MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2475–2484 Vol. 17, No. 5
0270-7306/97/$04.00+0

Expression of NFAT-Family Proteins in Normal Human T Cells

LYUDMILA LYAKH,[1] PARITOSH GHOSH,[2]† AND NANCY R. RICE[1]*

Molecular Biology of Retroviruses Section, Molecular Basis of Carcinogenesis Laboratory, ABL-Basic Research Program,[1] and Laboratory of Experimental Immunology, Division of Basic Science, SAIC,[2] NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

Received 25 July 1996/Returned for modification 27 September 1996/Accepted 12 December 1996

NFAT proteins constitute a family of transcription factors involved in mediating signal transduction. Using a panel of specific antisera in immunoprecipitation assays, we found that NFATp (135 kDa) is constitutively expressed in normal human T cells, while synthesis of NFATc (predominant form of 86 kDa) is induced by ionomycin treatment. NFAT4/x was very weakly expressed in unstimulated cells, and its level did not increase upon treatment with activating agents. NFAT3 protein was not observed under any conditions. Higher-molecular-weight species of NFATc (of 110 and 140 kDa) were also detected. In addition, translation of NFATc mRNA apparently initiates at two different AUG codons, giving rise to proteins that differ in size by 36 amino acids. Additional size heterogeneity of both NFATc and NFATp results from phosphorylation. In contrast to ionomycin treatment, exposure of cells to phorbol myristate acetate (PMA) plus anti-CD28 did not induce NFATc, indicating that under these conditions, interleukin-2 synthesis by these cells is apparently independent of NFATc. In DNA binding assays, both PMA plus anti-CD28 and PMA plus ionomycin resulted in nuclear NFAT. Surprisingly, the PMA-ionomycin-induced synthesis of NFATc that was detected by immunoprecipitation was not mirrored in the DNA binding assays: nearly all of the activity was due to NFATp. This is the first study of expression of all family members at the protein level in normal human T cells.

NFAT (nuclear factor of activated T cells) is implicated in regulation of interleukin-2 (IL-2) gene transcription (for reviews, see references 13 and 30). In addition, NFAT-binding sites have been identified in the regulatory regions of various other cytokine genes, including the IL-4 (3, 33, 38), tumor necrosis factor alpha (7, 23), and IL-3/granulocyte-macrophage colony-stimulating factor (4, 22) genes. Though originally found in T cells, NFAT DNA-binding activity and/or protein has now been found in other cell types, including B cells (2, 40, 41, 45), mast cells (29), natural killer (NK) cells (1), and a neuronal cell line and certain regions of the brain (8). Thus, NFAT is likely to play an important role in the regulation of a variety of genes in a number of different cell types.

To date, cDNAs from four different NFAT genes (NFATp, NFATc, NFAT3, and NFAT4/NFATx) have been cloned, and they constitute a related but quite divergent family (9, 11, 18, 21, 24, 26, 27). The family in turn is weakly related to the Rel/NF-κB family of transcription factors over a 300-amino-acid region called the Rel homology domain (RHD). NFAT sequences in this region govern DNA binding and association with the AP-1 transcription factor (12), and within the RHD, sequence conservation among the NFAT proteins is very high. Upstream of the RHD, NFAT proteins are less closely related, but they do share several serine- and proline-rich segments. Downstream of the RHD, NFAT proteins are variable in length and in sequence.

The hallmark of NFAT activity is its inducibility by agents that increase intracellular Ca^{2+} flux (e.g., ligands of the T-cell receptor or the calcium ionophore ionomycin) (for reviews, see references 5 and 35). The increased Ca^{2+} activates the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (phosphatase 2B), which directly or indirectly results in dephosphorylation and nuclear translocation of preexisting cytoplasmic NFAT (16, 17, 36). Since the process involves calcineurin, activation of NFAT is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (17, 36).

Until recently, NFAT was known primarily through DNA binding assays, but with molecular cloning of the genes, development of reagents capable of recognizing specific family members became possible. It is now known that NFATp mRNA is expressed constitutively in many different cell types (11, 21, 42), and preexisting NFATp protein translocates to the nucleus upon stimulation of the cell (16, 17). NFATc mRNA (26) is expressed less widely, and at least in some cell types (e.g., normal human NK cells and some Jurkat T-cell clones), it is not expressed unless cells are stimulated with immune complexes or with ionomycin and phorbol ester (1, 21, 26). Except for transient transfection studies, NFATc protein has not been described. NFAT3 mRNA (11) was found at significant levels in several organs, but little or none was seen in spleen, thymus, or peripheral blood lymphocytes. NFAT4 mRNA (9, 11, 21) is elevated in thymus, but is also detectable in various other organs. In Jurkat cells, it is expressed constitutively (21). Neither NFAT3 nor NFAT4 protein has been described. As a first step toward defining specific functions for the individual NFAT proteins, we have investigated the expression and DNA-binding properties of NFAT family members in both stimulated and unstimulated T cells. Since tumor cell lines may differ in significant ways from normal nontransformed cells, we have focused our efforts on freshly isolated human peripheral blood T cells (PBTs).

MATERIALS AND METHODS

Cell and tissue culture. Freshly purified PBTs were greater than 95% $CD3^+$ cells. Human PBTs were purified according to standard procedures, including Ficoll-Hypaque centrifugation, plastic adherence, nylon wool filtration, and Percoll gradient centrifugation. The Jurkat human T-cell line (clone LEI) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Phorbol myristate acetate (PMA; Sigma) was used at 5 to 10 ng/ml for PBTs and 20 ng/ml for Jurkat cells; ionomycin (Boehringer

* Corresponding author. Mailing address: Molecular Basis of Carcinogenesis Laboratory, ABL-Basic Research Program, P.O. Box B, Frederick, MD 21702-1201.

† Present address: Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33136.

Mannheim Biochemicals) was used at 0.25 to 1 μg/ml for PBTs and 2 μl/ml for Jurkat cells; purified anti-human CD28 antibody (anti-CD28; Pharmingen) was used at 100 to 500 ng/ml and CsA (Sandoz) at 500 ng/ml. Cells were preincubated with CsA for 1 to 1.5 h before addition of PMA and ionomycin.

Antisera. Rabbit antisera were raised against the following synthetic peptides: for NFATc, 801 (NH_2-CVSPKTTDPEEGFPRGLGA [residues 210 to 227 of human NFATc]) (26), 904 (NH_2-KSAEEEHYGYASSNVC [residues 38 to 52 of human NFATc]), 905 (NH_2-PSTSFPVPSKFPLC [residues 2 to 14 of human NFATc]), and 906 (NH_2-CVALKVEPVGEDLGSPP [residues 346 to 361 of human NFATc]); for NFATp, 1777 (NH_2-CSPPSGPAYPDDVLDYGLK [residues 53 to 70 of human NFATp]) (18) and 1616 (NH_2-VPAIKT EPSDEYEPSLIC [residues 680 to 696 of murine NFATp; in human NFATp, the underlined serines are threonines, and the underlined glutamic acid is aspartic acid [18]; a sequence similar to that of peptide 1616 is also found in the form of NFATc recently described by Park et al. [27], and it is therefore possible that antiserum 1616 cross-reacts with this longer form of NFATc]); for NFAT3, 890 (NH_2-CDSKVVFIERGPDGKLQWEE [residues 614 to 632 of human NFAT3]) (11) and 889 (NH_2-GRDLSGFPAPPGEEPPA [residues 886 to 902 of human NFAT3]); for NFAT4, 1689 (NH_2-DLQINDPEREFLERPSRDHL [residues 130 to 149 of human NFAT4]) (21); for NFATx, 1690 (NH_2-CLPSPESLDLG RSDGL [residues 1061 to 1075 of human NFATx]) (21); and for all NFATs, 796 (NH_2-SDIELRKGETDIGRKNTRC [residues 542 to 559 of human NFATc]) (26). Except for 889 and 1689, a cysteine residue was added at the N or C terminus of each peptide to facilitate coupling to hemocyanin as described previously (15). Monoclonal antibody 7A6, raised against recombinant NFATc, was a gift of G. Crabtree. NF-κB antisera 1226 (anti-p65) and 1141 (anti-p50) have been described previously (32).

Plasmids. Full-length human NFATc in plasmid pBJ5 ($p1SH107_c$) was a gift of G. Crabtree. The insert was excised with *Bam*HI and cloned into pRc/CMV (Invitrogen). Human NFATp, NFAT3, and NFAT4a, each in pRep4, were gifts of T. Hoey.

Transfection. Human 293 cells were seeded at 10^6/6-cm-diameter dish. Twenty-four hours later, they were transfected with NFAT expression vectors by the calcium phosphate method.

Oligonucleotides. Oligonucleotides were synthesized by an Applied Biosystems synthesizer and used in DNA binding assays without purification. For the electrophoretic mobility shift assay (EMSA), we used oligonucleotides containing the murine IL-4 NFAT site (5′-ATAAAATTTTCCAATGTAAA-3′), the murine IL-2 distal NFAT site (5′-GCCCAAAGAGGAAAATTTGTTTCATAC A-3′), and the human IL-2 distal NFAT site (5′-TAAGGAGGAAAAACTGT TTCATACA-3′). Complementary strands were renatured and labeled with [γ-^{32}P]ATP as previously described (31).

Metabolic labeling. PBTs (5×10^6/ml) or Jurkat cells (10^6/ml) were incubated in methionine- and cysteine-free RPMI 1640 medium (Sigma) containing 2% dialyzed fetal calf serum (Life Technologies, Inc.), ionomycin, PMA, CsA, anti-CD28, or no activating agent for 1.5 to 3 h. The cells were then resuspended in a fresh aliquot of the same medium containing 5% dialyzed fetal calf serum and incubated in the presence of [^{35}S]methionine and [^{35}S]cysteine (each at 100 μCi/ml) with or without activating agent(s) at 37°C for 1.5 to 2 h (PBTs) or 45 min (Jurkat cells). At 24 h after transfection, 293 cells were labeled for 1 h in the presence of [^{35}S]methionine and [^{35}S]cysteine (each at 100 μCi/ml).

Immunoprecipitations. To reduce nonspecific background, immunoprecipitations of lysates from PBTs and Jurkat cells were performed in two cycles. Whole-cell extracts were precipitated (with or without prior boiling), and precipitates were washed, boiled, and reprecipitated. Two different lysis buffers were used. The first, referred to as ELB, consists of 50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40. PBTs (50×10^6) were lysed in 0.3 to 1 ml of ELB, and insoluble material was removed by centrifugation at $15{,}000 \times g$ for 15 min. Lysis buffer also contained phosphatase inhibitors (1 mM sodium vanadate and 0.1 μg of calyculin A [Boehringer Mannheim Biochemicals] per ml) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin [Sigma] per ml, 5 μg of leupeptin per ml, and 5 μg of pepstatin per ml) or protease inhibitor cocktail (Boehringer Mannheim Biochemicals) diluted 1:50.

A different lysis procedure was used for Jurkat cells and in some PBT experiments. Cells were lysed in ELB buffer containing protease and phosphatase inhibitors plus 1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, and 0.5% sodium deoxycholate (ELB-SDS). Lysates were boiled, passed through a 22-gauge needle to shear the DNA, and clarified by centrifugation at $15{,}000 \times g$ for 15 min.

Regardless of lysis procedure, extracts were incubated overnight with antiserum and protein A-Sepharose (Pharmacia) after dilution with TNT buffer (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1% Triton X-100). Precipitates were washed three times with TNT buffer, boiled for 5 min in TNT containing 1% SDS, and centrifuged at $15{,}000 \times g$ for 3 min. The supernatants were collected, diluted at least fivefold with TNT buffer, and reprecipitated with a fresh aliquot of the same antiserum. The second cycle precipitates were washed and analyzed by 10% Tricine SDS-polyacrylamide gel electrophoresis (PAGE) (Novex).

Transfected 293 cells were lysed in ELB containing protease and phosphatase inhibitors. After boiling in the presence of 1% SDS, lysates were diluted with TNT buffer, and aliquots (1 to 3% of the total) were immunoprecipitated and analyzed by SDS-PAGE.

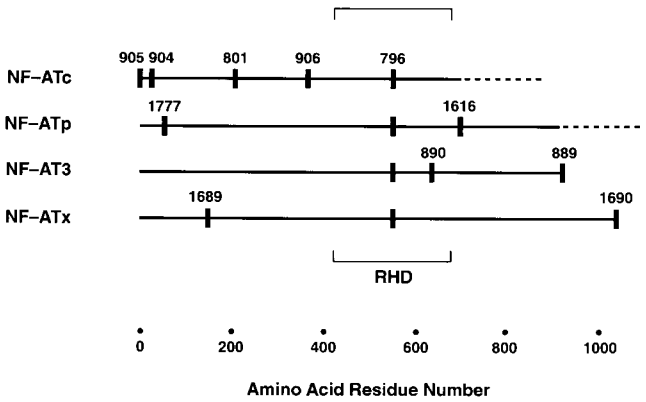

FIG. 1. NFAT antisera. The RHDs of the NFAT proteins are aligned. Boxes represent peptides against which antisera were raised. Peptide 796 is found in all four proteins. The solid line for NFATc represents one of the two splicing variants that have been described (26). The second NFATc variant is identical except for an additional 142 residues at the C terminus (denoted by the dashed line) and the replacement of residues 1 to 29 at the N terminus (27). Thus, peptide 905 is found in the first variant but not the second. Several 3′ splicing variants of NFATp have been found (18, 24). Two of them (denoted by the solid line) are very similar in size and differ only at their extreme C termini. The third is considerably longer (denoted by the dashed line). Several 3′ splice variants of NFAT4, called NFAT4a, -4b, and -4c by Hoey et al. (11) and NFATx by Masuda et al. (21), have also been described. NFAT4a and -4b have shorter C-terminal regions than NFATx. NFAT4c is identical to NFATx except at the extreme C terminus. Peptide 1689 is found in all forms, but peptide 1690 is unique to NFATx.

Preparation of cellular extracts and EMSA. For nuclear and cytoplasmic cell fractions, PBTs (25×10^6 to 125×10^6 cells) were rinsed in ice-cold hypotonic buffer (HB; 25 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$, 5 mM KCl) and lysed in 0.2 to 1 ml of HB containing 0.05% Nonidet P-40. After vortexing, the lysate was centrifuged at $500 \times g$ for 5 min; the supernatant constitutes the cytoplasmic fraction. After being washed once in 0.5 to 2.5 ml of HB, the pelleted nuclei were extracted with 30 to 150 μl of a solution containing 50 mM HEPES (pH 7.5), 300 mM NaCl, 5 mM EDTA, and 0.065% Nonidet P-40 at 4°C for 30 min with agitation. The extract was centrifuged at $15{,}000 \times g$ for 10 min at 4°C; the supernatant constitutes the nuclear extract. Protein concentrations were determined by using the Bio-Rad protein assay, with bovine serum albumin for standardization.

For EMSA, the binding reaction mixture was 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated double-stranded salmon sperm DNA, ^{32}P-labeled oligonucleotide (1 ng; $\sim 0.5 \times 10^5$ to 3×10^5 cpm), and cellular lysate (0.5 to 5 μg) in a total volume of 20 μl. The mixture was incubated at room temperature for 30 min. For supershift analysis, the reaction mixture minus ^{32}P-labeled DNA was preincubated with 1 μl of antiserum for 20 min on ice before addition of the ^{32}P-labeled DNA. Products were analyzed on 6% DNA retardation gels (Novex).

In vitro phosphatase treatment. Second-cycle ^{35}S-labeled immunoprecipitates were washed three times with TNT buffer, resuspended in dephosphorylation buffer (50 mM Tris-HCl [pH 8.5], 0.1 mM EDTA) with 20 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals), and incubated at 37°C for 60 min. After addition of gel-loading buffer, samples were boiled and analyzed by SDS-PAGE (Novex).

Human 293 cells expressing transfected NFATc were lysed in ELB buffer containing 1% SDS and 0.5% β-mercaptoethanol. Lysates were boiled, diluted fivefold, and immunoprecipitated with anti-NFATc 801. Immunoprecipitates were washed and treated with alkaline phosphatase as described above. They were then washed in 50 mM Tris-HCl (pH 7.0) and incubated with 2 μg of calcineurin (Upstate Biotechnology, Inc.) at 37°C for 45 min according to the manufacturer's protocol. After a final wash, immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis.

Immunoblotting. Immunoblots were incubated with anti-NFATc serum 801 diluted 1:1,000, and proteins were revealed with the Amersham ECL system.

RESULTS

NFAT antisera. To be able to distinguish the various NFAT family members, we raised antisera directed against synthetic peptides unique to each protein (Fig. 1). In addition, we raised an antiserum against a highly conserved peptide found in the

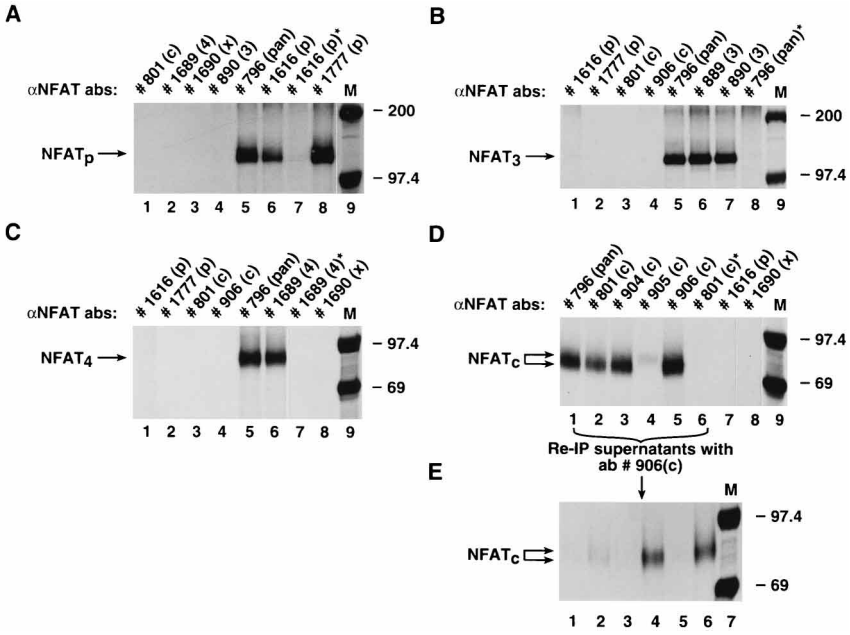

FIG. 2. Characterization of NFAT antisera. Human 293 cells were transfected with NFATp (A), NFAT3 (B), NFAT4a (C), or NFATc (D), and the cells were radioactively labeled 24 h later. (A to D) Lysates were precipitated with the indicated antisera (αNFAT abs), and precipitates were analyzed by SDS-PAGE. (E) Supernatants were collected from samples 1 to 6 shown in panel D and were reprecipitated (Re-IP) with anti-NFATc serum 906. Antiserum 796 is expected to recognize all NFAT family members and is designated 796(pan). Antisera 1616(p) and 1777(p) are directed at NFATp. Antisera 889(3) and 890(3) are directed at NFAT3. Antiserum 1689(4) is directed at all forms of NFAT4, while antiserum 1690(x) should recognize only NFATx. Antisera 801(c), 904(c), 905(c), and 906(c) are directed at NFATc. An asterisk indicates that the precipitation was carried out in the presence of cognate peptide. Sizes are indicated in kilodaltons.

RHD. This antiserum, 796, recognizes all of the known family members. Using lysates from human 293 cells transiently transfected with NFATc, NFATp, NFAT3, or NFAT4a, we tested each serum for the ability to immunoprecipitate its target protein in a specific manner (Fig. 2). As expected, antiserum 796 recognized all four NFAT family members (Fig. 2A to C, lanes 5; Fig. 2D, lane 1). This binding was specific, since it was prevented when precipitation was carried out in the presence of peptide 796 (Fig. 2B, lane 8). NFATp was precipitated by both NFATp antisera but not by sera directed at NFATc, NFAT3, or NFAT4 (Fig. 2A). Similarly, NFAT3 was recognized by both NFAT3 sera but not by antisera raised against NFATp or NFATc (Fig. 2B). NFAT4a was precipitated by serum 1689, which is expected to recognize all forms of NFAT4, but not by serum 1690, which is specific for the longest form, called NFATx (Fig. 2C, lanes 6 and 8). Neither NFATp- nor NFATc antisera precipitated NFAT4a (Fig. 2C, lanes 1 to 4). Finally, all NFATc antisera precipitated NFATc, but NFATp- and NFATx sera did not (Fig. 2D). In addition, recognition of each transfected protein was specific since it was prevented when precipitation was carried out in the presence of cognate peptide (Fig. 2A, lane 7; Fig. 2C, lane 7; Fig. 2D, lane 6). Thus, all of the sera specifically recognize their expected targets and do not cross-react with other family members.

We also tested the efficiency with which each antiserum precipitated its target. To do this, supernatants were collected following the immunoprecipitations shown in Fig. 2A to D and reprecipitated with one of the NFATp sera (for samples shown in Fig. 2A), with one of the NFAT3 sera (for samples shown in Fig. 2B), with anti-NFAT4a serum 1689 (for samples shown in Fig. 2C), and with one of the NFATc sera (for samples shown in Fig. 2D). In all cases, we found very little NFAT protein in the supernatants. An example of the results is shown in Fig. 2E. In four of the five samples, there was little or no NFATc left in the supernatants following the initial precipitation by either the pan-NFAT serum 796 or the NFATc antisera (Fig. 2E, lanes 1 to 3 and 5). The only exception was the supernatant following precipitation with anti-NFATc serum 905. In this case, substantial NFATc remained and was precipitated in the second step (lane 4). This result was expected, however, since antiserum 905 recognizes only a specific small fraction of all NFATc molecules (see below). Also as expected, NFATc was found in the supernatant when the first precipitation was carried out in the presence of competing peptide (lane 6). Thus, the antisera are not only specific but also efficient.

NFAT proteins in Jurkat cells. To test whether the antisera recognize their target proteins in T cells, we performed immunoprecipitation experiments with Jurkat cell lysates, since both NFATp and NFATc mRNAs have been detected in these cells (11, 22, 26). Jurkat cells were treated (or not) with PMA plus ionomycin (P+I) for 3 h, radioactively labeled, and precipitated with sera raised against NFATp, NFAT4/x, or NFATc. As in cells transfected with NFATp, anti-NFATp serum 1777 recognized a protein of about 135 kDa in both untreated and treated Jurkat cells (Fig. 3, lanes 4 and 10). NFAT4 antiserum 1689, which should detect all forms of NFAT4, and serum 1690, which should detect only the form called NFATx, both precipitated a protein of 155 kDa from untreated cells (lanes 5 and 6). Since the intensities of the two bands are similar, and since the size of the protein is consistent with that predicted by NFATx cDNA, we conclude that NFATx is the predominant form of NFAT4 expressed in these Jurkat cells. The intensities of the NFATp and NFATx bands were somewhat less in treated cells (lanes 10 and 11) than in untreated cells. This may be a toxic effect of the treatment and/or may be a downregulation of synthesis. NFATc antiserum 801 precipitated three proteins (140, 110, and 86 kDa) from untreated cells (lane 3),

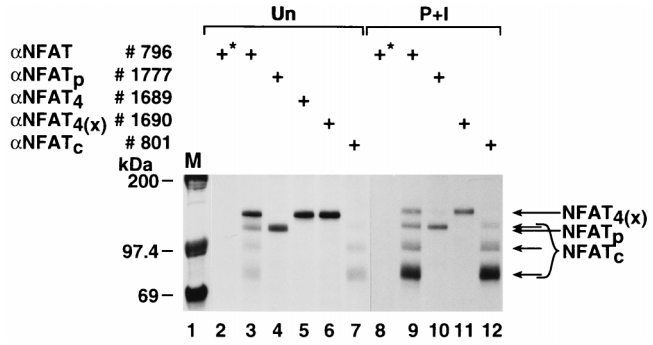

FIG. 3. Immunoprecipitation of NFAT proteins from Jurkat cells. Jurkat cells (clone LEI) were incubated in methionine- and cysteine-free medium that contained P+I or no activating agents (Un) for 2 h. They were resuspended in fresh aliquots of the same medium and incubated in the presence of [^{35}S]methionine and [^{35}S]cysteine for 45 min. Whole-cell lysates were boiled and immunoprecipitated with the indicated antiserum (αNFAT). Precipitates were washed, boiled, and reprecipitated with the same serum. These second precipitates were analyzed by SDS-PAGE. An asterisk indicates that the precipitation was carried out in the presence of cognate peptide. Mobilities of the proteins in lanes 2 to 7 cannot be compared precisely with those in lanes 8 to 12 since the two sets were analyzed on different gels.

and expression of the 110- and 86-kDa forms was greatly enhanced in the P+I-treated cells (lane 12), consistent with previous reports of the inducibility of NFATc (22, 26). The 86-kDa protein corresponds to the NFATc cDNA cloned by Northrop et al. (26) and is the same size as seen in the transfected 293 cells. The 110-kDa protein is predicted by the cDNA recently reported by Park et al. (27), and the 140-kDa protein is a new form of NFATc which has not been described previously (see below). Finally, the pan-NFAT serum 796 precipitated each of the NFATp, NFATc, and NFAT4 proteins, as expected (lanes 3 and 9), and this precipitation was prevented in the presence of cognate peptide (lanes 2 and 8). Thus, these NFAT antisera recognize their target proteins in Jurkat cells.

NFATp is expressed in untreated PBTs, and NFATc is inducible. Purified PBTs were treated with PMA or P+I for 2 h and then incubated in medium containing ^{35}S-labeled amino acids for 2 additional h in the continued presence of the activating agents. Control cells were radioactively labeled but not activated. Whole-cell extracts were immunoprecipitated and analyzed by SDS-PAGE (Fig. 4A). Like Jurkat cells, both untreated and treated PBTs contained a protein of 135 kDa which was precipitated by the pan-NFAT antiserum (Fig. 4A, lanes 2, 3, 7, and 11). Since this protein was also precipitated with anti-NFATp (lanes 5, 9, and 13) but not with anti-NFATc (lanes 4 and 12) or with anti-NFATx (lanes 6, 10, and 14), it is evidently NFATp. Both the pan-NFAT and NFATp antisera also precipitated a second protein with a molecular weight of about 100,000 from some samples (lanes 2, 3, and 5). We have not yet determined whether this protein is a splice variant of NF-ATp, a result of proteolysis of NF-ATp in the extracts, or a cross-reaction of the serum with a new form of NFATc protein recently described by Park et al. (27) (see below and Materials and Methods). In any case, the major form of NF-ATp in PBTs is the 135-kDa protein, and it is constitutively expressed. Its large size notwithstanding, this protein may correspond to a mixture of the two short alternatively spliced forms of NFATp (18), denoted by the solid line in Fig. 1 (see Discussion).

Treatment of cells with P+I, but not with PMA alone, resulted in a prominent broadly migrating band with an average molecular weight of 86,000. This protein was precipitated by the pan-NFAT serum and anti-NFATc (Fig. 4A, lanes 7 and 8) but not by anti-NFATp or anti-NFATx (lanes 9 and 10). It is therefore NFATc, whose size is consistent with the 716-amino-acid protein predicted by the sequence of Northrop et al. (26). A faint band at about the same position as NFATp was produced by anti-NFATc in cells treated with P+I (lane 8). This is not a result of cross-reaction of NFATp with the NFATc antiserum but rather represents the novel NFATc that was also seen in Jurkat cells (Fig. 2; see below). The induction of the NFATc proteins was inhibited by CsA (lanes 11 and 12). Thus, unlike the case for the Jurkat cells, there is little or no NFATc in untreated cells, but it is strongly induced by treatment with P+I.

In the experiment shown in Fig. 4A, anti-NFATx failed to detect a protein in either treated or untreated cells (lanes 6 and 10). This result differs from that seen with Jurkat cells, where NFATx was constitutively expressed at a high level. In other experiments or after long exposure of films, we do see expression of NFATx in both untreated and treated cells. For example, Fig. 4B shows a 155-kDa form of NFAT4 in ionomycin-treated PBTs. The protein was precipitated by anti-NFAT4 1689 (lane 1), and precipitation was prevented by competing peptide (lane 2). Additional experiments showed that this pro-

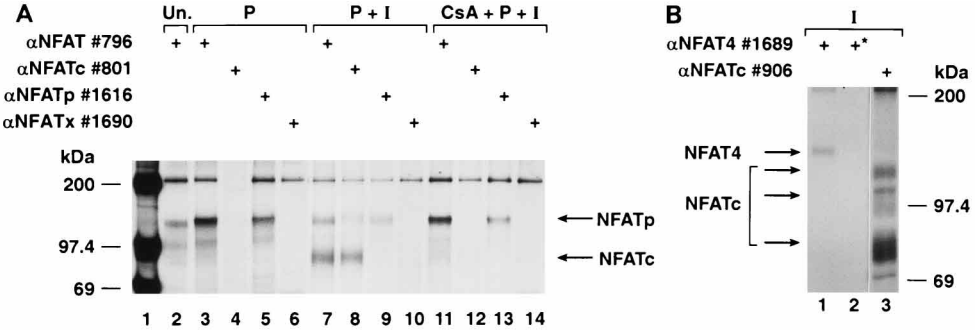

FIG. 4. Immunoprecipitation of NFAT proteins. (A) PBTs were incubated in methionine- and cysteine-free medium which contained PMA (P; 10 ng/ml), P+I (1 μg/ml), P+I plus CsA (500 ng/ml), or no activating agent (Un) for 2 h. The cells were then resuspended in a fresh aliquot of the same medium and incubated in the presence of [^{35}S]methionine and [^{35}S]cysteine for 2 h. Whole-cell extracts were prepared in ELB buffer and were immunoprecipitated as described in Materials and Methods. Precipitates were analyzed by SDS-PAGE. Anti-NFAT (αNFAT) serum 796 recognizes all NFAT proteins; anti-NFATc 801 recognizes NFATc; anti-NFATp 1616 recognizes NFATp; and anti-NFATx 1690 recognizes NFATx. (B) PBTs were incubated in methionine- and cysteine-free medium for 2 h, the last 30 min of which included ionomycin (I) at 0.5 μg/ml. The cells were then resuspended in a fresh aliquot of the same medium (including ionomycin) and incubated in the presence of ^{35}S-amino acids for 2 h. Whole-cell extracts were prepared in ELB-SDS; these were immunoprecipitated and analyzed by SDS-PAGE. Anti-NFAT4 1689 recognizes all forms of NFAT4; anti-NFATc 906 recognizes NFATc. An asterisk indicates that precipitation was carried out in the presence of cognate peptide.

tein was also precipitated by the antiserum specific for NFATx (data not shown). However, expression of NFAT4/x was always very low (compare its intensity with that of NFATc, precipitated from the same lysate [lane 3]), and its level was unaffected by activating agents. No shorter forms of NFAT4 (corresponding to the cDNAs called NFAT4a and -4b) were ever seen in PBTs or Jurkat cells.

Finally, as in Jurkat cells, anti-NFAT3 antisera 889 and 890 showed no reactivity with extracts from untreated PBTs or from cells treated with P+I (see below; also data not shown). Taken together, the results demonstrate that NFATp and NFATx are constitutively expressed in PBTs and that NFATc is induced by ionomycin (with or without PMA) in a CsA-sensitive manner. NFAT3 was not detected in PBTs.

It may be useful to comment on the level of the NFAT proteins observed in PBTs. We routinely used lysate from 20×10^6 to 80×10^6 cells for a single immunoprecipitation, and films were exposed for 1 to 3 weeks. In contrast, lysate from 3×10^6 Jurkat cells gave a strong signal in a few days. This is consistent with the difference in size of PBTs and Jurkat cells (protein content per cell is about 10 times higher in Jurkat cells) and with the resting state of the PBTs at the start of the experiments. The relatively weak signal in PBTs is not due to limiting antibody: immunoprecipitations (for both PBTs and Jurkat cells) were carried out with the same volume of antiserum that was sufficient to precipitate entirely the much greater amount of NFAT proteins from transiently transfected cells.

There are multiple forms of NFATc. While the 86-kDa protein was always the most prominent form precipitated by anti-NFATc 801, additional bands also appeared. As noted above, antiserum 801 detected additional proteins of 140 and 110 kDa in Jurkat cells (Fig. 3), and it precipitated a protein of 140 kDa from PBTs treated with P+I (Fig. 4A, lane 8). A 140-kDa protein and a broadly migrating species of about 110 kDa were also seen in ionomycin-treated PBTs by anti-NFATc 906 (Fig. 4B, lane 3). We therefore investigated whether these 140- and 110-kDa proteins might be alternate forms of NFATc. PBTs were treated with P+I, and whole-cell extracts were immunoprecipitated with the pan-NFAT serum 796, with anti-NFATc 801, or with three additional NFATc-specific antisera. Each of these sera precipitated not only the major 86-kDa form of NFATc but also two additional forms with apparent sizes of 140 and 110 kDa (Fig. 5A, lanes 1 to 5). Since these proteins were not precipitated by antisera directed at other NFAT family members (Fig. 3, lanes 10 and 11; Fig. 4A, lane 10; Fig. 5A, lane 6), they must be alternate forms of NFATc. The 110-kDa protein is presumably related to the cDNA recently reported by Park et al. (27). The 140-kDa form has not been described previously.

In addition to the higher-molecular-weight forms of NFATc, additional heterogeneity was suggested by the sequence of the cDNA that encodes the major 86-kDa protein (26). The first in-frame AUG codon lies in a context ill suited for translation initiation (cggAUGc), while the context of the second is closer to optimal (accAUGa) (14). This sequence predicts two translated products: a minor form initiated at the first AUG and a major form derived from the second. These two proteins would differ in size by the 36 amino acids upstream of the second initiation codon. To test this prediction, we used an antiserum (anti-NFATc 905) directed at the 14 N-terminal amino acids of the longer form. This serum should detect only the longer form, whereas a serum raised against any epitope downstream of the second methionine should detect both forms. In keeping with this prediction, antiserum 905 precipitated only a subset of the heterogeneous NFATc population, and that subset consisted of the largest molecules (Fig. 5A; compare lane 4 with lanes 3 and 5). This was true for all three sizes of NFATc protein. The same result was obtained in Jurkat cells (Fig. 5B; compare the 801 and 905 precipitates). Thus, some NFATc molecules contain the 905 peptide and some do not. This result is consistent with the possibility of two different initiation codons.

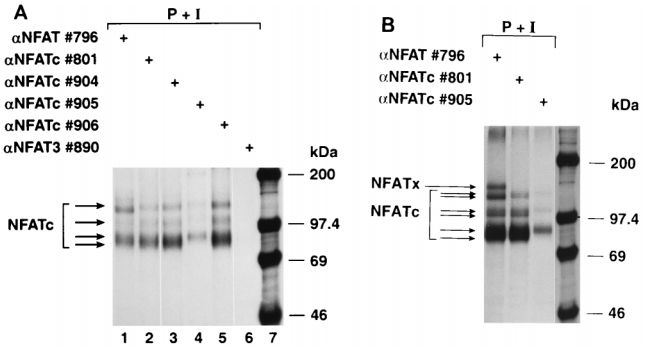

FIG. 5. There are multiple forms of NFATc. (A) PBTs were treated with PMA (10 ng/ml) and ionomycin (1 μg/ml) (P+I) for 1 h in methionine- and cysteine-free medium and then radioactively labeled for 2 h in the continued presence of P+I. Cells were lysed in ELB-SDS, and extracts were immunoprecipitated and analyzed by SDS-PAGE. Anti-NFAT (αNFAT) 796 recognizes all NFAT proteins; anti-NFATc 801, 904, 905, and 906 recognize NFATc; anti-NFAT3 890 recognizes NFAT3. (B) Jurkat cells were treated with P+I for 2 h and then radioactively labeled for 45 min in the continued presence of P+I. Whole-cell extracts were prepared in ELB-SDS, and immunoprecipitates were analyzed by SDS-PAGE. Antisera are identified above.

Although the 90-kDa protein precipitated by serum 905 was less heterogeneous in size than total NFATc, it did not migrate as a sharp band. One possible explanation is that phosphorylation of NFATc results in the diffuse migration pattern. To test whether NFATc is phosphorylated in cells treated with ionomycin alone or with P+I, NFATc immunoprecipitates (antiserum 801) were digested with alkaline phosphatase before electrophoresis. Digestion resulted in significantly increased mobility (Fig. 6A; compare lanes 2 and 4 with lanes 1

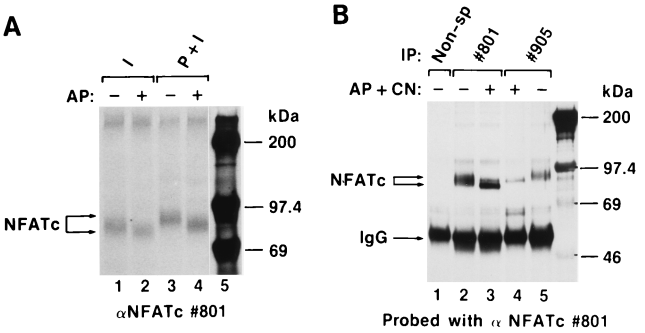

FIG. 6. Heterogeneity of NFATc. (A) PBTs were treated with ionomycin (I) (0.25 μg/ml) or PMA (0.5 ng/ml) plus ionomycin (P+I) for 1 h and then radioactively labeled in the continued presence of the activating agents for 2 h. Cells were lysed in ELB, and whole-cell extracts were immunoprecipitated with anti-NFATc (αNFATc) 801. Precipitates were treated with calf intestinal alkaline phosphatase (AP) (+) or were untreated (−) before SDS-PAGE. (B) Human 293 cells were transfected with a plasmid expressing NFATc. After 24 h, the cells were lysed in ELB-SDS, and the lysate was immunoprecipitated (IP) with anti-NFATc serum 801 or 905. Precipitates were untreated (−) or treated (+) with alkaline phosphatase (AP) and calcineurin (CN). The digested proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-NFATc serum 801. IgG, immunoglobulin heavy chain; Non-sp, nonspecific antiserum.

and 3), indicating that NFATc is a phosphoprotein. Treatment with P+I resulted in slower migration of NFATc relative to treatment with ionomycin alone (Fig. 6A, lanes 1 and 3), suggesting that the protein is more extensively phosphorylated when PMA is present. The same result has been seen with transiently transfected NFATc in Jurkat cells (26).

While phosphatase treatment increased NFATc mobility, it did not result in sharp bands. (Notice that the 140- and 110-kDa NFATc forms are practically undetectable in Fig. 6A. This is because we deliberately chose a light exposure of the film, in an attempt to resolve distinct bands within the broadly migrating 86-kDa protein.) This heterogeneity in size suggested that digestion was incomplete, even though the denatured protein was used as the substrate. We tested this possibility in assays using overexpressed NFATc derived from transient transfection of the human 293 cell line. We used the NFATc cDNA of Northrop et al. (26), which encodes 716 amino acids and on which our prediction of two initiation codons is based. As expected, expression of the NFATc plasmid resulted in a protein indistinguishable in size and heterogeneity from the major form seen in PBTs (Fig. 6B, lane 2). As with the PBTs, mobility increased but heterogeneity remained after alkaline phosphatase treatment (data not shown). However, if the protein was treated with both alkaline phosphatase and calcineurin, sharp bands resulted. Precipitation by antiserum 905, followed by digestion, yielded a single band of about 86 kDa (Fig. 6B, lane 4). Precipitation by antiserum 801, followed by digestion, yielded two bands, of about 86 and 82 kDa (Fig. 6B, lane 3). Thus, there are two forms of NFATc encoded by the cDNA in the expression vector: a minor longer form which contains the 905 peptide and a shorter major form which does not. The difference in their sizes agrees very well with the predicted 36-amino-acid difference in their lengths. These results confirm the use of two different initiation codons in translation of this NFATc cDNA. Similarly, the broad 86-kDa band seen in PBTs and Jurkat cells most likely results from a combination of primary sequence heterogeneity and phosphorylation-induced heterogeneity.

NFATc is not induced by treatment with PMA plus anti-CD28. Results presented above show that there is little or no NFATc in untreated PBTs but that its synthesis is strongly induced when cells are treated with ionomycin. We have reported previously that PBTs treated with PMA and anti-CD28 contain activated NFAT (6). To determine whether NFATc is induced by that treatment as well, we radioactively labeled treated cells and analyzed whole-cell extracts by immunoprecipitation. We found little or no induction of NFATc in cells stimulated with PMA plus anti-CD28 (Fig. 7A, lanes 2 and 3). There was a very low level of the 140-kDa NFATc detectable in untreated cells, and it increased only very slightly upon treatment. In contrast, incubation in P+I induced NFATc strongly (lane 4), as expected, and the induction was sensitive to CsA (lane 5).

As usual, NFATp was detectable in untreated cells (Fig. 7B, lane 1), and its synthesis typically increased somewhat in cells treated with PMA plus anti-CD28 (lane 2), an effect which was not prevented by CsA (lane 3). As in Jurkat cells, P+I treatment usually resulted in a lower level of NFATp synthesis (lane 4), which may be due to toxicity of the treatment or to actual downregulation of synthesis, or both. This effect was at least partially blocked by CsA (lane 5). Whatever the cause, the effect was not unique to NFATp, as synthesis of the NF-κB proteins p105 and p65 was also lower in the cells treated with P+I than in the cells treated with PMA and anti-CD28 (Fig. 7C). Comparison with NF-κB proteins is also useful in assessing the level of NFAT in PBTs. The NFAT films for Fig. 7A and B were exposed for 7 days, while a comparable signal for the NF-κB protein p105 (precipitated from the same volume of the same lysates) was obtained on overnight exposure.

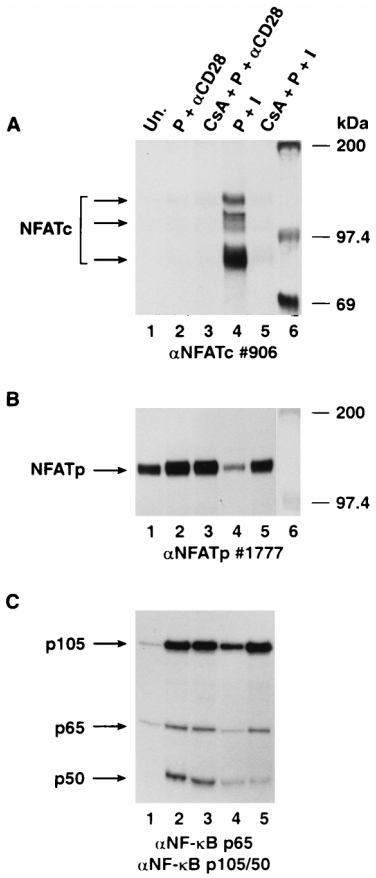

FIG. 7. Treatment of PBTs with PMA plus anti-CD28 does not induce synthesis of NFATc. PBTs were incubated in methionine- and cysteine-free medium for 3.5 h, the last 45 min of which included PMA (10 ng/ml) plus anti-CD28 (αCD28; 500 ng/ml) with or without CsA (500 ng/ml), PMA plus ionomycin (0.5 μg/ml) with or without CsA, or no activating agents. The cells were then labeled with ^{35}S-amino acids in fresh aliquots of the same medium, including inducers, for 1.5 h. The cells were lysed in ELB-SDS. Un., untreated. (A) Lysates were immunoprecipitated with anti-NFATc 906. (B) Supernatants from the precipitations shown in panel A were collected and reprecipitated with anti-NFATp 1777. (C) Supernatants from the precipitations shown in panel B were collected and reprecipitated with a combination of anti-p65 and anti-p105/p50.

To monitor the effectiveness of PMA plus anti-CD28 treatment, cells were routinely screened for IL-2 production. While incubation in PMA, ionomycin, or anti-CD28 alone resulted in no detectable IL-2, treatment with PMA plus anti-CD28 gave significant levels, as did P+I. Typical results following stimulation of the cells for 24 h were as follows: untreated cells, <1.5 U of IL-2/ml; PMA plus anti-CD28, 329 U/ml; PMA plus anti-CD28 plus CsA, 309 U/ml; P+I, 358 U/ml; and P+I plus CsA, <1.5 U/ml. Thus, as previously reported (6, 39), IL-2 synthesis is insensitive to CsA in cells treated with PMA plus anti-CD28, while its synthesis is completely CsA-sensitive in P+I-treated cells.

These results dramatize the very different effects of activating cultured cells with P+I compared with activating with PMA + anti-CD28. Both treatments induce synthesis of high levels of IL-2, but P+I activation utilizes a CsA-sensitive pathway and involves a strong induction of NFATc synthesis. In

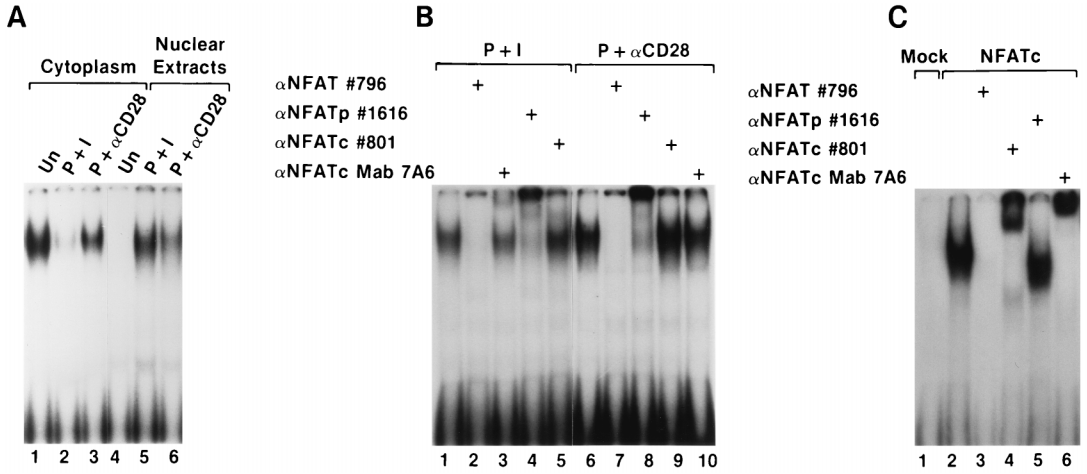

FIG. 8. Stimulation of PBTs leads to nuclear NFAT DNA-binding activity. (A) PBTs were treated with PMA (5 ng/ml) plus ionomycin (0.5 μg/ml) (P+I) or PMA plus anti-CD28 (100 μg/ml) (P + αCD28) for 4 h. Nuclear and cytoplasmic cell fractions were analyzed for the ability to bind a ^{32}P-oligonucleotide containing the NFAT-binding site from the murine IL-4 gene. Un, untreated. (B) Three micrograms of nuclear extract from cells treated with P+I and 5 μg from cells treated with PMA plus anti-CD28 were subjected to supershift analysis (same nuclear extracts as panel A). For lanes 2 to 5 and 7 to 10, NFAT-specific antibodies were added to the extracts prior to incubation with the ^{32}P-labeled murine IL-4 NFAT probe. Free probe is seen at the bottom of the gel. αNFATc MAb 7A6, monoclonal antibody 7A6 raised against NFATc. (C) 293 cells were transfected with a plasmid expressing NFATc, and whole-cell lysates were prepared 24 h later. Aliquots were analyzed by EMSA with or without NFAT-specific antibodies, using the ^{32}P-labeled murine IL-4 NFAT probe. In lane 1, the extract from mock-transfected cells was used as a negative control.

contrast, activation by PMA plus anti-CD28 is CsA insensitive and does not result in induction of NFATc.

Stimulation of PBTs leads to nuclear NFAT DNA-binding activity. To assess the effect of stimulation on NFAT DNA-binding activity, PBTs were treated with P+I or PMA plus anti-CD28, separated into nuclear and cytoplasmic fractions, and subjected to EMSA. As a probe, we used the murine IL-4 promoter NFAT-binding site, which can bind NFAT alone and does not require cobinding by AP-1. In untreated cells, all of the DNA-binding activity was located in the cytoplasm, as expected (Fig. 8A, lanes 1 and 4). After treatment of the cells with P+I for 4 h, nearly all of the DNA-binding activity was nuclear (lanes 2 and 5). In contrast, treatment of the cells with PMA plus anti-CD28 resulted in some nuclear DNA-binding activity (lane 6) but a high level of residual cytoplasmic NFAT as well (lane 3). Thus, both treatments result in NFAT translocation, but P+I appears to be more efficient, at least with the concentrations of reagents used in these assays.

The identities of the nuclear NFAT proteins were tested by supershift analysis (Fig. 8B). In agreement with the immunoprecipitation results, only NFATp was detected in nuclear extracts from cells treated with PMA plus anti-CD28. With the murine IL-4 NFAT-binding site as a probe, the binding activity (lane 5) was blocked completely by the pan-NFAT serum (lane 7) and was supershifted efficiently by anti-NFATp (lane 8). Neither anti-NFATc 801 nor monoclonal antibody 7A6 (raised against recombinant NFATc) had any effect (lanes 9 and 10).

In spite of the fact that both NFATp and NFATc were readily detectable in cells stimulated with P+I, nearly all of the DNA-binding activity was due to NFATp. This was true regardless of which NFAT site was used as probe and of which anti-NFATc serum was used for supershifting. For example, with the murine IL-4 NFAT site as probe, the pan-NFAT serum blocked binding completely (Fig. 8B, lanes 2), and anti-NFATp 1616 supershifted almost completely (lane 4). Anti-NFATc 801 and monoclonal antibody 7A6 shifted only a very small portion of the DNA-binding activity (lanes 3 and 5). With the distal NFAT-binding site from the human IL-2 promoter as a probe, the pan-NFAT serum again blocked binding completely, but four different anti-NFATc sera failed to shift or block significantly (data not shown). Thus, although NFATc was easily detectable by immunoprecipitation, and although essentially all NFATc was nuclear in these cells (data not shown), it did not appear to represent a significant fraction of the DNA-binding activity.

One possible explanation for the failure to detect DNA binding by NFATc is that the NFATc-specific antisera are ineffective supershifters. To test their abilities to supershift or inhibit an NFATc-containing DNA-binding complex, 293 cells were transiently transfected with an NFATc expression vector, and whole-cell extracts were analyzed in EMSA with the murine IL-4 probe. Overexpressed NFATc bound the DNA very well (Fig. 8C, lane 2), and, as in extracts from PBTs, the pan-NFAT serum blocked this binding completely (lane 3). As expected, anti-NFATp 1616 was unable to supershift the complex (lane 5), but both NFATc antibodies 801 and 7A6 shifted it completely (lanes 4 and 6). This was true of the three additional NFATc peptide antisera as well (data not shown). In addition, anti-NFATc 801 supershifted NFATc in extracts from Jurkat cells treated with P+I (data not shown). Thus, the NFATc antisera are capable of recognizing NFATc when it is bound to DNA, yet they detect little or no NFATc in DNA-binding complexes from P+I-treated PBTs. We conclude that nearly all of the DNA-binding activity in such cells is due to NFATp.

DISCUSSION

Given the central role played by NFAT in cytokine production, it is important to understand its regulation. This is likely to be a complicated task since four different NFAT family members have been identified, and at least three of these have alternatively spliced forms (11, 21, 24, 27). Many of these forms have been seen only at the cDNA level in libraries from established T-cell lines. To determine which NFAT proteins are present in normal cells, we have examined highly purified human PBTs in immunoprecipitation experiments.

In untreated T cells, the major form of NFAT was NFATp,

whose size was reduced from about 135 kDa to about 125 kDa by (possibly incomplete) phosphatase digestion (data not shown). Although this size is consistent with the longest of the alternatively spliced cDNAs described by McCaffrey et al. (24) and by Luo et al. (18), it is likely that it actually corresponds to the two shorter forms (denoted by the solid line in Fig. 1). In assays using antisera specific to each of the three NFATp forms, it has been shown that murine T cells express predominantly the two short forms, whose apparent sizes are about 140 kDa (42). In addition, when cDNA encoding the longest form (containing the region denoted by the dashed line in Fig. 1) was transfected into COS cells, the resulting protein migrated significantly more slowly than the predominant form in murine T cells (18). We assume that, like the mouse cells, human T cells are expressing the shorter NFATp, but we caution that we do not yet have an antiserum that can distinguish between the short and long forms. NFATp protein of approximate size 120 to 140 kDa has also been observed in human and murine T- and B-cell lines (25, 28, 34), in human NK cells and T-cell blasts induced by phytohemagglutinin (1), and in a neuronal cell line and some regions of the nervous system (8).

Unlike NFATp, little or no NFATc was detected in untreated cells. (Since our experiments involve metabolic labeling of cells, we cannot rule out the possibility that NFATc exists in resting T cells but turns over significantly more slowly than does NFATp.) However, like NFATc mRNA (1, 21, 26), we found that NFATc protein was strongly induced by treatment of the cells with ionomycin, with or without PMA. The major form of the protein produced a broad band in SDS-PAGE, with an average size of about 86 kDa. This protein has not been found previously in any cell type, but its size is consistent with that encoded by the NFATc cDNA described by Northrop et al. (26). We found that the size heterogeneity resulted from two factors: (i) phosphorylation and (ii) a difference in primary sequence. Transient transfection of NFATc cDNA resulted in two forms of the protein, both of which were phosphorylated. These two forms differed by about 4 kDa and resulted from initiation of translation at two different AUG codons. Since antiserum specific for the longer form also recognized only a subset of NFATc in PBTs, we think it likely that the two initiation codons are used there as well. Of course, we cannot exclude the possibility that in PBTs the two forms arise through alternative splicing. However, given the observed difference in their sizes and antibody reactivity, such alternatively spliced forms would have to be essentially the same as those translated from a single cDNA using two different AUG codons.

In addition to the major 86-kDa NFATc, denoted by the solid line in Fig. 1, we also detected two additional forms, of about 110 kDa and 140 kDa. The 110-kDa protein may be related to the NFATc cDNA recently described by Park et al. (27); the protein predicted by that cDNA would encompass both the solid and dashed lines in Fig. 1. However, that cDNA also differs at its extreme N terminus from the Northrop et al. cDNA, in that the Park et al. clone replaces the first 42 residues (counting from the methionine encoded by the first, weak AUG codon) with 29 different residues. Antiserum 905 would not recognize that protein. Therefore, since antiserum 905 does recognize a protein of about 110 kDa in both PBTs and Jurkat cells, there may be three NFATc proteins in this size range: one encoded by the Park et al. cDNA and two encoded by a cDNA of similar size which contains the Northrop et al. N terminus.

No cDNA corresponding to the 140-kDa NFATc has been described, but various cell types express an NFATc mRNA of 4.5 to 5.2 kb, which is large enough to encode it (11, 21, 27). Given the pattern set by the other NFAT proteins, it seems likely that the increased length of the 140-kDa NFATc results from further extension at the C terminus over that seen in the 110-kDa protein. To date, there is very little functional information about the region downstream of the RHD in the NFAT proteins. A transactivation domain has been found in this region in NFATp (19), but the 86-kDa form of NFATc, which terminates at essentially the C terminus of the RHD, can activate transcription in reporter assays (6, 11, 26). Interaction of NFATp with AP-1 appears to be governed by sequences within the RHD (12, 25), and interaction with calcineurin apparently occurs in the region upstream of the RHD (17, 20, 37, 43). It is therefore difficult to predict what functional differences exist among the various NFATc forms.

Of the other known NFAT family members, we were unable to detect NFAT3 in PBTs. This is not surprising, since NFAT3 mRNA has not been observed in spleen, thymus, or peripheral blood lymphocytes (11). We did detect one of the splice variants of NFAT4, called NFATx (21). Like NFATp, it was present in untreated cells but at a very low level that did not change upon treatment of the cells with P+I or PMA plus anti-CD28. The shorter forms of NFAT4, called NFAT4a and -4b, were not detected in PBTs or in Jurkat cells.

In summary, we detected NFATp, three forms of NFATc, and NFATx in PBTs. With the possible exception of NFAT4c (whose predicted size should be indistinguishable from that of NFATx), we believe that this is likely to be a complete list of the major NFAT proteins in these cells. All of the proteins precipitated by the pan-NFAT serum have been accounted for, using the NFATp-, NFATc-, and NFATx-specific sera. All other bands are either very faint, not reproducible, or clearly nonspecific.

Our results raise several interesting questions relevant to NFAT function. First, what are the transcription factors that regulate NFATc synthesis? We observed little or no NFATc protein in untreated cells but a strong CsA-sensitive induction of its synthesis in ionomycin-treated cells. These results suggest that preexisting NFATp, which translocates to the nucleus following treatment of cells with ionomycin (17, 36), might be important for NFATc mRNA synthesis. However, it was shown recently that NFATc can be found in T-cell lines established from NFATp-deficient mice, as judged by DNA binding and supershift assays using anti-NFATc monoclonal antibody 7A6 (44). An alternative regulatory mechanism might involve translational repression of a low level of preexisting NFATc mRNA in untreated cells and Ca^{2+}-induced derepression. NFATc protein itself might then upregulate NFATc gene transcription.

Second, is NFAT required for IL-2 synthesis by normal human PBTs? Moderate levels of IL-2 are synthesized following treatment of T cells with PMA plus anti-CD28 (references 6 and 39 and this report), and we have shown that only NFATp and trace levels of NFATx were detectable under these conditions. Yet IL-2 synthesis is normal in NFATp-negative cells treated with concanavalin A or anti-CD3 (10, 44), suggesting either that NFATp is not involved in IL-2 synthesis or that the normal role of NFATp can be filled by another NFAT family member in the deficient cells. It would be interesting to know whether NFATp-negative T cells can synthesize IL-2 in response to treatment with PMA plus anti-CD28.

Third, what triggers the nuclear translocation of NFATp in cells treated with PMA plus anti-CD28? Normally, NFAT translocation requires the Ca^{2+}-induced activation of calcineurin, which can be brought about by ligation of CD3 or by treatment with ionomycin. Since it involves calcineurin, this pathway is CsA sensitive. However, PMA–anti-CD28-induced

translocation of NFATp is CsA resistant, as is IL-2 synthesis in these cells (references 6 and 39 and this report). This finding suggests either that PMA plus anti-CD28 induces a different Ca^{2+}-independent phosphatase that can act on NFATp or that there are ways other than dephosphorylation to effect NFATp translocation.

Fourth, why were we unable to detect significant DNA binding by NFATc in extracts from P+I-treated cells? Judging from the immunoprecipitation experiments, there is as much NFATc as NFATp in those cells, yet nearly all of the DNA-binding activity was due to NFATp. This was true regardless of which of three different NFAT-binding sites was used as a probe. However, transfected NFATc was able to bind DNA, and the NFATc antisera were able to supershift the complex. Similarly, NFATc was detected in the DNA-binding complex from Jurkat cells treated with P+I. These results suggest that we would have detected NFATc in the T-cell extracts if it constituted a significant fraction of the total binding activity. Therefore, perhaps the relative intensities of the NFATp and NFATc bands in the immunoprecipitation experiments do not accurately reflect the absolute levels of the two proteins, and we have considerably overestimated the amount of NFATc present in the activated T cells. In a similar experiment, Aramburu et al. (1) found that CD16 ligand binding or P+I induced NFATc mRNA synthesis in human NK cells, but they were also unable to detect DNA binding by NFATc protein in nuclear extracts. DNA binding by NFATc was detected after CD3 ligation in murine T cells but not after treatment with ionomycin (16).

Finally, does PMA-induced phosphorylation of NFAT have functional consequences? NFATc migrated more slowly in extracts from cells treated with P+I than in extracts from ionomycin-treated cells. This was also true for NFATp (data not shown). This PMA-induced phosphorylation is not required for nuclear translocation of NFATp, which occurs in cells treated with ionomycin alone, but may possibly contribute to DNA binding, to transcriptional activity, or to interaction with other transcription factors.

ACKNOWLEDGMENTS

We are grateful to William Bere for T-cell purification, to Suzanne Specht for peptide coupling and antiserum production, to Gerald Crabtree for the NFATc expression vector and monoclonal antibody 7A6, to Timothy Hoey for human NFATp, NFAT3, and NFAT4a, and to Carol Shawver for preparation of the manuscript.

This research was sponsored in part by the National Cancer Institute, DHHS, under contract with ABL.

REFERENCES

1. **Aramburu, J., L. Azzoni, A. Rao, and B. Perussia.** 1995. Activation and expression of the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. J. Exp. Med. **182:**801–810.
2. **Choi, M. S. K., R. D. Brines, M. J. Holman, and G. G. B. Klaus.** 1994. Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. Immunity **1:**179–187.
3. **Chuvpilo, S., C. Schomberg, R. Gerwig, A. Heinfling, R. Reeves, F. Grummt, and E. Serfling.** 1993. Multiple closely-linked NFAT/octamer and HMGI(Y) binding sites are part of the interleukin-4 promoter. Nucleic Acids Res. **21:**5694–5704.
4. **Cockerill, P. N., A. G. Bert, F. Jenkins, G. R. Ryan, M. F. Shannon, and M. A. Vadas.** 1995. Human granulocyte-macrophage colony-stimulating factor enhancer function is associated with cooperative interactions between AP-1 and NFATp/c. Mol. Cell. Biol. **15:**2071–2079.
5. **Crabtree, G. R., and N. A. Clipstone.** 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. **63:**1045–1083.
6. **Ghosh, P., A. Sica, M. Cippitelli, J. Subleski, R. Lahesmaa, H. A. Young, and N. R. Rice.** 1996. Activation of nuclear factor of activated T cells in a cyclosporin A-resistant pathway. J. Biol. Chem. **271:**7700–7704.
7. **Goldfeld, A. E., P. G. McCaffrey, J. L. Strominger, and A. Rao.** 1993. Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor α gene promoter. J. Exp. Med. **178:**1365–1379.
8. **Ho, A. M., J. Jain, A. Rao, and P. G. Hogan.** 1994. Expression of the transcription factor NFATp in a neuronal cell line and in the murine nervous system. J. Biol. Chem. **269:**28181–28186.
9. **Ho, A. M., D. J. Thomas, L. A. Timmerman, X. Li, U. Francke, and G. R. Crabtree.** 1995. NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. J. Biol. Chem. **270:**19898–19907.
10. **Hodge, M. R., A. M. Ranger, F. C. de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher.** 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. Immunity **4:**397–405.
11. **Hoey, T., Y.-L. Sun, K. Williamson, and X. Xu.** 1995. Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity **2:**461–472.
12. **Jain, J., E. Burgeon, T. M. Badalian, P. G. Hogan, and A. Rao.** 1995a. A similar DNA-binding motif in NFAT family proteins and the Rel homology region. J. Biol. Chem. **270:**4138–4145.
13. **Jain, J., C. Loh, and A. Rao.** 1995b. Transcriptional regulation of the IL-2 gene. Curr. Opin. Immunol. **7:**333–342.
14. **Kozak, M.** 1987. An analysis of the 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15:**8125–8148.
15. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry. **18:**690–697.
16. **Loh, C., J. A. Carew, J. Kim, P. G. Hogan, and A. Rao.** 1996. T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NFAT1. Mol. Cell. Biol. **16:**3945–3954.
17. **Loh, C., K. T.-Y. Shaw, J. Carew, J. P. B. Viola, C. Luo, B. A. Perrino, and A. Rao.** 1996. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. J. Biol. Chem. **271:**10884–10891.
18. **Luo, C., E. Burgeon, J. A. Carew, P. G. McCaffrey, T. M. Badalian, W. S. Lane, P. G. Hogan, and A. Rao.** 1996. Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Mol. Cell. Biol. **16:**3955–3966.
19. **Luo, C., E. Burgeon, and A. Rao.** 1996. Mechanisms of transactivation by nuclear factor of activated T cells-1. J. Exp. Med. **184:**141–147.
20. **Luo, C., K. T.-Y. Shaw, A. Raghavan, J. Aramburu, F. Garcia-Cozar, B. A. Perrino, P. G. Hogan, and A. Rao.** 1996. Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. Proc. Natl. Acad. Sci. USA **93:**8907–8912.
21. **Masuda, E. S., Y. Naito, H. Tokumitsu, D. Campbell, F. Saito, C. Hannum, K.-I. Arai, and N. Arai.** 1995. NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. Mol. Cell. Biol. **15:**2697–2706.
22. **Masuda, E. S., H. Tokumitsu, A. Tsuboi, J. Shlomai, P. Hung, K.-I. Arai, and N. Arai.** 1993. The granulocyte-macrophage colony-stimulating factor promoter *cis*-acting element CLE0 mediates induction signals in T cells and is recognized by factors related to AP1 and NFAT. Mol. Cell. Biol. **13:**7399–7407.
23. **McCaffrey, P. G., A. E. Goldfeld, and A. Rao.** 1994. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-α gene transcription. J. Biol. Chem. **269:**30445–30450.
24. **McCaffrey, P. G., C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran, G. L. Verdine, A. Rao, and P. G. Hogan.** 1993. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science **262:**750–754.
25. **McCaffrey, P. G., B. A. Perrino, T. R. Soderling, and A. Rao.** 1993. NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. J. Biol. Chem. **268:**3747–3752.
26. **Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree.** 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature **369:**497–502.
27. **Park, J., A. Takeuchi, and S. Sharma.** 1996. Characterization of a new isoform of the NFAT (nuclear factor of activated T cells) gene family member NFATc. J. Biol. Chem. **271:**20914–20921.
28. **Park, J., N. R. Yaseen, P. G. Hogan, A. Rao, and S. Sharma.** 1995. Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. J. Biol. Chem. **270:**20653–20659.
29. **Prieschl, E. E., V. Gouilleux-Gruart, C. Walker, N. E. Harrer, and T. Baumruker.** 1995. A nuclear factor of activated T cell-like transcription factor in mast cells is involved in IL-5 gene regulation after IgE plus antigen stimulation. J. Immunol. **154:**6112–6119.
30. **Rao, A.** 1994. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. Immunol. Today **15:**274–281.
31. **Rice, N. R., and M. K. Ernst.** 1993. In vivo control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
32. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB

p50 has IκB-like functions. Cell **71:**243–252.
33. **Rooney, J. W., T. Hoey, and L. H. Glimcher.** 1995. Coordinate and cooperative roles for NFAT and AP-1 in the regulation of the murine IL-4 gene. Immunity **2:**473–483.
34. **Ruff, V. A., and K. L. Leach.** 1995. Direct demonstration of NFATp dephosphorylation and nuclear localization in activated HT-2 cells using a specific NFATp polyclonal antibody. J. Biol. Chem. **270:**22602–22607.
35. **Serfling, E., A. Avots, and M. Neumann.** 1995. The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. Biochim. Biophys. Acta **1263:**181–200.
36. **Shaw, K. T.-Y., A. M. Ho, A. Raghavan, J. Kim, J. Jain, J. Park, S. Sharma, A. Rao, and P. G. Hogan.** 1995. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. Proc. Natl. Acad. Sci. USA **92:**11205–11209.
37. **Shibasaki, F., E. R. Price, D. Milan, and F. McKeon.** 1996. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature **382:**370–373.
38. **Szabo, S. J., J. S. Gold, T. L. Murphy, and K. M. Murphy.** 1993. Identification of *cis*-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc. Mol. Cell. Biol. **13:**4793–4805.
39. **Thompson, C. B., T. Lindsten, J. A. Ledbetter, S. L. Kunkel, H. A. Young, S. G. Emerson, J. M. Leiden, and C. H. June.** 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc. Natl. Acad. Sci. USA **86:**1333–1337.
40. **Venkataraman, L., D. A. Francis, Z. Wang, J. Liu, T. L. Rothstein, and R. Sen.** 1994. Cyclosporin A-sensitive induction of NF-AT in murine B cells. Immunity **1:**189–196.
41. **Verweij, C. L., C. Guidos, and G. R. Crabtree.** 1990. Cell type specificity and activation requirements for NFAT-1 (nuclear factor of activated T-cells) transcriptional activity determined by a new method using transgenic mice to assay transcriptional activity of an individual nuclear factor. J. Biol. Chem. **265:**15788–15795.
42. **Wang, D. Z., P. G. McCaffrey, and A. Rao.** 1995. The cyclosporin-sensitive transcription factor NFATp is expressed in several classes of cells in the immune system. Ann. N. Y. Acad. Sci. **766:**182–195.
43. **Wesselborg, S., D. A. Fruman, J. K. Sagoo, B. E. Bierer, and S. J. Burakoff.** 1996. Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp). J. Biol. Chem. **271:**1274–1277.
44. **Xanthoudakis, S., J. P. B. Viola, K. T. Y. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, T. Curran, and A. Rao.** 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. Science **272:**892–895.
45. **Yaseen, N. R., A. L. Maizel, F. Wang, and S. Sharma.** 1993. Comparative analysis of NFAT (nuclear factor of activated T cells) complex in human T and B lymphocytes. J. Biol. Chem. **268:**14285–14293.