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Redundant and Unique Roles of Two Enhancer Elements in the TCRγ Locus in Gene Regulation and γδ T Cell Development

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Summary

Many mammalian genes, including those encoding antigen receptors, contain more than one enhancer element. Deleting one element often does not prevent expression, but functional redundancy has never been directly demonstrated by gene targeting of multiple elements. We demonstrate that simultaneous deletion of two enhancer/LCR-like elements in the TCR Cγ1 cluster, HsA and 3′$E_{cγ1}$, severely diminishes TCRγ transcription, selectively impairs development of γδ thymocyte subsets, but only modestly reduces TCRγ gene rearrangement, while deletion of each element separately has little effect. In contrast to these results in thymocytes, deletion of HsA alone reduces transcription of one Vγ gene specifically in peripheral γδ T cells. Thus, the two elements exhibit functional redundancy in thymocytes but also have unique functions in other settings.

Introduction

The development of B and T cells depends on the somatic assembly of immunoglubulin (Ig) and T cell receptor (TCR) genes, respectively, from V, D, and J gene segments and subsequent transcription of the genes. Receptor gene rearrangements are mediated by the lymphoid-specific V(D)J recombinase. Much evidence indicates that V(D)J rearrangement is controlled, at least in part, by *cis*-acting elements in the loci and corresponding transregulatory factors that also regulate transcription (Sleckman et al., 1996).

Many genes are regulated by multiple *cis*-acting elements in addition to the promoter, such as locus control regions (LCR), local enhancers, and silencers, which are often widely separated from each other in the locus (Blackwood and Kadonaga, 1998). LCRs often contain enhancers, but are attributed with the additional function of "opening" the chromatin locally, allowing access of the transcriptional machinery (Grosveld et al., 1987). Enhancers, by themselves, are often insufficient to overcome the repressive effects of chromatin (Blackwood and Kadonaga, 1998). It remains controversial whether LCRs and enhancers function in fundamentally different ways, as opposed to representing different ends of a functional continuum (Engel and Tanimoto, 2000).

Most immune receptor loci are known to contain multiple *cis*-acting elements that stimulate rearrangement and/or transcription (Sleckman et al., 1996). Variable effects on transcription or recombination of the corresponding gene have been reported when individual elements in the endogenous loci were replaced with a recombined loxP site by gene targeting (i.e., in cases where the selectable marker used for targeting was also deleted). Deletion of the only known enhancer element in the TCRβ locus abolished both germline transcription of the Dβ-Jβ gene segments and β gene rearrangement (Mathieu et al., 2000). Deletion of either the Igκ intronic enhancer or the 3′ enhancer had modest effects on rearrangement, whereas expression was largely normal in both cases (Xu et al., 1996; Gorman et al., 1996). In the Ig heavy chain locus, deletion of the intronic enhancer mildly impaired recombination and substantially reduced transcription (Sakai et al., 1999). Deletion of different 3′ enhancers had mild effects on these processes, but did strongly impair class switching (Manis et al., 1998; Pinaud et al., 2001). Deletion of the TCRα enhancer blocked rearrangement of TCRα genes and substantially impaired transcription of both TCRα and TCRδ genes (Sleckman et al., 1997). Deletion of the TCRδ enhancer blocked TCRδ rearrangement but not transcription (Monroe et al., 1999). A deletion of several hypersensitive sites in the TCRα LCR did not have a substantial impact on TCRα gene rearrangement or expression (Hong et al., 1997).

To explain the instances where the deletion of positive elements had little or no effect, it has been proposed that the multiple elements in the locus are at least partially redundant in function. Consistent with this possibility, examples have been reported of two elements acting at least partially redundantly in ectopically integrated transgenes (Baker et al., 1999). However, results with transgenes are in some instances not borne out by comparable analysis performed by gene targeting. Few if any gene targeting studies have demonstrated that simultaneous deletion of two *cis*-acting elements prevents gene expression of cellular development when separate deletion of either does not.

The murine TCR Cγ1 gene cluster comprises four closely linked Vγ gene segments, in the order Vγ5, 2, 4, and 3, which rearrange to a single common downstream J gene segment, Jγ1 (Figure 1A). The relative simplicity, compact size, and highly regulated rearrangement patterns characteristic of the cluster offer several advantages for the analysis of *cis*-acting regulatory elements (Raulet, 1989; Raulet et al., 1991; Vernooij et al., 1993). In early fetal thymocytes, rearrangements of Vγ3 and Vγ4 genes predominate, and the resulting $Vγ3^+$ and $Vγ4^+$ cells migrate to the skin or reproductive tissue, respectively. A switch occurs later in ontogeny, leading to the disappearance of Vγ3 and Vγ4 rearrangements and sharply increased Vγ2 and Vγ5 rearrangements. $Vγ2^+$ and $Vγ5^+$ cells that arise in the adult thymus migrate to the secondary lymphoid organs (Raulet et al., 1991; Goldman et al., 1993).

At least two *cis*-acting enhancer/LCR elements are present in the Cγ1 cluster. One is a T cell-specific transcriptional enhancer, 3′$E_{cγ1}$, located 3 kb downstream of the Cγ1 gene segment (Spencer et al., 1991). Similar

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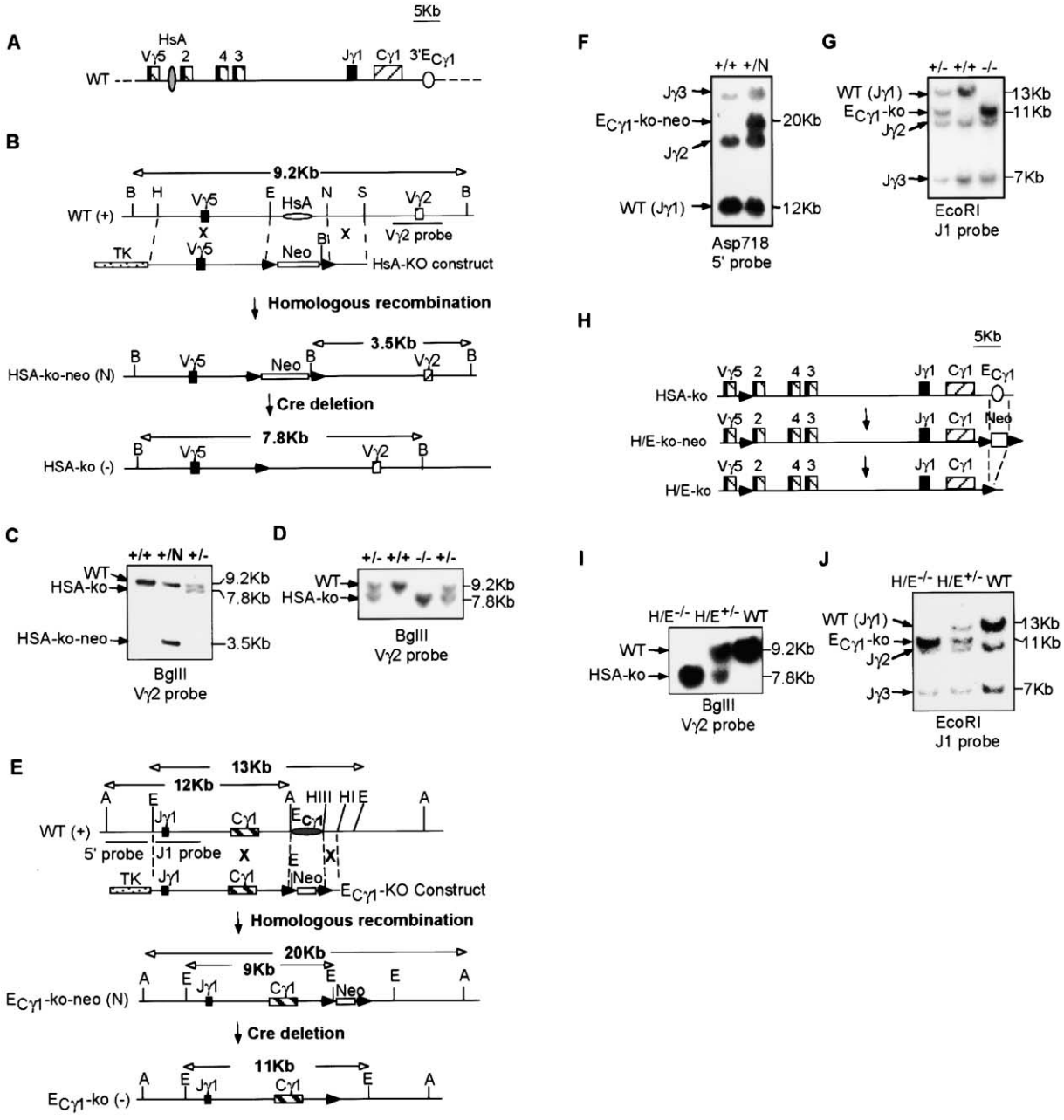

Figure 1. Gene Targeting Strategies for Deleting HsA and 3′$E_{c\gamma1}$, Separately and Together

(A) Organization of the Cγ1 cluster.

(B) HsA targeting scheme. Homologous recombination of the HsA-KO targeting construct yields the HsA-ko-neo chromosome indicated. Transient transfection with a Cre expression construct resulted in deletion of the *neo* cassette, yielding the HSA-ko chromosome in which HsA was replaced by a recombined loxP site. B, BglII; H, HindIII; E, EcoRI; N, NcoI; S, SpeI.

(C) An ES cell clone with one HsA-ko-neo chromosome (+/N) and a daughter clone with the HsA-ko chromosome (+/−), confirmed by Southern blotting (see [B] for strategy).

(D) Typing of tail DNA from pups of an intercross of HsA-ko heterozygous mice by Southern blotting.

(E) 3′$E_{c\gamma1}$ targeting scheme. A, Asp718; E, EcoRI; HIII, HindIII; HI, HpaI.

(F) Initially targeted $E_{c\gamma1}$-ko-neo ES clones were identified by Southern analysis of Asp718-digested genomic DNA with the 5′ probe (see [E]).

(G) Typing of tail DNA from pups of an intercross of $E_{c\gamma1}$-ko heterozygotes by Southern blotting (see [E] for strategy). Deletion of the *neo* cassette from the initially targeted $E_{c\gamma1}$-ko-neo chromosome occurred after crossing founder mice to Cre-transgenic mice, yielding the $E_{c\gamma1}$-ko chromosome in which 3′$E_{c\gamma1}$ was replaced by a recombined loxP site.

(H) HsA/3′$E_{c\gamma1}$ double targeting scheme.

(I and J) Southern blots to confirm deletion of HsA (I) and 3′$E_{c\gamma1}$ (J) in the H/E double-knockout mice. The schemes were as in (B) and (E), respectively.

enhancers were also found associated with the highly related Cγ2 and Cγ3 genes (Vernooij et al., 1993). A second element, HsA, was defined between the Vγ5 and Vγ2 genes, based on DNase I hypersensitivity (Baker et al., 1999). In transient transfection assays, $3'E_{C\gamma1}$ enhanced transcription specifically in T cell lines, whereas HsA was inactive. As tested by deleting the elements from a rearranged (Vγ2-Jγ1Cγ1) TCRγ transgene, $3'E_{C\gamma1}$ was required for transcription in immature but not mature T cells, whereas HsA was necessary and sufficient to achieve normal levels of transcription in mature T cells (Baker et al., 1999). HsA did not drive transcription in immature T cells in the absence of $3'E_{C\gamma1}$, but it prevented severe position effects that occurred with transgenes that contained $3'E_{C\gamma1}$ alone. No transcription was detected from integrated transgenes lacking both elements. These data suggested that $3'E_{C\gamma1}$ and HsA cooperate to produce the normal developmental pattern of Cγ1 gene transcription, with HsA possibly providing a chromatin-opening activity that buffers the gene from position effects. Together, these two elements possess many characteristics of an LCR (Baker et al., 1999). However, as these studies were performed with transgenes, they did not provide definitive evidence that the two elements cooperate in the endogenous locus, nor could they decisively define the normal functional roles of the elements. In this report, we have generated targeted deletions of $3'E_{C\gamma1}$ and HsA, separately and together, to investigate the roles of these elements in the endogenous locus.

Results

Generation of Knockout Mice Lacking HsA, $3'E_{C\gamma1}$, or Both Elements

To examine the role of HsA and $3'E_{C\gamma1}$ in the context of the endogenous TCRγ locus, the elements were deleted separately or together by gene targeting in ES cells, with subsequent introduction of the targeted genes into the mouse germline. In order to eliminate possible confounding effects of the selectable marker genes that were substituted for the elements in the process of gene targeting (Xu et al., 1996; Seidl et al., 1999), the marker genes were equipped with flanking loxP sites and subsequently deleted.

A targeted deletion of a 1.4 kb EcoRI-NcoI fragment containing HsA was generated in J1 ES cells of the 129/terSv strain. Homologous recombinants were selected and the *neo* cassette was subsequently deleted by transient transfection with a Cre expression plasmid (Figures 1B–1D) (Gu et al., 1993). The ES cells were used to prepare chimeric mice, which were crossed with either 129/SvJ or C57BL/6NCr (B6) mice. The heterozygous mice were subsequently intercrossed, and homozygous knockout mice ($H^{-/-}$ mice) and wild-type mice ($H^{+/+}$) were selected after typing. These crosses yielded mutant mice on either the pure 129 genetic background or the (B6x129) mixed genetic background, respectively (Figure 1D). Because polymorphisms in TCRγ genes may in some cases influence TCRγ gene expression or subset composition, it is important to point out that this breeding scheme with mixed background mice inadvertently selects for TCRγ loci of 129 origin in the homozygous mutant mice and of B6 origin in the homozygous wild-type littermates.

A slightly different strategy was used to generate mice in which $3'E_{C\gamma1}$ was deleted ($E^{-/-}$ mice). A 2.0 kb Asp718-HindIII fragment containing $3'E_{C\gamma1}$ was replaced by a *neo* cassette flanked by loxP sites in J1 ES cells (Figures 1E and 1F). Chimeric mice were generated and crossed to a CD1 strain that expresses a CMV-Cre transgene that directs Cre synthesis in germ cells, resulting in deletion of the *neo* cassette in vivo. The $E^{+/-}$ mice were crossed back to B6 mice, which were subsequently intercrossed to generate homozygous knockout mice on a mixed genetic background (Figure 1G).

To investigate the possibility that $3'E_{C\gamma1}$ and HsA function redundantly in supporting γδ T cell development, we also generated mice in which both elements were deleted ($H/E^{-/-}$ mice). Due to the close proximity of these two elements, the double knockouts could not be generated by interbreeding the single knockouts. Therefore, ES cells in which HsA had been deleted from one allele (HsA-ko) were transfected with the same $3'E_{C\gamma1}$ targeting construct used to generate $E^{-/-}$ mice ($E_{C\gamma1}$-KO construct). ES cell clones were selected in which $3'E_{C\gamma1}$ was replaced with the *PGK-neo* cassette on the same chromosome as the HsA mutation (H/E-ko-neo ES cells; Figure 1H) (see Experimental Procedures), and the *neo* cassette was subsequently deleted by transfection with the Cre expression plasmid. The resulting ES cells (H/E-ko; Figure 1H) were used to generate $H/E^{-/-}$ mice on the 129 and mixed genetic backgrounds, as described above for the $H^{-/-}$ mice. Deletion of both HsA and $3'E_{C\gamma1}$ was confirmed by Southern blot analysis as described for HsA or $3'E_{C\gamma1}$ knockout mice (Figures 1I and 1J).

Defective γδ T Cell Development in $H/E^{-/-}$ Mice but Not $H^{-/-}$ or $E^{-/-}$ Mice

None of the deletions resulted in detectable alterations in thymocyte cell numbers or αβ T cell development (data not shown). Subset analysis of adult $CD4^-CD8^-$ thymocytes in $H^{-/-}$ mice revealed a minor reduction in the percentage of $V\gamma2^+$ cells (30%–40%, Figures 2A and 2B; complete data summary in Table 1). The percentage of $V\gamma5^+$ cells in $H^{-/-}$ mice was unaffected in the 129 genetic background. $V\gamma5^+$ cells seemed to be more abundant in the $H^{-/-}$ mixed background mice, but this is likely due to allelic polymorphisms in TCRγ genes, since $H^{-/-}$ and $H^{+/+}$ contain different TCRγ alleles (see above) and we found a similar increase in wild-type strain 129 mice compared to B6 mice (Figure 2B; see above). In conclusion, the development of $V\gamma2^+$ thymocytes is only slightly impaired in $H^{-/-}$ mice, whereas $V\gamma5^+$ thymocytes were not affected. Analysis of fetal thymocytes indicated that development of $V\gamma3^+$ cells was also unaffected by the deletion of HsA (Table 1).

Development of T cells was also little affected in $E^{-/-}$ mice (Figure 2C and data not shown). The percentage of $V\gamma2^+$ cells was perhaps slightly reduced among adult DN thymocytes (by $<25\%$). $V\gamma3^+$ fetal thymocytes developed in normal proportions in these mice (Table 1). Although the percentage of $V\gamma5^+$ cells was comparable to that of the wild-type littermate (Figure 2C), it is likely that the enhancer mutation does slightly reduce the efficiency of Vγ5 cell development. This conclusion is suggested by the fact that the $E^{-/-}$ mice are homozygous for strain 129 alleles at the TCRγ locus and could be expected to have a greater frequency of $V\gamma5^+$ cells than

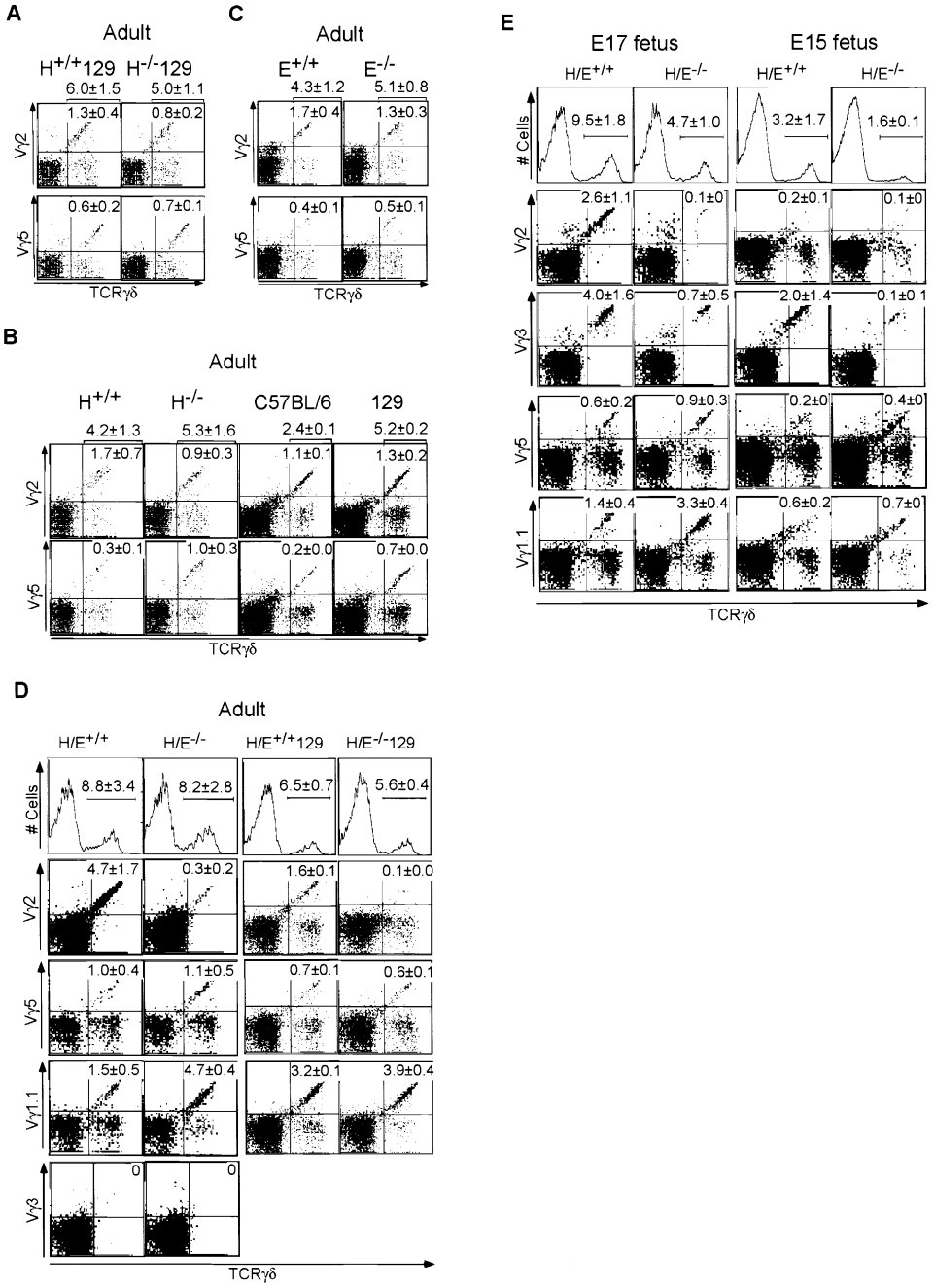

Figure 2. HsA and 3′$E_{c\gamma1}$ Function Redundantly in γδ T Cell Development

Gated $CD4^-CD8^-$ thymocytes were examined for expression of TCRγδ and specific Vγ proteins by four-color flow cytometry. Values within the plots represent the mean percentages ± SD of $CD4^-CD8^-$ thymocytes that express the Vγ, based on at least three independent determinations. In panels (A)–(C), values above the plots represent mean percentages ± SD of $CD4^-CD8^-$ thymocytes that express TCRγδ. It should be noted that the use of compensation settings in four-color flow cytometry sometimes resulted in the accumulation of $TCR\gamma\delta^+$ cells against the *x* axis, where they are difficult to visualize.

(A) Comparison of $H^{+/+}$ and $H^{-/-}$ mice on the 129 background.

(B) Comparison of $H^{+/+}$ and $H^{-/-}$ mice on a mixed (B6x129) background. Analysis of thymocytes from wild-type B6 and 129 inbred mice is shown for comparison.

(C) Comparison of 3′$E_{c\gamma1}{}^{+/+}$ and 3′$E_{c\gamma1}{}^{-/-}$ mice on a mixed background.

(D and E) γδ subsets among gated adult (D) or fetal (E) $CD4^-CD8^-$ thymocytes from 129 or mixed background (as indicated) $H/E^{+/+}$ and $H/E^{-/-}$ mice.

the wild-type littermate (with B6 TCRγ alleles) if the enhancer mutation was silent.

Simultaneous deletion of HsA and 3′$E_{c\gamma1}$ resulted in a dramatic (>15-fold) reduction in the percentage of $V\gamma2^+$ T cells among $CD4^-CD8^-$ thymocytes of both 129 and mixed background $H/E^{-/-}$ mice in comparison to $H/E^{+/+}$ littermates (Figure 2D). Thymus cellularity, αβ T cell development, and the total number of γδ T cells in

Table 1. Summary of Thymic Phenotypes of HsA ($H^{-/-}$), $3'E_{C\gamma1}$ ($E^{-/-}$), and HsA/$3'E_{C\gamma1}$ ($H/E^{-/-}$) Mutant Mice

Parameter[b]	Cells or Gene	Genotype		
		$H^{-/-}$	$E^{-/-}$	$H/E^{-/-}$
Development	Adult TCRγδ$^+$	1[a]	1	1
	Vγ2$^+$	1–2	1–2	>15
	Vγ3$^+$	1	1	>15
	Vγ4$^+$	nd[c]	nd	nd
	Vγ5$^+$	1	1–2	1
Rearrangements	Vγ2	1–2	1–2	2–3
	Vγ3	1	1	2–4
	Vγ4	1	1	2
	Vγ5	1	1	1
Transcripts[d]	Vγ2	1–2	1–2	15–20
	Vγ3	nd	nd	10–15
	Vγ4	nd	nd	5–10
	Vγ5	1	5	6–15

[a] Fold reduction compared to wildtype (1 = no change).
[b] Vγ2 and Vγ5 were analyzed in adult mice, and Vγ3 and Vγ4 were anlyzed in E15 and E17 fetuses.
[c] nd, not done.
[d] Transcript levels were adjusted based on the level of rearrangement.

the adult thymus were normal (Figure 2D and data not shown). The reduced percentage of Vγ2$^+$ cells was equally apparent in fetal thymocytes at day 17 postcoitum (E17), when the cells first become prominent (Figure 2E). In contrast, as shown by the analysis of strain 129 mutant mice, the percentage of Vγ5$^+$ cells was unchanged, and cells expressing Vγ1.1 (of another TCRγ cluster) were, if anything, slightly more abundant in the adult thymus (Figure 2D). A larger increase in Vγ1.1$^+$ cells was observed in the $H/E^{-/-}$ mice on the mixed genetic background (Figures 2D and 2E), but this difference was also evident when wild-type B6 and 129 strains were compared (data not shown), indicating that it was probably due to TCRγ gene polymorphisms between these strains.

The double mutation also had a marked effect on the development of Vγ3$^+$ cells, reducing the size of this subset by approximately 20-fold among E15 fetal thymocytes and 5- to 10-fold among E17 fetal thymocytes (Figure 2E, Table 1). Analysis of fetal thymocytes was performed in the mixed background mice because we were unable to obtain sufficient numbers of fetuses from the 129 strain mice, which breed poorly. However, the difference in Vγ3 usage cannot be attributed to TCRγ gene polymorphisms between 129 and B6 strains, because $H^{-/-}$ or $E^{-/-}$ mice, which also have TCRγ genes originating from the 129 mouse strain, have essentially normal numbers of Vγ3$^+$ fetal thymocytes (Table 1). The results demonstrate that HsA and $3'E_{C\gamma1}$ function redundantly in supporting development of Vγ3$^+$ and Vγ2$^+$ T cells, but do not play a crucial role in the development of Vγ5$^+$ or of cells expressing a Vγ gene from a different Cγ cluster (Vγ1.1$^+$ cells).

Redundant Regulation of TCR Cγ1 Genes by HsA and $3'E_{C\gamma1}$

We investigated whether the impaired development of TCRγδ subsets in the double-mutant mice was due to effects on TCRγ gene rearrangement. Southern blotting of genomic DNA with a Jγ1 probe detects Vγ rearrangements to Jγ1 (see Figure 1A) as well as to Jγ2 in another Cγ cluster (Takagaki et al., 1989). In adult thymocyte DNA, however, the only Jγ1 rearrangements of sufficient abundance to detect by this method are Vγ2 rearrangements. As expected from the developmental analysis, there was little or no reduction in Vγ2 rearrangements in adult thymocytes from $H^{-/-}$ or $E^{-/-}$ single-knockout mice (Figure 3A). Surprisingly, despite the substantial defect in the development of Vγ2$^+$ cells in $H/E^{-/-}$ mice, Vγ2 gene rearrangement was reduced by only 2.6-fold (an average of three determinations, based on phosphorimager analysis). Semiquantitative PCR analysis corroborated the small reduction in Vγ2 rearrangement in adult $H/E^{-/-}$ thymocytes on both the 129 and mixed backgrounds and revealed a similarly minor effect on Vγ2 rearrangements in E17 and E15 fetal thymocytes (Figures 3C and 3D).

Because γδ T cells are only a minor subset of the thymocytes examined in the previous experiment, we determined whether the mutations impair Vγ2 gene rearrangement in γδ T cells. For this analysis, rearrangement of the mutant allele was examined in purified 129 strain splenic γδ T cells (92% pure) from heterozygous mice that also harbored a wild-type allele. The HsA-deleted allele generated a shorter EcoRV fragment with a Vγ2 probe, allowing the two alleles to be distinguished by genomic Southern blotting. The analysis revealed a small (~2-fold) reduction in Vγ2 rearrangement of the H/E^- mutant allele compared to the wild-type allele and no change in the H^- allele (Figure 3B). The near normal rearrangement of the double-mutant allele suggests that the isolated $3'E_{C\gamma1}$ mutation also would not significantly impair rearrangement in γδ T cells, though this was not tested directly.

Vγ5 rearrangement was also not significantly affected by the H/E double mutation in adult 129 strain mice (Figure 3C, Table 1). In mixed background mice, the level of Vγ5 rearrangements appeared to increase in adult and fetal $H/E^{-/-}$ mice as well as in adult $H^{-/-}$ and $E^{-/-}$ mice (Figure 3C, Table 1, and data not shown). As no increase was observed in the $H/E^{-/-}$ 129 strain mice, it is likely that the apparent increase in mixed background mice is due to the allelic differences between 129 and B6 TCRγ genes discussed earlier. Finally, recombination of the Vγ3 and Vγ4 gene segments in E17, E15, and E14 fetal thymocytes was impaired only modestly (2- to 4-fold) in the H/E double-mutant mice (Figure 3D, Table 1). The single mutations had even smaller effects than the double mutation (Table 1 and data not shown).

Together, these data indicate that impaired rearrangement cannot account for much of the sharp reduction in the development of Vγ2$^+$ cells in the adult or of Vγ3$^+$ cells in the fetus. Therefore, TCRγ gene transcription was analyzed by semiquantitative RT-PCR of total RNA, using Vγ-specific primers in conjunction with a Jγ1 primer to detect only transcripts derived from assembled Vγ-Jγ gene segments. The primers spanned an intron in the gene, enabling us to distinguish products originating from spliced mRNA from products originating from contaminating genomic DNA. Initially, we analyzed RNA from adult $CD4^-CD8^-$ thymocytes that had also been depleted of mature TCRγδ$^+$ cells by cell sorting. These cells were examined in order to focus the

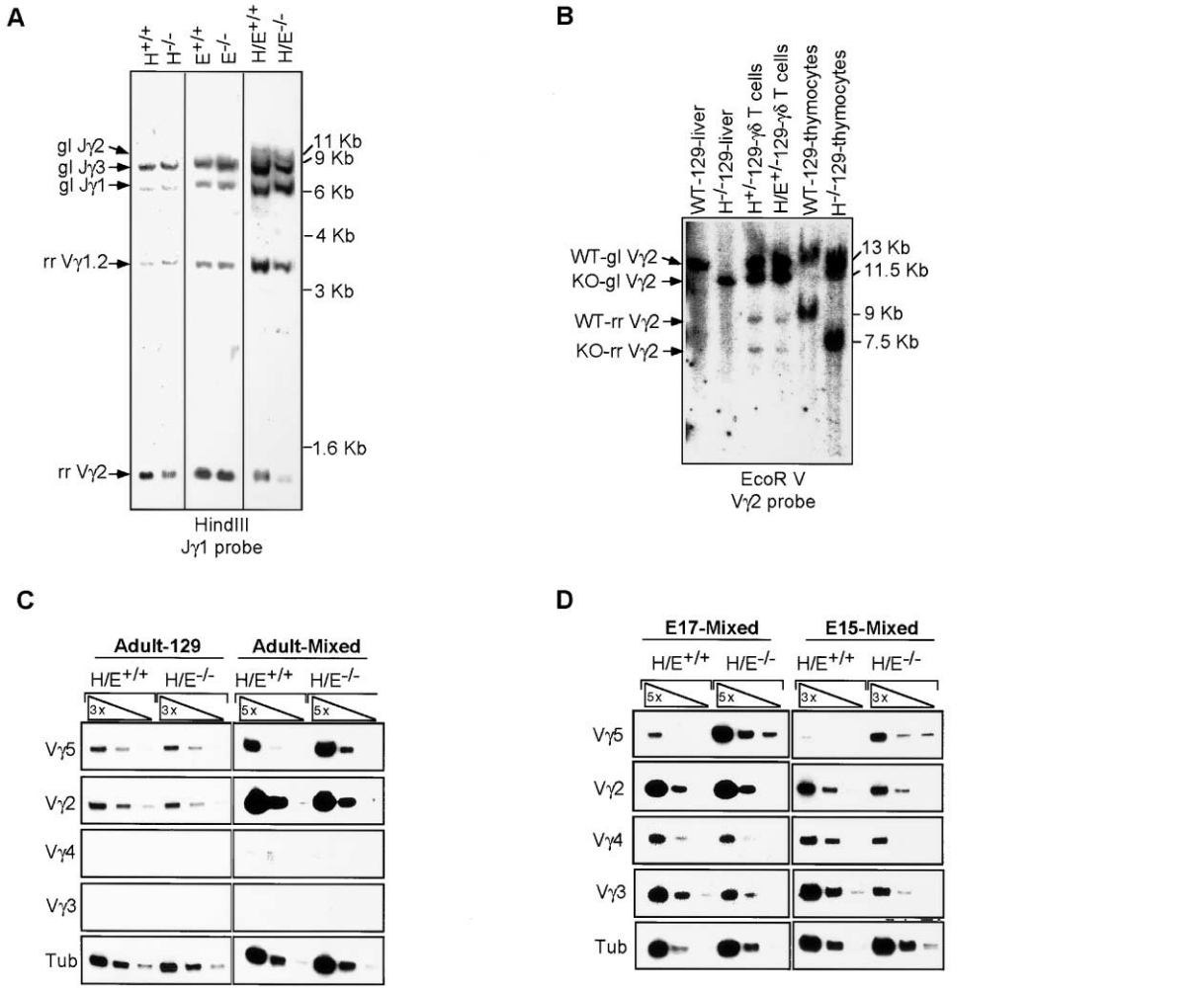

Figure 3. Rearrangement of Vγ Genes in HsA and 3′$E_{cγ1}$ Double- and Single-Knockout Mice

(A) Vγ2 rearrangements detected by Southern blotting with a Jγ1 probe in genomic DNA from adult thymocytes. Vγ2 rearrangement was reduced by an average of 2.6-fold in H/$E^{-/-}$ mice (n = 3). The level was estimated based on phosphorimager analysis as the ratio of the cpm in the rrVγ2 band to the sum of the cpms in the rrVγ2 and Jγ1 bands. The identities of the bands are indicated to the left. gl, germline; rr, rearranged.

(B) Vγ2 rearrangement in purified γδ T cells of heterozygous H/$E^{+/-}$ or $H^{+/-}$ mice detected by Southern blotting with the Vγ2 probe and compared to control samples. WT, wild-type; KO, knockout.

(C) Semiquantitative PCR analysis of Vγ-Jγ1 rearrangements (and β-tubulin as a loading control) in DNA from thymocytes of adult wild-type and H/$E^{-/-}$ mice. Results are shown with cells from 129 or mixed background mice, analyzed on different days. The serial dilution factor of the DNA samples is indicated in the triangles.

(D) Semiquantitative PCR analysis of Vγ rearrangements in E17 and E15 fetal thymocytes from mixed background mice.

analysis on precursor cells and preclude the influence of cellular selection for rare cell subsets on the results. H/$E^{+/+}$ and H/$E^{-/-}$ mice on the 129 background were compared. The analysis revealed a 30-fold reduction in the amount of Vγ2 transcripts in cells from the H/$E^{-/-}$ mice, as determined by dilution analysis (Figure 4A). Since the level of Vγ2 rearrangements was only modestly reduced in H/$E^{-/-}$ strain 129 mice, the reduction in transcript levels can be attributed largely to impaired transcription. The level of Vγ5 transcripts was also reduced in adult 129 strain H/$E^{-/-}$ mice by 7- to 8-fold (Figure 4A). As Vγ5 rearrangement levels were normal in these mice, the defect can be attributed to altered transcription. In contrast, no reduction in Vγ5 transcript levels was observed in 129 strain $H^{-/-}$ mice (data not shown). Indeed, since HsA is deleted when Vγ5 recombines with Jγ1 (Figure 1A), it appears likely that the defective Vγ5 transcription observed in H/$E^{-/-}$ mice is due primarily to the mutation in 3′$E_{cγ1}$.

As another approach to eliminate the possible influence of cellular selection of rare subsets, we bred H/$E^{-/-}$ mice to TCRδ knockout mice to generate H/$E^{-/-}$TCR$δ^{-/-}$ mice on a mixed genetic background (Itohara et al., 1993). Such mice do not express any cell surface TCRγδ receptors, thus preventing receptor-dependent cellular selection processes. Vγ2 rearrangements in $CD4^{-}CD8^{-}$ thymocytes were reduced by less than 3-fold in H/$E^{-/-}$TCR$δ^{-/-}$ mice as compared to H/$E^{+/+}$TCR$δ^{-/-}$ mice, confirming the modest effect of these mutations on Vγ2 rearrangement (Figure 4B). Vγ5 rearrangements

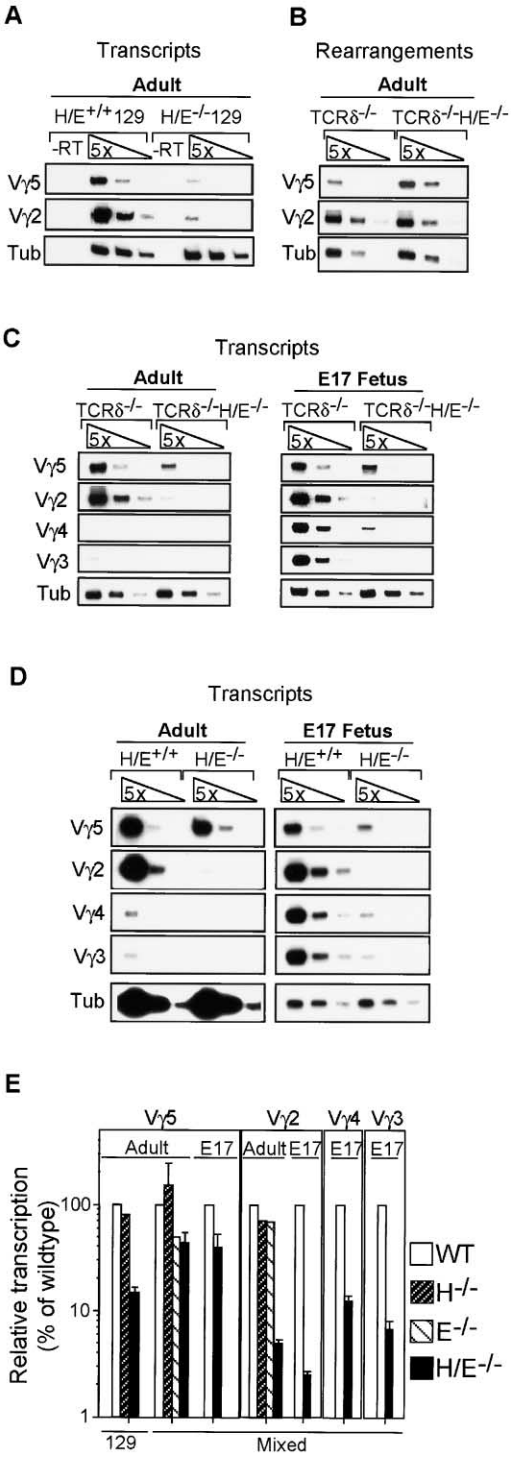

Figure 4. Reduced Transcription of Rearranged TCRγ Genes in γδ T Cell Precursors of HsA and 3′$E_{cγ1}$ Double-Mutant Mice

The serial dilution factors are indicated in the triangles above each dilution series.

(A) Comparison of Vγ transcript levels in RNA from $CD4^-CD8^-TCR\gamma\delta^-$ thymocytes of $H/E^{+/+}$ and $H/E^{-/-}$ mice on the 129 background. The highest sample concentration was also processed without addition of reverse transcriptase (−RT).

(B and C) Analysis of TCRγ rearrangements (B) and transcripts (C) in $H/E^{-/-}$ mixed background $TCR\delta^{-/-}$ mice by semiquantitative PCR and RT-PCR. The inability of $TCR\delta^{-/-}$ mice to express cell surface TCRγδ receptors prevents cellular selection processes. Nucleic acids from adult or E17 $CD4^-CD8^-$ thymocytes were analyzed.

(D) Comparison of Vγ transcript levels in RNA from mixed background $H/E^{+/+}$ and $H/E^{-/-}$ adult $CD4^-CD8^-$ thymocytes or E17 fetal thymocytes.

(E) Summary of relative transcript levels in single- and double-knockout mice. The values represent means ± SD (n = 2–3), except where no error bars are present (single determination). The level in wild-type mice was set to 100.

were elevated by 5-fold, presumably due to the aforementioned allelic polymorphism in TCRγ genes. Vγ2 transcripts were ~50-fold less abundant in the $H/E^{-/-}$ mice, and Vγ5 transcripts were 3-fold less abundant (Figure 4C). Adjusting for the levels of the corresponding rearrange-ments, this represents an ~17-fold reduction in Vγ2 transcripts and a 15-fold reduction in Vγ5 transcripts. Vγ3 and Vγ4 transcript levels in E17 fetal thymocytes were also strongly depressed in the $H/E^{-/-}TCR\delta^{-/-}$ triple-mutant thymocytes, by ~50-fold and 15-fold, respectively (Figure 4C).

Finally, we examined transcript levels in $CD4^-CD8^-$ thymocytes (i.e., from which γδ T cells had not been removed) from mixed background mice. A 20-fold reduction in Vγ2 transcripts was revealed in adult $H/E^{-/-}$ mice (Figures 4D and 4E). Importantly, the single mutations had little or no effect, demonstrating functional redundancy of the two elements in supporting Vγ2 transcription (Figure 4E). The overall level of Vγ5 transcripts was minimally reduced in the H/E mutant mice, but considering the increased level of Vγ5 rearrangement in these cells of mixed background (Figures 3C and 3D), it appears that the level of transcription per rearranged Vγ5 gene was reduced by ~6-fold. A similar reduction was observed in $E^{-/-}$ thymocytes, but not in $H^{-/-}$ thymocytes, corroborating the conclusion that 3′$E_{cγ1}$, but not HsA, plays some role in supporting Vγ5 transcription (Figure 4E).

We also examined E17 fetal thymocytes from mixed background mice. Taking into account the corresponding levels of rearrangement, substantial reductions were observed in the levels of all four Vγ transcripts (Figures 4D and 4E). Notably, Vγ3 and Vγ4 transcript levels were reduced by 15-fold and 8-fold, respectively.

Nonredundant Role of HsA in Maintenance of Peripheral $V\gamma2_+$ γδ T Cells

$H/E^{-/-}$ mice contained almost no $V\gamma2^+$ cells in the periphery, as might be expected (Figure 5A). Interestingly, in contrast to the situation in the thymus, a significant nonredundant role for HsA was evident in peripheral $V\gamma2^+$ cells. In $H^{-/-}E^{+/+}$ mice, there was a consistent 3- to 4-fold reduction in the number of $V\gamma2^+$ cells in the spleen or lymph nodes (Figures 5A and 5B). The reduction was apparent in both 129 and mixed background $H^{-/-}$ mice. The peripheral $V\gamma2^+$ cells that remained in $H^{-/-}$ mice also exhibited a 2-fold reduction in the average mean fluorescence intensity of staining with anti-Vγ2 antibody (Figures 5A and 5B). Furthermore, sorted peripheral $V\gamma2^+$ cells from $H/E^{-/-}$ mice contained an average of 4-fold less Vγ2 mRNA than $V\gamma2^+$ cells from wild-type littermates (n = 2, Figure 5C is one experiment). In con-

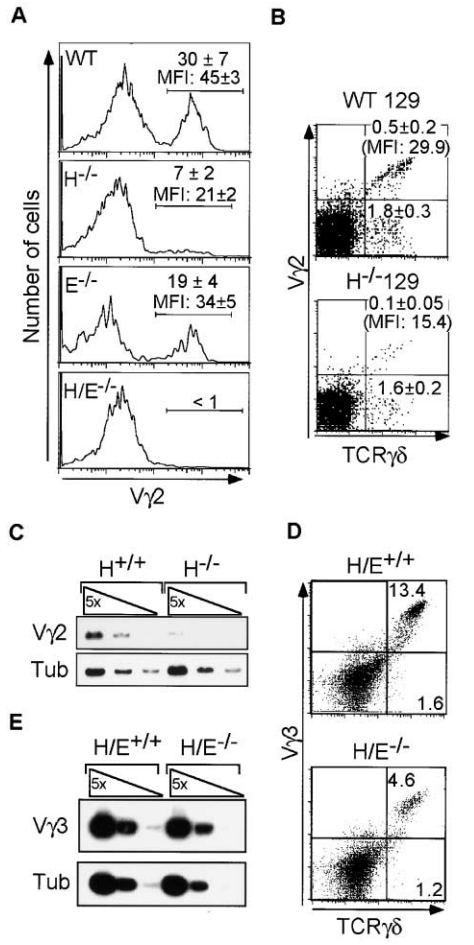

Figure 5. Effects of the Mutations on Peripheral γδ Cells

(A) Vγ2 expression by gated splenic γδ T cells from mixed background mutant and wild-type mice. The mean percent ± SD of $V\gamma2^+$ cells is indicated as is the mean fluorescence intensity of staining ($n \geq 3$).

(B) Analysis of $V\gamma2^+$ cells in the lymph nodes of $H^{+/+}$ and $H^{-/-}$ 129 strain mice. By gating on $CD4^-CD8^-$ lymph node cells and staining for TCRγδ versus Vγ2, the B cells (almost all the cells in lower left quadrants) serve as a reference population.

(C) Vγ2 transcript levels in sorted $V\gamma2^+$ γδ T cells from the spleens and lymph nodes of 129 strain $H^{+/+}$ and $H^{-/-}$ mice determined by semiquantitative RT-PCR.

(D) Dendritic epidermal T cell preparations from $H/E^{-/-}$ or wild-type mice on the mixed genetic background were analyzed by costaining with Vγ3 and TCRγδ antibodies. The γδ cell yields in different preparations vary, but the average difference was 2-fold, based on three determinations.

(E) Vγ3 transcript levels in DEC cell preparations from $H/E^{+/+}$ versus $H/E^{-/-}$ mice determined by RT-PCR.

trast, the number and cell surface receptor expression of $V\gamma2^+$ thymocytes was nearly normal (Figures 2A and 2B), as was the level of Vγ2 transcripts in $H^{-/-}$ thymocytes (Figure 4E). These results indicate that the level of transcription per peripheral $V\gamma2^+$ cell is reduced in the absence of HsA and demonstrate a nonredundant role for HsA in peripheral but not thymic γδ T cells. They further suggest that defects in both thymic and peripheral transcription of the Vγ2 gene contribute to the absence of $V\gamma2^+$ γδ T cells in the peripheral lymphoid organs of $H/E^{-/-}$ mice.

A comparable analysis of other peripheral γδ T cells yielded contrasting results. First, the percentage of $V\gamma3^+$ cells among dendritic epidermal cells was reduced by only 2-fold (an average of three determinations) in adult mice lacking both elements ($H/E^{-/-}$ mice) (Figure 5D), compared to a 20-fold reduction in the percentage of $V\gamma3^+$ cells in the E15 fetal thymus. These results suggest that a homeostatic mechanism may partially compensate for the reduced production of $V\gamma3^+$ γδ T cells in $H/E^{-/-}$ mice, while no such mechanism compensates for the reduced production of $V\gamma2^+$ cells. Furthermore, the level of cell surface Vγ3 expression (Figure 5D) and the level of Vγ3 transcripts among epidermal cells were nearly normal even in mice lacking both regulatory elements ($H/E^{-/-}$ mice) (Figure 5E). Therefore, neither HsA nor $3'E_{C\gamma1}$ are necessary to achieve near normal levels of Vγ3 transcription in these peripheral $V\gamma3^+$ cells. Second, the frequency of splenic $V\gamma5^+$ cells was nearly normal in $H/E^{-/-}$ mice, as was the level of cell surface expression (data not shown). These results suggest different requirements of HsA and $3'E_{C\gamma1}$ for expression of Vγ genes in different settings.

Discussion

Our results demonstrate functional redundancy between two widely separated *cis*-acting sequences in the regulation of the TCR Cγ1 gene cluster. The study should represent a stringent test of redundancy, as it was performed by targeting the endogenous locus with subsequent deletion of the selectable marker. In most respects, deletion of HsA or $3'E_{C\gamma1}$ separately had little effect. Simultaneously deleting both elements, however, resulted in substantial defects in transcription of assembled TCRγ genes in the thymus and modest reductions in recombination of certain Vγ genes. We also observed a reduction in germline transcription of Vγ genes in thymocytes that was comparable in magnitude to the reduction in rearrangement (data not shown). The defects in TCRγ rearrangement and transcription led to substantial defects in γδ T cell development in the thymus, most notably a profound reduction in the number of $V\gamma2^+$ T cells in the adult thymus and periphery and a severely reduced number of $V\gamma3^+$ T cells in the fetal thymus.

It has long been suggested that multiple enhancers function redundantly in gene regulation (Blackwood and Kadonaga, 1998). In addition to the examples discussed in the Introduction, the core Eμ enhancer and the flanking matrix attachment regions function in a partly redundant fashion, as shown by gene targeting of the endogenous rearranged chromosome in a hybridoma cell line (Hug et al., 1996; Wiersma et al., 1999). However, this study was possible only when the action of other elements in the locus were impaired by introduction of an exogenous selectable marker in the locus, possibly confounding the interpretation. In the case of the endogenous murine β-globin locus, deletion of the entire cluster of hypersensitive sites that encompass the LCR (5′HS1-6) had a major impact on the level of transcription (Epner et al., 1998), while separate deletions of several of the individual sites had little effect (Fiering et al., 1995; Hug et al., 1996; Bender et al., 1998). These results are certainly suggestive of redundancy but are not definitive on this point, since the 5′HS1-6 deletion included other

hypersensitive sites and other DNA that has not been tested separately for function by gene targeting.

It is surprising that HsA and 3′$E_{Cγ1}$ function redundantly since their sequences show no apparent similarity. Furthermore, 3′$E_{Cγ1}$ but not HsA was active in transient transfection assays, and deletion of each element yielded a distinct developmental pattern of expression in a transgene system (see below) (Baker et al., 1999). These considerations raise the possibility that the elements may interact differently with other locus elements and transcription factors, yet contribute similarly to gene recombination and expression in developing thymocytes.

Interestingly, the double mutation did not completely prevent rearrangement or expression of any of the genes, suggesting that yet other elements in the endogenous locus can partially substitute for HsA and 3′$E_{Cγ1}$. It is possible that some activity is provided by unidentified elements located between HsA and 3′$E_{Cγ1}$, as suggested by a low level of residual rearrangement in a minigene construct lacking both elements (Baker et al., 1999). However, other data raise the possibility that an additional important element(s) resides upstream of HsA. Insertion of a *PGK-neo* gene between an enhancer and the gene it regulates often inhibits enhancer function (Seidl et al., 1999). In the course of preparing the gene-targeted mice studied here, we examined mice in which HsA was replaced by the *PGK-neo* cassette. Development of V$γ2^+$ cells was severely reduced in these mice compared to mice in which both HsA and *PGK-neo* were deleted (data not shown). Conversely, however, replacement of 3′$E_{Cγ1}$ with *PGK-neo* did not significantly impact γδ T cell development (data not shown). These data suggest a role in Cγ1 recombination and/or transcription of unidentified elements upstream of HsA. It remains possible that compensating elements reside in the region downstream of 3′$E_{Cγ1}$, where enhancers were identified in other Cγ genes (Vernooij et al., 1993). Taken together, the results hint that rearrangement and transcription in the Cγ1 cluster are regulated by at least three *cis*-acting enhancer-like elements.

Interestingly, the double mutation did not alter the normal developmental order of Vγ gene rearrangement. This finding is reminiscent of the finding that the 5′LCR of the β-globin locus is necessary to achieve maximal transcription, but is dispensable to impose the normal developmental order of expression of β-globin genes (Epner et al., 1998).

While the two elements compensated for each other in many respects, each element also exhibited a unique activity. Mice lacking 3′$E_{Cγ1}$ but not HsA ($E^{-/-}$ mice) appeared to have a modest defect in Vγ5 transcription in the thymus. Since HsA is deleted when Vγ5 rearranges to Jγ1, a requirement for HsA was not expected. More notable was the finding that mice lacking HsA but not 3′$E_{Cγ1}$ ($H^{-/-}$ mice) had a reduced number of peripheral V$γ2^+$ cells, which were impaired in transcription of the Vγ2 gene. We hypothesize that the reduced number of V$γ2^+$ cells in these mice is a result of low cell surface expression of the receptor, leading to poor cell survival. In contrast, Vγ2 transcript levels were essentially normal in progenitor $CD4^-CD8^-$ thymocytes of $H^{-/-}$ mice, suggesting that V$γ2^+$ T cells develop a specific requirement for HsA as they fully mature and migrate to the periphery. These data are in accord with the study of transgenes containing a rearranged Vγ2 gene, where deletion of HsA resulted in reduced Vγ2 transcription in peripheral but not thymic V$γ2^+$ cells (Baker et al., 1999). Interestingly, HsA was not necessary for normal expression of Vγ3 in peripheral T cells. It is possible that the latter cells do not undergo the same alterations as V$γ2^+$ cells as they mature and emigrate to the periphery. Alternatively, unidentified elements located between Vγ3 and Vγ2 may provide compensatory enhancer function in mature γδ T cells.

It is instructive to consider instances where the gene targeting results differ with the previous analysis of TCRγ transgenes, because such differences may also suggest the existence of compensating elements in the endogenous locus. A notable difference was that deletion of 3′$E_{Cγ1}$ alone in the transgene, but not in the endogenous locus, resulted in significantly impaired Vγ2-Jγ1Cγ1 transcription in immature adult thymocytes. Another difference was that rearrangement was more dependent on the two elements in the case of the transgene studies. Probably the most interesting difference, however, was that HsA prevented position effects in the transgene experiments, but was not required for consistent expression of the endogenous locus in thymocytes. It is possible that other chromatin-opening elements exist in the endogenous locus but not the transgene or that the endogenous locus is well separated from neighboring regions of heterochromatin and does not require a local chromatin-opening element. The results are reminiscent of the finding that the β-globin locus maintains an open chromatin structure in mice lacking the β-globin 5′ LCR (Epner et al., 1998).

It is notable that the transcriptional and recombinational defects in the HsA/3′$E_{Cγ1}$ double-mutant mice did not correlate in all cases with the developmental defects. For example, whereas a substantial defect in Vγ5 transcription was apparent in precursor cells, V$γ5^+$ cells were present in normal numbers in the thymus and periphery in H/$E^{-/-}$ mice. It appears likely that the discrepancy is due to selective survival and/or expansion of a small number of cells that succeed in expressing Vγ5 at near normal levels. Compensation due to cellular selection is also suggested in the case of Vγ3, since the large developmental defect apparent in the E15 fetal thymus is less apparent by E17 and even less apparent among peripheral V$γ3^+$ cells in the epidermis. Cellular selection of a few V$γ2^+$ cells may be less efficient because mature V$γ2^+$ cells require HsA and/or 3′$E_{Cγ1}$ to maximally express their TCRγ gene (see above).

Significance of Gene Regulation by Multiple Enhancer Elements

Our results indicate that immune receptor loci are in some cases controlled by multiple enhancer-like elements with substantially redundant functions. The fact that deleting individual enhancers in other genes often has little or no effect on gene expression suggests that regulation by multiple functionally redundant enhancers may be common. The value of well separated functionally redundant *cis* elements in gene regulation may be several fold. First, a multiplicity of such elements means that loss of one element by mutation or deletion will not necessarily abrogate gene function. This may fit with the impression that genetic diseases arise more often

from mutations in coding sequences than from regulatory site mutations. Second, while such elements may function redundantly in some respects, they may also be endowed with unique stage-specific or cell type-specific activities, as seen for HsA in peripheral γδ T cells. Third, two separated enhancer elements, each of which functions bidirectionally, may provide maximal activity for genes located between them. Such a configuration may act by itself or in concert with boundary elements (Zhong and Krangel, 1997; Bell et al., 2001) to define regulatory domains. In short, a multiplicity of functionally similar regulatory elements may be one means of providing numerous levels of control, thus expanding the regulatory possibilities for a given gene.

Experimental Procedures

Mice and Gene Targeting

C57BL/6NCr and 129/SvJ mice were purchased from the National Cancer Institute. CD1 strain mice harboring a *CMV-Cre* transgene were obtained from Dr. A. Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). TCRδ$^{-/-}$ mice on the C57BL/6 background were purchased from the Jackson Laboratories (Bar Harbor, ME).

Gene targeting with the J1 ES cell line (Li et al., 1992) was performed as described in the Results section and Figure 1. For generation of 3′E$_{c\gamma1}$ knockout mice, targeted ES cells (containing *PGK-neo*) were selected with Ganciclovir and G418 and identified by PCR and Southern blotting. Deletion of *PGK-neo* in offspring from a cross between chimeric mice and CD1-Cre transgenic mice was demonstrated by Southern blotting with a Jγ1 probe and a *neo* probe. After crossing to B6 and intercrossing the heterozygous pups, E$^{-/-}$ and E$^{+/+}$ littermates were identified by Southern blotting with a Jγ1 probe. In most of the experiments shown for these and the other knockouts, littermates were compared in the same experiment, though in some cases we compared E$^{+/+}$ and E$^{-/-}$ lines that were generated from littermates.

HsA-targeted ES cell clones (HsA-neo-ko) were selected as above, identified by Southern blotting, and transiently transfected with a *CMV-Cre* expression plasmid as previously described (Torres and Kuhn, 1997). Clones in which *PGK-neo* was deleted (HsA-ko clones) were identified by Southern blotting with a *neo* probe as well a Vγ2 probe (Figure 1D). Chimeric mice were generated and crossed to 129 or B6 mice, and heterozygous pups were interbred to generate H$^{-/-}$ or control H$^{+/+}$ mice on either the 129 or mixed genetic backgrounds.

To generate HsA/3′E$_{c\gamma1}$ double-knockout mice, HsA-ko ES cell clones (Figure 1B) were transfected with the 3′E$_{c\gamma1}$ targeting construct, followed by selection as described above. Clones in which 3′E$_{c\gamma1}$ was replaced by a loxP-flanked *PGK-neo* gene were identified by PCR. It is expected that 3′E$_{c\gamma1}$ and HsA will be targeted on the same chromosome in approximately half of these clones. Such clones were identified by transfecting six of them with the CMV-Cre expression plasmid and analyzing about 200 subclones from each. When the two elements are targeted on the same chromosome, a large (35 kb) deletion should sometimes occur in which the recombined loxP sequence at the site where HsA was targeted recombines with one of the loxP sites incorporated into 3′E$_{c\gamma1}$. As determined by PCR and Southern blotting, two of the six parent clones yielded the predicted large recombinants at a frequency of about 30%. The remainder of the subclones from these two parent clones had deleted only the *neo* cassette and were selected for injection into B6 blastocysts. Chimeric mice were generated and crossed to 129 or B6 mice, and heterozygous pups were interbred to generate H/E$^{-/-}$ or control H/E$^{+/+}$ littermates on either the 129 or mixed genetic backgrounds, identified by PCR or Southern blotting.

Cell Preparation

Fetal thymocytes were staged by defining E0 as the morning on which vaginal plugs were detected. Dendritic epidermal cells were prepared from the skin of adult mice as previously described (Sullivan et al., 1985). Purified Vγ2^{+} γδ T cells were sorted from peripheral lymphocytes on a Beckman-Coulter Elite Cell Sorter. For preparation of CD4^{-}CD8^{-} double-negative (or CD4^{-}CD8^{-}γδTCR^{-} triple-negative) thymocytes, total thymocytes were incubated with biotinylated anti-CD4 and anti-CD8 antibodies (plus anti-TCRγδ antibody) followed by incubation with anti-biotin antibody coupled to magnetic beads, and nonstaining cells were sorted out on the AutoMACS (Mitenyi Biotec Inc, Auburn, CA). Post sort analysis revealed that the sorted cells were at least 96% pure. To purify γδ T cells, splenocytes that were nonadherent to nylon wool were stained with biotinylated anti-γδ TCR antibody followed by anti-biotin antibody conjugated with magnetic beads. The positively stained cells were sorted on the AutoMACS by two rounds of positive selection.

Antibodies and Flow Cytometry

FITC- or biotin-conjugated anti-TCRγδ (GL3) and Cy-Chrome-conjugated anti-CD3 antibodies were purchased from PharMingen (San Diego, CA). Red 613-conjugated anti-CD4 and Tricolor-conjugated anti-CD8 antibodies were purchased from GIBCO BRL (Gaithersburg, MD). Biotin-conjugated anti-Vγ3 TCR (F536) and anti-Vγ2 TCR (UC3-10A6) antibodies were prepared in this laboratory. FITC-conjugated anti-Vγ1.1 antibody was generously provided by Dr. P. Pereira (Pasteur Institute, Paris, France), and FITC-conjugated anti-Vγ5 antibody was generously provided by Dr. L. Lefrancois (University of Connecticut, Farmington, CT). For multicolor flow cytometry, cells were incubated with Tricolor anti-CD8, Red 613 anti-CD4, biotin- (or FITC-) conjugated anti-TCRγδ, and FITC- (or biotin-) conjugated anti-Vγ antibodies, followed by PE-streptavidin. The stained cells were analyzed on an EPICS XL instrument (Coulter, Hialeah, FL), and the data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

Primers, Probes, and Typing of Knockout Mice

L2, L4, L3, J1, 5′ tubulin, and 3′ tubulin primers have been described (Asarnow et al., 1989; Goldman et al., 1993). The sequence of L5 is 5′-CCTACTTCTAGCTTTCTTGC-3′. The probes for Southern blot analysis included a 2 kb XbaI-EcoRI fragment for Vγ2 and a 2.6 kb Asp718-EcoRI fragment upstream of Jγ1 gene (5′Jγ1 probe). Two Jγ1 probes were used: a 3 kb EcoRV fragment (Figure 1) and a 1.2 kb HindIII fragment (Figure 3).

Genomic Southern Blot Analysis, Semiquantitative PCR, and RT-PCR

The Southern blotting scheme with the Jγ1 probe was as described (Takagaki et al., 1989). Rearrangement was also detected in EcoRV digests using the Vγ2 probe depicted in Figure 1B. The semiquantitative PCR and RT-PCR assays to detect TCRγ gene rearrangements and transcripts have been described before (Baker et al., 1998). For the RT-PCR assay, samples of total cellular RNA were treated with DNase I to digest contaminating genomic DNA. RNA samples were reverse transcribed (RT) with Superscript II RNase H^{-} reverse transcriptase using oligo-dT primers. The RT products were serially diluted by either 5-fold or 3-fold, depending on the experiment (indicated in Figures), and subjected to semiquantitative PCR using different sets of primers. In all cases, the 5′ primers were in the leader exon, separated by an intron from the V coding exon. Thus, RT-PCR products from spliced mRNA templates will yield a smaller product than PCR products from contaminating genomic DNA. In the experiments shown, all the products were of the correctly spliced size, confirming that they originated from spliced mRNA.

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