A murine granzyme B promoter fragment that extends 243 base pairs upstream of the transcription start site confers high levels of luciferase reporter gene activity in transient transfection assays into T cells and mouse L cell fibroblasts. This promoter fragment contains canonical binding sites for the transcription factors AP-1, core binding factor (CBF), Ikaros, and the cyclic AMP responsive element binding protein (CREB). Oligonucleotides containing the granzyme B AP-1 or CBF elements form specific complexes with proteins present in nuclear extracts from activated CD8⁺ splenocytes, MTL cells, EL4 T cells, and L cells. A strong DNase1 hypersensitive site that coincides with the closely associated AP-1, CBF, Ikaros, and CRE elements is present in activated CD8⁺ T cells but not in resting T cells or L cells. Both in vitro and in vivo footprints are observed at these sequence elements in activated cytotoxic T cells (CTL) but not in resting T cells. The endogenous granzyme B gene is CTL-specific as no mRNA is detectable in EL4 or L cells. We propose that a condensed chromatin structure at the granzyme B promoter is responsible for transcription factor inaccessibility and repression of transcription in non-T cells.

The body's major defense against viral infections is mediated by cytotoxic T lymphocytes. These cells can sometimes mount an attack on healthy tissue and cause autoimmune diseases, and they are responsible for organ and tissue transplant rejection. Whether the response is appropriate or not, resting T lymphocytes are activated upon recognition of antigen, in the context of major histocompatibility complex molecules (Chien and Davis, 1993). One approach to understanding the events that occur is to study the specialized set of genes that are induced during the acquisition of killing potential. These include T cell-specific transcription factor binding sites such as Ikaros and core binding factor (CBF/PEBP2) (Haddad et al., 1993; Kamachi et al., 1990; Wang and Speck, 1992) as well as recognition sequences for the ubiquitous transcription factors AP-1 and the cyclic AMP response element binding factor (CREB). These sequences have been shown to be sufficient to induce reporter gene expression in immortalized T cell lines in which many of these transcription factors are constitutively active (Frégeau and Bleackley, 1993; Hanson and Ley, 1990; Hanson et al., 1993).

We are primarily interested in the events that take place at the endogenous granzyme B locus as resting lymphocytes make the transition to activated killers. We have developed a method for the transfection of reporter gene plasmids into primary mouse splenocytes and show that the minimal granzyme B promoter is able to induce significant levels of luciferase activity in activated CD8⁺ T cells. Electrophoretic mobility shift analysis was used to examine the DNA binding activities of transcription factors before and after CD8⁺ T cell activation. We have precisely established the sequences involved in transcription factor binding by in vitro footprinting studies using nuclear extracts derived from a cytotoxic T cell clone. DNase1 hypersensitivity analysis identified potentially important regulatory regions in the granzyme B promoter in CD8⁺ cells. Finally, we were able to observe these protein/DNA interactions in the endogenous promoter by in vivo footprinting analysis in resting and activated CD8⁺ T cells using the dimethyl sulfate (DMS)/LMPCR genomic footprinting method. Together these results have enabled us to probe the status of the endogenous gene before and after T cell activation and in a physiologically relevant system.

**EXPERIMENTAL PROCEDURES**

CDs—Primary splenocytes were obtained from 6- to 12-week-old Balb/c mice. Spleen tissue was ground through a fine wire screen in RPMI-1640 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 (Bleackley et al., 1982). The antigen- and IL-2-dependent CTL21.9 (Type 1) line was generated as described (Havele et al., 1986). EL4 is an IL-2-producing T lymphoma cell line (Petkau et al., 1986).
and L cells are a mouse fibroblast line. All cells were cultured in RHF M (RPMI supplemented with 20 µM HEPES (pH 7.5), 100 µM β-mercaptoethanol, and 10% fetal bovine serum (5% for L cells)). Type 1 cells, MTL 2.8.2 cells, and primary splenocytes were cultured in the presence of 60 units/ml recombinant IL-2. Primary splenocytes were stimulated with 5–10 µg/ml concanavalin A (Sigma) 1:300 to 1:500 dilution of rabbit polyclonal to the sheep and rat antibodies used. Promoter fragments (from our C11 gene clone), obtained by the bacterial AP-1 promoter (Promega), and 906 (from A. Puschel) has the bacterial lacZ promoter under the control of the SV2 viral promoter (Promega), and 906 (from A. Puschel) has the bacterial luciferase gene under the control of the SV2 viral promoter (Promega). Three 10–20-µl aliquots of cell lysates were measured for 20 s following the injection of luciferase reagent (Luciferase Assay, Promega) by a LUMAT LB9501 luminometer (Berthold Systems Inc.). β-Galactosidase assays were performed as described (Sambrook et al., 1989). Nuclear extracts from transfected cells types, the luciferase/b-galactosidase values are not relative between the different cell types. In Vivo DMS-Piperidine Treatment of DNA—Dimethyl sulfate (DMS)-piperidine treatment of DNA was performed as described in Mueller et al. (1992). Cells were pelleted at 300 × g for 5 min. Approximately 1 ml of medium was left behind to resuspend cells. They were then transferred to a microcentrifuge tube and incubated in a 37°C water bath. 10 µl of a 10% DMS/ethanol solution was added to the cells DMS treated with 2% for 15 min, and the remaining 2% DMS was added to 40 µl of cold PBS followed by centrifugation at 300 × g for 5 min at 4°C. The cell pellet was resuspended in 1–2 ml of cold PBS, 49 ml of ice-cold PBS was added, and cells were pelleted at 300 × g for 5 min at 4°C. The cell pellet was resuspended in 0.3 ml cold PBS and added to 2 ml of lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 100 mM Na2HPO4, 100 µg/ml proteinase K, and 25 µg/ml calf thymus DNA). The reaction was phenol-extracted, and the DNA was ethanol-precipitated. The fragments were visualized on a 7% polyacrylamide, 7 M urea sequencing gel.

In Vivo DMS-Piperidine Treatment of DNA—Dimethyl sulfate (DMS)-piperidine treatment of DNA was performed as described in Mueller et al. (1992). Cells were pelleted at 300 × g for 5 min. Approximately 1 ml of medium was left behind to resuspend cells. They were then transferred to a microcentrifuge tube and incubated in a 37°C water bath. 10 µl of a 10% DMS/ethanol solution was added to the cells DMS treated with 2% for 15 min, and the remaining 2% DMS was added to 40 µl of cold PBS followed by centrifugation at 300 × g for 5 min at 4°C. The cell pellet was resuspended in 1–2 ml of cold PBS, 49 ml of ice-cold PBS was added, and cells were pelleted at 300 × g for 5 min at 4°C. The cell pellet was resuspended in 0.3 ml cold PBS and added to 2 ml of lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 100 mM Na2HPO4, 100 µg/ml proteinase K, and 25 µg/ml calf thymus DNA). The reaction was phenol-extracted, and the DNA was ethanol-precipitated. The fragments were visualized on a 7% polyacrylamide, 7 M urea sequencing gel.

Transcriptional Regulation of Murine Granzyme B—Northern Blot Analysis—Northern blot analysis was performed as described (Lin et al., 1985). The DNA was phenol:chloroform-extracted 2–3 times and ethanol precipitated. A naked DNA control was prepared at the same time.

MTL 2.8.2s were grown on 24 × 24-cm plates to a final cell density of 2.5–4 × 10^7 cells/plate. The medium was removed, and 100 ml of prewarmed RHF M containing 0.1% DMS was added and incubated for 2 min. The cells were washed three times with prewarmed PBS. Approximately 8 ml of lysis buffer was layered over the cells, and the plates were swirled gently for 5 min. The lysed cells were scraped off the plates into 50-ml tubes and incubated at 37°C for 5 h. DNA was washed once with solution 2, and resuspended at 1.2 µg/ml concanavalin A for 20–24 h prior to transfection. Basically, 1.0 µl of genomic DNA sample was cut with EcoRI and electrophoretically separated on 1% agarose gels. Nucleic acids were transferred to Hybond-N nylon membranes (Amersham) by capillary transfer.
Granzyme B mRNA was expressed at high levels in CD8 lines, the IL-2-dependent MTL2.8.2 line, the IL-2 and alloantigen with a granzyme B cDNA. Fig. 1 depicts the relative message utilized in our experiments, we prepared total RNA and probed with a murine granzyme B cDNA and a human γ-actin cDNA. Fig. 2 depicts the induction profile of the granzyme B mRNA in the whole splenocyte population and of the mRNA in the CD8⁺ and CD4⁺/CD8⁻ depleted cells. We believe that the lower band is mature mRNA while the upper corresponds to a processing intermediate. Granzyme B mRNA appeared on day 1, was very high by day 3, and was not observed in the CD4⁺/CD8⁻ depleted cell fraction. Even though the control actin band is very low at day 1, the intensities of the 18 and 28 S RNAs were very similar in all lanes. Together, these Northern blot data indicate that this gene is efficiently transcribed, or the mRNA is sufficiently stable, only in cytotoxic T cells.

A 243-bp Promoter Fragment Confers High Levels of Reporter Gene Activity in T Cells and L Cells in Transient Transfection Analysis—To define important transcriptional regulatory elements in the granzyme B promoter, a series of deletion fragments was constructed and inserted upstream of a promoter-less luciferase gene. These constructs were then transfected into a variety of cell types, and the relative levels of reporter gene expression were examined. Two promoter fragments, one that extends 243 bp and another that extends 828 bp upstream from the transcription start site consistently produced the highest levels of luciferase activity in Type 1 CTL cells (Fig. 3A). The Rous sarcoma viral promoter was typically as active as the 828-bp granzyme B (C11 gene clone) promoter in T cells, and no luciferase expression was ever observed from the parental p19LUC plasmid. Larger 5' flanking sequences were examined (up to 5 kb) and were much less effective in activating luciferase gene expression than the 828-bp or the 243-bp fragments. These larger fragments were active in both T cells...
and non-T cells (data not shown). We decided to focus our study on the smaller, but highly active, 243-bp fragment. Fragments extending 108 bp, 169 bp, and 243 bp upstream of the transcription start site were transfected into MTL 2.8.2, EL4, L cells, and whole splenocytes. Splenocytes were activated by stimulation with IL-2, αCD3, and concanavalin A for approximately 20 h prior to transfection and were then re-exposed to stimulus for another 2 days. The immunomagnetic separation of the CD8⁺ fraction was performed prior to harvest on day 3 post-initial activation. Significant increases in luciferase activity were observed in all cell types, except EL4, as the 3'-CBF and AP-1 binding sites (contained within −169) and the 5'-CBF and Ikaros binding sites (contained within −243, see below) were included in the constructs (Fig. 3B). We did observe, however, a low but significant level of luciferase expression in EL4 cells upon stimulation with PMA/ionomycin. Apparently, two major elements, one between −108 and −169 and another between −169 and −243, seem to be very important for the high levels of reporter gene expression observed from the −243 fragment and for the inducibility by PMA/ionomycin in EL4 cells. These transfection studies indicate that the minimal granzyme B promoter confers high levels of expression in transient assays but is not necessarily restricted to T cells.

The Granzyme B −243 Promoter Contains Binding Sites for Four Known Transcription Factors—Within the −243 promoter fragment there exist consensus sequence binding sites for the transcription factors AP-1, core binding factor (CBF), Ikaros, and the cyclic AMP responsive element (CRE). These sites are located at approximately −108 (AP-1), −169 (AP-1), −200 (Ikaros), −180/−126 (CBF), −150 (AP-1) and at −90 (CRE) nucleotides relative to the transcription start site (Fig. 4).

Nuclear extracts were prepared from MTL 2.8.2 T cells, L cells, and PMA/ionomycin-stimulated and unstimulated EL4 cells and incubated with oligonucleotides containing the granzyme B AP-1 and CBF sequence elements. Electrophoretic mobility shift assays (EMSAs) showed that both binding sites formed specific complexes with nuclear proteins present in all of these cells (Fig. 5A). These results show that the AP-1 and CBF regulatory factors are present in the nuclei of cells that both express and do not express granzyme B.

We then performed mobility shift assays using nuclear extracts from purified CD8⁺ murine splenocytes to compare complexes in resting and activated cells. Fig. 5B shows that the granzyme B AP-1 oligonucleotide formed a complex with nuclear extracts from stimulated CD8⁺ cells whereas this complex was absent in resting splenocytes. It has been previously observed that c-Fos mRNA is absent in resting T cells (Jain et al., 1992). A supershift was observed in activated cells with a c-Fos antibody although the c-jun antibody used in this assay had a negligible effect on the complex in activated CD8, EL4, and L cells. These results indicate that in resting splenocytes the AP-1 complex is either absent or does not bind to DNA, and activation through the T cell receptor is required for effective DNA binding activity.

The granzyme B CBF oligonucleotide formed two complexes in CD8⁺ lymphocytes (Fig. 5C). In nuclear extracts obtained from resting splenocytes, a weak, indistinct complex was observed with a c-Fos antibody although the c-Jun antibody used in this assay had a negligible effect on the complex in activated CD8, EL4, and L cells. These results indicate that in resting splenocytes the AP-1 complex is either absent or does not bind to DNA, and activation through the T cell receptor is required for effective DNA binding activity.

The granzyme B CBF oligonucleotide formed two complexes in CD8⁺ lymphocytes (Fig. 5C). In nuclear extracts obtained from resting splenocytes, a weak, indistinct complex was observed with a c-Fos antibody although the c-Jun antibody used in this assay had a negligible effect on the complex in activated CD8, EL4, and L cells. These results indicate that in resting splenocytes the AP-1 complex is either absent or does not bind to DNA, and activation through the T cell receptor is required for effective DNA binding activity.
formed with the CBF oligonucleotide. Upon stimulation of the cells for 45 h with \(\alpha\)CD3, a slower mobility complex was observed. The complex was inhibited when the granzyme B CBF oligonucleotide was incubated with nuclear extracts in the presence of a 50 or 200 molar excess of unlabeled granzyme B CBF oligonucleotide. An interesting observation was made when antisera to either PEBP2 \(\alpha\)A (CBF \(\alpha\)-subunit) or PEBP2\(\beta\) (CBF \(\beta\)-subunit) was added to the reaction. The anti-\(\alpha\)A serum did not appear to affect the major complex; however, the anti-\(\beta\) serum was capable of disrupting the complex. The \(\alpha\)A anti-serum cross-reacts with all three of the known \(\alpha\) subunits,\(^2\) including \(\alpha\)B and \(\alpha\)C, which are expressed at high levels in T cells. We may be observing an as yet unknown variation of the \(\alpha\) subunit or an entirely different protein that is capable of binding to the CBF/PEBP2 binding site and interacting with the \(\beta\) subunit.

It has been determined previously that the Ikaros gene gives rise to a lymphoid-restricted family of functionally distinct transcription factor proteins which are involved throughout lymphocyte development (Georgopoulos et al., 1994; Molar and Georgopoulos, 1994). The Ikaros element in granzyme B formed a specific complex that was present in both unstimulated and \(\alpha\)CD3-stimulated CD8\(^{+}\) splenocytes (Fig. 5D). This complex was competed off with an excess of the granzyme B Ikaros oligonucleotide. A 200 molar excess of the mutant Ikaros oligonucleotide was added as indicated. A 50 or 200 molar excess of competitor binding sites was added for all shifts.

---

\(^{2}\) Y. Ito, personal communication.

---

**Fig. 5.** Nuclear extracts from T cells and non-T cells form complexes with oligonucleotide probes containing the granzyme B AP-1 and CBF binding sites. A, nuclear extracts prepared from MTL 2.8.2, EL4 (unstimulated or stimulated with 10 ng/ml PMA and 4 \(\mu\)M ionomycin), and L cells were incubated with the granzyme B AP-1 or CBF oligonucleotides and subject to electrophoresis on a nondenaturing polyacrylamide gel. DNA-protein complexes are marked. B, radiolabeled granzyme B AP-1 oligonucleotide incubated with 2 \(\mu\)g of nuclear extracts prepared from unstimulated and \(\alpha\)CD3-stimulated CD8\(^{+}\) splenocytes. For supershift binding assays, 0.5 \(\mu\)g of anti-c-Fos or anti-c-Jun antibody was added. C, the same nuclear extracts as in B were incubated with the radiolabeled granzyme B CBF oligonucleotide. 3 \(\mu\)l of the \(\alpha\)A or \(\beta\) antisera was added as indicated. D, the same extracts used in B and C were incubated with the granzyme B Ikaros oligonucleotide. A 200 molar excess of the mutant Ikaros oligonucleotide was added as indicated. A 50 or 200 molar excess of competitor binding sites was added for all shifts.
were included in the genomic granzyme B gene that contains the site of interest. The genomic granzyme B gene was probed in resting splenocytes and L cells. Together, this series of experiments indicates that the granzyme B promoter undergoes a structural modification upon T cell activation that allows transcription factors access to the locus, and this phenomenon is cell type-specific and activation-dependent.

Granzyme B Activation Correlates with Binding of Regulatory Proteins in Vivo—In vivo footprinting is a powerful assay that permits direct detection of protein/DNA interactions within the intact appropriate cell type. Moreover, this method can discriminate between accessible and inaccessible protein binding sites in the chromatin of living cells. In vivo footprint analysis in MTL 2.8.2 cells showed that in intact cells there exist several regions in the granzyme B promoter that were protected from dimethyl sulfate (DMS) methylation (Fig. 8A). One such footprint corresponds to the AP-1 binding site, and two others correspond to the CBF binding sites. The footprint at the CRE is indicated by a reduction of the band correlating to the G residue midpoint in the binding site. Two hypersensitive bands were observed corresponding to the A residues that flank the Ikaros element observed in MTL 2.8.2 were not apparent, probably due to the resolution limits of the gel. The in vivo footprints correlate well with the in vitro data and present a snapshot of the endogenous promoter as it is activating transcription. The absence of proteins bound in resting cells suggests that the transcription factors that are present do not have access to the DNA, and, moreover, it suggests a lack of repressor interactions. The resting cell profile was invariably identical with the naked DNA control in all experiments performed. In nonexpressing L cells, no protein-DNA interactions were observed (data not shown). These data indicate that the AP-1, CBF, and CRE binding sites are not occupied by their respective transcription factors in resting splenocytes and that T cell activation is required for DNA binding.

**DISCUSSION**

Each peripheral cytotoxic lymphocyte spends its life in a continuous search for the foreign antigen that can turn it into a potent killer. The acquisition of cytotoxic function requires de novo synthesis and assembly of the killing machinery. The granzyme B gene encodes one of the components of this machinery and is fully activated within 3 days. The major thrust of our experiments has been directed at understanding granzyme B gene induction in physiologically relevant primary lymphocytes.

We have used purified CD8+ splenocytes in reporter gene transfections, DNase1 hypersensitivity analysis, mobility shift assays, and in vivo footprinting. There was a recapitulation of general motifs that have been noted in regulation studies of other inducible or developmentally regulated genes. cis-Acting transcriptional enhancers of tissue-specific genes tend to consist of a cluster of ubiquitous and tissue-specific transcription factor binding sites. The granzyme B promoter region contains

allows DNase1 to penetrate into living cells and cleave exposed regions of DNA within intact nuclei. Regions of chromosomal DNA that are accessible to, or are bound by, transcription factors tend to be hypersensitive to DNase1 digestion. DNase1 hypersensitive sites can be visualized on Southern blots with probes designed to specifically end-label a restriction fragment that contains the site of interest. The genomic granzyme B gene is contained within a 4.4-kb EcoRI fragment whose 5′ end is 961 bp upstream of the transcription start site. A restriction fragment probe that extends from −828 to −546 relative to the transcription start site and does not contain repetitive sequence elements was used to end label this fragment.

We treated permeabilized, resting CD8+ splenocytes with increasing amounts of DNase1 and observed that the 4.4-kb EcoRI fragment gradually decreased in intensity with higher concentrations of DNase1 (Fig. 7A). No specific sub-bands appeared that would indicate a hypersensitive region. In activated T cells, however, an area just proximal to the transcription start site was highly sensitive to DNase1 cleavage. Following activation of CD8+ splenocytes for 3 days (IL-2, αCD3, concanavalin A, and αCD28), the 4.4-kb EcoRI fragment diminished with higher concentrations of DNase1 and a 750- to 900-bp sub-band appeared (Fig. 7B). The boundaries of this roughly 150-bp hypersensitive region directly correlate to the sequences which contain the AP-1, CBF, Ikaros, and CRE transcription factor binding sites. We were only able to detect a very faint hypersensitive site in L cell nuclei (Fig. 7C). Thus, even though there are nuclear factors in L cells that are capable of binding to granzyme B promoter sequences in vitro, they are not binding to the endogenous, chromosomal DNA. As a positive control, the constitutively active, c-Fos gene promoter was probed in resting splenocytes and L cells. Together, this series of experiments indicates that the granzyme B promoter undergoes a structural modification upon T cell activation that allows transcription factors access to the locus, and this phenomenon is cell type-specific and activation-dependent.

**FIG. 6.** In vitro DNase1 footprint analysis with MTL nuclear extracts shows clear footprints at the AP-1, CBF, and CRE elements. A less prominent footprint over the Ikaros element is also observed. 50 or 75 μg of MTL 2.8.2 nuclear extracts were incubated with an end-labeled granzyme B promoter fragment and subject to digestion with DNase1. The cleavage products were separated on a 7% 7 M urea-polyacrylamide gel. A control reaction containing the cleavage products of naked DNA and a Maxam-Gilbert G′A cleavage ladder were included in the two left lanes.

Each peripheral cytotoxic lymphocyte spends its life in a continuous search for the foreign antigen that can turn it into a potent killer. The acquisition of cytotoxic function requires de novo synthesis and assembly of the killing machinery. The granzyme B gene encodes one of the components of this machinery and is fully activated within 3 days. The major thrust of our experiments has been directed at understanding granzyme B gene induction in physiologically relevant primary lymphocytes.

We have used purified CD8+ splenocytes in reporter gene transfections, DNase1 hypersensitivity analysis, mobility shift assays, and in vivo footprinting. There was a recapitulation of general motifs that have been noted in regulation studies of other inducible or developmentally regulated genes. cis-Acting transcriptional enhancers of tissue-specific genes tend to consist of a cluster of ubiquitous and tissue-specific transcription factor binding sites. The granzyme B promoter region contains
binding sites for the widely expressed AP-1 and CRE transcription factors as well as for the T cell-specific core binding factor and Ikaros. These sequences can bind to nuclear proteins present in activated T cells and are sufficient to activate high levels of reporter gene expression in transient transfections. These sequences are important for granzyme B regulation in vivo as every binding site is fully accessible to, and bound by, transcription factors in activated CD8\(^+\) T cells. The spacing of these sequences is interesting in that the close proximity may be necessary for the concerted enhancer effect of several differentially regulated factors at this specific locus.

These trans-acting factors must be activated at the appropriate time by developmentally or externally derived signals. The Ikaros protein is expressed throughout T cell development. We have shown that it is present in resting CD8\(^+\) splenocytes and capable of binding DNA. Our data also show Ikaros to be bound in vivo in MTL 2.8.2 cells. We showed by EMSA and in vivo footprinting that the AP-1 transcription factor does not bind to its sequence element in the granzyme B promoter without prior activation through the T cell receptor. The transcription factor AP-1 is comprised of Fos and Jun heterodimers whose activity is regulated by de novo synthesis as well as

**Fig. 7.** DNaseI hypersensitivity analysis in resting and activated CD8\(^+\) splenocytes and L cells shows that the granzyme B promoter is hypersensitive to DNaseI digestion in activated T lymphocytes. Lysolecithin-permeabilized cells were treated with between 0 and 10.0 \(\mu\)g/ml DNaseI. Genomic DNA was cut with EcoRI, separated on 1.2% agarose gels (15 \(\mu\)g/lane), and transferred to nylon membranes. The blots were probed with a 279-bp 5'-granzyme B probe that indirectly end-labels a 4.4-kb EcoRI fragment whose 5' end is 961 bp upstream of the first transcribed nucleotide. A, the Southern blot of CD8\(^+\) unstimulated splenocytes (6-day exposure). Longer exposures of up to 2 weeks failed to reveal any hypersensitive sites. B, hypersensitive site formation in CD8\(^+\) splenocytes stimulated for 3 days with IL-2, \(\alpha\)CD3, concanavalin A, and \(\alpha\)CD28 (2-week exposure). C, the granzyme B locus in L cells (6-day exposure).
heterodimerization, the affinity of the binding site in MTL 2.8.2. These sites are indicated with specific genes such as the T cell receptor factor has been implicated in the regulation of many T cell-act as a potent transactivator of transcription. Core binding can bind efficiently to its cognate DNA sequence element and Rincon and Flavell (1994)). The phosphorylated AP-1 complex Crabtree and Clipstone (1994), Karin and Smeal (1992), and eukaryotic gene regulation. Activated trans-acting factors must be allowed access to their target cis-enhancer elements only in the appropriate tissues and at the appropriate times. The formation of nuclease hypersensitive sites in chromatin has been correlated with such important regulatory elements as enhancers, silencers, and locus control regions (reviewed in Felsenfeld (1993) and Gross and Garrard (1988)). For example, the human β-globin locus is composed of five developmentally regulated genes that are induced and expressed sequentially during embryonic, fetal, and adult development. The timely expression of these genes is controlled by a series of four stage-specific DNase1 hypersensitive sites that exist many kilobases upstream of the 5′-most gene in the cluster (Fraser et al., 1993). These hypersensitive sites consist of binding sites for ubiquitous and erythroid-specific transcription factors. Their stage-specific appearance has been shown to be dependent upon interaction with individual gene promoters of the globin genes (Reitman et al., 1993). It appears in this case that a cluster of binding sites alone is not sufficient to create a hypersensitive site, but an interaction with a distant element is necessary to induce structural changes in the locus.

The granzyme gene locus is potentially very interesting in this respect. We do not know whether the granzyme B proximal promoter sequences alone are sufficient to create the observed hypersensitive site in activated T cells or if another sequence element located elsewhere is required. The possibility may exist that all or a subset of the granzyme genes could be coordinately regulated by a higher level of control that involves the interaction of the individual promoters with a distant locus control region that would make the genes amenable to transcription upon the reception of the appropriate induction signal. We have looked for additional hypersensitive sites up to 3.5 kb 5′ of the transcription start site and have found none in this region.

There is increasing evidence that in vitro footprinting assays do not always reflect the true DNA/protein interactions occurring in the chromatin of intact cells. The state of chromatin condensation may sequester cis-elements and prevent binding of available transcription factors. In addition, the nonexpressing cells showed little evidence of a DNase1 hypersensitive site in the vicinity of the granzyme B gene. Interestingly, a hypersensitive site was apparent in activated but not resting CD8+ T cells. Hypersensitive sites are also observed within the Ikaros binding site in MTL 2.8.2. These sites are indicated with asterisks.

post-translational modification. Upon activation through the T cell receptor, they are phosphorylated through a protein kinase C/Ca++-mediated signal transduction cascade (reviewed in Crabtree and Clipstone (1994), Karin and Smeal (1992), and Rincon and Flavell (1994)). The phosphorylated AP-1 complex can bind efficiently to its cognate DNA sequence element and act as a potent transactivator of transcription. Core binding factor has been implicated in the regulation of many T cell-specific genes such as the T cell receptor α, β, and δ genes, the CD3 ε and δ genes (Hallberg et al., 1992; Hsiang et al., 1993; Prosser et al., 1992). CBF consists of two heterologous subunits, a DNA binding α subunit and a non-DNA binding β subunit (Wang et al., 1993; Zaiman et al., 1995). Through heterodimerization, the β subunit augments the DNA binding affinity of the α subunit. The α subunit has been shown to localize to the nucleus while the β subunit is found in the cytoplasm (Lu et al., 1995). Little is known about the signaling events that lead to the translocation of the α/β heterodimer into the nucleus. The hypothesis put forth suggests that the α subunit requires modification to make it more amenable to association with the β subunit. This modification would occur after the cell has been activated through the appropriate cell surface receptors. Results of mobility shifts show a weak complex in resting cells that could indicate a low affinity, partially dissociated α subunit complex. The complex in activated cells was much more intense and well defined.

Both CBF and AP-1 are able to act in combination with other transcription factors, most notably ets and NF-AT (nuclear factor of activated T cells) (Jain et al., 1993; Wotton et al., 1994). In the regulation of the IL-2 gene, NF-AT translocates to the nucleus in response to increases in intracellular levels of calcium. The nuclear NF-AT then unites with activated Fos/J un to form a complex with high affinity DNA binding and transactivation properties. Recently, it has been determined that there is more than one form of NF-AT (Northrop et al., 1994). Core binding factor is encoded by members of a multi-gene family, and one member of the α subunit family has been shown to be T cell-specific (Satake et al., 1995). An attractive model, similar to IL-2 gene induction, is that upon T cell activation CBF and AP-1 are modified by separate signaling pathways and then unite at the level of DNA binding and transactivation.

Chromatin is no longer viewed as a passive participant in eukaryotic gene regulation. Activated trans-acting factors must be allowed access to their target cis-enhancer elements only in the appropriate tissues and at the appropriate times. The formation of nuclease hypersensitive sites in chromatin has been correlated with such important regulatory elements as enhancers, silencers, and locus control regions (reviewed in Felsenfeld (1993) and Gross and Garrard (1988)). For example, the human β-globin locus is composed of five developmentally regulated genes that are induced and expressed sequentially during embryonic, fetal, and adult development. The timely expression of these genes is controlled by a series of four stage-specific DNase1 hypersensitive sites that exist many kilobases upstream of the 5′-most gene in the cluster (Fraser et al., 1993). These hypersensitive sites consist of binding sites for ubiquitous and erythroid-specific transcription factors. Their stage-specific appearance has been shown to be dependent upon interaction with individual gene promoters of the globin genes (Reitman et al., 1993). It appears in this case that a cluster of binding sites alone is not sufficient to create a hypersensitive site, but an interaction with a distant element is necessary to induce structural changes in the locus.

The granzyme gene locus is potentially very interesting in this respect. We do not know whether the granzyme B proximal promoter sequences alone are sufficient to create the observed hypersensitive site in activated T cells or if another sequence element located elsewhere is required. The possibility may exist that all or a subset of the granzyme genes could be coordinately regulated by a higher level of control that involves the interaction of the individual promoters with a distant locus control region that would make the genes amenable to transcription upon the reception of the appropriate induction signal. We have looked for additional hypersensitive sites up to 3.5 kb 5′ of the transcription start site and have found none in this region.

There is increasing evidence that in vitro footprinting assays do not always reflect the true DNA/protein interactions occurring in the chromatin of intact cells. The state of chromatin condensation may sequester cis-elements and prevent binding of available transcription factors. In addition, the nonexpressing cells showed little evidence of a DNase1 hypersensitive site in the vicinity of the granzyme B gene. Interestingly, a hypersensitive site was apparent in activated but not resting CD8+ lymphocytes. An inducible hypersensitive site has been observed at the human granzyme B promoter in a PEER T cell line when activated with TPA and dibutyryl-CAMP (Hanson et al., 1990). This site extends from approximately 30 to 400
nucleotides upstream of the transcription start site and roughly corresponds to the promoter sequences that are highly conserved between mice and humans. We were able to define more precisely the limits of the murine hypersensitive site in activated splenocytes and show that transcription factors form sequence-specific footprints at this site in vivo.

This series of experiments, together with the Northern analysis, transfection, and EMSA data, leads us to infer that the granzyme B locus may not be accessible to transcription factors in resting T cells or non-T cells. Ikaros and CREB proteins are present in resting cells but only upon activation of the T cells are the AP-1 and CBF capable of binding to their cognate sequence elements and, at this time, the promoter becomes accessible to them. There is presumably a highly coordinated mobilization of factors once the decondensation signal takes place. The promoter must be sensitive to multiple signaling events, and it is possible that the assembly of factors occurs only after all of the signaling events have been achieved. The IL-2 enhancer displays an all or nothing chromosomal binding phenomenon in EL4 cells stimulated with TPA/A23187 (Garritty et al., 1994). When cyclosporin A treatment was used to block the activation of the calcium-dependent NF-AT and NF-κB, transcription factors that normally bind to the IL-2 enhancer, no binding of even cyclosporin-insensitive factors was observed in vivo and the enhancer was unoccupied.

It has yet to be determined whether the transcription factor binding sites become accessible because of a shift in the nucleosome structure at the promoter or if the transcription factors themselves mediate the formation of the hypersensitive site. Alternatively, the granzyme B locus may be differentially methylated in different cell types, thus influencing DNA protein binding access. The granzyme B locus is relatively insensitive to DNase I digestion in L cell nuclei. This is not surprising because at no time in a fibroblast's existence does it produce granzyme B. Histone modifications, such as hyperacetylation of histone tails, or nucleosomal binding proteins such as nucleoplasmin and SWI/SNF (Chen et al., 1994; Kwon et al., 1994; Wolfe, 1994; Workman and Buchman, 1993) are potentially important players in gene induction. These proteins have been shown to facilitate transcription factor binding and nucleosome disruption and may be essential components of the chromatin rearrangement and transcriptional induction processes.

Whether these molecules or others play a role in granzyme B regulation in vivo awaits further investigation.

In this investigation we have confirmed that more than one level of regulation is required to permit the expression of granzyme B. Our proposed model of granzyme B regulation involves decondensation at the chromosomal locus in response to T cell activation. The integration of multiple signals would corroborate in the synthesis or activation of the necessary transcription factors and result in a staged assembly of factors at the newly accessible granzyme B promoter. The subsequent three dimensional structure would then activate the basal transcription machinery to initiate transcription and elongate the nascent RNA. This is similar to the model proposed for the regulation of IL-2 transcription (Garrity et al., 1994). Both systems require timely activation in response to lymphocyte stimulation and must be readily reversible in order to terminate the immune response.

Acknowledgments—We thank Yashashi Ito for providing the antisera to the CBF/PEBP2 subunits. We would like to thank Irene Shostak for maintaining cultured cell lines and isolating primary splenocytes and Roger Bradley for his expert preparation of the figures. We would also like to thank Michael J. Pinkowski, who was very helpful in performing FACS analysis.