Identification of a DNA Segment Exhibiting Rearrangement Modifying Effects upon Transgenic δ-deleting Elements

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Summary

Control of the rearrangement and expression of the T cell receptor α and δ chains is critical for determining T cell type. The process of δ deletion is a candidate mechanism for maintaining separation of the α and δ loci. Mice harboring a transgenic reporter δ deletion construct show α/β T cell lineage–specific use of the transgenic elements. A 48-basepair segment of DNA, termed HPS1A, when deleted from this reporter construct, loses tight lineage-specific rearrangement control of transgenic elements, with abundant rearrangements of transgenic δ-deleting elements now in γ/δ T cells. Furthermore, HPS1A augments recombination frequency of extrachromosomal substrates in an in vitro recombination assay. DNA binding proteins recognizing HPS1A have been identified and are restricted to early B and T cells, during the time of active rearrangement of endogenous TCR and immunoglobulin loci. These data are consistent with δ deletion playing an important role in maintaining separate TCR α and δ loci.

Two types of mature T cells possessing different tissue distributions and apparent functions have been described (1–9). These T cells have TCR α and β chains or TCR γ and δ chains, associated with T3. Molecular cloning and sequencing of all four TCR chains led to the discovery of the unique localization of the TCR δ chain on chromosome 14 in both mice and humans (10–16). The surprising feature of this locus is that its internal coding segments, D, J, and Cδ, are contained within the much larger TCR α chain locus. This unusual feature among rearranging loci in the immunoglobulin supergene family leads to the conundrum of how to regulate the recombination of the α and δ chain gene segments. In spite of the proximity of the two receptors, cross-utilization of segments between the receptors infrequently occurs (17, 18). These two receptor chains are expressed in a mutually exclusive manner in different subsets of T cells, suggesting that separate alleles do not behave independently. Furthermore, in γ/δ T cells, TCR α chain recombinations would delete the functional δ chain and could not be allowed. These results imply a mechanism to ensure that in γ/δ T cells, α gene rearrangement is inhibited and in α/β T cells, internal δ gene segments are not used.

During the cloning and sequencing of the human TCR δ chain, a novel rearrangement in early thymocytes was described that is a candidate mechanism for discrimination between using the TCR α versus TCR δ chains. This δ deletion rearrangement step occurs in immature thymocytes destined to become α/β-bearing T cells. An upstream δ-deleting element, δREC, preferentially recombines with a downstream δ-deleting element, ψJα, at very high frequency in polyclonal human thymus (13, 19), and to a lesser degree in murine thymus (20). This recombination deletes all internal δ coding segments, and occurs with high frequency on both alleles in mature α/β T cells, suggesting that the recombination would lead to subsequent rearrangements in the remaining TCR α chain locus.

A model was proposed in which δ deletion is a preliminary step in the formation of TCR α chains (19, 21). Use of the δ deleting elements would be an intermediary step in the implementation of a signal to rearrange and express TCR α chains, thus becoming an α/β-bearing T cell. This model does not dictate which receptor rearranges first, α or δ, nor in which order they rearrange. It only requires that deletion, when it occurs, occurs before Vα–Jα joining. The model would also necessitate that δ-deleting element recombinations would be restricted to the α/β T cell lineage, for obvious reasons.

Because δREC was frequently deleted on both alleles in mature α/β T cells, (13, 19, 20) assessing mature T cells for the occurrence of δ deletion was unproductive. Therefore, a δ deletion transgenic reporter construct was designed to molecularly "tag" mature α/β and γ/δ T cells for the occurrence of δ deletion (21). The construct contained the 5′ and 3′ δ-deleting elements, as well as some intervening TCR δ chain J and C segments. Since the transgene was independent of the endogenous α/δ locus, the status of transgenic δ-deleting elements could be assessed in α/β and γ/δ T cells. Analysis of mature transgenic α/β T cells demonstrated a high degree of rearrangement within this

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transgenic reporter construct, whereas mature transgenic γ/δ T cells were essentially devoid of δ-deleting element rearrangements (21). This lineage-specific use of δ-deleting elements suggested that the machinery responsible for δ deletion is present in developing α/β T cells and is absent from the γ/δ T cell population, supporting the model for δ deletion in the formation of α/β T cells. Furthermore, the transgenic reporter approach allows the manipulation of discrete DNA segments in the hope of identifying molecular mechanisms controlling the use of transgenic elements.

In this report, we extend the observations with these transgenic molecular tags, and identify a DNA binding complex and DNA recognition motif that are candidates for controlling the lineage-specific use of the δ-deleting elements.

Materials and Methods

PCR. Nonquantitative PCR was carried out essentially as described (22) using 0.5 μg of genomic DNA per reaction tube. The primers for all PCR reactions are listed in reference 21. Parameters for amplification of human δREC–Jα_x were 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s for 30 cycles. Internal human δREC probe was obtained by PCR amplification of cloned plasmid DNA with primers internal to those used for detecting rearrangements of transgenic human δREC. PCR product blots, representing 20% of reaction volume, were electrophoresed in 1% agarose gels, transferred, and hybridized as described above for high molecular weight DNA. Quantitative PCR for δREC–Jα_x rearrangements using competitor HδREC1 was as described (21).

Transgenic Mouse Construction. The human transgenic reporter construct (TG)2 was identical to TG1 as described (21), except for a 50-bp deletion within the 1.9-kb SalI to XhoI fragment, which was subcloned into pBluescript (Stratagene Corp., La Jolla, CA) and an oligonucleotide spanning this region, but that did not contain the 48-bp potential signal sequence region HPS1A, which was used to make the deletion by the oligonucleotide-directed site-specific mutagenesis method of Kunkel (23). A sequence confirmed deletion mutant was isolated, and the 1.85-kb SalI–Xho I HPS1A minus fragment was subcloned into the base transgene construct used to make TG1. PCR was used to confirm that isolated transgenic constructs contained the 48-bp deletion (data not shown). Transgenic founder lines were created by injecting construct DNA into day 1 fertilized embryos by the University of Alabama at Birmingham (Birmingham, AL) transgenic mouse facility.

Isolation and Expansion of γ/δ T Cells. γ/δ T cells were isolated from the thymus and/or spleen of four animals at a time. Single cell suspensions of thymocytes or splenocytes in DMEM were isolated on 100 × 15-mm petri dishes (Fisher Scientific, Pittsburgh, PA) that had been coated with the anti-δ TCR antibody (403A10; supplied by Osami Kanagawa, Washington University, St. Louis, MO). Plates were washed to remove remaining α/β T cells and immature T cells. DMEM with 10% fetal calf serum and 50 IU/ml *r*IL-2 was added, and the plates were incubated for 5–7 d. At this time, the cells were no longer adherent to the plates. Cells were collected, nuclei prepared (see extracting DNA for PCR below), 2 mg proteinase K was added, and the solution incubated at 52°C overnight. Extraction, precipitation, and analysis was as described (24).

Extracting DNA for PCR. Single cell suspensions from thymus or spleen were spun in eppendorf tubes at 1–2,000 *g*. Nuclei were prepared by 1% triton lysis, centrifuged, and solubilized in 1% SDS. DNA was cleaned by overnight digestion with proteinase K and phenol extraction. Liver DNA was prepared as described (22).

Nuclear Extract Preparation. Nuclear extracts were prepared essentially as described (25). For human thymus, a single cell suspension of thymocytes was made with a Dounce homogenizer in nuclear extract buffer I (15 mM Hepes, pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 mM $Na_2S_2O_5$, and 2 mM PMSF). After removing large debris and centrifugation, cells were resuspended in 20 ml buffer I. Nuclei were prepared by passing cells through a 27-guage needle five times with high velocity. The nuclei were centrifuged and resuspended in 10–20 ml buffer II (15 mM Hepes, pH 7.6, 115 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM $Na_2S_20_5$, and 2 mM PMSF). A 10% volume of 4M $(NH_4)_2SO_4$ was added to solubilize the nuclei, and the mixture rocked gently at 4°C for 30 min. DNA was spun out at 30,000 *g* for 1 h at 4°C. For each milliliter of the supernatant, 0.3 g solid $(NH_4)_2SO_4$ was added and the solution rocked gently for 15 min at 4°C. The precipitate was centrifuged at 10,000 *g* for 20 min and resuspended in 3–5 ml of buffer III (25 mM Hepes, pH 7.6, 50 mM KC1, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol). The extract was dialyzed against buffer III and stored at −80°C.

Cell Lines. Cell lines used for extract preparation were pre–T cells RPMI 8402 (26; gift of Stan Korsmeyer, Washington University, St. Louis, MO), CCRF-CEM (No. CCL 119; American Type Culture Collection, Rockville, MD), and 2052 (27; gift of Michael Lieber, Washington University, St. Louis, MO); pre–B cell line Nalm-6 (28; gift of Stan Korsmeyer); mature B cell line SU-DHL-6 (29; gift of Stan Korsmeyer); HELA cells (No. CCL2; American Type Culture Collection); and the erythroid leukemia line MEL (gift of Tim Ley, Washington University, St. Louis, MO). Mature B cells and T cells (unseparated tissues) were isolated as single cell suspensions from BALB/c mice of 3–6 mo of age. Line 38B9 (27) for in vitro recombination assays was a gift of John Kearney (University of Alabama at Birmingham, Birmingham, AL).

Mobility Shift Assay. The mobility shift assay used was a modification of Heberlein and Tjian (25). In brief, the desired probe was end labeled with ^{32}P, ethanol precipitated, washed extensively to remove unincorporated nucleotides, and counted. Reactions are performed in 1.5-ml eppendorf tubes by adding 10,000–50,000 cpm of probe (1–2 ng DNA) in 1–2 μl, 1.5 μl of 10 × gel shift buffer (40% glycerol, 10 mM EDTA, pH 8.0, 100 mM 2-mercaptoethanol, 100 mM Tris, pH 8.0, 500 mM NaCl), 2.5 μl 200 ng/μl poly dI.dC, x μl extract, and 10-x μl H_2O in a total volume of 15 μl. The reaction mixture was allowed to sit at room temperature for 30 min. A 4% nondenaturing acrylamide gel was used for fractionation of products in a running buffer of 1× TBE. Gels were run for 1–2 h at 100–150 volts. After electrophoresis, gels were transferred to Whatman 3-MM paper and dried under vacuum. Autoradiography was performed for 6–12 h.

In Vitro Recombination Assay. Plasmids pLH103 and pLH104 were created by replacement subcloning the heptamer-spacer-nonamer (h-s-n)[1] of δREC into the BamHI site of pJH299 (30). Oligonucleotides flanking the h-s-n of δREC with BamHI-clonable ends were used to generate BamHI-clonable DNA segments by PCR from cloned δREC DNA. For pLH103, the 5′ oligonucleotide was positioned to also include HPS1A in the PCR fragment;

[1]*Abbreviations used in this paper:* AMP, ampicillin; CAM, chloramphenicol; DTT, dithiothreitol; h-s-n, heptamer-spacer-nonamer; TG, transgenic reporter construct.

for pLH104, the 5′ primer was between HPS1A and the h-s-n. Subcloning each PCR product into the BamHI site of pJH299 resulted in a swap of recombination signals with 23-bp spacers, and like pJH299, pLH vectors rearrange by inversion. Each newly generated plasmid was confirmed by sequencing. The in vitro recombination system of Hesse et al. (27, 31) was performed essentially as described. In brief, 150 ng of plasmid was introduced into 2052 or 38B9 by osmotic transfection using dextran sulfate for 5 min at 37°C. Transfected cells were washed and then allowed to grow for 48 h in DMEM with 10% FCS for 2052 or RPMI with 20% FCS for 38B9, and then the plasmid DNA was harvested by alkaline lysis as described (32) except that the precipitation step after addition of acetate solution III was extended to 60 min. Before transforming into bacteria for selection, the plasmids were digested with DpnI to ensure that only the plasmids that actually replicated within the mammalian cells were tabulated (27). The DpnI digested plasmids were electroporated into DH5α cells using a manipulator (BTX Electrocell Manipulator 600; BTX, San Diego, CA) at the following settings: T = 2.5 kV/resistance high voltage, R5 (129 ohm), S = 1.3 − 1.5 kV, C not used in this mode, cuvette gap was 1 mm. 1–10% of the transformation mixture was plated onto 100 μg/ml ampicillin (AMP) LB plates, and the remainder of the transfection mixture was plated onto 100 μg/ml ampicillin + 11 μg/ml chloramphenicol (AMP–CAM) LB plates. Colonies were counted after 24 h from the AMP plates and 48 h from the AMP–CAM plates. Rearrangement frequencies were tabulated by comparing the number of replicated, unrearranged plasmids (AMP plates) to replicated, rearranged plasmids (AMP–CAM plates). To confirm that the colonies appearing on the AMP–CAM plates were true recombinants, 51 of pLH104 and 69 of pLH103 AMP–CAM–resistant colonies were sequenced. 88% of pLH104 AMP–CAM colonies and 74% of pLH103 AMP–CAM colonies were found to be true rearrangements. The AMP–CAM data in Table 4 has been adjusted by, and the statistical analysis reflects, the estimated number of recombinants based upon the sequencing results. Based upon the estimated frequency of rearrangements of 88 and 74%, respectively, and the number of rearrangements sequenced, we have 90% confidence of <10% error in the estimated rearrangement frequency.

Statistical Analysis. To assess the effect of cell type and plasmid type on rearrangement rate, logistic regression was used. The appropriateness of the model is established by likelihood ratio test ($P = 10^{-32}$) and that both plasmid type and cell type are significant factors in the model ($P = 8 \times 10^{-29}$ and $P = 3 \times 10^{-5}$, respectively). From this model it was found that plasmid pLH103 is 2.90 times more likely to rearrange than plasmid pLH104 (95% C-I for odds ratio 2.40–3.49) and B cells are 1.47 times more likely to rearrange than T cells (95% C-I for odds ratio 1.23–1.76). Statistical analysis of Table 3 for TGl and TG2 T cells populations with sample sizes of two and three, respectively, is not appropriate.

Results

Identification of a DNA-binding Protein Recognizing HPS1A. One possible mechanism for regulating lineage-specific use of the δ-deleting elements would be control of the process by sequence-specific DNA binding proteins. Any such protein(s) would be responsible for recognizing DNA elements near δREC, and, by DNA–protein or protein–protein interactions, regulate the use of δREC. Evidence for the role of any such protein(s) in δ deletion would be confirmed by constructing a second transgene identical to TG1 (21), but lacking the DNA recognition sequence.

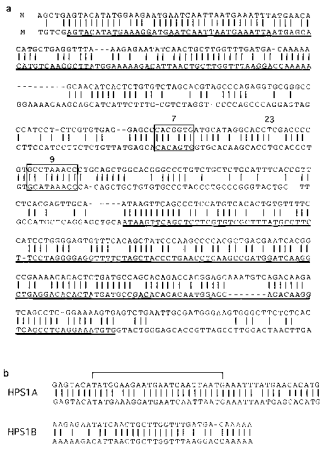

Figure 1. Sequence of human and murine δREC. (*a*) Comparison of human δREC and murine δREC1. Human sequence is shown on the top line, murine sequence on the bottom. Vertical lines represent identity between the two sequences; dashes represent gaps in the sequence placed by the analysis software for better fit. The canonical h-s-n recombinase recognition motif is boxed and labeled. The dark underlines outline the two areas of ~80% identity between human and mouse, HPS1 and HPS2, respectively. (*b*) HPS1 has been broken into the two areas HPS1A and HPS1B. Human sequence is on the top, murine sequence on the bottom. The bracket outlines the area of HPS1A that is protected during DNAse I footprinting (footprint not shown). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Y13607 and Y13608.

A candidate region for controlling lineage specificity in TG1 was discovered by first comparing sequences between human and murine δREC. The rationale was to search for areas of high DNA homology between δRECs, and then look for DNA binding proteins in the developing T cells of the thymus. Two areas of very high sequence homology, outside of the h-s-n canonical recombination motif, were noted 5′ and 3′ to the recombination signal (Fig. 1 *a*; 20). Nuclear extracts from murine thymus were used as probes to determine reactivity by mobility shift assay to both regions. DNA binding proteins were found in murine thymus to a 97-bp upstream region, designated HPS1, that is not detected in mature T cells of the spleen, nor in a HeLa cell nuclear extract (Fig. 2 *a*, *arrow*).

The 97-bp segment, HPS1, is underlined in Fig. 1 *a*. Two areas of near identity are seen within this region of DNA, HPS1A and HPS1B (Fig. 1 *b*). Each segment, 48 bp for HPS1A and 36 bp for HPS1B, was used as a probe for mobility shift assay and as cold competitor during a mobility shift assay with the whole 97-bp segment as probe. In Fig. 2 *c*, the 48-bp segment recognizes a similar complex in thymic extracts, as did the 97-bp segment. The 36-bp segment failed to detect any specific DNA binding proteins (data not shown). In addition, Fig. 2 *d* demonstrates that the reactivity in this 97-bp stretch of DNA has been localized to the 48 most 5′ bases of HPS1, termed HPS1A, by mobility shift competition assay. When using the 48 mer as cold competitor, the reactivity localized to the 97-bp segment was abolished. In contrast, when the 36-bp HPS1B segment was used as a cold competitor, essentially all reactivity to HPS1 remained.

DNaase footprinting analysis using a 230-bp segment of DNA containing HPS1A indicated that the area protected within this 48 mer was roughly 24 bp (Fig. 1 *b* and data not shown). This is a rather large footprint for a single DNA

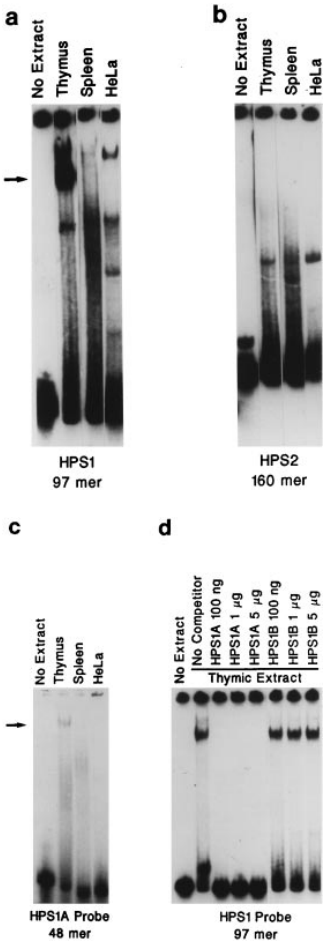

Figure 2. Mobility shift analysis for DNA binding proteins recognizing sequences around δREC. (*a*) Mobility shift analysis defining a protein complex, unique to the thymus (*arrow*), recognizing the upstream homology region HPS1. (*b*) Mobility shift gel using the downstream homology region HPS2 as probe. (*c*) Mobility shift gel showing reactivity of the identified DNA complex from thymus to the 48-bp subsegment HPS1A (*arrow*). (*d*) Competitive mobility shift assay using the 97-bp segment HPS1 as probe. Extracts used from thymus, spleen, and HeLa cells were crude and unpurified.

binding protein, suggesting this might be a multimember complex.

HPS1A Recognizing Proteins Are Found only in Lymphoid Cells and Tissues. To determine if the protein binding complex was restricted to the T cell lineage, mobility shift analysis was performed on several different cell types. Table 1 summarizes this data. It appears that the DNA binding complex recognizing HPS1A is present in pre–T cells (8402, CEM, and 2052) and pre–B cells (Nalm-6). Proteins recognizing the 48-bp segment were not found in mature B or T cells (spleen and SU-DHL-6), HeLa cells, or the erythroid leukemia line, MEL.

Table 1. *Cell Line and Tissue Survey for HPS1A-binding Proteins*

Present	Absent
Thymus	Spleen
8402	HeLa
CEM	Su-DHL-6
2052	MEL
Nalm-6	

Constructing δ-deleting Element TG2. Results from TG1 strongly suggested that regulatory signal sequences around δREC, if they exist, must be contained within the relatively small 1.9-kb DNA segment containing human δREC (Fig. 3 *a*; 21). Because of its proximity to the h-s-n and its temporal restriction to early T cells, HPS1A became the prime candidate for a region controlling lineage-specific use of δ-deleting elements. In an attempt to determine if HPS1A is important in the δ deletion process, TG2 was assembled using oligonucleotide-directed site-specific mutagenesis. The 1.9-kb SalI–XhoI δREC-containing fragment in Fig. 3 *b* was mutated to delete the 48-bp segment, HPS1A (*black box*). The newly generated 1.85-kb mutant fragment was ligated into the base construct (Fig. 3 *a*) to make TG2. TG2 was injected by the University of Alabama at Birmingham (Birmingham, AL) Transgenic Facility and seven founder lines containing the construct were created. All seven lines had expected transgenic bands by Southern analysis, and ranged in copy number from 2 to 20 copies (data not shown). The estimated copy number of each transgene is indicated in Table 2.

TG2 Animals Have δREC Recombinations in Both α/β and γ/δ T Cells by PCR. DNA was isolated from the thymus, spleen, and liver of all seven transgenic lines. None of the transgenic lines of TG2 contain detectable rearrangements by Southern blot analysis, even when the lanes are overloaded (data not shown). However, PCR performed on the same DNA sources easily detected δREC–ψJα and δREC–jα_{56-60} recombinants in 7/7 TG2 lines (Fig. 4 and Table 2). As was noted in TG1, δREC 3′ δ-deleting element recombinations are detected within liver DNA in those lines that have consistent δREC recombinations in thymus and spleen DNA. The likely source for these re-

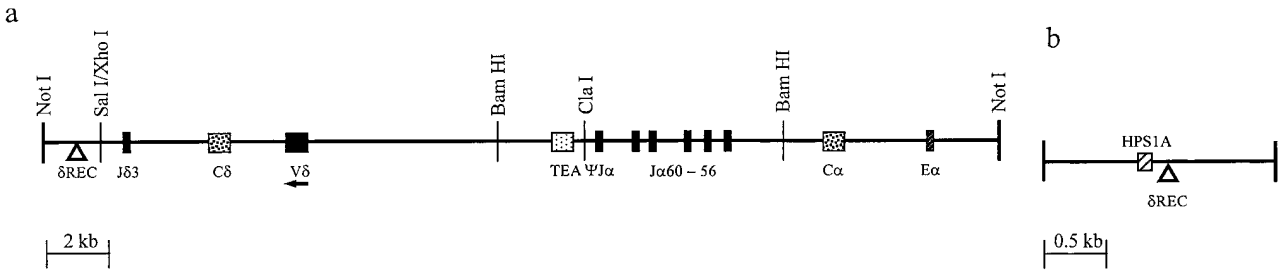

Figure 3. Schematic representation of the δ deletion transgenic constructs. (*a*) Map of TG1. The vertical bars represent cloning sites in the construction of TG1 (21). (*b*) Magnification of the 1.9-kb SalI–XhoI fragment of TG1. The black box represents the 48-bp segment HPS1A, the region of DNA deleted to make TG2.

Table 2. *δREC TG2 Recombination Results by PCR*

Clone	Estimated transgene copy number	Rearrangement α/β	Rearrangement γ/δ
3-1	20	++	+
4-4	15	++	+
4-5	10	+	+
5-4	10	++	+
5-5	5	++	+
6-1	20	+	+
6-3	10	++	+

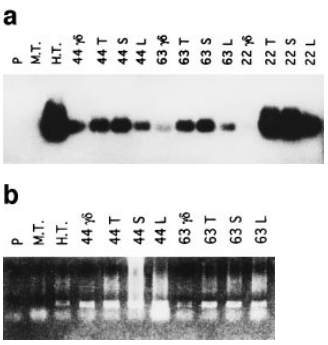

Figure 4. Southern blot of PCR products from (*a*) δREC to ψJα recombinations in TG2 (4-4 and 6-3) and TG1 (22) lines probed with an internal human δREC probe and (*b*) photograph of ethidium bromide–stained gel PCR products primed with germline human δREC primers. *P*, primers only; *M.T.*, murine thymus nontransgenic DNA negative control; *H.T.*, human thymus DNA positive control; *T*, polyclonal thymus DNA; *S*, polyclonal spleen DNA; γδ, polygonal γ/δ T cell DNA. Note that the diminished reactivity in 63 γδ lane in part *a* corresponds to less ethidium reactivity on 63 γδ DNA in part *b*. Ethidium bromide reactivity for TG1 line 22 DNA was reported in reference 27.

combinants is contamination from circulating blood T cells.

In contrast to TG1, δREC–ψJα and δREC–$Jα_{56–60}$ rearrangements are detected in the γ/δ populations of 7 out of 7 TG2 lines by PCR analysis (Fig. 4 and Table 2). It thus appears that the use of δ-deleting elements has been significantly altered in the construct that has deleted the 48-bp segment just 5′ to δREC.

Quantitative PCR Confirms Altered Use of δREC in TG2 Lines. Although the presence of δREC recombinants in γ/δ T cells of TG2 by noncompetitive PCR and the undetectable δREC recombinants in TG2 α/β T cell populations by Southern blot (data not shown) suggested altered transgenic δREC use in TG2 lines, these assays did not indicate the extent of the alteration. To better elucidate the differences between transgenic constructs, quantitative PCR was used to investigate δREC rearrangements. A competitor containing δREC and several Jα segments was constructed (21) and used with the enzyme immunoassay procedure described (33). IL-10 primers of construct pQPCR.MCC7 (33) are situated within a single exon of that gene and serve as a control for the number of alleles within each DNA preparation. Since the seven transgenic lines showing consistent δREC recombinations appeared to be identical, only three lines were used for quantitative PCR on tissues and purified γ/δ T cell populations (lines 4-4, 5-5, and 6-1). The thymus was used for a relatively pure population of T cells, predominantly α/β T cells. Table 3 summarizes the TG2 lines quantitative rearrangement data, and indicates that δREC recombinations are more frequent in the TG2 γ/δ

Table 3. *Quantitation of In Vivo Transgenic Rearrangements by PCR*

	δREC–Jα57	δREC–ψJα	δREC–Jα58	Total δREC rearrangements	
TG1					
14 Thymus	0.1 ± 0.08	0.2 ± 0.05	56 ± 23		56
14 γ/δ	<0.3 ± ND	<0.3 ± ND	0.3 ± 0.1		0.3
				Ratio α/β:γ/δ	188
22 Thymus	6 ± 2	25 ± 7	294 ± 74		325
22 γ/δ	<0.3 ± ND	2 ± 0.3	1 ± 0.4		3
				Ratio α/β:γ/δ	108
TG2					
4-4 Thymus	0.6 ± 0.2	0.4 ± 0.3	13 ± 3		14
4-4 γ/δ	12 ± ND	4 ± 1	50 ± 5		66
				Ratio α/β:γ/δ	0.2
5-5 Thymus	3 ± 0.4	36 ± 13	74 ± 19		113
5-5 γ/δ	0.5 ± 0.5	5 ± 3	23 ± 5		29
				Ratio α/β:γ/δ	4
6-1 Thymus	0.1 ± 0.05	0.1 ± 0.04	2.3 ± 0.4		3
6-1 γ/δ	6 ± 2	7 ± 0.6	75 ± 5		88
				Ratio α/β:γ/δ	0.03

All values normalized to 100,000 copies of IL-10 DNA.

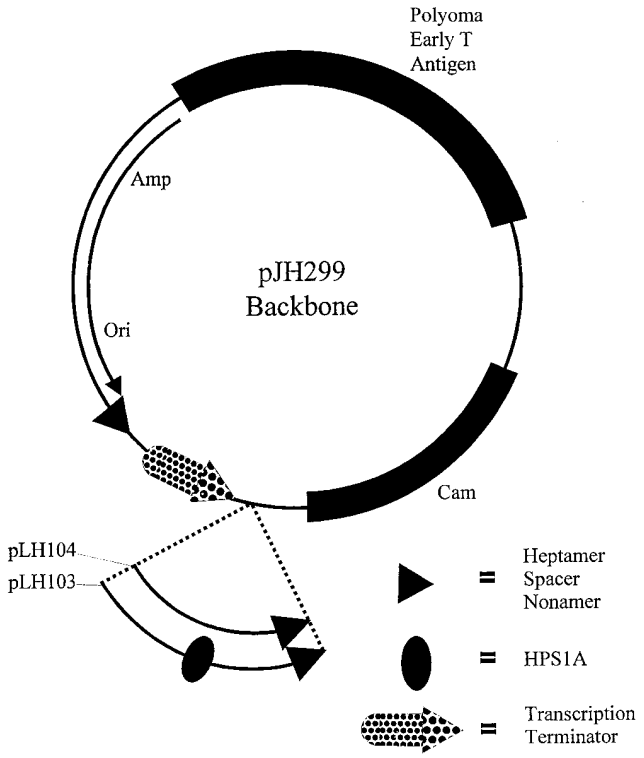

Figure 5. Schematic representation of the rearranging substrates pLH103 and pLH104. The plasmid pJH299 was modified to accept the δREC h-s-n with (pLH103) and without (pLH104) the adjacent HPS1A DNA segment.

T cell population when compared to TGl lines 14 and 22. The ratio of recombinations found in T cells of TG2 lines 4-4, 5-5, and 6-1 were 0.03–4-fold more frequent in the thymic T cell population than in the purified γ/δ T cell population. Conversely, in the TG1 lines, the ratio of δREC recombinations in α/β versus γ/δ T cells ranges from 109 to 186. This recombination ratio represents an ~100-fold lower α/β:γ/δ recombination frequency ratio in TG2 T cells than that seen in TG1 lines 14 and 22 T cells. In comparing the numbers from both TG1 and TG2 populations, the major change was the increased frequency of transgenic δREC recombinations in TG2 γ/δ T cells from <3 δREC rearrangements/100,000 alleles in TG1 lines to >25 δREC rearrangements/100,000 alleles in TG2 lines.

The data contained in Table 3 also suggests an altered use of transgenic δREC in polyclonal α/β T cell populations in TG2 lines compared to TG1 lines. On average, there are more rearrangements in TG1 thymus (190/100,000 alleles) versus TG2 thymus (33/100,000 alleles). This apparent reduced incidence of quantifiable rearrangements in TG2 α/β T cells (~fivefold decrease compared to TG1 lines) and the absence of detectable rearrangements in TG2 DNA by Southern blot analysis (data not shown) suggested that the DNA segment HPS1A also functions as an enhancer of recombination in α/β T cells. However, due to probable insertional effects within both transgenic lines, leading to a wide range of δREC recombinations within these lines (note the difference in thymic δREC recombinations in TG2 lines 4-4, 5-5, and 6-1), ~20–30 different founders for each construct would need to be analyzed to reach statistical significance. To circumvent the need for multiple transgenic founder lines, we used the in vitro recombination system of Hesse et al. (31) to follow the effects of HPS1A on recombination.

In Vitro Analysis of Rearranging Substrates Confirms a Lower Recombination Frequency when the DNA Segment HPS1A Is Absent. Plasmids pLH103 and pLH104 were generated from the base construct pJH299 (30) by the addition of the δREC h-s-n with and without the DNA segment HPS1A (Fig. 5). These rearranging substrates were independently transfected into the putative α/β T cell line 2052 (27) and the B cell line 38B9 (27) and analyzed for rearrangements (see Materials and Methods). Table 4 is a compilation of four separate experiments of each plasmid into both the T and B cell lines. Construct pLH103 (containing HPS1A) consistently produces more rearrangements in both cell lines, when compared to construct pLH104 (significant to $P << 0.00001$). The threefold increase in recombinations seen with pLH103 is very close to the ~fivefold increase in recombinations

Table 4. *Quantitation of In Vitro Rearrangements*

Cell line	Colony status	Plasmid		Fold increase pLH103/pLH104
		pLH103 (+ HPS1A)	pLH104 (− HPS1A)	
38B9	No. recombined* (AMP–CAM)	185	75	
(pre–B)	Total Dpnl-resistant colonies (AMP)	34,407	55,353	Combined 2.90‡
2052	No. recombined* (AMP–CAM)	119	98	
(pre–T)	Total Dpnl-resistant colonies (AMP)	19,151	32,455	

*69 pLH103 colonies and 51 pLH104 colonies were sequenced to obtain an estimate of the number of true recombinants (see Materials and Methods).
‡Significant by logistic regression model to $P << 0.00001$.

seen when comparing α/β T cells in TG1 lines to α/β T cells from TG2 lines (Table 3). Curiously, the B cell line 38B9 rearranges the substrates more frequently than the T cell line 2052. Nonetheless, the presence of HPS1A on nonchromosomal rearranging constructs in both T and B cells increases recombination by ~threefold.

Discussion

The unique positioning of the δ locus within the α TCR locus has clear implications for T cell lineage development. Mechanisms must exist to ensure the separation and control of recombinations within this combined locus. Two models have been proposed for α versus δ receptor development: (*a*) precommitment of T cell precursors, and (*b*) ordered rearrangement of receptors. Precommitment is defined as choosing which T cell type, α/β or γ/δ, to become by some unknown mechanism before rearranging the receptor genes (34–37). This choice could take place at any time before commencing TCR rearrangement, including the earliest stages of development in the bone marrow or fetal liver. This model implies that a signal for particular T cell lineage is generated, by other unknown mechanisms, and then implemented by controlling the use of TCR α or δ chain rearrangement and expression.

The second hypothesis, the ordered rearrangement hypothesis, states that control of the α/δ locus occurs through an ordered rearrangement of the δ chain first, and, if failed or inappropriate, then commencing α chain recombination (38, 39). This model implies that functional δ chain rearrangement and expression essentially precludes α chain recombination. Recent evidence from several groups favors this ordered rearrangement hypothesis (40, 41). Data presented seems to demonstrate a high degree of δ chain rearrangements have occurred in α/β T cells, a finding that is incompatible with a precommitment model.

At present, it is not clear how the data supporting each of these models will be reconciled. It is undoubtedly a complex process for choosing receptor type, and more data is needed before precise mechanisms can be defined. How then, does δ deletion fit into these paradigms? Several pieces of data suggest δ deletion plays a central role in the process of maintaining separate α and δ receptors. (*a*) The frequency of δ deletion, most notable in human thymus, is consistent with δ deletion often occurring on both alleles in cells destined to rearrange TCR α chains (19). (*b*) The high degree of evolutionary conservation in location, sequence identity, and apparent function indicates a significant role for δ deletion in T cell development (20). (*c*) The maintenance of lineage specificity for δ deletion in the first transgenic construct implies tight regulatory control over the use of δ-deleting elements (21). (*d*) A discrete population of developing CD3, CD4, CD8 negative thymocytes containing δREC–ψJα recombinations but not Vα–Jα joining implies that δ deletion occurs before TCR α chain recombination (21).

Corroborating data for δ deletion as an antecedent to Vα–Jα joining has been reported (42). In this in vitro system, immature thymocytes progress to undergo δREC rearrangements before forming functional TCR α chains. However, none of this data proves that δ deletion occurs before Vα–Jα joining on the same allele, in individual cells.

To try and understand the potential role of δ deletion in the formation of T cell receptors, we developed a transgenic reporter construct for the detection of δ-deleting element recombinations. This transgene was designed to molecularly "tag" cells having undergone δ deletion, for subsequent analysis within mature T cell populations. The model of δ deletion implies that use of deleting elements would be restricted to the α/β T cell population. If δ deletion was a random phenomenon, then rearrangements would be expected in all T cell lineages. Human TG1 displayed a remarkable degree of lineage specificity for the use of the δ-deleting elements (Table 3), as predicted by the model (21). This data strongly suggests that δ deletion is involved, in some fashion, with developing α/β T cells. It further implies that tight regulatory control of δ deletion in γ/δ T cells exists, even on random integration of transgenic δ-deleting elements. The data also suggested that DNA segments mediating the tight control of δ deletion would be contained within the transgene reporter construct, TG1.

The next obvious step was to define the molecular basis for lineage-specific control of this process. The identification of a DNA binding protein, specific to a region near δREC and restricted to rearranging cells, allowed the precise targeting of a DNA region that might be responsible for this lineage-specific control of transgenic elements. When TG2 was designed, one of three outcomes was anticipated: (*a*) no differences noted between TG1 and TG2, (*b*) loss of all or most recombination in α/β T cells of TG2, or (*c*) loss of lineage specificity with recombination seen in both α/β and γ/δ T cell populations of TG2. Either the loss of recombination or the loss of lineage specificity would substantiate the presence of signal sequences for control of δ deletion within the 48-bp segment, HPS1A, whose deletion was the only change between the two transgenic constructs. We report here the loss of tight lineage specificity for δ deletion, with frequent rearrangements present in TG2 γ/δ T cells. Moreover, the TG2 transgenic construct in α/β T cells also demonstrates alterations when HPS1A is removed, with a decreased frequency of transgenic δREC rearrangements (an ~fivefold decrease in peripheral T cells [Table 3] confirmed by in vitro recombinatorial analysis [Table 4]). Therefore, we propose that the 48-bp DNA element, HPS1A, is a recognition motif for a DNA binding protein complex that controls recombination at δREC.

Two lines of evidence supports HPS1A's role in δ deletion. As mentioned above, the removal of this recognition sequence in TG2 resulted in transgenic δREC rearrangements in both α/β and γ/δ T cells, and appears to alter the frequency of δREC recombinations in the α/β T cell subsets, and more importantly in in vitro recombination systems. This data suggests that this DNA motif has two functions: in α/β T cells, HPS1A seems to promote and facilitate recombination; in γ/δ T cells it seems to inhibit δREC recombination (see below).

Secondly, the presence of specific DNA binding proteins recognizing HPS1A is limited to early lymphoid lineages (pre–B and pre–T cells, Table 1) where active recombination of TCR genes is occurring (27). It is interesting that proteins recognizing HPS1A are also found in pre–B cells. Precedent for mechanisms separating two independent rearranging loci has been suggested between κ and λ immunoglobulin light chains. In this system, κ recombination precedes λ. When failed or inappropriate κ rearrangement has occurred, an element termed the κ-deleting element in humans (43–45) or RS in the mouse (41, 46, 47), deletes the Cκ segment and λ rearrangement commences. The deleting element resides ~10 kb 3′ to Cκ and is composed of the canonical recognition sequence of the putative recombinase complex, a h-s-n motif. In λ producing B cells, κ deletion has occurred on both κ alleles and appears to occur before the rearrangement and expression of λ light chains in most λ-producing B cells. A preliminary search around the κ-deleting element and RS sequences has not revealed any homology with δREC or HPS1A (data not shown). A more extensive search is currently underway.

The described effects of HPS1A on recombination of δREC are compatible with either positive or negative regulation. For example, in α/β T cells, the presence of proteins recognizing HPS1A may actively promote recombination of δREC, and the absence of such proteins in γ/δ T cells would be the basis for lineage specificity. This promotion of recombination, presumably through protein–protein interaction with the recombinase complex, would focus recombination at δREC and facilitate δREC rearrangements (we prefer "facilitation" to "enhancement" because of the relatively modest [~60–80%] decrease in transgenic δREC rearrangements when HPS1A is deleted). The positive regulatory model would have an absolute requirement for the presence of HPS1A-recognizing proteins before recombination could occur because of the scarcity of δREC rearrangements detected in the γ/δ population of TG1 (Table 3). If such a model were true, it would imply another DNA segment, other than the h-s-n of immunoglobulins and T cell receptors, is directing a recombinase mediated event. Two other such sites, termed KI and KII, were recently described in the murine κ light chain locus (48). Mutation of both elements resulted in an ~80% decrease in rearrangements into the *cis* κ chain locus. Interestingly, the decrease seen by removal of HPS1A closely matches the decrease seen when both κ locus elements are mutated. However, a comparison of DNA binding regions for KI, KII, and HPS1A shows no significant homology (data not shown) suggesting the two processes are not related.

Conversely, recombination of δREC may be a default pathway in T cell development, and proteins inhibiting this recombination in γ/δ T cells could result in the maintenance of lineage specificity. The default pathway in this negative regulatory model would need to be highly efficient to explain the high degree of δREC recombination found in α/β T cells (21). In addition, removal of a DNA recognition sequence (HPS1A) in the default pathway would also be required to effect the efficiency of δREC recombination, to explain the frequency change of transgenic δREC rearrangements seen in in vitro recombination assays and thymic α/β T cells of TG2 (Tables 3 and 4).

Although the data is not conclusive, we favor a model in which both positive and negative regulation occurs in the different lineages. The signal to become an α/β T cell would generate proteins that would interact with HPS1A, thereby promoting δREC recombinations leading to δ deletion. The signal to become a γ/δ T cell would result in the generation of proteins that would interact with HPS1A and prevent rearrangements involving δREC. It is also possible that positive or negative regulation of δREC recombination is one or more steps removed from interaction with HPS1A, protein–protein interactions with HPS1A binding proteins mediate the desired effect.

Nonetheless, the data from TG2 strongly suggests that HPS1A is the focal point for a signal that directs lineage-specific use of the δ-deleting elements. Understanding this signal will require the isolation, partial sequencing, and cloning of the DNA binding proteins recognizing HPS1A. We have successfully isolated the 70-kd member of this complex (data not shown). Partial amino acid sequencing has revealed no homology to known proteins, and we are actively pursuing the cloning of its gene.

δ deletion seems to play a central role in the process of maintaining separate TCR α and δ loci. The evolutionary presence of such seemingly redundant systems for deleting δ coding segments (Vα to Jα joining would also delete δ-coding segments) implies the choice between α or δ receptors needs to be tightly regulated, and that a complex interaction between inhibition and induction of recombination is involved in the process of choosing receptor type. For instance, the induction of δ chain rearrangement in γ/δ T cell precursors also requires the inhibition of Vα to Jα joining or the functional δ chain would be deleted. Therefore, the α/δ locus seems to be the focal point for the separation of α/β and γ/δ T cell lineages. Control of the rearrangement process within this combined locus appears to be the implementation of signals for determination of T cell type. Understanding δ deletion may lead to a description of the molecular events underlying T cell lineage commitment.

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