Mutational Analysis of the Murine Granzyme B Gene Promoter in Primary T Cells and a T Cell Clone*

(Received for publication, March 26, 1997, and in revised form, May 1, 1997)

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The granzyme B gene is induced in cytotoxic T lymphocytes in response to antigenic stimulation. Previous studies have identified several distinct regions in the granzyme B promoter which may be important in either the induction or the T cell specificity of the gene. These regions contain the canonical transcription factor binding sites AP1, cyclic AMP-responsive element (CRE), Ikaros, and core-binding factor (CBF/PEBP2). Each protein binding site was disrupted by site-directed mutagenesis to investigate its role in granzyme B promoter function. Mutations were introduced alone, or in various combinations, within the context of a 243-base pair promoter fragment known to confer high levels of reporter gene expression. Transfection assays revealed that all of the single binding site mutant promoters were capable of sustaining moderate to high levels of transcriptional activity in primary activated T lymphocytes, whereas certain mutants were more impeded in a T cell clone. A quadruple mutant promoter, with only the CRE binding site intact, showed background expression levels. This drop in expression was found to be mostly due to mutations in AP1 and the 3′ CBF binding sites. Their close proximity and requirement in promoter function suggest an important role for protein-protein interaction between these two factors.

In the immune system, the role of cytotoxic T lymphocytes (CTLs)¹ is to identify virus-infected or transformed cells and destroy them. T cells recognize foreign antigen on target cells via T cell receptor complexes in the context of major histocompatibility molecules (1). This recognition event induces a variety of molecular signal transduction pathways that activate the cell to proliferate (2). The expanding population of T cells acquires killing potential through the de novo induction of a specific subset of genes that encode the proteins that constitute the killing machinery (3). One form of T cell-mediated killing involves the production of specialized granules that accumulate in the cytoplasm following activation. These granules contain the pore-forming protein perforin (4) and a number of cytotoxic proteinases (granzymes) (5). Upon contact of an activated T cell with a target cell displaying foreign antigen, the granules and their contents are directionally exocytosed into the intercellular junction in a calcium-dependent process. Target cells are believed to take up granule components in a process that is facilitated by perforin. The exact cytotoxic nature of granzymes remains unclear, but granzyme B has been implicated as a mediator of DNA fragmentation once inside the target cell, through the activation of CPP32-like proteinases (6).

Following T cell activation, granzyme B gene expression is induced at the level of transcription, and its mRNA level peaks after 3 or 4 days. This pattern of expression correlates well with the onset of potent killing potential under a variety of conditions and is a useful marker of CTL activation in vivo (7). Previous studies have indicated that transcriptional regulation may be controlled by 5′-flanking promoter sequences (8). A series of promoter deletion fragments ranging from 108 bp to 5 kilobases were examined for their ability to confer transcriptional activity on a reporter gene following transfection into T cells. It was found that two promoter fragments consistently generated the highest reporter gene activity: –828 and –243. The 243-bp granzyme B promoter contains five regions in which we have found evidence of transcription factor binding both in vitro and in vivo. Several distinct regions of protection were evident in DNase I footprinting experiments with nuclear extracts derived from T cells. These coincide with consensus sequence binding sites for two known ubiquitous transcription factors, activating transcription factor/cyclic AMP-responsive element-binding proteins (ATF/CREB) and activator protein-1 (AP1), and two lymphoid-specific factors, core-binding factor (CBF/PEBP2) and Ikaros. In vivo evidence that these sites are important in promoter function was demonstrated by in vivo footprinting and DNase I hypersensitivity analyses (9) in T cell lines and in activated primary CD8⁺ T cells. Moreover, several of these transcription factor binding sites are evolutionarily conserved between the murine and human granzyme B promoters (10–12).

Several distinct signal transduction pathways are activated via the T cell receptor and costimulatory molecules. These signals are integrated in the nucleus in the form of a particular subset of activated transcription factors that converge on a specifically targeted promoter and activate transcription. By studying the specialized requirements for T cell-specific gene expression in terms of transcription factor necessity we can further understand the molecular events that are associated with T cell activation. To determine whether any of the above mentioned binding sites are required for granzyme B transcription, point mutations were systematically introduced into each,
either individually or in various combinations, and were assessed for their effect on the 243-bp promoter. We analyzed our promoter mutations by transient transfection assays in two distinct populations of T cells, freshly activated primary lymphocytes and an IL-2-dependent T cell clone (MTL). Primary lymphocytes were activated by a variety of nonchemical stimulation conditions and then transfected. MTL cells constitutively express the granzymes, and transfections were performed without any additional stimulation other than IL-2. Whether this immortalized state of activation reflects the state of primary activation is unknown; however, our transfection studies in these distinct cell types revealed some differences. These experiments enabled us to observe the effects of removing each transcription factor from the native promoter complex and assess its importance in the regulation of the granzyme B gene.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed using the Polymerase Chain Reaction (Amersham Corp.). All mutations were confirmed by sequencing.

Plasmids—Granzyme B promoter fragments were obtained by restriction enzyme digestion or polymerase chain reaction amplification. They were inserted upstream of the promoterless luciferase reporter gene of the basic pGL2 vector (Promega), and orientation was confirmed by sequencing or directional primer amplification. SVβ-gal contains the bacterial β-galactosidase gene under the control of the SV2 viral promoter (Promega). Plasmids were grown in DH5a Escherichia coli, and high quality supercoiled DNA was purified for transfection by CsCl density gradient centrifugation. DNA concentration and covalently closed circular content were determined by ethidium bromide fluorometric analysis in a Quioina-Turner model 450 fluorometer. The CD3+ and CD8+ T cell portion of a whole splenocyte population expanded in vitro was isolated by immunomagnetic separation from 6–12-week old BALB/c mice. Spleen tissue was ground through a fine wire screen in RHFM/IL-2 medium, and the cells were pelleted. Red blood cells were lysed with buffered ammonium chloride lysis buffer. The IL-2-dependent cytotoxic T cell line MTL 2.8.2 was generated from CBA/J mice as described (13). MTL 2.8.2 cells and primary splenocytes were cultured in RHF (RPMI supplemented with 20 mM HEPE (pH 7.5), 100 mM β-mercaptoethanol, and 10% fetal bovine serum) in the presence of 60 units/ml of human recombinant IL-2. Primary splenocytes were stimulated with 5 μg/ml concanavalin A (ConA; Sigma) and 1:750 to 1:1,000 dilution (determined empirically) of hamster anti-mouse CD3 monoclonal antibody supernant.

Transfections—Transient transfections were performed using a DEAE-dextran transfection procedure optimized for cytotoxic T cells (8). Primary splenocytes were cultured at 3.0 × 10^6 cells/ml in RHF plus 60 units/ml IL-2, 1:750 dilution CD3, and 5 μg/ml ConA for 20 h prior to transfection. Basically, 1.0 × 10^7 exponentially growing cells (MTL) or 2.0 × 10^7 (whole splenocytes) were washed twice in serum-free medium and resuspended in 1.0 ml of TBS (25 mM Tris-HCl (pH 7.5), 137 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.7 mM CaCl2, and 0.5 mM MgCl2 (pH 7.0)) with 500 μg/ml DEAE-dextran (Sigma), 15 μg of covalently closed circular luciferase reporter plasmid, and 5 μg of β-galactosidase control plasmid. The DNA was adsorbed for 15 min at room temperature. Cells were washed twice in serum-free medium and cultured at 2.5 × 10^7 cells/ml (MTL) or 2.0 × 10^6 cells/ml (splenocytes) in RHF plus 60 units/ml IL-2 and incubated at 37 °C in 5% CO2. Primary splenocytes were stimulated with additional ConA and ConA following transfection. The cells were harvested after 48 h, washed twice in phosphate-buffered saline, lysed in Triton lysis buffer (1% Triton X-100, 25 mM glycyglycine (pH 7.8), 15 mM MgSO4, 4 mM EDTA, 1 mM dithiothreitol), and luciferase and β-galactosidase assays were performed.

Luciferase and β-Galactosidase Assays—Three aliquots of cell lysates (100–200 μl) were measured for 20 s following the injection of Luciferase Reagent (Luciferase Assay, Promega) by a LUMAT LB5051 luminometer (Berthold Systems Inc.). β-Galactosidase assays were performed as described (14). Final activities are given as luciferase/β-galactosidase values.

RESULTS

It was previously established by in vitro footprinting that within the 243-bp granzyme B promoter, there existed five distinct regions that were protected from DNase I digestion (9).

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FIG. 1. Nucleotide sequence of the proximal granzyme B promoter and the nucleotide substitutions present in the transcription factor binding sites. Major potential transcription factor binding sites are shown declinded, and the transcription factor designations are shown above the sequences. Specific nucleotide substitutions are indicated by the arrows below the sequence for each binding site. The transcription start site is indicated by an arrow, and the translation initiation start site (ATG) is indicated in boldface.

These regions correspond to transcription factor binding sites for ATF/CREB, AP1, CBF, and Ikaros, and specific DNA-protein interactions were observed as in vivo footprints in activated CD8+ T cells and MTLs. This region corresponds to a strong DNase I-hypersensitive site that was only detectable in activated T cells. To determine whether any of these binding sites were important for the high levels of reporter gene expression observed from the 243-bp promoter, we constructed a series of promoters with three nucleotide substitutions in each binding site. This strategy conserved the spacing and the helical orientation of the binding sites. The bp changes were primarily transversions in the consensus sequence binding sites and were chosen on the basis of previous methylation interference data, in vivo footprinting data, and site-directed mutagenesis data collected either on the granzyme B promoter or on similar binding sites in other gene promoters (Fig. 1). In addition to the single binding site mutations, various double mutant promoters were constructed, as well as a quadruple mutant promoter in which the AP1, Ikaros, and both CBF sites were abolished, leaving only the CRE intact. Each mutant binding site, with the exception of the CRE and 5' CBF, was tested by electrophoretic mobility shift competition assays for the inability to compete for the wild type binding site with up to a 200 molar excess of mutant probe (data not shown). Following the mutagenesis reactions each construct was confirmed by sequencing and subcloned into a promoterless luciferase gene plasmid. Plasmid DNA was purified by cesium chloride density gradient centrifugation to obtain high quality supercoiled DNA. The concentration and percentage of covalently closed circular DNA was measured by fluorometric analysis prior to every transfection, and the covalently closed circular DNA content was not less than 90% for any test plasmid used.

Mutational Analysis of the Murine Granzyme B Promoter in Stimulated Primary Lymphocytes—We have shown that activated CD8+ cells isolated by immunomagnetic separation were able to drive high levels of reporter gene expression from the granzyme B promoter in transient transfections. Typically, the T cell portion of a whole splenocyte population expanded in culture to approximately 75% following stimulation with αCD3 or ConA for 3 days, as assessed by fluorescence-activated cell sorter analysis. The CD8+ population proliferated and ex-
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Activated Primary Lymphocytes

**Fig. 2.** Transient transfection analysis of granzyme B promoter mutations in primary lymphocytes stimulated with αCD3, ConA, and IL-2. Three deletion fragments and a series of transcription factor binding site mutants were cloned into the pGL2 promoterless luciferase reporter gene plasmid and were transfected along with an SVβ-galactosidase control plasmid into primary lymphocytes as described under “Experimental Procedures.” Cells were harvested after 2 days and assayed for reporter gene activity. The values represent an average of two independent transfections and are depicted as the relative light unit value of the luciferase assays divided by the A420 value of the β-galactosidase assays. The maximum averaged S.D. of the luciferase assays (performed in triplicate) for the two experiments was ± 0.335 (5′ CBF/Ikaros), and the minimum was ± 0.046 (pGL2). Promoter fragments and transcription factor binding sites are represented diagramatically on the left with boxes, and binding site mutations are indicated with an X.

The averaged corrected values for two independent primary cell transfection experiments are shown in Fig. 2. These cells were stimulated with αCD3, ConA, and IL-2. The values on the y axis represent the RLU of the luciferase assay divided by the absorbance of the β-galactosidase assays × 1,000. Because the β-galactosidase expression and incubation times of the assay differ between each transfection, the corrected values are only relative within a single experiment and are not compared between experiments or cell types. The promoterless pGL2 basic vector served as the negative control and gave luciferase values that were essentially background. The next three bars depict the promoter deletion series −108 to +29, −169 to +29, and −243 to +68. As transcription factor binding sites were added by increasing the length of the promoter to 243 bp, the luciferase activity increased substantially. The next eight bars represent the mutant promoter series in the context of the 243-bp promoter (−243 to +68). The largest effect for any single point mutation was observed for the CRE binding site, which lowered total promoter activity to 60% of wild type. A surprising finding was that the mutation of the AP1 binding site appeared to have relatively little impact on overall promoter activity (73% of wild type). Mutations in the 3′ CBF or Ikaros binding sites had slight negative effects on transcription, whereas the mutation of the 5′ CBF site had no effect on promoter activity. Overall, none of the single binding site mutations reduced activity to an extent where it was possible to conclude that any one factor was necessary for the expression of the 243-bp granzyme B promoter.

We then looked at the mutant binding sites in combination. Promoters lacking either or both the 5′ and 3′ CBF binding sites consistently showed normal expression in transient assays. The 5′ CBF/Ikaros double mutant contained the same binding site compliment as the −169 promoter and was approximately as active. When the AP1, Ikaros, and both CBF elements were all ablated the result was a very dramatic decrease in overall activity, comparable to the expression observed from the −108 promoter. Therefore, no other elements distal to the CRE were involved in promoter transcription.

**Primary Lymphocytes Treated with Individual Stimuli Displayed Similar Expression Patterns of the Mutant Promoters**—

Our primary cell cultures were initially stimulated with a combination of three potent activators of T cells (αCD3, ConA, and IL-2) in an effort to maximize granzyme B-luciferase reporter gene expression. This treatment, however, may mask contributions to overall promoter output by the individual transcription factors. The αCD3 antibody utilized in these experiments binds to the ε chain of the CD3/TCR complex (15). This antibody, in combination with IL-2, is a sufficient stimulus to generate potent cytolytic activity in primary lymphocyte cultures. ConA is a widely employed mitogen that interacts non-specifically with surface glycoproteins and is capable of activating T cells through the T cell receptor and likely through other glycosylated surface receptors. At high doses, IL-2 acti-
vates primary T lymphocytes to express perforin and granzyme B mRNAs, and these cells possess cytotoxic potential (16, 17). Instead of using all three stimuli simultaneously, αCD3, ConA, and IL-2 were added in culture individually, or in combination, and the mutant promoter fragments were transfected as above and assessed for reporter gene expression.

ConA alone or with added IL-2 appeared to be the best stimulus for this type of culture as the cells proliferated well and expressed high levels of luciferase activity from the granzyme B promoters. Fig. 3 depicts the average expression profiles of the wild type and mutant granzyme B promoters in ConA- and ConA + IL-2-stimulated whole primary lymphocytes. In cells treated with ConA alone (panel A), mutations in the CRE and AP1 binding sites lowered overall promoter expression to approximately 60% of wild type. The Ikaros mutation and either CBF binding site mutations were somewhat inhibitory to promoter activity. Only the 4x mutant promoter, with mutations in the AP1, Ikaros, and both CBF sites, significantly decreased promoter activity to near background levels, similar to the 108-bp promoter fragment (data not shown). No major effect of any individual binding site mutations was evident in these transfection experiments with ConA stimulation alone. Continuous exposure to 60 units/ml exogenous IL-2 in addition to ConA did appear to enhance the effects of certain mutations slightly. Under these conditions, mutations in the CRE and AP1 binding sites lowered overall promoter activity to 54 and 40% of wild type, respectively, the greatest extent observed in any of the primary cell transfections (Fig. 3B). The Ikaros and 5' CBF were dispensable to promoter function in these transfections, but the 3' CBF site may have been somewhat important as the expression of this mutant was only slightly higher than that of the CRE mutant.

Transfection experiments with cells stimulated with αCD3 alone resulted in a very good transfection efficiency, as determined by the high levels of β-galactosidase expression from the control plasmid, but very little granzyme B promoter activity was observed from any of the mutant or wild type promoters (data not shown). The combination of αCD3 and IL-2 did stimulate the cells enough to detect significant luciferase levels when approximately twice the normal number of cells were transfected. The expression pattern was very similar to that obtained with ConA stimulation. The CRE and AP1 sites were most important, but neither mutation lowered promoter activity to a significant extent, and the Ikaros and both CBF binding sites were dispensable (data not shown).

Finally, primary lymphocytes were treated with 5,000 units/ml human recombinant IL-2 and transfected with the longer 828-bp promoter after 20 h of initial stimulation. After further IL-2 treatment, no luciferase activity and only trace amounts of β-galactosidase activity were detectable after 1, 2, or 3 days post-transfection, and the cells failed to proliferate at any significant rate. It is likely that these cells were resistant to transfection and were unable to express the reporter gene.

Analysis of the Mutant Promoters in MTL 2.8.2 Cells—MTL 2.8.2 is an immortalized, IL-2 dependent, cytotoxic T cell clone that constitutively expresses the endogenous granzymes. These cells were transfected with the wild type and mutant promoter constructs and assayed for expression (Fig. 4). The absolute luciferase values were higher in MTL than in primary cells with the RLU reading for the full-length 828-bp promoter being greater than 10,000. The pGL2 promoterless vector served as the negative control and was essentially background. The activities of the minimal promoter deletion series are de-
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MTL 2.8.2.

**FIG. 4. Transfection analysis of granzyme B promoter mutations in an IL-2-dependent murine CTL line, MTL 2.8.2.** The same constructs as shown in Fig. 2 were transfected into MTL 2.8.2 cells cultured in IL-2-containing media (60 units/ml) and harvested after 2 days. The values represent the average of two independent transfections and are depicted as luciferase/β-galactosidase. The maximum averaged S.D. of the luciferase assays (performed in triplicate) for the two experiments was ± 1.69 (5' CBF), and the minimum was ± 0.061 (pGL2). Transcription factor binding sites are represented by boxes on the left, and mutations are indicated with an X.

Luciferase/β galactosidase

| pGL2(basic) |
|-------------|
| 0 10 20 30 |

AP1 In Conjunction with 3' CBF is Important in Conferring High Levels of Granzyme B Promoter Activity in T Cells—It was observed that when the AP1, Ikaros, and both CBF binding sites were abolished, expression dropped to near background levels. However, neither of the CBF sites appeared to be important for the observed high activity of the 243-bp fragment. To determine whether the AP1 and Ikaros binding sites were responsible for this dramatic loss of activity, a double mutant promoter was constructed in which both the AP1 and Ikaros sites were abolished. Additionally, when the AP1 and 3’ CBF sites were added to the –108 promoter (the –169 fragment), activity increased substantially, indicating that these two factors may be very important for promoter activity. A second double mutant promoter was constructed in which both the AP1 and 3’ CBF sites were abolished.

The reporter gene activity of these new mutant constructs was compared with the wild type promoter, each single mutant, and the 4X mutant promoter. The averaged values for four independent transfection experiments in activated primary cells and two independent transfections in MTL cells are shown in Fig. 5. In primary cells, the AP1 mutation appeared to reduce promoter activity to 61% of wild type, and Ikaros had no inhibitory effect (panel A). The AP1 mutant was much less active in MTL cells (37% of wild type), and some inhibition was observed for the Ikaros mutant (panel B). When both the AP1 and Ikaros binding sites are abolished, promoter expression was reduced to an extent in primary cells (47% of wild type) and was approximately the same as the AP1 mutant in MTL (40%). When the AP1 and 3’ CBF binding sites are abolished, the promoter is still active in MTL cells (73%), despite the absence of a functional AP1 binding site. However, expression is reduced to background levels (20%) in primary cells, indicating that together these sites are very important in conferring high transcriptional activity. From this series of transfections we conclude that the presence of functional binding sites for AP1 and the 3’ CBF is primarily responsible for the high level of expression observed from the granzyme B minimal promoter in primary lymphocytes. MTL cells, however, were primarily sensitive to mutations in the AP1 binding site alone but not in combination with the 3’ CBF.

**DISCUSSION**

The granzyme B gene is transcriptionally induced upon antigenic stimulation of cytotoxic T cells. We have previously identified several transcription factors that are involved in the induction or maintenance of an active granzyme B promoter and have shown that they bind to their cognate sequences in the endogenous promoter. In our present studies, primary murine lymphocytes stimulated via the T cell and IL-2 receptors were utilized as the principal experimental system. Results from transfection studies in this physiologically relevant system were compared with data obtained from transfections of the same reporters in a T cell clone. Primary lymphocytes express granzyme B mRNA in a stimulation-dependent manner. They require at least two independent signals for proliferation and CTL-specific gene expression. First, they are stimulated through the T cell receptor, by presented antigen, and second by IL-2 through the IL-2 receptor. MTL cells are differ-
ent in that they are an antigen-independent, IL-2 dependent, cytotoxic T cell line (13). MTL 2.8.2 cells were selected for antigen independence by culturing in the presence of high levels of IL-2 and the phorbol ester PMA (18), and they constitutively express all of the granzyme genes.

It was apparent from our previous transfection studies that reporter gene expression increased substantially as the length of the minimal promoter increased in length from 108 bp, to 169 bp, and to 243 bp. The shortest promoter included only the CRE binding site at $-90$. The next largest fragment included one CBF binding site at $-125$ and an AP1 at $-150$. The 243-bp promoter included another CBF binding site at $-180$ and Ikaros at $-200$. Promoter expression increased substantially in both primary lymphocytes and MTLs as more binding sites were appended; however, promoter activity decreased as the fragment was extended to $-402$ bp. Therefore, our studies focused on the sites contained within the 243-bp fragment.

The granzyme B CRE is a target for a ubiquitous family of basic leucine zipper-containing transcription factors (19). These proteins may become potent transcriptional activators following phosphorylation through the cAMP-stimulated protein kinase A signal transduction pathway. Stimulation through the T cell receptor results in increased levels of intracellular cAMP and the subsequent activation of transcription factors that are required for cAMP-responsive gene regulation. In the context of the short 108-bp promoter, the granzyme B CRE did not have significant transcriptional activation activity. However, it was quite important when included in the $-243$ promoter as its mutation reduced transcription between 36 and 60% of wild type in T cells. Thus, CREB's only exerted a substantial positive effect when linked in cis with the other transcription factors.

The ubiquitous transcription factor AP1 is composed of heterodimers of the fos and jun family of DNA-binding proteins. Upon T cell activation, fos and jun are induced by de novo synthesis, and their transcriptional activation potentials are stimulated by phosphorylation (20). The DNA binding activity of AP1 is low or absent in resting T cells (21). Upon stimulation through the T cell receptor, these proteins were observed to form a strong specific complex with an oligonucleotide probe containing the granzyme B AP1 binding site and a distinct in vivo footprint (9). Overall promoter expression in primary lymphocytes was lowered 73% to 61% of wild type when this site was altered in the $-243$ promoter. MTL cells were quite sen-
sitive to mutations in the AP1 binding site (64% to 39% of wild type expression). The results with MLT are similar to studies involving the human granzyme B promoter where the AP1 binding site was found to be essential for reporter gene expression in 12-O-tetradecanoylphorbol-13-acetate/dibutyryl cAMP-stimulated PEER cells (11). Analogously, the proximal AP1 binding site in the IL-2 gene promoter was necessary for high levels of reporter gene expression in both PMA-stimulated human peripheral blood cells and PMA-stimulated Jurkat T cells (22). The MLT line was developed from T cells treated with high levels of PMA and IL-2. The difference between our findings in primary cells and previous data in T cell clones may be explained by an inflated protein kinase C response due to stimulation by phorbol esters.

Core-binding factor, also known as polyoma enhancer-binding protein (PEBP2), is a heterodimer of an evolutionarily conserved family of three α subunits and a single β subunit (23–27). Following stimulation of T cells, the β-CBF subunit is believed to translocate from the cytoplasm into the nucleus where it augments the DNA binding activity of a lymphoid-specific α-CBF subunit (28). Weak CBF DNA binding activity was observed in resting T cells, but a distinct complex was observed to bind to the granzyme B 3′ CBF site in activated T cells. Although both CBF sites in the granzyme B promoter displayed prominent in vivo footprints in activated T cells, the mutation of either or both CBF sites had little or no negative effect on transcription in murine CTLs.

Ikaros is a zinc finger DNA binding protein that is expressed throughout hemopoietic development and is essential for the differentiation of the lymphoid lineage of cells (29, 30). This protein has been observed to bind in vitro to a nonconsensus DNA binding site in the granzyme B promoter in both resting and activated CTLs. The murine granzyme B Ikaros element is very similar to those found in the human granzyme B promoter and the CD3δ promoter. Mutations introduced into the Ikaros binding sites of both of these promoters significantly abrogated their expression in T cells (12, 29), whereas no negative effect was observed for mutations in the murine granzyme B Ikaros element.

In theory, a promoter fragment in which the 5′ CBF and Ikaros binding sites are abolished should only be as active as the 169-bp fragment, if they contained the same compliment of functional binding sites. Similarly, a promoter in which the AP1, Ikaros, and both CBF binding sites are abolished should reflect only the activity observed from the 108-bp promoter. In our primary cell transfection experiments the expression of the 5′ CBF/Ikaros double mutant was identical to that of the −169 promoter. The expression of the 4X mutant promoter was identical to the −108 promoter in both primary cells and in MLT cells. Therefore, we conclude that no transcription factors, other than the ones we have identified, are playing a significant role in the transcription of the minimal granzyme B promoter.

Primary lymphocytes must remain sensitive to a variety of signals so that inducible genes can be precisely controlled by subtly different stimuli. In contrast, cell lines are likely locked into invariably active signal transduction pathways. Recently, striking differences regarding the regulation of the IL-2 promoter were observed in transfection experiments performed in Jurkat T cells and primary lymphocytes, both activated with PMA + phytomyagglutinin. Several mutations were far more inhibitory to transcription in Jurkats than they were in primary cells (22). The most compelling differences between the results of our transfection studies performed in primary cells and those conducted in cell clones are that several transcription factors, such as AP1, CRE, and CBF, were not as important for transcription in primary cells as they have been reported to be under different conditions in cell lines (11, 12, 31). This may reflect the removal from normal growth and transcriptional control mechanisms which is typical of immortalized cell lines. MLTs were more sensitive to mutations in the CRE and AP1 binding sites than primary cells likely because they possess highly active forms of CREBs and AP1 subunits. Hence, the removal of these factors from the promoter complex would significantly affect expression.

These studies strongly suggest a functional interaction between the AP1 and CBF complexes. No other examples of interaction between these factors have been identified, but functional cooperativity between either AP1 or CBF and other transcription factors has been observed in many other promoters (32–38). In most instances, the binding sites for AP1 or CBF and another factor are separated by not more that 10 bp, and both intact sites are required for optimal DNA binding by either factor. This spacing appears to be critical as insertions of 5 or 10 bp between binding sites destroy cooperative binding and function as a unit. The granzyme B AP1 and 3′ CBF sites are separated by 20 bp, and both factors bind their respective elements independently of one another. The removal of either AP1 or CBF from the promoter complex has a limited negative effect, but the removal of both abrogates expression. This could denote a form of protein-protein interaction whereby the proteins associate in the absence of DNA binding, and only one binding site is required to tether both factors to the promoter. A similar situation is exemplified by the endothelin-1 promoter where the transcription factor GATA-2 potentiated AP1 activity despite the absence of a functional GATA binding site and vice versa (37). Similarly, the AP1 and GATA proteins were found to associate in the absence of DNA. Alternatively, AP1 and CBF could serve as a docking site for an adapter protein that requires at least one factor bound to the promoter for association.

We have successfully identified all of the cis-acting sequences that are responsible for the high levels of promoter activity observed from the 243-bp minimal promoter. These are the CRE, AP1, 3′ CBF, 5′ CBF, and Ikaros elements. T cell clones transcriptionally regulate granzyme B promoter expression in a manner that deviates from primary lymphocytes. Clones are differentially sensitive to certain mutations and thus may not contain the same compliment of transcription factors as primary cells. Although no single factor was necessary for expression, the combination of AP1 and CBF was essential for the granzyme B promoter. Further studies are required to delineate the nature of interplay between AP1 and CBF, but it is clear from our studies that murine granzyme B expression is regulated by transcription factors acting in concert.

Acknowledgments—We thank Brenda Duggan for help and technical support and Irene Shostak for maintaining cultured cell lines.

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