MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5486–5495
0270-7306/90/105486-10$02.00/0

Vol. 10, No. 10

Identification and Functional Characterization of the Human T-Cell Receptor β Gene Transcriptional Enhancer: Common Nuclear Proteins Interact with the Transcriptional Regulatory Elements of the T-Cell Receptor α and β Genes

LISA R. GOTTSCHALK AND JEFFREY M. LEIDEN*

Howard Hughes Medical Institute and Departments of Internal Medicine and Microbiology/Immunology, University of Michigan Medical Center, 1150 West Medical Center Drive, MSRB I, Room 4510, Ann Arbor, Michigan 48109-0650

Received 3 May 1990/Accepted 28 June 1990

A transcriptional enhancer has been mapped to a region 5.5 kilobases 3′ of the Cβ2 gene in the human T-cell receptor (TCR) β-chain locus. Transient transfections allowed localization of enhancer activity to a 480-base-pair *Hin*cII-*Xba*I restriction enzyme fragment. The TCR β enhancer was active on both the minimal simian virus 40 promoter and a TCR β variable gene promoter in both TCR α/β^+ and TCR γ/δ^+ T cells. It displayed significantly less activity in Epstein-Barr virus-transformed B cells and K562 chronic myelogenous leukemia cells and no activity in HeLa fibroblasts. DNA sequence analysis revealed that the enhancer contains a consensus immunoglobulin κE2 motif, as well as an AP-1-binding site and a cyclic AMP response element. DNase I footprint analyses using Jurkat T-cell nuclear extracts allowed the identification of five nuclear protein-binding sites, Tβ1 to Tβ5, within the enhancer element. Deletion and in vitro mutagenesis studies demonstrated that the Tβ2- Tβ3- and Tβ4-binding sites are each required for full transcriptional enhancer activity. In contrast, deletion of the Tβ1- and Tβ5-binding sites had essentially no effect on enhancer function. Electrophoretic mobility shift assays demonstrated that TCR α/β^+ and TCR γ/δ^+ T cells expressed Tβ2-, Tβ3-, and Tβ4-binding activities. In contrast, non-T-cell lines, in which the enhancer was inactive, each lacked expression of at least one of these binding activities. TCR α and β gene expression may be regulated by a common set of T-cell nuclear proteins in that the Tβ2 element binds a set of cyclic AMP response element-binding proteins that are also bound by the Tα1 element of the human TCR α enhancer and the decamer element present in a large number of human and murine TCR β promoters. Similarly, the Tβ5 TCR β-enhancer element and the Tα2 TCR α-enhancer element bind at least one common T-cell nuclear protein. Taken together, these results suggest that TCR β gene expression is regulated by the interaction of multiple T cell nuclear proteins with a transcriptional enhancer element located 3′ of the Cβ2 gene and that some of these proteins may be involved in the coordinate regulation of TCR α and β gene expression.

The majority of cytotoxic and helper T cells express a heterodimeric α/β T-cell receptor (TCR) that binds to antigenic peptides associated with major histocompatibility complex molecules on the surface of antigen presenting cells (for a review, see references 8 and 23). The distinct heterodimeric γ/δ TCR is expressed on a minority of circulating human T cells of unknown function (4, 5, 14, 38). The four TCR genes are similar to immunoglobulin genes in that each is composed of multiple germ line variable (V), diversity (D) (in the case of TCR β and δ genes), and joining (J) region gene segments that undergo rearrangement during T-cell ontogeny to form the mature TCR genes (for a review, see references 8 and 40). Previous studies have demonstrated that the rearrangement and expression of the murine TCR genes are highly regulated during thymic development (32, 37). Thus, the TCR γ and δ genes are rearranged and expressed relatively early (fetal days 14 to 16), followed by TCR β (fetal day 16) and, finally, TCR α gene rearrangement and expression (fetal day 17). Moreover, although B cells and T cells derive from common bone marrow precursors, the expression of immunoglobulin and TCR genes is generally lineage specific, i.e., most T cells rearrange and express TCR but not immunoglobulin genes. Finally, prior studies have suggested that a single recombinase activity is responsible for both immunoglobulin and TCR gene rearrangements and that, at least in the case of immunoglobulin genes, rearrangement is correlated with, and probably regulated by, transcription of the unrearranged immunoglobulin loci (42). Thus, a better understanding of the mechanisms regulating immunoglobulin and TCR transcription might be expected to lead to fundamental insights regarding the lineage-specific rearrangement and expression of B- and T-cell antigen receptor genes.

While a great deal is known about the structure of the four TCR genes, relatively little is known about the mechanisms that regulate the rearrangement and expression of these genes in the different T-cell subsets. Recent studies have described transcriptional enhancers in the human and murine TCR α loci (15, 41). The human TCR α enhancer, which is located 4.5 kilobases (kb) 3′ of the Cα gene, has been shown to be necessary for transcription from a Vα promoter in Jurkat T cells and to be active only in TCR α/β^+ T cells (15). Two previous studies also have identified a transcriptional enhancer element located 5 to 7.5 kb 3′ of the Cβ2 gene in the murine TCR β locus. Krimpenfort et al. (17) have reported that this enhancer displayed high-level activity in both B and T lymphocytes, while McDougall et al. (25) have suggested that the enhancer is T cell specific and relatively

* Corresponding author.

weak compared with the simian virus 40 (SV40) enhancer. Ratanavongsiri et al. (31) have suggested recently that this enhancer also displays activity in nonlymphoid cells. Thus, the cellular specificity and potency of the murine TCR β enhancer remain controversial. Moreover, a human TCR β enhancer has not yet been identified, and the molecular mechanisms responsible for regulating the activity of the TCR β enhancer have not yet been elucidated.

In this report, we have identified and characterized a human TCR β transcriptional enhancer located 5.5 kb 3′ of the Cβ2 gene. This enhancer, in combination with a TCR β promoter, displayed T-cell-specific activity and was shown to contain five nuclear protein-binding sites. Deletion and mutation analyses defined a core enhancer that is composed of three nuclear protein-binding sites, each of which is necessary for full enhancer activity. These three sites bind a set of nuclear proteins found in all the TCR α/β^+ and TCR γ/δ^+ T cells examined. In contrast, nonlymphoid cells, in which the enhancer was inactive, each lacked at least one of these DNA-binding activities. DNA sequence analyses and electrophoretic mobility shift assays (EMSAs) demonstrated that the TCR β enhancer contains binding sites for several nuclear proteins that also interact with the TCR β promoter and the TCR α enhancer and that may, therefore, be involved in coordinately regulating the expression of the TCR α and β genes.

MATERIALS AND METHODS

Cells and media. Human Jurkat TCR α/β^+ T cells, Molt4 TCR β^+ cells, PEER and Molt13 TCR γ/δ^+ T cells, Clone 13 Epstein-Barr-transformed B cells, and K562 chronic myelogenous leukemia cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) and 10% fetal calf serum (GIBCO) supplemented with 1% penicillin-streptomycin (GIBCO). Human HeLa cells were grown in αMEM (GIBCO) medium supplemented as described above.

Isolation of genomic clones and plasmid construction. A genomic clone containing the Cβ2 gene segment and 3′ flanking sequences was isolated from a human genomic library (12) by hybridization to a Cβ2 probe prepared from the previously described 12A1 human TCR β cDNA clone (19). A genomic clone containing a TCR Vβ promoter was isolated from a human peripheral blood genomic library (generously provided by John Lowe) by hybridization to a Vβ probe prepared from the previously described L17 human TCR β cDNA clone (20). Restriction enzyme mapping of these clones was performed by using standard techniques (22).

The plasmids pSPCAT (21) and pSV0CAT (11) have been described previously. A 480-base-pair (bp) *Hin*cII-*Xba*I fragment containing the human TCR β enhancer was subcloned in both orientations into the *Sma*I site 5′ of the minimal SV40 promoter and chloramphenicol acetyltransferase (CAT) gene in pSPCAT to produce the pSPCAT480 and pSPCATR480 plasmids. The pVβCAT and pRVβCAT plasmids were constructed by cloning a 1.7-kb *Nco*I fragment containing the L17 human Vβ promoter into the *Hin*dIII site of the pSV0CAT plasmid in 5′ to 3′ and 3′ to 5′ orientations with respect to the CAT gene. The same 480-bp *Hin*cII-*Xba*I TCR β enhancer fragment was cloned into the *Bam*HI site 3′ of the CAT gene in pVβCAT and pRVβCAT to produce the pVβCAT480 and pRVβCAT480 plasmids, respectively.

Transfections. Human Jurkat, Clone 13, K562, and HeLa cells were transfected with 10 μg of cesium chloride-purified DNA per 10^7 cells by using a modification of the DEAE-dextran method, as described previously (12). HeLa cells were also transfected by using a modification of the calcium-phosphate method, as described previously (30). PEER cells were transfected with 10 μg of DNA by electroporation (6) as described by Ho et al. (15) by using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 270 V and 960 μF. In order to control for differences in transfection efficiencies, all transfections contained 1.5 to 2 μg of the pRSVGH (36) or pRSVβgal (15) reference plasmids. All transfections were repeated at least three times.

CAT, β-galactosidase, and growth hormone assays. Cells were harvested 48 h after transfection by four cycles of freeze-thaw lysis, and the protein concentrations in cell extracts were determined by using a commercially available kit (Bio-Rad). CAT and β-galactosidase assays were performed as previously described (15). Growth hormone levels were determined from the same cell extracts as those used in the CAT assays by using a commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, Calif.).

DNA sequencing. DNA sequencing was performed on double-stranded DNA templates by the dideoxynucleotide chain termination protocol of Sanger et al. (35).

DNase I footprint analysis. Subfragments of the 480-bp *Hin*cII-*Xba*I fragment were end labeled with α-^{32}P-labeled deoxynucleotides by using the Klenow fragment of DNA polymerase I and purified by polyacrylamide gel electrophoresis. DNase I footprint analysis was performed as previously described (16), except 30 μg of Jurkat T-cell nuclear extract was used. Reaction products were fractionated on standard 8% sequencing gels. Standard Maxam and Gilbert (24) purine-sequencing reactions were run in parallel in order to identify protected sequences.

EMSAs. The following complementary oligonucleotides corresponding to the Tβ2 to Tβ5 and Tα1 nuclear protein-binding sites and to the decamer-containing Vβ promoter (VβP) sequence were synthesized with *Bam*HI and *Bgl*II overhanging ends on an Applied Biosystems model 380B DNA synthesizer.

Tβ2	CTGTTTATCTCTGAGTCACATCAGCACCAAGCCA
Tβ3	CAACAGGATGTGGTTTGACA
Tβ4	CCACCTTCAGAGCATCATGAGAACCACACTCACCGCATCCGGCACCCAA
Tβ5	AGAACTTCAGAGGGGAGGGG
Tα1	CTCCCATTTCCATGACGTCATGGTTACCA
VβP	TGAGGCTCAGTGATGTCACTGTGGGA

EMSAs were performed as previously described (16), except binding reactions using the Tβ3 oligonucleotide were performed in binding buffer with 10 mM NaCl. For cold competition experiments, 10 to 100 ng of unlabeled competitor oligonucleotide was included in the binding reactions.

Preparation of deleted and mutated TCR β enhancer elements. A TCR β-enhancer fragment containing the Tβ3 mutation was produced by site-directed oligonucleotide-mediated gapped heteroduplex mutagenesis as previously described (16) by using the following synthetic oligonucleotide: CTCTTACAGTCACACCAAGATCTTGGTTCACAT TTACTGGGTCC. A deletion mutant spanning bp 53 to 446 of the 480-bp *Hin*cII-*Xba*I enhancer fragment and lacking the Tβ1- and Tβ5-binding sites was prepared by the polymerase chain reaction (PCR) as previously described (28) by using the following synthetic oligonucleotide primers.

5′ primer	CCCGGATCCGGGCCATGAATGACAAGAAAATTGGTG
3′ primer	CCCGGATCCCTGCCAGGATGCTAACCAGGGGCCCAG

A second deletion mutant containing the Tβ2- to Tβ4-binding sites (bp 192 to 407) was prepared by PCR with the following primers:

5′ primer CCCGGATCCGGCTGCCGGGCTGTTTATCTCTGAGTC
3′ primer CCCGGATCCGCATAGGAGGGGGTTGGGTGCCGGATG

Mutations were introduced into the Tβ2-binding site by PCR using the following primer:

5′ primer CCCGGATCCCTGCCGGGCTCATTTACACTGTGTCTCATCAGCACC

A deletion mutant lacking 8 bp from the 3′ end of the Tβ4-binding site (i.e., encompassing bp 198 to 385) was prepared by digestion of the 483-bp *Hin*cII-*Xba*I enhancer fragment with *Hpa*II. Finally, a deletion mutant lacking the Tβ2-binding site (i.e., encompassing bp 237 to 407) was prepared by PCR using the following primer:

5′ primer CCCGGATCCAGCCACCTGCCCTAGCTCCATCTCTTAC

All deletions and mutations were confirmed by dideoxy-DNA sequence analyses.

RESULTS

A transcriptional enhancer 3′ of Cβ2 in the human TCR β locus. In order to identify transcriptional regulatory elements in the human TCR β locus, we tested a series of overlapping restriction enzyme fragments spanning 10 kb 3′ of the human Cβ2 gene segment for transcriptional enhancer activity. These fragments were cloned into the *Sma*I site 5′ of the minimal SV40 promoter and the bacterial CAT reporter gene in the pSPCAT plasmid (21) and transfected into human TCR α/β^+ Jurkat T cells. A 1.7-kb *Hin*dIII-*Bam*HI fragment located 4.4 kb 3′ of the Cβ2 gene segment was shown to increase transcription from the minimal SV40 promoter by eightfold in Jurkat cells (Fig. 1A). By using a similar transfection approach, the enhancer was further localized to a 480-bp *Hin*cII-*Xba*I fragment that was able to increase transcription from the minimal SV40 promoter by 20- to 40-fold when cloned in 3′ to 5′ and 5′ to 3′ orientations with respect to the SV40 promoter (Fig. 1A and B). This same 480-bp fragment also increased transcription 30-fold when cloned into the *Bam*HI site 3′ of the CAT gene in pSPCAT (data not shown). These results demonstrated that this fragment contains a transcriptional enhancer that functions in an orientation- and position-independent fashion in Jurkat T cells.

Promoter and cellular specificity of the TCR β enhancer. To test the effects of this transcriptional enhancer on a TCR β variable gene (Vβ) promoter, the 480-bp *Hin*cII-*Xba*I enhancer fragment was cloned into the *Bam*HI site of the pVβCAT plasmid in which CAT transcription is under the control of a TCR Vβ promoter (Fig. 1C). Interestingly, the Vβ promoter alone (pVβCAT) displayed little or no activity following transfection into Jurkat cells. The addition of the TCR β enhancer (pVβCAT480) resulted in a 79-fold increase in CAT activity. In contrast, the enhancer was unable to increase CAT activity when cloned into a control plasmid containing the Vβ promoter in a 3′ to 5′ orientation with respect to the CAT gene (pRVβCAT480) (Fig. 1C).

To characterize the cellular specificity of the TCR β enhancer, we compared the CAT activities produced by transfection of the enhancer-containing pSPCAT480 and pVβCAT480 plasmids with those produced by the pSPCAT and pVβCAT control plasmids following transfection into TCR γ/δ^+ PEER T cells, Epstein-Barr virus-transformed Clone 13 B cells, K562 chronic myelogenous leukemia cells, and HeLa fibroblasts. The TCR β enhancer significantly increased CAT activity from both promoters (30- to 80-fold) in TCR α/β^+ Jurkat and TCR γ/δ^+ PEER T cells (Fig. 2A and B). In contrast, the TCR β enhancer was unable to significantly increase CAT activity from either the minimal SV40 or the Vβ promoters in HeLa cells (Fig. 2A and B). It displayed low-level activity in Clone 13 B cells and in K562 cells, increasing CAT activity by 5- to 11-fold (Fig. 2A and B). The low levels of TCR β-enhancer activity observed in HeLa, Clone 13, and K562 cells were not due to differences in transfection efficiencies, because a control plasmid con-

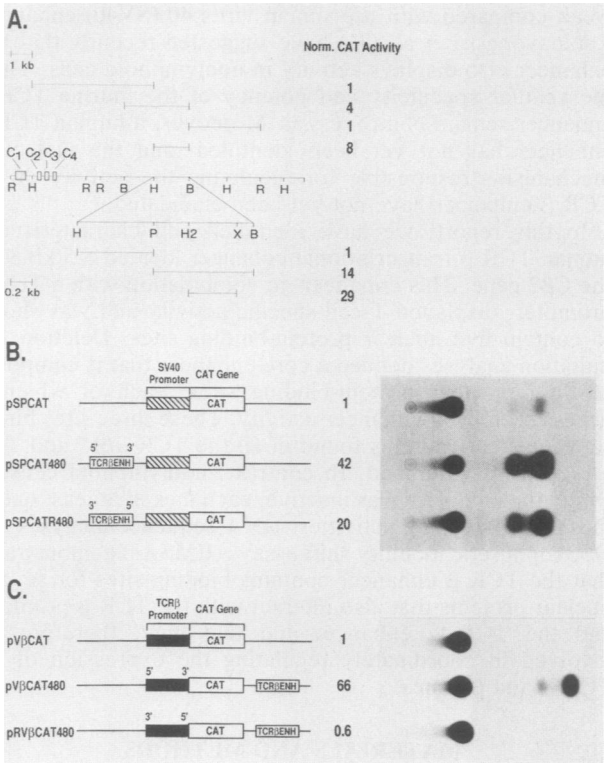

FIG. 1. Identification and localization of a transcriptional enhancer element 3′ of the human Cβ2 gene segment. (A) A partial restriction enzyme map of a human TCR β genomic clone. *Eco*RI (R), *Hin*dIII (H), *Bam*HI (B), *Pvu*II (P), *Hin*cII (H2), and *Xba*I (X) restriction endonuclease sites are shown. The four exons of the Cβ2 gene segment are depicted as open squares (C1 to C4). Overlapping restriction enzyme fragments from this clone (shown by bars) were subcloned into the *Sma*I site 5′ of the minimal SV40 promoter and CAT gene in pSPCAT (21) and tested for transcriptional enhancer activity following transfection into Jurkat T cells. Normalized CAT activity is shown at right and represents the CAT activity produced by each plasmid normalized to the CAT activity produced by the control plasmid, pSPCAT, which produced 0.4 to 1.9% acetylation in multiple transfections. (B and C) Activity of the TCR β enhancer on the minimal SV40 promoter and a TCR Vβ promoter in Jurkat T cells. The control plasmids, pSPCAT and pVβCAT, and the TCR β-enhancer-containing plasmids, pSPCAT480, pSPCATR480, pVβ CAT480, and pRVβCAT480, are shown schematically at the left. A total of 10 μg of each plasmid plus 2 μg of the pRSVGH or pRSVβgal reference plasmid was transfected into Jurkat T cells, and CAT activities and growth hormone levels or β-galactosidase activities were determined as described in Materials and Methods. The CAT activities of pSPCAT480 and pSPCATR480 corrected for transfection efficiencies and normalized to the activity produced by pSPCAT (percent acetylation, 1.7%) are shown as Norm. CAT Activity in panel B. The CAT activities of pVβCAT480 and pRVβCAT480 corrected for transfection efficiencies and normalized to that produced by the control plasmid, pVβCAT (percent acetylation, 0.08%), are shown in panel C. All transfections were repeated at least three times in separate experiments.

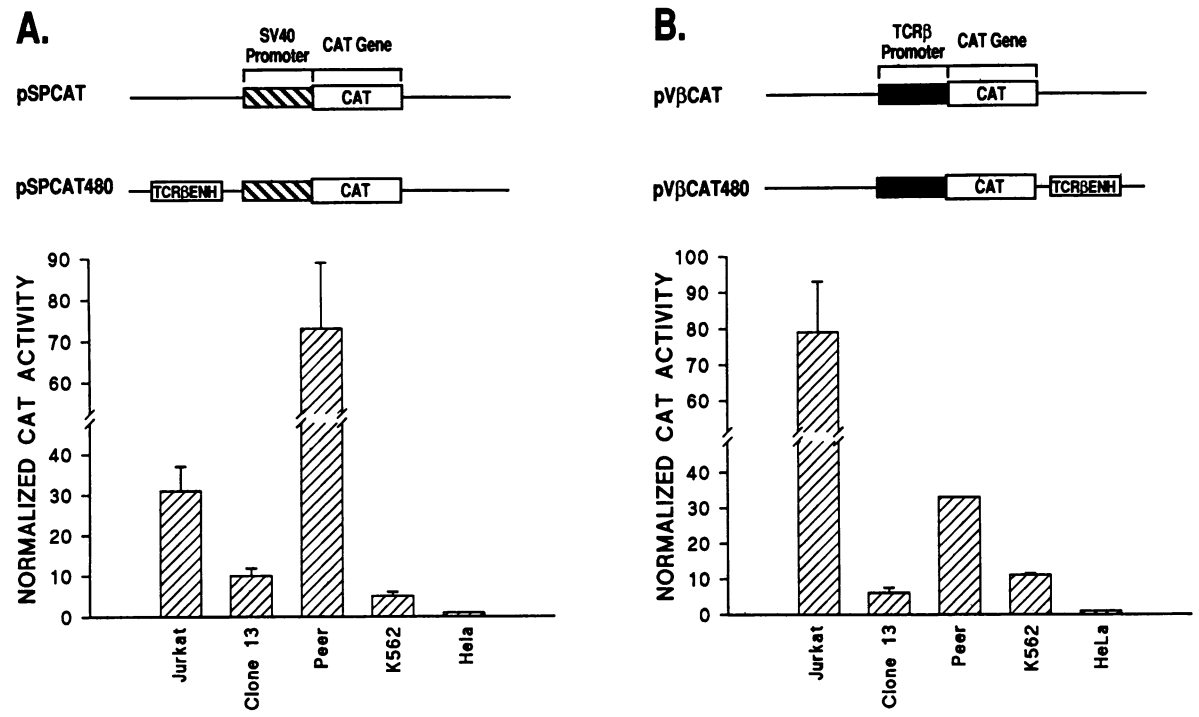

FIG. 2. Activity of the TCR β enhancer in different human cell lines. Ten micrograms of the pSPCAT or pSPCAT480 and pVβCAT or pVβCAT480 plasmids was transfected into TCR α/β^+ Jurkat T cells, Epstein-Barr virus-transformed Clone 13 B cells, TCR γ/δ^+ PEER T cells, K562 chronic myelogenous leukemia cells, and HeLa fibroblasts, and cell extracts were tested for CAT activity. Normalized CAT activity is the activity produced by the pSPCAT480 and pVβCAT480 plasmids normalized to the CAT activity produced by the control plasmids, pSPCAT and pVβCAT, following correction for transfection frequencies by using growth hormone levels or β-galactosidase activities as described in Materials and Methods. The results were confirmed by at least three separate transfections into each cell line.

taining the 4F2 heavy-chain enhancer (16) which was included in each transfection experiment produced high-level CAT activity in all three cell lines (data not shown). In addition, to rule out the possibility that the low level of enhancer activity observed in HeLa cells was due to an artifact of the transfection technique, HeLa cells were transfected by using two different techniques (DEAE-dextran and calcium-phosphate) with identical results. It is also worth noting that the Vβ promoter alone was slightly more active in Clone 13 B cells than in Jurkat T cells (data not shown). Taken together, these results suggest that the Vβ promoter alone is relatively inactive in T cells and, moreover, is unable by itself to confer T-cell specificity on TCR β gene expression. In contrast, the TCR β enhancer is able to confer high-level expression on a Vβ promoter in a relatively T-cell-specific fashion.

DNA sequence analysis of the TCR β enhancer. DNA sequence analysis (Fig. 3) of the 480-bp fragment containing the TCR β enhancer revealed that the enhancer included a consensus binding site for the AP-1 transcription factor (GTGACTCA, bp 213 to 220) (3, 18) in addition to a sequence identical at 10 of 11 bp to the κE2 immunoglobulin enhancer motif (GGCAGGTGGCT, bp 240 to 251) (7, 27) and a variant of the cyclic AMP response element (CRE) (TCACATCA, bp 217 to 225) (26). A comparison of the TCR β enhancer and the human TCR α and δ enhancers allowed the identification of a TCR β-enhancer sequence identical at 12 of 14 bp to the Tα2 nuclear protein-binding site of the TCR α transcriptional enhancer (CCCTCTGAAGTTCT, bp 452 to 466) (15) and at 8 of 9 bp to the δE7 nuclear protein-binding site of the human TCR δ enhancer (CCCTC TGAA, bp 457 to 466) (33). Finally, a comparison of the human TCR β enhancer and the murine TCR β enhancer (17) revealed a high degree (67%) of sequence identity.

Human TCR β enhancer contains multiple nuclear protein-binding sites. A variety of cellular and viral transcriptional enhancers have been shown to function by binding nuclear transcriptional regulatory proteins (for a review, see reference 29). To determine whether the TCR β enhancer contains nuclear protein-binding sites, the 480-bp *Hin*cII-*Xba*I enhancer fragment was subjected to DNase I footprint analyses using Jurkat nuclear extracts. Five nuclear protein-binding sites, Tβ1 to Tβ5, were identified (Fig. 3). Also, two additional sites (bp 59 to 68 and bp 98 to 108) were variably footprinted by Jurkat nuclear proteins. The Tβ2-binding site contained the AP-1 enhancer motif and the variant CRE, while the Tβ5-binding site was similar (12 of 14 bp) to the previously described Tα2 and δE7 nuclear protein-binding sites from the TCR α and δ gene transcriptional enhancers (15, 33). In contrast, the Tβ1-, Tβ3-, and Tβ4-binding sites did not correspond to previously identified enhancer motifs.

Deletion and mutation analyses of the TCR β enhancer. In order to determine the importance of each of the TCR β-enhancer nuclear protein-binding sites, a series of mutant TCR β-enhancer fragments were cloned into the *Bam*HI site of pVβCAT (Fig. 4B and C, left) and tested for transcriptional enhancer activity following transfection into Jurkat T cells. In an initial set of experiments, we demonstrated that deletion of both the Tβ1 and Tβ5 nuclear protein-binding sites had essentially no effect on TCR β transcriptional enhancer activity (Fig. 4B). Thus, these experiments defined the core TCR β enhancer as a 393-bp fragment (bp 53 to 406) that contained the Tβ2, Tβ3, and Tβ4 nuclear protein-binding sites. Interestingly, the murine and human Tβ2, Tβ3,

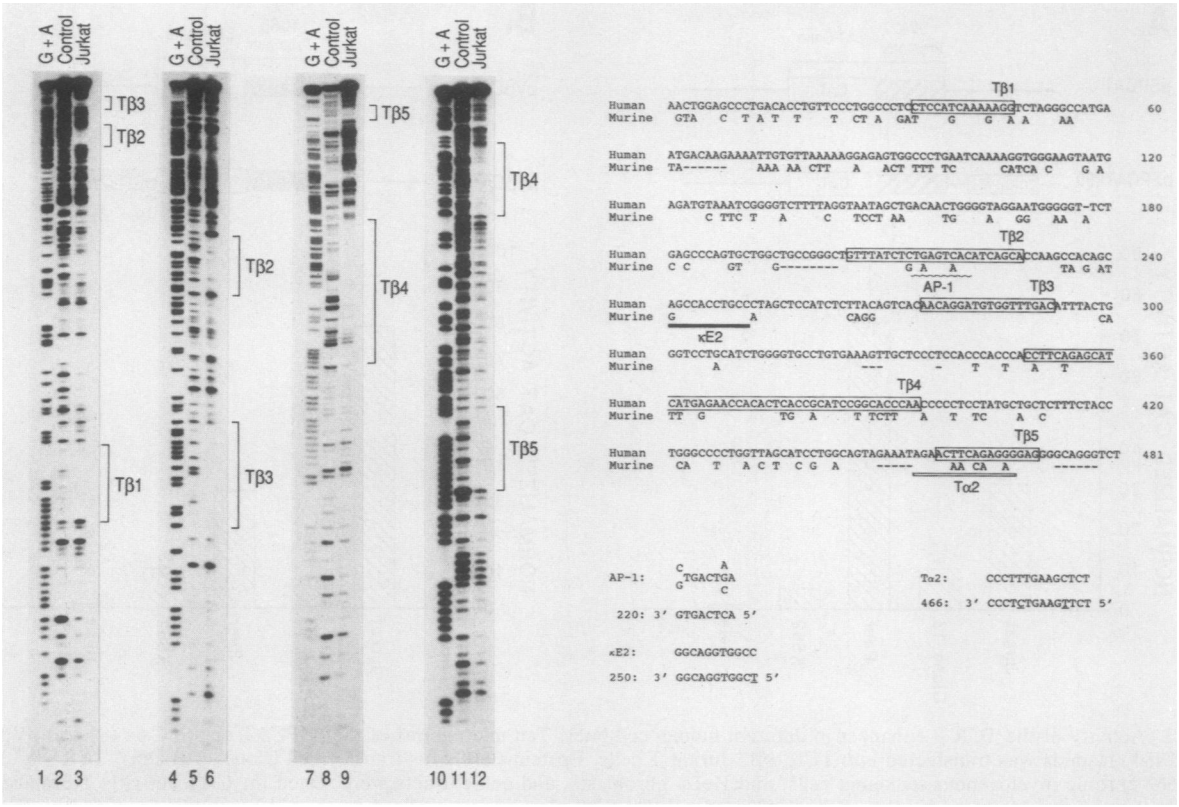

FIG. 3. DNA sequence and DNase I footprint analysis of the TCR β enhancer. The 480-bp *Hin*cII-*Xba*I fragment containing the TCR β enhancer was subjected to DNase I footprint analysis using Jurkat nuclear extracts (left panels) and to DNA sequence analysis (top right). The following fragments were end labeled and incubated with (Jurkat) or without (Control) Jurkat nuclear extract before partial digestion with DNase I: the 303-bp *Hin*cII-*Ava*II fragment (bp 1 to 303, lanes 1 to 3), the 303-bp *Ava*II-*Hin*cII fragment (bp 303 to 1, lanes 4 to 6), the 177-bp *Ava*II-*Xba*I fragment (bp 303 to 480, lanes 7 to 9), and the 177-bp *Xba*I-*Ava*II fragment (bp 480 to 303, lanes 10 to 12). Standard Maxam and Gilbert purine (G+A)-sequencing reactions of the same fragments were run in parallel. Protected sequences (Tβ1 to Tβ5) are shown in brackets adjacent to the autoradiograms and are boxed in the sequence at right. Differences in the sequence of the murine TCR β enhancer are shown below the sequence of the human TCR β enhancer, with dashes denoting deletions in the sequences. Sequences similar to the previously described AP-1 (wavy underline) (3, 18), κE2 (solid underline) (7), and Tα2 (double underline) (15) enhancer motifs are shown. Comparisons of the TCR β-enhancer sequence with the consensus AP-1, κE2, and Tα2 sequences are shown at bottom right, with differences between the sequences underlined.

and Tβ4 nuclear protein-binding sites share 72 of 88 bp (Fig. 3, right) supporting an evolutionarily conserved important functional role for these *cis*-acting sequence elements. In a further series of experiments, each of these core nuclear protein-binding sites was altered by site-directed mutagenesis (Tβ2 and Tβ3) or deletion (Tβ2 and Tβ4) or both, and the resulting fragments were then tested for enhancer activity in Jurkat T cells (Fig. 4C). The results demonstrated that mutation or deletion of any of the three core nuclear protein-binding sites resulted in significant (45 to 93%) reductions in enhancer activity. Taken together, these experiments suggested that in mature Jurkat T cells, the core TCR β enhancer is located within a 393-bp fragment that contains three nuclear protein-binding sites, each of which is required for full enhancer activity. Finally, although the Tβ1- and Tβ5-binding sites were not required for enhancer activity in mature Jurkat T cells, these results do not rule out an important functional role for these two sites at earlier stages of T-cell development or during physiologic processes such as T-cell activation by antigen.

EMSAs of the TCR β-enhancer nuclear binding proteins. To assess the number and specificity of binding of the TCR β-enhancer nuclear binding proteins, synthetic oligonucleotides corresponding to the Tβ2-, Tβ3-, and Tβ4-binding sites (see Materials and Methods) were used in EMSAs with Jurkat T-cell nuclear extracts. Jurkat nuclear extracts contained two protein complexes that bound specifically to each of the three oligonucleotides, resulting in alterations in their electrophoretic mobilities (Fig. 5A to C, arrows). The binding of each of these protein complexes was specific because in each case binding was inhibited in a dose-dependent manner by the addition of excess unlabeled specific competitor oligonucleotide but not by excess unlabeled nonspecific competitor oligonucleotide (Fig. 5A to C).

EMSAs were also performed by using nuclear extracts from other cell lines, including TCR β^+ Molt4 T cells, TCR γ/δ^+ PEER and Molt13 T cells, K562 chronic myelogenous leukemia cells, HeLa fibroblasts, and Epstein-Barr virus-transformed JY B cells (Fig. 5D). The results demonstrated that (i) none of the complexes was absolutely T cell specific and (ii) with the exception of the Tβ2A complex, which was absent in Molt4 nuclear extracts, each of the T cells expressed all of the binding activities. In contrast, each of the non-T cells lacked one or more of these binding activities. Interestingly, the HeLa and K562 cells in which the enhancer was essentially inactive were the only cell lines tested that lacked one or both of the Tβ3-binding activities.

TCR β enhancer binds a subset of nuclear proteins that also

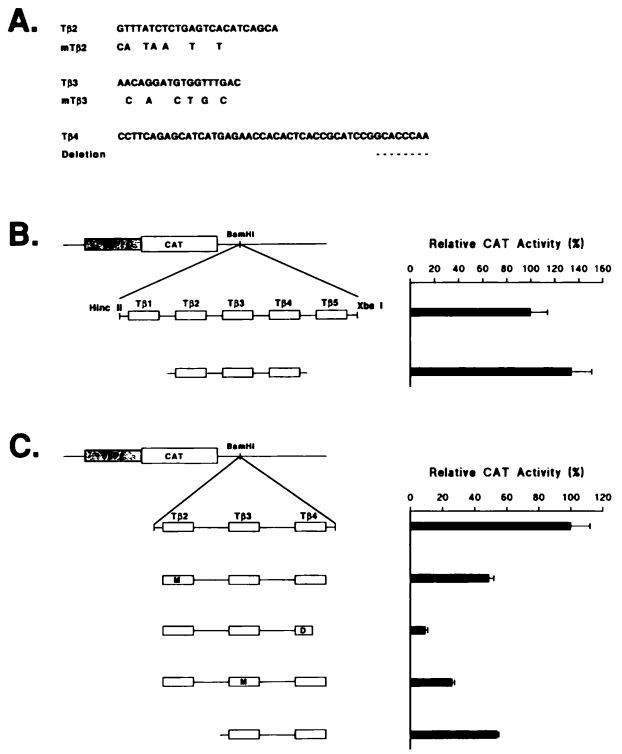

FIG. 4. Functional analysis of the nuclear protein-binding sites of the TCR β enhancer. (A) The nucleotide sequences of the Tβ2, Tβ3, and Tβ4 nuclear protein-binding sites as determined by DNase I footprint analysis (Fig. 3) are shown with mutations or deletions (dashes) or both noted below each sequence. (B) Localization of the core TCR β enhancer to a 393-bp fragment. A deletion mutant of the 480-bp *Hinc*II-*Xba*I TCR β enhancer that lacks the Tβ1 and Tβ5 nuclear protein-binding sites was prepared by using PCR as described in Materials and Methods. This 393-bp fragment was cloned into the *Bam*HI site of pVβCAT and assayed for transcriptional enhancer activity following transfection into Jurkat T cells. The data is shown as CAT activity, corrected for differences in transfection efficiencies, and normalized to the CAT activity produced by transfection of the wild-type 480-bp *Hinc*II-*Xba*I TCR β enhancer which produced 8.7% acetylation. (C) The effects of deletions and mutations of the Tβ2, Tβ3, and Tβ4 nuclear protein-binding sites on enhancer function. A fragment containing the core TCR β enhancer was prepared by using PCR. Mutations and deletions of the Tβ2-binding site were introduced into this core TCR β-enhancer fragment by using PCR as described in Materials and Methods. In addition, the Tβ3-binding site was subjected to in vitro mutagenesis by using a synthetic oligonucleotide corresponding to the mTβ3 sequence shown in panel A. Mutated binding sites are shown by a boxed M. In addition, the 480-bp *Hinc*II-*Xba*I enhancer was digested with *Hpa*II to yield a fragment that contained the Tβ2- and Tβ3-binding sites but lacked 8 bp from the 3′ end of the Tβ4-binding site (shown as a boxed D). The deleted and mutated enhancer fragments were cloned into the *Bam*HI site of the pVβCAT vector, and the resulting plasmids were transfected into Jurkat T cells. CAT activities, normalized to that produced by the core enhancer fragment containing the Tβ2 to Tβ4 nuclear protein-binding sites, are shown graphically to the right.

interact with TCR β promoters and the TCR α enhancer. As described above, an examination of the sequences of the TCR β-enhancer nuclear protein-binding sites revealed a number of interesting similarities with previously described nuclear protein-binding sites from TCR β promoters and the TCR α and δ transcriptional enhancers. Specifically, the Tβ2 nuclear protein-binding site contains a variant CRE which is highly related to the CRE contained within the Tα1 nuclear protein-binding site of the TCR α enhancer (Fig. 6A) (15). This sequence is also similar to the decamer sequence that has been shown to be located 50 to 70 bp 5′ of the cap site in a large number of murine and human TCR Vβ promoters (1, 2). Similarly, the Tβ5-binding site displays a high level of sequence identity with the Tα2-binding site of the TCR α enhancer (15) and the δE7 nuclear protein-binding site of the human TCR δ enhancer (33). In order to determine whether these similarities in *cis*-acting sequences were reflected in the ability of these elements to bind common sets of Jurkat nuclear proteins, EMSAs were performed by using labeled oligonucleotides corresponding to the Tβ2 (Fig. 6D)-, Vβ promoter (VβP; Fig. 6C)-, Tα1 (Fig. 6B)-, Tα2 (Fig. 6D)-, and Tβ5 (Fig. 6E)-binding sites in conjunction with a variety of related cold competitor oligonucleotides. Binding of Jurkat nuclear extracts to the radiolabeled Tβ2, Tα1, and Vβ promoter probes produced a similar band pattern that was inhibited by each of the other unlabeled oligonucleotides but not by unlabeled competitors containing mutations within the CRE (Fig. 6B to D). These results demonstrated that the Tβ2, Tα1, and Vβ promoter oligonucleotides all bind a common set of nuclear cyclic AMP response element-binding (CREB) proteins. In a parallel set of experiments, binding of a Jurkat nuclear protein complex to the Tβ5 element was shown to be inhibited by excess unlabeled Tα2 oligonucleotide but not by a mutant Tα2 oligonucleotide (Fig. 6E). Thus, these experiments demonstrated that several T-cell nuclear proteins are capable of interacting with transcriptional regulatory sequences from both the TCR α and β genes.

DISCUSSION

In the studies described in this report, we have identified a transcriptional enhancer element located 5.5 kb 3′ of the Cβ2 gene in the human TCR β locus. This enhancer functions in a position- and orientation-independent fashion, is required for significant transcription from a human TCR Vβ promoter, and displays preferential activity in human T cells, particularly in concert with a TCR Vβ promoter. DNA sequence and DNase I footprint analyses demonstrated that the enhancer has been highly conserved during mammalian evolution and contains at least five binding sites for nuclear proteins. Deletion and mutation analyses have localized the core TCR β enhancer to a 393-bp fragment that contains three nuclear protein-binding sites (Tβ2 to Tβ4), each of which is necessary for full enhancer activity. EMSAs demonstrated that TCR α/β^+ and γ/δ^+ T cells contain protein complexes that bind to each of the TCR β core enhancer motifs. In contrast, a variety of non-T cells were shown to lack at least one of these binding activities. One of the TCR β-enhancer nuclear protein-binding sites (Tβ2) contains a consensus AP-1 enhancer motif and a variant CRE that binds a set of T-cell nuclear CREB proteins that also interact with the Vβ promoter and the TCR α enhancer. In addition, the Tβ5 nuclear protein-binding site displays significant sequence identify to a core enhancer motif identified previously in the human TCR α and δ enhancers (15, 33) and binds a nuclear protein that also interacts with the Tα2 site of the TCR α enhancer (15).

Taken together, our results suggest that the TCR β-enhancer element described in this report plays an essential role in regulating both the T-cell specificity and the total level of human TCR β gene expression. In contrast, in transient expression assays, a human Vβ promoter alone

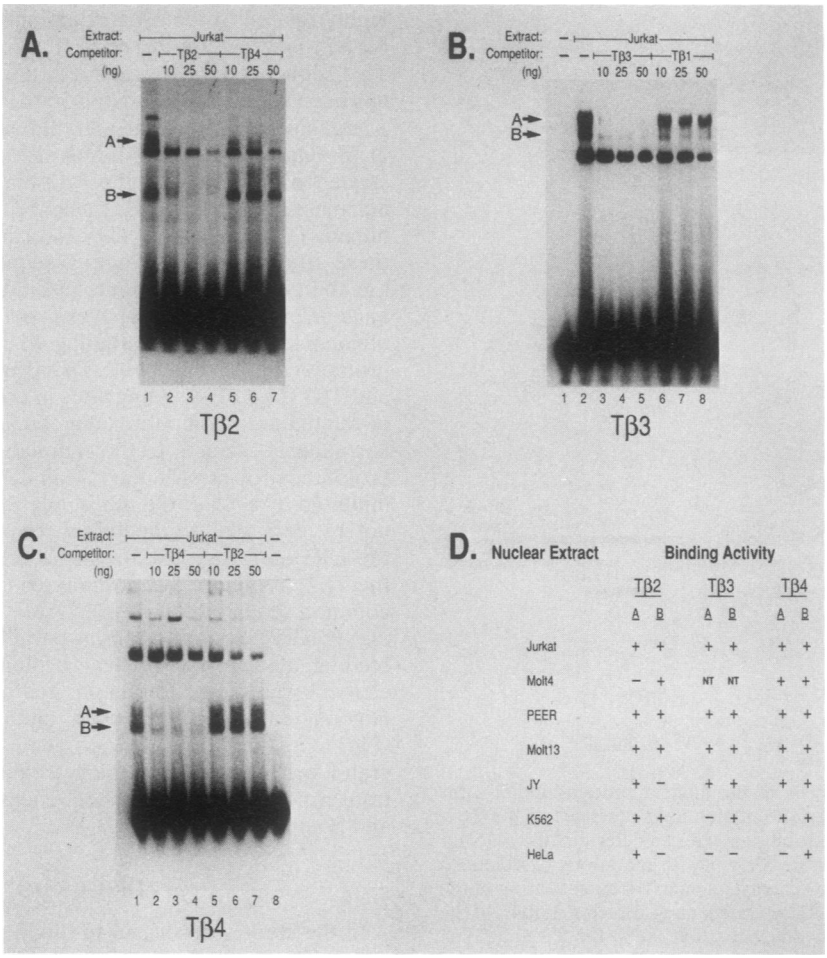

FIG. 5. EMSA of the TCR β nuclear binding proteins. (A through C) Radiolabeled, double-stranded synthetic oligonucleotides corresponding to the Tβ2-, Tβ3-, and Tβ4-binding sites as determined by DNase I footprint analysis (Fig. 3) were incubated with 2 μg of Jurkat nuclear extract, and the resulting complexes were resolved by electrophoresis in nondenaturing 4% polyacrylamide gels. For cold competition experiments, 10 to 50 ng of unlabeled competitor oligonucleotides was included in the binding reactions. Arrows denote the bands of altered mobility resulting from specific interactions of the Tβ2 to Tβ4 oligonucleotides with nuclear proteins. As described previously (16), the common band of altered mobility seen in each of the experiments represents nonspecific binding, since this complex was partially inhibited in each case by the addition of excess nonspecific cold competitor oligonucleotide and by excess poly(dI-dC) competitor. (D) Summary of the TCR β-enhancer nuclear protein-binding activities detected in different lymphoid and nonlymphoid cell nuclear extracts. NT, Not tested.

was relatively inactive in human T cells. However, it is of interest that the combination of the human TCR β enhancer and Vβ promoter appeared to confer a greater level of T-cell specificity than the same enhancer in combination with the heterologous minimal SV40 promoter (compare the Jurkat and Clone 13 lanes, Figs. 2A and B). Thus, the T-cell specificity of TCR β gene expression may require both promoter and enhancer elements. Our results are in agreement with those of previous studies of the murine TCR β enhancer (25) but contrast somewhat with those of Diamond et al. (9), who suggested that human Vβ-promoter elements alone confer T-cell specificity on TCR β gene expression, and with those of Ratanavongsiri et al. (31), who suggested that the murine TCR β enhancer is active in nonlymphoid cells. The reasons for these discrepancies remain unclear. However, it should be noted that these previous studies utilized different cell lines and Vβ promoters. Moreover, because the human TCR β enhancer had not yet been identified, it was not tested by Diamond et al. It is worth emphasizing that the low levels of enhancer activity observed in our studies of HeLa and K562 cells were not due to poor transfection efficiencies nor were they restricted to a single promoter.

Although our results suggest that the TCR β enhancer is responsible for conferring T-cell specificity on TCR β gene expression, the finding that the enhancer, in combination with either the SV40 or Vβ promoter, is equally active in TCR α/β^+ and TCR γ/δ^+ T cells implies that TCR β gene expression may not be differentially regulated in these two T-cell subsets. This is in accord with previous studies that have shown that significant TCR β gene expression can be detected in TCR γ/δ^+ T cells (38). It should also be noted that the cellular specificity of TCR β-enhancer activity is strikingly different from that of the previously described human TCR α enhancer which is active in TCR α/β^+ but not in TCR γ/δ^+ T cells (15). This suggests that the regulation of TCR α gene expression may be a critical step in controlling the differentiation of these two T-cell subsets.

An important unanswered question concerns the molecular mechanisms that account for the relative T-cell specificity of activity of the human TCR β enhancer. Our EMSA analyses demonstrated that none of the core enhancer-

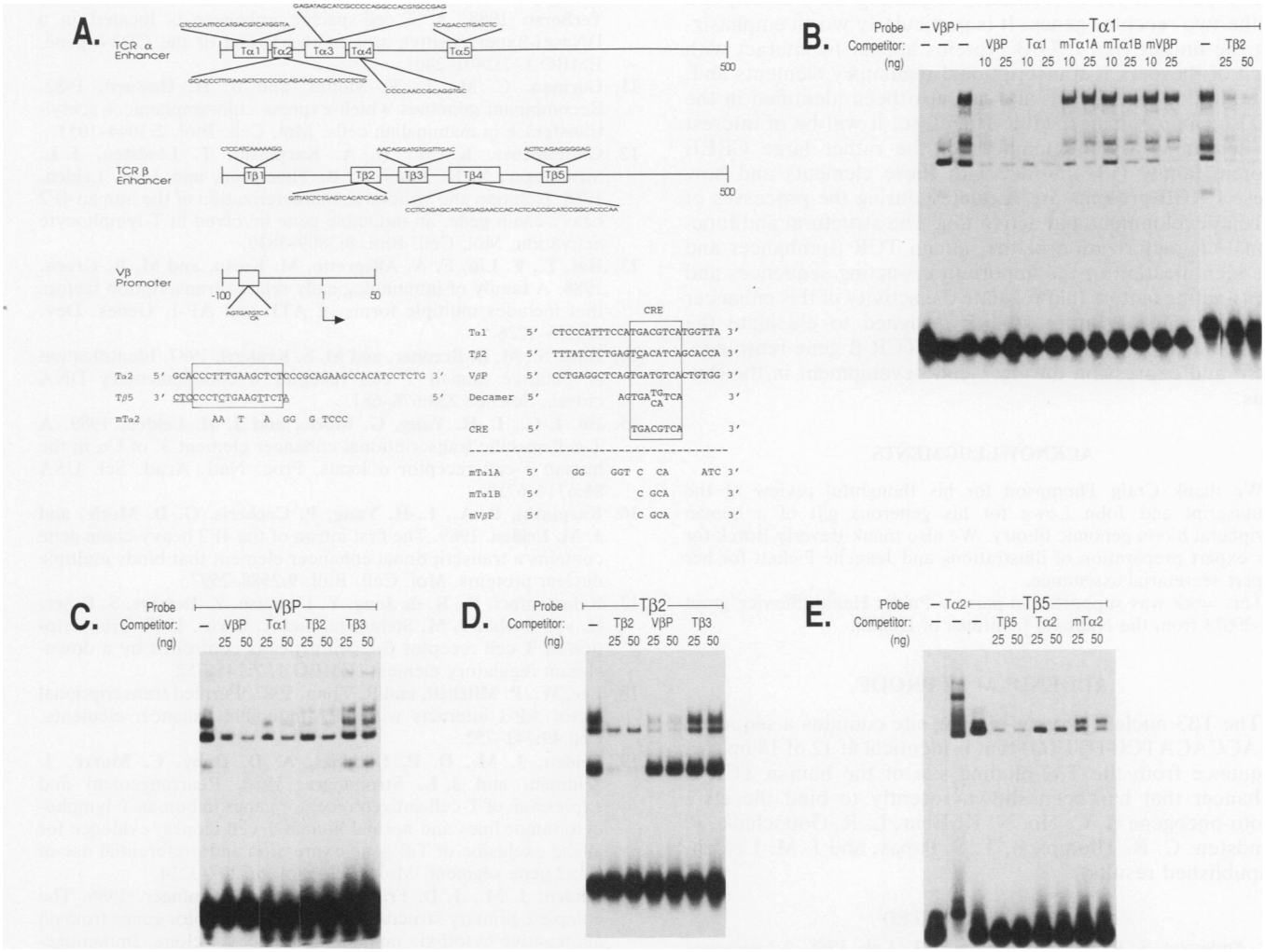

FIG. 6. TCR α and β enhancers and a Vβ promoter bind common sets of nuclear proteins. (A) A schematic representation of the human TCR α enhancer, TCR β enhancer, and Vβ promoter. The nucleotide sequences of the Tα1 to Tα5 (15) and Tβ1 to Tβ5 nuclear protein-binding sites as well as the consensus sequence of the Vβ promoter decamer are shown above and below the schematic maps. Comparisons of the nucleotide sequences of the Tα1, Tβ2, and L17 Vβ promoter sequences and the consensus sequences of the decamer (1, 2) and CRE (26) are shown in the bottom right panel. A comparison of the Tα2 and Tβ5 sequences are shown in the bottom left panel. Differences in the sequences are underlined. Sequences of the mutant synthetic oligonucleotides (mTα2, mTα1A, and mVβP) used in the EMSAs are also shown. (B to D) EMSAs of Jurkat nuclear protein binding to shared sequences in the TCR α and β transcriptional regulatory elements. Radiolabeled double-stranded oligonucleotides corresponding to the Tα1 (B), Tβ2 (D), and Tβ5 (E) nuclear protein-binding sites and to the decamer-containing Vβ promoter sequence (C) (see panel A) were incubated with 2 μg of Jurkat T-cell nuclear extract and 500 ng of poly(dI-dC) in the presence and absence of increasing amounts of unlabeled competitor double-stranded oligonucleotides as noted, followed by electrophoresis in nondenaturing 4% polyacrylamide gels.

binding proteins are expressed in an absolutely T-cell-specific fashion. However, it is worth noting that each of the T cells tested contained nuclear proteins that bound to each of the three core enhancer elements, while each of the non-T cells lacked one or more of these binding activities. Thus, perhaps high-level enhancer activity requires all three binding activities which are coexpressed only in T cells. This type of model is consistent with our deletion analyses that demonstrated that all three nuclear protein-binding sites are required for full enhancer activity and with the finding that the enhancer does display low-level activity in B cells that express two of the three core enhancer-binding activities. Finally, it is interesting that the Tβ3 nuclear protein-binding site is 100% conserved in the murine TCR β enhancer (17), that our mutagenesis experiments demonstrated that this site is critical for enhancer activity, and that the K562 and HeLa cells in which the enhancer is inactive both lack at least one of the Tβ3 nuclear binding activities. Thus, perhaps the lack of expression of Tβ3-binding proteins accounts for the inactivity of the TCR β enhancer in nonlymphoid cells.

The fact that the TCR α/β/CD3 complex is composed of seven polypeptides, each of which is required for successful cell surface expression (39), raises the interesting problem of how the synthesis of these proteins is coordinately regulated, especially during physiological changes such as T-cell activation during which profound changes in the cell surface density of the TCR/CD3 complex are known to occur (34). Our finding that common proteins interact with TCR β gene promoter and enhancer elements, as well as with the TCR α enhancer, raises the possibility that these common proteins

are responsible for coordinately regulating the transcription of the two receptor genes. It is particularly worth emphasizing the finding that CREB proteins appear to interact with each of these TCR transcriptional regulatory elements and, in addition, that a CRE site has also been identified in the CD3-δ-chain enhancer (10). Therefore, it will be of interest to determine which members of the rather large CREB protein family (13) interact with these elements and how these CREB proteins are regulated during the processes of T-cell development and activation. The structural and functional characterization of the human TCR β enhancer and the identification of the important *cis*-acting sequences and *trans*-acting factors that regulate the activity of this enhancer should facilitate future studies designed to elucidate the molecular mechanisms that control TCR β gene rearrangement and expression during T-cell development in the thymus.

ACKNOWLEDGMENTS

We thank Craig Thompson for his thoughtful review of the manuscript and John Lowe for his generous gift of a human peripheral blood genomic library. We also thank Beverly Burck for her expert preparation of illustrations and Jeanelle Pickett for her expert secretarial assistance.

This work was supported in part by Public Health Service grant AI-29673 from the National Institutes of Health.

ADDENDUM IN PROOF

The Tβ3 nuclear protein-binding site contains a sequence (AACCACATCCTGTTG) that is identical at 12 of 15 bp to a sequence from the Tα2-binding site of the human TCR α enhancer that has been shown recently to bind the ets-1 proto-oncogene (I.-C. Ho, N. K. Bhat, L. R. Gottschalk, T. Lindsten, C. B. Thompson, T. S. Papas, and J. M. Leiden, unpublished results).

LITERATURE CITED

1. **Anderson, S. J., H. S. Chou, and D. L. Loh.** 1988. A conserved sequence in the T-cell receptor β-chain promoter region. Proc. Natl. Acad. Sci. USA **85**:3551–3554.
2. **Anderson, S. J., S. Miyake, and D. Y. Loh.** 1989. Transcription from a murine T-cell receptor Vβ promoter depends on a conserved decamer motif similar to the cyclic AMP response element. Mol. Cell. Biol. **9**:4835–4845.
3. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell **49**:729–739.
4. **Bank, I., R. A. DePinho, M. B. Brenner, J. Cassimeris, F. W. Alt, and L. Chess.** 1986. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature (London) **322**:179–181.
5. **Brenner, M., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. Krangel.** 1986. Identification of a putative second T-cell receptor. Nature (London) **322**:145–149.
6. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
7. **Church, G. M., A. Ephrussi, W. Gilbert, and S. Tonegawa.** 1985. Cell-type specific contacts to immunoglobulin enhancers in nuclei. Nature (London) **313**:798–801.
8. **Davis, M. M., and P. J. Bjorkman.** 1988. T-cell antigen receptor genes and T-cell recognition. Nature (London) **334**:395–401.
9. **Diamond, D. J., F. B. Nelson, and E. L. Reinherz.** 1989. Lineage-specific expression of a T cell receptor variable gene promoter controlled by upstream sequences. J. Exp. Med. **169**:1213–1231.
10. **Georgopoulos, K., P. van den Elsen, E. Bier, A. Maxam, and C. Terhorst.** 1988. A T cell-specific enhancer is located in a DNaseI-hypersensitive area at the 3′ end of the CD3-δ gene. EMBO J. **7**:2401–2407.
11. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
12. **Gottesdiener, K. M., B. A. Karpinski, T. Lindsten, J. L. Strominger, N. H. Jones, C. B. Thompson, and J. M. Leiden.** 1988. Isolation and structural characterization of the human 4F2 heavy-chain gene, an inducible gene involved in T-lymphocyte activation. Mol. Cell. Biol. **8**:3809–3819.
13. **Hai, T., F. Liu, E. A. Allegretto, M. Karin, and M. R. Green.** 1988. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. Genes. Dev. **2**:1216–1226.
14. **Hata, S., M. B. Brenner, and M. S. Krangel.** 1987. Identification of putative human T cell receptor δ complementary DNA clones. Science **238**:678–681.
15. **Ho, I.-C., L.-H. Yang, G. Morle, and J. M. Leiden.** 1989. A T-cell-specific transcriptional enhancer element 3′ of Cα in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA **86**:6714–6718.
16. **Karpinski, B. A., L.-H. Yang, P. Cacheris, G. D. Morle, and J. M. Leiden.** 1989. The first intron of the 4F2 heavy-chain gene contains a transcriptional enhancer element that binds multiple nuclear proteins. Mol. Cell. Biol. **9**:2588–2597.
17. **Krimpenfort, P., R. de Jong, Y. Uematsu, Z. Dembic, S. Ryser, H. von Boehmer, M. Steinmetz, and A. Berns.** 1988. Transcription of T cell receptor β-chain genes is controlled by a downstream regulatory element. EMBO J. **7**:745–752.
18. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcriptional factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
19. **Leiden, J. M., D. P. Dialynas, A. D. Duby, C. Murre, J. Seidman, and J. L. Strominger.** 1986. Rearrangement and expression of T-cell antigen receptor genes in human T-lymphocyte tumor lines and normal human T-cell clones: evidence for allelic exclusion of Tiβ gene expression and preferential use of a Jβ2 gene segment. Mol. Cell. Biol. **6**:3207–3214.
20. **Leiden, J. M., J. D. Fraser, and J. L. Strominger.** 1986. The complete primary structure of the T-cell receptor genes from an alloreactive cytotoxic human T-lymphocyte clone. Immunogenetics **24**:17–23.
21. **Leung, K., and G. J. Nabel.** 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature (London) **333**:776–778.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Marrack, P., and J. Kappler.** 1987. The T cell receptor. Science **238**:1073–1079.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
25. **McDougall, S., C. L. Peterson, and K. Calame.** 1988. A transcriptional enhancer 3′ of Cβ2 in the T cell receptor β locus. Science **241**:205–208.
26. **Montiminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodmand.** 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83**:6682–6686.
27. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56**:777–783.
28. **Parmacek, M. S., and J. M. Leiden.** 1989. Structure and expression of the murine slow/cardiac troponin C gene. J. Biol. Chem. **264**:13217–13225.
29. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335**:683–689.
30. **Quakenbush, E., M. Clabby, K. M. Gottesdiener, J. Barbosa, N. H. Jones, J. L. Strominger, S. Speck, and J. M. Leiden.** 1987.

Molecular cloning of complementary DNAs encoding the heavy chain of the human 4F2 cell-surface antigen: a type II membrane glycoprotein involved in normal and neoplastic cell growth. Proc. Natl. Acad. Sci. USA **84**:6526–6530.

31. **Ratanavongsiri, J., S. Igarashi, S. Mangal, P. Kilgannon, A. Fu, and A. Fotedar.** 1990. Transcription of the T cell receptor β-chain gene is controlled by multiple regulatory elements. J. Immunol. **144**:1111–1119.
32. **Raulet, D. H., R. D. Garman, H. Saito, and S. Tonegawa.** 1985. Developmental regulation of T-cell receptor gene expression. Nature (London) **314**:103–107.
33. **Redondo, J. M., S. Hata, C. Brocklehurst, and M. S. Krangel.** 1990. A T cell-specific transcriptional enhancer within the human T cell receptor δ locus. Science **247**:1225–1229.
34. **Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman.** 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. Cell **30**:735–742.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
36. **Selden, R. F., K. B. Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore.** 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell. Biol. **6**:3173–3179.
37. **Snodgrass, H. R., P. Kisielow, M. Kiefer, M. Steinmetz, and H. von Boehmer.** 1985. Ontogeny of the T-cell antigen receptor within the thymus. Nature (London) **313**:592–595.
38. **Weiss, A., M. Newton, and D. Crommie.** 1986. Expression of T3 in association with a molecule distinct from the T-cell antigen receptor heterodimer. Proc. Natl. Acad. Sci. USA **83**:6998–7002.
39. **Weiss, A., and J. D Stobo.** 1984. Requirement for the coexpression of T3 and the T-cell antigen receptor on a malignant human T-cell line. J. Exp. Med. **160**:1284–1292.
40. **Wilson, R. K., E. Lai, P. Concannon, R. K. Barth, and L. E. Hood.** 1988. Structure, organization and polymorphism of murine and human T-cell receptor α and β chain gene families. Immunol. Rev. **101**:149–172.
41. **Winoto, A., and D. Baltimore.** 1989. A novel inducible and T cell-specific enhancer located at the 3′ end of the T cell receptor α locus. EMBO J. **8**:729–733.
42. **Yancopoulos, G. D., and F. W. Alt.** 1985. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. Cell **40**:271–281.