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 routine 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, p56⁷k (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 NFD105 (CD4+8+) were from Dr. Kenneth Rock (Harvard Medical School, Boston, MA); 32E9A10 (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 RLMI1 (CD4+8+) and EL4 (CD4+8+) were also used. All lymphoid cell lines were grown in RPMI 1640 medium supplemented with 10% inactivated FCS, 0.0002% B-ME, penicillin, and streptomycin. Non-lymphoid cell lines used were pre-B cell lines 70Z/3, 38B9, and HAFTL; B cell lines AJ9 and WEHI 231; and fibrosarcoma 164 (FS) were also used. All lymphoid cell lines were prepared by cutting plasmid P20 with restriction enzymes and digested with 40 μg of proteinase K, followed by precipitation with 3 M NaOAc, pH 5.2. The genomic DNA was purified by phenol-chloroform extraction, digested with the appropriate restriction endonucleases, and analyzed by electrophoresis through urea-containing sequencing gels.

Flow Cytometric Analysis of T Cell Lines. All murine thymoma cell lines were analyzed for CD4/LT4 and CD4/Lytr expression by flow cytometry using rat mAbs GK1.5 and 53-6.72, respectively. In 0.5 ml of RSB, 100 μl of nuclear suspension was treated with 6 x 10⁷ cells were washed in PBS and then treated with reticulocyte standard buffer (RSB) (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 5 mM MgCl2) 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 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, 1 mM EDTA, treated with 25 μg R.Nase 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 genomic CD8α 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 pGEM2zf(-) (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 Sall/XhoI ends (mutations are underlined): P3, TCGAGTGATAGATAGATAGACG; P3-M1, TCGAGTGATAGATCCTGACG; and P3-M2, TCGAGTCTAGAATCTCGAGAGC. The double-stranded oligonucleotides were cloned as dimers (head-to-tail) into the Sall 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 (GCAGAAGCTGTTGGGAGCGCGGTTC) 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 HaelII-Ddel fragment and probe 3 is a 48-bp Alul/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 μg 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 x 10⁶ 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).
Table 1. A Correlation between the Expression of CD8 and the Presence of HSS within the Murine CD8α Locus

<table>
<thead>
<tr>
<th>T cell line</th>
<th>CD4</th>
<th>CD8</th>
<th>DNase I HSS</th>
</tr>
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<tbody>
<tr>
<td>AKR1G1</td>
<td>+</td>
<td>+</td>
<td>-4.0</td>
</tr>
<tr>
<td>AKR33</td>
<td>+</td>
<td>+</td>
<td>-2.0</td>
</tr>
<tr>
<td>9A10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4F10</td>
<td>-</td>
<td>+</td>
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<tr>
<td>AKR5</td>
<td>+</td>
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<tr>
<td>RLM11</td>
<td>+</td>
<td>-</td>
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<td>EL-4</td>
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* 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.

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 CD8α 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.

expression, 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 (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 CD8α 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
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, AKRS, 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 non-radioactive 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 bar) regions of homology between shown sequences; (horizontal bar) a potential GATA site which does not score in this assay.

2.4). Complex 2 was competed efficiently by including nonradioactive probe 2 or 3 DNA in a binding reaction (Fig. 2A, lanes 2–5). A comigrating nonspecific complex was revealed when complex 2 was competed away (Fig. 2A, lanes 2–5). Conversely, complex 3 was competed by nonradioactive probe 2 and probe 3 DNA, albeit probe 2 competed somewhat less efficiently (Fig. 2A, lanes 11–12). Neither the probe 2 nor the probe 3 complex was competed by equivalent amounts of an irrelevant DNA (Fig. 2A, 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. 2B 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 assayng 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 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).}

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 (p/mGATA sense). GATA-3 is not detectable in HeLa cells as determined by Northern blots probed with mcs5b8 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 ΔS6-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 (ΔS6fas-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 se-
quency present within the P3 oligonucleotide (refer to Fig.
3 C). This idea was consistent with the results from the com-
petition experiments (Fig. 4) and was further confirmed by
the transfection experiments using an oligonucleotide con-
taining 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 ini-
tiation 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 previ-
ously identified sites in regulatory regions associated with
various globin genes, which bind the erythroid-specific tran-
scription 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 cel-
lar protein and in vitro translated GATA-3; (b) the indistin-
guishable sequence specificity of both proteins; (c) the pres-
ence 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–in-
dependent expression of globin genes, and the individual globin
gene promoters contain multiple binding sites for the ery-
throid-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 signi-
ficance of most of these sites in the context of the whole regu-
latory 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 assessed based on the ability of dimer-
ized 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 multimer-
ized elements by coexpression of the GATA-1 cDNA. Such
results with diverse GATA-containing regulatory sequences
suggest that the function of these elements may not be readily
determined by standard enhancer assays. Given the complex-
ities 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 acti-
-vates 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 tran-
scriptional 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 posi-
tively 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
enough 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 ex-
periments investigating the onset of GATA-3 mRNA expres-
sion 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 me-
chanism 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 func-
tional 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-
continued 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 e-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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