GATA Elements Are Necessary for the Activity and Tissue Specificity of the T-Cell Receptor Beta-Chain Transcriptional Enhancer

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Three high-affinity binding sites for the GATA family of transcriptional regulators have been identified within the T-cell receptor beta-chain (TCRβ) transcriptional enhancer, and their functional significance has been determined in an effort to understand the T-cell specificity of the enhancer more fully. One site, TE4, is important for activity of the enhancer in T cells. Neither site TE1 nor site TE2 can functionally replace a mutated TE4 site in T cells; however, the same protein, probably GATA-3, binds all three sites, as judged by electrophoretic mobility shift, oligonucleotide competition, and proteolytic clipping assays. These data suggest that additional proteins are critical for the ability of GATA-3 to activate the TCRβ enhancer. In fibroblasts, the GATA sequence at site TE1 appears to bind a negative regulator. Since this is not true in B cells, B cells and fibroblasts appear to have different mechanisms for negative regulation of the TCRβ enhancer.

The αβ T-cell receptor (TCR) is the major clonotypic antigen-specific receptor for T lymphocytes. Proper expression of TCR α and β chains is critical for normal T-cell development, since the receptor is required for positive and negative selection of developing thymocytes and for activation of peripheral T cells (5, 55). The genes encoding the α and β chains are similar to immunoglobulin genes with respect to organization and DNA rearrangement (5, 55). Furthermore, the same recombination machinery appears to be used for assembling TCR gene segments in T cells and immunoglobulin gene segments in B cells (47, 59). The T-cell specificity of rearrangement and expression of the TCRα and -β genes is in part regulated at the level of transcription (2, 57). TCRβ gene transcription appears to be primarily controlled by a T-cell-specific transcriptional enhancer located 3' of the Cβ2 coding region (12, 21, 32, 43, 49). Protein-binding sites have been identified in the TCRβ enhancer, and the functional significance of some sites has been established (26); however, the molecular mechanisms which confer T-cell specificity are not well understood.

GATA-3 is a transcription factor which has been shown to be important for determining the T-cell specificity of the TCRα and -β genes (15, 18, 20, 29). It is a member of the GATA family of transcription factors that recognize the consensus sequence WGATAR through a highly conserved C4 zinc finger domain (39). In vertebrates, four GATA members, GATA-1 through GATA-4, have been identified, and it has been shown that these proteins have somewhat overlapping but distinctive patterns of expression (39). The best-characterized member of this family is GATA-1, which is expressed in the erythroid, megakaryocytic, mast cell, and germ cell lineages (7, 8, 30, 46, 51). GATA-1 has been shown to regulate a number of erythroid cell-specific genes and to be required for normal erythropoiesis (41). Another family member, GATA-3, is the predominant GATA protein present in T cells, although high levels of GATA-3 are also detected in the developing central nervous system (15). GATA-3 mRNA is detectable early in thymus ontogeny, being present at day 12.5 in developing embryos (26). A number of enhancers that regulate T-cell-specific genes, including CD3δ and the TCRα, -β, -γ, and -δ genes, contain GATA sequences (26). These observations have led to the suggestion that GATA-3, like GATA-1, may be important for regulating lineage-specific gene expression and restricting hematopoietic cells to a particular development fate. The role of GATA-3 in determining the T-cell specificity of the TCRβ enhancer is not known, although several regions with good matches to the GATA consensus are present within the enhancer (12, 29). In this study, GATA-3-binding sites have been identified and their functional importance has been determined by mutational analysis. Three GATA-containing sites, TE1, TE2, and TE4, were shown to be recognized by proteins from crude T-cell nuclear extracts and to bind recombinant GATA-3 with high affinity. The GATA sequence at site TE4 is critical for the activity of the enhancer in T cells. Sites TE1 and TE2 are not functionally equivalent to TE4 and are unable to compensate for a mutated TE4 site despite evidence that indistinguishable proteins bind sites TE1 and TE4. In addition, site TE1, but not site TE2 or TE4, was shown to mediate negative regulation of the TCRβ enhancer in fibroblasts. Thus, the activation capability of GATA-3 for the TCRβ enhancer in T cells and the repressor activity of a GATA-binding protein in fibroblasts are both context dependent. These findings suggest that protein-protein interactions are critical for determining the action of GATA-binding proteins in the TCRβ enhancer.

MATERIALS AND METHODS

EMSAs and Op-Cu footprinting. Nuclear extracts from various cell lines were prepared as previously described (6, 48).
TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTE1</td>
<td>5' GACACACACATGTAGCTGACACTGACCTCG 3'</td>
</tr>
<tr>
<td>MTE2</td>
<td>5' CAGAGGCACCTGACACTGACCTGGAA 3'</td>
</tr>
<tr>
<td>MTE4</td>
<td>5' CGTTGACGTACTACGTGTTCGTAGTTCG 3'</td>
</tr>
<tr>
<td>pUC primer 1</td>
<td>5' GTAACACACGCGCCGAGCG 3'</td>
</tr>
<tr>
<td>pUC primer 2</td>
<td>5' AAGACGCTGACCTGACCG 3'</td>
</tr>
<tr>
<td>GATA site A</td>
<td>5' GATCATCTCTGACTACCTGCCTGAC 3'</td>
</tr>
<tr>
<td>GATA site B</td>
<td>5' ATTAGAGATTGCTCCTAG 3'</td>
</tr>
<tr>
<td>CRE site A</td>
<td>5' AAGCTGACTTCCTGACCG 3'</td>
</tr>
<tr>
<td>CRE site B</td>
<td>5' CTTTAGCTCTACCTGACCG 3'</td>
</tr>
<tr>
<td>TotA1</td>
<td>5' CTCATTCCATCGAGCTAGCTGGTACCA 3'</td>
</tr>
<tr>
<td>TotB1</td>
<td>5' TGGTAAACAGATGCATGTCCTAGATTAGGAG 3'</td>
</tr>
</tbody>
</table>

* Underlining indicates mutated bases.

Electrophoretic mobility shift assays (EMSA) were performed with 2 to 4 µg of protein from nuclear extracts or 1 µg of protein from extracts of bacteria expressing recombinant human GATA-3, 3 µg of poly(dl-dC), 10 mM Tris, 80 mM NaCl, 10% glycerol, and 10°C (1 ng) of probe. Binding reaction mixtures were incubated at room temperature for 20 min, loaded directly onto a 6% polyacrylamide gel, and electrophoresed at 200 V in 0.5 X Tris-borate-EDTA. For o-phospho-threonine-copper (Op-Cu) nucleosome footprinting, the reaction mixture described was increased 10-fold, and following electrophoresis, gels were treated with chemical endonuclease Op-Cu as previously described (23). The gel was visualized by autoradiography, and the free and bound complexes were cut out and eluted from the gel (31). The DNA was then separated on a 6% sequencing gel with G and G+A ladders and visualized by autoradiography.

Probes for EMSA and footprinting were generated from the murine TCRβ enhancer. Probes were generated by 5' end labeling with T4 polynucleotide kinase (31), end filling with the Klenow fragment of Escherichia coli polymerase (28), or PCR using a kinase-treated oligonucleotide (22). All probes were gel purified.

Site-directed mutagenesis. The TCRβ enhancer was subcloned into pUC19, and site-specific mutations were generated by PCR using previously described protocols (24, 40). Briefly, the procedure requires a single mutant primer and two flanking primers, which in this case annealed within the polylinker of pUC19. The oligonucleotides used in these experiments are shown in Table 1. Initially, the mutagenic primer and the downstream flanking primer (pUC19 primer 1) are used in a PCR to generate a primary product. Conditions for this initial PCR were as follows: 20 fmol of wild-type template, 1 µM each primer, 200 µM deoxynucleoside triphosphates, 10 mM Tris, 500 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin. PCRs were performed for 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min. The PCR product from this first reaction was gel purified and used in a second PCR as a primer with pUC19 primer 2 and the wild-type enhancer as the template. This second PCR product was then gel purified and subcloned into the appropriate vectors. All mutations were confirmed by DNA sequencing.

Transfections. For transfection experiments, the TCRβ enhancer and the mutant enhancers were subcloned into pA10LUC. This vector was constructed by replacing the chloramphenicol acetyltransferase gene in pA10CAT (11) with a luciferase reporter gene from p19LUC (54). The enhancer constructs were cloned downstream of the luciferase reporter gene. Oligonucleotides representing TE4 were multimerized and inserted 5' of the simian virus 40 promoter of pA10LUC or 5' of the herpes simplex virus thymidine kinase promoter of pBLCAT2 (27).

EL-4 cells were resuspended in RPMI supplemented with 5% fetal calf serum and 5 x 10^{-5} M mercaptoethanol at 1 x 10^{-3} to 3 x 10^{-3} cells per ml, and 0.3 ml of the cell suspension was electroporated by pulsing (960 µF, 220 V) from a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, Calif.). Cells were then resuspended in 5 ml of medium and incubated at 37°C in 5% CO₂ in air for 10 to 16 h before harvesting for luciferase assays. L cells and P3XAg8.63 cells were electroporated at 960 µF and 250 V and at 960 µF and 240 V, respectively. These cells were resuspended in Iscove modified Dulbecco medium supplemented with 10% fetal calf serum and cultured for 16 h.

For luciferase quantitation, cells were washed twice with phosphate-buffered saline and lysed with 0.5 ml of lysis buffer (1% Triton X-100, 25 mM glycylglycine [pH 7.8], 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol [DTT]). Lysates were cleared by centrifugation. To assay for luciferase activity, 500 µl of reaction buffer (15 mM KPO₄ buffer [pH 7.8], 2 mM ATP [pH 7.0], 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, 2 mM DTT) and 100 µl of substrate buffer (0.4 mM luciferin, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, 2 mM DTT) were added to 100 µl of the supernatant. Samples were measured by a luminometer.

All transfections were done in at least duplicate, and transfection efficiencies were controlled by cotransfecting a human growth hormone expression vector, pXGH5 (Nichols Institute Diagnostics, San Juan Capistrano, Calif.).

RESULTS

Three protein-binding sites within the TCRβ enhancer contain a GATA consensus sequence and bind recombinant GATA-3 efficiently. Sequence analysis revealed several potential GATA-binding sites within the TCRβ enhancer. To determine which GATA sequences were recognized by nuclear proteins, EMSAs were performed with probes spanning the 800-bp TCRβ enhancer fragment previously shown to contain enhancer activity (32). Nuclear extracts obtained from T cells, B cells, and fibroblasts were compared. Complexes TE1, TE2, TE4, and TE5 were unique to T-cell extracts (Fig. 1), although complexes with different mobilities bound to the same probes with non-T-cell extracts. Two additional complexes, TE3 and TE6, were observed in all extracts (Fig. 1 and data not shown).

When the six most prominent complexes were mapped by chemical nucleic footprinting, the consensus sequence for the GATA family of DNA-binding proteins, WGATAR, was observed in the sequences protected by the T-cell-specific complexes TE1, TE2, TE4, and TE5 (Fig. 1B).

The possibility that sites TE1, TE2, TE4, and TE5 represent in vivo GATA-3-binding sites was further investigated. First, the ability of recombinant GATA-3 to bind the sites was tested by EMSA. As shown in Fig. 2A, probes that included sites TE1, TE2, TE4, and TE5 gave specific gel shift complexes in the presence of recombinant GATA-3. The lower-mobility complexes observed with the TE2 and TE4 probes probably represent degraded GATA-3 present in the crude bacterial extracts. In addition, oligonucleotides representing each site were used as unlabeled competitors in an EMSA in which recombinant GATA-3 was bound to To3, an established GATA-3-binding site from the TCRα enhancer (15). TE1, TE2, and TE4 oligonucleotides competed very strongly for GATA-3 binding to To3 (Fig. 2B), indicating that these sites bind recombinant GATA-3 with a high affinity. TE5 appears to bind recombinant GATA-3 with a lower affinity, since it was a
weak competitor. Finally, oligonucleotides containing a GATA-3 consensus sequence (Table 1) were used as unlabeled competitors to determine if the TE1, TE2, and TE4 complexes formed from T-cell nuclear extracts contained GATA-binding proteins. GATA oligonucleotides competed very efficiently for all three of the complexes (Fig. 2C). Thus, GATA-binding protein is a component of the TE1, TE2, and TE4 EL-4 complexes. The T-cell specificity of these complexes is consistent with the possibility that the protein binding the sites is GATA-3 (Fig. 1A). Taken together, the in vitro binding studies show that three sites, TE1, TE2, and TE4, are recognized by T-cell-specific proteins in nuclear extracts, contain GATA sequences, and bind recombinant GATA-3 efficiently. The T-cell specificity and ability of GATA oligonucleotides to compete for complexes TE1, TE2, and TE4 suggest that GATA-3 in T-cell nuclear extracts binds these sites.

The TE4 GATA sequence is required for TCRβ enhancer activity in T cells; sites TE1 and TE2 are not functionally equivalent to site TE4. It is possible that the T-cell-specific activity of the TCRβ enhancer in vivo is mediated in part by GATA-3 binding at sites TE1, TE2, and TE4. Therefore, we designed experiments to determine if these three sites were important for TCRβ enhancer function and if they were functionally equivalent. Site-directed mutagenesis was performed on each site, using PCR (24, 40) (see Materials and Methods); the oligonucleotides used to generate the various mutations are shown in Table 1. The ability of each of the mutant sites to bind protein is shown in Fig. 3. Neither

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**FIG. 1.** Protein-binding sites within the TCRβ enhancer. (A) EMSAs using nuclear extracts prepared from NIH 3T3 cells, the plasmacytoma cell lines P3XAg8.63 and S107, and the T-cell lines EL-4, RS4.2, and SL12.2. End-labeled probes generated from the PvuII-NcoI TCRβ enhancer fragment were as follows: the TE1 probe is a 129-bp Alul-HindIII fragment, the TE2 probe is a 139-bp Sau3A-EarI fragment, the TE4 probe is a 89-bp Alul-MboI fragment, and the TE5 probe is a 136-bp Alul-NcoI fragment. T-cell-specific complexes are indicated by solid arrows; the TE6 complex is indicated by the open arrow. (B) Summary of binding sites within the TCRβ enhancer as determined by Op-Cu footprinting. T-cell-specific complexes are represented as hatched boxes; ubiquitous complexes are shown as stippled boxes. Potential GATA sequences are underlined. Binding sites described in previous studies (12, 49) that overlap the footprints are shown in parentheses. (C) Comparison of sequences from the footprinted sites of the murine TCRβ enhancer and known human TCRβ enhancer sequences (12, 43). Differences in the murine sequence are shown below the human sequence.
VOL. 14, 3-binding site from cleotides representing unlabeled competitors containing presence of context of the promoter lacking site TE1, TE2, TE4, mutant PvuI-NcoI extracts, labeled probes generated mutated TE1, extracts nuclear showed that gene luciferase recombinant GATA-3. (A) EMSAs using wild-type and mutant TE1, TE2, and TE4 probes with recombinant human GATA-3. (B) EMSAs with EL-4 nuclear extracts and wild-type and mutant probes.

minimal effect on enhancer activity, decreasing activity of the element by 25% (Fig. 4). Enhancer activity was not decreased further by a TE1 TE2 double mutation. However, the TE4 mutation had a significant effect on the TCRβ enhancer function, resulting in an 85% decrease in the activity of the enhancer. Combined mutations were also tested in the same vector to determine whether sites TE1 or TE2 have subtle functions which would be revealed in the absence of another

recombinant GATA-3 (Fig. 3A) nor proteins present in EL-4 nuclear extracts (Fig. 3B) bound to probes containing the mutated TE1, TE2, or TE4 site.

These mutations were tested for enhancer activity within the context of the 800-bp TCRβ enhancer by cloning wild-type and mutant PvuI-NcoI TCRβ enhancers into a site 3' of the luciferase gene under the control of a simian virus 40 early promoter lacking an enhancer. Transfection into EL-4 T cells showed that the TE1 and the TE2 mutations had only a

FIG. 4. Site TE4 is required for full TCRβ enhancer activity in T cells. The 800-bp PvuI-NcoI fragments of the wild-type (TCE) and mutant TCRβ enhancers were cloned 3' of a luciferase reporter gene in pA10LUC. EL-4 cells were transfected by electroporation with 10 µg of the various constructs. Each datum point represents at least three independent experiments.
site(s). TCRβ enhancers with combinations of mutated TE1 and TE4 sites or mutated TE1, TE2, and TE4 sites showed activity comparable to that of the enhancer with mutations in TE4 alone (Fig. 4). Therefore, these data show that although several TCRβ sites can bind GATA-3, they are not functionally equivalent. The TE4 GATA sequence is required for full activity of the TCRβ enhancer, whereas the TE1 and TE2 sites do not appear to be necessary, nor can they compensate functionally for the TE4 site.

T-cell nuclear proteins binding to sites TE1 and TE4 are biochemically indistinguishable. The experiments described above demonstrated that site TE4 is functionally distinct from sites TE1 and TE2. This functional difference could be caused by the presence of unique proteins in the TE4 complex. A more detailed biochemical analysis of the TE1 and TE4 complexes was performed to investigate this possibility. Initially, EMSAs were performed with EL-4 nuclear extracts in the absence or presence of different competitors. The abilities of different sites to cross-compete for binding of nuclear proteins were tested by using oligonucleotides representing various binding sites. Figure 5A shows that the TE4 site competes completely for the TE1 and TE2 complexes. Moreover, TE1 and TE2 site oligonucleotides competed for binding of T-cell nuclear proteins to TE4 (data not shown). These results confirm the data obtained with use of GATA oligonucleotide competitors (Fig. 2C) and suggest that TE1, TE2, and TE4 bind similar proteins.

In addition to a GATA sequence, the TE4 site has a potential CREB/ATF-binding site, and CREB family proteins have been shown to bind site β2 in the human TCRβ enhancer, which is homologous to murine site TE4 (1, 12, 49). It is possible that CREB/ATF family members binding to TE4 could account for the functional differences (9). However, cyclic AMP response element (CRE) oligonucleotides did not compete for either the TE1 or TE4 complex (Fig. 5B). In addition, the TE4 site was unable to compete for complexes that bind to a T01 probe which includes a CRE. Therefore, there is no indication that CREB family members are present in the TE4 complex. Thus, unlike the homologous site in the human enhancer, murine site TE4 does not appear to bind CREB/ATF proteins. Sites TE4 and β2 differ by two base pairs in their putative CREB-binding sequences (Fig. 1C), and this may account for the observed differences in their abilities to bind CREB/ATF proteins.

The protein-binding sites TE1 and TE4 were examined further in a proteolytic clipping assay (48). Upon addition of Staphylococcus aureus V8 protease to the EMSA reactions, cleavage products binding both probes appear to be similar when the difference due to probe length is discounted (Fig. 5C). Similar proteolytic products which retain binding ability indicate that identical or highly related proteins bind both sites. Thus, there is no evidence that the function of TE4 versus TE1 can be explained by differences in EL-4 nuclear proteins binding to the two sites in vitro. These data suggest that it is the context of TE4 that is different and that proteins bound at sites adjacent to the TE4 site may be required for its activity.

Site TE4 alone is not sufficient to function as a transcriptional enhancer. To determine directly if site TE4 could activate transcription in the absence of adjacent protein-binding sites, a 25-bp region representing the TE4 site was multimerized and cloned 5' of the simian virus 40 early promoter lacking enhancer sequences in the pA10LUC vector or the herpes simplex virus thymidine kinase promoter in the pBLCAT2 vector. Constructs with one, two, three, five, and six TE4 sites had no enhancer activity upon transfection into EL-4 cells (Fig. 6 and data not shown). These data are apparently inconsistent with a previous study by Marine and Winoto which showed that multimers of the TB2 GATA site from the human TCRβ enhancer activated a minimal fox promoter in MOLT 13 cells (29). The discrepancy between their results and ours may...
be due to differences in the cells used, differences in the promoter contexts of the multimers, or sequence differences between murine site TE4 and human site Tβ2, possibly related to differences in their ability to bind CREB/ATF proteins. Our data show that although the GATA sequence within TE4 is necessary for enhancer function, the TE4 site alone is not sufficient to activate transcription of an enhancer-dependent promoter. These data further support the notion that transcription factors bound at other sites, possibly adjacent to TE4, are necessary for TE4-dependent activation.

Site TE1 functions as a negative regulator of TCRβ function in fibroblasts but not in B cells. The PvuI-NcoI enhancer construct used in the experiments described above has been previously shown to be T-cell specific (32). Since tissue specificity of many enhancers, including T-cell-specific enhancers, is achieved by a combination of negative and positive elements (43, 57, 58), we wished to determine if site TE1, TE2, or TE4 was important for keeping the enhancer activity low in fibroblasts or B cells. Therefore, the constructs containing single mutations were transfected into P3XAg8.63, a plasmacytoma B-cell line, and L cells, a fibroblast line. Consistent with previous data from this laboratory, the wild-type TCR enhancer was not active in the B-cell line (Fig. 7A). In addition, none of the mutants showed significantly increased activity (Fig. 7A). Negligible enhancer activity was also observed when L-cell fibroblasts were transfected with the wild-type enhancer or with the mutant TE2 and mutant TE4 enhancer constructs (Fig. 7B). However, the TE1 mutant enhancer showed modestly increased activity (in excess of threefold) in fibroblasts relative to wild-type or to the other mutant enhancers (Fig. 7B). These results strongly suggest that a negative regulator present in fibroblasts binds to the TE1 site. Since mutation of the GATA consensus within site TE1 relieves repression, it seems likely that the negative regulator may be a member of the GATA family. The data further show that sites TE2 and TE4 cannot functionally replace site TE1 for mediating the repressor activity. Finally, the data suggest that regulation of the TCRβ enhancer is different in B cells and fibroblasts because the TE1 mutation does not activate the enhancer in B cells.

**DISCUSSION**

This study examined the functional significance of GATA elements in determining the T-cell specificity the TCRβ enhancer; three important findings have emerged. First, only one of three high-affinity GATA-3-binding sites is critical for activation of the enhancer in T cells. Second, another GATA site is important for repression of enhancer activity in fibroblasts but not in B cells, demonstrating that different mechanisms regulate the enhancer in fibroblasts and B cells. Finally, for both activation and repression of the TCRβ enhancer, GATA-binding proteins are context dependent, emphasizing the importance of protein-protein interactions for the mechanism of action of GATA-binding proteins.

**TCRβ activity in T cells.** Identification of site TE4 as an important functional element of the TCRβ enhancer in T cells is consistent with previous work which defined a 350-bp core as the minimal TCRβ enhancer (12, 43, 49). TE4 is the only site identified in this work that is within the minimal enhancer, and it corresponds to previously described sites β1 and β2 in the murine enhancer and Tβ2 in the human enhancer (12, 49). Analysis of the TE4 GATA mutation confirms and extends previous deletional and mutational analyses in this region (12, 21, 29, 42, 49). We examined the TE4 mutation within the context of an 800-bp enhancer fragment to determine whether other sites could compensate for TE4 function. This was particularly important since the TCRβ enhancer includes other GATA sequences, TE1 and TE2, which bind recombinant GATA-3 with a high affinity and which are recognized by proteins from T-cell nuclear extracts. Our data show that TE4 is functionally distinct and that sites TE1 and TE2 are not equivalent to TE4, since they were unable to compensate for the TE4 mutation. Although mutation of the GATA consensus
within TE1 and TE2 had a negligible effect on the activity of the TCRB enhancer, it is possible that these sites are important during T cell development at a stage not represented by EL-4 cells. In addition, these studies do not address the functional significance of nonconsensus or low-affinity GATA sites within the TCRB enhancer (19, 33).

Our results further demonstrate that mutation of the TE4 GATA consensus sequence and loss of ability to bind recombinant GATA-3 correlate with loss of TE4 function, suggesting that GATA-3 is necessary for TE4-dependent activation in vivo. Four facts support this hypothesis: (i) GATA-3 is the only known GATA-binding protein present in T cells (15, 18, 37, 39), (ii) TE4 binds recombinant GATA-3 strongly, (iii) GATA site oligonucleotides compete completely for the TE4 complex, and (iv) the TE4 complex is T cell specific, consistent with the T-cell-specific expression of GATA-3.

TE1 and TE2 also bind recombinant GATA-3 and a tissue-specific protein complex from nuclear extracts. EMSAs with different competitor oligonucleotides suggest that these sites are also binding GATA-3. Furthermore, proteins binding to TE1 and TE4 appear to be similar, as judged from partial proteolytic digestion. Thus, it seems likely that in vivo, GATA-3 binds site TE1 and TE2 as well as site TE4. However, TE1 and TE2 do not function like TE4, strongly suggesting that a different context for the sites is critical in determining their functions. According to the context model, GATA-3 bound at TE4 interacts with proteins bound to nearby sites and those interactions permit transcriptional activation, whereas GATA-3 bound at TE1 or TE2 is surrounded by different proteins that do not allow transcriptional activation. This model is supported by our demonstration that multimers of the TE4 site are not able to activate transcription alone. The ability of neighboring proteins to influence the function of the lymphoid cell-specific factor LIF-1 has been recently demonstrated (4, 10).

There are potentially a number of proteins that could be required for GATA3-dependent activation at the TE4 site. An obvious candidate was the CREB family of activators since the TE4 footprinted region includes sequences that match the consensus for a CRE which serves as a binding site for ATF/CREB family proteins (13, 16, 36). CREs are found within the Vβ promoter and the enhancers for TCRa, CD3δ and CD2 (1, 9, 12, 25, 49). However, oligonucleotide competitions and proteolytic clipping experiments show no evidence of CREB proteins binding to TE4. This is in contrast to the homologous sequence in the human TCRβ enhancer, TB2, which binds CREB/ATF (12). The role, if any, of CREB proteins at the TE4 site is unclear. Other protein-binding sites that may influence TE4 include an Ets site and a CBF site within the core enhancer, a C/EBP site, a TFE-3 site, and binding sites for the high-mobility group family members and POU domain proteins (14, 32, 34, 35, 38, 42, 45, 49, 50, 52, 53, 56). Additional work will be necessary to test the context model and to identify proteins which are required for GATA-3 function.

Negative regulation of the TCRβ enhancer in non-T cells. The TCRβ enhancer is T cell specific, having virtually undetectable activity in lineages such as fibroblasts and B cells (32, 49). Although some of the T cell specificity can be explained by the chromatin structure of the TCRβ enhancer in T cells and the presence in T cells of T cell-specific activators such as GATA-3, it seems likely that negative regulators may also be required to keep the enhancer silent in non-T cells. Silencing elements have been identified in the TCRa and -γ enhancers, and a role for negative elements in the TCRβ enhancer has been suggested in previous studies (17, 57, 58).

When GATA site mutations were tested for activity in B cells and fibroblasts, the data showed that mutation of the TE1 site resulted in an increase in enhancer activity in fibroblasts but not in B cells, thus directly demonstrating negative regulation of the TCRB enhancer. Mutation of sites TE2 and TE4 had no effect on enhancer activity in either cell type. The results suggest that a negative regulator binds the TE1 site in fibroblasts, although the identity of this protein is not currently known. EMSAs and chemical nuclease footprinting show a protein present in crude nuclear extracts from fibroblasts which binds TE1 and TE4, generating complexes different in mobility and footprint patterns from the T-cell complexes (Fig. 1A and data not shown). Since these complexes are also competed for with GATA oligonucleotides and abolished by mutation of the GATA consensus sequence (data not shown), the negative regulator may be a GATA family member. Although GATA family members have been demonstrated to act as positive factors, a recent study demonstrated that ectopic expression of GATA-2 could inhibit erythroid differentiation, indicating that in certain situations, GATA proteins may act as negative regulators (3). It is also possible that the GATA elements of the TCRB enhancer may recruit a negative regulator, element, similar to that proposed for the glycophorin B promoter (44).

These data also reveal another functional difference between GATA sites, since TE4 cannot replace TE1 for negative regulation, even though TE1 and TE4 appear to bind indistinguishable proteins.

Our data also suggest that the mechanisms for negative regulation of the TCRB enhancer are different in B cells and fibroblasts, since mutation of the TE1 site increased enhancer activity in fibroblasts but not in B cells. It is interesting to speculate that this difference may be related to the need to control rearrangement of T-cell receptor and immunoglobulin genes. There is evidence that enhancer-dependent transcription in the immunoglobulin and T-cell receptor loci may target the V(D)J recombination machinery to the correct genes in B cells and T cells, respectively (59). Thus, it may be that special mechanisms are necessary in B cells to silence TCRB transcription to ensure that β-chain genes are not rearranged in B cells.

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Our data also suggest that the mechanisms for negative regulation of the TCRβ enhancer are different in B cells and fibroblasts, since mutation of the TE1 site increased enhancer activity in fibroblasts but not in B cells. It is interesting to speculate that this difference may be related to the need to control rearrangement of T-cell receptor and immunoglobulin genes. There is evidence that enhancer-dependent transcription in the immunoglobulin and T-cell receptor loci may target the V(D)J recombination machinery to the correct genes in B cells and T cells, respectively (59). Thus, it may be that special mechanisms are necessary in B cells to silence TCRβ transcription to ensure that β-chain genes are not rearranged in B cells.

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These data also reveal another functional difference between GATA sites, since TE4 cannot replace TE1 for negative regulation, even though TE1 and TE4 appear to bind indistinguishable proteins.

Our data also suggest that the mechanisms for negative regulation of the TCRβ enhancer are different in B cells and fibroblasts, since mutation of the TE1 site increased enhancer activity in fibroblasts but not in B cells. It is interesting to speculate that this difference may be related to the need to control rearrangement of T-cell receptor and immunoglobulin genes. There is evidence that enhancer-dependent transcription in the immunoglobulin and T-cell receptor loci may target the V(D)J recombination machinery to the correct genes in B cells and T cells, respectively (59). Thus, it may be that special mechanisms are necessary in B cells to silence TCRβ transcription to ensure that β-chain genes are not rearranged in B cells.
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