Functional GATA-3 Binding Sites within Murine CD8α Upstream Regulatory Sequences

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Summary

Genes encoding the accessory molecules CD8 and CD4 are activated early in thymocyte development, generating $CD4^+8^+$ double positive intermediates, which give rise to two functionally distinct mature T cell subsets that express either CD4 or CD8. The mechanisms that govern the activation or suppression of the CD8 gene are likely to be central to the T cell development program. To identify the key regulatory factors, we have initiated an analysis of the transcriptional regulation of the murine CD8α gene. We have identified three $CD8^+$ cell-specific DNase I hypersensitive sites (HSS) located upstream of the murine CD8α gene. In vitro mobility shift analysis of the −4.0-kb HSS region has revealed multiple binding sites for the T cell–restricted transcription factor GATA-3. In vitro translated murine GATA-3 binds specifically to both CD8 GATA sites, and coexpression of this factor in transient transfection assays transactivates a reporter construct containing these sequences. These results provide the first evidence for the role of a T cell–restricted factor in the regulation of either CD8 or CD4 genes.

The glycoprotein molecules CD4 and CD8, in conjunction with the TCR, play an important role in T cell antigen recognition and thymocyte development. The TCR mediates T cell recognition by contacting the antigenic peptide and polymorphic regions of the MHC, whereas CD4 and CD8 act as coreceptors to stabilize the TCR–MHC interaction by associating with the TCR (1–3) and monomorphic regions of the MHC molecule (4, 5). In addition, CD4 and CD8 may provide transducing signals in developing and mature T cells through the lymphocyte-specific tyrosine kinase, $p56^{lck}$ (6–9). Both the adhesion and signal transduction functions of the CD4 and CD8 molecules are likely to play a role in the maturation and selection of the T cell repertoire (10–13).

CD4 and CD8 expression follow a complex, developmentally regulated pattern that has been best characterized during fetal thymic ontogeny (14). The earliest T cell precursors that populate the thymus do not express the TCR and have been termed double negative (DN)[1] thymocytes since they lack expression of both CD4 and CD8 molecules on the cell surface. DN thymocytes differentiate into double positive (DP) thymocytes that coexpress CD4 and CD8, together with TCR. At this stage of maturation, thymocytes bearing TCRs that can appropriately interact with MHC class I or II molecules are positively selected and further differentiate to become single positive (SP) cells that express either CD8 or CD4, respectively. Studies with transgenic mice expressing specific TCRs have provided compelling evidence that the TCR specificity determines the phenotype of developing thymocytes (15–18). Furthermore, indirect evidence indicates that the inactivation of either CD4 or CD8 during the DP to SP transition may occur in response to TCR-mediated signals (19, 20). Therefore, an analysis of CD4 or CD8 gene expression is expected to provide insight into the molecular mechanisms that positively activate the transcription of the coreceptor genes early in T cell development and those that negatively regulate their expression in cells destined to become mature CD4 or CD8 SP T cells.

Surprisingly, very little is known about the regulation of the CD4 or CD8 genes. Recently, Sawada and Littman (21) have identified a T cell–specific enhancer flanking the murine CD4 gene and Siu et al. (22) have demonstrated that the CD4 promoter mediates in part, the stage and tissue specificity of CD4 gene expression. We have initiated an analysis of the transcriptional regulation of the murine CD8α gene by searching for functional T cell–specific nuclear factor (NF) binding sites within developmentally regulated DNase I hypersensitive sites (HSS) flanking the gene. The DNase I hypersensitivity assay has been successfully used to identify several tissue-specific enhancers (21, 23–26) and the complex locus control region (LCR) of the human β-globin locus (27–29). We have identified three $CD8^+$ T cell–specific

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; DN, double negative; DP, double positive; HSS, hypersensitive site; LCR, locus control region; SP, single positive.

DNase I HSS upstream of the CD8α gene. An investigation of protein–DNA interactions by in vitro mobility shift analysis has identified multiple binding sites for a T cell–restricted transcription factor GATA-3 (30–34), within a HSS domain located 4 kb 5′ of the CD8 gene. We show that coexpression of the murine GATA-3 gene results in transcriptional activation of a reporter plasmid dependent on CD8 GATA sequences, suggesting that these sites are functional. Our results provide the first evidence for a T cell–restricted transcription factor regulating coreceptor gene expression.

Materials and Methods

Cell Lines. Murine thymoma cell lines AKR1G1 ($CD4^+8^+$) and NFC105 ($CD4^+8^+$) were from Dr. Kenneth Rock (Harvard Medical School, Boston, MA); 322E-9A10 ($CD4^+8^+$) and S802MC4F10 ($CD4^-8^+$) were from Dr. Ellen Ritchie (University of Texas, Smithville, TX); AKR5 ($CD4^+8^-$) and AKR33 ($CD4^+8^+$) were from Dr. Harald von Boehmer (Basel Institute for Immunology, Basel, Switzerland); and RLM11 ($CD4^+8^-$) and EL-4 ($CD4^-8^-$). Murine cell lines used were pre-B cell lines 70Z/3, 38B9, and HAFTL; B cell lines AJ9 and WEHI 231; and fibroblast cell line NIH3T3. Human cell lines HeLa (cervical carcinoma) and fibrosarcoma 164 (FS) were also used. All lymphoid cells were grown in RPMI 1640 medium supplemented with 10% inactivated FCS, 0.0002% β-ME, penicillin, and streptomycin. Nonlymphoid cells were grown in DME medium supplemented with 10% calf serum, penicillin, and streptomycin.

Flow Cytometric Analysis of T Cell Lines. All murine thymoma cell lines were analyzed for CD4/L3T4 and CD8/Lyt2 expression by flow cytometry using rat mAbs GK1.5 and 53-6.72, respectively. Cells were labeled using primary Abs derived from cell culture supernatant followed by FITC goat anti–rat IgG (Tago, Inc., Burlingame, CA).

DNase I Hypersensitivity Assay. Nuclei were isolated as follows: 6×10^7 cells were washed in PBS and then treated with reticulocyte standard buffer (RSB) (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$) with 0.5% NP-40 on ice for 5 min. The nuclei were pelleted, washed in RSB without NP-40, and resuspended in 0.5 ml of RSB. 100 μl of nuclear suspension was treated with various amounts of DNase I for 2 min at 37°C. The reaction was quenched with 120 μl of RSB containing 2% SDS and 10 mM EDTA, and digested with 40 μg of proteinase K, followed by purification with several phenol and chloroform extractions and precipitation with 3 M NaOAc, pH 5.2. The genomic DNA was dissolved in 100 μl of TE 10 mM Tris, ImM EDTA, treated with 25 μg RNase for 3 h, and precipitated after several extractions with phenol and chloroform. 12–15 μg of DNase-treated DNA was digested with the appropriate restriction endonucleases, and analyzed by Southern blotting using random-primed DNA probes.

Plasmids. Plasmid HN2 contains the murine genomic CD8α gene and was a gift from Dr. J. Parnes (Stanford University Medical Center, Stanford, CA) (35). The mammalian expression constructs R/mGATA sense and R/mGATA antisense contain the murine GATA-3 cDNA driven by the Rous sarcoma virus promoter and enhancer (31). Plasmid mc5b8 contains the murine GATA-3 cDNA clone (31). P14 contains the 9.0-kb genomic BamHI fragment from the genomic clone, G3-1-1A, cloned into the BamHI site of pGEM3zf(−) (Promega Corp., Madison, WI). P20 contains a 478-bp Sau3AI DNA fragment encompassing the −4.0-kb HSS, cloned into the BamHI site of Bluescript (M13−) (Stratagene, La Jolla, CA). The insert was completely sequenced and restriction sites were identified to generate subfragments for in vitro mobility shift assays.

Wild type and mutant oligonucleotides from probe 3 were synthesized with SalI/XhoI ends (mutations are underlined): P3, TCGAGTGATAGAATAGATAGCAG; P3-M1, TCGAGTGATAGAATCTGCAGCAG; and P3-M2, TCGAGTTCTAGAATCTGCAGCAG. The double-stranded oligonucleotides were cloned as dimers (head-to-tail) into the SalI site of Δ56fos-CAT, in which the bacterial chloramphenicol acetyl transferase reporter gene (CAT) (36) is transcribed from a minimal c-*fos* gene promoter and named (P3)-CAT, (P3-M1)-CAT, and (P3-M2)-CAT, respectively. Each construct was sequenced using a primer (GCAGAGCTGGGTGGGAGCGCGGTC) corresponding to nucleotides +72 to +95 of the c-*fos* promoter by the dideoxy-chain termination method.

Cloning and Sequencing of Upstream CD8 Sequences. A 730-bp HindIII-EcoRI CD8 probe, derived from plasmid HN2, was used to screen a mouse genomic library from a T cell hybridoma line derived from: AKR × B10.A (Dr. A. Winoto, University of California, Berkeley, CA). A 9.0-kb BamHI fragment, encompassing the −4.0 and −2.0-kb HSS regions, was subcloned from the positive phage clone G3-1-1A. The 5′ DNase I hypersensitive site regions were mapped, subcloned, and sequenced using the dideoxy-chain termination method.

Probes. Probe 2 is a 61-bp HaeIII/DdeI fragment and probe 3 is a 48-bp AluI/Sau96I fragment from plasmid P20. The GATA-3 binding sites identified within these fragments are separated by 56 nucleotides, with the probe 2 site located further 5′ relative to the CD8α gene.

In Vitro Analysis of DNA–Protein Interactions. The DNA probes were prepared by cutting plasmid P20 with restriction enzymes and endlabeling with polynucleotide kinase. In vitro binding reactions were performed as previously described (37) using 2 ng of labeled DNA probe, 10 μg of crude nuclear extract (38), and 2–3 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ). For competition experiments, increasing amounts of unlabeled DNA probe were included in the binding reaction in a 15 μl final volume. The resulting protein DNA complexes were separated on a 4% polyacrylamide gel. The GATA-3 protein was generated by in vitro transcribing mRNA from plasmid mc5b8 (Stratagene) and in vitro translating the protein in wheat germ extract (Promega Corp.).

Methylation Interference Assay. Methylation interference was performed as previously described (39). Briefly, an end-labeled DNA probe was partially methylated with dimethyl sulfate and used in preparative in vitro binding assays. DNA retained in the nucleoprotein complex and in the free fragment was isolated, cleaved with piperidine, and analyzed by electrophoresis through urea-containing sequencing gels.

Cotransfections and CAT Enzyme Analysis. 10 μg of reporter construct ([P3]-CAT, [P3-M1]-CAT, [P3-M2]-CAT, or Δ56fos-CAT) and 5 μg of transactivator (R/mGATA sense or R/mGATA antisense) were cotransfected into $1–2 \times 10^6$ HeLa cells. Transient transfections were carried out using the calcium phosphate method (40) and were repeated at least twice in duplicate. Cell extracts were prepared from transfected cells 48 h later and 80–100 μg of cell extract was assayed for 1.5 h as previously described (41).

Results

CD8 Expression–specific Hypersensitive Sites 5′ to the Murine CD8α Gene. To identify potential transcriptional regulatory domains important for CD8 gene expression, we analyzed the chromatin structure of this locus by partial DNase I digestion. Nuclei prepared from T cells expressing, or not

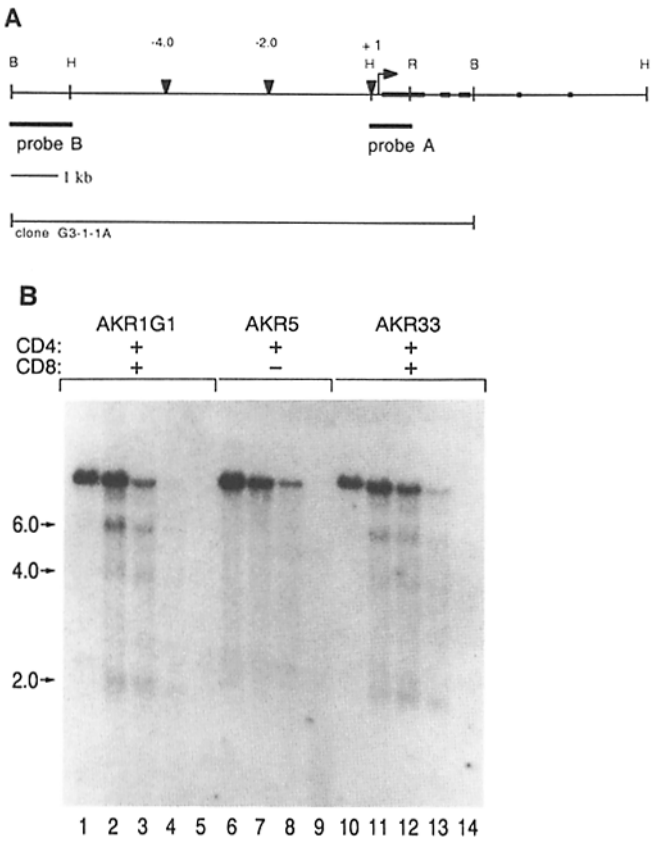


Figure 1. Three CD8 expression-specific HSS map upstream of the murine CD8α gene. (*A*) Restriction map of the murine CD8α gene and a schematic representation of the −4.0 and −2.0 -kb hypersensitive sites contained within the genomic clone G3-1-1A. The three hypersensitive sites were identified using a 730-bp HindIII-EcoRI fragment (*probe A*) and verified using a 1.2-kb BamHI-HindIII fragment (*probe B*). The 9.0-kb clone G3-1-1A was obtained using probe A. (*Boxed regions*) CD8 exons; and restriction sites for BamHI (B), HindIII (H), and EcoRI (R). (*B*) Analysis of DNase I HSS in the 5′ flanking region of the murine CD8 gene. Nuclei from the T cell lines: AKR1G1 ($CD4^+8^+$, lanes *1–5*), AKR5 ($CD4^+8^-$, lanes *6–9*), and AKR33 ($CD4^+8^+$, lanes *10–14*), were digested with 0.1 μg (lanes *2*, *6*, and *11*), 0.2 μg (lanes *3*, 7, and *12*), 0.3 μg (lanes *4*, *8*, and *13*), or 0.4 μg (lanes *5*, *9*, and *14*) of DNase I in a final volume of 0.1 ml for 2 min at 37°C. Lanes *1* and *10* are not treated with DNase I. The DNA was purified, digested with BamHI, electrophoresed through a 1% agarose gel, transferred to nitrocellulose, and probed with probe A. (*Arrows*) HSS.

expressing, the CD8 gene were treated with increasing concentrations of DNase I followed by analysis of BamHI-digested genomic DNA by Southern blotting (Fig. 1 *B*). The 730-bp HindIII-EcoRI fragment (Fig. 1 *A*, probe A) detects a 9.0-kb BamHI genomic fragment. In the $CD4^+8^+$ DP T cell lines, AKR1G1 and AKR33, three additional bands were observed in the lanes containing DNase I-digested DNA, indicating the presence of DNase I HSS. Two of these HSS mapped 4.0 and 2.0 kb upstream of the CD8α coding sequences, whereas the third site mapped close to the CD8α gene transcription initiation start site and is likely to correspond to the promoter region. The Southern blot was reprobed with an upstream 1.2-kb BamHI-Hind III fragment

Table 1. *A Correlation between the Expression of CD8 and the Presence of HSS within the Murine CD8α Locus*

			DNase I HSS		
T cell line	CD4	CD8	−4.0	−2.0	+1
AKR1G1*	+	+	+‡	+	+
AKR33	+	+	+	+	+
9A10	+	+	±	+	±
4F10	−	+	+	+	+
AKR5	+	−	−	−	−
RLM11	+	−	−	−	−
EL-4	−	−	−	−	−

* T cell lines were analyzed for CD4 and CD8 surface expression by antibody staining and flow cytometry.
‡ DNase I HSS were identified as described in Materials and Methods.

(probe B in Fig. 1 *A*) to confirm the location of each hypersensitive site (data not shown). To determine if the presence of these HSS correlated with CD8 gene expression, a panel of T cell lines displaying different expression patterns of CD4 and CD8 were examined by the DNase I assay (Table 1). All three HSS were observed only in cell lines that expressed the CD8 gene, and not in $CD4^+8^-$ SP cell lines, nor in EL-4 cells that express neither CD4 nor CD8. The CD4 and CD8 expression pattern was confirmed by flow cytometry (summarized in Table 1). The consistent link between CD8 cell surface expression and the identified hypersensitive sites suggested that these sequences may play an important role in the regulation of the CD8α gene.

Interaction of a T Cell-specific Factor with the −4.0-kb HSS. Although DNase I HSS often mark the location of functional enhancers, e.g., the Ig λ chain gene enhancer (23) and the human CD2 enhancer (24), there are other cases where a combination of HSS is required to detect significant transcriptional enhancer activity. The best characterized example is the LCR of the β-globin locus, where four HSS spread over 20 kb are required to confer high level, position independent, tissue-specific expression on the β-globin gene or a linked heterologous gene (27–29). However, each hypersensitive region analyzed contains multiple binding sites for an erythroid-specific transcription factor (42–46), that has now been shown to be critical for erythroid differentiation (47). We postulated that one way to identify factors necessary for CD8 gene expression would be to search for T cell-specific factors that bound within the CD8 hypersensitive domains.

Of the three newly identified DNase I HSS, one corresponds to the promoter region of the CD8 gene and two others represent potential upstream regulatory elements. To identify nuclear factors that may be important for the regulation of the CD8 gene, we carried out in vitro DNA-protein binding assays. A 9.0-kb fragment of genomic DNA containing both upstream HSS was cloned and 50–100-bp DNA fragments spanning ~500 bp around the two CD8 upstream

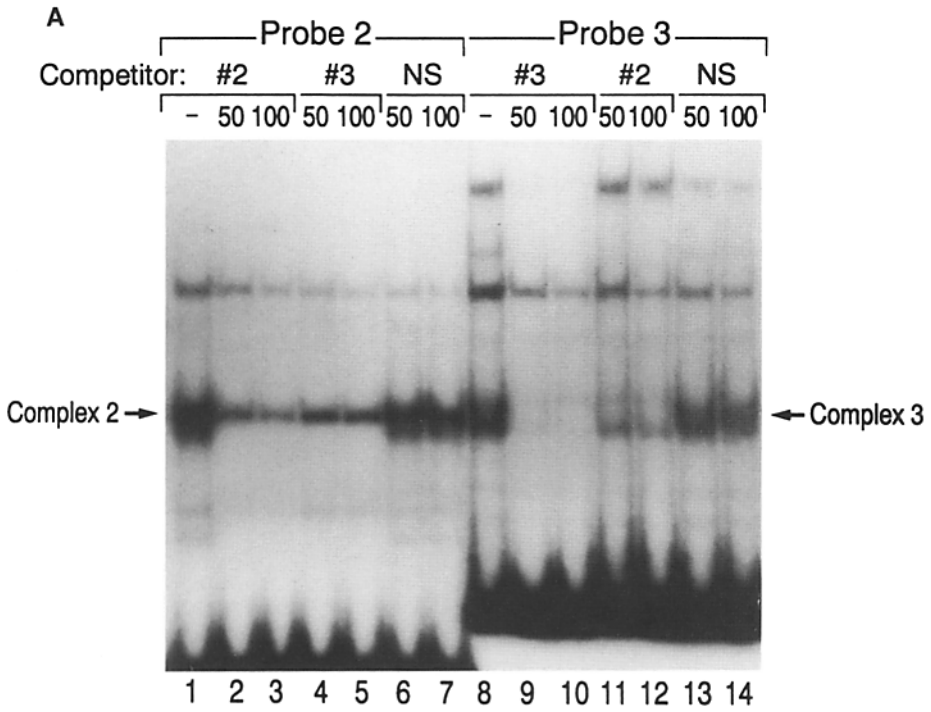
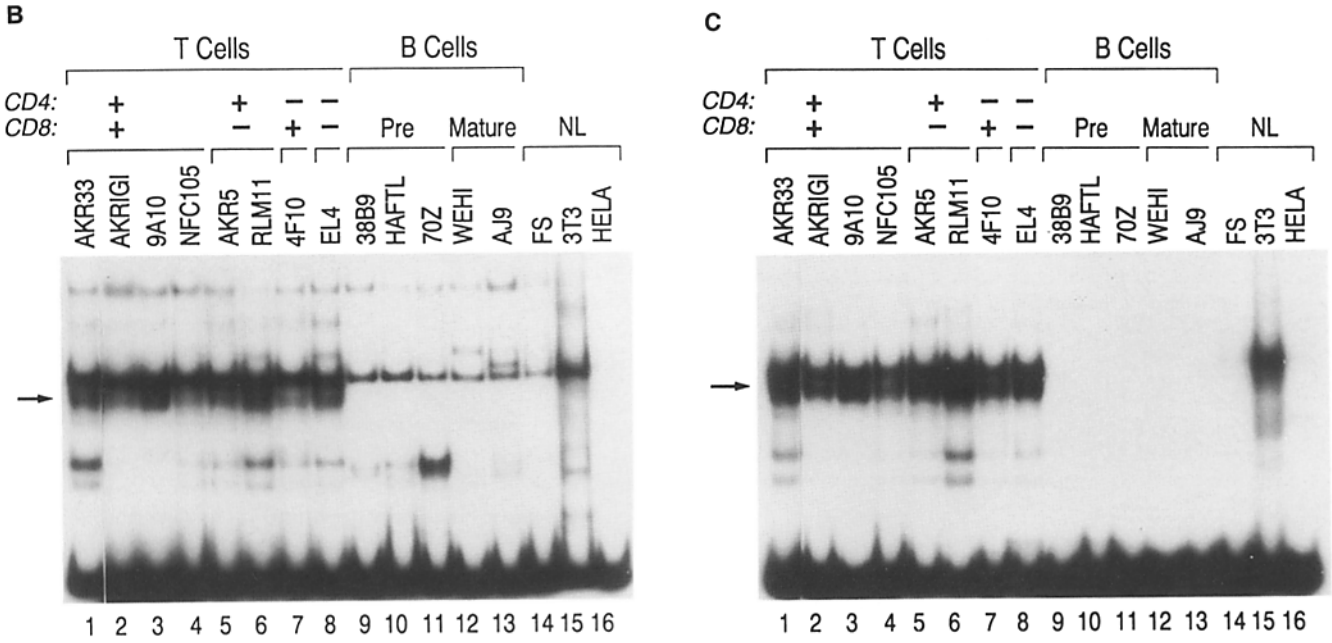


Figure 2. A T cell–specific nuclear protein binds to two adjacent DNA fragments from the −4.0-kb HSS. (*A*) Nuclear protein extracts were prepared from a $CD4^+8^-$ T cell line, AKR5, and tested in a gel mobility shift assay for binding to radioactively labeled DNA probes 2 (lanes *1–7*) and 3 (lanes *8–14*). Bindings were performed in the absence of competitor DNA (lanes *1* and *8*) or in the presence of 50- or 100-fold molar excess of unlabeled nonradioactive DNA: probe 2 (lanes *2*, *3* and *11*, *12*), probe 3 (lanes *4*, *5* and *9*, *10*), or nonspecific DNA (lanes *6*, *7* and *13*, *14*). To examine the tissue distribution of complex 2 (*B*) and complex 3 (*C*), nuclear extracts were prepared from various murine B and T cell lines and tested by gel mobility shift assay using a radioactively labeled AluI/AluI subfragment of probe 2, or probe 3, respectively. The cell lines used and their expression patterns are noted above each lane and the specific complexes are indicated with arrows. Probe 2 generates an additional nonspecific (upper) complex that is present in all cell extracts examined. To separate the specific and nonspecific complexes, this gel was run for a longer time.



HSS were radiolabeled, incubated with nuclear extracts, and analyzed by electrophoresis through nondenaturing polyacrylamide gels. Nucleoprotein complexes were identified by their slower mobility compared with the free probe during electrophoresis and sequence specificity of the interaction was confirmed by competition experiments.

One DNA fragment from the −2.0-kb HSS region showed several specific nucleoprotein complexes by gel shift assay, suggesting a cluster of binding sites within this fragment. However, preliminary analysis of the tissue distribution of the factors generating these complexes did not reveal T cell–specific protein–DNA interactions and therefore, we have not further analyzed this fragment (data not shown). Two adjacent DNA fragments, probes 2 and 3, from within the −4.0-kb HSS domain, revealed specific nucleoprotein complexes (complexes 2 and 3, respectively, are indicated by arrows, Fig. 2 *A*) in extracts derived from both $CD4^+8^+$ DP and $CD4^+8^-$ SP T cell lines. Since the complexes had similar mobility, we tested whether they represented binding of the same nuclear factor to both probes by crosscompetition experiments (Fig.

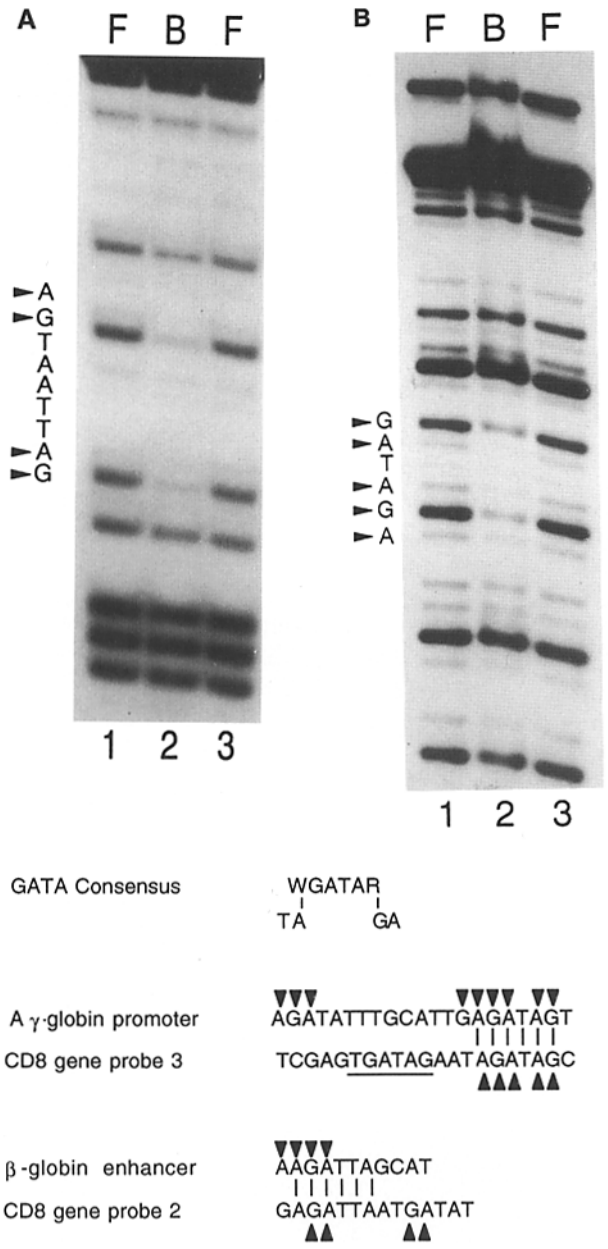


Figure 3. Methylation interference assay detects sequence homology to the GATA binding motif. Nuclear protein extract from AKR5 was incubated with partially methylated probe 2 (*A*) or probe 3 (*B*) and electrophoresed through a 4% nondenaturing acrylamide gel. Complex 2 or 3 was excised from the gel, as well as the free fragment. The eluted DNA was cleaved with piperidine and analyzed on a 12% sequencing gel. (►) G or A residues which make close contact with the nuclear protein (*F*, free probe; *B*, bound probe). (*C*) Sequence comparison between the region of protein contact in complexes 2 and 3, and GATA sequences from globin genes (42, 46). (►) Nucleotide residues detected by methylation interference; (*vertical bars*) regions of homology between shown sequences; (*horizontal bar*) a potential GATA site which does not score in this assay.

2 *A*). Complex 2 was competed efficiently by including nonradioactive probe 2 or 3 DNA in a binding reaction (Fig. 2 *A*, lanes *2–5*). A comigrating nonspecific complex was revealed when complex 2 was competed away (Fig. 2 *A*, lanes *2–5*). Conversely, complex 3 was competed by nonradioactive probe 2 and probe 3 DNA, albeit probe 2 competed somewhat less efficiently (Fig. 2*A*, lanes *11–12*). Neither the probe 2 nor the probe 3 complex was competed by equivalent amounts of an irrelevant DNA (Fig. 2*A*, lanes *6*, *7* and *13*, *14*, respectively), showing that the nucleoprotein complex represented the specific interaction of nuclear factor(s) with DNA. Furthermore, these results indicated that the same factor may bind to both probes.

To determine the tissue distribution of the probe 2/3 binding protein and to further confirm their similarity, we examined a panel of extracts derived from B and T lymphoid cell lines and several nonlymphoid cell lines. Both probes detected a factor in all T cell lines assayed, irrespective of their status of CD4 or CD8 expression (Fig. 2, *B* and *C*, lanes *1–8*). This factor was absent in the three pre-B, two mature B, and in the nonlymphoid cell lines HeLa or FS (Fig. 2 *B* and *C*, lanes *9–16*). Although both probes generated a complex with NIH3T3 cell extracts (Fig. 2, *B* and *C*, lane *15*), we believe that the factor responsible is different from that detected in T cell extracts based on the slower mobility of this complex and the lack of GATA-3 mRNA in these cells (data not shown, see below). Equivalent amounts of cell extract were analyzed as determined by assaying for the ubiquitous octamer binding protein (data not shown). Thus, probes 2 and 3 bind a protein that is restricted to T lymphoid cells.

GATA-3 Binding Sites within the −4.0-kb Hypersensitive Region. To precisely map the site of DNA–protein interactions, we used methylation interference assays. This assay detects G and A residues whose methylation interferes with DNA–protein interaction and therefore, are absent from DNA eluted from the nucleoprotein complex band of a preparative electrophoretic mobility shift gel. On the noncoding strand of probe 2, two G and two A residues were significantly depleted in the bound DNA lane (Fig. 3 *A*) identifying a sequence element GATTAATGA. On the noncoding strand of probe 3, bands corresponding to five purine residues were diminished in the bound DNA lane (Fig. 3 *B*), identifying the sequence AGATAG. The sequences which scored in this assay bear significant homology to sequences identified in the human β-globin enhancer (42) and the Aγ-globin promoter (46) (Fig. 3). These sequences bind the erythroid-specific transcription factor NF-E1, EryF-1, or GATA-1 (30, 42, 43). We conclude that a T cell–specific nuclear factor recognizes a site similar to that seen by the GATA-1 protein, and binds to two sites within the −4.0-kb HSS upstream of the murine CD8 gene.

Recently, a search for genes encoding GATA-1 related proteins by low stringency hybridization has led to the identification of two related genes, GATA-2 and GATA-3. GATA-1 is expressed exclusively in erythroid cells, GATA-2 is expressed at low levels in erythroid cells and other cell types, and GATA-3 is expressed predominantly and abundantly in T lymphoid cells and to a lesser extent in fetal brain (30–34). This pattern of expression suggested the possibility that the T cell–restricted factor detected in nuclear extracts was the GATA-3 protein.

To determine if GATA-3 protein can bind to the CD8 GATA sites in probes 2 and 3, we synthesized this factor by in vitro

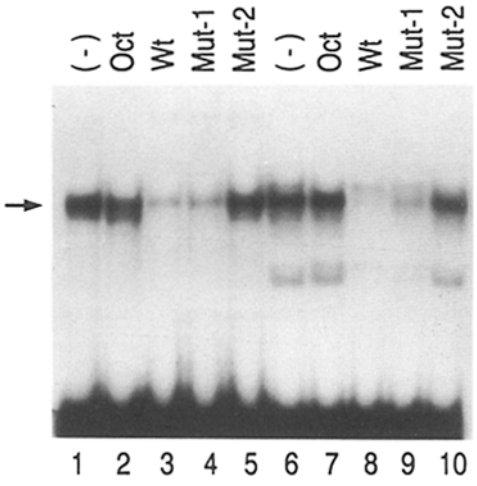


Figure 4. GATA-3 protein binds specifically to probe 3 and comigrates with the cellular nucleoprotein complex 3. Nuclear protein extract from 9A10, a DP T cell line (lanes *1–5*), or in vitro translated GATA-3 (lanes *6–10*) was incubated with radioactively labeled probe 3 and examined by the electrophoretic mobility shift assay. Competitions were performed with 100-fold molar excess of nonspecific DNA derived from the octamer binding site in lanes *2* and 7; a dimer of the P3 oligonucleotide containing the wild-type probe 3 GATA sequence (lanes *3* and *8*); a dimer of the P3-M1 oligonucleotide containing mutations in a single GATA site (lanes *4* and *9*); or a dimer of the P3-M2 oligonucleotide containing two mutated GATA sites (lanes *5* and *10*).

transcription and translation of the murine GATA-3 gene and carried out binding analyses. Incubation of either radiolabeled probe 2 (data not shown) or probe 3 DNA with wheat germ extracts containing in vitro translated GATA-3 protein, generated a nucleoprotein complex (Fig. 4, lane *6*) which comigrated with the cellular protein in complex 3 (Fig. 4, lane *1*). Both the cellular complex and the in vitro translated GATA-3 protein complex were competed specifically by oligonucleotides containing the probe 3 GATA site (Fig. 4, lanes *3* and *8*), but not by irrelevant DNA containing the octamer binding site (Fig. 4, lanes *2* and 7). Surprisingly, an oligonucleotide containing a mutation in the GATA site identified by methylation interference (P3-M1) was also able to compete for GATA-3 binding (Fig. 4, lanes *4* and *9*). Competition analyses using lower levels of competitor DNA showed that the M1 mutation competed two–threefold less efficiently than the wild-type competitor (P3) (data not shown), suggesting the presence of an adjacent, lower affinity GATA binding site within the oligonucleotide sequence. Oligonucleotides containing a second mutation (P3-M2) in the adjacent GATA consensus site in probe 3 did not compete for cellular complex 3 and in vitro translated GATA-3 binding (Fig. 4, lanes *5* and *10*). In addition, neither probe 2 nor probe 3 generated a complex when incubated with wheat germ translation extracts alone or with in vitro translated p50, a component of the transcription factor NF-κB (data not shown). Taken together, the results of the in vitro binding analysis indicate that the T cell–restricted GATA-3 protein interacts with several elements located within the −4.0-kb DNase I HSS and suggests that this factor may play a role in CD8α gene expression.

Functional Analysis of the CD8 GATA Binding Site. GATA-1 has been identified as the critical factor required for erythroid-specific expression of various globin and nonglobin genes and erythroid differentiation (47, 49). Recently, functional GATA-3 binding sites have been identified in the regulatory sequences of T cell–specific genes such as the TCRs α, β, and δ (31–34). In all cases, the functional significance of the GATA elements have been inferred from the observation that multimerized GATA sites activate transcription from a minimal promoter in the presence of coexpressed GATA protein. To determine if the CD8α gene GATA sites were active in such an assay, an oligonucleotide containing CD8 GATA sequences from probe 3 was cloned as a dimer upstream of a minimal c-*fos* gene promoter driving the CAT gene. As a control, an oligonucleotide containing a mutation in the GATA site identified by methylation interference was also cloned as a dimer into the same vector. For transactivation assays, each plasmid was cotransfected into nonlymphoid HeLa cells with a plasmid expressing the murine GATA-3 cDNA (R/mGATA sense). GATA-3 is not detectable in HeLa cells as determined by Northern blots probed with mc5b8 cDNA (data not shown). The transfected cells were harvested 48 h later and cellular extracts assayed for CAT enzyme activity. Plasmid (P3)-CAT, containing the wild-type GATA site from probe 3, showed 16-fold greater activity in the presence of the GATA-3 expression vector compared with a vector containing the gene in the opposite orientation (Fig. 5). Plasmid Δ56-CAT lacking CD8 GATA sites and plasmid (P3-M1)-CAT carrying a single mutated GATA site were only 1.5- and 6.1-fold more active in the presence of the GATA-3 cDNA expressing vector. The

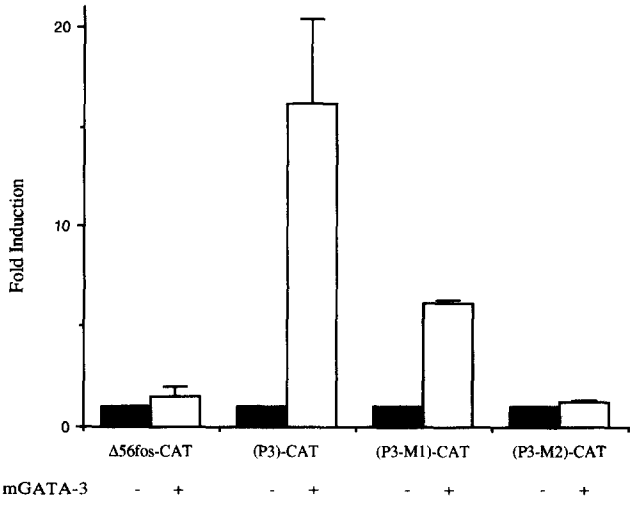


Figure 5. GATA-3 transactivates CD8 GATA sites in a cotransfection assay. HeLa cells were transiently transfected with 10 μg of reporter plasmid (Δ56fos-CAT, [P3]-CAT, [P3-M1]-CAT, or [P3-M2]-CAT) and 5 μg of transactivator mGATA-3 sense (+) or antisense (−). Fold induction is calculated as the ratio of percent chloramphenicol conversion with mGATA-3 sense to mGATA-3 antisense.

relative high residual GATA-3–dependent activity of the (P3-M1)-CAT could be due to the adjacent GATA consensus sequence present within the P3 oligonucleotide (refer to Fig. 3 *C*). This idea was consistent with the results from the competition experiments (Fig. 4) and was further confirmed by the transfection experiments using an oligonucleotide containing a mutation in the second GATA site cloned into the CAT containing plasmid, (P3-M2)-CAT. In this case, the GATA-3 inducible activity was drastically affected (Fig. 5). Thus, the GATA sites in the CD8α gene −4.0-kb HSS are functional by the same criteria that has been used for such sequences from the globin genes (30, 44, 45, 48) or the TCR α, β, and δ genes (31–34).

Discussion

We report the first analysis of factors that may regulate the transcription of the murine CD8α gene. We proceeded by searching for CD8 expression–specific DNase I HSS in the CD8α locus, analyzing such regions for interesting (T cell–specific) regulatory protein binding sites, and finally, testing those sites for their ability to act as transcriptional activators. Three DNase I HSS were identified in DP and $CD4^-8^+$ SP T cell lines. Multiple binding sites for a T cell–specific factor were detected within the distal HSS, which maps approximately 4.0-kb upstream of the transcription initiation start site of the murine CD8α gene. High resolution mapping of the binding sites for these factors by methylation interference assays showed them to be homologous to previously identified sites in regulatory regions associated with various globin genes, which bind the erythroid-specific transcription factor, GATA-1. A T cell–restricted member of the GATA gene family, called GATA-3, has been cloned and shown to recognize similar consensus sequences found within the TCR α, β, and δ chain gene enhancers. We show that in vitro translated GATA-3 protein binds to multiple CD8 GATA sites and propose that the T cell–specific factor we detect in nuclear extracts is the cellular GATA-3 protein based on the following evidence: (*a*) the equivalent mobilities of the cellular protein and in vitro translated GATA-3; (*b*) the indistinguishable sequence specificity of both proteins; (*c*) the presence of GATA-3 mRNA in all T cell lines tested (Landry, D. B., and R. Sen, unpublished data) and; (*d*) the absence of other GATA factors in T lymphocytes (Leonard, M. W., and J. D. Engel, unpublished data).

GATA binding proteins were first identified as erythroid-specific factors that bound within the regulatory sequences of chicken and human globin genes (42–46). Both the LCR, which regulates copy number–dependent, integration site–independent expression of globin genes, and the individual globin gene promoters contain multiple binding sites for the erythroid-specific GATA-1 protein. Although a critical role for GATA-1 in erythroid differentiation has been unequivocally demonstrated by gene disruption methodology (47), it has proved difficult to directly demonstrate the functional significance of most of these sites in the context of the whole regulatory sequence. To circumvent this problem, GATA sites have been analyzed individually or as multimers cloned upstream of a minimal TATA box containing promoter. For example, the functional significance of GATA sites in the TCR α, β, and δ enhancers have been judged based on the ability of dimerized sequences to activate transcription (31–34). Similarly, GATA motifs from DNase I HSS associated with the β-globin LCR have also been studied by transactivation of multimerized elements by coexpression of the GATA-1 cDNA. Such results with diverse GATA-containing regulatory sequences suggests that the function of these elements may not be readily determined by standard enhancer assays. Given the complexities of GATA site–dependent transcription, we assayed the CD8 GATA sites from the −4 kb HSS as a dimer upstream of a minimal c-*fos* gene promoter. This CD8 sequence activates transcription strongly when cotransfected with the GATA-3 gene into nonlymphoid cells, suggesting that the sites are functional and therefore, may play a role in the transcriptional regulation of the murine CD8α gene.

Does this T cell–restricted factor satisfy the various criteria expected of a critical regulator of CD8 gene expression during thymocyte differentiation? The developmental pattern of CD8 expression suggests at least two levels of regulation. First, the CD8 gene is activated as thymocytes progress from DN to DP cells and second, it is inactivated in cells that are positively selected by class II MHC molecules. The GATA-3 binding sites that we have identified are located within a CD8-specific DNase I HSS, consistent with a role for this factor in the regulation of CD8 gene expression. However, two lines of evidence indicate that the properties of GATA-3 are not sufficient to accommodate all the regulatory complexities of the CD8 gene. First, the factor we detect in nuclear extracts (likely to be the product of the GATA-3 gene) is present in both DP and $CD4^+8^-$ SP T cell lines, suggesting that the lack of CD8 expression in the later cells is not due to the absence of this factor. Second, our preliminary results of experiments investigating the onset of GATA-3 mRNA expression during fetal thymocyte ontogeny shows that this gene is transcribed as early as day 13/14 of gestation (Landry, D. B., and R. Sen, unpublished data), preceding the onset of CD8 protein expression which occurs around day 16/17 of gestation (50). We speculate that T cell specificity, as well as stage specificity within the lineage, may be achieved by GATA-3 protein acting in concert with other currently unidentified regulatory proteins. This model provides a mechanism by which different stage-specific T cell genes may be regulated by both GATA-3 and other stage-specific factors.

It is interesting to point out some parallels between the regulation of the β-globin locus and the CD8 gene. First, the β-globin locus is believed to be regulated in part by the erythroid-specific factor GATA-1, since functional GATA-1 binding sites have been found within the β-globin LCR HSS and promoter HSS regions. Similarly, we have identified functional GATA-3 binding sites within the CD8-specific −4.0-kb HSS and, as well as, within the CD8α promoter HSS region (Mittal, P., D. Landry, and R. Sen, unpublished results). In both cases, additional tissue-specific factors are likely to be required. Second, switching to other β-type globin genes occurs by the suppression of the earlier genes despite the con-

tinued presence of GATA-1. Similarly, whereas GATA-3 may play a role in the activation of the CD8α gene, it continues to be expressed in the later $CD4^+8^-$ SP lineage. Finally, the LCR of the β-globin locus is spread over many kilobases of DNA and is composed of four tissue-specific DNase I HSS, the most proximal of which is located only 6.0-kb upstream of the ε-globin gene. A combination of these four HSS is required to achieve high-level, tissue-specific expression of globin genes. In the CD8 locus, the HSS we have characterized is approximately 4.0-kb upstream from the transcription initiation site of the CD8α gene, and we speculate that this HSS will act in concert with other presently unidentified regulatory HSS.

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References

1. Gallagher, P.F., B.F. De St. Groth, and J.F.A.P. Miller. 1989. CD4 and CD8 molecules can physically associate with the same T cell receptor. *Proc. Natl. Acad. Sci. USA.* 86:10044.
2. Saizawa, K., J. Rojo, and C.A. Janeway. 1987. Evidence for a physical association of CD4 and the CD3:α:β T-cell receptor. *Nature (Lond.).* 328:260.
3. Takada, S., and E.G. Engleman. 1987. Evidence for an association between CD8 molecules and the T cell receptor complex on cytotoxic T cells. *J. Immunol.* 139:3231.
4. Norment, A.M., R.D. Salter, P. Parham, V.H. Engelhard, and D.R. Littman. 1988. Cell–cell adhesion mediated by CD8 and MHC class I molecules. *Nature (Lond.).* 336:79.
5. Doyle, C., and J.L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature (Lond.).* 330:256.
6. Veillette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine protein kinase p56lck. *Cell.* 55:301.
7. Rudd, C.E., J.M. Trevillyan, J.D. Dasgupta, L.L. Wong, and and S.F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190.
8. Rudd, C.E. 1990. CD4, CD8 and the TCR-CD3 complex: a novel class of protein-tyrosine kinase receptors. *Immunol. Today.* 11:400.
9. Molina, T.J., K. Kishihara, D.P. Siderovski, W. van Ewjik, A. Narendran, E. Timms, A. Wakeham, C.J. Paige, K.-U. Hartmann, A. Veillette, et al. 1992. Profound block in thymocyte development in mice lacking p56lck. *Nature (Lond.).* 357:161.
10. Seong, R.H., J.W. Chamberlain, and J.R. Parnes. 1992. Signal for T-cell differentiation to a CD4 cell lineage is delivered by CD4 transmembrane region and/or cytoplasmic tail. *Nature (Lond.).* 356:718.
11. Ramsdell, F., and B.J. Fowlkes. 1989. Engagement of the CD4 and CD8 accessory molecules is required for T cell maturation. *J. Immunol.* 143:1467.
12. Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1990. CD8 is required during positive selection of $CD4^-8^+$ T cell. *J. Exp. Med.* 171:427.
13. Teh, H.S., A.M. Garvin, K.A. Forbush, D.A. Carlow, C.B. Davis, D.R. Littman, and R.M. Perlmutter. 1991. Participation of CD4 coreceptor molecules in T cell repertoire selection. *Nature (Lond.).* 349:241.
14. Blackman, M., J. Kappler, and P. Marrack. 1990. The role of the T cell receptor in positive and negative selection of developing T cell. *Science (Wash. DC).* 248:1335.
15. Teh, S.H., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the αβ T cell receptor determining the CD4/CD8 phenotype of T cells. *Nature (Lond.).* 335:229.
16. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature (Lond.).* 335:271.
17. Kaye, J., M.L. Hsu, M.E. Sauron, S.C. Jameson, N.R.J. Gascoigne, and S.M. Hedrick. 1989. Selective development of $CD4^+$ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature (Lond.).* 341:746.
18. Berg, L.J., A.M. Pullen, B.F. de St. Groth, D. Mathis, C. Benoist, and M.M. Davis. 1989. Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell.* 58:1035.
19. Robey, E.A., B.J. Fowlkes, J.W. Gordon, D. Kioussis, H. von Boehmer, F. Ramsdell, and R. Axel. 1991. Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell.* 64:99.
20. Borgulya, P., H. Kishi, U. Muller, J. Kirberg, and H. von Boehmer. 1991. Development of the CD4 and CD8 lineage of T cells: instruction versus selection. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:913.
21. Sawada, S., and D.R. Littman. 1991. Identification and characterization of a T-cell–specific enhancer adjacent to the murine CD4 gene. *Mol. Cell. Biol.* 11:5506.

22. Siu, G., A.L. Wurster, J.S. Lipstick, and S.M. Hedrick. 1992. Expression of the CD4 gene requires a Myb transcription factor. *Mol. Cell. Biol.* 12:1592.
23. Hagman, J., C.M. Rudin, D. Haasch, D. Chaplin, and U. Storb. 1990. A novel enhancer in the immunoglobulin lambda locus is duplicated and functionally independent of NFκB. *Genes & Dev.* 4:978.
24. Greaves, D.F., F.D. Wilson, G. Land, and D. Kioussis. 1989. Human CD2 3′ flanking sequences confer high-level, T cell-specific, position independent gene expression in transgenic mice. *Cell.* 56:979.
25. Vidal, M., R. Morris, F. Grosveld, and E. Spanopoulou. 1990. Tissue-specific control elements of the Thy-1 gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:833.
26. Gross, D.S., and W.T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57:159.
27. Grosveld, F., G. Blom van Assendelft, D.R. Greaves, and G. Kollies. 1987. Position-independent high level expression of the human β-globin gene in transgenic mice. *Cell.* 51:975.
28. Blom van Assendelft, G., O. Hanscombe, F. Grosveld, and D.R. Greaves. 1989. The β-globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. *Cell.* 56:969.
29. Talbot, D., P. Collins, M. Antoniou, M. Vidal, F. Grosveld, and D.R. Greaves. 1989. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. *Nature (Lond.).* 338:352.
30. Yamamoto, M., L.J. Yo, M.W. Leonard, H. Beug, S.H. Orkin, and J.D. Engel. 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes & Dev.* 4:1650.
31. Ko, L.J., M. Yamamoto, M.W. Leonard, K.M. George, P. Ting, and J.D. Engel. 1991. Murine and human T-lymphocyte GATA-3 factors mediate transcription through a *cis*-regulatory element within the human T-cell receptor δ gene enhancer. *Mol. Cell. Biol.* 11:2778.
32. Ho, I.C., P. Vorhees, N. Marin, B. K. Oakley, S.F. Tsai, S.H. Orkin, and J.M. Leiden. 1991. Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor α gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1187.
33. Joulin, V., D. Bories, J.F. Elevuet, M.C. Labiastie, S. Chretien, M.G. Matter, and P.H. Romeo. 1991. A T cell specific TCR δ DNA binding protein is a member of the human GATA family. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1809.
34. Marine, J., and A. Winoto. 1991. The human enhancer-binding protein Gata3 binds to several T-cell receptor regulatory elements. *Proc. Natl. Acad. Sci. USA.* 88:7284.
35. Zamoyska, R., A.C. Vollmer, K.C. Sizer, C.W. Liaw, and J.R. Parnes. 1985. Two Lyt-2 polypeptides arise from a single gene by alternative splicing patterns of mRNA. *Cell.* 43:153.
36. Gilman, M.Z., N. Wilson, and R.A. Weinberg. 1986. Multiple protein-binding sites in the 5′ flanking region regulate *c-fos* expression. *Mol. Cell. Biol.* 6:4305.
37. Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequence. *Cell.* 46:705.
38. Dignam, J.D., R.M. LeBowitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475.
39. Jamieson, C., and R. Sen. 1990. Methylation interference assay. *DNA and Protein Engineering Techniques.* 2:73.
40. Introduction of DNA into Mammalian Cells. 1988. *In* Current Protocols in Molecular Biology. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. Greene Publishing Associates and Wiley-Interscience, New York. 9.1.1–9.1.4.
41. Nelson, B., L. Hellman, and R. Sen. 1988. The NF-κB-binding site mediates phorbol ester–inducible transcription in nonlymphoid cells. *Mol. Cell. Biol.* 8:3526.
42. Wall, L., E. deBoer, and F. Grosveld. 1988. The human β-globin gene 3′ enhancer contains multiple binding sites for an erythroid-specific protein. *Genes & Dev.* 2:1089.
43. Evans, T., M. Reitman, and G. Felsenfeld. 1988. An erythroid-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc. Natl. Acad. Sci. USA.* 85:5976.
44. Tsai, S.F., D.I.K. Martin, L.I. Zon, A.D. D'Andrea, G.G. Wong, and S.H. Orkin. 1989. Cloning of the cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature (Lond.).* 339:446.
45. Trainor, C.D., T. Evans, G. Felsenfeld, and M.S. Boguski. 1990. Structure and evolution of the human erythroid transcription factor. *Nature (Lond.).* 344:92.
46. Martin, D.I.K., S.F. Tsai, and S.H. Orkin. 1989. Increased γ-globin expression in a nondeletion HPFH mediated by an erythroid-specific DNA-binding factor. *Nature (Lond.).* 338:435.
47. Pevny, L., M.C. Simon, E. Robertson, W.H. Klein, S.-F. Tsai, V. D'Agati, S.H. Orkin, and F. Constantini. 1991. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature (Lond.).* 349:257.
48. Martin, D.I.K., and S.H. Orkin. 1990. Transcriptional activation and DNA binding in the erythroid factor GF-1/NF-E1/Eryf-1. *Genes & Dev.* 4:1886.
49. Orkin, S.H. 1990. Globin gene regulation and switching: circa 1990. *Cell.* 63:665.
50. Husmann, L.A., R.P. Shimonkevitz, I.N. Crispe, and M.J. Bevan. 1988. Thymocyte subpopulations during early fetal development in the Balb/c mouse. *J. Immunol.* 141:736.