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NFATx, a Novel Member of the Nuclear Factor of Activated T Cells Family That Is Expressed Predominantly in the Thymus

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The nuclear factor of activated T cells (NFAT) regulates cytokine gene expression in T cells through *cis*-acting elements located in the promoters of cytokine genes. Here, we report the cDNA cloning, chromosomal localization, and initial characterization of a transcription factor related to NFATp and NFATc. The novel molecule, designated NFATx, exhibits in its middle a region very similar to the Rel homology domain in NFATc and NFATp. The amino-terminal region of NFATx also shows significant similarities to corresponding sequences in NFATc and NFATp and contains three copies of a conspicuous 17-residue motif of unknown function. We provide evidence showing that NFATx can reconstitute binding to the NFAT-binding site from the interleukin 2 promoter when combined with AP1 (c-Fos/c-Jun) polypeptides and that NFATx is capable of activating transcription of the interleukin 2 promoter in COS-7 cells when stimulated with phorbol ester and calcium ionophore. NFATx mRNA is preferentially and remarkably found in the thymus and at lower levels in peripheral blood leukocytes. The expression pattern of NFATx, together with its functional activity, strongly suggests that NFATx plays a role in the regulation of gene expression in T cells and immature thymocytes.

Upon recognition of foreign antigens, T lymphocytes secrete an array of cytokines that carry out many of the biological effector activities that enable lymphocytes to mount an effective immune response (2). Consequently, considerable effort has gone into trying to unravel the mechanisms that regulate the coordinate production of cytokines (interleukin 2 [IL-2], IL-3, IL-4, IL-5, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor [GM-CSF], gamma interferon, and tumor necrosis factor alpha) by the T cells (20, 31, 43). Regulation, for the most part, occurs at the level of initiation of transcription, and it is likely that *cis*-acting elements conserved in sequences of several cytokine gene promoters [e.g., CLE1(CK1), CLE2(CK2), and CLE0] play a role in coordinate induction by binding common or related transcription factors (2, 25, 36).

One factor that has been implicated in the transcriptional induction of several cytokine genes is NFAT (nuclear factor of activated T cells) (37). NFAT, which was identified in the IL-2 promoter system, is composed of members of the AP1 family of transcription factors (10, 14) and a preexisting cytoplasmic polypeptide designated $NFAT_c$ (10) or $NFAT_p$ (24). The cytoplasmic NFAT component is present in uninduced cells and is translocated to the nucleus upon calcium mobilization (10). This translocation seems to depend on the activity of the protein phosphatase calcineurin and to be sensitive to the action of the immunosuppressants cyclosporin A and FK506 (for reviews, see references 8 and 35). These and other characteristics of NFAT have now been shown to closely parallel the characteristics of nuclear factors that bind to functionally important elements in other cytokine promoters. In addition to the IL-2 promoter, NFAT or related factors have been reported to regulate GM-CSF, IL-3, IL-4, and tumor necrosis factor alpha gene promoters through partially related NFAT-binding sites, CLE0 elements, P sites, and κ sites (7, 11, 21, 22, 39).

We have been characterizing one such element, CLE0, from the murine GM-CSF gene promoter (21, 25), which is completely conserved in the human GM-CSF promoter. We recently showed that CLE0 was a target for two distinct induction signals, phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187), and that CLE0 was recognized by a cyclosporin A-sensitive factor identical or related to NFAT (21). In agreement with the regulation by NFAT (8, 35), we showed that overexpression of calcineurin phosphatase potentiated activation of the GM-CSF promoter and recovered transcription inhibited by cyclosporin A (42). Furthermore, we purified a 120-kDa protein component of NFAT to homogeneity and demonstrated that, in combination with AP1 proteins (c-Fos/c-Jun), the purified protein reconstituted both NFAT binding and the cyclosporin A-sensitive CLE0 binding (41).

In this study, we used sequence information derived from the purified 120-kDa protein and cloned human cDNAs encoding the 120-kDa protein and closely related factors. We chose to screen for homologs of the NFAT cytoplasmic component because the differences between the IL-2 NFAT-binding sites and CLE0 elements implied the existence of NFAT-related factors (20). In addition, biochemical evidence from renaturation of NFAT-binding activities from sodium dodecyl sulfate (SDS)-acrylamide gels had also suggested heterogeneity of NFAT (24, 30). Here, we present the cloning and initial characterization of a novel member of the NFAT family to which we refer as NFATx. We show that NFATx specifically binds the NFAT-binding sequence and that it is capable of activating the IL-2 promoter in non-T cells. This molecule is expressed predominantly, but not exclusively, in the thymus and expands the NFAT family of transcription factors that potentially play a role in the expression of cytokine genes in T cells and immature thymocytes.

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MATERIALS AND METHODS

Protein purification and peptide sequence determination. The 120-kDa component of NFAT was purified to near homogeneity as described by Tokumitsu et al. (41) from nuclear extracts derived from Jurkat T cells that had been stimulated for 2 h with PMA at 50 ng/ml and A23187 at 1.0 μM. Fractions containing the 120-kDa protein were combined and subjected to SDS-polyacrylamide gel electrophoresis, with the corresponding band excised and subjected to LysC endoproteinase digestion. The peptides were purified by microbore reversed-phase high-pressure liquid chromatography, and amino acid sequencing was done with Applied Biosystems (Foster City, Calif.) 476A and 477A gas phase sequencers.

PCR and cDNA cloning. A 437-bp DNA fragment was generated from Jurkat cDNA by PCR using degenerate oligonucleotides encoding the peptides KV LEIPLEK and QPNMLFVEIPEYRN derived from the 120-kDa protein. The oligonucleotides used were 5′ GA(A/G)AT(A/C/T)CCI(C/T)TIGA(A/G)CC 3′ and 5′ TA(C/T)TCIGG(A/G/T)AT(C/T)TCIAC(A/G)AA 3′ for the sense and antisense primers, respectively (I is deoxyinosine). The 437-bp fragment was purified and cloned into the pCRII vector (Invitrogen Inc., San Diego, Calif.), and its nucleotide sequence was determined (Sequenase; U.S. Biochemical, Cleveland, Ohio). The 437-bp fragment was then used to screen Jurkat cDNA libraries in λZiplox (GIBCO BRL, Gaithersburg, Md.) at low stringency. For NFATx, additional screening of the cDNA libraries was done at high stringency with a ~330-bp probe spanning the first 110 bp from the 5′ end of the sequence in Fig. 1. Plasmids from positive cDNA clones were excised according to GIBCO BRL protocols, and their inserts were characterized by restriction enzyme and nucleotide sequence analyses. Nucleotide sequences on both strands were determined by using the Applied Biosystems 373A DNA sequencing system with fluorescent dye terminators and confirmed when necessary by the Sequenase DNA sequencing system (U.S. Biochemical) with radioisotope labeling.

Northern blots. Poly(A)$^+$ RNAs were prepared from Jurkat T cells by using the Fast Track kit from Invitrogen Inc. Jurkat T cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 4 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Northern (RNA) blots of poly(A)$^+$ RNA from different human tissues were purchased from Clontech Inc. (Palo Alto, Calif.). For hybridization probes, we used DNA fragments derived from NFAT cDNA clones. For NFATx, we used a 537-bp *Bgl*II fragment (positions 2698 to 3235 in Fig. 1B). Other probes spanning the NFATx sequence (positions 1 to 1139 and positions 1156 to 2614 in Fig. 1B) were used for confirmation, giving essentially identical results. For NFATp, we used a fragment that corresponds to the 437-bp fragment shown in Fig. 1A. For NFATc, we used a 665-bp fragment which corresponds to positions 182 to 847 in the NFATc sequence reported by Northrop et al. (29). The human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe was obtained from Clontech Inc. Electrophoresis of RNA, blotting, hybridization, stripping, and random labeling were performed according to standard procedures (34).

FISH. Fluorescence in situ hybridization (FISH) was performed as described previously (33, 40). Prometaphase chromosome preparations for high-resolution FISH analysis were obtained from peripheral lymphocyte cultures of healthy donors by thymidine synchronization followed by incorporation of bromodeoxyuridine. For FISH analysis, two cDNA fragments for NFATx (fragments A and B [positions 1 to 1396 and positions 1575 to 3699, respectively, in Fig. 1B) were used. The cDNA fragments were labeled by nick translation with biotin-16-dUTP. Hybridization probes were efficiently detected by the amplification procedure using goat anti-biotin antibody and fluorescein isothiocyanate-anti-goat immunoglobulin G. The chromosomes were counterstained with propidium iodide in antifade mounting medium and examined under a Nikon Microphot FXA microscope. The photographs were taken with Kodak color 100 film through a B-2A filter for fluorescein isothiocyanate signals on the propidium iodide-stained R-banded chromosomes and a UV-2A filter for G-banded chromosomes.

EMSA. Electrophoretic mobility shift assays (EMSAs) were carried out with ~10-μg protein extracts as described previously (21). The oligonucleotides used in mobility shift assays contained the following sequences (only one strand is shown; sequence overhangs are lowercased): NFAT site (9), 5′gatcGGAGGA AAAACTGTTTCATACAGAAGGCGT3′; AP1 site (28), 5′gatcTCAACAGA GGGGACTTTCCGAGAGGCC3′; murine NFAT site (24), 5′gatcGCCCAAA GAGGAAAATTTGTTTCATACAG3′; GM43,47 (which corresponds to the CLE0 oligonucleotide with a double substitution at positions −43 and −47) (21), 5′gatcGTCACCATTAATCCTTTACTCTAACTGT3′.

Extracts from transfected COS-7 cells were prepared by transfecting the cells by electroporation with 10 μg of the appropriate plasmid DNA in a DEAE-dextran (10 μg/ml) solution at pulse conditions of 960 μF and 220 V using a Gene Pulser apparatus (Bio-Rad, Hercules, Calif.). Cells were cultured at 37°C and harvested 56 h after electroporation. Nuclear and cytosolic extracts of transfected COS-7 cells were prepared as described by Watanabe et al. (45).

Fractions containing purified AP1 were obtained as described previously (21). Recombinant purified c-Jun and c-Fos expressed as histidine fusion proteins in *Escherichia coli* (1) were kindly provided by T. Kerppola and T. Curran (Roche Institute of Molecular Biology, Nutley, N.J.).

Plasmids and DNA transfections. pME-NFATc and pME-NFATx were constructed by inserting the corresponding cDNAs downstream of the SRα promoter in the expression vector pME18S (16). The NFATc cDNA was assembled from two overlapping clones and includes 326 bp of the 5′ untranslated sequence and the poly(A)$^+$ tail at the 3′ end of the sequence. The NFATx cDNA insert was assembled from three overlapping clones and corresponds to sequences (positions 1 to 3676) in Fig. 1. Plasmid pIL2Luc1 (42) was derived from pIL2-CAT1 (obtained from G. Crabtree, Stanford University) and contains positions −567 to +47 of the IL-2 gene. pCMVβ (19) is a *lacZ* reporter vector that is transcribed by the cytomegalovirus promoter and was obtained from Clontech Inc.

Transfections were carried out as described above by electroporation in the presence of DEAE-dextran (10 μg/ml). Semiconfluent COS-7 cells (100-mm dish) were transfected with 6 μg of pIL2Luc1 and 12 μg of pME-NFATx or pME18S. For pCMVβ transfections, 2 μg of plasmid was used. Luciferase activity was measured by a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, Calif.) in the presence of coenzyme A using a luciferase assay system (Promega, Madison, Wis.). β-Galactosidase was also measured by luminescence with the chemiluminescent substrate Galacton (Tropix, Bedford, Mass.). Protein concentration was measured by using bicinchoninic acid (Pierce, Rockford, Ill.) as suggested by the supplier.

Nucleotide sequence accession number. The EMBL and GenBank accession number for NFATx is U14510.

RESULTS

Cloning of NFATx. We previously purified to homogeneity a 120 kDa protein that reconstituted NFAT binding in combination with AP1 (c-Jun/c-Fos) (41). As a source, we used nuclear extracts derived from human Jurkat T cells that had been stimulated for 2 h with phorbol ester and calcium ionophore. Using this method, we prepared ~5 μg of the 120-kDa protein, subjected it to endoproteinase LysC treatment, and obtained partial amino acid sequences from some of the resulting peptides. We next designed degenerate oligonucleotides from two of the peptide sequences (KVLEIPLEK and QPNMLFVEIPEYRN) and amplified a 437-bp fragment from Jurkat cDNA. Nucleotide sequencing of this fragment revealed that, in addition to the expected flanking peptide sequences, the PCR fragment encoded the sequence KVVFTEK, which corresponded to the sequence of another peptide derived from the purified 120-kDa protein (Fig. 1A). Soon after, we realized that this nucleotide sequence was 86.5% identical (Fig. 1A) to sequences of the murine gene encoding the transcription factor NFATp (23). At the amino acid level, the sequences were 95.9% identical (139 of 145 amino acids), strongly indicating that the 120-kDa protein we purified was encoded by the human NFATp gene.

Suspecting the existence of additional NFAT-related proteins (20), we screened Jurkat cDNA libraries at low stringency with the 437-bp fragment and isolated several clones that were classified by restriction enzyme and sequencing analyses into three different genes. One set of clones encoded a protein, referred to as NFATp, of a >90% amino acid identity to the murine NFATp protein. Another set of cDNA clones encoded the recently described NFATc protein (29). The third set of cDNA clones encoded a novel but related protein, to which we refer as NFATx. We then focused our attention on the previously uncharacterized NFATx molecule.

The NFATx cDNA sequence revealed an open reading frame encoding a protein of 1,075 amino acids with a deduced molecular weight of 115,592 (Fig. 1B). The Rel-homology domain of about 290 residues, which was recognized in NFATc (29) and murine NFATp (23), was also present in the middle of NFATx (Fig. 2). The similarity of NFATx in this domain was remarkable, showing a 69% amino acid identity to NFATc and a 66% amino acid identity to both the human NFATp and the murine NFATp (Fig. 2A). The similarity was particularly high (~78% identity to NFATc and NFATp) in the first 200 residues of this region (Fig. 2A), which in the Rel domain contains sequences that direct specific DNA binding (4, 13, 18). This indicated that NFATx would likely bind to DNA sequences

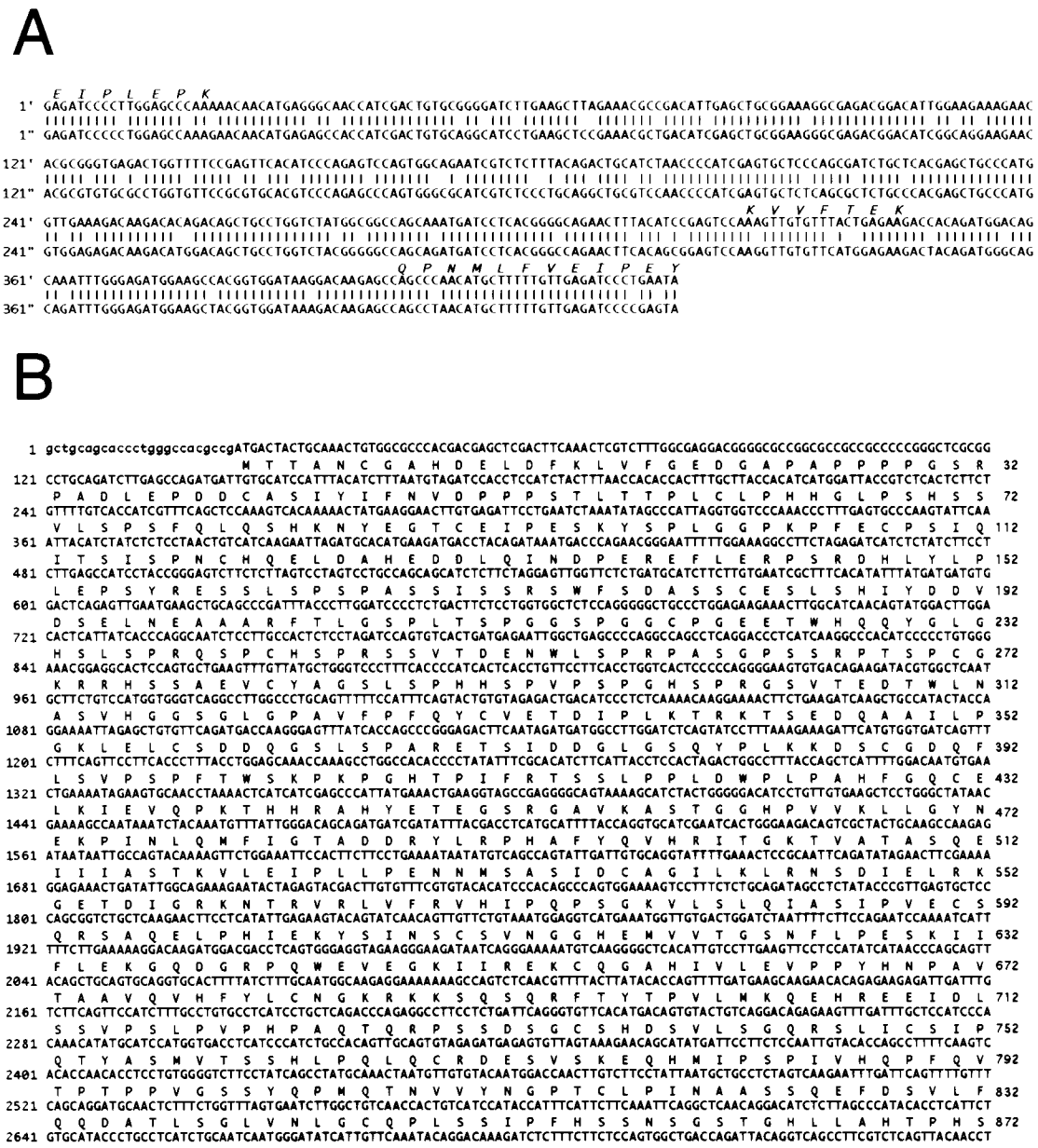

FIG. 1. (A) Comparison of the nucleotide sequence of the 437-bp PCR fragment encoding part of the 120-kDa NFAT component (upper strand) with analogous sequences from the murine NFATp cDNA (23) (lower strand). Amino acid sequences derived from peptides of the 120-kDa protein are italicized. (B) Nucleotide and deduced amino acid sequences of NFATx. Nucleotide sequences are numbered on the left and amino acid sequences are numbered on the right. The stop codon is represented by a period. Noncoding sequences are lowercased.

similar to the binding sites of NFATp and NFATc. Also, we noticed that the Rel homology domain of NFATx, analogously to those of other Rel proteins (4, 13), contained a putative nuclear localization signal (KRKK for NFATx and KRKR for NFATp and NFATc) at its carboxy terminus (Fig. 2A).

Besides the Rel homology domain, sequences at the amino terminus of the NFATx also exhibited similarity to corresponding sequences in NFATc and NFATp. The similarities, albeit low, were significant, with >30% of the residues being identical to those in NFATc, NFATp, and murine NFATp (data not

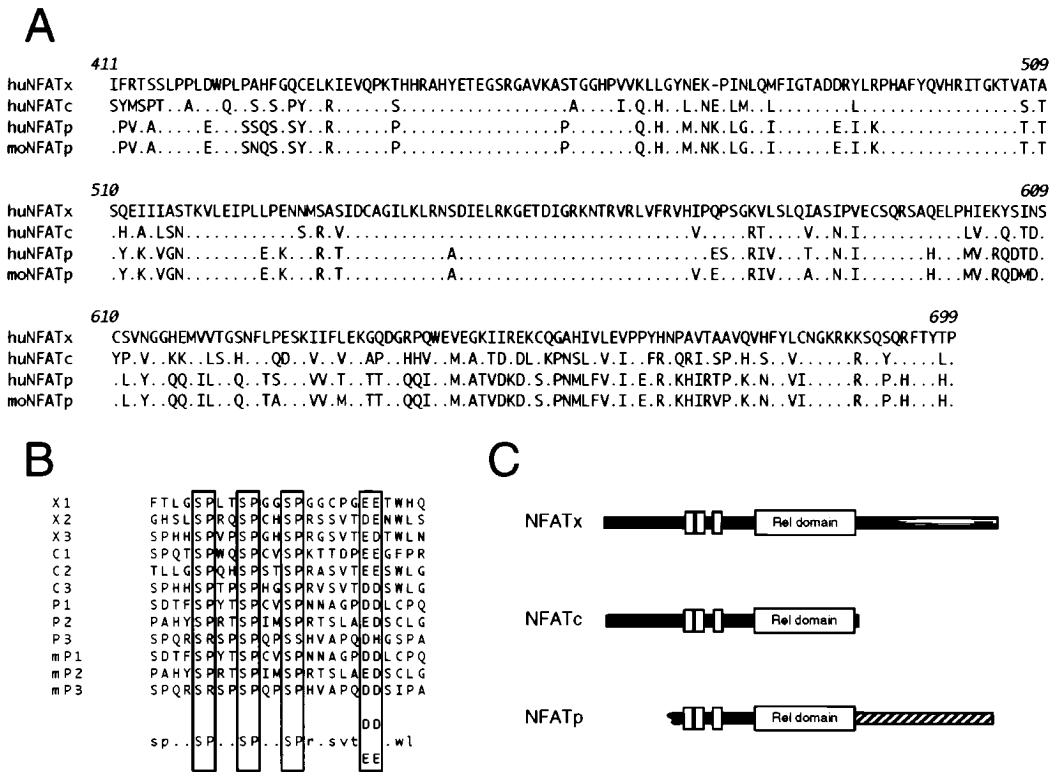

FIG. 2. (A) Comparisons of amino acid sequences in the Rel homology domains of human (hu) and murine (mo) members of the NFAT family of transcription factors. Periods denote sequence identity with NFATx. Numbering is with respect to NFATx. (B) Alignment of SP boxes present in the amino termini of NFAT proteins. SP boxes for NFATx (X), NFATc (C), NFATp (P), and murine NFATp (mP) are numbered 1 to 3, with 1 and 3 representing, respectively, the closest and farthest boxes with respect to the amino terminus of the protein. The sequences of X1, X2, and X3 start at NFATx amino acid residues 203, 232, and 288, respectively (see Fig. 1B). Highly conserved residues are boxed. A consensus sequence is shown below. Residues that are >55% conserved between the SP boxes from the human NFAT proteins are lowercased. Less conserved residues are represented by periods. (C) Schematic representation of the structure of NFATx compared with those of NFATc and murine NFATp. Rel homology domains are indicated. SP boxes are represented by small rectangles. Solid bars, >30% identical residues. The available sequence for murine NFATp is derived from a partial clone (23).

shown). This amino-terminal region, rich in prolines and serines, showed no similarities to other sequences in the databases. However, this region contained a conspicuous SPXXS PXXSPXXXXX(D,E)(D,E) motif, here referred to as the SP box, that was repeated three times in NFATx, as well as in NFATc and NFATp (Fig. 2B). The core sequences in this box were highly conserved; moreover, some positions intercalated in or surrounding this motif appeared to prefer certain amino acid residues (lowercased in Fig. 2B). The function of the SP box is unknown, and similar sequences were not found for other proteins in the databases.

NFATx is preferentially expressed in the thymus. With the availability of human cDNA probes for the three members of the NFAT family, we next determined the tissue-specific expression pattern of the NFATx, NFATc, and NFATp genes by Northern analysis. For NFATx, a major hybridizing band with an apparent size of 7.0 kb was seen specifically in RNAs from the thymus and, at lower levels, in leukocytes (Fig. 3A). Prolonged exposure of the blot revealed weak NFATx expression in the testis, ovary, spleen, muscle, and kidney and very weak expression in the prostate, small intestine, colon, placenta, lung, and liver (Fig. 3A). However, it is possible that the weak expression in some tissues is due to contaminating leukocytes circulating in the tissues.

For NFATc, we observed two hybridizing bands with apparent sizes of 5.2 and 2.9 kb (Fig. 3A). Both bands were clearly seen in all tissues tested except the brain, liver, and kidney. Interestingly, the intensities of the two bands were different in different tissues. The intensity of the 5.2-kb band was strong in the tissues where it was expressed, except in the placenta and pancreas. The 2.9-kb band, on the other hand, showed more variability in its expression pattern. This band was preferentially expressed in the thymus, leukocytes, and muscle and at lower levels in the spleen, testis, and heart (Fig. 3A). The pattern of expression shown by the 2.9-kb band seems to correspond to the NFATc tissue distribution reported previously (29) with ribonuclease protection assays. The relationship between the 5.2- and the 2.9-kb bands remains to be understood, and further characterization is necessary to explain the different tissue distribution.

As for NFATp, an ~8.0-kb band was detected in all tissues tested, although it was expressed at very low levels in the brain. NFATp expression was high in leukocytes, the spleen, and the placenta and very weak in the heart and colon. Again, weak signals may have been caused by contaminating leukocytes in the tissues.

We next investigated the effect of stimulation of Jurkat cells on the expression of NFATx compared with the expression of NFATc and NFATp (Fig. 3B). As previously shown by Northrop et al. (29), the expression of NFATc was induced by the action of the calcium ionophore and further increased with the addition of phorbol ester (Fig. 3B). Both bands, the 5.2 kb and 2.9 kb, showed a similar pattern of expression upon stimulation of the Jurkat cells (Fig. 3B). The expression of NFATx, on the other hand, was not significantly affected. Similarly, the expression of NFATp was for the most part unaffected, al-

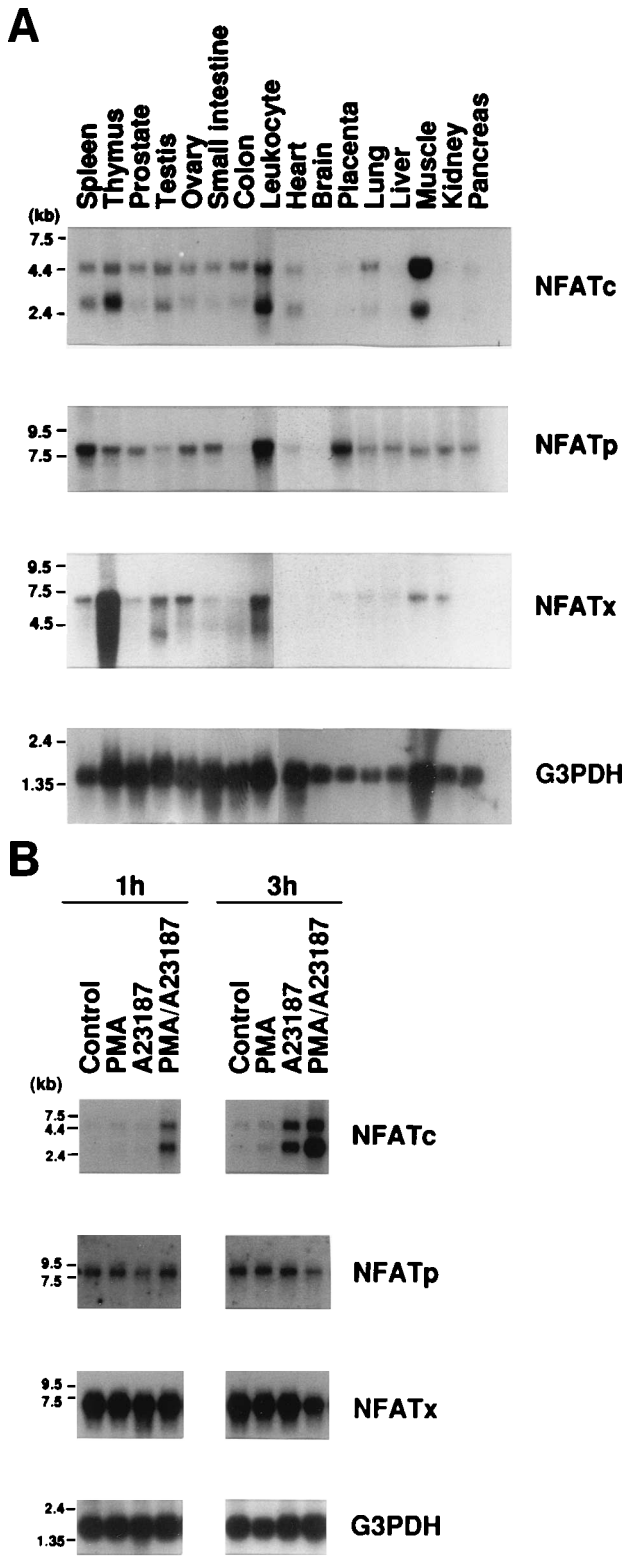

FIG. 3. Expression of NFATc, NFATp, and NFATx mRNAs in different human tissues and in Jurkat T cells. (A) Northern blots were hybridized with probes from cDNAs corresponding to NFATc, NFATp, NFATx, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The positions of size markers are indicated on the left. (B) Northern analyses using poly(A)$^+$ RNA extracted from Jurkat cells that were treated for 1 or 3 h with PMA (50 ng/ml), calcium ionophore A23187 (1.0 μM), or a combination of both.

though a consistent downregulation of the message was observed upon PMA-A23187 stimulation (data not shown and Fig. 3B).

Chromosomal location of NFATx. We next decided to determine the chromosomal location of the gene encoding NFATx. By FISH, we located the majority of the doublet signals in chromosome 16q21→q22 (Fig. 4). We used two different NFATx cDNA fragments as hybridization probes (see Materials and Methods) and obtained similar results. By similar techniques, NFATc and NFATp were mapped in different chromosomes (data not shown). Thus, three genes encoding different members of the NFAT family are located in different chromosomes of the human genome.

NFATx specific binding to the NFAT-binding site. The high homology shown by NFATx in the Rel domain of NFATc and NFATp suggested that NFATx analogously bound to the NFAT-binding site. To test this, we transfected an expression vector containing the full-length cDNA of NFATx (pME-NFATx) into COS-7 cells and prepared nuclear and cytosolic extracts from the transfected cells. We also transfected an expression vector containing full-length NFATc (pME-NFATc) for comparison purposes and the empty expression vector (pME18S) as a mock control. EMSAs were then carried out with a double-stranded oligonucleotide containing the NFAT-binding site from the human IL-2 promoter as a probe (Fig. 5). The results demonstrated the presence of DNA-binding activities in extracts from cells transfected with both NFATx and NFATc expression vectors (Fig. 5A). This activity was absent from cells transfected with the expression vector alone (Fig. 5A, lanes 1 and 7). Interestingly, the mobility shift elicited by NFATx is slightly slower than that elicited by NFATc (Fig. 5A, lanes 3 to 6, 10, and 12), and neither corresponds identically to the shift elicited by nuclear extracts from stimulated Jurkat cells (Fig. 5A, lane 13).

In agreement with previous characterization of NFAT in T cells (10, 14), exogenous AP1 polypeptides were necessary to reconstitute NFAT binding by the cytoplasmic extracts from NFAT-transfected cells (Fig. 5A, lanes 9 to 12). The exogenous AP1 we used was purified from Jurkat cells, but recombinant c-Jun and c-Fos purified from *E. coli* cells were equally capable of providing the AP1 component to reconstitute NFAT binding (Fig. 5B). Nuclear extracts, on the other hand, did not require exogenous AP1 (Fig. 5A, lanes 3 and 5), probably because of endogenous AP1 present in the nuclei of COS-7 cells.

The binding to the NFAT site by NFATx was sequence specific. Oligonucleotides containing the NFAT or AP1 binding site were able to compete for reconstituted binding activity, while oligonucleotides containing an irrelevant sequence were not (Fig. 5C, lanes 5 to 8). Moreover, extracts from NFATx-transfected COS-7 cells did not bind the IL-2 κB site even in the presence of exogenous AP1 (Fig. 5C, lane 10). Similar results were obtained with extracts from COS-7 cells overexpressing NFATc (Fig. 5C, lane 1 to 5 and 9).

Notably, the binding specificities shown by NFATc and NFATx, although similar, were not identical. This was manifested when the murine, as opposed to the human, NFAT-binding site from the IL-2 promoter was used for the mobility shift assays (Fig. 6) (see Materials and Methods for nucleotide sequences of both sites). Unlike with the human probe, two specific band shifts have been observed with EMSA using the murine NFAT-binding site. This can be explained partly because of the difference in sequence (15). The slowly migrating band corresponds to the shift seen with the human NFAT-binding site, while the fast-migrating band is due to NFAT binding in the absence of AP1 (14, 15). Interestingly, cytosolic

FIG. 4. Refined mapping for a cDNA fragment of NFATx (fragment A, positions 1 to 1396 in Fig. 1B) to chromosome 16q21→q22 by FISH. (a and c) FITC signals (arrows) on R-banded chromosomes 16 visualized through a Nikon B-2A filter; (b and d) G-banding pattern on the same chromosomes as seen through a UV-2A filter.

extracts from NFATc- but not NFATx-transfected COS-7 cells were able to bind the murine NFAT-binding site in the absence of exogenous AP1 (Fig. 6; compare lanes 2 and 7). This binding by NFATc was specific to the NFAT sequence and could not be inhibited by the addition of oligonucleotides containing the AP1 site (Fig. 6, lanes 2 to 5). Thus, although the significance of this phenomenon is uncertain, NFATx and NFATc appear to have different requirements for binding the murine NFAT-binding site.

NFATx can activate the IL-2 promoter in COS-7 cells. We next checked the effect of NFATx on the activity of the IL-2 promoter in COS-7 cells. The expression vector containing the full-length NFATx cDNA (pME-NFATx) or the expression vector itself (pME18S) was cotransfected with the plasmid pIL2Luc1, which contains the reporter luciferase gene under the control of the IL-2 gene promoter (positions −567 to +47). The transfected COS-7 cells were divided into two sets and incubated at 37°C for ~40 h, and one set was mock stimulated while the other set was stimulated with PMA (50 ng/ml) and A23187 (10 μM) for 8 h before harvesting. Cell extracts were then prepared, and the luciferase activity was measured and normalized to protein mass. The results demonstrated that the IL-2 promoter activity increased about six times when cotransfected with pME-NFATx after PMA-A23187 stimulation (Fig. 7). The stimulation of cells was necessary for observing the transactivation because very little effect was seen in the absence of phorbol ester and calcium ionophore. In contrast, the IL-2 promoter had no significant activity in COS-7 cells, with or without stimulation, when cotransfected with the expression vector pME18S (Fig. 7). Also, pME-NFATx had no effect, relative to pME18S, on the β-galactosidase activity directed by the cytomegalovirus promoter from the plasmid pCMVβ (data not shown).

DISCUSSION

The loose similarity among several *cis*-acting elements that bound NFAT complexes suggested the existence of multiple NFAT-related factors (20, 31). This was substantiated by the cloning of NFATc and murine NFATp cDNAs, which encoded homologs of the cytoplasmic component of NFAT (23, 29). In this report, we extend this family of transcription factors with the identification of a third member, referred to as NFATx.

Beginning with partial amino acid sequences derived from purified NFATp, we cloned cDNAs from Jurkat cDNA libraries encoding human NFATp, NFATc, and the previously uncharacterized NFATx. Three distinct regions were observed in the primary structure of NFATx relative to NFATc and NFATp (Fig. 2): an amino-terminal region, a carboxy-terminal region, and a middle region containing the Rel homology domain (4).

The amino-terminal region showed significant homology (>30% identical residues) to analogous sequences from NFATc and NFATp but no similarities to sequences from the databases. This region is characterized by the presence of three repeats containing the sequence SPXXSPXXSPXXXXX(D,E)(D,E), here referred to as SP boxes. Although their significance is unknown, the SP boxes are conserved in NFATc and NFATp (Fig. 2B), suggesting some degree of biological relevance. Northrop et al. (29), in their characterization of NFATc, reported the dominant negative effect on IL-2 promoter activity caused by a plasmid encoding the amino-terminal sequences of NFATc. In light of the fact that the dominant negative mutant included the SP boxes, there exists the possibility that the mutant inhibits the action of not only NFATc but also other members of the NFAT family. The importance of the SP boxes for the mechanism of inhibition by the dominant

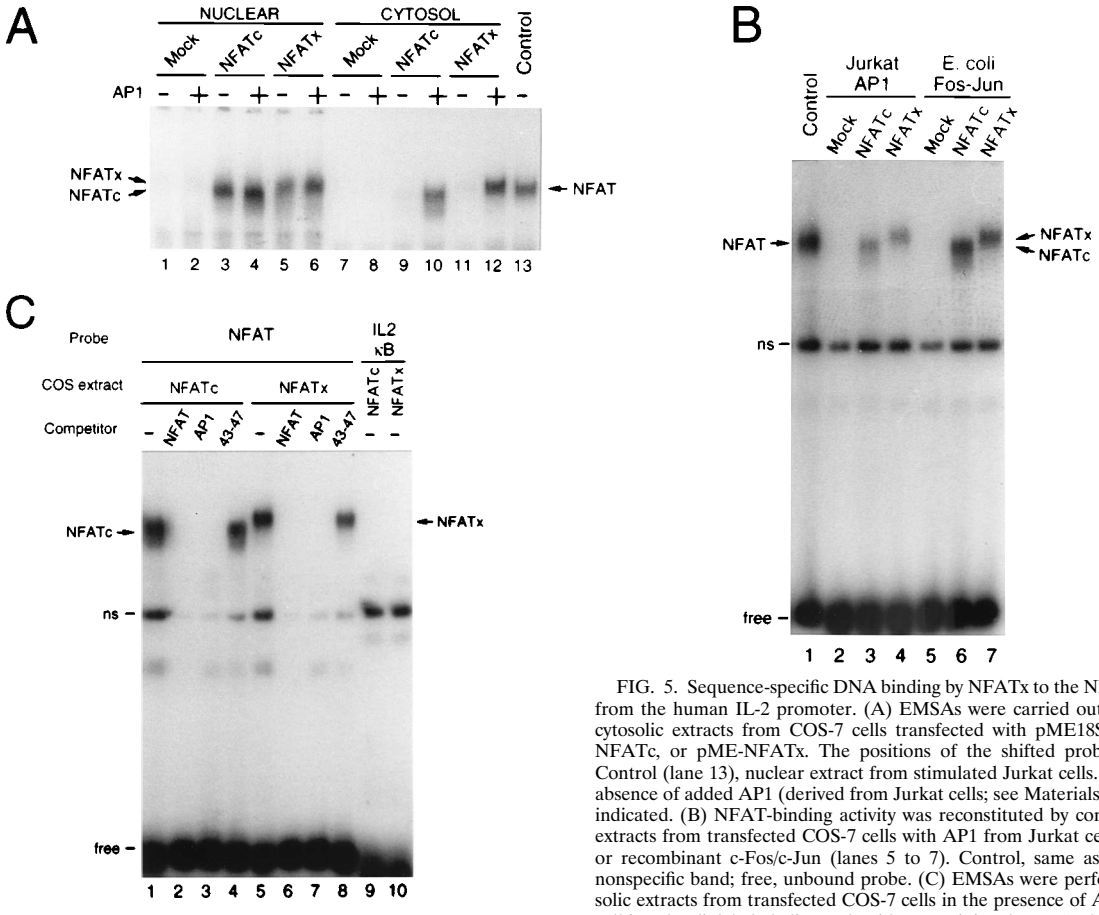

FIG. 5. Sequence-specific DNA binding by NFATx to the NFAT-binding site from the human IL-2 promoter. (A) EMSAs were carried out with nuclear or cytosolic extracts from COS-7 cells transfected with pME18S (Mock), pME-NFATc, or pME-NFATx. The positions of the shifted probe are indicated. Control (lane 13), nuclear extract from stimulated Jurkat cells. The presence or absence of added AP1 (derived from Jurkat cells; see Materials and Methods) is indicated. (B) NFAT-binding activity was reconstituted by combining cytosolic extracts from transfected COS-7 cells with AP1 from Jurkat cells (lanes 2 to 4) or recombinant c-Fos/c-Jun (lanes 5 to 7). Control, same as in panel A. ns, nonspecific band; free, unbound probe. (C) EMSAs were performed with cytosolic extracts from transfected COS-7 cells in the presence of AP1 (from Jurkat cells) and radiolabeled oligonucleotides containing the NFAT-binding site (lanes 1 to 8) or the IL-2 κB site (lanes 9 and 10). Unlabeled oligonucleotides bearing the NFAT-binding site (lanes 2 and 6), the AP1-binding site (lanes 3 and 7), or a mutated CLE0 site 43-47 (21) (lanes 4 and 8) were added to the assays as competitors at a 50-fold molar excess.

negative mutant needs to be assessed to clarify this point. We also note that, although the SP box consensus sequence was absent in other sequences from the databases, the sequence SPXX has been reported to appear frequently in many gene-regulatory proteins, including the products of homeotic genes, segmentation genes, steroid hormone receptors genes, and certain oncogenes (38).

As for the other two regions, the carboxy-terminal region of NFATx shows no significant similarity to the analogous sequence in NFATp or anything else in the databases, and it is absent in NFATc (Fig. 2). On the other hand, the Rel homology domain is the most conserved region within members of the NFAT family (>66% identical residues in a stretch of 290 amino acids). However, NFAT proteins exhibit less than 20% identity to Rel proteins, making them a distant subfamily of the group of proteins containing Rel domains. Nevertheless, sequences analogous to residues in the Rel domain that have been implicated in DNA binding, dimerization, and nuclear localization (4, 13) may impart similar functions to the NFAT proteins. Interestingly, residues from the Rel proteins that have been demonstrated to directly contact DNA are located at the amino terminus of the Rel domain (18). It is this region that has the highest sequence identity within the NFAT proteins (Fig. 2A), suggesting that the different NFAT proteins bind similar motifs. In fact, we were able to demonstrate reconstitution of binding by NFATx in COS-7 extracts to the NFAT-binding site (Fig. 5).

It is not yet known whether NFATx is present in NFAT complexes in Jurkat cells. However, previous biochemical studies of the cytoplasmic component of NFAT by elution and renaturation from SDS-polyacrylamide gels showed it to be heterogeneous, having more than one molecular mass range (M_r, 90,000 to 140,000) (24, 30). Arguably, NFATx could represent one or more of the molecules detected in the above assays. Furthermore, Northrop et al. (30) showed that the shifts in mobility in EMSA shown by the reconstituted NFAT complexes were slightly different, depending on the molecular size ranges of the renatured proteins. Specifically, NFAT from higher-molecular-mass fractions tended to run more slowly in EMSA than proteins renatured from lower molecular size ranges. This fits nicely with the slower mobility shift shown by NFATx (M_r, 116,000) than the shift exerted by NFATc (M_r 82,000) (Fig. 5). In addition, antisera against NFATc or NFATp do not completely block or supershift all of the NFAT present in the mobility shift assays (23, 29). Thus, NFATx may represent part of the heterogeneous NFAT complex present in T cells.

Concomitant to the NFATx binding to NFAT-binding sites, we showed that overexpression of NFATx in COS-7 cells activated the IL-2 promoter. We observed a sixfold stimulation

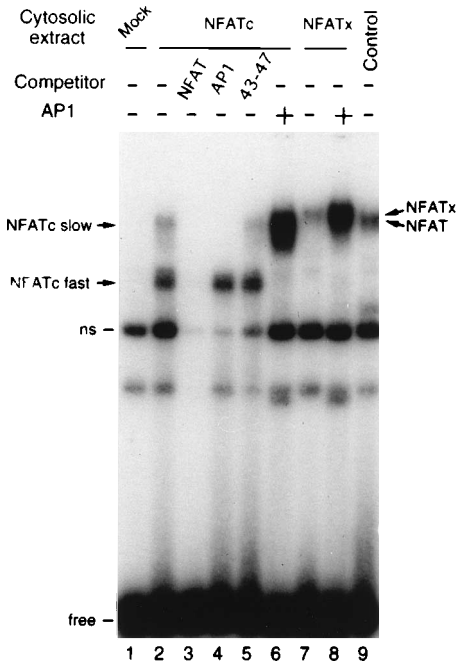

FIG. 6. NFATc, but not NFATx, can bind the murine IL-2 NFAT-binding site in the absence of added AP1 proteins. EMSAs were carried out with the indicated cytosolic extracts from transfected COS-7 cells in the presence (+) or absence (−) of added AP1. Unlabeled competitor oligonucleotides were added as for Fig. 5C. The positions of the shifted bands are indicated. Control (lane 9), nuclear extract from stimulated Jurkat cells.

by pME-NFATx over mock (pME18S)-transfected cells with PMA-A23187 (Fig. 7). The reason for the requirement for PMA-A23187 stimulation in COS-7 cells is unclear, but it may represent regulatory mechanisms similar to those in Jurkat T cells (8). In fact, transient transfection in Jurkat T cells showed that the IL-2 promoter activity is increased two or three times when NFATx is overexpressed only after activation by both PMA and A23187 (27).

A similar transactivation activity has been shown for NFATc. Northrop et al. (29) reported an 8- to 10-fold increase by transfected NFATc in COS cells on the IL-2 promoter using a similar expression vector to drive NFATc synthesis. In our experiments, we found an 8- to 22-fold stimulation using pME-NFATc (27). However, the constitutively high expression of NFATp and NFATx mRNAs in Jurkat cells (Fig. 3) argues for an early effect by NFATp, and perhaps NFATx, relative to NFATc. Thus, the regulation of IL-2 promoter activity by NFAT seems to be complex, with components regulated temporally, perhaps in a manner similar to the regulation of the NF-κB-binding sites by the different Rel proteins (5, 12, 26). Characterization of all the members of the NFAT family may be necessary to understand transcriptional regulation through NFAT DNA-binding motifs. Moreover, the heterogeneity of NFAT and the complexity of IL-2 gene regulation are further increased with the detection of spliced variants affecting the coding region of NFATx and NFATp (23, 27).

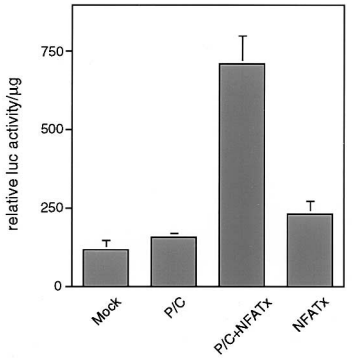

FIG. 7. Transactivation of the IL-2 promoter by NFATx in COS-7 cells. Semiconfluent cells were cotransfected with 6 μg of the reporter plasmid pIL2Luc1 and 12 μg of the expression vector pME18S or pME-NFATx. Transfected cells were stimulated with PMA and calcium ionophore (P/C) for 8 h before harvesting. Luciferase (luc) activity was assayed in whole-cell extracts and normalized to protein mass. The data are averages for three independent experiments.

Although the different NFAT proteins are capable of binding the same NFAT-binding site, their binding sequence specificities are not identical. We show, for example, that full-length NFATc can bind the murine NFAT site in the absence of AP1 while full-length NFATx cannot (Fig. 6). It may be that the difference in binding can be due to the extra carboxy-terminal region in NFATx, which is missing in NFATc. Although a truncated murine NFATp protein has been shown to bind the NFAT-binding sequence in the absence of AP1 (23), it remains to be demonstrated whether full-length NFATp is capable of such binding. Nevertheless, it appears that some binding sites allow NFAT binding in the absence of AP1 while other sites require the AP1 component for binding (15, 39). This, perhaps, is an indication of preferences by a particular NFAT for particular sites and a potential device for regulation at the different *cis*-acting elements that bind NFAT. Moreover, the opportunities for additional regulation at NFAT-binding sites in various cytokine gene promoters are expanded when the diverse members of the AP1 family are considered. Further work is necessary to clarify the relative roles of the different components of NFAT.

An intriguing facet of NFATx is its remarkable expression in the thymus (Fig. 3), which implies some functional activity by NFATx in events that occur in this tissue. NFAT complexes, in fact, have been implicated in the intrathymic development of T cells, demarcated by differences in competence to produce IL-2 (6, 32). In addition, it has been demonstrated that certain subsets of thymocytes can express large amounts of other cytokines as well, including IL-4, IL-5, IL-10, gamma interferon, and tumor necrosis factor alpha (3, 44, 46). NFATx, by its virtue of binding NFAT-binding sites, may play a role in the regulation of cytokine expression in the thymus.

Another activity in the thymus in which NFAT complexes have also been proposed to play a role is the antigen-induced cell death of thymocytes involving the Nur77 transcription factor (8). Interestingly, NFATx mRNA is highly expressed in the $CD4^+$ $CD8^+$ thymocytes (17), which constitute the subset that undergoes programmed cell death during negative selection. The NFATx cDNA cloning, then, might provide tools for further investigating programmed cell death during T-cell development.

In summary, we have identified and characterized NFATx as an additional member of the NFAT family of transcription factors. NFATx is capable of reconstituting NFAT complexes with AP1 on NFAT-binding sites and transactivating the IL-2 promoter. It then appears that the NFAT transcription complex contains several members of related molecules, complicating analyses of the relative effects of a particular NFAT on the IL-2 and other cytokine promoters. The availability of the NFAT cDNA clones, however, should help us determine the roles of different NFAT members in the coordinate and dif-

ferential expression of cytokines during T-cell activation. Finally, the predominant NFATx expression in the thymus may reflect an additional role for this transcription factor in the programmed development of T cells.

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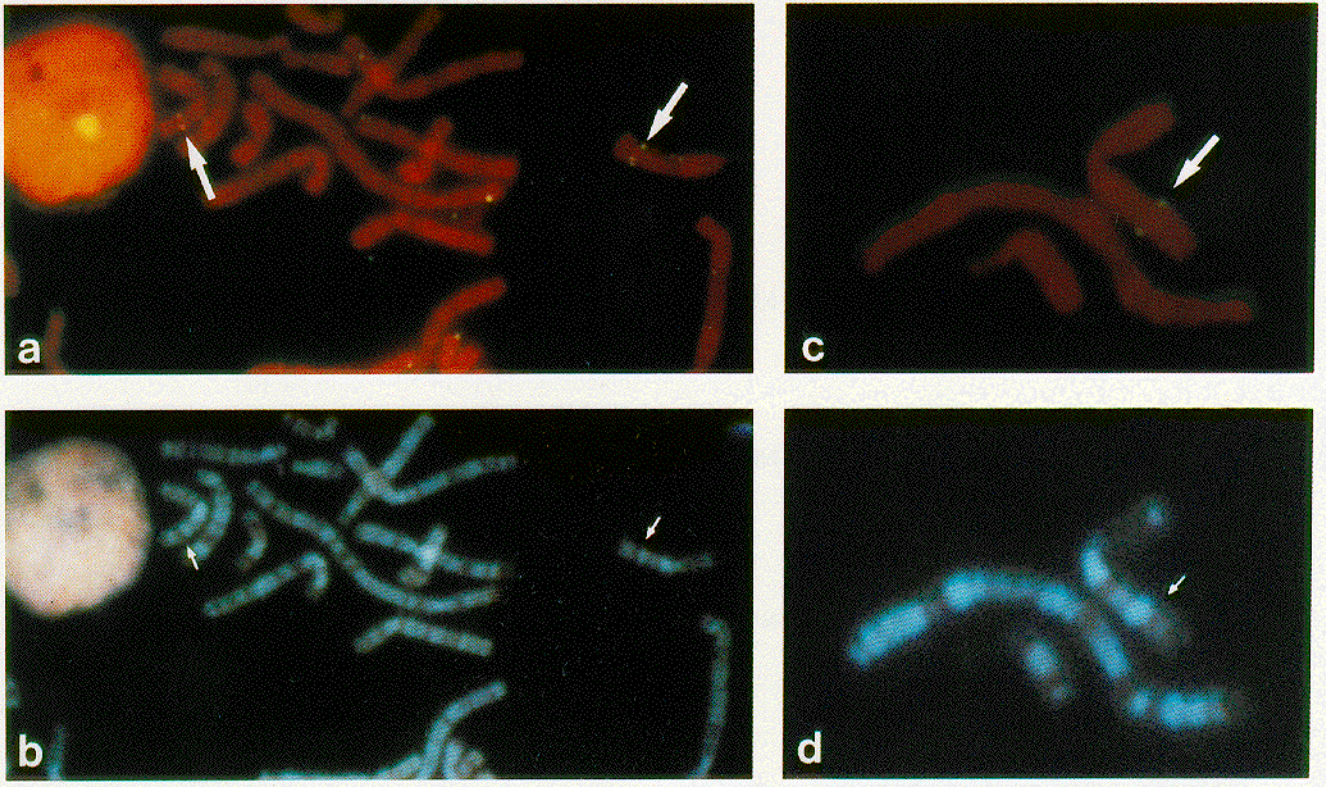
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