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Transcription from a Murine T-Cell Receptor Vβ Promoter Depends on a Conserved Decamer Motif Similar to the Cyclic AMP Response Element

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We identified a regulatory region of the murine Vβ promoter by both in vivo and in vitro analyses. The results of transient transfection assays indicated that the dominant transcription-activating element within the Vβ8.3 promoter is the palindromic motif identified previously as the conserved Vβ decamer. Elimination of this element, by linear deletion or specific mutation, reduced transcriptional activity from this promoter by 10-fold. DNase I footprinting, gel mobility shift, and methylation interference assays confirmed that the palindrome acts as the binding site of a specific nuclear factor. In particular, the Vβ promoter motif functioned in vitro as a high-affinity site for a previously characterized transcription activator, ATF. A consensus cyclic AMP response element (CRE) but not a consensus AP-1 site, can substitute for the decamer in vivo. These data suggest that cyclic AMP response element-binding protein (ATF/CREB) or related proteins activate Vβ transcription.

The surface expression of a competent antigen receptor is essential for the proper development and functioning of thymus-derived lymphocytes. As T cells mature within the thymus, the specific interactions of the T-cell receptor (TCR) with products of the major histocompatibility complex are central to the processes by which self-tolerance and major histocompatibility complex restriction are generated (36). Subsequently, peripheral T cells recognize foreign antigen in the context of self-major histocompatibility complex products and are activated for host defense functions via specific TCR binding. The TCR mediates ligand binding through the clone-specific α/β heterodimer, which exists in noncovalent association with the invariant chains of CD3 (47).

The α and β chains of the TCR are similar to the immunoglobulin molecules in both protein structure and gene organization (28). These proteins contain an amino-terminal variable portion and a carboxy-terminal constant (C) region. The variable portion of the β chain is encoded in the germ line in separate variable (V), diversity (D), and joining (J) gene segments, while the α chain is generated from V and J segments alone. During T-cell differentiation, functional α- and β-chain V-region genes are assembled at specific developmental stages by the somatic recombination of these germ line elements. Analysis of fetal thymic development has provided a model of this regulated progression in which distinct molecular events appear to control the rearrangement of individual TCR chains (53).

The rearrangement process also displays strict tissue specificity. TCR and immunoglobulin gene elements are thought to utilize a common "recombinase," based on the ability of the shared recognition sequences (nonamer and heptamer) to mediate recombination in both B and T cells (57). Nevertheless, endogenous V elements of immunoglobulin and TCR chains remain in germ line configuration except in the appropriate tissue. One model of regulated recombination proposes that transcriptional activity at a target locus might impart an altered, i.e., "accessible," chromatin configuration required for rearrangement (1, 2). A detailed knowledge of transcriptional regulation in rearranging genes should aid in formally testing such a model.

The regulation of immunoglobulin gene expression has been studied in great detail. The promoters of both heavy- and light-chain genes were shown to require an octanucleotide motif (15, 17, 37, 43) which functions as the binding site of a B-cell specific transcription activator (30, 40, 51, 54). In addition, enhancer regions have been located within the J–C intron of both heavy- and kappa light-chain genes (7, 19, 45), and the essential *cis*- and *trans*-acting elements have been identified (16, 35, 44, 49). Similar detailed analyses of TCR gene regulation have yielded only limited results. Enhancer regions have been identified downstream of the constant regions of murine α- and β-chain genes (27, 39, 55). Specific *trans*-acting factors and potential protein-binding sites, however, have been only partially characterized. Furthermore, although in vitro analysis of nuclear protein binding to the human Vβ8.1 promoter revealed multiple binding sites, no functional significance could be attributed to these potential regulatory elements (46). Functional dissection of the same promoter indicated a potential enhancing region located between 500 and 800 bases upstream of the transcription start site, though no specific sequence elements were identified (14).

Recently, we and others have reported that sequence comparison of several murine Vβ promoters reveals a well-conserved decanucleotide sequence (consensus AGTGA CATCA) positioned at approximately −70 base pairs (bp) relative to the transcription start site (3, 34). This motif was also found in certain human and rabbit Vβ genes (29, 52). The position, conservation, and sequence similarity to AP-1- and cyclic AMP response element-binding protein (ATF/CREB)-binding sites suggested that the Vβ decamer might participate in transcriptional regulation. In the current report, we indicate that the conserved Vβ decamer is required for high-level expression of a murine Vβ gene, that the

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decamer serves as the binding site of a transcription activator, and that the decamer-binding protein(s) displays properties similar to those of ATF/CREB.

MATERIALS AND METHODS

Plasmid constructions. The intact Vβ locus used for transient transfection analysis was constructed in three parts. First, the functionally rearranged Vβ8.2-Dβ2-Jβ2.4 was cloned from the T-cell clone 2C (50) as a 7-kilobase *Sac*I fragment in LambdaZap (Stratagene) and the VDJ was subcloned into pBluescript as a 900-bp *Eco*RI-*Cla*I fragment. Next, the promoter and first exon of Vβ8.3, isolated as a 1.3-kilobase *Bam*HI-*Eco*RI fragment from phage A6-1 (11), was subcloned upstream of the rearranged Vβ8.2. Promoter deletions were generated by removing the regions between the upstream *Bam*HI site and the *Bgl*II site at −521, the *Acc*I site at −239, the *Ssp*I site at −164, or the *Hpa*I site at −92. Two additional deletions, to −84 and −33, were generated by exonuclease III digestion (ExoIII/Mung kit; Stratagene) of plasmids previously truncated to the *Bgl*II site at −521. The Exo/Mung process created a *Sac*I site at the 5′ end of the −33 construct by introducing a G residue at −34. Mutations were introduced into the region between −95 and −33 by subcloning synthetic oligonucleotides (67mers) into this *Sac*I site. Mutations A to F are base for base substitutions of 5′-GGATCCTAGG-3′ for the native sequence (see Fig. 2). The same strategy was used to create mutant promoters with a consensus AP-1 or cyclic AMP response element (CRE) site by deletion or transition, respectively, of position −56. The sequences of wild-type and mutant promoters created by oligonucleotide insertion were determined by the chain termination method of Sanger et al. (48). Finally, the Cβ2 and flanking regions were added as a *Cla*I-*Sal*I fragment taken from the 3′ end of phage A6-2 (11). A 695-bp *Stu*I-*Nco*I fragment containing a β-chain enhancer (27) was taken from cosmid C45 (50) and added downstream of Cβ2. The polyoma origin and truncated histone 4 (H4) were taken from plasmid MO/MO GPT (21), a gift from R. Grosschedl.

Transient transfection. All cells were maintained in RPMI with 10% fetal calf serum, and transfections were performed by using the DEAE-dextran method. Briefly, 3×10^7 cells were washed twice, suspended at 10^7/ml in TS buffer (21) and 0.5 mg of DEAE-dextran and 10 μg of plasmid DNA per ml, incubated for 30 min at room temperature, washed twice, and suspended in RPMI–10% fetal calf serum at 3×10^5/ml. Cells were cultured at 37°C and harvested after 48 to 72 h. Total RNA was isolated by the acid-guanidinium thiocyanate method as described previously (9).

S1 nuclease analysis. A 50-μg portion of total cellular RNA was hybridized to 10^5 cpm (10 ng) of 5′-end-labeled Vβ8.3 probe (*Rsa*I-*Hpa*I, +61 to −92) in a 15-μl volume containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 1 mM EDTA, and 0.4 M NaCl (pH 6.4) at 45°C for 12 to 16 h. For the *Hpa*II-*Hpa*II H4 probe (21), 10 μg of RNA and hybridization at 55°C were used. Hybrids were digested with 150 U of S1 nuclease (Sigma Chemical Co.) at 37°C for 30 min, and the protected fragments were analyzed by electrophoresis through 8% denaturing polyacrylamide gels, autoradiography, and densitometric scanning.

Nuclear extracts. Nuclear extracts were prepared by the procedure of Heberlein and Tjian (23), except that cells were initially incubated in buffer I without sucrose for 15 min on ice and ruptured by passage (three times) through a 25-gauge needle. Nuclear extracts were dialyzed against 100 volumes of HEMG–0.1 M KCl (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1 M KCl). Nuclear extract from EL-4 cells was partially purified by heparin-agarose chromatography. Extract was loaded on the column and washed in HEMG–0.1 M KCl, and bound proteins were eluted with HEMG–0.4 M KCl. Peak fractions were pooled and dialyzed against 200 volumes of HEMG–0.1 M KCl.

DNase I footprinting. The 5′ ^{32}P-end-labeled probes were prepared by isolating a fragment containing the Vβ8.3 region from −164 to +18. Coding strand probe was labeled at a polylinker *Bam*HI site 5′ to −164, and the noncoding strand was labeled at the *Nru*I site at +18. Labeled probe (2 ng) was incubated in a 30-μl volume containing 15 μl of nuclear extract (or HEMG–0.1 M KCl for control), 2 μg of poly(dI-dC), and 2% polyvinyl alcohol. Footprinting with purified ATF (22), a gift from T. Hai and M. Green, used 12.5 μl of purified protein (approximately 78 ng), 0.2 μg of poly(dI-dC), and $MgCl_2$ at 5 mM. Binding reactions proceeded for 20 min at room temperature followed by treatment with freshly diluted DNase I (Worthington Diagnostics) for 60 to 90 s. The reactions were terminated by the addition of 35 μl of stop solution (20 mM EDTA, 1% sodium dodecyl sulfate, 0.2 M NaCl, 250 μg of tRNA per ml). The samples were purified by phenol-chloroform extraction and ethanol precipitation and fractionated on 10% denaturing polyacrylamide gels. The G+A sequence ladder was generated by the method of Maxam and Gilbert (38).

Gel mobility shift and methylation interference assays. Gel mobility shift assays were carried out essentially as described by Sen and Baltimore (49), using the following probes: Vβ8.3-51mer, a 51-bp *Alu*I fragment from −82 to −32 subcloned into the *Sma*I site of pBluescript and excised with *Bam*HI and *Eco*RI; Vβ8.3-30mer, a double-stranded synthetic oligonucleotide with the sequence from −72 to −43; Vβ8.3-67mers, double-stranded synthetic oligonucleotides of wild-type or mutant sequence extending from −95 to −34 with *Sac*I-compatible overhangs. Mutant 67mers contain substitution of the sequence 5′-GGATCCTAGG-3′ for the wild-type sequence in the regions A to F (see Fig. 2). End-labeled probe (0.1 to 0.5 ng, 10^4 cpm) and various amounts of cold competitor were mixed with 5 μg of nuclear extract in a 20-μl reaction [10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 50 mM NaCl, 2.5 μg poly(dI-dC), 4% glycerol] and incubated for 25 to 30 min at room temperature. Reactions were loaded directly onto 4% polyacrylamide gels and run in 1× TBE (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) for 3 h. Gels were dried prior to autoradiography.

For methylation interference assays, Vβ8.3-51mer probe was end labeled by treating with kinase after digestion with *Bam*HI (coding strand) or *Eco*RI (noncoding strand). A 10-ng amount of probe, partially methylated with dimethyl sulfate as described before (49), was incubated with 125 μg of BW5147 nuclear extract and 30 μg of poly(dI-dC) in a large-scale reaction. Following resolution on a 4% polyacrylamide gel, complexes were visualized by autoradiography and excised, and the DNA was eluted, purified, cleaved with piperidine, and subjected to electrophoresis on denaturing 15% polyacrylamide gels (38).

RESULTS

A critical regulatory region is located between −84 and −33 in the Vβ8.3 promoter. We used transient transfection and

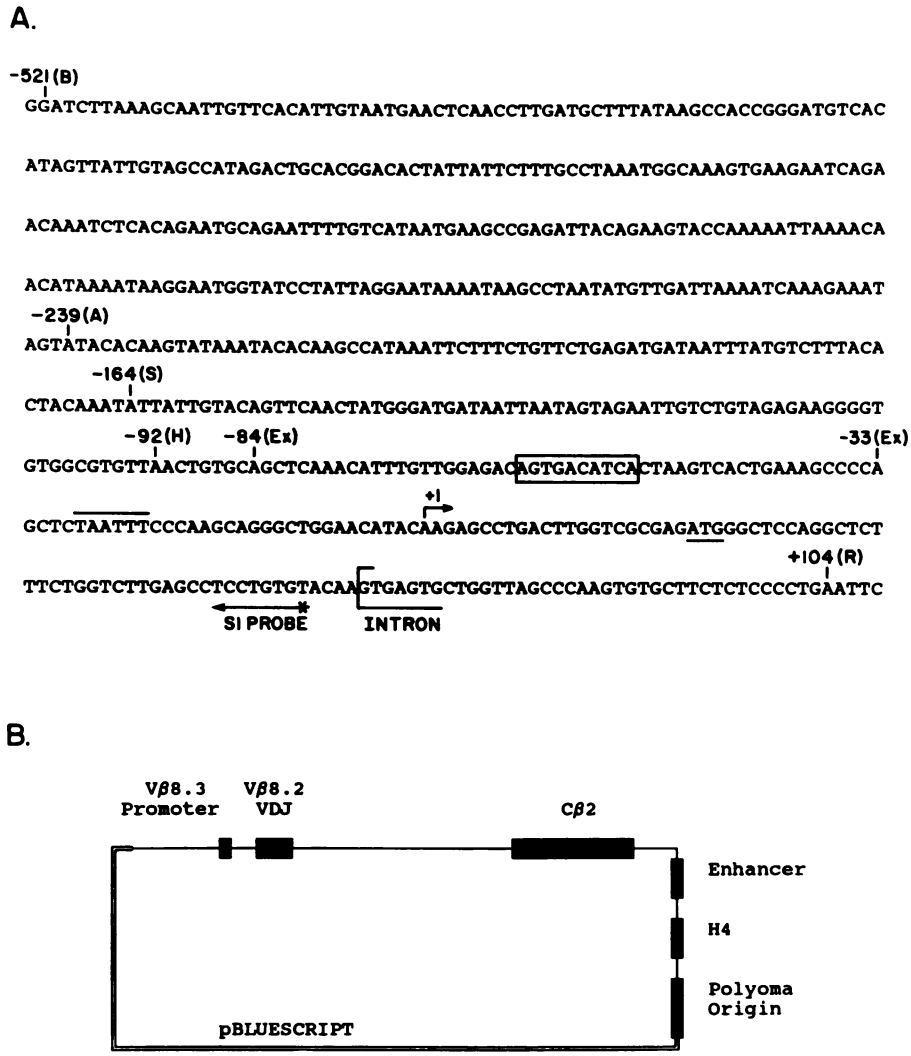

FIG. 1. Vβ8.3 promoter and plasmid constructs. (A) Nucleotide sequence of the Vβ8.3-5′ region from −521 to +104. Indicated are the conserved Vβ decamer motif (boxed), putative TATA element (overline), transcription initiation site (+1), initiating methionine codon (underline), donor splice site and first exon (bracket), and the *Rsa*I site used to end label the S1 nuclease protection probe (*). The 5′-end points of promoter deletion constructs are indicated above the sequence. Enzymes used to generate the deletions are as follows: B, *Bgl*II; A, *Acc*I; S, *Ssp*I; H, *Hpa*I; Ex, exonuclease III. The *Eco*RI (R) site at +104 is common to the first intron of both Vβ8.3 and Vβ8.2. (B) Schematic representation of the transient transfection substrates. H4, Truncated histone 4 gene used as transfection control. Enhancer, 695-bp *Stu*I-to-*Nco*I fragment from the 3′-flanking region of Cβ2.

S1 nuclease protection assays to identify control elements in a murine Vβ promoter. To analyze the promoter in the context of an intact β locus, we constructed a plasmid in which the Vβ8.3 promoter and first exon were placed upstream of the functionally rearranged Vβ8.2-Dβ2-Jβ2.4-Cβ2 (Fig. 1). The Vβ8.3 promoter was used to avoid the alternative splicing events which we described previously for the Vβ8.2 gene (10). S1 nuclease protection of correctly initiated RNA from the Vβ8.3 promoter revealed protected products clustered in a 4-bp span. We indicated the initiation site (+1) as the nucleotide corresponding to the 5′ end of the longest protected product. Initial constructs contained the 7 kilobases downstream of Cβ2 shown to be essential for high-level β expression in transgenic mice (27). Although elimination of this region reduced transcription by about 2.5-fold in transfected EL4 and YAC-1 cells, full activity was restored by the addition of a 695-bp *Stu*I-*Nco*I β-chain enhancer fragment (data not shown). The polyoma origin, which allows for plasmid replication in murine cells, was required to obtain detectable RNA levels from all constructs. The plasmids also contain a control promoter and gene (truncated H4) for use in determining the transfection efficiency of each plasmid (21).

A series of 5′-to-3′ deletions of the Vβ8.3 promoter was generated and tested for transcriptional capacities by transient transfection into T cells (BW5147 and YAC-1). Initial experiments showed that deletion of the region between −1300 and −521 did not affect the level of Vβ8.3 transcription (data not shown). Further deletion of the sequences between −521 and −84 resulted in a promoter with nearly full activity (Fig. 2A). Quantitation by densitometric scanning of autoradiographs from four separate transfections indicated that the −92 and −84 promoters yield, on average, >80% of the level of correctly initiated transcripts produced by the full (−521) promoter. Deletion of the sequences between −84 and −33, however, reduced the transcriptional

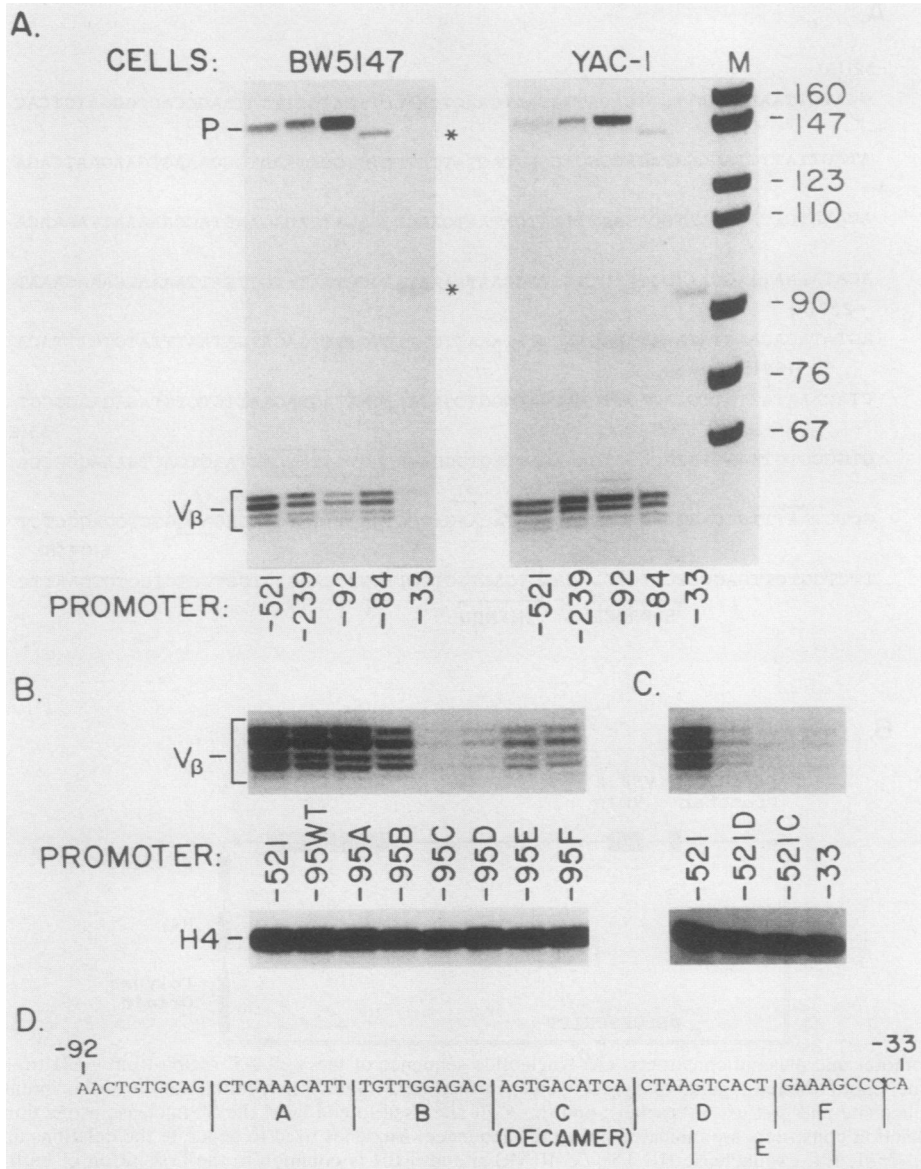

FIG. 2. Deletion and mutation of the Vβ8.3 promoter, revealing a critical element. (A) S1 nuclease protection of RNA from BW5147 and YAC-1 cells transfected with Vβ8.3 promoter deletion constructs. The 5′-end points (−521 to −33) of the promoters tested are indicated below each lane. Protected fragments corresponding to correctly initiated Vβ8.3 transcripts are indicated by the bracket. Protected fragments corresponding to readthrough transcripts are detected at the full length of the probe (P) or at positions marked by the asterisks. Lane M, End-labeled *Msp*I-digested pBR322 DNA as size marker, with sizes indicated in base pairs. (B) S1 nuclease protection of RNA from BW5147 transfected with wild-type or mutated Vβ8.3 promoter constructs. Promoters extending 5′ to −95 were generated by oligonucleotide insertion as described in Materials and Methods. A through F refer to the regions shown in panel D. Protected fragments corresponding to correctly initiated Vβ8.3 and truncated H4 transcripts are indicated. (C) S1 nuclease protection as in panel B, using promoters extending 5′ to −521. (D) Nucleotide sequence of Vβ8.3 from −92 to −33. A through F indicate the regions of the promoter into which the sequence 5′-GGATCCTAGG-3′ was substituted for the native sequence in synthetic oligonucleotides. Region C contains the conserved Vβ decamer motif.

capacity to <10% of the full promoter. Thus, the essential proximal promoter elements for Vβ8.3 appear to reside between −84 and −33. The conserved Vβ decamer is located within this region at −62 to −53. The −33 construct, which retains a putative TATA element (TAATTT, −28 to −23), was capable of low levels of correct transcription and may therefore define the "core" Vβ promoter.

Mutation of the conserved Vβ decamer greatly reduces transcriptional capacity. The specific sequences within the proximal promoter which are required for full expression were identified by mutational analysis. A series of mutations was introduced into the critical region by adding synthetic oligomers upstream of −33. The sequence GGATCCTAGG was substituted separately for each of the regions labeled A to F in Fig. 2D. The resulting constructs, which extend 5′ to −95, were assayed for transcriptional competence by transfection into BW5147 cells. The S1 protection products of these promoters, as well as those of the truncated H4 control, are shown in Fig. 2B. Two mutant promoters, −95A and −95B, maintained the same level of correctly initiated

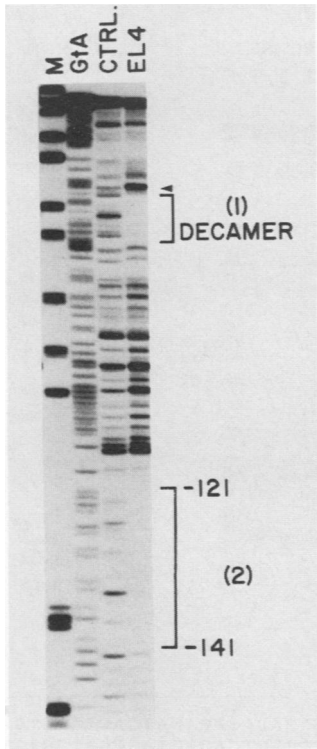

FIG. 3. Nuclear factor binding at the Vβ decamer and an upstream site. DNase I footprint assay of the Vβ8.3 promoter-coding strand probe. Protected regions are shown with brackets, and the boundaries of upstream region 2 are indicated. Region 1 contains the Vβ decamer, and the DNase I-hypersensitive site at −44 is indicated by the arrowhead. M, End-labeled *Msp*I-digested pBR322 DNA as size markers; G+A, Maxam and Gilbert sequencing ladder of the coding strand probe; CTRL, DNase I-treated sample without nuclear factors; EL4, DNase I-treated sample incubated with EL-4 nuclear extract partially purified by heparin-agarose chromatography.

RNA as the wild type, −95WT. Mutation C, which eliminates the conserved Vβ decamer, produced the greatest effect on the Vβ promoter. This mutation reduced the transcriptional activity to the same level as deletion to −33, i.e., <10% of −95WT. Mutation D, located immediately downstream of the decamer, resulted in a promoter with only 15% of the activity of −95WT. Mutations E and F, positioned further downstream of the decamer, maintained approximately 35% of full activity. The effects of mutations C and D were also tested in the context of a larger promoter by adding the sequences between −521 and −95 (*Bgl*II-*Hpa*I) upstream of −95C and −95D. Intact upstream sequences did not compensate for mutations of the decamer or flanking sequence (Fig. 2C). Elimination of the decamer from either the minimal (−95) or the full (−521) promoter resulted in a 10- to 20-fold reduction in transcriptional capacity. We conclude, therefore, that the proximal Vβ8.3 promoter contains a basal enhancing element which includes the decamer and the downstream sequences.

The Vβ decamer is the binding site of a specific nuclear factor. To determine specific sequences which serve as binding sites for nuclear factors, we first performed DNase I footprint analysis of the Vβ8.3 proximal promoter. Figure 3 shows the protection by partially purified EL4 nuclear extract of a probe labeled on the coding strand at a polylinker site immediately upstream of −164. Within the region defined as critical by transfection assays (−84 to −33), a single protected area can be identified. The conserved Vβ decamer is contained within the footprint, which is characterized by enhanced DNase cleavage at or near position −44 on the coding strand. The boundaries of the DNase protection are indicated for both coding and noncoding strands in Fig. 4D. A similar pattern of footprinting over the decamer and flanking sequence, derived with nuclear extracts from several cell types, has been reported previously for the human Vβ8.1 promoter (46). Our results confirm that extracts from both T cells (EL4, BW5147, and YAC-1) and non-T cells (L cells and a B-cell line, WEHI279) contain proteins which can footprint the decamer of Vβ8.3 (data not shown). An additional protected area, −141 to −121 on the coding strand, can also be identified in this assay. Since deletion of the region containing this site had a modest effect on the transcriptional competence of the Vβ8.3 promoter (Fig. 2), its role in Vβ regulation has not been investigated further for this report.

The importance of the decamer sequence in nuclear protein binding to the proximal promoter was confirmed in gel mobility shift assays. Probes containing the wild-type and mutant sequences tested in transfection assays (Fig. 2) were analyzed for their ability to form specific DNA-protein complexes. The wild-type probes 51mer, 67mer WT, and 30mer WT formed several prominent complexes when incubated with crude BW5147 nuclear extract (Fig. 4A). Only complexes I to IV are specific to the Vβ sequence, as determined by competition and methylation interference assays (see below). Mutant probes 67mer A and 67mer B, which are fully functional in transfection constructs, formed the same complexes as the wild-type probes. Mutant 67mer D, which generates a barefly functional promoter construct, formed low but detectable amounts of the wild-type complexes. Similarly, probes containing mutations E and F formed intermediate amounts of specific complexes (data not shown). Only mutant 67mer C, in which the decamer has been eliminated, failed to form any specific DNA-protein complexes. The ability of wild-type and mutant probes to compete for specific DNA-protein complexes was also tested (Fig. 4B). Complexes formed with the 30mer WT are effectively inhibited by 67mer WT, much less efficiently by mutant 67mer D, and not at all by the decamer-minus mutant 67mer C. Thus, all specific DNA-protein complexes which form in vitro with the proximal Vβ8.3 promoter were dependent on a functional decamer sequence. Moreover, the wild-type and mutant sequences demonstrated a direct correlation between protein-binding capacity in vitro and the promoter function in vivo.

Fine mapping of nuclear protein contact with the Vβ8.3 proximal promoter was determined by methylation interference assay. Both coding and noncoding contact residues were determined with the 51mer probe and crude BW5147 nuclear extract. Complex V, which is formed with all probes independent of probe sequence, showed the same cleavage pattern as free DNA. Complexes I, II, and III, however, all displayed a specific methylation interference pattern (Fig. 4C). Methylation of coding strand G residues −61 and −59, or noncoding strand G residues −57, −54, and −52, prevented specific protein binding. These residues are contained within the DNase I footprint region (Fig. 4D) and are part of or immediately flank the decamer. The reduction of transcription capacity by promoters containing mutations C and D can therefore be explained by the positions of critical contact residues within these regions. No other G residues showing methylation interference could be identified within

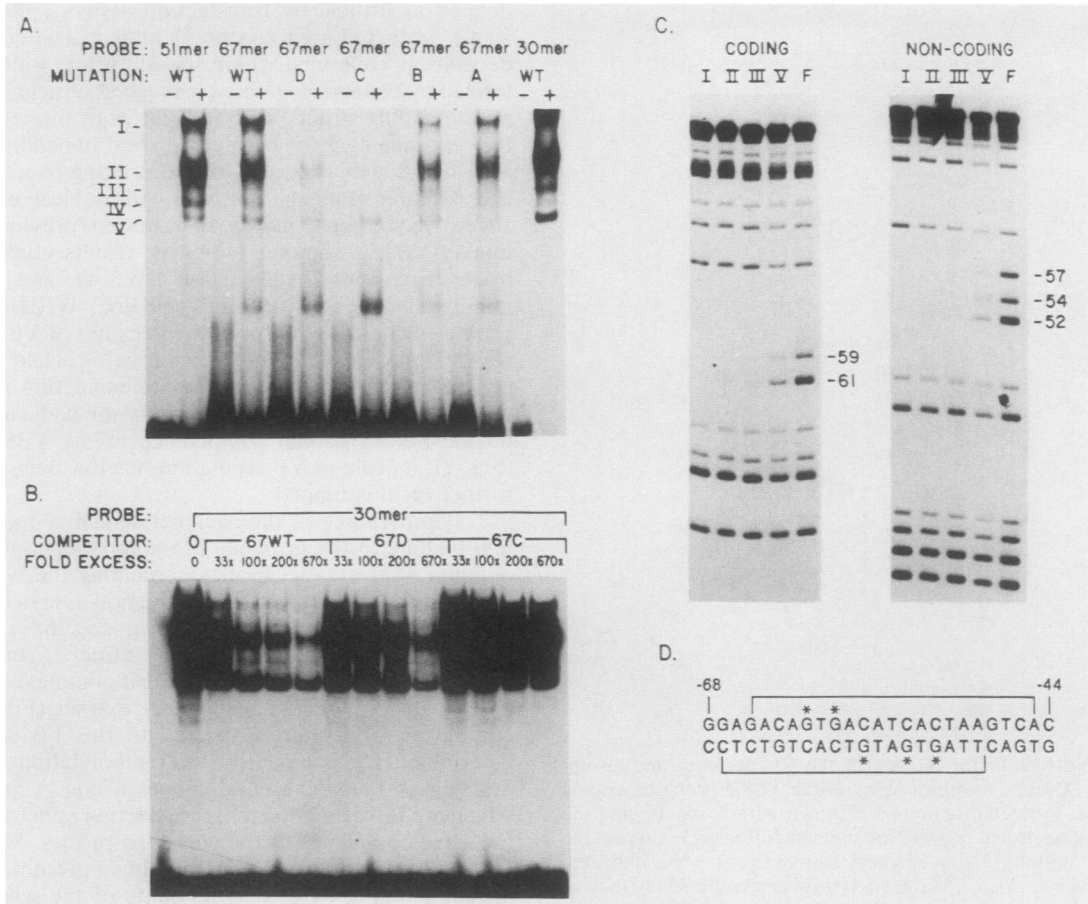

FIG. 4. Nuclear factor binding to the Vβ8.3 proximal promoter specific for the decamer motif. (A) Gel mobility shift assays, using wild-type (WT) and mutant (A to D) Vβ8.3 probes as described in Materials and Methods. Positions of the 10-bp mutations are indicated in Fig. 2. End-labeled probes were incubated without (−) or with (+) BW5147 nuclear extract and subjected to gel electrophoresis. Reproducible protein-DNA complexes are indicated as I to V. Free DNA is at the bottom of the photograph. (B) Competition for nuclear factor binding to the Vβ8.3 promoter. Wild-type or mutant 67mer oligonucleotides were added at the indicated molar excess to binding reactions with end-labeled Vβ8.3-30mer as probe. (C) Methylation interference analysis. The Vβ8.3-51mer subclone was end labeled on the coding or noncoding strand and assayed for methylation interference as described in Materials and Methods. Complexes I, II, III, and V, as well as free DNA, were analyzed. G residues showing methylation interference are indicated at −59 and −61 (coding) or −57, −54, and −52 (noncoding). (D) Summary of protein binding at the Vβ8.3 decamer. Regions protected in DNase I footprint assays of coding and noncoding strands are shown by brackets. G residues showing methylation interference are marked by asterisks.

the proximal promoter. The regions covered by mutations E and F lack detectable contact residues or DNase footprints and were therefore not identified as protein-binding sites by our assays. The mechanism by which these mutations affect transcriptional capacity requires additional study.

Since the decamer motif of the Vβ promoters was first identified by sequence comparison of several genes (3), and since the Vβ8.3 gene contains an exact match to the consensus sequence, we were interested to determine whether the native sequences found in other Vβ genes would bind nuclear factors in the same manner as Vβ8.3. The synthetic 30mer oligonucleotide probes shown in Fig. 5 were tested for binding in the gel mobility shift assay. With crude BW5147 nuclear extract, every Vβ sequence tested was capable of forming DNA-protein complexes with mobilities similar or identical to those formed with the Vβ8.3 probe (Fig. 5). These data suggest that most Vβ promoters, despite a lack of absolute sequence conservation, are bound by the same factor(s). The relative affinity for specific protein binding appears to differ depending on the sequence. The precise residues which determine the affinity of each decamer probe were not determined. As shown for Vβ8.3, however, sequences within the decamer itself, the immediate flanking sequences, or flanking sequence beyond the 30 bases in the probes may contribute to the relative affinity of each Vβ gene for the specific binding factor(s).

The Vβ decamer is an ATF-binding site in vitro. The core sequence of the consensus Vβ decamer, TGACATCA, is a partial palindrome similar to previously described regulatory elements. These include the consensus AP-1-binding site, TGAGTCA, and the consensus CREB- or ATF-binding site, TGACGTCA (13). More importantly, the contact residues identified by methylation interference are the same for the decamer and CRE/ATF sites. The only exception is that the Vβ motif contains a central -CA- rather than the invariant -CG- of the CRE (4, 24, 33). These similarities prompted us to investigate the possibility that the Vβ decamer-binding protein(s) might be related to factors which bind AP-1 or CREB/ATF sites. Synthetic probes (30mers) containing the AP-1 site and flanking sequence of the human collagenase gene (5), the CRE and flanking sequence of the rat somatostatin gene (41), or the ATF-binding site of the adenovirus

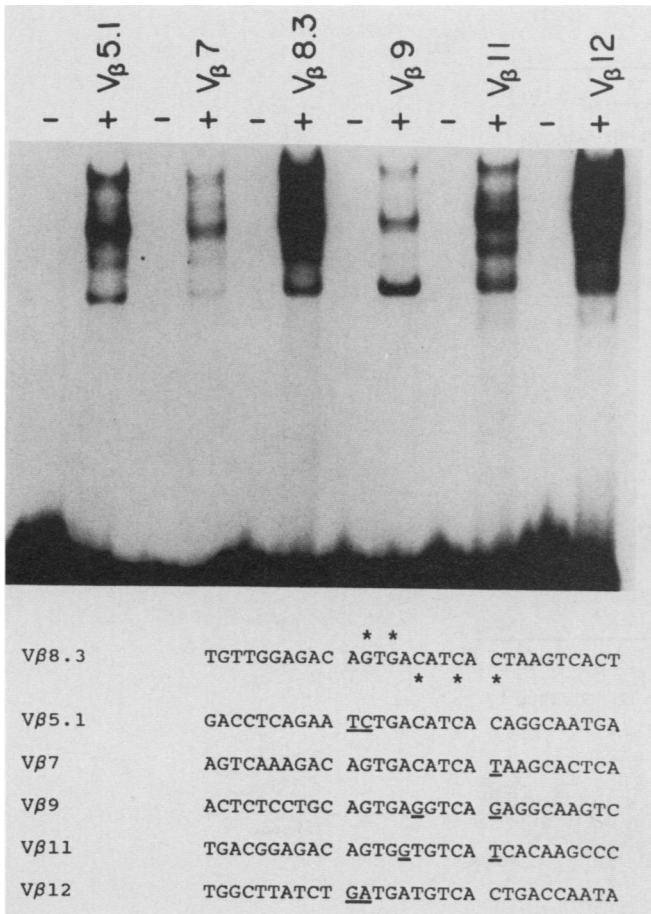

FIG. 5. Decamer motifs from different Vβ genes showing similar binding. Gel mobility shift assay, using end-labeled 30mer probes derived from the sequences shown at the bottom. The Vβ8.3 sequence is shown on the top line, and the positions of contact G residues are indicated (*). Residues at which the Vβ sequences differ from Vβ8.3 are indicated by underlining. Probes were incubated without (−) or with (+) BW5147 nuclear extract, as described in Materials and Methods.

E4 gene (32) were synthesized and tested in the gel mobility shift assay for specific DNA-protein complex formation with crude BW5147 extract. Our results confirm previous reports (13, 24) that the CRE and ATF sites bind nuclear proteins in a similar or identical fashion, but differ in binding properties compared with an AP-1 site (Fig. 6A; data not shown). Interestingly, the pattern of complexes formed with the Vβ8.3 probe is also similar or identical to the CRE/ATF pattern, but distinct from that formed with AP-1. Furthermore, cold Vβ8.3 decamer or somatostatin CRE probes are nearly identical in their ability to compete for protein binding to either labeled probe. The AP-1 probe, however, is 5- to 10-fold less efficient as a competitor in the same assay (Fig. 6A).

More direct evidence of the ability of proteins which recognize CRE/ATF sites to interact with the Vβ decamer is presented in Fig. 6B. A preparation of nuclear factors purified by affinity chromatography, using the E4 ATF site (22), a kind gift from T. Hai and M. Green, was tested in a DNase I footprint assay with the Vβ8.3 promoter probe. The purified ATF preparation yielded a footprint over the decamer which is identical to that obtained with the partially purified EL4 extract, both footprints showing the enhanced cleavage at position −44. Whereas the EL4 extract showed binding activity at the upstream site (−141 to −121), the purified ATF showed no binding in this region. These data indicate that the Vβ8.3 decamer acts in vitro as a high-affinity ATF-binding site, possessing an apparently lower cross-reactivity with a consensus AP-1 site.

Consensus CRE substitutes for the Vβ decamer in vivo. To test in vivo the decamer's similarity to an ATF/CRE site and distinction from an AP-1 site, two final mutations were introduced into the Vβ8.3 proximal promoter. The palindromic core of the decamer (TGACATCA) was mutated to an ATF/CRE site (TGACGTCA) by a single A-to-G transition or to an AP-1 site (TGACTCA) by a single-base-pair deletion. Gel mobility shift assays, using as probes the 67mers containing these mutations in the Vβ8.3 context, revealed distinct DNA-protein complexes consistent with the CRE/ATF and AP-1 patterns of Fig. 6A (data not shown). The mutant promoters were tested by transient transfection, and the results of the S1 protection assay are shown in Fig. 7. Mutation of the decamer to the AP-1 site reduced transcription from the Vβ8.3 promoter to the level of the −33 construct, whereas mutation to the CRE increased the transcriptional capacity. This enhancement was seen after transfection into either BW5147 or EL4 cells and represents an approximately threefold-greater level of correctly initiated transcripts. Thus, these single-base-pair mutations maintain the direct correlation between in vitro binding and in vivo function as shown with the more extensive mutations A to F.

DISCUSSION

We report here that a conserved element in the murine Vβ promoter is essential for the high-level expression of this gene. Mutation of this single element, either by complete substitution (−95C) or by a single-base-pair deletion (−95AP1), reduced the basal transcription by 10-fold. In contrast, Diamond et al. (14) report that elimination of the decamer in the human Vβ8.1 promoter did not appreciably affect the enhancing capacity of a dominant control region located between −510 and −800. In their system, the activity of the core promoter (5′ to −45) was stimulated 10-fold by the upstream element, but only 2-fold by the decamer-containing fragment (−350 to −45). Since the authors did not quantitate correctly initiated transcripts, however, the level of reporter gene activity in their system may not accurately reflect true promoter activity, and the importance of the decamer may be underestimated. Furthermore, we found no evidence for a distal upstream enhancer in the Vβ8.3 region. The lack of extensive homology between different Vβ promoters (3) may indicate that certain regulatory elements will be unique to individual genes or subfamilies. The conservation of decamer motifs possessing similar protein-binding capacities, however, suggests a common function for this element in several Vβ genes.

The decamer motif of Vβ genes and the CRE identified in many cyclic AMP genes share several features. First, the core of each element is the 8-bp palindrome TGA--TCA. Whereas the consensus Vβ sequence contains a central -CA- dinucleotide, the CRE contains an invariant -CG-. The significance of the sequence difference has yet to be determined. It is interesting to note that the high-affinity AP-1 site found in the cJUN promoter is actually an 8-bp palindrome, TGACATCA, which is identical to the core of the Vβ decamer (6). The possibility that JUN/AP-1 factors may also interact with the decamer still remains. Nevertheless, it is clear that the Vβ decamer and CRE elements are very

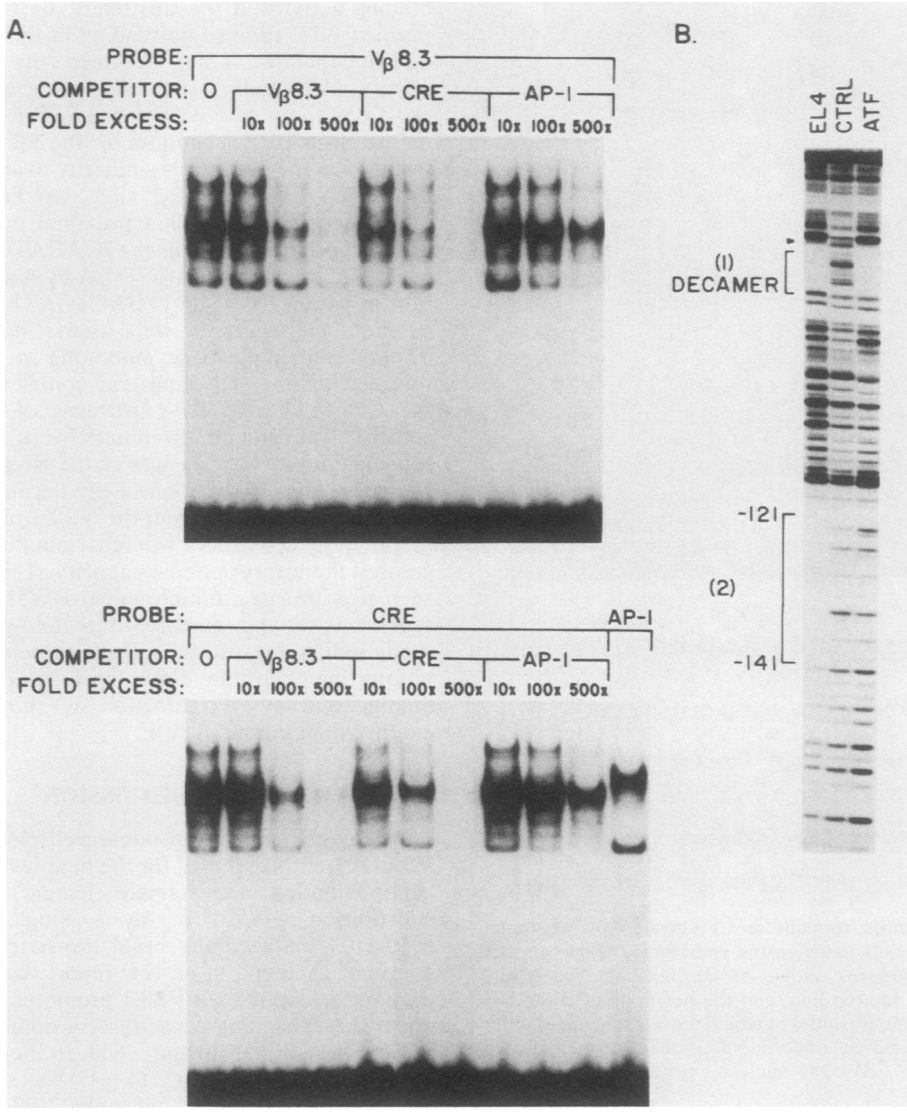

FIG. 6. Similarity of the Vβ8.3 decamer motif to the consensus CRE and its binding of purified ATF in vitro. (A) Gel mobility shift assays, using end-labeled 30mer probes containing the Vβ8.3 decamer (top) and the somatostatin CRE or collagenase AP-1 site (bottom). Unlabeled competitor was added to each binding reaction at the molar excess indicated above each lane. The AP-1 probe was tested without competitor (bottom, far right). (B) DNase I footprint analysis, using EL-4 nuclear extract and affinity-purified ATF. Protected regions are indicated as in Fig. 3. ATF was purified by T. Hai as described previously (22). CTRL, DNase I-treated sample without nuclear factors.

similar in protein-binding properties. The multiple sequence-specific DNA-protein complexes formed with a functional CRE (4, 24) are also formed with the Vβ8.3 sequence. Moreover, the somatostatin CRE and Vβ decamer compete equally well with each other for complex formation. The similarity in binding is further revealed by an overlapping pattern of contact residues detected by methylation interference (33). Finally, affinity-purified ATF/CREB is able to footprint the Vβ decamer in the same fashion as a T-cell nuclear extract. These data, coupled with the ability of a consensus CRE to substitute for the decamer in vivo, strongly suggest that decamer-binding proteins are identical or closely related to ATF/CREB.

The involvement of a ubiquitous, inducible transcription activator in Vβ expression has several implications. Decamer-binding factors, presumably ATF/CREB-related proteins, are detected in extracts of both T and non-T cells. In addition, the mobilities of the protein-DNA complexes are not distinct in the various cell types. Thus, in contrast to the immunoglobulin octamer and B-cell-specific octamer-binding factor, a T-cell-specific form of decamer-binding factor(s) cannot be identified under the conditions of our assays. Yet the β chain and other TCR components are expressed only in T lymphocytes. Rearranged β chains introduced into the germ line of transgenic mice follow this pattern, with expression in non-T cells being typically low and variable (27, 50). The promoter and enhancer elements contained in the transgene constructs are therefore sufficient to confer tissue-specific transcription. Whereas the human Vβ8.1 promoter displayed T-cell specificity (14), the murine Vβ3 promoter did not (39). Our Vβ8.3/8.2 locus, which contains a β-chain promoter and enhancer, appears to show the same tissue preference as the larger transgenic constructs. Transfections into tissue-culture T cells showed high levels of Vβ expression. Transfection into a B cell line (WEHI-279), a pre-B cell line (70Z/3), and L cells, however,

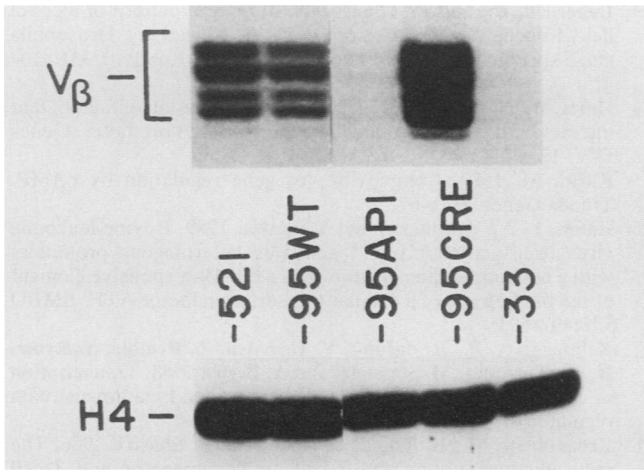

FIG. 7. Consensus CRE interchangeable with the Vβ8.3 decamer. S1 nuclease protection of RNA from BW5147 cells transfected with the indicated wild-type or mutant promoter constructs. Protected fragments corresponding to correctly initiated Vβ and H4 transcripts are indicated. The Vβ8.3 decamer was mutated to a consensus AP-1 or CRE site by deletion or transition of the A residue at −56, as described in Materials and Methods.

failed to yield any Vβ transcripts, despite detectable levels of the control H4. The only non-T cell to yield detectable Vβ transcription was the myeloma S194, which also yields extremely high levels of control H4 (data not shown).

Several mechanisms can be postulated by which the Vβ decamer could contribute to the apparent tissue-specific expression of these genes. Affinity purification of ATF does reveal multiple proteins capable of interacting with the recognition motif in vitro (22). This may allow for the differential expression of various forms of these factors in distinct tissues. Furthermore, the activity of ATF/CREB factors can be modulated, either in response to certain viral proteins (26, 32) or by the activity of cellular protein kinases (56). The deduced amino acid sequence of a CREB cDNA clone reveals the presence of several distinct phosphorylation sites (20), suggesting that the protein's activity could be altered by specific phosphorylation patterns. The leucine zipper domain may also allow for the formation of cell-type-specific heterodimers of ATF/CREB with other transcription activators, possibly forms of JUN/AP-1 (25). Thus, the possibility exists for a T-cell-specific form or modification of ATF/CREB which is not easily detected by in vitro binding assays.

Alternatively, the Vβ decamer may act in conjunction with other regulatory elements to create a functionally T-cell-specific locus. The CRE of the human glycoprotein hormone α-subunit promoter contributes to the tissue-specific basal expression of that gene, but only when combined with a separate promoter element (12). Similarly, it has been postulated that the structure of the immunoglobulin promoter prevents activation in non-B cells by the ubiquitous octamer-binding protein (31). The additional protein-binding sites identified by us and others (46) may combine with the decamer to regulate Vβ expression via a unique interaction of ubiquitous and specific factors. Finally, developmentally determined nucleosome or DNA methylation patterns may restrict the accessibility of the decamer for a ubiquitous factor in nonexpressing cells. An example of such regulation has been demonstrated with the binding of specific nuclear factors to the rat tyrosine aminotransferase gene (8). Further experiments are required to determine whether, and by what mechanism, the decamer and ATF/CREB contribute to tissue specificity.

The possibility that forms of decamer-binding proteins may participate in T-cell-specific expression is strengthened by our observation that Vβ decamer or CRE motifs can be identified in a number of T-cell genes. These include TGA CATCA in the upstream region of human CD8 (D. Littman, personal communication); TGACATCA in the 5′-untranslated portion of murine Lyt2 (42); TGACATCA in the upstream region of human TdT (J. Tillinghast, personal communication); TGACATCA in the enhancer region of murine T3 delta chain (18); TGACGTCA in the enhancer region of murine TCR α chain (55); and TAACATCA in the enhancer region of murine TCR β chain (27). DNase I footprint assays demonstrate that the TCR α-chain enhancer motif is able to bind nuclear proteins in vitro (55). Furthermore, our preliminary experiments indicate that all of these motifs share a similar or identical gel mobility shift pattern with the Vβ decamer and the consensus CRE (data not shown).

A final striking feature of TCR expression is the induction of V-segment rearrangements at discrete developmental stages. The developmental cues which initiate these rearrangement events are unknown, though it is clear that they are unique to maturation within the thymus. B-cell differentiation, which yields functional immunoglobulin genes, fails to activate TCR V segments for recombination. The accessibility model of targeted recombination proposes that the chromatin structure of each rearranging locus is determined by cell type and developmental stage (1). The binding of specific transcription activators, perhaps in conjunction with increased polymerase activity, is a mechanism by which the chromatin structure could be altered. In this light, it is interesting to note that the Vβ decamer may interact with factors whose activity can be induced, particularly by elevated cyclic AMP levels. The stage-specific activation of Vβ transcription and rearrangement may involve modification of decamer-binding factors in response to specific developmental cues. Investigation of the molecular events which accompany lymphocyte differentiation will be facilitated by cells which can be induced in vitro along a normal developmental pathway.

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