Oncogene (2001) 20, 2476–2489

www.nature.com/onc

Partners in transcription: NFAT and AP-1

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Combinatorial regulation is a powerful mechanism that enables tight control of gene expression, via integration of multiple signaling pathways that induce different transcription factors required for enhanceosome assembly. The four calcium-regulated transcription factors of the NFAT family act synergistically with AP-1 (Fos/Jun) proteins on composite DNA elements which contain adjacent NFAT and AP-1 binding sites, where they form highly stable ternary complexes to regulate the expression of diverse inducible genes. Concomitant induction of NFAT and AP-1 requires concerted activation of two different signaling pathways: calcium/calcineurin, which promotes NFAT dephosphorylation, nuclear translocation and activation; and protein kinase C (PKC)/Ras, which promotes the synthesis, phosphorylation and activation of members of the Fos and Jun families of transcription factors. A fifth member of the NFAT family, NFAT5, controls the cellular response to osmotic stress, by a mechanism that requires dimer formation and is independent of calcineurin or of interaction with AP-1. Pharmacological interference with the NFAT:AP-1 interaction may be useful in selective manipulation of the immune response. Balanced activation of NFAT and AP-1 is known to be required for productive immune responses, but the role of NFAT:AP-1 interactions in other cell types and biological processes remains to be understood. *Oncogene* (2001) **20,** 2476–2489.

Keywords: nuclear factor of activated T cells; Fos; Jun; transcriptional regulation

Introduction

Regulation of gene expression in eukaryotes is achieved by coordinate assembly of multiple transcription factors and DNA-binding proteins onto specific DNA regulatory regions that control gene transcription. The resulting multiprotein-DNA complexes have a precise sterospecific architecture and have been termed enhanceosomes. The presence of diverse transcription factors within enhanceosomes ensures a strict temporal and tissue-specific control of gene expression, since each of the factors needs to be present within the relevant cell type and also correctly activated to permit enhanceosome assembly and consequent gene transcription. The resulting requirement for integration of multiple signaling pathways, leading to optimal activation of each of the transcription factors within the enhanceosome complex, explains how ubiquitous signaling intermediates promote the initiation of very specific biological responses in response to stimulation of different receptors within the same cell, or the same receptors expressed on different cell types.

Assembly of synergistic transcription factors on promoters or enhancers can occur as a simple independent binding of different proteins to adjacent binding sites, or involve a further degree of control in what have been termed composite DNA elements. Binding on these composite elements involves not only functional synergy but the existence of cooperative physical interactions between two or more different transcription factors. The result is a DNA-bound complex containing several proteins, that overall is much more stable than the complexes formed by any of its individual components bound independently to the composite DNA site.

Among the pairs of unrelated transcription factors that have been found to bind cooperatively to composite DNA sites, one of the best well-known examples is the ternary NFAT:AP-1 complex that contains proteins of the nuclear factor of activated T cells (NFAT) and AP-1 families. Composite NFAT:AP-1 sites have been described in numerous genes involved in the productive immune response. The activation of NFAT proteins is regulated by calcium and calcineurin, whereas that of AP-1 proteins is regulated by other pathways including protein kinase C (PKC) and Ras. Cooperation between these two unrelated families of transcription factors constitutes an important mechanism by which these two distinct signaling pathways are integrated to produce a biological response. Depending on whether or not both transcription factors are concomitantly activated, distinct sets of genes may be activated, eliciting different patterns of cellular response.

The NFAT family

Five different members of the NFAT family of transcription factors have been identified so far (Table 1). The classical members of this family comprise NFAT1 (also known as NFATp or NFATc2) (McCaffrey *et al*., 1993), NFAT2 (NFATc or NFATc1) (Northrop *et al*., 1994), NFAT3 (NFATc4) Hoey *et*

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Table 1 The NFAT family

NFAT protein	Other names	Domain organization (TAD Regulatory DNA binding C-Terminal)
NFAT1	NFATp, NFATc2	
NFAT2	NFATc, NFATc1	
NFAT3	NFATc4	
NFAT4	NFATx, NFATc3	
NFAT5	NFATz, NFATL1, TonEBP	

NFAT protein(s)	Phenotype of knockout mice
NFAT1	Moderately enhanced B and T cell responses with a bias towards increased production of IL-4 and other Th2 cytokines.
NFAT2	Embryonic lethal (defect in cardiac valve development). In RAG blastocyst complementation system B and T cells show reduced proliferative response and moderately impaired Th2 response with decreased IL-4 production.
NFAT3	Not reported yet
NFAT4	Mildly impaired development of CD4 and CD8 single positive thymocytes due to increased apoptosis of double positive thymocytes. Mild hyperactivation of peripheral T cells.
NFAT5	Not reported yet.
NFAT1+NFAT2	In fetal liver chimeras, T cells show impaired effector functions with severely reduced production of Th1 and Th2 cytokines. Paradoxically B, cells are hyperreactive.
NFAT1+NFAT4	Lymphoproliferative disorder due to TCR hyperreactivity and defects in T cell apoptosis. Increased Th2 responses with allergic blefaritis and interstitial pneumonitis.

al., 1995) and NFAT4 (NFATx or NFATc3) (Hoey *et al*., 1995; Masuda *et al*., 1995). Recently a new NFAT member with special characteristics was cloned in two independent laboratories, who named it NFAT5 and TonEBP respectively (Lopez-Rodriguez *et al*., 1999b; Miyakawa *et al*., 1999). All five NFAT proteins share a Rel-like homology region and recognize similar DNA binding sites in the regulatory regions of numerous genes (Rao *et al*., 1997). However, NFAT5 possesses several specific features that differentiate it from the classical NFAT1–4 members, amongst them its inability to form cooperative complexes with AP-1 proteins on composite DNA elements (Lopez-Rodriguez *et al*., 1999b). Therefore, NFAT5 will be discussed separately at the end of this review, and the term NFAT will be used, in many but not all cases, to refer only to the four conventional NFAT proteins.

Each of the conventional NFAT proteins contains a moderately-conserved regulatory domain in its N-terminal region, that controls NFAT cellular distribution and transcriptional activation (Figure 1) (Aramburu *et al*., 1998; Beals *et al*., 1997b; Crabtree, 1999; Kiani *et al*., 2000; Luo *et al*., 1996a; Zhu *et al*., 1998). This domain is highly phosphorylated on multiple serine residues in resting cells; upon cell activation it is dephosphorylated by the calcium/calmodulin-dependent phosphatase calcineurin, the major upstream regulator of NFAT (Aramburu *et al*., 1998; Beals *et al*., 1997a; Jain *et al*., 1993a; Liu *et al*., 1999; Luo *et al*., 1996a; Shibasaki *et al*., 1996). Immediately adjacent to the regulatory domain lies the highly-conserved NFAT DNA binding domain, which is distantly related in its primary sequence but shows a strong structural similarity to the DNA binding domains (Rel homology region) of the Rel/NF-κB family of transcription factors (Chen *et al*., 1998; Jain *et al*., 1995a; Nolan, 1994; Zhou *et al*., 1998). Indeed, as discussed in detail below, NFAT DNA-binding domains cannot only bind cooperatively to DNA with AP-1, but also can form Rel/NF-κB-like dimers on certain types of NFAT-binding DNA elements (Kinoshita *et al*., 1997; Macian and Rao, 1999; McCaffrey *et al*., 1994). In pairwise comparisons of the different NFAT and NF-κB family members, the DNA-binding domain shows 60–70% sequence identity among the classical NFAT proteins, ~40% sequence identity between these proteins and NFAT5, and only 15–17% sequence identity between individual members of the NFAT and NF-κB/Rel families. Transcriptional activation domains have been mapped to both the amino- and carboxy- terminal regions of different NFAT proteins (Avots *et al*., 1999 ; Luo *et al*., 1996a).

NFAT1, 2 and 4 are expressed mainly in cells of the immune system where they play a key role in regulating a large number of inducible genes during the immune response. The known NFAT target genes in the immune system are described in a later section. Mice that are deficient for both NFAT1 and NFAT4 show a striking allergic phenotype, suggesting a role for these two proteins in suppressing production of the

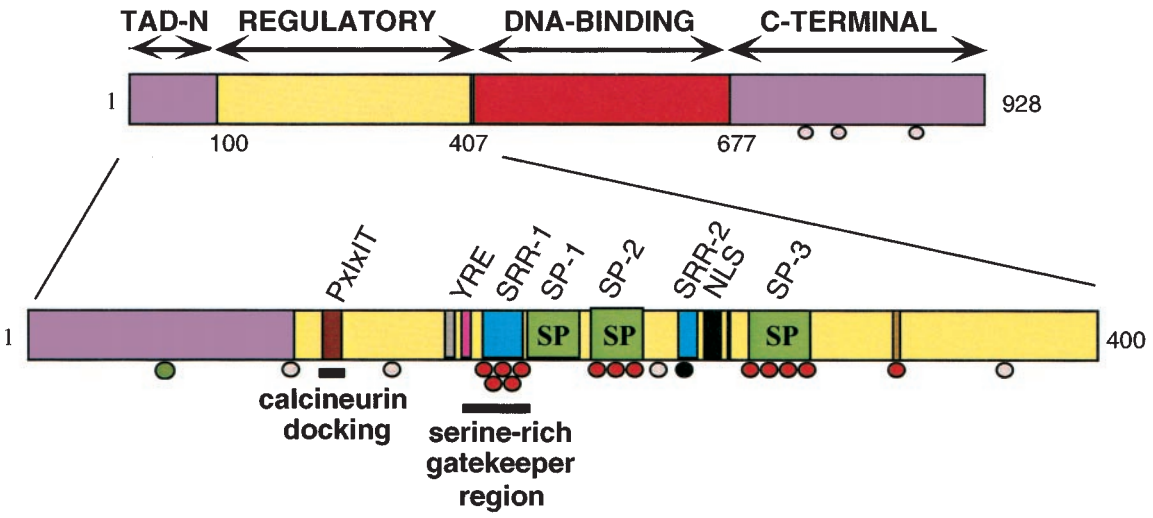

Figure 1 Domain structure and phosphorylation of NFAT proteins. The N-terminal transactivation domain (TAD-N), regulatory domain, DNA binding-domain and C-terminal domain of NFAT proteins are indicated. Some forms of the proteins have altered N-termini or are truncated at their C-termini due to alternative splicing. The regulatory domain is enlarged to show conserved sequence motifs as colored boxes: two serine-rich regions (SRR-1 and SRR-2), three SPxx repeat motifs (SP-1, -2, and -3), a major calcineurin docking site (PxIxIT), a nuclear localization sequence (NLS), and a conserved YRE/D sequence that forms part of the gatekeeper region (YRE). Phosphorylated residues are shown as filled circles based on data from NFAT1 (Okamura *et al*., 2000): conserved phosphoserines that become dephosphorylated upon activation are shown in red, non-conserved phosphoserines in gray, the conserved phosphoserine situated N-terminal to the NLS in black; and the inducible phosphorylation site in the N-terminal transactivation domain in green

specific cytokines that control the development of allergy (Ranger *et al*., 1998b). In contrast, T cells deficient in both NFAT1 and NFAT2 are essentially incapable of cytokine production, but B cells from these mice show an unprecedented phenotype of hyperproliferation, suggesting a role for NFAT in suppressing B-cell responses (Peng *et al*., 2001) (Table 1).

All five NFAT proteins are also expressed in various non-lymphoid tissues, where they are involved in the regulation of diverse cellular functions in organs other than the immune system. The functions of NFAT proteins in extra-immune tissues have been largely inferred from the phenotypes of gene-disrupted mice lacking individual or multiple family members (see Table 1), and the identity of only a few target genes is known. Thus targeted disruption of the NFAT2 gene results in intrauterine death of the NFAT2-deficient embryos due to a defect in cardiac valve formation (Delapompa *et al*., 1998; Ranger *et al*., 1998a). In muscle tissue NFAT2 is thought to cooperate with GATA2 to induce myocyte hypertrophy (Musaro *et al*., 1999). NFAT3 is expressed in hippocampal neurons where it induces transcription of NFAT dependent genes in response to depolarization (Graef *et al*., 1999). A calcineurin-dependent pathway that induces cardiac hypertrophy and involves a possible NFAT3-GATA4 cooperation has also been described (Molkentin *et al*., 1998). Finally, NFAT proteins have been implicated in the regulation of chondrogenesis and adipogenesis (Ho *et al*., 1998; Ranger *et al*., 2000). Whether these extra-immune NFAT-regulated processes require NFAT:AP-1 cooperation, or whether new transcriptional partners such as GATA proteins are required for NFAT activity outside the immune system, remains to be elucidated.

Signaling pathways that modulate NFAT activity

Combinatorial regulation is a powerful mechanism that allows the generation of specific, tightly-controlled responses through integration of independent signaling pathways that activate different transcription factors. For NFAT and AP-1, target genes that require the cooperative NFAT:AP-1 interaction will be expressed only when there is coordinate activation of calcium and MAP kinase/protein kinase C signaling pathways, that are required to turn on NFAT and AP-1 respectively. While NFAT activation requires calcium signaling, activation of AP-1 proteins is induced by engagement of a different signaling pathway that involves PKC, the small G protein Ras, and several MAP kinases including JNK and ERK. The specific signaling cascades involved are reviewed in detail elsewhere in this issue, and will not be considered here except to note that in T cells, the relevant protein kinase C appears to be PKCθ (Altman *et al*., 2000; Sun *et al*., 2000).

Calcium and store-operated calcium entry

Activation of NFAT proteins is achieved through activation of signaling pathways that produce a rise in intracellular free calcium levels. The increase in intracellular calcium concentrations induces activation of the phosphatase calcineurin, which dephosphory-

lates NFAT proteins (Loh *et al*., 1996; Luo *et al*., 1996b; Shibasaki *et al*., 1996). This process of calcium mobilization and calcineurin activation is triggered in cells of the immune system by engagement of surface receptors such as the antigen receptor in T and B cells (TCR and BCR), the Fcγ receptor in macrophages and NK cells and the Fcε receptor in mast cells and basophils (reviewed in Kiani *et al*., 2000; Rao *et al*., 1997). Because of the presence of constitutively active intracellular kinases that rephosphorylate and inactivate NFAT, calcineurin is continuously needed to maintain NFAT proteins in an active state, and both the nuclear translocation of NFAT and its activation of gene transcription are reversed by treatment of T cells with the calcineurin inhibitors cyclosporin A and FK506 (Loh *et al*., 1996; Shibasaki *et al*., 1996; Timmerman *et al*., 1996). As a result, NFAT activation occurs optimally under conditions of capacitative calcium entry and low sustained increases of intracellular calcium, while large transient peaks of calcium entry, that activate other signaling intermediates and transcription factors such as JNK and NF-κB, do not induce effective (i.e. sustained) nuclear translocation and activation of NFAT proteins (Dolmetsch *et al*., 1997). The importance of capacitative calcium entry is demonstrated by the severe combined immunodeficiency phenotype of patients with primary impairments of capacitative calcium entry, who exhibit severe defects in NFAT activation and production of multiple cytokines (Feske *et al*., 2000; Fischer *et al*., 1994). This mechanism, therefore, represents a first level of signaling specificity, in that it facilitates specific decoding of the information contained in the amplitude and duration of the calcium signal.

Calcineurin

A conserved motif in the regulatory domain (PxIxIT) is critical for the interaction of NFAT and calcineurin (Aramburu *et al*., 1998, 1999; Garciacozar *et al*., 1998; see Figure 1). However, the surface of NFAT-calcineurin interaction may be more extensive than that encompassed by the PxIxIT sequence alone (Liu *et al*., 1999; Park *et al*., 2000). The sequence of the PxIxIT motif is SPRIEIT in NFAT1 and NFAT2, CPSIQIT in NFAT3, and CPSIRIT in NFAT4; i.e. the variable residues in the PxIxIT motif are polar in all four NFAT proteins. Mutation of the polar residues to alanine in NFAT1 greatly decreases intereaction of this protein with calcineurin, and a peptide spanning the SPRIEIT sequence blocks the interaction between all four NFAT proteins and calcineurin (Aramburu *et al*., 1998; Garciacozar *et al*., 1998). *In vitro* selection of a high-affinity calcineurin-binding peptide from a degenerate peptide library bearing the SPRIEIT motif led to isolation of a peptide (PVIVIT) in which the polar residues had been substituted with bulky β-branched non-polar resides (Aramburu *et al*., 1999). This peptide was approximately 30–50-fold more potent than the SPRIEIT peptide in blocking NFAT-calcineurin binding and calcineurin-mediate dephosphorylation of all four NFAT proteins; however it did not block the calcineurin active site or the ability of calcineurin to dephosphorylate several protein and peptide substrates other than NFAT. When expressed as a GST fusion protein in cells, the VIVIT peptide selectively blocked NFAT activation and the expression of NFAT-dependent genes, without blocking the activation of other transcription factors (e.g. NF-κB) or affecting the expression of genes that were calcineurin-dependent but not dependent on NFAT. Finally, substitution of the VIVIT sequence into wildtype NFAT1 yielded a protein that was highly sensitive to the basal levels of calcineurin activity found in resting cells, since it was partially dephosphorylated and activated under these conditions (Aramburu *et al*., 1999).

Upon targeting of calcineurin to the regulatory domain of NFAT proteins, the phosphatase removes a large number of phosphates from the heavily phosphorylated regulatory domains, causing a pronounced and characteristic mobility shift of the proteins in SDS gels. Detailed analysis of NFAT1 (Okamura *et al*., 2000) indicates that 14 phosphoserine residues, all of which are located in highly conserved sequence motifs in the NFAT family, are stoichiometrically phosphorylated in resting cells, and that 13 of these are dephosphorylated upon stimulation (Figure 1). The strong conservation of these 13 phosphoserine residues in the NFAT family argues strongly for a conserved mechanism of NFAT regulation by dephosphorylation and rephosphorylation.

Mechanism of NFAT activation

The mechanism by which dephosphorylation mediates NFAT regulation has been elucidated by detailed analysis of NFAT1 and NFAT2 (Beals *et al*., 1997a; Neal and Clipstone, 2001; Okamura *et al*., 2000). In NFAT1, removal of five phosphates from a conserved serine-rich sequence located immediately adjacent to the PxIxIT calcineurin-binding motif exposes a nuclear localization signal (NLS) in the regulatory domain and renders an additional eight phosphoserine residues in the regulatory domain significantly more accessbile to calcineurin (Okamura *et al*., 2000). Complete dephosphorylation of all 13 residues further results in masking of a nuclear export signal (NES), which binds the nuclear export receptor Crm1, and promotes the full translocation of NFAT1 to the nucleus. It is not entirely clear whether the nuclear export signal is intrinsically encoded in the primary sequence of NFAT proteins, since the NES sequence identified in NFAT2 (Klemm *et al*., 1997) is not fully conserved in other family members, or whether it is conferred by NES-bearing proteins such as 14.3.3 which bind to phosphorylated regions of NFAT (Chow and Davis, 2000). Nevertheless the data for NFAT1 are consistent with the hypothesis that full dephosphorylation promotes a conformational switch that activates multiple functions of NFAT1, including its nuclear localization, DNA binding, and transcriptional activity (Okamura *et al*., 2000; Park *et al*., 1995; Shaw *et al*., 1995).

Extensive dephosphorylation is also necessary to fully activate the DNA-binding and transcriptional functions of NFAT2 (Neal and Clipstone, 2001; Porter *et al*., 2000); thus artificial localization of phosphorylated NFAT2 to the nucleus is not likely to be sufficient for full transcriptional activity as suggested by one report (Timmerman *et al*., 1996). Transcription mediated by nuclear NFAT4 is also likely to involve extensive dephosphorylation of its regulatory domain. Although one report suggested that phosphatase-dead calcineurin activated NFAT4 by binding to a nuclear export signal and competing away the nuclear export factor Crm1, thus interfering with futile shuttling of NFAT4 from the cytoplasm to the nucleus, the dominant-negative calcineurin used in this study retained $\sim$10% of wildtype phosphatase activity (Zhu and McKeon, 1999). Thus taken together, the data are consistent with the plausible hypothesis that dephosphorylation plays a conserved role in activating all four calcinuerin-regulated NFAT proteins at multiple levels including localization to the nucleus, optimal DNA binding, and optimal transcriptional activity.

Constitutive kinases that oppose calcineurin and inactivate NFAT

There are conflicting data about which kinases oppose calcineurin activity to rephosphorylate and therefore inactivate different members of the NFAT family. The expectation is that such kinases would be constitutively active, since they are required to maintain NFAT in its phosphorylated state in resting cells. Moreover, one would expect that such constitutive NFAT kinases would phosphorylate exactly those residues known to be dephosphorylated by calcineurin following cell activation; based on the findings with NFAT1 discussed above, these residues are conserved in all four calcineurin-regulated members of the NFAT family. However this criterion is met only by GSK3, which phosphorylates the conserved SPxx repeat motifs present in the regulatory domains of all four calcineurin-regulated NFAT proteins including NFAT2 (Beals *et al*., 1997b) and NFAT3 (Graef *et al*., 1999). These motifs are known targets for dephosphorylation by calcineurin (Okamura *et al*., 2000) (Figure 1). Another constitutive kinase, casein kinase 1, has been reported to phosphorylate residues in the regulatory domain of NFAT4 that are not fully conserved in other members of the NFAT family (Zhu *et al*., 1998).

Cross-talk between NFAT and AP-1 signaling pathways: NFAT regulation by MAP kinases and other mitogen-stimulated kinases

Although the major pathways for activation of the NFAT and AP-1 transcription factors are distinct, there is evidence that cross-talk may occur. As reviewed elsewhere in this issue, AP-1 activation is regulated at multiple levels by activation of MAP kinases. MAP kinases are also involved in regulating NFAT activation, although the data (which are often based on NFAT and MAP kinase overexpression) tend to be contradictory and are not always internally consistent. The MAP kinase p38 has been suggested to phosphorylate and prevent the nuclear localization of NFAT1 (del Arco *et al*., 2000; Porter *et al*., 2000), and p38 and ERK have been reported to phosphorylate and deactivate NFAT2 (Porter *et al*., 2000), but the target residues for these kinases have not been identified and shown to be phosphorylated *in vivo*. Likewise JNK2 was reported to phosphorylate NFAT4 and control its cellular distribution (Chow *et al*., 1997), although this finding was directly contradicted by another report (Zhu *et al*., 1998). Recently, it was reported that JNK1 phosphorylates specific residues in the PxIxIT calcineurin targeting motif of NFAT2, thus inhibiting the NFAT2-calcineurin interaction (Chow *et al*., 2000); however the corresponding region of NFAT1 is not phosphorylated in stimulated cells (Okamura *et al*., 2000), suggesting that either this region is selectively accessible in NFAT2 or that this method of regulation might not play a major role at endogenous levels of NFAT and JNK expression.

Nevertheless, T cells from JNK1$^{-/-}$ mice show increased nuclear localization of NFAT2 but not NFAT1, suggesting that JNK directly or indirectly suppresses the activation of this NFAT family member (Dong *et al*., 1998). Consistent with this finding T cells engineered to lack all JNK activity show increased rather than decreased production of IL-2 (Dong *et al*., 2000). An additional point is that JNK resembles NFAT proteins in being activated by increased intracellular calcium in T and B cells; however, the magnitude and duration of intracellular calcium oscillations needed for optimal JNK activation appear to be different from those required for optimal NFAT activation (high transient calcium spikes for optimal JNK activation, compared to low sustained increases in $[Ca^{2+}]_i$ levels for optimal NFAT activation) (Dolmetsch *et al*., 1997; Werlen *et al*., 1998). The calcium requirement for JNK activation in T and B cells appears to be mediated by calcineurin, as it is blocked by the calcineurin inhibitor cyclosporin A (Dolmetsch *et al*., 1997; Werlen *et al*., 1998).

A new phosphorylation site that seems to be important for NFAT activity has been described in the transactivation domain of NFAT1 (Garcia-Rodriguez and Rao, 2000; Okamura *et al*., 2000). This site is phosphorylated in cells stimulated with both PMA and ionomycin, but not in cells stimulated with ionomycin alone. The kinase that mediates the phosphorylation of this site has not yet been identified but clearly responds to mitogenic stimuli and not to the classical calcium signals that activate NFAT.

Signaling pathways that may repress NFAT:AP-1 cooperation

Control of NFAT:AP-1 interactions may also be achieved by specific repressor proteins that target this interaction and can control their activity. Thus ICER, a cAMP-inducible transcriptional repressor, binds specifically to NFAT:AP-1 composite sites and may

form inactive complexes with NFAT (Bodor and Habener, 1998). Once bound to DNA, ICER acts as a transcriptional repressor that controls expression of genes such as IL-2 and IL-4 by targeting and inhibiting NFAT : AP-1 cooperation. In a similar mechanism, activation of the IL-2 promoter is repressed by $p21^{SNFT}$, a bZIP protein that forms complexes with NFAT and Jun on composite DNA elements and prevents Fos from being incorporated into the ternary complex (Iacobelli *et al*., 2000). $p21^{SNFT}$ also heterodimerizes with Jun and inhibits Jun transcriptional activity. HPK1, a serine/threonine kinase of the germinal center kinase family, has also been identified as an inhibitor of Jun activation during TCR stimulation (Liou *et al*., 2000). Each of these inhibitors might regulate one or more distinct cellular processes, including productive immune response, apoptosis or T cell development and differentiation, by targeting and inhibiting cooperative NFAT:AP-1 transcriptional activity.

NFAT-dependent gene transcription

Diversity of transcriptionally-active complexes containing NFAT

Cooperative complexes of NFAT and AP-1 The basic region-leucine zipper (bZIP) proteins of the Jun (cJun, JunB and JunD) and Fos (cFos, FosB, Fra1 and Fra2) families are AP-1 transcription factors that bind as homo- or heterodimers to AP-1 regulatory elements present in many genes. As reviewed elsewhere in this issue (see review by Chinenov and Kerppola in this issue), Fos and Jun proteins bind DNA through their conserved basic region and interact with each other through their leucine zipper domains (Glover and Harrison, 1995).

Cooperation between NFAT and AP-1 transcription factors has been demonstrated in the promoter/enhancer region of several genes in immune cells (reviewed in Rao *et al*., 1997). As discussed in this issue (see review by Chinenov and Kerppola in this issue) and elsewhere (Rao *et al*., 1997), a wide range of composite binding sites has been observed, and comparable degrees of cooperativity appear to be achieved *in vivo* by pairing weak NFAT sites with moderately strong AP-1 sites (as in the GM330 element of the GM-CSF enhancer and the ARRE-1 site of the IL-2 promoter), moderately strong NFAT sites with weak AP-1 sites (as in the ARRE-2 site of the murine IL-2 promoter), or weak AP-1 sites with weak NFAT sites (as in the ARRE-2 site of the human IL-2 promoter) (see Figure 2). The binding of AP-1 and NFAT to their individual binding sites in these composite elements is characterized by relatively high dissociation rates, but a strong cooperative complex with greatly increased stability is formed when all three

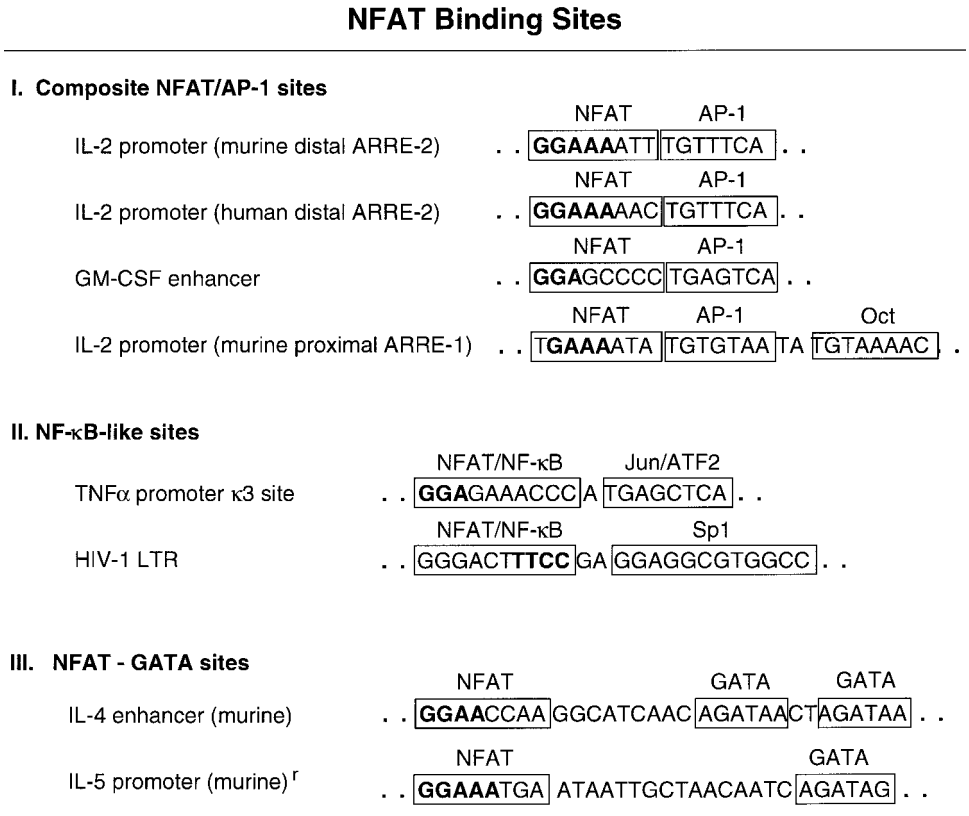

Figure 2 NFAT binding sites. Representative examples of the different groups of NFAT binding sites discussed in the text are shown. The core of the NFAT binding site is shown in bold. r:Sequence of the murine IL-5 promoter is from the non-coding strand

proteins bind the composite DNA site (Chen *et al*., 1998; Jain *et al*., 1993b; Peterson *et al*., 1996). Although the protein-protein contacts involve an extended network of backbone and side-chain contacts (see below), they are not strong enough to stabilize the NFAT:AP-1 complex in the absence of DNA.

The structurally best-characterized example of cooperative binding of NFAT and AP-1 occurs at the composite ARRE-2 site of the IL-2 promoter, which contains a binding site for NFAT (GGAAAA) and a weak AP-1 binding site (TGTTTCA) separated by two base pairs (Figure 2). In the mouse, the intervening bases are TT, and the site supports weak binding of NFAT in the absence of AP-1; in contrast the presence of the AC intervening sequence in the human element greatly reduces the independent binding of NFAT (Jain *et al*., 1993b). The DNA binding domains of NFAT and the bZIP regions of Fos and Jun are necessary and sufficient to achieve cooperative association of the ternary NFAT:AP-1 complex on the ARRE-2 site (Chen *et al*., 1998; Jain *et al*., 1993a). The complex is sustained by an extended contact surface between the amino-terminal domain of the NFAT Rel homology region and the leucine zipper regions of Fos and Jun (Figure 3a), and facilitated by the bending of both the AP-1 heterodimer and the DNA towards the NFAT DNA binding domain (Chen *et al*., 1998; Diebold *et al*., 1998) (see review by Chinenov and Kerppola in this issue). Although Fos-Jun dimers can normally bind in both possible orientations on AP-1 sites, the cooperative complex of NFAT and AP-1 has a very specific orientation, in which Jun binds the AP-1 half-site closer to NFAT (Chen *et al*., 1998; 1995; Diebold *et al*., 1998; see review by Chinenov and Kerppola in this issue). Although most contacts involve the amino-terminal domain of the NFAT Rel homology region the carboxy-terminal domain of this region makes some contacts with Fos (Figure 3) (Chen *et al*., 1998).

Two major foci in the amino-terminal domain of the NFAT Rel-homology region are responsible for holding the ternary complex together (Figure 4). The E′F loop in the NFAT Rel-homology region, supported by a small hydrophobic patch centered on Phe573 in NFAT1, contacts the fork region of Jun close to the DNA surface, while the CX loop contacts Fos in successive layers along the zipper helix. The residues of NFAT proteins that are involved in making contacts with DNA and with Jun are very conserved in the four classical members of the NFAT family of transcription factors, while the residues involved in Fos contact are more variable (Chen *et al*., 1998). The protein-protein contacts mediated by the E′F and CX loops of the NFAT Rel-homology region are essential for the cooperative binding of NFAT with AP-1, as shown by the fact that despite the extended network of contacts, NFAT:AP-1 cooperation can be completely disrupted by making limited substitutions in the NFAT-Fos-Jun interface (Chen *et al*., 1998; Macián *et al*., 2000; Sun *et al*., 1997). Specifically, substitutions of Arg468 and Ile469 on the CX loop and Thr535 in the E′F loop of NFAT1 with alanine, alanine and glycine respectively eliminate all cooperative interactions between NFAT and Fos/Jun or Jun-Jun dimers, without altering NFAT DNA binding activity. Similar results are obtained when Phe473 is mutated to Ala,

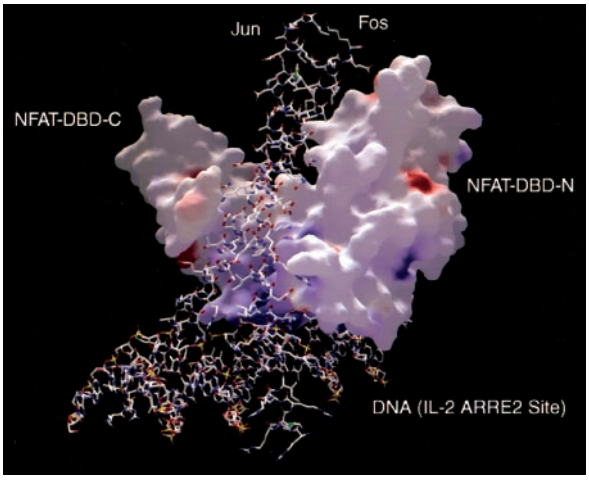

Figure 3 Representation of the structure of the ternary complex formed on the ARRE-2 site of the IL-2 promoter by NFAT, Fos and Jun. The colors on the surface representation of the NFAT DNA binding domain represent electrostatic potential (blue, positive; red, negative). NFAT-DBD-N: amino-terminal domain of the NFAT DNA binding domain. NFAT-DBD-C: carboxy-terminal domain of the NFAT DNA binding domain

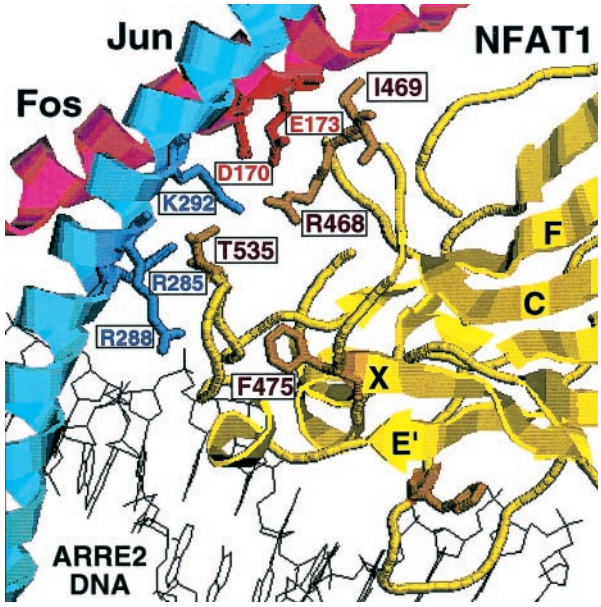

Figure 4 The NFAT/AP-1 interface. Side chains of critical residues maintaining the interaction between E′F and CX loops of NFAT (in yellow) and Fos (in red)/ Jun (in blue) are highlighted

thus disrupting the structure of the E′F loop (Maciàn *et al*., 2000).

Binding of NFAT dimers to Rel/NF-κB-like binding sites In addition to forming composite complexes with AP-1 dimers, the calcium-regulated NFAT proteins can bind as dimers to Rel/NF-κB-like elements in the regulatory regions of certain NFAT target genes (Kinoshita *et al*., 1997; Macián and Rao, 1999; McCaffrey *et al*., 1994). These dimers are weak and non-cooperative, and form only on DNA; in solution, the NFAT proteins are strictly monomeric except at unphysiologically high concentrations. NFAT5 represents a special case, since this protein is an obligatory dimer both in solution and in its DNA-bound form; as with Rel/ NF-κB proteins, dimerization is necessary for DNA-binding and transcriptional function (discussed below) (Lopez-Rodriguez *et al*., 1999a). Despite the ability of NFAT proteins to form dimers, there are no reports of cross-dimerization between members of the NFAT and NF-κB/ Rel families.

Examples of AP-1-independent, NF-κB/Rel-like binding sites for NFAT proteins are provided in Figure 2. A good example is the k3 element of the TNF-α promoter, which binds NFAT dimers (McCaffrey *et al*., 1994) as well as certain Rel-containing dimers (Goldfeld *et al*., 1993) *in vitro*. Similarly, the tandem NF-κB sites in the HIV-1 long terminal repeat, which are clearly capable of binding p50/ RelA dimers, also support the weaker binding of NFAT proteins (Kinoshita *et al*., 1997; Macián and Rao, 1999; McCaffrey *et al*., 1994). Mutations of the 5′ half-sites of the HIV-1 LTR NF-κB elements, which eliminate NF-κB binding, have little or no effect on binding of NFAT proteins, suggesting that the NFAT dimer nucleates preferentially on the 3′ half-site of these Rel/ NF-κB-like elements.

NFAT:GATA cooperation There is also considerable evidence for functional cooperation between NFAT and GATA proteins, although it is not yet clear whether this is due to a physical mechanism of cooperative stabilization of NFAT-GATA complexes on DNA. NFAT3 and GATA4 have been shown to interact in yeast two-hybrid assays, and NFAT3-GATA4 cooperation may be important for cardiomyocyte growth and cardiac hypertrophy. NFAT2 and GATA2 are co-expressed in nuclei of developing myoblasts differentiating in response to IGF-1, and can be co-immunoprecipitated from skeletal muscle extracts. A distal enhancer in the IL-4 gene binds both NFAT1 and GATA3, and cooperation between these proteins may be required for enhancer activity. The activity of the IL-5 promoter may also require both NFAT and GATA3. Thus the theme of NFAT-GATA cooperation is observed in a large number of different cell types in the context of diverse biological outcomes, and an investigation into the underlying mechanism will be of considerable interest. In particular, it would be interesting to determine whether the E′F loop, which mediates NFAT:DNA and NFAT:Jun contact, plays a role in NFAT:GATA cooperation. This loop shows structural variability between different members of the NFAT and Rel families, and may be involved in cooperative interactions of Rel proteins with different biological partners.

Genes regulated by NFAT in cooperation with AP-1

NFAT proteins have been implicated in regulating transcription of a very large number of inducible genes in immune-system cells (reviewed in Rao *et al*., 1997). These include the IL-2 gene; the linked IL-3 and GM-CSF genes; the linked IL-4, IL-5, and IL-13 genes that are coordinately expressed by differentiated T helper type 2 (Th2) T cells; the IFNγ gene encoding the defining cytokine expressed by Th1 cells; the gene encoding the proinflammatory cytokine TNFα; the genes encoding the cell-surface receptors CD40L, FasL, CD5, Igκ, and CD25; the chemokine genes IL-8 and MIP-1α, and the cyclooxygenase 2 (Cox2) gene. The many *ex vivo* studies documenting this point have been validated by the finding that T cells lacking the two major NFAT family members expressed in immune-system cells, NFAT1 and NFAT2, are effectively incapable of producing many of these inducible genes (Peng *et al*., 2001). In other cases expression of the VIVIT peptide, which inhibits NFAT:calcineurin binding and therefore NFAT activation, has been shown to inhibit transcription of endogenous inducible genes in Jurkat T cells.

For the majority of these genes a dependence on NFAT:AP-1 cooperation has been established, in part by using an elegant strategy involving use of a mutant NFAT1 protein that cannot cooperate with AP-1 (Macián *et al*., 2000). A notable exception is the TNF-α gene, discussed in the following section. Most of the known NFAT binding sites in these genes are in fact composite NFAT:AP-1 DNA elements, which as discussed above are composed of an NFAT site whose core sequence (GGAAA) is positioned ~3 base pairs from the start of an often weak AP-1 site (see Figure 2). The characteristics of selected NFAT:AP-1 target genes and individual NFAT:AP-1 composite elements in these genes are briefly reviewed below.

IL-2 NFAT proteins are necessary for IL-2 gene expression, as inferred from the fact that IL-2 expression is strongly inhibited by the VIVIT peptide (Aramburu *et al*., 1999) and by a dominant-negative fragment containing the calcineurin-binding region of NFAT4 (Chow *et al*., 1999), and that T cells lacking both NFAT1 and NFAT2 do not produce IL-2 in response to TCR stimulation (Peng *et al*., 2001). Cooperation of NFAT with AP-1 is required for IL-2 gene transcription, as apparent from studies using a mutant NFAT1 protein unable to interact with Fos/Jun heterodimers (Macián *et al*., 2000). The IL-2 promoter contains four (or perhaps five) NFAT binding sites, of which two (or three) are composite NFAT:AP-1 binding elements (Jain *et al*., 1995b; Rooney *et al*., 1995b; see

Figure 2). The distal NFAT site of the IL-2 promoter, located at − 280 bp relative to the transcription start site, is a true composite element, since simultaneous binding of NFAT and AP-1 to this element greatly increases the stability of the ternary complex. The proximal site at −135 bp is also likely to be a composite NFAT:AP-1 site. The site at −90 bp is interesting because it contains a composite NFAT:AP-1 element in which the NFAT binding site (GAAA) is very weak and does not conform to the consensus (GGAAA), and because the adjacent AP-1 site is located immediately 5′ to an Oct binding site (see Figure 2). A site located at −45 bp relative to the transcription start site appears to be a high-affinity NFAT binding site that does not require cooperation with AP-1. Finally, the CD28 response element of the IL-2 promoter has the characteristics of a dimeric NFAT/NF-κB/ Rel site; it has been reported to bind both NFAT dimers and NF-κB/ Rel dimers and to function cooperatively with AP-1 proteins bound to the adjacent AP-1 site (Himes *et al*., 1996; Rooney *et al*., 1995b).

GM-CSF and IL-3 The GM-CSF and IL-3 genes are closely linked, being separated by only 10 kbp in both humans and mice. Upstream of the GM-CSF gene lies an enhancer element that controls GM-CSF expression and contains four NFAT sites, three of which support cooperative binding with AP-1 (Cockerill *et al*., 1995). The cell-type specificity of GM-CSF expression differs from that of IL-3: GM-CSF is expressed by lymphocytes as well as myeloid cells, while IL-3 is exclusively expressed by lymphoid cells. This difference has been attributed to the fact that IL-3 gene expression appears to be controlled by a separate upstream enhancer, that contains NFAT: Oct elements rather than NFAT:AP-1 composite sites (Bert *et al*., 2000; Cockerill *et al*., 1999; Duncliffe *et al*., 1997). However the use of a mutant NFAT1 protein incapable of AP-1 interaction shows clearly that both IL-3 promoter activity and expression of the endogenous IL-3 gene required cooperative interactions between NFAT and AP-1, thus placing IL-3 firmly in the category of NFAT:AP-1-dependent genes (Macián *et al*., 2000).

IL-4, IL-5 and IL-13 These three cytokines are signature cytokines of the Th2 lineage of T cells, which are produced when naïve T cells are exposed to antigen in the presence of IL-4. Each of these cytokine genes has been proposed to require NFAT:AP-1 cooperation. However, Th2 differentiation is also known to require GATA3 (reviewed in Avni and Rao, 2000) and it is plausible that the regulatory regions of these genes also support cooperative NFAT:GATA3 interactions. *IL-4*. At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Burke *et al*., 2000; Rooney *et al*., 1995a; Takemoto *et al*., 1997). A distal IL-4 enhancer located 3′ of the IL-4 gene has been shown to contain tandem binding sites for NFAT and GATA proteins, and to bind both NFAT1 and GATA3 in chromatin immunoprecipitation assays (Agarwal *et al*., 2000; Ho *et al*., 1996; Hodge *et al*., 1996). Cooperation between NFAT and other proteins (c-Maf, NIP45) on the IL-4 promoter has also been proposed (Agarwal *et al*., 2000; Ho *et al*., 1996; Hodge *et al*., 1996). *IL-5*. Several studies have reported that IL-5 transcription is NFAT-dependent and, although interaction with other factors such as GATA-3 has been suggested (Zhang *et al*., 1999), the existence of cooperation between NFAT and AP-1 on putative composite sites on the IL-5 promoter has been described (reviewed in Rao *et al*., 1997). *IL-13*. While the IL-13 promoter was induced in an AP-1-dependent fashion by a mutant NFAT protein incapable of making cooperative interactions with AP-1, expression of the endogenous IL-13 gene required NFAT:AP-1 cooperation, suggesting the existence of distal regulatory regions that contain composite NFAT:AP-1 sites (Macián *et al*., 2000).

IFN-γ This cytokine defines the T helper 1 (Th1) lineage. Its NFAT dependence is inferred from the fact that T cells lacking both NFAT1 and NFAT2 show no IFN-γ expression (Peng *et al*., 2001). Chromatin immunoprecipitation assays confirm that NFAT1 binds selectively *in vivo* to the IFN-γ promoter region, which is not accessible to NFAT binding in Th2 cells (Agarwal *et al*., 2000; Ho *et al*., 1996; Hodge *et al*., 1996); the likely site in the IFN-γ promoter has an adjacent AP-1 site and is likely to support cooperative interactions of NFAT and AP-1 (reviewed in Rao *et al*., 1997). However the IFN-γ promoter alone shows weak inducibility that is not restricted to IFN-γ producing Th1 cells, and thus distal regulatory regions may be required. A T-box transcription factor, T-Bet, has recently been shown to be important for Th1 lineage commitment and IFN-γ production (Szabo *et al*., 2000), and it would be worthwhile to investigate the possibility of NFAT: T-Bet cooperation at regulatory regions of the IFN-γ gene.

FasL Two NFAT sites have been described in the Fas ligand promoter (Latinis *et al*., 1997). In T cells, FasL expression is induced by engagement of both TCR and the costimulatory receptor CD28 (Norian *et al*., 2000). Full stimulation of the FasL promoter appears to be induced by cooperation of calcineurin and PKCθ and involves activation of NFAT and AP-1 (Villalba *et al*., 1999). Similar cooperation between PKCθ and calcineurin is been reported to induce JNK and activate IL-2 gene transcription (Werlen *et al*., 1998).

CD25 NFAT has been implicated in the regulation of CD25 expression (Schuh *et al*., 1998). NFAT1 and NFAT2 bind to two different sites within the CD25 promoter with adjacent non-consensus AP-1 sites. Electrophoretic mobility shift assays show that complexes that bind these sites contain NFAT and AP-1.

Cox2 The promoter of the cyclooxygenase 2 (Cox2) gene, an inducible early response gene in T cells,

contains two NFAT sites, one of which resembles an NFAT:AP-1 composite site (Iniguez *et al*., 2000). Induction of COX-2 expression requires stimulation with PMA and ionomycin and is inhibited by cyclosporin A and dominant negative forms of NFAT and Jun. These results and the detection in gel shift experiments of complexes containing NFAT and AP-1 suggest that NFAT : AP-1 cooperation regulates COX-2 gene transcription (Iniguez *et al*., 2000).

Genes independent of NFAT:AP-1 cooperation

TNFα To determine the importance of NFAT:AP-1 cooperation for gene transcription in T cells, experiments were carried out using an NFAT mutant protein unable to interact with AP-1 (Fos/Jun and Jun/Jun dimers) (Macián *et al*., 2000). These studies revealed that whereas NFAT:AP-1 cooperation was essential for NFAT-dependent transcription of certain genes (IL-2, IL-3, IL-4, MIP-1α and FasL), the mutant NFAT protein was fully capable of inducing transcription of the TNF-α gene implying a lack of requirement for cooperative interactions with Fos and Jun. As discussed above, the predominant NFAT site in the TNFα promoter is the quasi-palindromic κ3 site, which resembles an NF-κB/ Rel binding site (Goldfeld *et al*., 1993; McCaffrey *et al*., 1994). Thus the finding that the TNFα gene falls into the NFAT:AP-1-independent category raises the interesting possibility that NFAT:AP-1-independent genes may characteristically contain such dimeric binding sites for NFAT. Alternatively, NFAT may utilise other non-AP-1 partners such as GATA proteins (see previous section) at the regulatory regions of NFAT:AP-1-independent genes.

It is important to note that while strict *physical* cooperativity between NFAT and AP-1 is not required, AP-1 family members do cooperate *functionally* with NFAT to promote TNFα gene transcription (Falvo *et al*., 2000; Tsai *et al*., 1996). Immediately adjacent to the κ3 site of the TNFα promoter is a binding site for ATF2-Jun heterodimers, factors which cooperate functionally with NFAT dimers bound to the κ3 site. Depending on the cells and stimulation conditions, these dimers cooperate with other transcription factors bound to the proximal TNFα promoter, to form enhanceosome complexes that drive TNFα gene transcription (Falvo *et al*., 2000; Tsai *et al*., 1996; see review by Chinenov and Kerppola in this issue).

NFAT target genes in non-immune cells It is likely that many NFAT-dependent genes in cell types outside the immune system are regulated by NFAT nuclear partners other than AP-1. Suggested genes include the atrial natriuretic factor gene and the b-type natriuretic peptide gene in cardiomyocytes (Molkentin *et al*., 1998), genes controlling the slow twitch programme in skeletal muscle fibres (Chin *et al*., 1998) and the gene encoding the inositol trisphosphate receptor in hippocampal cells (Graef *et al*., 1999).

Biological outcomes of NFAT activation in the presence or absence of AP-1

It seems very likely that even in a single cell type, NFAT proteins elicit different programmes of gene expression depending on whether AP-1 proteins or other cooperating transcription factor are activated at the same time. Some biological responses that could potentially be regulated in this manner are described below.

T cell development in the thymus

Development of T cells in the thymus involves opposing processes of negative and positive selection that eliminate strongly self-reactive cells that might mediate autoimmune responses, and permit survival and maturation of cells capable of recognizing foreign antigens in the context of self major histocompatability complex (MHC) proteins, respectively. High-affinity interaction of immature thymocytes with MHC/antigen complexes lead to negative selection while low affinity interactions result in positive selection and maturation. Several findings provide evidence for the involvement of NFAT in positive selection: NFAT4 is the major NFAT protein present in the thymus, and NFAT4$^{-/-}$ mice show a moderate impairment of positive selection (Oukka *et al*., 1998); inhibition of NFAT activation with the calcineurin inhibitor cyclosporin A blocks positive selection (Gao *et al*., 1988); and expression of a constitutively-active form of calcineurin in a transgenic mouse increases sensitivity to a signaling through the TCR and results in increased positive selection (Hayden-Martinez *et al*., 2000). While positive selection shows a preferential requirement for calcium signaling, negative selection requires stimulation of both the TCR and the costimulatory receptor CD28, which induces JNK activation (Rincon *et al*., 1998). CD28-deficient mice show defects in negative selection, and inhibiting the interaction of CD28 with its ligands, B7.1 and B7.2 (CD80 and CD86), results in decreased thymocyte apoptosis (Noel *et al*., 1998; Samoilova *et al*., 1997). It is therefore possible that difference in the balance of NFAT and AP-1 activation and cooperation may determine the initiation of programmes of gene expression in immature thymocytes, that lead to either cell survival (positive selection) or cell death (negative selection). Supporting this hypothesis, cooperation between NFAT and AP-1 is required for FasL expression, and potentiation of activation-induced cell death in T cells, a process analogous to negative selection, is only induced by NFAT1 in the presence of cooperative interaction with Fos and Jun (Macián *et al*., 2000).

Th1-Th2 differentiation in the periphery

As outlined earlier, naïve T cells respond to antigen stimulation by differentiating into distinct subsets of effector cells that are characterized by differing patterns of cytokine expression. Th1 cells produce IFN-γ while

Th2 cells express IL-4, IL-5, and IL-13. As described in the previous section, NFAT proteins play a role in transcription of all of these cytokine genes. Chromatin immunoprecipitation experiments have shown that NFAT1 binds to sites in the IL-4 promoter and a distal IL-4 enhancer in Th2 cells but not in Th1 cells; conversely NFAT1 binds to sites in the IFN-γ promoter in Th1 cells but not in Th2 cells, suggesting that the accessibility of these regulatory regions to NFAT is determined by changes in chromatin structure occurring during T cell differentiation (Agarwal *et al*., 2000). As also described above, NFAT:AP-1 cooperation is also required for transcription of both Th1 and Th2 cytokine genes; thus the NFAT:AP-1 interaction may be globally important for T helper cell differentiation without necessarily playing a selective role. It is possible, however, that the Th1-Th2 decision depends on the availability of specific AP-1 partners for NFAT: specifically, it has been suggested that Th2 cells preferentially upregulate a particular AP-1 family member, JunB, which participates in IL-4 promoter induction (Li *et al*., 1999). A region of the IL-4 promoter that confers Th2 specificity in reporter assays and transgenic mice has been shown to contain a composite NFAT:AP-1 site (Wenner *et al*., 1997), although the particular AP-1 proteins binding to this site have not been identified. Furthermore, JNK1- and JNK2-deficient mice show impaired Th1 development, suggesting that depending on the conditions, impaired AP-1 activation may preferentially affect with the Th1 or Th2 arm of T helper cell development (Dong *et al*., 1998, 2000; Yang *et al*., 1998).

Lymphocyte tolerance/ anergy

Anergic T cells show a profound impairment of AP-1 activation due to a block in Ras activation that results in defective downstream activation of the ERK and JNK protein kinases (Fields *et al*., 1996; Li *et al*., 1996). Nevertheless anergic cells, even under resting conditions, maintain levels of calcium mobilization that are able to activate NFAT proteins (Healy *et al*., 1997). Similar results were obtained in an *in vivo* model of B cell tolerance. Thus the productive immune response in lymphocytes is an important example of the importance of cooperativity between NFAT and AP-1, while an anergic/ tolerant state may be induced when the balance of these two transcription factors is altered.

NFAT5

NFAT5 is an outlying member of the NFAT family

NFAT5, the most recent addition to the NFAT/ Rel family of transcription factors, was isolated by three laboratories on the basis of its high degree of sequence similarity ($\sim$43%) to the DNA-binding domain (Rel homology region) of NFAT proteins (Lopez-Rodriguez *et al*., 1999b; Pan *et al*., 2000; Trama *et al*., 2000). In addition, NFAT5 was independently cloned in a yeast one-hybrid assay as TonEBP, a protein binding to the tonicity response element (TonE) of the betaine transporter gene (Miyakawa *et al*., 1999).

Isolation of full length cDNA encoding NFAT5 showed that this new Rel protein possessed characteristics that were distinct from those of NFAT1-4 (Lopez-Rodriguez *et al*., 1999b). First, the DNA-binding domain of NFAT5 does not support cooperative interactions with Fos and Jun (see below). Second, NFAT5 lacks the conserved regulatory domains of the calcium-regulated NFATs: neither the short (180 amino-acid) serine-rich N-terminal region nor the long ($\sim$900 amino-acid) C-terminal region of NFAT5 possesses the serine-rich and SPxx-repeat motifs characteristic of the NFAT regulatory domains. Furthermore, immunocytochemical and biochemical analysis showed that NFAT5 is completely or partially localized to the nucleus in most cell types, and neither its subcellular distribution nor its phosphorylation state are detectably altered by calcineurin (Lopez-Rodriguez *et al*., 1999b).

NFAT5 mediates the cellular response to osmotic stress

There is considerable evidence that NFAT5 is responsible for mediating the spectrum of cellular responses to osmotic stress. In osmotically-stressed cells, NFAT5 levels increase, it becomes localized to the nucleus, and its migration in SDS gels is altered in a manner reminiscent of phosphorylation (Lopez-Rodriguez *et al*., 1999b; Miyakawa *et al*., 1999). Moreover, NFAT5 binding elements are present in the regulatory regions of a variety of osmotically-regulated genes (e.g. aldose reductase, the betaine transporter and the sodium-myo-inositol transporter), which together mediate the cell's transcriptional response to osmotic stress (see Table 2). The proteins encoded by these genes are responsible for the synthesis or transport of compatible organic osmolytes such as betaine, taurine and myo-inositol, which buffer the cell against osmotic stress (reviewed in Burg *et al*., 1997).

DNA-binding by NFAT5

The DNA-binding domain of NFAT5 resembles those of the classical NFAT proteins, but differs in two major respects. First, it does not bind cooperatively with Fos and Jun to composite NFAT:AP-1 sites (Lopez-Rodriguez *et al*., 1999b). Second, it is dimeric in solution, and dimer formation is obligatory for DNA binding (Lopez-Rodriguez *et al*., 2001, submitted).

Comparison of the DNA binding domains of NFAT5 with those of NFAT1–4 (Chen *et al*., 1998; Zhou *et al*., 1998) shows strong conservation of essentially all the residues involved in contacting DNA, but effectively no conservation of residues involved in making contacts with Jun (Lopez-Rodriguez *et al*., 1999b). Accordingly, the DNA binding

Table 2 Binding sites for NFAT5/TonEBP in osmotically-responsive genes

Betaine transporter (BGT)	
Dog BGT1	TACTTGGTGGAAAAGTCCAGCTGGTGATTCACCCT
Inositol transporter (SMIT)	
SMIT TonE-A	GCAAGTGGAAAACTACCAAGA
SMIT TonE-B2	TTAGCTGGAAAATTCCAAACA
SMIT TonE-C1	AGAGGTGGAAAATTACAGGCA
SMIT TonE-C2	TGGCATGGAAAGTTACTCAAA
Aldose reductase (AR)	
Human AR: ORE-A	AGTTACATGGAAAAATATCTGGGCTAGTCTGTTCT
Mouse AR: ORE-A	ACTCAAGTGGAAAATATCTGTTCTTTATAAATTTT
Rat AR: ORE-A	ATCCAAGTGGAAAATATCTGTTCTTTATAAATTTT
Human AR: ORE-B	TCCCTCCTGGAAAATTTATACAGAACAGACTAGCC
Mouse AR: ORE-B	CACCCCAGGGAAAATTTATAAAGAACAGATATTTT
Rat AR: ORE-B	CATCCCTGGGAAAATTTATAAAGAACAGATATTTT
Human AR: ORE-C	CACCAAATGGAAAATCACCGGCATGGAGTTTAGAG
Mouse AR: ORE-C	CACCGACTGGAAAATCACCAGAATGGGATTTAGAG
Rat AR: ORE-C	CACCAACTGGAAAATCACCAGAATGGCACTTAGAG
Rabbit AR: ORE-C	CAACGGAAAATCACCAG

domain of NFAT5 unambiguously selected consensus NFAT-binding sequences (TGGAAA) from a random library or double stranded oligonucleotides, but was not capable of cooperative binding with AP-1 proteins on a well-characterized composite NFAT : AP-1 site (Lopez-Rodriguez *et al*., 1999b). Furthermore, mutation of candidate DNA contact residues abrogated the binding of NFAT5 to DNA (Lopez-Rodriguez *et al*., 2001, submitted). Despite its strong sequence selectivity, however, the DNA-binding affinity of NFAT5 is weaker than that of NFAT1 (Lopez-Rodriguez *et al*., 1999a,b), possibly reflecting a requirement for a partner protein other than AP-1.

Despite the similar sizes of their recombinant DNA-binding domains, the NFAT5: DNA complex migrates with lower affinity than the NFAT1:DNA complex in native gels when monomeric DNA sites are used (e.g. the ARRE2 site of the IL-2 promoter). In contrast on palindromic DNA elements such as the proximal NF-κB site of the HIV-1 long terminal repeat, the single NFAT5:DNA complex migrates with a mobility equivalent to that of the dimeric complex of NFAT1 with DNA (Lopez-Rodriguez *et al*., 1999a). The isolated DNA binding domain of NFAT5 is also capable of interacting with full-length NFAT5 in cell lysates, although it shows no interaction with NFAT1 or RelA present in the same lysates (Lopez-Rodriguez *et al*., 1999a). When overexpressed in HeLa cells fused to a nuclear export sequence, the NFAT5 DNA binding domain retained endogenous NFAT5 in the cyoplasm (Lopez-Rodriguez *et al*., 1999a), overriding its natural localization in the nucleus of these cells (Lopez-Rodriguez *et al*., 1999b). Together these results suggested that NFAT5 was dimeric either in the presence or absence of DNA.

More recently (Lopez-Rodriguez *et al*., 2001, submitted), gel filtration studies have confirmed that the DNA binding domain of NFAT5 forms a dimer in solution while the DNA binding domain of NFAT1 is monomeric. Co-expression of Myc- and HA- tagged forms of NFAT5 in cells revealed that full-length NFAT5 is also dimeric in both resting and osmotically-stressed cells. Alignment of the C-terminal regions of the Rel homology regions of Rel/ p50 and NFAT5 indicates that the dimer contact residues conserved throughout the NF-κB/ Rel family are also conserved in NFAT5. Mutations of these conserved residues abrogated not only dimerization but also the DNA-binding of NFAT5, indicating that like NF-κB/ Rel proteins, NFAT5 binds DNA as an obligate dimer and the conserved residues in the dimerization interface play an essential role. When co-expressed with an NFAT5 reporter, the isolated DNA binding domain of NFAT5 behaved as a specific and powerful dominant negative capable of blocking NFAT5-mediated reporter activity, but not NFAT or NF-κB reporter activity. This dominant-negative function was strongly disrupted by mutations that impaired dimerization, suggesting that the transcriptional function of NFAT5 required the formation of a dimeric transactivation domain facilitated by the strong dimerization of its DNA binding domain (Lopez-Rodriguez *et al*., 2001, submitted).

Final remarks

It is clear that the calcium-regulated NFAT proteins play a major role in regulating inducible gene expression in the immune system, and that they also have important biological functions in other cell types, both during development and in the adult animal. However much still remains to be understood. While many of the target genes regulated by NFAT proteins during the productive immune response have been identified, their target genes in other tissues and during other biological processes remain largely unknown. Whether these proteins function with AP-1 or non-AP-1 partner proteins in non-immune cell types also remains unclear. If the bulk of NFAT functions in non-immune cells were controlled by partner proteins other than AP-1, it might be feasible to manipulate the immune response pharmacologically by inhibiting the NFAT:AP-1 interaction.

Acknowledgments
We thank Lin Chen for Figure 3. This work was supported by NIH grants CA42471 and AI40127 (to A Rao) and by a special fellowship from the Leukemia and Lymphoma Society (to C Lopez-Rodriguez).

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