) or ionomycin (Iono) for 30 min, in the presence or absence of CsA, MeBm<sub>2</sub>t<sup>1</sup>-CsA (MeBm<sub>2</sub>t), MeAla<sup>6</sup>-CsA (MeAla), FK506, or 15-O-demethyl-FK520 (FK520Me) and RNAse mapping of TNF- $\alpha$  and  $\gamma$ -actin mRNAs was carried out as described in Materials and Methods.

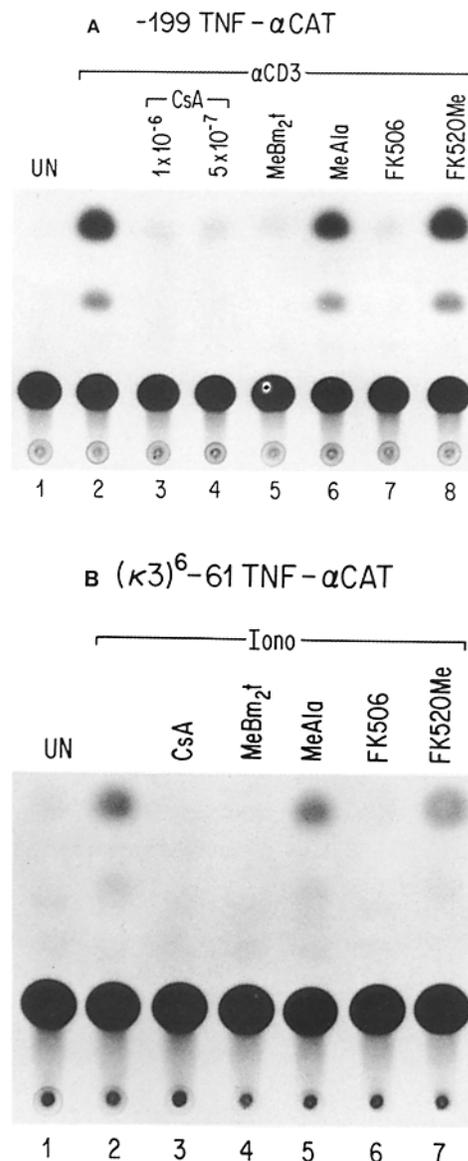
duction pathway leading to the activation of the TNF- $\alpha$  gene in T cells.

A region of the TNF- $\alpha$  promoter containing -199 nucleotides (nt) relative to the mRNA cap site is sufficient for maximal induction of a linked chloramphenicol acetyl transferase (CAT) gene in stimulated T cells (3). To test whether the elements regulated by calcineurin were present in this region, we transfected the -199 TNF- $\alpha$  CAT plasmid into Ar-5 cells and treated the cells with the various drugs before stimulation with anti-CD3. CsA, FK506, and the active CsA analogue MeBm<sub>2</sub>t potently inhibited CAT activity induced by anti-CD3 (Fig. 2 A, lanes 3–5 and 7), whereas the inactive analogues MeAla<sup>6</sup>-CsA and FK520Me did not (Fig. 2 A, lanes 6 and 8). Similar results were obtained when ionomycin was used to induce the cells (data not shown).

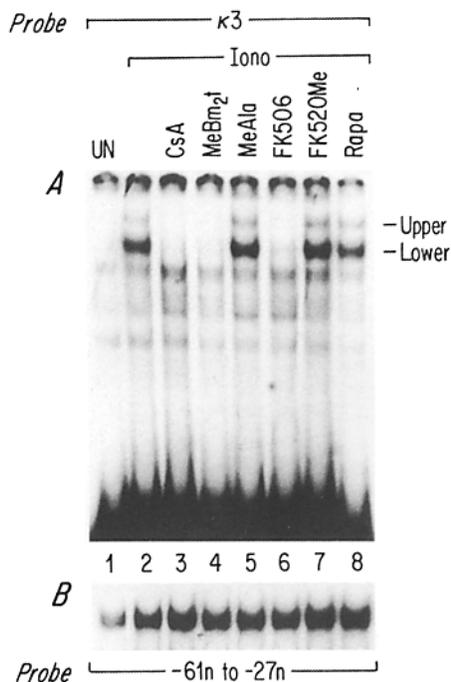
The  $\kappa$ 3 element of the TNF- $\alpha$  promoter, located between -106 to -87 nt relative to the TNF- $\alpha$  transcription start site, is the major mediator of the ionomycin-inducible, CsA-sensitive induction of the gene in T cells (3). To determine whether transcription driven solely by the  $\kappa$ 3 element also depended on calcineurin phosphatase activity, a CAT plasmid containing six copies of the  $\kappa$ 3 element linked to the minimal TNF- $\alpha$  gene promoter was transfected into Ar-5 cells and CAT activity in cells stimulated with ionomycin in the presence or absence of the inhibitors was assessed (Fig. 2 B). Again these experiments showed that the active CsA analogue MeBm<sub>2</sub>t<sup>1</sup>-CsA was a strong inhibitor to inducible CAT activity driven by the  $\kappa$ 3 multimer (Fig. 2 B, lane 4), whereas the inactive analogues MeAla<sup>6</sup>-CsA (Fig. 2 B, lane 5), and FK520Me (Fig. 2 B, lane 7) were not. Moreover, rapamycin did not block induction of CAT activity by ionomycin (data not shown). Identical results were obtained when the cells were stimulated with anti-CD3 (data not shown). Taken together, these data implicate the  $\kappa$ 3 element as a major target of calcineurin during the activation of TNF- $\alpha$  gene transcription in activated T cells.

The  $\kappa$ 3 element forms two DNA-protein complexes in EMSA with proteins that are present in cytosolic extracts from unstimulated T cells or nuclear extracts from stimulated T cells (3). The appearance of these inducible complexes is rapid,

does not require de novo protein synthesis, and is blocked by CsA (3). To test the effect of the CsA and FK506 analogues on binding of nuclear proteins to the  $\kappa$ 3 element, nuclear extracts were prepared from Ar-5 cells that had been pretreated with the analogues before stimulation by ionomycin and an EMSA was performed using the  $\kappa$ 3 element as a probe.



**Figure 2.** (A) Inhibition of  $\alpha$ CD3 induction of the human -199 TNF- $\alpha$ /CAT gene fusion in Ar-5 T cells. Autoradiogram shows results of CAT assays of extracts prepared from Ar-5 cells transfected with the human -199 TNF- $\alpha$ /CAT reporter gene fusion. (B) Inhibition of ionomycin induction of the TNF- $\alpha$  promoter element  $\kappa$ 3 fused to a minimal TNF- $\alpha$  promoter in Ar-5 T-cells. Autoradiogram shows results of CAT assays of extracts prepared from Ar-5 cells transfected with the  $\kappa$ 3 multimer construct ( $\kappa$ 3)<sup>6</sup>-61 TNF- $\alpha$ CAT. Cells were transfected and mock induced (UN) or induced with  $\alpha$ CD3 or ionomycin (Iono) for 18 h in the presence or absence of CsA, MeBm<sub>2</sub>t<sup>1</sup>-CsA (MeBm<sub>2</sub>t), MeAla<sup>6</sup>-CsA (MeAla), FK506, or 15-O-demethyl-FK520 (FK520Me) as indicated. The results are representative of three independent experiments.

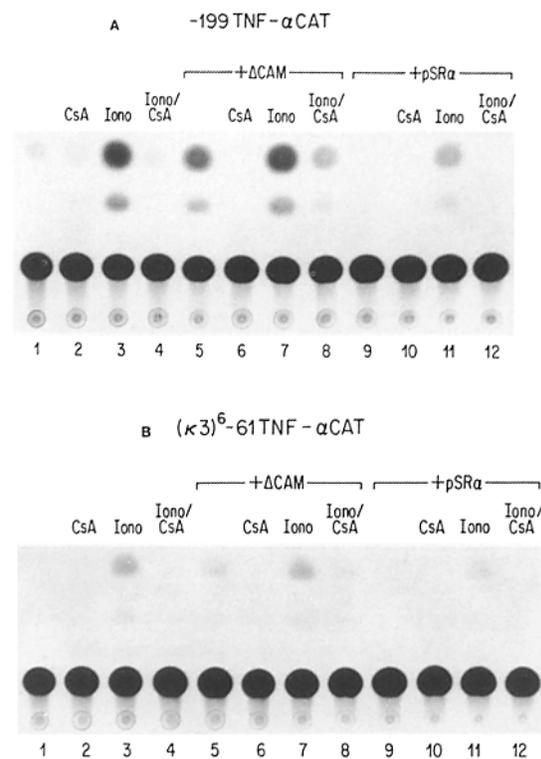


**Figure 3.** Inhibition of ionomycin induction of nuclear  $\kappa 3$  binding complexes. Gel shift experiments with nuclear extracts from ionomycin stimulated and unstimulated Ar-5 T cells using chemically synthesized  $\kappa 3$  (A) or -61n to -27n (B) oligonucleotides as probes. Nuclear extracts were prepared from Ar-5 T cells that had been induced with ionomycin for 30 min in the presence or absence of CsA, MeBm<sub>2t</sub><sup>1</sup>-CsA (MeBm<sub>2t</sub>), MeAla<sup>6</sup>-CsA (MeAla), FK506, or 15-O-demethyl-FK520 (FK520Me), or rapamycin (Rapa) as indicated and described in Materials and Methods. The -61n to -27n probe (B) binds to a constitutive factor and serves as an internal control for the experiment displayed in A.

As shown in Fig. 3, MeBm<sub>2t</sub><sup>1</sup>-CsA (Fig. 3, lane 4), CsA (Fig. 3, lane 3), and FK506 (Fig. 3, lane 6) blocked induction of the nuclear  $\kappa 3$  binding factors in ionomycin-stimulated Ar-5 cells, whereas MeAla<sup>6</sup>-CsA (Fig. 3, lane 5), FK520Me (Fig. 3, lane 7) or rapamycin (Fig. 3, lane 8) do not. Thus, induction of the  $\kappa 3$  binding complex, induction of TNF- $\alpha$  mRNA levels, and induction of CAT activity driven by the -199 TNF- $\alpha$  promoter or the  $\kappa 3$  multimer all show the same pattern of sensitivity to the CsA and FK506 analogues. These data suggest strongly that calcineurin is necessary for the modification event that allows activation of the  $\kappa 3$  binding factor, which in turn is required for TNF- $\alpha$  gene transcription in stimulated T cells. However, they do not rule out the involvement of other calcium-mediated pathways.

To test whether calcineurin-mediated signaling might be sufficient for activation of the  $\kappa 3$  binding factor and TNF- $\alpha$  gene expression in T cells, a second approach was employed. A plasmid that encodes a COOH-terminally truncated calcineurin A chain, which possesses constitutive phosphatase activity (11), was cotransfected with the -199 TNF- $\alpha$  CAT construct into Ar-5 cells. Under these conditions, we observed substantial induction of CAT activity in the absence of any stimulation of the T cells (Fig. 4 A, lane 5). Induction

of TNF- $\alpha$  CAT activity by the coexpressed constitutively active calcineurin was blocked by the addition of CsA at the time of transfection (Fig. 4 A, lane 6), as expected since the catalytic activity of the truncated calcineurin A chain is still inhibited by CsA/CyP complexes (26). Addition of ionomycin at 24 h after transfection resulted in increased expression of CAT activity (Fig. 4 A, lane 7), by an average of 30% in four independent experiments (as quantified by Betascope analysis). This ionomycin-dependent increase in CAT activity was blocked by addition of CsA 10 min before addition of ionomycin (Fig. 4 A, lane 8), suggesting that the effect of ionomycin was to potentiate the activity of endogenous calcineurin rather than to activate independent noncalcineurin-mediated pathways. The residual level of CAT activity seen in Fig. 4 A, lane 8 most likely reflects the accumulation of CAT protein in the 24 h before the addition of ionomycin and CsA. In control experiments, cotransfection of the pSR $\alpha$  expression vector caused a slight decrease in the level of induction of CAT activity (Fig. 4 A, lanes 9–12) when com-



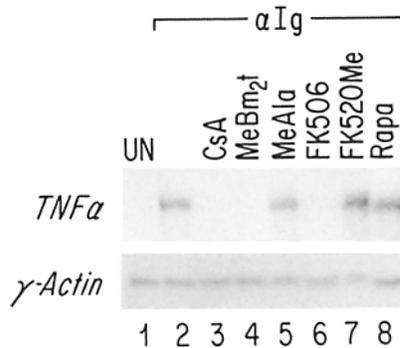
**Figure 4.** Constitutive expression of calcineurin activates TNF- $\alpha$  gene expression and the  $\kappa 3$  element in Ar-5 T cells. (A) Autoradiogram shows results of CAT assays of extracts prepared from Ar-5 cells transfected with the -199 TNF- $\alpha$ CAT gene fusion alone or cotransfected with a plasmid that constitutively expresses the catalytic subunit of calcineurin ( $\delta$ CAM) or a control plasmid (pSR2 $\alpha$ ) as indicated. (B) Autoradiogram shows the results of CAT assays of extracts from cells transfected with ( $\kappa 3$ )<sup>6</sup>-61 TNF- $\alpha$ CAT alone or cotransfected with  $\delta$ CAM or pSR2 $\alpha$  as indicated. Cells were transfected and either mock induced, treated with CsA at the time of transfection, induced with ionomycin (Iono), or treated with CsA 10 min before induction with ionomycin (Iono/CsA). The cells were treated with ionomycin for 18 h before harvest. The results are representative of three independent experiments.

pared with the level of CAT activity in cells transfected with the -199 TNF- $\alpha$  CAT plasmid alone (Fig. 4 A, lanes 1-4). This effect therefore emphasizes the activation of the TNF- $\alpha$  promoter in cells cotransfected with the constitutively active calcineurin (Fig. 4 A, lanes 5-8).

To test whether the  $\kappa 3$  element was indeed the target of calcineurin we cotransfected the  $\kappa 3$  multimer CAT plasmid either with the constitutively active calcineurin or the vector alone (Fig. 4 B). The results paralleled those obtained using the -199 TNF- $\alpha$  CAT construct; the  $\kappa 3$  multimer construct was inducible by calcineurin alone (Fig. 5 B, lane 5) and this induction was blocked by CsA (Fig. 5, lane 6).

To determine whether induction of TNF- $\alpha$  gene expression in activated B cells also requires calcineurin phosphatase activity, we stimulated Akata human B cells with anti-IgG in the presence of the CsA and FK506 analogues. In B cells as in T cells, CsA (Fig. 5, lane 3), MeBm<sub>2</sub>t<sup>1</sup>-CsA (Fig. 5, lane 4), and FK506 (Fig. 5, lane 6), all potently inhibited TNF- $\alpha$  mRNA induction whereas MeAla<sup>6</sup>-CsA (Fig. 5, lane 5), FK520Me (Fig. 5, lane 7) and rapamycin (Fig. 5, lane 8), did not. Taken together, these results demonstrate that calcineurin participates in the induction of TNF- $\alpha$  gene expression in activated B cells as well as in activated T cells.

In conclusion, the data presented establish a pivotal role for calcineurin in the induction of TNF- $\alpha$  gene transcription in both T and B cells stimulated through their surface receptors for antigen. Recently, we have demonstrated that antibodies to NFATp supershift both the upper and lower  $\kappa 3$  binding complexes in T cell nuclear extracts and that both purified and recombinant NFATp binds specifically to the  $\kappa 3$



**Figure 5.** Inhibition of Anti-IgG induction of TNF- $\alpha$  gene expression in Akata B cells. Cells were stimulated with anti-IgG for 30 min, in the presence or absence of CsA or FK506 or the various analogues and RNase mapping of TNF- $\alpha$  and  $\gamma$ -actin mRNAs was carried out as described above

element (McCaffrey, P., A. E. Goldfeld, and A. Rao, manuscript submitted for publication). Taken together with the data presented here, a plausible hypothesis is that calcineurin-mediated dephosphorylation of NFATp or an immunologically cross reactive protein, and its binding to the  $\kappa 3$  TNF- $\alpha$  promoter element, is sufficient to induce the immediate-early expression of the gene in T cells. An interesting question for future investigation is whether similar to its function in T cells, calcineurin activates NFATp or other proteins in B cells, which then bind to the  $\kappa 3$  element to mediate TNF- $\alpha$  gene transcription.

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