LINEAGE-SPECIFIC EXPRESSION OF A T CELL RECEPTOR VARIABLE GENE PROMOTER CONTROLLED BY UPSTREAM SEQUENCES

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Expression and surface assembly of antigen receptors occur during the differentiation of lymphoid progenitors into mature B and T lymphocytes, endowing each lymphocyte with a unique antigen recognition capacity. The specific polymorphic antigen binding structure on B lymphocytes is surface Ig, while the CD3-Ti TCR subserves that purpose on T cells (1–3). The latter is a heterodimeric structure non-covalently associated with monomorphic CD3 subunits (γ, δ, ϵ, and ζ), which assemble on the surface of T cells and interact with foreign antigens in association with MHC molecules.

From prior studies it has been shown that the assembly of Ig genes takes place early in B cell development (4) in an ordered fashion, culminating in the rearrangement and expression of H chain genes (5), and subsequently, L chain genes (6–8), so that a functional Ig molecule is produced. The molecular architecture of the Ig H chain (IgH) and L chain (IgL) loci has been intensively studied (9). Transfection analysis of both IgH and IgL genes revealed an element in addition to the V_H or V_L promoters that contributed to the expression of these genes. The latter has been generally referred to as an enhancer, which was shown to possess similar properties to the well-described viral enhancer elements (10). This element was shown to be located in the major intron between the J region and the $C\mu$ or C_k genes (11–15).

More recently, the TCR loci have been characterized, and the genes encoding the Ti antigen/MHC binding subunits present on the majority (95%) of peripheral T lymphocytes (α and β), as well as those encoding surface receptors (γ and δ) on a minor population (5%), have been cloned. One major conclusion from those studies is that the organization of IgH and TCR genes that are composed of multiple V, D, J, and C segments (except TCR-α) is very similar in both mouse and human (16, 17). To define elements that confer a strict tissue specificity to the expression of the β subunit of the TCR, we have begun an analysis of a TCR V region gene promoter. This analysis will aid in the understanding of the molecular basis for T lineage differentiation and serve as a first step in identifying nuclear factors that bind to the DNA elements that we have characterized.

In this report we show that the promoter of a functionally rearrangement $V\beta$ gene

(*Vβ8.1;* references 18–20) acts in a tissue-specific manner upon transfection analysis. Additionally, we present evidence for a strong upstream element of 230 bp, which is active in either orientation in conferring to a heterologous promoter, T cell–specific expression. No previously characterized expression element or transcription factor binding site was localized in this region of the promoter, including those sequences responsible in part for cell-specific expression of Ig genes.

Of considerable importance is the additional finding that the $V_\beta 8$ promoter is nonfunctional in B cells, the most closely related hematopoietic cell type to T cells. Our transfection results point to the promoter as a restricting element in the T lineage expression of the *Vβ8.1* gene, which is consistent with the observation that rearranged or germline configured V_β or V_H genes are not expressed in the inappropriate cell type. Since we and others (21, 22) find no enhancer element equivalent to the Ig expression enhancer in the major intron between the J region and $C_{\beta 1}$, it is likely that the V_β promoter plays a key role in tissue-specific expression of the β chain of the TCR.

Materials and Methods

Cells and Media.   The human cell lines Jurkat subline 77 (a gift of G. Crabtree, Stanford Medical School) and JY (a gift of T. Springer, Harvard Medical School) were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) and 10% FCS (Flow Laboratories, Inc., McLean, VA) supplemented with penicillin-streptomycin (Gibco Laboratories) and glutamine (Gibco Laboratories). Murine cell lines EL-4 (a gift of T. Taniguchi, Osaka University) and J558 (American Type Culture Collection, Rockville, MD) were grown, respectively, in RPMI 1640 (Gibco Laboratories) and DMEM (Gibco Laboratories) supplemented identically as in the case of human cells. Fibroblasts of human (Hela; a gift of P. Sharpe, M. I. T.) and monkey origin (Cos; a gift of B. Seed, Harvard Medical School) were cultured in DMEM (Gibco Laboratories) and supplemented as described above.

Plasmid Construction.   The charon 30 phage $\lambda\beta 3$ (23) was cleaved with the enzyme Sma HI, and a 3.5-kb fragment containing the *Vβ8.1* gene was identified by hybridization. The subcloned DNA in pUC 12 was further cleaved using Bgl II and Xba I and subcloned into the expression vector pOCAT-2 (a gift of D. Moore, Harvard Medical School; reference 24). A series of deletions was generated by restriction enzyme digestion, which are referred to with the prefix BP800, BP570, etc. Their derivation is shown in Table I. Plasmids that contain the 309-bp fragment containing the RSV enhancer (25) 5' of the inserted promoter also contain the prefix R such as in RBP800 (see Table I). Plasmids with the prefix IgHE (H chain enhancer) contain the 1.0-kb Xba I fragment derived from $pSV_2 \gamma_{2b}VC$ (12), and are described more fully in Table I.

An additional set of plasmids were made using the expression vector pUTKAT (a gift of D. Moore; reference 24), which contains the 250-bp herpes simplex thymidine kinase promoter fragment (TK;[1] reference 26). The largest of these BT800s was derived by cleaving the 817-bp Xba I to Bgl II $V_\beta 8.1$ promoter fragment with Nsi I at −45 (Fig. 1 *B*), filling in the ends with four deoxynucleotide triphosphates and the klenow fragment of DNA polymerase I, and subsequent ligation to Xba I cleaved and filled in pUTKAT. The remainder of the *Vβ8.1* upstream DNA-TK promoter fusion plasmids (BT800, BT570, etc.) are described in Table I. Similarly, pUTKAT derivatives containing either the RSV enhancer fragment (RTK) or the IgHE fragment (IgHETK) are so designated.

Plasmids in which the deletions created are internal are designated with the 5' and 3' boundaries of the deletion (i.e., BT or BP 8051, see Table I). The BP prefix indicates a 3' boundary

at +17, while the BT prefix indicates a 3' boundary at -45, unless designated otherwise (Table I and Fig. 5).

Transfection Analysis. Lymphoid cells were transfected with plasmid DNA by a modification of Fujita et al. (27). Briefly, suspension cells (Jurkat, JY, J558) were adjusted to a concentration of 3×10^5 with fresh medium for 12–16 h before transfection. 1.5×10^7 cells were pelletted, then washed with 1× transfection buffer (27), and resuspended in transfection buffer and DEAE-dextran (J77, 500 μg/ml; JY and J558, 300 μg/ml) with DNA (20 μg/ml) in 1 ml. The concentration of DEAE-Dextran that gave the most efficient conversion of ^{14}C-chloramphenicol (see below) was used in each case. In the case of J77 and J558, after a period of 90 min at 37°C (resuspension every 30 min), 0.1 ml DMSO was added and the cells were further incubated at room temperature (RT) for 3 min. The cells were then diluted in 10 ml of 1× transfection buffer, pelleted at 1,200 rpm, and further washed with RPMI 1640 without serum. The washed and pelleted cells were then resuspended in 10 ml of complete medium, and incubated for ~40 h at 37°C.

A slightly modified method of DEAE-dextran-mediated transfection was used for EL-4 cells. Since the subline of cells we used attached to plastic (tissue culture 100 × 20-mm plates, Falcon Labware, Oxnard, CA), the transfection took place directly on the plates. 12–16 h before the transfection, cells were detached from large flasks by a 5' treatment with PBS-EDTA (0.6 mM, pH 8.0) at 37°C, and ~3 × 10^6 cells were plated on the 100 × 20-mm dish in fresh medium. The medium was aspirated, and the plates washed with transfection buffer, then 2.5 ml of a solution of 100 μg/ml DEAE-dextran and 8 μg/ml plasmid DNA in transfection buffer was applied for 30–60 min. The plates were subsequently washed with 10 ml of transfection buffer followed by serum-free RPMI 1640. The cells were incubated for 2 d in 10 ml of fresh complete medium.

Transfection of fibroblasts using CaPO$_4$, (L, Hela, Cos) was done according to established methods using 5 μg of DNA per 100-mm dish containing 7.5×10^5 cells (28).

Chloramphenicol Acetyl-transferase (CAT) Assay. Cells were harvested by centrifugation (suspension) or by previous treatment with PBS-EDTA (adherent), then washed two times in PBS. Freeze-thaw lysis in 0.25 M Tris HCL, pH 7.4, and subsequent assays were done according to Gorman et al. (29) using ^{14}C-chloramphenicol (0.2 μCi at 40–60 Ci/mol; New England Nuclear, Boston, MA)) and 0.8 mM acetyl-coenzyme A (Sigma Chemical Co., St. Louis, MO). The time of assay varied depending on the cell line (Jurkat, EL-4, and J558, 6 h; JY, Hela, and Cos, 1–2 h). A 10-min incubation of certain extracts at 60°C inactivated an activity (presumably a de-acetylase), which enhanced the conversion of chloramphenicol to its acetylated forms. This effect was proportional to the activity before inactivation, and therefore, did not change the relative activities of the test plasmids. Every experimental plasmid was tested in duplicate in three or more separate transfections with each cell line. The various forms of acetylated chloramphenicol were quantitated by excising the radioactive spots and scintillation counting in Betafluour (New England Nuclear).

S1 and RNAse Protection. S1 probes (shown in Fig. 2 *A*), and subsequent hybridization and protection methods were done according to Diamond and Goodman (30). Maxam-Gilbert sequencing of the end-labeled probe was done according to Maniatis et al. (31). Products of the S1 and sequencing reactions were subjected to electrophoresis on 8% polyacrylamide/8 M urea gels. The gels were autoradiographed at -70°C with Cronex Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

For RNAse protection, an Nco I fragment (600 bp) encompassing the $V_\beta 8.1$ promoter proximal sequences from pV$_8\beta$ (see Table I) was subcloned at the Sma HI site by blunt-end ligation into ptz19R (United States Biochemical Corp., Cleveland, OH) in both orientations. The plasmid ptzV$_\beta$ was linearized with Eco R1, and after purification was resuspended in 10 mM Tris and 1 mM EDTA, pH 8.0 (TE), at 1 mg/ml. Labeled ssRNA transcripts were prepared as described (32), and hybridization protection was carried out according to Melton et al. (33). The reaction products were subjected to electrophoresis on an 8% polyacrylamide/ 8 M urea gel along size end-labeled pUC 12 Hpa II-digested size markers.

Miscellaneous Techniques. Plasmids were purified two times over CsCl ethidium-bromide gradients, and in every case the structure of the plasmid was verified by extensive restriction enzyme digestion. In many cases the boundaries of the newly inserted fragment were se-

quenced to verify orientation and the identity of the insert. The 855 bp of the 5' end and flanking sequences of the $V_\beta 8.1$ gene were sequenced on both strands by the Sanger method of dideoxynucleotide termination sequencing.

Protein concentration was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). Each sample in a particular assay contained the identical amount of protein, although between experiments the amount of protein varied.

Results

Sequence Analysis of a $V_\beta 8.1$ Promoter.   The sequence of the $V_\beta 8.1$ promoter is shown in Fig. 1 *A*, where several important sequence motifs are underlined. Examination of the proximal sequence (with respect to mRNA start site) reveals two potential transcription factor binding sites, the CCAAT box (34, 35) at −149 and a presumptive AP-1 binding site (−69 to −53; reference 36). Within the latter lies a decanucleotide (AGTGA TCA) of unknown function found in 13 of 14 V_β promoters examined (37). However, detailed comparisons of the upstream sequence (+1 to −800) of the $V_\beta 8.1$ promoter with other TCR V and V_β promoters failed to show any striking sequence similarities (use of the local program; reference 38). This is not surprising, since a comparision of Ig V_H promoters (39) revealed only a striking similarity at the position of the octamer and TATA motif (40, 41).

In a further sequence comparison with a region downstream of $C_\beta 2$ identified by Krimpenfoort et al. (21) to be a regulatory element for TCR β chain transcription (C_β enhancer; $C_\beta E$), we found only limited areas of close sequence similarity. However, certain stretches of sequence showed near identical patterns at several different positions (V_β, −669 to −680/$C_\beta E$, 812−822 10/11; V_β, −752 to −761/$C_\beta E$, 890−899 10/10; V_β, −225 to −239/$C_\beta E$ 479−483 14/14 plus two gaps; V_β, −751 to −771/$C_\beta E$, 471−491 18/18 plus four gaps). Numbering of V_β promoter according to Fig. 1 *A* and $C_\beta E$ according to Krimpenfoort et al. (21).

S1 Mapping Reveals Location of Proximal Transcription Signals.   To determine the transcriptional start site and signals for the $V_\beta 8.1$ promoter in Jurkat cells, we performed protection studies using cytoplasmic RNA from a variety of cells and analyzed the products on 8% polyacrylamide/8 M urea gels along side a partial Maxam-Gilbert sequencing ladder as shown by the autoradiograph of the gel in Fig. 2 *A*. The S1-protected fragment of 55 bp aligns with a C residue 29 bp downstream of a Goldberg-Hogness box (GATA; reference 41). The protected fragment is of the identical size in the T cell lines Jurkat and JM, both of which express the $V_\beta 8.1$ gene (Fig. 2 *B*, lanes *1* and *2*), although it is not present in another T cell clone, RFL 3.51 (Fig. 2, lane *3*), which utilizes a V_β segment from a different family (*$V_\beta 2.3$;* unpublished results). Given that the assay is accurate to within ±1 bp and adenosine is a favored base for eukaryotic transcription initiation (41), we have placed the start at the A (+1) as shown. We confirmed this finding by using the technique of RNase protection of a ^{32}P complementary RNA probe and the same RNA samples as in Fig. 2 B (Fig. 2 C, lanes *8-11*). Note, as expected, use of a sense ssRNA probe failed to yield a protected fragment (Figure 2 *C*, lanes *3-6*).

The 0.8-kb $V_\beta 8.1$ Promoter Fragment Is Sufficient for T Cell Expression.   To characterize the $V_\beta 8.1$ promoter further we used the properties of the heterologous reporter gene (CAT) to analyze expression in a variety of cultured cells. We subcloned 820 bp of the 5' flanking sequence between the Xba I site at +20 and the Bgl II site at −800 into the pOCAT vector (24) upstream of the CAT coding sequence (Fig. 3 *A*). These
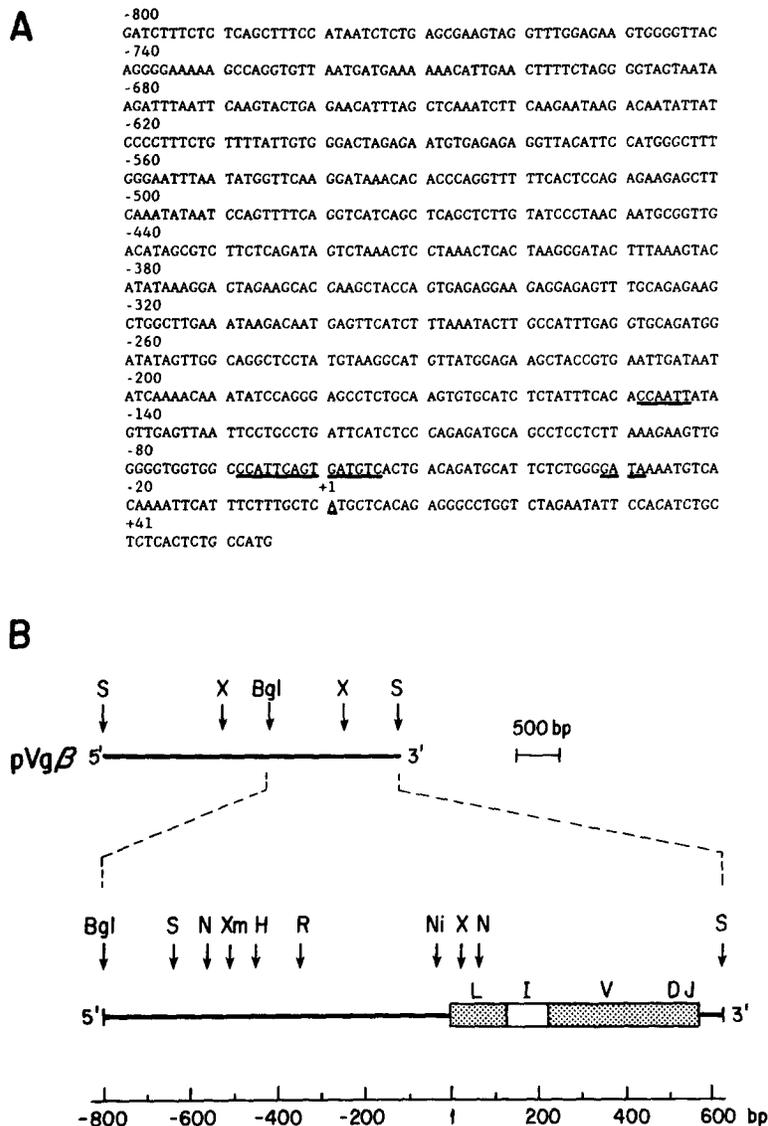
A

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-800
GATCTTTCTC TCAGCTTTCC ATAATCTCTG AGCGAAGTAG GTTTGGAGAA GTGCGGCTTAC
-740
AGCGGAAAAA GCCAGGTGTT AATGATGAAA AAACATTGAA CTTTTCTAGC GGTAGTAATA
-680
AGATTTAATT CAAGTACTGA GAACATTTAG CTCAAATCTT CAAGAATAAG ACAATATTAT
-620
CCCCTTTCTG TTTTATTGTC GGACTAGAGA ATGTGAGAGA GGTTACATTC CATGGGCTTT
-560
GGGAATTTAA TATGGTTCAA GGATAAACAC ACCCAGGTTT TTCACTCCAG AGAAGAGCTT
-500
CAAATATAAT CCAGTTTTCA GGTCATCAGC TCAGCTCTTG TATCCCTAAC AATGCGGTTG
-440
ACATAGCGTC TTCTCAGATA GTCTAAACTC CTAAACTCAC TAAGCGGATAC TTTAAAGTAC
-380
ATATAAAGGA CTAGAAGCAC CAAGCTACCA GTGAGAGGAA GAGGAGAGTT TGCAGAGAAG
-320
CTGGCTTGAA ATAAGACAAT GAGTTCATCT TTAAATACTT GCCATTTGAG GTGCAGATGG
-260
ATATAGTTGG CAGGCTCCTA TGTAAGGCAT GTTATGGAGA AGCTACCGTG AATTGATAAT
-200
ATCAAAACAA ATATCCAGGG AGCCTCTGCA AGTGTGCATC TCTATTTCAC ACCAATTATA
-140
GTTGAGTTAA TTCCTGCCTG ATTCATCTCC CAGAGATGCA GCCTCCTCTT AAAGAAGTTG
-80
GGGGTGGTGG CCCATTCAGT GATGTCACTG ACAGATGCAT TCTCTGGGCA TAAAATGTCA
-20                   +1
CAAAATTCAT TTCTTTGCTC ATGCTCACAG AGGGCCTGGT CTAGAATATT CCACATCTGC
+41
TCTCACTCTG CCATG
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B

FIGURE 1. (A) Sequence of the 5' flanking DNA of the Vβ8.1 promoter. Underlined sequences are discussed in the text. +1 indicates the putative start site for mRNA synthesis. However, the upstream C was determined experimentally in this paper (Fig. 2). The A at +53 is the first base of the initiator methionine residue within the leader protion of the TCR β chain protein. (B) Restriction map of the rearranged Vβ8.1 segment subcloned from phage DNA. pVgβ is described in Table I. The abbreviations for the restriction endonucleases are as follows: Bgl, Bgl II; H, Hinc II; N, Nco I; Ni, Nsi I; R, Rsa I; S, Sma I; X, Xba I; Xm, Xmn I. The boxes that contain letters represent the leader exon (L), first intron (I), variable segment (V), diversity segment (D), and joining segment (J), which comprise a mature Vβ gene segment. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00797.
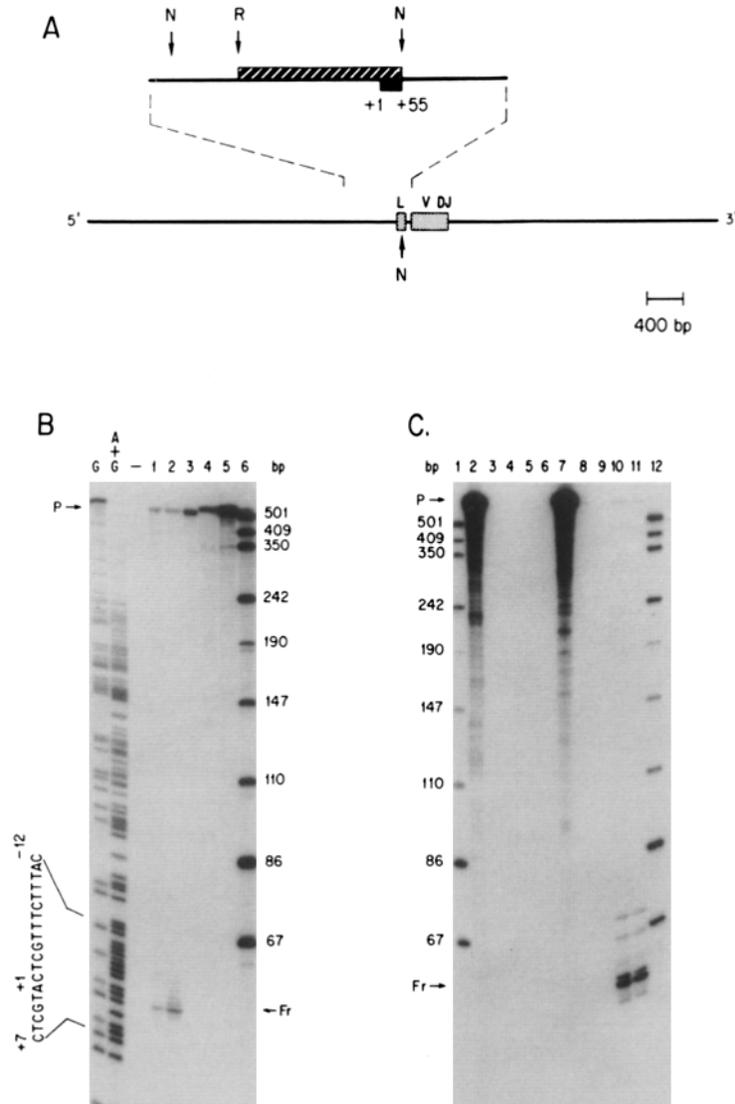
FIGURE 2.   S1 and RNase protection analysis of cellular RNA by the exon containing the L DNA segment of the *Vβ8.1* V gene. (*A*) Map of the region used in the protection analysis. The striped bar represents the fragment either double stranded (S1) or single stranded (RNase), which was radioactively end (S1) or uniformly labeled (RNase), and hybridized to cellular RNAs. Numbering is identical to Fig. 1 *A*, and symbols are defined exactly as in Fig. 1 *B*. The darkened box represents the protected region of the probe after hybridization and enzymatic cleavage. (*B*) Results of S1 analysis of RNA from Jurkat (lane *1*), JM (lane *2*), RFL 3.51 (lane *3*), yeast (lane *4*) cell. To the left of lanes *1* and *2* are shown the results of Maxam-Gilbert sequencing reactions on the end-labeled probe used in the RNA protection analysis (S1). Shown in lane *6* are Hpa II–digested and end-labeled pUC 12 molecular weight markers, whose sizes are defined on the extreme right in base pairs. The sequence of the indicated region containing the transcriptional start site is shown on the left as determined in Fig. 1 *A*. *P* corresponds to the labeled hybridization probe, and *Fr* indicates the protected fragment. (*C*) Results of RNase protection of cellular RNAs from Jurkat (lanes *6* and *11*), JM (lanes *5* and *10*), EL-4 (lanes *4* and *9*), and yeast RNA (lanes *3* and *8*) using an ssRNA probe that was generated from the sense (lanes *1-5*) or antisense (lanes *6-11*) strand from the plasmid ptzV$_8$β or its reverse ptzV$_8$β' (Table I). Shown in lanes *1* and *12* are end-labeled and Hpa II–digested pUC 12 DNA molecular weight markers.
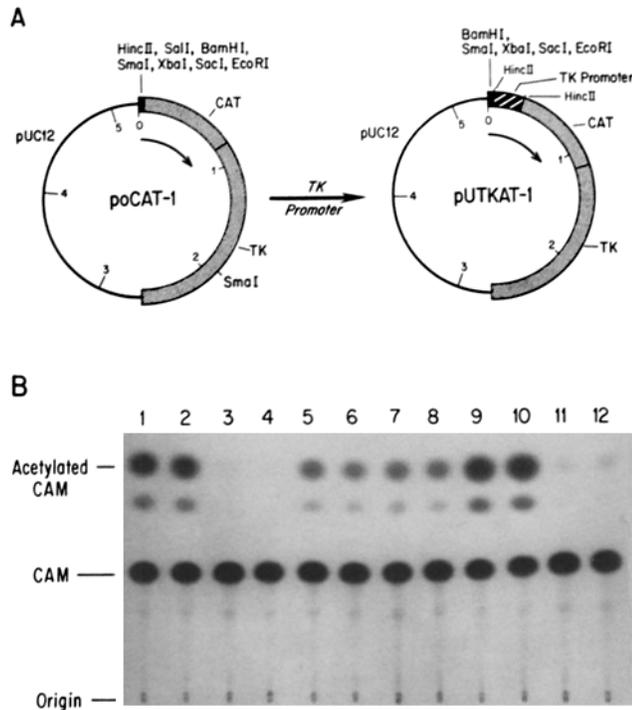
A

B

FIGURE 3. (A) Expression vectors used in the analysis of the *Vβ8.1* promoter. Prost and Moore (24) have described pUC 12 derivatives that can accept either proximal promoter fragments (pOCAT-1) or distal promoter fragments using the TK promoter proximal signals (pUTKAT-1). The TK mRNA 3' untranslated region was fused to the CAT gene to provide a eukaryotic polyadenylation signal sequence. (B) Results of transfection of several *Vβ8.1* promoter plasmids into EL-4 cells. As described in Materials and Methods, a CAT assay was performed for 6 h, and the products that were separated on TLC plates were analyzed by scintillation counting and quantitated as shown in Fig. 5 A. Plasmids transfected are the full-length promoter fragment, BP800 (lanes 1 and 2), ΔBP800 (lanes 3 and 4), BP045 (lanes 5 and 6), pUTKAT (lanes 7 and 8), RBP800 (lanes 9 and 10), and ΔRBP800 (lanes 11 and 12).

plasmids were transfected using DEAE-dextran (see Materials and Methods) into EL-4 (a murine T cell tumor) and Jurkat (human T cell tumor; data not shown) cells. As shown in Fig. 3 B (lanes 1 and 2 vs. 7 and 8) it is clear that the BP800 plasmid stimulates greater CAT activity in T cells than pUTKAT, which contains the TK promoter.

To further define the sequences necessary for T cell–specific Vβ promoter activity, a series of 5'→3' deletions were constructed and assayed in both Jurkat and EL-4 cells.[2] A schematic illustrating the structure of these deletions as well as the others presented in this report are shown in Fig. 4, A and B. This deletion series demonstrates that the majority of the promoter activity resides upstream of −350, predominantly between −800 and −510 (Fig. 4 A). In fact, an internal deletion of sequences between −510 and −45, which leaves the upstream sequences intact, results in a plasmid with T cell Vβ promoter activity equivalent to the intact promoter plasmid BP800 (Fig. 4 A).

Deletion of Transcriptional Start Site and Upstream GATA Sequence Abrogates Transcription from the Vβ8.1 Promoter. To determine whether the CAT protein acetylating activity resulted from mRNA that was initiated in plasmid BP800 using the endogenous

[2] We have subsequently found the murine line to be more active for Vβ8 promoter–stimulated CAT activity than most human cell lines (JM, REX, HBPALL; data not shown), so that the effect of deletions that reduce the strength of the promoter are more easily seen in EL-4 than in Jurkat cells. However, the relative effects of the deletions are identical between EL-4 and Jurkat, as presented in the data on Jurkat transfections shown in tabular form.
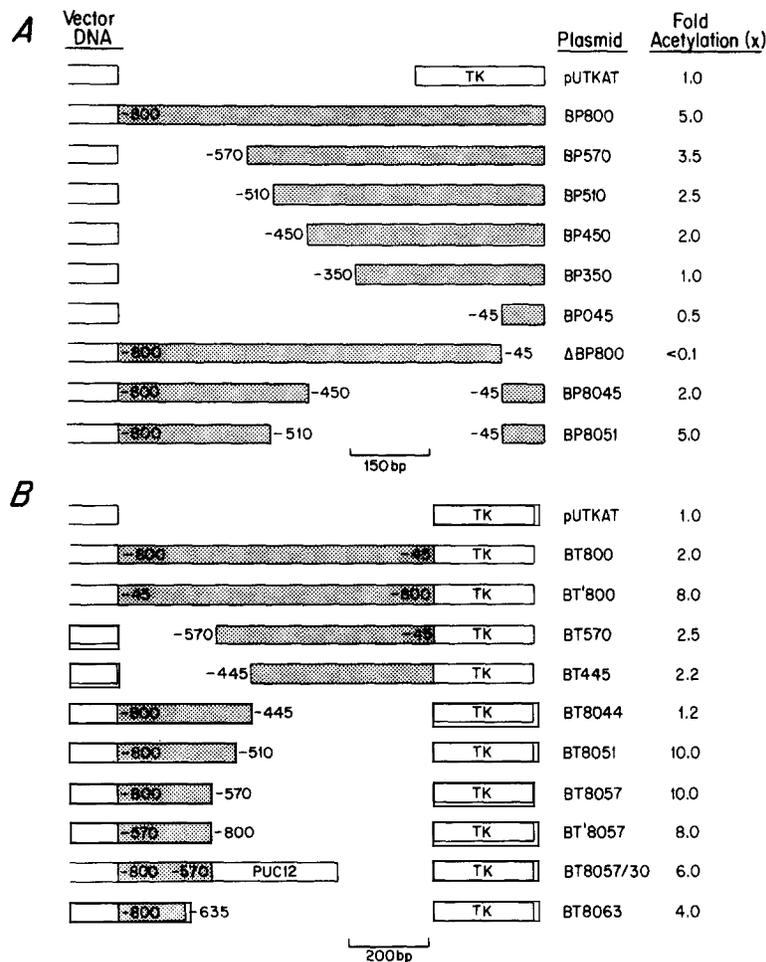
FIGURE 4   Summary of activities of the deleted forms of the Vβ8.1 promoter in T cells. (A) Shown are plasmids that were tested in both EL-4 and Jurkat cells a minimum of three separate times. Derivation of each plasmid is shown in Table I, with Vβ promoter region sequences stippled. The fold acetylation quantities were determined by averaging the results of each replicate after quantitation by scintillation counting. The variation between experiments did not exceed 20%. The fold differences shown are for EL-4 transfections, and are applicable to the Jurkat cell line, except that the activity of the Vβ8.1 promoter-containing plasmids are reduced by a factor of two. (B) Same as Fig. 4 A, except the plasmids compared are all derivatives of the TK promoter fused with upstream fragments of the Vβ8.1 promoter. Derivation of each plasmid is shown in Table I.

start site (determined above), we excised the proximal promoter sequences using the combination of Nsi I and Xba I digestion (Fig. 1 B and Table I). This deletion plasmid (ΔBP800) was transfected into Jurkat and EL-4 cells along with another deletion plasmid (ΔRBP800) similarly prepared, which contains the RSV enhancer (Fig. 3 B and Table I). Note that essentially no CAT activity arises from these plasmids, as compared with the undeleted plasmids shown in Fig. 3 B (lanes 3 and 4, 11 and 12 vs. 1 and 2, 9 and 10). A deletion from −800 to −45 did not completely abolish

TABLE I

Derivation of Plasmids Used in this Study

Plasmid	Method of construction
pOCAT	(24)
pUTKAT	(24); contains the TK promoter in the polylinker as a 250-bp Hinc II fragment.
RTK	RSV LTR fragment (309 bp), ends blunted using Klenow and four deoxynucleotide triphosphates, Bam HI linkers attached, cloned into the polylinker of pUTKAT.
IgHETK	pSV$_2\gamma_{2b}$VC digested with Xba I, the 1.0-kb fragment blunt ended with Klenow, cloned into the Xba I site of pUTKAT.
pV$_g\beta$	$\varnothing\lambda\beta$3 digested with Sma HI, subcloned into the Sma HI site of pUC 12.
ptzV$_g\beta$	Isolate 600-bp Nco I fragment from pV$_g\beta$, blunt end, ligate to Sma HI cleaved ptz19R.
BP800	pV$_g\beta$ was digested with Bgl II and Xba I, the 817-bp fragment cloned into the polylinker of pOCAT.
IgHEBP800	IgHETK digested with Hinc II to remove TK promoter, the Bgl II-Xba I fragment from pV$_g\beta$ was blunt ended, and ligated into the Hinc II site.
RBP800	The Xba I-Bgl II fragment was ligated to Xba I-digested pOCAT. The 309-bp Bam HI fragment from pRTK was ligated to the Bgl II end of the promoter fragment, Bam HI cut back of multiple inserts and digestion of polylinker, was followed by isolation of the linear plasmid over agarose, and subsequent ligation gave the expected product.
BP570	pV$_g\beta$ digested with Nco I and Xba I, the 587-bp fragment was blunt ended with Klenow and cloned into pUTKAT.
BP510	BP800 was partially digested with Xmn I and completely with Sac I, the ends were made blunt with Klenow, and the plasmid was closed by ligation.
BP450	BP800 was digested with Hinc II, and the 467-bp fragment was subcloned into pOCAT at the Hinc II site.
BP350	The Bgl II-Xba I fragment from pV$_g\beta$ was digested with Rsa I, and cloned into pOCAT, which was partially digested with Sma HI and completely with Xba I.
BP045	BP800 was digested with Sac I and Nsi I to remove the internal 755-bp fragment, and the plasmid was closed by ligation.
ΔBP800 ΔRBP800	BP800 or RBP800 was digested with Xba I and Nsi I to remove a 72-bp fragment, made blunt with Klenow, and closed by ligation.
BP8045	BP800 was digested with Nsi I and partially with Hinc II to remove a 405-bp fragment, the ends were blunted, and the plasmid closed by ligation.
BP8051	BP800 was digested completely with Nsi I and partially with Xmn I to remove an internal 455-bp fragment, the ends were blunted, and the plasmid closed by ligation.
BT800 BT'800	Isolate the Nsi I-Bgl II fragment from pV$_g\beta$, blunt end, and ligate to Xba I-digested and blunt-ended pUTKAT.
BT570	Isolate the Nco I-Nsi I fragment of PB800, and ligate it to Xba I-digested and blunt-ended pUTKAT.
BT445	Isolate the Hinc II-Nsi I fragment from BP800, blunt end, and ligate it to Xba I-digested and blunt-ended pUTKAT.
BT8044	Isolate the Bgl II-Hinc II fragment from BP800, blunt end, ligate to blunt-ended and Xba I-digested pUTKAT.
BT8051	Isolate the Bgl II-Xmn I fragment from BP800 blunt end, and ligate to blunt-ended and Xba I-digested pUTKAT.
BT8057 BT'8057	Isolate the Bgl II-Nco I fragment of BP800, blunt end, and ligate to blunt-ended and Xba I-digested pUTKAT.
BT8057/P30	Isolate the 300-bp Rsa I-digested fragment from pUC12, ligate it to Sal I-digested and blunt-ended BT8057.
BT8063	Isolate the Bgl II-Ssp I fragment of BP800, blunt end, and ligate to Xba I-digested and blunt-ended pUTKAT.

CAT activity (Fig. 3 *B*, lanes *5* and *6*) as compared with the ΔBP800 plasmid (Fig. 3 *B*, lanes *3* and *4*). Collectively, these results suggest that the initiation site we experimentally determined (Fig. 2 *B*) is correct, and presumably utilized in the plasmids described here.

Vβ8.1-TK Promoter Fusion Plasmids Are Active in T Cells.   The identification of a discrete region containing T cell promoter activity lead us to construct a series of plasmids using the upstream region of the *Vβ8.1* promoter beyond −5 fused to the TK promoter in the plasmid pUTKAT (Table I and Figure 4 *B*). These plasmids were transfected into both Jurkat and the EL-4 cell line (Fig. 4 *B*). A surprising result occurred when we examined the results of transfection analysis of plasmids containing sequences between −800 and −570 fused in either orientation to the TK promoter in pUTKAT as shown in Fig. 5, lanes *1-4*, and summarized in Fig. 4 *B*. There is little difference in the CAT activities generated by plasmids with the 230-bp 5' flanking sequence in either orientation, although the stimulation above pUTKAT is 10-fold when the $V_\beta 8$ promoter upstream fragment is in the natural orientation (Fig. 5, lanes *1* and *2* vs. *7* and *8*). The addition of a 300-bp Rsa 1 fragment from pUC 12 between the TK promoter and the *Vβ8.1* promoter 5' flanking sequence only slightly decreases its ability to stimulate CAT activity (Fig. 5, lanes *5* and *6*). Further truncation (fragment −800 to −635; Fig. 4 B) reduces the CAT activity, suggesting that we have entered an important segment of the 230-bp fragment. Since we have not made additional 3' deletions of the V_β promoter fragment, the precise 5' boundary of the upstream element is still unknown. These results provide evidence for a T cell promoter element (TPE) contained within the 230-bp upstream sequence of the $V_\beta 8$ promoter.

It is striking that the precise deletion of 60 bp between −450 and −510 (compare BP8045 vs. BP8051 or BT8044 vs. BT8051 in Fig. 4) activates the proximal sequences of either TK or the $V_\beta 8$ promoter. The molecular basis for the negative activity of this sequence is not understood, although its activity is equivalent within the context of the $V_\beta 8$ or TK promoter proximal DNA (Fig. 4). A further point is that the interaction of the −45 to −800 fragment with its natural proximal sequence ($V_\beta 8$) is more efficient than when it is apposed to the TK promoter (Fig. 4, *A* and *B*). Presumably, certain sequence-specific alignments of DNA elements are more produc-
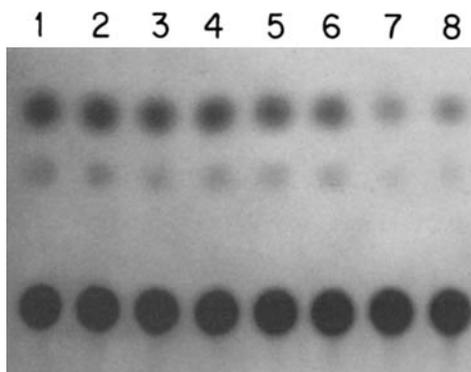

Figure 5. Upstream 230-bp fragment confers T cell activation on the TK promoter. Methods of transfection (legend to Fig. 3 *A*) and CAT analysis were done on EL-4 cells as previously described. The plasmids transfected are BT8057 (lanes *1* and *2*), BT'8057 (lanes *3* and *4*), BT-8057/30 (lanes *5* and *6*), and pUTKAT (lanes *7* and *8*).
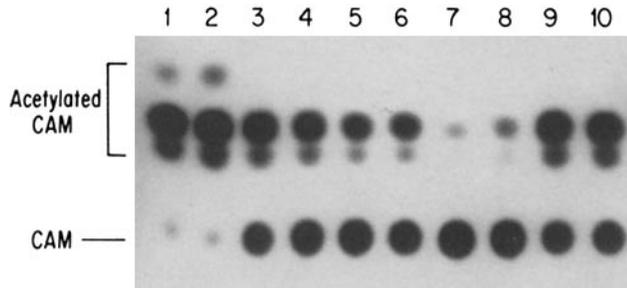
FIGURE 6.   Expression of $V\beta8.1$ promoter plasmids in Cos cells. Transfection and CAT assays for adherent cells are described in Materials and Methods. Similar results were obtained for Hela cells (data not shown). Plasmids transfected are pRTK (lanes *1* and *2*), BT8044 (lanes *3* and *4*), BT8057 (lanes *5* and *6*), BP800 (lanes *7* and *8*), and pUTKAT (lanes *9* and *10*).

tive in the context of the $V\beta8$ promoter elements, which is not unlike the situation with other tissue-specific promoters, including *IFN-β* (42).

Transfection of Vβ8.1 Promoter Plasmids into Nonlymphoid Cells.   The foregoing experiments have demonstrated that flanking DNA from the *Vβ8.1* promoter stimulate the TK promoter in T cells, however, the tissue specificity of this effect has not yet been defined. Therefore, we transfected plasmids that showed the highest activity in T cells into several fibroblasts' cell lines (COS and Hela cells; Fig. 6 and Table II) using CaPO$_4$ (27). The results demonstrate that plasmids that contain either proximal or upstream *Vβ8.1* promoter elements such as BP800 (Fig. 6, lanes *7* and *8*) or BP8051 (Table II) are inactive with respect to TPE activity in fibroblasts.

At the same time, we tested a series of *Vβ8.1*-TK promoter fusion plasmids in fibroblasts using CaPO$_4$ transfection. If we compare in each case the activity of a fusion plasmid containing upstream *Vβ8.1* promoter flanking DNA to the parental TK promoter plasmid, it is clear that in fibroblasts, no increase in CAT activity can be detected (Fig. 6, lanes *3-6* vs. *9-10* and Table II). The small amount of CAT activity observed with BP plasmids may be attributed to the presence of basal transcriptional elements such as a putative CAAT box, AP-1 recognition site, and the universal TATA element (see Fig. 1 *A*). Experiments with the *IL-2* promoter using this vector system reveal a similar basal CAT activity in all cells tested (data not

TABLE II

Quantitative Analysis of CAT Assay Results from Transfected Vβ8.1 Promoter Plasmids in Fibroblasts and B Cells

	Cell lines			
Plasmids	JY	J558	Hela	CV1 (Cos)
pUTKAT	X*	X	X	X
RTK	10X	10X	20X	NL‡
BP800	0.1X	0.04X	0.1X	0.1X
BP8051	0.05X	0.04X	ND	0.1X
BT8057	1.2X	1.3X	ND	0.5X

Results shown are the mean of four separate transfections. Each cell type was transfected with plasmids separately in duplicate.

* X, percent acetylation of pUTKAT, with other values normalized to it.

‡ NL, nonlinear acetylation of CAM approaching 100%.

shown). Note, by way of contrast, the large stimulation of cat activity by the pRTK plasmid (Fig. 6, lanes *1* and *2*).

Transfection in Lymphoid Cells Other Than T Cells.   To more precisely define the tissue specificity of the *Vβ8.1* promoter and determine whether it functions in cells of the B lineage, two different B cell lines JY and J558, which are, respectively, a human EBV-transformed mature B cell and a murine B cell myeloma were examined. The full-length *Vβ8.1* promoter (BP800; Fig. 7, lanes *3* and *4*), the deleted version, which still contains the TPE (BP8051; Fig. 7, lanes *5* and *6*), and the TPE alone fused to TK (BT8057; Figure 7, lanes *7* and *8*) were separately transfected into both murine and human B cell lines. The results are unequivocal; in both non-T cell lines the *Vβ8.1* promoter proximal elements are inactive (Fig. 7 and Table II). Furthermore, in contrast to the situation in T cells, the 230-bp TPE fragment does not further stimulate TK promoter activity in B cells. Note that we have included the RTK plasmid (Fig. 7, lanes *9* and *10*) in this experiment, which is several-fold more active than the TK plasmid (Fig. 7, lanes *1* and *2*) alone, thus indicating that it is possible to activate the TK promoter in these cell lines (see also Table II).

We conclude that the *Vβ8.1* promoter contains sequences upstream of the start site of transcription that impart a T lineage lymphoid-specific promoter selectivity. The magnitude of the selectivity in murine lymphoid cells is 30–50-fold, and 20–30-fold in human cells (Fig. 4 and Table II). This would seem to be enough to account
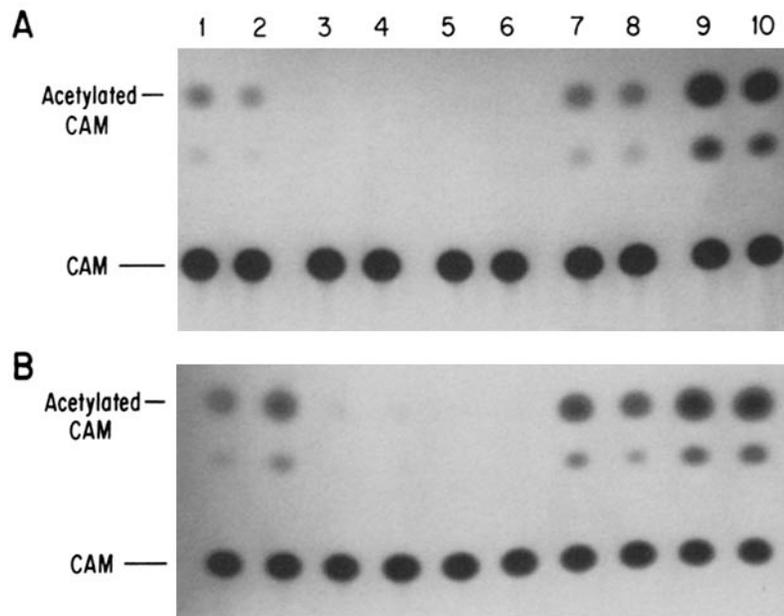

FIGURE 7.   *Vβ8.1* promoter plasmids are inactive in either murine or human B cells. Transfections and CAT assays were carried out as described in Materials and Methods and the legend to Fig. 3 *B*. J558 (*A*), a murine B cell line, or JY (*B*), a human EBV-transformed B cell line were transfected with plasmids pUTKAT (lanes *1* and *2*), BP800 (lanes *3* and *4*), BP8051 (lanes *5* and *6*), BT8057 (lanes *7* and *8*), and pRTK (lanes *9* and *10*). Quantitation of the results by scintillation counting are shown in Table II.

TABLE III

Quantitative Analysis of CAT Assay Results from Transfected Vβ8.1
Promoter Plasmids: Effect of RSV and IgH Enhancers in B and T Cells

	Cell lines			
Plasmids	EL-4	J558	Jurkat	JY
			%	
IgHEBP800	70*	4.8	54	1.9
RBP800	19	0.4	36	1.5
IgHETK	21	61	24	20
RTK	21	15.3	97.5‡	60

The numbers shown are the percentages of acetylated CAM determined after
scintillation counting of the cut-out spots from TLC plates. Each point is the
average of two separate determinations that varied ± 5%. For comparison,
data on the percentage acetylation with BP800 (EL-4, 12%; J558, 0.1%; Jur-
kat, 13%; and JY, 1.3%) and pUTKAT (EL-4, 2.4%; J558, 2.5%; Jurkat,
5.2%; and JY, 13%) in the cell lines tested are presented here.
* The reaction of CAM to its acetylated form in this instance is nonlinear be-
cause of the strength of the interaction between the enhancer and the promoter,
thus underestimating the difference between EL-4 and J558.
‡ Nonlinear acetylation reaction.

for the T cell specificity of the rerranged endogenous Vβ8.1 gene as determined by
Northern blot analysis of TCR-specific mRNA (16, 17).

IgH Chain Enhancer Does not Stimulate Vβ8.1 Expression in B Cells. The striking re-
sult that demonstrated a T vs. B selectivity of the Vβ8.1 promoter could be further
tested by taking advantage of the transcriptional enhancer elements that are active
in either lymphoid cells exclusively (H chain enhancer, IgHE; references 12, 13, and
43) or throughout all eukaryotic cells such as the RSV enhancer. To this end, either
the 1.0-kb murine IgHE-containing fragment (12) or the previously described RSV
enhancer (25) was placed 5' of both the TK and Vβ8.1 gene promoters (see Table
I), and they were separately transfected in duplicate into both murine and human
B and T cells along with several other previously described plasmids. As shown in
Table III, the IgHE is very active in stimulating expression from the TK promoter
in all the cell lines tested, especially J558. The RSV enhancer is most active in human
cell lines, but substantial activity occurs in both murine cell lines tested (Table III).

A different story emerges when we examine the results of transfection into lym-
phoid cells of Vβ8.1 promoter plasmids containing the RSV or IgHE enhancer ele-
ments upstream of -800 (Table III). The Vβ8.1 promoter is activated in T cells by
both of these elements, however, the results in B cells indicate a very feeble interac-
tion. These results are in sharp contrast to the experiments with TK promoter (Table
III), indicating that a TCR promoter is resistant to activation by these enhancers
in B cells.

Discussion

Several genes that are active in lymphoid cells have been characterized with re-
spect to expresson elements located 5' of the transcription start or in downstream
introns. The IgH and IgL gene loci contain both types of elements functioning to
regulate mRNA transcription. MHC class I or class II genes also contain 5' (34,

44) or intronic regulatory elements (45). In addition, IFN-γ is capable of up-regulating the promoter-associated elements of MHC genes (44-47).

Studies of the DNA sequence elements that contribute to the regulation of TCR expression have been more limited. Two reports demonstrated enhancer-like sequences within the intron (47) of the human α chain locus or 3' of the C$\beta2$ gene in the mouse (CβE,(21,22). Luria et al. (47) also have presented evidence for tissue specificity of a 0.70-kb V$_\alpha$ promoter fragment in T cells. Given that Ti β subunit gene transcription precedes (48) Ti α, we considered it particularly important to undertake the present analysis. Our study of the V$\beta8.1$ promoter has demonstrated both the presence of an autonomous element referred to as TPE (Fig. 5), and the tissue-specificity of the whole 0.80-kb fragment containing the promoter sequences (Figs. 4 B, and 6 and 7).

As a result of previous studies with TCR and MHC genes that localized expression elements within intronic sequences (see above), we first concentrated on defining regulatory sequences downstream of the promoter within the intron between the rearranged J of the V$\beta8.1$ V gene and C$\beta1$ (see reference 49 for a map of this region). However, transfection analysis of plasmids containing the $\sim$4.0 kb of the intron cloned in pUTKAT revealed no T cell–specific stimulation of the TK promoter (data not shown) in Jurkat or EL-4 cells. Instead, we found that a 0.80-kb promoter–containing fragment was active both in human and murine T cells (Fig. 4).

Sequence analysis of the V$\beta8.1$ promoter fragment revealed similarities only to basal transcription elements (see Results), but no tissue-specific elements (TCR V$_\alpha$ and V$_\beta$ promoters, Ig V$_H$ and V$_L$ promoters, IL-2 promoter, MHC class I and class II promoters, and IgH enhancer including the octamer) from a diverse set of genes was located in this region. However, comparison of the sequences of the recently described CβE and the V$\beta8.1$ promoter showed significant similarities, even though it was a human/mouse comparison (see Results and reference 21). However, a counterpart to the murine 3' Cβ enhancer has not yet been identified in human. Nevertheless, a combination of expression elements that serve to enhance transcription is found both in the IgH (23, 50) and IgL (14, 51) loci of the mouse or the globin (52, 53) and H5 histone (54) loci in the chicken, which gives added significance to the similarities that were found in the sequence comparison of the V$\beta8.1$ promoters 5' flanking DNA with the murine CβE (see Results).

To prove that the TPE is separate from and independent of the V$\beta8.1$ proximal sequences, we subcloned DNA upstream of −45 5' to the TK promoter in pUTKAT, for example, as shown by BT8051. In this series of experiments we demonstrated that the TPE could confer upon the TK promoter T cell–specific expression, and that as little as 230 bp was required. Interestingly, DNA between −445 and −510 seemed to act to repress the TPE (Fig. 4 B). This small DNA element is also capable of repressing the TPE when the V$\beta8.1$ promoter proximal signals are used (i.e., BP8045 vs. BP8051; Fig. 4 A). Such "negative" activity has been observed in the promoters of MHC class I genes (55), IFN-β (42) IFN-α (56), and the IgH enhancer (57). The molecular basis for the negative influence of this sequence remains to be determined.

The sequence shown, by Anderson et al. (AGTGAT/CG/ATCA) (37), to be common to many murine V$_\beta$ promoters as well as V$\beta8.1$ (earlier pointed out by Siu et al. [20], for V$_\beta8$ promoters as part of a larger 16-bp region of similarity) is not obligatory

for the function of the TPE described herein. In fact, the plasmids BP350 or BP045 are very similar in their expression properties in T cells, even though BP350 contains the decanucleotide whereas BP045 does not. Earlier studies of this region using the DNAse I footprint technique (49) localized a segment of 23 bp protected by extracts from lymphoid and nonlymphoid cells. Based on the nucleotide sequence, a candidate for the protein responsible for this observed protection is the transcription factor AP-1, which is found in most eukaryotic cells (see Results and Fig. 1 A). Thus, the T cell lineage specificity of this interaction remains to be determined.

Using the above transient transfection methods we also made a series of observations concerning the fine specificity of $V_\beta 8.1$ expression in lymphoid cells. We found that none of the plasmids driven by the $V_\beta 8.1$ promoter or TPE-TK constructs that were preferentially expressed in T cells were active in B cells (Fig. 7). More importantly, using either a lymphoid-specific enhancer active in B and T cells (50) or a ubiquitous functional enhancer (RSV), we were unable to activate the $V_\beta 8.1$ promoter in B cells (although activation occurred in T cells; Table III). In a similar study of V_H promoter specificity (50), it was found that enhancer-activated expression of a V_H promoter does not occur in fibroblasts or EL-4 cells. Similarly, the regulated T cell-specific IL-2 promoter could not be activated by an enhancer in non-T cells (26, 58).

The possible implications of these results for transcriptional specificity and the control of rearrangement of receptor genes in B and T cells should be considered. Several types of evidence have accumulated that demonstrate that V_H genes (59), which are either unrearranged or specifically rearranged, are restricted in expression to B cells. To date, only transcripts arising from non-V start sites within the IgH locus have been found in T cells (60–62).[3]

Circumstantial evidence from chromatin accessibility studies (66, 67) highlights the role of DNAse I-accessible chromatin as a factor in the process of gene rearrangement. These arguments have been formalized by Alt and his colleagues (4, 68–70) by a careful analysis of an artificial recombination substrate in B and T cells. They have found that increased transcription is associated with substrate rearrangement, and significantly that TCR V region gene segments ($D\beta$ and $J\beta$) can recombine appropriately in B cells. Furthermore, a $V\beta$-$D\beta$-$J\beta$-IgH enhancer recombination substrate undergoes $D\beta$ to $J\beta$ rearrangements in both B and T cells, but only undergoes $V\beta$ to $DJ\beta$ rearrangements in T cells. These findings demonstrated that the IgH enhancer was sufficient for $D\beta J\beta$ rearrangements but that elements associated with the $V\beta$ (possibly the promoter) controlled tissue-specific $V\beta$ to $DJ\beta$ rearrangement (L. Ferrier and F. W. Alt, personal communication).

Given the correlation between transcription and rearrangement, our data suggest that the reason for the inactivity of the substrate in B cells is the lack of transcriptional activity from the $V\beta$ promoter (see Fig. 7, A and B). Experiments in cultured B and T cells with recombination substrates utilizing the $V_\beta 8.1$ promoter could directly test the hypothesis that rearrangement is dependent upon transcription of the utilized V gene. More important would be to determine whether the activity of the TPE in conjunction with downstream transcriptional start signals would be

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[3] An example exists in the literature (63–65) of a V_H gene transcribed in a T cell tumor after rearrangement caused it to fuse with part of the $J_\alpha C_\alpha$ locus on chromosome 14. In this case, expression elements from the newly juxtaposed locus may have overridden the normal control elements 5' of the V_H promoter in these morphologically transformed human T cell tumors.

adequate to activate rearrangement within a recombination substrate. Finally, experiments using cell fusions of B and T cells followed by assay of either the endogenous or introduced V_β promoters for expression would help determine whether there exists an inhibitory substance in B cells that extinguishes expression of specific TCR promoters.

Summary

We have characterized the sequence contribution of DNA 5' of a functionally rearranged TCR promoter ($V\beta8.1$) on its T lineage–specific expression through the use of the chloramphenicol acetyl-transferase (CAT) reporter gene. A 230-bp fragment located 570 bp upstream of the determined transcription start site of the $V\beta8.1$ promoter confers a T lineage specificity of expression to a heterologous promoter. The inability of the $V\beta8.1$ promoter and its associated elements to function in B cells suggests the existence of a mechanism to prevent inappropriate V_β gene expression in B cells. Of considerable interest is the fact that both a B cell–specific and a nontissue-specific enhancer element were incapable of stimulating significant expression of this promoter in B cells. We discuss the implication of these results on the process of rearrangement of both Ig and TCR genes, and the differentiation of the lymphoid system.

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