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Recombinant NFAT1 (NFATp) Is Regulated by Calcineurin in T Cells and Mediates Transcription of Several Cytokine Genes

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Transcription factors of the NFAT family play a key role in the transcription of cytokine genes and other genes during the immune response. We have identified two new isoforms of the transcription factor NFAT1 (previously termed NFATp) that are the predominant isoforms expressed in murine and human T cells. When expressed in Jurkat T cells, recombinant NFAT1 is regulated, as expected, by the calmodulin-dependent phosphatase calcineurin, and its function is inhibited by the immunosuppressive agent cyclosporin A (CsA). Transactivation by recombinant NFAT1 in Jurkat T cells requires dual stimulation with ionomycin and phorbol 12-myristate 13-acetate; this activity is potentiated by coexpression of constitutively active calcineurin and is inhibited by CsA. Immunocytochemical analysis indicates that recombinant NFAT1 localizes in the cytoplasm of transiently transfected T cells and translocates into the nucleus in a CsA-sensitive manner following ionomycin stimulation. When expressed in COS cells, however, NFAT1 is capable of transactivation, but it is not regulated correctly: its subcellular localization and transcriptional function are not affected by stimulation of the COS cells with ionomycin and phorbol 12-myristate 13-acetate. Recombinant NFAT1 can mediate transcription of the interleukin-2, interleukin-4, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor promoters in T cells, suggesting that NFAT1 contributes to the CsA-sensitive transcription of these genes during the immune response.

The nuclear factor of activated T cells (NFAT) was originally identified in T cells as an essential transcription factor for interleukin-2 (IL-2) gene expression (54). NFAT DNA-binding activity has since been detected in B cells, mast cells, and natural killer cells (1, 3, 5, 19, 46, 63, 67). In addition to binding to the IL-2 promoter, NFAT binds to sites in the regulatory regions of several other cytokine genes, including the genes encoding tumor necrosis factor alpha (TNF-α) (14, 31), IL-4 (6, 49, 58), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (8, 24, 29). These observations have suggested that NFAT plays a major role in coordinating cytokine gene transcription during the immune response (reviewed in reference 47).

A component of the NFAT complex is present in resting T cells (11) and can be detected by its ability to bind autonomously to certain NFAT sites (23, 33, 48). This component (NFATp or NFATc) appears in the nucleus following stimulation with T-cell receptor ligands, calcium ionophores, or other agents that induce an increase in cytoplasmic free calcium, and it confers the DNA-binding specificity of NFAT (33). However, full transactivation by NFAT requires the participation of a nuclear component that is synthesized in response to stimulation with phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (11) and includes members of the AP-1 (Fos/Jun) family of transcription factors (2, 22, 42, 49). AP-1 proteins bind cooperatively with NFATp/c to NFAT sites in the regulatory regions of cytokine genes and stabilize the NFAT complex (21). Calcium ionophores such as ionomycin, together with PMA, mimic stimulation through the T-cell receptor, inducing cytokine gene expression and activating transcription of reporter genes controlled by NFAT sites in a variety of cytokine genes (30, 65).

A central aspect of the regulation of NFAT is that the appearance of NFAT DNA-binding activity in the nuclei of activated T cells is blocked by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (3, 30). CsA and FK506 form complexes with their intracellular receptors (immunophilins), and the drug-immunophilin complexes inhibit the phosphatase activity of calcineurin (protein phosphatase 2B), a ubiquitous calcium- and calmodulin-dependent phosphatase (reviewed in reference 26). CsA and FK506 inhibit the transcription of several cytokine genes (60), at least partly by inhibiting the calcineurin-dependent translocation of the preexisting subunit of NFAT to the nucleus of activated T cells (55). Overexpression of wild-type calcineurin or a constitutively active form of calcineurin substitutes for the calcium requirement in T-cell stimulation and reduces the sensitivity of gene expression to inhibition by CsA and FK506 (7, 15, 25, 43, 62, 65).

The hunt for the NFATp/c component of NFAT led to the isolation of cDNAs encoding four distinct classes of proteins: NFATp, NFATc, NFAT3, and NFATx-NFAT4-NFATc3 (NFATx/4/c3) (17, 18, 28, 32, 41). These proteins define a novel family of transcription factors whose conserved DNA-binding domains show a weak similarity to the DNA-binding domains of Rel family proteins (18, 20, 40). The DNA-binding domains of all four NFAT family proteins are capable of binding cooperatively with Fos and Jun family proteins at the IL-2 promoter NFAT site (18, 20, 28). The mRNAs encoding all

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four proteins are detected in human lymphocytes but differ in their patterns of expression in other cells and tissues and in their representation in resting and activated cells (18, 28, 41), suggesting that each protein may subserve specific functions. The individual roles of NFAT family proteins in regulating gene expression in lymphoid and nonlymphoid cells remain to be determined.

The terms NFATp and NFATc were originally used to describe DNA-binding activities present in cellular extracts; hence, more than one NFAT family protein may have been present in the original NFATp (preexisting) and NFATc (cytoplasmic) preparations (11, 33). Therefore, on the basis of the nomenclature used by Hoey et al. (18), here we rename NFATp, the first isolated NFAT family member (32), as NFAT1. We use this nomenclature since it makes no assumptions about the subcellular localization of this protein or its level of expression in resting cells.

We report here the cloning of two new isoforms of NFAT1 that are the predominant isoforms expressed in T cells. The subcellular localization and transactivation functions of recombinant NFAT1 are regulated by calcineurin in T cells, as expected for the preexisting, cytoplasmic component of NFAT (7, 26, 47) and as previously shown for endogenous murine NFAT1 (51, 55). In contrast, recombinant NFAT1 behaves aberrantly in COS cells, indicating that NFAT1 is not regulated in all cell types. We show that recombinant NFAT1 mediates transcription of the IL-2, IL-4, TNF-α, and GM-CSF promoters in T cells, suggesting that NFAT1 participates in the CsA-sensitive transcription of these cytokine genes during the immune response.

MATERIALS AND METHODS

Library screening and sequence analysis. The cloning strategy for murine NFAT1 is shown in Fig. 1A. Isolation of clone Q1B1 from a murine Ar-5 T-cell λZAPII cDNA library with probe a has been described elsewhere (32). Probe b, a 573-bp *Eco*RI (5′ site in the polylinker of pBluescript) fragment corresponding to the 5′ end of the clone Q1B1, was used to screen the cDNA library and an amplified murine λ 129 SV genomic library (Stratagene) as previously described (32). A genomic fragment hybridizing with the probe was sequenced and found to encode two tryptic peptides of purified murine NFAT1, which were not found in the original partial cDNA sequence (Fig. 2B). A 300-bp *Pvu*I-*Bgl*I probe (probe c) from this genomic fragment was used to isolate four clones corresponding to the 5′ end of NFAT1 cDNA. All of the 5′ cDNA clones overlap the original partial cDNA sequence, but none of the isolated clones spans the full coding sequence. Several of the 3′ cDNA clones have long 3′ untranslated sequences, but none contains a polyadenylation site or poly(A) tail. This is not surprising, since the mRNA of NFAT1 is ~8 kb long (32). The cDNA clones were excised into pBluescript SK(−) as instructed by the supplier, and the inserts were aligned by restriction mapping. Both strands of several cDNA and genomic clones spanning the full coding sequence of NFAT1 were sequenced with a Sequenase kit (U.S. Biochemical). Most of the 3′ untranslated region of clone Q1B1 was removed by excising the fragment between the *Avr*II site in the cDNA (3′ of the stop codon) and the *Eco*RV site in the polylinker of pBluescript. The full-length cDNA was constructed by replacing the *Not*I (in pBluescript)-to-*Bsm*I fragment of this plasmid with the *Not*I-*Bsm*I fragment of an overlapping 5′ clone (U1), resulting in pNFAT1-A, which has the full-length coding sequence for the murine NFAT1-A with 54-bp 5′ and 154-bp 3′ untranslated regions.

The cloning strategy for human NFAT1 is shown in Fig. 1B. Probe b was used to screen a Jurkat cDNA library, and one clone (21B2) with 1,466 bp of human NFAT1 cDNA was isolated. Comparison with murine cDNA clones indicated that this human clone corresponds to the 5′ end of NFAT1 cDNA. The same cDNA library was screened again with probe d (736 bp; *Nhe*I site to the 3′ end of 21B2), and several overlapping clones which span the region between nucleotides 997 and 2229 of the human cDNA were isolated. Rescreening with probe e (629 bp; *Bgl*II site to the 3′ end of clone 42) isolated additional clones, most (five of seven) of which ended close to the end of the Rel similarity region. However, one clone (118A) appeared to be a shorter variant with a 3′ region similar to the 3′ terminus of murine NFAT1-C, while another (103A) contained sequence corresponding to the murine isoform C.

Sequence analysis was done with the GCG program package (Genetics Computer Group, Inc).

RT-PCR. Cytosolic RNA was isolated from unstimulated Jurkat cells as previously described (32). Primers (1, 5′-AAGAGCCAGCCCAACATGC-3′; 2, 5′-CAGCCTTACTACCCCCAGCAC-3′; 3, 5′-CGCTCTAGAAGGAGGTCCTGAAAACT-3′; and 4, 5′-CGCAAGCTTGGGAGATGAACATGAAAG-3′; added restriction enzyme sites are underlined) were synthesized on the basis of the sequence of clone 103A. Primers 3 and 4 were used to make cDNA from Jurkat cytosolic RNA; the cDNA made was used as the template for PCR with primer 3 as the 3′ primer and primer 1 or 2 as the 5′ primer, using a SuperScript reverse transcription-PCR (RT-PCR) kit (Life Technology, Inc.). The B- and C-like PCR products (Fig. 1B) were subcloned into plasmid pBluescript KS(−) and sequenced.

Plasmids. Expression plasmids encoding full-length murine NFAT1 isoforms were constructed by using plasmid pLGP3 (39). An intermediate plasmid was made by subcloning the *Eco*RI-*Avr*II fragment of the partial murine NFAT1-A cDNA (32) into pLGP3. To generate the C termini for the B and C isoforms, PCR products were made by using *Taq* polymerase (Perkin-Elmer) and the corresponding cDNA clones as templates. The common 5′ primer used was 5′-CTGAGCCCGGGCGC-3′, and the specific 3′ primers were 5′-CGACGGAAGCTTGATCAAAGACCAGTCAC-3′ for isoform B and 5′-CGACGGAAGCTTGCGTCGCGAGATTATGT-3′ for isoform C. The 3′ primers have a *Hin*dIII site (underlined) introduced for cloning. The *Sma*I-*Hin*dIII fragment of the intermediate plasmid was replaced by the *Sma*I-*Hin*dIII fragments of the PCR products encoding the C termini of the B and C isoforms, resulting in intermediate plasmids for isoform B and C. Plasmids pLGPmNFAT1-A, -B, and -C were constructed by subcloning the *Spe*I (polylinker)-*Bgl*II fragment of pNFAT1-A into these intermediate plasmids between the *Eco*RI (polylinker of pLGP3) and *Bgl*II sites.

A derivative of pEFBOS (36), plasmid pEFBOSCX, which has the *Xba*I-*Xba*I stuffer fragment replaced by a polylinker with *Cla*I, *Sal*I, *Bam*HI, and *Xba*I sites, was a gift from D. A. Cantrell, and pACTAG2 (59) was a gift from M. Tremblay. The *Hin*dIII-*Xba*I fragment encoding three copies of the hemagglutinin (HA) epitope tag (YPYDVPDYA) from pACTAG2 was subcloned into the *Sal*I and *Xba*I sites of pEFBOSCX, resulting in pEFTAG. The *Aat*II-*Hin*dIII fragments of NFAT1-A, NFAT1-B, and NFAT1-C from the pLGP3 expression plasmids were subcloned into the *Xho*I site of pEFTAG in frame with the HA tag, resulting in pEFTAGmNFAT1-A, pEFTAGmNFAT1-B, and pEFTAGmNFAT1-C, respectively. The first three residues of NFAT1 were replaced by amino acids AQCGRSS from pEFTAG.

The pBLCAT5/NFAT3X reporter plasmid was constructed by subcloning three copies of the murine IL-2 promoter NFAT site from plasmid pMILNFAT-CAT (23) upstream of the minimal thymidine kinase promoter in pBLCAT5 plasmid (57), a gift from E. Serfling. Plasmid 5′IL2-CAT, which contains sequence from −576 to +42 of the human IL-2 promoter (56), was a gift from G. R. Crabtree. Plasmid TNFα-Luc, which contains the sequence from −614 to +20 of the human TNF-α promoter in pGL2, was a gift from S. L. McKnight. Plasmid pSVO-801 (61), which contains the chloramphenicol acetyltransferase (CAT) reporter gene driven by the murine IL-4 promoter (−801 to +58), was a gift from L. H. Glimcher. Plasmid pHGM, which contains the region from −627 to +29 bp of the human GM-CSF promoter (8), was from P. N. Cockerill. Plasmid pSRα-ΔCaM-AI (43) was a gift from R. L. Kincaid; plasmids pSRα-CnB and pSRα (34) were gifts from F. McKeon.

Transfection, CAT, and luciferase assays. Jurkat and COS cells were obtained from the American Type Culture Collection; aliquots of log-phase cells were frozen and stored in liquid nitrogen. Jurkat and COS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM glutamine, and 50 μM 2-mercaptoethanol (Life Technology, Inc.). The untransformed murine T-cell clone Ar-5 was cultured in IL-2-containing medium as described previously (23). Jurkat cells were transfected by electroporation in serum-free medium in 0.4-cm cuvettes with settings of 250 V and 960 μF, using a Bio-Rad Gene Pulser. COS cells were transfected by the DEAE-dextran method (38). One day after transfection, cells were treated overnight by adding ionomycin or PMA to cell culture media; solvent was added as a control. Cells were harvested 2 days after transfection, and CAT assays were performed as described previously (23) and quantified with a PhosphorImager (Molecular Dynamics). Routinely, 5 μl from 100 μl of cell extract was used for a 30-min CAT assay. Luciferase assays were done with a luciferase assay system (Promega). Transfection efficiencies were determined by cotransfecting a Rous sarcoma virus-human growth hormone (hGH) plasmid (52) and measuring the hGH concentration in the cell culture medium with a radioimmunometric assay kit (Hybritech). CAT activities were normalized for transfection efficiency or protein amount for aliquots of the same transfection. Protein concentrations were determined with the Bradford assay (Bio-Rad), with bovine serum albumin as a standard.

Antibodies and Western blotting (immunoblotting). Polyclonal and monoclonal antibodies against peptides or recombinant protein fragments of NFAT1 were raised and purified as described previously (16, 64). Antibody against the HA epitope (12CA5) was from Boehringer Mannheim. Western blotting of whole-cell sodium dodecyl sulfate (SDS) lysates was done as described previously (16, 55).

Immunocytochemistry. Cells were transfected as described above. Jurkat cells were allowed to attach to poly-D-lysine-coated coverslips 2 days after transfection, and COS cells were grown overnight on uncoated coverslips 1 day after transfection. Attached cells were either left unstimulated or stimulated with 3 μM ionomycin for another 15 min at 37°C. CsA (1 μM) was added 10 min

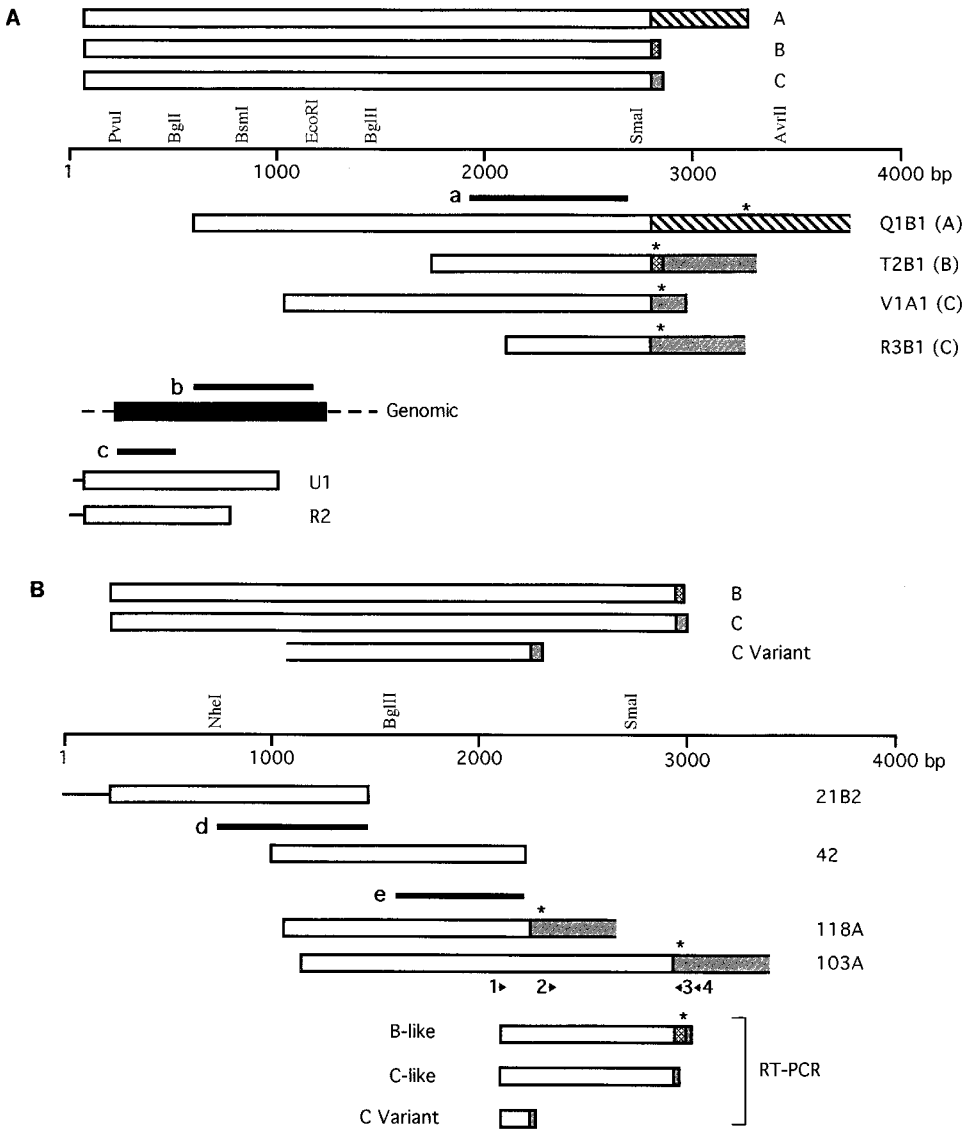


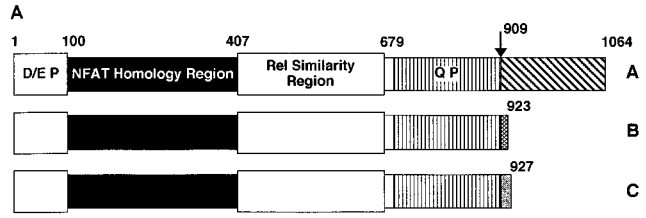
FIG. 1. Strategies for cloning murine (A) and human (B) NFAT1. The ruler in each panel shows the length starting from the 5′ end of the compiled cDNA sequence and the restriction sites used for generating probes and subcloning. The coding regions of the NFAT1 isoforms are shown schematically above the ruler, with the open box representing the common region and the filled boxes with different patterns representing the alternatively spliced 3′ regions encoding the different C termini. The partial sequence of the short human C variant is represented by the open-ended box. The probes used for screening are shown below the ruler as thick lines designated by lowercase letters. For clarity, only representative clones isolated by using each probe are shown below the probe with their designations. Each murine isoform is represented by at least two independently isolated clones. Open boxes represent the common coding region, whereas the filled boxes indicate the alternatively spliced cDNA forms, with the same pattern representing the same sequence. Open-ended boxes indicate cDNA clones longer than shown. Asterisks indicate stop codons. Thin lines represent the 5′ untranslated region. A fragment of a murine genomic clone is indicated in panel A, with the exon shown as a black box and the introns shown as dashed lines. The arrowheads in panel B indicate the primers used for RT-PCR, pointing in the direction of polymerase reaction, and the primers are numbered as in Materials and Methods. The RT-PCR products from Jurkat cells are indicated in panel B. When primer 2 was used as the 5′ primer, only PCR products corresponding to the fragments of isoforms B and C were detected.

before treatment and was present during stimulation in indicated samples. Cells were fixed immediately after treatment and then stained with antibody 12CA5 to the HA epitope tag as previously described (55). After incubation with secondary antibody, cells were stained with 10 μg of Hoechst dye 33342 (Sigma) per ml for 2 min, washed, and mounted for microscopy.

GenBank accession numbers. The GenBank accession numbers for the NFAT1 sequences described in this report are U02079 (murine NFAT1-A), U36575 (murine NFAT1-B), U36576 (murine NFAT1-C), U43341 (human NFAT1-B), and U43342 (human NFAT1-C).

RESULTS

Isolation of cDNA clones encoding murine and human NFAT1. Figure 1A summarizes the alignment of a subset of representative cDNA clones and a murine genomic clone used to deduce the primary sequence of murine NFAT1. The compiled murine cDNA sequence has an open reading frame encoding all of the tryptic peptides obtained from purified murine NFAT1 (32), two of which (Y-38 to E-45 and A-51 to D-90) are located N terminal to the previously published protein sequence. The cDNA clones fall into three classes designated NFAT1-A, NFAT1-B, and NFAT1-C, which are related by alternative splicing at a common site in the coding sequence (Fig. 2). The cDNAs for NFAT1-B and NFAT1-C also share extensive sequences after the common splicing site (Fig. 1A).



B

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m MDVPEPQPDPDGGDGPGHEPGGSPQDELDFSILFDYDYLNPIEEEPIAHKAISSPSGLAY 60
h -NA--R--Q-----A----------E----N----N---VA-P---P-- 60
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C

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                910 AATSESWVGTERYIERKFWKKTLVQPGLLPSFLLLGSLSAGPRSQTPSERKPIE
murine A            EDVPLSCSQIAWCCQHPLGTCPVLPGPLAVEWWEGQLGRGLEPIPWAPDSAGSL
                    HEVDSVGLAGVVGMVLLTLMHHFSMDQNQTPSPHWQRHKEVASPGWI 1064

murine B    910 ELIDTHLSWIQNIL 923
human  B    908 ELIDTHLSWIQNIL 921

murine C    910 VNEIIRKEFSGPPSRNQT 927
human  C    908 VNEIIRKEFSGPPARNQT 925
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FIG. 2. Primary structure of NFAT1. (A) Schematic diagram of the protein structure of NFAT1. Numbers indicate the amino acid positions of murine NFAT1. Regions defined by sequence analysis are shown in boxes. D/E P, the N-terminal acidic, proline-rich region; QP, the glutamine- and proline-rich region. The arrow at aa 909 indicates the common splicing site. The short forms are not shown. (B) Comparison of the deduced amino acid sequences of the C isoforms of murine (top) and human (bottom) NFAT1. Residues that are identical in the murine and human sequences are indicated by dashes; gaps are indicated by dots. The two tryptic peptides derived from the N-terminal region mentioned in Materials and Methods are underlined under the murine sequence. The four spaced leucines are overlined, and the Rel similarity region is underlined. The arrowhead above the murine sequence indicates the common splicing site of A, B, and C isoforms, whereas the arrow below the human sequence indicates the splicing site for the short variant found in human cDNA. (C) Amino acid sequences of the C termini of murine and human isoforms after the common splicing site. The short human variant has the same C terminus as human NFAT1-C.

NFAT1-B has an insertion of 85 bp between the common splicing site and the sequence shared with NFAT1-C (Fig. 1A). An in-frame stop codon terminates the open reading frame of NFAT1-B within this insertion. Starting from the first ATG codon, NFAT1-A has 1,064 amino acid residues, NFAT1-B has 923 residues, and NFAT1-C has 927 residues, with predicted molecular masses of 115,045, 99,699, and 100,077 Da, respectively, and they differ after residue 909.

Overlapping cDNA clones encoding the human homolog of NFAT1-C were isolated by screening a human Jurkat T-cell cDNA library (Fig. 1B). To determine whether the B isoform was also expressed in human cells, we used RT-PCR to analyze the cytosolic RNA of Jurkat cells. Assuming that murine and human mRNAs are organized similarly, that is, that the B and C isoforms share a region of 3′ untranslated sequence, we used two antisense primers downstream of the stop codon of NFAT1-C and sense primers in the coding region for RT-PCR. Sequence analysis of the PCR products showed that both the human B and C isoforms of NFAT1 were represented. Human NFAT1-B and NFAT1-C have 921 and 925 residues, with predicted molecular masses of 99,817 and 100,178 Da, respectively.

The first ATG in the cDNA was assigned as the start codon, although there are no in-frame stop codons in the upstream sequences of 70 bp (murine) or 220 bp (human) in the isolated cDNAs. In support of this assignment, the human and murine cDNA sequences diverge upstream of the first ATG codon. Furthermore, when human NFAT1-B and NFAT1-C were expressed in COS cells with an engineered ATG codon at the 5′ end of the isolated cDNAs, the resulting proteins migrated on SDS-polyacrylamide gels with a higher apparent molecular weight than T-cell NFAT1 (data not shown). In contrast, COS cell expression of NFAT1-B or NFAT1-C starting with the assigned ATG codon yielded proteins migrating with the same apparent molecular weight as endogenous T-cell NFAT1 (see Fig. 4).

We also isolated several cDNA inserts, representing a short variant of human NFAT1 cDNA, from the Jurkat cDNA library (Fig. 1B). These cDNAs encode an apparent splicing variant of the C isoform, lacking sequences between the 3′ end of the Rel similarity region and the common splicing site of the three isoforms identified in mouse cells. However, similar variants were not isolated from the murine T-cell cDNA library, and there is no evidence that a corresponding short form of the protein is expressed in murine and human T cells (see below).

Structure of NFAT1. Figure 2A shows a schematic diagram of the predicted structure of murine and human NFAT1. The human and murine proteins show 90% identity in their amino acid sequences (Fig. 2B and C). The region spanning the first 100 amino acids (aa) of NFAT1 (designated D/E P in Fig. 2A) is rich in acidic (19%) and proline (17%) residues and shows little sequence similarity to the corresponding regions of other NFAT family members (18, 28, 41). The central region of NFAT1 contains the DNA-binding domain (aa 410 to 679 of murine NFAT1), which shows a strong homology within the NFAT family (18, 28, 41) and a weak resemblance to the Rel homology region as noted before (18, 20, 40). NFAT1 contains a C-terminal region of over 200 aa (designated QP in Fig. 2A) that is rich in glutamine (15%) and proline (11%). This region does not exist in NFATc (41) and has little sequence similarity to the C-terminal regions of NFAT3 or NFATx/4/c3 (17, 18, 28). NFAT1-B and NFAT1-C have very short unique sequences after the splicing site, whereas NFAT1-A has 155 amino acid residues of unique sequence (Fig. 2C).

Sequence comparison of NFAT family proteins revealed another conserved region (approximately aa 102 to 406 of

FIG. 3. Sequence alignment of the NHR of human NFAT1, NFATc, NFAT3, and NFATx/4/c3 (labeled NFAT4). Residues conserved in all four sequences or showing only K/R or D/E substitutions are highlighted as white letters against a black background. Dashes represent gaps introduced into the sequences to optimize sequence alignment. The lines under the sequences indicate the SP motifs and other motifs mentioned in the text. The numbers on the right indicate the amino acid numbers of the last residues of the respective lines. All isoforms of NFAT1 possess the same NHR sequences, as do all isoforms of NFAT4 (including NFATx and NFATc3).

human NFAT1) (Fig. 3) just N terminal to the DNA-binding domain. This region, designated the NFAT homology region (NHR), is rich in serine and proline and is found only in NFAT family proteins. The NHR shows ~30 to 40% pairwise sequence identity between NFAT1 and other NFAT family members, and the conserved residues are clustered in conserved sequence motifs (underlined in Fig. 3). Among these motifs are the three repeats of the SP motif with a consensus sequence SPxxSPxxSPxxxxx(D/E)(D/E) as noted before (28). The second and third SP motifs are incomplete in NFAT3. The spacing between these repeats is not tightly conserved. N terminal to the first SP motif is a serine-rich motif with a consensus sequence of SPxS(s)SxSSxSxxS(D/E) (residue 168 to 181 in human NFAT1). Flanking the third SP motif are two motifs rich in basic residues, one with a consensus sequence of RxxSPxxKR(R/K)xS (residue 244 to 255 in human NFAT1) and the other with a consensus sequence of IPxKxxKT(S/T)xD (residue 318 to 328 in human NFAT1). The NHR begins with a motif of PxIxIT (aa 111 to 116 in human NFAT1) which is followed by a conserved YR(E/D) motif. Strong homology is also found in the C terminus of the NHR which is adjacent to the Rel similarity region.

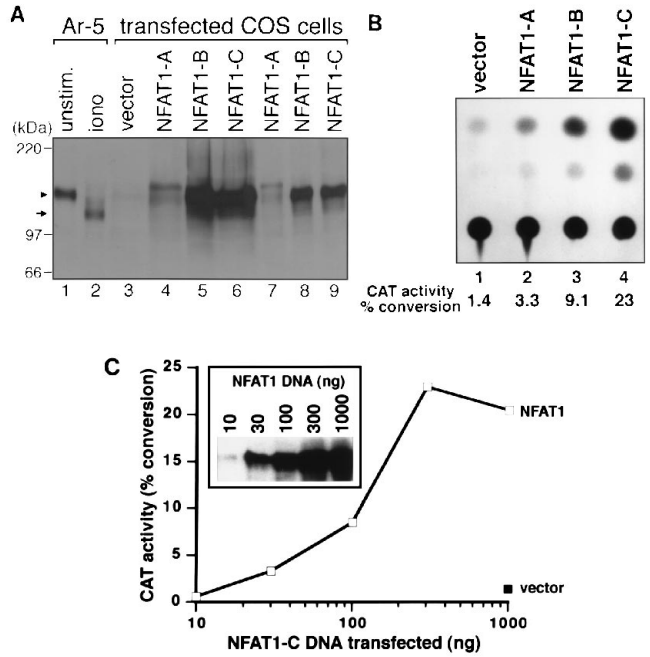
The NHR of NFAT1 also contains a sequence of four leucine residues (L-121 to L-142; overlined in Fig. 2B) spaced in a heptad repeat that is not found in the other NFAT family proteins. Although the interspersed glycine and proline residues within this sequence suggest that it does not form a helical leucine zipper, it is intriguing that both RelA and RelB have similar leucine zipper-like motifs that are required for their transactivation activities (10, 50). The corresponding regions in other NFAT family proteins are rich in acidic residues.

Expression of NFAT1 protein. Experiments in which immunoprecipitation of NFAT1 with one antiserum was followed by Western blotting with an antiserum recognizing a different region of the protein confirmed that isoforms B and C of NFAT1 contained the N-terminal peptide and the DNA-binding domain (64). These two isoforms, which comigrate on SDS-gels, constitute the bulk of the NFAT1 detected by Western blotting in human and murine T-cell lines. Both isoforms are expressed in murine spleen and thymus and in some nervous system cells but are not detected in a variety of other organs or tissues (16, 64). NFAT1-B and NFAT1-C have been found in several lymphoid and myeloid lineage cells, including T cells, B cells, natural killer cells, mast cells, monocytes, and macrophages (55, 64), suggesting that NFAT1 may play a general role in regulating the transcription of cytokine genes (47).

Western analysis of extracts from unstimulated human Jurkat cells, using antisera against the N-terminal and C-terminal peptides or the DNA-binding domain of NFAT1, did not reveal a protein corresponding in size (~80 kDa) to the isoform that would be encoded by the shorter variant NFAT1 cDNA, nor was such a protein detected in human Epstein-Barr virus-transformed B cells, in murine Ar-5 T cells, or in Ar-5 cells stimulated for up to 5 h with a variety of stimuli, including ionomycin, immobilized anti-CD3 antibodies, or IL-2 (3a). Therefore, the variant human cDNA may represent a splicing variant that is not normally expressed as protein, and we focused our subsequent analyses on the long isoforms. However, it is noteworthy that NFAT4 cDNAs (18) display alternative splicing at the corresponding site and that NFATc (41) has only a short C-terminal sequence following the DNA-binding domain, like the predicted variant NFAT1 protein.

Transactivation and regulation of NFAT1 in COS cells. We first examined the function of NFAT1 by transient transfection of COS cells, which do not express detectable endogenous NFAT1 (Fig. 4A, lane 3). NFAT1-B and NFAT1-C expressed in COS cells are similar in apparent molecular weight to NFAT1 from resting murine Ar-5 T cells, which migrates as a single band of ~140 kDa (Fig. 4A; compare lanes 5, 6, 8, and 9 with lane 1). NFAT1-A is expressible in COS cells but at a much lower level and is larger than the other two isoforms and the endogenous NFAT1 in Ar-5 cells, most likely because of its long unique C terminus, which may also contribute to its lower level of expression (Fig. 4A, lanes 4 and 7). NFAT1 in stimulated Ar-5 T cells showed a decrease in apparent molecular weight to ~120,000 (lane 2), which is caused by dephosphorylation (51, 55); likewise, COS cell-expressed NFAT1-B and NFAT1-C showed a similar decrease in apparent molecular weight upon treatment with calcineurin in vitro (26a). The single bands corresponding to the phosphorylated and dephosphorylated forms of NFAT1 are recognized by antibodies directed against the unique C termini of isoforms B and C but not by antibodies directed against several unique C-terminal peptides of isoform A (64). Together, these results suggested that the A isoform is a minor component in T cells; therefore, for subsequent analyses, we focused on isoforms B and C.

The transactivation activity of each of the NFAT1 isoforms was assayed by measuring CAT activity in COS cells cotransfected with a NFAT1 expression plasmid and a CAT reporter gene driven by three copies of the IL-2 promoter NFAT site. Each isoform of NFAT1 was able to stimulate CAT gene expression (Fig. 4B, lanes 2 to 4). In several independent transfection experiments, NFAT1-C stimulated CAT gene expression by 5- to 16-fold over the basal level. The level of transactivation by NFAT1-C increased as a function of the level of NFAT1-C protein expressed in COS cells (Fig. 4C). The low transactivation activity of NFAT1-A (Fig. 4B) could reflect its low level of expression (Fig. 4A). It is not possible, however, to compare the relative levels of transactivation by the different isoforms, since transactivation depends not only



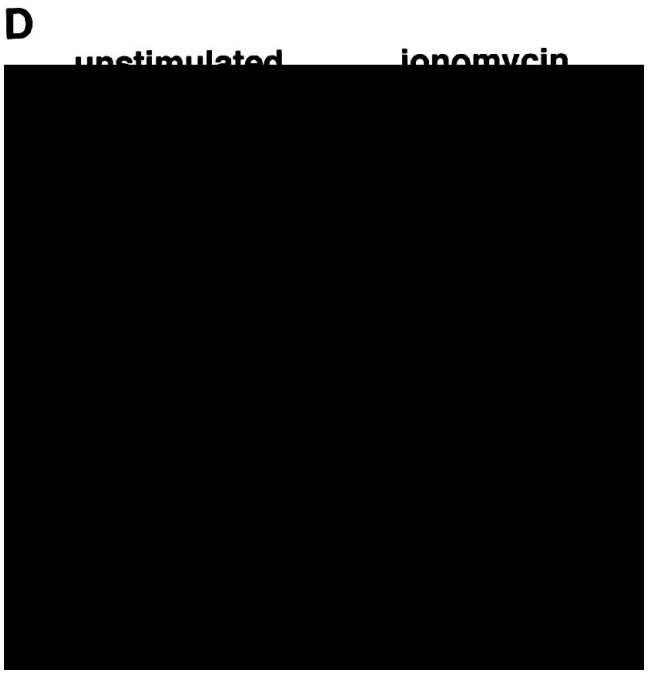


FIG. 4. Expression and transactivation activity of NFAT1 in COS cells. (A) Expression of the NFAT1 isoforms. COS cells were cotransfected with 1 μg of the reporter plasmid pBLCAT5/NFAT3X and 1 μg of expression plasmid for each NFAT1 isoform or vector control. Two days after transfection, cells were lysed and NFAT1 expression was assessed by Western analysis using anti-67.1 antibody. Lanes 1 and 2 contain lysates from resting and ionomycin-stimulated Ar-5 T cells (2×10^5 cells), respectively. The arrowhead indicates NFAT1 from resting Ar-5 cells, whereas the arrow indicates dephosphorylated NFAT1 from ionomycin-stimulated Ar-5 cells. Lysates of 4.8×10^5 (lanes 3 to 6) or 1.2×10^5 (lanes 7 to 9) transfected COS cells were loaded. The data shown are representative of at least three separate transfection experiments. (B) Transactivation mediated by NFAT1 in COS cells. COS cells were transfected as in panel A, and cell extracts were made 2 days later for assay of CAT activity, which is shown as percent conversion under each lane. The data shown are representative of at least three separate transfection experiments. (C) Transactivation as a function of the level of NFAT1 expressed in COS cells. One microgram of empty vector pLGP3 (filled square) or increasing amounts of pLGPmNFAT1-C (open squares) DNA was cotransfected into COS cells with 1 μg of pBLCAT5/NFAT3X. The total amount of DNA transfected was kept constant at 1 μg per transfection by using the empty vector DNA. Two days after transfection, half of the cells were lysed for Western analysis (insert) as in panel A, and half were used for CAT assays as in panel B. (D) Subcellular distribution of NFAT1 in COS cells. COS cells were transfected as in panel A with 1 μg of pEFTAG mNFAT1-C plasmid; 1 day after transfection, cells were transferred to coverslips and grown overnight. Cells were either unstimulated or stimulated with 5 μM ionomycin for 20 min and then fixed for immunocytochemistry as described in Materials and Methods. The data shown are representative of at least three separate transfection experiments.

on the expression of the isoform but also on the level of nuclear localization and appropriate posttranslational modification (55). These functions do not appear to be appropriately regulated in COS cells: as reported for NFAT3 (18), transactivation by NFAT1 in COS cells was not altered by stimulation with ionomycin and PMA (data not shown). The recombinant NFAT1 was found in the cytoplasm of the majority of the transfected COS cells and to some extent in the nuclei of some cells (Fig. 4D). Neither the subcellular distribution of the NFAT1 nor the number of cells containing NFAT1 in their nuclei was detectably altered by ionomycin stimulation, suggesting that NFAT1 is not regulated by a calcium-stimulated signaling pathway in COS cells. Although COS cells have been widely used in studying transactivation by recombinant NFAT family proteins (17, 18, 28, 41), our results suggest that COS cells lack the requisite mechanisms for regulating NFAT1 subcellular localization and transactivation.

Transactivation and regulation of NFAT1 in Jurkat cells. We next examined the transactivation activity of NFAT1 in Jurkat T cells, which show calcium- and calcineurin-dependent regulation of the endogenous cytoplasmic component of NFAT (7, 30, 65). Overexpression of NFAT1 in unstimulated Jurkat cells resulted in increased expression of a CAT reporter gene driven by three copies of the IL-2 promoter NFAT site (Fig. 5A). This small increase in basal transactivation activity is most likely due to some spillover of the overexpressed NFAT1 into the nucleus, since the majority of the recombinant NFAT1 is in the cytoplasm (see below). Importantly, the transcriptional activity of NFAT1 is inducible: stimulation of the NFAT1-transfected cells with ionomycin plus PMA increased expression of the CAT reporter gene by 6- to 30-fold (Fig. 5A; compare lanes 3, 5, and 7 with lanes 4, 6, and 8). The increase in CAT activity upon stimulation of cells transfected with vector alone (Fig. 5A, lanes 1 and 2) can be attributed to endogenous NFAT. However, the increase in NFAT-dependent transactivation mediated by overexpressed recombinant NFAT1 is sufficiently large by comparison that we were able to investigate the regulation of NFAT1 in Jurkat cells independently of the endogenous NFAT family proteins present in these cells.

To further study the regulation of recombinant NFAT1, we examined the individual effects of ionomycin and PMA on transactivation by NFAT1-C (Fig. 5B). Ionomycin or PMA alone did not stimulate detectable endogenous NFAT transactivation activity, but together they did (Fig. 5B, lanes 1 to 4). Ionomycin alone reproducibly stimulated transactivation mediated by the recombinant NFAT1 by two- to threefold in Jurkat cells (Fig. 5B, lane 6), whereas PMA alone caused only a marginal increase (Fig. 5B, lane 7). Ionomycin plus PMA gave maximal stimulation of the NFAT activity by 10- to 30-fold over that of the unstimulated cells (Fig. 5B, compare lanes 5 and 8). Furthermore, the stimulation of NFAT1 activity by ionomycin was blocked by pretreatment of cells with CsA (Fig. 5C). The recombinant NFAT1 was present at equivalent levels under all conditions, as judged by Western blotting (data not shown). The NFAT site that we used is a composite site con-

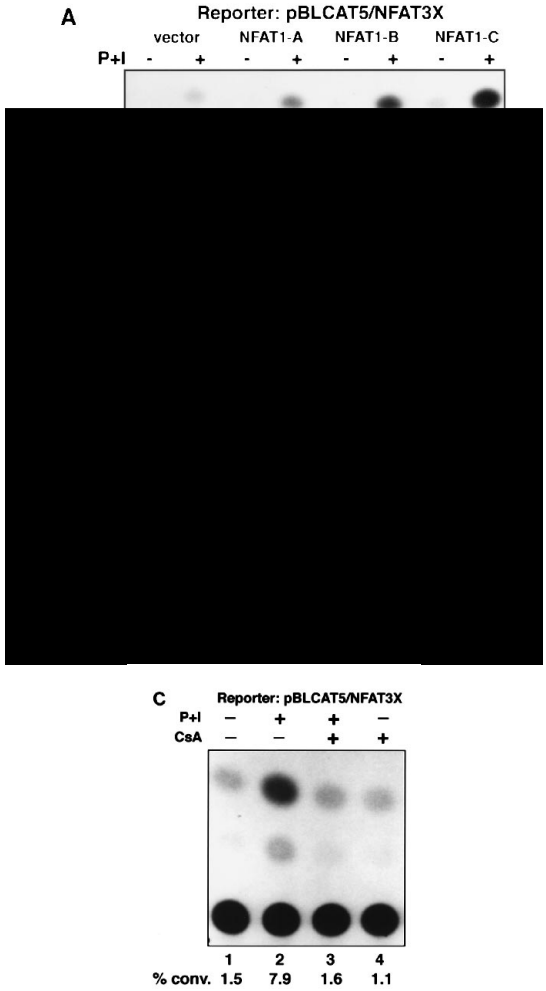


FIG. 5. Transactivation by NFAT1 in Jurkat T cells. (A) Transactivation by NFAT1 isoforms in resting (−) and stimulated (+) Jurkat cells. Jurkat cells were cotransfected with 1 μg of pBLCAT5/NFAT3X reporter plasmid and 10 μg of pLGP3 empty vector (lanes 1 and 2), pLGPmNFAT1-A (lanes 3 and 4), pLGPmNFAT1-B (lanes 5 and 6), or pLGPmNFAT1-C (lanes 7 and 8), and each transfection mixture was aliquoted into two flasks. One day after transfection, cells were left untreated (lanes 1, 3, 5, and 7) or treated overnight with 1 μM ionomycin (I) and 10 nM PMA (P) (lanes 2, 4, 6, and 8). CAT activity in cell extracts was assayed the next day and is shown as percent conversion under each lane. (B) Transactivation by NFAT1 in response to different stimuli. Jurkat cells were transfected with 1 μg of pBLCAT5/NFAT3X and 10 μg of pEFTAG (lane 1 to 4) or 10 μg of pEFTAGmNFAT1-C (lanes 5 to 8), and each transfection mixture was aliquoted into four flasks. One day after transfection, cells were either left untreated (−) or treated overnight with 1 μM ionomycin (I), 10 nM PMA (P), or both (P+I). CAT activity in cell extracts was assayed the next day. The percent conversion normalized for total protein amount in each sample is shown under each lane. (C) CsA inhibits NFAT1 transactivation. Jurkat cells were transfected with 1 μg of pBLCAT5/NFAT3X and 10 μg of pEFTAG mNFAT1-C as in panel B and aliquoted into four flasks. One day after transfection, cells were either left untreated (lanes 1 and 2) or treated with CsA (lanes 3 and 4) for 1 h and then either unstimulated (lanes 1 and 4) or stimulated (lanes 2 and 3) overnight with ionomycin and PMA (P+I). CAT activity was measured as in panel B. Data in all panels are representative of at least three separate experiments.

taining both NFAT1 and AP-1-binding sites (2, 4, 47). The dual requirement for ionomycin and PMA stimulation suggests that transactivation from this site is a result of cooperation of NFAT1 with Fos and Jun proteins (4, 21, 47). These results indicate that activation of recombinant NFAT1 accurately represents the activation of the cytoplasmic component of NFAT in Jurkat cells.

Stimulation of NFAT1-mediated transactivation activity by calcineurin. The ability of CsA to inhibit transactivation mediated by NFAT1 indicated that recombinant NFAT1 is regulated by calcineurin in Jurkat cells. To examine this hypothesis further, we transiently transfected Jurkat cells with expression plasmids encoding NFAT1, a constitutively active form of the calcineurin A chain lacking its autoinhibitory domain, the calcineurin B chain, and the CAT reporter plasmid (Fig. 6). We included the calcineurin B chain in the experiments because it is required for the stability and catalytic activity of the calcineurin A chain, whether full length or truncated to remove the autoinhibitory domain (34, 45). Overexpression of calcineurin stimulated CAT expression, presumably by activating endogenous NFAT, and CAT levels were further increased by treatment with ionomycin and PMA. In Jurkat cells overexpressing NFAT1, active calcineurin strongly increased CAT expression in the absence of stimulation, and stimulation with ionomycin and PMA further enhanced transactivation. Treatment with CsA blocked both the effect of calcineurin and the stimulation by PMA and ionomycin. These experiments strengthen the conclusion that activation of recombinant NFAT1 is controlled by calcineurin.

Regulation of the subcellular localization of recombinant NFAT1 in T cells. We investigated the subcellular localization of recombinant NFAT1 by immunocytochemistry. A plasmid encoding full-length HA-tagged murine NFAT1 was transiently transfected into Jurkat cells. The epitope tag did not appear to change the transactivation activity of the recombinant NFAT1 (Fig. 5A and B), and the transactivation activity of the tagged recombinant NFAT1 was enhanced by the constitutively active calcineurin (Fig. 6). The recombinant NFAT1 protein was visualized by using monoclonal antibody 12CA5 against the HA epitope, and the nuclei of Jurkat cells were stained with Hoechst dye 33342. In resting Jurkat cells, the recombinant NFAT1 was cytoplasmic, with minimal antibody staining in the nucleus, as shown by the different staining patterns of antibody 12CA5 and Hoechst dye 33342 (Fig. 7, top panels). Ionomycin stimulation caused translocation of HA-tagged NFAT1 into the nucleus in the majority of transfected cells, as visualized by the overlapping staining of 12CA5 and Hoechst dye in the center of the cells (center panels), and this translocation was completely blocked by pretreatment of the

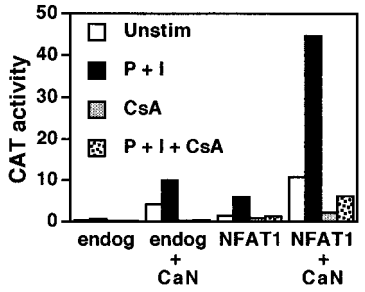


FIG. 6. Calcineurin increases transactivation mediated by NFAT1. Jurkat cells were transfected with 1 μg of pBLCAT5/NFAT3X reporter and empty expression vectors (endog), 10 μg each of pSRα-ΔCaM-AI and pSRα-CnB (endog + CaN), 10 μg of pEFTAGmNFAT1-C (NFAT1), or 10 μg each of pEFTAGmFNAT1-C, pSRα-ΔCaM-AI, and pSRα-CnB (NFAT1 + CaN). The total amount of DNA per transfection was kept constant by using appropriate empty vectors. Each transfection mixture was aliquoted into four flasks, and the cells were stimulated the next day as indicated. For CsA treatment, CsA was added to cell culture media immediately after transfection at a final concentration of 1 μM. Bars represent CAT activity in percent conversion measured as in Fig. 4C. Data shown are representative of three experiments. Unstim, unstimulated; P + I, PMA plus ionomycin.

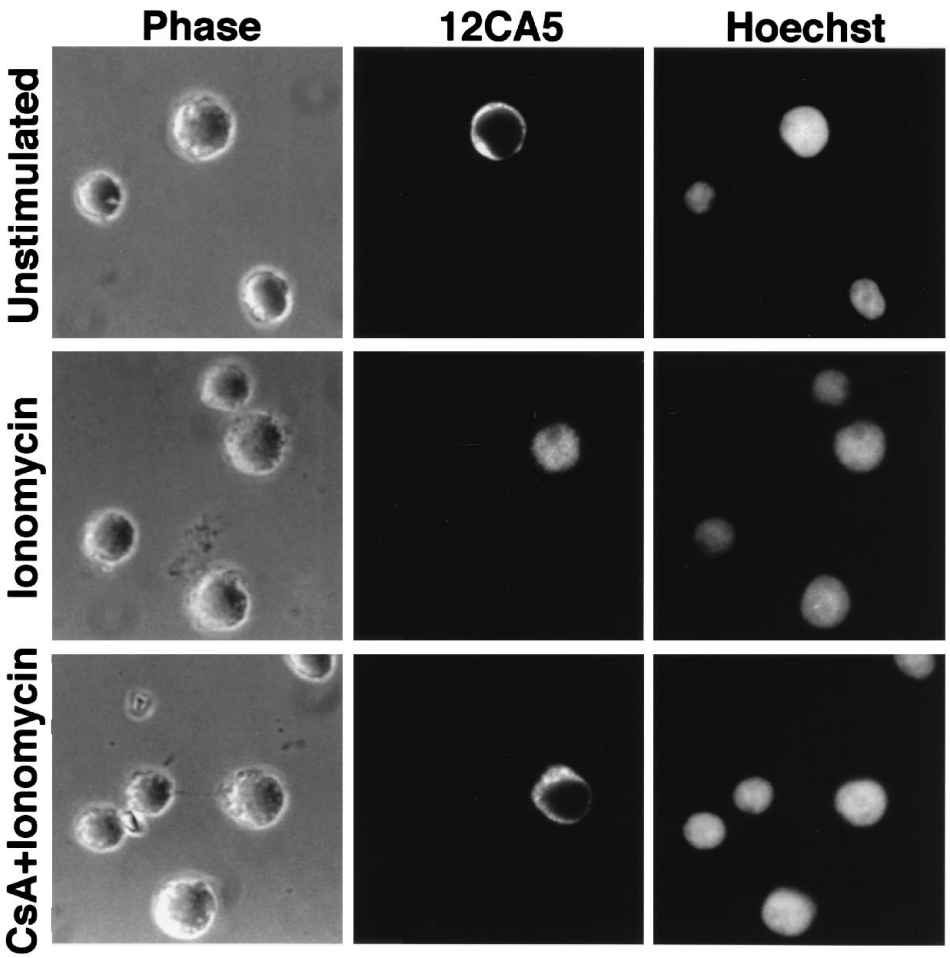


FIG. 7. Regulation of subcellular localization of NFAT1. Jurkat cells were transfected with 10 μg of pEFTAGmNFAT1-C as described in Materials and Methods. Two days after transfection, recombinant NFAT1 was visualized with antibody 12CA5 to the HA tag (12CA5), and the nuclei were stained with Hoechst dye 33342 (Hoechst). Routinely, transfection efficiency was 1 to 5%, as estimated from the total number of cells stained. Untransfected cells are visible by phase-contrast microscopy (Phase) and with Hoechst staining but are invisible with 12CA5 staining. Shown are representative fields (magnification, ×100) of cells left unstimulated (Unstimulated), stimulated with ionomycin for 15 min (Ionomycin), or CsA treated and then ionomycin stimulated in the presence of CsA (CsA+Ionomycin). The condition of cells was checked under phase-contrast field (Phase).

cells with CsA (bottom panels). The same results were observed in murine T cells transfected with the same plasmid (data not shown). The same subcellular distribution was observed for recombinant NFAT1 without the HA tag, which can be visualized by using antibodies directed against NFAT1 in transfected Jurkat T cells because of the low level of endogenous NFAT1 expression (data not shown). Our results, which have been reproduced in two different Jurkat cell lines, confirm that the subcellular distribution of recombinant NFAT1 in Jurkat cells is regulated by a calcium- and calcineurin-dependent pathway, as observed for endogenous NFAT1 in murine T cells and primary lymphoid cells (51, 55).

Transactivation mediated by NFAT1 at different cytokine gene promoters. We examined the role of NFAT1 in the expression of different cytokine genes by cotransfecting Jurkat cells with an NFAT1-expressing plasmid and reporter plasmids in which expression of the CAT gene was controlled by the IL-2, IL-4, TNF-α, or GM-CSF promoter. Transactivation of the human IL-2 promoter was detected in cells stimulated with ionomycin plus PMA but not in unstimulated cells, and cells transfected with the NFAT1 expression plasmid showed a much higher level of inducible CAT expression than cells transfected with the empty expression vector (Fig. 8A). Furthermore, induction of CAT gene expression was blocked by CsA (Fig. 8A). The expression of hGH controlled by the Rous sarcoma virus promoter was not influenced by coexpression of NFAT1 (data not shown); these results ruled out the possibility that induction of IL-2 promoter was due to gross increase in transcription by NFAT1. Similarly, overexpression of NFAT1 increased transcription driven by the promoters of murine IL-4 (Fig. 8B), human TNF-α (Fig. 8C), and human GM-CSF (Fig. 8D) in cells stimulated with ionomycin plus PMA. These results have been seen in multiple transfection experiments using different plasmid preparations (Table 1). They demonstrate that in T cells, recombinant NFAT1 is capable of contributing to inducible transcription from several cytokine promoters, suggesting that NFAT1 participates in regulating the expression of many different cytokines during the immune response.

DISCUSSION

We have isolated two new isoforms of NFAT1 that are the predominant isoforms present in immune system cells. When expressed in Jurkat T cells, these proteins (NFAT1-B and

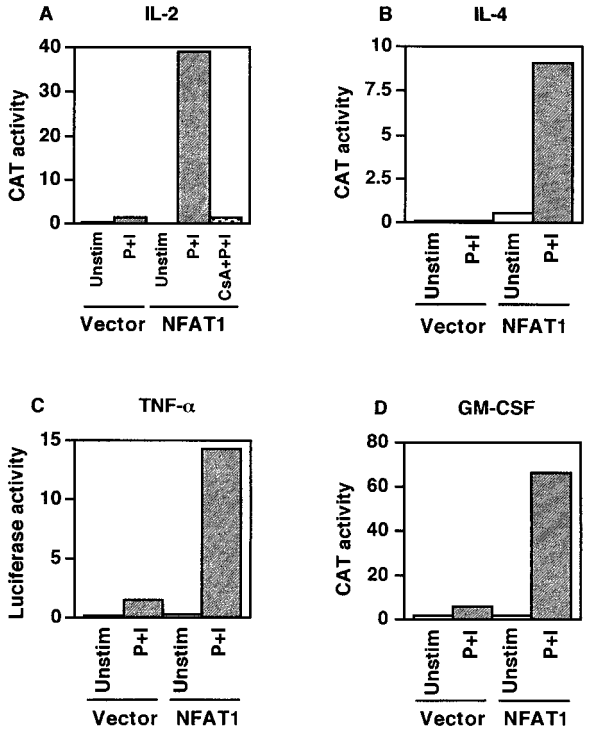


FIG. 8. NFAT1 transactivates the IL-2 (A), IL-4 (B), TNF-α (C), and GM-CSF (D) promoters. Jurkat cells were transfected with 2 μg of 5′IL2-CAT reporter plasmid (A) or 5 μg of pSVO-801 (B), TNFα-Luc (C), or pHGM (D) and 10 μg of an NFAT1 expression vector (NFAT1) or an empty vector control (vector) and then aliquoted into three or two flasks. One day after transfection, cells were left unstimulated (Unstim), stimulated with PMA and ionomycin (P+I), or pretreated with 1 μM CsA for 1 h and then stimulated overnight with PMA and ionomycin (CsA+P+I). Bars depict CAT activity (in percent conversion) or luciferase (in 10^3 light units) activity measured 1 day after stimulation. The transfection efficiency was determined by measuring the hGH expression from a cotransfected Rous sarcoma virus-hGH plasmid.

NFAT1-C) exhibit the properties expected for the cytoplasmic component of NFAT. Like endogenous NFAT1 (55), the recombinant proteins localize in the cytoplasm of resting cells and translocate to the nucleus upon stimulation with ionomycin. As expected, however (30), their full transcriptional activity at multimerized NFAT sites requires additional stimulation with PMA. Transactivation mediated by recombinant NFAT1 is potentiated by coexpression of a constitutively active form of calcineurin, as previously noted for transactivation by endogenous NFAT (7, 15, 25, 62, 65). Recombinant NFAT1 increased transcription of several cytokine promoters (IL-2, IL-4, GM-CSF, and TNF-α) previously shown to possess NFAT-binding sites (reviewed in reference 47), suggesting that NFAT1 contributes to the inducible expression of these cytokine genes, and possibly other CsA-sensitive genes, during the immune response. However, NFAT1 may not be absolutely required or may be compensated by other NFAT family proteins for the expression of these cytokines, since the levels of IL-2 and TNF-α produced by T cells of NFAT1-deficient mice appear normal (17a, 66).

A previous report showed that after subcellular fractionation of resting Jurkat and Raji cell lines, NFAT1 was present in both the cytoplasmic and nuclear fractions and its subcellular distribution was not affected by CsA treatment of the cells (44). The reason for the apparent constitutive nuclear localization of NFAT1 in these cell lines is not clear; although the authors showed that their nuclear extracts were not contaminated with an abundant cytoplasmic protein, it is possible that their lysis and extraction conditions permitted cytoplasmic to nuclear redistribution of NFAT1. Alternatively, cell culture conditions may have resulted in partial nuclear localization of NFAT1, or the cell lines used may have had specific alterations in the NFAT activation pathway. A precedent is provided by the murine lymphoma EL4 cell line, which has a high constitutive level of nuclear NFAT and harbors a mutation in the calcineurin Aα chain that results in constitutive calcineurin phosphatase activity (12). These questions could be resolved by immunocytochemical analysis of NFAT1 localization in these cell lines. Nevertheless, these cells have been valuable for establishing a second important mechanism by which calcineurin regulates NFAT1 function, namely, an increase in DNA-binding activity following dephosphorylation (44). This mechanism has also been borne out in the untransformed murine T-cell clone Ar-5 (55), as well as in the Jurkat lines used in this study (27a).

NFAT1 possesses two regions of sequence similarity with the other three NFAT family proteins and two regions of unique sequence. The 283-aa DNA-binding domain of NFAT1 is highly conserved (66 to 72% sequence identity) with the other NFAT family proteins (18, 28, 41). A novel NHR just N terminal to the DNA-binding domain also shows significant conservation of sequence. In contrast, the sequence of the N-terminal ~100 aa is unique in each NFAT family protein; it is rich in acidic residues and proline in the case of NFAT1, NFAT3, and NFAT4/NFATx and is proline rich but not especially acidic in the case of NFATc (18, 28, 41) (Fig. 2B). The region immediately C terminal to the DNA-binding domain, when present, is also unique to each NFAT family protein (18, 28, 41). The functions of these unique and conserved regions (other than the DNA-binding domain) remain to be determined, but some possibilities are discussed below.

As noted previously (18), the DNA-binding domains of the four NFAT family proteins show a higher level of amino acid

TABLE 1. Enhancement of the transcription of cytokine promoters by overexpression of NFAT1 in Jurkat cells

Reporter Exp	Relative transactivation activity[a]		
	Vector, Unstimulated	NFAT1-C	
		Unstimulated	P+I
IL-2			
I	0.49	0.67	37
II	0.49	0.36	31
III[b]	0.14	<0.01	28
IL-4			
I[b]	0.88	6.9	113
II	0.25	0.88	4.1
III	<0.01	2.8	8.8
TNF-α			
I[b]	0.11	0.18	9.4
II	0.19	0.24	8.1
III	0.48	0.59	3.1
GM-CSF			
I[b]	0.30	0.30	11
II	0.69	0.69	2.9
III	0.27	0.17	5.1
IV	0.44	0.39	27

[a] Jurkat cell transfection and CAT assays were done exactly as for Fig. 8. Values are relative CAT/luciferase activities normalized to the CAT/luciferase activity of PMA-plus-ionomycin (P+I)-stimulated cells transfected with expression vector and reporter plasmid (set as 1.0).

[b] The raw data for CAT or luciferase activity are plotted in Fig. 8.

identity in their N-terminal ~190 aa than in their C-terminal portions. The region of high sequence similarity corresponds to the minimal DNA-binding subdomain of NFAT1 (187 aa) that we previously mapped by deletion analysis (20). In its turn, this minimal DNA-binding domain of NFAT1 corresponds exactly to the N-terminal specificity subdomain of the p50 NF-κB DNA-binding domain (13, 37). Indeed, the conserved DNA recognition loop (RFRYxCEG) near the N terminus of the Rel homology region, which makes multiple DNA contacts in p50 NF-κB (13, 37), corresponds to a completely conserved sequence (RAHYETEG) of NFAT proteins that we have shown by mutational analysis is required for DNA binding by NFAT1 (20). In the presence of Fos and Jun family proteins, recombinant fragments containing the DNA-binding domains of all four NFAT family proteins bind with similar affinities to the IL-2 promoter NFAT site (18), and the highly conserved minimal DNA-binding domain is sufficient for this cooperative interaction (20). In contrast, in the absence of Fos and Jun proteins, there appear to be differences in the binding-site selectivity of the four NFAT proteins for the IL-2 and IL-4 promoter NFAT sites (17, 18). The differences in binding-site selectivity suggest that in vivo, different NFAT proteins may selectively mediate the transcription of different genes. This question cannot be readily addressed by using transient-transfection systems, since the overexpressed proteins are likely to mediate transactivation even from sites to which they bind with low affinity.

The N and C termini of NFAT1 show features of transactivation domains. Besides the heptad repeat of leucines mentioned above, the unique N-terminal region of NFAT1 (D/E P in Fig. 2A) contains two acidic hydrophobic patches, ^{25}QDEL DFSILFDYEY^{39}L and ^{60}YPDDVMDYG^{69}L, that resemble those shown to be critical for transactivation by acidic activation domains (9, 53). Similar acidic hydrophobic patches are present in NFAT3 (^{8}DEELEFKLVFGE^{20}E) and NFAT4/NFATx (^{9}HDELDFKLVFGE^{21}D) (18, 28). The conserved serine- and proline-rich NHR may also contain a transactivation domain; alternatively, this conserved region may mediate regulatory interactions or other functions common to the NFAT family. The C-terminal region (QP in Fig. 2A) is rich in glutamine and proline and so may also constitute a transactivation domain (35).

Transactivation by NFAT1 is strongly regulated in T cells, at the levels of subcellular localization as well as cooperation with nuclear transcription factors. Although recombinant NFAT1 translocates to the nucleus in T cells in response to stimulation with ionomycin alone, it mediates only a low level of transactivation from multiple copies of the IL-2 promoter NFAT site under these conditions. Additional stimulation with PMA is required for optimal transactivation. The most likely possibility is that the requirement for dual stimulation with PMA and ionomycin reflects the requirement for synthesis and posttranslational modification of AP-1 proteins, which are known to stabilize the binding of NFAT1 and other NFAT family proteins at this site (reviewed in reference 47). It is also possible that stimulation with PMA and/or ionomycin is needed for the intrinsic transactivation functions of NFAT1. In the case of the IL-2, IL-4, and GM-CSF promoters, full activation would require the cooperation of NFAT1 with Fos and Jun as well as the additional participation of other transcription factors (for instance, NF-κB) that are activated by stimulation with ionomycin and/or PMA.

The mechanism of subcellular localization of NFAT family proteins is a question of considerable interest. One potential mechanism is that NFAT family proteins are actively retained in the cytoplasm of resting T cells and released from the retention mechanism upon stimulation. Alternatively, the proteins may be actively transported into the nucleus upon activation, by a calcium- and calcineurin-dependent mechanism that is present in T cells but not in COS cells. The conserved NHR in NFAT family proteins could function as a calcineurin-sensitive regulatory region to control the subcellular localization of NFAT family proteins; indeed, the N-terminal region of NFAT1 has been shown to bind calcineurin directly (27, 64a). These hypotheses can now be experimentally tested by using recombinant NFAT1 proteins that are correctly regulated in T cells.

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