TRANSCRIPTION FACTORS OF THE NFAT FAMILY: Regulation and Function

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ABSTRACT

As targets for the immunosuppressive drugs cyclosporin A and FK506, transcription factors of the NFAT (nuclear factor of activated T cells) family have been the focus of much attention. NFAT proteins, which are expressed in most immune-system cells, play a pivotal role in the transcription of cytokine genes and other genes critical for the immune response. The activity of NFAT proteins is tightly regulated by the calcium/calmodulin-dependent phosphatase calcineurin, a primary target for inhibition by cyclosporin A and FK506. Calcineurin controls the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells by interacting with an N-terminal regulatory domain conserved in the NFAT family. The DNA-binding domains of NFAT proteins resemble those of Rel-family proteins, and Rel and NFAT proteins show some overlap in their ability to bind to certain regulatory elements in cytokine genes. NFAT is also notable for its ability to bind cooperatively with transcription factors of the AP-1 (Fos/Jun) family to composite NFAT:AP-1 sites, found in the regulatory regions of many genes that are inducibly transcribed by immune-system cells. This review discusses recent data on the diversity of the NFAT family of transcription factors, the regulation of NFAT proteins within cells, and the cooperation of NFAT proteins with other transcription factors to regulate the expression of inducible genes.

INTRODUCTION

Proteins belonging to the NFAT (nuclear factor of activated T cells) family of transcription factors play a central role in inducible gene transcription during the immune response (1–4). Despite their name, NFAT proteins are expressed...
Table 1  Stimuli that elicit NFAT activation

<table>
<thead>
<tr>
<th>Receptor/Function</th>
<th>Stimulus/Drug</th>
<th>Cell type tested</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Immunoreceptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cell receptor (TCR)</td>
<td>Ag/MHC, αCD3</td>
<td>T cells</td>
<td>(1–4, 52)</td>
</tr>
<tr>
<td>B cell receptor</td>
<td>αIg</td>
<td>B cells</td>
<td>(177–179)</td>
</tr>
<tr>
<td>CD40</td>
<td>CD40L plus IL-4</td>
<td>B cells</td>
<td>(178)</td>
</tr>
<tr>
<td>FcgRI</td>
<td>Ag/IgE</td>
<td>Mast cells, basophils</td>
<td>(173, 180, 181)</td>
</tr>
<tr>
<td>FcgRIIA (CD16)</td>
<td>Ag/IgG, αCD16</td>
<td>NK cells</td>
<td>(44)</td>
</tr>
<tr>
<td><strong>G protein-coupled receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m3 Muscarinic acetylcholine receptor</td>
<td>Carbachol</td>
<td>Stably transfected Jurkat T cells and PC12 pheochromocytoma cells</td>
<td>(66, 86, 182)</td>
</tr>
<tr>
<td>H1 histamine receptor</td>
<td>Histamine</td>
<td>Endothelial cells (human umbilical vein)</td>
<td>J. Carew &amp; A. Rao, unpublished</td>
</tr>
<tr>
<td>Thrombin receptor</td>
<td>Thrombin</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pharmacological agents</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>Ionomycin, A23187</td>
<td>T cells, B cells, mast cells, macrophages, NK cells, endothelial cells, PC12 cells</td>
<td>(1–4, 9, 22, 44, 50, 52, 173, 177–181) and J. Kim &amp; P. G. Hogan, unpublished</td>
</tr>
<tr>
<td>Inhibitor of endoplasmic reticulum Ca^{2+}-ATPase</td>
<td>Thapsigargin</td>
<td>T cells</td>
<td>(71)</td>
</tr>
</tbody>
</table>

not only in T cells, but also in other classes of immune-system cells. NFAT proteins are activated by stimulation of receptors coupled to calcium mobilization, such as the antigen receptors on T and B cells, the Fcg receptors on mast cells and basophils, the Fcy receptors on macrophages and NK cells, and receptors coupled to certain heterotrimeric G proteins (see Table 1). Receptor stimulation and calcium mobilization result in activation of many intracellular enzymes including the calcium- and calmodulin-dependent phosphatase calcineurin, a major upstream regulator of NFAT proteins (5, 6). Stimulated cells inducibly transcribe a large array of activation-associated genes, many of which are potential targets for NFAT; the genes encode transcription factors, signaling proteins, cytokines, cell surface receptors, and other effector proteins (7–10).

As substrates for calcineurin, NFAT proteins are major targets of the immunosuppressive drugs cyclosporin A (CsA) and FK506 that have revolutionized transplant surgery since the introduction of CsA in 1983 (11, 12). The toxicity of these drugs, which arises from their ability to inhibit calcineurin in cells outside the immune system (13, 14), has precluded their use in other clinical situations for which immunosuppressive therapies might be warranted (such as allergy, inflammation, and autoimmune disease). Compounds that target NFAT directly, without affecting the phosphatase activity of calcineurin,
may lack the toxicity of CsA and FK506 while retaining their broad immunosuppressive effects. Conceivably, more selective immunomodulatory effects might be achieved by ablating the functions of individual NFAT proteins in specific cell types or on selected inducible genes.

This review describes recent advances in our understanding of the structure, regulation, and function of NFAT proteins. Because of space limitations, we do not discuss the potential involvement of NFAT proteins in many biological phenomena, including apoptosis, thymocyte development, and immunodeficiency diseases. For an introduction to earlier work on NFAT and calcineurin, we refer the reader to several excellent reviews (1–7, 11, 15–19).

**STRUCTURE AND DISTRIBUTION OF NFAT PROTEINS**

*Isolation and Nomenclature*

NFAT was first identified in T cells as a rapidly inducible nuclear factor binding to the distal antigen receptor response element, ARRE-2, of the human IL-2 promoter (20). The induction of NFAT in T cells required calcium-activated signaling pathways and was blocked by CsA and FK506 (21). The NFAT complex that formed on this site contained a cytoplasmic component that was expressed in resting T cells but not in fibroblasts, and a nuclear component that was expressed in both T cells and fibroblasts upon stimulation with phorbol esters (22). The nuclear component was identified as the ubiquitous transcription factor AP-1, which consists of dimers of Fos- and Jun-family proteins (reviewed in 1). The cytoplasmic component (also termed preexisting, from its presence in resting cells) was purified based on its ability to bind independently, in the absence of AP-1 proteins, to the distal NFAT site of the murine IL-2 promoter (23–25) and as an NFAT:AP-1 complex to the CLE0 element of the human GM-CSF promoter (26). It appears in the nucleus as a result of calcium mobilization and calcineurin activation (22, 27) and migrates on SDS gels with an apparent molecular weight of 120–140 kDa (23, 26, 28, 29).

Over the next few years, studies from several laboratories indicated that the preexisting/cytoplasmic component of NFAT was a mixture of proteins belonging to a novel family of transcription factors [reviewed in (2–4); see Figure 1 and Table 2]. The first member of the family (NFATp, later renamed NFAT1) was purified from cytoplasmic extracts of a murine T cell clone by affinity chromatography using the distal NFAT site of the murine IL-2 promoter (23, 24) and cloned from murine (Ar-5) and human (Jurkat) T cell cDNA libraries (24, 30). A distinct protein belonging to the same family, NFATc, was purified similarly from bovine thymus, and cDNA encoding it was isolated from a Jurkat T cell cDNA library (25). cDNA clones encoding two other NFAT proteins, NFAT3 and NFAT4, were isolated from Jurkat T cell and human
Table 2  Proposed nomenclature for NFAT family proteins and chromosomal location of the genes

<table>
<thead>
<tr>
<th>Proposed</th>
<th>Nomenclature</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Current</td>
<td>Human</td>
</tr>
<tr>
<td>NFAT1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFAT1a</td>
<td>NFATp (24), NFATc2 (183), NFAT1A (30)</td>
<td>20q13.1-13.31 (183, 184)</td>
</tr>
<tr>
<td>NFAT1b</td>
<td>NFAT1b (30)</td>
<td></td>
</tr>
<tr>
<td>NFAT1c</td>
<td>NFAT1c (30)</td>
<td></td>
</tr>
<tr>
<td>NFAT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFAT2a</td>
<td>NFATc (183), NFATc2 (183), NFATc.α (34)</td>
<td>18q23-qter (183)</td>
</tr>
<tr>
<td>NFAT2b</td>
<td>NFATc.β (34)</td>
<td></td>
</tr>
<tr>
<td>NFAT3</td>
<td>NFAT3 (31)</td>
<td></td>
</tr>
<tr>
<td>NFAT4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFAT4x</td>
<td>NFATx (32), NFATc3 (33)</td>
<td>16q21-22 (32)</td>
</tr>
<tr>
<td>NFAT4a, b, c</td>
<td>NFAT4a, b, c (31)</td>
<td>8D (33)</td>
</tr>
</tbody>
</table>

*The *Nfat1* locus maps between *Ada* and *Gnas* (expressed as genetic distance in centiMorgans): centromere-*Ada*-3.1-*Nfat1, Cebpb*-4.8-*Gnas* (184).

*The human NFAT2 gene is closely linked to STS marker D18S497 (183).

Peripheral blood lymphocyte (PBL) cDNA libraries by cross-hybridization to an NFAT1 probe (31). cDNA clones encoding the NFATx isoform of NFAT4 were isolated from Jurkat T cell and murine thymus cDNA libraries (32, 33). Most recently, cDNA encoding a variant of NFATc, NFATc.β, was isolated from a Raji B cell library (34).

A unifying nomenclature for NFAT proteins is proposed in Figure 1 and Table 2, which also lists the chromosomal location of the NFAT genes. The present complicated nomenclature arises largely from the isolation, in different

**Figure 1**  Schematic alignment of NFAT proteins predicted from their cDNAs. For nomenclature, see Table 2. The region of highest homology within NFAT proteins is the DNA-binding domain (DBD), which shows similarity to the Rel homology region of Rel-family transcription factors. A second region of homology is the NFAT homology region (NHR), which binds to calcineurin (Cn) and is found only in NFAT family proteins. The N- and C-terminal transactivation domains (TADs) are indicated. The acidic/hydrophobic motifs in the N-terminal TADs are schematized as a dot. The C-terminal LDQTYLDDVNEIIRKEFS motif, where present, is indicated as a thick bar. For protein isoforms, identical shading patterns represent identical sequences. The boundary of each region is labeled above the sequences with numbering referring to position in the human protein. The lengths indicated are also for the human proteins, except for NFAT1a, which was cloned from mouse. The GenBank accession numbers for these sequences are U00279 (murine NFAT1a), U43341 (NFAT1b), U43342 (NFAT1c), U08015 (NFATc.α), U59736 (NFATc.β), L41066 (NFAT3), U14510 (NFATx), L41067 (NFAT4c), U28807 (NFATc3, the murine homolog of NFATx).
laboratories, of multiple isoforms and species variants of the same protein; and
from the fact that the names NFATp (preexisting) and NFATc (cytoplasmic),
which were originally used to describe NFAT DNA binding activity, were car-
ried over to the recombinant proteins. Because it is conceivable that some
members of the NFAT family will be neither preexisting nor cytoplasmic in
certain cell types, we suggest the simplified nomenclature used by Hoey et al
(31) (NFAT1, NFAT2, NFAT3, NFAT4), which follows that employed for other
transcription factors such as those belonging to the STAT and GATA families
(35, 36). For simplicity, the proposed nomenclature in Figure 1 and Table 2 is
used in the remainder of this review. As we discuss in a later section, DNA-
protein complexes containing NFAT proteins come in many forms, and thus
we use NFAT as a general abbreviation for NFAT-family proteins, as well as
occasionally in its original sense to refer to transcriptionally competent, NFAT-
containing complexes such as NFAT:AP-1.

Sequence Homologies, Functional Domains

The predicted primary structures of the four NFAT proteins are shown schemati-
cally in Figure 1. Multiple isoforms, most likely derived by alternative splicing,
have been described for NFAT1, NFAT2, and NFAT4. Two major regions of
sequence homology, comprising the DNA-binding domain (DBD) (37) and the
NFAT homology region (NHR) (30), are represented in all the isoforms. The
DNA-binding domain, which lies between amino acid residues ≈400 and ≈700
in the known isoforms, is highly conserved within the NFAT family and shows
moderate sequence similarity to the DNA-binding domains of Rel-family pro-
teins (37–39). The NFAT homology region of ≈300 amino acids, which is
located just N-terminal to the DNA-binding domain, shows a lesser degree of
pairwise sequence identity but strong conservation of several sequence motifs
characteristic of the NFAT family (30, 32, 33). The region of NFAT homol-
ogy defines a calcineurin-activated regulatory domain that binds calcineurin,
dephosphorylated when calcineurin is activated, and controls the calcineurin-
regulated nuclear translocation of NFAT1 (40). The properties of these two
domains are described in more detail in later sections.

The regions of NFAT proteins located outside the DNA-binding and regu-
laratory domains show relatively little sequence conservation. The ≈100 amino
acid region at the very N-terminus of each protein, which behaves as a strong
transactivation domain (TAD) in NFAT1 (41), is not conserved in sequence.
The most obvious instance of sequence conservation in the region C-terminal
to the DBD is in a region spanning the C-terminal splice site, where NFAT3
and NFAT4x contain a sequence highly homologous to the NFAT1c sequence
LDQTYLDDVNEIIRKEFS (Figure 1). A detailed discussion of the transacti-
vation domains of NFAT proteins is deferred to a later section.
Tissue Distribution, Potential Target Genes

The transcript sizes and reported sites of expression of NFAT mRNAs are listed in Table 3. Only in the case of NFAT2a and NFAT2b have distinct transcripts been correlated with protein isoforms (34). The mRNA expression data should be interpreted with some caution: There are significant differences in the expression patterns of NFAT mRNAs reported by different laboratories (Table 3) that may reflect variable contamination with circulating lymphocytes or resident mast cells. Moreover, analysis of NFAT1 indicates that protein and mRNA

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**Table 3** Tissue distributions of NFAT family members

<table>
<thead>
<tr>
<th>Member</th>
<th>mRNA size</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFAT1</td>
<td>~8 kb (h, m)</td>
<td>Thymus, spleen, heart, testis, brain&lt;sup&gt;1&lt;/sup&gt; (25) PBL, placenta, muscle, spleen, thymus, pancreas&lt;sup&gt;a&lt;/sup&gt; (31) spleen, leukocyte, placenta&lt;sup&gt;a&lt;/sup&gt; (32) B cells, mast cells, T cells&lt;sup&gt;b&lt;/sup&gt; (173, 177)</td>
</tr>
<tr>
<td></td>
<td>2.7 kb (h)</td>
<td>PBL, testis, thymus, spleen, small intestine, prostate, colon&lt;sup&gt;b&lt;/sup&gt; (34), NK cells&lt;sup&gt;b&lt;/sup&gt; (44) spleen, thymus, skin tumor&lt;sup&gt;1&lt;/sup&gt; (25) muscle, thymus, spleen, PBL, testis&lt;sup&gt;b&lt;/sup&gt; (31) muscle, thymus, leukocyte; low in others&lt;sup&gt;a&lt;/sup&gt; (32)</td>
</tr>
<tr>
<td>(NFAT&lt;sub&gt;c&lt;/sub&gt;α)</td>
<td>4.5 kb (h)</td>
<td>spleen, testis, ovary, small intestine, thymus, prostate, colon, PBL&lt;sup&gt;b&lt;/sup&gt; (34) Muscle, leukocyte, thymus, spleen, testis, ovary, colon&lt;sup&gt;b&lt;/sup&gt; (32) muscle: low in thymus, spleen, testis, PBL&lt;sup&gt;b&lt;/sup&gt; (31)</td>
</tr>
<tr>
<td>NFAT2a</td>
<td>4.5 kb (h)</td>
<td>spleen, testis, ovary, small intestine, thymus, prostate, colon, PBL&lt;sup&gt;b&lt;/sup&gt; (34) Muscle, leukocyte, thymus, spleen, testis, ovary, colon&lt;sup&gt;b&lt;/sup&gt; (32) muscle: low in thymus, spleen, testis, PBL&lt;sup&gt;b&lt;/sup&gt; (31)</td>
</tr>
<tr>
<td>NFAT2b</td>
<td>7.0 kb (h)</td>
<td>Thymus, leukocyte, low in others&lt;sup&gt;a&lt;/sup&gt; (32) Not determined</td>
</tr>
<tr>
<td>NFAT3</td>
<td>3 kb (h)</td>
<td>Placenta, lung, kidney, testis, ovary, heart, colon;</td>
</tr>
<tr>
<td></td>
<td>4.5 kb (h)</td>
<td>low in brain, spleen, thymus, PBL&lt;sup&gt;b&lt;/sup&gt; (31)</td>
</tr>
<tr>
<td>NFAT4</td>
<td>7.0 kb (h)</td>
<td>Thymus, leukocyte, low in others&lt;sup&gt;a&lt;/sup&gt; (32) Not determined</td>
</tr>
</tbody>
</table>

Abbreviations: h, human; m, mouse; w, protein detected by Western blotting with specific antibodies; e, protein detected by specific antibody supershift in EMSA; i, protein detected by immune staining; r, mRNA detected by ribonuclease protection assays; n, mRNA detected by Northern blotting; p, mRNA detected by RT-PCR.
Table 4  Involvement of calcineurin and NFAT proteins in expression of selected inducible genes

<table>
<thead>
<tr>
<th>Cell type tested</th>
<th>Effects of CsA or FK506</th>
<th>NFAT implicated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β macrophages (bacteria)</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(185)</td>
</tr>
<tr>
<td>IL-1β mast cells (FceRI)</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(186)</td>
</tr>
<tr>
<td>IL-2 T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(2–4, 112, 187)</td>
</tr>
<tr>
<td>IL-3 T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(187, 188)</td>
</tr>
<tr>
<td>IL-3 eosinophils, neutrophils</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(189)</td>
</tr>
<tr>
<td>IL-4 T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(152, 180, 190)</td>
</tr>
<tr>
<td>IL-4 mast cells</td>
<td>inhibition</td>
<td>yes and references therein</td>
<td></td>
</tr>
<tr>
<td>IL-5 T cells</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(191)</td>
</tr>
<tr>
<td>IL-5 T cells (Ag/APC)</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(188)</td>
</tr>
<tr>
<td>IL-5 T cells (ConA/αTCR)</td>
<td>no inhibition</td>
<td>n.t.</td>
<td>(192)</td>
</tr>
<tr>
<td>IL-5 T cells (PMA/dbcAMP)</td>
<td>inhibition</td>
<td>yes</td>
<td>(193, 194)</td>
</tr>
<tr>
<td>IL-5 mast cells (Ag/IgE)</td>
<td>inhibition</td>
<td>yes</td>
<td>(173, 195)</td>
</tr>
<tr>
<td>IL-6 mast cells (FceRI)</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(186, 196)</td>
</tr>
<tr>
<td>IL-8 T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(155)</td>
</tr>
<tr>
<td>IL-10 T cells (Ag/APC)</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(188)</td>
</tr>
<tr>
<td>IL-10 spleen cells</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(197)</td>
</tr>
<tr>
<td>IL-13 T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(198)</td>
</tr>
<tr>
<td>IL-13 T cells (αCD28/PMA ± αCD3)</td>
<td>stimulation</td>
<td>n.t.</td>
<td>(199)</td>
</tr>
<tr>
<td>GM-CSF T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(58, 59, 141, 187, 200–202)</td>
</tr>
<tr>
<td>endocytotic cells</td>
<td>partial inhibition</td>
<td>yes</td>
<td>(9)</td>
</tr>
<tr>
<td>NK cells (Ag/IgG)</td>
<td>inhibition</td>
<td>yes</td>
<td>(44)</td>
</tr>
<tr>
<td>eosinophils, neutrophils</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(189)</td>
</tr>
<tr>
<td>IFN-γ T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(203–206)</td>
</tr>
<tr>
<td>TGF-β T cells</td>
<td>stimulation</td>
<td>n.t.</td>
<td>(207, 208)</td>
</tr>
<tr>
<td>TNF-α T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(57, 136, 153, 187)</td>
</tr>
<tr>
<td>TNF-α B cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(57, 137)</td>
</tr>
<tr>
<td>macrophages (bacteria)</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(185)</td>
</tr>
<tr>
<td>mast cells (Ag/IgE)</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(186, 196)</td>
</tr>
<tr>
<td>NK cells (Ag/IgG)</td>
<td>inhibition</td>
<td>yes</td>
<td>(44)</td>
</tr>
<tr>
<td><strong>B. Surface Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L T cells</td>
<td>inhibition</td>
<td>yes</td>
<td>(150, 151, 209)</td>
</tr>
<tr>
<td>IL-2Rα T cells (CD25)</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(210)</td>
</tr>
<tr>
<td>CD69 T cells</td>
<td>partial inhibition</td>
<td>n.t.</td>
<td>(211)</td>
</tr>
<tr>
<td>CTLA-4 spleen cells</td>
<td>n.t.</td>
<td>altered in</td>
<td>(73, 176)</td>
</tr>
<tr>
<td>FasL spleen cells</td>
<td>n.t.</td>
<td>decreased in</td>
<td>(176)</td>
</tr>
<tr>
<td>T cell hybridoma</td>
<td>inhibition</td>
<td>n.t.</td>
<td>(212)</td>
</tr>
</tbody>
</table>

(Continued)
expression do not always correlate: Although NFAT1 mRNA has been variably detected in brain, heart, and skeletal muscle, NFAT1 protein expression has not been detected in bulk extracts of these tissues (42, 43). Consistent findings are that NFAT1 and NFAT2 mRNAs are expressed in peripheral lymphoid tissue (spleen, PBL), and that NFAT4 mRNA is expressed at high levels in the thymus, suggesting a role in T cell development. NFAT3 mRNA is expressed at low levels in lymphoid tissues (31), and thus this protein may be preferentially expressed outside the immune system. NFAT2 mRNA is upregulated in activated T cells and NK cells via a CsA-sensitive mechanism that may involve an NFAT-regulated positive feedback loop (25, 32, 44).

At the protein level, NFAT2 and the major NFAT1 isoforms NFAT1b and NFAT1c are expressed in peripheral T cells and T cell lines (Table 3), but their relative expression may vary in different cell lines or T cell subsets. NFAT1 is also expressed in other classes of immune-system cells (B cells, mast cells, NK cells, some monocytes and macrophages), consistent with NFAT activation and CsA-sensitive gene expression in these cells (Table 1). Additionally, NFAT1 is expressed in olfactory epithelium (45) and in some endothelial cell lines (9, 42).

Some potential target genes for NFAT proteins are listed in Table 4. NFAT involvement is reasonably well established for the IL-2, IL-4, GM-CSF, and
TNF-α cytokine genes in T cells. There is good but less extensive evidence for NFAT regulation of the IL-3, IL-5, IL-8, interferon-γ (IFN-γ) and CD40L genes in T cells, the TNF-α gene in B cells, and the IL-4 and IL-5 genes in mast cells. In many of the other examples listed in Table 4, inducible gene expression is inhibited by CsA and FK506, suggesting but by no means proving the participation of calcineurin and NFAT. High concentrations of these immunosuppressants are known to affect cell metabolism by interfering with the chaperone (peptidyl prolyl isomerase) functions of their immunophilin receptor (46). Further, calcineurin is known to modulate the functions of transcription factors other than NFAT (reviewed in 3). A recent example relates to the CsA-sensitive induction of Nur77 mRNA in activated T cells, which is thought to be mediated by RSRF (MEF2), a nuclear factor distinct from NFAT (47).

REGULATION OF NFAT ACTIVATION

Early Stages of NFAT Activation: Role of Calcineurin

The early activation of NFAT proteins has been investigated for NFAT1 (43, 48–52), recombinant NFAT2 (25), and recombinant NFAT4 (33, 53). Three different steps of activation have been defined: dephosphorylation, nuclear translocation, and increase in affinity for DNA (Figure 2). The consensus from these studies is that, in resting cells, NFAT proteins are phosphorylated, reside in the cytoplasm, and show low affinity for DNA. Stimuli that elicit calcium mobilization (see Table 1) result in the rapid dephosphorylation of NFAT proteins and their translocation to the nucleus; the dephosphorylated proteins show increased affinity for DNA. Each step of activation is blocked...
by CsA or FK506, implying that calcineurin is involved, and suggesting that
dephosphorylation is the initial step of activation. It should be noted that
cytokine and LPS receptors, which are not linked to Ca\(^{2+}\) mobilization, are not
expected to activate calcineurin or NFAT.

The activation of NFAT proteins follows precisely the activation of cal-
ccineurin. In T cells, if intracellular free calcium levels ([Ca\(^{2+}\)]) remain el-
evated, calcineurin activity remains high, and NFAT1 remains activated and
nuclear for many hours (43, 51, 52). Conversely if calcineurin activity is
inhibited by removal of the calcium-mobilizing stimulus, or by addition of
the calcineurin inhibitors CsA and FK506 to stimulated T cells, NFAT1 re-
verts to its original phosphorylated state within 5–15 min, returns to the cyto-
plasm, and again shows a low affinity for DNA (43, 51, 52). Similar results
have been obtained for recombinant NFAT4 expressed in fibroblasts (53). The
dephosphorylation/rephosphorylation and nuclear import/export cycle can be
repeated many times by removal and readdition of ionomycin (51, 53), in-
dicating that the activation of NFAT1 and NFAT4 is regulated by a dynamic
interplay between calcineurin and constitutively active kinases. In an analo-
gous physiological situation, nuclear NFAT1 returns slowly to the cytoplasm
in antigen-stimulated cells as a result of feedback mechanisms that cause a de-
cline in capacitative calcium entry and the eventual repletion of calcium stores
(52).

Overexpression of mutant calcineurins has pronounced effects on NFAT
function. Constitutively active calcineurin substituted partially for the cal-
cium requirement for NFAT-dependent gene expression (54–60). Conversely,
an inactive calcineurin, mutated in an essential active site histidine, inhibited
the movement of recombinant NFAT4 from the cytoplasm to the nucleus of
stimulated fibroblasts (53). Further, a calcineurin A chain fragment containing
only the B subunit-binding region suppressed NFAT-dependent reporter gene
expression in Jurkat T cells, apparently by competing with the endogenous A
chain for the regulatory B chain and thereby reducing the calcineurin activity
in stimulated cells (61). However, mice deficient in the expression of the major
calcineurin A\(\alpha\) isoform showed only a moderate impairment of the secondary
immune response, suggesting that NFAT activation could be mediated by other
calcineurin isoforms (62).

**Requirement for Capacitative Calcium Entry**

A prolonged elevation of [Ca\(^{2+}\)], levels, beyond the initial transient resulting
from the emptying of calcium stores, is required in T cells to maintain cal-
cineurin and NFAT proteins in an activated state. This plateau phase of calcium
mobilization is the outcome of capacitative calcium entry, a process that cou-
Ples the depletion of intracellular calcium stores to the influx of extracellular
calcium through specialized calcium channels (reviewed in 63–65). Capacitative calcium entry can be elicited by stimulation of each of the receptors listed in Table 1, as a result of their ability to activate phospholipase C, increase the intracellular concentration of inositol trisphosphate, and deplete intracellular calcium stores (5, 6, 66). It can also be elicited pharmacologically by treating cells with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase needed for repletion of calcium stores, or with calcium ionophores such as ionomycin, which deplete the stores directly (67, 68). Addition of SKF96365, an inhibitor of capacitative calcium entry, to stimulated T cells resulted in an immediate deactivation of NFAT1 (52) that resembled the deactivation caused by the calcineurin inhibitors CsA and FK506 (51).

The importance of capacitative calcium entry for NFAT activation is further emphasized by the finding that among mutant T cells selected for their inability to promote NFAT-dependent expression of a toxin gene, a high proportion were defective in their ability to increase \([\text{Ca}^{2+}]_i\) in response to ionomycin or thapsigargin (69, 70). Moreover, parallel measurements of \([\text{Ca}^{2+}]_i\) and NFAT-dependent reporter gene expression in individual T cells indicated that effective activation of the transcriptional functions of NFAT proteins required capacitative calcium entry (71). In cells stimulated with thapsigargin or immobilized anti-CD3, NFAT-dependent reporter gene expression was observed only in the cells that also showed a sustained increase in \([\text{Ca}^{2+}]_i\). In T cells that were less effectively stimulated, as with soluble rather than immobilized anti-CD3, or under conditions that would decrease the driving force for calcium influx, only the transient peak of calcium mobilization was apparent, and NFAT-dependent transactivation was less effective or did not occur (71).

**Dephosphorylation of NFAT Proteins by Calcineurin**

It is likely that NFAT proteins are directly dephosphorylated by calcineurin in activated cells. When tested in vitro, calcineurin dephosphorylated purified NFAT1 (23), NFAT1 in immunoprecipitates (40, 43), recombinant NFAT1 in COS cell extracts (40), and endogenous NFAT1 in T cell nuclear or cytoplasmic extracts (49). The phosphatase inhibitor okadaic acid, at a concentration (300 nM) known to inhibit protein phosphatase-1 (PP1) and PP2A completely, did not inhibit the dephosphorylation of recombinant NFAT1 by added calcineurin in COS cell extracts (40). This result argues against the involvement of a phosphatase cascade initiated by calcineurin and resulting in PP1 or PP2A activation, such as that previously described in hippocampus (72).

The evidence relating dephosphorylation to changes in NFAT function is strong but still circumstantial. Dephosphorylation is a major biochemical change detected in NFAT1, NFAT2, and NFAT4 after cell stimulation (25, 33, 43, 50–53) and clearly precedes the nuclear import of endogenous NFAT1 (50),
suggesting that dephosphorylation is required for nuclear import. Dephosphorylation may also be required for optimal DNA binding. Stimulation of T cells with ionomycin or through the T cell receptor (TCR) results in a substantial increase in the intrinsic DNA-binding affinity of NFAT1 (50–52) and NFAT2 (73) for the distal NFAT site of the murine IL-2 promoter. Treatment of cell extracts with calcineurin or alkaline phosphatase also results in increased DNA binding of NFAT1 (49), suggesting that phosphorylated residues directly or indirectly mask a region of DNA contact in NFAT1. A model incorporating these findings is discussed below (Figure 2).

**Targeting of Calcineurin to the NFAT Regulatory Domain**

There is evidence that NFAT proteins interact directly with calcineurin within the cell. NFAT1 in T cell extracts binds to calcineurin immobilized on calmodulin-sepharose beads (48, 51). Binding can be demonstrated using both the phosphorylated form of NFAT1 from resting cells and the physiologically dephosphorylated form from stimulated cells (48, 51). The ability of bacterially expressed recombinant NFAT1 to bind to calcineurin in vitro (40) shows that the interaction is not mediated by other proteins in cell lysates.

Analysis of recombinant NFAT fragments shows that the NFAT-calcineurin interaction involves the N-terminal ≈400 residues of NFAT1, NFAT2, and NFAT4 (40, 53), and thus the interaction maps to the conserved NHR (30). The conserved residues in this region are clustered in several short sequence motifs (30), which include the three SP motifs (consensus SPxxSPxxSPxxxx[D/E][D/E]) noted earlier (32, 33). In NFAT1 and NFAT4, the N-terminal region containing the conserved residues functions as a calcineurin-activated regulatory domain: It contains phosphorylated serine residues that are dephosphorylated by calcineurin, and it regulates the subcellular localization of covalently linked protein domains in a calcium- and calcineurin-dependent way (40, 53). The regulatory domain also contains a candidate nuclear localization sequence (NLS) whose mutation prevents the nuclear import of full-length NFAT1 (40). When overexpressed, a fragment of NFAT2 containing this regulatory domain has a “dominant negative” effect, in that it downregulates NFAT-dependent transactivation mediated by endogenous or recombinant NFAT proteins (25, 74).

The NFAT-calcineurin interaction appears to be another example of targeting interactions involving protein phosphatases (40). For instance, the catalytic subunit of PP1, which shows little substrate specificity in vitro, is targeted to substrates in glycogen, in myofibrils, and in sarcoplasmic reticulum by distinct targeting subunits (75). Calcineurin itself is directed to the inositol trisphosphate and ryanodine receptors by an interaction with FKBP12 (76), and to the RII subunit of protein kinase A and relevant subsynaptic substrates by its interaction with the A-kinase anchor protein AKAP-79 (77, 78).
Models and Speculations

A model for NFAT activation is depicted in Figure 2. NFAT proteins are targeted via their conserved regulatory domains to inactive calcineurin in resting cells; depending on the affinity, these interactions may be either transient or stable. Increases in \([\text{Ca}^{2+}]\), lead to the activation of calmodulin (79); binding of calmodulin to a region near the C-terminus of calcineurin displaces the autoinhibitory domain of calcineurin and exposes the calcineurin active site (80). Activated calcineurin dephosphorylates associated NFAT proteins at multiple sites in their regulatory domains. The NLS in the regulatory domain, and potentially a DNA recognition region in the DNA-binding domain, become accessible in the dephosphorylated proteins. One possibility (not shown) is that the residues immediately adjacent to the NLS or to the region of DNA contact are directly modified with phosphates and thus masked. Alternatively, dephosphorylation may expose these regions by producing an intrinsic conformational change in NFAT proteins (Figure 2). The model also depicts a calcineurin-dependent interaction of the regulatory domain with a larger protein complex (possibly calcineurin itself) in the cytoplasm. This is inferred from the fact that although they are small enough to diffuse through nuclear pores, N-terminal fragments of NFAT1 and NFAT4 are retained in the cytoplasm of resting cells and move to the nucleus only upon activation (40, 53). Dephosphorylated NFAT proteins are still capable of binding calcineurin and may continue to bind it in activated cells.

The rapid reversal of NFAT activation by CsA and FK506 implies a continuing interplay between NFAT kinases and calcineurin (51, 53). Both sets of enzymes can act on NFAT proteins in the nucleus: Activated calcineurin maintains nuclear NFAT proteins in their activated state, while the kinases rephosphorylate the proteins when calcineurin is inhibited with CsA or FK506. An interesting possibility is that the enzymes move to the nucleus upon activation, as demonstrated for calcineurin overexpressed with recombinant NFAT4 in fibroblasts (53).

OTHER SIGNALING PATHWAYS THAT INFLUENCE NFAT ACTIVATION

Many signaling pathways and proteins, other than calcineurin, modulate NFAT function in T cells (see Figure 3). Since most of the experiments involve measuring reporter gene expression driven by a composite NFAT:AP-1 site, their interpretation is necessarily complex, and it is not always apparent whether calcineurin, NFAT proteins, or AP-1 proteins are affected. For the sake of brevity, we have limited our discussion to the effects of pathways normally
Figure 3  A simplified view of some signaling pathways reported to influence NFAT-dependent transcription (see text). Molecules discussed in the text are boxed. Unidentified signaling molecules and pathways are indicated by question marks. Key: CaMK, multifunctional Ca$^{2+}$/calmodulin-dependent protein kinases; CAML, calcium-signal modulating cyclophilin ligand; Cn, calcineurin; ΔCnA/B, constitutively active calcineurin lacking the autoinhibitory domain; JNK, c-Jun N-terminal kinase; DN, dominant negative; SRF, serum response factor.
activated in antigen-stimulated cells, concentrating on cases in which NFAT function has been specifically addressed, and where there is an indication of the mechanisms involved. Experiments involving transgenic or “knockout” mice are not described except where a mechanistic interpretation is possible.

**TCR-Proximal Steps: Lck, ZAP-70, Vav**

The earliest consequence of TCR cross-linking is the activation of tyrosine kinases including Lck and ZAP-70, and the tyrosine phosphorylation of multiple intracellular substrates including Vav (81, 82). Stimulation of T cells with a combination of a calcium ionophore and PMA bypasses these early steps but mimics the effects of TCR cross-linking on gene transcription (6). Both Lck and ZAP-70 are required for calcium mobilization in T cells (82), and overexpression of wild-type or constitutively active Lck upregulated NFAT-dependent reporter gene expression in Lck JCam.1 cells (83). Likewise, overexpression of kinase-deficient ZAP-70 inhibited NFAT activity in Jurkat T cells stimulated with anti-TCR antibodies but did not affect NFAT activity in PMA/ionomycin-stimulated cells, suggesting that an early TCR-proximal step was involved (84).

ZAP-70 interacts with Vav, an SH2 domain–containing protein that is rapidly phosphorylated on tyrosine residues upon T cell activation (85). In turn, Vav interacts through its SH2 domain with SLP-76, a tyrosine-phosphorylated protein that also binds the adapter protein Grb2 (85a). Overexpression of Vav and/or SLP-76 augmented basal and TCR-stimulated NFAT-dependent reporter gene expression in Jurkat cells (85a, 86). The effect of Vav was specific for T cell receptor stimulation, since NFAT activation through the muscarinic receptor was not augmented by Vav. Likewise, there was no augmentation in cells lacking Lck, CD45, or the TCR β chain, and Vav overexpression did not result in increases in [Ca^{2+}], or in the levels of tyrosine-phosphorylated proteins. Vav-induced augmentation was blocked by the calcineurin inhibitors CsA and FK506 and by overexpression of dominant negative forms of Ras and Raf, suggesting that Vav functioned upstream of these molecules or in a parallel pathway.

**PMA-Activated Signaling Pathways**

Single-cell measurements indicate that PMA enhances the calcium sensitivity of NFAT-dependent reporter gene expression, as assessed by the number of cells expressing the reporter gene at different [Ca^{2+}] levels (71). The magnitude of this effect is striking: In the presence of PMA, an average threefold increase in the sustained plateau level of [Ca^{2+}] results in reporter gene expression in half of the responsive cells (71). The most likely effect of PMA is to induce the de novo synthesis and posttranslational modification of AP-1 and other transcription factors (6), thereby increasing the transcriptional effectiveness of nuclear NFAT proteins. PMA-activated kinases may also directly phosphorylate
NFAT2 (25), although there are no data on whether this influences NFAT2 function. In contrast, PMA stimulation does not, under the conditions so far tested, alter the mobility of NFAT1 on SDS gels, its subcellular distribution, or the function of its major transactivation domain (41, 52).

Mechanistically, the major known effect of PMA in T cells is to activate protein kinase C, Ras, and Raf; the effects of activated and dominant negative versions of these proteins on NFAT-dependent transactivation in T cells have been previously reviewed (6). A major conclusion is that Ras is indispensable for NFAT function: Activated Ras substitutes effectively for PMA, and together with constitutively active calcineurin it provides a full stimulus for NFAT-dependent reporter gene expression, whereas dominant negative Ras inhibits NFAT activation in response to TCR stimulation (6, 87, 88). Multiple Ras effectors including Raf and the Ras-related GTPases Rac and Rho have been described (88–94). The Raf pathway is clearly important for AP-1 induction, via activation of the Raf substrate MEK1, a dual specificity kinase, and its substrates the MAP kinases ERK-1 and ERK-2 (reviewed in 95). In T cells, however, constitutively active Raf or MEK1 was not as effective as activated Ras in substituting for PMA and synergizing with ionomycin to stimulate NFAT-driven transcription. Consistent with these findings, dominant negative versions of these proteins only partially inhibited Ras-mediated activation (88, 96). A constitutively active form of another Ras effector, Rac-1, activated AP-1-driven reporter gene expression maximally by a pathway not involving ERK but did not activate an NFAT-driven reporter gene efficiently, even in combination with ionomycin and constitutively active MEK1 (88). Based on these results, it has been proposed that yet another Ras effector pathway exists in T cells and is needed for maximal expression of a reporter gene driven by NFAT:AP-1 (88; see Figure 3). It will be interesting to determine whether this effector pathway targets NFAT proteins, AP-1 proteins, or both.

Pathways Related to Calcium Mobilization

In addition to activating calcineurin, increases in $\left[\text{Ca}^{2+}\right]$ lead to the activation of other calmodulin-dependent enzymes (79). Among these, two multifunctional $\text{Ca}^{2+}$/calmodulin-dependent protein kinases (CaMK) may regulate NFAT activity. A constitutively active form of a T cell CaMKII, CaMKIIγB, partially inhibited both NFAT- and AP-1-driven reporter gene expression in Jurkat T cells and blocked the ability of constitutively active calcineurin to augment IL-2 promoter function (97, 98). The opposite effect was observed for constitutively active CaMKIV/Gr, which enhanced NFAT activity in large part by activating AP-1 (99). The enhancement by CaMKIV/Gr, which was not blocked by dominant negative Ras, may be mediated through upregulation of the transcriptional function of serum response factor (SRF) and the consequent induction of c-Fos.

Curiously, both CaMKs are activated by CaM and Ca\(^{2+}\) during T cell activation, exhibit broad substrate specificity, and phosphorylate the same minimal consensus substrate sequence (101), suggesting that the opposite effects of the two CaMKs may involve their targeting to different intracellular substrates.

Overexpression of a cyclophilin-binding protein, CAML, significantly increased the basal level of intracellular calcium in transfected T cells and partially substituted for the calcium requirement for NFAT-dependent transcription (102). This effect required extracellular calcium and was inhibited by CsA or FK506, suggesting that CAML functioned by facilitating calcium entry into cells (102).

**Costimulatory Pathways**

The T cell surface molecule CD28 delivers a costimulatory signal necessary for maximal activation through the TCR (103). CD28 stimulation results in a striking augmentation of IL-2, TNF-\(\alpha\), GM-CSF, and IFN-\(\gamma\) production (104), which has its basis in a large increase in steady state mRNA levels (105). A significant component of the effect is posttranscriptional (106), possibly arising from mRNA stabilization (105). However, CD28 stimulation also results in three- to eightfold increases in reporter gene expression driven by the IL-2, IL-3, GM-CSF, and IFN-\(\gamma\) promoters (107, 108). The CD28 response elements (CD28REs) in these genes have been mapped (107, 108) and shown to bind inducible factors variously identified as Rel proteins (109–111), NFAT proteins (112, 113), and \(\approx 35\)-kDa protein(s) visualized by UV cross-linking to a labelled oligonucleotide probe but not yet characterized molecularly (108, 114). It is interesting that in both the IL-2 and GM-CSF promoters, the sequences that confer responsiveness to HTLV-1 Tax map to the CD28REs (113, 115).

The nature of the signaling pathways involved in CD28 signaling has been studied in many laboratories (116). Stimulation of T cells with anti-CD28 antibodies elicits a spectrum of responses, in part resembling those elicited by activation of the TCR, but differing from those resulting from stimulation with the physiological ligand of CD28, B7.1 (117). The consensus is that the CD28 pathway is not CsA sensitive (104, 118).

In considering whether NFAT proteins play a role in CD28-mediated costimulatory pathways, two observations are relevant. First, at least some NFAT proteins may be activated via CD28-dependent, calcineurin-independent pathways, as judged by the CsA-insensitive induction of low levels of NFAT-dependent reporter gene expression and nuclear NFAT DNA-binding activity in T cells stimulated with anti-CD28 and PMA (74). Second, NFAT proteins can bind to the CD28REs of both the IL-2 and GM-CSF promoters, which bind Rel-family proteins (108–111, 115). Thus a plausible mechanism linking CD28 stimulation to cytokine gene transcription would be activation of NFAT proteins through
a distinct pathway that may synergize with the calcium/calcineurin pathway, followed by binding of the activated proteins to CD28REs and conventional NFAT sites in the promoter and enhancer regions of NFAT-inducible genes. The potential interplay between NFAT and Rel proteins at the CD28REs and other κB-like sites is discussed in a later section.

Other Signaling Pathways

A variety of biological and pharmacological agents including protein kinase A (PKA) activators (119–123), glucocorticoids (124–126), transforming growth factor-β (TGF-β) (127), retinoic acid (128), and vitamin D3 (129) are known to inhibit IL-2 production by activated T cells. Of these, PKA activators and vitamin D3 have been reported to affect NFAT function.

PKA In a mouse T cell hybridoma stimulated with thapsigargin, dibutryl cAMP inhibited NFAT:AP-1-dependent reporter gene expression, and the inhibition was substantially reversed by PMA, suggesting either that AP-1 proteins were limiting in the treated cells or that a PKA-mediated inhibition of NFAT function could be compensated for by an increase in AP-1 (71). Whether PKA activators inhibit AP-1 function, NFAT function, or both remains unclear. In several studies in which EL-4 mouse thymoma cells were used, PKA activators had no effect on NFAT DNA-binding activity (119, 121, 122); this finding could reflect the fact that NFAT is constitutively nuclear in EL-4 cells (130), in which the calcineurin A chain bears a spontaneous activating mutation in its autoinhibitory domain (60). In primary human T cells, however, prostaglandin E2 (PGE2) significantly inhibited the induction of both NFAT:AP-1 and AP-1 DNA-binding activity (123); whereas in primary CD4+ murine T cells, cholera toxin, one of whose several effects is to elevate cAMP (131), inhibited the induction of NFAT but not AP-1 DNA-binding activity (120). The inhibitory effects of PKA may not involve AP-1, however. An established target for PKA is the transcription factor CREB, which becomes phosphorylated on serine-133; based on the findings that CREB is phosphorylated at the same site, presumably by other kinases, upon stimulation through the TCR (132, 133), and that thymocytes from transgenic mice expressing a dominant negative version of CREB showed significantly decreased induction of several Fos- and Jun-family mRNAs (132), it has been suggested that CREB is involved in upregulating AP-1 activity in stimulated T cells. Clearly, further experiments are necessary to elucidate the molecular effects (if any) of PKA on the phosphorylation status and function of NFAT proteins in cells. If the evidence favors a consistent effect of PKA on NFAT activity, a further point that will need to be considered is that PKA activators upregulate IL-4 and IL-5 production while inhibiting IL-2 production (120, 121).
NFAT FAMILY TRANSCRIPTION FACTORS

VITAMIN D3 The natural immunosuppressant vitamin D3 downregulates IL-2 production by activated T cells. The inhibitory effects of vitamin D3 are mediated through the vitamin D3 receptor, which binds as a heterodimer with its partner the retinoid X receptor to sequences overlapping the distal NFAT site of the human IL-2 promoter, thereby preventing the binding of the NFAT:AP-1 complex to the same site (129).

GLUCOCORTICOIDS NFAT and NFAT:AP-1 complexes do not appear to be direct targets for inhibition by glucocorticoids, because neither the induction of NFAT DNA-binding activity nor the level of NFAT:AP-1-dependent reporter gene expression was affected by dexamethasone treatment of Jurkat T cells (124–126).

RETINOIC ACID, TGF-β The inhibitory effects of retinoic acid and TGF-β have been mapped to the octamer element of the IL-2 promoter (127, 128), which is now known to be a composite NFAT:AP-1:Oct site (112). However, neither compound influenced NFAT:AP-1-dependent reporter gene expression, suggesting that neither directly affected the function of NFAT.

DNA BINDING AND TRANSACTIVATION

DNA Binding

The DNA-binding domains of NFAT proteins are likely to be similar in three-dimensional structure to the DNA-binding domains of Rel-family proteins, and to utilize corresponding loops to make some of their contacts with DNA (37–39). The domains (≈300 amino acids) are highly conserved within each family (60–70% pairwise identity for NFAT proteins; 41–61% for mammalian Rel proteins) and show a low but significant level of sequence identity (≈17%) in pairwise comparisons between individual members of the NFAT family and the Rel family (31). The minimal DNA-binding domain (≈190 amino acids) of NFAT1 (37) corresponds almost exactly to the N-terminal specificity domain of NF-κB p50, which makes the majority of base-specific contacts with DNA (134, 135). This domain contains the highly conserved recognition loop (RFRYxCEG) of Rel-family proteins, represented in the NFAT family as the equally highly conserved RAHYFETEG sequence in which each of the underlined residues is likely to contact DNA (37, 39).

A striking difference between NFAT and Rel proteins is that whereas Rel proteins are dimeric in solution and bind DNA as obligate dimers, NFAT proteins are monomeric in solution and when bound to DNA (31). NFAT proteins can also bind to certain κB-like sites (see below): in this case, an NFAT monomer binds to the 5’ half-site, and a second NFAT monomer binds to the symmetrical
Table 5  Site selectivities of NFAT proteins

<table>
<thead>
<tr>
<th>NFAT</th>
<th>IL-2 (distal NFAT)</th>
<th>IL-4 (P1)α</th>
<th>TNF-α (κ3)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFAT1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>(31, 33, 34)</td>
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<td>++</td>
<td>+++</td>
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<td>(31–34)</td>
</tr>
<tr>
<td>NFAT3</td>
<td>+</td>
<td>+++</td>
<td>n.t.</td>
<td>(31)</td>
</tr>
<tr>
<td>NFAT4</td>
<td>±</td>
<td>+++</td>
<td>−</td>
<td>(31–33)</td>
</tr>
</tbody>
</table>

αResults from Hoy et al (31) using bacterially-expressed recombinant DNA-binding domains. Using full-length recombinant proteins expressed in COS cells, Ho et al (33) found that NFAT2 bound least well to the IL-4 P1 site (binding decreases in the order NFAT1 > NFAT4 > NFAT2).

Although all four NFAT proteins are capable of binding cooperatively with AP-1 proteins to the distal IL-2 promoter NFAT site (31, 32, 34), they show characteristic site preferences when tested for DNA binding in the absence of AP-1 (Table 5). Of the four NFAT proteins, NFAT1 displays the highest affinity for each of the three sites shown in Table 5, binding in the order IL-4 P1 > distal IL-2 > TNF-α κ3. In contrast, NFAT4 binds poorly or not at all to the distal IL-2 site (31–33), and neither NFAT2 nor NFAT4 bind under standard conditions to the TNF-α promoter κ3 site (33, 34). As a consequence, NFAT2b fails to activate the TNF-α promoter when transiently expressed in T or B cell lines, while NFAT1 is highly active in this respect (34). Curiously, overall TNF-α production is not impaired in NFAT1-deficient mice (73), possibly because the TNF-α promoter contains additional NFAT sites capable of binding the other NFAT proteins (137), or because the other NFAT proteins do function at the κ3 site in the genomic context.

**Transactivation Domains**

Analysis of NFAT1 suggests that both the N- and C-terminal regions of NFAT proteins contain transcriptional activation domains (30). The N-terminal TAD of NFAT1 has been localized to the N-terminal ≈100 residues of NFAT1, which like the corresponding regions of NFAT2b, NFAT3, and NFAT4 is rich in acidic

3′ half-site, without stabilization of the binding by protein-protein interactions between the monomers (136). This property has led to the classification of NFAT proteins as “monomeric Rel proteins” (39). The monomeric nature of NFAT proteins explains why the minimal DNA-binding fragment of NFAT1, which lacks the region corresponding to the C-terminal dimerization domain of Rel proteins, can bind independently to DNA (37). Moreover, it provides a rationale for why the N-terminal two thirds of the Rel similarity region of NFAT proteins is more highly conserved in sequence than the C-terminal one third (31).
residues and proline (30–32, 34). In this region, NFAT1 contains at least one acidic/hydrophobic patch, 23 QDELDFSILFDYEYL 29, that resembles those implicated in transactivation by acidic activation domains (138, 139). Similar acidic/hydrophobic patches are present in NFAT2b ( 4 LEDQEFDFEFLFEF 17 ), NFAT3 ( 8 DEELEFKLVFGE 20 ), and NFAT4 ( 9 HDELDFKLVFGE 21 ) (31, 32, 34). Despite the very limited sequence conservation, therefore, the N-terminal regions of all these proteins could conceivably function as acidic transactivation domains. The corresponding region of NFAT2a, which lacks the acidic/hydrophobic patches, is also capable of transactivation (C Luo, unpublished), perhaps through interaction with a distinct set of nuclear proteins.

When expressed as a GAL4 fusion protein, the region of NFAT1c located C-terminal to the DNA-binding domain is also capable of transactivation (41). Again, this region shows only limited sequence similarity to the corresponding regions of NFAT2b, NFAT3, or NFAT4, except for the LDQTYLDDVNEIRKEFS sequence mentioned previously, which spans a C-terminal splice site and is represented in NFAT3 and NFAT4x (see Figure 1). Although more experiments are needed to establish the significance of this conserved segment, it is interesting that the NFAT1c isoform, which possesses this sequence, is reproducibly more active in transactivation assays than the NFAT1b isoform, which differs in this portion of the C-terminal sequence (41).

Cooperation with AP-1
NFAT proteins show a characteristic ability to cooperate with AP-1 in DNA binding and transactivation (reviewed in 1). The minimal DNA-binding domain of NFAT1 suffices for cooperative binding with AP-1 dimers to the distal NFAT site of the murine IL-2 promoter (37). The interaction between NFAT proteins and AP-1 involves binding of these unrelated transcription factors to adjacent sites on DNA and results in an approximately 20-fold increase in the stability of the NFAT1:AP-1:DNA complex compared with the DNA-protein complex containing NFAT1 alone (23, 27, 140). In DNA-protein complexes containing NFAT1, Fos, and Jun, the Fos-Jun dimer shows a remarkable orientational preference that is not apparent in the absence of NFAT1, in that the AP-1 half-site proximal to the bound NFAT1 monomer is occupied exclusively by Jun (140). The paradigm of NFAT-AP-1 cooperation has been recognized in a number of different cytokine promoter/enhancer regions (reviewed in 1) (Table 6). Note that cooperative binding implies not merely that two transcription factors can occupy adjacent sites on DNA, but that the DNA-protein complex containing both transcription factors is significantly more stable or of higher affinity than those containing the individual proteins (27, 140, 141).

When expressed without its transactivation and regulatory domains in T cells, the DNA-binding domain of NFAT1 is found in the nucleus and can mediate
Table 6  Selected NFAT binding sites in gene regulatory regions

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Species</th>
<th>Site</th>
<th>Sequences</th>
<th>References</th>
</tr>
</thead>
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<td>IL-2</td>
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<td>AGGAAAAT</td>
<td>TGGTTC</td>
</tr>
<tr>
<td></td>
<td>h</td>
<td>−280</td>
<td>AGGAAAAAC</td>
<td>TGGTTC</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>−135</td>
<td>AGGAAAAC</td>
<td>AAAGGTA</td>
</tr>
<tr>
<td></td>
<td>h</td>
<td>−135</td>
<td>AGGAAAAT</td>
<td>GAAGGTA</td>
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<td>TG1GTA</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>−45</td>
<td>TGGAAAAT</td>
<td>ATATTG</td>
</tr>
<tr>
<td>IL-4</td>
<td>m</td>
<td>P1</td>
<td>TGGAAAAT</td>
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<td>P</td>
<td>TGGAAAAT</td>
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<td>m</td>
<td>P</td>
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<td>TGAGTT</td>
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<tr>
<td></td>
<td>h</td>
<td>P</td>
<td>TGGAAAAT</td>
<td>TGAGTT</td>
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<td>CGGAGCCCC</td>
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<td>g</td>
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<tr>
<td></td>
<td>h</td>
<td>GM550</td>
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<td>CD40L</td>
<td>m, h</td>
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<td>GTACTCA</td>
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Consensus

<table>
<thead>
<tr>
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<tr>
<td>GAAAAATN</td>
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Group II

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>NFAT</th>
<th>AP-1</th>
<th>References</th>
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<tbody>
<tr>
<td>hTNF-α</td>
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</tr>
<tr>
<td>hIL-8</td>
<td>−81/−65</td>
<td>GGAAGAACCC</td>
<td>ATGAGCTCA</td>
<td>(ATF-2/Jun) (136, 154)</td>
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<tr>
<td>E-selectin</td>
<td>PDI</td>
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<td></td>
<td>(9, 157)</td>
</tr>
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<td>HIV-1 LTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hGM-CSF</td>
<td>CK-1 (CD28RE)</td>
<td>GGAGCTTCC</td>
<td>AGGGAGCTGC</td>
<td>(Sp1) (201)</td>
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<td>mIL-2</td>
<td>CD28RE/AP-1</td>
<td>AAGAGATTC</td>
<td>ACAGTTC</td>
<td>(AP-1) (112, 113)</td>
</tr>
</tbody>
</table>

For each element, a continuous sequence is divided by inspection into adjacent NFAT and AP-1 sites in Group I, and a κB-like site and the adjacent sequence in Group II. Introduced gaps are indicated by dashes. Nucleotides known or presumed to interact with NFAT proteins are shown in boldface type. Known and probable binding sites for AP-1/CREB/ATF proteins are underlined, with nucleotides fitting the consensus shown in boldface type. The Group I NFAT consensus is based on the sequences shown. The human IL-4 P element and the IL-13 element could equally well be classified in Group II. m, mouse; h, human; r, sequence shown is for the noncoding strand.
strong NFAT-dependent transactivation in cells stimulated with ionomycin and PMA (41). Under these circumstances, mutation of either the NFAT site or the adjacent AP-1 site abrogated reporter gene expression, indicating that transcription required recruitment of AP-1 proteins by the NFAT DNA-binding domain. Recruitment of unrelated transcription factors by strong cooperative interactions involving DNA-binding domains could be a general theme in transcriptional regulation: The isolated DNA-binding domains of GATA proteins and MyoD1 induce cell differentiation (142, 143), and protein-protein interactions have been detected between the DNA-binding domains of AP-1 and Ets proteins (144), AP-1 and NF-κB (145), C/EBP and NF-κB (146), and c-Jun and the glucocorticoid receptor (147).

NFAT BINDING SITES IN GENE REGULATORY REGIONS

Table 6 presents a compilation of known and potential NFAT binding sites in the regulatory regions of selected inducible genes. The largest and best-documented class consists of sites on which NFAT proteins form a cooperative complex with AP-1 or other bZIP proteins (Group I). NFAT proteins also bind to sites that are, or resemble, binding sites for conventional Rel-family proteins (Group II). Sites that bind NFAT proteins with relatively high affinity in the apparent absence of an adjacent AP-1 site have also been described (112).

NFAT:AP-1 Sites

This subject has been extensively reviewed (1, 141), and only salient points are emphasized here. Composite elements that support the cooperative binding of NFAT and AP-1 proteins have been noted in the promoter regions of the IL-2, IL-4, IL-5, and CD40L genes, and in the GM-CSF enhancer (Table 6, Group I). Based on a comparison of these sequences, the NFAT binding site is presented as a 9-bp element positioned next to a 7–8-bp element capable of binding dimers of AP-1, CREB/ATF, or other bZIP-family proteins. The spacing of the NFAT and AP-1 elements appears to be narrowly specified, with no more than a single base pair insertion or deletion permitted between the two sites. Sites in which the GGAAA core sequence is preceded by a T rather than an A appear to bind NFAT proteins more strongly. The identified AP-1/CRE sites range from strong elements, as in the GM330 AP-1 and GM420 CRE sites, to barely recognizable variants of the consensus sequence, as in the IL-4 and CD40L sites. The relative strengths of the NFAT and AP-1 elements can vary reciprocally over a wide range: The −90 IL-2 site does not bind NFAT proteins independently and binds AP-1 proteins only moderately well, but supports a strong cooperative interaction between these two proteins. Conversely the CD40L element pairs a strong NFAT site with a relatively weak AP-1 site. Note that the CTLA-4
sites in Table 6 were identified by inspection, within the region shown to be functional by promoter deletions. Also note that NFAT:AP-1 cooperation at the IFN-γ, IL-13, and CTLA-4 sites has not yet been established.

The possibility that NFAT binding to Group I-like sites in vivo always involves AP-1 or bZIP proteins deserves serious consideration. In the absence of NFAT, AP-1 proteins do not bind detectably to the distal NFAT site of the murine IL-2 promoter (23, 140), a moderately high-affinity NFAT-binding site that is the prototype for a cooperative NFAT:AP-1 site (23). It is possible, therefore, that even strong NFAT sites with no obvious adjacent AP-1 sites, such as the −45 site of the IL-2 promoter (Table 6), do in fact support the formation of cooperative NFAT:AP-1 complexes in the context of larger transcriptional complexes assembled on genomic promoters and enhancers. Conversely, reporter gene expression driven by multiple AP-1 sites has been reported to be selectively sensitive to CsA and FK506 in T cells (148, 149); this may reflect, in part, the ability of AP-1 proteins to recruit NFAT proteins to adjacent sites.

An important technical point when assessing protein binding to these sites by electrophoretic mobility shift assay is that the use of insufficiently long oligonucleotides may lead to the classification of an NFAT site as independent of AP-1 (see 150 and 151 for the CD40L site), or an AP-1 site as independent of NFAT. Carrying this reasoning further, if either half of a composite NFAT:AP-1 site is of low affinity, the weak binding of the relevant protein to that site may be entirely missed if the adjacent site or the partner protein that binds to it is not represented in the assay.

The relative order of the NFAT and AP-1 sites is fixed as shown in Table 6: reversal of the order, such that the NFAT site lies 3′ to the AP-1 site on the DNA strand represented, permits independent but not cooperative binding (J Jain, A Rao, unpublished). By this analysis, the interaction of the bZIP protein c-Maf with NFAT1 on the IL-4 promoter (152) is unlikely to involve the same type of protein-protein interactions as in prototype NFAT:AP-1 complexes (140), since it occurs on the sequence AGTTGCTGAACCAAGGGAAAAATGAGTT (non-coding strand), with the order of NFAT (bold and underlined) and c-Maf (underlined) sites reversed with respect to the canonical NFAT:AP-1 site.

**κB-Like Sites**

NFAT proteins also bind several sequences that resemble binding sites for Rel family proteins (Group II). The κ3 element in the TNF-α promoter, which behaves as a strong CsA-sensitive element in stimulated T cells (153), is the best-documented example of a functional site in this group (136). As mentioned above, NFAT1 appears to bind with much higher affinity than NFAT2 and NFAT4 to this site (33, 34). Detailed analysis indicates that the GGA sequence (bold in Table 6) is a nucleating site that is preferentially occupied at low NFAT1
concentration; mutation of this sequence eliminates binding to the site (136). At higher concentrations, a second molecule of NFAT1 binds to the other half-site, contacting the GGG sequence on the opposite strand, but the monomers bound to the two half-sites make few if any cooperative interactions with each other. An adjacent site that binds ATF-2/Jun dimers is required for maximal function of the \( \kappa 3 \) element in reporter assays, although there are no stabilizing protein-protein interactions between NFAT1 and ATF-2/Jun bound to the adjacent sites (154).

The IL-8 site, which was originally identified by inspection as a \( \kappa B \)-like site (155), may also fit the criteria for classification in Group II. The distance between the presumed CRE and the GGAA sequence identified by mutation as functional (155) matches the spacing in the TNF-\( \alpha \) \( \kappa 3 \) element more closely than the spacing in any of the Group I sites. The IL-8 site does not bind any of the known Rel proteins in electrophoretic mobility shift assays using nuclear extracts from stimulated Jurkat T cells, but the complex formed reacts with anti-NFAT1 antibodies (S Okamoto, K Matsushima, personal communication). Moreover, production of IL-8 and induction of the IL-8 promoter are both strongly inhibited by FK506 in Jurkat T cells (155), in which NF-\( \kappa B \) induction is not sensitive to CsA or FK506 (21), implying that this site behaves in vivo as a functional NFAT site.

The \( \kappa B \) sites in the E-selectin promoter and the HIV-1 LTR are other examples of \( \kappa B \)-like sites that bind NFAT1 in vitro (Table 6). Where known, the nucleotides (TTCC) that are complementary to those contacted by bound NFAT1 are shown in bold, and comprise the nucleating site. Both sites are well established as Rel binding sites (156, 157), but it is not known whether NFAT binding at these sites has a functional effect.

**CD28 Response Elements**

The CD28REs of the IL-2 and GM-CSF promoters bind Rel-family proteins as well as NFAT proteins (109–113, 115), and we have therefore classified them as \( \kappa B \)-like elements in Group II. The CD28RE of the GM-CSF promoter is the same as its CK-1 site (115). Like the IL-8 site, however, these elements have characteristics of both Group I and Group II sites, although the Rel-like characteristics may predominate.

The binding of NFAT proteins to the IL-2 promoter CD28RE has been suggested to involve their cooperation with AP-1 proteins bound to a strong adjacent AP-1 site (112). Based on our earlier discussion of composite NFAT:AP-1 sites, this would only be possible if the NFAT protein bound to the weak NFAT site, AAGAAA, at the 5’ end of the element; however, the spacing between the NFAT and AP-1 sites is not optimal for cooperation (Table 6). The functional sequence in the CD28RE is thought to be the GGAA sequence on the noncoding
strand (TTCC in Table 6), which could serve as the nucleating sequence for binding of NFAT proteins to a κB-like site (see discussion above): Mutation of this site diminishes or eliminates IL-2 promoter function in response to anti-CD3 (112, 158). Indeed, NFAT proteins appear to be capable of binding to the CD28RE independently of AP-1, as observed in nuclear extracts from Jurkat T cells stably expressing HTLV-1 Tax (113). Given the available evidence, it is plausible that NFAT proteins could bind in either mode, to one or both ends of the CD28REs. It remains to be determined, however, whether either mode of NFAT binding is functional in terms of CD28 costimulation.

Interactions Among NFAT and Rel Proteins on κB-Like Sites

An interesting question is the degree to which NFAT and Rel proteins contribute to transactivation at κB-like sites or CD28REs. Mixed dimers of NFAT and NF-κB proteins have not been observed, and so any given κB-like site is expected to bind either NFAT or NF-κB, and not a combination of both proteins. The relative contributions of NFAT and Rel proteins to transactivation from the site will depend in part on the concentrations of NFAT, AP-1, and Rel proteins in the nucleus at any given time of stimulation, and in part on relative affinities of these proteins for the site in question. Since NFAT proteins are activated primarily by calcium-mobilizing stimuli, whereas Rel proteins are activated by many types of stimuli (159, 160), the cell type and mode of stimulation employed will be crucial. Bound NFAT and Rel proteins may have similar or antagonistic effects on promoter function.

An interesting example of an interaction between NFAT and NF-κB is provided by their competition on the P sequence of the human IL-4 promoter (TGGAAATTTTC, Table 6), which binds both NFAT and NF-κB (161). The corresponding P1 sequence in the mouse IL-4 promoter (TGGAAATTTTT, Table 6) differs by two nucleotides from the human sequence, and hence binds NFAT with fourfold higher affinity and NF-κB with fourfold lower affinity than does the human sequence. In consequence, the activity of the human IL-4 promoter was repressed by PMA stimulation and by coexpression of RelA, while that of the mouse IL-4 promoter was not. Moreover, substitution of the mouse sequence for the human P element eliminated the sensitivity of the human IL-4 promoter to PMA stimulation and to Rel A (161), consistent with the proposed competition between NFAT and NF-κB for binding to the P site.

ROLE OF NFAT PROTEINS IN GENE TRANSCRIPTION

Multiple NFAT Binding Sites in Gene Regulatory Regions

A notable feature of NFAT-dependent promoters and enhancers is the presence of multiple NFAT binding sites. This theme, first recognized in the IL-2 (162,
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163) and IL-4 (164, 165) promoters, has since been extended to the GM-CSF enhancer (141), two more sites in the IL-2 promoter (112), the TNF-α promoter (137), and a newly identified enhancer located 14-kb upstream of the IL-3 gene (P Cockerill, personal communication). Each of these regulatory regions contains three to five sites for NFAT binding, within a total length of 200 to 300 base pairs. The implication is that higher-order, synergistic interactions among NFAT-containing complexes are required for effective transcription. A similar hypothesis was proposed based on studies of reporter expression, using constructs driven by multiple NFAT:AP-1 sites (71, 166). T cells stably expressing such constructs showed reporter expression in only a proportion of stimulated cells, and changes in the strength or effectiveness of the stimulus changed the number of expressing cells rather than the level of reporter expression, suggesting that NFAT-dependent transcription was initiated in the stimulated cells only when they attained a threshold level of nuclear NFAT proteins that permitted tandem occupancy of multiple sites. In vivo footprinting of the IL-2 promoter suggested that these long-range interactions were not limited to NFAT, but involved the synergistic assembly of all the relevant transcription factors into a coordinate promoter complex (167). Indeed, transcriptional activation in many systems requires the ordered assembly of a cooperative transcription complex, containing transcription factors, coactivators, and the core transcriptional machinery (168).

Cell Specificity of Cytokine Gene Expression

Each cell type in the immune system produces a characteristic pattern of cytokines during an immune response (169). For instance, IL-2 is produced by T cells, IL-4 by T cells and mast cells, IFN-γ by T cells and NK cells, and TNF-α by virtually every cell type. Other inducible genes such as CD40L, CTLA-4, and FasL are also selectively expressed. The cytokine profiles of T cells differentiating down the Th1 and Th2 pathways have been well documented; Th1 cells preferentially produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (169–171). In many pathological situations, the balance between Th1 and Th2 patterns of cytokine production determines the clinical outcome of the immune response (103, 170).

The proto-oncogene c-Maf, a member of the bZIP family, is held to be responsible for the selective expression of IL-4 in Th2 cells (152). Within the T cell lineage, c-Maf is expressed in Th2 but not in Th1 cells. Coexpression of c-Maf and NFAT1 in Th1 cells and B cells results in strong synergistic activation of the IL-4 promoter, reflecting their interaction with the P0 NFAT site and an adjacent c-Maf binding site. As discussed above, the orientation and spacing of the NFAT and Maf-binding sites within this region suggest that the NFAT:Maf interaction differs substantially from the conventional NFAT:AP-1
interaction, and protein-protein interactions between NFAT and c-Maf remain to be demonstrated. If the interactions are in fact cooperative, it is likely that the protein-protein contacts involved will differ from those observed on composite NFAT:AP-1 sites.

What determines the cell-specific expression of inducible genes? As noted above, the sequences of the NFAT sites and their arrangement relative to sites for other transcription factors show considerable variability. For instance, the interaction of NFAT and Oct proteins differs on the IL-2 and IL-4 promoters, being cooperative in the former case and competitive in the latter (172); both NF-κB and NFAT:AP-1 are required for the function of the human GM-CSF promoter (59); and GATA as well as NFAT proteins have been implicated in IL-5 gene induction (173–175). Selective gene expression is likely to be determined by the particular NFAT proteins and cooperating transcription factors expressed in individual cell types, or by cell-specific nuclear coactivators (or corepressors) that mediate interactions between these transcription factors and the basal transcription complex.

Target Genes for NFAT Proteins: Lessons from NFAT1-Deficient Mice

The specialized functions of NFAT1 in the immune response have been explored by targeted disruption of the NFAT1 gene (73, 176). In both cases the targeted exon was in the DNA-binding domain, and its disruption resulted either in the expression of a deleted version of the protein without DNA-binding activity (176), or in no protein expression (null phenotype) (73). Except for a moderate degree of splenomegaly, the NFAT1-deficient mice developed normally and were immunocompetent, as they showed no impairment in IL-2, IL-4, TNF-α, and IFN-γ production by stimulated spleen cells. Surprisingly, however, certain primary and secondary immune responses were markedly enhanced, with a tendency toward the development of a late Th2-type response in at least three experimental situations: increased intrapleural accumulation of eosinophils and increased serum IgE levels in an in vivo model of allergic inflammation (73); increased serum IgE levels in response to immunization with TNP-ovalbumin (176); and more efficient differentiation toward a Th2 phenotype in spleen cells stimulated in vitro with IL-4 and anti-CD3 (176).

Given the evidence that NFAT proteins are essential transcription factors for the expression of cytokine genes, the overall immunocompetence of NFAT1-deficient mice suggests that many genes that are targets for NFAT1 may be redundantly controlled by another NFAT protein. However, the unusual phenotype of enhanced immune responsiveness is consistent with the possibility that some genes are unique or preferred targets for NFAT1. In normal mice, activation of NFAT1 may elevate the expression of inhibitory cytokines or cell-surface
receptors in certain cells, or decrease the expression of activating effector proteins, thus dampening the overall magnitude of the immune response and perhaps limiting the late expression of Th2-type cytokines. By analogy, protein kinase C (PKC), which is activated early in stimulated T cells, has both positive effects and feedback inhibitory effects on the subsequent response: It modifies and activates the positive regulators Ras and Raf but also decreases [Ca$^{2+}$] by inhibiting phospholipase C-$\gamma$ and activating plasma membrane calcium pumps (131). Alternative explanations for the phenotype of NFAT1-deficient mice are that changes in early cytokine production (176) alter the balance of cytokines produced at later times, thus skewing the overall pattern of the response; or that unless counterbalanced by the presence of NFAT1, the other NFAT proteins will favor transcription of the cytokine genes characteristic of Th2 cells.

Given the fact that NFAT proteins or their mRNAs have been detected outside the immune system, some target genes for NFAT1 and other NFAT proteins are likely to be expressed in nonlymphoid cells. For instance, mRNAs encoding all four NFAT proteins are expressed in testis and/or ovary and in skeletal or cardiac muscle, suggesting a possible involvement in reproduction and in muscle development or function. New members of the NFAT family may also be discovered, with presently unsuspected roles.

**FUTURE DIRECTIONS**

The molecular characterization of NFAT proteins has greatly advanced our understanding of inducible gene transcription in the immune system. However, the basis for the cell-specific expression of genes induced via the receptor/calcium/calcineurin pathway remains to be understood. This will involve defining the functions of individual NFAT proteins, both biochemically and by targeted gene disruption; exploring the mechanisms by which calcineurin, kinases, and other signaling molecules regulate the phosphorylation status, subcellular localization and transactivation functions of NFAT proteins; and determining whether NFAT-mediated transcription involves cooperation with cell-specific transcription factors or coactivator proteins. Other questions are whether NFAT proteins play a role in other biological and immune processes, such as lymphocyte development and programmed cell death; or in disease processes such as immunodeficiency or autoimmune diseases or oncogenesis.

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