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A Conserved Tissue-Specific Structure at a Human T-Cell Receptor β-Chain Core Promoter

JÖRN-PETER HALLE, PHILIPP HAUS-SEUFFERT, CLAUDIA WOLTERING, GERTRAUD STELZER, and MICHAEL MEISTERERNST*

Laboratorium für Molekulare Biologie-Genzentrum der Ludwig-Maximilians-Universität München, D-81377 Munich, Germany

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The T-cell receptor (TCR) β-chain promoters have been characterized as nonstructured basal promoters that carry a single conserved ubiquitous cyclic AMP-responsive element. Our investigation of the human TCR β gene uncovers a surprisingly complex and tissue-specific structure at the TCR Vβ 8.1 promoter. The core of the promoter (positions −42 to +11) is recognized by the lymphoid cell-specific transcription factors Ets-1, LEF1, and AML1 as well as by CREB/ATF-1, as is demonstrated in gel shift and footprinting experiments. With the exception of LEF1, these factors activate transcription in T cells. Binding sites at the core region show little conservation with consensus sites. Nonetheless, CREB, Ets-1, and AML1 bind and activate cooperatively and very efficiently through the nonconsensus binding sites at the core promoter region. Moderate ubiquitous activation is further induced by CREB/ATF and Sp1 factors through proximal upstream elements. The tissue-specific core promoter structure is apparently conserved in other T-cell-specifically expressed genes such as the CD4 gene. Our observations suggest that both the enhancer and the promoter have a complex tissue-specific structure whose functional interplay potentiates T-cell-specific transcription.

T-cell-specific expression of the T-cell receptor (TCR)–CD3 complex and the CD4-CD8 coreceptors depends on distal enhancer elements (8, 18, 22, 29, 31, 38, 46, 64, 78, 80), which is reminiscent of the B-cell-specific regulation of transcription of the immunoglobulin genes (4). The enhancers contain multiple binding sites for ubiquitous, lymphoid cell- and T-cell-specific factors (reviewed in references 9 and 40). Many T-cell- and lymphoid cell-specific factors were initially identified because they bind to functional important elements of the TCR enhancers. Examples are members of the family of high-mobility-group (HMG) box-containing factors TCF1 and LEF1 (T-cell factor 1 and lymphoid enhancer binding factor 1) (74, 75, 77), the zinc finger protein Ikaros (17, 49), and the runt domain factor PEBP2α (polyomavirus enhancer binding protein 2α). The latter, also called CBFα (core binding factor α), is the murine homolog of human AML1 (52), which is involved in the induction of acute myeloid leukemia (reviewed in references 30 and 50). In addition, the lymphoid cell-specific factors Ets-1, Elf-1, and GATA3 as well as the ubiquitous CREB/ATF family bind to and activate the TCR enhancers (16, 20, 28, 43, 79, 80).

Expression of the TCR genes is dependent on distal enhancers (22, 38, 40, 46). Like other tissue-specific and inducible enhancer elements (for reviews, see references 72 and 73), the TCR enhancers assemble the transcription factors in a complex multiprotein structure (19, 20). The corresponding promoters are thought to contribute little to tissue-specific transcription of TCR genes (40). For example, sequence comparisons of the different β-chain promoters revealed only one conserved element (1, 59, 69). It resembles a cyclic AMP-responsive element (CRE) and is recognized by different members of the ubiquitous CREB/ATF family of transcription factors (2, 16, 39). Mutational analysis of a murine TCR Vβ promoter suggested that the CRE is the only promoter element that is required for transcriptional activation (2). With the exception of the CRE, TCR promoters seemed to be unrelated, based on previous reports (1, 59, 69). On certain individual promoters, functional binding of ubiquitous transcription factors such as Sp1 (37) and TFII-I (42) could be demonstrated. In other cases, the involvement of additional lymphoid cell-specific factors has been predicted but not definitively demonstrated (7, 11, 48, 58, 60).

The TCR enhancers can function through a variety of heterologous and ubiquitous promoters (20, 22). These findings appear to be consistent with a general role of promoters that assemble the basal machinery, being rather nonstructured and neither contributing substantially to tissue specificity nor displaying specificity in promoter-enhancer interactions. In contrast to these descriptions, our investigations of the human TCR Vβ 8.1 promoter suggest that it is both tissue-specifically transcribed and highly structured. We identified several nonconsensus elements at the core region and immediately upstream of it that are recognized and activated by both ubiquitous and lymphoid cell-specific factors. Cooperativity on one hand between factors acting through the core promoter regions and on the other hand between the promoter and the enhancer establishes a mechanism for potentiation of tissue-specific transcription.

MATERIALS AND METHODS

Constructs. All constructs were analyzed by DNA sequencing. Sequences are available upon request. TCR Vβ 8.1 promoter (11, 66) fragments and mutations in the promoter were produced by PCR and cloned into the *Xho*I and *Hin*dIII sites of pGL2-Basic (Promega) upstream of the firefly luciferase reporter gene (Fig. 1). The human TCR β-chain enhancer (positions 150 to 481, numbered according to reference 22) was produced by PCR and cloned into the *Bam*HI site downstream of the luciferase gene. CD4 core promoter sequences (positions −42 to +20) (62) were synthesized and cloned into the *Sma*I and *Bgl*II sites of pGL2-Basic. The vector pGL2-promoter (Promega) contained the simian virus 40 (SV40) promoter. An internal control plasmid was constructed by cloning the β-galactosidase cDNA into *Hin*dIII and *Bam*HI sites of pRc/CMV (Invitrogen). For production of the RNase protection assay probe, the TCR Vβ 8.1 promoter (positions −83 to +11) was inserted into the *Not*I site of pRc/CMV. The cyto-

* Corresponding author. Mailing address: Laboratorium für Molekulare Biologie-Genzentrum der Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, D-81377 Munich, Germany. Phone: 49-89-74017203. Fax: 49-89-74017448. E-mail: meister@lmb.uni-muenchen.de.

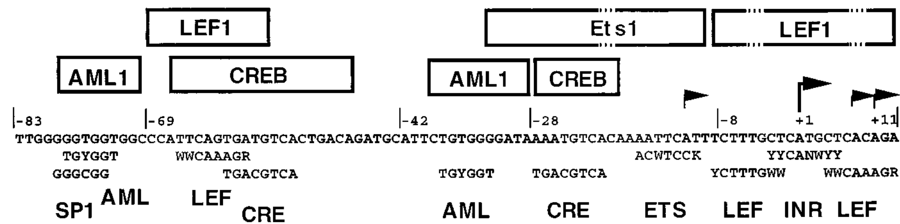

FIG. 1. Summary of structural information of the human Vβ 8.1 promoter, as deduced from data presented in this study. Binding sites for defined regulatory factors as evidenced by footprinting and gel shift analyses (Fig. 4C, 4D, and 5A) are marked by boxes. Consensus sequences of regulatory elements (reviewed in reference 9) and the initiator (INR) (33) are indicated below the corresponding sites. Major (+1) and minor (+7, +9, and −12) transcription start sites as seen in vivo and in vitro (Fig. 6D) are indicated by arrows.

megalovirus (CMV) and TCR Vβ promoters have the same orientation in this construct. An antisense transcript of both promoters was generated with Sp6 polymerase.

Reporter gene assay. Jurkat and HeLa cells were cultivated in RPMI medium containing 10 and 5% fetal calf serum (PAN Systems), respectively. Cells were collected at densities of 5×10^5 to 8×10^5 (Jurkat) or 2×10^5 to 3×10^5 (HeLa) per ml and washed once in phosphate-buffered saline. In standard reactions, approximately 1.6×10^7 Jurkat and 8×10^6 HeLa cells were mixed with 10 μg of luciferase reporter and 2 μg of CMV–β-galactosidase expression plasmids in 400 μl of RPMI medium lacking serum. After 20 min, cells were electroporated in 0.4-cm cuvettes at 960 μF and 250 V (Gene Pulser; Bio-Rad). After an additional 20 min, the cells were reseeded in 10 ml of RPMI medium containing 10% fetal calf serum. For maximum TCR β-gene activity (56), 10 ng of phorbol-12-myristate (Sigma) per ml in dimethyl sulfoxide was added 4 h after electroporation. At 36 to 40 h after electroporation, cells were harvested, washed once in phosphate-buffered saline, and lysed in 150 μl of luciferase lysis buffer (Promega) for 20 min at room temperature. Supernatants were clarified by centrifugation (2 min, 14,000 rpm); 40 μl was mixed with 100 μl of luciferase assay solution (Promega) and analyzed in a Topcounter (Canberra-Packard). β-Galactosidase activity was determined by incubating 30 μl of the supernatant with 270 μl of β-galactosidase assay solution (63) and measurement of optical density at 420 nm. Luciferase units were normalized to β-galactosidase values except when Sp1 expression vectors were cotransfected, which significantly stimulated the CMV promoter; in the latter case, the luciferase values were normalized to total protein concentrations (Fig. 3). All values were corrected by subtracting background activities of a vector lacking the promoter. Data in the figures are the means of at least three experiments ± standard errors of means. When expression plasmids were present in cotransfection studies, total DNA was kept constant with the corresponding empty expression vectors.

RNase protection assays. Poly(A)$^+$ RNA was isolated from cells 24 h after transfection of DNA plasmids by using Qiagen direct mRNA mini kits. The RNA was hybridized to a ^{32}P-labeled probe complementary to the TCR Vβ 8.1 promoter luciferase and CMV–β-galactosidase reporters. RNase protection assays were performed as described previously (63).

In vitro transcription reactions. Preparation and purification of recombinant or native general transcription factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II, and the conditions used for the transcription reactions have been described previously (26, 47, 70). Transcriptions were conducted in the presence of 100 μM all four nucleotides, and transcripts were analyzed by primer extension with an end-labeled primer complementary to the luciferase gene 28 bp downstream of the promoter cloning site with sequence CTTTATGTTTTTGGCGTCTTCCA essentially as described previously (32).

Expression and purification of activator proteins. All recombinant proteins were purified from *Escherichia coli* BL21(DE3). An *Nco*I-*Bam*HI fragment of the CREB cDNA (21) was cloned into pET11d. Recombinant CREB encomprising amino acids 3 to 341 was enriched from *E. coli* extracts by ammonium sulfate precipitation (40%) and subsequently heat treated for 10 min at 55°C. Full-length Ets-1 was expressed and purified as described previously (51). Glutathione *S*-transferase (GST)–Ets-1 (20) was purified on glutathione columns by standard methods. An *Nco*I-*Hin*dIII fragment of AML1 cDNA (52, 20) was cloned in pET28b (Novagen), and the carboxy-terminal His-tagged protein was purified on Ni-nitrilotriacetic acid columns (Qiagen). The human LEF1 cDNA (77) was amplified by PCR with a primer generating an amino-terminal *Nde*I site and cloned into the *Nde*I site of pET11d. LEF1 carrying an amino-terminal His tag was purified on Ni-nitrilotriacetic acid columns.

Footprints and EMSA. The footprints and the electromobility shift assays (EMSA) were performed on purified PCR fragments of the promoter produced with one labeled primer. A labeled coding strand was used except in the CREB footprint, in which the noncoding strand was labeled. Reaction mixtures included 40 fmol of the DNA fragment, 1 μg of poly(dI-dC), and the amounts of proteins indicated in the figures in 20 μl of 25 mM HEPES (pH 8.2)–10% glycerol–4 mM $MgCl_2$–65 mM KCl–5 mM dithiothreitol–0.2 mM phenylmethylsulfonyl fluoride–500 ng of bovine serum albumin per μl–0.002% Nonidet P-40 and were incubated for 30 min at 25°C. In EMSAs, the reactions were directly loaded onto a 4% polyacrylamide gel (50:1 acrylamide to bisacrylamide) in 30 mM Tris-borate buffer (pH 8.2) and run at 5 V/cm. In footprinting experiments, DNase I (Boehringer Mannheim) was added in amounts and for periods indicated in the figure legends. Reactions were stopped, purified, and analyzed on denaturing 8% (19:1) acrylamide-bisacrylamide gels according to standard procedures.

RESULTS

Structure and tissue specificity of the TCR Vβ 8.1 promoter. In an attempt to understand tissue-specific regulation of the TCR β gene, we have reinvestigated the structure and function of the human Vβ 8.1 promoter, which is utilized in the recombined TCR β-chain gene locus in the human CD4$^+$ Jurkat T-cell line (11). The promoter region was initially defined with deletion constructs in transient transfection assays in Jurkat cells (Fig. 2). Promoter activity resided within an approximately 94-bp fragment spanning positions −83 to +11 relative to the major start site of transcription (Fig. 1) (11). Constructs comprising positions −42 to +11 mediated approximately 50% of the overall promoter activity (Fig. 2A) and were sufficient to initiate transcription from the major transcription start site in vivo, as shown in RNase protection assays (Fig. 2B; compare −42/+11 with −83/+11). This region (positions −42 to +11) is subsequently termed the core promoter. Utilizing the deletion constructs, we then investigated the tissue specificity of core and upstream promoter regions by comparing their activities in Jurkat and HeLa cells. The Vβ promoter is active in Jurkat and HeLa cells, although lower luciferase units were measured in HeLa cells. This finding points to the functions of both ubiquitous and lymphoid cell-specific factors. Preferential transcription in T cells is suggested by normalization of luciferase units to either cotransfected CMV promoter/enhancer-driven β-galactosidase activities or activities of the ubiquitously transcribed SV40 promoter. When the SV40 promoter is used as a standard, the promoter is about three times more active in Jurkat cells (Fig. 2A). Normalization to a CMV promoter-driven β-galactosidase gene yielded levels sixfold above the corresponding activities in HeLa cells.

Lymphoid cell specificity resided entirely within positions −42 to +11 at the core promoter region (Fig. 2A). The core promoter could be further subdivided in at least two lymphoid cell-specific subregions. Deletion of the region from −42 to −29 further reduced promoter activity in Jurkat but not HeLa cells (Fig. 2A). Low but reproducible activity was measured solely in Jurkat cells with a minimal promoter construct that included sequences surrounding the initiation start site (positions −8 to +11). A third tissue-specific element, with weak activity, is located between positions −28 and −10, as will be demonstrated below. T-cell-specific activity has previously been reported for the CD4 core promoter (62), which served here as a positive control (Fig. 2A).

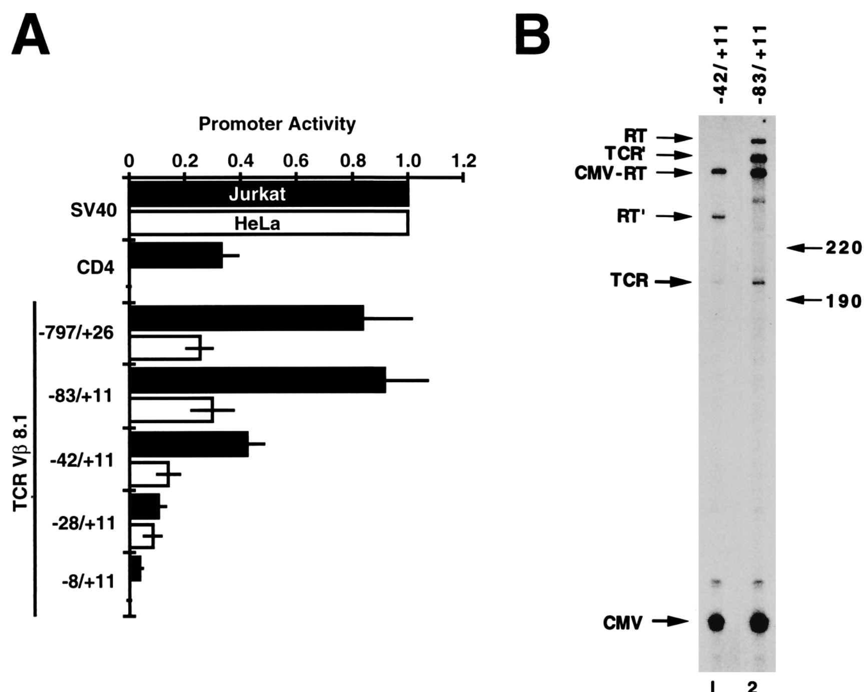

FIG. 2. The TCR Vβ 8.1 promoter is more active in T cells. (A) Deletion constructs of the Vβ 8.1 promoter were analyzed in transient transfection assays in Jurkat (filled bars) and in HeLa cells (open bars). Positions included in constructs relative to the major transcription start site are indicated. CMV–β-galactosidase served as an internal control within one cell type, and the SV40 promoter was used as a standard to normalize activities in HeLa and Jurkat cells. The CD4 promoter (62) construct comprised core promoter sequences from −42 to +20. (B) Analysis of TCR Vβ 8.1 mRNA in vivo by RNase protection. RNA generated by transfected core (−42/+11; lane 1) and full-length promoters (−83/+11; lane 2) in Jurkat cells was analyzed. Protected fragments originating from Vβ promoters (TCR), cotransfected CMV–β-galactosidase (CMV), read-through transcripts of full-length (RT), core Vβ (positions −42 to +11) (RT′), and CMV (CMV-RT) promoters and the positions of RNA size markers (sizes in base pairs) are indicated. The protected transcripts of the TCR Vβ 8.1 promoter were approximately 200 bp, in agreement with the calculated 198 bp for a transcript starting from position +1.

Proximal upstream regions contain binding sites for ubiquitous factors. The CRE conserved in the TCR Vβ promoters is located between positions −53 and −60 at the Vβ 8.1 promoter (Fig. 1). Despite the conservation of the CRE, it had very little effect on promoter activity, as concluded from comparisons of the −69/+11 and −42/+11 deletion constructs (Fig. 3) and various point mutations (data not shown). The effect of the upstream region, which was seen in both HeLa and Jurkat cells (Fig. 2A), was entirely mediated through an element further upstream (positions −83 to −70) which resembled an Sp1 site (Fig. 3). Similar GT boxes (Fig. 1) have

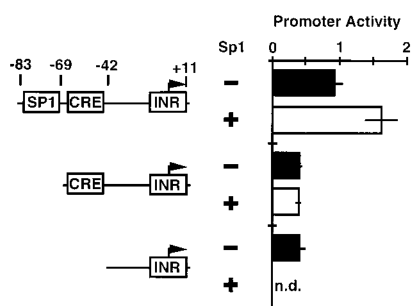

FIG. 3. Sp1 but not CREB activates through upstream regulatory sequences. Deletion constructs comprising positions indicated were analyzed in Jurkat cells in the absence (black bars) or presence (open bars) of 2 μg of cotransfected Sp1 expression vector (CMV-hSp1) (25) if indicated. The promoter was in *cis* and in *trans* dependent on Sp1 and the Sp1 element, respectively, but not on the CREB/ATF site (CRE). Positions relative to the initiation site (INR) are indicated. n.d., not determined.

been characterized as binding sites for Sp1 family members (67) in the TCR Vα (37) and uteroglobin (24) promoters. In support of a function of Sp1 through the Vβ promoter, overexpression of Sp1 in cotransfection experiments moderately but reproducibly stimulated the promoter, and this activation was dependent on the presence of the upstream Sp1 site (Fig. 3). Hence, Sp1 may contribute to ubiquitous activity of the Vβ promoter.

CREB activates the promoter through a cryptic core promoter element. Cotransfection of CREB or ATF-1 expression vectors also elevated promoter activity (Fig. 4A). Surprisingly, the approximately fivefold stimulation of the promoter by CREB did not require the upstream CRE but was solely dependent on the core promoter region, as demonstrated by comparisons of the different deletion constructs (Fig. 4A). Indeed, a partially conserved CREB/ATF binding site is found between positions −28 and −21. The core CRE contains three point mutations in the CREB consensus sequence, retaining one perfectly conserved half site (Fig. 1). Lack of sequence conservation may have prevented the discovery in previous investigations of the core CRE, which is therefore termed a cryptic CRE.

Activation of the core promoter by CREB and ATF-1 was activator specific. AP1, which recognizes related sequences, analyzed through cotransfection of c-*jun* and c-*fos* expression vectors, did not stimulate the promoter (Fig. 4B). Activation by CREB required the protein kinase A-inducible domain, while a mutant that carries a serine-to-alanine mutation at residue 133 (CREB-M1) (21) moderately but reproducibly repressed the promoter (Fig. 4B). This dominant negative effect is indicative of competition of the overexpressed mutant with endog-

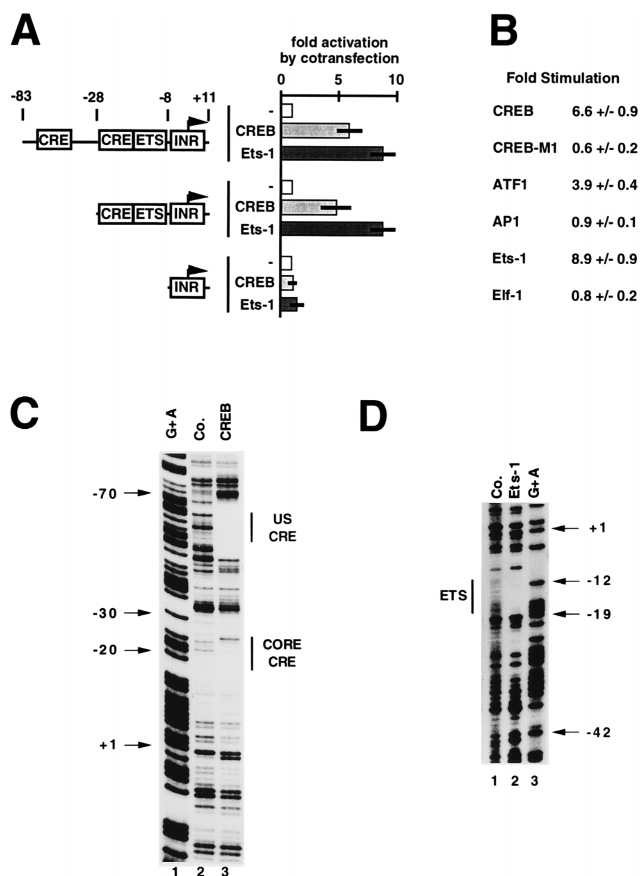

FIG. 4. The Vβ promoter strongly responds to Ets-1 and CREB/ATF-1. (A) Core promoter-dependent activation is demonstrated in cotransfections with 1 μg of expression vectors encoding CREB (pRSV-CREB) (21) or Ets-1 (pEVRF-Ets-1) (20) in Jurkat cells. Activation is dependent on the cryptic CREB (CRE) and Ets-1 (ETS) binding sites located at the core promoter. INR, initiator region. (B) Activation is specific for CREB, ATF-1 (ATF-1 cDNA [82] in pRc/CMV) and Ets-1 and requires the CREB activation domain. CREB-Ser133Ala carrying a mutation in the protein kinase A site is inactive (pRSV-CREB-M1) (21), as are AP1 (c-*jun* and c-*fos*; pRSV-cJ and pTK-FOS24) (36) and Elf-1 (pCMV-Elf-1) (10). Expression plasmids (1 μg) were cotransfected with standard concentrations of full-length Vβ −83/+11 promoter in Jurkat cells. (C) Analysis of protein-DNA interactions in DNase I footprints (10 ng of DNase I for 1 min). Recombinant CREB (1.5 μg; lane 3) binds to the upstream CREB/ATF site (US; positions −61 to −54) and the cryptic core promoter CREB/ATF site (positions −28 to −21), the latter requiring 20-times-higher CREB concentrations. Lane 1, G+A reaction; lane 2, control (Co.). (D) Recombinant GST–Ets-1 (5 μg; lane 2) protected positions −9 to −32 in DNase footprinting experiments. A typical hypersensitive site of ETS domain proteins (23) is visible at position −19. Lane 1, control; lane 3, G+A reaction.

enous CREB/ATF-1 and provides indirect evidence that CREB/ATF proteins function through the core promoter at physiological concentrations at least in certain cell lines. The function of CREB/ATF-1 transcription factors through the core may also explain the activity exhibited by the region spanning positions −28 to −9 in HeLa cells (compare −28/+11 and −8/+11 in Fig. 2A).

Specific binding of CREB to the promoter was also demonstrated in in vitro footprinting experiments. Recombinant nonphosphorylated CREB expressed in and purified from *E. coli* bound to both the upstream and the low-affinity core CRE (Fig. 4C), although the conserved upstream CRE was protected at 20-fold-lower concentrations. These data not only suggest a function of CREB through the Vβ core promoter but may also indicate that CREB proteins activate more efficiently in close proximity to the initiator region (Fig. 1). Beyond this promoter function of CREB, we have evidence that the upstream CRE mediates enhancer function, which may provide a reasonable explanation for its conservation in the different Vβ promoters (27).

Overexpression of Ets-1 efficiently activates the core promoter. Our initial characterization of the promoter (Fig. 2A) indicated the participation of additional factors at core promoter regions, which are restricted to Jurkat cells. Tissue-specific core promoter activity has previously been described in the context of the CD4 promoter, which is activated by the lymphoid cell-specific Ets-1 protein (62). We could reproduce this finding by showing (i) that the CD4 promoter is inactive in HeLa cells but active in T cells (Fig. 2A) and (ii) that overexpression of Ets-1 switches the promoter on in HeLa cells (27). Here we tested the effects of Ets-1 on Vβ promoter activity, although homologies to Ets-1 consensus sites were weak. However, Ets-1 could very efficiently (up to 10-fold) activate the Vβ promoter (Fig. 4A). Activation depended on the core promoter region which contains a cryptic Ets-1 site (Fig. 1A). Thus, both Ets-1 and CREB activate transcription very efficiently through Vβ core promoter regions. Ets-1 but not the closely related factor Elf-1 stimulated the promoter (Fig. 4B).

To characterize Ets-1 binding, footprinting experiments were conducted with recombinant protein (Fig. 4D). Ets-1 interacted specifically with the core region exhibiting the characteristic hypersensitive site within the protected region (23). Specific binding to the core promoter required high concentrations of expressed Ets-1. This may be partially explained by the known low affinity of Ets-1 for its own recognition site in the absence of other factors (20, 35). In addition, the affinity for the nonconserved Vβ core promoter element between positions −17 and −9 may be particularly low. In contrast to CREB, the concentrations of Ets-1 apparently do not suffice to significantly activate the promoter, as this region of the promoter displayed very little T-cell specificity in Jurkat cells (Fig. 2A). However, it has been reported that Ets-1 concentrations vary during differentiation (6, 44) and activation (5, 55) of T cells, leaving open the possibility that Ets-1 activates the Vβ promoter at some point during development. In any event, we note a potent capacity of Ets-1 to induce the promoter through core promoter elements.

Conserved AML1 and LEF sites at the core promoter. In Jurkat cells, factors other than Ets-1 must be responsible for tissue-specific core promoter effects. Sequence analysis of the core promoter revealed partially conserved binding sites for AML1/PEBP2α/CBF (hereafter termed AML1) between positions −42 and −29 and for TCF/LEF immediately upstream of the initiation site of transcription (Fig. 1). Footprinting experiments proved that recombinant LEF1 and AML1 proteins could indeed bind to the predicted sites (Fig. 5A). Both factors also protected sites in the upstream promoter region against DNase I digestion. These recognition sites overlap the Sp1 and CREB/ATF sites, respectively. Expression of AML1 in Jurkat cells activated the promoter up to threefold (Fig. 5B). This activation was dependent on the core but not on the upstream binding site, which overlaps the Sp1 site. Hence, the lymphoid cell-specific AML protein functions through the core promoter.

Mutations in the upstream LEF1 binding site had no influence on promoter activity (Fig. 6A). In contrast, mutations in the two core promoter LEF1 sites reduced promoter activity (Fig. 6A). The importance of these sites is suggested by their conservation within several TCR Vβ promoters (Fig. 6B). Also, the lymphoid cell-specific CD4 core promoter is remarkably related to the TCR Vβ 8.1 core promoter. Homologies at

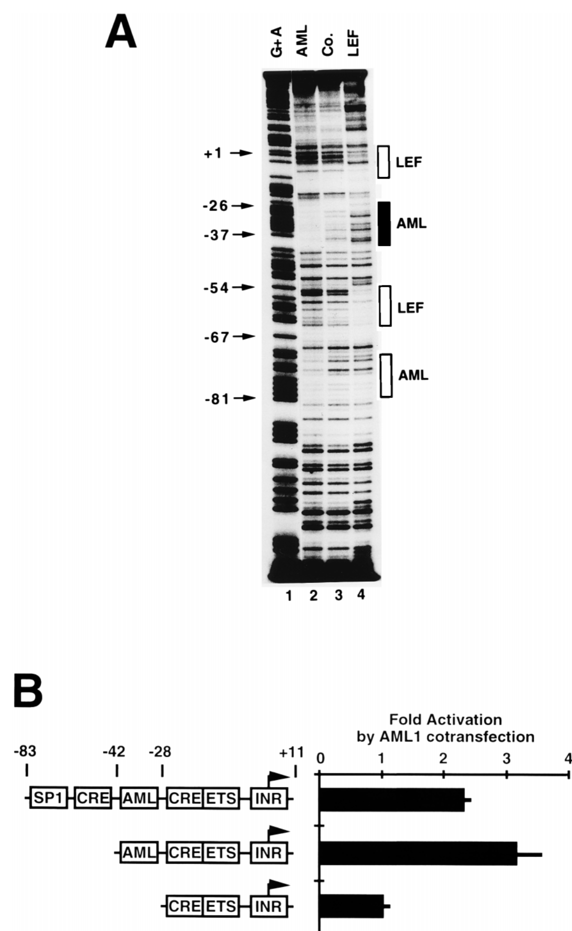

FIG. 5. AML1 (PEBP2α) and LEF bind to and AML1 activates the Vβ promoter. (A) Binding of recombinant DNA binding domain of AML1 (lane 2, 40 ng; lane 3, control [Co.]) and full-length LEF1 (lane 4, 200 ng) was analyzed in DNase I footprinting experiments (10 ng of DNase I for 1 min). On the left, the positions relative to the transcription start site are indicated (lane 1, G+A reaction). AML1 protected positions −39 to −29 and less efficiently positions −79 and −70, and LEF1 protected positions −69 to −58 and positions −8 to +5, as indicated on the right. On the other DNA strand, LEF1 protected positions −8 to +11 (data not shown). (B) Cotransfection of 2 μg of pCG-PEBP2α (20), comprising amino acids 35 to 501 of AML1 (52), activated dependent on the AML1 site (AML) located between positions −42 and −29. Activation of AML1 through the upstream binding site, which was found in in vitro footprinting experiments and which overlaps with the Sp1 recognition site, was not detectable. INR, initiator region.

the CD4 promoter include the initiation start site and the Ets-1, AML1, and both putative LEF1 binding sites but not the core CRE (Fig. 6C). Absence of the latter provides an explanation for the lack of activity of the CD4 promoter in HeLa cells (Fig. 2A). Transient LEF1 expression did not stimulate the promoter in T cells, and we did not yet succeed in reconstituting the activity of the minimal core promoter (positions −8 to +11) in HeLa cells. Hence, we cannot exclude the involvement of other activators. These, however, could also be other members of the TCF/LEF family.

The location of the conserved region in the immediate vicinity of the initiator raises the possibility that it might affect the activity of general transcription factors. The Vβ core promoter contains two basal elements, a residual TATA box and an initiator region. The latter closely resembles the initiator consensus sequence (33) with the notable exception of a G residue at position +3 (Fig. 1A). Mutations within the initiator

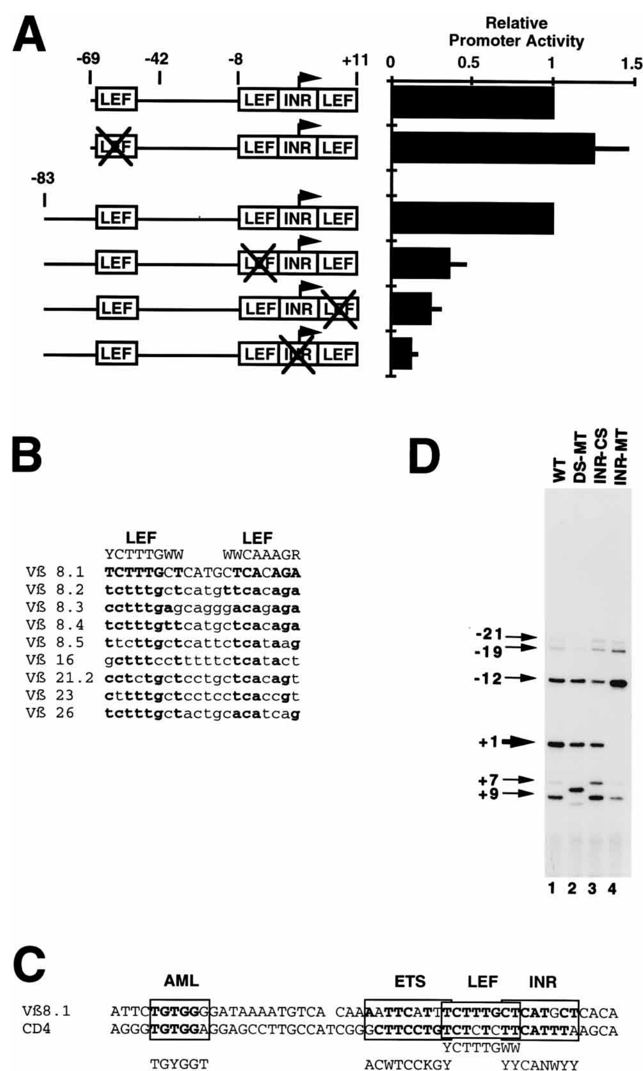

FIG. 6. A conserved functional TCF/LEF-like element at the core promoter. (A) Mutations within both core promoter LEF1 binding sites (LEF), designed to not change the partially overlapping upstream CRE and the initiator (INR) consensus sequences (Fig. 1), but not within the upstream site reduce promoter activity. Mutations were TTCAGTGA to TTCgaTGA (in the −69/+11 construct), TCTTTGCT to TCTggGCT and CTCACAGA to CTCcataA in the three putative LEF boxes, and, to inactivate the initiator, $CTCA_{+1}TGCT$ to $gggA_{+1}TGCT$, all in the −83/+11 context. (B) The sequences surrounding the initiator are conserved in different TCR Vβ promoters, as shown by alignment (1, 69) of the initiator regions and comparison to the consensus sequence of LEF1. Homologies to the consensus sequences are in boldface. (C) Several regulatory elements are conserved between the TCR Vβ and the CD4 core promoter, as shown by alignment of promoters and comparison of consensus sequences of activator recognition sites. Homologies to the consensus sequences are shown in boldface. (D) In vitro analysis of core promoters in a purified transcription system. The wild-type promoter (WT; lane 1), the downstream LEF1 mutation (DS-MT; lane 2), a conversion of the initiator to the consensus sequence ($CTCA_{+1}TtCT$) (INR-CS; lane 3), and an initiator down-mutation as shown in panel A (INR-MT; lane 4) were analyzed by primer extension. Positions refer to the major in vivo transcription start site. Transcription start sites upstream and downstream of +1 are observed as minor transcription start sites that do not substantially contribute to the promoter activity in nuclear extracts in vitro (27) and in vivo (Fig. 1 and reference 11). Note that due to the translation initiation codon at positions +1 to +3, which is out of frame with the luciferase open reading frame and with the TCR β coding region in the native context transcripts starting from upstream sites will probably not contribute to protein expression.

consensus sequence strongly reduced promoter activity (Fig. 6A). The initiator but not the TATA box is important for basal promoter activity. The basal elements alone are not sufficient to confer measurable promoter activity in T cells (27). However, sequences surrounding the initiator might also affect the function of other general factors such as TFIIE (32). We have investigated this issue by analyzing core promoter constructs in a purified HeLa-derived in vitro transcription system. This system contains a complete set of general transcription factors but is devoid of regulatory factors. Transcripts started from several sites in purified systems (Fig. 6D). These were generated by RNA polymerase II, as demonstrated by inhibition with α-amanitin (data not shown). Upstream start sites, i.e., at position −12, probably do not contribute to gene activity in vivo, as the promoter contains an ATG start codon at position +1 (Fig. 1). Mutations in the downstream LEF1 site barely affected transcription from the major start site (Fig. 6D), though they led to qualitative changes at the minor transcription start site at position +9. Similarly, mutations in the second LEF1, the AML, the CREB, and the Ets elements did not affect basal promoter activity (27). In contrast, the mutation within the initiator consensus sequence (positions −3 to +1 from CTCA to GGGA) abolished starts of transcription from position +1 (lane 4). Generation of an initiator consensus sequence, by changing position +3 from G to T, did not influence basal transcription (lane 3). These data show that mutations in the LEF1 sites indeed impair the function of regulatory but not of general factors. While not yet identifying unambiguously the specific activators acting through the LEF1 binding sites, our data provide indications for a T-cell-specific core promoter structure that is conserved in several promoters.

Cooperativity in binding and activation of core promoters. The complex structure of the TCR Vβ 8.1 promoter is remarkably reminiscent of the corresponding enhancer complexes (20, 40). Many of the activators which are shown here to bind to the TCR promoter are also part of the TCR enhancer complexes. The arrangement is not identical but in some situations is similar. For example, ETS elements are usually located in the vicinity of AML1 recognition sites. On the enhancers, these transcription factors bind cooperatively and activate synergistically (20, 79). Similarly, we could show that Ets-1 binding to the cryptic core sites is enhanced, if CREB binds to the adjacent core CRE (Fig. 7A; compare lane 2 with lane 5). Surprisingly, the Ets-CREB complex comigrated with the Ets-1 complex seen at higher concentrations (compare lane 3 with lane 5). Also, the CREB–Ets-1 complex displayed reduced mobility at high Ets-1 concentrations (compare lanes 5 and lane 6). Ets-CREB complexes were supershifted with antibodies against both proteins (data not shown). Hence, the low mobility of Ets-1 alone may be explained by the binding of two Ets-1 molecules at higher concentrations (lane 3).

Cooperativity in binding to the T-cell enhancers has been described in the cases of AML1 and Ets-1 (20, 79). In agreement with these observations, we could also observe cooperative binding of the AML1 DNA binding domain and Ets-1 at the TCR Vβ 8.1 promoter both in EMSAs (data not shown) and in footprinting experiments (Fig. 7B). At limiting factor concentrations, allowing no individual footprints, the combination of Ets-1 and AML1 produced a footprint at the core promoter region (compare lanes 4 and 5 with lane 6). Similarly, binding of CREB to the core promoter assisted Ets-1 binding (compare lanes 2 and 3). In combination, the three factors moderately but significantly protected the core promoter more efficiently (lane 7). We could not detect cooperative binding of CREB and the AML1 DNA binding domain in footprinting experiments (data not shown). However, we cannot exclude

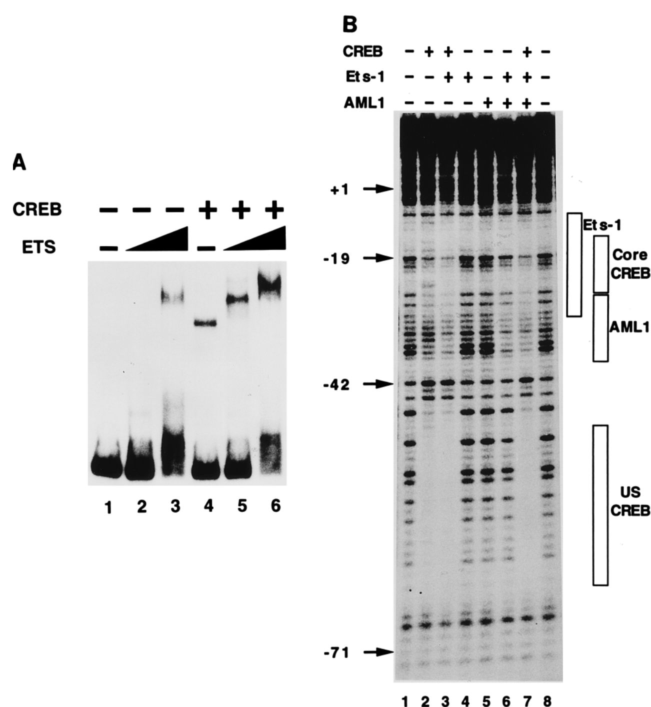

FIG. 7. Cooperative binding of activators to the core promoter. (A) Gel mobility shift experiments using a core promoter oligonucleotide comprising positions −42 to +11. Increasing amounts of Ets-1 (0 ng [lane 1 and 4], 200 ng [lanes 2 and 5], and 1 μg [lanes 3 and 6]) were analyzed in the absence (lanes 1 to 3) or presence (lanes 4 to 6 [100 ng]) of CREB. CREB and Ets-1 bind cooperatively at lower Ets-1 concentrations (compare lanes 2 and 5), leading to a complex with reduced mobility (lane 5). (B) Footprinting analyses with CREB, Ets-1, and AML1 at limiting activator concentrations. CREB (0.5 μg; lanes 2, 3, and 7), Ets-1 (1 μg; lanes 3, 4, and 6), and the AML1 DNA binding domain (10 ng; lanes 5 to 7) were analyzed on a full-length promoter fragment including positions −83 to +11 and additional restriction sites on both ends; DNase I (40 ng) was applied for 10 s. Binding sites as deduced from single activator footprints (Fig. 4C, 4D, and 5A) are indicated. CREB (lane 2) completely protected the upstream CRE (US) and to a lesser extent the core CRE. Two hypersensitive sites within the core promoter are seen. Addition of limiting amounts of Ets-1 (lane 3), which did not lead to a footprint alone (lane 4), enhanced the core promoter footprint and led to disappearance of the hypersensitive sites induced by CREB. Similarly, the hypersensitive site at −19 observed in the Ets-1 footprint at saturating concentrations (Fig. 4D) disappeared in the CREB–Ets-1 footprint. At limiting concentrations, combinations of AML1 and Ets-1 but not the individual factors protected the core promoter against DNase I digestion (lanes 4 to 6). Addition of CREB to the AML1–Ets-1 complex further enhanced core promoter footprints and protected the upstream CRE (lane 7).

that the full-length AML1 protein cooperates with CREB. It is also possible that cooperativity is further amplified at the level of transcriptional activation. In Jurkat nuclear extracts, the promoter is completely protected at low overall protein concentrations, indicative for cooperativity between the natural factors (data not shown).

Cooperativity between Ets-1, CREB, and AML1 was also seen in cotransfection experiments in Jurkat cells. Ets-1 and CREB stimulated the full-length promoter 8- and 6-fold, respectively, while cotransfection of both expression vectors yielded a 26-fold activation, thereby exceeding additive effects (Fig. 8A). Cooperativity was even more pronounced if limiting amounts of Ets-1 and CREB expression vectors were cotransfected (2.5- and 2-fold, respectively, versus 7.5-fold for the combination [Fig. 8B]). Similarly, combinations of AML1 with Ets-1 and CREB exceeded additive effects (Fig. 8A and B). The combination of the three activators could stimulate the promoter up to 70-fold.

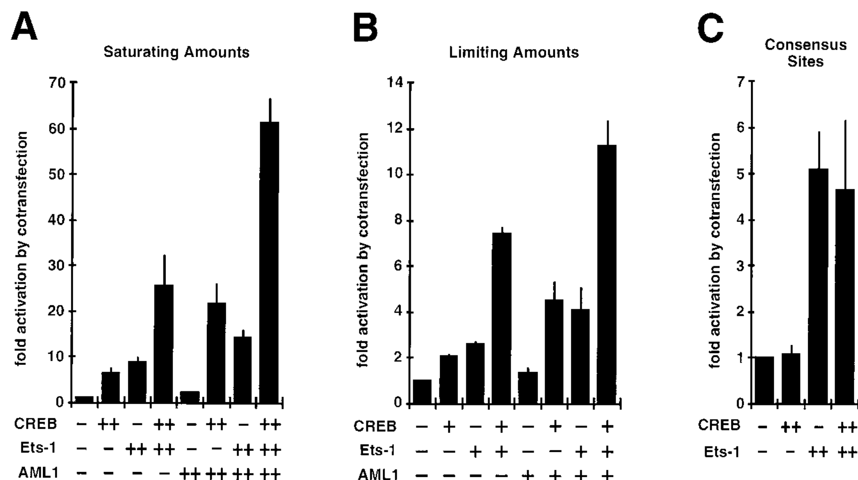

FIG. 8. Cooperative activation by activators that function through the low-affinity binding sites on the core promoter. (A) Ets-1 cooperatively activates with both CREB and AML1. Combinations of 1 μg of CREB, 1 μg of Ets-1, and 2 μg of AML1 expression vectors were cotransfected with the full-length promoter construct (−83/+11). (B) Cooperativity at limiting amounts of expression vectors. Transfections were performed as for panel A, but amounts of the individual expression vectors were reduced fivefold. (C) Consensus core promoter binding sites abolish cooperativity in cotransfection experiments. The core promoter CREB and Ets-1 site were mutated to consensus binding sites (from AAATGTCA to TGACGTCA and from AAAATTCATT to ACACTTCCTG, respectively), and stimulation by CREB and Ets-1 was analyzed as for panel A.

It should be noted, however, that this cooperativity is not sufficient to generate saturating numbers of core promoter complexes. In fact, the promoter responds to overexpression of the different factors under conditions under which the enhancer is not further activated (27). This finding argues for the importance of high-affinity binding sites on enhancers, despite a certain degree of cooperativity that stabilizes the Ets-CREB-AML-promoter complexes in the presence of low-affinity recognition sites. When the low-affinity CREB and Ets-1 core promoter binding sites were changed to consensus, high-affinity sites, the promoter activity increased 10-fold in Jurkat cells (27). This most likely indicated enhanced binding of endogenous factors to the improved sites. Indeed, cotransfection even of high amounts of the CREB expression vector did not further stimulate the promoter, arguing for complete occupancy of the up-mutated CRE. Similarly, relative activation by Ets-1 cotransfection was reduced by the improved core promoter binding sites (compare Fig. 8A and C). Interestingly, the consensus sites abolished the cooperativity of CREB and Ets-1 observed on the wild-type promoter. Hence, low-affinity core promoter sites allow for cooperative concentration-dependent regulation of the promoter activity, whereas high-affinity sites elicit a constitutive activity.

Tissue-specific promoter structure is important for enhancer and overall gene activity. In agreement with the lack of expression of the TCR β gene in the absence of the enhancer, the promoter accounts for only about 1% of the overall gene activity (Fig. 9). A construct comprising positions −83 to +11 is 100-fold stimulated by the native enhancer cloned at a distal position. This experiment demonstrated that the 94-bp promoter construct was necessary and sufficient to facilitate maximal enhancer function. Deletion of the regulatory elements of the promoter not only reduced the promoter activity (Fig. 2A) but also strongly impaired relative enhancer and absolute gene activity (Fig. 9). Furthermore, core promoter regions comprising positions −42 to −9 efficiently raised enhancer activity. Hence, the regulatory regions at the promoter, including T-cell-specific elements (Fig. 9 and reference 27), cooperate with the enhancer, with overall gene activity exceeding synergistic levels. This observation provides evidence for the relevance of the promoter structure in the presence of the enhancer and establishes a mechanism for potentiation of tissue-specific expression of the TCR β gene.

DISCUSSION

Many tissue-specifically expressed genes are regulated by highly structured cell-specific distal enhancer elements. Enhancers act through promoters that are frequently controlled by ubiquitous activators and general transcription factors. Likewise, the family of TCR β genes appeared to employ nonstructured, ubiquitous, weak basal promoters that contain a single conserved regulatory CRE. Our analysis of the human TCR Vβ 8.1 promoter suggests that this picture is superficial, at least in the case of the Vβ 8.1 promoter. In contrast to previous investigations, we find a complex and tissue-specific structure that is important for promoter activity, structurally resembles the TCR enhancers, and potentiates both promoter

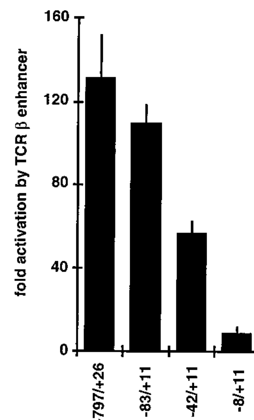

FIG. 9. Efficient enhancer function requires regulatory promoter regions. Promoter constructs were analyzed in combination with the TCR β enhancer in a distal position (2.5 kbp from the promoter). The relative stimulation of transcription by the enhancer is given.

and enhancer activity. The core region of the promoter, here defined as positions −42 to +11, harbors several cryptic regulatory elements. Certain activators such as Ets-1 and CREB are shown to function surprisingly well through the low-affinity binding sites at core promoters in the vicinity of the start site of transcription. Interestingly, the tissue-specific structure of the Vβ promoter appears to be conserved in the CD4 promoter and in part also in other TCR Vβ and the Vα promoters. Thus, these observations might be relevant in the context of other genes.

CREB activates efficiently through cryptic binding sites at the core promoter. The structure and tissue specificity of the Vβ 8.1 and other promoters may have been underestimated in earlier investigations for two reasons. First, many of the functional elements may contain nonconsensus low-affinity binding sites for regulatory factors that are not detected by sequence comparisons but are cooperatively bound by regulatory proteins. Second, promoter activity is mostly low, reaching approximately 1% of the overall gene activity in the presence of distal enhancers, making it difficult to measure the influence of the individual sites at the promoters, whose characterization is now facilitated by more sensitive reporters. Generally, low promoter activity is consistent with the in vivo observations explaining, for example, lack of TCR expression in the absence of enhancers in animals.

In addition to identifying several tissue-specific elements, we could also confirm the contribution of ubiquitous proteins such as Sp1 and CREB to promoter activity. The CRE is highly conserved, and also Sp1 recognition sites have been described in the context of others such as the TCR Vα promoter (37). CREB/ATF factors bind to the upstream CRE that is conserved between most TCR Vβ promoters (1, 2, 16, 39, 59). Surprisingly, CREB and also ATF-1 activate transcription efficiently through the core region but barely affect promoter activity through the conserved upstream CRE. The conserved high-affinity element could also not modulate promoter activity in *cis*. This may be understandable in the light of recent investigations which showed that the upstream CRE is more important for enhancer than for promoter activity (27). Potentiation of enhancer activity provides a very efficient mechanism to raise overall gene activity, which exceeds by far the activity of the isolated intact promoter. Similar functions were assigned to the Sp1 site and other regulatory elements in the core promoter (27).

Nonetheless, the capacity of the low-affinity CRE at the core promoter is surprising, considering that the inactive high-affinity CRE is located only 30 bp further upstream (Fig. 1A). Similarly, Ets-1 functioned more efficiently in the proximity of the start site of transcription at both the Vβ and CD4 promoters (27). Although we cannot fully exclude the involvement of other regulatory components in activation of Ets and CREB through sites between positions −30 and −10 relative to the major start site of transcription, at the present time there is no evidence for it. No additional ubiquitous regulatory elements were detected in core promoters, and CREB and Ets-1 activated transcription equally well in HeLa and T cells.

On certain genes, activators such as E2F (68) have been shown to function through the core region, while the vast majority of regulatory sites are located upstream of core promoters. The basal transcription machinery assembles at core promoters covering large regions extending from positions −40 to +20, or even further downstream, in part on both sides of the DNA (recently reviewed in reference 53). This implies that core activators might sterically interfer with preinitiation complex assembly and thereby inhibit transcription. However, for reasons that remain to be elucidated, CREB and Ets-1 induce transcription very efficiently at core promoters.

Tissue specificity of the Vβ 8.1 promoter. The Vβ promoter is targeted by several tissue-specific factors, including Ets-1, LEF1, and AML1. The relevance of individual elements in vivo is difficult to predict. Although we have not yet performed these studies in different lymphoid cell lines, several reasons argue for their relevance. Ets-1, LEF1, and AML1 proteins are expressed early in the thymus and throughout T-cell development (9). All factors are integral parts of individual, and some are integral parts of all, members of the distal TCR enhancer complexes (9, 20, 40). Moreover, Ets-1 and AML1 activate the Vβ promoter when introduced in nonlymphoid cells. The position of the Ets-1 site is precisely conserved in the CD4 promoter, and ETS-like elements have been found in the TCR Vβ 2 and the TCR Vδ 1 promoters (48, 58).

Specific binding, the conservation of the recognition site, and the T-cell specificity of the elements surrounding the start site of transcription suggest a role of the group of LEF/TCF HMG box proteins in regulation of the Vβ promoter. Different members of the HMG box proteins have been isolated; some are found in various tissues, which could complicate the analysis of defined proteins such as LEF1 (12, 74, 75, 77). It is also possible that LEF1 requires other components present in the genomic environment or exhibits a more complex architectural role which might not be detectable in transient transfection assays (20, 65).

As described above, we also found ubiquitous proteins that raise promoter activity. However, it is possible that the influence of lymphoid factors was underestimated by using the SV40 promoter as a standard. The SV40 promoter is activated by Sp1 (13), which has been reported to be present in high concentrations in T cells (61). Further in support of this assumption, normalization of luciferase activities to CMV promoter activities yielded even higher activities of the Vβ promoter in T cells. Earlier investigations, indicating T-cell-specific elements at the Vβ promoter without defining factors acting through these upstream elements (11, 60), were not confirmed under our experimental conditions.

Cooperativity at the core promoter and between promoter and enhancer. Ets-1, CREB, and AML1 bind and activate transcription cooperatively at the Vβ core promoter. This is reminiscent of the TCRα and other TCR enhancers (20, 79) on which Ets-1 and AML1 bind cooperatively to adjacent sites. Here we also describe cooperativity between Ets-1 and CREB in both binding and transcriptional activation. This was not evident in earlier direct protein-protein interaction studies, conducted in the absence of DNA, in which Ets-1 interacted specifically with ATF-2 (20). While we cannot exclude a role of ATF-2 at the promoter, our data suggest that cooperativity between Ets-1 and CREB requires the presence of the two adjacent DNA recognition sites. Somewhat surprising, Ets-1 but not by Elf-1 acted through the core promoters, which is not expected from the binding site at the promoter (76). This may indicate that regions other than the DNA binding domains are involved in cooperative interactions at core promoters. Alternatively, Elf-1 may not have the capacity to activate transcription at core promoters.

Cooperativity probably facilitates factor binding to the low-affinity sites and thereby potentiates tissue-specific transcription. For example, CREB is ubiquitously expressed, Ets-1 is expressed in both T and B cells, and the hematopoietic expression of AML1 is restricted to thymocytes (9). Interactions between the proteins that promote complex assembly might also support looping of distal enhancers toward promoters (3, 41, 45, 54, 57, 71). Given the increasing numbers of regulatory

elements located in the core promoter regions of enhancer-regulated genes (14, 15, 34, 62, 81), cooperation between activators on the core promoter and the enhancer may not be restricted to TCR genes.

Indeed, the promoter structure is important for enhancer activity, as the complete but not a minimal promoter is efficiently activated by the distal TCR Vβ enhancer in Jurkat cells. While we have not investigated physical enhancer-promoter interactions, potentiation of enhancer activity by the regulatory promoter regions clearly underlines the relevance of this promoter study and establishes an intriguing mechanism for amplification and regulation of T-cell-specific transcription.

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