MOLECULAR AND CELLULAR BIOLOGY, Sept. 1993, p. 5450–5460
0270-7306/93/095450-11$02.00/0

Vol. 13, No. 9

A Novel POU Domain Protein Which Binds to the T-Cell Receptor β Enhancer†

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Received 19 April 1993/Returned for modification 25 May 1993/Accepted 21 June 1993

POU domain proteins have been implicated in the regulation of a number of lineage-specific genes. Among the first POU domain proteins described were the immunoglobulin octamer-binding proteins Oct-1 and Oct-2. It was therefore of special interest when we identified a novel lymphoid POU domain protein in Southwestern (DNA-protein) screens of T-cell λgt11 libraries. This novel POU protein, TCFβ$_1$, binds in a sequence-specific manner to a critical motif in the T-cell receptor (TCR) β enhancer. Sequence analysis revealed that TCFβ$_1$ represents a new class of POU domain proteins which are distantly related to other POU proteins. TCFβ$_1$ is encoded by multiple exons whose organization is distinct from that of other POU domain proteins. The expression of TCFβ$_1$ in a tissue-restricted manner and its ability to bind to multiple motifs in the TCR β enhancer support a role in regulating TCR β gene expression. The expression of TCFβ$_1$ in both B and T cells and the ability of recombinant TCFβ$_1$ to bind octamer and octamer-related motifs suggest that TCFβ$_1$ has additional roles in lymphoid cell function. The ability of TCFβ$_1$ to transactivate in a sequence-specific manner is consistent with a role for regulating lymphoid gene expression.

The mature T-cell receptor (TCR) β gene is generated by developmentally regulated rearrangement of germ line gene segments in the thymus (2, 39, 45). Gene-targeted disruption of the TCR β gene has demonstrated that β gene expression is essential for subsequent thymocyte development (33). This dominant role for TCR β gene expression in T-cell development suggests a need to clarify the molecular mechanisms regulating β gene expression. Transcription of the TCR β gene is regulated by multiple *cis*-acting elements (1, 16, 20, 28, 30, 38). One of these elements, the TCR β enhancer region, enhances lymphoid cell-specific transcription in tissue culture (20, 28) and is essential for expression of β transgenes in mice (20). In addition, the β enhancer is essential for rearrangement of TCR β gene substrates in transgenic mice (7, 53). The minimal TCR β enhancer has been mapped (9, 48), and the multiple motifs which bind nuclear proteins have been identified (9, 48).

POU proteins are a subset of homeobox proteins which in addition to the homeobox contain a POU-specific domain (12). POU proteins regulate lineage-specific expression of a number of genes by binding octamer- or octamer-related motifs (12, 40). The immunoglobulin (Ig) heavy-chain gene promoter contains octamer motifs which are essential for B-cell-specific Ig gene expression in tissue culture (5, 55) and transgenic mice (17). Octamer motifs have also been found to be critical for expression of other lymphoid genes such as the Igκ (22) and interleukin-2 (52) genes. Two octamer-binding, lymphoid POU domain proteins have been identified (4, 42, 47). Oct-1 has been implicated in regulation of small nuclear RNA promoter activity via a selective activation domain (49) and in association with a coactivator in Ig gene expression (24). The Oct-2 POU protein was initially implicated in regulation of Ig gene expression (34, 42), although recent experiments cast doubt on a selective role of Oct-2 in regulating Ig gene expression (24). It was therefore of special interest when we identified TCFβ$_1$, a novel POU domain protein from a T-cell cDNA library, by its ability to bind E4, a critical motif in the TCR β enhancer. The E4 motif (30) has also been termed Tβ$_2$ (9) or βE1 (48). The characteristics of this novel lymphoid POU domain protein as described in this report suggest that we have identified a new class of POU domain proteins.

MATERIALS AND METHODS

Screening λgt11 libraries. A Jurkat λgt11 library was screened with a multimerized 3× E4A probe (E4A sense strand, 5′ TCTGGGTGTTTATCTGTAAGTA 3′; antisense strand, 5′ TACTTACAGATAAACACCCAGA 3′). The plasmid containing the E4 motif was linearized and labeled by a Klenow fill-in reaction, and the insert was gel purified. The probe was then run through an Elutip-d column; to decrease false negatives, the probe was filtered through a 0.45-μm-pore-size Gelman Acrodisc membrane. A modified version (43, 44) of the original renaturation-denaturation λgt11 screening protocol was used. The positive clones in question were plaque purified four times before they were analyzed for sequence specificity. The clones were screened with four probes. The E3A and E4A motifs are from the TCR β enhancer, whereas the AP-1 and *ets* motifs are from the murine TCR Vβ$_2$ promoter (see Tables 1 and 2 for sequences). The E3 region has also been termed Tβ$_3$ (9) or βE4 (48); the E4 region has also been termed Tβ$_2$ (9). The AP-1 and the *ets* motifs correspond to the −85 to −73 and −75 to −62 regions of the murine TCR Vβ$_2$ promoter (31) and bind JunB/c-Fos and Ets-2, respectively (31).

Generation of lambda lysogenic extracts and Southwestern (DNA-protein) analysis. The lambda lysogenic extracts were made essentially as described previously (43, 44). Briefly,

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† Publication 62 from the La Jolla Institute for Allergy and Immunology.

TABLE 1. TCR β enhancer motifs used in this study

Name	Sequence[a]
E4 motif	GCATCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACATCAGCACCAAGTAAGAATGG
βE1	5′ TCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACA 3′
E4A	5′ TCTGGGTGTTTATCTGTAAGTA 3′
βE2	5′ TATCTGTAAGTAACATCAGCACCAAGTAAGAATGG 3′
E4B	5′ GTAAGTAACATCAGCACCAAG 3′
βE3	5′ GTAAGAATGG
E3 motif	CCACCTGCCATAGCTCCATCTCCAGGAGTCACAACAGGATGTGGTTTGACATTTACCAGGT
βE3	CCACCTGCCATAGCTCCATCTCCAGGAGTC 3′
E3A	5′ CCAGGAGTCACAACAGGATGTGGTTTG 3′
E3B	5′ CAACAGGATGTGGTTTGACATTTAC 3′
E2 motif	CCTACATCTGGGGTGCCTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTTGGGAA
βE5	5′ CTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTT 3′
E1 motif	CCACACTTGCCACATCCTGTCTTCAAACCCTTCTCATGCAGCCCTTTCTACCTCAGCCTCT

[a] Sequences of the TCR β enhancer (9, 20, 48), the βE oligonucleotides (30, 48), the E3 oligonucleotides (30, 48), and the E4 oligonucleotides (30) have been described previously. For the sake of brevity, the complementary strands are not shown.

the bacterial cell pellet from a 2-ml isopropylthiogalactopyranoside (IPTG)-induced culture was resuspended in 140 μl of sample buffer (100 mM Tris-HCl [pH 6.8], 200 mM β-mercaptoethanol, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol), boiled for 3 min at 90°C, and run on 10% denaturing polyacrylamide gels. The proteins were transferred to nitrocellulose sheets (0.45-μm pore size; Sartorius) at 100 V for 1 h as described previously (50). The nitrocellulose was then subjected to denaturation-renaturation and probed as described previously (43, 44).

In vitro transcription and translation and reverse gel shift assays. The $TCF\beta_1$ insert was cloned into an ATG Bluescript vector. The ATG codon was introduced in the Bluescript vector by using adaptor double-stranded oligonucleotides (5′ AGCTTCAACCAGCCTCCCGCGACGATGG 3′). The template was linearized and in vitro transcribed (31). The in vitro-transcribed $TCF\beta_1$ RNA was then in vitro translated in reticulocyte lysates in the presence of [^{35}S]methionine. This labeled protein was used in reverse gel shift assays. The linearized plasmid DNA (with or without the E4A motif) was incubated with 1 μl of a 50-ml reticulocyte lysate in the presence of 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 50 ng of poly(dI-dC). The reactions were run on 4% nondenaturing polyacrylamide gels in 0.25× Tris-borate-EDTA.

Bacterial overexpression of recombinant $TCF\beta_1$ protein. The $TCF\beta_1$ insert was cloned in frame into the *Eco*RI site of the pRSET B vector (Invitrogen). For expressing $TCF\beta_1$ in bacteria, plasmids were used to transform *Escherichia coli* BL21(DE21) containing plasmid pLysS (46). The bacterial culture was grown to an optical density of 1 at 37°C, and protein expression was induced by adding 1 mM IPTG for 4 h at 37°C. The bacterial pellet was resuspended in 20 mM Tris (pH 7.4)–500 mM NaCl–10% glycerol–1 mM EDTA–0.1% Nonidet P-40–1 mM phenylmethyl sulfonyl fluoride–5 mg of leupeptin per ml–1% (vol/vol) aprotinin. The $TCF\beta_1$

TABLE 2. Wild-type and mutant motifs used in DNA binding assays

Name	Sequence[a]
βE1 and βE5 oligonucleotides	
βE1	
Wild type[b]	5′ TCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACA 3′
Mutant 1	5′ TCTCACCCCAGGGAGTTTTGTTTATCTGTAAGTAACA 3′
Mutant 2	5′ TCTCACCCCAGGTCTGGCGTGGGCTCTGTAAGTAACA 3′
Mutant 3[c]	5′ TCTCACCCCAGGTCTGGCTGTTGCGATGTAAGTAACA 3′
Mutant 4	5′ TCTCACCCCAGGTCTGGCTGTTTATCTTGCCTGCACA 3′
βE5	
Wild type[d]	5′ CTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTT 3′
Mutant	5′ CTGTGAATGCTCCCCCACGACAGAACATTCTGAGCATTTT 3′
Motifs from the TCR Vβ2 promoter[e]	
AP-1	5′ TATGAGCTTAGTCAGTTCA 3′
ets	5′ TATGTTTCCTGAGGAAGCA 3′
POU protein-binding motifs[f]	
Octamer	
Wild type	5′ ATGAATATGCAAATCAGGTGA 3′
Mutant	5′ ATGAATATGCCCCTCAGGTGA 3′
Pit	
Wild type	5′ CCTGATTATATATATATTCATGAAGGTG 3′
Mutant	5′ CCTGATTATATATAGCGGACTGAAGGTG 3′

[a] Mutated regions are underlined. For the sake of brevity, the complementary strands are not shown.
[b] An effective competitor for all proteins which bind the E4A motif in a sequence-specific manner; described previously (48).
[c] Inactive in transcriptional assays in T cells (26).
[d] Described previously (48).
[e] The AP-1 motif extends from −85 to −73 and the *ets* motif extends from −75 to −62 in the TCR $V\beta_2$ promoter (31).
[f] Described previously (6). The octamer motifs are from the Ig promoter.

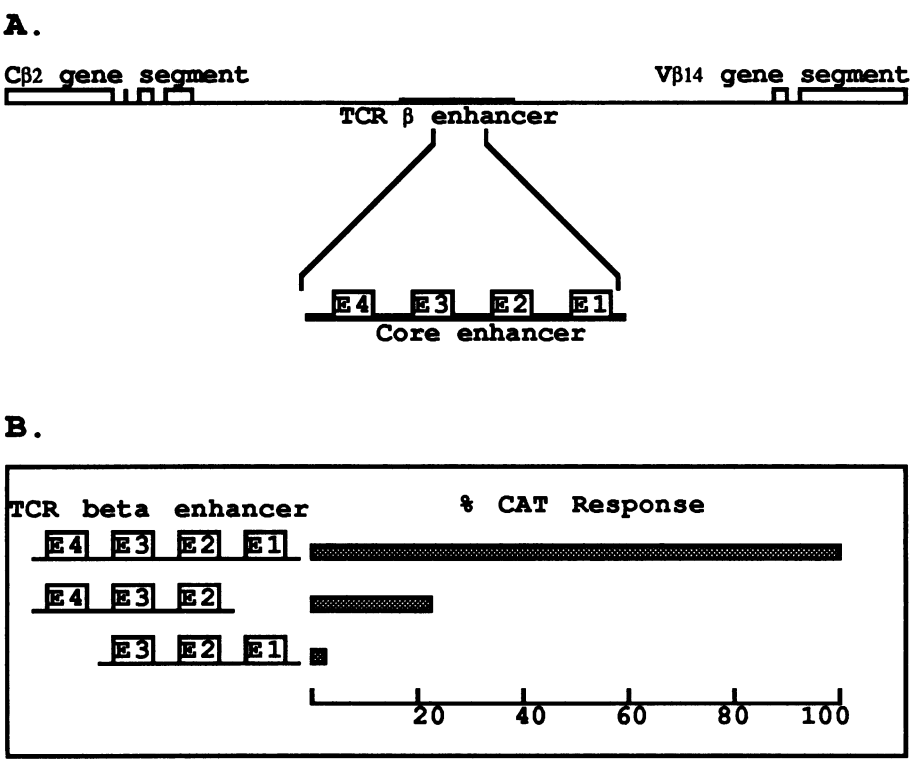

FIG. 1. Role of the E4 motif in TCR β enhancer activity. (A) Schematic cartoon of the protein-binding sites in the core TCR β enhancer. The E4 motif (30) has also been termed $T\beta_2$ (9) or βE1 (48) in other studies. (B) Deletional analysis of the functional core of the TCR β enhancer. The different fragments of the TCR β enhancer were cloned upstream of the TK promoter in the pBLCAT2 vector. Data represent percent activity of the β enhancer fragment (bp 521 to 780); the activity of the TK promoter (1.4%) was enhanced by this β enhancer fragment to 85%. Deletion of the E1 region decreased CAT activity to 21%, whereas deletion of the E4 region decreased CAT activity to 0.5%. Similar results were seen in multiple experiments ($n > 4$).

protein was preferentially precipitated with 30% ammonium sulfate. This $TCF\beta_1$ preparation was found to be >95% pure.

Gel shift assays. End-labeled octamer or βE1 motifs (10,000 cpm) were incubated with ~1 μl of pure recombinant $TCF\beta_1$ in the presence of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.8), 4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM spermidine, 100 mg of bovine serum albumin per ml, 2 mM dithiothreitol, 15% glycerol, and 2 μg of poly(dI-dC). The DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide (0.25× Tris-borate-EDTA) gel.

Cells and transfection. Cells were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and glutamine. The human cell lines Jurkat, HUT-74, MOLT3, MOLT4, Raji, and Daudi were obtained from the American Type Culture Collection. T cells were transfected by the DEAE-dextran method as described previously (30, 31, 38). Experiments were repeated more than four times. The chloramphenicol acetyltransferase (CAT) activities of the lysates were determined with equal amounts of protein under conditions such that the assay is in the linear range. The indicator plasmid pCH110 was cotransfected to normalize for differences in transfection efficiencies (38). The effect of deleting the E4 motif on β enhancer activity was determined by cloning the deleted fragments of the TCR β enhancer upstream of the thymidine kinase (TK) promoter in the pBL2CAT vector. The TCR β enhancer fragments with intact E4, E3, E2, and E1 motifs (nucleotides [nt] 521 to 780) was generated by the polymerase chain reaction. The sequence numbers refer to the original description of the TCR β enhancer (9). The E1 and E4 regions were deleted from the β enhancer fragment by the polymerase chain reaction and partial *Alu*I digestion, respectively.

Northern (RNA) and Southern analyses. Total RNA from a panel of cell lines was prepared by solubilizing cells in guanidinium thiocyanate as described previously (25). Poly(A)$^+$ RNA (~2 to 5 μg per lane) was run on a 1% agarose gel. Poly(A)$^+$ RNA from a panel of human tissues were obtained from Clontech. The Northern filters were hybridized with ^{32}P-labeled $TCF\beta_1$ cDNA probes in the presence of 50% formamide, 6× SSPE, 5× Denhardt's solution 0.1% SDS, and 100 μg of denatured salmon sperm DNA per ml at 42°C. Filters were washed with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 55°C for 1 h. In some cases, a $TCF\beta_1$ antisense RNA probe extending from nt 675 to 975 was also used. Identical results were obtained with either type of $TCF\beta_1$ probe. Human high-molecular-weight genomic DNA was prepared as described previously (25). Twenty micrograms of genomic DNA was cut to completion with the appropriate restriction enzyme. The digested DNA was run on 0.7% agarose gels, transferred to nitrocellulose, and hybridized as described above.

RNase protection analysis. The antisense RNA probe corresponding to $TCF\beta_1$ sequence from bp 675 to 975 was generated and used in RNase protection assays as described previously (25). The human thymus poly(A)$^+$ RNA was

obtained from Clontech. The 340-bp-long probe yields a protected band of 300 nt.

Genomic cloning of the TCFβ$_1$ gene. A Charon 4A human genomic DNA library was screened with a TCFβ$_1$ cDNA probe, and the inserts were cloned into plasmid vectors for detailed analysis. The exon-intron organization was determined by sequencing with TCFβ$_1$-specific cDNA primers. The introns were characterized by restriction mapping and Southern analysis with exon-specific TCFβ$_1$ probes as described previously (38). The restriction map of the two overlapping lambda clones completely reproduces the TCFβ$_1$ Southern pattern with uncloned genomic DNA, thus suggesting that the entire genomic TCFβ$_1$ gene has been cloned and characterized.

Cotransfection analysis of the TCFβ$_1$ gene. Transactivation characteristics were determined in a cotransfection assay using reporter constructs with multimerized 6× Pit motifs from the prolactin promoter (15) or multimerized 6× inactive dpm8 motifs (49) cloned at −52 from the transcriptional start site of a minimal β-globin promoter. Winship Herr, Cold Spring Harbor Laboratory, kindly provided these plasmids. The cDNAs of the different POU domain proteins were expressed from the cytomegalovirus promoter in the pCG vector (49). HeLa cells were cotransfected with reporter (2 μg), expression plasmid (4 μg), and transfection control plasmid (1 μg), and RNA was harvested from the transfected cells 60 h later as described previously (49). The RNA was used to estimate the activity of the reporter construct by RNase protection assays using a β-globin probe which generates a 350-bp protected band from appropriately initiated transcripts (49). The intensity of the signal was quantitated by scanning densitometric analysis. The indicator plasmid pα 4×(A+C) was cotransfected with reporter and expression plasmids to simultaneously determine transfection efficiency. The activity of the α-globin plasmid was determined by RNase protection assays using an α-globin probe (49). The RNase protection assays of both β-globin and α-globin transcripts were performed simultaneously for convenience and accuracy. The corrected and normalized reporter activities are presented.

Nucleotide sequence accession number. The TCFβ$_1$ cDNA sequence has been deposited in the GenBank/EMBL library under accession number L14482.

RESULTS

Cloning of a gene which encodes a TCR β enhancer-binding protein. The functional core of the TCR β enhancer has multiple motifs which bind nuclear proteins (9, 48). The E4 motif maps to the 5′ end of the minimal β enhancer (Fig. 1A). Deletion of this region dramatically reduces β enhancer activity (Fig. 1B). Similar results have been obtained in an independent deletional analysis of the β enhancer (9). These data suggest that the E4 motif is crucial for β enhancer activity. This conclusion is consistent with the mapping of E4 in vitro footprints in both mouse and human genes (9, 48), the ~85% conservation of the E4 region between mouse and human genes (9, 48) and the reduction in enhancer activity by substitutional mutagenesis of the E4 motif (9).

To identify genes which encode E4-binding proteins, a Jurkat T-cell λgt11 library was screened with a multimerized E4A probe (see Table 1 for sequences). In situ analysis identified nine clones which bind the E4 motif in a sequence-specific manner. This finding was confirmed when the lambda lysogenic extracts were tested by Southwestern analysis. The protein encoded by one such clone bound to the E4A motif in a sequence-specific manner (Fig. 2A). It did not bind to the AP-1 or *ets* motif or to the E3A motif from the TCR β enhancer. All of the E4A-binding clones bound in a similar sequence-specific manner. The insert was then cloned into an ATG Bluescript vector. The ^{35}S-labeled protein was transcribed and translated in vitro and visualized on denaturing protein gels by autoradiography. A protein with a relative molecular size of ~50 kDa could be visualized in the lysates programmed with sense RNA but not in unprogrammed lysates or those programmed with inappropriate RNA (Fig. 2B). The labeled protein was then tested in a reverse gel shift assay and shown to bind the E4A motif in

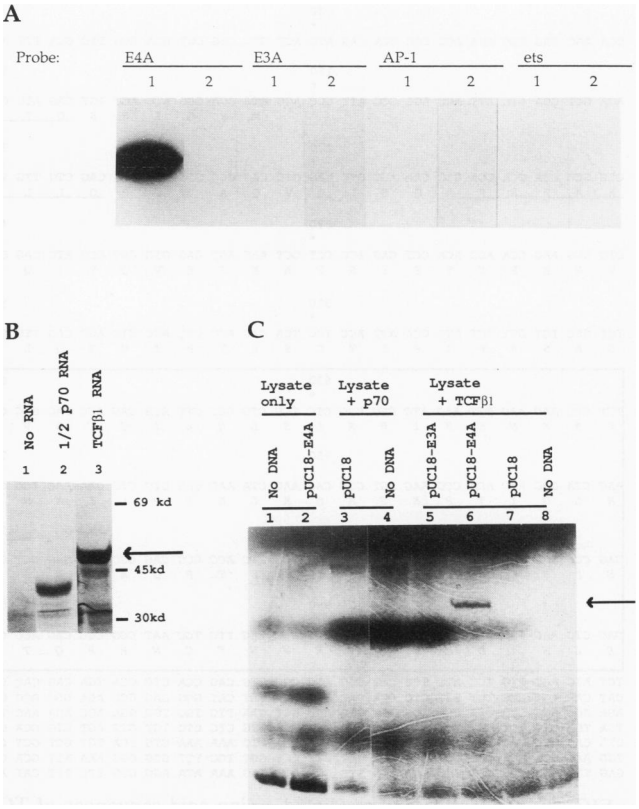

FIG. 2. Sequence-specific binding of TCFβ$_1$ to the E4 motif in the TCR β enhancer. (A) Southwestern analysis of the λgt11 TCFβ$_1$ clone. Lambda lysogenic extracts from the TCFβ$_1$ λgt11 clone (lanes 1) and an irrelevant λgt11 recombinant (lanes 2) were assayed for the ability to bind to four motifs in a Southwestern assay. These motifs included the TCR β enhancer motifs E4A and E3A. The AP-1 and *ets* motifs are from the Vβ$_2$ promoter and bind recombinant JunB–c-Fos heterodimers and the Ets-2 transactivator, respectively. The sequences of these motifs are listed in Tables 1 and 2. (B) Visualization of the [^{35}S]methionine-labeled TCFβ$_1$ protein generated by in vitro transcription and translation. The reticulocyte lysates were programmed with either TCFβ$_1$ RNA or a control RNA encoding the C-terminal half of the p70 lupus autoantigen (30). Reticulocyte lysates programmed with the two RNAs and unprogrammed lysates were all run on 10% denaturing polyacrylamide gels. The TCFβ$_1$ gene encodes a protein which migrates with a mobility of ~50 kDa. (C) TCFβ$_1$ binds to the E4A motif in a sequence-specific manner in a reverse gel shift assay. The ability of unprogrammed reticulocyte lysates or those programmed with an irrelevant RNA (p70) or TCFβ$_1$ RNA to bind plasmid DNAs was determined in a reverse gel shift assay. The plasmid DNAs included pUC18, the E4A motif cloned in pUC18, and the E3A motif cloned in pUC18. The arrow identifies the specific E4A-TCFβ$_1$ DNA-protein complex.

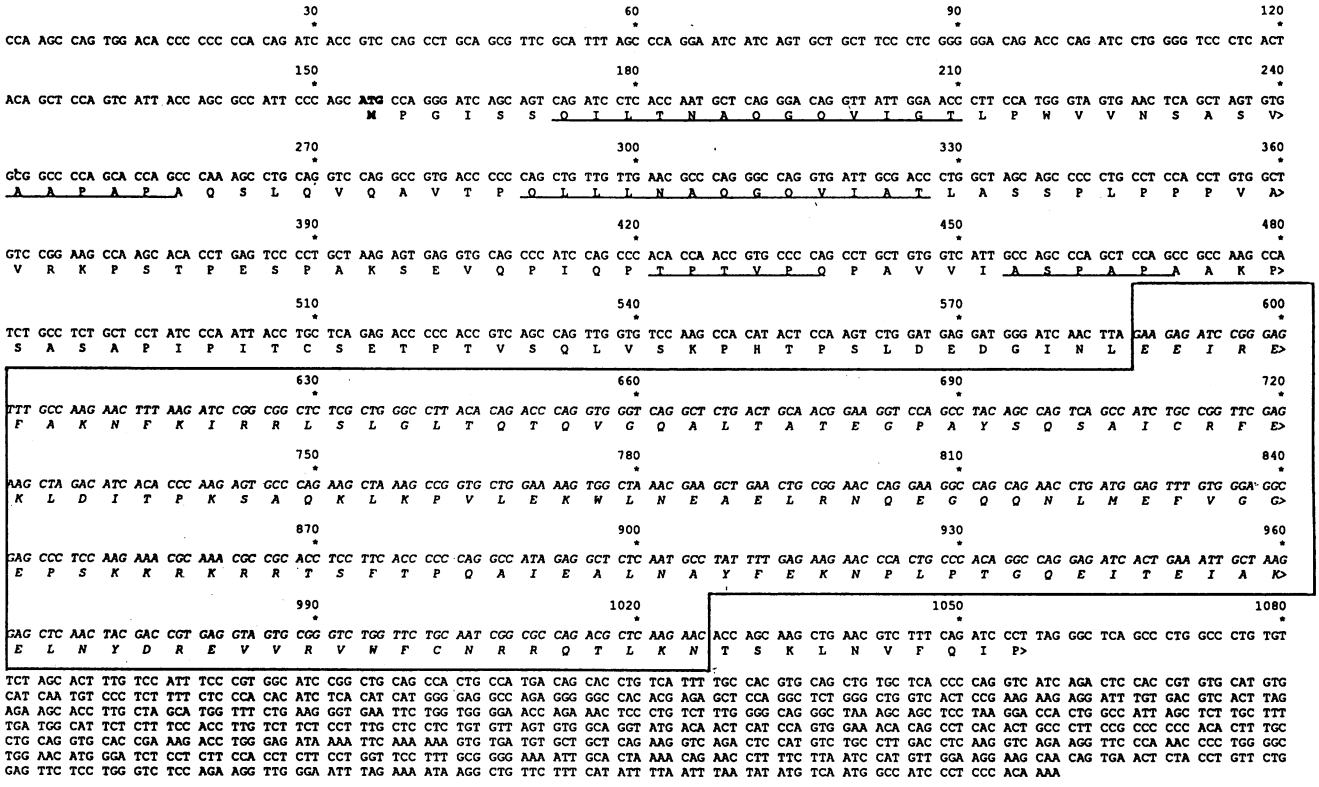

FIG. 3. Nucleotide and predicted amino acid sequences of TCFβ_1. The presumptive initial methionine is shown in boldface type. The conserved POU domain of the TCFβ_1 protein is boxed. The direct repeats in the N-terminal region of the TCFβ_1 protein are underlined. We have sequenced 10 cDNA clones (~1.4 kb long) from Jurkat, human tonsil, and human thymus cDNA libraries to verify the identical TCFβ_1 coding sequence. The 3′ end of the cDNA sequence (terminal 546 nt) was obtained from one Jurkat cDNA clone by polymerase chain reaction amplification using λ-specific primers. The complete cDNA sequence thus obtained completely matched the corresponding genomic sequence.

a sequence-specific manner (Fig. 2C), thus suggesting that the gene encoding a E4 motif-binding protein had been cloned.

TCFβ_1 sequence and relationship with other POU domain proteins. Sequence analysis revealed that all of the clones encode the same gene, which was named the TCFβ_1 gene. The TCFβ_1 cDNA sequence and a conceptual translation are shown in Fig. 3. Data base searches revealed that the TCFβ_1 gene was a novel gene. A conceptual translation of the transcript identified a region homologous to the POU domain in the carboxyl end of the protein (Fig. 4). This similarity extended through the POU-specific domains A and B and the POU homeodomain. The WFC motif, which is conserved in all POU domain proteins within the POU homeodomain, is also present in the TCFβ_1 protein.

Although TCFβ_1 is clearly a POU domain protein, it has significantly more divergence (~26%) at the consensus POU residues than does any other POU protein (<8%). Since the POU domains of all known POU proteins are more closely related to each other than TCFβ_1 is to any one of them, we suggest that TCFβ_1 is a novel POU protein which belongs to an as yet undescribed POU subfamily. In accordance with the nomenclature described earlier (40), we have tentatively classified TCFβ_1 as the only known member of POU domain subfamily VI. The divergence in amino acid identity of TCFβ_1 from other known POU proteins is most significant in the POU-specific domain A region. TCFβ_1 shows ~48% identity with other POU members, whereas the other POU proteins are ~70% identical when compared with each other in the POU-specific domain A region.

The sequence of TCFβ_1 differs dramatically outside the POU domain. Three direct repeats, QLLLNAQGQVIATL, AAPAPA, and TPTVPQ, can be identified in the proline-rich N-terminal portion of the TCFβ_1 protein. Direct repeats have also been identified in the proline-rich activation domains of some transactivators, such as AP-2 and CTF-1 (29, 54). A proline-rich activation domain has also been identified in the Oct-2 protein (34). The N-terminal region of TCFβ_1 also has a high β-pleated sheet content, as suggested by Chou-Fasman and Robson-Garnier algorithm analysis of the TCFβ_1 amino acid sequence. Two other POU domain transactivators, Pit-1 and Oct-3/4 have a similar high β-pleated sheet content in this region. These structural characteristics suggest that TCFβ_1 is a novel DNA-binding protein which regulates transcription.

Genomic organization of the human TCFβ_1 gene. The two partially overlapping genomic clones which encode TCFβ_1 were obtained by screening a human DNA Charon 4A library with the TCFβ_1 cDNA probe. TCFβ_1 is encoded by six coding exons (Fig. 5) in a region spanning <15 kb of the human genome. Exon 6 encodes most of POU-specific domain B and the POU homeodomain. POU-specific domain A and the 5′ end of POU-specific domain B are encoded in exon 5. It is of interest that the splice donor at the end of exon 2 is not the prototypic GT but rather GC (Table 3). Although this is a potential site for alternative splicing,

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GENE  CLASS ---------- POU Specific Domain ---------- ----- Linker -----
             --POU Specific Domain A--      ----POU Specific Domain B---
Pit-1    (I) RELEQFANEFKVRRIKLGYTQTNVGEALAAVHGSEF   SQTTICRFENLQLSFKNACKLKAILSKWLEEAE QVGALYNEKVGANE
Oct1    (II) EELEQFAKTFKQRRIKLGFTQGDVGLAMGKLYGNDF   SQTTISRFEALNLSFKNMCKLKPLLEKWLNDAE NLSSDSSLSSPSALNSPGIEGLS
Oct2    (II) EELEQFARTFKQRRIKLGFTQGDVGLAMGKLYGNDF   SQTTISRFEALNLSFKNMCKLKPLLEKWLNDAE TMSVDSSLPSPNQLSSPSLGFEPAG
cfla1 (III) DDLEAFAKQFKQRRIKLGFTQADVGLALGTLYGNVF   SQTTICRFEALQLSFKNMCKLKPLLQKWLEEAD STTGSPTSIDKIAAQG
Brn-1 (III) DDLEQFAKQFKQRRIKLGFTQADVGLALGTLYGNVF   SQTTICRFEALQLSFKNMCKLKPLLNKWLEEAD SSTGSPTSIDKIAAQG
unc-86 (IV) RQLETFAEHFKQRRIKLGVTQADVGKALAHLKMPGVGSLSQSTICRFESLTLSHNNMVALKPILHSWLEKAE EAMKQKDTIGDINGILPNT
Brn-3  (IV) RELEAFAERFKQRRIKLGVTQADVGSALANLKIPGVGSLSQSTICRFESLTLSHNNMIALKPILQAWLEEAE GPQREKMNKPELFNGG
i-pou  (IV) RELEAFAERFKQRRIKLGVTQADVGKALANLKLPGVGAVSQSTICRFESLTLSHNNMIALKPILQAWLEEAE AQAKNKRRDPDAPSVLPAG
Oct3/4  (V) KELEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVF   SQTTICRFEALQLSLKNMCKLRPLLEKWVEEAD NNENLQEICKSETLVQA
TCFβ1  (VI) EEIREFAKNFKIRRLSLGLTQTQVGQALTATEGPAY   SQSAICRFEKLDITPKSAQKLKPVLEKWLNEAE LRNQEGQQNLMEFVGGEPS
Consensus    LE FA  FK RRI LG TQ DVG ALA L    F   SQSTICRFE L LS KNM KLKPLL KWLEEAE
                                     MG      V     T           N A A   I  A  ND D

GENE  CLASS ---------- POU HOMEO DOMAIN ----------
             -Basic-   ---Helix1--      --Helix2--     --Helix3 WFC--
Pit-1    (I) RKRKRRTTI SIAAKDALERHFGEHSKP SSQEIMRMAEELNLE KEVVRVWFCNRRQREKR
Oct1    (II) RRRKKRTSI ETNIRVALEKSFLENQKP TSEEITMIADQLNME KEVIRVWFCNRRQKEKR
Oct2    (II) RRRKKRTSI ETNVRFALEKSFLANQKP TSEEILLIAEQLHME KEVIRVWFCNRRQKEKR
cfla1 (III) RKRKKRTSI EVSVKGALEQHFHKQPKP SAQEITSLADSLQLE KEVVRVWFCNRRQKEKR
Brn-1 (III) RKRKKRTSI EVSVKGALESHFLKCPKP SSQEITNLADSLQLE KEVVRVWFCNRRQKEKR
unc-86  (IV) DKKRKRTSI AAPEKRELEQFFKQQPRP SGERIASIADRLDLK KNVVRVWFCNQRQKQKR
Brn-3   (IV) EKKRKRTSI AAPEKRSLEAYFAVQPRP SSEKIAAIAEKLDLK KNVVRVWFCNQRQKQKR
i-pou   (IV) EKK  RTSI AAPEKRSLEAYFAVQPRP SGEKIAAIAEKLDLK KNVVRVWFCNQRQKQKR
Oct3/4   (V)  RKRKRTSI ENRVRWSLETMFLKCPKP SLQQITHIANQLGLE KDVVRVWFCNRRQKGKR
TCFβ1  (VI) KKRKRRTSF TPQAIEALNAYFEKNPLP TGQEITEIAKELNYD REVVRVWFCNRRQTLKN
Consensus   RKRKKRTSI     K  LE  F   PKP S   IT  A  L LE KEVVRVWFCNRRQK KR
            KRKRR         R           QR T    A       MK  N I      Q
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FIG. 4. Sequence comparison of the POU domain of TCFβ$_1$ with other POU domain proteins. The region compared extends from the POU-specific domains A and B to the POU homeodomain. The POU domain proteins are classified as described by Rosenfeld (40). For the sake of brevity, additional class III (27) and class V (13) POU domain proteins are not shown.

analysis of >20 cDNA clones from Jurkat and human tonsil libraries (data not shown) suggests that it does not occur in lymphoid cells. The organization of the TCFβ$_1$ gene is distinct from that of the single encoding exon found in class III POU domain proteins (27) but similar to that of multiple exons encoding class II POU domain proteins (10). The organization of the multiple exons which encode class II (10), class IV (51), and class VI (Fig. 5) POU domain proteins reveals distinct differences even in the conserved POU domain regions. In Oct-2, exon-intron junctions have been mapped in the middle of POU-specific domain A, at the end of POU-specific domain B, and between helix 1 and helix 2 of the POU homeodomain (10). On the other hand, a single intron-exon junction has been mapped to the basic region of the POU homeodomain in i-pou (51) or POU-specific domain B in TCFβ$_1$ (Fig. 5) POU domain protein. This nonconservation of genomic organization in conserved regions probably reflects the fact that different classes of POU domain proteins arose by convergent evolution.

Tissue distribution of TCFβ$_1$. A potential regulator of TCR β gene expression should be expressed in human T cells. A panel of human T-cell lines (Jurkat, HUT-74, MOLT3, and MOLT4) was found to express TCFβ$_1$ when poly(A)$^+$ RNA was subjected to Northern analysis (Fig. 6A). Under these stringency conditions, Southern analysis revealed that the TCFβ$_1$ cDNA probe detects a single-copy gene (Fig. 6B). TCFβ$_1$ is also expressed in human thymus, as determined by Northern (Fig. 6D) and RNase protection (Fig. 6C) analyses. In addition to being expressed in T lymphocytes, TCFβ$_1$ is

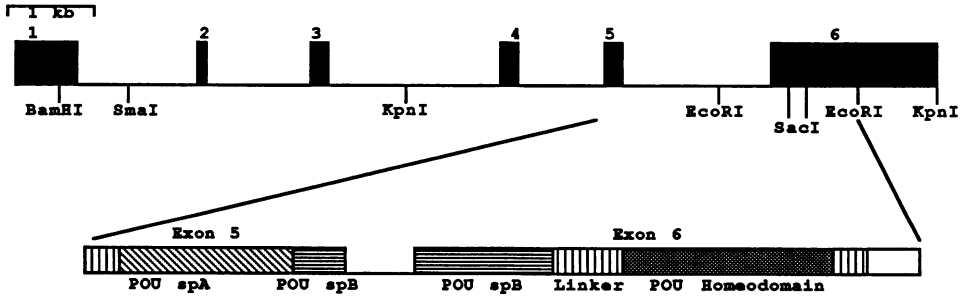

FIG. 5. Genomic organization of the human TCFβ$_1$ gene. The exon-intron boundaries were determined by restriction enzyme analysis, Southern analysis, and sequencing with TCFβ$_1$-specific primers. Exon 1 encodes the 5′ untranslated region of the TCFβ$_1$ gene. The intron between exons 1 and 2 is ~1.4 kb long, the intron between exon 2 (123 bp) and exon 3 (200 bp) is ~1.2 kb long, and the intron between exons 3 and 4 (144 bp) is ~2.4 kb long. The introns between exons 4 and 5 and exons 5 and 6 are ~1 and 1.6 kb long, respectively. Exon 5, which encodes POU-specific domain A (spA) and the 5′ part of POU-specific domain B (spB), is ~165 bp long, whereas exon 6, which encodes the rest of POU-specific domain B, the linker region, the POU homeodomain, and the 3′ untranslated region, is ~1,600 bp long.

TABLE 3. Exon-intron boundaries of the human TCFβ$_1$ gene

Boundary	Sequence[a]
Exon 1	GCCCAGGAATCGTAAGCTG···
cDNA	GCCCAGGAATCATCAGTGCTGC
Exon 2	···ACCCACAGATCAGTGCTGC
Exon 2	CTCAGGGACAGGCAGTGGC···
cDNA	CTCAGGGACAGGTTATTGGAAC
Exon 3	···CCTCCCAGGTTATTGGAAC
Exon 3	CTAAGAGTGAGGTACAGGG···
cDNA	CTAAGAGTGAGGTGCAGCCCAT
Exon 4	···TTTCACAGGTGCAGCCCAT
Exon 4	GTTGGTGTCCAGTAAGTGG···
cDNA	GTTGGTGTCCAAGCCACATACT
Exon 5	···CTGCCTAGAGCCACATACT
Exon 5	GCCATCTGCCGGTGAGTAA···
cDNA	GCCATCTGCCGGTTCGAGAAGC
Exon 6	···GCCCACAGGTTCGAGAAGC

[a] The conserved splice donor and acceptor are underlined.

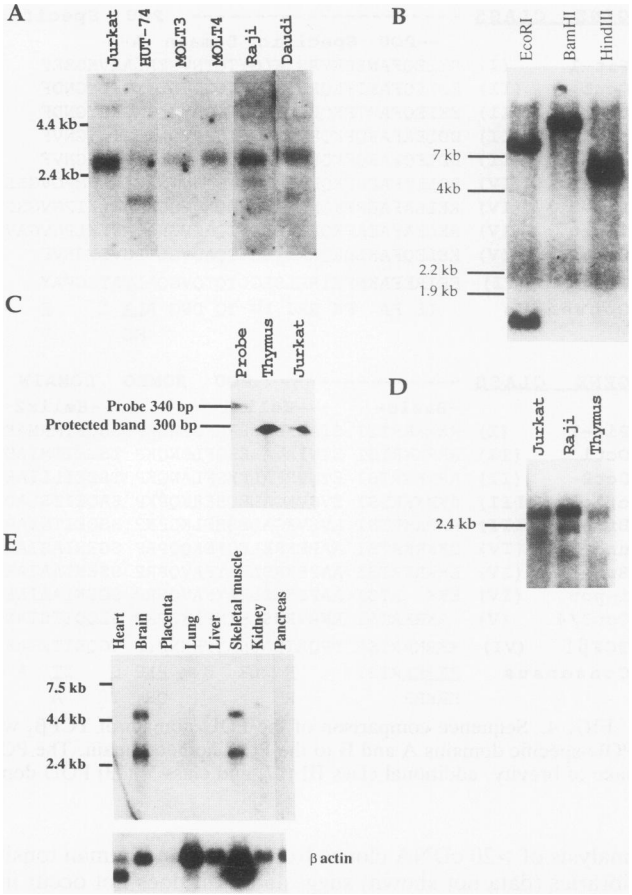

FIG. 6. Expression of the TCFβ$_1$ gene in lymphoid cells. (A) Expression of TCFβ$_1$ in human T- and B-cell lines. One microgram of poly(A)$^+$ RNA was run in each lane. The probe was a full-length Jurkat TCFβ$_1$ cDNA clone. The major transcript is ~2.8 kb. HuT-72 also reproducibly expresses a smaller transcript. (B) Southern analysis of the TCFβ$_1$ gene. The restricted genomic DNA (20 μg per lane) was transferred to nitrocellulose membranes, and the Southern blots were probed with a full-length TCFβ$_1$ cDNA. (C) Expression of TCFβ$_1$ in human thymus, determined in an RNase protection assay. The antisense RNA probe corresponded to nt 675 to 975 of the TCFβ$_1$ cDNA (see Fig. 3A). The probe was 340 nt long, whereas the protected band is 300 nt long. The thymus poly(A)$^+$ RNA was obtained from Clontech. (D) Expression of TCFβ$_1$ in human thymus. One microgram of poly(A)$^+$ RNA was run in each lane, and the Northern blots were probed with a TCFβ$_1$ cDNA probe. The thymus poly(A)$^+$ RNA was obtained from Clontech. The major TCFβ$_1$ transcript in the thymus is ~2.8 kb long. (E) Expression of TCFβ$_1$ in normal human tissues. A human multiple-tissue Northern blot of poly(A)$^+$ RNAs was purchased from Clontech. The probe was a TCFβ$_1$ antisense RNA probe (nt 675 to 975). Identical results were obtained with a labeled TCFβ$_1$ cDNA probe (data not shown). A human β-actin probe was used as a control. In addition to the 2.8-kb TCFβ$_1$ transcript, transcript of ~5 kb is easily detectable in brain and skeletal muscle.

expressed in the human B-cell lines Raji and Daudi (Fig. 6A). The expression of TCFβ$_1$ in both B and T cells is consistent with the reported activity of the minimal TCR β enhancer in some B-cell lines (20, 48). The expression of TCFβ$_1$ in B cells is also of special interest because of the demonstrated role of octamer-binding POU domain proteins in regulating Ig gene expression (5, 17, 24, 34, 42). The major TCFβ$_1$ transcript in lymphoid cells is ~2.8 kb long. HuT-72 T cells also express a smaller TCFβ$_1$ transcript. It is possible that this smaller transcript arises by alternative splicing, although no cDNAs have been identified to support such a claim. The tissue distribution of the TCFβ$_1$ gene was determined by Northern analysis of poly(A)$^+$ RNA from a panel of human tissues. The expression of TCFβ$_1$ in human brain, and skeletal muscle but not in liver, kidney, heart, placenta, or pancreas (Fig. 6E) demonstrates that TCFβ$_1$ is expressed in a lineage-restricted manner. In addition to the 2.8-kb TCFβ$_1$ transcript, 5-kb TCFβ$_1$ transcript is readily identifiable in brain and skeletal muscle.

Mutational analysis of the TCR β enhancer motifs recognized by TCFβ$_1$. To analyze the DNA binding characteristics of TCFβ$_1$, we generated bacterially expressed recombinant TCFβ$_1$. The purified TCFβ$_1$ was visualized on SDS-polyacrylamide gels (Fig. 7A) and found to be >95% pure. As expected, this TCFβ$_1$ preparation binds the βE1 oligonucleotide from the TCR β enhancer (Fig. 7B). The βE1 oligonucleotide completely overlaps the E4A motif, and the flanking sequences stabilize the DNA-protein complex in gel shift assays (data not shown). A panel of βE1 mutants was then analyzed for the ability to abolish the βE1-TCFβ$_1$ DNA-protein complex in gel shift assays. Sequences of the mutants are shown in Table 2. As shown in Fig. 7B, mutants 2 and 3 had a dramatically reduced affinity, whereas mutants 1 and 4 competed as effectively as the wild-type βE1 motif did. The inactive βE1 mutants (mutants 2 and 3) are substituted in the region which is 100% conserved between human and mouse genes (9, 20, 48). The reported inability of mutant 3 to drive transcription in T cells (26) is consistent with the notion that TCFβ$_1$ regulates β enhancer activity.

Earlier studies with the TCR β enhancer have suggested that β enhancer-binding proteins bind to multiple motifs in the β enhancer (9, 48). To ascertain whether TCFβ$_1$ binds multiple motifs, the ability of a panel of TCR β enhancer motifs to abolish the TCFβ$_1$-βE1 DNA-protein complex was determined (Fig. 8). The ability of TCR β enhancer motifs βE2 and βE5 to compete in a gel shift assay suggests that TCFβ$_1$ binds to multiple motifs in the β enhancer. This conclusion is supported by the identification of a mutant βE5 motif (see Table 2 for sequence) which is a less effective competitor than the wild-type βE5 motif (Fig. 8).

TCFβ$_1$ binds to octamer and octamer-related motifs. The

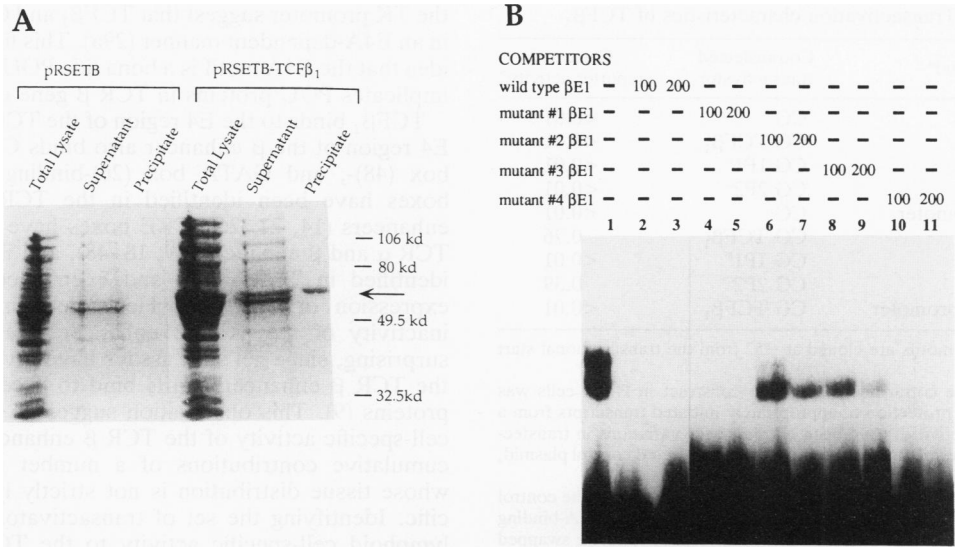

FIG. 7. Characterization of the binding of TCFβ$_1$ to the TCR β enhancer. (A) Generation of bacterially expressed recombinant TCFβ$_1$. The pRSET B vector containing the TCFβ$_1$ insert was used to transform *E. coli* BL21(DE3) containing plasmid pLysS. The TCFβ$_1$ protein was expressed by induction with IPTG and was purified by ammonium sulfate precipitation. The total bacterial lysate, supernatant, and resuspended precipitated TCFβ$_1$ protein were run on parallel lanes on a 10% denaturing polyacrylamide gel. (B) Recombinant TCFβ$_1$ binds the βE1 motif in the TCR β enhancer in a sequence-specific manner. The βE1 motif, which completely overlaps the E4A motif, was used as a probe and incubated with purified recombinant TCFβ$_1$. Wild-type βE1 and a panel of mutant βE1 motifs were used as competitors (100 to 200 ng) in a gel shift assay. Wild-type and βE1 mutants 1 and 4 were effective competitors, whereas mutants 2 and 3 were less effective. Mutant 3 is also inactive in transcriptional assays (26).

mutational analysis also suggests that mutating an AT-rich region to a GC-rich region abrogated binding of TCFβ$_1$ for its cognate motif in the TCR β enhancer. The original lymphoid POU domain proteins Oct-1 and Oct-2 were cloned on the basis of their ability to bind octamer and octamer-related motifs. Similar AT-to-GC mutations in the octamer motif abrogated binding by other POU domain proteins. We therefore wanted to determine the ability of TCFβ$_1$ to bind octamer motifs in a gel shift assay. The Ig octamer probe binds in a sequence-specific manner to TCFβ$_1$. The binding of TCFβ$_1$ to the octamer probe can be competed for with a wild-type octamer motif but not a mutant octamer motif (Fig. 9). Similar results were obtained with the Pit motif (15) from the prolactin promoter (data not shown). These results were confirmed in a gel shift assay with the β enhancer βE1 probe. The wild-type octamer effectively competed, whereas the mutant octamer motif did not (Fig. 9). The ability of TCFβ$_1$ to bind the octamer and octamer-related Pit motifs, not

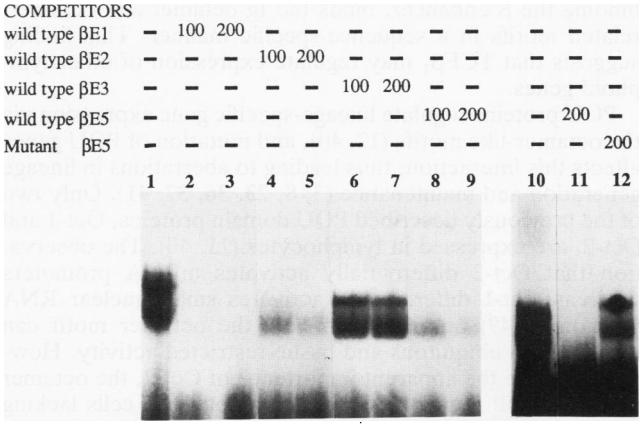

FIG. 8. Recombinant TCFβ$_1$ binds multiple motifs in the TCR β enhancer. The βE1 motif was used as a probe and incubated with purified recombinant TCFβ$_1$. The ability of wild-type βE1 (100 to 200 ng) and other TCR β enhancer motifs (βE2, βE3, and βE5) to abolish the βE1-TCFβ$_1$ DNA-protein complex was determined in a gel shift assay. The mutant βE5 motif is a less effective competitor than the wild-type βE5 motif. See Table 2 for sequences.

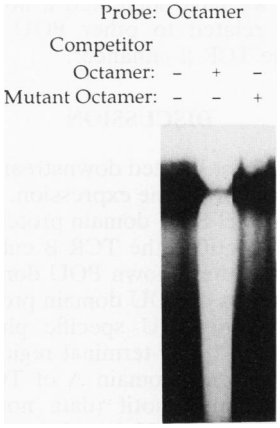

FIG. 9. Bacterially expressed recombinant TCFβ$_1$ binds the Ig octamer motif in a sequence-specific manner. The octamer motif was labeled, and the ability of octamer and mutant octamer motifs to abolish the DNA-protein complex in a gel shift assay was determined. The wild-type octamer motif competed, whereas the mutant octamer motif did not. Consistent with this conclusion, when the TCR β enhancer βE1 motif was used as a probe, the wild-type octamer motif competed, whereas the mutant octamer motif did not (data not shown).

TABLE 4. Transactivation characteristics of TCFβ1

Reporter plasmid[a]	Cotransfected transactivator	Corrected reporter activity[b]
β-Globin promoter	CG	<0.01
	CG TCFβ1	<0.01
	CG 1P1[c]	<0.01
	CG 2P2[c]	<0.01
6× Pit β-globin promoter	CG	<0.01
	CG TCFβ1	0.76
	CG 1P1[c]	<0.01
	CG 2P2[c]	0.39
6× dpm8 β-globin promoter	CG TCFβ1	<0.01

[a] The multimerized motifs are cloned at −52 from the transcriptional start site.

[b] The activity of the cotransfected reporter construct in HeLa cells was determined by RNase protection of appropriately initiated transcripts from a β-globin promoter. Activities have been corrected for variations in transfection efficiencies by determining the activity of a cotransfected control plasmid, pα 4×(A+C) (49).

[c] The cDNAs of the POU domain proteins are expressed under the control of the cytomegalovirus promoter in the CG vector (49). The DNA-binding POU domains of Oct-1 and Oct-2 in 1P1 and 2P2, respectively, are swapped with the Pit-1 POU domain (49).

present in the TCR β enhancer, suggests that TCFβ1 may have additional functional roles in lymphoid cells.

Transactivation characteristics of TCFβ1. The transactivation capability of TCFβ1 was determined in cotransfection analysis using a reporter plasmid in which multimerized Pit motifs are cloned upstream of a minimal β-globin promoter (49). The ability of TCFβ1 to transactivate was determined by RNase protection analysis of appropriately initiated transcripts. The activity of the α-globin transfection control plasmid was also determined by RNase protection assays. The TCFβ1 cDNA when overexpressed from a cytomegalovirus promoter in HeLa cells transactivated in a Pit motif-dependent manner (Table 4). When reporter plasmids with either no motifs or an inactive motif (dpm8) were cotransfected with the TCFβ1 expression plasmid, no transactivation was seen. This finding suggests that TCFβ1 is a transactivator.

In conclusion, we have identified a novel POU domain protein distantly related to other POU domain proteins which binds to the TCR β enhancer.

DISCUSSION

The TCR β enhancer located downstream of the Cβ2 gene segment is essential for β gene expression. In this report, we have identified a novel POU domain protein, TCFβ1, which binds to a critical motif in the TCR β enhancer. TCFβ1 is distantly related to other known POU domain proteins and represents a new class of POU domain proteins. The DNA-binding POU domain (POU specific plus POU homeodomain) is present in the C-terminal region of the protein. Deletion of POU-specific domain A of TCFβ1 reduced its affinity for the octamer motif (data not shown), which suggested that like other POU domain proteins (40), TCFβ1 requires the POU-specific domains for high-affinity DNA binding. A role for TCFβ1 in regulating TCR β enhancer activity is suggested by its comparative inability to bind a mutant motif which is inactive in transcriptional assays (26). The ability of TCFβ1 to bind multiple motifs in the TCR β enhancer and a lineage-restricted expression further support a role in regulating β gene expression. Preliminary cotransfection studies using a multimerized E4A motif upstream of the TK promoter suggest that TCFβ1 and Oct-2 transactivate in an E4A-dependent manner (29a). This finding supports the idea that the E4A motif is a bona fide POU-binding motif and implicates POU proteins in TCR β gene expression.

TCFβ1 binds to the E4 region of the TCR β enhancer. The E4 region of the β enhancer also binds CRE (9, 18, 48)-, E box (48)-, and GATA box (26)-binding proteins. GATA boxes have been identified in the TCR α, β, γ, and δ enhancers (14, 21, 26), CRE boxes have been identified in TCR α and β enhancers (9, 18, 48), and E boxes have been identified in TCR α, β, and δ enhancers (21, 48). The expression of TCFβ1 in HeLa cells in contrast to the inactivity of the TCR β enhancer in HeLa cells is not surprising, since gel shift assays have revealed that none of the TCR β enhancer motifs bind to T-cell-specific nuclear proteins (9). This observation suggested that the lymphoid cell-specific activity of the TCR β enhancer was due to the cumulative contributions of a number of transactivators whose tissue distribution is not strictly lymphoid cell specific. Identifying the set of transactivators which confer a lymphoid cell-specific activity to the TCR β enhancer is essential to our understanding of β gene expression.

The presence of GATA-3, which is expressed in T cells but not in B cells or macrophages, raises the possibility that GATA-3 is important for regulating enhancer activity. It is of interest to note that GATA-3 and TCFβ1 bind to motifs which are partially overlapping. Mutation of the β enhancer motif, which abrogates GATA-3 binding, also decreases binding of TCFβ1 to the E4 motif. It remains to be clarified whether this reflects a functional interaction (cooperative or antagonistic) between TCFβ1 and GATA-3 or suggests differential involvement of the two transactivators at distinct stages of T-cell maturation.

The octamer and octamer-related motifs regulate transcription of both ubiquitous and lymphoid cell-specific genes. POU domain proteins bind the octamer and related motifs. The octamer motif is essential for Ig gene expression, since a nonbinding mutation of the octamer motif in the V_H promoter decreases lymphoid Ig gene expression ~30-fold in the presence of an intact Ig enhancer in transgenic mice (17). Octamer motifs have also been implicated in induction of interleukin-2 gene expression by signals from the TCR (52). TCFβ1 is expressed in both T and B cells and, in addition to binding the β enhancer, binds the Ig octamer and octamer-related motifs in a sequence-specific manner. This finding suggests that TCFβ1 may regulate expression of other lymphoid genes.

POU proteins regulate lineage-specific gene expression via the octamer-like motifs (12, 40), and mutation of POU genes affects this interaction, thus leading to aberrations in lineage generation and maintenance (3, 8, 23, 36, 37, 41). Only two of the previously described POU domain proteins, Oct-1 and Oct-2, are expressed in lymphocytes (11, 40). The observation that Oct-2 differentially activates mRNA promoters whereas Oct-1 differentially activates small nuclear RNA promoters (49) helps explain how the octamer motif can display both ubiquitous and tissue-restricted activity. However, despite the apparent importance of Oct-2, the octamer motif can still regulate mRNA promoters in T cells lacking Oct-2 (52). To account for these observations, Oct-1 protein has been suggested to regulate mRNA promoters (24, 35). However, the identification of a unique Oct-1 activation domain which preferentially regulates small nuclear RNA promoters (49) but not mRNA promoters puts some constraints on such explanations. The identification of a novel POU domain protein, TCFβ1, as described in this report

suggests a more complex regulation of transcription by lymphoid POU domain proteins than previously thought. Future studies of the physiological functions of the TCFβ_1 protein will clarify the relative importance of different POU domain proteins in the generation and maintenance of the lymphoid lineage.

ACKNOWLEDGMENTS

The kind gift of plasmids by Winship Herr is gratefully acknowledged. We acknowledge technical help by Jody Flatt and Pat Fitzgerald, advice from Gottfried Baier, endless discussions with Doug Green, and valuable comments on the manuscript from Bob Margolis.

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