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The NFAT-1 DNA Binding Complex in Activated T Cells Contains Fra-1 and JunB

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Activation of T cells induces transcription of the interleukin-2 (IL-2) gene. IL-2 expression is regulated through the binding of transcription factors to multiple sites within the IL-2 enhancer. One such *cis*-acting element within the IL-2 enhancer is the NFAT-1 (nuclear factor of activated T cells) binding site. NFAT-1 binding activity is absent in resting cells but is induced upon T-cell activation. The induction of NFAT-1 binding activity can be inhibited by cyclosporin A, potentially accounting for the ability of cyclosporin A to inhibit IL-2 production by T cells. We have previously reported that the NFAT-1 binding complex is composed of at least two proteins and that the 5′ portion of the NFAT-1 sequence acts as a binding site for one or more proteins from the Ets family of transcription factors. We now report that the 3′ portion of the NFAT-1 sequence contains a variant AP-1 binding site. NFAT-1 binding can be specifically inhibited by oligonucleotides containing a consensus AP-1 site. Moreover, mutation of the AP-1 site at the 3′ end of the NFAT-1 sequence inhibits both NFAT-1 binding and the ability of the NFAT-1 binding site to activate expression from a reporter plasmid upon T-cell activation. Since AP-1 sites bind dimeric protein complexes composed of individual members of the Fos and Jun families of transcription factors, we used antibodies specific for individual Fos and Jun family members to determine whether they are present in the NFAT-1 binding complex. These experiments demonstrated that the NFAT-1 binding complex contains JunB and Fra-1 proteins. Northern (RNA) blot analyses demonstrate that both *fra*-1 and *junB* mRNAs are induced upon T-cell activation, although *fra*-1 mRNA is present even in quiescent T cells. Of interest, *junB* is not expressed in quiescent T cells, and it is induced with kinetics that are similar to those for the induction of IL-2 mRNA expression. Taken together, these results suggested that the JunB-Fra-1 heterodimer is the inducible nuclear component of the NFAT-1 binding activity and that JunB expression regulates the formation of the heterodimer. In addition, these data indicated that specific heterodimers of Fos and Jun family members may have selective roles in the induction of transcription during cellular activation.

The T-cell-derived lymphokines play important roles in regulating immune responses. One of the lymphokines produced by activated T cells is interleukin-2 (IL-2). IL-2 sustains the proliferation of T cells activated through their antigen-specific receptor. Therefore, induction of IL-2 is an important early step in T-cell-mediated immune responses. Resting T cells do not transcribe the IL-2 gene. The induction of IL-2 gene expression during T-cell activation occurs at the transcriptional level, and it is regulated by a T-cell-specific transcriptional enhancer located in the 5′ flanking region of the gene (5, 10, 22, 24, 35). Thus, the IL-2 enhancer has been used as a model system for the study of transcriptional regulation during T-cell activation.

Previous studies have demonstrated that the 320-bp IL-2 enhancer contains a number of distinct nuclear protein-binding sites that play a role in regulating IL-2 transcription during T-cell activation (6, 28). Several of these nuclear protein-binding sites have been shown to bind known transcription factors such as AP-1, Oct-1, and NF-κB, while other sites bind complexes of unknown proteins (7). One such binding complex, NFAT-1, is found exclusively in activated T cells (36). The induction of NFAT-1 binding activity and IL-2 production is inhibited by cyclosporin A, possibly accounting for the ability of cyclosporin A to block the induction of IL-2 upon T-cell stimulation.

We have recently reported that the 5′ end of the NFAT-1 binding site contains a sequence motif that can function as a binding site for a subset of the Ets family of transcription factors (38). Detailed analyses of the NFAT-1 DNA-protein contact points by methylation interference experiments revealed that in addition to two contact sites which corresponded to the Ets binding site, there is at least one additional DNA-protein contact point 3′ of the Ets binding site (see Fig. 2). Mutational analysis of the sequences at this 3′ contact point suggested that nucleotides in this region were important for the function of the NFAT-1 element within the IL-2 enhancer. In addition, UV cross-linking studies of the NFAT-1 complex suggested that the NFAT-1 complex was composed of at least two distinct proteins. Therefore, we sought to determine whether the sequences at the 3′ end of the NFAT-1 site represent a binding site for an additional component of the NFAT-1 complex. An examination of the sequence at the 3′ end of the NFAT-1 site revealed a motif that differed from a consensus AP-1 site by 1 nucleotide. Thus, the present studies were designed to test

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the possibility that the 3′ end of the NFAT-1 site contains a functionally important AP-1 site.

AP-1 binding activity has been shown to be composed of dimers of proteins from the Fos and Jun families of transcription factors (29). To date, three members of the Jun family of transcription factors, c-Jun, JunB, and JunD, have been identified, while four members of the Fos family, c-Fos, FosB, Fra-1, and Fra-2, have been identified (4, 13, 25, 29–31, 34, 42). AP-1 binding activity, which has been implicated in the activation of many genes, can result from the formation of either Jun-Jun or Fos-Jun dimers (29). Although the *cis* element that has been defined as the AP-1 binding site is TGANTCA, previous reports have suggested that at least some Fos-Jun heterodimers are more promiscuous in their binding (2, 33). To date, it is not clear whether specific homo- and heterodimeric AP-1 complexes are involved in the transcriptional activation or silencing of distinct genes which are expressed upon cellular activation. However, it has been shown previously that the kinetics of expression of Fos and Jun family members following activation is highly ordered in most cell types. The induction of c-Fos, c-Jun, FosB, and JunB occurs early upon cellular stimulation and is followed by induction of the expression of Fra-1 and Fra-2 (1, 4, 13, 17, 25, 32). The third member of the Jun family, JunD, appears to be constitutively expressed in many cell types (1, 13, 17, 30).

In this report, we show that the inducible NFAT-1 binding complex can be inhibited by an NFAT-1 probe by a consensus AP-1 oligomer and that mutation of the putative AP-1 site at the 3′ end of the NFAT-1 binding motif inhibits both NFAT-1 binding and the ability of the NFAT-1 motif to activate gene expression upon T-cell activation. Using antibodies specific for individual Fos and Jun family members, we show that the NFAT-1 binding complex contains JunB and Fra-1.

MATERIALS AND METHODS

Cells and cell lines. T lymphocytes were isolated and cultured as previously described (38). Activation of cells was performed by treatment for 6 to 8 h (unless otherwise specified) with phorbol myristate acetate (PMA) (10 ng/ml) or phorbol dibutyrate (100 ng/ml) and ionomycin (0.4 μg/ml). Cyclosporin A (1 μg/ml) was added 15 min prior to stimulation of cells with PMA and ionomycin. EL4 murine thymoma cells were utilized for the transfection studies. These cells were cultured as previously described (15).

Preparation of nuclear extracts. Nuclear extracts were prepared from T cells as previously described (38). Protein concentrations of extracts were determined by the Bradford assay (3) with a protein assay kit (Bio-Rad, Richmond, Calif.).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as previously described, with the following changes (38). The DNA binding buffer for NFAT-1, NFATpAP-1, and NFATmAP-1 binding contained 250 ng of poly(dI-dC), 50 mM KCl, 10 mM Tris (pH 7.5), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 1.25 mM dithiothreitol, 1.1 mM EDTA, and 15% (vol/vol) glycerol. Binding to NF-κB was performed with a similar buffer that also contained 50 mM NaCl. In each binding reaction mixture, 1.0 to 1.5 μg of nuclear extracts was used. All probes used for binding or competition were gel purified and then annealed. Probes contained either *Bam*HI-*Bgl*II ends (NFAT-1, mOCTA, NFATmAP-1, NFATpAP-1) or AG ends [AP-1, NF-kB, and mutated AP-1(mAP-1)] which were utilized for labeling by Klenow fill-in with ^{32}P-labeled nucleotides. The sequences of the oligonucleotide probes used in these studies were as follows: NFAT-1, AGAAAGGAGGAAAAACTGTTTCATACAGA AGGCGTT; NFATmAP-1, AGAAAGGAGGAAAAACCTG GAATTACAGAAGGCGTT; NFATpAP-1, AGAAAGGAG GAAAAACTGACTCATACAGAAGGCGTT; AP-1, GTGA CTCAGCGCG; mAP-1, GGAATTAGGCGCG; NF-κB, TC TCAGAGGGGACTTT; and mOCTA, TAGACACACAG GATTTGTATATTTCATGAA. Underlined portions of sequences denote regions of mutation. For the probe NFATpAP-1, the mutation was a change of the variant AP-1 site to the consensus AP-1 site of the simian virus 40 enhancer. Supershift experiments were performed by the addition of rabbit sera to the EMSA reaction mixtures. Unless otherwise specified, 1 μl of antisera was added to each reaction mixture. Preclearing immunoprecipitations of nuclear extracts were performed by the addition of 1 μl of rabbit sera to 10 μl (2 to 4 μg of protein) of nuclear extract. The extracts were incubated on ice for 2 h prior to the addition of protein A-Sepharose beads (Pharmacia). The reaction mixtures were then incubated for 2 h at 4°C with agitation, and the beads were pelleted with a microcentrifuge. Five microliters of supernatant was then used in simultaneous EMSAs on the NFAT-1 and NF-κB probes as described above.

The sera were raised against Fos and Jun family members, and they selectively reacted against specific family members as described previously (19–21). The anti-Jun family serum reacts against all three Jun proteins. An anti-Fos serum which reacts against all four Fos family members equally is referred to as anti-Fos family and a serum which reacts predominantly against Fos and FosB and recognizes Fra-1 and Fra-2 only weakly is referred to as anti-Fos/FosB in the remainder of this article. Rabbit sera that were raised against cardiac troponin C (anti-CTNC) served as a negative control.

Transfections and CAT assays. Transfection by the DEAE-dextran method and PMA (50 ng/ml) and ionomycin (1.4 μg/ml) stimulation of EL4 cells were performed as previously described (15). The plasmids employed for transfection were pSPCAT (23), pSPNFATCAT (38), and pSPNFATmAP-1CAT. pSPNFATmAP-1CAT was constructed by linking three copies of the oligonucleotide NFATmAP-1 together and ligating the resulting oligomer into the *Sma*I site of pSPCAT. The insert was confirmed by dideoxy DNA sequencing. Ten micrograms of plasmid was cotransfected with 2 μg of pRSVβgal as a control for transfection efficiency. Protein concentrations were determined by the Bradford assay (Bio-Rad), and β-galactosidase assays were performed as described previously (14). Protein concentrations used in chloramphenicol acetyltransferase (CAT) assays were normalized on the basis of β-galactosidase activities to correct for transfection efficiency. CAT assays were performed as previously described (14).

Isolation of RNA and Northern (RNA) blot analysis. RNA was isolated from resting T cells and from T cells activated for various times with phorbol dibutyrate plus ionomycin. RNA was extracted from cells by a guanidinium isothiocyanate method and purified through a cesium chloride gradient (37). Loading of RNA samples onto the agarose-formaldehyde gels was equalized against the rRNA. Gels were blotted onto nitrocellulose and baked under vacuum for 2 h at 80°C (37).

Northern blots were probed sequentially with the cDNAs for the murine *fra*-1, murine *junB*, and human HLA class I genes. The *fra*-1 and *junB* 1.4-kb fragments were isolated

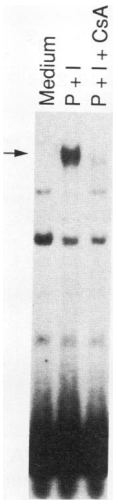

FIG. 1. Regulation of NFAT-1 binding activity. EMSAs were performed with the NFAT-1 probe to demonstrate the inducibility and cyclosporin A (CsA) sensitivity of NFAT-1 binding. Nuclear extracts from primary human T cells were cultured in medium alone, stimulated with PMA plus ionomycin for 6 h (P + I), or stimulated with PMA plus ionomycin for 6 h following a 15-min pretreatment with cyclosporin A (P + I + CsA). The arrow denotes the NFAT-1 complex, which is induced upon cell activation. Induction of the NFAT-1 complex is inhibited by cyclosporin A pretreatment. Other bands on the autoradiograph are not specific, as determined by competition studies or reproducibility (unpublished observations). These data are in agreement with previously published data obtained with the T-cell line Jurkat (7).

from the *Eco*RI site of pBluescript KS (Stratagene) from the plasmids p*fra*-1 and p*junB*, respectively. The HLA fragment was isolated as previously described (24). Isolated inserts were labeled by nick translation and used at 10^6 cpm/ml of hybridization buffer. Blots were prehybridized (50% formamide, 5× SSC [1× SSC equals 0.15 M NaCl and 0.015 M sodium citrate], 1× Denhardt solution, 25 mM sodium phosphate [pH 6.5], 250 μg of RNA per ml) at 42°C for 2 h and hybridized overnight in prehybridization solution containing labeled probe and 10% dextran sulfate. Final blot washes were 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 56°C for 30 min for *junB* and HLA and 2× SSC–0.1% SDS at 50°C for 30 min for *fra*-1.

RESULTS

Identification of a potential AP-1 site in NFAT-1. Previous studies have defined the NFAT-1 sequence as an element which becomes protein bound upon activation of T cells (28, 36). The NFAT-1 binding activity is inhibited by treatment with cyclosporin A, potentially accounting for the inhibition of IL-2 production in cyclosporin-treated cells (7, 28). An example of the induction of NFAT-1 binding activity upon T-cell activation and its inhibition by cyclosporin A is shown in Fig. 1. Previous studies from this and other laboratories have identified three nucleotides in the NFAT-1 site which are protected from methylation as a result of the NFAT-1 binding activity (26, 38). We have previously shown that two of these contact points at the 5′ end of the NFAT-1 site correspond to an Ets binding site that is capable of binding the Elf-1 transcription factor (38). However, the 3′ contact site in the NFAT-1 element would not be expected to be protected by Ets protein binding. We have, therefore, examined the 3′ sequences in the NFAT-1 binding site more closely to see whether they contain potential binding sites for known transcription factors. As shown in Fig. 2, the 3′ contact site is in the center of an element which displays considerable similarity to known AP-1 binding sites (33).

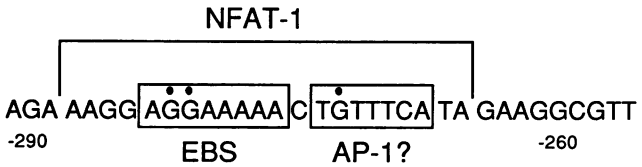

FIG. 2. NFAT-1 site of the IL-2 enhancer. The sequence shown in this figure corresponds to the NFAT-1 probe used in the EMSAs in this article. NFAT-1 is the region that has been previously described as the NFAT-1 site of the IL-2 enhancer (36). The boxed area labeled EBS has been described as an Ets binding domain (38). The second boxed area is the putative AP-1 site that is investigated in this article. The solid circles above the G residues in the boxed area represent DNA contact points defined by methylation interference assays (38).

AP-1 oligonucleotides are capable of blocking NFAT-1 binding. To determine whether there is an AP-1 component to the NFAT-1 binding complex, inhibition of NFAT-1 binding was examined with an oligonucleotide containing a consensus AP-1 site (Fig. 3). The nonlabeled AP-1 oligonucleotide was able to completely inhibit NFAT-1 binding, although it was fivefold less efficient at inhibition than the NFAT-1 oligonucleotide itself. This level of inhibition was much greater than would be expected as a result of nonspecific inhibition of DNA binding activity. To confirm this, we examined additional oligonucleotide competitors for their abilities to inhibit NFAT-1. The mOCTA sequence, which contains no known protein-binding site (Fig. 3A) and a consensus NF-κB binding site (Fig. 3B), had no effect on NFAT-1 binding. Mutation of the consensus AP-1 site was shown to abolish its ability to inhibit NFAT-1 binding (Fig. 3B). Inhibition of NFAT-1 with the AP-1 probe produced a new band with mobility faster than that of the normal NFAT-1 band shift. The significance of this band is uncertain at the present time, since it is also occasionally seen in nuclear extracts of resting and activated T cells to which cold competitor oligonucleotides have not been added. Under these conditions, this lower band is not effectively inhibited by the NFAT-1 oligonucleotide. Taken together, these data were consistent with the hypothesis that there is an AP-1 component to the NFAT-1 DNA binding complex. Furthermore, it appears that the AP-1 binding activity may play an important role in the ability of the complex to bind to DNA, since the AP-1 oligonucleotide can efficiently inhibit the NFAT-1-binding activity.

To independently verify these conclusions, nuclear extracts from activated T cells were tested for their abilities to bind to NFAT-1 and to an NFAT-1 probe with a mutated AP-1 site (NFATmAP-1) (Fig. 4). The NFATmAP-1 site was unable to bind the NFAT-1 from activated T-cell extracts, a result consistent with the hypothesis that AP-1 binding activity is part of the NFAT-1 complex and that the AP-1 binding site is located at the 3′ end of the NFAT-1 site.

The putative AP-1 binding site is essential for NFAT-1 function. To determine whether the putative AP-1 site is also important for NFAT-1 function, we examined the abilities of NFAT-1 oligonucleotides with mutations in their putative AP-1 sites to function as inducible enhancer elements when transfected into the EL4 T-cell line. Three copies of either

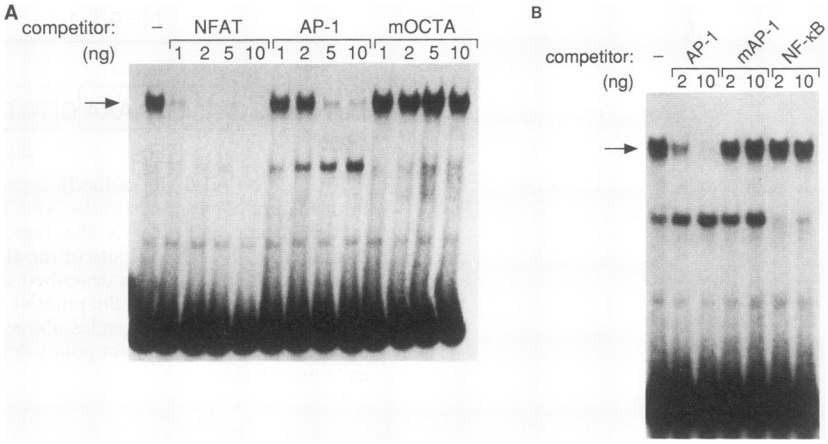

FIG. 3. Competition of NFAT-1 binding with AP-1 oligonucleotides. (A) EMSAs using a radiolabeled NFAT-1 oligonucleotide and nuclear extracts from activated T cells in the presence of increasing concentrations of unlabeled NFAT-1, AP-1, and mOCTA oligonucleotides are shown. Competing oligonucleotides are listed, and amounts are indicated above the autoradiograph. NFAT-1 binding (arrow) was inhibited by the NFAT-1 and AP-1 oligonucleotides but not by the nonspecific mOCTA oligonucleotide. (B) EMSAs using a radiolabeled NFAT-1 oligonucleotide and nuclear extracts from activated T cells with AP-1, mAP-1, and NF-κB are shown. Competition studies as described for panel A reveal inhibition of NFAT-1 binding (arrow) by a cold AP-1 oligonucleotide but not by a mutated form of the oligonucleotide (mAP-1) or an unrelated oligonucleotide, NF-κB.

NFAT-1 or NFATmAP-1 were cloned into the pSPCAT reporter construct containing the CAT gene under the control of the minimal simian virus 40 promoter. The resulting reporter constructs were transfected into EL4 T cells, and following transfection, cells were split into two groups. One group was cultured in medium alone, while the other was stimulated for 12 h with a combination of PMA and ionomycin. Consistent with previous results, a plasmid containing three copies of the wild-type NFAT-1 site was shown to be induced eightfold upon T-cell activation (Fig. 5). However, neither the plasmid containing only a minimal simian virus 40 promoter or that containing three copies of NFATmAP-1 displayed any induction of CAT activity upon T-cell stimulation.

JunB and Fra-1 are present in the NFAT-1 binding complex. Since the NFAT-1 binding site appears to contain a functional AP-1 motif, we next performed experiments to determine whether members of the Fos and Jun families of transcription factors were present in the NFAT-1 DNA binding complex and, if so, to identify the specific family members in the complex. For these experiments, a variety of antibodies raised against Fos and Jun proteins were employed in supershift EMSAs. Antisera that recognized all known Jun family members were shown to reproducibly supershift the NFAT-1 complex (Fig. 6, left panel). Antisera that specifically bind to JunB also supershifted the NFAT-1 complex. In contrast, JunD-specific antisera had no effect on NFAT-1 binding. Control rabbit antisera raised against CTNC also had no effect on the NFAT-1 band shift.

To date, JunB has not been demonstrated to form ho-

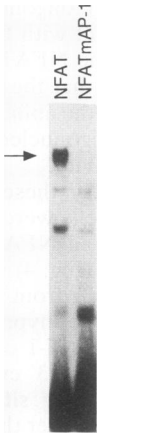

FIG. 4. Mutation of the putative AP-1 site of NFAT-1 abolishes binding. EMSAs with nuclear extracts from PMA plus ionomycin-induced T cells bound to either radiolabeled NFAT-1 (left lane) or radiolabeled NFATmAP-1 (right lane) were performed.

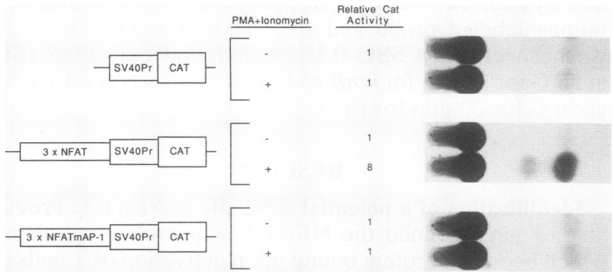

FIG. 5. Functional characterization of the putative AP-1 site within NFAT-1. EL4 cells were transfected with a vector containing a minimal simian virus 40 promoter (pSPCAT) (23), pSPCAT altered by the addition of three copies of NFAT-1 (pSPNFATCAT) (38), or three copies of NFAT-1 with a mutated AP-1 site (pSPNFATCATmAP-1). Following transfection, half of the cells from each transfection were stimulated with PMA plus ionomycin for 12 h while the rest were cultured in medium alone, and extracts were prepared. CAT assays were performed on extracts normalized for transfection efficiency as previously described (14). The plasmids used in the transfection are listed to the left of the autoradiograph. PMA plus ionomycin stimulation is denoted by a plus. Fold induction is based on the amount of CAT activity that was induced by PMA plus ionomycin for each transfection. Quantitation was performed with a Betagen scanner. Data are representative of four separate experiments.

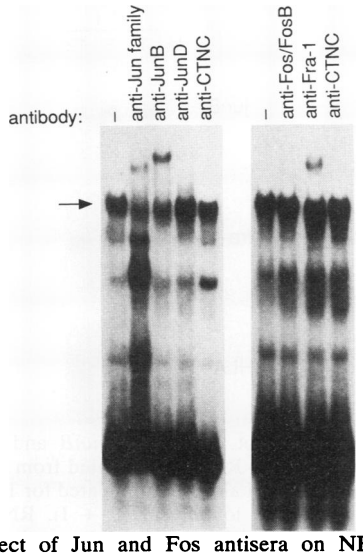

FIG. 6. Effect of Jun and Fos antisera on NFAT-1 binding activity. EMSAs were performed with the NFAT-1 oligonucleotide, nuclear extracts from activated T cells, and Fos and Jun antisera (19, 20) as described in Materials and Methods. One microliter of the listed antibody was added to each of the EMSAs. Supershifts of the NFAT-1 binding complex (arrow) were detected with the anti-Jun, anti-JunB, and anti-Fra-1 sera. Anti-CTNC was used as a control for nonspecific effects. Binding to NF-κB was not affected by any of the Fos and Jun antisera (data not shown).

modimers. This suggested that a Fos family member may also be present in the NFAT-1 complex. An antiserum which recognizes all four members of the Fos family was found to supershift the NFAT-1 complex (data not shown). This indicated that a Fos family member was also binding to the NFAT-1 complex. To determine whether a specific Fos family member was involved, additional antisera were used in supershift EMSAs. Antisera which recognize c-Fos and FosB had no effect on the NFAT-1 binding complex. In contrast, a Fra-1-specific antiserum was found to supershift the NFAT-1 complex (Fig. 6, right panel). Together, these data suggested that NFAT-1 contains a JunB–Fra-1 heterodimer. Consistent with this hypothesis, we found that both Fra-1 and JunB antisera could supershift and/or block NFAT-1 binding in a dose-dependent fashion (Fig. 7A). This is further confirmed by the fact that preclearing the nuclear extracts of Fra-1 or JunB prior to EMSA analysis ablates NFAT-1 activity without affecting NF-κB binding (Fig. 7B). Preclearing nuclear extracts with antisera that reacted with broad specificity for Fos or Jun family members, such as anti-Jun, could also deplete NFAT-1 binding activity. In contrast, preclearing with antisera with specificity for other single family members such as JunD had no effect on binding. These findings suggested that the majority of the NFAT-1 complexes contain both Fra-1 and JunB. The simplest explanation for this observation is that most NFAT-1 complexes contain a JunB–Fra-1 heterodimer. However, since 5 to 10% of the NFAT-1 activity remained following our preclearing experiments, this may represent other AP-1 factors that make up a minor component of the complexes present in in vitro binding assays. It remains possible that the NFAT-1 complex that stimulates IL-2 transcription in vivo is only a minor species of the NFAT-1 complex detected by an EMSA.

The restricted use of a Fra-1–JunB heterodimer as the AP-1 component in the NFAT-1 complex results from the sequence of the NFAT-1 site. It is possible that our inability to detect additional AP-1 components within the NFAT-1 complex

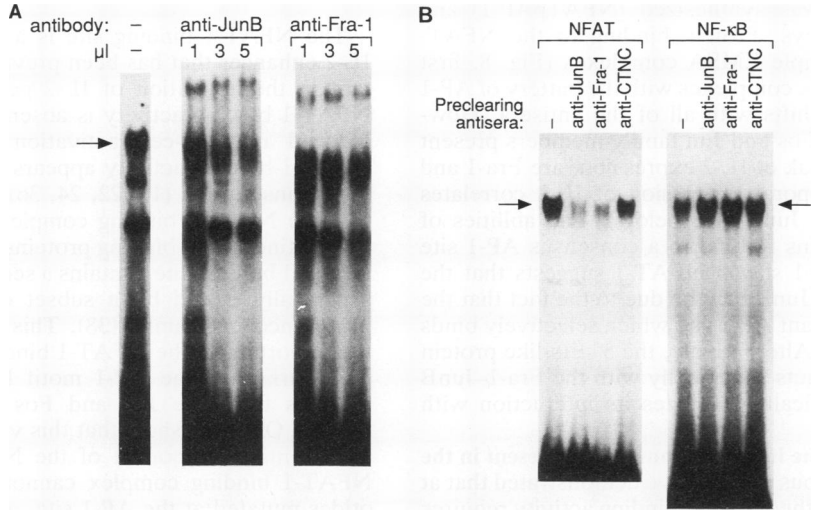

FIG. 7. Quantitative analysis of Fra-1 and JunB present in the NFAT-1 DNA binding complex. (A) Dose-dependent effect of anti-JunB and anti-Fra-1 sera on NFAT-1 binding. The leftmost lane is an EMSA using the NFAT-1 oligonucleotide and nuclear extracts from activated T cells. The NFAT-1 complex is the band indicated by an arrow. In all other lanes, identical EMSAs were performed with increasing amounts of Fra-1- and JunB-specific antisera. For each antiserum, a progressive increase in the amount of antiserum led to a corresponding decrease in the NFAT-1 complex. Part of this decrease results from the supershifted complexes seen in the figure, while the rest results from antibody-induced inhibition of DNA binding. Similar amounts of anti-CTNC had no effect on NFAT-1 DNA binding (data not shown). (B) Effect of preclearing nuclear extracts of Fra-1 and JunB by immunoprecipitation. EMSAs were performed with nuclear extracts that were precleared by immunoprecipitation with no antiserum (lane −), anti-Fra-1, anti-JunB, and anti-CTNC. Following precipitation of immune complexes with protein A-Sepharose, the extracts were incubated with the NFAT-1 and NF-κB probes. Fra-1 and JunB could inhibit NFAT-1 binding activity (left panel) without affecting NF-κB binding (right panel). The no-antiserum control and the nonspecific anti-CTNC had no effects on NFAT-1 or NF-κB binding. This figure is representative of three independent experiments.

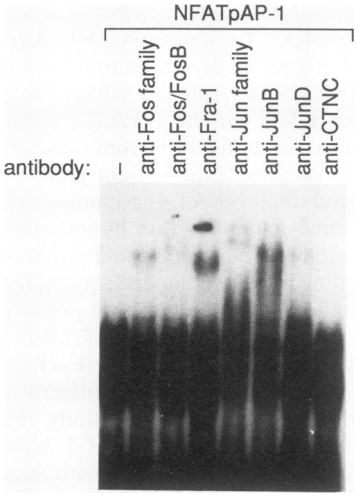

FIG. 8. Multiple complexes form on the NFATpAP-1 oligonucleotide. The NFAT-1 site was modified to contain a consensus AP-1 site in place of the variant site that is present in the normal sequence. EMSAs yielded multiple specific DNA binding complexes, with the most prominent being the doublet shown in this figure (first lane). The upper band of the doublet is similar in size to that produced by the normal NFAT-1 DNA binding complex. Supershift analysis of the NFATpAP-1 binding complexes revealed that all specific antisera could shift the DNA-protein complexes further. This figure is representative of four independent experiments.

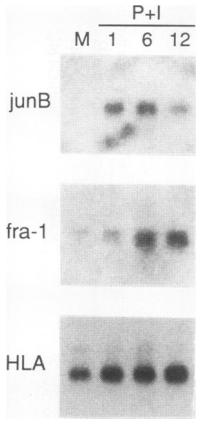

FIG. 9. Northern blot analysis of *junB* and *fra*-1 expression during T-cell activation. RNA was isolated from primary human T cells cultured in medium alone or stimulated for 1, 6, and 12 h with phorbol dibutyrate and ionomycin (P + I). RNA samples were equalized for 28S rRNA, separated by gel electrophoresis, and transferred to nitrocellulose. The resulting blots were hybridized with radiolabeled probes specific for *junB*, *fra*-1, and HLA class I (HLA), and the resulting autoradiograms are shown. The data are representative of three separate experiments.

might have been due to the inability of these components to bind to the variant AP-1 site within NFAT-1. To test this possibility, an oligonucleotide that contains the NFAT-1 site in which the variant AP-1 site has been converted to a consensus AP-1 site was synthesized (NFATpAP-1) and used in supershift assays. Protein binding to the NFATpAP-1 resulted in multiple EMSA complexes (Fig. 8, first lane). Treatment of these complexes with the battery of AP-1 antisera yielded supershifts with all of the antisera. However, the predominant Fos and Jun family members present in the cell at 6 h, the peak of IL-2 expression, are Fra-1 and JunB. In fact, the temporal expression of IL-2 correlates with that of Fra-1 and JunB (see below). The abilities of multiple Fos-Jun proteins to bind to a consensus AP-1 site but not the variant AP-1 site in NFAT-1 suggests that the specificity of Fra-1 and JunB may be due to the fact that the NFAT-1 3′ site is a variant AP-1 site which selectively binds this AP-1 heterodimer. Alternatively, the 5′ Ets-like protein factor in NFAT-1 interacts specifically with the Fra-1–JunB heterodimer and specifically stabilizes its interaction with the variant AP-1 site.

JunB may represent the inducible component present in the NFAT-1 complex. Previous results have demonstrated that at least one component of the NFAT-1 binding activity requires new protein synthesis following T-cell activation (9, 36). To test whether either Fra-1 or JunB requires transcriptional activation for its expression during T-cell activation, we examined the expression of *fra*-1 and *junB* by Northern blot analysis with RNA obtained from resting T cells and T cells at various times after induction with a phorbol ester and ionomycin. As seen in Fig. 9, we were unable to detect the expression of *junB* in resting T cells. However, *junB* was rapidly induced upon T-cell activation, and its expression was maintained for at least 6 to 12 h after activation. This pattern of *junB* expression correlated closely with the kinetics of IL-2 gene expression (18, 22, 24). In contrast, although a severalfold induction of *fra*-1 occurred upon T-cell activation, we consistently observed Fra-1 expression in resting T cells. Of interest, the expression of neither *junB* nor *fra*-1 mRNA was affected by pretreatment of T cells with cyclosporin A (data not shown).

DISCUSSION

The NFAT-1 binding site is a *cis*-acting element of the IL-2 enhancer that has been previously shown to be important in the activation of IL-2 gene transcription (36, 38). NFAT-1 binding activity is absent in resting T cells and is induced upon T-cell activation (36). The induction of NFAT-1 binding activity appears to parallel the kinetics of IL-2 transcription (18, 22, 24, 36). Previous data suggested that the NFAT-1 binding complex is composed of at least two distinct DNA binding proteins and that the 5′ end of the NFAT-1 binding site contains a sequence motif which can be specifically bound by a subset of proteins from the Ets proto-oncogene family (38). This report demonstrates that the 3′ portion of the NFAT-1 binding complex is composed of a variant of the AP-1 motif known to bind dimers of proteins from the Jun and Fos families of transcription factors. Our data show that this variant AP-1 binding site is an essential component of the NFAT-1 binding site. The NFAT-1 binding complex cannot bind to NFAT-1 nucleotides mutated at the AP-1 site, and multimerized NFAT-1 binding motifs containing mutations in the AP-1 component fail to function as an inducible enhancer motif upon T-cell activation. Finally, using antibodies specific for individual Fos and Jun family members, we have shown that the majority of NFAT-1 binding complexes detected in an EMSA contain JunB and Fra-1 molecules. These data suggest that NFAT-1 binding activity is composed of a heterodimer of JunB–Fra-1 in association with a member of the Ets proto-oncogene family.

We had previously reported that mutation of the 5′ portion

of the NFAT-1 site produced an oligonucleotide which could not inhibit NFAT-1 binding in an EMSA reaction (38). This appears to be due to the fact that the NFAT-1 complex has only low affinity for the variant AP-1 site in the absence of the 5′ Ets binding site in NFAT-1. In contrast, as we show here, a consensus AP-1 site can compete effectively for the ability to form an NFAT-1 binding complex, presumably because of its ability to bind directly to dimers containing Fra-1 and JunB. On the basis of these data, it would appear that NFAT-1 complex formation is required for Fra-1–JunB binding to the variant site present in NFAT-1.

UV cross-linking studies have previously revealed that there are at least two components which can be bound to an NFAT-1 oligonucleotide by UV cross-linking, one of 69 and one of 98 kDa (38). The size of the 69-kDa protein is close to that predicted for a cross-linked JunB–Fra-1 heterodimer. At the present time, it is unclear whether the quantitative presence of Fra-1 and JunB in the NFAT-1 complex results from the fact that this heterodimer has specific protein-protein interactions with the Ets component of the NFAT-1 complex or because this Fos-Jun heterodimer has a fine sequence specificity different from those of other AP-1 binding dimers. Differences in the fine sequence specificities of individual members of a transcription factor family have recently been demonstrated for the Ets family of proto-oncogenes (27, 39, 41). It is, therefore, quite likely that there may also be differences in fine sequence specificities of individual heterodimers of the Fos-Jun family. Consistent with this possibility, multiple Fos-Jun family members can bind to NFAT-1 when the variant AP-1 site is converted to a consensus AP-1 site. In addition, the predominant presence of JunB and Fra-1 in the NFAT-1 oligonucleotide complex may also result from the fact that JunB–Fra-1 is the predominant heterodimer formed in the cells at the time of IL-2 transcription. This time point of T-cell activation (6 to 8 h) is later than the time of peak c-*fos* mRNA expression in similarly treated cells (24) or in an activated murine clone (17). Moreover, in fibroblasts, an ordered formation of AP-1 heterodimers occurs, with JunB–Fra-1 being a major heterodimer present during a similar period following stimulation (19–21). Because our experiments were performed with normal T cells from multiple donors, it is unlikely that the reproducible presence of Fra-1–JunB in the NFAT-1 binding complex is an artifact of working with a cell clone. The absence of other AP-1-forming dimers within the NFAT-1 complex may be due to the fact that there is a concentration-dependent threshold for the participation of an AP-1 dimer in the formation of the NFAT-1 complex (8). Whatever the reason, it appears that JunB and Fra-1 are the primary AP-1 components at the time during which IL-2 is transcribed following T-cell activation (18, 22, 24). We have examined T-cell nuclear extracts isolated from cells stimulated for as little as 2 h and still find that Fra-1 and JunB are the predominant AP-1 components of the NFAT-1 complex.

While this article was in preparation, a report suggesting that NFAT-1 contains Fos and Jun was published (16). In general, our data support and extend this observation. However, given our results, it is puzzling that c-*fos* transfection into a T-cell clone was found to augment inducible NFAT-1 activity, since we are unable to demonstrate c-Fos binding in the NFAT-1 complex isolated from normal T cells. However, there may be multiple reasons for this discrepancy. For example, the overexpression of an individual Fos component at high levels may substitute for the binding of the actual Fos component in NFAT-1. Alternatively, overexpression of c-Fos may displace the JunB–Fra-1 heterodimer from certain binding sites, thereby making JunB–Fra-1 more available to participate in the NFAT-1 binding complex.

Previous data concerning the NFAT-1 complex have suggested that it is composed of a nuclear component which is transcriptionally induced and a constitutively expressed T-cell component which undergoes a modification during T-cell activation that allows it to complex with the newly synthesized nuclear component (9). It is the formation of a complex from these components that is inhibited by cyclosporin A (9). On the basis of what is known of the Fos and Jun families of transcription factors, it appears that Fra-1 and JunB together compose the nuclear component of the NFAT-1 DNA binding complex. Fos and Jun family members have been previously shown to contain nuclear localization signals, and they are ubiquitously expressed in numerous cell types (29). We were able to demonstrate that the *junB* gene is not expressed in quiescent T cells. Instead, it undergoes rapid induction following T-cell activation. In contrast, although Fra-1 is induced severalfold upon T-cell activation, it is reproducibly found at the RNA level in quiescent T cells. Taken together, these data suggest that JunB is the anisomycin-sensitive component of the NFAT-1 complex (36).

During the last several years, other examples of complex formation between an AP-1 dimer and an Ets family member have been reported (12, 40). Ets-1 has been shown to bind to the PEA3 binding site in the polyoma enhancer and to transactivate this enhancer cooperatively with the products of c-*fos* and c-*jun* genes (40). One difference between the NFAT-1 binding site and the PEA3 binding site is the spatial orientations of the Ets and AP-1 binding sites. In PEA3, the Ets and AP-1 binding motifs overlap, while in NFAT-1, the domains are 1 bp apart, with the contact points as defined by methylation interference being 8 bp apart. Our finding that a consensus AP-1 oligonucleotide is capable of blocking NFAT-1 complex assembly and binding suggests that protein-protein interactions appear to be important in NFAT-1 complex formation. While we have previously shown that recombinant Elf-1 is capable of binding to the NFAT-1 site in vitro, we have thus far not been able to show that cellular Elf-1 participates in the NFAT-1 complex (38). Thus, another Ets-related protein which shares Elf-1's restricted DNA binding specificity may be the in vivo component of the NFAT-1 DNA binding complex. This Ets-related protein may not bind to the NFAT-1 site in the absence of NFAT-1 complex formation and presumably has the ability to complex with Fra-1 and JunB, the predominant components of AP-1. The amino- and carboxy-terminal domains that flank the DNA binding domains of Ets proteins vary considerably in size and appear to play an important role in regulating the accessibility of the DNA binding domain for DNA binding interactions of Ets proteins (27, 39, 41). These differences in size may thus account for the varying distances in the interaction of Ets family members with other transcription factors relative to their DNA binding sites. Alternatively, a third protein which does not contact DNA may act as a bridge between the Ets family member and the Fra-1–JunB heterodimer in the NFAT-1 complex. This third protein might not be detected in a DNA cross-linking study. Consistent with this possibility, a 29-kDa protein which has been shown to increase NFAT-1 binding activity from nuclear extracts in vitro has recently been isolated (11). This protein was found in the nuclear fraction, and it does not bind directly to DNA. Resolution of these issues must await the conclusive demonstration of the additional components

which make up the NFAT-1 complex and the ability to reconstitute the NFAT-1 complex in vitro.

In summary, our data demonstrate that the NFAT-1 binding site contains a variant of the AP-1 consensus motif. This motif is required for the ability of the NFAT-1 binding site to transactivate a reporter construct when transfected into activated T cells. In nuclear extracts isolated from time points during which active IL-2 transcription is occurring, the NFAT-1 complex appears to be predominantly composed of a heterodimer of Fra-1 and JunB. The reasons for the specificities of the Fra-1 and JunB members of the Fos-Jun family for binding to the imperfect AP-1 site in NFAT-1 remain to be determined. Thus, our data add to the growing evidence that specific transcription factor interactions play an important role in the regulation of activational gene expression. The interaction of individual proteins that compose the NFAT-1 binding complex and their protein-protein and protein-DNA interactions may help us to understand mechanisms by which transcription factors cooperate in regulating gene expression.

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