

The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes

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Cyclosporin A and FK506 are immunosuppressive compounds that have similar inhibitory effects on the expression of several lymphokines produced by T lymphocytes. Despite their similar effects the drugs bind to two different cytosolic proteins, cyclophilin and FKBP respectively which raises the possibility that they have different modes of action. Using constructs in which mRNA production controlled by a specific transcription factor could be readily measure we found that both cyclosporin A and FK506 completely inhibited transcription activated by NF-AT, NFIL2 A, NFIL2 B and partially inhibited transcription activated by NF κ B. Cyclosporin A and FK506 inhibited only transcriptional activation that was dependent on Ca²⁺ mobilization. However, cyclosporin A and FK506 did not inhibit Ca²⁺ mobilization dependent expression of *c-fos* mRNA indicating that only a subset of signalling pathways regulated by Ca²⁺ is sensitive to these drugs. Furthermore, we did not observe any qualitative differences between the effect of cyclosporin A and FK506 on six different transcription factors which suggests that these drugs may interfere with the activity of a novel Ca²⁺ dependent step that regulates several transcription factors.

Key words: cyclosporin A/FK506/T lymphocyte activation/transcription

Introduction

Activation of T lymphocytes is a complex process requiring recognition of antigen by the T cell antigen receptor as well as concomitant signals from antigen presenting cells in the form of the cytokines interleukin 1 or interleukin 6. These events lead to the activation of second messenger pathways such as tyrosine kinases and protein kinase C and to an increase in intracellular Ca²⁺ (Weiss *et al.*, 1984; Wiskocil *et al.*, 1985; Mustelin *et al.*, 1990). These events on the cell membrane initiate a sequential activation of a group of genes that in turn give rise to proliferation and immunologic function (see Crabtree, 1989 for review). Cyclosporin A, a cyclic undecapeptide, inhibits T cell activation at an early step and thereby interferes with numerous immunoregulatory functions carried out by activated T cells through

lymphokines such as interleukins 2, 3 and 4, γ interferon, GM-CSF and TNF α (Elliot *et al.*, 1984; Bickel *et al.*, 1987; Arai *et al.*, 1989; Tocci *et al.*, 1989). Treatment with cyclosporin A does not prevent the increase in intracellular Ca²⁺ and kinase activation, however, the transcriptional activation of certain genes is inhibited. Recently we found that cyclosporin A, at concentrations with no detectable toxic effect on cells, inhibits the activation of specific transcription factors that regulate genes such as interleukin 2 which are expressed early in T lymphocyte activation (Emmel *et al.*, 1989). These findings placed the action of cyclosporin A at or proximal to the transcription factors involved in gene activation but distal to the known cytoplasmic events in the pathway.

Cyclosporin A binds and inhibits the activity of cyclophilin (Haendler *et al.*, 1987), a peptidyl-prolyl *cis-trans* isomerase (Fisher *et al.*, 1989; Takahashi *et al.*, 1989) that may play a role in the folding of proteins *in vivo* (Lang *et al.*, 1987; Lin *et al.*, 1988). In *Drosophila melanogaster* a gene homologous to cyclophilin, *ninaA*, affects the expression of rhodopsin by a post-translational mechanism which may occur via a putative isomerase activity of *ninaA* protein on the folding of rhodopsin (Schneuwly *et al.*, 1989; Shieh *et al.*, 1989). However, cyclosporin A may mediate its effect by a mechanism that is not directly linked to the inhibition of the isomerase activity of cyclophilin. In *Neurospora crassa* and *Saccharomyces cerevisiae* a protein homologous to cyclophilin mediates the toxic effects of cyclosporin A apparently by forming a toxic complex with cyclosporin A. This is supported by the finding that in *N. crassa* heterokaryons having one mutant allele of cyclophilin and one normal allele die in the presence of cyclosporin A (Tropschug *et al.*, 1989). Furthermore, all the cyclosporin A resistant mutants either lack cyclophilin or have a mutation that prevents binding. Similar inhibitory complexes of cyclophilin and cyclosporin A may mediate the effect of cyclosporin A in the activation of T lymphocytes.

Recently another potent immunosuppressant was discovered, FK506, that has biologic effects very similar to cyclosporin A but is a macrolide with a different chemical structure than cyclosporin A (Sawada *et al.*, 1987). Remarkably, FK506 also binds to a peptidyl-prolyl *cis-trans* isomerase, FKBP, and inhibits its activity (Harding *et al.*, 1989; Siekierka *et al.*, 1989), however, FKBP has no sequence similarity to cyclophilin (Maki *et al.*, 1990; Standaert *et al.*, 1990). This raises the possibility of dissecting the signalling pathways leading to activation of individual transcription factors by virtue of sensitivity to cyclosporin A or FK506. We found that the effects of the two drugs were similar on each of the four transcription factors that were inhibited and on each of the two transcription factors that were not inhibited. Furthermore, the distinguishing feature of the transcription factors affected by cyclosporin A and FK506 is that they require Ca²⁺ mobilization for their activation. These results suggest that

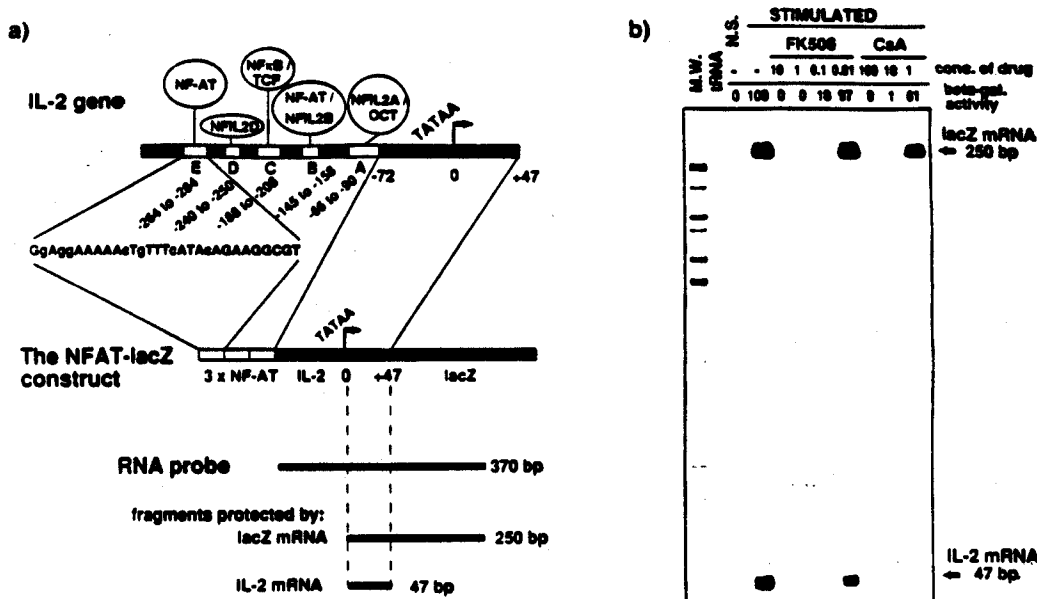


Fig. 1. (a) The map of the 5' region of the interleukin 2 (IL-2) gene and the structure of the NFAT-lacZ construct. Sites which bind proteins believed to contribute to transcriptional control of interleukin 2 are noted with the names of the putative binding proteins (Durand et al., 1988). The NFAT-lacZ construct contains the *E. coli lacZ* β -galactosidase gene attached to position +47 of the interleukin 2 which precedes the translational start site for interleukin 2. A trimer of the NF-AT binding site (-286 to -257 of interleukin 2) is ligated to a minimal interleukin 2 promoter at -72. All three NF-AT sites are oriented 5'-3'. The location of the RNA probe (370 bp) relative to the NFAT-lacZ construct is presented as a black line and the lower lines present fragments of the RNA probe protected by lacZ RNA (250 bp) and interleukin 2 RNA (47 bp). Lower case letters indicate NF-AT contact sites determined by a methylation interference assay (Durand et al., 1988). (b) FK506 and cyclosporin A inhibit the transcriptional activity of NF-AT and transcription of interleukin 2. Jurkat cells stably transfected with the NFAT-lacZ construct were stimulated for 5 h with ionomycin (2 μ M) and phorbol myristate acetate (32 nM) in the presence of the indicated concentrations of FK506 or cyclosporin A (nM). Transcription of lacZ mRNA from the NFAT-lacZ construct was measured by a ribonuclease protection assay using the RNA probe described in (a). Interleukin 2 mRNA can be measured on the same lane of the gel since the ribonuclease protection probe contains 47 bp from the 5' untranslated region of interleukin 2. β -galactosidase activity is presented as percent of maximal activity and it correlates with the intensities of the protected bands representing lacZ mRNA. NS, no stimulation; CsA, cyclosporin A. Molecular weight markers (M.W.) are *MspI* digested pBR322 DNA (New England Biolabs).

cyclophilin and FKBP affect a common signal transduction pathway that requires an increase in intracellular Ca²⁺ for the activation of several transcription factors involved in gene regulation during T lymphocyte activation.

Results

Cyclosporin A and FK506 inhibit the activity of several transcription factors

Changes in gene expression which mediate T cell activation are related to induction of several transcription factors such as *c-fos* (Reed et al., 1986) and NF κ B (Leung and Nabel, 1988). Activation of the interleukin 2 gene which is a growth factor for T lymphocytes requires the activity of several transcription factors as illustrated in Figure 1a (Shaw et al., 1988; Serfling et al., 1989). To study the influence of cyclosporin A and FK506 on the activity of individual transcription factors induced during T lymphocyte activation the human leukemic cell line Jurkat was stably transfected with plasmids that have multiple copies of specific binding sites for transcription factors controlling the expression of *Escherichia coli lacZ* β -galactosidase gene. We chose to analyze the transcription factor binding sites NFIL2A, NFIL2B and NF-AT from the interleukin 2 gene. Although interleukin 2 gene has been reported to contain NF κ B and AP-1 binding sites these binding sites may also be occupied by other transcription factors and therefore we chose to analyze the NF κ B binding site from the immunoglobulin λ

light chain and the AP-1 binding site from the metallothionein gene which are more thoroughly characterized. The interleukin 2 minimal promoter was used in most of the constructs since it has very low activity by itself but still gives high expression when linked to enhancer elements (Verweij et al., 1990). The AP-1 construct was ligated to SV40 minimal promoter which contains six SP-1 sites and it therefore presents transcription controlled by both AP-1 and SP-1 binding factors. The NFIL2A construct contained the TATA box from the γ fibrinogen promoter but very similar results can be obtained with a construct that has NFIL2A binding sites linked to interleukin 2 minimal promoter (unpublished observations). Three copies of each binding site linked to a promoter construct were used except in the case of NFIL2A and NFIL2B constructs where four copies were used because they gave a higher level of expression. The transfected plasmids contain a hygromycin resistance gene and stable transfectants were selected in the presence of hygromycin. Hygromycin resistant clones that had little or no constitutive expression but expressed high levels of β -galactosidase upon stimulation with phorbol ester and calcium ionophore were selected and cloned with the fluorescence activated cell sorter (FACS) using the fluorogenic substrate for β -galactosidase, fluorescein-digalactoside (Nolan et al., 1988).

NF-AT directed transcription was assayed in a clone of Jurkat cells having one copy of a construct with three NF-AT binding sites controlling the expression of the lacZ gene

(Figure 1a). *lacZ* RNA in this cell line was detected only after stimulation (Figure 1b). This inducible expression of *lacZ* mRNA was inhibited in a dose dependent manner by both cyclosporin A and FK506. The size of the RNA probe fragment protected by *lacZ* mRNA in the ribonuclease protection assay indicated that the NF-AT binding sites ligated to promoter sequences from interleukin 2 (-72 to +47) directed correctly initiated transcripts at the interleukin 2 gene initiation site (Figure 1a). Interleukin 2 mRNA can be measured in the same lane of the gel since the ribonuclease protection probe contains 47 bp from the 5' untranslated region of interleukin 2 (Figure 1a and b). Furthermore, since the ribonuclease probe used to measure interleukin 2 and *lacZ* transcripts is identical, direct comparisons can be made between the activity of the transfected gene and the activity of the endogenous interleukin 2 gene. The intensities of the bands representing *lacZ* mRNA correlate well with the measured β -galactosidase enzymatic activities (Figure 1b).

Similar results were obtained when the transcriptional activity of the entire interleukin 2 enhancer was investigated by analysis of induced β -galactosidase activity encoded by *lacZ* (Figure 2). Both cyclosporin A and FK506 also inhibited in a dose dependent manner the ability of the NFIL2A, NFIL2B and NF κ B binding sites to activate transcription (Figure 2). The transcription from a construct in which sites for both AP-1 and SP-1 are controlling transcription of *lacZ* was not significantly affected by cyclosporin A or FK506. A 50% inhibition of transcription from constructs sensitive to these drugs was achieved with ~10 nM cyclosporin A and 0.1 nM FK506 (Figure 2). These dose-response relationships are consistent with previous reports that FK506 is 100-fold more effective than cyclosporin A and are similar to the concentrations required to block T cell activation (Sawada *et al.*, 1987).

FK506 inhibits the DNA binding activity of NF-AT

Electrophoretic mobility shift assays were performed to examine the effect of cyclosporin A and FK506 on the binding activity of NF-AT, NFIL2B and AP-1 transcription factors (Figure 3). NF-AT binding activity was inhibited by cyclosporin A as demonstrated previously by Emmel *et al.* (1989) and it was also inhibited by FK506 (Figure 3a). Electrophoretic mobility shift assay with the NFIL2B binding site gave rise to an inducible retarded band that was not inhibited by cyclosporin A or FK506 although the transcriptional activity was inhibited (Figure 3b). The DNA binding activity of AP-1 was not inhibited by FK506 (Figure 3c).

Only Ca^{2+} regulated activities are inhibited by cyclosporin A and FK506

T lymphocytes require the concordant actions of two signals derived from the antigen receptor and interleukin 1 or interleukin 6 for activation. These pathways can to some extent be mimicked by stimulation with a combination of calcium ionophore and phorbol ester. Transcription of *lacZ* mRNA directed by three copies of the NF κ B site (Figure 4a) was stimulated with phorbol ester alone and this phorbol ester induced activity was completely resistant to FK506 (Figure 4b). However, when cells were stimulated with phorbol ester together with ionomycin the transcriptional activity of NF κ B was further enhanced 4-fold. The increased level of NF κ B mediated transcription induced by calcium ionophore was almost completely inhibited by FK506.

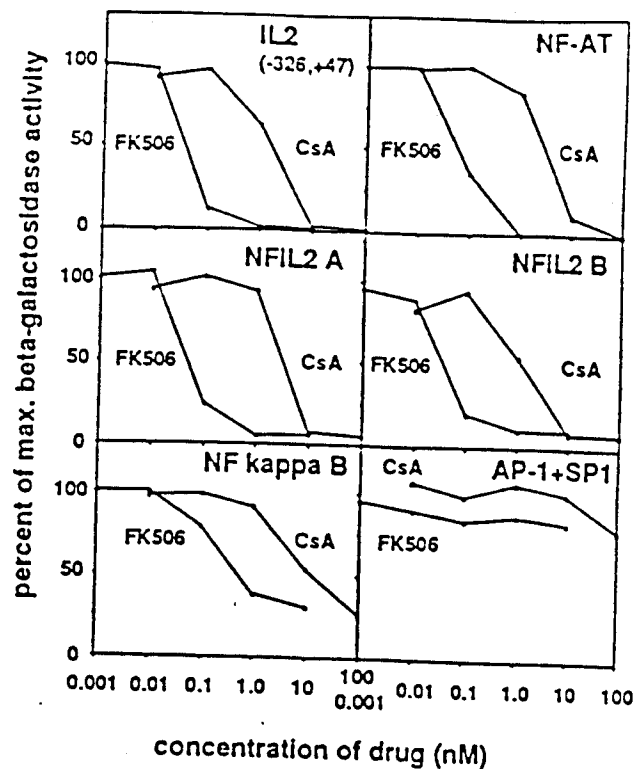


Fig. 2. Dose-response to cyclosporin A and FK506 of transcription mediated by individual transcription factors. Jurkat cell lines were stimulated for 5 h with 2 μ M ionomycin and 32 nM phorbol myristate acetate in the presence of the indicated concentrations of cyclosporin A or FK506. Graphs in different boxes present β -galactosidase activities of different clones stably transfected with constructs containing *E. coli lacZ* β -galactosidase gene in the transcriptional control of various transcription factors. IL2, *lacZ* transcription controlled by bases -326 to +45 from interleukin 2 gene (proteins contributing to transcription from this region are described in Figure 1A); NF-AT, *lacZ* transcription controlled by three copies of NF-AT binding site (-286 to -257 of interleukin 2) linked to -72 of the interleukin 2 gene promoter (described in Figure 1A); NFIL2A, *lacZ* transcription controlled by four copies of NFIL2A binding region (-93 to -65 of interleukin 2) linked to a minimal γ -fibrinogen promoter; NFIL2B, *lacZ* transcription controlled by four copies of NFIL2B binding region (-159 to -134 of interleukin 2) linked to the interleukin 2 promoter; NF κ B, *lacZ* transcription controlled by three copies of the NF κ B site of the mouse κ light chain gene linked to the interleukin 2 promoter; AP-1+SP-1, *lacZ* transcription controlled by three copies of the AP-1 binding site from the human metallothionein gene linked to the SV40 promoter that contains six SP-1 sites (Lee *et al.*, 1987). β -galactosidase activities are presented as percent of the maximal activity of each cell line.

(Figure 4b). These effects on the transcriptional activity directed by the NF κ B site were paralleled by similar changes in DNA binding activity (Figure 4c). The effect of cyclosporin A on the transcriptional activity of NF κ B was similar to the effect of FK506 (Figure 5a). The sensitivity of NF κ B mediated transcription to calcium ionophore treatment may be specific for T lymphocytes since in a pre-B cell line 70Z/3 we did not detect any augmentation of the transcriptional activity of NF κ B by ionomycin treatment (Figure 5b). Furthermore, NF κ B was not sensitive to cyclosporin A or FK506 in 70Z/3 cells (Figure 5b).

An association between Ca^{2+} mobilization and the effect of cyclosporin A and FK506 was also observed with other transcription factors. A small but significant transcriptional activity of NF-AT was generated by ionomycin treatment alone and this activity was inhibited by cyclosporin A and FK506 (Figure 5a). Transcriptional activities of the

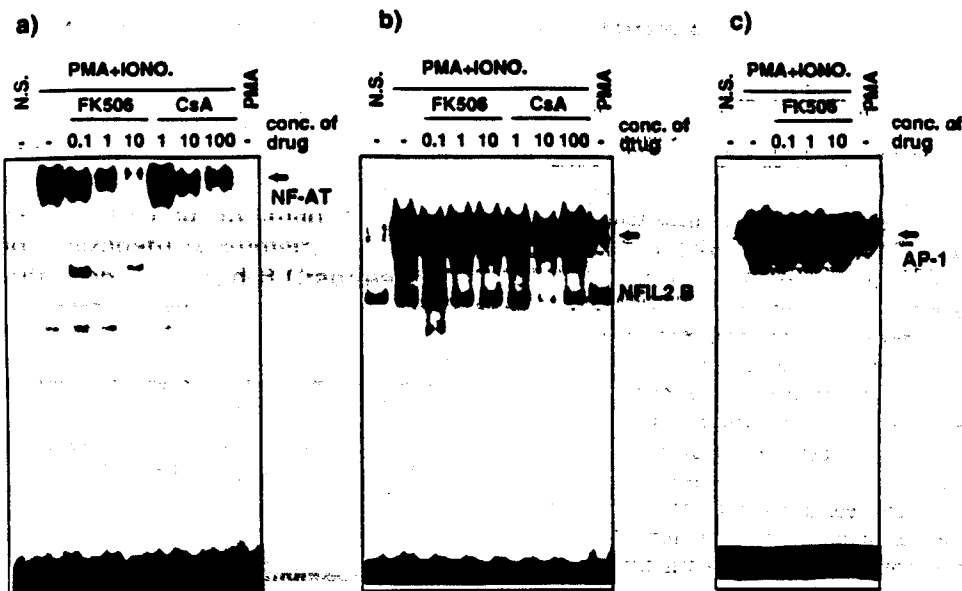


Fig. 3. The effect of FK506 and cyclosporin A on the development of DNA binding activities of NF-AT (a), NFIL2B (b) and AP-1 (c). Jurkat cells were stimulated for 5 h in the presence of the indicated concentrations of cyclosporin A and FK506 (nM) and the DNA binding activity was measured with an electrophoretic mobility shift assay. NS, no-stimulation; PMA+IONO., stimulated with 2 μ M ionomycin and 32 nM phorbol myristate acetate; CsA, cyclosporin A; PMA, stimulated with 32 nM phorbol myristate acetate. Inducible bands are indicated with arrows.

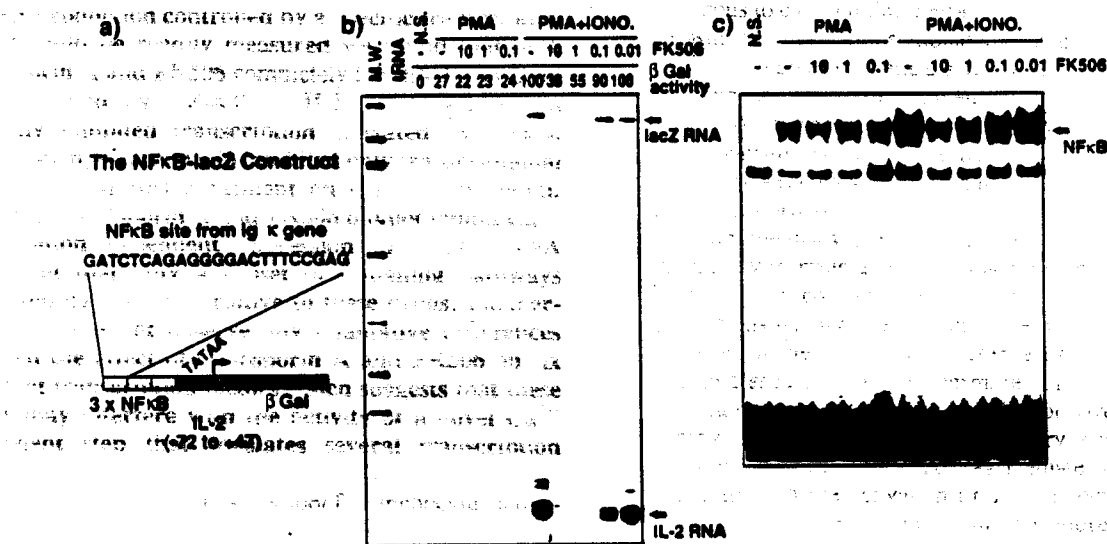


Fig. 4. (a) The NF κ B-lacZ construct has three copies of the NF κ B binding site linked to the interleukin 2 promoter (-72 to +45) directing transcription of the *E. coli lacZ* β -galactosidase gene. (b) Ca²⁺ dependent transcriptional activity of NF κ B is inhibited by FK506. Jurkat cells stably transfected with the NF κ B-lacZ construct were stimulated for 5 h either with phorbol myristate acetate alone or together with calcium ionophore in the presence of the indicated concentrations of FK506 (nM). Transcription from the NF κ B-lacZ construct was measured by a ribonuclease protection assay. The size of the probe sequence protected by lacZ mRNA (173 bp) indicates correct initiation from the transcription initiation site inside the promoter fragment from the interleukin 2 gene. β -galactosidase activity is presented as percent of maximal activity. NS, no stimulation; PMA, 32 nM phorbol myristate acetate; PMA+ionomycin, 32 nM phorbol myristate acetate and 2 μ M ionomycin. Molecular weight markers (M.W.) are *Msp*I digested pBR322 DNA (New England Biolabs). (c) Ca²⁺ dependent DNA binding activity of NF κ B is inhibited by FK506. Jurkat cells were stimulated as described above and the DNA binding activity was measured using an electrophoretic mobility shift assay. The NF κ B specific bands are indicated by an arrow. The abbreviations are as in the legend to Figure 4B.

interleukin 2 control region from -326 to +47, NFIL2A and NFIL2B required stimulation with both ionomycin and phorbol ester as did the full transcriptional activity of NF-AT and all these activities were completely inhibited by cyclosporin A and FK506 (Figure 5a).

Ca²⁺ mobilization dependent activation of c-fos mRNA expression is not inhibited by cyclosporin A or FK506

Not all signalling pathways regulated by increased intracellular Ca²⁺ were inhibited by cyclosporin A or FK506.

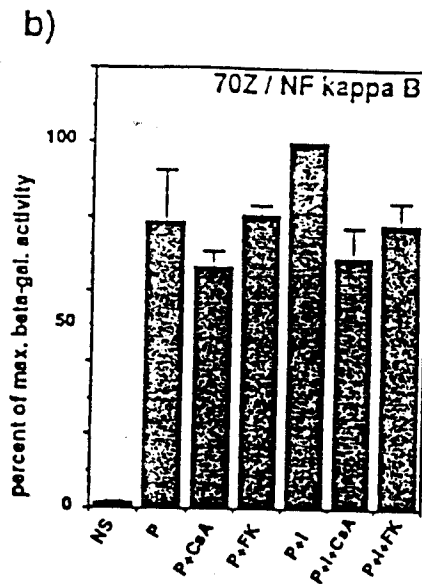
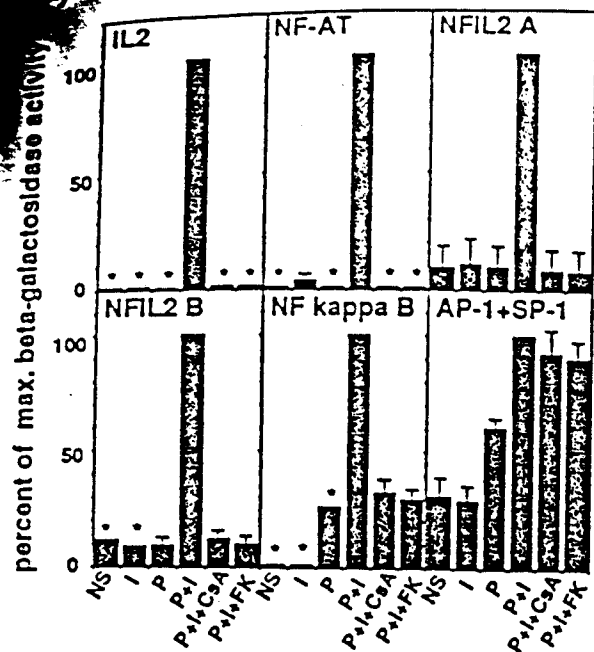


Fig. 5. (a) Stimulation requirements for the activation of transcription by NF-AT, NFIL2A, NFIL2B, NFκB, AP-1 and SP-1 transcription factors and by a control region of interleukin 2 gene. Jurkat cells stably transfected with constructs described in the legend to Figure 2 containing various regions controlling the transcription of *E. coli lacZ* were stimulated for 5 h in the presence of the indicated drugs. The bars present the mean of β-galactosidase activities of three different clones derived from independent transfections except in the case of NFIL2B (two independent clones) and AP-1 (one clone). β-galactosidase activity is presented as the percentage of the maximal activity of each cell line. Error bars present standard deviations, a star indicates a standard deviation <3%. NS, no stimulation; I, 2 μM ionomycin; P, 32 nM phorbol myristate acetate; CsA, 100 nM cyclosporin A; FK, 10 nM FK506. (b) NFκB activity in a pre-B cell line 70Z/3 is not sensitive to calcium ionophore, cyclosporin A or FK506 treatment. The bars present the mean of β-galactosidase activities of two different clones. The two clones were stably transfected with the NFκB-*lacZ* construct (Figure 4a) and the clones were derived from independent transfections. Error bars present standard deviations. Abbreviations are explained in the legend to Figure 5a.

Ca²⁺ ionophore enhanced the expression of *c-fos* mRNA by ~3-fold over the induction seen with phorbol ester alone. However, cyclosporin A and FK506 had no effect on the level of *c-fos* expression (Figure 6). In addition, FK506 did not affect the calcium ionophore dependent DNA binding activity of AP-1 (Figure 3c). The AP-1 binding site is recognized by a heterodimer of *c-jun* and *c-fos* which leads to the activation of transcription (Halazonetis *et al.*, 1988). The finding that the Ca²⁺ regulated transcriptional activity of AP-1 was not affected by cyclosporin A or FK506 is consistent with the finding that the Ca²⁺ mobilization dependent expression of *c-fos* mRNA was not inhibited by FK506 (Figure 6).

Requirement of protein synthesis for transcriptional activities inhibited by cyclosporin A and FK506

We investigated whether protein synthesis is required for the signalling pathway inhibited by cyclosporin A and FK506. Ribonuclease protection assays were performed to quantitate *lacZ* mRNA transcribed under the control of NFIL2A, NFIL2B and NFκB transcription factors in the presence of a protein synthesis inhibitor, anisomycin. Transcriptional activities of NFIL2A and NFIL2B were inhibited by anisomycin as quantitated by *lacZ* mRNA transcribed under the control of NFIL2A or NFIL2B transcription factors (Figure 7b and c). Figure 7 also shows that interleukin 2 mRNA expression induced by Ca²⁺ mobilization and phorbol ester is blocked by inhibition of protein synthesis as has previously been demonstrated (Shaw

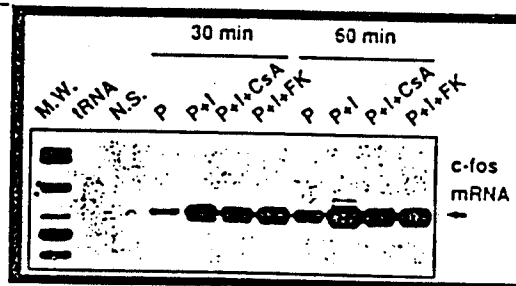


Fig. 6. Cyclosporin A or FK506 do not inhibit calcium ionophore mediated signals to *c-fos* mRNA expression. Jurkat cells were stimulated for 30 and 60 min in the presence of cyclosporin A and FK506. The expression of *c-fos* mRNA was analyzed by ribonuclease protection. NS, no stimulation; P, 32 nM phorbol myristate acetate; P+I, 32 nM phorbol myristate acetate and 2 μM ionomycin; P+I+CsA, P+I and 100 nM cyclosporin A; P+I+FK, P+I and 10 nM FK506. Molecular weight markers (M.W.) are *MspI* digested pBR322 DNA (New England Biolabs).

et al., 1988). The transcriptional activity of NFκB produced by phorbol ester treatment was not inhibited by anisomycin (lanes 3 and 4, Figure 7a) but the Ca²⁺ dependent transcriptional activity produced by treatment with phorbol ester together with ionomycin was significantly inhibited by anisomycin (lanes 7 and 8, Figure 7b). However, FK506 was able further to inhibit the transcriptional activity of NFκB even in the presence of anisomycin (lanes 8 and 10, Figure 7a).

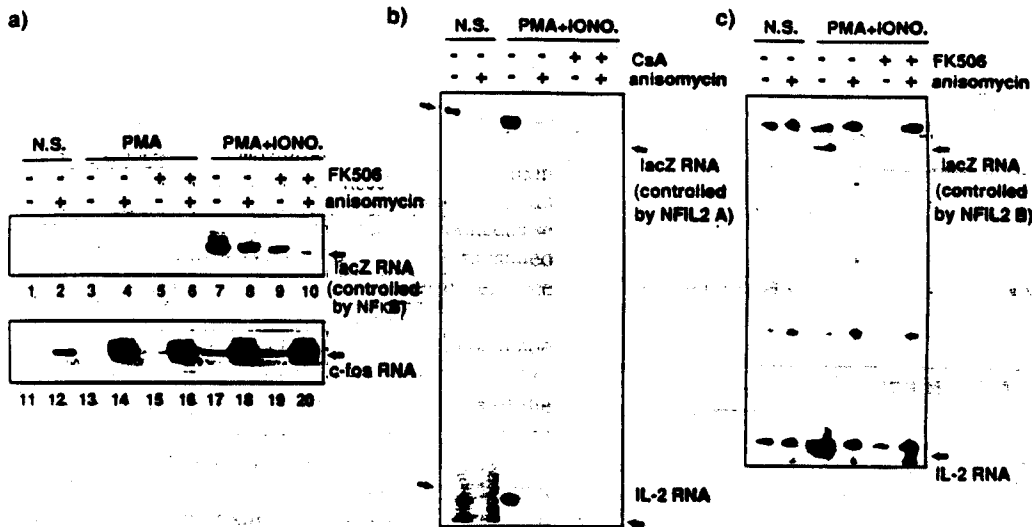


Fig. 7. (a) Ca^{2+} dependent transcriptional activity of NF κ B requires new protein synthesis. Jurkat cells that contained stable integrants of the NF κ B-*lacZ* construct (described in Figure 4a) were stimulated for 90 min with phorbol ester or with phorbol ester together with calcium ionophore in the presence or absence of FK506 and anisomycin. The amount of *lacZ* and *c-fos* mRNA was quantitated by a ribonuclease protection assay. In each instance anisomycin treatment increased the amount of *c-fos* mRNA indicating inhibition of protein synthesis (Greenberg *et al.*, 1986). NS, no stimulation; PMA, 32 nM phorbol myristate acetate; PMA+ionophore, 32 nM phorbol myristate acetate and 2 μ M ionomycin; FK506, 10 nM FK506; anisomycin, 100 μ M anisomycin. (b and c) Protein synthesis is required for the transcriptional activity of NFIL2A and NFIL2B. (b) Jurkat cells with stable integrants of *lacZ* transcribed under the control of four copies of the NFIL2A binding site linked to the γ fibrinogen promoter and (c) cells with stable integrants of *lacZ* transcribed under the control of four copies of the NFIL2B binding site linked to interleukin 2 minimal promoter were stimulated for 90 min with phorbol ester and calcium ionophore in the presence or absence of anisomycin and cyclosporin A or FK506. *lacZ* mRNA was quantitated by a ribonuclease protection assay. Increased levels of *c-fos* RNA were also seen in those samples treated with anisomycin (data not shown). CaA, 100 nM cyclosporin; other abbreviations are as for (a) above.

Discussion

We have shown that both cyclosporin A and FK506 inhibit the activity of several transcription factors involved in the activation of T lymphocytes and that the effects of these compounds are indistinguishable except for a consistent 100-fold difference in their effective concentration. Fifty percent inhibition of transcription was observed at concentrations of cyclosporin A and FK506 that are close to their binding constants to cyclophilin and FKBP, respectively, supporting previous reports which suggest that cyclophilin and FKBP mediate the effects of cyclosporin A and FK506, respectively (Handschumacher *et al.*, 1984; Harding *et al.*, 1989; Siekierka *et al.*, 1989).

All the transcriptional activities that were inhibited by cyclosporin A and FK506 required increased intracellular Ca^{2+} . The strong correlation between a requirement for Ca^{2+} mobilization and sensitivity to cyclosporin A and FK506 suggests that these drugs interfere with the activity of a Ca^{2+} regulated signalling pathway. However, only a subset of Ca^{2+} regulated signalling pathways is affected by cyclosporin A or FK506 since the Ca^{2+} regulated expression of *c-fos* mRNA was not inhibited by these drugs.

The Ca^{2+} mobilization-dependent transcriptional activity of NF κ B was inhibited by cyclosporin A and FK506 and it was also partially inhibited by a protein synthesis inhibitor. Previously the transcriptional activity of NF κ B has been reported to be independent of new protein synthesis (Sen and Baltimore, 1986). Signal transduction through NF κ B can be initiated by the activation of protein kinase C which leads to the dissociation of NF κ B from I κ B in the cytoplasm and to the translocation of active NF κ B into the nucleus, a process which does not require new protein synthesis

(Bauerle and Baltimore, 1988). It is possible that co-stimulation with calcium ionophore and phorbol ester leads additionally to the transcription of new NF κ B or to the transcription of a factor similar to NF κ B. This effect may be specific for T lymphocytes since we did not detect such a Ca^{2+} dependent augmentation in transcriptional activity of NF κ B in the pre B cell line, 70Z/3. Although the activity of some subspecies of protein kinase C are sensitive to Ca^{2+} (Nishizuka, 1988) the finding that the Ca^{2+} dependent activity of NF κ B required new protein synthesis indicates that a direct activation of protein kinase C by increased intracellular Ca^{2+} does not completely explain the observed Ca^{2+} dependent activity of NF κ B in T lymphocytes. Recently, Schmidt *et al.* (1990) have also reported that the activation of NF κ B is inhibited by cyclosporin A in a signal dependent manner.

Since cyclosporin A and FK506 inhibit the peptidyl-prolyl *cis-trans* isomerase activities of cyclophilin and FKBP respectively, the inhibited isomerase activities might mediate the effects of cyclosporin A and FK506. Proline *cis-trans* isomerism has been proposed to regulate other processes, namely the activity of transport proteins (Brandl and Deber, 1986), binding of concanavalin A to sugar residues (Brown *et al.*, 1977) and binding of prothrombin fragment 1 to phospholipid (Marsh *et al.*, 1979). Peptidyl-prolyl *cis-trans* isomerase activity has been reported to affect the folding of proteins *in vitro* (Lang *et al.*, 1987; Lin *et al.*, 1988) and thus it may be involved in the refolding of new proteins *in vivo* after synthesis on the ribosome. If this is the case, the effect of cyclosporin A and FK506 may therefore be associated with events that require new protein synthesis. Although transcriptional activities of NF-AT (Shaw *et al.*,

1988). NFIL2A and NFIL2B did require new protein synthesis we did observe a small inhibitory effect of FK506 in the presence of the protein synthesis inhibitor, anisomycin, on the transcriptional activity of NF κ B. This suggests that the inhibitory effect of FK506 on transcription may not always require new protein synthesis. However, it is still possible that anisomycin did not completely inhibit protein synthesis.

Recently we have shown that rapamycin, which is structurally related to FK506, binds to FKBP with a binding affinity similar to that of FK506 and also inhibits the isomerase activity of FKBP (Bierer *et al.*, 1990). In contrast to FK506, rapamycin does not inhibit T cell activation induced by signals from the antigen receptor but, on the contrary, when added in 500-fold excess reverses the effect of FK506 on interleukin 2 production (Dumont *et al.*, 1990) and on all the transcription factors that were inhibited by FK506 (P. Matilla and G. Crabtree, unpublished results). These results do not favor the involvement of the isomerase activity in signal transduction in T lymphocytes but rather can be explained by a model in which FK506 forms an inhibitory complex with FKBP that interferes with signal transduction of T lymphocytes. A similar model which includes a complex of a protein homologous to cyclophilin and cyclosporin A has previously been demonstrated for cyclosporin A mediated toxicity in *N. crassa* and *S. cerevisiae* (Tropschug *et al.*, 1989). Since cyclophilin and FKBP are proline isomerases and thus may be involved in the folding of several proteins they may have an intrinsic characteristic of interacting with many different proteins. Binding to cyclosporin A and FK506 could give them a new specificity to interfere with protein(s) important in signal transmission in T lymphocytes.

Cyclophilin and FKBP may directly interact with all four different transcription factors inhibited by cyclosporin A and FK506. This is made less likely by the observation that we did not find a single transcription factor among six tested (NF-AT, NFIL2A, NFIL2B, NF κ B, AP-1 or SP-1) that was inhibited by one but not by both cyclosporin A and FK506. This suggests that a novel Ca²⁺ dependent step proximal to the transcription factors in signal transduction of T lymphocytes is the target of both cyclosporin A and FK506.

Materials and methods

Plasmids

The *E. coli lacZ* β -galactosidase gene was a *Bam*HI fragment of PMC1871 (Shapira *et al.*, 1983) and was initially cloned into the *Bam*HI site of SP65 (Promega). The initiation of translation was derived from the MoMuLV envelope gene and was cloned by inserting a synthetic sequence CTAGAC-TGACATGGCGGATC between the *Xba*I and *Bam*HI sites of SP65. This construct, called SP6- β gal, was constructed by Constance Cepco (Harvard Medical School, Boston) and Jack Price (Mill Hill MRC, England). Polyadenylation sequence and small t intron of SV40 was derived from a *Sac*I and *Hind*III fragment of pko-neo (van Doren *et al.*, 1984). An *Xho*I linker was inserted inside this fragment into a *Bam*HI site 3' of the polyadenylation sequence. The fragment was blunt ended by Klenow fragment and ligated to the *Sma*I site of SP65. The resulting plasmid, called SP6- β galpolyA, originated from Jacik Showronski (Cold Spring Harbour, New York). The hygromycin resistance gene under the transcriptional control of herpes simplex virus thymidine kinase promoter was derived as a blunt ended *Nar*I-*Nru*I fragment from pHYg (Sugden *et al.*, 1985). This fragment was ligated to the *Sma*I site of pTZ19 (Pharmacia) and a *Bgl*II linker was inserted to a *Sac*I site filled in by Klenow fragment of the pTZ19. The *lacZ* with the inserted initiation of translation and polyadenylation sequences was cut out from SP6- β galpolyA with *Hind*III and *Xho*I and ligated between the *Hind*III and *Sac*I sites of the modified pTZ19. The resulting plasmid

is called pLacZH and it has the *lacZ* gene 5' of the hygromycin resistance gene. The orientations of transcription of both genes are the same.

The fragment carrying the *lacZ* and hygromycin resistance genes was cleaved from pLacZH and *Hind*III and *Bgl*II and inserted in the place of chloramphenicol acetyltransferase gene which was removed with *Hind*III and *Bam*HI from the following plasmids: pIL2CAT (Durand *et al.*, 1988) which has sequences -326 to +47 from interleukin 2 gene controlling the expression of chloramphenicol acetyltransferase gene, NFAT.22.6 which has three copies of the NF-AT binding site linked to the interleukin 2 promoter (-72 to +47 of interleukin 2), pA 4.1 which has four copies of the NFIL2A binding site linked to γ fibrinogen minimal promoter (Durand *et al.*, 1988), X₃CAT which has three copies of the AP-1 binding site from the metallothionein gene enhancer linked to the SV40 minimal promoter (Lee *et al.*, 1987), KBCAT which has three copies of the human immunoglobulin κ light chain κ B site ligated to -72 of interleukin 2 minimal promoter and NFIL2BCAT which has four copies of NFIL2B binding site from interleukin 2 gene linked to interleukin 2 minimal promoter. The resulting plasmids were called pIL2ZH, NFATZH, NFIL2AZH, X₃ZH, KBZH and NFIL2BZH respectively. NFAT.22.6 was constructed by Cor Verweij by inserting three copies of the NF-AT binding sequence (-286 to -257 of interleukin 2 gene) to the *Xho*I site of p22/6 which was a deletion mutant from pIL2CAT and had sequences between -296 and -72 relative to interleukin 2 transcription start site deleted and a *Xho*I linker inserted in place of the deletion (Verweij *et al.*, 1990). KBCAT was constructed by annealing oligonucleotides gatCTCAGAGGGGACTTCCGAG and gatCTCGGAAAGTCCCCTCTGA and ligating the annealed product with itself. The ligation product was filled in with Klenow enzyme and ligated to the filled in *Xho*I site of p22/6. The resulting plasmid contained three copies of the κ B site, the two 5' ones in the reverse orientation in respect to the orientation of the κ B sites in the κ immunoglobulin gene. NFIL2BCAT was constructed by annealing oligonucleotides tcgaCAATCCAAAGAGTCATCAGAAGAGGAC and tcgaGTCCTCTTCTGATGACTCTTTGGAATTG and ligating the double stranded sequence into the *Xho*I site of p22/6. NFIL2BCAT has four copies of the NFIL2B binding site linked to the minimal interleukin 2 promoter in the same orientation as the NFIL2B site in the interleukin 2 gene. The orientations of the inserted genes were confirmed with sequencing. The resulting plasmids were linearized for electroporation with *Kpn*I which cuts immediately 3' of the hygromycin resistance gene.

Transfections and selection of clones

Cell lines with stable integrants of the constructs were established by electroporating linearized plasmids into Jurkat cells and selecting with hygromycin. The cells were maintained in complete RPMI 1640 (Gibco) supplemented with 10% v/v fetal calf serum, 100 U/ml penicillin and 50 U/ml streptomycin in a 5% CO₂/95% air atmosphere. Ten million cells were electroporated with 20 μ g of linearized plasmid in 1 ml RPMI 1640 using a 250 V and 960 μ F electric shock (Gene Pulser, Bio-Rad). Immediately after electroporation cells were suspended in 24 ml of tissue culture media and 1 ml aliquots were added into a 24 well plate. The following day hygromycin was added in 1 ml of culture media to each well to give a final concentration of 200 μ g/ml of hygromycin (Calbiochem, CA). Hygromycin containing media was changed periodically until resistant cells grew out which usually occurred in 2 weeks. Clones expressing high levels of *lacZ* after stimulation were selected and cloned using fluorescence activated cell sorter (FACS) and the fluorogenic substrate fluorescein-di-galactoside (Nolan *et al.*, 1988). The following cell clones were used: IL2.2A, IL2.7B, IL2.11A (transfected with IL2ZH); NFAT.4B, NFAT.5A (transfected with NFATZH); NFIL2A.4.4, NFIL2A.11.5, NFIL2A.20.9 (transfected with NFIL2AZH); NFIL2B.6.1, NFIL2B.11.2 (transfected with NFIL2BZH); KB.2.2, KB.5.2, KB.7.2 (transfected with KBZH) and X.7.1 (transfected with X₃ZH). The cell line J.NFATZ.1 that was transfected with a construct containing three copies of NF-AT binding sites controlling the expression of *lacZ* is described by Fiering *et al.* (1990). Cell lines J.NFATZ.1, KB.5.2, NFIL2B.11.5 and NFIL2B.6.1 were used for ribonuclease protection assays.

Stimulation of cells

All stimulations were done with exponentially growing cells whose density was not greater than 0.5 million cells/ml. Cells were stimulated in fresh tissue culture media at a density no higher than 0.7 million cells/ml. Stock solutions of phorbol myristate acetate (Sigma) and ionomycin (Calbiochem) were prepared in dimethylsulfoxide and stock solutions of cyclosporin A, FK506 and anisomycin were prepared in ethanol and stored at -20°C.

Ribonuclease protection assay

KSGal which was a template for the *lacZ* RNA probe was constructed by

a *XhoI*-*PvuII* fragment from NFATZH which contains promoter sequences from interleukin 2 (from -72 to +47) and *lacZ* sequences. *KSgl* was linearized with *KpnI* and the RNA probe was synthesized with T7 RNA polymerase. The probe was 310 bp long and a 172 bp fragment was protected by *lacZ* mRNA. This probe also protected a 47 bp fragment of endogenous interleukin 2 mRNA. The probe was used to detect *lacZ* and interleukin 2 mRNA in all cell lines except in J:NFATZ.1 which contained a different translational initiation site for *lacZ*. The template used for the RNA probe for detecting *lacZ* RNA in this cell line is described by Fiering et al. (1990). The probe was 370 bp long and the fragment protected by *lacZ* mRNA was 250 bp long. The template for *c-fos* riboprobe was constructed by cutting a 200 bp *NsiI*-*Tth1111* fragment from the 3' untranslated region of the human *c-fos* gene and cloning this fragment into *PstI* and *SmaI* sites of SP63 (Promega) by first filling in the *Tth1111* site by Klenow enzyme. The resulting plasmid was linearized with *HindIII* and the RNA probe was synthesized with SP6 RNA polymerase. The resulting RNA probe was 250 bp long and the size of the fragment protected by *c-fos* mRNA was 200 bp.

Ribonuclease protection was carried out essentially as described by Melton et al. (1984). Hybridizations were done at 42°C in 80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl overnight in 30 µl using 10 µg of RNA. The samples were digested by adding 300 µl of 5 µg/ml RNase A, 120 U/ml RNase T1, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl and letting the digestion proceed for 1 h at 37°C. The digestion was stopped by adding 20 µl of 10% SDS and the samples were subsequently treated with proteinase K, phenol and chloroform extracted, precipitated with ethanol, dissolved in 4 µl of 80% formamide and electrophoresed in a 6% denaturing polyacrylamide gel.

β-Galactosidase assay

β-Galactosidase activity was measured as the fluorescence generated by the cleavage of 4-methylumbelliferyl-β-D-galactoside (Sigma) at the β-galactosidase bond. Fifty thousand cells were stimulated in 200 µl fresh culture media in 96 well trays for 5 h at 37°C. After stimulation the cells were centrifuged, supernatant removed and 180 µl reaction media (100 mM Na₂HPO₄, pH 7.0, 10 mM KCl, 1 mM MgSO₄, 0.1% Triton X-100, 0.5 mM 4-methylumbelliferyl-β-D-galactoside) was added. After 1 h at room temperature the reaction was stopped by adding 75 µl of stop buffer (300 mM glycine, 15 mM EDTA, pH 11.3) and the fluorescence at 460 nm was measured using 355 nm excitation with a Titertek Fluoroscan II.

Electrophoretic mobility shift assay

Nuclear extracts were made as previously described (Durand et al., 1988) with minor modifications. Fifty million cells per experimental point were harvested and washed once in ice cold phosphate buffered saline and the following steps were done at 4°C. The cells were pelleted and washed once in 1 ml of buffer A (10 mM HEPES pH 7.8, 15 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 1.0 mM PMSF) and once in 1 ml buffer B (buffer A plus 0.2% NP-40). Pelleted nuclei were resuspended in 324 µl of buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1.0 mM PMSF, 10% v/v glycerol), and 36 µl 3 M (NH₄)₂SO₄ pH 7.9 was added. Following gentle mixing for 30 min, the viscous solution was centrifuged at 100 000 r.p.m. for 10 min in a Beckman TLC 100 rotor. An equal volume of 3 M (NH₄)₂SO₄ was added to the supernatant and precipitated proteins were pelleted at 50 000 r.p.m. for 10 min and resuspended in 50–100 µl of buffer C. Protein samples were dialyzed by passage over a P6DG column (Bio-Rad). Concentrations of proteins were determined by the Bradford assay (Bradford, 1976).

Electrophoretic mobility assays were done essentially as described (Fried and Crothers, 1981; Garner and Revzin, 1981). Binding reactions for NF-AT and NF-κB were carried out in 20 µl in a solution consisting of 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5% glycerol and 1.7 µg poly(dI-dC). Binding reactions for NFIL2B were carried out in 50 mM NaCl, 25 mM Tris borate pH 7.5, 1 mM EDTA, 5% glycerol and 1.7 µg poly(dI-dC) and binding reactions for AP-1 were carried out in a solution consisting of 50 mM-HCl pH 7.5, 13 mM MgCl₂, 1 mM EDTA, 5% glycerol and 1.7 µg poly dI-dC. Five µg of protein was used for each experimental point except for NF-AT binding reactions in which 10 µg of protein was used. The protein solutions were incubated for 60 min on ice with 0.1–0.5 ng of end-labeled double stranded oligonucleotides. The probe for NF-AT was derived by annealing oligonucleotides gatcTAAGGAG-GAAAACTGTTTCATG and gatcCATGAAACAGTTTTCCTCCTTA. The probe for AP-1 was derived by annealing oligonucleotides tcgaGTGACTCAGCGG and tcgaCCGCTGAGTCAC which represents the sequence of the AP-1 binding site from the human metallothionein enhancer (Lee et al., 1987). The probes for NFIL2B and NF-κB were derived from the oligonucleotides that were used for the construction of NFIL2ZH and KBZH. Radioactive nucleotides were filled in to the annealed oligonucleotides by Klenow enzyme. The samples were electrophoresed at

room temperature in 4.5% polyacrylamide gels in 90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA except the samples for NFIL2B and AP-1 which were electrophoresed in the above buffer diluted 4-fold.

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