

## Identification of a T-cell-specific transcriptional enhancer located 3' of *C $\gamma$ 1* in the murine T-cell receptor $\gamma$ locus

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**ABSTRACT** A transcriptional enhancer element has been localized 3 kilobases 3' of the murine T-cell receptor *C $\gamma$ 1* locus using a chloramphenicol acetyltransferase reporter gene construct. As a monomer the enhancer functions only in PEER  $\gamma\delta$  cells and Jurkat  $\alpha\beta$  cells of the T-cell lines tested. However, a tetramer of the enhancer functions in virtually all T-cell lines tested, including  $\alpha\beta$  T-cell lines, but not in other cell types. These results suggest that elements other than the enhancer are responsible for the failure of rearranged *C $\gamma$ 1* genes to be expressed in  $\alpha\beta$  T cells. The enhancer has been localized to a 200-base-pair *Rsa* I restriction fragment, which contains sequence motifs similar to those found in the other T-cell receptor enhancers but not in the immunoglobulin enhancers.

Whereas the majority of T cells express a CD3-associated T-cell antigen receptor (TCR) composed of  $\alpha$  and  $\beta$  chains (1, 2), a smaller subset expresses the more recently characterized CD3-associated  $\gamma\delta$  TCR. Although the function of  $\gamma\delta$  T cells is still speculative, they possess several features distinct from  $\alpha\beta$  T cells (3–5).  $\gamma$  and  $\delta$  rearrangements are the first to occur in murine ontogeny, as early as embryonic day 13 or 14 (6–8), to generate the first T cells to appear in ontogeny. The early fetal thymic  $\gamma\delta$  cells home to the epidermal epithelium and constitute the Thy-1<sup>+</sup> dendritic epidermal cells (9). Later waves of  $\gamma\delta$  T cells home to different epithelial tissues as well as the secondary lymphoid organs (10–12).

Strikingly, within each wave of migrating T cells, distinct sets of *V $\gamma$*  and *V $\delta$*  gene segments are utilized (13–16). It is possible that differential *V*-gene usage by discrete sets of  $\gamma\delta$  cells is regulated, at least in part, at the level of gene rearrangement. Studies of immunoglobulin genes suggest that prior transcription of unrearranged gene segments may regulate the frequency of rearrangement (17). Hence, an understanding of the control of  $\gamma$  gene transcription may lead to insights into the programed rearrangement of *V $\gamma$*  and *V $\delta$*  genes and the genesis of distinct sublineages of  $\gamma\delta$  cells.

Moreover, the developmental decision to differentiate along the  $\gamma\delta$  lineage or  $\alpha\beta$  lineage may be controlled at least partly at the level of transcription. Although *C $\gamma$ 1* (and other *C $\gamma$* ) genes are rearranged in most peripheral  $\alpha\beta$  T cells, the corresponding transcripts are usually absent (6, 13). Thus, transcriptional regulation may play an important role in the developmental decision between  $\alpha\beta$  and  $\gamma\delta$  lineages.

Toward the goal of understanding  $\gamma$  gene transcriptional regulation, we have isolated a tissue-specific transcriptional enhancer located 3 kilobases (kb) downstream of *C $\gamma$ 1*. The minimal enhancer was active in some, but not all, transfected T-cell lines. However, multimers of the enhancer, or of a specific site within the enhancer, were active in virtually all T-cell lines tested but not in B cells or nonlymphoid cells. Potential roles of the *C $\gamma$ 1* enhancer in regulating T-cell development are discussed.

## MATERIALS AND METHODS

**Murine TCR  $\gamma$  Genomic Clones.** A genomic clone containing the 17-kb *V $\gamma$ 2-J $\gamma$ 1C $\gamma$ 1* *Eco*RI fragment was described previously (13). An overlapping genomic clone containing an additional 12.5 kb downstream was isolated from a BALB/c genomic library in EMBL3 by hybridization with the 1.7-kb *Bgl* II-*Sal* I fragment 2.5 kb 3' of *C $\gamma$* .

**Plasmids.** Plasmids J21 and J21MoEn have been described (18). In p $\beta$ -GAL-A the chicken  $\beta$ -actin promoter was cloned 5' of the  $\beta$ -galactosidase gene and a simian virus 40 (SV40) poly(A) sequence in Bluescript pKS (Stratagene). In the remaining plasmids, the various  $\gamma$  gene fragments were subcloned into the downstream polylinker of J21.

**Cell Lines.** All cells were maintained in 10% fetal calf serum, RPMI 1640 medium, 50  $\mu$ M 2-mercaptoethanol, and antibiotics.

**Transfections.** All cells were transfected using the DEAE-dextran method as described (19) with the following modifications. For all  $\gamma\delta$  T cells and two  $\alpha\beta$  T cells, Hut78 and S49.1,  $4 \times 10^7$  cells were used per transfection, whereas  $\approx 10^7$  NIH 3T3 cells were used per transfection. For the remaining cell lines  $2 \times 10^7$  cells per transfection were used. For all transfections,  $\approx 3 \mu$ g of DNA per  $10^7$  cells was used. Equal molar amounts of DNA, adjusted to equal weight using sonicated herring sperm DNA, were used. All transfections were done in duplicate and experiments were repeated at least twice.

In some experiments, 3  $\mu$ g of p $\beta$ -GAL-A was cotransfected along with the J21 constructs as an internal control for transfection efficiency. The cytoplasmic extracts were prepared as before and  $\beta$ -galactosidase activity was assayed by a standard protocol.

**Chloramphenicol Acetyltransferase (CAT) Assays.** The CAT assay was performed essentially as described (19) with the following modifications. After a 40- to 48-hr incubation following transfection, the cells were harvested and 50–200  $\mu$ g of proteins of cell extract (depending on transfection efficiency) was assayed for its ability to convert [<sup>14</sup>C]chloramphenicol to the acetylated form in a 1- to 2-hr incubation period. In the case of some poorly transfected cell lines (PEER, Molt-13  $\gamma\delta$  cells, and the DN  $\gamma\delta$  hybridomas), the incubation period was extended to 4–6 hr and extra acetyl-CoA was added at 2-hr intervals. Quantitation of acetylation ratios was performed by liquid scintillation spectroscopy in Ecolume (NEN) fluor of the excised spot from thin-layer chromatography sheets.

**DNase I Footprinting.** Footprinting was done using a standard protocol (20) with heparin fractions of the nuclear extracts indicated. Briefly, nuclear extracts in buffer C/0.1 M KCl (20) were bound to heparin-Sepharose CL-6B (Pharmacia), eluted with 0.5 M KCl, and dialyzed against buffer C/0.1 M KCl.

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Abbreviations: CAT, chloramphenicol acetyltransferase; TCR, T-cell antigen receptor; SV40, simian virus 40; MLV, murine leukemia virus. \*Present address: 489 LSA, University of California, Berkeley 94720.

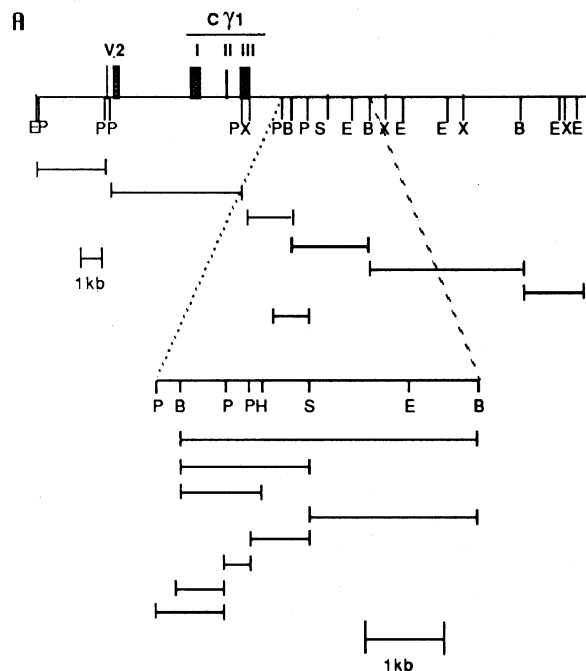
**RESULTS**

We used a transient transfection assay to screen the *C $\gamma$ 1* locus for enhancer activity. Supercoiled plasmids carrying the reporter gene, CAT, and fragments of the *C $\gamma$ 1* locus were transfected into cell lines. Two days later cell extracts were tested for CAT activity. Restriction fragments from 2.5 kb to 8 kb in length spanning 30 kb of a rearranged *V $\gamma$ 2-J $\gamma$ 1C $\gamma$ 1* gene were subcloned into the downstream polylinker of the test plasmid J21. In J21, a minimal *fos* promoter (-71 to +109) drives CAT expression. Stimulation of CAT activity indicates the presence of a transcriptional enhancer. We found only one fragment, a 3.9-kb *Bgl* II-*Bgl* II fragment located 2.5 kb downstream of *C $\gamma$ 1*, with reproducible enhancer activity in the human  $\gamma\delta$  cell line PEER (Fig. 1A). By testing restriction fragments of the 3.9-kb fragment, we found that most of the activity resided in a 560-bp *Bgl* II to *Pvu* II fragment (.56BP) (Fig. 1A). This fragment could be further trimmed to a 200-bp *Rsa* I-*Rsa* I minimal enhancer fragment (.20RR), which had indistinguishable activity from .56BP (Fig. 2). Further removal of the 5' or 3' half of .20RR reduced the activity severalfold (Fig. 2). The enhancer functioned in both orientations, although the activity was slightly stronger

when the enhancer was subcloned downstream of the CAT gene in the reverse transcriptional orientation (Fig. 1B).

To define the cellular specificity of the  $\gamma$  enhancer, we transfected a number of T and non-T cells with J21 constructs that include the minimal enhancer (Table 1). The enhancer was active in the PEER  $\gamma\delta$  cell line and the Jurkat  $\alpha\beta$  cell line and inactive in the three B cells tested (A20, M12.1, and BJAB), in the fibroblast NIH 3T3 cell line, and in the cervical carcinoma HeLa cell line. These results suggested that the enhancer is T-cell specific. Surprisingly, however, the enhancer had little or no activity in several other  $\gamma\delta$  cell lines (Molt-13, DN7.1, DN7.3, DN2.3, and DN1.1) or in several other  $\alpha\beta$  cell lines (BW5147, S49.1, EL-4, and Hut78). Larger fragments containing the enhancer (3.9BB and 1.0BH) were also inactive in Molt-13 and DN7.1 cell lines (not shown). This pattern of expression was highly reproducible in numerous transfection experiments.

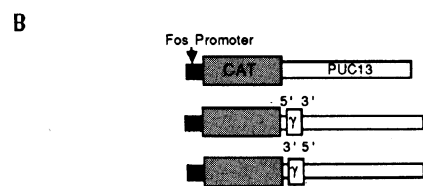
Previous studies have shown that multiple tandem copies of enhancers generally yield stronger activity than enhancer monomers (21). We therefore tested a tetramer of the .20RR minimal enhancer fragment, subcloned downstream of the CAT gene in J21, for activity in several cell lines. The tetramer was active in all four T-cell lines tested, including



Plasmid	Peer $\gamma\delta$ cells Relative activity		
	Expt. 1	Expt. 2	Expt. 3
J21-3.5PP	4.7 <sup>1</sup>	--	--
J21-7.0PP	1.5	--	--
J21-2.5XB	--	--	0.7
J21-3.9BB	--	18.5	--
J21-8.0BB	0.6	--	--
J21-3.0BB	0.8	--	--
J21-0.9PPR	52.7	--	--
J21-MoEn	80.0	42.6	50.2
J21	1.0	1.0	1.0

Plasmid	Expt. 4	Expt. 5	Expt. 6
	J21-3.9BB	5.9	11.5
J21-1.7BS	7.1	28.1	--
J21-1.0BH	--	--	16.0
J21-2.2SB	0.4	--	--
J21-0.85PS	0.6	--	--
J21-0.28PP	--	--	1.0
J21-0.56BP	6.8	20.8	--
J21-0.9PPR	15.5	--	--
J21-MoEn	15.5	63.5	--
J21	1.0	1.0	1.0



Plasmid	Relative activity	CAT Assay
J21	1.0	
J21-0.56BP	10.0	
J21-0.56BPR	18.4	

FIG. 1. Identification and mapping of a transcriptional enhancer element at the 3' end of the murine *C $\gamma$ 1* locus. (A) Partial restriction endonuclease map using *Eco*RI (E), *Pvu* II (P), *Xba* I (X), *Bgl* II (B), *Sal* I (S), and *Hind*III (H). Not all of the *Pvu* II, *Hind*III, and *Xba* I sites are shown. The five exons of *V $\gamma$ 2-J $\gamma$ 1C $\gamma$ 1* are shown as stippled boxes. Test fragments shown as bars were subcloned into the polylinker 3' of the CAT gene in the J21 vector and appear across from their relative activity in representative experiments. Average activity values of duplicate transfections were normalized to those produced by transfection with the control plasmid J21, which were 0.3%, 1.5%, 0.3%, 0.4%, 0.1%, and 0.5% for experiments 1-6, respectively. The 900-base-pair (bp) *Pvu* II fragment in J21-0.9PPR is in reverse orientation. (B) Representative CAT assay displaying the activity of the TCR  $\gamma$  enhancer when cloned downstream of the minimal *fos* promoter (-71 to +109) and CAT reporter gene and transfected into PEER cells. The control J21 as well as the TCR  $\gamma$  enhancer-containing plasmids are shown schematically. In J21-BP.56 and J21-BP.56R, the 560-bp *Bgl* II to *Pvu* II fragment (A) is cloned downstream of CAT into the unique *Bgl* II site of J21 in the orientations indicated. Relative CAT activities were calculated as before relative to that of J21 (1.5% conversion). The thin-layer chromatography spots corresponding to [<sup>14</sup>C]chloramphenicol (Chl) and acetylated [<sup>14</sup>C]chloramphenicol (Ac-Chl) are indicated. Superscript 1, this enhancement was not reproduced in other experiments.

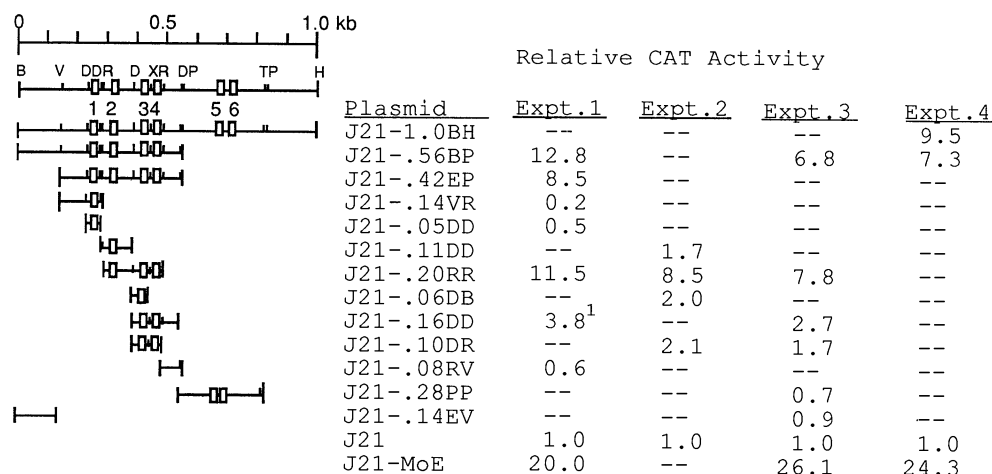


FIG. 2. Deletional analysis of the  $\gamma$  enhancer. Fragments are shown schematically by bars and named on their right by size (in kb) and restriction endonuclease end-points. The restriction enzymes used are *Bgl* II (B), *Dde* I (D), *Eco*RV (V), *Hind*III (H), *Pvu* II (P), *Rsa* I (R), *Sty* I (T), and *Bst*BI (X). The regions that correspond to NF $\gamma$ -6 are numbered and represented schematically by open boxes. Activity normalized to activity of cells transfected with J21 (0.70%, 0.8%, 0.28%, and 0.55% for experiments 1-4, respectively). Superscript 1, the 160-bp *Dde* I fragment was in reverse orientation in experiment 1.

the Molt-13 and DN7.1 cell lines, in which the monomer was inactive (Table 1). We also tested a tetramer of an internal 60-bp fragment of the enhancer, .06DB, which had little or no activity by itself (Fig. 2) but includes a major protein-binding site as determined by DNase I footprinting analysis (see below). The .06DB tetramer is strongly active in 9 of 10 T-cell lines, including 6 of 6  $\gamma\delta$  cell lines and 3 of 4  $\alpha\beta$  cell lines, the exception being EL-4 (Table 1). Thus, tetramerization of the enhancer leads to activity in almost all of the T-cell lines in which the monomer is inactive. Neither the .20RR tetramer nor the .06DB tetramer is active in the non-T-cell lines tested, including three B-cell lines and the nonlymphoid NIH 3T3 and HeLa cell lines (Table 1). Therefore, the tetramerized enhancer functions as a strong T-cell-specific enhancer.

Transgenic studies suggest that the *C $\gamma$*  enhancer identified herein is important for gene activation in  $\gamma\delta$  cells *in vivo*. Mice transgenic for a rearranged 15-kb *Eco*RI-*Sal* I *V $\gamma$ 2-J $\gamma$ 1C $\gamma$*  fragment, including the enhancer, express high levels of the

transgene in  $\alpha\beta$  and  $\gamma\delta$  T cells (22). In contrast, three independent lines of mice transgenic for an 11.7-kb *Nco* I-*Nco* I *V $\gamma$ 2-J $\gamma$ 1C $\gamma$*  fragment, which ends 1 kb upstream of the enhancer, fail to express the transgene in either  $\gamma\delta$  or  $\alpha\beta$  cells (D.M.S., E. Selsing, and D.H.R., unpublished results). Although the 11.7-kb fragment also lacks 2.3 kb of upstream sequence present in the active 15-kb fragment, these upstream sequences failed to yield significant or reproducible enhancer activity in the transient transfection assay (Fig. 1). The transgenic results are therefore consistent with a necessary role for the downstream enhancer described here in *C $\gamma$*  gene expression.

It was surprising that the enhancer monomer failed to support CAT gene activation in several  $\gamma\delta$  T-cell lines, including several that express endogenous *C $\gamma$*  genes [i.e., the DN7.1, DN7.3, and DN2.3 T hybridomas (16)]. To ask if this might reflect a preferential interaction of the enhancer with a homologous promoter, we replaced the *c-fos* promoter

Table 1. Activity of DNA segments dissected from  $\gamma$  enhancer

Cell line	Moloney	.42EP	.20RR	.20RR tetramer	.06DB	.06DB tetramer
$\gamma\delta$ T						
PEER	22.2	—	6.8	135.1	1.8	83.4
Molt-13, exp. 1	25.1	—	1.0	10.9	1.0	50.2
Molt-13, exp. 2	91.8	1.0	—	—	—	183.2
DN7.1	9.2	1.2	—	4.1	—	10.2
DN2.3	62.8	—	—	—	—	31.4
DN7.3	73.2	—	—	—	—	17.3
DN1.1	28.6	1.0	—	—	—	12.2
$\alpha\beta$ T						
Jurkat, exp. 1*	15.8	—	3.5	31.6	1.5	31.0
Jurkat, exp. 2	14.3	—	5.0	42.2	3.1	43.9
BW5147	13.4	—	—	—	—	9.7
S49.1	16.8	—	—	—	—	15.3
EL-4	11.0	—	—	—	—	1.3
B						
A20, exp. 1*	—	—	1.0	0.5	—	—
A20, exp. 2	20.0	—	—	—	—	1.0
M12.4.1	6.0	0.6	—	—	—	0.6
Nonlymphoid						
HeLa, exp. 1	17.7	—	—	1.1	—	—
HeLa, exp. 2	9.0	0.7	—	—	—	1.0
NIH 3T3	12.2	0.6	—	—	—	1.0

The constructs containing single or four-tandem copies of the enhancer-containing fragments (see Fig. 2) were transfected into different cell lines. Relative enhancer activity was determined quantitatively by scintillation spectroscopy of the excised spot from thin-layer chromatography sheets. The stimulation of transcription by the fragments is normalized to that of the J21 vector, which is set to 1. —, Not determined.

\*p $\beta$ GAL-A was cotransfected with CAT constructs in the same experiment (see text). The CAT activity was then normalized to the  $\beta$ -galactosidase activity.

of J21 with a  $V\gamma 4$  promoter (a 470-bp *Bgl* II to *Bst*NI fragment). Use of the  $V\gamma 4$  promoter did not reveal enhancer activity in the Molt-13  $\gamma\delta$  T-cell line or in the murine  $\gamma\delta^+$  hybridomas, even in those hybridomas that express the endogenous  $V\gamma 4$ -*J\gamma 1C\gamma 1* gene (e.g., DN7.1) (data not shown). This was not due to the inactivity of the  $V\gamma 4$  promoter in these cells, since it functioned well with the Moloney murine leukemia virus (MLV) enhancer.

It is possible that sites flanking .06DB, within .20RR, partially inhibit activity in some T-cell lines, as measured in the transient transfection assay. The relative activity of the tetramer of .20RR (the minimal enhancer) in the Molt-13 and DN7.1 cell lines was lower than that of the tetramer of the smaller .06DB fragment. In contrast, the .20RR tetramer was as active as or more active than the .06DB tetramer in PEER and Jurkat cell lines (Table 1). Further studies will be necessary to determine the significance of this partial inhibition.

To further characterize the enhancer, a 420-bp *Eco*RV to *Pvu* II fragment containing the enhancer was assayed for nuclear protein binding using the DNase I footprinting technique. Of six sites (NF $\gamma$ 1–NF $\gamma$ 6) that were reproducibly protected from DNase I digestion when incubated with the nuclear extracts from T cells, three (NF $\gamma$ 2–NF $\gamma$ 4) are within the minimal enhancer; of these, the most extensively protected site was NF $\gamma$ 3 (Fig. 3). NF $\gamma$ 1, NF $\gamma$ 5, and NF $\gamma$ 6 are

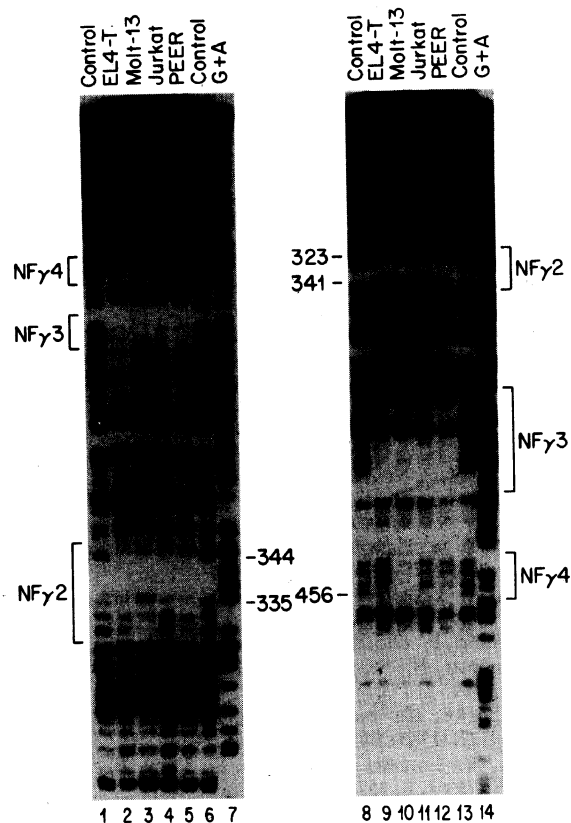


FIG. 3. DNase I footprinting of the  $\gamma$  enhancer. For the positive strand (lanes 1–7), DNA was labeled at the *Eco*RV site and then cut by the *Pvu* II restriction enzyme. The reverse was performed for the negative strand (lanes 8–14). A ladder of G + A Maxam–Gilbert sequencing reactions was run in parallel (lanes 7 and 14). The control samples were not incubated with nuclear extracts (lanes 1, 6, 8, and 13). The cited positions correspond to the *Bgl* II–*Hind*III sequence (see Fig. 4). Lanes 2–5, NF $\gamma$ 3 is strongly protected by all T-cell extracts tested as is position 344 (NF $\gamma$ 2). Position 335 is protected in all extracts except Molt-13 (lane 3), in which it is hypersensitive. Lanes 9–13, NF $\gamma$ 3 is strongly protected by all T-cell extracts as is position 456 (NF $\gamma$ 4). Position 323 (NF $\gamma$ 2) is clearly protected by the Molt-13 and PEER extracts (lanes 10 and 12, respectively). Positions 341 (NF $\gamma$ 2) and 387 (NF $\gamma$ 3) are hypersensitive sites. These patterns of protection and hypersensitivity were reproduced in each of three experiments.

outside of the minimal enhancer fragment and are therefore inessential for maximal enhancer activity.

The 1.0-kb *Bgl* II to *Hind*III fragment containing the enhancer was sequenced (Fig. 4A). Within the minimal enhancer fragment we discerned various motifs (Fig. 4B and legend). Notably, an 11-bp motif within NF $\gamma$ 3 and a similar motif within NF $\gamma$ 4 are similar to sequences previously identified in the other three TCR genes' enhancers (i.e.,  $\delta E3$ ,  $T\alpha 2/NF\alpha 5$ , and  $\beta$  core), in the CD3  $\delta$  and  $\epsilon$  enhancers, and in the core region of several viral enhancers, including those of SV40, polyoma, and Moloney MLV (27–34). Mutational analysis of the viral core sequences and the corresponding sequence in the TCR  $\alpha$  gene demonstrates that those sequences are essential for full enhancer function (29, 33, 35).

## DISCUSSION

Similar to many other T-cell-specific genes, including TCR  $\alpha$  and  $\beta$  and CD3  $\delta$  and  $\epsilon$ , a T-cell-specific enhancer is found at the 3' end of the TCR *C\gamma 1* gene segment. The immunoglobulin genes, IgH and Ig $\kappa$ , are also known to contain 3' enhancers as well as weaker enhancers in their joining-constant (J–C) introns. An enhancer appears in the J–C intron of the TCR  $\delta$  gene but intronic enhancers are not detected in the other TCR genes; the report of an intronic TCR  $\alpha$  enhancer has not been confirmed in a more recent study (18, 36). The location of enhancers controlling TCR and immunoglobulin genes 3' of the J gene segment fits with the model that nonrearranged variable gene segments must be brought into the "activation domain" of a downstream enhancer for full activity (37–40).

Unexpectedly, the  $\gamma$  enhancer monomer was not demonstrably active in many of the T-cell lines tested, but tetramerization of the enhancer revealed activity in virtually all T-cell lines. The TCR  $\delta$  enhancer monomer also fails to function in some  $\gamma\delta$  cells (A. Winoto, personal communication). Given that at least two enhancers appear to control the IgH and Ig $\kappa$  gene loci, it is possible that additional  $\gamma$  enhancer elements, outside the regions assayed, are required for activity in some  $\gamma\delta$  cells. However, transgenic data (see *Results*) suggest that the enhancer described here is sufficient for expression in many  $\gamma\delta$  cells *in vivo*.

Alternatively, the  $\gamma$  enhancer may simply be weak in some  $\gamma\delta$  cells, though of sufficient strength to support the accumulation of the relatively low levels of  $\gamma$  mRNA (41). This possibility is supported by our finding that tetramerization of the minimal enhancer or a site within the minimal enhancer leads to strong enhancer activity in all  $\gamma\delta$  T-cell lines tested. Finally, it is possible that sites within the minimal enhancer are the target of factor(s) that inhibit enhancer activity in some T-cell lines, at least when the sequences are introduced into cells by the transient transfection procedure. Determination of the relative contribution of these and other mechanisms to the pattern of cell-type specificity we have observed requires further investigation.

When tetramerized, the enhancer displayed T-cell specificity but not  $\gamma\delta$  T-cell specificity—i.e., it functioned in  $\gamma\delta$  and  $\alpha\beta$  T-cell lines. Thus, there is no evidence that the specificity of the enhancer accounts for the fact that rearranged *C\gamma 1* genes are not expressed in  $\alpha\beta$  T cells. Cis-acting regulatory elements flanking the enhancer may suppress  $\gamma$  expression in  $\alpha\beta$  T cells (42). Similar cis-acting elements that prevent  $\alpha$  gene expression in  $\gamma\delta$  T cells have been reported (43). Such elements may play a role in the separation of the  $\gamma\delta$  and the  $\alpha\beta$  lineages during ontogeny.

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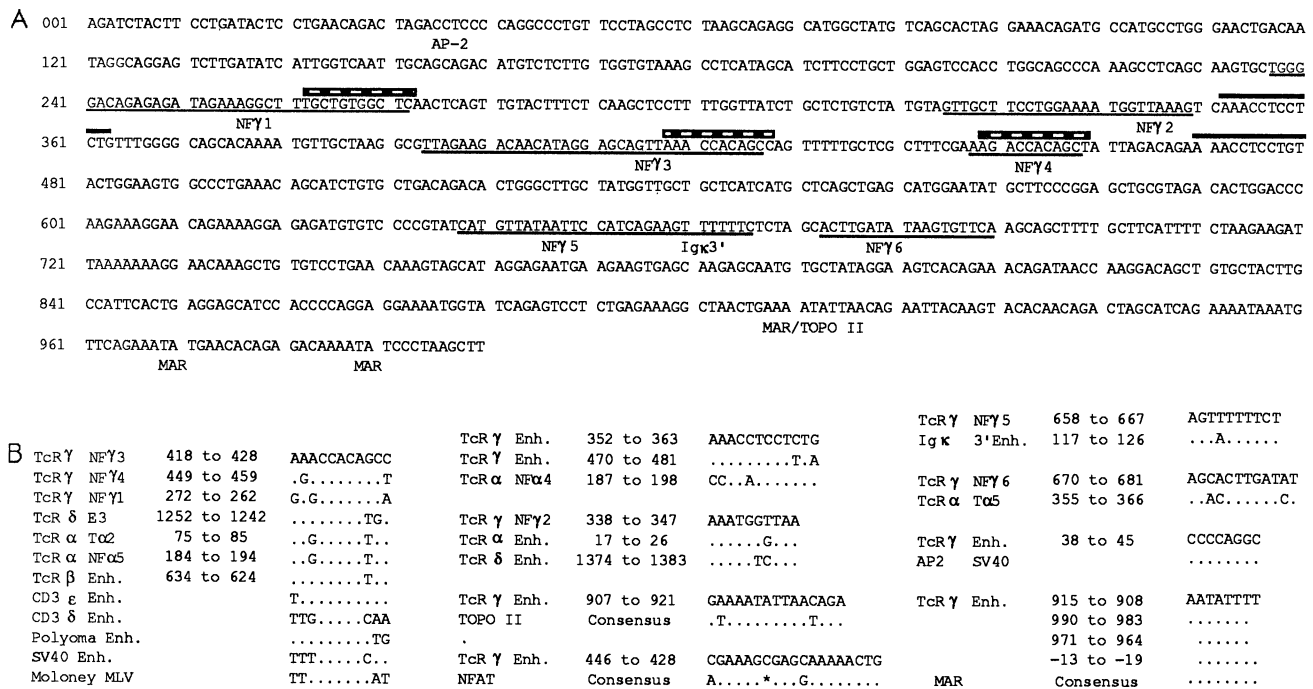


FIG. 4. Sequence of the 1-kb *Bgl* II-*Hind*III fragment containing the  $\gamma$  enhancer with sequence comparisons. (A) Underlined sequences indicate regions protected from DNase I by nuclear proteins. Thick overlined sequences indicate 12-bp repeats, and the overlined checkered boxes indicate 11-bp "core" repeats. Sequences displaying homology to the previously described AP-2, NFAT, and MAR binding motifs are labeled (23-25). (B) Sequence similarities with other enhancers. In addition to those cited in the text, similarities to the AP-2 binding site (23), the Ig $\kappa$  3' enhancer (26), and the human TCR $\alpha$  enhancer (T $\alpha$ 5) (27) are presented. The NFAT and MAR consensus motifs are listed. Dots indicate identity, and the asterisk indicates the absence of a base. Enh., enhancer; TOPO, topoisomerase.

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