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Functional Analysis of the Vγ3 Promoter of the Murine γδ T-Cell Receptor

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The initial day 14 wave of fetal thymocytes express a γδ T-cell receptor (TCR). This surface TCR is generated by preferential rearrangement of Vγ3 and Vδ1 recombination segments. To delineate the role of regulatory sequences in this expression, we have analyzed the Vγ3 promoter control region under the regulation of its cognate Cγ1 enhancer. Transcription initiates 25 bases downstream from a TATTAA sequence at a consensus initiator motif. The minimal 5′ promoter sequences supporting expression by transient analysis extend −243 nucleotides from the +1 start site. Three regulatory sequences in this region have been defined by deletion and mutagenesis: a consensus CTF/NF-1 site at −55, an Ets homology sequence at −65, and a degenerate, but crucial, SP-1 site at −100. The presence of additional sequences downstream of the start site which extend through the leader intron were necessary for expression. In contrast to other TCR or immunoglobulin variable regions, one or more strong upstream suppressor sequences resembling silencer elements have been observed. A 311-bp fragment, positions −586 to −897, exhibited strong repressing activity regardless of orientation when placed upstream of heterologous promoters.

The ordered appearances of T-cell receptor (TCR) Vγ genes during ontogeny provide the developing embryo with a functional category of T cells which are distinctive in their thymic origin, timing, and adult localization (1, 20, 29, 39, 57, 59, 64). Most notably, the homogeneous, nonallotypic appearance of the canonical Vγ3-to-Jγ1 rearrangement (50) is closely coupled temporally to the emergence from fetal day E13 to E18 of intraepithelial lymphocytes (s-IELs) (28) found exclusively in the adult epidermis (5). This rearrangement is essentially undetectable in the adult thymus (24, 65). The closely linked Vγ4 gene, located 1.3 kb upstream of Vγ3, follows a similar chronology. Following productive rearrangement of Vγ4-Jγ1, thymocytes expressing this receptor emerge during a slightly later wave (28, 65). In the adult, however, these (r-IELs) reside in mucosal epithelium such as that found in the female reproductive tract (40).

A similar situation is observed at the δ locus (12), where tightly regulated V gene usage is also the case in the early fetus. The attending use of Vδ1-Dδ2-Jδ2-Cδ rearrangements, when paired as functional heterodimer receptors with Vγ3-Jγ1-Cγ1 gene products, establishes an invariant pattern of developmental control at these diverse loci. Coordination of these events does not require surface expression, since disruption of the δ gene in mutant mice did not alter the normal pattern of early thymic rearrangements (41). The continued regulation of these early thymic γδ T-cell events without the selection afforded by receptor expression, such as is observed in the autoreactive recognition elimination of TCR αβ cells (80), is consistent with their proposed role. Because of the tissue-localized nature and homogeneous receptor expression of these T cells, proposed functions are believed to mediate a response to a limited host antigen(s) resulting from epithelial invasion of pathogens or trauma (30, 58). Indeed, s-IELs have been shown to exhibit cytotoxic T-lymphocyte activity (65) and also to respond in vitro to a factor(s) produced by stressed keratinocytes (30). Thus, recognition of such self-antigens may preclude the normal pattern of thymic T-cell affinity selection.

A targeted gene rearrangement is therefore a more plausible model with which to guide analysis of the control mechanisms involved in Vγ gene expression (24, 41, 65). Such V gene targeting has been proposed to involve accessibility and/or transcription of the recombining segments (17–19, 55). Accessibility of chromatin regions to the recombinase complex machinery is, in part, believed to be mediated by DNA-binding factors. In addition to possibly inducing higher-order chromatin alterations, factor-DNA complex formation has other well-defined local effects, such as DNA bending (21). Certainly, sequence-specific DNA-binding factors may further act as modulators to sustain transcriptional activity. Transcription preceding rearrangement, termed germ line or sterile transcription, has been reported for certain immunoglobulin V_H and V_κ and for TCR Vα and Vγ genes. Given the vast distance between the V segments and their respective enhancers in the unrearranged context, such transcription is clearly enhancer independent (24, 65). Nonetheless, active locus enhancer function is required for initiating the rearrangement event and continued transcriptional maintenance (17). Alternatively, sterile transcription could be serving only as a marker and could therefore be a consequence and not the cause of the accessible chromatin state.

The Cγ1 enhancer appears to convey a well-defined, T-cell-specific activity upon heterologous promoters but inherently no γδ T-cell specificity (46, 76). A transcriptional enhancer silencer has been proposed to explain lineage-restricted specificity of the Cγ1 locus (38). Control of specific Vγ gene targeting would appear to reside in promoter-associated sequences and thus mediate some or all of the mechanisms discussed.

We have analyzed Vγ3 gene regulation in conjunction with its cognate enhancer activity and have defined a minimal promoter region which appears to impart transcriptional

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activity in transient reporter gene assays. However, this active region is strongly repressed if additional upstream sequences, some of which may be tissue specific, are included. This activity may be modulated as a result of chromatin structure. The negative-acting sequences exhibit properties reminiscent of silencers, such as heterologous promoter repression regardless of orientation.

MATERIALS AND METHODS

Plasmid constructs. A 560-bp *Bgl*II Cγ1 enhancer-containing fragment from J21.56BPR (76), kindly provided by D. H. Raulet (University of California, Berkeley), was cloned into the *Bam*HI polylinker site of pGEM-7Z(+) (Promega). Several clones with one, two, or four fragment inserts were isolated. These inserts were digested with *Cla*I-*Sac*I and ligated to the downstream polylinker site of pBL-CAT2 reporter gene vector (52) to generate pBL-CAT-$C\gamma1_{enh}(n)°$ (n = 1 to 6). A 2.4-kb *Eco*RI-*Eco*RV fragment from the functionally rearranged Vγ4,3-Jγ1-Cγ1 genomic clone of the murine dendritic epidermal cell (dEC) clone 1D2 (49) in cosmid vector pWE5.5-15 was subcloned into pGEM-7Z(+) in the *Eco*RI-*Sma*I sites. This Vγ4- and partial Vγ3-containing region was then used in construction of Vγ promoter vectors by cloning into pBL-CAT2 or pBL-CAT2-$C\gamma1_{enh}$-2° vectors. The 311-bp *Hin*dIII repressor fragment was directly cloned into the upstream polylinker site of J21.56BPR.

Promoter truncations were constructed by use of indicated restriction sites or by PCR with the following primers: 5′ V3 (−223) GCTCTAGAGGGAATGGTATCTGTTG, V3(−147) GCTCTAGAAATGTCATAAAATGACCC, V3(−85) GCTC TAGAAATAAGTCTTGCCTTCC, V3(−47) GCTCTAGAG CTAGGGCTGCATATTAATC, and 3′ V3(+208) CGGGA TCCTGAGATATCCAGGAG.

PCR site-directed mutagenesis was carried out as previously described (31). The primer used for CTF/NF-1 mutation was GTCTTGCCTTCCTCCCCGTGCCCTTTGCACT AGAGCTAGGGCTGC, and that used for Ets mutation was GTCTTGCCCCCCCCCCCTGGCCCTTTGCCATAGAGC TAGGCTGC. The primer used for Sp-1 GC box mutation was ACAGCTCACTAAATGAAGTTGCTAACAGCTCAG AAATAAG. All PCR-generated constructs were confirmed by sequencing all of the Vγ3 promoter region.

Transfections and CAT assays. All cell transfections were carried out with ~2×10^7 cells and Qiagen-isolated plasmids in a Bio-Rad Gene Pulser apparatus. Peer cells were transfected with Vγ3-Jγ1-Cγ1 *Eco*RI-isolated genomic fragment and with RSV-neo selection vector for stable integrants. Transient transfections for chloramphenicol acetyltransferase (CAT) reporter assays employed 6 pmol of plasmid DNA and 10 μg of β-galactoside-expressing vector pCH-110 (Pharmacia). Cell lysates were prepared at 48 h posttransfection by four cycles of freezing on dry ice-ethanol and thawing at 37°C for 5 min (each cycle). CAT activity was assayed by measuring extent of [^{14}C]chloramphenicol acetylation as described previously (25). β-Galactosidase activity was measured by using the chromogenic substrate chlorophenol red β-D-galactopyranoside (CPRG; Boehringer-Mannheim) to normalize CAT values for transfection efficiency (36). Reported CAT values were obtained from two or more independent transfections.

RNA and Northern blot analysis. Total RNA from cells (JAC-3 or Peer) was extracted as previously described (13). Primer extension reaction was performed as described elsewhere (16) by using 30 μg of total JAC-3 RNA primed with antisense L-1 primer. Northern (RNA) blot analysis of stably transfected and untransfected Peer RNAs was accomplished by a previously described method (71). Random-primed ^{32}P-labeled Vγ3-specific probe (49) or a *Bam*HI-*Hin*dIII fragment of RSV-neo was utilized to detect the appropriate mRNAs.

EMSAs. Crude nuclear extracts were prepared by the electrophoretic mobility shift assay (EMSA) method of Dignam (15). Double-stranded oligonucleotide probes were 5′ ^{32}P end labeled and gel purified prior to use. A total of 4×10^4 cpm per probe was incubated with 20 to 25 μg of total protein for 20 min at room temperature. Complexes were analyzed in native 5% acrylamide gels run with 0.5× Tris-borate-EDTA (TBE) buffer at constant power.

Nucleotide sequence accession number. The 1,314-bp DNA sequence between the Vγ4 recombination signal sequence and the Vγ3 AUG initiation codon has been assigned GenBank accession number L20100.

RESULTS

Determination of Vγ3 transcriptional start site. Determination of the initiation site of transcription for Vγ3 was necessary to begin an analysis of the *cis*-acting sequences essential for regulation of expression. First, we determined the 1,314-bp DNA sequence between the Vγ4 recombination signal sequence and the Vγ3 AUG initiation codon. Total RNA was then isolated from murine cell line JAC-3. This interleukin-2-independent subline was derived from the mature γδ TCR-expressing dEC line 1D2 (49) by stable transfection of a constitutive human interleukin-2 expression vector. A primer extension reaction was performed by using antisense primer L1, complementary to the Vγ3 leader peptide exon (Fig. 1A). A major start site was detected 26 bp downstream from a TATA-like sequence, TATTAAT, to yield a 5′ untranslated sequence (UT) of 32 bases (Fig. 1B). The sequence surrounding the initiation site matched in seven of eight positions with the consensus sequences for the initiator element

YAYTcYYY
CACTGTCT
+1

(74). Therefore, as with other V regions, Vγ3 has a conventional proximal promoter architecture.

Vγ3-Jγ1-Cγ1 functionally rearranged gene activity in Peer cells. A genomic 16.5-kbp *Eco*RI fragment was obtained from the murine dEC clone 1D2 which includes the functionally rearranged Vγ3-Jγ1-Cγ1 gene and extends from ~0.5 kbp 5′ of Vγ4 to ~3.9 kbp downstream of the Cγ1 3′ enhancer (the putative γδ 3′ silencer would not be included). This fragment was utilized to generate a stably transfected Peer T-cell line. This clone and fragments thereof were used exclusively in all subsequent promoter experiments and constructions.

The human acute lymphoblastic leukemia (ALL) T-cell line Peer was chosen for stable integration because of previous reports indicating strong activity from 3′ Cγ1 enhancer reporter gene constructs in this line (65, 76). Cotransfections with the expression vector RSV-neo and use of G418 antibiotic selection (500 and 300 μg/ml) gave resistant cells which were assayed by Northern analysis for Vγ3-Jγ1-Cγ1 and Neo mature transcripts (Fig. 2). Only the transfected Peer (lanes 1 and 2) and endogenous γ chain-expressing JAC-3 cells (lane 4) gave positive signals. Furthermore, the sizes and alternatively polyadenylated forms (1.5 and 1.7 kb) of Cγ1 were indistinguishable from those

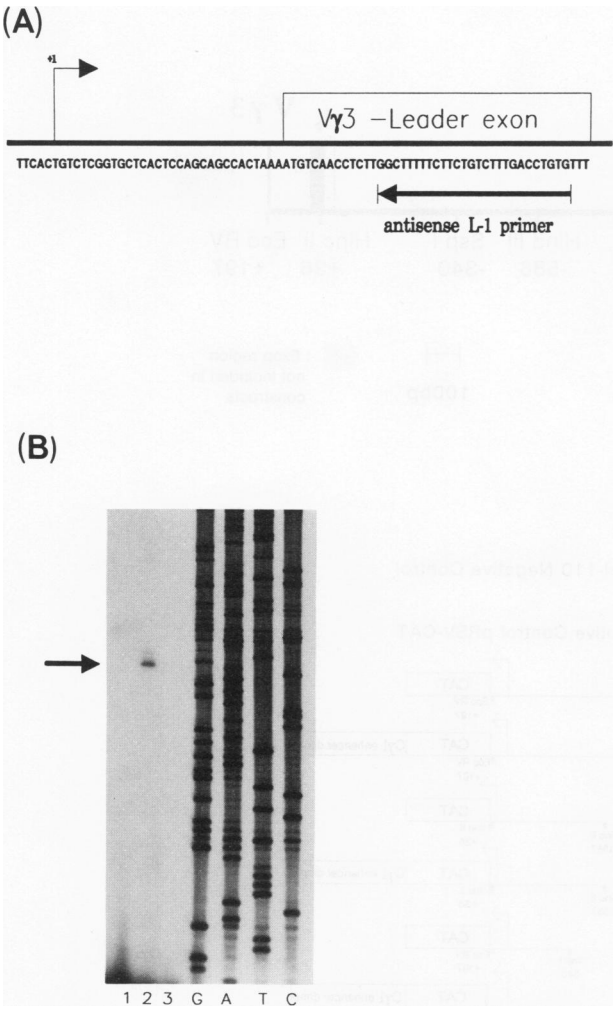


FIG. 1. Nucleotide sequence of Vγ3 leader exon, 5′ UTR, and primer extension analysis. (A) Diagram indicating transcriptional +1 nucleotide at the initiator element and oligonucleotide primer sequence from the leader exon minus strand utilized to determine the transcriptional initiation site. (B) Primer extension transcriptional start analysis of Vγ3-Jγ1-Cγ1 functionally rearranged loci from 1D2/JAC-3 dEC line. Lane 1, L-1 primer only without RNA; lane 2, total RNA (30 μg) from JAC-3 annealed with L-1 primer and extended by using avian myeloblastosis virus reverse transcriptase; lane 3, L-1 primer with 30 μg of yeast tRNA. The sequencing ladder was obtained by using L-1 primer and Vγ3 genomic sequence subclone (lanes G, A, T, and C).

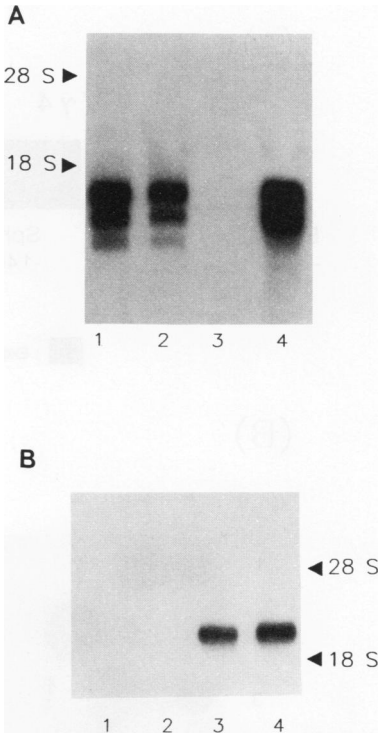


FIG. 2. Northern blot analysis of Peer stable transformants. (A) Northern blot of ~10 μg of total RNA fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon membrane, and hybridized to a gel-purified Vγ3-specific DNA fragment probe (49). Functionally rearranged Vγ3-Jγ1-Cγ1 genomic clone-transfected bulk Peer cells selected by G418 (G418 bulk-Peer) for RSV-neo cotransfected vector. Lane 1, 300 μg/ml of G418 bulk-Peer; lane 2, 500 μg/ml of G418 bulk-Peer; lane 3, untransfected Peer cells; lane 4, JAC-3 dEC. (B) Parallel lanes probed for the neomycin resistance gene. Lane 1, JAC-3; lane 2, untransfected Peer; lane 3, 500 μg/ml of G418 bulk-Peer; lane 4, 300 μg/ml of G418 bulk-Peer.

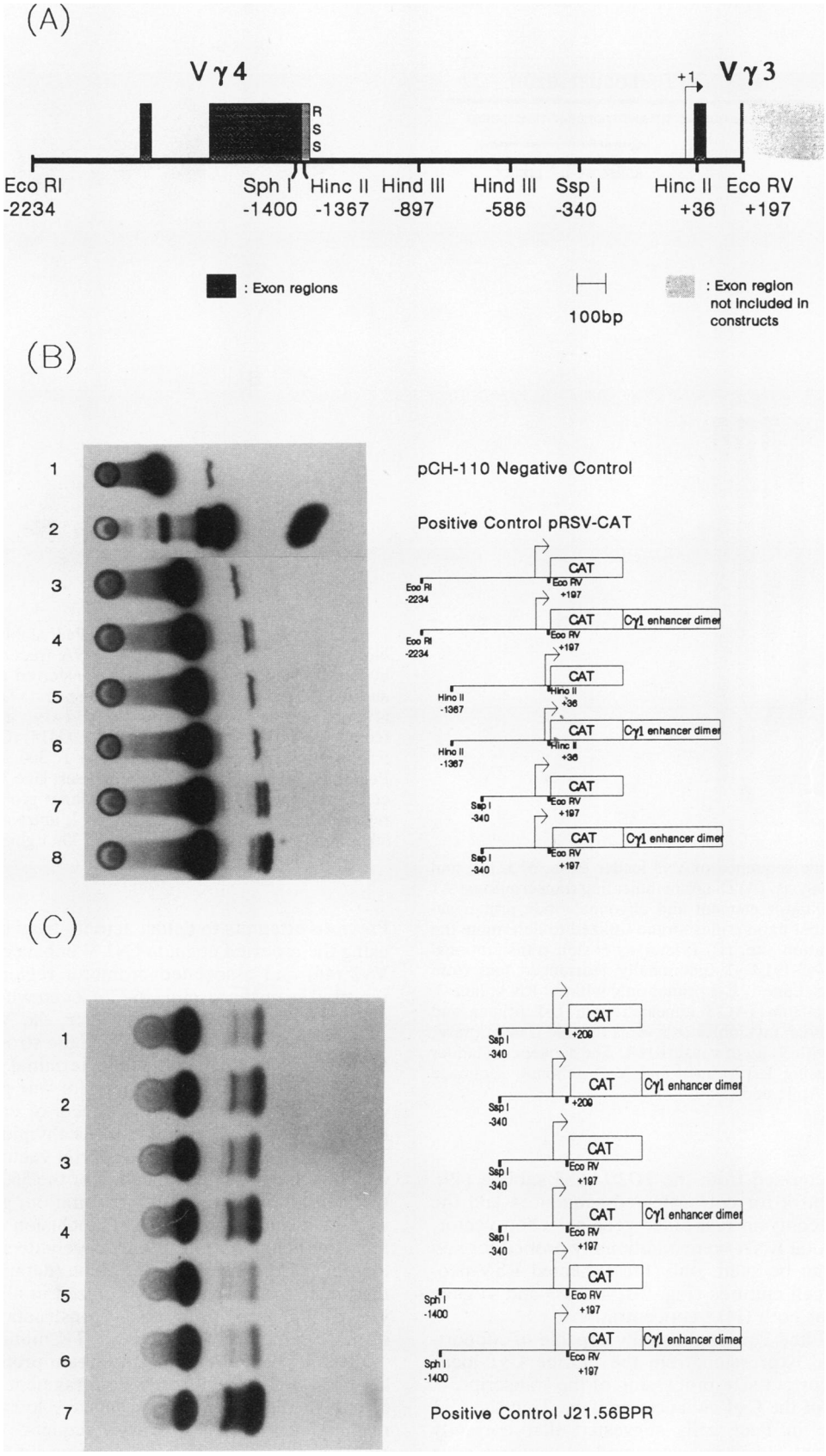
previously characterized from the 1D2/JAC-3 subline (49). As a positive control for cotransfected sequences and the presence of the neomycin resistance gene selection vector, parallel lanes of total RNA were additionally probed for *neo* transcripts. As can be seen, only the expected RSV-neo-transfected Peer cell cultures (Fig. 2B, lanes 3 and 4) gave positive signals for both G418 concentrations.

The human cell line Peer is thus fully capable of supporting transcriptional expression from the murine Cγ1 locus with apparently correct size processing of the transcript.

Optimalization of the Cγ1 enhancer activity. That the Cγ1 locus was active in Peer cells suggested that correctly dissected functional elements assembled in reporter gene constructs could be utilized in analysis of promoter activity. Previous attempts to detect activity from such constructs by using the reported cognate Cγ1 3′ enhancer with Vγ4 (76) or Vγ2 (46) and associated promoter region sequences were reported as unsuccessful (46, 76), even when assayed in cells expressing γδ receptors. However, the Vγ4 promoter was active in constructs which utilized the strong T-cell enhancer from murine leukemia virus long terminal repeat (LTR) (76).

To maximize optimal activity for our promoter analysis, we systematically tested the effects of enhancer multimerization. The herpes simplex virus thymidine kinase (HSV-TK) minimal promoter CAT reporter vector pBL-CAT2 (52), which included at the 3′ polylinker the 560-bp Cγ1 enhancer fragment(s) in a head-to-tail orientation, gave highest activity with a dimer configuration. Inclusion of the tetramer or hexamer configurations progressively decreased activity below that of the TK promoter alone (data not shown). Thus, the dimer configuration was utilized in all subsequent analyses of Vγ3 promoter fragment constructions by substitution of these sequences for the HSV-TK minimal promoter.

Identification of a strong, upstream promoter repressor. A 2.4-kb *Eco*RI-*Eco*RV restriction fragment encompassing the 5′ end of the genomic rearranged γ locus (Fig. 3A) is the maximal amount of upstream sequence included in both stable Peer transfectants and in transient CAT reporter gene constructs. This fragment includes the Vγ4 coding regions,


(A)
Vγ4
+1
Vγ3
R
S
S
Eco RI -2234
Sph I -1400
Hinc II -1367
Hind III -897
Hind III -586
Ssp I -340
Hinc II +36
Eco RV +197
: Exon regions
100bp
: Exon region not included in constructs
(B)
1
2
3
4
5
6
7
8
pCH-110 Negative Control
Positive Control pRSV-CAT
CAT
Eco RV +197
Eco RI -2234
Cγ1 enhancer dimer
Hinc II +36
Hinc II -1367
Ssp I -340
(C)
1
2
3
4
5
6
7
CAT
+209
Ssp I -340
Cγ1 enhancer dimer
Eco RV +197
Sph I -1400
Positive Control J21.56BPR

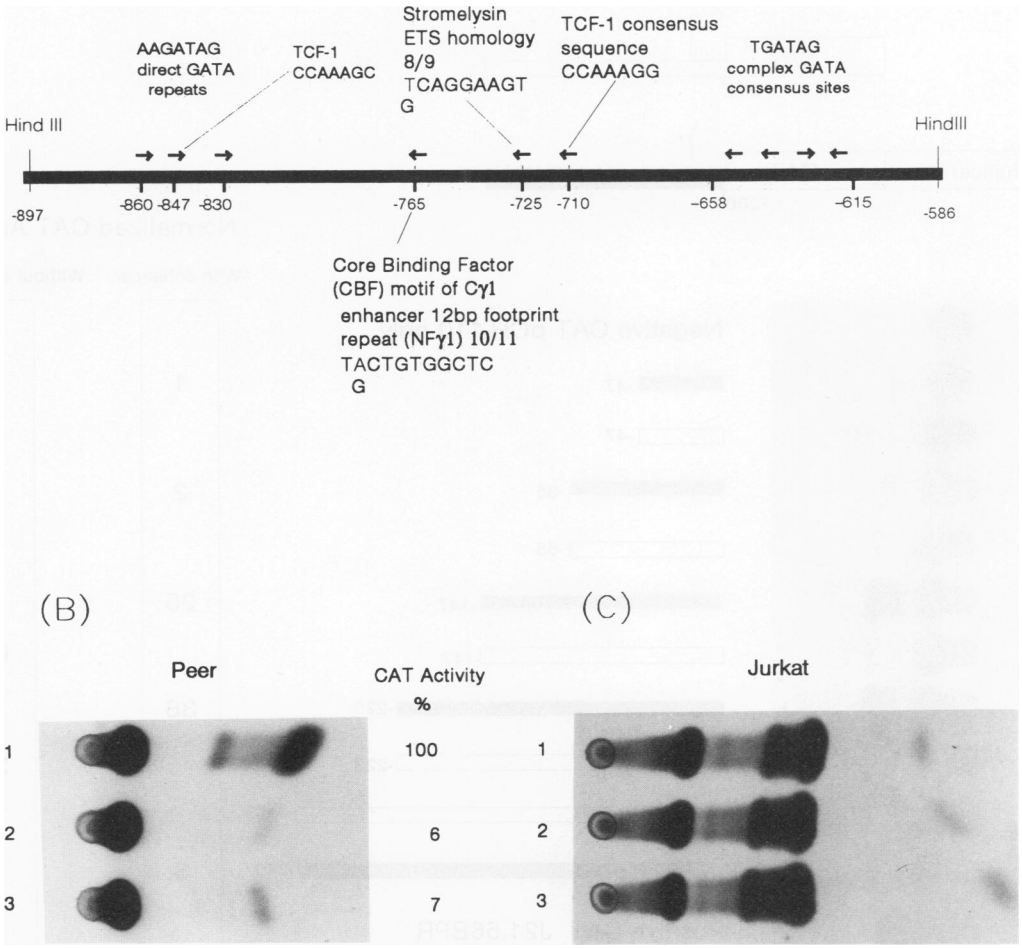


FIG. 4. Repressor fragment diagram and heterologous promoter activity in T cells. (A) Site map of potential *cis* regulatory sequences with recognizable homology to known DNA-binding nuclear factors and sites (34). (B) The strong repressing effect of the *Hin*dIII 311-bp fragment from the Vγ4-Vγ3 intergenic region when located upstream of a heterologous minimal c-Fos promoter, J21.56BPR vector (76) (lane 1) in sense (lane 2) or antisense orientation (lane 3). (C) Lack of observable inhibition with the repressor constructs in Jurkat αβ T cells.

the intergenic region, and the Vγ3 coding regions to position +197 bp. In addition, truncation constructs at the 5′ sites −1.4, −1.3, and −0.34 kb from +1 and 3′ to +36, +197, or +208 bp were generated from restriction sites for analysis of promoter activity.

As in the previous reports (46, 76), no significant activity was generated by the 5′ −2.23 kb to +197 region or from the restriction site truncations extending from −1.3 kb to +36 and −1.4 kb to +197 (Fig. 3B and C). Low activity of the −340 to +197 or +208 fragment was reproducibly obtained in the presence or absence of the Cγ1 enhancer. This suggested the possibility of repressive effects being mediated by the deleted upstream sequences. A further suggestive observation was that a 311-bp *Hin*dIII fragment, extending from −586 to −897 (Fig. 4A), contained several consensus protein binding sites previously implicated in mediating transcriptional repression (7, 21, 34) and/or activation effects (8, 33, 35, 47, 79). These include a unique complex of six direct and inverted consensus repeats of the GATA binding motif, as well as sequences resembling those of LEF/TCF-1, Ets, and the CBF core element found in several T-cell-specific enhancers (26, 34, 37, 67, 68, 78, 81, 82).

The CAT gene reporter construct J21.56BPR (76), used as our source of the 560-bp Cγ1 3′ enhancer fragment, was used as a heterologous promoter test vector. To determine whether any activity was associated with the 311-bp *Hin*dIII fragment, the sequence was introduced into the polylinker *Hin*dIII site immediately upstream of the c-Fos minimal

FIG. 3. Restriction map of Vγ4,3 region and reporter gene activities of various promoter fragments. (A) Intron-exon structure of the 2.432-kb Vγ4- and Vγ3-containing fragment. (B) CAT assays, with or without enhancer dimer, indicating low or no activity from the 5′ end point restriction fragments −2234, −1367, and −340 and +197 or +36 3′ end points. Lane 1, negative control CAT assay, pCH-110 normalizing β-galactosidase expression vector (relative activity, 1×); lane 2, pRSV-CAT positive control vector (relative activity, 160×); lanes 3 to 8, relative activities of constructs as diagrammed: lane 3, 1.5×; lane 4, 2.1×; lane 5, 1.8×; lane 6, 2.4×; lane 7, 3×; lane 8, 4×. (C) Increased activities of the Vγ3 5′ upstream region when truncated at −340 are not affected with the 3′ end point boundaries at either +197 or +208 (lanes 1 to 4). Activity of a −1400 to +197 construct (lanes 5 and 6). In comparison to the −340 to +197 or +208 constructs, a lower activity results from inclusion of upstream sequences.

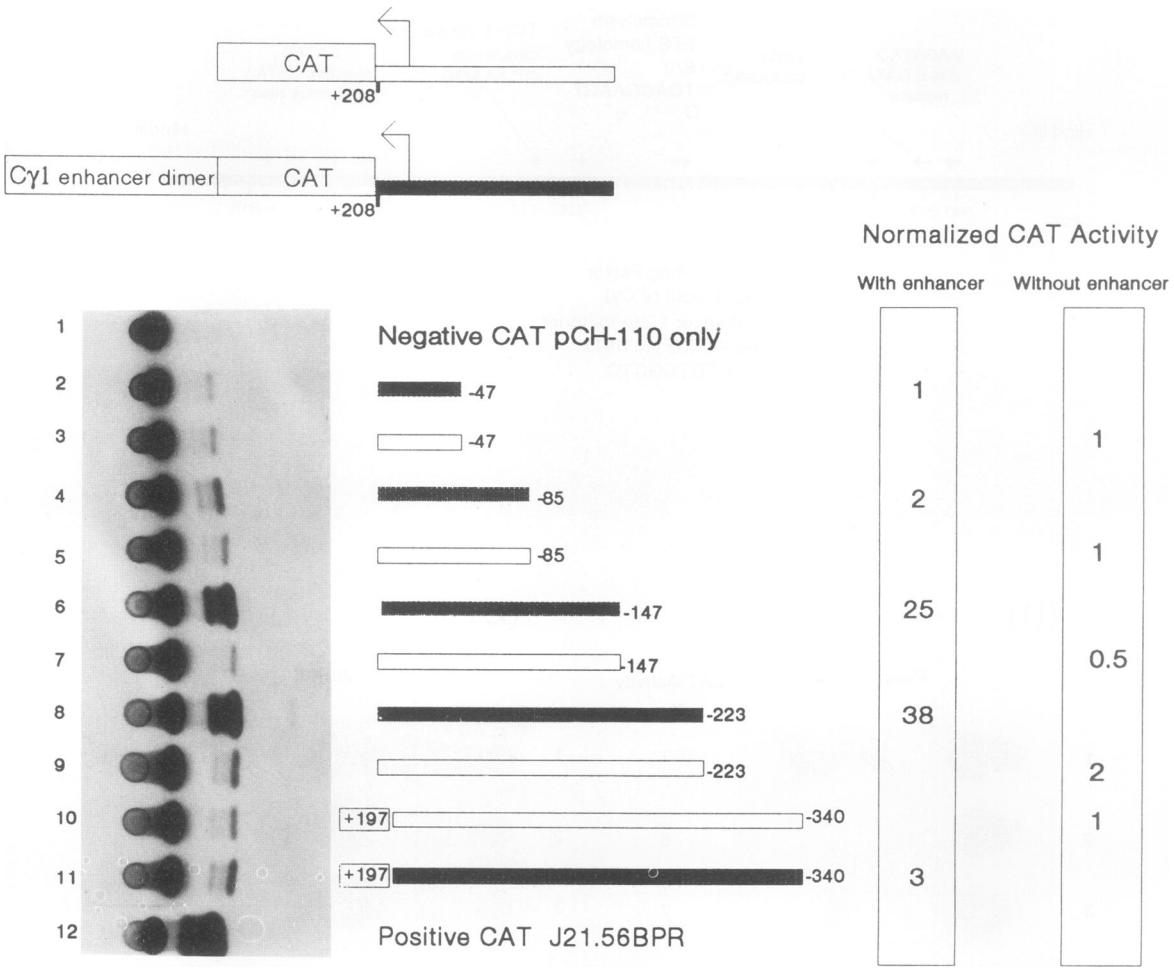


FIG. 5. Minimal active promoter fragment 5′ truncations. The Vγ3 promoter PCR truncation fragments extending from −223, −147, −85, or −47 to +208 and −340 to +197 with their relative CAT values as enhancerless basal activities (lanes 3, 5, 7, 9, and 10) and with the enhancer dimer activities (lanes 2, 4, 6, 8, and 11). Control assays: negative CAT pCH-110 normalizing β-Gal expression vector (lane 1) and positive J21.56BPR Cγ1 enhancer CAT vector (lane 12).

promoter. A strong repression, ≥90% of J21-0.56BPR CAT activity, was observed in Peer cells with a single-copy insert regardless of the fragment orientation (Fig. 4B). These results were reproduced in comparable Cγ1 enhancer constructs that utilized the minimal HSV-TK promoter (data not shown). Similar repression of the basal activity from the enhancerless HSV-TK promoter constructs also was observed (data not shown). Thus, the effect mediated by the 311-bp fragment appeared to be enhancer independent. Additional testing of the ±311 J21-0.56BPR constructs in the αβ T-cell line Jurkat revealed no repressing activity (Fig. 4C). This confirms the specificity of the 311-bp fragment and indicates that its activity might be cell type restricted.

Delineation of the Vγ3 minimal promoter. The significant repression engendered by sequences between −586 and −897 prompted a search for additional negative regions downstream of the weak −340 constructs. By using PCR, we generated a further set of 5′ truncations at positions −223, −147, −85, and −47. As shown in Fig. 5, significant activity now was observed only in the −223 and −147 constructs which contained the Cγ1 enhancer compared with the weak signals from −340 5′ ends. To accommodate optimal PCR oligonucleotide primer-annealing temperatures, the 3′ end of PCR-generated promoter fragments was extended to position +208. This nucleotide is 27 bp into the mature peptide-encoding, second exon of Vγ3. As observed in Fig. 3C (lanes 1 to 4), the additional 11 nucleotides were inconsequential in their effect on activity of the −340 5′ truncation constructs. To further delineate necessary sequences 3′ to the start site, truncations utilizing maximally active 5′ endpoints −147 and −223, but deleting positions 3′ of +36 (at the leader peptide exon second codon, ATG TC/), were generated. No CAT activity in Peer cells was recovered from the constructs with this truncation (Fig. 6), implicating sequences between +36 and +208 as critical. Further analysis of this downstream region was not carried out. However, there are two distinguishable sequence features in this region: an ~80-bp sequence (including 34 bp of the leader exon extending from +42 to +122) that is ~60% (48 of 80) T and a consensus, extended dyad, E box motif (TCCAGATGGA) which spans the second exon splice-acceptor site.

Mutational analysis of positive-acting sequences within the minimal 5′ promoter. Sequence and transcriptional start analysis had revealed typical initiator (Inr) and TATA box-like sequences. Thus, we attempted to characterize several additional consensus or unusual sequences which were

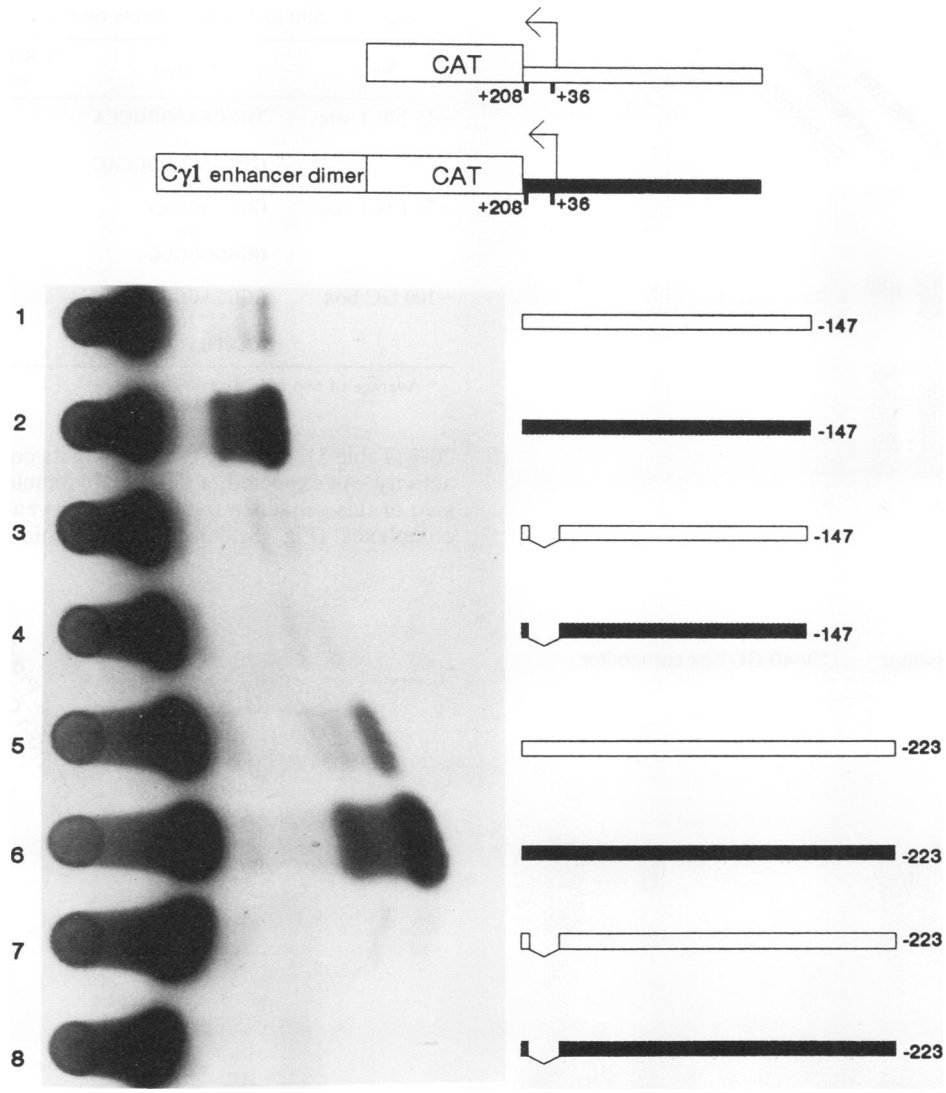


FIG. 6. Delineation of Vγ3 leader exon-intron sequences necessary for promoter activity. The 5′ end point constructs which gave the most-active reporter signals (−147 and −223) were deleted 3′ from +36 to +208, and their CAT activities were analyzed. As for Fig. 5, activities with enhancerless (lanes 1, 3, 5, and 7), and enhancer (lanes 2, 4, 6, and 8) dimer constructs were examined.

present within the active, minimal upstream promoter region.

An 8-nucleotide (nt) direct repeat (ACAGCTCA) spaced by 16 nt is located at −118 to −86. These sequences were good candidates for contributing to the minimal promoter, as the activity at the −86 truncation is severely reduced relative to that of the strong −143 construct (Fig. 5). Duplex oligonucleotide probes overlapping this region produced apparent specific DNA-binding complexes with crude nuclear extracts from JAC-3 in EMSA. Unexpectedly, specific sequence mutation-EMSA competitions indicated that these complexes did not result from binding to the direct repeats. Instead, the complexes were merited to the intervening 16 bp which contained a degenerate SP-1-like GC box (GGG CaGGa) centered at −100 (Fig. 7A). To formally test the role of this motif, the 8 bp of the GC box were replaced in the −223 CAT vector with nucleotide transition mutations while leaving the direct repeats intact. The mutation essentially eliminated promoter function (17% of wild type) (Table 1).

Although critical to the promoter, the degenerate GC box bound had significantly lower protein binding affinity (Fig. 7B, lanes 6 to 14) than the conventional GC box III (GGGC GAGG) of the simian virus 40 (SV40) early promoter (lanes 2 to 5). Qualitatively, shifted complexes otherwise appeared to be indistinguishable, with only minor high mobility differences (Fig. 7B, lane 2 versus lane 6). Similar EMSA complex profiles were observed regardless of tissue origin of the nuclear extract (data not shown).

A CTF/NF-1 site is centered at position −55. The sequence

$$\underline{TGGC}_{CCTTTGCCA}$$
$$ACCGGGAAA_{\underline{CGGT}}$$

exactly conforms to the reported high-affinity binding-recognition consensus, $^{TGGC}NNNNN_{CGGT}$, such as that found in the adenovirus origin of replication (42, 69). Mutagenesis of the site reduced the activity of the −223 wild-type vector to

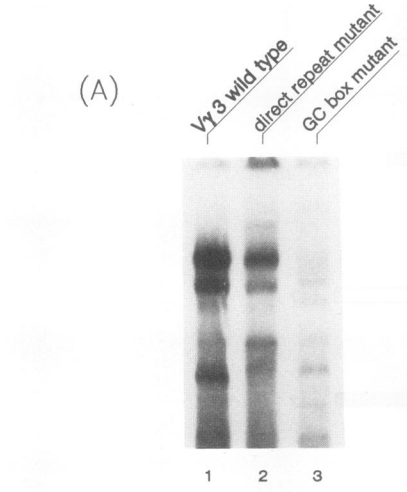


TABLE 1. Site mutational effects on minimal promoter activity

Site	Change	% Relative CAT activity[a]	SD (%)
−55 NF-1 site	TGGCAAAGGGCCA	100	
	to		
	GTGCAAAGGGCAC	21	±5
−70 Ets-1 site	GGGGAGGAA	100	
	to		
	GGGGGGGGG	51	±7
−100 GC box	GGGCAGGACCAT	100	
	to		
	AAATGAAGTTGC	17	±2

[a] Average of two independent assays.

20% (Table 1), formally establishing its contribution to basal activity. As expected, a duplex oligonucleotide probe inclusive of this sequence (−33 to −78) revealed several EMSA complexes (Fig. 8, lane 3). The uninhibited adenovirus

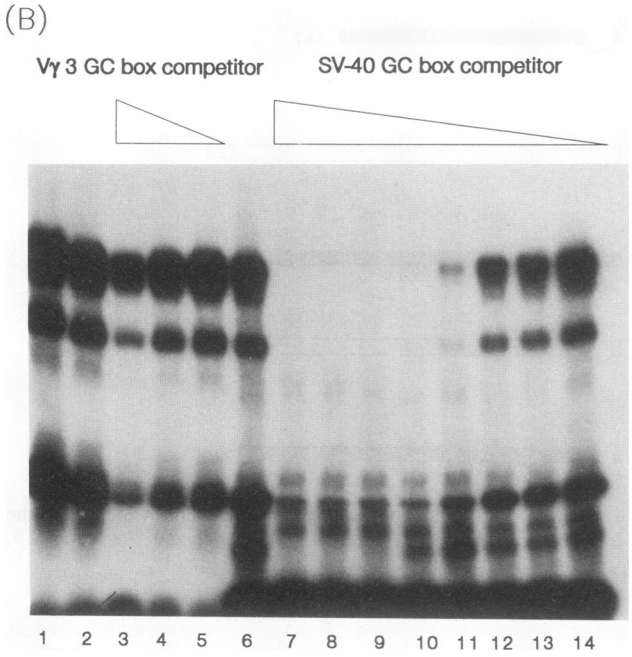


FIG. 7. EMSA analysis of binding to the −100 degenerate GC box. (A) Lane 1, wild-type duplex oligonucleotide from −130 to −80, which includes the −100 GC box *cis* sequence (GGGCAGGA) that is flanked by the direct repeats (ACAGCTCA) at −90 and −114, incubated with nuclear extracts from dEC cell clone 1D2; lane 2, scrambled mutation of direct repeats at −90 and −114 to CACA GACT; lane 3, transition mutations of −100 GC box to AAAT GAAG. (B) The −100 GC box interactions were directly compared to those of the SV40 early promoter, GC box III, (GGGCGGAG) probe (75), with 20 μg (lane 1) or 10 μg (lane 2) of 1D2/JAC-3 nuclear extract. The corresponding Vγ3 unlabeled competitor was used with decreasing molar excess concentrations of 2,000× (lane 3), 1,000× (lane 4), and 50× (lane 5) against the GC box III probe by using 10 μg of nuclear extract. Lane 6, Vγ3 −100 GC box probe incubated with 1D2/JAC-3 nuclear extract (~20 μg). Competition with GC box III duplex oligonucleotides in decreasing molar excess concentrations of 2,000× (lane 7), 1,000× (lane 8), 500× (lane 9), 100× (lane 10), 50× (lane 11), 10× (lane 12), 5× (lane 13), and 1× (lane 14) with 20 μg of extract.

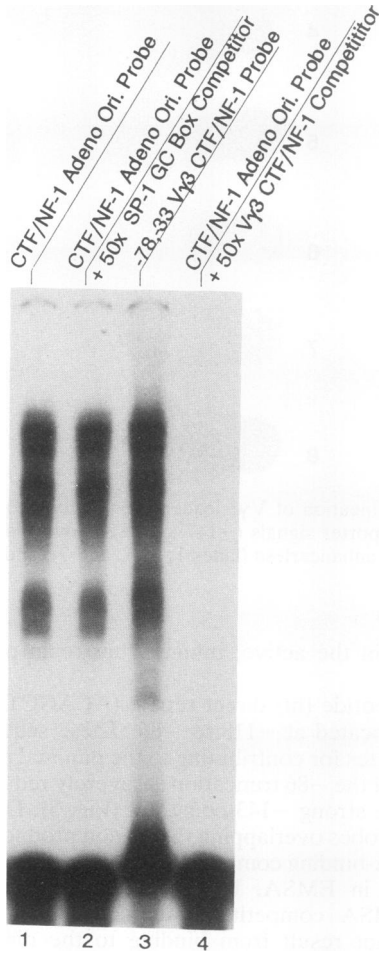


FIG. 8. EMSA analysis of binding to the −55 CTF/NF-1 site. JAC-3 γδ dEC nuclear extract binding to a consensus CTF/NF-1 site from the adenovirus origin of replication (lane 1) competed with unlabeled 50× molar excess of a SV40 early promoter GC box III (lane 2) or the (−33 to −78) Vγ3 CTF/NF-1 site (lane 4). Lane 3, corresponding (−33 to −78) Vγ3 CTF/NF-1 probe and its associated complexes.

CTF/NF-1 probe has a similar EMSA profile (Fig. 8, lane 1) and was cross-inhibited specifically at equivalent concentrations with the unlabeled Vγ3 CTF/NF-1 sequence (Fig. 8, lane 4) but not with the irrelevant Sp-1 GC box III probe (Fig. 8, lane 2). The CTF/NF-1 site was distinctive in that the specific EMSA binding profiles, as determined by the irrelevant Sp-1 GC box or specific adenovirus CTF/NF-1 competitions, differed radically depending on the nuclear extract derivation (data not shown).

A third region from −62 to −70 shares significant homology with one of a series of previously reported Ets-1/PU-1 sites within the human T-cell leukemia virus type I (HTLV-I) LTR (11). This sequence (TTCCTCCCC) abuts the −55 CTF/NF-1 site. Mutation of this site reduced the CAT activity of the −223 vector to 50% (Table 1). Although no strong EMSA signals were observed from the Ets/HTLV-I homology site, it was included in the Vγ3 CTF/NF-1 probe used (Fig. 8, lane 3). Indeed, an additional higher-mobility band(s) that persisted after competitions with the adenovirus CTF/NF-1 sequence which does not include a similar Ets-like binding site was detected with this probe (data not shown). A role for Ets-1 or related factors in Vγ3 expression is consistent with its high level of expression in lymphoid cells (8, 33).

DISCUSSION

The Vγ3 minimal promoter as a regulator of expression. Developmental selection of the variable gene segments (Vγ3 and Jγ1) comprising the embryonic day 14 γδ TCR is likely to occur as a consequence of promoter-specific regulatory sequences (24, 41, 65). In this report we have analyzed, by transient expression assays, a minimal region of the Vγ3 promoter that, along with its cognate enhancer, exhibits the required potential for T-cell-specific activity. This sequence includes at least three sites which appear important for full promoter activity.

Interactions at the minimal Vγ3 promoter. A CTF/NF-1 consensus binding site interacts with DNA-binding factors which are expressed in all or most tissues. The CTF/NF-1 family of factors represents a series of discrete genes as well as alternatively spliced variants (72). This gene family is recognized for its transcriptional regulatory and replication-associated activities (22, 43, 56). Cellular distribution of these factors is diverse. Accordingly, we observed wide differences in CTF/NF-1 DNA-binding complexes among crude nuclear protein extracts of murine αβ, γδ, and human γδ T cells. These complexes did not reflect variations due to NF-1 sequence recognition. Elimination of CTF/NF-1 binding interactions at this site reduced promoter activity fivefold.

A survey of Sp-1 mRNA tissue expression in the mouse from early development to adult has demonstrated that the highest levels detected were in the thymus (70). The degenerate GC box Sp-1 binding site at −100 may also be regarded as a site that interacts with ubiquitously expressed factors (9, 44, 45). The relevance of this interaction was demonstrated by the >80% reduction in promoter activity when the GC box site was mutated. The site is unusual in that it is bounded by 8-bp direct repeats which initially were thought to be the factor(s) recognition motif. However, the observed pattern of EMSA DNA-protein binding complexes was not due to primary recognition of these repeats, although they might contribute to the affinity. Qualitatively, the −100 GC box was comparable to the SV40 Sp-1 site profile. Quantitatively, the SV40 site displayed significantly greater affinity.

The finding of other members of the Sp-1 multigene family of factors which recognize a GT box sequence with high affinity implicate a higher intricacy of associations at nonconsensus GC box sites (47). Regulation of gene expression by factor phosphorylation is a well-recognized mechanism. Indeed, Sp-1 itself has been shown to act as a phosphorylation substrate (42, 73). We have observed changes in complex binding affinity to the Vγ3 GC box probe by alterations of T-cell stimulation (data not shown). The resultant effects on DNA-binding affinity, factor interactions, and/or their function in transcriptional activity could affect levels of receptor expression maintenance.

The significant homology of the proximal −65 ETS site to the HTLV-I LTR sequence and the widespread T-cell regulatory functions associated with this large family of factors (27, 32, 84) compelled us to analyze its possible role. The strong interaction of the abutting CTF/NF-1 site prevented definitive EMSA interpretation of the −65 site. Attempts to dissect the sequence by use of probes specifically competing for relatives within the Ets family, such as PU.1, were not conclusive (data not shown). Only after specific competition eliminated the strong CTF/NF-1 factor complexes were additional bands which could be specifically self-inhibited with mutant CTF/NF-1 probes containing a wild-type −65 site revealed (data not shown). The mutational data support a role for this sequence in Vγ3 expression. Possible cooperative interactions with the NF-1 site could result in the modest 50% modulatory effect observed.

The Vγ3 3′ regulatory region. As a novel feature of the Vγ3 promoter, we have delineated an additional regulatory domain which appears essential for antigen receptor expression. Truncation of regions 3′ to the transcriptional start site sequence, located through the first exon coding region and through the subsequent intron, totally abolished promoter activity. Although not common, enhancers can be located within transcription units. Several antigen receptor loci, including immunoglobulin H (6, 23), immunoglobulin κ (62, 63), and TCR δ (10, 66), carry enhancers in their J-C introns. None, however, carry regulatory elements in their V coding exons or leader introns. An E box dyad overlaps the second exon splice acceptor site and a sequence usually rich in T (~60%) stretches from the first exon into the intron. It will be important to establish whether this region functions at the level of transcriptional initiation or elongation or whether it influences the stability of transcripts.

The Vγ4,3 intergenic repressing region. A 311-bp sequence (−586 to −897) strongly repressed heterologous promoters (TK or Fos) in Peer cell transient transfection assays. The effect was observed in the presence or absence of the cognate Cγ1 enhancer. It appears to be only a portion of the negative modulatory region between these two V genes. Although not tested on heterologous promoters, sequences 3′ (−340 to −223) reduced basal activity of the Vγ3 promoter. However, the negative effect was not seen in enhancerless constructs. Differential enhancer-dependent versus-independent repression could play a crucial role in regulation of germ line versus mature loci expression.

The DNA-binding GATA family is largely conserved across evolution and has been associated with numerous developmental and regulatory functions (3, 4, 7, 35, 53). The −897 to −586 region contains six consensus recognition sequences for this family of factors. Four of these sites are identical and lie within a ~50-bp region (−615 to −658). The unprecedented clustering of these consensus sites resembles a similar region in the human β-globin locus at the erythroid cell-specific hypersensitive site HS3 (60). The 50-bp Vγ3

GATA tetrad appears to interact specifically with T-cell nuclear extracts (14), and this EMSA interaction could be mediated by as few as two GATA molecules (54). The lymphoid cell-specific factor, GATA-3, is known to bind and/or to be functionally critical in the αβ, and γδ TCR enhancers (37, 48, 53, 65, 76). GATA-3 mRNA is expressed prior to and during the point of γδ s-IEL thymic T-cell development, embryonic day 14 (51).

Potential for developmental regulation at the Vγ4,3 loci. In our bulk Peer stable transfection, inclusion of upstream sequences did not appear to influence transcription when stably integrated into cells, whereas their presence in transient assays repressed activity. However, unlike in Peer transient expression assays, the 311-bp fragment in either orientation failed to repress a heterologous promoter in the αβ TCR cell Jurkat. The contrasting results obtained in the stable versus transient Peer transfectants are reminiscent of similar reports for other loci such as the chick lysozyme gene (77) and the human β-globin gene cluster (61). Elements in these loci were also functionally dependent on their state of cellular chromatin assimilation. How such interactions, if present at the Vγ4,3 loci, influence chromatin structure and function remains to be elucidated. The lack of repressor function of the 311-bp fragment in Jurkat transient cell assays signifies that this effect is not merely due to out-of-context organization but may be additionally influenced by tissue-specific factors.

These observations suggest possible mechanisms whereby, through targeted promoter function, developmental timing of V-gene-specific expression could be mediated. The presence of 3′ constant region silencers which down-regulate TCR γ and α enhancer function in inappropriate tissues have been reported (38, 83). A putative Cγ1 silencer was postulated to explain Cγ1 γδ T-cell restriction or lack thereof in transgenic mice (38). The α silencer was demonstrated to prevent the expression of heterologous promoter constructs in γδ T cells but not in αβ T cells (83). Unlike the constitutive αβ or γδ down-regulation which silencers such as these impart, we envision the Vγ4,3 intergenic repressor as a regulator of cell lineage through control of variable region usage. Two scenarios may be conceived. (i) The repressor of Vγ4,3 could be active during all early thymocyte development. Availability of Vγ3 transactivators differentially derepresses the silencing effect on Vγ3 but not on Vγ4, allowing germ line transcription and targeted recombination to occur. At day 15 to 16, availability of Vγ4 transactivators now turn on Vγ4 and inactivate Vγ3. (ii) Alternatively, embryonic thymocytes initially do not express lineage repressor activity. With the possible exception of putative Ets recognition sequences, no other identifiable homologies are shared among Cγ1-associated V region 5′ promoter sequences. This is not the case with Vα or Vβ promoters (2), suggesting that the regulation of discrete Vγ genes is individually controlled. Our identification of critical Vγ3 regulatory sites, generally consisting of ubiquitous recognition sequences, reduces the possibility that unique lineage-specific factors target all Vγ promoters. Thus, during embryonic development the staged expression of requisite ubiquitous transactivators with which sufficient Vγ3 activity could be obtained would first initiate Vγ3 germ line transcripts. Alternatively, initial Vγ3 basal activity may simply be stronger than Vγ4 activity if presence of a crucial Vγ4 ubiquitous factor was limiting or delayed. Nevertheless, because the Vγ4 recombination signal sequence (RSS) heptamer site (in boldface) contains a minus strand overlapping consensus GATA site (TT GATAGAGTGT), Vγ4-RSS would remain inaccessible to recombinase because of its involvement in a GATA-3 complex. Recombination is then defaulted to Vγ3, whose RSS sequence is accessible. At day E15 to E16, elevated initiation at Vγ4 occurs, and increased competition with Vγ3 accompanied by elimination of the RSS GATA blockade yields Vγ4 functional rearrangements. A substantial drop in GATA-3 levels during dEC development is consistent in part with our observation that GATA-3 mRNA levels in mature JAC-3 cells appear significantly lower than in other human and murine αβ T cells, or human γδ Peer cells (14). Finally, establishment of adult thymocyte lineage repressor function allows for the mature γδ Vγ2,5 loci usage profile to supersede the Vγ4,3 utilization. Regardless, the need for lineage repressor activity in αβ T cells is obviated by the putative Cγ1 silencer (38).

We favor the second alternative, since approximately 20% of Vγ3 chains sequenced from day 14 mRNA carry the Vγ4 5′ leader sequence (24). This implies that, as predicted, both Vγ3 and Vγ4 promoters can be activated at the same time and still retain targeted rearrangement.

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