MOLECULAR AND CELLULAR BIOLOGY, Apr. 1995, p. 2071–2079 Vol. 15, No. 4
0270-7306/95/$04.00+0

Human Granulocyte-Macrophage Colony-Stimulating Factor Enhancer Function Is Associated with Cooperative Interactions between AP-1 and NFATp/c

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Received 8 August 1994/Returned for modification 26 September 1994/Accepted 10 January 1995

The promoter of the human granulocyte-macrophage colony-stimulating factor gene is regulated by an inducible upstream enhancer. The enhancer encompasses three previously defined binding sites for the transcription factor NFAT (GM170, GM330, and GM550) and a novel NFAT site defined here as the GM420 element. While there was considerable redundancy within the enhancer, the GM330, GM420, and GM550 motifs each functioned efficiently in isolation as enhancer elements and bound NFATp and AP-1 in a highly cooperative fashion. These three NFAT sites closely resembled the distal interleukin-2 NFAT site, and methylation interference assays further defined GGA(N)$_9$TCA as a minimum consensus sequence for this family of NFAT sites. By contrast, the GM170 site, which also had conserved GGA and TCA motifs but in which these motifs were separated by 15 bases, supported strong independent but no cooperative binding of AP-1 and NFATp, and this site functioned poorly as an enhancer element. While both the GM330 and GM420 elements were closely associated with the inducible DNase I-hypersensitive site within the enhancer, the GM420 element was the only NFAT site located within a 160-bp *Hin*cII-*Bal*I fragment defined by deletion analysis as the essential core of the enhancer. The GM420 element was unusual, however, in containing a high-affinity NFATp/c-binding sequence (TGGAAAGA) immediately upstream of the sequence TGACATCA which more closely resembled a cyclic AMP response-like element than an AP-1 site. We suggest that the cooperative binding of NFATp/c and AP-1 requires a particular spacing of sites and that their cooperativity and induction via independent pathways ensure very tight regulation of the granulocyte-macrophage colony-stimulating factor enhancer.

The expression of the closely linked GM-CSF and IL-3 genes is induced in T cells at the transcriptional level in response to activation of the T-cell receptor and other surface molecules (19, 23). GM-CSF and IL-3 form part of the cytokine network directing the proliferation, differentiation, and function of hemopoietic cells (23). In humans, the expression of IL-3 is largely restricted to T cells, while the expression of GM-CSF is much more widespread, with GM-CSF typically being expressed in tissues at sites of inflammation.

In human T cells, the IL-3/GM-CSF locus is regulated by a CsA-sensitive enhancer that increases the inducible activities of both the GM-CSF and IL-3 promoters (8). The enhancer coincides with an inducible DH site located 3 kb upstream of the GM-CSF gene, within the 10.5-kb region that separates the IL-3 and GM-CSF genes (Fig. 1A). The enhancer increases the activity of the GM-CSF promoter by an order of magnitude and responds to signals that mimic activation of the T-cell receptor. Transfection studies with Jurkat T cells indicate that activation of the enhancer depends upon both induction of Ca^{2+} flux and mobilization of protein kinase C and that this activation is blocked by CsA (8). This same pattern of CsA-sensitive induction exists for the transcription factor NFAT (nuclear factor of activated T cells) (28, 30, 31), which was previously shown to mediate the CsA-sensitive activation of the IL-2 promoter (21). It is therefore significant that the only transcription factor-binding sites previously identified within the enhancer are three NFAT-binding sites and a weak AP-1-binding site (8) (Fig. 1B).

NFAT is a heterogeneous complex that typically contains members of both the NFAT and AP-1 families of transcription factors (4, 8, 17, 18, 24, 26), and the induction of NFAT consequently requires at least two distinct activation pathways (17, 33). The NFAT family currently consists of two closely related proteins, termed NFATp and NFATc (22, 25), that share a conserved DNA-binding domain which itself resembles the conserved DNA-binding domain of the Rel/NF-κB family of transcription factors (24, 28). NFATp and NFATc are expressed in the cytoplasm in T cells and translocate to the nucleus in response to increases in cytosolic Ca^{2+} through a process requiring the CsA-inhibitable phosphatase calcineurin (7, 27). Although NFATp is expressed constitutively in the cytoplasm, NFATc is reported to be induced at the transcriptional level upon T-cell activation (25). NFATp and NFATc have similar sizes and can bind independently to the IL-2 NFAT site at a low affinity to generate indistinguishable complexes in gel mobility shift assays. In the nucleus, NFATp and NFATc typically associate with NFAT sites in conjunction with members of the AP-1 family of proteins (4, 18, 26), even though NFAT sites sometimes have very poor AP-1-binding sites (28). AP-1 ordinarily binds to the consensus sequence TGAGTCA as a heterodimer of a member of the Fos family

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and a member of the Jun family of proteins (1, 9). The AP-1 proteins are induced principally via the Ras and protein kinase C pathways, thus accounting for the dependence of NFAT upon both the Ca^{2+} and protein kinase C pathways, which are normally induced upon T-cell receptor activation (21, 24, 33).

The three previously defined NFAT sites found in the GM-CSF enhancer each resemble the distal IL-2 promoter NFAT site in that they have loosely conserved purine-rich segments positioned upstream of AP-1-like motifs (8, 28). The IL-2 promoter and GM-CSF enhancer NFAT sites are different, however, in their relative affinities for NFATp/c and AP-1 (8). Our previous binding studies (8) demonstrated that while the human distal IL-2 NFAT element is a moderate-affinity binding site for NFATp or NFATc, it lacks the ability to bind AP-1 independently. In contrast, the GM170, GM330, and GM550 NFAT sites in the GM-CSF enhancer each function as moderate- to high-affinity AP-1 sites, retaining the ability to bind AP-1 in the absence of NFATp or NFATc (8). The GM330 and GM550 sites do not, however, appear to be efficient NFATp/c-binding sites (8).

In this study we examined DNA elements that contribute to enhancer function. We defined the essential core of the enhancer and showed that it contained potential binding sites for the T-lymphotropic transcription factor CBF (16) and a novel NFAT site that encompassed a CRE-like sequence in place of the more usual AP-1 motif. Three of the four NFAT sites in the enhancer bound AP-1 and NFATp in a highly cooperative fashion. This class of NFAT sites appeared to require a particular spacing between AP-1- and NFATp/c-binding sites for cooperative binding and enhancer function.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: DH, DNase I hypersensitive; CsA, cyclosporin A; CRE, cyclic AMP response element; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; IL-2, interleukin-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; CBF, core-binding factor; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Oligonucleotides. Oligonucleotide duplexes used as probes and competitors and in the construction of reporter plasmids had the following sequences, with complementary single-stranded regions used for cloning shown in lowercase for the upper strand only: GM170, gatcCTGTAGGAAACAGGGGCTTGAGTCACTCCAG; GM330, gatcCCCCCATCGGAGCCCCTGAGTCAGCATGGCG; GM400, gatcTCTAGGAATGCCTGCCTGTGTCATGGCCTCA; GM420, gatcCCATCTTTCTCATGGAAAGATGACATCAGGG; GM420 dCRE, gatcCCATCTTTCTCATGGAAAGATAGCACTAGGG; GM430, gatcTCACACATCTTTCTCATGGAAAGATGA; GM550, gatcTGAAAGGAGGAAAGCAAGAGTCATAATAAGA; IL-2 NFAT, gatcCGAAAGGAGGAAAAACTGTTTCATACAGAAG; and AP-1, gatcTGGATCACCCGCAGCTTGACTCATCCTTGCA.

Plasmid construction. All of the CAT reporter plasmids were based on the plasmid pHGM (8), which has a 600-bp fragment of the human GM-CSF promoter upstream of the CAT gene. Enhancer fragments and oligonucleotides were inserted into appropriate sites of the multiple cloning site located upstream of the promoter in the same orientation in relation to the promoter as that in which they exist in the GM-CSF enhancer.

Recombinant proteins. wbFos and wbJun were prepared as described previously (1). The plasmid pQE-31#1, used to make a 293-amino-acid truncated fragment of NFATp, was a gift from A. Rao and was engineered by deleting into the *Xho*I-*Sma*I fragment of the mouse NFATp gene in pNFATpXS (22) from the 3′ end. All three recombinant proteins were expressed in *Escherichia coli* with a His_6 tag, solubilized in guanidine HCl, and purified by chromatography on Qiagen Ni^{2+}-nitrilotriacetic acid-agarose according to the manufacturer's instructions.

Antibodies. The antibody R59 was raised against recombinant truncated NFATp prepared from pNFATpXS (22). The antibody 67.1 was raised against peptide 72 of purified mouse NFATp (22). These antibodies were a gift from A. Rao and cross-react between human and mouse NFATp. The 7A6 monoclonal antibody raised against human NFATc residues 1 to 654 (25) was provided by G. Crabtree.

Transfection assays. Enhancer activities were determined by transfecting 10 μg of each CAT reporter construct into 5×10^6 Jurkat T cells and measuring CAT expression after stimulation with 3 μM A23187 (calcium ionophore) and 20 ng of PMA per ml for 6 h in the presence and absence of 0.1 μM CsA, as described previously (8).

Gel electrophoretic mobility shift assays. Gel shift assays and preparation of nuclear extracts were as previously described (8), except that assays employed 5 μg of nuclear extract and 2 μg of poly(dI-dC) in a 15-μl volume. In some instances nuclear extracts were enriched by binding to heparin-Sepharose, washing with 0.15 M KCl, and eluting with 0.45 M KCl before the final dialysis. Assays of nuclear proteins used 5 μg of nuclear extracts prepared from unstimulated cells and cells stimulated for 2 to 3 h with 20 ng of PMA per ml and 2 μM A23187 in the presence and absence of 0.1 μM CsA. Assays of recombinant proteins used 8 ng of wbJun and 5 ng of wbFos as the source of AP-1 (1) and 0.5 ng of truncated NFATp with 100 ng of poly(dI-dC) and 0.2 ng of probe. When indicated, 0.05 μl of 67.1 antiserum, 0.2 μl of R59 antiserum, 0.2 μl of preimmune antiserum, or 0.1 μl of 7A6 ascites was included.

DNase I footprinting. Probes were prepared by 5′ end labelling the *Hin*cII-*Bal*I fragment at either end with [^{32}P]ATP. Nuclear extracts enriched by heparin-Sepharose chromatography were prepared as described above from Jurkat cells stimulated for 2 h with 20 ng of PMA per ml and 2 μM A23187. Approximately 5 ng of probe was incubated for 30 min at 22°C in a 100-μl volume of 20 mM HEPES (pH 7.9)–50 mM KCl–10 mM NaCl–1 mM dithiothreitol–10% glycerol with 25 μg of nuclear extract, 1 μg of poly(dI-dC), and 5 μg of tRNA and then digested with 1 U of DNase I per ml for 30 s at 22°C after addition of $MgCl_2$ to 3 mM and $CaCl_2$ to 0.1 mM. A protein-free DNase I control employed 0.1 U of DNase I per ml. Purified DNA recovered from each digest was examined on a DNA sequencing gel alongside a G+A DNA sequencing reaction.

Methylation interference assays. Probes were prepared from *Xho*I-*Nru*I or *Eco*RV-*Hin*dIII fragments of plasmids in which single copies of the GM170, GM330, GM420, GM430, and GM550 oligonucleotides were inserted at the *Bgl*II site of pHGM. Probes were ^{32}P end labelled by using either the *Hin*dIII or *Xho*I 5′ end and partially methylated at guanine residues with dimethyl sulfate. Methylation interference assays were performed essentially as described previously (3) with modifications (32), with stimulated Jurkat cell nuclear extracts enriched by heparin-Sepharose chromatography. Briefly, gel mobility shift assays were performed as described above (with a fivefold scale-up), the gels were electrotransferred to NA-45 membranes, NFAT complexes and free probe bands were excised, and DNA was eluted and cleaved with 10% piperidine–1 M NaCl–5 mM EDTA before purification and electrophoresis on 10% acrylamide gels containing 8 M urea, 50 mM Tris acetate, 20 mM Na acetate, and 2 mM EDTA, pH 8.

RESULTS

Definition of the GM-CSF enhancer core. A 716-bp *Bgl*II fragment encompasses all of the GM-CSF enhancer elements required for efficient activation of the GM-CSF promoter in transfection assays in the Jurkat human T-cell line (8) (Fig. 1A). We previously defined three NFAT-binding sites in the enhancer, which were named according to their positions in the 716-bp sequence (GM170, GM330, and GM550; Fig. 1B), and a weak AP-1-binding site (termed GM400) that is unlikely to make a significant contribution to enhancer activity (8). To ascertain the relative contributions that different elements of the enhancer make to its inducible activity, stepwise deletions were made into the enhancer from either direction (Fig. 1B). These deletions were designed to remove each of the NFAT sites in turn. Enhancer fragments subcloned into the GM-CSF promoter/CAT reporter gene plasmid pHGM (8) were tested for inducible enhancer activity in transient transfection assays with Jurkat T cells (Fig. 1B). Following transfection, cells were stimulated with a combination of phorbol ester and calcium ionophore to mimic T-cell receptor activation.

Initial deletion analyses indicated that the 425-bp *Bam*HI-*Bal*I subfragment that included the three NFAT sites and the weak AP-1 site (pBB425; Fig. 1B) retained full enhancer activity. Since the 425-bp *Bam*HI-*Bal*I fragment of the enhancer had, if anything, a slightly greater activity than the previously defined 716-bp *Bgl*II fragment, all subsequent enhancer activities have been normalized as a percentage of the activity obtained with the plasmid pBB425 and compared with the activity obtained with pHGM, which contains the promoter alone. The fold induction of each construct relative to an unstimulated control is also shown in Fig. 1B.

Only marginal decreases in activity occurred upon deletion of either the GM170 region or the GM550 region from

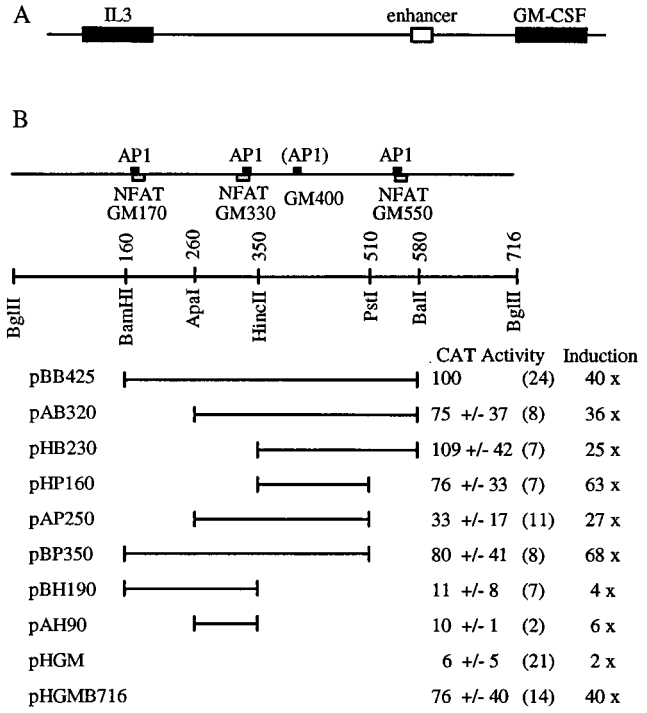

FIG. 1. Expression of GM-CSF enhancer/CAT reporter plasmids in Jurkat cells. (A) Location of the GM-CSF enhancer in the 10.5-kb intergenic region between the IL-3 and GM-CSF genes. (B) Deletion analysis of the 716-bp *Bgl*II fragment of the GM-CSF enhancer. Enhancer fragments coupled to the GM-CSF promoter in the CAT reporter plasmid pHGM were transfected into Jurkat cells. CAT activities of cell lysates were determined after stimulation of the cells for 6 h with 20 ng of PMA per ml and 3 μM A23187. Enhancer subclones were named by using the initial letters of the enzyme sites used for cloning and the approximate lengths of the fragments. The activity of each plasmid was expressed relative to that of pBB425, which was included in each of a total of 15 independent transfection experiments. Activities are given together with the standard deviation, and the number of times each plasmid was assayed is shown in parentheses. The fold induction represents the response of each plasmid relative to that of an unstimulated control for each plasmid.

pBB425, since the plasmids lacking either one of these sites (pAB320 and pBP350) had activities in the same range as those of pBB425 and pHGMB716. Deletion of both the GM170 and GM330 regions from pBB425 also had little effect, indicating that most of the activity of the enhancer was confined to the 230-bp *Hin*cII-*Bal*I fragment (pHB230; Fig. 1B), which retains only one of the three previously defined NFAT sites (GM550). The plasmid pHB230 was 18 times as active as pHGM and supported a 25-fold induction of CAT activity in response to stimulation with PMA and A23187. In contrast, the plasmid pBH190, which encompassed the upstream half of the enhancer, was only twice as active as pHGM even though it encompassed two of the three NFAT sites. Surprisingly, only a one-third decrease in activity was observed when the GM550 site was also deleted from pHB230 to create pHP160. Thus, the 160-bp *Hin*cII-*Pst*I fragment defined here as the essential core of the enhancer retained 76% of the activity obtained with pBB425 but included none of the previously defined NFAT sites. This core fragment was also tightly regulated, since it supported a 63-fold induction of CAT activity upon stimulation, and its activity was reduced by 80% when stimulated in the presence of CsA. Longer enhancer fragments were, however, slightly more sensitive to CsA, since the activities of the plasmids pHGMB716, pBB425, pAB320, pAP250, and pBP350 were reduced by 90 to 98% in the presence of CsA (data not shown).

It is likely that a high degree of redundancy operates in the utilization of activation elements within the enhancer, since the GM170 and GM550 regions did make small contributions to the activity of the enhancer core in plasmids that were already missing one or two NFAT sites. Thus, approximately twofold decreases in activities were observed when the 100-bp *Bam*HI-ApaI fragment was deleted from pBP350 to create pAP250 and when the 70-bp *Bal*I-*Bgl*II fragment was deleted from pAB320 to create pAP250 and from pHB230 to create pHB160. These differences were consistently observed in independent assays.

There may also exist an inhibitory element in the 90-bp *Apa*I-*Hin*cII fragment. The plasmids pBH190 and pAH90 had little activity on their own even though they encompass NFAT sites, and the plasmid pAP250 was only 43% as active as the enhancer core plasmid (pHP160) even though it included the GM330 NFAT site in addition to the core. This observation was reproducible, since pAP250 was consistently much less active than pHP160 in the three assays in which both plasmids were assayed on the same occasion and could thus be compared directly.

The essential core of the enhancer lies within a DH site. The earliest detectable event during the activation of the IL-3/GM-CSF locus is the appearance of the CsA-suppressible DH site within the enhancer (8). To determine whether the DH site was closely associated with either the essential core of the enhancer or any of the NFAT sites, the position of the DH site was finely mapped in nuclei isolated from activated Jurkat cells. Sites of DNase I cleavage were mapped from both directions by Southern blot hybridization analysis by utilizing *Bgl*I sites located 1.9 and 3.6 kb upstream of the GM-CSF gene (Fig. 2).

When mapped from downstream with probe A, the DH site appeared as a broad region approximately 150 to 250 bp across located principally within the essential core of the enhancer. The most intense DNase I cutting occurred between positions 350 and 400 in the sequence of the 716-bp *Bgl*II fragment, and a second zone of DNase I cutting was greatest between positions 450 and 500 (Fig. 2A, arrows). A somewhat protected region existed between positions 400 and 450, which became more visible when mapped in even finer detail from the downstream *Bam*HI site (data not shown).

As might be expected in a region where DNase I cuts frequently, the mapping of DNase I cleavage sites was biased towards the direction from which they were mapped. Thus, probe B revealed the strong site between positions 350 and 400 and an additional frequently cut region between positions 250 and 300. The DH site again appeared as a broad region about 150 bp across with two high-intensity bands (Fig. 2B, arrows) flanking a protected section that in this instance spanned the GM330 element. Consequently, the DH site probably existed as a 250-bp DH region defined by the *Apa*I and *Pst*I sites, with two protected regions coinciding with the GM330 element and a site near position 425.

Two nuclear factors associate with the essential core of the enhancer. Since previously identified transcription factor binding sites could only partially account for the induction of the DH site and the properties of the enhancer, DNase I footprint analysis (10) was used to seek additional regulatory elements within the essential core of the enhancer. Probes were made by labelling the *Hin*cII-*Bal*I fragment of the enhancer at either end and assayed with nuclear extracts prepared from activated Jurkat cells. Two regions within the essential core were partially protected in similar positions on both strands of the sequence (labelled FP on the upper strand shown in Fig. 3). One of these regions encompassed the sequence TGGAAA

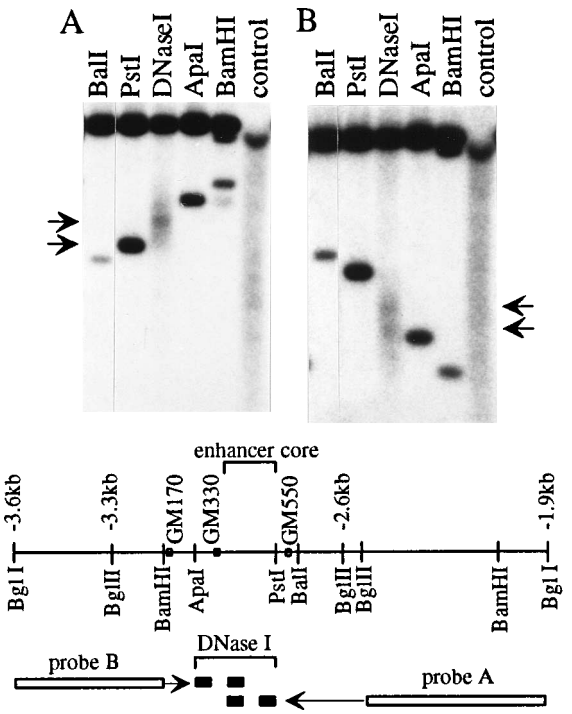

FIG. 2. Fine mapping of the DH site in nuclei isolated from Jurkat cells activated for 7 h with 2 μM A23187 and 20 ng of PMA per ml and digested with 5 μg of DNase I per ml. The DH site was mapped as described previously (8) from downstream (A) and upstream (B) by Southern blot hybridization analysis with probes A and B, respectively, and utilizing *Bgl*I sites located 1.9 (A) and 3.6 (B) kb upstream of the GM-CSF gene. All DNA samples were digested to completion with *Bgl*I. The marker lanes contain intact purified Jurkat cell DNA partially digested with *Bal*I, *Pst*I, *Apa*I, or *Bam*HI. The control lane contains a DNase I digest of intact purified DNA. The same filter was used for both panels. Probes A and B (open boxes) were *Bgl*II-*Bam*HI and *Bgl*II-*Bgl*I fragments originating from the lambda clone J1-16 (8). Areas of enhanced DNase I cleavage within the DH site are indicated by arrows and displayed as solid boxes. The location of the 160-bp *Hin*cII-*Bal*I enhancer core fragment is bracketed.

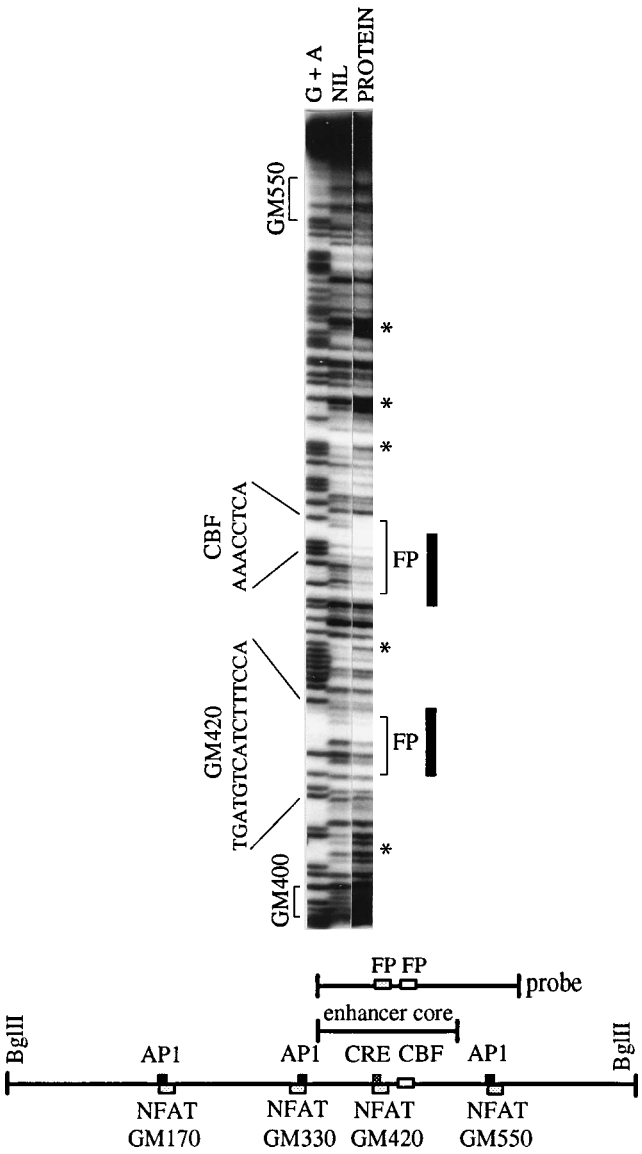

FIG. 3. DNase I footprint analysis of nuclear protein-binding sites within the enhancer core. The data were obtained with a probe labelled on the upper strand, and brackets indicate footprinted (FP) regions protected from DNase I digestion. Solid boxes indicate the relative positions of complementary regions protected on the lower strand (data not shown). Asterisks highlight regions of enhanced DNase I cleavage. The three lanes represent a G+A sequencing reaction, the DNase I digestion products of protein-free DNA (NIL), and a DNase I digestion of probe complexed with activated Jurkat cell nuclear protein.

GATGACATCA, which was subsequently found to be homologous to NFAT sites present elsewhere in the enhancer and in the IL-2 promoter. This element was centered at approximately position 420 in the sequence of the 716-bp *Bgl*II fragment of the enhancer and therefore has been defined here as the GM420 element, in keeping with previously identified motifs in the enhancer. Significantly, this element lies within the DH site between two of the regions of highest-frequency DNase I cleavage (Fig. 2A). Like other NFAT sites, the GM420 element contains a conserved GGA core within a purine-rich region (28). Interestingly, a highly homologous second purine-rich region (GAGAAAGATG) exists as an inverted repeat just downstream of the GM420 element, but this repeated sequence does not contain a GGA core. The GM420 element is unusual, however, in that the conserved purine motif lies upstream of a CRE-like sequence (TGACATCA) (5) rather than upstream of an AP-1 element as found in the other previously identified sites.

The second protected region was centered at position 450 and encompassed two overlapping sequences (TGCCCA CAAACCTCA) that resembled binding sites for CBF, a transcription factor implicated in the regulation of several T-cell-specific genes (16), including that for IL-3 (6). These newly identified CBF- and NFAT-like binding sites may account for the activity of the essential core of the enhancer and cooperate with the other three NFAT sites in the enhancer (summarized in Fig. 3). The GM550 and GM400 regions were not protected in the above-described assay, most likely because they encompass low-affinity factor-binding sites.

The GM420 element encompasses a strong NFATp/c-binding site. To examine the binding of NFATp/c to the GM420 region, we designed a truncated probe (GATCTCACACATC TTTCTCATGGAAAGATGAGATC) (termed here GM430) that included both the conserved purine motif TGGAAAGA and the inverted repeat sequence GAAAGATG (underlined) but lacked an intact CRE. We tested binding of the GM430 probe in gel mobility shift assays with nuclear extracts prepared from the Jurkat and HSB-2 human T-cell lines. In each case the GM430 probe associated with an inducible CsA-inhibitable

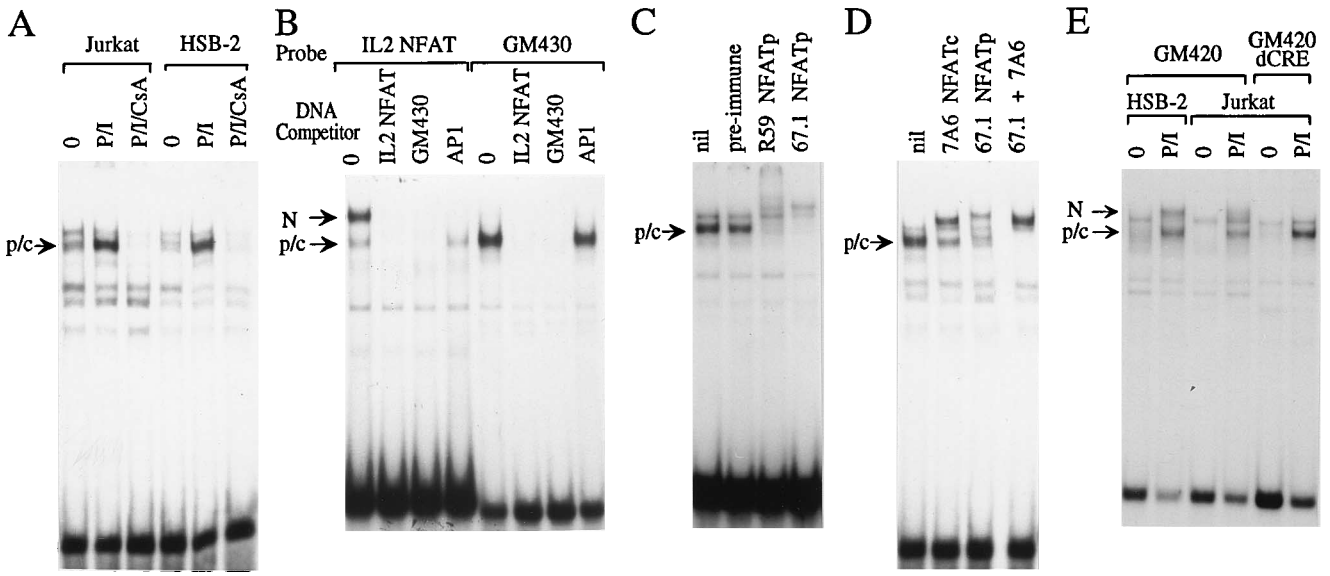

FIG. 4. Gel electrophoretic mobility shift assays of the GM420 NFAT site. (A) GM430 binding with nuclear extracts prepared from unstimulated cells (0) and cells stimulated with PMA and A23187 in the presence (P/I/CsA) and absence (P/I) of CsA. Bands migrating below the GM430 NFATp/c complex appear to represent nonspecific constitutive factors, while the complex migrating above NFATp/c is a factor unrelated to NFATp/c that often appears constitutively. (B) Assays of stimulated Jurkat cell nuclear extract binding to the GM430 and IL-2 NFAT probes in the presence and absence of 25 ng of duplex oligonucleotide competitors. (C) Assays of stimulated Jurkat cell nuclear extract binding to the GM430 probe in the presence and absence (nil) of preimmune serum, R59 antiserum directed against recombinant NFATp, and 67.1 antiserum directed against an NFATp peptide. Both NFATp antisera produced a supershifted complex characteristic of specific NFATp binding. (D) Assays of stimulated HSB-2 cell nuclear extract binding to the GM430 probe in the presence and absence of R59 and 67.1 NFATp and 7A6 NFATc antisera. (E) GM420 and GM420 dCRE binding with nuclear extracts prepared from unstimulated cells and cells stimulated with PMA and A23187 (P/I). Note that a constitutively expressed factor unrelated to NFAT binds to the GM420 probe and migrates between the NFATp/c and NFAT complexes. NFAT (N) and NFATp/c (p/c) complexes are indicated with arrows.

NFATp/c-like factor (p/c in Fig. 4A), which comigrated with the NFATp/c complex associated with the IL-2 NFAT site (Fig. 4B). Significantly, the GM430 sequence bound NFATp/c to approximately a 5- to 10-fold-greater extent than the IL-2 sequence.

The GM430 motif was formally identified as an NFATp/c-binding site on the basis of several operational criteria. The GM430 sequence efficiently inhibited NFAT and NFATp/c binding to the IL-2 NFAT site, and the IL-2 NFAT site inhibited binding of NFATp/c to the GM430 sequence (Fig. 4B). This inhibition was specific since an AP-1 competitor blocked the formation of just the upper IL-2 NFAT complex and did not affect NFATp/c binding to the GM430 probe. The GM430 NFATp/c complexes that formed with Jurkat cell extracts were also either blocked or supershifted by two different specific NFATp antibodies but not by preimmune serum (Fig. 4C) or by NFATc antibodies (data not shown). This suggested that our Jurkat NFATp/c complexes contained predominantly NFATp, since the 67.1 NFATp antibody was raised against a peptide not conserved between NFATp and NFATc. The GM430 probe did, however, have the capacity to associate with both NFATp and NFATc, since both complexes were detected when HSB-2 extracts were employed. Hence, NFATp/c complexes formed with HSB-2 extracts were partially supershifted by either NFATp or NFATc antibodies and were completely supershifted by a combination of the two antisera (Fig. 4D).

We anticipated that the full-length GM420 element would function as a composite site. Since other NFAT sites typically bind NFATp/c-like factors in conjunction with AP-1 and since CREs are closely related to AP-1 sites (5, 14, 29), we tested the ability of the full-length GM420 element to form NFAT-like complexes with nuclear extracts prepared from activated Jurkat and HSB-2 cells. As anticipated, the GM420 probe formed more-slowly migrating inducible NFAT-like complexes resembling those formed with the IL-2 NFAT site, in addition to forming NFATp/c complexes (Fig. 4E). The CRE was clearly required for the upper NFAT complex formation, since a mutation of the CRE from TGACATCA to TAGCACTA (GM420 dCRE; Fig. 4E) eliminated NFAT but not NFATp/c complex formation. We have not yet determined the composition of the upper complex, but since CREs are closely related to AP-1 sites, it could contain various combinations of AP-1 and CREB/ATF family proteins.

Like the GM420 element, the three previously identified GM-CSF enhancer NFAT sites all form complexes resembling the upper IL-2 NFAT complex (8). It was therefore of interest to determine if NFATp/c contributed to the formation of the upper NFAT complex with each site. Each of the four NFAT sites was tested in gel mobility shift assays with activated Jurkat cell nuclear extracts, which were shown above to contain predominantly NFATp rather than NFATc (Fig. 5). In each case the upper NFAT complex was specifically eliminated when the R59 antibody directed against recombinant NFATp was included, and a characteristic supershifted complex was seen with the 67.1 NFATp peptide antibody. Furthermore, the analysis of HSB-2 extracts with NFATc and NFATp antibodies indicated that each of the four NFAT sites could associate with either NFATp or NFATc (data not shown).

For each of the NFAT probes we also investigated the components of the inducible Jurkat extract complexes appearing below the NFAT band that comigrated with NFATp/c and AP-1 (Fig. 5). Previous studies suggested that these bands contained both AP-1 and NFATp/c complexes in the case of GM170 but just AP-1 in the cases of GM330 and GM550 (8). This view was confirmed in the present study, since the lower GM330 and GM550 complexes were unaffected by NFATp

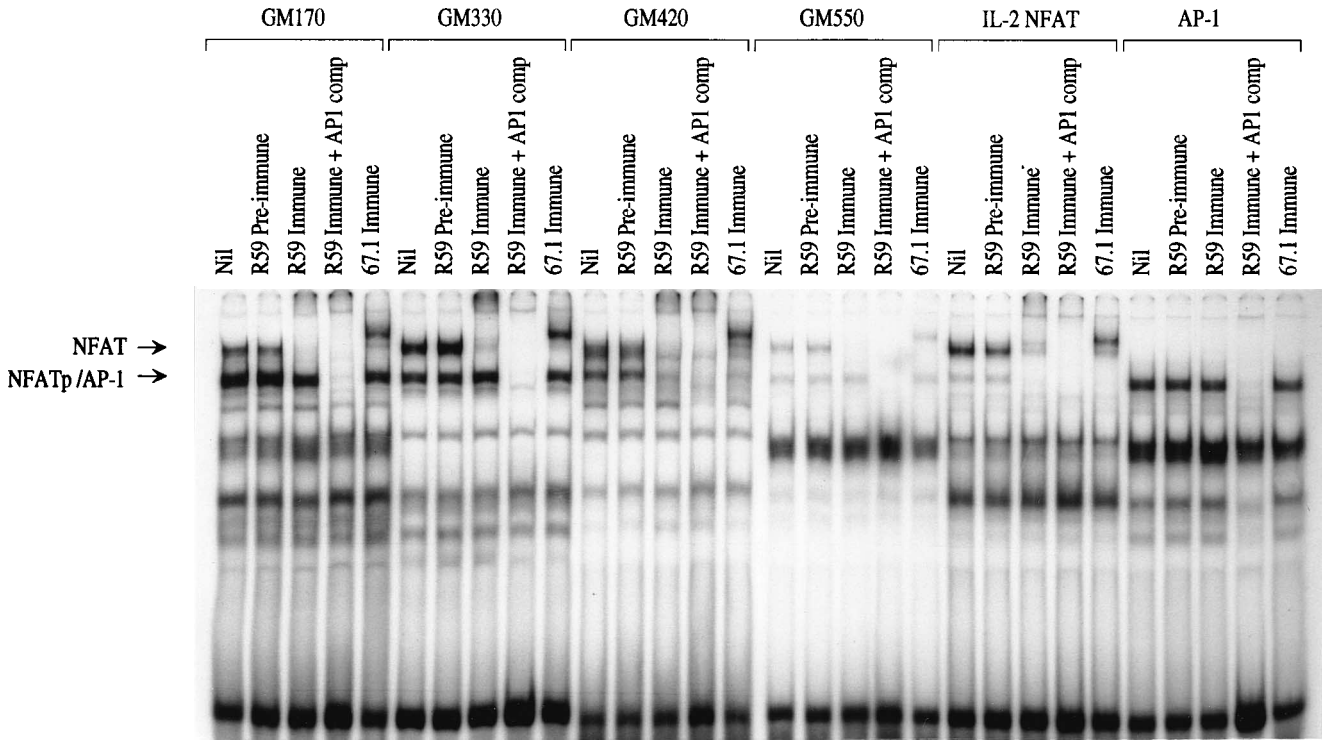

FIG. 5. Gel electrophoretic mobility shift assays of stimulated Jurkat cell nuclear extract binding to NFAT sites in the presence and absence (Nil) of preimmune antiserum, R59 antiserum raised against recombinant NFATp with or without additional AP-1 oligonucleotide competitor (AP1 comp), or 67.1 antiserum raised against an NFATp peptide. The upper arrow indicates the mobility of NFAT complexes containing NFATp/c and AP-1. The lower arrow indicates the mobility expected for individual NFATp, NFATc, and AP-1 complexes.

antibodies but were completely inhibited upon inclusion of an AP-1 oligonucleotide competitor (Fig. 5) (8). In the case of the GM170 probe, the lower complex was reduced in the presence of NFATp antibodies (Fig. 5) or competitor (8) and was completely inhibited when AP-1 competitor and NFATp/c antibodies were combined. The effects of the antibodies appeared to be specific, since they had no effect upon AP-1 binding to an AP-1 probe. The accumulated data therefore suggest that the GM170 element has roughly equal and moderately high affinities for both AP-1 and NFATp, while the GM330 and GM550 elements are very-low-affinity NFATp-binding sites. These studies also suggested that the GM420 probe was a weak AP-1-binding site, since a faint AP-1-like band remained after inclusion of NFATp antibodies and this band was eliminated by an AP-1 competitor. The ability of the four GM-CSF NFAT sites to form AP-1 complexes distinguished them from the IL-2 NFAT site, which independently associated with NFATp/c but not AP-1.

NFATp and AP-1 bind cooperatively to three of the four NFAT sites. Binding studies described above and elsewhere (8) suggest that AP-1 and NFATp/c bind together to form NFAT complexes with the GM-CSF enhancer. To verify this hypothesis and to determine whether the binding of AP-1 and NFATp to each site is cooperative, we examined the binding of bacterially expressed recombinant proteins to NFAT sites in the GM-CSF enhancer and IL-2 promoter (Fig. 6). Heterodimers of truncated cFos and cJun proteins containing the DNA-binding and leucine zipper domains were employed as the source of AP-1 (1) and assayed either in isolation or together with a truncated NFATp protein (22) corresponding to the conserved Rel domain (22, 25) in gel mobility shift assays.

The four GM-CSF enhancer NFAT sites showed widely

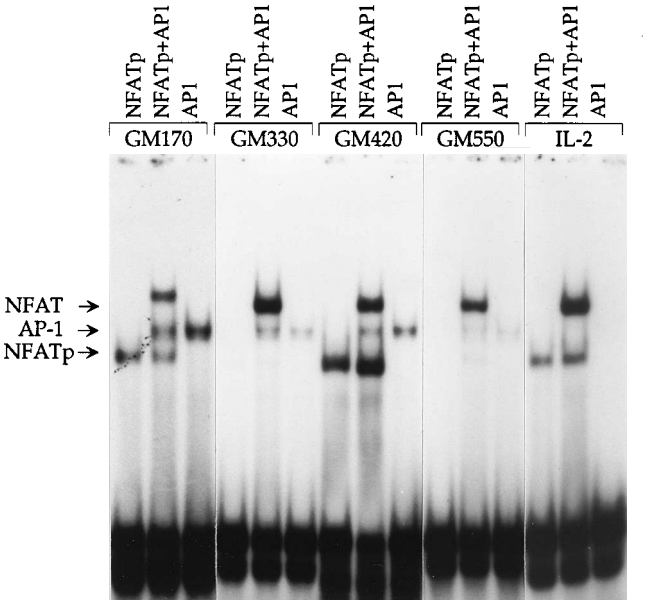

FIG. 6. Gel electrophoretic mobility shift assays of NFAT-like complexes formed with recombinant NFATp and AP-1. Binding reaction mixtures contained 8 ng of wbJun and 5 ng of wbFos (AP1) and/or 0.5 ng of truncated NFATp. Each reaction mixture contained 0.2 ng of probe and 100 ng of poly(dI-dC), and autoradiographs with different exposures have been matched to compensate for differences in specific activities of probes.

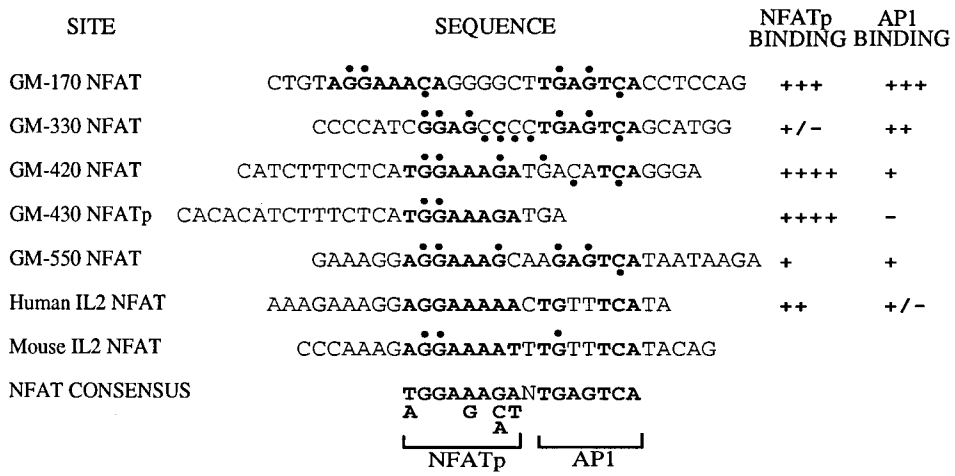

FIG. 7. Methylation interference assays of NFAT binding. Methylated DNA probes were prepared from plasmids containing the GM170, GM330, GM420, GM430, and GM550 oligonucleotides. Bound NFAT and NFATp/c complexes were recovered from gel mobility shift assays which employed 25 μg of heparin-Sepharose-purified nuclear extract prepared from Jurkat cells stimulated with 20 ng of PMA per ml and 2 μM A23187. DNA recovered from NFAT or NFATp/c complexes was electrophoresed alongside unbound probe recovered from the same gel. Assays were performed on both strands of each sequence, and G residues required for binding are indicated by circles above and below each sequence. Alongside each sequence is shown its relative binding affinity for AP-1 and NFATp, as estimated from accumulated binding and competition data. The consensus sequence derived from this subfamily of NFAT sites has taken into consideration the methylation interference data and the ability of each site to bind AP-1 and NFATp both cooperatively and independently. No methylation interference assays were performed with the human IL-2 NFAT site, and the mouse IL-2 NFAT data are taken from reference 28.

varying affinities for both NFATp and AP-1. As previously observed with nuclear extracts, the GM420 element was a high-affinity NFATp site and the GM170 and IL-2 NFAT sites were intermediate-strength NFATp sites, while the binding of NFATp to the GM330 and GM550 sites was with very low affinity. All four GM-CSF enhancer NFAT sites had the capacity to bind AP-1 in the absence of NFATp, while the IL-2 NFAT site did not. The GM170 element appeared to have the highest affinity for AP-1, in agreement with results of previous experiments that used nuclear extracts as the source of AP-1 (8) (Fig. 5).

The cooperativity of binding of AP-1 in combination with NFATp was very striking in the cases of the GM330, GM420, GM550, and IL-2 NFAT elements, where the binding of AP-1 as part of the NFAT complex was, respectively, 16-, 4-, 10-, and 400-fold greater than that obtained with AP-1 alone. Significantly, even though there was no independent binding of one of the two components for the GM330, GM550, and IL-2 sites, NFAT-like complex formation resulted in greatly enhanced binding of both components. Although recombinant AP-1 and NFATp also combined to form an NFAT-like complex with the GM170 element, there was no evidence for cooperativity in their binding, and the NFAT-like complex had a reduced mobility relative to the other four probes. This was unexpected, since the GM170 element was the only one of the sites examined that bound both the AP-1 and the NFATp components with moderate to high affinities.

To more directly compare the affinities of each of the NFAT sites for NFATp and AP-1, each element was also tested as an inhibitor of recombinant NFATp and AP-1 binding to high-affinity GM430 NFATp- and stromelysin AP-1-binding sites. The ability of each element to function as an NFATp or AP-1 competitor (data not shown) directly reflected the binding activities previously observed with nuclear extracts (Fig. 5) (8), and a summary of the relative affinities of each element for AP-1 and NFATp is included in Fig. 7. The GM430 and GM420 elements functioned equally well as high-affinity NFATp competitors, being about four times as efficient as the intermediate-affinity GM170 and IL-2 NFAT sites. The GM330 and GM550 sites, which supported no detectable NFATp/c binding, functioned poorly as NFATp competitors. As expected, the GM170 and GM330 elements functioned as efficient AP-1 competitors, while the GM420 and GM550 elements, which do not exactly match the AP-1 consensus, were about 50% as efficient as competitors.

NFAT binding requires a GGA core sequence and an AP-1 element. The most highly conserved features of the NFAT sites described here are a GGA core within a purine-rich segment and a downstream AP-1-like motif. The requirement for these regions in each of the four sites was tested by DNA methylation interference assays (3) of Jurkat cell NFAT complexes, and the data obtained from both strands are summarized in Fig. 7. These assays clearly demonstrated that both G residues in the conserved GGA core were required for NFAT binding to all four sites and for NFATp/c binding to the truncated GM430 element. The repeated GAAAGATG motif present on the opposite strands of the GM420 and GM430 probes did not appear to participate in binding. It was also evident that G residues in both halves of the AP-1-like motif contributed to NFAT complex formation in each case. Significantly, the CRE-like motif present in the GM420 probe gave a pattern similar to that obtained with the AP-1 elements present in the other NFAT sites. The finding that G methylation interfered with both NFATp/c and AP-1 binding indicated that these complexes must interact at least in part with the major groove of the DNA helix. A best-fit NFAT consensus sequence has also been derived here from the accumulated binding and methylation interference data obtained with both nuclear extracts and recombinant proteins.

Three of the four NFAT-like elements function efficiently as enhancer elements. The apparent redundancy of activation elements in the GM-CSF enhancer makes identification of individual functional elements very difficult. Therefore, to examine the potential of the NFAT-like motifs to function as enhancer elements, each site was tested as a multimer in isolation from the enhancer. Tandem arrays of three copies of each of the four individual NFAT sites were placed upstream of the GM-CSF promoter and tested for enhancer activity as

TABLE 1. Transient transfection assays of enhancer function of NFAT site multimers in Jurkat cells[a]

Construct	CAT activity[b]	Induction (fold)	CAT activity with CsA[c]
pHGMBB425	100 (8)	87	3
pGM170-3	11 ± 5 (7)	4	6
pGM330-3	79 ± 33 (7)	25	21
pGM400-3	5 ± 2 (4)	2	
pGM420-3	176 ± 55 (7)	160	3
pGM550-3	81 ± 63 (6)	37	6
pHGM	3 ± 3 (5)	3	

[a] Three head-to-tail copies of each oligonucleotide duplex were inserted into the appropriate *Bam*HI or *Bgl*II site upstream of the GM-CSF promoter in pHGM in their natural orientations relative to the promoter. Plasmids are named according to whether they contain three copies of the GM170, GM330, GM420, or GM550 NFAT site or the GM400 AP-1 site. CAT activities (relative to that of pBB425) and inductions (relative to that of an unstimulated plasmid control for each plasmid) obtained in response to activation by PMA and A23187 are expressed as in Fig. 1.

[b] Mean ± standard deviation. The number of times each plasmid was assayed is shown in parentheses.

[c] Activity obtained when stimulation was in the presence of 0.1 μM CsA.

described above (Table 1). Three of the DNA multimers (GM330, GM420, and GM550) supported inducible transcriptional activity almost as great or greater than that with the 425-bp *Bam*HI-*Bal*I fragment. The enhancer activity of each multimer was also suppressed by CsA, although this suppression was incomplete in the case of the GM330 element. Significantly, the most active element was the GM420 motif, which represented the highest-affinity NFATp/c site and formed part of the enhancer's essential core. In contrast, the GM170 element had very modest activity in this context, suggesting that a lack of cooperative binding between AP-1 and NFATp/c translates to a lack of independent function. The weak GM400 AP-1-binding site (8) had no activity when tested as an enhancer element.

DISCUSSION

The GM-CSF enhancer encompassed four NFAT sites, three of which (GM330, GM420, and GM550) were highly inducible by PMA and A23187 and were likely to account for much of the enhancer's CsA-sensitive activity. The inducible DH site within the enhancer encompassed the GM330 and GM420 elements and two overlapping CBF-like sites. The most significant of these sites was the GM420 element, which functioned as a high-affinity NFATp/c site and was the only NFAT site present in the functional enhancer core. Since NFATp is induced in the nucleus within 15 min of activation by Ca^{2+} (12), we speculate that high-affinity binding of NFATp/c to this site is one of the earliest events in the activation of the locus and one that leads to cooperative binding of AP-1 and triggers formation of the DH site. The induction of the DH site in this fashion would thus facilitate binding of other factors, such as CBF, to adjacent sites and increase the likelihood of NFATp/c gaining access to flanking lower-affinity NFATp/c sites. Since both PMA and A23187 are required for the induction of the DH site, its maintenance is likely to rely on cooperative binding of AP-1 and NFATp/c.

The NFAT sites listed in Fig. 7 that function as enhancer elements and bind NFATp/c and AP-1 in a cooperative fashion constitute a conserved family having the minimum consensus sequence $GGA(N)_9TCA$ and an optimal consensus that might resemble TGGAAAAATTGAGTCAG. Most likely, just half of the palindromic AP-1 motif would be sufficient for function within an NFAT site, and this could be equally well provided by an AP-1 site or a CRE. While the NFATp/c- and AP-1-binding motifs are themselves in some cases poorly conserved, the spacing between the two is remarkably constant. In addition, both components have to contact the DNA to form the NFAT complex, and the lack of cooperativity seen for the GM170 element suggests that a particular spacing of the binding sites is required for a cooperative interaction to occur. Indeed, there is exactly one turn of the DNA helix separating the GG sequence from the TCA sequence in the consensus sequence $GGA(N)_9TCA$, which allows for direct contact between the NFATp/c and AP-1 complexes if both associate via the major groove. The observation that the GM170 motif was nonfunctional as an enhancer, even though it bound both AP-1 and NFATp/c with a high affinity, suggested that a cooperative interaction was essential for enhancer function. Conversely, each of the functional NFAT sites described here had the capacity to bind AP-1 and NFATp in a highly cooperative manner, and this cooperativity was enforced by either the AP-1- or the NFATp-binding site being a very weak binding site.

The ideal consensus binding sequence for NFATp/c remains to be determined, but high-affinity NFATp/c sites usually contain the sequence GGAAA. The sequence TGGAAA is likely to be favored over AGGAAA, since the GM420 motif is a much stronger NFATp/c-binding site than the GM550 motif and the sequence TGGA also occurs in IL-4 and tumor necrosis factor alpha gene NFAT sites that bind NFATp/c independently (28). A comparison of several high-affinity NFATp/c-binding sites (28) (Fig. 7) suggests TGGAAAAAT as an optimal NFATp/c-binding site. The 3′ T appears to be favored in the case of the mouse and human IL-2 NFAT sites (28), and a T also occurs in this position in the GM420 element.

The NFAT sites described here most likely represent a highly specific subfamily, as many NFAT sites that do not conform to the same rigidly conserved structure have now been identified. Several cytokine gene promoters have NFAT sites termed CLEO elements (19, 20), in which GGAAA core sequences directly abut weak AP-1 sites, but there is no strong cooperativity in the binding of AP-1 and NFAT to these sites (unpublished observations). In the IL-4 promoter, NFATp/c-binding sites are closely linked to Oct-1-binding sites rather than AP-1-binding sites (28). One other example of an NFATp/c site upstream of a CRE exists in the case of the tumor necrosis factor alpha promoter (12), but the spacing between the two sites is greater than that in the GM420 sequence, and AP-1 and NFATp/c do not appear to bind cooperatively to this site.

It is significant that NFAT sites can encompass either AP-1 sites or CREs. This raises the possibility that NFATp/c may bind to NFAT sites in conjunction with either AP-1 or CREB/ATF family proteins, and these two families of proteins may compete for NFAT-binding sites. The CRE consensus sequence TGACGTCA is remarkably similar to the AP-1 consensus sequence TGAGTCA, and the Fos/Jun family is closely related to the CREB/ATF family of leucine zipper proteins (5, 15). Consequently, Fos and Jun proteins can also associate with CREs, and Jun/ATF heterodimers can bind either CREs or AP-1 sites (5, 14, 29). Since both Jun/Jun and Jun/Fos dimers can form NFAT complexes, we speculate that Jun/ATF heterodimers may also associate with the CRE-like sequence in the GM420 element to form NFAT-like complexes. Although we have no evidence to date for CREB/ATF involvement in NFAT-like complex formation, we have data from gel mobility shift assays indicating that both AP-1 (Fig. 6) and CREB/ATF proteins (data not shown) can associate with the

GM420 sequence. It is also intriguing that the same GM420 CRE-like sequence TGACATCA arises in other genes expressed in T cells, such as the T-cell receptor, CD3, and CD8 genes (2, 11, 13).

These studies indicated that GM-CSF enhancer function relies on converging signalling pathways that activate an array of composite transcription factor-binding sites. The cooperative nature of factor binding to these sites ensures not only that the binding of both components of the NFAT complex is greatly enhanced but that activation of these sites is very tightly regulated. Since the two components of the NFAT complex require two distinct activation pathways, it follows that the GM-CSF enhancer is unlikely to be inappropriately activated via one pathway alone.

ACKNOWLEDGMENTS

We are indebted to A. Rao for providing antisera and DNA clones for NFATp, to G. Crabtree and S. Ho for providing NFATc antisera, and to D. Cohen and T. Curran for providing wbFos and wbJun DNA clones and protein. We thank A. Sapa for technical assistance.

This work has been supported by the National Health and Medical Research Council of Australia and the Anti-Cancer Foundation of the Universities of South Australia.

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