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Immunology

Identification of a T-cell-specific enhancer at the locus encoding T-cell antigen receptor γ chain

(transcriptional enhancer)

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ABSTRACT **The γδ and αβ T-cell antigen receptor (TCR) heterodimers are expressed in a lineage-specific, mutually exclusive manner. Regulation of expression occurs at the transcriptional level. A 13-kilobase (kb) stretch of DNA encompassing variable–joining–constant segments $V_{\gamma 4}$–$J_{\gamma 1}$–$C_{\gamma 1}$ of the murine γ-chain gene was examined for the presence of transcriptional enhancing elements by a transient transfection assay. DNA fragments from this region were inserted into a test plasmid containing a heterologous promoter fused to the human growth hormone gene. An 1800-base-pair (bp) fragment located 3 kb 3′ to C_γ exon III was found to display enhancing activity in several T-cell lines. Maximum enhancing activity could be localized further to fragments as small as 400 bp in some cell lines. Nucleotide sequence analysis of this 400-bp segment revealed homologies to previously described core enhancer elements and to other TCR gene enhancers. The TCR γ-chain gene enhancer is active in both γδ and αβ T cells, indicating that it is not primarily responsible for lineage-specificity of expression, but it is inactive in non-T-cells.**

T-cell receptor (TCR) genes undergo somatic rearrangement similar to immunoglobulin genes. They encode clonally variable heterodimers that are expressed in association with the invariant CD3 complex on the surface of T cells, where they are responsible for determining antigen specificity. There are two types of TCR, which are encoded by distinct loci and expressed on distinct T-cell lineages, αβ and γδ. αβ T cells constitute the vast majority of adult peripheral T cells and are well-characterized in terms of function, antigen specificity, and major histocompatibility complex restriction. The function(s) of γδ T cells, on the other hand, remains unresolved, although their distinct tissue distribution, more limited diversity, and general lack of CD4 and CD8 expression suggest that it is not the same as that of αβ T cells (1, 2).

The transcription of genes encoding TCR γδ chains* is regulated quite differently from that of genes encoding TCR αβ chains.* They are expressed earlier during thymic ontogeny (3, 4) and generally fail to be transcribed in αβ T cells later in life, even if productively rearranged (5, 6). There is one exception to the latter rule—namely, the subset of γ-chain genes utilizing the variable–joining–constant region gene segments $V_{\gamma 2}$–$J_{\gamma 2}$–$C_{\gamma 2}$; however, these are almost never expressed at the cell surface (6–8). The choice between γδ and αβ T-cell lineages appears to be determined at the level of transcription—i.e., by the presence or absence of particular trans-acting factors—rather than at the level of gene rearrangement as has also been suggested (9). Thus, in transgenic mice made with previously rearranged TCR γ- and δ-chain genes, located on two 40-kilobase (kb) cosmid clones, αβ T cells occurred in normal numbers and failed to show transcription of the transgenes (10). Interestingly, when a shorter 13-kb TCR γ-chain gene was used instead, the transgene was transcribed both in γδ and αβ T cells. This suggests that positive elements that activate the γ-chain gene in both αβ and γδ T cells are contained within the 13-kb construct and that negative elements, which specifically suppress transcription in αβ T cells, are located in the additional flanking regions of the 40-kb construct.

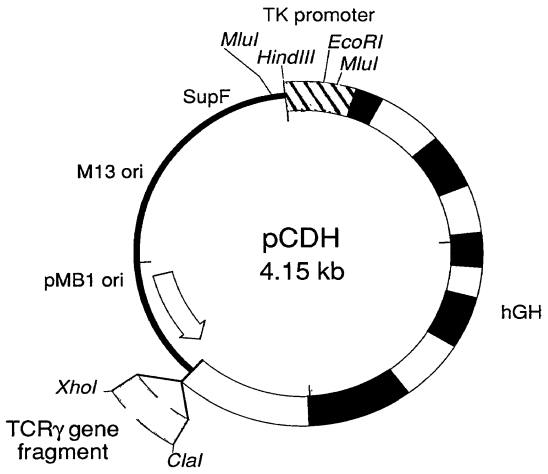

FIG. 1. Map of hGH expression plasmid pCDH. Base pairs 1–2500 are derived from pTKGH, and base pairs 2501–4150 are from pCDM8. Solid black boxes indicate the hHG exons. TCR γ-chain gene fragments are inserted between the *Xho* I and *Cla*I restriction sites, and the *TK* promoter is indicated by a hatched box.

In the present study, we analyzed the 13-kb segment for the putative positive DNA elements—i.e., enhancers—and found such an element 3 kb 3′ to C_γ exon III. This enhancer is active in the γδ T-cell lines Peer and MOLT-13 as well as in the αβ T-cell lines Jurkat and EL4 but is inactive in various non-T-cell lines. In some T-cell lines, the TCR γ-chain gene enhancer (TCR γ enhancer) was found to be stronger than the previously described TCR α enhancer (11).

MATERIALS AND METHODS

Plasmids. pCDM8 (12) and pTKGH (Nicholls Institute, San Juan Capistrano, CA) were subjected to digestion with *Nco* I and *Eco*RI (partial), respectively, followed by treatment with Klenow fragment of DNA polymerase I to fill in the resulting single-stranded "overhangs" and by redigestion with *Hin*dIII. Blunt-end/*Hin*dIII fragments of 1649 base pairs (bp) and 2500 bp generated by these manipulations were

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Abbreviations: TCR, T-cell receptor; V, variable; J, joining; C, constant; hGH, human growth hormone; TK, thymidine kinase; TCR γ or α enhancer, TCR γ- or α-chain gene enhancer.

*Genes encoding the TCR α, β, γ, and δ chains have been assigned the names *TCRA, TCRB, TCRG*, and *TCRD* in the human genome and *Tcra, Tcrb, Tcrg*, and *Tcrd* in the mouse.

isolated from pCDM8 and pTKGH, respectively, and ligated first with either *Xho* I or *Cla* I linkers and then with each other. The resulting human growth hormone (hGH) expression plasmid, pCDH (Fig. 1), contains the bacterial origin of replication and *supF* sequences from pCDM8 (but none of its eukaryotic transcriptional regulatory elements) and the hGH gene (*GH*) under the control of the minimal thymidine kinase gene (*TK*) promoter from pTKGH. *Cla* I and *Xho* I linkers provide unique sites for the directional insertion of gene fragments to be tested for enhancer activity. In the bacterial strain MC 1061 containing the p3 helper plasmid, pCDH confers both tetracyclin and ampicillin resistance. When inserting gene fragments excised from pUC plasmids into pCDH, coselection with ampicillin and tetracyclin eliminates pUC-derived plasmids, making prior separation of the insert from pUC unnecessary. Vectors similar to pCDH were generated containing the c-*fos* (13) and Moloney murine leukemia virus promoters (a gift from the laboratory of N. Hopkins). Manipulations required to generate these vectors were similar to those described above except for the initial

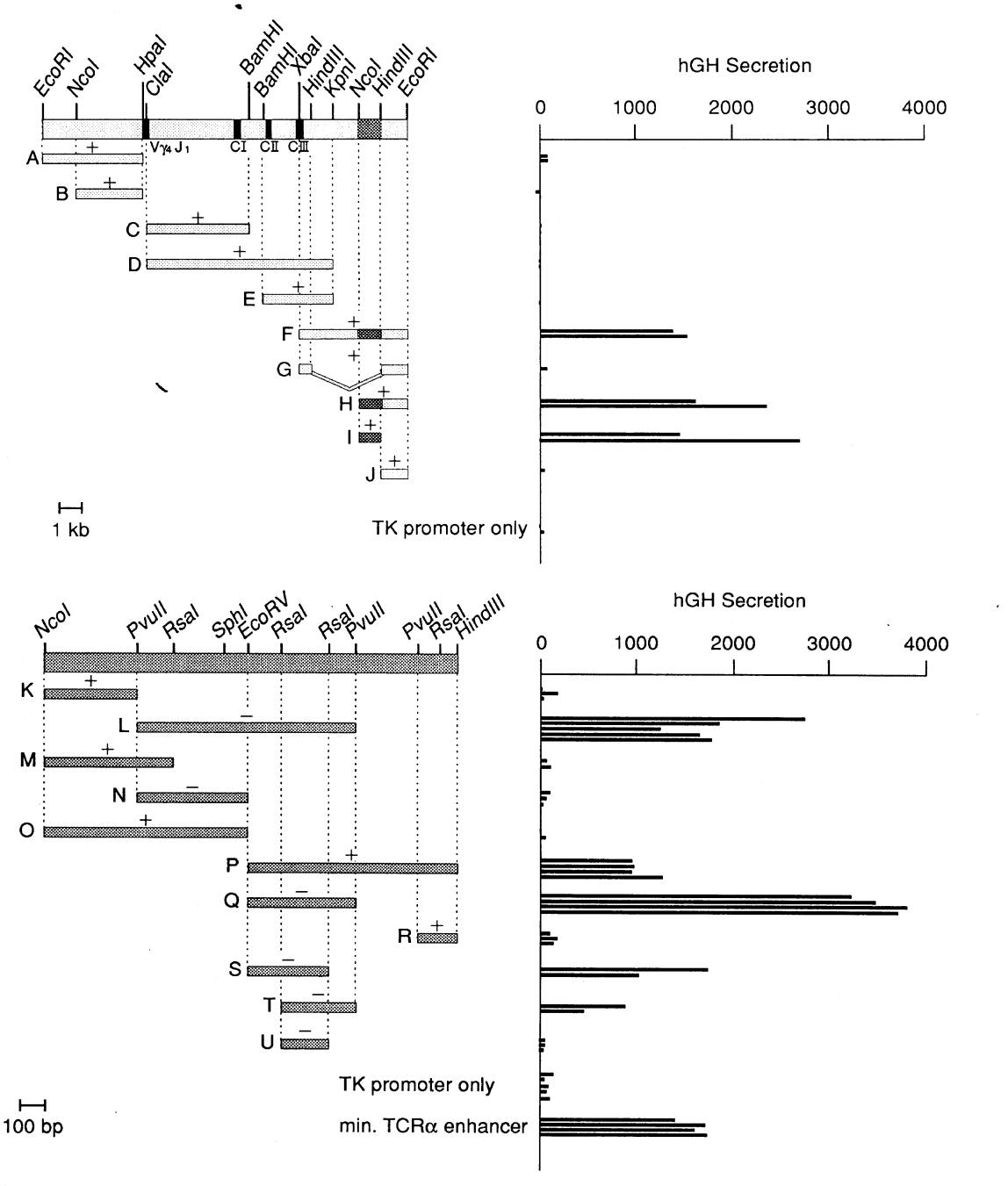

FIG. 2. (*Upper*) Identification of enhancing elements within the $V_{\gamma 4}$–$J_{\gamma 1}$–$C_{\gamma 1}$ gene. (*Left*) A restriction map of the region examined together with the location of restriction fragments tested is shown, where + and − denote the orientation of each fragment in pCDH; + means that the 5′ end is adjacent to the hGH gene. Constructs were transfected into the γδ T-cell line Peer, and their ability to direct hGH secretion was determined by an immunoradiometric assay. (*Right*) The hGH assay values (in cpm) corresponding to each fragment are indicated on the bar graph to the right of each fragment. Each bar corresponds to the average of three transfections. At least two sets of data are shown for each construct, although if values are very close to background values, the corresponding bar may not be visible. Values for background binding were determined for each set of transfections from mock-transfected samples and samples transfected with promoterless hGH constructs and were subtracted from measurements of test constructs to give the values shown (in some cases negative values result from this procedure). Dark shading (*Upper Left*) indicates the minimal region, corresponding to fragment I, that confers enhancing activity. (*Lower*) Mapping of the enhancing activity to smaller regions within fragment I. The same methods were used as in *Upper*.

step in which the small (200–300 bp) promoter fragments were inserted at the *Bam*HI site upstream of the promoterless hGH gene in pØGH (Nicholls Institute). $V_{\gamma 4}$–$J_{\gamma 1}$–$C_{\gamma 1}$ gene fragments were derived from the cosmid clone p_γ-4 (10). Initially, the central *Eco*RI fragment shown in Fig. 2 *Upper Left* was subcloned into pUC19. By insertion of *Cla* I and *Xho* I linkers at appropriate restriction sites, a series of *Cla* I–*Xho* I segments was generated for insertion into pCDH. The TCR α enhancer used as a control was derived from J21 0.2 BgPv (a gift of A. Winoto).

Transfections and hGH Assays. The following cell lines were used: EL4, murine αβ T-cell line; Jurkat, human αβ T-cell line; MOLT-13, human γδ T-cell line; Peer, human γδ T-cell line; P815, murine mastocytoma; Raji, human B-cell line. Cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 15% (vol/vol) fetal calf serum, 2 mM glutamine, 100 units of penicillin per ml and 100 μg of streptomycin per ml. Transfections were carried out by electroporation with a BRL Cell-Porator set at 400 V, 800 μF, and low resistance. Cells were electroporated at densities of 10^6–10^7 per ml in a total of 1 ml of their original culture medium with 15 μg of plasmid. They were then transferred to 3 ml of fresh medium and cultured for 5–6 days. hGH secretion was measured by using the Allegro hGH Transient Gene Expression Assay system according to the manufacturer's instructions (Nicholls Institute). All transfections were performed at least twice, in each case with triplicate samples (also at least two separate DNA preparations were tested for each construct).

Sequence Analysis. Nucleotide sequencing was carried out by the dideoxy chain-termination method with a Sequenase 2 kit (Pharmacia).

RESULTS

A 13-kb segment of DNA containing the rearranged $V_{\gamma 4}$–$J_{\gamma 1}$–$C_{\gamma 1}$ gene from the cosmid clone p_γ-4 (10) was examined for enhancing activity. For this purpose subfragments of 2.5–7 kb (fragments A–F in Fig. 2 *Upper Left*) were inserted into the vector pCDH 3′ to the reporter gene (human *GH* under the control of the enhancerless *TK* promoter), and transcriptional activity was assayed by transient transfection of each construct into the human γδ T-cell line Peer. Fragments A–E, encompassing the region from 4 kb upstream of $V_{\gamma 4}$ to 1.5 kb downstream of C_γ exon III, showed no enhancing activity, whereas fragment F spanning 4.7 kb from C_γ exon III to the 3′ *Eco*RI site gave 7-fold induction relative to a control construct containing only the *TK* promoter and hGH gene. By the analysis of various subfragments of fragment F, the enhancing activity was mapped to 400 bp constituting fragment Q (Fig. 2 *Lower Left*) located about 3 kb 3′ to C_γ exon III (Fig. 2). The level of enhancement conferred by fragment Q was higher than that conferred by the larger fragments from which it was derived (1.5- to 2-fold higher than fragments F, H, I, and L and 3- to 4-fold higher than fragment P). Further dissection of fragment Q resulted in the reduction (fragments S and T) or complete loss (fragment U) of enhancing activity. The minimal 230-bp murine TCR α enhancer (11), used as a control, exhibited levels of activation comparable to our 300-bp fragment S (Fig. 2 *Lower Right*). The TCR γ enhancer was active in either orientation (compare, for instance, activities of fragments I and L in Fig. 2) and in combination with all promoters tested [c-*fos* and murine Moloney leukemia virus promoters in addition to *TK* promoter (data not shown)].

The activities of fragments I, L, and Q were tested in a number of other cell lines (Fig. 3). Fragment I showed activity in the T-cell lines MOLT-13 (human γδ), Jurkat (human αβ), and EL4 (murine αβ). Fragments L and Q were active in the first two lines but interestingly not in EL4 (or only slightly). No enhancement was observed with any fragment in the human B-cell line Raji or the murine mastocytoma line P815 (Fig. 3 *Upper*).

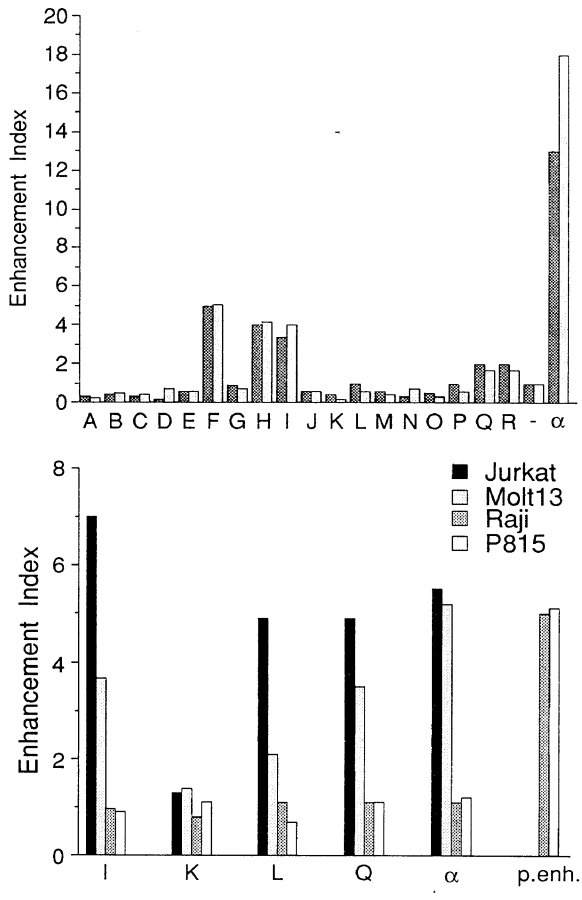

FIG. 3. Determination of enhancing activity of some gene fragments tested in Fig. 2 in the cell lines EL4 (*Upper*; values from two separate experiments shown as shaded or unshaded bars) and Jurkat, MOLT-13, Raji and P815 (*Lower*; values shown are averages of two experiments). Except in the case of the cell line EL4, the test constructs used in these experiments contained the c-*fos* rather than the *TK* promoter. Values were determined as in Fig. 2 *Right* and then normalized for each cell line with respect to an enhancerless construct (assigned a value of 1 to facilitate comparison). Constructs denoted α and p.enh. represent positive controls containing the minimal TCR α enhancer (gift of A. Winoto) and polyoma virus enhancer (from the pCDM8 vector), respectively.

The DNA sequence of fragment Q is shown in Fig. 4. It contains several perfect repeats, including two of 9 bp and one each of 8 bp and 7 bp. The 8-bp repeat shares considerable homology with the polyoma virus core enhancer sequence, AAAACCACAC. In addition, sequences can be identified with homology to the lymphoid-specific octamer motif, ATTTGCAT (14), and the immunoglobulin κ-chain gene enhancer element κE2 (15). Comparison with other TCR gene enhancer sequences reveals a striking homology (66% identity over 38 bp) with the murine TCR β enhancer (Fig. 4*B*). DNase I footprinting of the TCR β enhancer has shown this region to contain a nuclear protein binding site (βE4) (16). The same region shows strong homology to sections of the murine TCR α enhancer (78% identity over 18 bp, with a 1-bp gap) and the human TCR δ enhancer (88% identity over 17 bp), which also contains a nuclear protein binding site (δE5) (17).

DISCUSSION

The transcriptional enhancer identified here maps 3 kb 3′ to $C_{\gamma 1}$, matching the positions of the TCR α, β, and δ enhancers

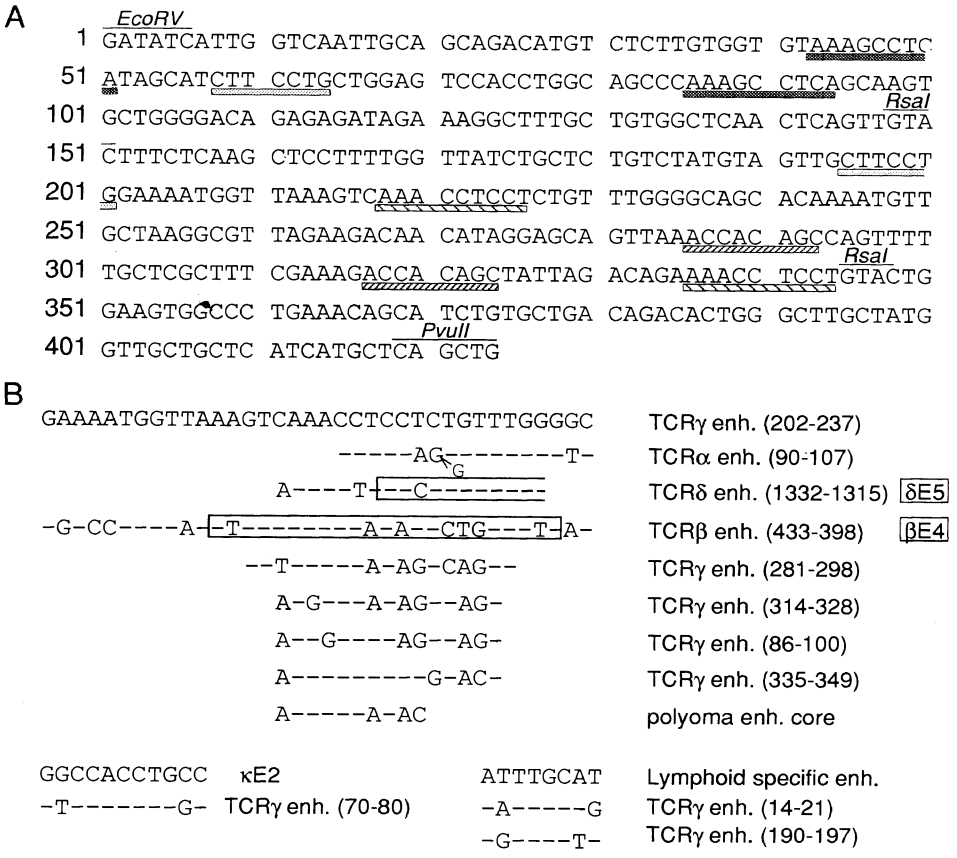

FIG. 4. (*A*) Sequence of fragment Q from Fig. 2, the minimal TCR γ enhancer. Repeats are indicated by matching the underlining. (*B*) Alignment of sections of the TCR γ enhancer with homologous sequences from other TCR gene enhancers (enh.) (9, 14, 15) and previously described core enhancer sequences (12, 13). Dashes indicate identity.

relative to their respective genes (3 kb 3′ to C_α, 5.5 kb 3′ to $C_{\beta 2}$, and 6 kb 3′ to C_δ, respectively) (11, 16–21). At the nucleotide level, however, homology between the TCR γ enhancer and the other sequences is very limited. In this context, the 38-bp homology between the TCR γ and β enhancers, mentioned above, stands out strongly. An important shared function for this region in TCR transcriptional regulation is supported additionally by the shorter, though still significant, homologies to the TCR α and δ enhancer sequences, as well as by the correlation with nuclear protein binding sites for two of the three homologous sequences. Mutational analysis of the TCR γ enhancer and DNase I footprinting studies should cast some light on this issue.

The γ-chain genes, except for those containing the V_2 gene segment, are transcribed only in γδ T cells and not in αβ T cells, indicating lineage-specific regulation (5–8). In this regard, it is interesting that the TCR γ enhancer is considerably weaker relative to the TCR α enhancer when compared in the murine αβ line EL4 but is stronger or equivalent in the other cell lines examined—i.e., human γδ T cells (Peer and MOLT-13) and human αβ T cells (Jurkat). Fine mapping of the enhancing activity also gives somewhat different results for EL4. Thus, the 400-bp fragment Q shows maximal or near-maximal enhancement in all other cell lines but displays lower than maximal enhancement in EL4 by a factor of 2 to 3, suggesting that nearby elements provide important synergistic functions in the latter case but not in the former ones. In the absence of data from additional cell lines, the simplest interpretation for these observations is that the murine $C_{\gamma 1}$ enhancer is generally less active in αβ T cells than in γδ T cells in the mouse, owing to the absence of certain positive trans-acting factors or the presence of certain negative trans-acting factors in the former as opposed to the latter cell type. However, in human lines these cell lineage-specific differences are obscured by species-specific differences (e.g., human negative factors may not work on a murine enhancer).

As indicated above, transgenic studies imply that transcription of TCR γ-chain loci may also be regulated by cis-acting silencing elements (10). In the case of the TCR α-chain locus, several such elements have been identified in the vicinity or immediately adjacent to the TCR α enhancer (22). They appear to suppress its activity specifically in γδ T cells. Intriguingly, the presence of a silencer is suggested in our experiments by the 3- to 4-fold difference in activity between fragment Q and fragment P (Fig. 2 *Lower Right*), the latter containing only an extra 400 bp. However, since the effect is observed in γδ T cells, its relevance to lineage-specific regulation is unclear.

An additional regulatory problem for the TCR γ-chain locus is presented by the apparent need to prevent coexpression of two types of V_γ segments in one cell. $C_{\gamma 1}$ can be expressed together with any of four V_γ segments, $V_{\gamma 4}$–$V_{\gamma 7}$, depending on the course of somatic rearrangement. Cell fusion experiments show that γδ T cells expressing $V_{\gamma 4}$ or $V_{\gamma 7}$, but not those expressing $V_{\gamma 5}$ or $V_{\gamma 6}$, can activate a rearranged but previously silent $V_{\gamma 4}$ gene in an αβ T-cell fusion partner (6, 23). Since somatic rearrangement of each of these V_γ segments results in an identical location vis-a-vis the $C_{\gamma 1}$ enhancer, we assume that the $C_{\gamma 1}$ enhancer can activate each of their associated promoters. (The $V_{\gamma 1}$ and $V_{\gamma 2}$ genes, on the other hand, which rearrange to $J_{\gamma 4}$–$C_{\gamma 4}$ and $J_{\gamma 2}$–$C_{\gamma 2}$, respectively, and are not located close to the $V_{\gamma 4}$–$V_{\gamma 7}$ gene cluster, are probably controlled by different enhancers.) Specificity of expression of the $V_{\gamma 4}$–$V_{\gamma 7}$ genes is likely to be imposed then by elements near their respective V_γ promoters. In this context, it may be relevant that a 3.5-kb fragment containing the putative $V_{\gamma 4}$ transcriptional initiation site

(fragment A in Fig. 2 *Upper Left*) showed no transcriptional activity in Peer or MOLT-13 cells in our assay system, even when linked to the strong heterologous polyoma virus enhancer (data not shown). Perhaps this is due to the absence of certain promoter-specific factors present only in cells of the appropriate γδ T-cell lineage.

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