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Identification and Characterization of a Novel Nuclear Factor of Activated T-cells-1 Isoform Expressed in Mouse Brain*

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The nuclear factor of activated T-cells (NFAT) family transcription factors play a key role in the control of cytokine gene expression in T-cells. Although initially identified in T-cells, recent data have unveiled unanticipated roles for NFATs in the development, proliferation, and differentiation of other tissues. Here we report the identification, cDNA cloning, and functional characterization of a new isoform of NFAT1 highly expressed in mouse brain. This isoform, which we named NFAT1-D, is identical to NFAT1 throughout the N-terminal regulatory domain and the portion of the Rel domain which includes the minimal region required for specific binding to DNA and interaction with AP-1. The homology stops sharply upstream of the 3′-boundary of the Rel homology domain and is followed by a short unique C-terminal region. NFAT1-D was expressed at high levels in all brain districts and was found as a constitutively active transcription complex. Transfection of a NFAT/luciferase reporter in the neuronal cell line PC12, which also expresses NFAT1-D, showed that these cells expressed a constitutive NFAT activity that was enhanced after nerve growth factor-induced differentiation but was resistant to the immunosuppressant cyclosporin A. NFAT1-D was, however, inducibly activated in a cyclosporin A-sensitive manner when expressed in T-cells, suggesting that the activity of NFAT proteins might be controlled by their specific cellular context.

Initially described as a transcriptional complex that bound a T-cell antigen receptor (TCR)[1] response element on the interleukin (IL)-2 gene enhancer, nuclear factor of activated T-cells (NFAT) is a family of transcription factors crucially involved in the regulation of cytokine gene expression in T-cells (1). NFAT activity is strongly unregulated after TCR triggering; however, receptor engagement can be bypassed by a combination of phorbol esters and calcium ionophores, which activate protein kinase C and induce a rise in intracellular calcium ions, respectively. This dual requirement reflects the subunit composition of NFAT factors, which includes a cytoplasmic and a nuclear component. In resting cells, NFAT is found as a cytosolic protein phosphorylated on serine residues. After an elevation in intracellular calcium ions, the calmodulin-dependent phosphatase calcineurin is activated and dephosphorylates NFAT, exposing a nuclear localization sequence near the N terminus of NFAT and resulting in its translocation to the nucleus (2–5). This process is exquisitely sensitive to the immunosuppressants cylosporin A (CsA) and FK506, which interact with specific cytosolic receptors and form complexes that bind with high affinity to calcineurin and lock it in an inactive conformation (6–8). In the nucleus NFAT assembles in cooperative DNA-binding complexes with dimers of the AP-1 family of transcription factors (4, 9). Complementation studies using constitutively active mutants of signaling proteins have shown that the calcium/calcineurin and the Ras/protein kinase C/mitogen-activated protein kinase pathways are integrated at the level of NFAT proteins (10, 11).

The NFAT family of transcription factors includes to date five members, NFAT1 (also named NFATp or NFATc2), NFAT2 (also named NFATc or NFATc1), NFAT3 (also named NFATc4), NFAT4 (also named NFATc3), and the recently identified atypical member NFAT5 (12–18). All NFAT proteins share a conserved DNA binding domain, which shows a weak similarity with the DNA binding domain of Rel family proteins (RHD) and permits interaction with Fos/Jun heterodimers at composite DNA binding sites on a number of cytokine gene enhancers (1). The N-terminal region shows significant homology among NFAT1–4 and is characterized by a number of features important for regulation, including a nuclear localization sequence, the sites of interaction with calcineurin, and a highly conserved SP repeat region (SP*XX*SP*XX*SP*XXXXX*(D/E)(D/E), which is likely to be the target of kinase/phosphatase activity (1). Furthermore, the N-terminus of NFAT1 has been functionally characterized as a transactivation domain and, like the corresponding regions of NFAT2–4, contains at least one acidic/hydrophobic patch that resembles those implicated in transactivation by acidic activation domains (1). In agreement with a regulatory role for the N-terminal NFAT homology region, NFAT5, which lacks this region, has a calcineurin-independent constitutive nuclear localization in a number of

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[1] The abbreviations used are: TCR, T-cell antigen receptor; IL, interleukin; NFAT, nuclear factor of activated T-cells; CsA, cyclosporin A; luc, luciferase; GST, glutathione *S*-transferase; RT, reverse transcription; PCR, polymerase chain reaction; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescent protein.

cell types, including T-cells (14). NFAT family members differ widely at their C termini, as they are expressed mostly as multiple isoforms generated by either alternative splicing or, as recently shown for NFAT2, by alternative polyadenylation (19). The difference in transactivation activity among NFAT isoforms appears related to the presence, in specific isoforms, of an additional transactivation domain in the C-terminal region (20).

Although initially characterized in T-cells, NFAT family members are in some instances broadly expressed not only in other cells of the immune system, but also in ontogenically distinct tissues. While NFAT2 expression is mostly restricted to T and B-cells and NFAT4 to thymocytes (12, 18), NFAT1, NFAT3, and NFAT5 are found in many tissues in both man and mouse (12–14, 21, 22), and unanticipated roles for these factors have been unveiled in development, proliferation, and differentiation of a number of tissues. For example, NFAT1 has been shown to be a repressor of chondrogenesis (23), whereas NFAT2 was found to be essential for heart development (24, 25). Furthermore, a number of data suggest a potential role for NFAT factors in myogenesis and adipogenesis (26, 27). Specific NFAT family members are also expressed in the brain. NFAT1 has been detected in the mouse olfactory bulb and in a neuronal cell line (22), and NFAT3- and NFAT5-specific mRNA has also been detected at high levels in the brain (13, 14). Recently NFAT3 has been shown to translocate to the nucleus and activate NFAT-dependent transcription in response to electrical activity or potassium depolarization in pyramidal neurons, suggesting a role for NFAT3 in hippocampal synaptic plasticity and memory (28). Hence, NFAT factors might be implicated in brain development and function. Here we report the cDNA cloning and characterization of a new NFAT1 isoform highly expressed and constitutively active in mouse brain, initially suggested by the presence of high levels of constitutive luciferase activity in the brain of a NFAT/luciferase (NFAT/luc) reporter transgenic mouse.

MATERIALS AND METHODS

Plasmids, Antibodies, and Glutathione S-Transferase Fusion Proteins—The NFAT/luc reporter contains a trimer of the distal NFAT binding site on the IL-2 enhancer upstream of the gene encoding firefly luciferase, as well as the bacterial *neo* gene for G418 selection of eukaryotic cells (29). The plasmid pRDII, containing the bacterial gene encoding chloramphenicol acetyltransferase under the control of a multimer of a nuclear factor-κB binding site (30), was used as a transfection control. A pGEX construct containing the cDNA encoding the Rel homology domain of NFAT1 and the retroviral vector pEGZ-HA-NFATp containing the gene encoding murine NFAT1-C were a kind gift of Edgar Serfling and Andris Avots. The plasmid pcDNAamp/NFAT1-D contains the cDNA encoding NFAT1-D under the control of the cytomegalovirus early enhancer in the mammalian expression vector pcDNAamp (Invitrogen, Groningen, Holland). The full-length NFAT1-D cDNA was obtained by RT-PCR from mouse brain. The primers were designed to add an *Hin*dIII site 5′ of the ATG initiation codon and an *Xba*I site 3′ of the stop codon for directional cloning into pcDNAamp. The nucleotide sequence of the cDNA was checked by automatic sequencing. The plasmid pGFP/NFAT1-D was obtained by subcloning the *Hin*dIII-*Xba*I fragment from pcDNAamp/NFAT1-D into the corresponding sites of the polylinker of pEGFP-C3 (CLONTECH, Palo Alto, CA).

Polyclonal antibodies against NFAT1 were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An anti-NFAT1 mAb, which recognizes a specific epitope in the N-terminal region of NFAT1 (22), was purchased from Affinity Bioreagents (Golden, CO). The anti-NFATx and anti-NFATc1 mAbs were a kind gift from Gerald R. Crabtree. The cDNA encoding amino acid residues 619–673 of NFAT1-D, corresponding to the unique C-terminal region, was obtained by RT-PCR from total brain RNA. The primers were designed to provide the RT-PCR product with a *Bam*HI restriction site at the 5′-end and by an in-frame termination codon and an *Eco*RI restriction site at the 3′-end. The RT-PCR product was purified, digested with *Bam*HI and *Eco*RI, and cloned in the corresponding sites of the polylinker of the bacterial expression vector pGEX-2T (Amersham Pharmacia Biotech Italia srl, Milan, Italy). The glutathione *S*-transferase fusion protein was affinity purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech Italia) according to the manufacturer's instructions and used to raise a polyclonal anti-NFAT1-D antiserum in rabbits. The anti-human CD3ϵ mAb OKT3 and anti-mouse CD3ϵ mAb 145-2c11 were affinity purified on a protein G column (Amersham Pharmacia Biotech Italia) from hybridoma supernatants (ATCC). The anti-murine Vβ8.1 mAb F23.1 (31) was kindly provided by Ed Palmer.

Generation and Analysis of Transgenic Mice—The 2.8-kilobase *Not*I-*Xho*I restriction fragment isolated from the plasmid NFAT/luc was microinjected into fertilized C57BL/6xDBA eggs and transgenic mice generated as described previously (32). Founder mice were back-crossed to C57BL/6 mice. Three positive founder lines were established. Total genomic DNA purified from the tail of the founders was assayed for the presence of the NFAT/luc transgene by Southern blot using as a probe a *Not*I-*Xho*I restriction fragment from NFAT/luc labeled with [^{32}P]dCTP by random priming. Alternatively, the transgene was identified by PCR of genomic DNA using luciferase-specific primers. Luciferase activity in the transgenic progeny was determined as described (33) on peripheral blood lymphocytes purified by Ficoll gradient centrifugation and subsequent overnight incubation in medium to remove adherent monocytes and macrophages and was then subsequently activated with a combination of 50 ng/ml PMA and 250 ng/ml A23187. Identification of the transgenic progeny from the NFAT/luc line harboring a functional insertion of the transgene was routinely carried out using the latter methodology. T-cell activation by CD3 cross-linking on solid substrate was carried out as described previously (29) using the anti-mouse CD3ϵ mAb 1452c11. Mouse organs (recovered from mice bled previously under anesthesia to remove contamination by circulating lymphocytes) were assayed for luciferase activity after homogenization in a Polytron homogenizer in 3% Triton X-100, 20 mM Tris-HCl, pH 8, 150 mM NaCl (in the presence of 0.2 mg/ml sodium orthovanadate, 1 μg/ml leupeptin, aprotinin, and pepstatin, and 10 mM phenylmethylsulfonyl fluoride). Protein concentrations in the cleared lysates were determined using a kit from Pierce (Rockford, IL) and bovine serum albumin as a standard. Luciferase activities were determined using a kit from Promega Italia srl (Milan, Italy) and normalized to the protein content.

cDNA Cloning and Analysis, Northern Blots, and RT-PCR—A uni-Zap-XR mouse brain library (Stratagene GmbH, Heidelberg, Germany) was screened using a probe derived from the Rel domain of mouse NFAT1. Basically, 10^6 phage particles were plated onto NZY soft agar plates together with infection-competent *Escherichia coli* (XL-1 strain pretreated with 10 mM $MgCl_2$, 0.2% maltose) and grown overnight at 37 °C. Phage plaques were transferred in duplicate to nitrocellulose membranes and lysed by treatment for 2 min with 1.5 M NaCl, 0.5 M NaOH followed by 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 (5 min), and washed in 0.2 M Tris-HCl, pH 7.5, 2 × SSC. Filters were baked at 80 °C for 3 h, prehybridized overnight at 42 °C in 5 × SSPE, 5 × Denhardt's solution, 30% deionized formamide, 1% SDS, and 200 μg/ml denatured salmon sperm DNA, and subsequently probed by hybridization overnight at 42 °C in the same solution using a fragment of DNA generated by PCR from the Rel domain of NFAT1 and labeled by random priming with [^{32}P]dCTP. Filters were washed once in 2 × SSPE at room temperature for 10 min, twice in 0.5 × SSPE, 0.5% SDS at 55 °C for 20 min, and once in 0.5 × SSPE, 0.5% SDS at room temperature for 10 min and then exposed to x-ray film overnight at −80 °C with an intensifying screen. Positive plaques were isolated and phage particles recovered in SM buffer containing 1% chloroform. These were then used in a secondary and tertiary screening to obtain pure positive clones. The cDNA was recovered by the plasmid rescue technique and then sequenced.

Total RNA was extracted from mouse brain homogenized in a Dounce homogenizer, using guanidinium isothiocyanate. Northern blot analysis was carried out by standard protocols using a PCR probe corresponding to the unique 3′-portion of the NFAT1-D cDNA and labeled with [^{32}P]dCTP by random priming. The multiple mouse tissue Northern blot was purchased from CLONTECH and hybridized first with the same NFAT1-D-specific probe and subsequently with an actin probe according to the manufacturer's instructions. RT-PCR of total brain RNA or of RNA extracted from mouse splenocytes or a mouse T-cell hybridoma was carried out on a Perkin Elmer Life Sciences 2400 thermal cycler (Norwalk, CT) using primers specific for the unique 3′-portion of NFAT-1D cDNA and kits from Takara Shutzo Co. (Shiga, Japan) and Sigma Aldrich srl (Milan, Italy). The specificity of the PCR products was confirmed by automatic sequencing. Alternatively, to clone the 5′-portion of NFAT1-D, reverse transcription of total RNA and subsequent PCR amplification were carried out in one step using the One-Step RT-PCR Kit (CLONTECH) according to the manufacturer's

instructions. NFAT1-D-specific first strand cDNAs were generated using two different primers mapping to the unique 3′-terminus of the coding region (5′-GGCAGAATGTTACAGAGC-3′) and to the 3′-untranslated region (5′-TGTTACAGAGCCAGCAGC-3′), respectively. A 660-base pair fragment spanning NFAT1 cDNA from the ATG initiation codon to codon 220 in the conserved N-terminal domain was subsequently amplified by PCR. PCR products were separated by agarose gel electrophoresis and recovered using a kit from Qiagen GmbH (Hilden, Germany). Automatic sequencing was performed on both strands of the RT-PCR products. The T to C transition at codon 78 was confirmed on independent RT-PCR products. A full-length cDNA was subsequently obtained by RT-PCR and completely sequenced. The cDNA sequence encoding NFAT1-D has been submitted to GenBank (accession number AF289078).

Nuclear Extracts and Gel Mobility Shift Assays—Nuclear extracts were obtained from mouse tissues and from mouse C2C12 muscle cells. Tissues and cells were washed twice with phosphate-buffered saline and homogenized in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) with a Dounce homogenizer (pestle B). The homogenate was centrifuged for 30 s in a microfuge. The pellet containing the nuclei was resuspended in 3 volumes of ice-cold buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and the tube was rocked at 4 °C for 15 min. The nuclear extract was centrifuged for 2 min at maximum speed at 4 °C, and the supernatant obtained was frozen in aliquots at −80 °C. The probe and competitors for gel mobility shift assays were obtained by annealing the following oligonucleotides: NFAT-F (GGGAAAGAAAGGAGGAAAAAGTGTTTCATACAG) and NFAT-R (TGGTTCTGTATGAAACACTTTTTCCTCCTTTCTT) for the probe (NFAT); NFATm-F (GGGAAAGAAAGGAGAAAAAAATTTTTAATACAG) and NFATm-R (TGGTTCTGTATTAAAAATTTTTTTCTCCTTTCTT) for the cold competitor NFATm; B7 (AGCTAAGCATGAGTCAGACAC) and B8 (GATCGTGTCTGACTCATGCTT) for the aspecific competitor (Asp). Binding reactions and electrophoresis of the complexes were performed as described previously (34). 0.5 μl of an antibody raised against the NFAT1 Rel homology domain (anti-mouse NFATp, Upstate Biotechnology), anti-NFATc1/NFAT2 (kindly provided by Gerald R. Crabtree), or anti-NFAT1-D was added to the binding mixture as indicated.

Cell Lines and Transfections, Luciferase Assays, Confocal Microscopy—The mouse T-cell hybridoma 58hCD4 expressing the 3BBM74 TCR (35), kindly provided by Ed Palmer, and the human T-lymphoma line Jurkat were grown in RPMI supplemented with 7.5% defined bovine serum (HyClone Laboratories, Logan, UT). The neuronal line PC12 (ATCC) was grown in 5% bovine serum and 5% equine serum (HyClone Laboratories). Jurkat cells were transiently transfected with DEAE/dextran and activated either by CD3 cross-linking on a secondary antibody-coated plate using the anti-CD3 mAb OKT3 or by a combination of 50 ng/ml PMA and 250 ng/ml A23187 as described previously (29). Transfection control samples were activated with 50 ng/ml PMA. CsA (Sandoz, East Hanover, NJ) was added at a final concentration of 500 ng/ml 15 min before activation. Cells were collected 8 h after activation and processed for luciferase and chloramphenicol acetyltransferase assays as described (29, 33). Relative luciferase activities were normalized to protein content of each sample and to the levels of chloramphenicol acetyltransferase activity in the transfection controls. All samples were in duplicate, and transfections were repeated three to five times. PC12 cells were either transiently transfected by DEAE/dextran as above or stably transfected by electroporation and selected in G418 as described previously for Jurkat cells (36). PC12 differentiation was achieved by plating the cells on collagen-coated plastic in the presence of 10 ng/ml nerve growth factor (a kind gift of Stefano Alemà) for 1 week. Confocal microscopy was carried out on Jurkat cells transiently transfected with the pEGFP/NFAT1-D construct, either as such or after treatment for 10, 20, and 40 min with 500 ng/ml A23187, in the presence or absence of 500 ng/ml CsA, using a Leica Microsystems confocal microscope (Heidelberg, Germany).

Immunoprecipitations and Immunoblots—Brain lysates were obtained by homogenization in a Dounce homogenizer in 1% Triton X-100, 20 mM Tris-HCl, pH 8, 150 mM NaCl in the presence of 0.2 mg/ml sodium orthovanadate, 1 μg/ml leupeptin, aprotinin, and pepstatin, and 10 mM phenylmethylsulfonyl fluoride. T-cell hybridoma lysates were obtained as described (29). When required, T-cells were activated by anti-TCR antibody cross-linking in solution as described (29). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and probed by immunoblot. Peroxidase-labeled secondary antibodies were revealed by chemiluminescence using reagents from Pierce. NFAT1-D was immunoprecipitated from brain lysates using the polyclonal anti-NFAT1-D antiserum and protein A-Sepharose (Pharmacia Amersham Biotech) as described (29). Prestained molecular weight markers were purchased from Life Technologies Italia srl.

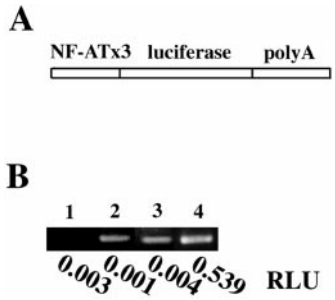

FIG. 1. **Generation and identification of NFAT/luc transgenic mice.** *Panel A*, scheme of the NFAT/luc transgene. *Panel B*, ethidium bromide staining of the PCR products obtained by amplification of genomic DNA from a nontransgenic mouse (*lane 1*) and from three founders (*lanes 2–4*), using primers specific for the luciferase gene. The identity of the PCR products, which migrated at the expected size of 900 base pairs, was confirmed by nucleotide sequencing. The *numbers* below each *lane* indicate the luciferase values obtained on purified peripheral blood lymphocytes from the same mice after activation with a combination of PMA and A23197 for 16 h.

RESULTS AND DISCUSSION

Generation and Characterization of a NFAT-Luc Reporter Mouse—Reporter mice have been shown to be a powerful tool for the study of development, activation, and differentiation of immune cells *in vivo* (37–40). To generate reporter transgenic mice for NFAT, a construct encoding firefly luciferase under the control of a trimer of the distal NFAT binding site on the IL-2 gene enhancer was used for microinjection (Fig. 1*A*). This construct has been used extensively in Jurkat cells both for transient transfection assays and for generating stable transfectants, and has been shown to be strongly inducible in response to TCR agonists, as well as to a combination of phorbol esters and a calcium ionophore (10 and references therein). Five positive founders were identified by Southern blot analysis of genomic DNA (data not shown). Of these, three were found to have a germline transmission of the transgene, as shown by both Southern blot and PCR analysis of genomic DNA of the F1 progeny (Fig. 1*B* and data not shown). Productive insertion of the transgene was assayed by quantitating luciferase activity in peripheral blood lymphocytes maximally stimulated *in vitro* with a combination of PMA and A23187. As shown in Fig. 1*B*, induction of NFAT-dependent luciferase activity was detected in only one of the three founders, from which a stable transgenic line was derived and used for subsequent studies.

The extent and time course of NFAT activation in transgenic thymocytes, splenocytes, and lymph node T-cells were determined following activation for different times with either immobilized anti-CD3 mAb or by a combination of PMA and A23187. As shown in Fig. 2, NFAT activation was transient and peaked sharply at 12 h in response to either stimulation, the only exception being an anticipated response of thymocytes to pharmacological stimulation. Qualitatively and quantitatively similar results were obtained reproducibly on thymocytes and splenocytes from these mice, whereas the response of lymph node T-cells was quantitatively variable, possibly reflecting priming by previous exposure of the mice to environmental antigens (data not shown). Nevertheless, NFAT activation in T-cells faithfully reproduces the transient response of NFAT to T-cell-activating stimuli, indicating that this mouse line can be used as a tool for the analysis of physiological responses to antigen in normal mouse lymphocytes.

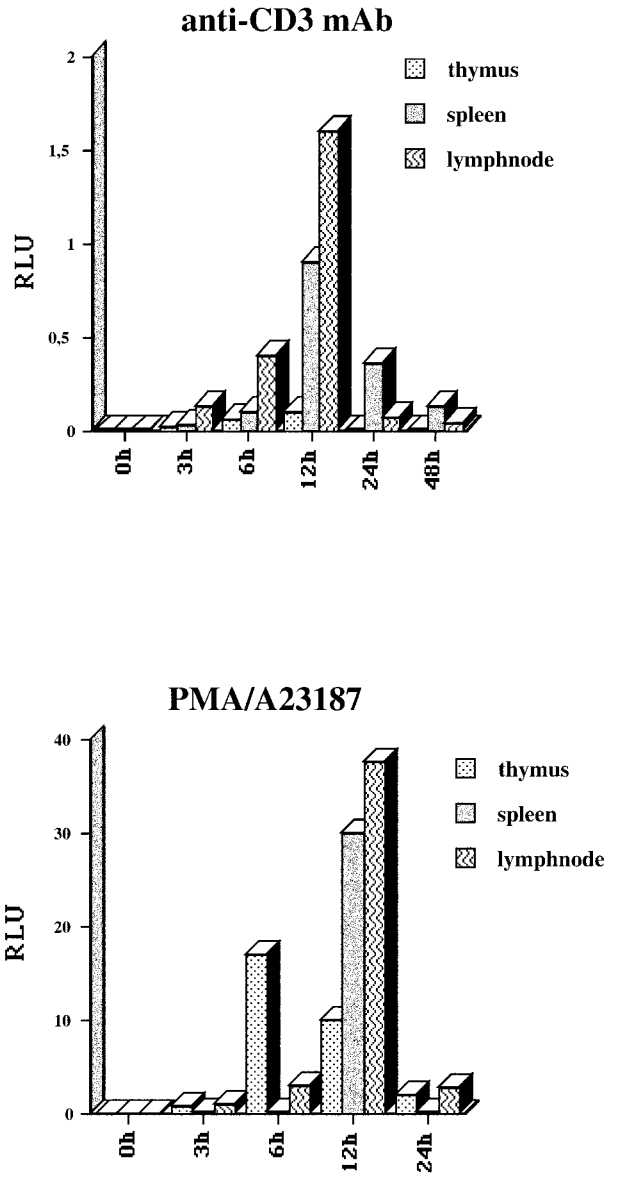

FIG. 2. **Time course of NFAT-dependent luciferase expression in lymphoid organs of NFAT/luc transgenic mice.** The relative luciferase activity in lymphocyte extracts from thymus, spleen, and lymph node of a representative NFAT/luc mouse is shown. Cells were activated for the times indicated either by cross-linking the TCR·CD3 complex using an anti-CD3 mAb (*top panel*) or with a combination of PMA and A23187 (*bottom panel*).

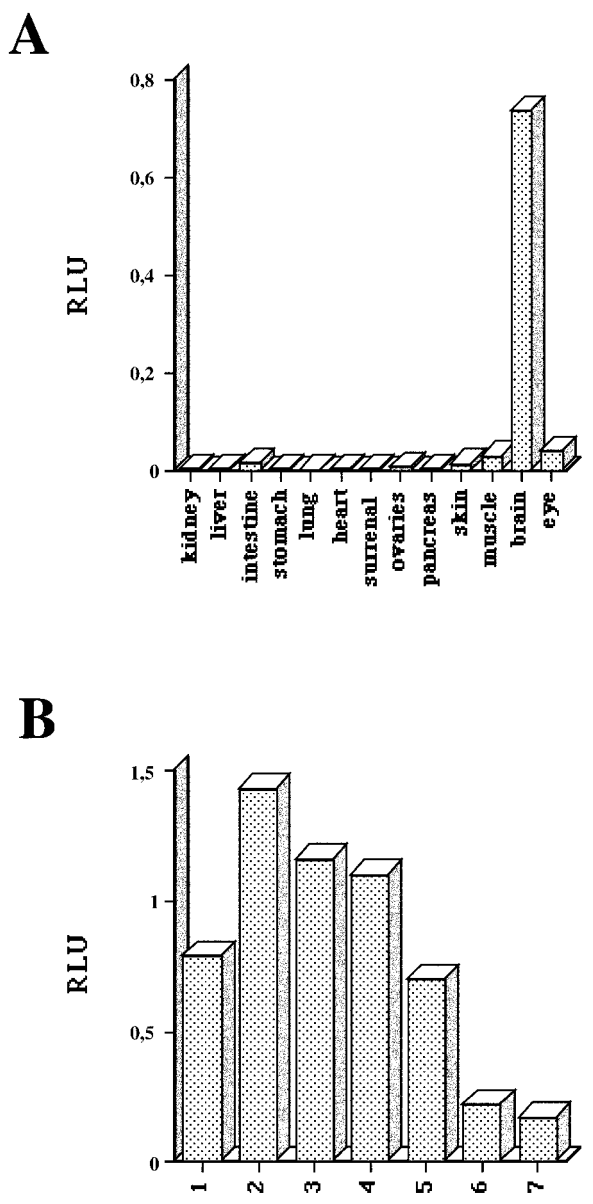

FIG. 3. **Luciferase activity in different organs of NFAT/luc mice.** *Panel A*, relative luciferase activity in extracts from different organs of a representative NFAT/luc mouse. *Panel B*, relative luciferase activity in extracts from sequential sections of the brain of a representative NFAT/luc mouse, dissected along the antero-posterior axis (*samples 1–7*). *1*, olfactory bulb; *2* and *3*, sequential sections of the forebrain; *4*, midbrain; *5*, pons; *6*, cerebellum; *7*, medulla oblongata.

Expression of a Constitutive NFAT Activity in Mouse Brain—NFAT family members are expressed broadly in many tissues; however, expression in tissues other than lymphocytes has in many cases been shown only at the RNA level and has mostly not been related to activity. Because all NFAT factors recognize similar DNA binding motifs, we reasoned that expression of luciferase activity in NFAT/luc mice in body districts other than the immune system might be indicative of expression of functional NFAT proteins. We therefore assayed a panel of tissues from these mice for luciferase activity. As shown in Fig. 3*A*, high levels of luciferase activity were reproducibly found in the brain. Furthermore, low but significant luciferase activity was found consistently in the eye. On the other hand, in two out of eight mice analyzed, a high level of luciferase activity was found in the skin, whereas no activity was detectable in the skin of the remaining mice (Fig. 3*A* and data not shown), suggesting that the NFAT activity detected in the skin might either be inducible or be expressed by migrating cells, such as dendritic cells. This phenomenon is being characterized and will be the topic of another paper. Here we have characterized further the NFAT activity found in the brain.

Expression of transgene-encoded luciferase in the brain might reflect insertional activation of the transgene, unrelated to NFAT. To rule out this possibility, NFAT activity in nuclear extracts of total brain and isolated cerebellum from nontransgenic mice was assayed by electrophoretic mobility shift. As shown in Fig. 4*A*, a gel shift of a labeled oligonucleotide spanning the distal NFAT binding site on the IL-2 gene enhancer was observed using nuclear extracts from both brain and cerebellum. As expected, a weak binding activity, strongly upregulated after stimulation by a combination of PMA and A23187, was detected in nuclear extracts from mouse splenocytes. The gel shift of the labeled nucleotide in the presence of nuclear extracts from brain and cerebellum was abolished by

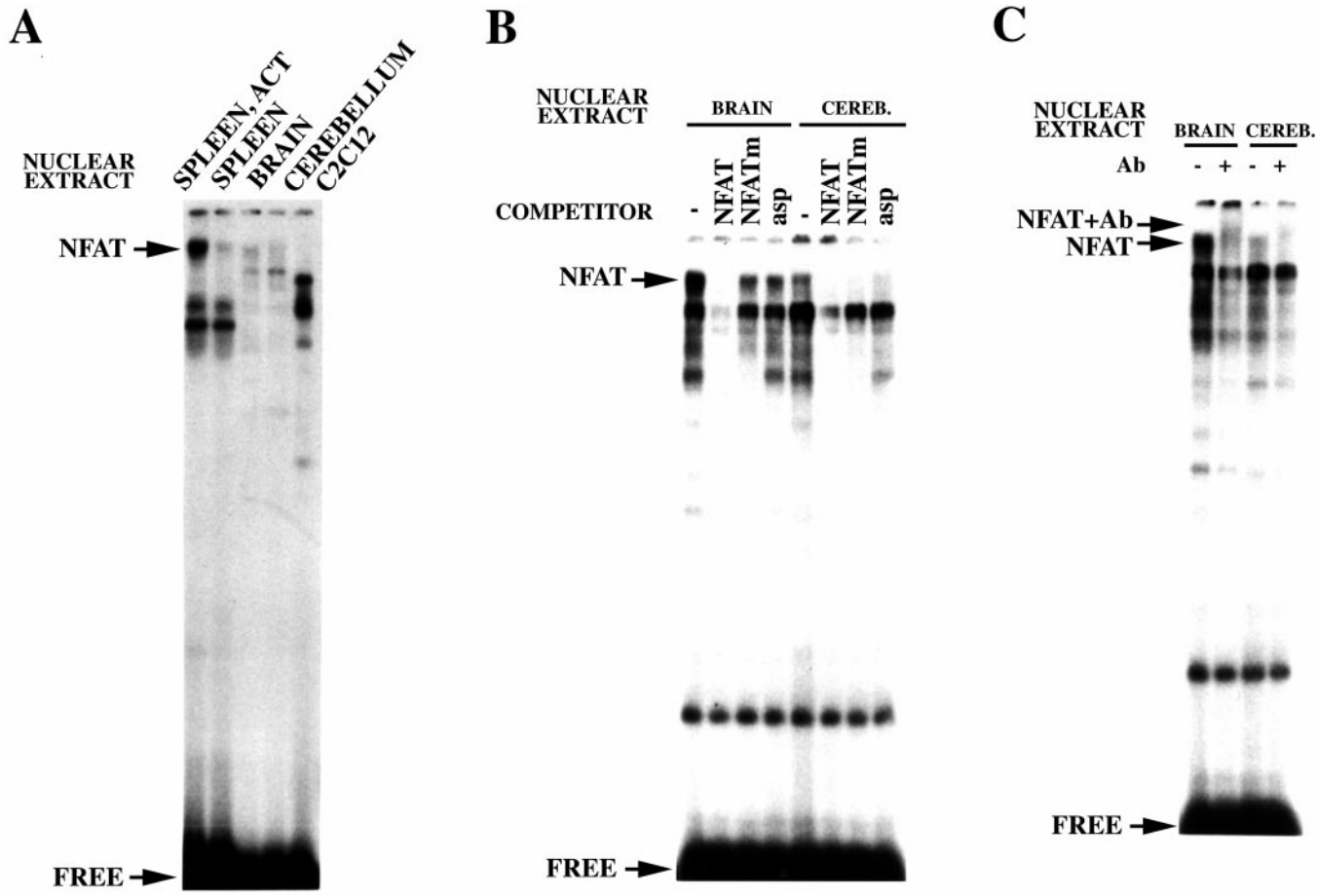

FIG. 4. **Characterization of NFAT activity in mouse brain.** *Panel A*, nuclear extracts used in the electrophoretic mobility shift assay were obtained from normal mouse brain and cerebellum and from resting splenocytes or splenocytes maximally activated with a combination of PMA and A23187. A nuclear extract from untreated C2C12 cells was used as a negative control. The oligonucleotide corresponds to the distal NFAT binding site on the murine IL-2 gene promoter. *Panel B*, the specificity of the NFAT binding activity in the brain and cerebellum was assessed using as competitor an excess of either unlabeled specific oligonucleotide or a specific oligonucleotide carrying substitutions at positions critical for DNA binding (*NFATm*), or an aspecific oligonucleotide (*asp*). *Panel C*, the specificity of the NFAT binding activity in the brain and cerebellum was assessed in a supershift assay using a polyclonal antibody specific for the Rel homology domain of NFAT1.

the addition of excess unlabeled oligonucleotide but not by a similar oligonucleotide mutated at critical residues for NFAT binding nor by an unrelated oligonucleotide (Fig. 4*B*). Furthermore, the specific oligonucleotide was supershifted by an antibody to the Rel homology domain of NFAT1, which is highly conserved among all NFAT family members (Fig. 4*C*). A faster migrating species whith a similar binding specificity for NFAT was observed both in brain and cerebellum (Fig. 4), possibly reflecting the existence of additional NFAT members or NFAT-related proteins in these tissues. Hence the luciferase activity detected in the brain of NFAT/luc mice reflects a constitutive NFAT activity in mouse brain.

Identification and Cloning of a Novel NFAT1 Isoform in Mouse Brain—Expression and costitutive activity of NFAT1 in the mouse olfactory bulb has been reported previously (22). NFAT3 has also been shown to be expressed in hippocampal neurons, albeit inducibly (28). Furthermore, the mRNA encoding a novel atypical family member, NFAT5, has also been detected in brain (14). Quantitation of luciferase activity along the antero-posterior axis of the brain of these mice showed (Fig. 3*B*) that luciferase was expressed not only in the olfactory bulb (*bar 1*) but in all brain districts, with the lowest levels in the hindbrain (*bars 5–7*), suggesting that the constitutive NFAT activity responsible for luciferase expression in the brain of NFAT/luc mice might result from the combined activity of different family members and/or to a previously unidentified NFAT factor.

To assess this possibility, we screened a mouse brain cDNA library using the Rel homology domain of NFAT1 as a probe. Three positive clones (λ1 and the identical clones H1/H7) were isolated independently, and their nucleotide sequences were determined. As shown in Fig. 5, the longest sequence compiled from overlapping clones contained an open reading frame of 458 codons. The N-terminal portion of the predicted translation product of the open reading frame showed a 100% identity with NFAT1, with the exception of one nonconservative substitution at residue 287 of the published sequence resulting from a single base change. This region included part of the NFAT homology domain and most of the Rel homology domain (residues 216–618 of NFAT1). The homology stopped abruptly downstream of the minimal DNA binding domain in the Rel homology region (41). The 55-amino acid residue C-terminal peptide encoded by the open reading frame did not show significant homology with any other sequence in the GenBank and EMBL data bases (Fig. 5*A*). The cDNA ended with a poly(A) tail (Fig. 5*A*). Furthermore, a cDNA clone of the same length as λ1 was isolated from a brain cDNA library from a different mouse strain, ruling out the possibility of a recombination artifact (data not shown). The 100% identity of the 5′-portion of the open reading frame with the central portion of NFAT1 suggested that we had isolated a partial cDNA encoding a novel isoform of NFAT1. To assess this point, we carried out a RT-PCR using for first strand cDNA synthesis a primer mapping in the unique 3′-portion of the cDNA, and for amplification a pair of primers designed to span

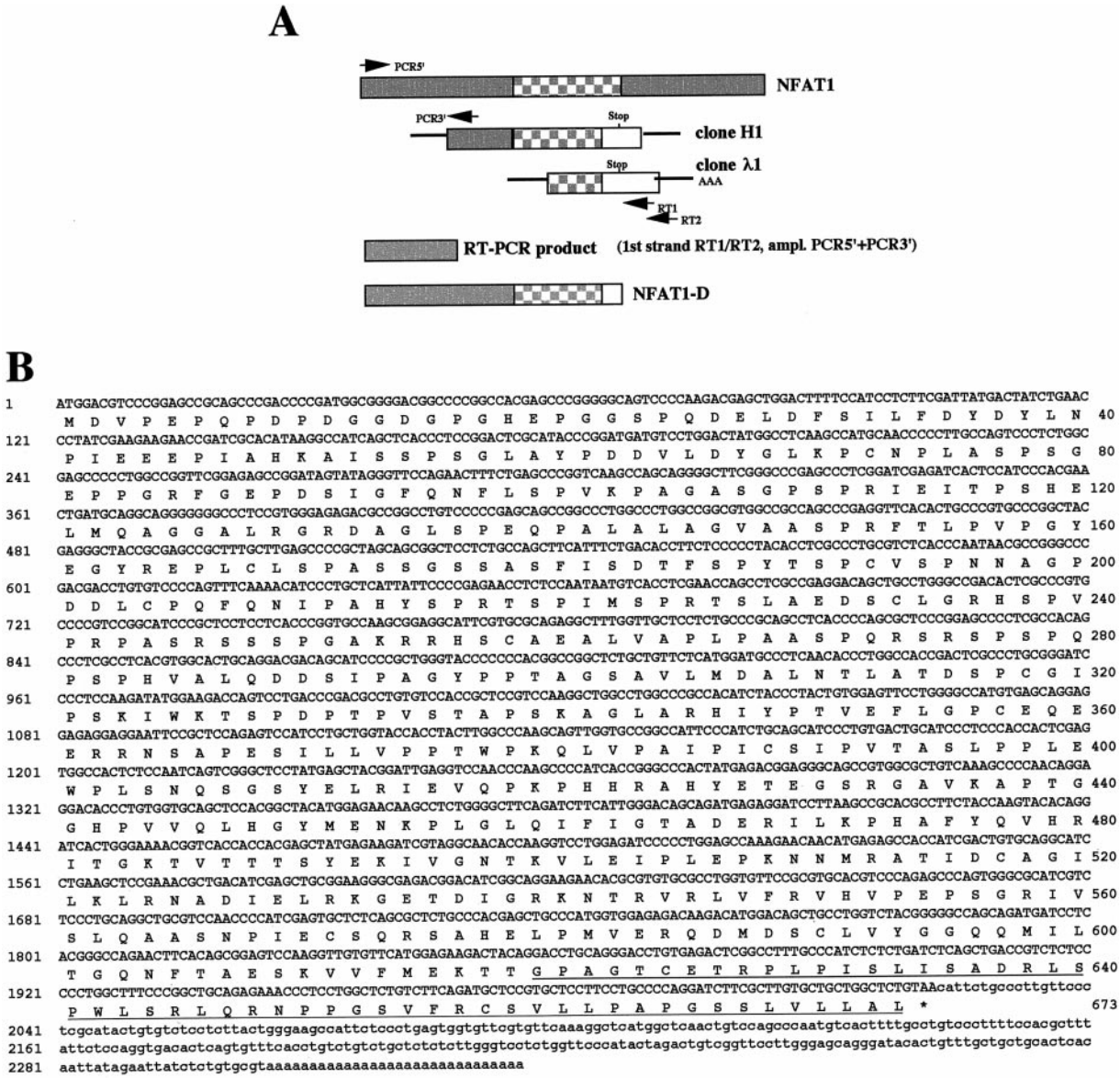

FIG. 5. **Cloning and nucleotide sequence of mouse NFAT1-D cDNA.** *Panel A*, cloning of NFAT1-D cDNA. Partial cDNA clones were obtained from a mouse brain cDNA library. Clones H1/H7 and λ1 are partially overlapping. The 5′-portion of the cDNA was obtained by RT-PCR using as primer for first strand synthesis RT1 or RT2, which map in the unique region of NFAT1-D, and as amplification primers two oligonucleotides mapping in the 5′-region of NFAT1. A scheme of the full-length NFAT1-D cDNA showing its homologies with NFAT1 (*gray* and *checkered boxes*) and its unique 3′-region (*white box*) is shown at the *bottom* of the *panel*. *Panel B*, nucleotide sequence and deduced amino acid sequence of NFAT1-D cDNA. The unique region is *underlined*. Nucleotide sequences are *numbered* on the *left*, and amino acid sequences are on the *right*. The *star* denotes the stop codon.

NFAT1 cDNA from the initial ATG to codon 223 (Fig. 5A). The RT-PCR gave a fragment of the expected size (not shown), and nucleotide sequence determination confirmed the identity of this fragment with the 5′-end of the cDNA encoding NFAT1, with the exception of a T to C transition at codon 78, resulting in a non conservative substitution as compared with the published sequence. A full-length cDNA was subsequently obtained by RT-PCR. The full-length cDNA sequence and predicted translation product are shown in Fig. 5B.

The cDNA encodes a protein of 673 amino acid residues, which is identical to NFAT1 up to residue 618 (with the exception of the substitutions L78P and P287L) and thus represents a novel NFAT1 isoform, presumably generated by alternative splicing, as is the case for most known isoforms of NFAT family members (1). Because there are three known isoforms of NFAT1, named from A to C, we propose to name this new isoform NFAT1-D (or NFATc2-D, or NFATp-D according to the alternative nomenclatures). The region of homology of NFAT1-D with the other NFAT1 isoforms spans the N-terminal NFAT homology region, which is responsible for transactivation and interaction with calcineurin and contains the phosphorylatable serine residues that modulate NFAT activity through the opposite activities of GSK-3 and calcineurin (3, 4, 42). It also includes the minimal region of the Rel homology domain required for DNA binding (41). Although NFAT1-A, B, and C differ at their C-termini, the homology among these isoforms extends beyond the Rel homology domain into the C-terminal transactivation domain. On the contrary, NFAT1-D lacks the whole C-terminal transactivation domain as well as the last 19 residues of the Rel homology domain, suggesting that NFAT1-D might be subject to a distinct regulation strategy.

Expression of NFAT1-D in Brain and PC12 Cells—Northern blot analysis of total brain RNA using as a probe a PCR amplification product spanning the unique 3′-portion of the NFAT1-D cDNA showed that NFAT1-D is expressed in brain

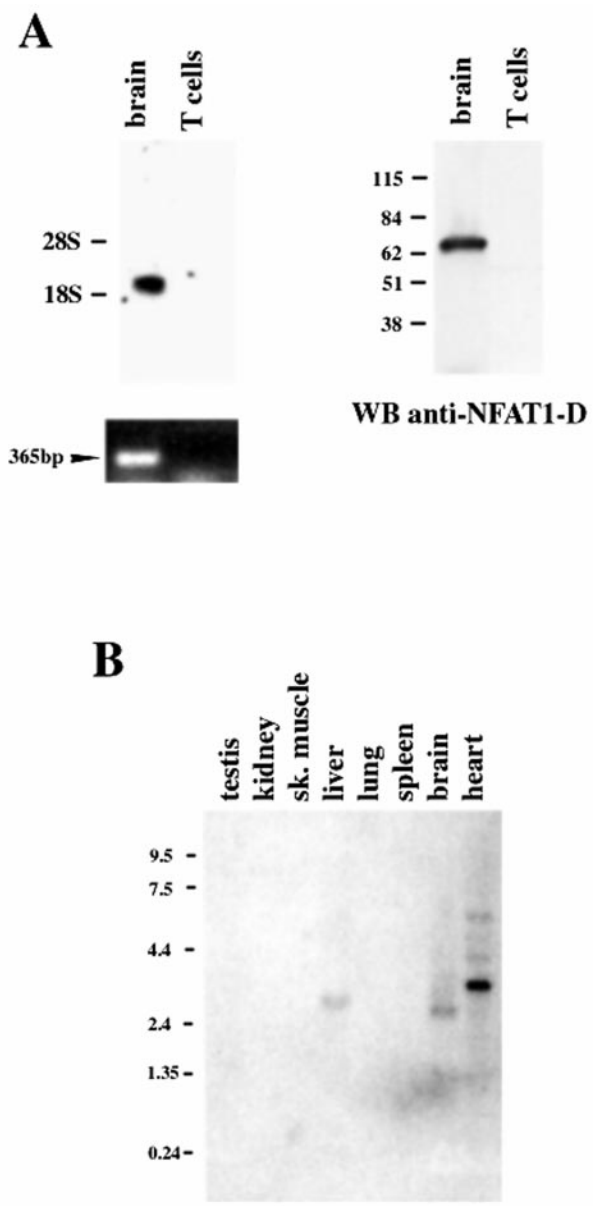

FIG. 6. **Expression of NFAT1-D in mouse tissues.** *Panel A*, *left*, Northern blot (*top*) and RT-PCR (*bottom*) analysis of NFAT1-D mRNA expression in mouse brain and in a mouse T-cell hybridoma activated with a combination of PMA and A23187. The RT-PCR product (whose identity was confirmed by nucleotide sequencing) was obtained by amplification of the unique 3′-region of NFAT1-D and was used as a probe for the Northern blot shown above. The migration of 28 S and 18 S rRNA in the same gel is shown. *Right*, immunoblot analysis of cell extracts from similar samples using a polyclonal antiserum raised against a glutathione *S*-transferase fusion protein containing the unique C-terminal region of NFAT1-D. The migration of molecular mass markers is indicated. *Panel B*, Northern blot analysis of NFAT1-D mRNA expression in mouse tissues. The migration of molecular mass markers is shown. Reprobing with actin cDNA confirmed that similar amounts of RNA were loaded in each lane (data not shown).

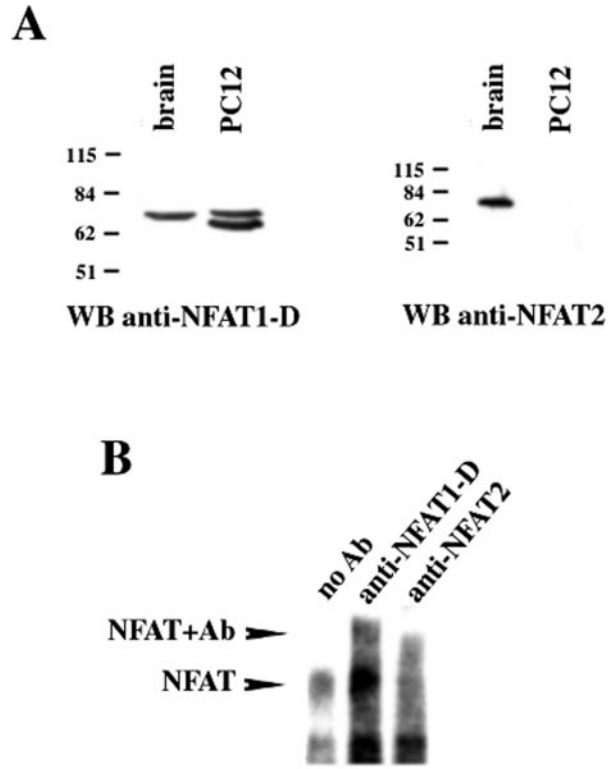

FIG. 7. **Functional characterization of NFAT1-D in mouse brain.** *Panel A*, immunoblot analysis of brain or PC12 extracts using either the anti-NFAT1-D antiserum or an anti-NFAT2 monoclonal antibody. *Panel B*, supershift assay using mouse brain nuclear extracts and antibodies specific for either NFAT1-D or NFAT2.

but not in T-cells (Fig. 6*A*). This was confirmed by RT-PCR analysis of brain and T-cell RNA using primers specific for the same region of the cDNA (Fig. 6*A*). Furthermore, hybridization of poly(A)$^+$ RNA from a panel of adult mouse tissue revealed that, in addition to the brain, NFAT1-D-specific RNA was expressed in heart and liver but was undetectable in lung, skeletal muscle, testis, kidney, and spleen (Fig. 6*B*). The transcript found in the brain was of the expected size of ~2.6 kilobases, whereas a single 2.8-kilobase transcript was found in the liver, and four longer transcripts of about 3.4, 4.0, 4.4, and 5.5 kilobases were found in the heart, suggesting the existence of multiple NFAT1-D mRNA isoforms.

Immunoblot analysis of brain lysates using an antiserum to a glutathione *S*-transferase fusion protein containing the unique C-terminus of NFAT1-D showed that NFAT1-D was translated as a protein of the expected molecular mass of 70 kDa (Fig. 6*A*). The identity of the protein was further confirmed by immunoprecipitation of NFAT1-D from brain lysate using the antiserum specific for the unique C-terminus, followed by immunoblot with a monoclonal antibody specific for an epitope mapping close to the N terminus of NFAT1 (data not shown). All brain districts were found to express NFAT1-D (data not shown), in agreement with the luciferase expression data shown in Fig. 2. Furthermore, immunoblot analysis, as well as RT-PCR, showed that the neuronal line PC12 also expressed NFAT1-D (Fig. 7*A* and data not shown).

To assess whether other NFAT family members might contribute to the NFAT activity found in the brain, brain expression of some other NFAT factors was tested by immunoblot. As shown in Fig. 7*A*, NFAT2 was also expressed in the brain but not in PC12 cells, suggesting that cells other than neurons, such as glial cells, might be responsible for its expression. Alternatively, neuronal NFAT2 activity might have been turned off in PC12 cells following transformation. As expected, no NFAT4 was found either in brain or in PC12 cells (data not shown). To assess whether NFAT1-D or NFAT2, or both, contributed to the expression of the luciferase activity in NFAT/luc mice, a supershift experiment using brain nuclear extract was carried out in the presence of either a monoclonal antibody against NFAT2 or the polyclonal antiserum specific for NFAT1-D. As shown in Fig. 7*B*, both antibodies induced supershift of the labeled oligonucleotide, indicating that in addition to NFAT-1D, NFAT2 and potentially other NFAT family members are constitutively active in mouse brain. This possibility is

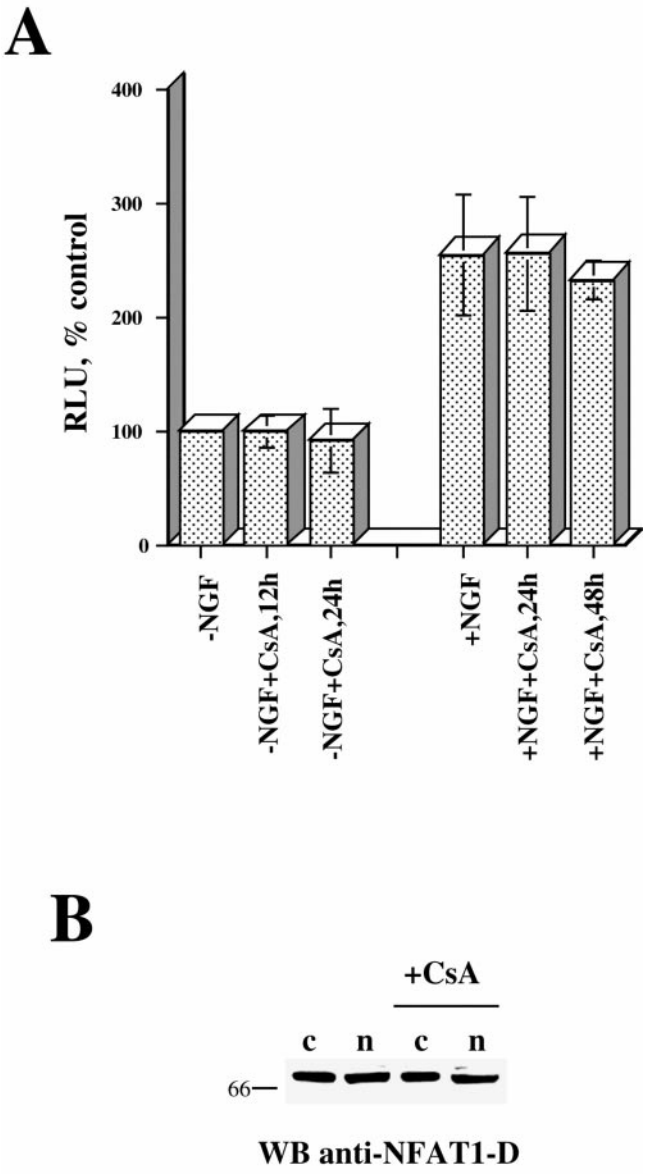

FIG. 8. **Regulation of NFAT1-D activity in neuronal cells.** *Panel A*, relative luciferase activity in extracts of three PC12 clones stably transfected with the NFAT/luc construct. Basal levels of luciferase activity in the three clones were 17.7, 3.7, and 2.9 RLU/10^6 cells, respectively. Activities are presented as the percent of untreated stably transfected PC12 cells. Cells were either left untreated or treated with 500 ng/ml CsA for the indicated times. The same experiment was carried out on the same cells after differentiation by treatment for 7 days with nerve growth factor on collagen-coated plates. *NGF*, nerve growth factor. *Panel B*, immunoblot analysis of nuclear (*n*) and cytosolic (*c*) extracts of PC12 cells, either untreated or treated with 500 ng/ml CsA for 24 h. Equal amounts of proteins were loaded in each *lane*. The migration of molecular mass markers is indicated.

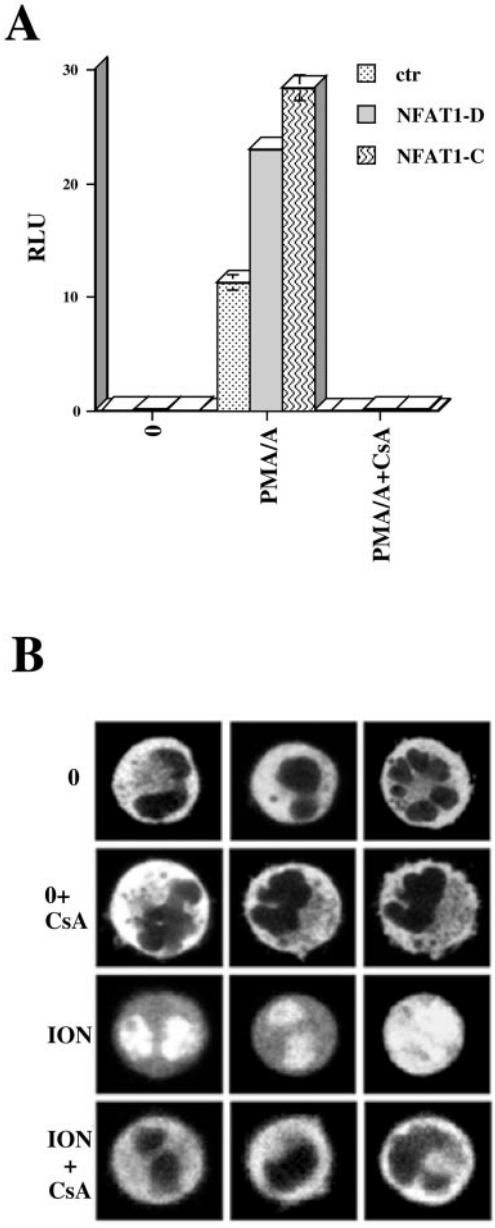

FIG. 9. **Regulation of NFAT1-D activity in T-cells.** *Panel A*, relative luciferase activity in Jurkat T-cells transiently transfected with either empty vector or vectors encoding NFAT1-C or NFAT1-D, and activated for 8 h with a combination of PMA and A23187 in the presence or absence of 500 ng/ml CsA. Luciferase activities have been normalized to the chloramphenicol acetyltransferase activities in transfection controls. The data show the results of two independent experiments, each with duplicate samples. *Panel B*, confocal microscopy of Jurkat cells transiently transfected with a NFAT1-D/GFP fusion protein and activated for 40 min with A23187 (*ION*) in the absence or in the presence of 500 ng/ml CsA.

supported by the observation that a combination of the two antibodies failed to shift the NFAT complex completely (data not shown).

Differential Regulation of NFAT Activity in Neuronal Cells and in T-cells—The electrophoretic mobility shift assays showed a constitutive binding activity of both NFAT1-D and NFAT2 in brain. A constitutive activity of NFAT1, correlated to the expression of a NFAT1 isoform of a molecular mass identifying it as NFAT1-A, was also described in the mouse olfactory bulb (22). In T-cells, the activity of both NFAT1 and NFAT2 is inducible only in response to T-cell-activating stimuli. Specifically, increased concentrations of intracellular calcium ions result in activation of calcineurin, which permits nuclear translocation of NFAT by dephosphorylation of the negatively regulatory serine residues in the N-terminal NFAT homology domain (6). This process is exquisitely sensitive to the immunosuppressant CsA (7, 8). To investigate the regulation of NFAT activity in neuronal cells, NFAT-driven luciferase expression was tested in the neuronal cell line PC12. These cells express both NFAT1-D (Fig. 7*A*) and an NFAT1 isoform of a molecular mass identifying it as NFAT1-A (22). PC12 cells were either transiently or stably transfected with the NFAT/luc construct. 8 out of 12 PC12 clones stably transfected with NFAT/luc expressed significant levels of luciferase, indicating that NFAT activity is constitutive in neuronal cells, in agree-

ment with the constitutive expression of an NFAT1 binding activity in nuclear lysates of PC12 cells (22). Similar data were obtained in transiently transfected cells (data not shown). Treatment of stably transfected PC12 cells with CsA for up to 72 h did not affect NFAT activity, as shown by the unaltered levels of luciferase expression (Fig. 8*A*), whereas the same treatment completely suppressed the inducible NFAT activity in a similar Jurkat T-cell stable transfectant (Ref. 10 and data not shown). On the other hand, a significant enhancement in NFAT-dependent luciferase expression was measured in stably transfected PC12 cells that had been induced to differentiate by treatment with nerve growth factor. Adding CsA to differentiated PC12 cells did not alter luciferase activity (Fig. 8*A*). In support of these data, immunoblot analysis of nuclear and cytosolic extracts of PC12 cells, either untreated or treated for 24 h with CsA, revealed significant levels of NFAT1-D in the nucleus, regardless of the presence of CsA (Fig. 8*B*), again suggesting that at least some NFAT factors in neuronal cells are regulated differently compared with T-cells.

To understand whether NFAT1-D regulation depends on its cellular context, the cDNA encoding full-length NFAT1-D was cloned into a mammalian expression vector under the control of a strong constitutive promoter and transiently cotransfected in Jurkat T-cells together with the NFAT/luc reporter. A similar construct encoding NFAT-1C, which is known to be regulated in T-cells, was used as a control. As shown in Fig. 9*A*, ectopic expression of NFAT1-D, while not affecting the basal transcription of the reporter gene, resulted in significant up-regulation of the levels of luciferase activity induced by a combination of PMA and a calcium ionophore and dependent on endogenous NFAT proteins. This enhancement was completely abrogated by treatment with CsA, indicating that, when expressed in T-cells, NFAT1-D is regulated like endogenous NFAT proteins. As expected, similar results were obtained by overexpression of NFAT1-C (Fig. 9*A*).

To confirm further the differential regulation of NFAT1-D in neuronal cells and T-cells, the cDNA encoding NFAT1-D was tagged with green fluorescent protein and transiently transfected in Jurkat cells. Cells were subsequently activated with the calcium ionophore A23187 either in the absence or in the presence of CsA. The subcellular localization of the NFAT1-D/GFP fusion protein was analyzed by confocal microscopy. The results are presented in Fig. 9*B*. As opposed to neuronal cells, when expressed in T-cells NFAT1-D was confined to the cytosol in the absence of stimulation, whereas it rapidly translocated to the nucleus in response to increased intracellular calcium flux. The inducible nuclear translocation of NFAT1-D was completely abrogated by CsA (Fig. 9*A*). Hence the activity of NFAT1-D is not intrinsically constitutive, but it is regulated in a tissue-specific manner. Interestingly, although expression of both NFAT1 and NFAT3 has been detected in many cell types, the activity of these factors appears to be consistently dependent on calcineurin activation through a calcium-dependent, CsA-sensitive mechanism, as reported for skeletal muscle cells, vascular smooth muscle cells, cardiomyocytes, adipocytes, and hippocampal neurons (21, 26–28, 43). A similar regulation of NFAT2 has been described in cardiac endothelial cells (24, 25). On the other hand, NFAT1-A (22), NFAT1-D, and NFAT2 all appear to be constitutively active in the brain. A different balance in the positive and negative components regulating NFAT factors, primarily calcineurin and GSK3, might underlie the high basal NFAT activity in mouse brain.

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