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Activation of the Granulocyte-Macrophage Colony-Stimulating Factor Promoter in T Cells Requires Cooperative Binding of Elf-1 and AP-1 Transcription Factors

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The granulocyte-macrophage colony-stimulating factor (GM-CSF) gene has been studied extensively as a model system of transcriptional induction during T-lymphocyte activation. The GM-CSF gene is not expressed in resting peripheral blood T cells but is rapidly induced at the transcriptional level following activation through the cell surface T-cell receptor. A highly conserved 19-bp element located immediately 5′ of the human GM-CSF TATA box (bp −34 to −52), herein called purine box 1 (PB1), has been shown to bind a T-cell nuclear protein complex and to be required for transcriptional induction of the GM-CSF gene following T-cell activation. The PB1 sequence motif is highly conserved in both human and murine GM-CSF genes. In this report, we demonstrate that the PB1 element alone confers inducibility on a heterologous promoter following transfection into human Jurkat T cells. In addition, we identify a major PB1 nuclear protein-binding complex that is not present in resting peripheral blood T cells but is rapidly induced following T-cell activation. Sequence analysis revealed that PB1 is composed of adjacent binding sites for Ets and AP-1 transcription factors. In vitro mutagenesis experiments demonstrated that both the Ets and AP-1 sites are required for binding of the inducible PB1 nuclear protein complex and for the transcriptional activity of this element and the GM-CSF promoter in activated T cells. Using antibodies specific for different Ets and AP-1 family members, we demonstrate that the major inducible PB1-binding activity present in activated T-cell nuclear extracts is composed of the Elf-1, c-Fos, and JunB transcription factors. Taken together, these results suggest that cooperative interactions between specific Ets and AP-1 family members are important in regulating inducible gene expression following T-cell activation.

The activation of normal peripheral blood T cells results in the precisely orchestrated transcriptional induction of more than 100 new genes, many of which are important mediators of the inflammatory response to foreign pathogens (7). Activation-specific T-cell genes include those encoding cell surface molecules that induce important changes in T-cell adhesion as well as a number of lymphokines, such as IL-2 (interleukin-2), IL-3, gamma interferon, and GM-CSF (granulocyte-macrophage colony-stimulating factor), which can function in both autocrine and paracrine pathways to control the proliferation and function of multiple inflammatory cell types (7). Given the importance of inducible T-cell genes in modulating the inflammatory response, the elucidation of the molecular mechanisms underlying inducible transcription in T cells may yield important insights into both normal and pathophysiological T-cell function.

Several genes have been characterized extensively as model systems for understanding inducible transcription in T cells. These include the IL-2 (2, 4, 8, 10), IL-3 (27, 30), GM-CSF (5, 6, 24), and human immunodeficiency virus long terminal repeat (1, 18, 20, 22) genes. The GM-CSF gene is among the best characterized of these inducible T-cell genes. GM-CSF is a potent cytokine that stimulates the proliferation and maturation of several hematopoietic lineages, including neutrophils, macrophages, and eosinophils (6, 11, 23, 35). GM-CSF is not expressed in resting T cells but is induced at the transcriptional level during the first 20 h following T-cell activation (6). Previous studies have demonstrated that the transcriptional induction of the human GM-CSF gene in T cells is mediated at least in part by a 90-bp element located directly upstream of the transcriptional start site (bp −53 to +37) (29). DNase I footprint analyses of this promoter element demonstrated binding of a T-cell nuclear protein complex to a 19-bp purine-rich region (herein called purine box 1 [PB1]) located between bp −34 and −52 (28). Subsequent in vitro mutagenesis experiments demonstrated that this region is required for the inducible expression of the intact GM-CSF promoter in T cells. Interestingly, the PB1 sequence motif is highly conserved in both the murine and human GM-CSF genes (24) and is also present in the 5′ flanking regions of the human and murine IL-5 genes (29).

Although these previous studies demonstrated that the PB1 element is required for inducible expression of the GM-CSF gene in T cells, it remained unclear whether this element alone was sufficient to confer inducible expression on a minimal promoter in T cells. More importantly, the identities of the transcription factors that bind to PB1 to mediate GM-CSF transcriptional activation remained unclear. In the studies described in this report, we demonstrate that (i) the PB1 element alone, when multimerized upstream of a minimal promoter, functions as an inducible transcriptional element in T cells, (ii) PB1 is composed of adjacent Ets and AP-1 binding sites, both of which are required for

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binding of an inducible T-cell nuclear complex and for the transcriptional activity of PB1 in activated T cells, (iii) both the Ets and AP-1 binding sites are also required for the inducible activity of the GM-CSF promoter in T cells, and (iv) the major inducible PB1-binding complex in activated T-cell nuclear extracts contains Elf-1, c-Fos, and JunB. Taken together, these results suggest that cooperative binding of specific Ets and AP-1 family members to inducible promoters plays an important role in regulating activation-specific gene expression in T cells.

MATERIALS AND METHODS

Cells and cell lines. Human peripheral blood lymphocytes (PBL) were isolated from buffy coats obtained by leukapheresis of healthy 21- to 31-year-old donors by density gradient centrifugation as described previously (18). Purified cells were >99% lymphocytes as determined by Wright staining. Viability was greater than 99% as measured by trypan blue exclusion. PBL were cultured at a concentration of 2×10^6/ml in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) (GIBCO). PBL were activated by treatment for 6 to 8 h with phorbol myristate acetate (PMA; 10 ng/ml) plus ionomycin (0.4 μg/ml). Human Jurkat and murine EL4 T-cell tumor cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin-streptomycin (GIBCO) as described previously (18).

Plasmids. The GM-CSFCAT, mElf-1CAT, and mAP-1CAT plasmids were made by cloning three copies of the following synthetic oligonucleotides into the *Sma*I site of pSPCAT (31): GM-CSFCAT, ACAGTTAGAGGAAATGAT TAATGGTG; mElf-1CAT, ACAGTTATGCCAAATGATT AATGGTG; and mAP-1CAT, ACAGTTAGAGGAAATAT TGGATGGTG.

The GM-CSFPrLuc plasmid was made by cloning the human GM-CSF promoter (bp −55 to +39) into the *Xho*I-*Hin*dIII sites of the pGL2-Basic vector (Promega, Madison, Wis.). The GM-CSFPrmElf-1Luc and GM-CSFPrmAP-1Luc plasmids are identical to GM-CSFPrLuc except that they contain mutations in the Elf-1 and AP-1 sites as shown in Fig. 1.

The pRSVβgal reference plasmid, in which the bacterial *lacZ* gene is under the control of the Rous sarcoma virus long terminal repeat, has been described previously (9).

Nuclear extracts. Normal human PBL were washed with phosphate-buffered saline and incubated in buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) for 10 min at 4°C. Cells were collected by centrifugation at 1,500 × *g* for 2 min and lysed by incubation in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) at 4°C for 20 min. Lysates were cleared by centrifugation at 15,000 × *g* for 2 min at 4°C, aliquoted, and frozen at −70°C. Protein concentrations were determined by using a commercially available kit (Bio-Rad, Richmond, Calif.).

EMSAs. The following double-stranded oligonucleotides containing overhanging *Bam*HI-*Bgl*II ends were synthesized on an Applied Biosystems model 380B DNA synthesizer and labeled with ^{32}P-nucleotides by fill-in with the Klenow fragment of DNA polymerase I prior to use in electrophoretic mobility shift assays (EMSAs): GM-CSF, ACAGT TAGAGGAAATGATTAATGGTG; mElf-1, ACAGTTATG CCAAATGATTAATGGTG; mAP-1, ACAGTTAGAGGAA ATATTGGATGGTG; Tα3, GAGATAGCATCGCCCCAG GCCACGTGCCGAG; Tα1, CTCCCATTTCCATGACGTC ATGGTTACCA; and mNIP, GATCCACCTTCTTCACT TGTTCCCA.

Mixtures for binding reactions using T-cell nuclear extracts contained 2.0 μg of T-cell nuclear protein, 30,000 dpM (0.1 to 0.5 ng) of radiolabeled oligonucleotide probe, 250 ng of poly(dI-dC) in 50 mM KCl, 10 mM Tris (pH 7.5), 10 mM HEPES, 1.25 mM dithiothreitol, 1.1 mM EDTA, and 15% (vol/vol) glycerol in a final reaction volume of 15 μl. Following incubation at room temperature for 30 min, DNA-protein complexes were fractionated by electrophoresis in nondenaturing 5% polyacrylamide gels at 120 V for 4 h at 4°C in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). For competition experiments, 5 or 50 ng of unlabeled competitor oligonucleotide was added to the binding reactions before the addition of the radiolabeled oligonucleotide probe. For antibody supershift experiments, 1 to 2 μl of the appropriate antibody was added to nuclear extract and incubated at 4°C for 15 min prior to use in EMSAs as described above. All gels were dried and subjected to autoradiography using intensifying screens as described previously (31).

Transfections and CAT assays. Exponentially growing cultures containing 10^7 Jurkat T cells were transfected with 5 μg of reporter plasmid and 1 μg of the pRSVβgal reference plasmid, using DEAE-dextran as described previously (31). Transfections of murine EL4 cells were identical except that they were done with 10 μg of reporter plasmid. Thirty-six hours after transfection, cultures were treated with medium alone or PMA (50 ng/ml) plus ionomycin (1.5 μg/ml). Cells were harvested 10 to 12 h after stimulation, and cell extracts normalized for protein content by using a commercially available kit (Bio-Rad) were assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activities as described previously (31).

Antibodies. Preparation and specificities of the anti-Elf-1 (α-Elf-1) (18), α-c-Fos (17), α-JunB (17), α-JunD (17), α-Fra-1 (17), and α-cyclin D1 (21) rabbit antisera have been described previously. The α-Ets-1 and α-c-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The α-Jun family (α-Jun) and α-fos family (α-Fos) rabbit antisera recognize all known members of the Jun and Fos protein families, respectively (24). The α-cyclin D1 rabbit antiserum was the generous gift of C. Sherr (21).

RESULTS

PB1 contains adjacent Ets and AP-1 binding sites and binds an inducible T-cell nuclear protein complex. Sequence analysis of the PB1 element (bp −41 to −59; antisense strand) of the murine GM-CSF promoter revealed the presence of adjacent potential binding sites for Ets (A/CGGAA/T) and AP-1 (TGANTCA) transcription factors (Fig. 1). These sites have been highly conserved in the human GM-CSF promoter and are also present with a similar spacing in the 5′ flanking regions of the human and murine IL-5 promoters (Fig. 1B). Interestingly, the NFAT sites of the IL-2 promoter also contain adjacent Ets and AP-1 binding sites, both of which have been shown previously to be essential for binding of NFAT and for the inducible function of this element in activated T cells (2, 31). However, in the case of the NFAT element, the Ets and AP-1 binding sites are separated by 3

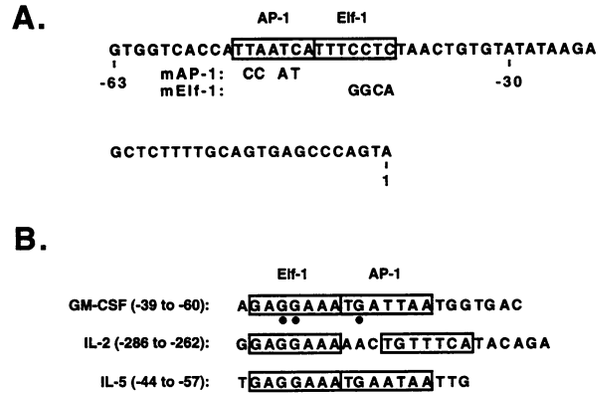

FIG. 1. Location and sequence of the PB1 element from the murine GM-CSF promoter. (A) Nucleotide sequence of the proximal murine GM-CSF promoter. The top line shows the sequence of nucleotides −63 to +1 (24). The potential AP-1 and Elf-1 binding sites which together comprise PB1 are boxed and labeled. The nucleotide substitutions made in the mutant AP-1 (mAP-1) and Elf-1 (mElf-1) sequences are shown below the promoter sequence. The nucleotides involved in binding the inducible PB1 nuclear protein complex (see Fig. 2) from activated normal human T cells were determined by methylation interference and are marked (●) in panel B. (B) Comparison of purine-rich nuclear protein-binding sites from the GM-CSF promoter (PB1) (29), the IL-2 promoter/enhancer (NFAT) (31), and the IL-5 promoter (25).

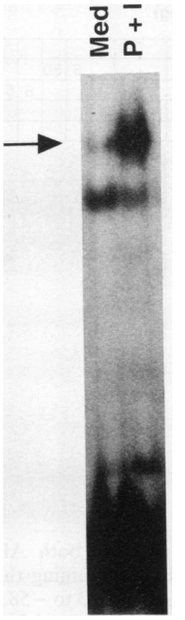

FIG. 2. Binding of an inducible T-cell nuclear protein complex to the PB1 motif from the murine GM-CSF promoter. A radiolabeled oligonucleotide containing the PB1 element of the murine GM-CSF promoter (bp −33 to −58; see Fig. 1A) was used in EMSAs with nuclear extracts prepared from resting normal human peripheral blood T cells (Med) or duplicate cultures of T cells activated for 8 h with PMA plus ionomycin (P + I). The inducible PB1-binding activity is indicated by an arrow.

bp, whereas these sites are directly adjacent to each other in the GM-CSF and IL-5 promoters (Fig. 1B). The significance of this difference is discussed further below.

To determine whether PB1 binds an inducible T-cell nuclear protein complex and, if so, whether the Ets and AP-1 sites are required for binding, we performed a series of EMSAs using a radiolabeled PB1 probe and nuclear extracts from resting or activated normal human peripheral blood T cells (Fig. 2 and 3). As shown in Fig. 2, PB1 binds two major nuclear protein complexes. The complex displaying slower electrophoretic mobility (Fig. 2, arrow) was barely detectable in resting peripheral blood T-cell nuclear extracts but was significantly induced following 8 h of activation with PMA plus ionomycin. This inducible PB1-binding complex represented a sequence-specific DNA-binding activity because it was efficiently competed for by excess unlabeled PB1 competitor oligonucleotide but not by two unrelated control oligonucleotide competitors, Tα3 and mNIP (Fig. 3). Binding of this inducible complex (Fig. 3, arrow) required both the Ets and AP-1 sites, since mutations in either site abolished the ability of the resulting oligonucleotide to compete for binding of this complex to the wild-type labeled PB1 probe (Fig. 3; compare the GM-CSF competitor with the mAP-1 and mElf-1 competitors). In addition, methylation interference experiments demonstrated that G residues in both the Ets and AP-1 sites were required for binding of the inducible PB1 complex (Fig. 1B). Thus, these experiments demonstrated that PB1 binds an inducible T-cell nuclear protein complex and that this binding activity requires both the Ets and AP-1 motifs of PB1. Moreover, the finding that oligonucleotides containing mutations in either the Ets or AP-1 site were unable to efficiently compete for binding of the PB1 complex suggests that PB1 complex formation requires cooperative binding of proteins to the Ets and AP-1 sites.

In addition to the inducible binding complex described above, PB1 bound a nuclear protein complex that displayed more rapid mobility by EMSAs (Fig. 2). The significance of this complex remains unclear. It is present in equal concentrations in both resting and activated T-cell nuclear extracts (Fig. 2). Moreover, it was efficiently competed for by both the wild-type unlabeled PB1 oligonucleotide and the unlabeled PB1 oligonucleotide containing mutations in the Ets site (mElf-1) but was not competed for by the PB1 oligonucleotide containing mutations in the AP-1 site (Fig. 3, mAP-1) or by the unrelated Tα3 or mNIP oligonucleotide (Fig. 3). Thus, it appears to represent a constitutive activity which binds to the AP-1 site of PB1. Of note, none of the bands in these EMSAs are competed for by oligonucleotides containing mutations in the AP-1 site, suggesting that binding of proteins to the Ets site of the PB1 oligonucleotide requires simultaneous binding of proteins to the AP-1 site. In addition to these two major PB1-binding activities, a third minor complex of slower electrophoretic mobility was detected in some EMSAs (Fig. 3). The identity and sequence specificity of this binding activity remains unclear, as it was not consistently demonstrated in T-cell nuclear extracts.

PB1 confers inducible expression on a heterologous promoter following T-cell activation. To determine whether PB1 alone is able to function as an inducible element in T cells, we cloned three copies of the PB1 oligonucleotide 5′ of a minimal simian virus 40 (SV40) promoter and bacterial CAT reporter gene and transfected the resulting plasmid into human Jurkat T cells. Thirty-six hours after transfection, the cultures were divided in half; one half was treated with medium alone for 10 h, while the other half was activated by treatment with PMA plus ionomycin for 10 h. As shown in Fig. 4, the SV40 promoter alone was inactive in both resting and activated T cells. In contrast, the reporter plasmid containing three copies of the wild-type PB1 element 5′ of

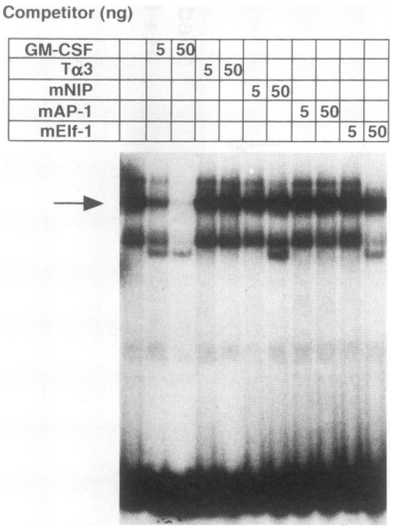

FIG. 3. PB1 binding requires both AP-1 and Elf-1 motifs. A radiolabeled oligonucleotide containing the PB1 element from the murine GM-CSF promoter (bp −33 to −58; see Fig. 1A) was used in EMSAs with nuclear extracts prepared from normal human T cells activated for 8 h with PMA plus ionomycin. Unlabeled competitor oligonucleotides (5 or 50 ng) corresponding to the wild-type GM-CSF probe (GM-CSF) or mutants containing nucleotide substitutions in the AP-1 (mAP-1) or Elf-1 (mElf-1) motif (see Fig. 1A) were added to the binding reaction mixtures as shown. Tα3 and mNIP are unrelated control competitor oligonucleotides. The inducible PB1-binding activity corresponding to that shown in Fig. 2 is denoted with an arrow. Note that this binding activity was competed for only by the wild-type unlabeled GM-CSF oligonucleotide, not by mutant oligonucleotide competitors containing nucleotide substitutions in either the AP-1 or Elf-1 motif. In contrast, the noninducible PB1-binding activity that displays more rapid electrophoretic mobility was competed for by both wild-type and mElf-1 oligonucleotides but not by the mAP-1 competitor.

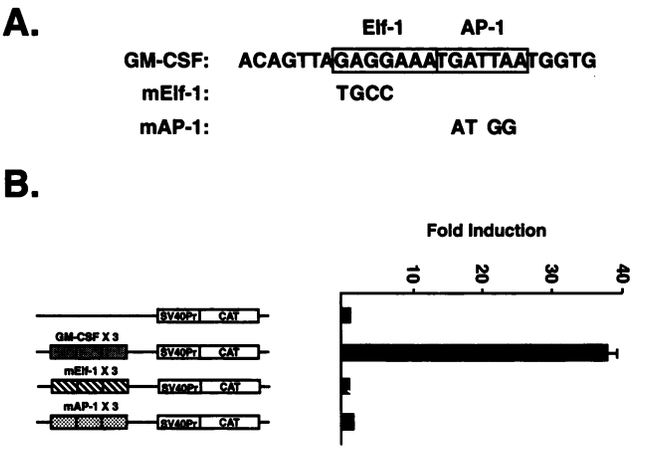

FIG. 4. Inducible transcriptional activity of the PB1 element requires both Elf-1 and AP-1 motifs. (A) Nucleotide sequences of the wild-type (GM-CSF) and mutant AP-1 (mAP-1) and Elf-1 (mElf-1) oligonucleotides. The Elf-1 and AP-1 motifs are boxed and labeled. (B) Transient transfections using PB1 reporter constructs. Jurkat human T cells were transfected with CAT reporter constructs containing three copies of the wild-type or mutant PB1 oligonucleotides cloned 5′ of the minimal SV40 promoter (SV40pr) (left). As a negative control, the pSPCAT reporter containing the minimal SV40 promoter but lacking all GM-CSF sequences was used in a parallel transfection. All transfection mixtures also contained the pRSVβgal reference plasmid to correct for differences in transfection efficiencies. Thirty-six hours after transfection, the cultures were divided in half. Half of each culture was left unstimulated, while the other half was activated by treatment with PMA plus ionomycin for 10 h. Cells were harvested, and CAT and β-galactosidase activities were determined from extracts normalized for protein content. Fold induction = CAT activity in activated cultures/CAT activity in unstimulated cultures after normalization for differences in transfection efficiencies. All transfections were repeated three to five times. The standard error bars for the pSPCAT, mElf-1CAT, and mAP-1CAT transfections are too small to be shown on this scale.

the SV40 promoter was inactive in resting T cells but was induced 36-fold following T-cell activation. To determine whether the Ets and AP-1 sites were important for the inducible activity of PB1, we cloned three copies of PB1 oligonucleotides containing mutations in either the Ets (mElf-1) or AP-1 (mAP-1) site (Fig. 4A) upstream of the minimal SV40 promoter and repeated the transfections into Jurkat cells. Mutations of either the AP-1 or Ets site that abolished binding of the inducible PB1-binding complex (Fig. 3) also abolished the ability of PB1 to function as an inducible transcriptional element in T cells (Fig. 4B). Therefore, both the Ets and AP-1 sites in PB1 are required for binding of an activation-specific T-cell nuclear protein complex and for the inducible transcriptional activity of this element in T cells.

The Ets and AP-1 sites of PB1 are both required for transcriptional induction of the GM-CSF promoter in T cells. Previous studies have demonstrated that the sequences located between bp −53 and +37 of the human GM-CSF promoter confer inducible transcriptional activity on a reporter plasmid following T-cell activation (28). To determine whether both the Ets and AP-1 sites of PB1 are required for transcriptional induction of the GM-CSF promoter in T cells, we constructed a series of reporter plasmids in which the firefly luciferase gene is under the transcriptional control of the wild-type human GM-CSF promoter (bp −55 to +39) (GM-CSFPrLuc) or the GM-CSF promoter containing mutations in the Elf-1 (GM-CSFPrmElf-1Luc) or AP-1 (GM-CSFPrmAP-1Luc) site of PB1 (Fig. 1). These reporter constructs were transfected into EL4 T cells, and the cultures were divided in half. One half was treated with medium alone, while the other half was activated for 12 h by treatment with PMA plus ionomycin. In agreement with previous results (28), the wild-type GM-CSF promoter was induced approximately sixfold following T-cell activation (Fig. 5). Mutation of either the Elf-1 or AP-1 site decreased inducibility to 1.2- or 1.4-fold, respectively. Thus, both the Elf-1 and AP-1 sites are required for the inducible transcriptional activity of the GM-CSF promoter in T cells.

The inducible PB1-binding complex contains specific AP-1 and Ets family members. The results of experiments described above are consistent with a model in which the cooperative binding of Ets and AP-1 transcription factors to PB1 is required for the inducible expression of the GM-CSF gene following T-cell activation. However, binding-site sequence analysis alone is insufficient to determine the identities of the cognate transcription factors that bind to a given sequence motif. Therefore, it remained formally possible that one or more non-Ets and/or non-AP-1 family members were binding to PB1 to mediate its inducible transcriptional activity. Moreover, both the Ets and AP-1 transcription factor families contain multiple members. Thus, the identities of the putative Ets and AP-1 PB1-binding factors remained unclear.

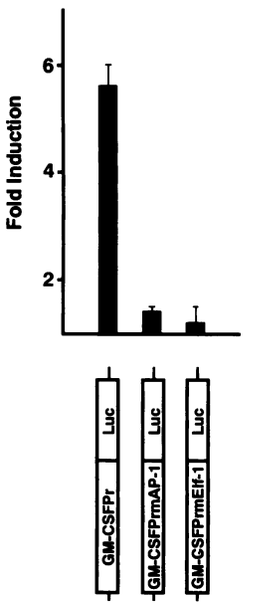

FIG. 5. Both AP-1 and Ets binding sites are required for inducible expression of the GM-CSF promoter in T cells. EL4 T cells were transfected with luciferase reporter constructs containing the wild-type GM-CSF promoter (bp −55 to +39) (GM-CSFPrLuc) or the identical promoter with mutations in the Elf-1 (GM-CSFPrmElf-1Luc) or AP-1 (GM-CSFPrmAP-1Luc) site of PB1 as shown in Fig. 1. All transfections also contained the pRSVβgal reference plasmid. Thirty-six hours after transfection, the cultures were divided in half. One half was treated with medium alone, while the other half was activated with PMA plus ionomycin. Twelve hours after stimulation, cells were harvested and cell extracts equalized for protein content were assayed for luciferase and β-galactosidase activities. The results are expressed as fold induction (= luciferase activity in the stimulated culture/luciferase activity in the unstimulated culture) following correction for differences in transfection efficiencies. All transfections were repeated at least three times, and results are shown as mean ± standard error.

To further address these issues, we examined whether antibodies specific for individual Ets and AP-1 family members would bind to the major inducible PB1-binding complex present in activated T-cell nuclear extracts. As shown in Fig. 6, the binding of this complex was completely abolished by α-Elf-1 antibodies (Fig. 6A and C, α-Elf-1) as well as by antisera that recognize all Jun or all Fos family members (Fig. 6B, α-jun Family and α-fos Family). These results suggested that all of the complexes contain Elf-1 and one or more AP-1 family members. A number of control experiments were performed to confirm that these results represented specific inhibition of binding activity. First, it has previously been shown that the α-Elf-1 antiserum used in these studies does not cross-react with Ets-1, Ets-2, or GABPα (18, 32). In addition, the α-Elf-1 antiserum does not affect the binding or mobility of AP-1 nuclear complexes to consensus AP-1 sites (data not shown). As shown in Fig. 6, neither the α-Elf-1, α-Jun family, nor α-fos family antiserum inhibited the binding of CREB/ATF proteins to the previously described (14) Tα1 site of the human T-cell receptor α enhancer. Finally, we have shown that incubation of these antisera with in vitro-translated Elf-1, c-Fos, and JunB proteins under conditions identical to those used in the EMSAs shown in Fig. 6 does not result in significant proteolytic cleavage of any of these proteins (data not shown). Thus, the finding that these antisera quantitatively abolished the PB1-binding activity demonstrated that this complex contains Elf-1 or a highly related Ets family member and one or more AP-1 family members.

To more precisely determine which AP-1 family members were contained in the inducible PB1-binding complex, we used rabbit antisera specific for individual Jun and Fos proteins in EMSAs with the PB1 oligonucleotide probe (Fig. 6B). Antisera specific for c-Fos and JunB both abolished most of the inducible PB1-binding activity (Fig. 6B, α-c-fos and α-junB). In contrast, antisera specific for Fra-1 and JunD (Fig. 6C) as well as control antisera against cardiac troponin C (Fig. 6C, α-cTnC), cyclin D1 (Fig. 6B, α-D1), c-Myc (Fig. 6B, α-c-myc), and Ets-1 (Fig. 6B, α-ets-1) had little or no effect on this binding complex. Taken together, these results suggested that the major inducible PB1-binding activity in activated T cells contains Elf-1, c-Fos, and JunB. However, because there was a small amount of residual binding activity that was not abolished by the α-c-Fos or α-JunB antiserum, we cannot rule out the possibility that there are less abundant complexes composed of Elf-1 and other AP-1 family members. It should be emphasized that previous studies have demonstrated the specificity of the antisera used in these experiments (2, 17). As shown in Fig. 6B, these antisera did not inhibit binding of CREB/ATF proteins to the control Tα1 oligonucleotide (14) from the human T-cell receptor α enhancer. Thus, it is unlikely that the observed activities of these antisera resulted from cross-reactivity with other known Ets or AP-1 family members or from nonspecific effects on DNA binding. However, we cannot rule out the possibility of cross-reactivity with related but uncloned Ets or AP-1 transcription factors.

DISCUSSION

The GM-CSF gene has been studied extensively as a model of inducible gene expression following T-cell activation. Unlike the case for other lymphokine genes such as the IL-2 gene (1, 8, 10) or immediately-early genes such as c-*myc* (19), which are regulated by complex multipartite promoters and enhancers, inducible GM-CSF gene expression is controlled by a relatively simple 57-bp element located immediately 5′ of the transcriptional start site (29). Previous studies have identified a 19-bp motif within this region which binds a T-cell nuclear protein complex and which is required for the inducible expression of the intact 626-bp promoter in T cells (28, 29). This motif is highly conserved in both the human and murine GM-CSF genes (24, 28). In this report, we have demonstrated that this motif, which we have called PB1, binds an inducible complex from activated T-cell nuclear extracts and can function as an inducible transcriptional element in T cells when multimerized 5′ of a heterologous promoter. More importantly, we have shown that PB1 is composed of adjacent Ets and AP-1 binding sites, both of which are required for binding of the inducible T-cell nuclear complex and for the inducible transcriptional activity of PB1 in activated T cells. Both sites are also required for the inducible transcriptional activity of the intact GM-CSF promoter in T cells. Using antibodies specific for individual Ets and AP-1 family members, we have shown that the major inducible PB1-binding activity present in activated T-cell nuclear extracts contains Elf-1, c-Fos, and JunB.

Our results are in accord with the previous in vitro mutagenesis experiments of Nimer et al. (29). Although these previous studies did not identify the Ets and AP-1 binding sites, they did show that mutations or deletion of

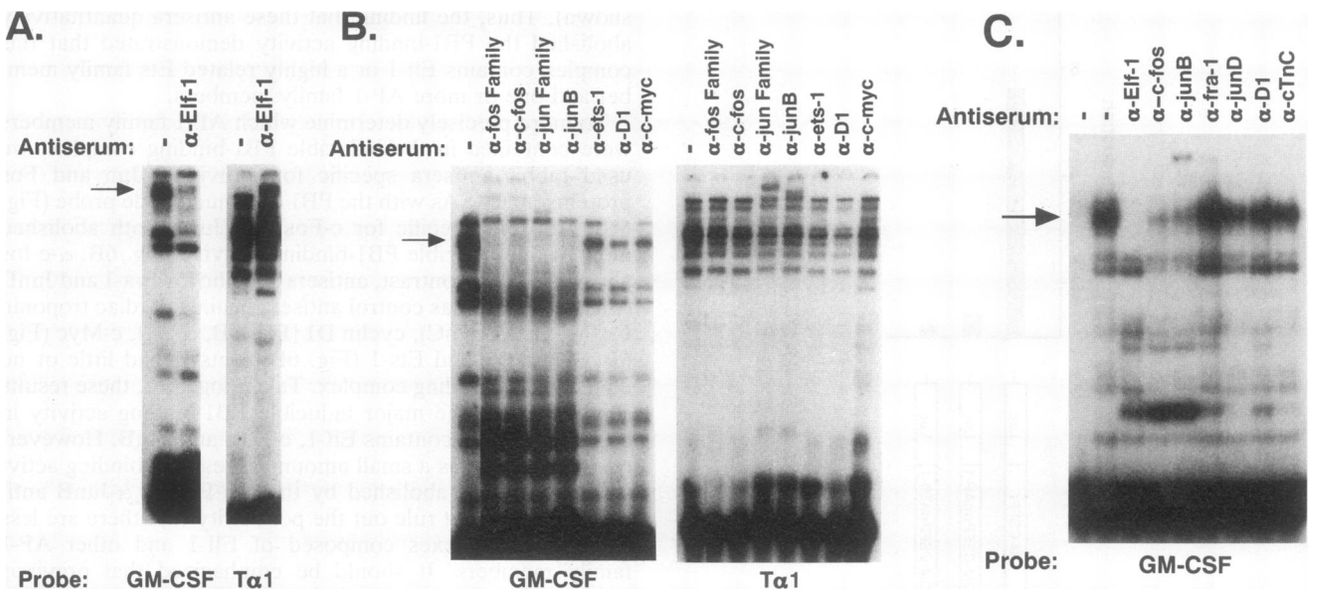

FIG. 6. The inducible PB1-binding activity of T cells contains Elf-1, c-Fos, and JunB. (A) Role of Elf-1 in the inducible PB1-binding activity of T cells. Radiolabeled probes containing the PB1 sequence from the murine GM-CSF promoter (bp −33 to −58; see Fig. 1A) or the control Tα1 sequence from the human T-cell receptor α enhancer (14) were used in EMSAs with nuclear extracts prepared from normal human T cells activated for 8 h with PMA plus ionomycin. Where indicated, α-Elf-1 antiserum was added to the binding reaction mixtures. The inducible PB1-binding activity is denoted by an arrow. (B and C) Role of AP-1 family members in the inducible PB1-binding activity of T cells. EMSAs using the radiolabeled PB1 and Tα1 oligonucleotides and antisera that recognize all Jun (α-jun Family) or all Fos (α-fos Family) family members or specific Fos and Jun family members (α-c-fos, α-fra-1, α-junB, and α-junD) were performed as described above. The α-cyclin D1 (α-D1), α-c-Myc, α-Ets-1, and α-cardiac troponin C (α-cTnC) antisera were used as negative controls. The inducible PB1-binding activity is denoted by an arrow.

PB1 nucleotides included in the Ets and AP-1 sites (as identified in this study) abolished inducible GM-CSF promoter activity whereas mutations or deletions not involving the Ets and AP-1 sites had no effect on the promoter in activated T cells.

Recent studies have suggested that adjacent Ets and AP-1 binding sites in the NFAT element of the IL-2 enhancer also play an important role in regulating inducible IL-2 gene expression following T-cell activation (2, 15). However, there are several important differences in the structures and functions of the NFAT and PB1 elements. First, the spacings of the Ets and AP-1 sites are different (directly adjacent in PB1 and separated by 3 bp in NFAT; Fig. 1). Second, previous studies have demonstrated that the major NFAT-binding complex in activated human T-cell nuclear extracts contains Fra-1 and JunB (2), whereas the present study demonstrates that the major PB1-binding activity from these same cells contains c-Fos and JunB. Of note, the two studies used identical antibody preparations, and therefore it is unlikely that differences in the antibody preparations could account for these results. Finally, although we have shown in this study that an α-Elf-1 antiserum abolishes the major inducible PB1-binding activity from activated T-cell nuclear extracts, this same antiserum has no effect on the NFAT-binding complex from the same nuclear extracts (data not shown). Given these data, it seems likely that the NFAT element of the IL-2 promoter binds a nuclear protein complex which is distinct from that bound by the PB1 element of the GM-CSF promoter. Confirmation of this model awaits the precise identification of the transcription factors that bind to the NFAT site.

The finding that the inducible T-cell PB1-binding activity is composed of both Ets and AP-1 family members suggests that multiple mechanisms may regulate the binding and transcriptional activity of this complex. Thus, for example, previous studies have shown that both c-Fos and JunB are transcriptionally activated during T-cell activation (7). In addition, dephosphorylation of Jun family members may play an important role in their activation (3). Finally, we have recently shown that the transcriptional activity of Elf-1 activity is regulated by binding to and release from the retinoblastoma protein and that this binding reaction is itself regulated by activation-specific phosphorylation of the retinoblastoma protein following T-cell activation (32). These results are consistent with the observation that multiple signaling pathways are required to induce lymphokine gene expression in T cells (4, 8, 10). They also suggest that simple overexpression of a single Ets or AP-1 transcription factor will be insufficient to mimic the transcriptional effects of T-cell activation on GM-CSF promoter activity. Similarly, it may be difficult to accurately reconstruct the inducible PB1-binding activity in vitro by using recombinant proteins. Nevertheless, identification of the components of the inducible PB1-binding activity and elucidation of the functionally important protein-protein interactions and posttranslational modifications of the complex members constitute an important first step in understanding the molecular mechanisms that regulate both gene expression and cell cycle progression following T-cell activation.

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ADDENDUM IN PROOF

Since the submission of the manuscript, we have compared the signalling requirements of the PB1 and NFAT nuclear protein complexes. Unlike NFAT, which requires stimulation with both PMA and ionomycin and is cyclosporin A sensitive, the PB1 binding activity described in this report is induced by treatment of normal T cells with PMA alone and is resistant to treatment with cyclosporin A. These differences in signalling requirements further suggest that NFAT and PB1 are composed of distinct nuclear proteins.

REFERENCES

1. **Arya, S. K.** 1988. Human and simian immunodeficiency retrovirus: activation and differential transactivation of gene expression. AIDS Res. Hum. Retroviruses **4**:175–186.
2. **Boise, H. L., B. Petryniak, X. Mao, C. H. June, C. Y. Wang, T. Lindsten, R. Bravo, K. Kovary, J. M. Leiden, and C. B. Thompson.** 1993. The NFAT-1 DNA binding complex in activated T cells contains Fra-1 and JunB. Mol. Cell. Biol. **13**:1911–1919.
3. **Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter.** 1991. Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. Cell **64**:573–584.
4. **Cantrell, D. A., and K. A. Smith.** 1984. The interleukin-2 system: a new cell growth model. Science **224**:1312–1316.
5. **Chan, J. Y., D. J. Slamon, S. D. Nimer, D. W. Golde, and J. C. Gasson.** 1986. Regulation of expression of human granulocyte-macrophage colony-stimulating factor (GM-CSF). Proc. Natl. Acad. Sci. USA **83**:8669–8673.
6. **Clark, S. C., and R. Kamen.** 1987. The human hematopoietic colony-stimulating factors. Science **236**:1229–1237.
7. **Crabtree, G. R.** 1989. Contingent genetic regulatory events in T lymphocyte activation. Science **243**:355–361.
8. **Durand, D. B., J. P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**:1715–1724.
9. **Fleischmann, J., D. W. Golde, R. H. Weisbart, and J. C. Gasson.** 1986. Granulocyte-macrophage colony-stimulating factor enhances phagocytosis of bacteria by human neutrophils. Blood **68**:708–711.
10. **Fujita, T., H. Shibuya, T. Ohashi, K. Yamanishi, and T. Taniguchi.** 1986. Regulation of human interleukin-2 gene: functional DNA sequence in the 5′ flanking region for the gene expression in activated T lymphocytes. Cell **46**:401–407.
11. **Gasson, J. C.** 1991. Molecular physiology of granulocyte-macrophage-colony-stimulating factors. Blood **77**:1131–1145.
12. **Gasson, J. C., R. H. Weisbart, S. E. Kaufman, S. C. Clark, R. M. Hewick, G. G. Wong, and D. W. Golde.** 1984. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. Science **226**:1339–1342.
13. **Grabstein, K. H., D. L. Urdal, R. J. Tushinski, D. Y. Mochizuki, V. L. Price, M. A. Cantrell, S. Gillis, and P. J. Conlon.** 1986. Introduction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science **232**:506–508.
14. **Ho, I.-C., L.-H. Yang, G. Morle, and J. M. Leiden.** 1989. A T-cell-specific transcription enhancer element 3′ of Cα in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA **86**:6714–6718.
15. **Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao.** 1992. Nuclear factor of activated T cells contains fos and jun. Nature (London) **356**:801–804.
16. **Kovary, K., and R. Bravo.** 1991. The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. **11**:4466–4472.
17. **Kovary, K., and R. Bravo.** 1992. Existence of different Jun and Fos proteins during G_0-to-G_1 transition and during exponential growth in mouse fibroblasts: differential role of Fos proteins. Mol. Cell. Biol. **12**:5015–5023.
18. **Leiden, J. M., C. Y. Wang, B. Petryniak, D. M. Markovitz, G. J. Nabel, and C. B. Thompson.** 1992. A novel Ets-related transcription factors, Elf-1, binds to human immunodeficiency virus type 2 regulatory elements that are required for inducible *trans* activation in T cells. J. Virol. **66**:5890–5897.
19. **Lindsten, T. C., C. H. June, and C. B. Thompson.** 1988. Multiple mechanisms regulating c-myc gene expression during normal T-cell activation. EMBO J. **7**:2787–2794.
20. **Markovitz, D. M., M. Hannibal, V. L. Perez, C. Gauntt, T. M. Folks, and G. J. Nabel.** 1990. Differential regulation of human immunodeficiency virus (HIVs): a specific regulatory element in HIV-2 responds to stimulation of the T-cell antigen receptor. Proc. Natl. Acad. Sci. USA **87**:9098–9102.
21. **Matsushime, H., M. E. Ewen, D. K. Strom, J. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr.** 1992. Identification and properties of an atypical catalytic subunit ($p34^{PSK}$/cdk4) for mammalian D type G1 cyclins. Cell **71**:323–334.
22. **McCune, J. M.** 1991. HIV-1: the infective process in vivo. Cell **64**:351–363.
23. **Metcalf, D.** 1985. The granulocyte-macrophage colony-stimulating factor. Science **229**:16–22.
24. **Miyatake, S., T. Otsuka, F. Lee, and K. Arai.** 1985. Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: comparison of the mouse and human genes. EMBO J. **4**:2561–2568.
25. **Miyatake, S., J. Shlomai, K. Arai, and N. Arai.** 1991. Characterization of mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoter: nuclear factors that interact with an element shared by three lymphokine genes—those for GM-CSF, interleukin-4 (IL-4), and IL-5. Mol. Cell. Biol. **11**: 5894–5901.
26. **Nabel, G., and D. Baltimore.** 1987. A inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
27. **Niemeyer, C. M., C. A. Sieff, B. Mathey-Prevot, J. Z. Wimperis, B. E. Bierer, S. C. Clark, and D. G. Nathan.** 1989. Expression of human interleukin-3 (multi-CSF) is restricted to human lymphocytes and T cell tumor cell lines. Blood **73**:945–961.
28. **Nimer, S., D. A. Elaine, E. A. Morita, M. J. Martis, and W. Wachsman.** 1988. Characterization of the human granulocyte-macrophage colony-stimulating factor region by genetic analysis: correlation with DNase I footprinting. Mol. Cell. Biol. **8**:1979–1984.
29. **Nimer, S., J. Farser, J. Richards, M. Lynch, and J. Gasson.** 1990. The repeat sequence CATT(A/T) is required for granulocyte-macrophage colony-stimulating factor promoter activity. Mol. Cell. Biol. **10**:6084–6088.
30. **Shomaker, S. G., R. Hromas, and K. Kaushansky.** 1990. Transcriptional regulation of interleukin-3 gene expression in T lymphocytes. Proc. Natl. Acad. Sci. USA **87**:9650–9654.
31. **Thompson, C. B., C. Y. Wang, I. Ho, P. R. Bohjanen, B. Petryniak, C. H. June, S. Miesfeldt, L. Zhang, G. J. Nabel, B. Karpinski, and J. M. Leiden.** 1992. *cis*-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. Mol. Cell. Biol. **12**:1043–1053.
32. **Wang, C. Y., C. B. Thompson, B. Petriniak, W. G. Kaelin, and J. M. Leiden.** 1993. Regulation of an Ets-related transcription factor, Elf-1, by binding to the retinblastoma protein. Science **260**:1330–1335.
33. **Weisbart, R. H., D. W. Golde, S. C. Clark, G. G. Wong, and J. C. Gasson.** 1985. Human granulocyte-macrophage colony-stimulating factor is a neutrophil activator. Nature (London) **314**:361–363.
34. **Weisbart, R. H., L. Kwan, D. W. Golde, and J. C. Gasson.** 1987. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. Blood **69**:18–21.
35. **Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, D. P. Luxenberg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang, and S. C. Clark.** 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science **228**:810–815.