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Enhancer Control of Local Accessibility to V(D)J Recombinase

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We have studied the role of transcriptional enhancers in providing recombination signal sequence (RSS) accessibility to V(D)J recombinase by examining mice carrying a transgenic human T-cell receptor (TCR) δ gene minilocus. This transgene is composed of unrearranged variable ($V_\delta1$ and $V_\delta2$), diversity ($D_\delta3$), joining ($J_\delta1$ and $J_\delta3$), and constant (C_δ) gene segments. Previous data indicated that with the TCR δ enhancer (E_δ) present in the $J_\delta3$–C_δ intron, V(D)J recombination proceeds stepwise, first V to D and then VD to J. With the enhancer deleted or mutated, V-to-D rearrangement is intact, but VD-to-J rearrangement is inhibited. We proposed that E_δ is necessary for J segment but not D segment accessibility and that J segment inaccessibility in the enhancerless minilocus resulted in the observed V(D)J recombination phenotype. In this study, we tested this notion by using ligation-mediated PCR to assess the formation of recombination-activating gene (RAG)-dependent double-strand breaks (DSBs) at RSSs 3′ of $D_\delta3$ and 5′ of $J_\delta1$. In five lines of mice carrying multicopy integrants of constructs that either lacked E_δ or carried an inactivated E_δ, the frequency of DSBs 5′ of $J_\delta1$ was dramatically reduced relative to that in the wild type, whereas the frequency of DSBs 3′ of $D_\delta3$ was unaffected. We interpret these results to indicate that E_δ is required for $J_\delta1$ but not $D_\delta3$ accessibility within the minilocus, and we conclude that enhancers regulate V(D)J recombination by providing local accessibility to the recombinase. *cis*-acting elements other than E_δ must maintain $D_\delta3$ in an accessible state in the absence of E_δ. The analysis of DSB formation in a single-copy minilocus integrant indicates that efficient DSB formation at the accessible RSS 3′ of $D_\delta3$ requires an accessible partner RSS, arguing that RSS synapsis is required for DSB formation in chromosomal substrates in vivo.

The process of V(D)J recombination assembles the variable (V), diversity (D), and joining (J) gene segments at T-cell receptor (TCR) and immunoglobulin (Ig) loci to produce the highly diverse and clonally distributed antigen receptors expressed on T and B lymphocytes (1, 2, 16, 30, 47, 52). V(D)J recombination is directed by recombination signal sequences (RSSs) consisting of conserved heptamer and nonamer motifs, separated by either a 12- or a 23-bp spacer, that flank all V, D, and J gene segments. Recombination invariably occurs between one gene segment flanked by an RSS with a 12-bp spacer and another flanked by an RSS with a 23-bp spacer. RSSs are both necessary and sufficient to direct V(D)J recombination on artificial substrates in immature T and B lymphocytes, and they serve as sites of cleavage by the recombinase. Cleavage results in the formation of a blunt double-stranded break (DSB) at the signal end (which carries the RSS) and hairpin formation at the coding end (which carries the adjacent coding sequence) (42–44, 48). Signal ends are subsequently joined in precise fashion to form a signal joint, whereas coding ends are processed and joined in an imprecise fashion to form the coding joint.

The essential components of the recombinase machinery include the lymphoid-specific and developmentally regulated proteins RAG-1 and RAG-2 (37, 46), as well as ubiquitous DSB repair proteins (22, 59). Coexpression of RAG-1 and RAG-2 occurs in immature T and B cells (as well as in germinal-center B cells [18, 21]) and is sufficient for site-specific recombination of extrachromosomal substrates in nonlymphoid cells (37, 46). Further, RAG-1 and RAG-2 are essential for V(D)J recombination at endogenous TCR and Ig loci in vivo, as assessed by gene targeting (34, 51). Importantly, recent studies have shown that recombinant RAG-1 and RAG-2 are sufficient for DSB formation at RSSs in vitro (33), proving that these proteins are enzymatic participants in the initial steps of the V(D)J recombination reaction.

In contrast to the highly restricted pattern of RAG expression, the other proteins so far shown to be required for V(D)J recombination are components of the general DSB repair pathway (22, 59). A multisubunit DNA-dependent protein kinase (DNA-PK) has been demonstrated to be essential for V(D)J recombination (4, 24, 36, 53, 55, 62). Three different proteins appear to be part of this complex: Ku70, Ku80, and the catalytic DNA-PK subunit, which is defective in severe combined immunodeficiency mice. Although not required for initial DSB formation, the multisubunit DNA-PK is required for proper formation of coding and signal joints that are the end products of the V(D)J recombination reaction.

Although functional RSSs and the above-mentioned enzymatic activities appear sufficient to direct the process of V(D)J recombination on extrachromosomal substrates, there is an additional level of developmental control exerted at endogenous antigen receptor loci (1, 2, 16, 30, 47, 52). For example, fibroblasts transfected with RAG-1 and RAG-2 fail to rearrange their endogenous Ig and TCR genes. Furthermore, developing B and T cells display tightly regulated rearrangement of individual antigen receptor loci. Fully rearranged TCR genes are formed only in developing T cells, and fully rearranged Ig genes are formed only in developing B cells. Among Ig loci, IgH rearranges prior to Igκ and Igλ, and among TCR loci, β, γ, and δ rearrange prior to α. Furthermore, rearrangements within the IgH and TCR β loci occur in an ordered fashion, with D-to-J rearrangement preceding V-to-DJ rearrangement.

Developmental control has been hypothesized to depend

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upon accessibility of RSSs to cleavage by the recombinase (1, 2, 52). Strong evidence supporting this hypothesis has recently been obtained by Stanhope-Baker et al. (54), who demonstrated lineage-specific and developmental stage-specific differences in the accessibility of TCR and Ig locus RSSs to exogenously introduced RAG proteins in isolated nuclei. Because the developmental pattern of accessibility paralleled the developmental pattern of V(D)J recombination, these results suggest that an open or accessible chromatin configuration is critical for V(D)J recombination at endogenous antigen receptor loci in vivo.

cis-acting elements such as enhancers and promoters have been demonstrated to play an important role in V(D)J recombination (52), presumably by regulating accessibility to the recombinase machinery. For example, elimination of enhancer elements from chromosomally integrated V(D)J recombination substrates inhibits V(D)J recombination in transgenic mice and in transfected cell lines (14, 26, 27, 39), and substitution of one enhancer for another within such constructs can modify the developmental onset of V(D)J recombination (7, 28, 38). Moreover, deletion of enhancers or promoters from endogenous TCR and Ig loci via homologous recombination inhibits V(D)J recombination as well (5, 6, 9, 17, 49, 56, 58, 60). Enhancers have previously been shown to induce local accessibility within chromosomal substrates, as measured in vitro by DNase I sensitivity (15, 40) and by the binding of T7 polymerase to a chromosomally integrated T7 promoter (23). It therefore seems reasonable to attribute enhancer effects on V(D)J recombination to their ability to regulate the accessibility of RSS substrates to RAG-1 and RAG-2. However, a direct role for enhancers in altering RSS accessibility to the recombinase has not been formally demonstrated. The possibility that enhancers regulate a late stage in the V(D)J recombination reaction, for example, the joining phase, has not been ruled out.

This study exploits an unrearranged human TCR δ gene minilocus system in transgenic mice to test the role of enhancers in regulating accessibility to V(D)J recombinase. The transgenic construct includes human $V_\delta1$, $V_\delta2$, $D_\delta3$, $J_\delta1$, $J_\delta3$, and C_δ gene segments, as well as the human TCR δ enhancer (E_δ) within the $J_\delta3$–C_δ intron. This transgene, or a version in which the human TCR α enhancer (E_α) is substituted for E_δ, undergoes V(D)J recombination in the thymus, through a VD intermediate, to generate fully rearranged VDJ products (27, 28). In the absence of a functional enhancer, V-to-D recombination proceeds while VD-to-J recombination is largely blocked (20, 27, 29, 41). To explain this behavior, it was hypothesized that a functional enhancer is required for J segment accessibility to the recombinase but is not required for D segment accessibility. However, as these studies only measured the end product of the V(D)J recombination reaction, a direct role of the enhancer in RSS accessibility could not be firmly established.

Visualization of the first step of the V(D)J recombination reaction, DSB formation, allows for a more direct measure of substrate accessibility. This can be accomplished by use of ligation-mediated-PCR (LM-PCR) as described by Roth et al. (44) and Schlissel et al. (48). Using this approach, we demonstrate that an enhancer can determine the accessibility of individual RSSs within a transgenic V(D)J recombination substrate and thereby dictate the pattern of transgene rearrangement. As such, our results support the notion that enhancer control of RSS accessibility dictates the developmental pattern of V(D)J recombination at antigen receptor loci. Our results also indicate that efficient cleavage at an accessible RSS depends upon the presence of an accessible partner RSS, indicating that RSS synapsis is required for RAG-dependent DSB formation in vivo.

MATERIALS AND METHODS

Transgenic mice. Transgenic lines A and H (27), J (28), P and Q (20), and U and Z (29) have been described previously. Transgenic line S, which carries a version of the TCR δ minilocus with a mutation in the c-Myb binding site of E_δ, has not been reported previously. This line was determined to be single copy by comparison of tail DNA samples of this and previously identified single-copy integrants on slot blots (Schleicher & Schuell, Keene, N.H.) probed with radiolabeled C_δ and $D_\delta3$ DNA fragments.

Oligonucleotides. The oligonucleotides used were linker 2 (5′-CCGGGAGA TCTGAATTCCAC-3′), linker A (5′-GCGGTGACCCGGGAGATCTGAATT C-3′), linker B (5′-GAATTCAGATC-3′), JD1DSBext (5′-CTGGAGTAGTCA TGGAGCCA-3′), JD1DSBint (5′-GCCAGCTTTCCAACACAGAG-3′), DD3DSBext (5′-CTCTTCAAATCCATCCTCCC-3′), DD3DSBint (5′-TGTAT TCCCCAAATGTGCTG-3′), JD3DSBext (5′-TTTCCATCATGAAGGCAGC TA-3′), JD3DSBint (5′-AGCCCTGCCCTAGTATCTCTT-3′), DD3GL (5′-CC CCTGCAGTTTTTGTACAG-3′), DD3NDSBint (5′-GCCTTTTCTGCCTAA CCCAC-3′), and DD3NDSBext (5′-CTCCATGAGACGTTTAAGTACC-3′). Oligonucleotides $V_\delta1$, $J_\delta1$, C_δA, and C_δB were as described previously (25, 27).

Purification of genomic DNA. Genomic DNA was harvested from 3- to 4-week-old mice for use in LM-PCR as described elsewhere (3). Briefly, total splenocytes or thymocytes were washed twice in 10 ml of 1× phosphate-buffered saline (PBS) and were resuspended in 300 μl of 1× PBS. The cells were then brought to a volume of 3 ml with lysis solution (300 mM NaCl, 25 mM EDTA, 50 mM Tris-Cl [pH 8.0], with sodium dodecyl sulfate added to 0.2% [wt/vol] and proteinase K added to 0.2 mg/ml immediately prior to use) and were incubated for 3 to 5 h at 37°C, with mixing by gentle inversion (30 to 60 times). The reaction was stopped by addition of 1.25 volumes of buffered phenol, mixing by inversion, and centrifugation at room temperature to separate phases. The aqueous phase was reextracted with phenol and was then extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with 1 volume of chloroform-isoamyl alcohol (24:1). One volume of anhydrous ethyl ether was then added, and the phases were separated by gravity. The top phase was discarded, and the ether residue was evaporated in the fume hood. The DNA was harvested by adding 1 volume of isopropyl alcohol and immediately spooling. The DNA was resuspended in 3 ml of TE (10 mM Tris [pH 7.5]–1 mM EDTA), following which 330 μl of 3 M sodium acetate (pH 7.0) and 6.7 ml of cold 100% ethanol were added. After mixing, the DNA was respooled and resuspended in 3 to 5 ml of TE by gentle rocking overnight at 23°C. The DNA was then stored at 4°C.

DSB analysis by LM-PCR. The LM-PCR technique was modified from previous descriptions (35, 44, 48). The asymmetrical linker was formed by incubating 20 nmol each of the linker A and linker B oligonucleotides in 100 μl of 250 mM Tris (pH 8.0) for 5 min at 90°C, followed by 5 min at 60°C. The mixture was then slowly cooled to 23°C and stored at −20°C until it was thawed on ice for use.

Genomic DNA (1 to 3 μg) was ligated to 20 pmol of the asymmetrical linker in 50 μl of a solution containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (wt/vol) polyethyleneglycol 8000, and 2.5 U of T4 ligase (Gibco BRL, Gaithersburg, Md.) for 12 to 16 h at 16°C. The reaction was stopped by adding 1 volume of 10 mM Tris (pH 8.3)–50 mM KCl–0.1% Triton X-100 and heating at 95°C for 15 min. Linker-ligated DNA was stored at −20°C.

Serial fourfold dilutions of the linker-ligated DNA from transgenic thymi were prepared by dilution into mock-linker-ligated nontransgenic thymus DNA in order to keep the total concentration of DNA in each PCR mixture constant. DSBs were then detected by nested PCR. First-round PCR mixtures included 20 pmol of linker 2 and 20 pmol of the relevant external primer (JD1DSBext for $J_\delta1$ DSBs, DD3DSBext for DSBs 3′ of $D_\delta3$, and DD3NDSBext for DSBs 5′ of $D_\delta3$) along with 100 ng of target DNA. Hot-start PCRs were carried out in a volume of 25 μl containing 50 mM KCl, 10 mM Tris (pH 8.3), 3 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) dCTP, dGTP, dATP, and dUTP, 1 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.), and 0.25 U of uracil *N*-glycosylase (Perkin-Elmer, Norwalk, Conn.). First-round PCR was carried out for 15 cycles (45 s at 94°C, 60 s at 56°C, and 120 s at 72°C), with a final 10-min extension step at 72°C. The first-round reaction product was chloroform extracted, and 1μl was carried into the second round of PCR. Second-round PCR was carried out with 20 pmol of linker 2 and 20 pmol of the relevant internal primer (JD1DSBint for $J_\delta1$ DSBs, DD3DSBint for DSBs 3′ of $D_\delta3$, and DD3NDSBint for DSBs 5′ of $D_\delta3$) for 30 cycles without the use of uracil *N*-glycosylase, but under otherwise identical conditions.

PCR products were kept frozen at −20°C until they were chloroform extracted. Twenty percent of each PCR product was then electrophoresed on a 1.5% agarose gel, transferred and UV crosslinked to a nylon membrane (Micron Separations Inc., Westborough, Mass.), and hybridized with a 1.3-kb ^{32}P-labeled probe that spanned the area from 320 bp 5′ of $D_\delta3$ to $J_\delta1$. The probe was labeled by random hexamer priming (13).

PCR analysis of V(D)J recombination products. Thymus genomic DNA (5 to 25 ng) was amplified for 25 cycles with 20 pmol of each of the primer pairs $V_\delta1$ plus $J_\delta1$ and C_δA plus C_δB, and PCR products were detected following electrophoresis, blotting, and hybridization with radiolabeled C_δ or $V_\delta1$ probes as described previously (27).

For DNA sequence analysis of VD and VDD rearrangement products, thymus genomic DNA (0.5 μg) was amplified for 35 cycles with primers $V_\delta1$ and DD3GL. PCR products were digested with *Pst*I and *Sal*I and cloned into *Pst*I-

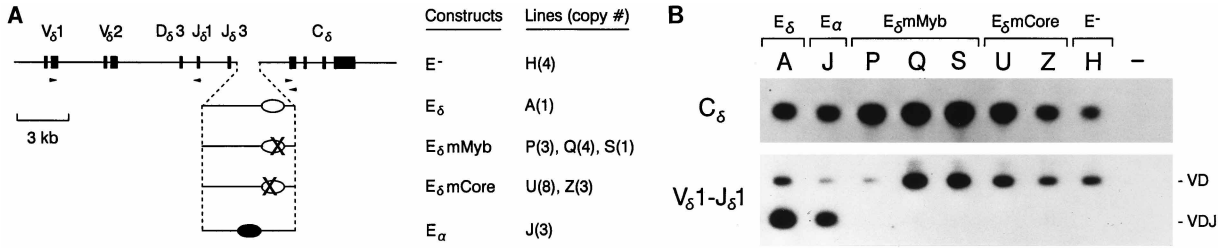

FIG. 1. VD and VDJ rearrangement within the TCR δ minilocus in transgenic mice. (A) The various lines of transgenic mice contain versions of a TCR δ minilocus in which the enhancer in the $J_\delta3$–C_δ intronic region is manipulated. Lines A and J contain wild-type copies of E_δ and E_α, respectively. Lines P, Q, and S contain mutations in the c-Myb binding site of E_δ, whereas lines U and Z carry mutations in the CBF/PEBP2 (core) binding site of E_δ. Line H lacks an enhancer. Transgenic copy numbers are given in parentheses. Primers used for PCR analysis of VD and VDJ rearrangement events are indicated by arrowheads. (B) Thymocyte genomic DNA was amplified by PCR with the indicated primer pairs. Southern blots were probed with radiolabeled C_δ and $V_\delta1$ DNA fragments. The positions of the 1.2-kb VD and 0.3-kb VDJ rearrangement products detected by PCR with $V_\delta1$ and $J_\delta1$ primers are indicated. PCR with C_δ primers served as an internal control. All mice were between 3 and 5 weeks of age.

and *Sal*I-digested pBluescript KS+. Double-stranded templates were sequenced by the dideoxy chain termination method (8, 45) with Sequenase (Amersham-USB, Cleveland, Ohio).

RESULTS

V(D)J recombination phenotypes of TCR δ minilocus constructs studied. We analyzed the properties of eight different lines of transgenic mice, carrying five different minilocus constructs, in this study (Fig. 1A). Two lines, A and J, carry a functional enhancer, E_δ and E_α, respectively (27, 28). Six lines, P, Q, S, U, Z, and H, lack a functional enhancer (20, 27, 29). Of these, line H lacks the entire 1.4-kb E_δ (E^-); lines P, Q, and S have mutations in the E_δ c-Myb binding site (E_δmMyb); and lines U and Z have mutations in the E_δ core-binding factor/polyomavirus enhancer-binding protein 2 (CBF/PEBP2) site (E_δmCore).

We used PCR analysis to display the previously established V(D)J recombination phenotypes of lines A, J, P, Q, U, Z, and H, as well as the V(D)J recombination phenotype of newly identified line S (Fig. 1). This PCR analysis relies on the use of $V_\delta1$ and $J_\delta1$ primers to amplify, in a single reaction, both fully rearranged VDJ products as 0.3-kb species and partially rearranged VD products as 1.2-kb species. As a control, PCR was also performed with a pair of C_δ primers, which amplifies a fragment that arises independent of transgene rearrangement. PCR products were detected on a Southern blot probed with radiolabeled $V_\delta1$- and C_δ-specific DNA fragments. In this and all other experiments presented, the combinations of PCR primers and radiolabeled probes detect products of the human TCR δ minilocus but do not detect products of the endogenous murine TCR δ locus. As shown previously, lines carrying a functional enhancer displayed both VD and VDJ rearranged products (Fig. 1B). By contrast, all lines in which the enhancer had been eliminated or mutated displayed VD rearranged products but only low-level VD-to-J rearrangement. The abundance of VDJ rearranged products in these lines is typically 0.1 to 1% of the level observed in lines carrying a functional enhancer (20, 27, 29).

Analysis of minilocus DSBs by LM-PCR. The distinct V(D)J recombination phenotypes of the various transgenic lines are likely the result of enhancer control of substrate accessibility to the recombinase. We hypothesized that accessibility at $J_\delta1$, but not $D_\delta3$, required the presence of E_δ. In the absence of E_δ, $J_\delta1$ would no longer be accessible to the recombinase, and selective inaccessibility at $J_\delta1$ would result in selective failure of the VD-to-J step of transgene rearrangement. Proof of this hypothesis would require a means to more directly measure accessibility at $D_\delta3$ and $J_\delta1$. To do so, we used LM-PCR to detect the formation of DSBs at RSSs immediately 3′ of $D_\delta3$ and 5′ of $J_\delta1$. If failure of VD-to-J rearrangement resulted from selective inaccessibility at $J_\delta1$, we predicted that DSB formation might be high at $D_\delta3$ but very low at $J_\delta1$.

In the LM-PCR technique, all blunt DSBs contained within harvested genomic DNA are ligated to an asymmetrical linker (Fig. 2A). Nested gene-specific primers (primers 2 and 3 or primers 4 and 5) and a linker-specific primer (primer 1) were then used in PCRs to amplify products reflecting DSB formation at RSSs 3′ of $D_\delta3$ and 5′ of $J_\delta1$. In order to obtain semiquantitative information, serial fourfold dilutions of target genomic DNA were analyzed in the PCRs. Furthermore, primers 4 and 6 were used in control PCRs to compare the amount of template DNA containing unrearranged $J_\delta1$ gene segments in different DNA preparations, as only templates that are germ line in this region can serve as substrates for DSB formation. All PCR products were electrophoresed through agarose gels and were detected by blotting and hybridization with a single radiolabeled probe.

To ensure that the DSBs detected by this technique were RAG dependent, we initially addressed the tissue specificity of DSB formation. Minilocus V(D)J recombination occurs in a T cell-specific fashion (27). As thymocytes express RAG proteins whereas splenic RAG expression appears restricted to germinal-center B cells (18, 21), RAG-dependent TCR δ minilocus DSBs should be present in thymocytes but not in splenocytes. Therefore, thymocytes and splenocytes from E_δ line A were analyzed by LM-PCR. At both $D_\delta3$ and $J_\delta1$, DSBs were readily detected in thymocytes but were undetectable in splenocytes (Fig. 2B). By normalizing the quantities of germ line template analyzed in these samples, we estimated that the DSB frequency 3′ of $D_\delta3$ in thymocytes was at least 256-fold greater than that in splenocytes from the same animal. DSB formation at the RSS 5′ of $J_\delta1$ was also highly specific, as thymocytes displayed a DSB frequency that was at least 64-fold greater than that in splenocyte samples. PCR analysis of genomic DNA that had been mock-linker ligated was included as a control. As expected, DSB PCR signals were absolutely dependent on linker ligation, whereas template PCR signals were independent of linker ligation. No signal was detected from nontransgenic thymus DNA.

Because PCR signals were routinely undetectable in the third or fourth dilution of line A thymocyte DNA even after prolonged autoradiographic exposure (or additional cycles of PCR amplification), we think it likely that the signals detected in the most dilute DNA samples arise from only one (or a few) broken-ended molecules. Based on this assumption, we estimate the absolute frequency of DSBs at $D_\delta3$ and $J_\delta1$ to be

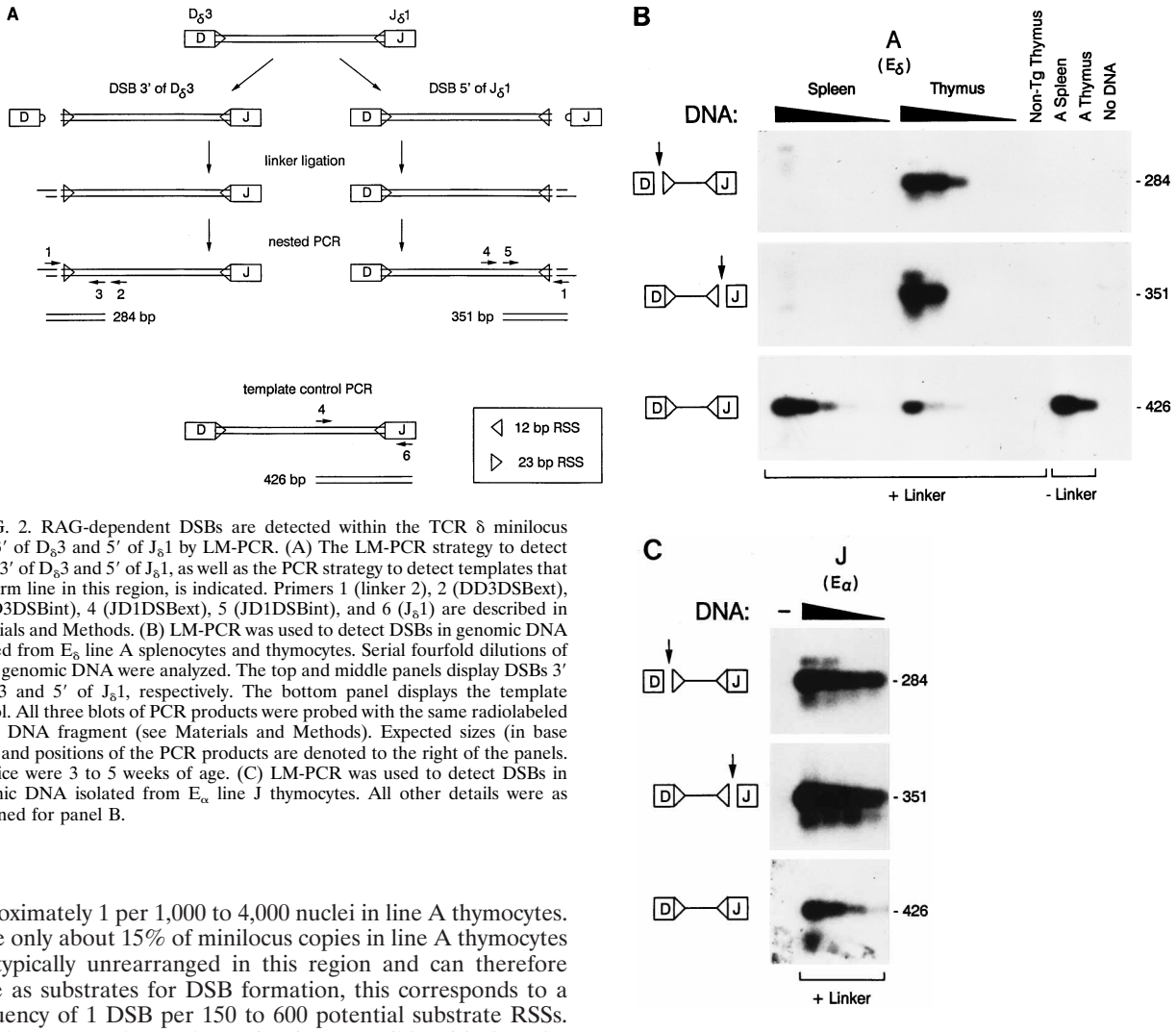

FIG. 2. RAG-dependent DSBs are detected within the TCR δ minilocus both 3′ of $D_\delta 3$ and 5′ of $J_\delta 1$ by LM-PCR. (A) The LM-PCR strategy to detect DSBs 3′ of $D_\delta 3$ and 5′ of $J_\delta 1$, as well as the PCR strategy to detect templates that are germ line in this region, is indicated. Primers 1 (linker 2), 2 (DD3DSBext), 3 (DD3DSBint), 4 (JD1DSBext), 5 (JD1DSBint), and 6 ($J_\delta 1$) are described in Materials and Methods. (B) LM-PCR was used to detect DSBs in genomic DNA isolated from E_δ line A splenocytes and thymocytes. Serial fourfold dilutions of target genomic DNA were analyzed. The top and middle panels display DSBs 3′ of $D_\delta 3$ and 5′ of $J_\delta 1$, respectively. The bottom panel displays the template control. All three blots of PCR products were probed with the same radiolabeled 1.3-kb DNA fragment (see Materials and Methods). Expected sizes (in base pairs) and positions of the PCR products are denoted to the right of the panels. All mice were 3 to 5 weeks of age. (C) LM-PCR was used to detect DSBs in genomic DNA isolated from E_α line J thymocytes. All other details were as explained for panel B.

approximately 1 per 1,000 to 4,000 nuclei in line A thymocytes. Since only about 15% of minilocus copies in line A thymocytes are typically unrearranged in this region and can therefore serve as substrates for DSB formation, this corresponds to a frequency of 1 DSB per 150 to 600 potential substrate RSSs. This frequency of DSB formation is compatible with the relatively high level of VDJ rearranged products because the former is an instantaneous or snapshot measure of V(D)J recombination, whereas the latter is a cumulative measure.

Thymocytes and splenocytes from E_α line J displayed a pattern of DSB formation quite similar to that of E_δ line A, with high levels of DSBs detected both 3′ of $D_\delta 3$ and 5′ of $J_\delta 1$ (Fig. 2C). Thus, in two different transgenic lines that carry a functional enhancer in the $J_\delta 3$–C_δ intron and that undergo efficient VD-to-J rearrangement, RAG-dependent DSB formation was readily detectable both downstream of $D_\delta 3$ and upstream of $J_\delta 1$. Taken together, the results of these experiments confirmed the specificity of the LM-PCR assay in this transgenic system and showed that in transgenes with a functional enhancer, both $D_\delta 3$ and $J_\delta 1$ are accessible to the recombinase.

Dependence of $J_\delta 1$ RSS accessibility on a functional enhancer. The high-level DSB formation at both $D_\delta 3$ and $J_\delta 1$ in E_δ line A was then compared to DSB formation in transgenic lines carrying constructs in which E_δ had been mutated or deleted. E_δmMyb lines P and Q were found to display high-frequency DSB formation 3′ of $D_\delta 3$ (Fig. 3A and B). The elevated levels of DSB PCR signals in lines P and Q relative to those in E_δ line A roughly parallel the differences in germ line template between the lines, suggesting that the actual DSB frequencies 3′ of $D_\delta 3$ are quite comparable in all three lines on a per template basis. The differences in template DNA between the lines can be accounted for by higher transgene copy numbers and decreased VD-to-J recombination in lines P and Q.

In contrast to the high-level DSB formation 3′ of $D_\delta 3$ in E_δmMyb lines P and Q, DSB formation immediately 5′ of $J_\delta 1$ was greatly decreased in these lines compared to that in E_δ line A. No DSB PCR signal was detected at this site in line P, while a very low signal was detected in only the most concentrated sample of line Q. Accounting for the template differences between the various DNA samples, we estimate from this experiment that the DSB frequency 5′ of $J_\delta 1$ in E_δ line A is 1,024-fold greater than that in line P and 64-fold greater than that in line Q. Although there was some variation in these values from experiment to experiment, the differences between E_δ line A and the E_δmMyb lines were always 64-fold or greater. DSB formation at the RSS 5′ of $J_\delta 3$ displayed a similar dependence on the presence of a functional enhancer (data not shown). The observation that $D_\delta 3$ DSBs closely parallel the template amount, whereas $J_\delta 1$ and $J_\delta 3$ DSBs are greatly decreased specifically in E_δmMyb lines, provides strong evidence that the intronic E_δ is required for accessibility of $J_\delta 1$ and $J_\delta 3$ but not of $D_\delta 3$.

E_δmCore transgenic lines displayed a pattern of accessibility similar to that observed in the E_δmMyb lines (Fig. 3A and B). Accounting for differences in the abundance of germ line tem-

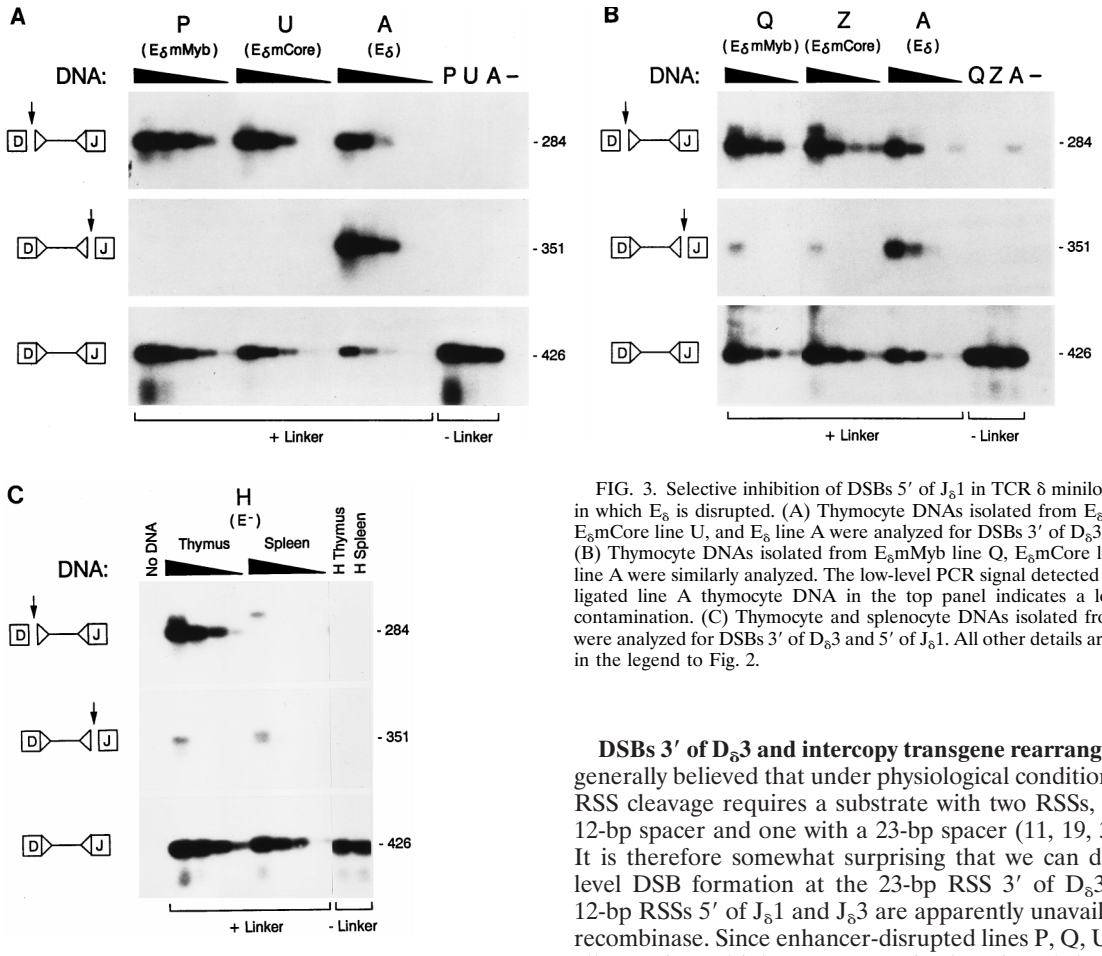

FIG. 3. Selective inhibition of DSBs 5′ of $J_\delta1$ in TCR δ minilocus constructs in which E_δ is disrupted. (A) Thymocyte DNAs isolated from E_δmMyb line P, E_δmCore line U, and E_δ line A were analyzed for DSBs 3′ of $D_\delta3$ and 5′ of $J_\delta1$. (B) Thymocyte DNAs isolated from E_δmMyb line Q, E_δmCore line Z, and E_δ line A were similarly analyzed. The low-level PCR signal detected in non-linker-ligated line A thymocyte DNA in the top panel indicates a low-level PCR contamination. (C) Thymocyte and splenocyte DNAs isolated from E^- line H were analyzed for DSBs 3′ of $D_\delta3$ and 5′ of $J_\delta1$. All other details are as explained in the legend to Fig. 2.

plates, the DSB frequencies 3′ of $D_\delta3$ in lines U and Z were comparable to that in E_δ line A, whereas the DSB frequencies 5′ of $J_\delta1$ were greatly reduced. The $J_\delta1$ DSB frequency in E_δ line A was estimated to be 256-fold greater than that in line U and 64-fold greater than that in line Z.

We also examined DSB formation in E^- line H (Fig. 3C). Thymocytes from line H displayed a decrease in $J_\delta1$ accessibility relative to $D_\delta3$ accessibility similar to those seen in the E_δmMyb and E_δmCore lines. Once again the DSB abundance 3′ of $D_\delta3$ paralleled the amount of template DNA in lines H and A. However, accounting for template differences, we estimate that DSBs 5′ of $J_\delta1$ are 64-fold more abundant in E_δ line A than in E^- line H. This difference is, if anything, an underestimate, since $J_\delta1$ DSBs were reproducibly detected at similar frequencies in line H thymocytes and splenocytes. This suggests that the low-level $J_\delta1$ DSBs are not RAG dependent. One possible explanation is that low-frequency breakage may occur preferentially in this region during DNA preparation and may reflect a unique property of the transgene integration site. The detection of breakage introduced by a presumably nonspecific mechanism precisely at the $J_\delta1$ RSS can be explained by the structure of the linker 2 oligonucleotide used for PCR amplification. This oligonucleotide includes a CAC sequence at its 3′ end which overlaps all RSS sequences and should therefore provide specificity for DSBs at RSSs (see reference 48). Consistent with this explanation, PCR analysis of $J_\delta1$ DSBs in line H using the linker A primer that lacks the terminal CAC revealed a much more heterogeneous breakage pattern (data not shown).

DSBs 3′ of $D_\delta3$ and intercopy transgene rearrangement. It is generally believed that under physiological conditions, efficient RSS cleavage requires a substrate with two RSSs, one with a 12-bp spacer and one with a 23-bp spacer (11, 19, 31, 50, 57). It is therefore somewhat surprising that we can detect high-level DSB formation at the 23-bp RSS 3′ of $D_\delta3$ when the 12-bp RSSs 5′ of $J_\delta1$ and $J_\delta3$ are apparently unavailable to the recombinase. Since enhancer-disrupted lines P, Q, U, Z, and H all contain multiple, concatamerized copies of the transgene, one explanation for our results would be the pairing of the 23-bp RSS 3′ of $D_\delta3$ in one copy of the transgene with an accessible 12-bp RSS in another copy.

A logical candidate would be the accessible 12-bp RSS 5′ of $D_\delta3$. The arrangement of RSSs flanking D_δ gene segments (12-bp RSS–D–23-bp RSS) permits D-to-D rearrangement between tandemly arrayed D segments (for example, V–D1–D2–J, V–D1–D2–D3–J) at the endogenous murine and human TCR δ loci (10, 32). Although only one D_δ segment was included in the minilocus, an analogous rearrangement event in which $D_\delta3$ in one copy of the minilocus is joined to $D_\delta3$ in another copy should be permitted. Attempted $D_\delta3$-to-$D_\delta3$ rearrangement could explain the abundance of DSBs 3′ of $D_\delta3$ in lines lacking a functional enhancer.

To assay for D-to-D joining, we amplified putative VD rearrangement products from E_δmCore line U and by sequence analysis asked whether any actually reflected VDD rearrangement events. Two of seven rearrangement events sequenced appeared to reflect VDD rearrangement, whereas the remainder appeared to reflect VD rearrangement (Fig. 4). The apparent presence of a second copy of $D_\delta3$ in these sequences is highly unlikely to have arisen by chance. Given the presence of one $D_\delta3$ sequence adjacent to the 3′ RSS, the probability of a chance 12 of 12 match to $D_\delta3$ occurring a second time in a sequence of 16 nucleotides, as is found in the first sequence listed, is less than 1 in 10^6. The detection of VDD rearranged products provides direct evidence for rearrangement events between accessible gene segments in different copies of the transgene and indicates that such rearrangement events be-

	Vδ1	N	Dδ3	N	P	Dδ3	RSS
Germline:	GTGCTCTTGGGGAACT		ACTGGGGGATACG			ACTGGGGGATACG	CACAGTGCTACAAAACC
(V-D-D)	GTGCTCTTGGGG		ACTGGGGGATAC	ACGC		GGGGGATACG	CACAGTGCTACAAAACC
(V-D-D)	GTGCTCT	CGAA	GGGGA	A		ACTGGGGGgTACG	CACAGTGCTACAAAACC
(V-D)	GTGCTCTTGGGGAAC				GT	ACTGGGGGATACG	CACAGTGCTACAAAACC
(V-D)	GTGCTCTTGGGGAAC			CC	T	ACTGGGGGATACG	CACAGTGCTACAAAACC
(V-D)	GTGCTCTTGGGGAA			AAA		ACTGGGGGATACG	CACAGTGCTACAAAACC
(V-D)	GTGCTCTTGGGGAACT			CA			ACAAAACC
(V-D)	GTGCTCTTGGGGAACT					TGGGGGATACG	CACAGTGCTACAAAACC

FIG. 4. Intercopy VDD rearrangement within the minilocus. Genomic DNA from E_δmCore line U thymocytes was amplified by PCR using the primers $V_\delta1$ and DD3GL. The sequences of seven cloned PCR products are organized to highlight the presumed contributions of the germ line $V_\delta1$ and $D_\delta3$ gene segments, as well as N and P nucleotides. The single lowercase g residue probably corresponds to a *Taq* polymerase error.

tween accessible RSSs are permitted even when they are separated by regions of inaccessible chromatin. Furthermore, this result provides a potential explanation for high-level DSB formation 3′ of $D_\delta3$ in the face of inaccessible J_δ RSSs.

To test whether the formation of DSBs at the 23-bp RSS 3′ of $D_\delta3$ was truly dependent on the availability of an accessible 12-bp RSS elsewhere in the minilocus, we examined DSB formation in E_δmMyb line S, which contains a single-copy minilocus integration (Fig. 5A). This line displayed a dramatic reduction in DSB formation 5′ of $J_\delta1$, much as had been observed for the other enhancer-disrupted lines. After normalization for template abundance, we estimated that the frequency of DSB formation 5′ of $J_\delta1$ was at least 256-fold greater in line A than in line S.

However, unlike the other enhancer-containing and enhancer-disrupted lines, in which DSB formation 3′ of $D_\delta3$ was high and varied largely as a function of template abundance, in line S DSB formation 3′ of $D_\delta3$ was dramatically reduced. Thus, despite the fact that template levels were much higher in line S than in line A, DSBs were detected at significantly reduced levels in line S compared to line A. After normalization for template abundance, we estimated the DSB frequency 3′ of $D_\delta3$ to be 64- to 256-fold higher in line A than in line S. In fact, the reductions in DSB formation 3′ of $D_\delta3$ and 5′ of $J_\delta1$ appear to be quite similar in this line.

There are two possible interpretations of this result. A reduction in DSB formation 3′ of $D_\delta3$ might be due to inaccessibility of this gene segment, perhaps because the minilocus had integrated into a relatively inactive region of chromatin in line S. Alternatively, $D_\delta3$ might be accessible, and the reduction in DSB formation might result from the lack of an accessible partner RSS. The first explanation was considered unlikely because VD rearranged products were readily detected in this line (Fig. 1B), implying that the $V_\delta1$ and $D_\delta3$ gene segments must be accessible to the recombinase. However, to more carefully test this point, we measured DSB formation at the RSS 5′ of $D_\delta3$ (Fig. 5B and C). This analysis required a new nested PCR strategy to detect DSB formation, as well as a new PCR strategy to measure germ line templates that are potential substrates for DSB formation at this site (Fig. 5B). The results were striking (Fig. 5C). Unlike DSBs 3′ of $D_\delta3$, DSBs 5′ of $D_\delta3$ were abundant in line S. Moreover, the relative abundance of DSBs 5′ of $D_\delta3$ in lines A and S roughly paralleled the observed differences in template amount between these lines. The dramatic difference in DSB formation 5′ and 3′ of $D_\delta3$ in line S cannot easily be attributed to a difference in accessibility between these two sites, as $D_\delta3$ is only 13 bp. Rather, we conclude that DSB formation at the accessible RSS 3′ of $D_\delta3$ is critically dependent on the presence of an accessible partner RSS, which is lacking in line S.

DISCUSSION

It is now well established that enhancers can impart developmental control to the process of V(D)J recombination, and it has been proposed that they impart developmental control by determining substrate accessibility to RAG-1 and RAG-2 (1, 2, 52). Indeed, RSS accessibility, as measured by cleavage by exogenously introduced RAG proteins in isolated nuclei, is developmentally regulated (54), and enhancers are capable of modifying chromatin accessibility as measured by other criteria (15, 23, 40). However, to date, a direct connection between enhancer function and RSS accessibility has not been established. Our experiments demonstrate that inactivation or elimination of an enhancer from a transgenic minilocus reporter construct results in a blockade of V(D)J recombination that can be attributed to selective inhibition of RAG-dependent RSS cleavage. Our system is unique because in the absence of a functional enhancer, the V-to-D step of transgene rearrangement proceeds relatively unimpeded and because, although the VD-to-J step is dramatically inhibited, this inhibition occurs despite normal levels of RAG-dependent RSS cleavage 3′ of $D_\delta3$. Rather, inhibition of VD-to-J rearrangement is associated with a selective blockade of RAG-dependent RSS cleavage 5′ of $J_\delta1$. Given that all other aspects of minilocus VDJ recombination proceed normally, this selective blockade of RSS cleavage is most readily interpreted as a highly localized inhibition of accessibility to RAG proteins within the minilocus construct. We conclude, therefore, that a functional enhancer is critical for local accessibility to the recombinase.

A critical assumption in our experiments is that DSB frequency parallels, and is therefore a useful measure of, RSS accessibility. In particular, it is essential that changes in RSS accessibility affect the DSB frequency at the affected RSS but not at other RSSs within the minilocus. However, at first glance, this expectation runs contrary to the prevailing notion that RSS cleavage depends upon prior synapsis of 12-bp and 23-bp RSSs. The notion that synapsis is required for cleavage is supported by the measurement of V(D)J recombination in transfected plasmid substrates in vivo, which demonstrates that recombination is inhibited between RSSs that are positioned so close together that constraints on DNA bending would

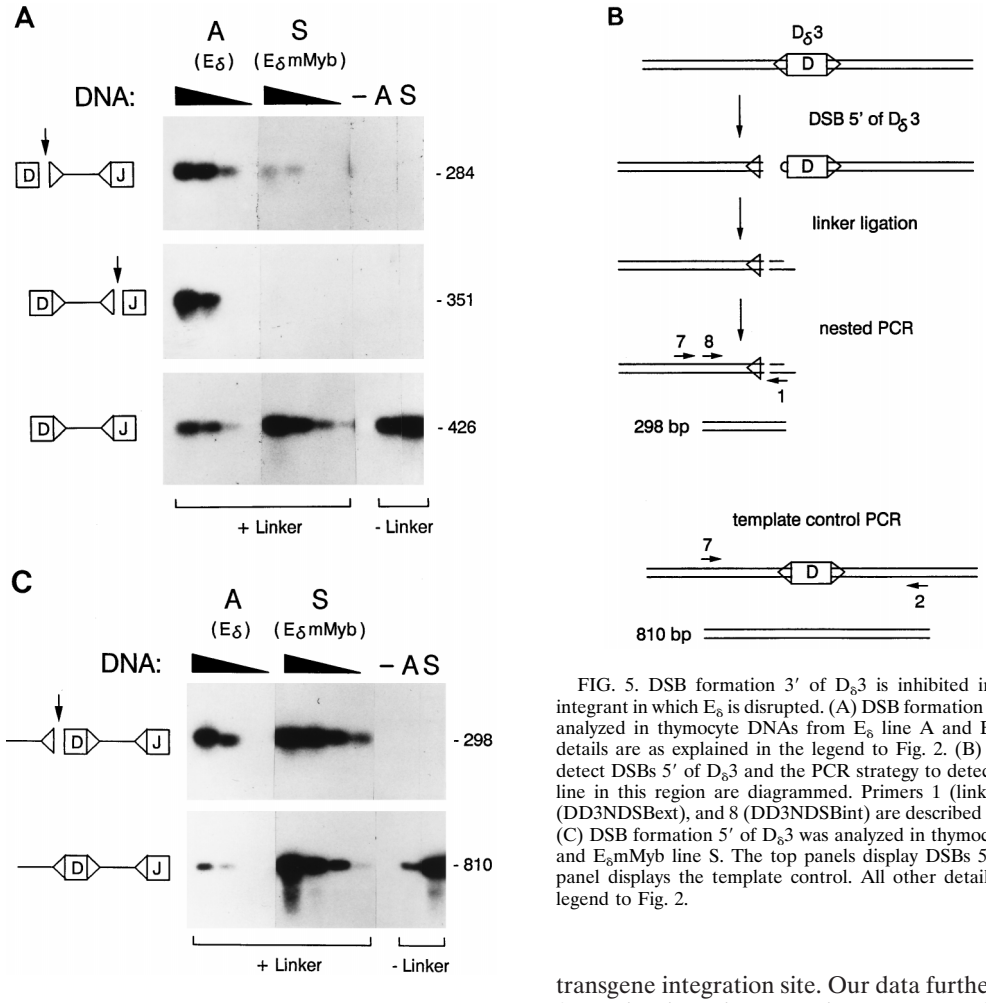

FIG. 5. DSB formation 3′ of $D_\delta 3$ is inhibited in a single-copy minilocus integrant in which E_δ is disrupted. (A) DSB formation 3′ of $D_\delta 3$ and 5′ of $J_\delta 1$ was analyzed in thymocyte DNAs from E_δ line A and E_δmMyb line S. All other details are as explained in the legend to Fig. 2. (B) The LM-PCR strategy to detect DSBs 5′ of $D_\delta 3$ and the PCR strategy to detect templates that are germ line in this region are diagrammed. Primers 1 (linker 2), 2 (DD3DSBext), 7 (DD3NDSBext), and 8 (DD3NDSBint) are described in Materials and Methods. (C) DSB formation 5′ of $D_\delta 3$ was analyzed in thymocyte DNAs from E_δ line A and E_δmMyb line S. The top panels display DSBs 5′ of $D_\delta 3$, and the bottom panel displays the template control. All other details are as explained in the legend to Fig. 2.

block appropriate synapsis (50). Further support is provided by the measurement of open-and-shut junctions in transfected plasmid and retroviral substrates in vivo, which indicates that this cleavage, modification, and rejoining reaction is dramatically inhibited in substrates carrying a single RSS (19, 31). The rare events that are detected are thought to reflect synapsis with cryptic or noncanonical RSSs elsewhere in the substrate (31). Finally, a requirement for RSS synapsis is indicated by recent studies of RSS cleavage by RAG proteins in vitro, which demonstrate that under the appropriate conditions, RSS cleavage is highly dependent on the presence of two RSSs, one with a 23-bp spacer and one with a 12-bp spacer (11, 57). As such, it would be predicted that selective inaccessibility at the 12-bp RSSs 5′ of $J_\delta 1$ and $J_\delta 3$ might lead to reduced DSB formation not only at these RSSs but also at the accessible 23-bp RSS 3′ of $D_\delta 3$. This clearly was not the case in the majority of our transgenic lines.

Our data indicates that the DSB frequency at the RSS 3′ of $D_\delta 3$ is maintained despite inaccessible J_δ RSSs because synapsis can occur between the 23-bp RSS 3′ of $D_\delta 3$ in one copy of the minilocus and the 12-bp RSS 5′ of $D_\delta 3$ in another copy. Thus, DSB frequency appears to be a reasonable measure of RSS accessibility in any multicopy minilocus integrant. Consistent with this notion, the measured DSB frequency 3′ of $D_\delta 3$ was, on a per template basis, quite similar in all multicopy lines. Thus, this frequency was not significantly influenced by transgene copy number variation between three and eight or by transgene integration site. Our data further indicates that DSB formation is an inappropriate measure of RSS accessibility in a single-copy minilocus integrant, because the RSS synapsis that is apparently required for efficient DSB formation cannot take place. As such, our data provides strong support for the notion that synapsis is important for the production of DSBs in chromosomal RSSs in vivo. We cannot be certain whether the synapsis requirement is an absolute one, as the low-level residual DSB formation that we do detect may reflect the availability of cryptic or noncanonical RSSs that may interact with the lone accessible canonical RSS at reduced frequencies. We also cannot be certain that the accessibility of paired RSSs is the only factor (other than the recombinase itself) that influences DSB formation. It is possible, for example, that chromosomally associated factors influence DSB formation by actively recruiting the recombinase. If this were shown to be the case, our conclusions would have to be reevaluated.

Since the accessibility of $D_\delta 3$ (and, although not directly measured, $V_\delta 1$ and $V_\delta 2$ as well) is maintained even in the absence of a functional enhancer within the $J_\delta 3$–C_δ intron, there must be additional regulatory elements located within the 5′ region of the minilocus that function to provide accessibility in an E_δ-independent fashion. Because the frequency of RSS cleavage 3′ of $D_\delta 3$ is nearly 2 orders of magnitude greater than the frequency of RSS cleavage 5′ of $J_\delta 1$ in an enhancer-disrupted minilocus, our data argues for a very steep gradient of accessibility across a relatively short (1-kb) region of DNA. In other words, the effect of any regulatory element that maintains $D_\delta 3$ accessibility cannot extend as far as $J_\delta 1$. We have

speculated that this steep gradient of accessibility could result from the effects of a boundary or enhancer-blocking element located between $D_\delta 3$ and $J_\delta 1$ (27). We have recently asked whether this DNA segment could block the effects of an enhancer in a stably integrated reporter construct but have found this not to be the case (61). Drawing the conclusion that a boundary does not exist, we suggest that accessibility within the 5′ region of the minilocus is not determined by a potent regulatory element (i.e., a strong enhancer) whose global influence would have to be sharply terminated between $D_\delta 3$ and $J_\delta 1$. Rather, we suggest that V and D segment accessibility is more likely maintained by closely associated regulatory elements whose activities are weak and whose regulatory influences extend over relatively short distances, perhaps corresponding to the displacement of only a few nucleosomes.

The $V_\delta 1$ and $V_\delta 2$ promoters are obvious, although unproven, candidate local regulators of germ line $V_\delta 1$ and $V_\delta 2$ segment accessibility, respectively. These elements may also promote accessibility of the $D_\delta 3$ 3′ RSS on VD rearranged templates, because they would lie only several hundred base pairs away following rearrangement. However, since our data argues that these elements do not extend their influence an additional 1 kb to $J_\delta 1$ on VD rearranged templates, they are unlikely to extend their influence over several kilobases to modulate germ line $D_\delta 3$ accessibility on unrearranged templates. This leads us to propose that there must be an additional regulatory element, probably located immediately 5′ of $D_\delta 3$, that is responsible for inducing germ line $D_\delta 3$ accessibility. Independent regulation of D_δ accessibility is also suggested by the developmentally regulated usage of different D_δ gene segments that is apparent at the endogenous human and murine TCR δ loci. $D_\delta 3$ is used selectively early in development, whereas $D_\delta 1$, $D_\delta 2$, and $D_\delta 3$ are all used at later stages (10, 12, 25). With these considerations in mind, we are currently testing the notion of E_δ-independent and V_δ promoter-independent regulation of $D_\delta 3$ accessibility by analysis of DSB formation in additional transgenic constructs, and we will attempt to identify the putative regulator of germ line $D_\delta 3$ accessibility in similar fashion.

In summary, our analysis of DSB formation in versions of a transgenic TCR δ minilocus construct provides evidence that enhancers can impart discrete and dramatic changes in RSS accessibility to RAG proteins in vivo, suggests the presence of novel elements that provide highly localized accessibility to RSSs even in the absence of an enhancer, and provides strong support for the notion that RAG-dependent DSB formation requires synapsis between accessible RSSs in chromosomal substrates in vivo.

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