Transcriptional Regulation of the T Cell Antigen Receptor ζ Subunit: Identification of a Tissue-restricted Promoter

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Summary

The cell surface expression of T cell antigen receptors (TCR) is regulated in part by the limiting synthesis of the ζ subunit. Utilizing fragments from the 5′ region of the human ζ gene, two discrete regions that promote transcription were characterized. Both of these elements are located within 125 bases of the most 3′ site of transcription initiation. The more proximal (3′) promoter exhibits activity in lymphoid as well as nonlymphoid cells. In contrast, the more distal (5′) promoter element functions in a tissue-restricted fashion. The tissue-specific promoter is localized to a 29-base fragment. The sequence of this region is remarkable for a stretch of 11 consecutive purines that are required for activity. This element constitutes the only known tissue-specific promoter for an invariant TCR subunit. Consistent with the unique role served by the ζ subunit in assembly of the TCR, this study demonstrates that the expression of the ζ gene is regulated in a fashion distinct from other TCR components.

Recognition of foreign antigens by T cells is dependent on the expression of the multi-subunit TCR. On most cells, the antigen recognition component of this receptor consists of clonally derived TCR-α/β heterodimers (for a review see reference 1). These exist in stable association with a set of invariant subunits that include the three related members of the CD3 complex and the ζ subunit (for a review see reference 2). In mature T cells, ζ is synthesized in limiting amounts, and largely regulates the assembly of complete receptors and their steady state cell surface expression (3, 4). Partial receptors, lacking ζ, assemble and traverse the Golgi apparatus, but are largely targeted for degradation in lysosomes (5). The importance of the regulated transcription of ζ in T cell development has been established by the findings that ζ-deficient mice exhibit profound defects in T cell development (6), whereas mice which might overexpress this subunit demonstrate premature inactivation of recombinant activating genes (RAG) 1 and 2 accompanied by abnormal T cell development (7). ζ is unique among the TCR components in being expressed in human NK cells where it assembles with the Fc receptor for IgG (8, 9). It is interesting to note that tumor-bearing mice with impaired immune function exhibit a selective loss of the ζ subunit, with its apparent replacement by the related Fcϵ-γ subunit (10). A similar loss of ζ is seen in tumor-infiltrating T cells from patients with malignancies (11, 12).

The TCR genes are in general characterized by weak, tissue nonspecific, nonclassical promoters, which exhibit little activity in the absence of 5′ or 3′ enhancers (13–17). The ζ gene also lacks classical promoter elements (18, 19) and has multiple transcription start sites (18, 19). The analysis of ζ gene expression has been limited to the finding of a correlation between methylation patterns and ζ mRNA levels during murine thymocyte development (20). We have evaluated the 5′ region of the human ζ gene for elements that are responsible for its regulated and tissue-specific expression.

Materials and Methods

Plasmids. Promoter constructs were generated by cloning fragments from a human genomic clone, HGZ7 (19), into the promoterless and enhancerless vector pGL-2 Basic (Promega, Madison, WI), upstream of the luciferase gene. Constructs with 3′ ends at −32 (relative to the most 3′ site of transcription initiation) were generated by the use of naturally occurring restriction sites and subcloning through pGem 3Zf+ (Promega) before insertion into the polylinker of pGL-2 Basic from KpnI to HindIII. Constructs with 3′ ends at +58 were generated by PCR amplification using genomic constructs as templates (19), products were cloned into pGL-2 Basic from XhoI to HindIII. A construct that contained a fragment from −485 to +58 was generated by ligating the KpnI/PstI fragment from a vector containing −485/−32 into a vector already containing −158/+58 after cutting with KpnI and PstI. Fragments from −307 to −207, −257 to −168, −207 to −109, −169 to −59, and −109 to −9 were generated by PCR and cloned into pGL-2 basic from SacI to XhoI. Constructs containing fragments from −159 to −97, −159 to −126, −140 to −110, and −125 to −97 were generated by annealing overlapping double-stranded oligonucleotides with XhoI compatible ends and cloning into pGL-2 basic.

To generate a 22-bp mutation from −119 to −98 in −307/+58

(−307/+58.Δ22) a sense oligonucleotide (GTGAACGATCTTAAGCTAGCTAGCATCCTTGGCCCTGTCGGCAGGTG), containing the mutation, and a 3′ antisense oligonucleotide (TATATAAGCTTTCCCTCAGAAAGAGGCTGG) were utilized in the PCR with −307/+58 as a template. Similarly, an antisense oligonucleotide containing the 22-base substitution (GCTAGCTAGCTTAAGATCGTTCACAGGCAGGAAGCCCTG) and a 5′ sense oligonucleotide (TATATCTCGAGCCATCGAGAACTTGTATTTGCC) were utilized for amplification. The products of these amplifications were reamplified with the 5′ sense and 3′ antisense oligonucleotides (21). The resulting fragment was cloned into pGL-2 Basic. Site-directed mutagenesis was performed using the Transformer Mutagenesis Kit (Clontech, Palo Alto, CA). CMV-β-Gal was provided by Vinay Jain and Ian Magrath (National Institutes of Health).

Transfections and Activity Assays. Luciferase reporter gene constructs were purified by columns (Qiagen Inc., Chatsworth, CA) followed by cesium chloride banding; 15 μg of plasmid was used in all transfection assays. 500 ng of a β-Gal construct containing the CMV promoter was cotransfected in all studies. Transfection of T and B cells was carried out by electroporation of 10^7 cells in 200 μl of complete media (3) with 10 mM Hepes in a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) at 0.23 kV for Jurkat and BJAB, and 0.20 kV for MOLT-4, with a capacitance setting of 960 μF. HeLa cells were transfected by calcium phosphate precipitation (22). After 18–24 h, cells were pelleted and lysed in 50 μl of 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 10% glycerol, and 1% Triton X-100. Postnuclear supernatants were assayed using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The luciferase activity in 10 μl of lysate was determined after addition of 100 μl of luciferase assay reagent (20 mM tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP, pH 7.8). β-Gal activity was determined by incubation of 10 μl of lysate with 167 μl of β-Gal reagent 1 (10 mM $NaHPO_4$, 1 mM $MgCl_2$, and 0.01 mg/ml of a β-Gal substrate, AMPGD [Tropix, Bedford, MA]) after the injection of 100 μl Emerald Enhance (Tropix). Background luciferase or β-Gal activities from mock-transfected controls were first subtracted from the luciferase or β-Gal activities of test constructs. The luciferase activity from a given sample was then normalized to the β-Gal activity of the same sample, multiplied by 1,000 and divided by the normalized luciferase value for pGL-2 Basic.

Results and Discussion

Transcription from the human ζ gene is initiated from multiple start sites, the two most prominent of which are at base +1 and base −61 (19). Sequences from the 5′ region of the ζ gene were cloned upstream of the luciferase reporter gene in the promoterless and enhancerless plasmid pGL2-Basic and analyzed in transient transfection assays in the ζ-expressing human T cell tumor line Jurkat (23). Equivalent luciferase activity was found with fragments that extended from −1471 to −32 (−1471/−32) and from −485 to −32 (−485/−32) (Fig. 1 *A*). A third construct, −685/−32, had comparable activity (data not shown). A fragment that extended from −485 to +58 (−485/+58) was more active than −485/−32, indicating that sequences around the 3′ transcription start site are required for full promoter activity. To further delineate the minimum requirements for full activity, other constructs with 3′ end points at +58 were evaluated. A −307/+58 fragment had activity equivalent to −485/+58. Additional 5′ deletions to −159 and to −69 resulted in stepwise decreases in activity relative to the fully active −307/+58 construct, with −69/+58 having ∼25% of maximal activity. These findings suggest that there are multiple elements within the 5′ region of the ζ gene between −307 and +58 that contribute to transcriptional activity. They also establish the ex-

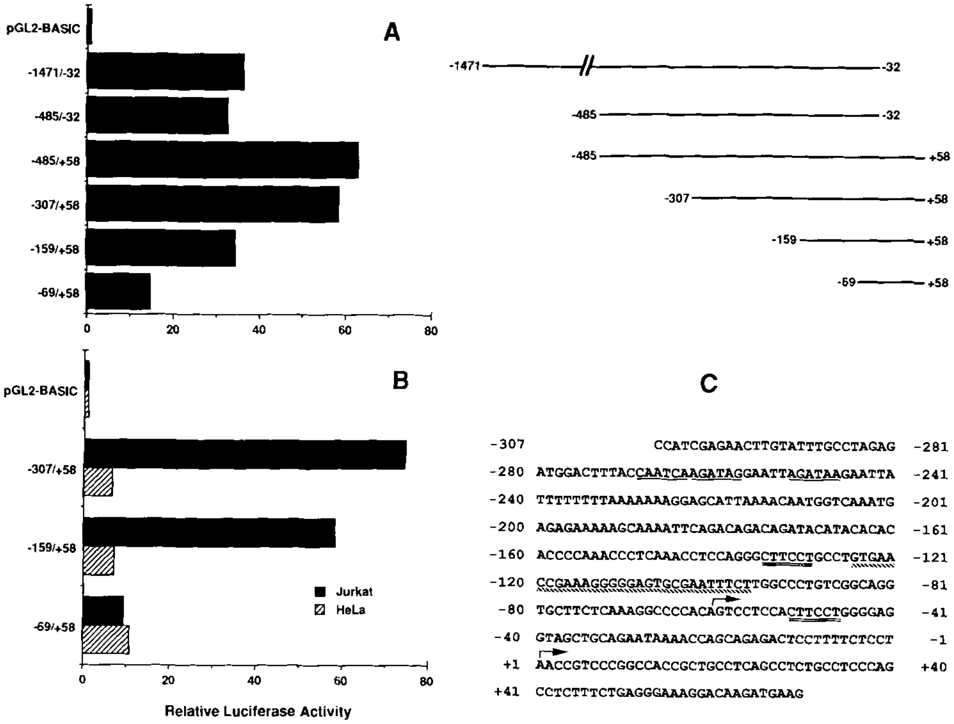

Figure 1. Deletional analysis of the ζ 5′ regulatory region. Luciferase activity of the indicated fragments of the ζ gene cloned in front of the luciferase reporter gene are shown normalized to a vector containing the luciferase gene alone (*pGL-2 BASIC*). (*A*) Jurkat cells, (*B*) Jurkat and HeLa, and (*C*) nucleotide sequence of the region from −307 to +58. (*Arrows*) The major transcription start sites. Three GATA-3 sites at −269, −263, and −252 are underlined, two minus strand Ets consensus sequences at −135 and −52 are underlined twice. (∖∖∖∖∖∖∖) The region termed Pz2 (see text) is indicated. These sequence data are available from EMBL/GenBank/DDBJ under accession number U 14115.

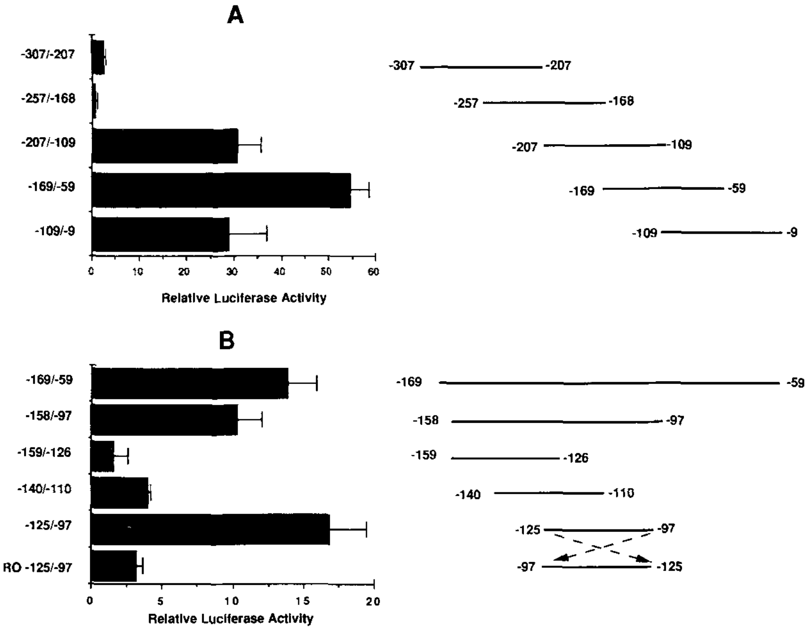

Figure 2. Localization of an upstream promoter in Jurkat cells. (*A*) Comparison of five overlapping fragments spanning the region from −307 to −9 (indicated schematically to the left of the graph). (*B*) Evaluation of multiple fragments contained within the −169/−59 fragment. Error bars indicate standard deviation of the mean for triplicates of a single experiment.

istence of at least one element in the region from −69 to +58 that can function as a core promoter. Because of this, the −69/+58 region shall be referred to as promoter of ζ #1 (Pz1).

The activity of constructs were next compared in Jurkat and HeLa (24) cells. Pz1 (−69/+58) was active in both cell types (Fig. 1 *B*). Unlike Jurkat, no increase was found in HeLa with constructs that extended to −159 and to −307. This tissue-specific increase in activity in the 5′ region of the ζ gene constitutes the only example of tissue-restricted activity in the 5′ region of a gene for an invariant TCR subunit. The nucleotide sequence of the region implicated in regulating transcription from the ζ gene is shown in Fig. 1 *C*. Of note are binding sites for the T cell–specific transcription factor GATA-3 (17, 25, 26), and for consensus sequences for members of the Ets family of transcription factors (27, 28).

To ascertain whether there are elements other than Pz1 that function as promoters, five overlapping fragments of ∼100 bp spanning the region from −307 to −9 were cloned into pGL2-Basic (Fig. 2 *A*). The two most 5′ fragments (−307/−207 and −257/−168), which include the GATA-3 consensus sequences, had no significant activity in Jurkat, whereas the three more 3′ fragments (−207/−109, −169/−59, and −109/−9) were all active. Only the most 3′ fragment (−109/−9), which overlaps Pz1, was active in HeLa (data not shown). The −169/−59 fragment consistently had greater activity than the overlapping −207/−109 fragment. One interpretation of this data is that a single tissue-specific promoter element in the overlap between −207/−109 and −169/−59 is responsible for activity from both fragments, with bases unique to −169/−59 required for full activity. To test this, −158/−97 was generated and found to have activity comparable to −169/−59 (Fig. 2 *B*). Further characterization was carried out by the generation of three plasmids containing smaller overlapping fragments spanning −158 to −97 (Fig. 2 *B*). The most 5′ of these (−159/−126) had little activity over pGL2-Basic, the middle one (−140/−110) consistently had low but detectable activity, whereas the most 3′ fragment (−125/−97) demonstrated activity comparable to the larger −169/−59 and −158/−97 constructs. The activity of −125/−97 decreased by >80% when its orientation was reversed (*RO−125/−97*). These findings establish −125/−97 as a second core promoter element for ζ (Pz2).

To determine whether Pz2 is a tissue-specific promoter, its activity relative to Pz1 and to −169/−59 was assessed in HeLa, Jurkat, another ζ-expressing T cell (MOLT-4 [29]), and a human B cell lymphoma (BJAB [30]) (Fig. 3). Whereas the activity of the downstream promoter (Pz1) was within twofold for all cell lines, Pz2 was 8–10-fold more active in

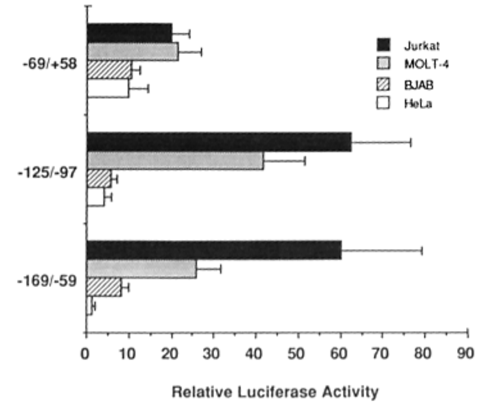

Figure 3. Tissue-specific expression from the 5′ region of the ζ gene. The relative luciferase activity from Pz1 (−69/+58), Pz2 (−125/−97), and −169/−59 are compared. This figure represents the cumulative results of multiple experiments using these four cell lines. Shown is the mean ± the SE of the mean. The number of points used for each cell type ranged from 8 to 13.

T cells than the non-T cells, and as already noted, the larger fragment, −169/−59, also demonstrated a tissue-restricted pattern of activity.

To evaluate the transcriptional activity of 5′ regions of the ζ gene, ζ promoter constructs were compared with a plasmid in which the expression of the luciferase gene was driven by the SV40 early promoter. The −307/+58 fragment and Pz2 (−125/−97) had activities of 70 and 30%, respectively, relative to the SV40 construct, with −169/−59 being similar in activity to Pz2 (data not shown). This demonstrates that the 29-bp Pz2 region has substantial activity. To determine its functional relevance in the context of the fully active 5′ region, a construct was generated in which the 22 bases from −119 to −98 of −307/+58 were mutated (−307/+58.Δ22; Fig. 4 *A*). Consistent with a role for this region in transcription from the ζ gene, this substitution resulted in a 65% decrease in activity when assayed in Jurkat. It is of note however, that −307/+58.Δ22 was still more active than the Pz1 element, suggesting that regions other than Pz1 and Pz2 influence transcription from the ζ gene.

The sequence of the Pz2 region is remarkable for a core of 11 consecutive purines (bases 118–108). To assess the requirements for activity from Pz2, a systematic mutagenesis of sets of three consecutive bases within Pz2 was undertaken (Fig. 4, *B* and *C*). Changes in the 11-base purine stretch resulted in marked reductions in activity (constructs *M2–M4*, Fig. 4 *B*). In contrast, mutations at either the 5′ (*M1*) or 3′ (*M6*) end of Pz2 had no substantial effect on activity (Fig. 4 *C*). Alteration of the nucleotide sequence immediately 3′ to the purine stretch (M5) reproducibly resulted in a decrease in activity of 40–70%. It is apparent from this analysis that this purine stretch is important for the promoter activity of Pz2. It is interesting to note that the 5′ region of the mouse ζ gene contains a region that is highly homologous to Pz2, with 11 consecutive purines, 10 of which are identical to those in Pz2 (Hsu, V. and R. Klausner, personal communication). Gel mobility retardation assays for the Pz2 region have been carried out using T cell extracts, and specific bands identified which comigrated with bands from extracts of HeLa cells and B cells (data not shown). This may be indicative of the complexity of interactions involved in regulating transcription from this region.

The requirements for transcription from classical, TATA-containing, promoters has been extensively analyzed, and it is clear that a complex array of factors are involved in the initiation of transcription (31, 32). Less is known about nonclassical TATA-less promoters. Recent studies (33–36) suggest that transcription factors that bind to enhancers might also function to facilitate transcription initiation, and may be involved in the tissue-restricted activity of some TATA-less promoter elements (37). The *trans*-acting factors responsible for transcription for Pz2 remain to be determined. Potential candidates include proteins that bind to purine-rich regions of DNA such as NFAT (38, 39), Ikaros (40, 41), and members of the Ets family of transcription factors (27, 42, 43). All of these are known to be involved in the regulation of lymphoid genes.

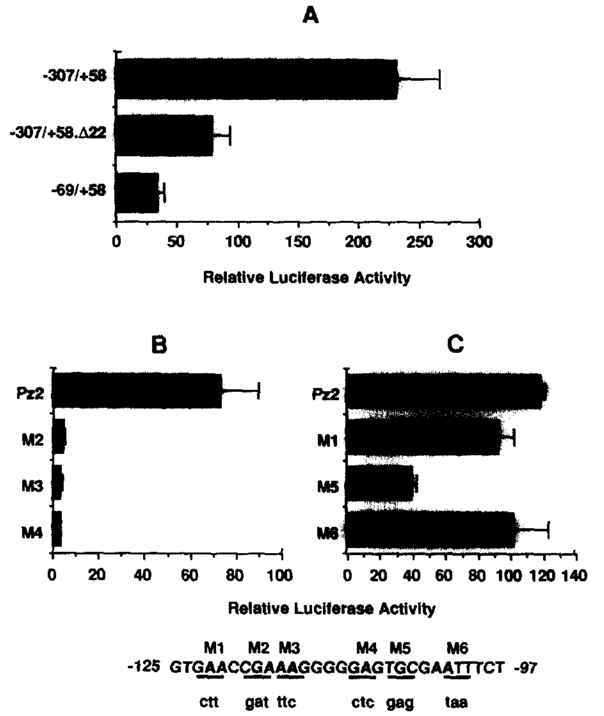

Figure 4. Analysis of the Pz2 promoter. (*A*) Evaluation of a 22-base substitution of bases −119 to −98 in the −307/+58 construct. (*B* and *C*) Relative luciferase activity of each of six mutations of the Pz2 promoter. The position of each of the mutations is indicated above the Pz2 sequence and the actual mutations are indicated below. Error bars indicate ± SD from triplicate samples. The data in each panel are from a single representative experiment.

The genes encoding the clonally derived TCR subunits and the invariant CD3 complex members are generally characterized by nontissue-specific core promoters with minimal activity, and by enhancers that play a dominant role in determining the specificity and extent of transcription (13–17). Consistent with the limiting role of ζ in receptor assembly, and its unique expression pattern, ζ is regulated in a distinct fashion. The identification of Pz2 constitutes the only example of a tissue-specific promoter for an invariant TCR subunit. Our analysis also demonstrates that, in addition to Pz1 and Pz2, there are other elements in the 5′ region of the ζ gene that have positive effects on transcription. Furthermore, the isolated 5′ region of the ζ gene (−307/+58) has some activity in nonlymphoid cells, whereas ζ gene expression is limited to T cells and NK cells. It is apparent therefore, that inhibitory mechanisms are also employed to maintain the tissue-restricted expression of ζ.

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