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A Novel Element Upstream of the Vγ2 Gene in the Murine T Cell Receptor γ Locus Cooperates with the 3′ Enhancer to Act as a Locus Control Region

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Summary

Transgenic expression constructs were employed to identify a cis-acting transcription element in the T cell receptor (TCR)-γ locus, called HsA, between the Vγ5 and Vγ2 genes. In constructs lacking the previously defined enhancer ($3'E_{C\gamma1}$), HsA supports transcription in mature but not immature T cells in a largely position-independent fashion. $3'E_{C\gamma1}$, without HsA, supports transcription in immature and mature T cells but is subject to severe position effects. Together, the two elements support expression in immature and mature T cells in a copy number–dependent, position-independent fashion. Furthermore, HsA was necessary for consistent rearrangement of transgenic recombination substrates. These data suggest that HsA provides chromatin-opening activity and, together with $3'E_{C\gamma1}$, constitutes a T cell–specific locus control region for the TCR-γ locus.

Key words: T cell receptor γ • locus control region • V(D)J recombination • transcription • enhancer

T and B cell antigen receptor genes are assembled from variable (V), joining (J), and in some cases diversity (D) gene segments by the process of V(D)J recombination, which targets recombination signal sequences adjacent to each rearranging gene segment (1, 2). Many studies have demonstrated a strong correlation between the timing or lineage specificity of V(D)J recombination and prior transcription of the unrearranged genes ("germline transcription") (1). Furthermore, cis-acting DNA elements that regulate transcription play a decisive role in V(D)J recombination (1). At least one and sometimes two elements with classical enhancer activity have been identified at each Ig and TCR locus (3). Transgenic recombination substrates that lack enhancers almost never rearrange normally (1). Deletion of the single enhancers identified at the TCR-β and TCR-α loci caused a profound decrease in V(D)J recombination at these loci, as well as reduced receptor gene transcription (4–6). In loci that have multiple enhancers, deletion of one enhancer has variable effects. For example, deletion of the intronic κ enhancer caused a substantial reduction in rearrangement without substantially affecting transcription of the alleles that did undergo rearrangement (7). Deletion of the 3′ κ enhancer caused a modest reduction in both κ gene rearrangement and transcription (8). Much evidence suggests that enhancers also play a role in regulating the timing and cell specificity of rearrangement (1, 9, 10). In addition, elements in or near the promoters of V genes can also regulate the timing and cell specificity of V gene rearrangements (11).

Evidence suggests that sequences in addition to typical enhancers are often required to obtain high-level transcription in transgenic mice. Sequences proximal to the IgH intronic enhancer exhibit chromatin-opening activity that is important for normal transcription of transgenic heavy chain genes (12). Similarly, consistent high-level expression of TCR-α transgenes required the presence of elements downstream of the defined enhancer element (13). In both cases, the combination of the enhancer and the additional required sequences exhibited features of a locus control region (LCR),[1] a type of regulatory cassette first defined in the β globin locus (14; for review see reference 15). LCRs promote consistent, tissue-specific transcription in chromatin templates, a property that classical enhancers by themselves often lack. Operationally, an LCR is defined as a cis-acting element that confers tissue-specific, high-level, copy number–dependent, integration site–independent expression of a linked gene in transgenic mice. Although LCRs often contain elements that function as enhancers in transient transfection assays, sequences in addition to these en-

[1]*Abbreviations used in this paper:* DN, double-negative; DP, double-positive; LCR, locus control region; rr, rearranged; RT, reverse transcriptase; SP, single-positive.

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hancer elements are often necessary for LCR activity (12, 16–18). It is believed that LCRs function to initiate an open chromatin configuration in the appropriate cell type, thereby isolating linked genes from possible negative effects of neighboring chromatin. Based on their chromatin-altering properties, LCRs could potentially play a role in the control of V(D)J recombination.

The Cγ1 cluster of the murine TCR-γ locus is relatively small, spanning 40–50 kbp, and includes four V region gene segments (Vγ5, Vγ2, Vγ4, and Vγ3) that rearrange to a single J gene segment (Jγ1). The choice of Vγ gene for rearrangement is highly developmentally regulated, with Vγ3 and Vγ4 predominating in the early fetal thymus and Vγ2 predominating later in development (19). A T cell–specific transcriptional enhancer, denoted here as $3'E_{C\gamma1}$, was previously localized 3 kb downstream of Cγ1 by its capacity to activate reporter gene expression from a minimal promoter in transient transfection experiments (20, 21). The $3'E_{C\gamma1}$ element was the only enhancer element detected with the transient transfection assay within a 17-kb stretch of DNA derived from a rearranged Vγ2 gene. As a monomer, $3'E_{C\gamma1}$ was active only in a few T cell lines; however, when multimerized, it was active in nearly every T cell line tested.

Although several γ gene constructs that contain $3'E_{C\gamma1}$ underwent transcription in transgenic mice (22–25), it has not been directly established that $3'E_{C\gamma1}$ promotes transcription in templates that are integrated in chromatin, nor has it been established that $3'E_{C\gamma1}$ is the only enhancer-like element in the locus. Furthermore, the role of $3'E_{C\gamma1}$ and other putative enhancer elements in regulating recombination at the Cγ1 locus has not been examined. In this study, we describe a novel enhancer-like element between the Vγ5 and Vγ2 genes that plays a clear role in driving γ transgene transcription and recombination and that, in combination with $3'E_{C\gamma1}$, possesses many properties of an LCR.

Materials and Methods

Mice. The rearranged (rr)V2H$^+$E$^+$ transgene was previously described as the γ transgene containing the EcoRI–SalI fragment of the G8 TCR-γ gene (22, 25). The rrV2H$^+$E$^-$ transgene was identical except that the 2.8-kb KpnI–SalI fragment containing $3'E_{C\gamma1}$ was removed from the 3′ end. The rrV2H$^-$E$^+$ transgene lacked the 1.5-kb EcoRI–NcoI fragment containing HsA at the 5′ end. The rrV2H$^-$E$^-$ transgene lacked both of these fragments. The γD(H$^+$E$^+$) transgene was assembled from BALB/c DNA derived from phage clones. It included the 5-kb EcoRI fragment containing Vγ2 and HsA and a 15.5-kb MboI fragment containing Jγ1, Cγ1, and $3'E_{C\gamma1}$ that extended from 4.8 kb upstream of Jγ1 to 4 kb downstream of Cγ1. Compared with germline DNA, the transgene lacked 18.5 kb of DNA between Vγ2 and Jγ1, including the Vγ3 and Vγ4 genes. The Vγ2 gene in the γD transgenics contained an XhoI linker at the ClaI site in the coding region that disrupted the reading frame and allowed discrimination between transgenes and endogenous genes. The γD(H$^+$E$^-$) transgene was identical to γD(H$^+$E$^+$) except it lacked the 2.6-kb KpnI–MboI 3′ fragment containing $3'E_{C\gamma1}$. The γD(H$^-$E$^+$) transgene lacked the 3.5-kb EcoRI–XbaI 5′ fragment containing HsA. The γD(H$^-$E$^-$) transgene lacked the 2.6-kb 3′ fragment containing $3'E_{C\gamma1}$ and a 1.5-kb EcoRI–NcoI fragment containing HsA. The transgene constructs, free of vector DNA, were injected into fertilized (C57BL/6 × CBA/J)F_2 eggs. Transgenic founders were either analyzed directly or were backcrossed repeatedly to B6 mice (rrV2 lines) or CBA/J mice (γD lines; purchased from the National Cancer Institute, Bethesda, MD) to generate transgenic lines. Mice were bred and maintained in specific pathogen-free facilities at the University of California at Berkeley.

Assay for DNase I Hypersensitive Sites. The DNase I hypersensitive assays were performed on thymocytes and LPS blasts as described (26) except that the cells were lysed in a saponin solution (27). The quantities of DNase I (Type IV; Sigma Chemical Co.) per tube were as follows: 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25, and 50 μg. The control tube contained water. The DNase I hypersensitive assays on liver were performed as previously described (28). LPS blasts were made by incubating spleen cells, from which CD4$^+$ and CD8$^+$ cells had been depleted by complement lysis, with 40 μg/ml LPS (*Salmonella typhosa*; Difco Labs., Inc.) at a concentration of 2×10^6 cells/ml for 3 d. More than 90% of the resulting cells stained positive for the B cell marker B220.

Cell Preparations for Nucleic Acid Analysis. Peripheral T cells were prepared from a mixture of spleen and lymph node cells by passing the cells over nylon wool columns. To purify α/β and γ/δ T cells, the isolated peripheral T cells were combined with thymocytes, and the mixture was partially depleted of CD4 and CD8 cells by complement lysis followed by cell sorting with an Epics Elite flow cytometer (Coulter Immunology) using anti-γδ (GL3–FITC) and anti-αβ (H57.597–biotin) antibodies. Fetal thymus was timed by designating the day of the plug as day 0. The whole fetal thymus, including the capsule, was used to isolate RNA. CD4$^-$CD8$^-$ double-negative (DN) thymocytes were prepared by complement lysis of whole thymocytes with anti-CD4 (RL172) and anti-CD8 (3.168.8 or AD415) antibodies and a mixture of guinea pig complement (GIBCO BRL) and rabbit complement followed by isolation of live cells on a Ficoll gradient. CD4$^+$CD8$^+$ thymocytes (double-positives [DPs]) and CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ thymocytes (single-positives [SPs]) were sorted on an Epics Elite flow cytometer (Coulter Immunology) using anti-CD4 and anti-CD8 antibodies. The enriched DN populations of the γD lines employed for the analysis of transcription in different developmental stages (see Fig. 4) were not sorted and hence were only ~50% pure. For semiquantitative transcription analysis of γD lines, DN, DP, and SP thymocytes were sorted to >99% purity using anti-CD4 and anti-CD8 antibodies.

Nucleic Acid Preparation. Total RNA was prepared by the single step method using water-saturated phenol as described (29). 20 μg of tRNA was added as a carrier. Genomic DNA was prepared from defined numbers of cells as described (29). Lambda DNA (2.5 μg; New England Biolabs, Inc.) was added as carrier.

RNase Protection Assay. RNase protection assays (30) were performed with a riboprobe generated using T7 RNA polymerase and a linearized pKS Bluescript™ vector (Stratagene Inc.) construct containing the KpnI–BsrI fragment (273 bp) spanning the V–J junction of the G8 γ gene. The control riboprobe specific for γ actin mRNA (31) was generated using SP6 RNA polymerase. Densitometric analysis was performed using a PhosphorImager® (Molecular Dynamics).

Semiquantitative PCR. Serial threefold dilutions of DNA were prepared in the presence of 50 μg/ml bacteriophage lambda

DNA (New England Biolabs, Inc.), and PCR reactions were performed as described (11) with the L2 and J1 primers (32). The transgene contained an XhoI linker in the V2 coding sequence; thus, digestion of the products with XhoI distinguished the endogenous product from the transgene product. The sample dilutions were compared with a standard curve prepared with DNA from the DN2.3 hybridoma (33), which contains two Vγ2 rearranged genes and four tubulin genes. A β tubulin PCR was used to normalize the samples. The bands were visualized by autoradiography, and their intensities were measured on a PhosphorImager®. Reverse transcriptase (RT)-PCR was employed for a parallel analysis of transcript levels in the γD transgenic lines. The procedure was done as described using either oligo-dT or J1 primer for reverse transcription and the L2 and J1 primers (or tubulin primers) for PCR (32), with or without RT, except that 1 μCi α-[^{32}P]dCTP was added during the PCR amplification step, and 28 cycles of amplification were performed. The PCR products were digested and analyzed as described for the genomic PCR.

Antibodies and Flow Cytometry. Anti–Vγ2 TCR (UC3-10A6) and anti–δ TCR (GL3) were purified and conjugated with biotin and FITC, respectively. Anti-CD8α–Tricolor was purchased from Caltag Labs., and anti-CD4–Red 613 was from GIBCO BRL. Unseparated thymocytes from adult mice were stained with all four antibodies in the first step and streptavidin–PE (Molecular Probes, Inc.) in the second step. Gated TCR-γ/δ$^+$ CD4$^-$CD8$^-$ thymocytes were examined for Vγ2 expression on an Epics XL-MCL flow cytometer (Coulter Immunology).

Results

Recombination and Transcription Occur in the Absence of 3′E$_{Cγ1}$. Initially, we compared the in vivo activity of two γ transgene constructs consisting of Vγ2, 4, and 3 gene segments upstream of the Jγ1-Cγ1 genes, all in their germline configurations. The two constructs were identical except that one lacked a 2.8-kb 3′ fragment that contains 3′E$_{Cγ1}$ (Fig. 1 A shows a map of the germline Cγ1 locus). We found that several independent transgenic lines of each type consistently underwent rearrangement of Vγ2 to Jγ1 in thymocytes and that both constructs were efficiently transcribed (data not shown; see below for a similar analysis). These data indicated that 3′E$_{Cγ1}$ is not absolutely required for either transgene rearrangement or expression and raised the possibility that the constructs contained a second cis-acting enhancer-like element. A clue to the site of such an element came from a previous study of a transgene construct (19L5) containing a rearranged Vγ2–Jγ1Cγ1 gene lacking 3′E$_{Cγ1}$ that was not expressed in vivo (reference 20 and Raulet, D., unpublished data). Compared with the constructs above, 19L5 lacked a 1.5-kb segment of DNA upstream of Vγ2 on its 5′ end. These data raised the possibility that a relevant enhancer element might lie on this 1.5-kb DNA segment 5′ of Vγ2.

DNase I Hypersensitive Sites in the Cγ1 Locus. Examination of the region upstream of Vγ2 demonstrated a clear DNase I hypersensitive site, denoted HsA, in adult thymocytes (Fig. 1 B). HsA mapped to the region 3 kb upstream of Vγ2, corresponding to the 5′ region that was absent from the 19L5 construct compared with the rearrangement constructs described above. The site was not DNase hypersensitive in B lymphocytes (LPS blasts, 90% B cells) or liver cells (Fig. 1 B).

In a parallel analysis, the 3′E$_{Cγ1}$ region was weakly hypersensitive in normal adult thymocytes (data not shown). The DNase hypersensitivity of 3′E$_{Cγ1}$ (designated HsE) was more clearly demonstrated in a transgenic line with 15 copies of an integrated TCR-γ transgene, called γB, that consists of 40 kb of contiguous germline DNA from the γ locus (Fig. 1 C; reference 11). HsE was also hypersensitive to DNase I in B cells of the transgenic mice but was not hypersensitive in liver cells. Several other hypersensitive sites, most of them weak, were also detected in the transgene, but these have not been corroborated in nontransgenic cells (data not shown).

HsA and 3′E$_{Cγ1}$ Function to Enhance γ Gene Expression In Vivo. To systematically investigate the transcription-enhancing activities of HsA and 3′E$_{Cγ1}$ in vivo, we compared four transgene constructs containing a prerearranged Vγ2–

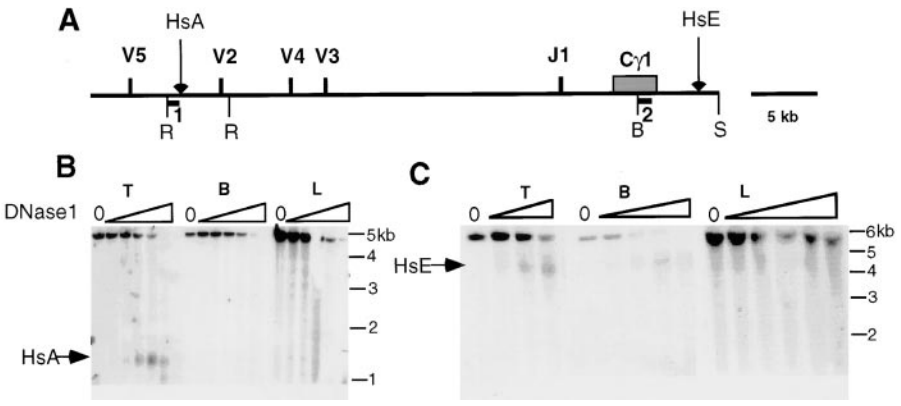

Figure 1. DNase I hypersensitive (Hs) sites in the TCR-γ locus. (A) Map of the γ locus. Restriction enzyme sites: R, EcoRI; B, BamHI; and S, SalI. Probes 1 and 2 employed for Southern analysis are indicated, as are the locations of HsA and HsE. (B) Assay for Hs sites upstream of Vγ2. DNA from nuclei treated with twofold increasing amounts of DNase I starting with 0.2 μg was digested with EcoRI. Southern blots were hybridized with probe 1. Unfractionated thymocytes (T), LPS B cell blasts (B), and liver cells (L) were compared. (C) Assay for Hs sites downstream of Cγ1. Assay was performed as for B except that the cells were from a mouse containing 15 copies of a contiguous γ locus transgene (γB), the DNA was digested with BamHI and SalI, and probe 2 was employed. Molecular weight markers (shown as kb) are indicated to the right of each blot.

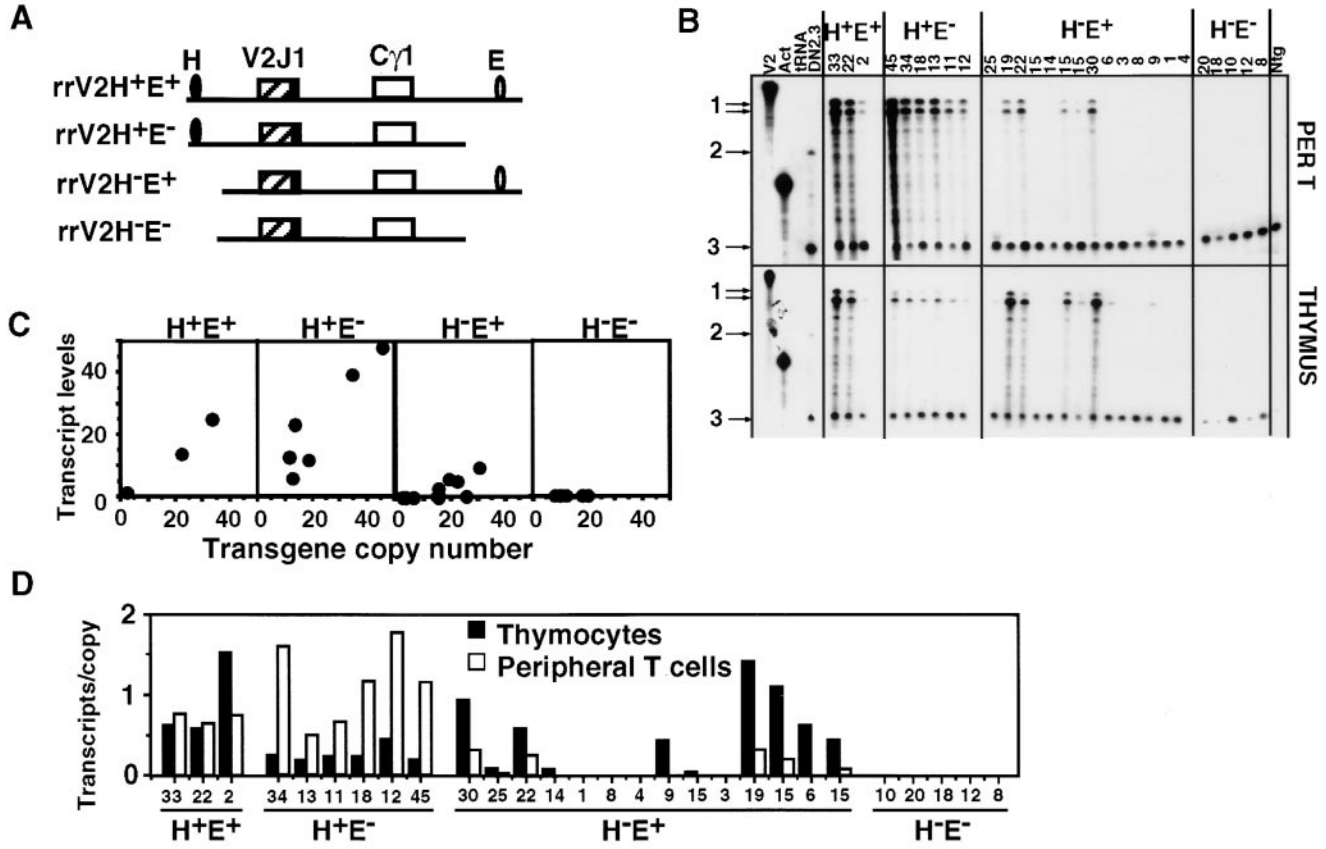

Figure 2. (A) Schematic depiction of rrV2 constructs. HsA (H) and $3'E_{C\gamma1}$ (E) are indicated. (B) RNase protection assay with RNA from peripheral T cells (top panel) and thymocytes (bottom panel) from each transgenic line. The transgene copy number of each line is indicated above each lane. Lanes containing undigested Vγ2 (V2) and actin (Act) probes, tRNA, and RNA from the DN2.3 cell line are indicated. The identities of the protected transcripts are as follows: 1, transgenic Vγ2; 2, endogenous Vγ2; 3, actin. Endogenous Vγ2 is not expressed in α/β lineage cells, which comprise the majority of peripheral T cells and thymocytes (see text). The double bands in the transgene Vγ2 probably represent spliced and unspliced transcript. Ntg, nontransgenic. (C) Relationship between normalized transcript levels in peripheral T cells and transgene copy numbers. One unit of transcript is defined as the level from one rearranged Vγ2 gene in the DN2.3 cell line. (D) The levels of transcript, corrected for transgene copy number, in thymocytes and peripheral T cells for each transgenic line. The transgene copy number for each line is shown below the bars.

Jγ1Cγ1 gene (Fig. 2 A). The $rrV2H^+E^+$ construct containing both HSA and $3'E_{C\gamma1}$ was previously described (25). The $rrV2H^+E^-$ construct contained HsA but lacked the 2.8-kb 3′ fragment containing $3'E_{C\gamma1}$, $rrV2H^-E^+$ contained $3'E_{C\gamma1}$ but lacked a 1.5-kb 5′ fragment containing HsA, and $rrV2H^-E^-$ lacked both the 5′ and 3′ fragments. Founders were either killed and analyzed directly or bred to generate transgenic lines. Transgene copy numbers were determined by Southern blot analysis. In the cases where founder mice were analyzed directly, we determined transgene copy number in the cells being examined to minimize the effects of the transgene mosaicism that sometimes occurs in founder animals.

A quantitative RNase protection assay was used to measure Vγ2 transcripts in RNA from peripheral T cells and thymocytes from the transgenic mice. The riboprobe spanned the unique V–J junctional region of the transgene, allowing the specific detection of transgene-encoded transcripts as full length protected products. γ Transcript levels were normalized by inclusion of a control γ actin probe in each reaction. The results demonstrated that the transgene was efficiently transcribed in peripheral T cells from all three $rrV2H^+E^+$ lines and from all six $rrV2H^+E^-$ lines (Fig. 2 B). Similarly, transgene transcription was detected in thymocytes from all of these transgenic lines (Fig. 2 B). Transcription was T cell–specific, as no transcripts could be detected in B cells or kidney cells from several representative transgenic lines (data not shown). In contrast to the transgenes containing HsA, transgene transcription could not be detected in either peripheral T cells or thymocytes of the five $rrV2H^-E^-$ transgenic lines. Thus, the fragment containing HsA consistently enhances T cell–specific γ gene expression in chromatin templates in the absence of $3'E_{C\gamma1}$.

In contrast to the consistent expression of transgenes containing HsA, sporadic expression was observed in the case of the $rrV2H^-E^+$ transgene, which contained $3'E_{C\gamma1}$ but not HsA. Of the 14 lines tested, expression was detected in 5 or 6 lines in peripheral T cells and in 7 or 8 lines in thymocytes (Fig. 2 B). No expression of the transgene was detected in B cells or kidney cells from several repre-

sentative transgenic lines (data not shown). Sporadic expression of the rrV2H$^-$E$^+$ transgene was dependent on the 3′ fragment containing 3′E$_{C\gamma1}$ because, as already discussed, the rrV2H$^-$E$^-$ transgene lacking this fragment was not expressed in five independent lines. Hence, 3′E$_{C\gamma1}$ can enhance transcription in chromatin templates but is subject to transgene position effects.

To allow quantitative comparisons, transgene expression levels in peripheral T cells were plotted against transgene copy number. One unit of transcripts was defined as the level of transcripts directed by an endogenous rearranged Vγ2 gene. This value was determined by parallel analysis of the DN2.3 γ/δ cell line, which contains two rearranged Vγ2 alleles (Fig. 2 B). In transgenic lines that contained HsA (rrV2H$^+$E$^+$ and rrV2H$^+$E$^-$), the graphs revealed a roughly proportional relationship between the number of integrated transgene copies and the levels of transgene expression (Fig. 2 C). Furthermore, the slope of the graphs was ~1, indicating that the level of transcripts per transgene copy was roughly the same as the level directed by an endogenous Vγ2 gene. Even an rrV2H$^+$E$^+$ transgenic line with only two transgene copies exhibited a similar level of transgene expression per copy as the endogenous gene (Fig. 2 D). In contrast, the rrV2H$^-$E$^+$ transgene, which lacked HsA but contained 3′E$_{C\gamma1}$, was transcribed at detectable levels in less than half of the lines (Fig. 2, C and D). The lines where transcripts were detectable were all high-copy lines. No transcripts were detected in the lines harboring the rrV2H$^-$E$^-$ construct. These results demonstrated that the transgenes that contained HsA exhibited position-independent, roughly copy number–dependent transcription of the transgene in peripheral T cells. In contrast, the transgene that contained 3′E$_{C\gamma1}$ but not HsA exhibited severe position effects.

Transgene Expression in γ/δ Cells. Transgene expression in γ/δ cells was investigated by determining transcript levels in sorted γ/δ cells in one transgenic line of each type and by assessing the effect of the transgene on the percentage of Vγ2$^+$ cells among thymic γ/δ cells in several lines. Abundant transgene transcripts were present in γ/δ T cells from the three lines examined, representing an rrV2H$^+$E$^+$ line, a high-expressing rrV2H$^-$E$^+$ line, and an rrV2H$^+$E$^-$ line (Fig. 3 A). This result was also confirmed by RT-PCR assay using purified peripheral γ/δ T cells (data not shown). Similar levels of transcript were found in sorted α/β T cells from the three lines. The expression of the transgene in α/β T cells is probably due to the absence from the transgene of a transcriptional silencer that inhibits expression of endogenous γ genes in α/β T cells (24, 34) (see Discussion).

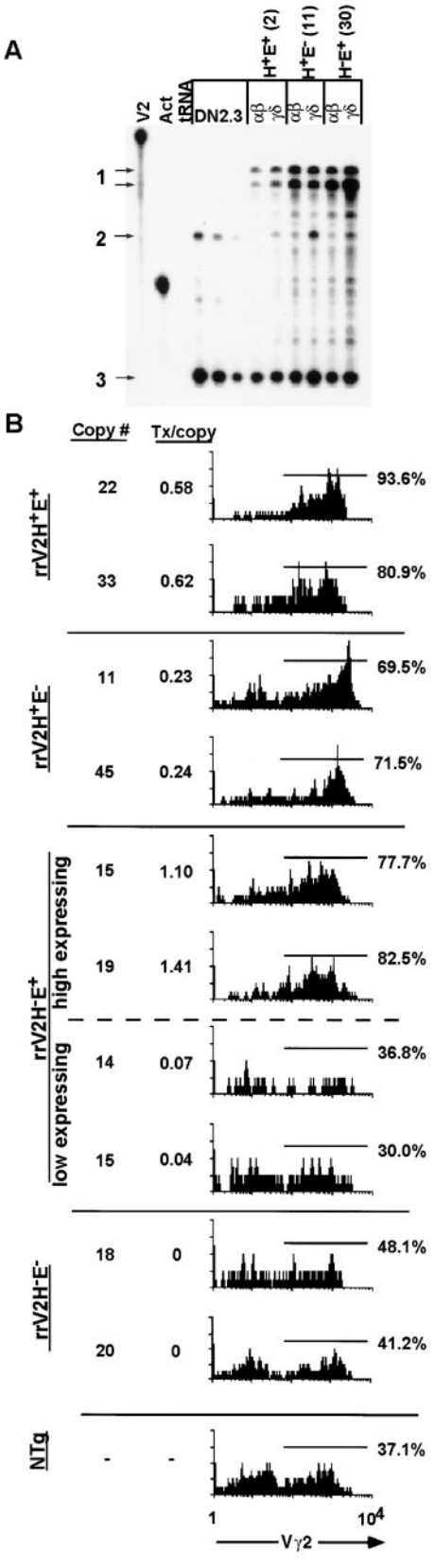

Figure 3. Expression of the transgenes in γ/δ cells. (A) Transgene expression in sorted α/β and γ/δ T cells from one transgenic line representing each construct. The transgene copy number is noted in parentheses. The assay employed three different concentrations of DN2.3 RNA. Identities of the protected bands are listed in the legend for Fig. 2 B. (B) Effect of the transgenes on the percentage of Vγ2$^+$ γ/δ cells in the thymus. Gated CD4$^-$CD8$^-$ TCR-γ/δ$^+$ adult thymocytes were examined for Vγ2 expression. The transgene copy numbers and RNA expression levels in the thymus are indicated next to each histogram. Vγ2 expression by nontransgenic thymic γ/δ T cells (enriched CD4$^-$CD8$^-$ cells gated on CD4$^-$CD8$^-$ TCR-γ/δ$^+$ cells) is shown for comparison.

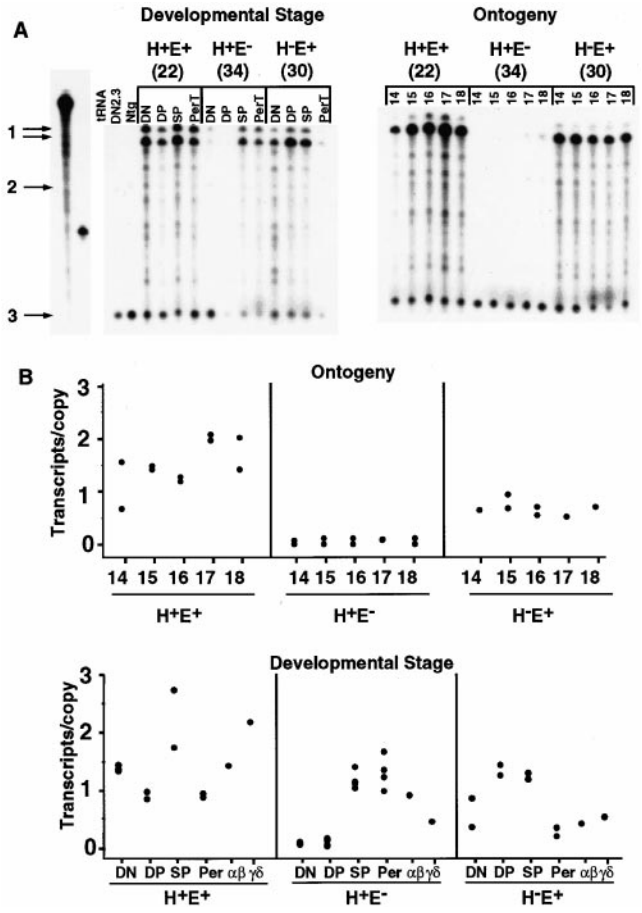

Figure 4. Developmental activities of HsA and $3'E_{C\gamma1}$. (A) RNase protection assay on RNA from fetal thymocytes collected at the indicated different days of ontogeny (right) and T cell populations representing various developmental stages (left). One line representing each construct is shown, with the transgene copy number noted in parentheses. The populations are: $CD4^-CD8^-$ thymocytes (DN); $CD4^+CD8^+$ thymocytes (DP); a mixture of $CD4^+CD8^-$ and $CD8^+CD4^-$ thymocytes (SP); and peripheral T cells (PerT). The remaining details of the figure are identical to those in Fig. 2 B. (B) The levels of transcript per transgene copy (same units as Fig. 2) are shown for the populations representing various stages of development and ontogeny. Each point represents an individual line. Varying numbers of lines were tested at each stage/population. Only one H^-E^+ line was tested at E14, 17, and 18, and the analyses of γ/δ versus α/β expression were only performed on one representative line of each type. Four H^+E^- lines were assayed for DN and DP populations.

Flow cytometry was employed to determine the percentage of $V\gamma2^+$ thymic γ/δ cells in transgenic lines of each type (Fig. 3 B). We chose lines that had the most similar transgene copy numbers to minimize the effect of gene dosage. In nontransgenic mice, ~35–50% of thymic γ/δ cells expressed Vγ2. The percentage was unaffected in two $rrV2H^-E^-$ lines (41–48%) but was elevated to 80–94% in the two $rrV2H^+E^+$ transgenic lines. The percentage was also elevated in two $rrV2H^+E^-$ lines (~70%) and two $rrV2H^-E^+$ lines that exhibited high levels of transgene transcripts in the thymus (~80%). In contrast, two $rrV2H^-E^+$ lines that were expressed poorly at the mRNA level also showed no enhancement in the percentage of $V\gamma2^+$ cells (30–37%). Thus, transgene expression at the mRNA level in bulk populations correlated with Vγ2 surface expression in γ/δ cells. Furthermore, the position effects exhibited by the $rrV2H^-E^+$ transgene in bulk populations were recapitulated in the analysis of γ/δ cells.

HsA and $3'E_{C\gamma1}$ Are Developmentally Regulated. Normalization of the transcript levels determined by RNase protection to transgene copy number demonstrated that the $rrV2H^+E^-$ transgene was expressed at higher levels in peripheral T cells than in thymocytes in all six transgenic lines, by an average of 4.5-fold (Fig. 2 D). In contrast, the $rrV2H^-E^+$ transgene was expressed at lower levels in peripheral T cells than in thymocytes in all the lines where expression could be detected, by an average of fivefold. In the $rrV2H^+E^+$ lines, the transcript levels in thymocytes were similar to the levels in peripheral T cells, with one low-copy line exhibiting marginally higher (twofold) expression in thymocytes. These data suggested that HsA and $3'E_{C\gamma1}$ are differentially regulated in peripheral T cells and thymocytes.

To clarify the developmental activity of the two elements, we examined representative lines for transgene expression during thymocyte ontogeny and in subsets of adult thymocytes (Fig. 4). The transgene with both elements, $rrV2H^+E^+$, was expressed well in fetal thymocyte populations from day 14–18 of gestation. Similarly, two $rrV2H^-E^+$ lines that exhibited transgene expression in adult thymocytes also exhibited substantial transgene expression in fetal thymocytes. In contrast, the $rrV2H^+E^-$ transgene was expressed very poorly in fetal thymocytes in both lines tested. We conclude that the HsA element displays poor enhancing activity in fetal thymocytes, whereas the $3'E_{C\gamma1}$ element, when not subject to position effects, evinces relatively strong activity in fetal thymocytes.

Transgene expression levels were also determined in adult immature $CD4^-CD8^-$ (DN) thymocytes, immature $CD4^+CD8^+$ (DP) thymocytes, and a mixture of the relatively mature SP $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (Fig. 4). The $rrV2H^+E^+$ transgene was expressed well in all of these cell populations. Consistent with the ontogeny data, all four $rrV2H^+E^-$ transgenics tested exhibited poor expression in immature DN and DP thymocytes but strong expression in SP thymocytes and peripheral T cells. In contrast, in two lines where the $rrV2H^-E^+$ transgene was expressed well in unseparated thymocytes, expression was relatively strong in the DN, DP, and SP populations but weak in peripheral T cells (Fig. 4). These results suggest that $3'E_{C\gamma1}$, when not subject to negative position effects, functions well as an enhancer in immature thymocytes. In contrast, HsA by itself does not enhance transcription in immature thymocytes. As expected, little or no transgene expression was observed in DN thymocytes from two $rrV2H^-E^+$ lines in which the transgene was expressed poorly in unseparated thymocytes and from the one $rrV2H^-E^-$ line tested (data not shown).

Role in Gene Rearrangement. Four new transgenic recombination substrates were prepared to examine the role of HsA and $3'E_{C\gamma1}$ in γ gene recombination (Fig. 5 A).

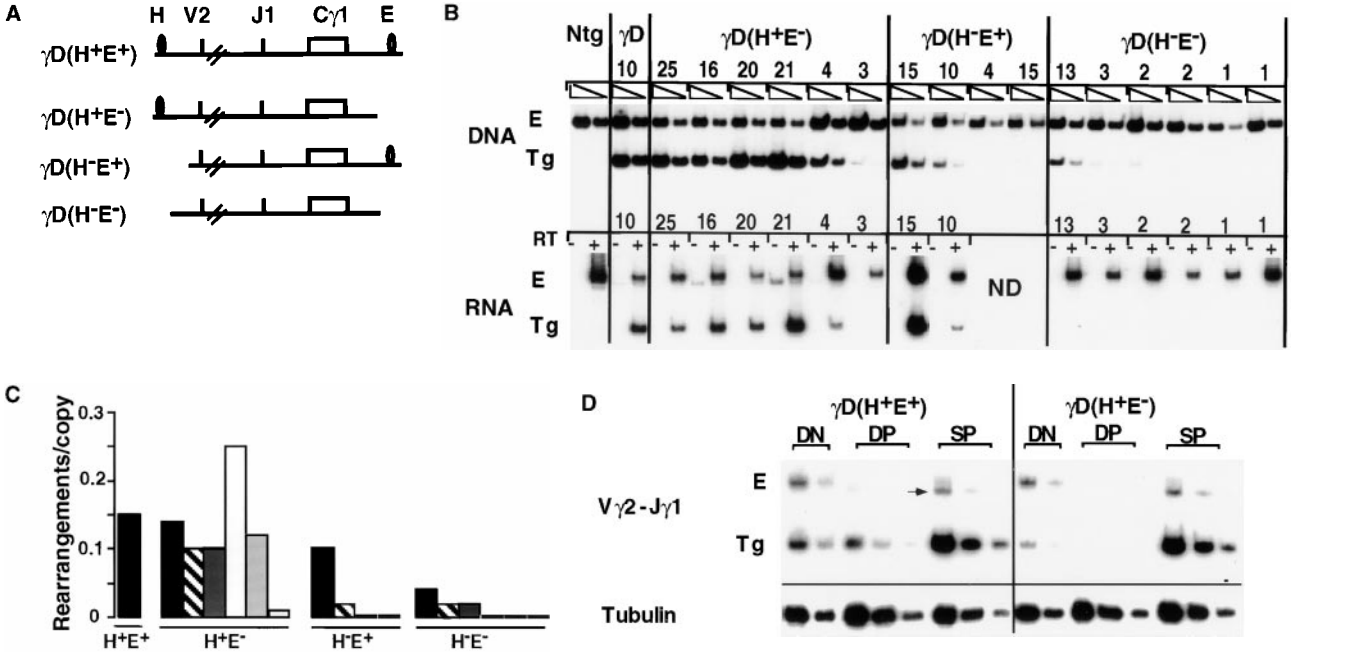

Figure 5. Role of HsA and $3'E_{C\gamma1}$ in γ gene recombination. (A) Schematic representation of the γD transgene recombination substrates. Hatch marks indicate the internal sites where some genomic DNA was omitted from the constructs. (B) Transgene rearrangement and transcription in enriched populations of DN thymocytes (~50% pure). Rearrangements of Vγ2 to Jγ1 were assayed by PCR with L2 and J1 primers on genomic DNA from the indicated transgenic lines. PCR products emanating from the transgene (Tg) or endogenous (E) genes were distinguished by digestion with XhoI, a site which had been inserted in the transgene Vγ2 gene. The transgene copy number is indicated above each lane. DNA from 5000 and 1667 cells was amplified for each line. Transcripts of the rearranged genes were assayed in parallel by RT-PCR. (C) Normalized rearrangement levels per transgene copy are shown. Each bar represents an individual line. The values are based on a more complete titration performed separately (see Materials and Methods). (D) Transgene transcription in sorted (99% pure) DN, DP, and SP thymocytes from γD(H^+E^+) and γD(H^+E^-) lines. A representative RT-PCR assay using Vγ2 rearrangement–specific PCR primers with tubulin PCR as a loading control is shown. PCR was performed on samples serially diluted threefold. Only two lower-sample concentrations are shown for DN populations. Transcripts derived from the transgene and endogenous genes were distinguished as in B. Endogenous Vγ2 genes are not expressed in DP and SP thymocytes. Lower bands below the endogenous transcript (arrow) represent XhoI-digested genomic transgene DNA contaminant. No significant signals were observed in PCR without RT step in each sorted population.

The γD construct consisted of a 5-kb genomic fragment containing HsA and Vγ2 attached to a 15.5-kb genomic fragment containing Jγ1, Cγ1, and $3'E_{C\gamma1}$; γD(H^+E^-) was identical to γD except it lacked 2.6 kb of DNA containing $3'E_{C\gamma1}$; γD(H^-E^+) contained the $3'E_{C\gamma1}$ fragment but lacked 3.5 kb of DNA containing HsA; and γD(H^-E^-) lacked the $3'E_{C\gamma1}$ fragment as well as a 1.5-kb fragment of DNA encompassing HsA. In all of the transgenes, the Vγ2 gene contained a frameshift mutation to prevent expression of a functional protein. Rearrangement and expression of the transgenes was determined by semiquantitative PCR or RT-PCR, respectively, in thymocyte populations that had been enriched in DN cells (~50% DN thymocytes).

Vγ2–Jγ1 transgene rearrangements were easily detected in the single γD transgenic line that was examined (Fig. 5, B and C). For comparative purposes, a separate analysis showed that the level of transgene rearrangement was one half to one third that of endogenous Vγ2 gene rearrangement levels after normalizing for gene copy. Approximately similar levels of rearrangement were detected in five of the six γD(H^+E^-) transgenic lines, which lacked $3'E_{C\gamma1}$; one line exhibited low levels of rearrangement. These findings corroborated the initial data in which $3'E_{C\gamma1}$ was not necessary to support γ gene rearrangement in transgenic substrates. The role of HsA in stimulating recombination was suggested by the results with the γD(H^-E^-) transgene, which was identical to γD(H^+E^-) except that it lacked the HsA fragment. Rearrangement was undetectable in three of these transgenic lines and reduced by a factor of three to five in the remaining three lines. Hence, although low levels of rearrangement occurred in some transgenic lines in the absence of both HsA and $3'E_{C\gamma1}$, the fragment containing HsA stimulated high levels of rearrangement.

Rearrangement of the γD(H^-E^+) transgene was approximately normal in one transgenic line, undetectable in two lines, and reduced severalfold in a fourth line (Fig. 5, B and C). This pattern of rearrangement, indicating clear position effects, cannot be clearly distinguished from the pattern observed in the γD(H^-E^-) lines. Therefore, it is unclear from these data whether the $3'E_{C\gamma1}$ element plays a discrete role in stimulating γ gene rearrangement (see Discussion).

Transcripts of the rearranged genes were detected by RT-PCR in each of the γD, γD(H^+E^-), and γD(H^-E^+) lines where recombination was detected (Fig. 5 B), and the relative levels were roughly correlated with the extent of rearrangement. In contrast, no such transcripts were de-

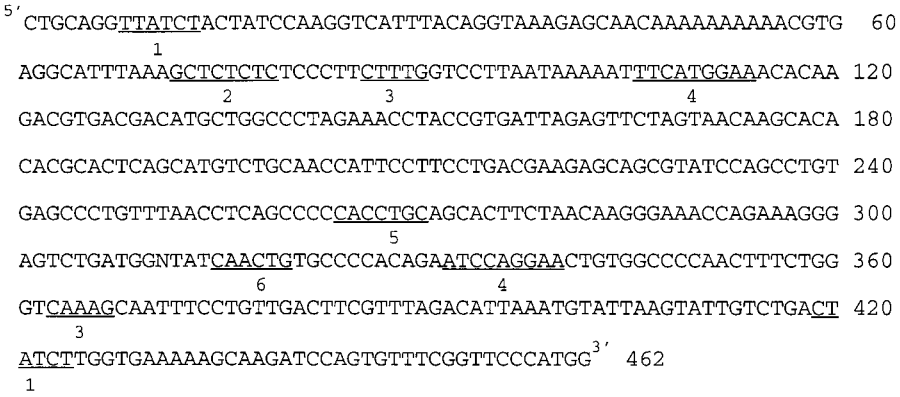

Figure 6. Sequence of the PstI/NcoI fragment containing HsA. Consensus transcription factor binding sites are underlined and are numbered as follows: 1, gata 3; 2, gaga; 3, lef/tcf; 4, stat; 5, ebox; 6, myb.

tected in the γD(H^-E^-) lines that exhibited low levels of rearrangement, supporting the earlier conclusion that transcription of the rearranged genes requires HsA and/or $3'E_{C\gamma1}$. To confirm that the developmental pattern of transgene transcription in the γD lines paralleled that of the rrV2 lines, sorted DN, DP, and SP thymocytes from γD(H^+E^+) and γD(H^+E^-) lines were assayed for transcripts of the rearranged transgene by semiquantitative RT-PCR (Fig. 5 D). The results demonstrate that transgene expression was high in each population from the γD(H^+E^+) line but was lower in DNs, undetectable in DPs, and high in SPs from the γD(H^+E^-) line, consistent with the results from the rrV2 lines. The weak signal in the DNs of the γD(H^+E^-) line is likely derived from the 5–10% of γ/δ T cells present in this population, as the transgene transcripts were hardly detectable in the $CD3^-CD4^-CD8^-$ population of this line (data not shown).

Sequence of HsA. With the use of multiple restriction enzyme digests, we localized the DNase I hypersensitive site associated with HsA to a 462-bp PstI–NcoI fragment (data not shown). Although we have not proven that this small fragment contains the functional site defined by the transgenic studies, other studies have shown a colocalization of cis-acting functional sites and DNase I hypersensitive sites (35). The sequence of this fragment revealed several consensus sites for known transcription factors, including sites for ebox proteins, myb, gata 3, lef/tcf, stat, and gaga factors (Fig. 6).

Discussion

In Vivo Enhancer Activity of $3'E_{C\gamma1}$. The data indicate that $3'E_{C\gamma1}$ functions as an enhancer in vivo. In terms of enhancing transcription, $3'E_{C\gamma1}$ seems to play a more important role than HsA in immature thymocytes. DNase I hypersensitivity of $3'E_{C\gamma1}$ in thymocytes was easily detected in transgene templates. The hypersensitivity in B cells may be due to the absence from the transgene of "silencer elements" present in the endogenous locus, although we emphasize that the transgenes were not expressed in B cells. The endogenous $3'E_{C\gamma1}$ site was clearly hypersensitive in a dendritic epidermal γ/δ T cell line (Goldman, J., and D.H. Raulet, unpublished data) but was difficult to detect in thymocytes, perhaps because endogenous γ gene expression is silenced in most thymocytes. Overall, the $3'E_{C\gamma1}$ element has the properties of a typical non-LCR enhancer element in that it is active in transient transfection assays, exhibits DNase I hypersensitivity, and enhances transcription in vivo but is subject to transgene position effects.

HsA Functions as an Enhancer In Vivo. The HsA element is a T cell–specific enhancer-like element that promotes transcription of rearranged γ genes in mature T cells. In addition, HsA stimulates recombination of transgenic γ rearrangement substrates. HsA was hypersensitive to DNase I in thymocytes but not in B cells or liver cells.

Although HsA exhibited clear enhancer activity in mature T cells when integrated as a multicopy transgene, it was devoid of enhancer activity in transient transfection assays in the PEER and Jurkat cell lines, in which $3'E_{C\gamma1}$ was active (data not shown). Both of these cell lines are unlikely to represent immature cells where HsA is nonfunctional, because the Jurkat line, at least, appears to be relatively mature based on its capacity to produce cytokines after TCR cross-linking. It is possible that HsA only functions with a homologous (γ gene) promoter element or only in the context of chromatin. Other instances have been reported where an element enhanced transcription when integrated in chromatin but not in transient transfection assays (18, 36, 37).

HsA in Combination with $3'E_{C\gamma1}$ Has Properties of an LCR. HsA, when combined with $3'E_{C\gamma1}$ as in the rrV2H^+E^+ transgene, confers efficient transgene expression in cells that normally express γ genes, including DN thymocytes and purified γ/δ cells, but does not drive expression in non-T cells. The enhanced percentage of $V\gamma2^+$ thymic γ/δ cells with the various lines of transgenic mice provides further evidence that the transgenes are indeed expressed in γ/δ cells. Significantly, expression of the rrV2H^+E^+ transgene was independent of transgene position effects, and the level of transgene expression was proportional to the number of transgene copies. Thus, the combination of HsA and $3'E_{C\gamma1}$ exhibits several characteristics of LCRs. We have not demonstrated that these elements are effective in single transgene copies, as none of the relevant lines contained just a single copy. However, an rrV2H^+E^+ line with two transgene copies exhibited high levels of transgene expression.

Although the rrV2H$^+$E$^+$ transgene was regulated appropriately in most respects, it was inappropriately expressed in α/β lineage T cells, unlike endogenous γ genes of this type. Previous studies provided evidence that the absence of expression of endogenous γ genes in α/β lineage cells is due to an associated "silencer" element (24). Although the silencer has not been subsequently defined or localized in detail, we have recently shown that transgenes containing an additional 10 kb of flanking 3′ DNA compared with the rrV2H$^+$E$^+$ transgene, when present at low copy number, are strongly downregulated in α/β but not γ/δ T cells (reference 34 and Kang, J., and D.H. Raulet, unpublished data). These data are consistent with the conclusion that transgene expression in α/β T cells observed here is due to a lack of cell type–specific repressive elements.

Transgenes containing only HsA or 3′E$_{Cγ1}$ were clearly expressed inappropriately. The transgene containing 3′E$_{Cγ1}$ but not HsA exhibited severe position effects and was not expressed in a copy number–dependent fashion, suggesting that 3′E$_{Cγ1}$ by itself is insufficient to open the chromatin. The transgene containing HsA but not 3′E$_{Cγ1}$ was poorly expressed in some cells in which endogenous γ genes are expressed well, such as DN thymocytes and fetal thymocytes. Nevertheless, HsA by itself did stimulate transcription in peripheral T cells in every line tested and was expressed in a roughly copy number–dependent fashion in peripheral T cells. The effects of HsA suggest that it may isolate linked genes from the inhibitory effects of neighboring chromatin. The putative chromatin-opening activity of HsA is probably operative even in cells where HsA alone functioned poorly as an enhancer. In total or DN thymocytes, HsA without 3′E$_{Cγ1}$ was expressed poorly, and 3′E$_{Cγ1}$ without HsA was subject to position effects; together, the elements supported high-level position-independent expression in both populations (Figs. 2 and 4). Thus, HsA may relieve position effects in immature thymocytes, cooperating with 3′E$_{Cγ1}$ to yield maximal levels of expression. Consistent with the role of HsA as a chromatin-opening element, we found that the 3′E$_{Cγ1}$ site was not DNase I hypersensitive in an rrV2H$^-$E$^+$ transgenic line that expressed the transgene poorly but was hypersensitive in an rrV2H$^+$E$^+$ transgenic line (data not shown).

Other LCRs have been shown to involve cooperative elements that enhance transcription and exhibit chromatin-opening activity (12, 13, 16, 17, 37–40). Hence, chromatin-opening elements may be at least partially separable from classical enhancers in several LCRs, including the TCR-γ LCR.

Enhancer Activities of HsA and 3′E$_{Cγ1}$ Are Differentially Developmentally Regulated. In the absence of 3′E$_{Cγ1}$, HsA drove transcription in mature SP thymocytes and peripheral T cells but not immature thymocytes. This was true of all four lines tested. In contrast, 3′E$_{Cγ1}$ by itself, when not subject to position effects, drove expression in DN, DP, and SP thymocytes but did so less well in peripheral T cells. Correspondingly, 3′E$_{Cγ1}$ functioned relatively well in fetal thymocytes. It will be of considerable interest to address the developmental roles of these two elements in vivo, where different sets of Vγ genes are used in the fetal and adult stages. Other instances have been reported of lymphocyte receptor genes with multiple, developmentally regulated enhancer elements. For example, both the CD4 and CD8 loci contain elements that seem to function differently in mature versus immature T cells (41–47).

Roles of HsA and 3′E$_{Cγ1}$ in V(D)J Recombination. In addition to promoting transcription of γ genes, our results with the γD series of recombination substrates indicate a role for HsA in supporting rearrangement of γ genes, even in the absence of 3′E$_{Cγ1}$. These results are of interest given the fact that HsA is a poor enhancer in immature thymocytes, the population in which rearrangement presumably takes place. It will be of interest to assess in future studies whether rearrangement promoted by HsA in the absence of 3′E$_{Cγ1}$ primarily involves the chromatin-opening activity of HsA or is associated with prior transcription of unrearranged Vγ genes. For technical reasons, we have been unable to address whether such germline transcription occurs from the transgenes.

The γD(H$^-$E$^-$) transgene underwent weak and sporadic rearrangement, as did the γD(H$^-$E$^+$) transgene. These data alone were therefore insufficient to assess the role of 3′E$_{Cγ1}$ in stimulating γ gene rearrangement. The finding that transgene rearrangement occurs to a limited extent in some lines lacking both elements is surprising, as enhancer elements are usually required for V(D)J recombination. It is possible that the transgene integrated into especially open chromatin in these lines. Alternatively, it remains possible that elements in the transgenes other than 3′E$_{Cγ1}$ and HsA participate in stimulating γ gene rearrangement. However, it is notable that no transcription of the rearranged transgenes was detected, confirming the importance of 3′E$_{Cγ1}$ and HsA in transcription.

The Unique Location of HsA. All of the enhancer elements identified to date in antigen receptor loci are located either downstream of the constant regions or within the J–C introns. The location of HsA between Vγ2 and Vγ5 is therefore a novel scenario for antigen receptor genes. A ramification of the inter-V region location of HsA is that rearrangements of Vγ5 to Jγ1 will delete the element. Therefore, HsA must be unnecessary for supporting transcription of rearranged Vγ5 genes. One possibility is that HsA is only necessary to initially open the chromatin surrounding the Vγ genes and that subsequent maintenance of an open configuration is controlled by other elements and/or factors. Alternatively, there may exist additional elements upstream of the Vγ5 gene that support chromatin opening in the relevant cells. As the Vγ5 gene is unusual in that it is believed to undergo rearrangement preferentially in intestinal epithelial lymphocytes rather than thymocytes (48), it would not be surprising if the gene was regulated differently than the other Vγ genes. Finally, it is possible that the endogenous γ locus is sufficiently open in the absence of HsA for at least some cells to efficiently transcribe γ genes. It will be of interest to explore these possibilities by deleting HsA and/or 3′E$_{Cγ1}$ at their endogenous locations.

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