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Identification of human granzyme B promoter regulatory elements interacting with activated T-cell-specific proteins: Implication of Ikaros and CBF binding sites in promoter activation

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ABSTRACT **Granzyme B serine protease is found in the granules of activated cytotoxic T cells and in natural and lymphokine-activated killer cells. This protease plays a critical role in the rapid induction of target cell DNA fragmentation. The DNA regulatory elements that are responsible for the specificity of granzyme B gene transcription in activated T-cells reside between nt −148 and +60 (relative to the transcription start point at +1) of the human granzyme B gene promoter. This region contains binding sites for the transcription factors Ikaros, CBF, Ets, and AP-1. Mutational analysis of the human granzyme B promoter reveals that the Ikaros binding site (−143 to −114) and the AP-1/CBF binding site (−103 to −77) are essential for the activation of transcription in phytohemagglutinin-activated peripheral blood lymphocytes, whereas mutation of the Ets binding site does not affect promoter activity in these cells.**

Activation of normal peripheral blood T lymphocytes is characterized by transcriptional induction of various genes which participate in the acquisition of effector cell function. Maturation of cytotoxic T lymphocytes (CTLs) is initiated by interaction of the T-cell antigen receptor (TCR) with foreign antigen presented in the context of class I major histocompatibility molecules on the surfaces of target cells. Activation triggers nuclear and cytoplasmic processes that lead to lysis of the target cells by the CTLs. The molecular mediators of this cell lysis involve components of cytoplasmic granules which are released by exocytosis into the space between the effector and target cells. These cytoplasmic granules contain perforin (pore-forming protein), as well as a variety of serine proteases referred to as granzymes or fragmentins (1–7). Purified perforin can disrupt target cell membranes but does not induce target cell DNA fragmentation, which characterizes CTL-mediated cell killing (8). Purification of granzymes has shown that these granule proteases play a role in activating DNA fragmentation and apoptosis (9, 10). Recently, production of homozygous null mutants in the granzyme B (GrzB) gene has demonstrated that alloreactive CTLs require GrzB for the rapid induction of DNA fragmentation and apoptosis of target cells (11). In both human and mouse, the GrzB gene is not expressed in resting T cells but is induced at the transcriptional level following T-cell activation (12, 13).

Studies of transcriptional activation of various T-cell-specific genes, such as those encoding interleukin 2 (IL-2) and TCR (reviewed in ref. 14), have identified regulatory factors which initiate the specific transcription of activated T-lymphocyte genes. The region including nt −1170 to +31 of the human GrzB gene controls inducible T-cell-specific transcription of a reporter gene in transgenic mice (15). Sequences between nt −609 and −80 relative to the transcriptional start site (+1) of the GrzB promoter are necessary and sufficient for activation of transcription in the PEER T-cell leukemia cell line treated with phorbol 12-myristate 13-acetate and dibutyryl cAMP (16). AP-1 binding sites and cAMP response elements in the −226/−154 region synergistically activate transcription starting from the GrzB promoter in activated PEER cells (17). We have reported that a proximal promoter element (−148 to +60) in the human GrzB gene controls specific transcription in phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) and in CD3-activated Jurkat cells (18). In this study we localize functional DNA sequences and factors that play an essential role in controlling human GrzB gene transcription in PHA-activated PBLs. The −143/−114 region of this gene promoter contains a critical Ikaros binding site, mutation of which abrogates promoter activity in activated PBLs. A second element, located in the −103/−77 region of the promoter, contains a binding site for the core-binding factor (CBF), binds AP1-related transcription factors, and stimulates GrzB transcription in activated PBLs.

MATERIALS AND METHODS

Cell Culture, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assays. These have been described (18).

Plasmid Constructs. Plasmid pBLCAT3, containing the CAT gene without any promoter, was kindly provided by G. Schütz (19). The constructs CAT3A and A11TF have been described (18). The mutated constructs CAT3AM1, CAT3AM2, and CAT3AM5 were prepared by PCR amplification of the corresponding region on CAT3A; the oligonucleotide primers were as follows: CAT3AM1, 5′-TGCTCTAGATCAGGCAGAGGCAGTGGGGTGGCAGCAT-3′ (upper strand) and 5′-CGCGGATCCAGCAGAAGCAGGATTGGTTGCATC-3′ (lower strand); CAT3AM2, 5′-TGCTCTAGAATCAGGCAGAGGCAGTGGGGGTGG-3′ (upper strand), 5′-GGTGTTGTGAGACGACACTCTTTTTCTGTAAATGCTGCCCACCCC-3′ (lower strand), 5′-GAGTGTCGTCTCACAACACCAACCAGAGGGGAACATGAAG-3′ (upper strand), and 5′-CGCGGATCCAGCAGAAGCAGGATTGGTTGCATC-3′ (lower strand); CAT3AM5, 5′-TGCTCTAGAATCAGGCAGAGGCAGTGGGGGTGG-3′ (upper strand) and 5′-GGGGGATCCGCT-

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Abbreviations: CAT, chloramphenicol acetyltransferase; CTL, cytotoxic T lymphocyte; EMSA, electrophoretic mobility-shift assay; GrzB, granzyme B; IL, interleukin; PHA, phytohemagglutinin; PBL, peripheral blood lymphocyte; TCR, T-cell antigen receptor.

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GCTGCTGTTGTTGCCTCC-3′ (lower strand). Underlining denotes regions of mutation. Amplification products were cloned upstream from the CAT reporter gene between the *Xba* I and *Bam*HI sites in the promoterless vector pBLCAT3. The mutations were confirmed by dideoxy DNA sequencing of the constructs.

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer and purified by PAGE. Sequences (5′ to 3′) were as follows: GB1 (−143 to −114), TCAGGCAGAGGCAGTGGGGGTGGGCAGCAT; GB1M, TCAGGCAGAGGCAGTGGGGTGGCAGCAT; GB2 (−103 to −77), TCTGTGATGAGACACCACAAAACCAGA; GB2M, TCTGTGATGAGACAAAAAAAAACCAGA; GB2A (−103 to −84), TCTGTGATGAGACACCACAA; GB2B (−95 to −69), GAGACACCACAAAACCAGAGGGGAACA; GB2BM, GAGACAAAAAAAAACCAGAGGGGAACA; GB5 (−20 to +6), GAGAGCAAGGAGGAAACAACAGCAGC; GB5M, GAGAGCAAGGAGGCAACAACAGCAGC; IK, CATGAATGGGGGTGGCAGAGA; IKM, CATGAATGGGGTGGCAGAGA; CBF, GATCCACAACAGGATGTGGTTTGACATTTA; CBFM, GATCCACAACAGGATGTGAGATCTCATTTA; AP-1, CGTGACTCAGCGCGG; ETS, ATAAACAGGAAGTGGT; ETSM, ATAAACACCAAGTGGT; NFAT-1, AGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGTT. IK is an Ikaros protein binding site derived from the mouse CD3 δ-chain gene enhancer (20); IKM is the mutated IK probe; CBF refers to the CBF binding site described in the human TCR β-chain gene enhancer (21); CBFM is the mutated CBF probe; the AP-1 site is derived from the human metallothionein IIA gene promoter (22); ETS and ETSM contain a high-affinity Ets binding site and a mutated Ets binding site, respectively (23). NFAT-1 is the NF-AT binding site of the human IL-2 enhancer (24). Underlining denotes regions of mutation.

Electrophoretic Mobility-Shift Assay (EMSA). Nuclear extracts from both resting PBLs and 48-hr PHA-activated PBLs were prepared (25). Single-stranded oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase and annealed with a 2-fold excess of the unlabeled complementary strand. The probe was incubated in a 20-μl binding reaction mixture containing 1 μg of poly[(dI-dC)·(dI-dC)] and 2 μg of nuclear extract in EMSA buffer (40 mM KCl/20 mM Hepes, pH 7.5/1 mM $MgCl_2$/0.1 mM EGTA/0.5 mM dithiothreitol/4% Ficoll). The DNA–protein complexes were separated by 5% PAGE.

Antibodies. The c-Fos (antibody K-25) is an affinity-purified rabbit polyclonal antibody raised against a peptide (aa 128–152) which maps to a highly conserved domain of human c-Fos p62 (Santa Cruz Biotechnology). K-25 reacts with vertebrate c-Fos, FosB, Fra-1, and Fra-2 proteins, as shown by immunoprecipitation, Western blotting, and cell staining. The c-Jun antibody, kindly provided by Michael Karin, was raised against the DNA-binding domain of the Jun protein family (26). For supershift experiments, the Fos antibody K-25 was added to the EMSA reaction mixture for 15 min on ice. For supershift experiments, the Jun antibody was preincubated with nuclear extract for 12 hr on ice.

Methylation Interference. Duplex oligonucleotides labeled on one strand were prepared as described above. The binding reaction mixture (25 μl) contained 500,000 cpm of end-labeled methylated probe and 25 μg of nuclear extract diluted in EMSA buffer. Free and bound DNA were treated as described (27).

RESULTS

EMSA analysis of the −148/+6 GrzB promoter region identified three major DNA binding sites that interacted specifically with nuclear factors from PHA-activated PBLs (Fig. 1).

The −143/−114 GB1 Region Contains an Ikaros Binding Site and Contributes to the Regulation of the GrzB Gene Promoter in Activated PBLs. A strong specific complex, C1 (Fig. 2*A*), was detected by the GB1 probe in nuclear extract from PHA-activated PBLs, but not in extract from resting PBLs. At least four weaker specific complexes, C2–C5, were detected with the GB1 probe; one of these, C4, was also present in nuclear extract from resting PBLs (Fig. 2*A*). Sequence analysis of the −143/−114 GB1 region revealed a G-rich element at −129 to −123 of the GrzB promoter. Such a TGGGGGT site was previously described in the mouse CD3δ enhancer and binds a zinc finger transcription factor called Ikaros, whose expression is restricted to lymphoid cells in the adult mouse (20). The IK oligonucleotide, corresponding to the Ikaros site of the CD3δ enhancer, efficiently competed with the GB1 probe for binding of the five protein complexes. An oligonucleotide bearing a deletion within the G box of the CD3δ enhancer's Ikaros site (IKM), previously reported to abrogate Ikaros binding (20), did not interfere with the formation of complexes detected by the −143/−114 GB1 site. The same results were observed with the GB1M probe, which bears the same mutation as the IKM probe. Using the IK probe, we detected strong specific binding of a factor present in PHA-activated PBLs, but not in resting PBLs (C1, Fig. 2*B*). Formation of this complex was inhibited by a 20-fold molar excess of unlabeled GB1 oligonucleotide. In contrast, a 100-fold molar excess of unlabeled GB1M or IKM oligonucleotide was ineffective in reducing binding to the labeled IK probe. Formation of the four other complexes seen with the IK probe was also efficiently blocked by the unlabeled GB1 and IK oligonucleotides but not by the unlabeled GB1M and IKM oligonucleotides. We then investigated the functional effect of a deletion within the GB1 site on the transcriptional activity of the GrzB promoter in PHA-activated PBLs. Deletion of the GB1 site in the G box, shown above to abrogate DNA binding, was introduced into CAT constructs which contained the −148/+60 promoter region fused to the CAT reporter gene (CAT3AM1). This deletion, when introduced into the Ikaros G-box binding site, abrogates the enhancer effect on CD3δ gene transcription (20). The wild-type promoter construct (CAT3A) and the CAT3AM1 mutated construct were introduced into PHA-activated PBLs. This mutation suppressed GrzB promoter activity in activated PBLs by a factor of 10 (Fig. 3), suggesting that members of the Ikaros family play a critical role in controlling GrzB transcription during T-cell activation.

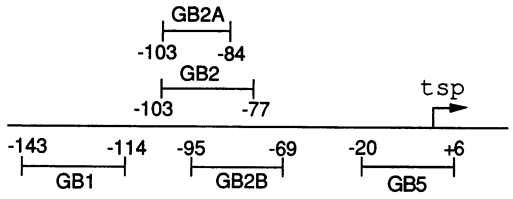

FIG. 1. DNA binding sites used for EMSAs are positioned within the GrzB promoter relative to the transcriptional start point (tsp).

The −103/−77 GB2 Element Binds AP-1- and CBF-Related Transcription Factors and Is Essential for GrzB Promoter Activity in Activated PBL. The −103/−77 DNA sequence of the GrzB promoter, known as GB2, was examined for its ability to bind nuclear factors present in both PHA-activated and resting PBLs. Two complexes, NFGB2A and NFGB2B, specifically interacted with this DNA element in activated PBLs (Fig. 4*A*). While analyzing the specificity of factors which bound to this element, we found that an unlabeled oligonucleotide containing an AP-1 site efficiently competed for the formation of the NFGB2A complex found in extracts from PHA-activated PBLs (Fig. 4*A*) and PHA/phorbol 12-myristate 13-acetate-activated PBLs (data not shown), but not from resting PBLs. This effect was specific and could be mapped to the 5′ end of the site by competition experiments using the GB2A probe (Fig. 4*A*). Since

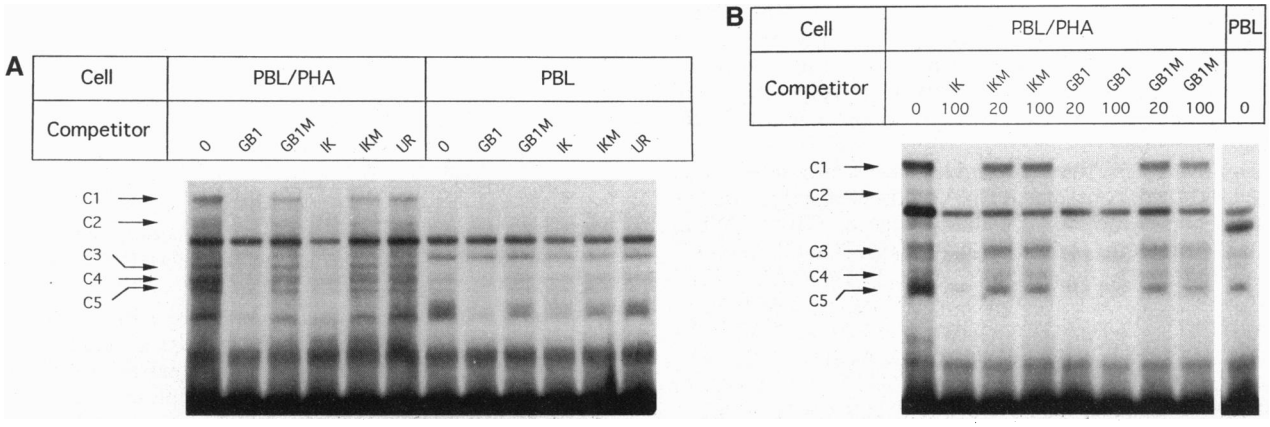

FIG. 2. The −143/−114 GB1 region of the human GrzB promoter contains an Ikaros binding site. EMSAs were carried out with nuclear extracts from resting PBLs and PHA-activated PBLs. (*A*) Binding of nuclear factors to the −143/−114 GB1 probe. A 100-fold molar excess of unlabeled DNA competitors was included in the assay to test the sequence specificity of the complexes. Competitors are indicated above the lanes; 0, no competitor; UR, an unrelated oligonucleotide. Arrows indicate the specific retarded DNA–protein complexes C1–C5. (*B*) Binding of nuclear factors to the CD3δ Ikaros binding site (IK probe). Competitors used are indicated above their respective molar excess compared with the radiolabeled IK probe.

the consensus AP-1 binding site did not completely match the −103/−77 region, we investigated the possibility of AP-1 binding to this site, using antisera directed against Fos and Jun. The Fos antiserum was able to supershift the NFGB2A complex, while the antiserum directed against Jun both inhibited the binding and supershifted this complex (Fig. 4*B*). These results suggest that the NFGB2A complex includes a factor that is closely related to AP-1.

Binding of the NFGB2B complex was assigned to the −95/−69 GB2B region (Fig. 4 *A* and *C*). The NFGB2B complex was formed with extract from activated PBLs, but not with extract from resting PBLs (Fig. 4*C*). The binding of factors in the NFGB2B complex was further investigated by methylation interference, revealing a pattern of methylation of three guanines on the lower strand of the −95/−69 GB2B site (data not shown), which is reminiscent of the binding site TGTGGT for the CBF found in the enhancer of several T-cell-specific genes, including the TCR genes (28). Formation of the NFGB2B complex was inhibited by the addition of unlabeled CBF oligonucleotide (Fig. 4*C*), which corresponds to the CBF site of the TCRβ enhancer (21). Oligonucleotides designed with a mutation in the CBF site reported to abolish CBF binding (CBFM) (21) or in the GB2B site (GB2BM) were unable to compete for the formation of the NFGB2B complex (Fig. 4*C*). Methylation interference analysis using the −103/−77 GB2 region revealed that guanine residues involved in CBF binding also played a role in the formation of the upper complex, which contains the AP-1 component, since this site was protected when the upper NFGB2A complex was analyzed (Fig. 4*D*). Mutation in the −103/−84 region, which contains the guanines involved in the formation of NFGB2A and NFGB2B complexes, was introduced into the CAT −148/+60 construct (CAT3AM2), resulting in a significant decrease in transcription of the GrzB promoter in PHA-activated PBLs (Fig. 3). These data suggest that the −103/−84 region is essential for the full effect of the GrzB promoter in these cells.

The −20/+6 Region of the GrzB Promoter Binds an Ets-Related Factor, but Mutation of the Ets Binding Site Does Not Affect Promoter Activity in Activated PBLs. The −20/+6 region of the GrzB promoter contains sequences in the −13/−5 region that are reminiscent of consensus binding sites for factors of the Ets family. EMSAs using extract from activated PBLs revealed a specific low-migrating complex that interacted with the −20/+6 GB5 oligonucleotide (Fig. 5). This lower complex was observed with nuclear extracts from both resting and PHA-activated PBLs (data not shown). Competition was observed for the formation of the lower complex by the ETS oligonucleotide, but not by mutated sites ETSM or GB5M (Fig. 5). Methylation interference analysis revealed that guanines present in the core GGAA Ets binding site interfered with binding of this factor to the GB5 probe and that the GB5 oligonucleotide specifically bound recombinant Ets protein (data not shown). The −13/−5 region of the GrzB promoter also contains an NF-AT binding site, but binding of the NF-AT factor to the −20/+6 site was excluded (data not shown). Mutation of the −20/+6 Ets site, which interferes with Ets binding, was introduced into CAT constructs of the GrzB promoter (CAT3AM5) and did not affect promoter activity in PHA-activated PBLs (Fig. 3). These results suggest

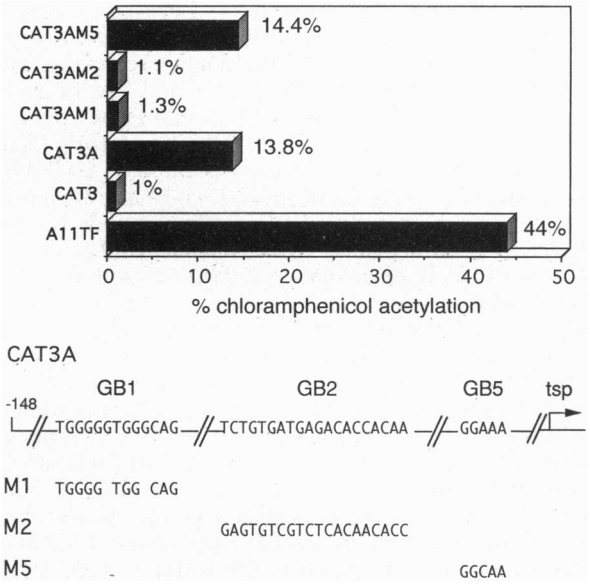

FIG. 3. Relative activity of GrzB promoter constructs containing mutations within the GB1, GB2, and GB5 binding sites was analyzed by transient CAT expression PHA-activated PBLs. The CAT3A construct contains the −148/+60 region of the functional GrzB promoter; CAT3AM1 is the same as CAT3A except for two 1-bp deletions in the GB1 Ikaros binding site; CAT3AM2 is mutated within the GB2 region; in CAT3AM5, a mutation was introduced in the Ets binding site within the GB5 region. CAT3 is a promoterless vector used as negative control and A11TF is an enhancer construct used as positive control; tsp, transcription start point. Values of % chloramphenicol acetylation are means from at least five experiments.

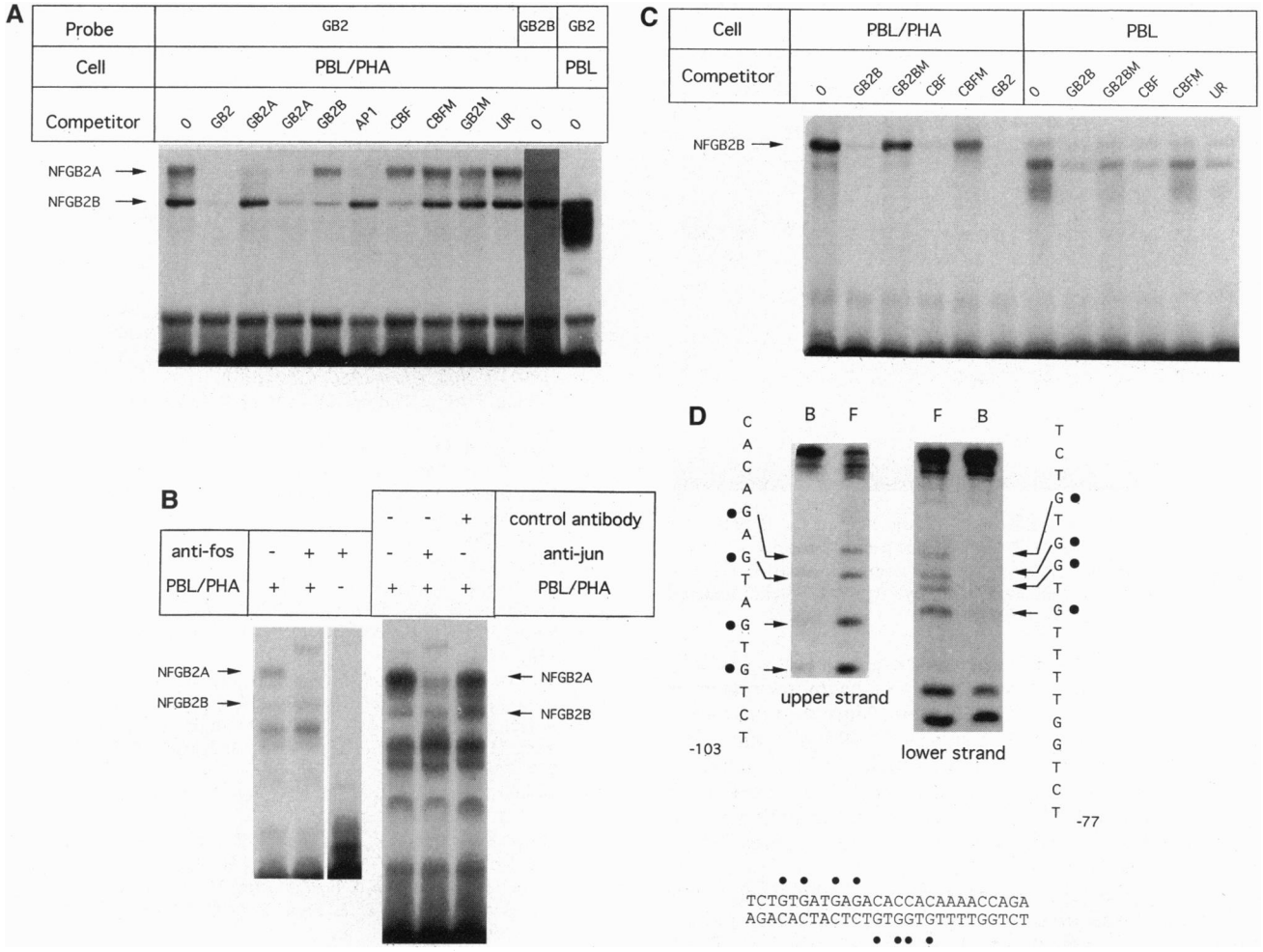

FIG. 4. Nuclear factors specifically found in PHA-activated PBLs bind to the −103/−77 GB2 region. (*A*) End-labeled GB2 and GB2B probes were used in an EMSA with nuclear extracts prepared from PBLs and PHA-activated PBLs. Competitors are indicated above the lanes; 0, no competitor; UR, unrelated oligonucleotide. A 100-fold molar excess of DNA competitors was included except for GB2A, right lane, where a 250-fold excess was used. Arrows indicate specific DNA–protein complexes NFGB2A and NFGB2B. (*B*) Presence of Fos and Jun proteins in the NFGB2A complex found in nuclear extract of PHA-activated PBLs. Supershift of the NFGB2A complex is seen with both Fos (*Left*) and Jun (*Right*) antibodies. (*C*) GB2B probe was end-labeled and used in an EMSA with nuclear extracts prepared from PBLs and PHA-activated PBLs. Competitors (100-fold molar excess) are indicated above the lane. (*D*) Methylation interference analysis of the NFGB2A complex with the GB2 oligonucleotide treated with dimethyl sulfate. B, bound; F, free probe. Protected guanine (G) residues are indicated (•).

that although this site can bind Ets-related proteins, this interaction is not functionally significant in the induction of the GrzB promoter during T-cell activation.

DISCUSSION

The GrzB transcription rate is increased during activation of T cells. We have previously shown that major regulatory elements that control the activation of transcription are located in the −148/+60 region of the human GrzB promoter (18). A cAMP response element with some analogy to a 10-bp sequence found upstream from the TCR β-chain genes, located at −67 to −60 of the GrzB promoter, was shown not to be crucial for GrzB transcription during T-cell activation (17) and was thus not investigated in this study. We have focused on (*i*) the fine mapping of regulatory elements which bind transactivating factors specifically found in nuclear extract of PHA-activated PBLs and (*ii*) mutational analysis of regulatory sites in order to assess their roles in the activation of GrzB transcription. Our results show that the −143/−114 GB1 region contains an Ikaros binding site whose integrity is essential for GrzB promoter activity in activated PBLs. At least five specific complexes specifically interact with the GB1 site and could represent the binding of isoforms of the Ikaros protein (29–31). The Ikaros factor was originally isolated as a T-cell-specific gene that encoded a zinc finger protein which bound to an enhancer element of the CD3δ gene (20). More recent studies have shown that this gene plays a key role in the development of mature T- and B-lymphocytes and natural killer cells (29). Our results implicate an Ikaros binding site in the regulation of a human T-cell-specific gene that encodes the GrzB serine protease, thereby reinforcing the hypothesis of a pivotal role for this factor in lymphoid-specific gene expression.

A second site, located in the −103/−77 region of the GrzB promoter, is able to bind two factors that can be assigned to AP-1- and CBF-related proteins. Methylation interference analysis shows that formation of the two protein complexes at this site is not independent. Furthermore, mutation of the sole 3′ CBF binding site in the −103/−77 region also abrogates formation of the NFGB2A complex containing AP-1 (data not shown). Nuclear proteins that bind to CBF motifs appear to play a general role in the T-cell-specific expression of cellular genes (21, 28, 32, 33). Mutation of the −103/−77 region containing the AP-1 and CBF binding sites has a drastic effect on GrzB promoter activity in activated PBLs, suggesting that these factors are crucial in activating GrzB transcription in these cells.

Several members of the *ets* protooncogene family have been shown to control differential expression of T-cell genes, such

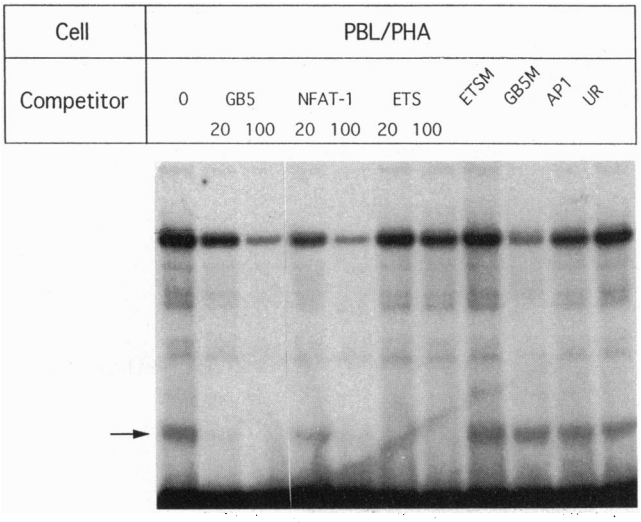

FIG. 5. The −20/+6 GB5 region of the human GrzB promoter contains an Ets binding site. The GB5 probe was end-labeled and used in an EMSA with nuclear extract prepared from PHA-activated PBLs. Competitors and their molar excess relative to the GB5-radiolabeled probe are indicated above each lane.

as those encoding TCR, CD4, IL-3, Lck, and perforin (34–38). One binding site, located in the −20/+6 promoter region, is indeed able to bind Ets-related protein, but mutational analysis of the −20/+6 Ets binding site shows no significant effect of the mutation on GrzB promoter activity in PHA-activated PBLs. Ets-related proteins do not seem to be essential for GrzB transcription during T-cell activation, but their possible implication in the downregulation of GrzB expression in resting T-cells remains to be explored.

In summary, this report describes three regulatory elements in the human GrzB promoter, two of which are essential for stimulating GrzB transcription during T-cell activation. These two regulatory sites which control GrzB promoter activity in activated T-cells, the Ikaros binding site and the CBF binding site, have previously been implicated in the control of T-cell-specific expression of various genes, such as the TCR and CD3δ genes. These findings emphasize the role of these regulatory sites in the cascade of events leading to T-cell differentiation. The cooperativity of these factors in activating and/or suppressing GrzB expression during the various stages of T-cell commitment leading to CTL activation remains to be elucidated.

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