The nuclear factor of activated T cells (NFAT) group of transcription factors regulates gene expression in immune and non-immune cells. NFAT-mediated gene transcription is orchestrated, in part, by formation of a composite regulatory element. Here we demonstrate that NFAT interacts with transcription factor CCAAT-enhancer-binding protein (C/EBP) to form a composite enhancer complex, to potentiate expression of the peroxisome proliferator-activated receptor-γ2 gene. Formation of a ternary NFAT-C/EBP-DNA complex is required for the transcriptional cooperation. A similar NFAT-C/EBP composite element is found in the regulatory region of the insulin-like growth factor 2, angiotensin-converting enzyme homolog, and transcription factor POU4F3 genes. Thus, the NFAT-C/EBP composite element represents a novel regulatory enhancer to direct NFAT-mediated gene transcription.

Nuclear factor of activated T cells (NFAT)\(^1\) was first identified as an important regulator for cytokine gene expression (1, 2). Subsequent isolation of cDNAs revealed a broad distribution of NFAT in multiple tissues. For example, NFAT regulates expression of type I tumor necrosis factor-α (TNF-α) (3). NFAT regulates expression of b-type natriuretic peptide during cardiac hypertrophy (4). NFAT also modulates skeletal muscle fiber type by directing selective gene expression in slow oxidative myofibers (e.g., myoglobin gene and slow fiber-specific troponin I gene) (5). NFAT also regulates expression of fatty acid-binding protein and transcription factor peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) during adipogenesis (6, 7). Recent microarray analyses have further revealed a wealth of novel NFAT target genes (8–10). As elucidation of the physiological functions of NFAT progresses, many more novel NFAT target genes will be identified. However, the molecular basis that governs the expression of these NFAT target genes remains to be determined.

NFAT interacts with transcription factor AP-1 (Fos and Jun proteins) to form a cooperative composite enhancer (NFAT/AP-1) and to regulate expression of many cytokine genes (11). The cooperative induction is mediated, in part, by protein-protein and protein-DNA interactions. For example, the well characterized antigen receptor-responsive element (ARRE) from the interleukin (IL)-2 gene contains binding sites for NFAT and AP-1 (12–14). The DNA binding domains of NFAT and AP-1 are required for the cooperative interaction on the ARRE enhancer (15, 16). Structural analysis reveals multiple contacts between the DNA binding domains of NFAT and AP-1 (17–19). Conserved residues are responsible for the protein-protein and protein-DNA interactions. Thus, intimate associations between NFAT, AP-1, and DNA are necessary for the transcription cooperation of the IL-2 gene.

Transcription factor peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) plays an important role in adipogenesis (20–23). Multiple transcription factors have been shown to regulate the PPAR-γ2 gene expression. For example, members of the CCAAT/enhancer-binding protein (C/EBP) family induce expression of PPAR-γ2 during adipocyte differentiation (24, 25). Several C/EBP-binding elements have been identified on the PPAR-γ2 gene promoter (26–30). Recently, two distinctive NFAT-binding sites (proximal and distal) are identified on the PPAR-γ2 gene (7). The NFAT- and C/EBP-binding sites are located in the immediate upstream regulatory region of the PPAR-γ2 promoter. Whether NFAT cooperates with C/EBP to regulate the PPAR-γ2 gene expression remains uncertain.

The significance of the PPAR-γ2 NFAT regulatory elements is further implicated by recent DNase I-hypersensitive site studies (31). Two DNase I-hypersensitive sites (DHS1 and DHS2) are found in the PPAR-γ2 gene. DNase I-hypersensitive sites represent regions that are “accessible” for transcription factor binding in the nucleosome-packaged chromatin (32–35). Binding of transcription factors to the DNase I-hypersensitive sites may facilitate chromatin rearrangement and/or transcriptional activation of promoters that are actively engaged in gene expression. Thus, identification of proteins that bind to DNase I-hypersensitive sites is important to understand transcriptional regulation. The PPAR-γ2 distal NFAT element is located between the DHS1 and the DHS2 DNase I-hypersensitive site. The PPAR-γ2-proximal NFAT element is located within DHS1. The proximity of these NFAT elements to the DNase I-hypersensitive sites suggests that understanding the molecular basis of NFAT-mediated transcription will shed new light on the regulation of PPAR-γ2 gene.

The purpose of this study is to examine the molecular basis of NFAT-mediated gene expression. We report that NFAT cooperates with C/EBP to regulate PPAR-γ2 gene expression. The transcription cooperation is mediated, in part, by the formation of a composite NFAT-C/EBP complex on the PPAR-γ2-proximal NFAT element. Promoter analysis reveals that a similar
NFAT/C/EBP complex element regulates transcription of insulin-like growth factor 2, angiotensin-converting enzyme homolog, and transcription factor POUSF4 genes. Thus, the NFAT/C/EBP complex represents a novel composite regulatory element to direct NFAT-mediated gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—HepG2 hepatoma cells were obtained from the Cell Culture Core Facility of the Liver Research Center, Albert Einstein College of Medicine. BHK fibroblasts and HepG2 hepatoma cells were cultured in minimal essential medium. COS and 3T3/L1 cells were cultured in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were transfected by using LipofectAMINE (Invitrogen). 3T3/L1 cells were subjected to differentiation in the presence of insulin (5 μM), dexamethasone (1 μM), and the phosphodiesterase inhibitor isobutylmethylxanthine (0.5 mM).

Reagents—The PPARγ2 promoter luciferase reporter plasmids and the expression vectors for calcineurin, C/EBP, and NFATc4 have been described (7, 36–40). The sequences for the IGF2, ACEH, and POUSF4 genes have been reported (GenBank™ accession number U71085, AF366532, and AF044575, respectively). The IGF2 and ACEH promoters were amplified from genomic DNA and subcloned into pGL3 basic luciferase reporter plasmid using XhoI and XbaI sites. Double-stranded oligonucleotides encoding a triple repeat of the POUSF4 NFAT/C/EBP element were subcloned upstream of the pGL3 promoter luciferase reporter plasmid. The NFATc4 Rel domain was amplified by PCR and subcloned into the pCDNA3 plasmid using NofI and XbaI sites. Mutations on the NFATc4 Rel domain were generated by overlapping PCR.

The NFATc Rel domain was also subcloned into pSET plasmid using PvuII site for recombinant protein expression.

Chromatin Immunoprecipitations—Nuclear factors that were associated with chromatin in differentiated and undifferentiated 3T3/L1 cells were cross-linked to DNA using formaldehyde (1%). Cells were harvested, and cross-linked chromatin was sheared by sonication. Sonicated chromatin in differentiated and undifferentiated 3T3/L1 cells was used for chromatin immunoprecipitations on the NFATc4 Rel domain were generated by overlapping PCR. The NFATc4 Rel domain was also subcloned into pSET plasmid using PvuII site for recombinant protein expression.

DNA Precipitation Assays—Double-stranded biotinylated oligonucleotides were incubated with HepG2 or 3T3/L1 cell extract for 12 h before precipitation with 20 μl of streptavidin-Sepharose (2 h). After three washes in Triton/lysis buffer, precipitated DNA and its associated proteins were separated in SDS-PAGE, and immunoblot was performed to detect endogenous NFAT and C/EBP. For competition analysis, the excess amounts of non-biotinylated oligonucleotides (100 pmol) were added to the precipitation assays.

Gel Mobility Shift Assays—Nuclear extracts were prepared from cultured cells as described (7). Double-stranded oligonucleotides [PPARγ2-proximal NFAT, 5′-ATTACAGGGAAAAATTTGCGCAGCTGCTC-3′; NFAT mutated PPARγ2-proximal NFAT, 5′-ATTACACGGATTATCGCGCAGCTGCTC-3′; C/EBP mutated PPARγ2-proximal NFAT, 5′-ATTACAGGGAAAATATTTCGCTGCTC-3′; both NFAT and C/EBP mutated PPARγ2-proximal NFAT, 5′-ATTACAGGGAAAATATTTCGCTGCTC-3′; both NFAT and C/EBP mutated PPARγ2-proximal NFAT, 5′-ATTACAGGGATTATCGCGCAGCTGCTC-3′; both NFAT and C/EBP mutated PPARγ2-proximal NFAT, 5′-ATTACAGGGATTATCGCGCAGCTGCTC-3′] were added to the precipitation of streptavidin-Sepharose (2 h). After three washes in Triton/lysis buffer, precipitated DNA and its associated proteins were separated in SDS-PAGE, and immunoblot was performed to detect endogenous NFAT and C/EBP. For competition analysis, the excess amounts of non-biotinylated oligonucleotides (100 pmol) were added to the precipitation assays.

In Vitro Translation—T7-coupled in vitro transcription-translation (Promega Inc.) was performed in the presence of [35S]methionine and -cysteine (PerkinElmer Life Sciences). Translated NFATc4 was used in binding assays (41) with recombinant GST or GST-C/EBPβ. Wild-type, NFAT-mutated, or C/EBP-mutated PPARγ2-proximal NFAT oligonucleotides were added as indicated. Bound NFATc4 was visualized by autoradiography, and quantitated by PhosphorImager analysis. Co-immunoprecipitations—Cells extracts prepared using Triton/lysis buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glyceroophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonfyl fluoride, 10 μg/ml leupeptin) were incubated (5 h at 4 °C) with antibodies prebound to protein G-Sepharose. After three washes with Triton/lysis buffer, the bound proteins were separated in SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). Antibodies to detect FLAG-NFATc4 (Sigma), NFATc2, and C/EBP (Santa Cruz Biotechnology) were used for immunoblot analysis. Enhanced chemiluminescence was performed to visualize NFAT and C/EBP.

RESULTS

NFAT Cooperates with C/EBP to Increase PPARγ2 Gene Transcription—We demonstrated previously (7) that NFAT increases PPARγ2 gene expression. Two NFAT-binding elements (proximal and distal) are located in the upstream regulatory region of the PPARγ2 gene (Fig. 1A). Several C/EBP-binding sites are found on the PPARγ2 gene (26–30) (Fig. 1A). We tested whether NFAT cooperates with C/EBP to regulate the PPARγ2 gene expression. PPARγ2 promoter luciferase reporter plasmid was co-transfected with C/EBP expression vectors (C/EBPβ, C/EBPα, or C/EBPβ) into BHK cells. Expression of C/EBP increased the PPARγ2 gene transcription (Fig. 1B). Administration of ionomycin and PMA, to activate the NFAT signaling pathway, also increased the PPARγ2 gene transcription. Importantly, treatment of ionomycin plus PMA cooperated with C/EBP and led to a further increase in the PPARγ2 gene transcription. Increased PPARγ2 promoter activity was blocked by expression of the dominant-negative NFAT inhibitor (Fig. 1C), which has been shown to inhibit specifically NFAT-mediated gene transcription (42–46). Thus, these data indicate that NFAT cooperates with C/EBP to regulate the PPARγ2 gene transcription.

Calcineurin phosphatase binds to and dephosphorylates NFAT (1, 2, 47). Dephosphorylated NFAT then translocates into the nucleus to mediate gene transcription. Targets of the calcineurin-mediated dephosphorylation include the conserved Ser phosphorylation sites found in four NFAT members (7, 48–51). Previous studies (7, 41, 48–50, 52) showed that expression of constitutive active calcineurin promotes NFAT nuclear localization and increases NFAT-mediated gene transcription. Replacement of the conserved Ser residues with Ala, to prevent phosphorylation, also causes constitutive nuclear localization of NFAT and increases NFAT-mediated gene transcription (7, 48–51). We tested whether co-expression of activated cal-
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A schematic representation of the PPARγ2 gene promoter. Identified NFAT- (filled triangles) and C/EBP (open ovals)–binding sites are illustrated. DNase I-hypersensitive sites (DHS1 and DHS2) are also shown (shaded boxes). Deletion to remove upstream regulatory regions of the PPARγ2 luciferase reporter plasmid is also indicated. B and C, BHK cells were transfected with PPARγ2 promoter (−1 to −200) luciferase reporter plasmid and expression vectors for different C/EBP members (C/EBPα, C/EBPβ, or C/EBPδ) (B). Expression vector for dominant-negative NFAT (dnNFAT) was transfected as indicated (C). The cells were stimulated without (Untreated) and with ionomycin (I, 2 μM) plus PMA (P, 100 nM) for 16 h before harvest. Transfection efficiency was monitored by measurement of β-galactosidase activity. D and E, BHK cells were transfected with PPARγ2 promoter (−1 to −200) luciferase reporter plasmid and expression vectors for different C/EBP members (C/EBPα, C/EBPβ, or C/EBPδ) and/or constitutive active calcineurin (ΔCN) (D). Expression vectors for different constitutive nuclear NFAT members (cnNFATc1, cnNFATc3, or cnNFATc4) were also transfected as indicated (E). Cells were harvested 36 h after transfection. Luciferase and β-galactosidase activities were measured. F, different PPARγ2 promoter luciferase reporter plasmids were co-transfected with C/EBPα in BHK cells as indicated. The cells were stimulated without (Untreated) and with ionomycin (I, 2 μM) plus PMA (P, 100 nM) for 16 h before harvest. Transfection efficiency was monitored by measurement of β-galactosidase activity.

NFAT and C/EBP Interact with the PPARγ2 Promoter in Vivo—Sequence analysis indicated that a C/EBP-binding site is located immediately adjacent to the PPARγ2-proximal NFAT site (Fig. 1). The configuration of the NFAT-C/EBP element on the PPARγ2-proximal NFAT site is analogous to the established composite NFAT−AP-1 enhancer found on the IL-2 gene (see Fig. 4). Because the proximal NFAT element is critical for the transcription cooperation of the PPARγ2 gene and is located within the DHS1 hypersensitive site, we hypothesized that NFAT interacts with C/EBP, to form a novel composite enhancer, to mediate transcription cooperation of the PPARγ2 gene.

To examine in vivo binding of NFAT and C/EBP to the PPARγ2 promoter, we performed chromatin immunoprecipitations (Fig. 2A). 3T3/L1 cells were subjected to differentiation in the presence of insulin, dexamethasone, and IBMX. After 6 days in differentiation media, 3T3/L1 cells became lipid-laden adipocytes. Chromatins from undifferentiated and different stages of differentiating 3T3/L1 cells, after cross-linked with formaldehyde, were harvested. Isolated chromatins were immunoprecipitated with C/EBPβ, NFATc2 or NFATc4 antibodies. The PPARγ2 promoter in the precipitates was detected by PCR amplification. Chromatin immunoprecipitations indicated that neither C/EBPβ nor NFAT interacted with the PPARγ2 promoter in undifferentiated (day 0) 3T3/L1 cells. Two days after initiation of adipocyte differentiation, the PPARγ2 promoter was detected in the C/EBPβ, but not NFAT, precipitates. On day four of adipocyte differentiation, both C/EBPβ and NFAT (NFATc2 and NFATc4) interacted with the PPARγ2 promoter. Interestingly, interaction of NFAT and C/EBP with
A

![Diagram A](image1.png)

B

![Diagram B](image2.png)

C

![Diagram C](image3.png)

**Fig. 2. NFAT and C/EBP interacts with the PPARγ2 promoter in vivo.** A, binding of NFAT and C/EBPβ to the PPARγ2 promoter at different stages of adipocyte differentiation. 3T3/L1 cells were subjected to adipocyte differentiation in the presence of insulin, dexamethasone, and isobutylmethylxanthine. Chromatin immunoprecipitations were performed to precipitate NFAT (NFATc2 and NFATc4) and C/EBPβ-regulated promoters. DNA isolated was amplified by PCR using oligonucleotides encoding the PPARγ2 promoter. B and C, extracts prepared from HepG2 or 3T3/L1 cells were incubated with biotinylated PPARγ2-proximal or -distal NFAT oligonucleotides. Protein-DNA complexes were precipitated by streptavidin-Sepharose and resolved in SDS-PAGE, and immunoblotting analyses were performed to detect bindings of endogenous NFAT and C/EBP (B). Excess amount of non-biotinylated PPARγ2-proximal elements were used in competition analysis to indicate specificity of NFAT-C/EBP interaction (C).

The PPARγ2 gene promoter precedes the full induction of PPARγ2 protein expression, which occurs after day four of adipocyte differentiation (20). Nonetheless, these data indicate temporal requirements of NFAT and C/EBP in the PPARγ2 gene transcription. Association of NFAT with the PPARγ2 promoter in the early stage supports previous studies that NFAT inhibition blocks adipocyte differentiation (6).

We further examined whether NFAT and C/EBP were present in the proximal NFAT element by DNA precipitation assays. Biotinylated oligonucleotides encoding the PPARγ2-proximal and distal NFAT elements were incubated with extracts isolated from HepG2 or 3T3/L1 cells, which express NFAT, C/EBP and PPARγ endogenously. The biotinylated oligonucleotides and their associated proteins were then precipitated using streptavidin-Sepharose and separated on SDS-PAGE. Immunoblotting analysis indicated the presence of NFATc2 in the DNA precipitates (Fig. 2B). C/EBPα and C/EBPβ were detected in the PPARγ2-proximal, but not the distal, NFAT complex. These data suggest that the PPARγ2-proximal and distal NFAT elements form different NFAT complexes (7).

We also performed competition analysis to demonstrate specificity of NFAT-C/EBP interaction (Fig. 2C). Incubation with excess amount of wild-type, but not the mutated, non-biotinylated PPARγ2-proximal oligonucleotides abolished both NFAT and C/EBP binding. Together, these data indicate that C/EBP binds to the PPARγ2-proximal NFAT element.

**The PPARγ2-proximal NFAT Composite Element Binds NFAT and C/EBP—**Next, we performed gel mobility shift assays to examine binding of C/EBP on the PPARγ2-proximal NFAT element (Fig. 3). Nuclear extracts were prepared from cells transfected with NFATc4, C/EBPα, and NFATc4 plus C/EBPα. Immunoblotting analysis revealed similar expression of NFATc4 and C/EBPα in different nuclear extracts (Fig. 3B). NFATc4 or C/EBPα interacted with the PPARγ2-proximal NFAT element and formed distinctive protein-DNA complexes (Fig. 3B). Co-expression of NFATc4 with C/EBPα markedly increased the formation of protein-DNA complexes. The NFAT-C/EBP-DNA complex exhibited slight decrease in electrophoretic mobility as compared with the NFAT-DNA complex (see asterisks). Importantly, there is a cooperative interaction between NFATc4 and C/EBPα upon binding to the PPARγ2-proximal NFAT element. Antibody supershift analysis further revealed specificity and cooperativity between NFAT and C/EBP in the formation of the NFAT-C/EBP-DNA complex. These data indicate that NFAT and C/EBP form a cooperative complex on the PPARγ2-proximal NFAT element.

Previous studies indicated that mutation at the NFAT-binding site of the IL-2 NFAT composite element reduces AP-1 binding (16, 53–55). These results indicate that although consensus AP-1-binding site is found on the IL-2 NFAT composite element, AP-1 requires the presence of bound NFAT to promote DNA binding. To further examine interaction between NFAT and C/EBP on the PPARγ2-proximal NFAT element, we performed mutagenesis to abolish either the NFAT- or the C/EBP-binding site (Fig. 3A). Gel mobility shift assays indicated that mutation at the cognate NFAT-binding site abolished NFAT interaction (Fig. 3B). In addition, the NFAT mutated PPARγ2-proximal NFAT element failed to bind C/EBP. These data indicate that C/EBP requires the presence of bound NFAT to stabilize DNA binding.

We also examined formation of NFAT-DNA complexes from the C/EBP mutated PPARγ2-proximal NFAT element (Fig. 3B). Mutation at the cognate C/EBP site abolished C/EBP interaction. In addition, the C/EBP mutated PPARγ2-proximal NFAT element failed to exhibit cooperation in the formation of NFAT-DNA complex. Antibody supershift analysis further supported the loss of cooperation. Together, these data indicate that simultaneous binding of NFAT and C/EBP are required for the cooperative interaction on the PPARγ2-proximal NFAT element.

Previous studies established that recombinant NFAT and AP-1 proteins support cooperative interaction on the IL-2 NFAT element (14, 15, 56). To further confirm the cooperative binding of NFAT and C/EBP, we performed gel mobility shift assays using recombinant NFATc4 and C/EBPβ proteins (Fig. 3C). Recombinant NFATc4 or C/EBPβ proteins interacted with the PPARγ2-proximal element (see asterisks). NFATc4 and C/EBPβ also formed multimers on the PPARγ2-proximal element (see arrowheads). Combination of minimal amount of the NFATc4 protein with the C/EBPβ protein markedly increased the formation of NFAT-C/EBP-DNA complexes. Importantly, the NFAT-C/EBP-DNA complex exhibited a decrease in electrophoretic mobility as compared with the NFAT-DNA or the C/EBP-DNA complexes (see asterisks). Also, there is a cooperative interaction between NFATc4 and C/EBPβ upon binding to the PPARγ2-proximal NFAT element. These data further indicate that NFAT and C/EBP form a cooperative complex on the PPARγ2-proximal NFAT element.

Next, we examined the dissociation of the NFAT-C/EBP complexes on the PPARγ2-proximal NFAT element (Fig. 3D). Pre-
The PPARγ2-proximal NFAT composite element binds NFAT and C/EBP. A and B, co-expression of C/EBPα with NFATc4 increases formation of NFAT-DNA complexes on the PPARγ2-proximal NFAT element. The NFAT- (filled box) and the C/EBP (shaded box)-binding sites on the PPARγ2-proximal NFAT composite element are shown (A). Mutations to abolish NFAT or C/EBP binding are also illustrated (underlined). Nuclear extracts prepared from cells expressing NFATc4, C/EBPα, or NFATc4 and C/EBPα were incubated with 32P-labeled double-stranded oligonucleotides encoding the PPARγ2-proximal NFAT composite element (B). Gel mobility shift assays were performed in the presence (+) or absence (−) of antibody against NFAT or C/EBPα. Oligonucleotides with mutated NFAT-binding site, mutated C/EBP-binding site,
assembled NFAT-C/EBP-DNA complexes were competed with excess amount of unlabeled wild-type, NFAT-mutated, or C/EBP-mutated PPAR2-proximal NFAT elements. The PPAR2 distal NFAT element and the consensus C/EBP-binding site were also used to test NFAT and C/EBP binding, respectively. Competition analysis indicated that the wild-type and the NFAT-mutated PPAR2-proximal NFAT elements promoted dissociation of NFAT-C/EBP complex to a similar extent. Both oligonucleotides required about 10 min to dislodge 50% off the preassembled NFAT-C/EBP-DNA complex. The consensus C/EBP-binding site required about 5 min to attain 50% dissociation. In contrast, the C/EBP-mutated PPAR2-proximal NFAT element and the PPAR2 distal NFAT element required over 20 min to remove 50% of the NFAT-C/EBP-DNA complex. These data indicate that C/EBP determines the dissociation of the NFAT-C/EBP complex on the PPAR2-proximal NFAT element.

Gel mobility shift assays indicate that both NFAT- and C/EBP-binding sites are required for stable formation of the NFAT-C/EBP complex on the PPAR2-proximal NFAT element (Fig. 3). In contrast, C/EBP is the determining factor for the dissociation of the NFAT-C/EBP-DNA complex. Next, we examined the contribution of the NFAT- and the C/EBP-binding site of the proximal NFAT element on the transcriptional cooperation of the PPAR2 promoter (Fig. 3E). Mutation at the NFAT-binding site of the PPAR2-proximal NFAT element abolished transcriptional cooperation. Mutation at the C/EBP-binding site of the PPAR2-proximal NFAT element also abolished transcriptional cooperation. These data suggest that simultaneous binding of NFAT and C/EBP on the proximal NFAT element is required for the transcriptional cooperation of the PPAR2 gene.

**Preferential Binding of NFAT and C/EBP on the PPAR2-proximal and the IL-2 NFAT Elements, Respectively**—Co-expression of NFAT and C/EBP markedly increases formation of the NFAT-C/EBP-DNA complex on the PPAR2-proximal NFAT element (Fig. 3), indicating a cooperative interaction of NFAT and C/EBP upon DNA interaction. To examine further cooperative binding between NFAT and C/EBP on the PPAR2 NFAT element, we combined nuclear extracts, prepared from NFATc4, C/EBP, or c-Jun-expressing cells, to perform gel mobility shift assays (Fig. 4A). Increasing the amount of C/EBP proteins enhanced the formation of NFAT-DNA complexes on the PPAR2-proximal element. However, increasing the amount of c-Jun had minimal effect on the formation of NFAT-DNA complexes. Increasing the amount of NFATc4 proteins also increased the amount of NFAT-DNA complexes in the presence of C/EBP but not C/EBP. Using the ARRE NFAT site of the IL-2 gene as a probe increased the amount of NFATc4-enhanced formation of the NFAT-DNA complexes in the presence of c-Jun but not C/EBP. Increasing the amount of c-Jun, but not C/EBP, also increased formation of NFAT-DNA complexes on the IL-2 NFAT element. These data indicate that NFAT preferentially interacts with C/EBP on the PPAR2-proximal NFAT element. Conversely, NFAT prefers to interact with c-Jun on the IL-2 ARRE NFAT site.

To examine further preferential binding of C/EBP and AP-1 on different NFAT elements, we performed competition using unlabeled AP-1 and C/EBP consensus oligonucleotides (Fig. 4B). The C/EBP oligonucleotides reduced formation of NFAT-DNA complexes on the PPAR2-proximal NFAT element more effectively than the AP-1 oligonucleotides. Conversely, the AP-1 oligonucleotides markedly reduced formation of NFAT-DNA complexes on the IL-2 NFAT element. These data further reveal preferential interaction of C/EBP and AP-1 on the PPAR2-proximal and the IL-2 NFAT element, respectively.

**NFAT Interacts with C/EBP—NFAT may interact with C/EBP to promote formation of a ternary complex with the PPAR2-proximal NFAT element.** We prepared cell extracts from HepG2 or 3T3/L1 cells to examine whether NFAT interacts with C/EBP endogenously. Co-immunoprecipitation assays indicated that NFATc2 interacted with C/EBPα or C/EBPβ in vivo (Fig. 5A). Thus, there are interactions between NFAT-DNA, C/EBP-DNA, and NFAT-C/EBP to promote formation of a ternary complex on the PPAR2-proximal NFAT element.

Next, we mapped the C/EBP binding domain on NFAT (Fig. 5B). A series of NFATc4 deletion mutants were co-expressed with C/EBPα in COS cells. Immunoblot analysis indicated expression of NFATc4 proteins (Fig. 5B). Co-immunoprecipitation assays indicated that NFATc4 interacted with C/EBPα. Deletion of the COOH-terminal domain (residues 451–902) of NFATc4 had minimal effect on the interaction with C/EBPα. Further deletion to residues 365 of NFATc4, to remove the Rel homology DNA binding domain, abolished C/EBPα interaction. These data indicate that the Rel homology domain of NFATc4 is important for the interaction with C/EBPα.

We further generated the NFATc4 mutant that contains the Rel homology DNA binding domain to examine NFAT-C/EBP interactions (Fig. 5C). Co-immunoprecipitation assays revealed the presence of NFATc4 Rel proteins in the C/EBPα precipitates. These data confirm that the NFAT Rel homology domain interacts with C/EBP.

We also mapped the NFAT-binding site on the C/EBP proteins. We performed binding assays using recombinant NFAT and in vitro translated C/EBP (Fig. 5D). Deletion analysis indicated that removal of the conserved C/EBP DNA binding domain abolished NFAT interaction. These data indicate that the DNA binding domains of NFAT and C/EBP are required for interaction upon binding to the PPAR2-proximal element.

To determine whether NFAT directly associates with C/EBP, we performed binding assays using recombinant C/EBP and in vitro translated NFAT (Fig. 5E). Recombinant GST was used as a control. Binding assays indicated that NFATc4 was detected or both NFAT- and C/EBP-binding sites mutated were also used to examine NFAT-DNA complex formation. The positions of the NFAT-DNA complexes are indicated by asterisks. NFAT and C/EBP also formed multimers on the PPAR2-proximal element (arrowheads). The amounts of NFAT-DNA complexes were quantified by PhosphorImager analysis. Expression of FLAG epitope-tagged NFATc4 and C/EBPα in transfected COS cells was detected by M2 and C/EBPα antibody, respectively. C, cooperative DNA binding using recombinant NFATc4 and C/EBPβ proteins. Different concentrations of NFATc4 and C/EBPβ proteins were incubated with the PPAR2-proximal element in gel mobility shift assays. The positions of the NFAT-DNA, C/EBP-DNA, and NFAT-C/EBP-DNA complexes are indicated by asterisks. NFAT and C/EBP also formed multimers on the PPAR2-proximal element (arrowheads). The amounts of NFAT-DNA complexes were quantified by PhosphorImager analysis. D, dissociation of the NFAT-C/EBP complexes. 32P-Labeled PPAR2-proximal NFAT element was incubated with nuclear extracts prepared from cells expressing NFATc4 and C/EBPα for 30 min. Excess amounts of unlabeled oligonucleotides were used as competitors and incubated with the preassembled NFAT-C/EBP-DNA complexes for the indicated times (0, 2, 5, 10, and 20 min). The NFAT-C/EBP complexes were separated by non-denaturing polyacrylamide gel electrophoresis, and the amounts of remaining complexes were quantitated by PhosphorImager analysis and plotted against time of competition. E, mutation at the NFAT- or the C/EBP-binding site on the proximal NFAT element abolished transcription cooperation of the PPAR2 promoter. Site-directed mutagenesis was performed to abolish (X) NFAT (filled triangle) or C/EBP (open oval) binding to the proximal NFAT element of the (–1 to –273) PPAR2 promoter. Expression constructs for constitutive nuclear NFATc4 and C/EBPα were co-transfected with PPAR2-luciferase reporter plasmids to examine transcription cooperation. Cells were harvested 36 h after transfection. Luciferase and β-galactosidase activities were measured.
in the C/EBP but not GST precipitates. These data indicate that NFAT directly associates with C/EBP.

Gel mobility shift assays indicate formation of a cooperative ternary NFAT-C/EBP-DNA complex on the PPARγ2-proximal NFAT element. Increasing amounts of C/EBPα or c-Jun were premixed with the NFATc4 nuclear extract (left panels). Conversely, increasing amounts of NFATc4 were premixed with the C/EBPα or the c-Jun nuclear extracts (right panels). The combined nuclear extracts were incubated with 32P-labeled PPARγ2-proximal (top panels) or IL-2 (bottom panels) NFAT oligonucleotides to examine formation of the NFAT-DNA complex in gel mobility shift assays. B, the PPARγ2-proximal and the IL-2 NFAT elements were incubated with HepG2 nuclear extracts. Excess amounts of unlabeled oligonucleotides encoding the canonical binding site for AP-1 or C/EBP were used as competitors. Gel mobility shift assays were performed to examine formation of NFAT-DNA complexes.

The Rel Domain of NFATc4 Is Critical for Interaction with C/EBP—Previous structural analysis indicated that Arg468, Ile469, and Thr535 on the Rel homology domain of NFATc2 interact with AP-1 (Fos-Jun) to promote formation of a ternary complex on the IL-2 NFAT element (17–19). Similar amino acid residues are found in the Rel domain of other NFAT members (Arg488, Tyr489, and Thr555 of NFATc3; and Arg474, Asn475, and Thr541 of NFATc4), suggesting conserved interactions with AP-1 (11, 15). Because AP-1 and C/EBP are basic leucine zipper type transcription factors, we tested whether similar amino acid residues on the NFAT Rel domain are required for the interaction with C/EBP (Fig. 6A). Replacement of Arg474 and Asn475 with Ala and Thr541 with Gly of NFATc4 abolished NFAT-C/EBP interaction. The R474A/N475A/T541G NFATc4 also exhibited reduced binding to c-Jun in co-immunoprecipitation assays. These data indicate conserved interactions in NFAT-C/EBP and NFAT-AP-1 complexes.

Reduced interaction between NFAT and C/EBP by mutation at the Rel domain may affect formation of the ternary NFAT-C/EBP-DNA complex. We performed gel mobility shift assays to determine the relative binding affinity of wild-type and R474A/N475A/T541G NFATc4 to the PPARγ2-proximal NFAT element (Fig. 6B). Immunoblot analysis indicated similar expression of NFATc4 and C/EBP proteins. Binding of the wild-type and R474A/N475A/T541G NFATc4 to the C/EBP-mutated
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PPARγ2-proximal NFAT element was also similar, supporting previous studies (15, 19) that distinct residues on the Rel domain interact with Fos, Jun, and DNA. Saturation binding assays, however, indicated that the wild-type NFATc4 exhibited about 4-fold higher affinity than the R474A/N475A/T541G NFATc4 toward binding to the PPARγ2-proximal NFAT element. Increased amounts of NFAT-C/EBP DNA complexes were also detected when increasing the amount of the wild-type, but not the mutated R474A/N475A/T541G, NFATc4 was combined with C/EBP prior to gel mobility shift assays (Fig. 6C). Together, these data confirm that formation of a cooperative complex on the PPARγ2-proximal NFAT site requires protein-protein interaction between NFAT and C/EBP.

We further examined transcription activity mediated by NFATc4 cooperated with C/EBPα and increased PPARγ2 gene transcription (Figs. 1 and 6D). Co-expression of the constitutive nuclear NFATc4 with C/EBPα also increased reporter gene activity mediated by multiple repeats of the PPARγ2-proximal NFAT composite element. To facilitate nuclear accumulation of the C/EBP binding defective R474A/N475A/T541G NFATc4, conserved Ser residues (Ser168 and Ser170) were replaced with Ala to prevent phosphorylation and to promote nuclear localization. In the absence of C/EBP, expression of S168A/S170A/R474A/N475A/T541G NFATc4 directed NFAT-mediated activity to a similar extent as the constitutive nuclear NFATc4. However, S168A/S170A/R474A/N475A/T541G NFATc4 had minimal effect to further increase C/EBPα-activated PPARγ2

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NFAT1, NFAT2, NFAT3, and NFAT4) (1, 46). The NFATs bind as a hetero-dimeric complex. FLAG epitope-tagged wild-type and mutated R474A/N475A/T541G NFATc4 were co-expressed with C/EBPα or c-Jun in COS cells. NFATc4 proteins were immunoprecipitated (IP) from COS cell extracts without (−) and with (+) M2 monoclonal antibody (anti-NFAT). C/EBPα or c-Jun in the immunoprecipitates was detected by immunoblot (IB) analysis. Expression of NFATc4 proteins is also indicated. B, NFAT-C/EBP interactions are important for the formation of a cooperative complex. FLAG epitope-tagged wild-type and mutated R474A/N475A/T541G NFATc4 were co-expressed with C/EBPα in COS cells. Nuclear extracts were prepared and incubated with increasing amounts of 32P-labeled PPARγ-2-proximal NFAT element (40, 80, 120, 160, and 200 fmol). The amount of NFAT-C/EBP complexes and remaining probe were quantitated by PhosphorImager analysis. The ratio of bound/free was plotted against the amount of bound to assess relative binding affinity. Expression of NFATc4 and C/EBPα is shown. Binding of wild-type and mutated R474A/N475A/T541G NFATc4 to the C/EBP mutated PPARγ-2-proximal NFAT element is also indicated. C, increasing amounts of wild-type and mutated R474A/N475A/T541G NFATc4 were premixed with the C/EBPα nuclear extracts. The combined nuclear extracts were incubated with 32P-labeled PPARγ-2-proximal NFAT oligonucleotides to examine formation of NFAT-DNA complex in gel mobility shift assays. Expression of NFATc4 proteins is also shown. D, NFAT-C/EBP interactions are important for transcription cooperation of the PPARγ-2 gene. Expression vectors for constitutive nuclear NFATc4 (cnNFATc4) and C/EBPα were co-transfected with the PPARγ-2 (−1 to −273) luciferase reporter plasmid to examine transcription cooperation. Luciferase reporter plasmid containing a triple repeat of the PPARγ-2-proximal NFAT-C/EBP element was also examined. The effect of R474A/N475A/T541G mutation on cnNFATc4-mediated transcription was also examined. Cells were harvested 36 h after transfection. Luciferase and β-galactosidase activities were measured. p values are compared with cnNFATc4-mediated transcription activity.

gene transcription. Together, these data establish that transcription cooperation of the PPARγ-2 gene by NFAT and C/EBP requires interactions between NFAT-DNA, C/EBP-DNA, and NFAT-C/EBP.

**NFAT-C/EBP Composite Elements Regulate Other Gene Expressions**—The NFAT-C/EBP composite enhancer element represents a novel regulatory motif to direct NFAT-mediated gene transcription. Similar NFAT-C/EBP-binding elements are present in the regulatory regions of the IGF2, ACEH, and transcription factor POU4F3 (POU4F3) genes (Fig. 7A). Gel mobility shift assays revealed binding of NFAT and C/EBP on these regulatory elements (Fig. 7, B and C). Importantly, either co-expression or combination of NFAT and C/EBP further increased formation of NFAT-DNA complexes, indicating a cooperative effort of NFAT and C/EBP in gene regulation.

We also examined the function of these NFAT-C/EBP complexes using reporter gene assays (Fig. 7D). Expression of constitutive nuclear NFAT or C/EBP increased IGF2 and ACEH gene transcription. Co-expression of constitutive nuclear NFAT and C/EBP further increased IGF2 and ACEH gene transcription. Co-expression of constitutive nuclear NFAT and C/EBP also increased gene transcription mediated by a triple repeat of the POU4F3 NFAT-C/EBP element was also examined. The effect of R474A/N475A/T541G mutation on cnNFATc4-mediated transcription was also examined. Cells were harvested 36 h after transfection. Luciferase and β-galactosidase activities were measured. p values are compared with cnNFATc4-mediated transcription activity.

**DISCUSSION**

**NFAT Partners**—NFAT mediates gene transcription in immune and non-immune cells. Characterization of the antigen receptor-response element (ARRE) indicated that a cytoplasmic (NFATc) and a nuclear (NFATn) component cooperate together to regulate IL-2 gene transcription (13, 45). The NFATc component was subsequently identified as a family of transcription factors (NFATc1, NFATc2, NFATc3, NFAT4, NFAT5; and NFATc4, NFAT3) (1,
2). The NFATc group of proteins translocates into the nucleus in response to elevated intracellular calcium. NFATc then co-operates with NFATn to mediate gene transcription. The Fos and Jun groups of transcription factors (AP-1 proteins) were identified as the NFATn component (12, 14). Sequence analysis indicates a close proximity of the NFAT- and the AP-1-binding sites on the ARRE element. Molecular and structural analyses further demonstrate intimate associations to promote cooperative binding of NFAT and AP-1 (15, 17–19). Thus, NFAT and AP-1 forms a composite enhancer complex to mediate IL-2 gene transcription.

In this report, we demonstrate that NFAT interacts with C/EBP to form a composite element to regulate several gene transcriptions. Thus, C/EBP represents a novel NFATn component. Interaction between NFAT and C/EBP is analogous to the NFAT/Rel domain. Indeed, similar amino acid residues on the NFAT/Rel domain are required for the interaction with C/EBP or AP-1. These data demonstrate an intimate association between NFAT, C/EBP, and DNA. Further structural analysis will reveal whether a ternary complex is formed by NFAT/C/EBP/DNA.

C/EBP and AP-1 are both bZIP-type transcription factors. Conserved interactions of C/EBP and AP-1 with the NFAT Rel domain suggest that other bZIP transcription factors may complex with NFAT to mediate transcription cooperation. These observations also suggest that the DNA sequence immediately adjacent to the NFAT site may govern preferential formation of NFAT/C/EBP, NFAT/AP-1, or NFAT-bZIP complexes.

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erate with NFAT to regulate gene expression (57–59). Whether NFAT interacts with ATF2, c-Maf, or other bZIP factors to form a similar cooperative complex will require identification of optimal binding sequence for their DNA interaction. Alternatively, these bZIP factors may heterodimerize with Fos, Jun, or C/EBP to promote DNA binding and thus circumvent the requirement of a cognate binding sequence.

Other transcription factors, including Oct1, MEF2, GATA, and Sp1, have been reported to cooperate with NFAT to mediate gene transcription (4, 60–64). Their mode of cooperation with NFAT, however, seems to be different from the NFAT/C/EBP or the NFATAP-1 complex. Although NFAT interacts with Oct1, MEF2, GATA, or Sp1, cooperative association of NFAT with these factors upon DNA binding has not been demonstrated. The lack of cooperative interaction is probably because they are further apart in their cognate binding sequences. Delineating the molecular mechanisms of these NFAT cooperative associations remains to be ascertained.

**NFAT/C/EBP and NFATAP-1 Composite Regulatory Elements—** NFAT was first identified as an important regulator in IL-2 gene expression (12, 45, 54). Many other genes were subsequently identified as NFAT targets (1, 2, 47, 65). Cooperative interaction between NFAT and AP-1 is critical for expression of these genes. Multiple members of the Jun (c-Jun, JunB and JunD) and the Fos (c-Fos, FosB, Fra1, and Fra2) group of transcription factors are indicated in the formation of the NFATAP-1 complex (12, 14, 53). Homo- and heterodimerizations between different Fos and Jun members further provide a wide selection of AP-1 proteins to interact with NFAT. Thus, the NFATAP-1 regulatory element is likely composed of different AP-1 dimers.

The C/EBP group of transcription factors (C/EBPα, C/EBPβ, C/EBPδ, NF-IL6, LAP, CRP2; C/EBPγ, Ig/EBP; C/EBPβ, CRP3; C/EBPε, CRP1; and C/EBPζ, CHOP, Gadd153) is expressed in a variety of tissues (66–71). Homo- and heterodimerizations of C/EBP lead to transcriptional regulation in multiple biological processes. Alternative usage of different translational start sites further generates transcriptional activators or repressors of C/EBP (72, 73). Analogous to the NFATAP-1 complex, the NFATAP-1 complex is probably composed of a wide array of C/EBP homo- and heterodimers. Interactions with C/EBP transcriptional activators or repressors suggest an additional level of regulation in NFAT/C/EBP-mediated gene transcription. Thus, different partners modulate NFAT-mediated gene transcription.

The NFAT-dependent DNA binding of C/EBP (or AP-1) indicates that activation of both NFAT and C/EBP (or AP-1) is required for gene transcription. Dephosphorylation mediated by the calcium-activated calcineurin phosphatase is important for the NFAT activation (1, 2, 47). Conversely, phosphorylation regulates C/EBP and AP-1. The extracellular signal-regulated kinase-mitogen-activated protein kinase pathway, including the extracellular signal-regulated kinase-activated p90 ribosomal S6 kinase, phosphorylates C/EBPβ and C/EBPδ (74–78), whereas the c-Jun NH2-terminal kinase-mitogen-activated protein kinase pathway is important for the c-Jun activation (79–81). The p38 mitogen-activated protein kinase pathway regulates C/EBPβ CHOP Gadd153 (82). Integration of multiple signaling pathways that lead to the activation of NFAT, C/EBP, or AP-1 suggests that NFAT/C/EBP- or NFATAP-1-mediated gene transcription is critical in multiple biological processes. These observations also suggest that NFAT is a ubiquitous regulator.

It is likely that the NFAT/C/EBP and the NFATAP-1 composite regulatory elements will direct transcription of different subsets of NFAT target genes. These NFAT target genes may be differentially transcribed because of restricted tissue expression of C/EBP and AP-1. In addition, temporal expression of C/EBP and AP-1 may provide another level of regulation. For example, different C/EBP members regulate gene transcription at various stages of adipocyte differentiation (24, 25). Different signaling pathways may feed into the NFAT/C/EBP or the NFATAP-1 complex to accomplish differential expression of NFAT target genes as well. Further studies to identify the distinct pools of NFAT targets that are transcribed under cooperation with C/EBP or AP-1 will shed light on the molecular basis of NFAT-mediated gene transcription.

In this study, we identify IGF2, ACEH, and POUF3 as novel targets of NFAT. Sequence analysis indicates that the NFAT/C/EBP-binding site is located in close proximity to a DNase I-hypersensitive site on the IGF2 gene (83, 84). DNase I-hypersensitive sites on the ACEH and the POUF3 genes remain to be determined. Because the NFAT/C/EBP complex of the proximal NFAT element is located within the DHS1 of the PPARγ2 gene (Fig. 1), it is tempting to speculate that NFAT and C/EBP are common residents of DNase I-hypersensitive sites. Future studies to test this hypothesis are warranted.

In conclusion, we have identified C/EBP as a new NFATn component. Formation of NFAT/C/EBP composite enhancer complex expands the repertoire of gene transcription mediated by NFAT.

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