Flanking nuclear matrix attachment regions synergize with the T cell receptor δ enhancer to promote V(D)J recombination

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Previous studies have identified nuclear matrix attachment regions (MARs) that are closely associated with transcriptional enhancers in the IgH, Igκ, and T cell receptor (TCR) β loci, but have yielded conflicting information regarding their functional significance. In this report, a combination of *in vitro* and *in situ* mapping approaches was used to localize three MARs associated with the human TCR δ gene. Two of these are located within the Jδ3–Cδ intron, flanking the core TCR δ enhancer (Eδ) both 5′ and 3′ in a fashion reminiscent of the Ig heavy chain intronic enhancer-associated MARs. The third is located about 20 kb upstream, tightly linked to Dδ1 and Dδ2. We have previously used a transgenic minilocus V(D)J recombination reporter to establish that Eδ functions as a developmental regulator of V(D)J recombination, and that it does so by modulating substrate accessibility to the V(D)J recombinase. We show here that the Eδ-associated MARs function synergistically with the core Eδ to promote V(D)J recombination in this system, as they are required for enhancer-dependent transgene rearrangement in single-copy transgene integrants.

The assembly of antigen receptor genes in developing T and B lymphocytes occurs by the process of V(D)J recombination (1–3). This process is carried out by an enzymatic machinery that includes the lymphoid-specific recombinase activating gene (RAG)-1 and RAG-2 proteins, as well as a series of ubiquitously expressed DNA repair proteins. RAG-1 and RAG-2 catalyze the initial steps of the V(D)J recombination reaction by recognizing and then introducing a single-stranded nick at the 5′ border of recombination signal sequences that flank all T cell receptor (TCR) and Ig variable (V), diversity (D), and joining (J) gene segments. The developmental regulation of V(D)J recombination is mediated at least in part by the restriction of RAG-1 and RAG-2 expression to defined stages of T and B lymphoid development. However, locus-specific and gene segment-specific patterns of V(D)J recombination are thought to be established by regulating the accessibility of chromosomal recombination signal sequences to the recombinase (4–6). Although a precise biochemical definition of accessibility is lacking at this time, there is a good understanding of the cis-acting elements within TCR and Ig loci that serve as developmental regulators of accessibility. Specifically, a host of studies involving the manipulation of transgenic V(D)J recombination reporter substrates or endogenous TCR and Ig loci have established that transcriptional enhancers and promoters play critical roles in this process (4–6). However, these elements by themselves may not be sufficient to provide accessibility to the recombinase.

The nuclear matrix is a proteinaceous network that includes the nuclear lamina and extends throughout the nucleus, providing a structural framework for the organization of both chromatin fibers and the enzymatic machineries involved in chromatin metabolism (7–12). Chromatin is periodically anchored to the nuclear matrix so as to form looped structures, with an average loop size of 86 kb. Specific regions of DNA, known as matrix attachment regions (MARs), serve to anchor the loops to the matrix. MARs are generally A/T-rich, tend to have a high unwinding potential, and may contain sequences homologous to topoisomerase II sites. They have, in some instances, been identified at the borders of transcriptionally active chromatin domains. However, in other instances, they have been identified in intragenic regions, often in close association with transcriptional enhancers, transcriptional promoters, and origins of replication. DNA replication, transcription, and RNA processing may all occur in association with the nuclear matrix, and MAR sequences have been attributed a wide range of activities, including transcriptional activation, transcriptional repression, and boundary functions (7–12).

MARs have been identified in close association with the intronic transcriptional enhancers of the IgH (13), Igκ (14), and TCR β (15) loci. The core Ig heavy chain intronic enhancer (Eμ) is flanked by MARs both 5′ and 3′. These MARs have been shown to mediate transcriptional repression in non-B cells (16–18). However, in B cells of transgenic mice, they were shown to synergize with Eμ to activate transcription and to extend a region of accessible chromatin within a reporter substrate (19, 20). Despite these observations, elimination of the IgH MARs from the endogenous locus provides no evidence for significant roles in either transcription or V(D)J recombination (21). Similarly, gene targeting failed to reveal a role for the TCR β MAR (15, 22). The MAR associated with the intronic Igκ enhancer was shown to increase the proportion of B cells that express and somatically hypermutate a transgenic reporter substrate at high levels (23). Recent gene targeting confirmed the positive influence on somatic hypermutation, revealed a MAR-dependent suppression of κ rearrangement in pro-B cells, but detected no obvious effect on κ expression (24). Clearly, a coherent picture of MAR function at Ig and TCR loci has yet to emerge.

In previous studies addressing cis-acting regulators of TCR δ gene rearrangement, we used a transgenic V(D)J recombination reporter substrate composed of elements of the human TCR δ locus to establish a role for the intronic TCR δ enhancer (Eδ) as a developmental regulator of V(D)J recombination (25, 26). We showed, in addition, that Eδ regulates V(D)J recombination in this system by modulating the accessibility of particular recombination signal sequences within the reporter to RAG-1 and RAG-2 (27). More recently, we have screened for additional cis-acting elements that might collaborate with Eδ to regulate V(D)J recombination. As a result of this screen, we now report the identification of three MARs in the vicinity of the human Cδ gene segment, one associated with Dδ1 and Dδ2, and a pair that flank the core Eδ in a fashion that is highly reminiscent of the pair that flank the core Eμ. A functional test of the Eδ-associated MARs indicates that they can synergize with the

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Abbreviations: RAG, recombinase activating gene; TCR, T cell receptor; MAR, matrix attachment region; Eδ, TCR δ enhancer; Eμ, Ig heavy chain intronic enhancer; BEAD-1, blocking element alpha/delta-1.

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enhancer to promote V(D)J recombination in the context of a transgenic V(D)J recombination reporter substrate.

Materials and Methods

Plasmids and DNA Fragments. Plasmids pBK10.9, pKB3.0, pBK3.1, and pKB6.4, covering 23.4 kb of the human TCR α/δ locus, were generated by ligation of *Bam*HI–*Kpn*I fragments of cosmid K7A (28) into *Bam*HI- and *Kpn*I-digested pBluescript. Plasmid pBB1.6 was generated by ligation of a 1.6-kb *Bam*HI fragment obtained from the same cosmid into *Bam*HI-digested pBluescript. A 3.5-kb *Bam*HI fragment containing Dδ1 and Dδ2 was excised from cosmid K3B (28) and ligated into *Bam*HI-digested pBluescript to generate pDδBm3.5. A 3.8-kb *Bam*HI–*Bgl*II fragment containing Jδ3 and Eδ was excised from pBK10.9 with *Not*I and *Bgl*II and ligated into *Not*I- and *Bam*HI-digested pBluescript to generate pEδ3.8. Plasmid pEδ0.4 includes a blunted 370-bp *Dra*I–*Xba*I core Eδ fragment cloned into the *Sma*I site of pBluescript (29). A 1.0-kb DNA fragment (fragment l) upstream of the core Eδ was generated by PCR amplification using primers EδXba3′F (5′-ACTACACTAAGAGTTG-GAGCA-3′) and EδDra5′R (5′-GCTAAATTCATCAG-GCAGTTG-3′) and pEδ3.8 as a template.

The MAR-deleted TCR δ minilocus construct was generated as follows. The 370-bp core Eδ was released from pE-P-Neo-scs′ (30) using *Xba*I and *Eco*RV, and was ligated into *Xba*I- and *Sma*I-digested pBK10.9, thereby replacing the MAR-Eδ-MAR region with the core Eδ. The resulting plasmid was digested with *Xba*I and *Kpn*I to release a 7.1-kb fragment containing the core Eδ along with Cδ. The Eδ minilocus plasmid (25) was also digested with *Xba*I and *Kpn*I to release the entire MAR-Eδ-MAR-Cδ region, and the 7.1-kb core Eδ–Cδ fragment was ligated in its place.

Nuclear Matrix Preparation and *in Vitro* MAR Assay. Histone and DNA-depleted nuclear matrices were prepared from 2×10^8 Jurkat cells according to the method of Cockerill and Garrard (14). The *in vitro* MAR assay was performed as described (14) using nuclear matrix from 1×10^7 cells, 200,000 cpm of ^{32}P-end-labeled DNA fragments, and 200 μg/ml sonicated *Escherichia coli* genomic DNA in an 80-μl binding reaction. End labeling was accomplished by using either the Klenow fragment of *E. coli* DNA polymerase or T4 polynucleotide kinase. Binding reactions were processed as described (14), and input and matrix-bound DNA fragments were identified by agarose gel electrophoresis followed by autoradiography of the dried gel.

***In Situ* MAR Assay.** Isolation of nuclei from 1×10^8 Raji cells and lithium 3,5-diiodosalicylate-extraction of histones to prepare nuclear halos were performed according to Mirkovitch *et al.* (31) and Fleenor and Kaufman (32). Nuclear halo aliquots equivalent to 1.5×10^7 cells were digested with *Bam*HI and *Bgl*II, and soluble and insoluble DNA fractions were purified as described (32). A total of 2.5 μg of DNA from each fraction was electrophoresed on a 1.0% agarose gel, transferred to a nylon membrane, and probed with DNA fragments that were ^{32}P-labeled by random priming. The DNA probes were Eδ (0.4-kb *Dra*I–*Xba*I fragment), blocking element alpha/delta-1 (BEAD-1) (2.5-kb *Eco*RI–*Nsi*I fragment), and Dδ1/2 (3.5-kb *Bam*HI fragment).

Production and Analysis of Transgenic Mice. Minilocus DNA was purified as described previously (25) and microinjected into fertilized (C57BL/6 × SJL) F_2 eggs by the Duke University Comprehensive Cancer Center Shared Transgenic Mouse Resource (Durham, NC). Transgene integrants were characterized for copy number and structure by analysis of undigested tail DNA on slot blots and of *Eco*RI- or *Bam*HI-digested tail DNA on genomic Southern blots by using ^{32}P-labeled human Cδ, Vδ1, and Eδ and murine Cα probes. Transgenes were maintained on a mixed C57BL/6 × SJL/J background. PCR analysis of minilocus rearrangement in genomic DNA prepared from thymocytes of 4- to 5-wk-old mice was performed as described previously (25).

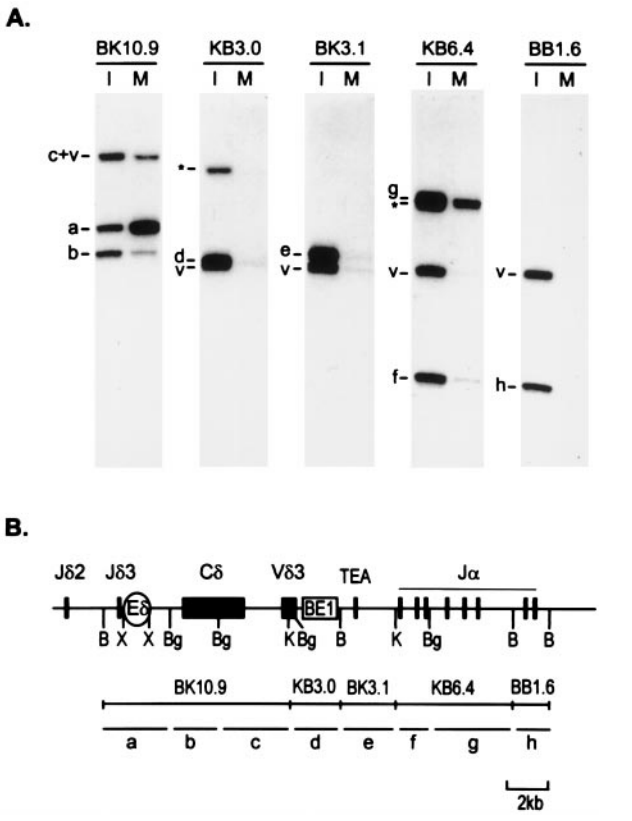

Fig. 1. Identification of nuclear matrix binding activity upstream of human Cδ by using an *in vitro* MAR assay. (*A*) DNA fragments generated from plasmids pBK10.9 (digested with *Bam*HI and *Bgl*II), pKB3.0 (digested with *Bam*HI and *Kpn*I), pBK3.1 (digested by *Bam*HI and *Kpn*I), pKB6.4 (digested with *Bam*HI, *Bgl*II and *Kpn*I), and pBB1.6 (digested with *Bam*HI) were end-labeled and tested for nuclear matrix binding as described in *Materials and Methods*. Input (I) and matrix-bound (M) fractions were analyzed by agarose gel electrophoresis and autoradiography. Lanes I represent 10% of the material added to the binding reaction, whereas lanes M represent the total bound material recovered. The vector fragment in each digest is indicated by ''v'', and partial digestion products are indicated by ''*''. (*B*) Schematic map of the human Cδ region and the plasmids and fragments tested in the *in vitro* assay. BE1 denotes the BEAD-1 element (30), and TEA denotes the T-early-alpha exon. B, *Bam*HI; X, *Xba*I; Bg, *Bgl*II; K, *Kpn*I.

Results

To identify MARs associated with the TCR δ gene, we initially assembled a small panel of plasmids containing a series of contiguous genomic segments that span a 25-kb region extending from Jδ3 into the Jα region (Fig. 1). End-labeled restriction fragments were incubated with a nuclear matrix preparation *in vitro* to identify fragments with affinity for the matrix. Plasmid BK10.9 released a 3.8-kb *Bam*HI–*Bgl*II fragment (fragment a) that was preferentially recovered in the matrix-bound fraction relative to fragments b and c+v that were also released from this plasmid [compare lane I (input) to lane M (matrix), Fig. 1*A*], suggesting the presence of a MAR in this fragment. Of note, fragment a also includes Eδ. Similar analysis revealed no obvious matrix association for any of the fragments released from additional plasmids KB3.0, BK3.1, and BB1.6. However, a 4.7-kb *Bgl*II–*Bam*HI fragment (fragment g) released from KB6.4

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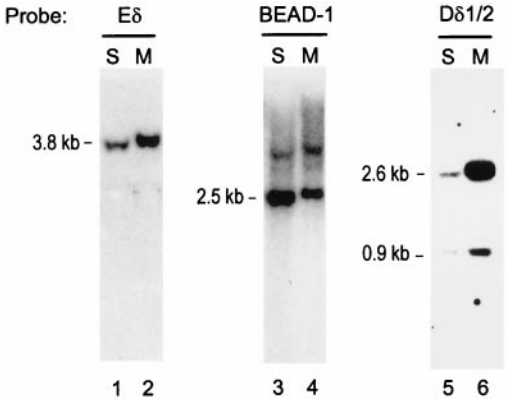

Fig. 2. Confirmation of MARs by using an *in situ* MAR assay. Soluble (S) and matrix-associated (M) DNAs were isolated from *Bam*HI plus *Bgl*II-digested nuclear halos and were analyzed on a Southern blot that was serially probed with radiolabeled Eδ, BEAD-1, and Dδ1/2 probes. Hybridization signals were quantified by PhosphorImager.

showed some enrichment in the matrix-bound fraction, suggesting the possibility of a weak MAR in this fragment as well.

We sought to confirm the finding of Eδ-associated MAR activity by using an assay that identifies regions of DNA that are directly associated with the nuclear matrix *in situ*. The human B cell lymphoma Raji contains the entire TCR α/δ locus in germ-line configuration on both chromosomes. Raji nuclei were used to prepare histone-depleted nuclear halos, which were digested with restriction enzymes *Bam*HI and *Bgl*II to release DNA fragments that are not directly associated with the nuclear matrix. Soluble (S) and matrix-associated (M) DNA fractions were then recovered and analyzed for specific DNA sequences by genomic Southern blot (Fig. 2). Hybridization with a ^{32}P-labeled Eδ probe revealed an apparent 3.2-fold enrichment of the 3.8-kb *Bam*HI–*Bgl*II fragment in the matrix-associated fraction relative to the soluble fraction (compare lanes 1 and 2). In contrast, reprobing of the blot with a ^{32}P-labeled BEAD-1 probe that detects a 2.5-kb *Bgl*II–*Bam*HI fragment contained within fragment d (Fig. 1*B*) revealed an apparent 2.8-fold enrichment in the soluble fraction (compare Fig. 2, lanes 3 and 4). The 3.8-kb *Bam*HI–*Bgl*II fragment was therefore enriched in the nuclear matrix approximately 9-fold relative to the control DNA fragment, confirming the results of the *in vitro* MAR assay.

The *in vitro* MAR assay was used to fine map MAR activity within fragment a (Fig. 3 *A–D*). Plasmid pEδ3.8 was digested to release intact insert fragment a or three subfragments of a, a 1.1-kb fragment i containing Jδ3, a 1.4-kb fragment j containing Eδ, and a 1.3-kb fragment k. As observed previously, fragment a associated preferentially with the nuclear matrix relative to vector fragment v (Fig. 3*A*). Among the subfragments, j and k both bound to the nuclear matrix, with binding of fragment j exceeding that of fragment k (Fig. 3*B*). In contrast, fragment i displayed no matrix binding activity relative to control fragment v. We then asked whether MAR activity within fragment j could be separated from Eδ. To do so, a plasmid containing the core 370-bp Eδ fragment was digested to liberate insert fragment m and vector fragment v. Fragment l, corresponding to the 1-kb region immediately 5′ of the core Eδ, was generated by PCR and mixed with restriction fragments m and v so that all fragments were present at similar molarity. Fragment l, but not fragment m, displayed potent *in vitro* nuclear matrix binding activity. Hence the core Eδ does not detectably bind to the nuclear matrix. Rather, the core Eδ is flanked by a relatively strong MAR on the 5′ side (fragment l) and a somewhat weaker MAR on the 3′ side (fragment k). This organization is remarkably similar to that at

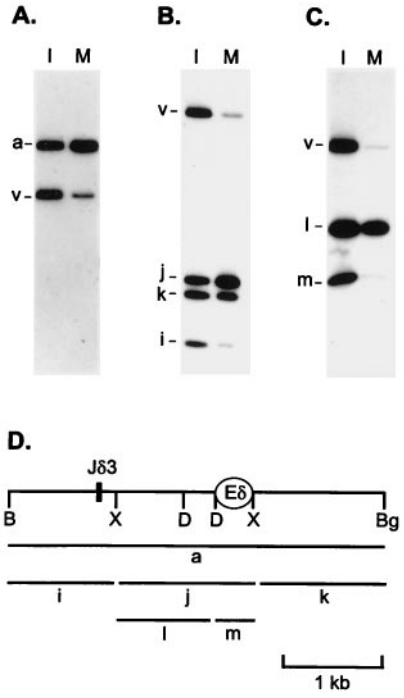

Fig. 3. Localization of two MARs flanking the core Eδ by using an *in vitro* MAR assay. DNA fragments released from pEδ3.8 by digestion with *Bam*HI and *Sal*I (*A*) or *Bam*HI, *Xba*I, and *Sal*I (*B*) were tested for *in vitro* binding to the nuclear matrix as in Fig. 1. The *Sal*I site is contributed by the plasmid vector. (*C*) Alternatively, DNA fragments were released from pEδ0.4 by digestion with *Bam*HI and *Sal*I and were mixed with PCR-generated fragment l. Fragments were end-labeled and tested for nuclear matrix binding *in vitro*. (*D*) Schematic map of the Eδ region and the fragments tested in the *in vitro* MAR assay. B, *Bam*HI; X, *Xba*I; D, *Dra*I; Bg, *Bgl*II.

the IgH locus, where the core Eμ is flanked by MARs both 5′ and 3′.

In screening for additional TCR δ-associated MARs, we also identified matrix binding activity in a region about 20 kb upstream of Eδ. Plasmid pDδBm3.5, which contains a 3.5-kb *Bam*HI fragment carrying the Dδ1 and Dδ2 gene segments, was digested to release intact insert (fragment n) and vector (fragment v). Fragment n bound selectively to the nuclear matrix *in vitro* (Fig. 4*A*). To better map MAR activity, the insert was further digested to produce the 2.0-kb fragment o, which carries the Dδ gene segments, and the 1.5-kb fragment p. Relative to fragment v, both fragments bound to the nuclear matrix, but enrichment in the bound fraction was particularly strong for fragment o (Fig. 4*B*). To confirm these results by using the *in situ* MAR assay, the Southern blot in Fig. 2 was reprobed to detect the Dδ1–Dδ2 region. A 2.6-kb fragment was enriched 13.4-fold in *Bam*HI- and *Bgl*II-digested matrix-associated DNA (37.5-fold relative to the non-matrix-associated BEAD-1 region), whereas a 0.9-kb fragment was enriched 5.3-fold in this fraction (14.8-fold relative to BEAD-1). Taken together, the *in vitro* and *in situ* mapping data indicate that a strong MAR is associated with Dδ1 and Dδ2.

Because MARs are found in close association with Eδ, we wondered whether they might impact Eδ function or Eδ-dependent processes. In previous studies (25), we developed a human TCR δ minilocus V(D)J recombination reporter substrate that includes Vδ1, Vδ2, Dδ3, Jδ1, Jδ3, Eδ, and Cδ (Fig. 5*A*). This reporter rearranges efficiently in thymocytes of transgenic mice. A specific step of transgene rearrangement, VD to J, is Eδ-dependent, as it is dramatically inhibited by deletion of a 1.4-kb fragment containing Eδ (25). Of note, both Eδ-

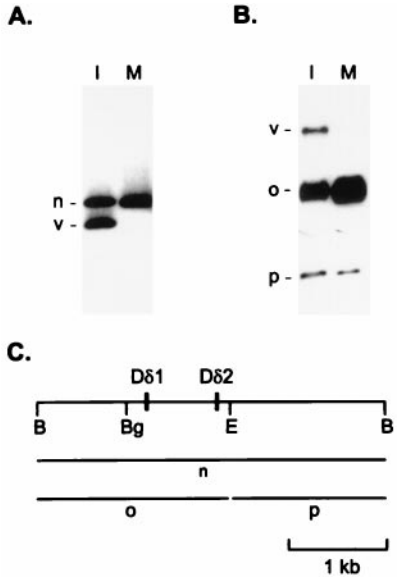

Fig. 4. Localization of a MAR associated with Dδ1 and Dδ2 by using an *in vitro* MAR assay. DNA fragments produced by *Bam*HI digestion (*A*) or *Bam*HI plus *Eco*RI digestion (*B*) of plasmid pDδBm3.5 were end-labeled and tested for nuclear matrix binding. (*C*) Schematic map of the Dδ1/Dδ2 region. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI.

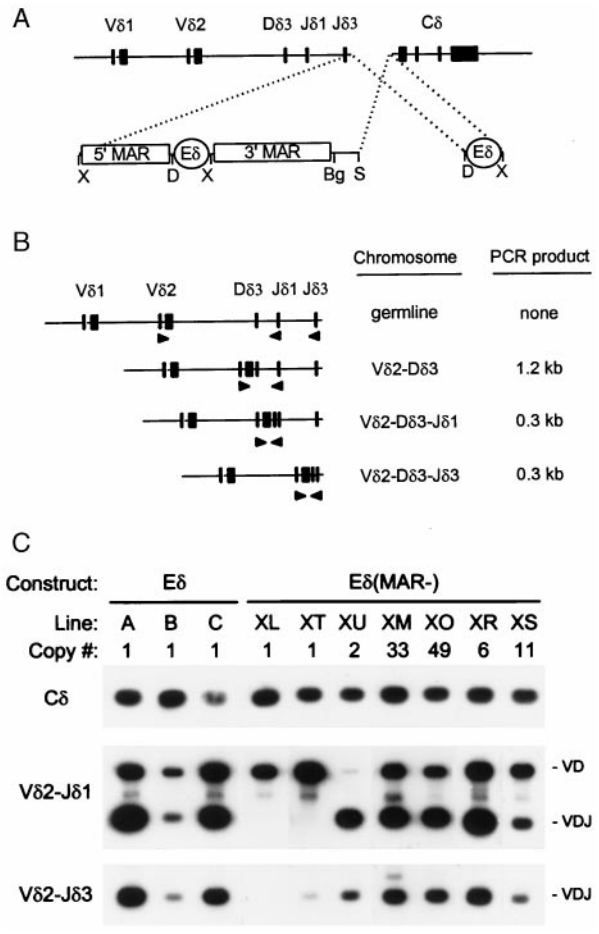

Fig. 5. Flanking MARs synergize with Eδ to stimulate V(D)J recombination. (*A*) Schematic representation of the Eδ wild-type and Eδ(MAR−) transgenic V(D)J recombination reporter substrates. X, *Xba*I; D, *Dra*I; Bg, *Bgl*II; S, *Sma*I. (*B*) PCR strategy to detect transgene Vδ2 rearrangement events. (*C*) Thymocyte genomic DNA preparations from transgenic lines carrying an Eδ minilocus or an Eδ(MAR−) minilocus were amplified by PCR using the Cδ primer pair, the Vδ2–Jδ1 primer pair, or the Vδ2–Jδ3 primer pair. Southern blots were probed with radiolabeled Cδ or Vδ2 DNA fragments. Transgene copy numbers were determined by comparison of human Cδ and control murine Cα hybridization signals on Southern blots of *Eco*RI-digested tail DNA.

associated MARs are contained within the wild-type construct, and the Eδ deletion removed not only the core Eδ, but the 5′ MAR as well. Nevertheless, as VD to J rearrangement is similarly inhibited by the introduction of discrete mutations into the core Eδ with flanking MARs intact (33, 34), it is clear that VD to J rearrangement requires core Eδ function, and that the MARs by themselves cannot provide this function. To determine whether the flanking MARs might synergize with Eδ to promote transgene VD to J rearrangement, we generated seven lines of transgenic mice containing a TCR δ minilocus with the core Eδ intact but flanking MARs deleted (Fig. 5*A*). Two of the Eδ(MAR$^-$) lines (XL and XT) were determined to carry single-copy integrations of the transgene; one of these (XT) was truncated at its 5′ end so that it lacked Vδ1, but retained Vδ2. The remaining lines (XU, XM, XR, XO, XS) had copy numbers ranging from 2 to 49, with the transgene integrated at a single site as a head-to-tail tandem array in each instance (data not shown). Three previously described Eδ lines (A, B, and C; Fig. 5*C*) served as controls (25). All of the Eδ lines carry single-copy integrations, with that in line C truncated in a fashion similar to that in line XT.

V(D)J recombination within the minilocus was assessed by a semiquantitative PCR assay using V- and J-specific primers, as described previously (25). Because two of the single-copy lines lacked Vδ1, we assessed rearrangements involving the Vδ2 gene segment. PCR using primer pair Vδ2–Jδ1 should amplify fully rearranged VDJ products of 0.3 kb and partially rearranged VD products of 1.2 kb, whereas PCR using primer combination Vδ2–Jδ3 should amplify fully rearranged VDJ products of 0.3 kb (Fig. 5*B*). Consistent with previous results, the Vδ2–Jδ1 primer pair detected fully rearranged VDJ products at high levels in Eδ lines A and C, and at a substantially lower level in Eδ line B (Fig. 5*C*; the comparatively low level of rearrangement in line B presumably reflects integration of the construct into inhibitory chromatin in this line). Similar analysis of Eδ(MAR$^-$) lines revealed two very distinct phenotypes that segregated according to copy number. Both single-copy lines displayed readily detectable VD products but essentially undetectable V(D)J products, much like previously analyzed lines carrying mutations in the core Eδ (33, 34). In striking contrast, the five multicopy lines displayed patterns of VD and VDJ rearranged products quite similar to those in the Eδ lines. Results for the Vδ2–Jδ3 primer pair closely paralleled those for the Vδ2–Jδ1 primer pair, with the exception that in this case, fully rearranged VDJ products were detectable at low levels in Eδ(MAR$^-$) line XT. Taken together, the results indicate that the Eδ-associated MARs have a major influence on Eδ-dependent V(D)J recombination events in single-copy transgene integrants, but have little or no influence on these events in multicopy integrants.

Discussion

In this report, we used a combination of *in vitro* and *in situ* approaches to localize three MARs associated with the human TCR δ gene. Two of these are located within the Jδ3–Cδ intron, flanking the core Eδ both 5′ and 3′. As MARs have also been identified in close association with the intronic enhancers of the IgH, Igκ, and TCR β loci, the juxtaposition of enhancers and MARs seems a highly conserved feature of antigen receptor genes that should reflect a significant functional relationship. We tested this notion by analyzing the function of the Eδ-

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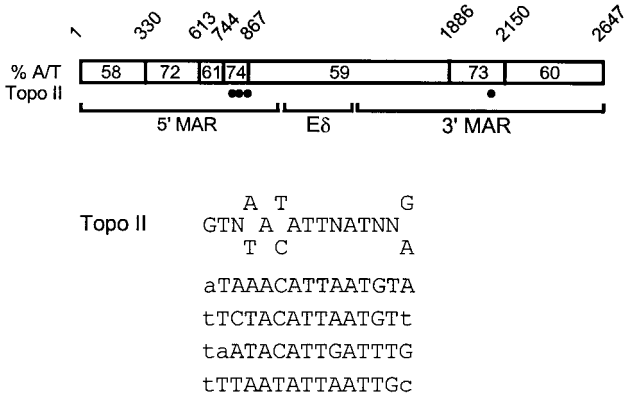

Fig. 6. Sequence features of the MAR-Eδ-MAR region. Numbering runs from the *Xba*I site upstream of the 5′ MAR to the *Bgl*II site downstream of the 3′ MAR. Regional A/T content and near-consensus Topoisomerase II sites (filled dots) are identified. The topoisomerase II consensus sequence (35) and four close matches are provided, with mismatched residues represented by lowercase lettering.

associated MARs within the context of a transgenic V(D)J recombination reporter substrate, and we provide evidence that these MARs function synergistically with the core Eδ to promote V(D)J recombination in this system. To our knowledge, this is the first report documenting a role for MARs as positive regulators of V(D)J recombination.

A striking aspect of our data is the dramatic variation in the V(D)J recombination phenotypes of the Eδ(MAR$^-$) transgenic lines. This variation cannot readily be attributed to integration site effects. Negative chromosomal position effects should affect not only the enhancer-dependent VD to J step of transgene rearrangement, but the enhancer-independent V to D step as well. Detection of V to D rearrangement at high levels in all transgenic lines provides an effective internal control that argues for transgene integration into permissive chromatin in every instance. This leads us to conclude that the V(D)J recombination phenotype of Eδ(MAR$^-$) transgenic lines depends on minilocus copy number, and that the influence of MAR deletion on V(D)J recombination is apparent only in single-copy integrants of the transgene.

The core Eδ flanking regions identified in this study have sequence properties consistent with those of other MAR elements. MARs typically display an A/T content of 70% or more and often contain consensus sites for topoisomerase II binding and cleavage (11). The core Eδ itself sits within a 1019-bp region with an A/T content of 59% (Fig. 6). Within the 5′ flank, there is a 283-bp region that is 72% A/T and a 123-bp region that is 74% A/T. The 3′ flank contains a 264-bp region that is 73% A/T. The A/T-rich segment immediately 5′ of the core Eδ displays one sequence with a 14/15 match, and two sequences with 13/15 matches, to the topoisomerase II consensus (35), and the A/T-rich segment in the 3′ flank displays a single site with a 13/15 match to the consensus. Two lymphoid-specific MAR binding proteins have been identified, SATB1, which is expressed predominantly in the thymus, and Bright, which is B cell-specific (36, 37). Both proteins contact DNA in the minor groove with only limited contact to the DNA bases and limited sequence specificity, but appear to interact with sequences displaying a central A/T-rich core embedded within a region in which only A, T, and C residues are present on one strand (ATC sequence). Many sequences fitting this description can be identified within the 5′ and 3′ Eδ flanks, including a sequence as long as 33 bp in the 5′ flank, and sequences as long as 43 and 30 bp in the 3′ flank. Whether any serve as bona fide binding sites for known MAR binding proteins remains to be determined.

As noted previously, different assay systems have provided discrepant data regarding the functional importance of MARs in TCR and Ig loci. For example, the IgH and Igκ MARs have dramatic functional consequences when tested in transgenic reporters (19, 20, 23). Although deletion of the Igκ MAR from the endogenous locus reveals a phenotype at least partially overlapping with that identified in transgenic mice (24), there appears to be no obvious function revealed by deletion of the endogenous IgH MARs (21). Although transgenic reporters could provide misleading information because they might not accurately recreate the natural chromosomal environment, such reporters could actually be more sensitive indicators of bona fide activities of MARs that are masked at the endogenous locus because of functional redundancy. Therefore, it is of interest to note the presence of a potent MAR associated with Dδ1 and Dδ2, approximately 20 kb 5′ to the MARs that flank Eδ. If additional, relatively close MARs were also present in the IgH locus, their presence might explain the null phenotype in gene-targeted mice.

Previous studies have indicated that the MARs flanking the core Eμ are required for high level, integration site-independent and copy number-dependent Eμ-driven transcription in B cells of transgenic mice (19). In other words, the MAR-Eμ-MAR region has LCR-like properties that the core Eμ, by itself, lacks. We do not think that the Eδ-associated MARs function in precisely the same way. First, as evidenced by the variable levels of transgene rearrangement occurring in wild-type lines A, B, and C, even an intact MAR-Eδ-MAR region apparently cannot overcome chromosomal position effects. Second, as noted above, the V to D rearrangement internal control indicates that the Eδ(MAR$^-$) transgene has integrated into permissive chromatin in all instances. Thus, our results point to a requirement for synergy between MARs and the core Eδ, even in the context of permissive chromatin.

The nature of the synergy between Eδ and the flanking MARs is unclear. Previous data indicated that the core Eμ could establish a local region of accessible chromatin in the absence of flanking MARs, but that the flanking MARs were necessary for the enhancer to establish a larger accessible chromatin domain and to stimulate transcription from a linked promoter (20). Reports of high mobility group-I/Y-mediated displacement of histone H1 at MARs (38) and an association of SWI/SNF chromatin remodeling components with the nuclear matrix (39) suggest potential mechanisms for MAR-dependent alterations in chromatin structure. We have previously shown that enhancer control of V(D)J recombination within the TCR δ minilocus results from enhancer control of Jδ gene segment accessibility to the recombinase (27). Hence, the Eδ-associated MARs could modulate V(D)J recombination by either directly or indirectly influencing chromatin structure in the region. If these MARs indeed function to propagate a region of accessible chromatin from Eδ to the Jδ segments, as described for the Eμ-associated MARs, we might expect the effects of MAR deletion to be distance dependent. Thus, MAR deletion might have a greater effect on rearrangements involving Jδ1 than on rearrangements involving Jδ3, because in the Eδ(MAR$^-$) minilocus, Jδ3 is only 250 bp upstream of the core Eδ, whereas the distance to Jδ1 is 2 kb. Low level detection of fully rearranged Vδ2–Dδ3–Jδ3 products, but not Vδ2–Dδ3–Jδ1 product in transgenic line XT is consistent with this notion (Fig. 5*C*). However, because a similar result was not obtained for transgenic line XL, the results do not unambiguously distinguish between a propagated effect or another mechanism for synergy.

It is intriguing that the failure of VD to J recombination characteristic of the single-copy integrants is in some way compensated in the multicopy integrants. Although the basis for

this is not clear, the compensation phenomenon suggests that the transgene carries a distinct regulatory element that, in two or more copies, functions redundantly with the MARs, or otherwise obviates the need for MARs. This element is unlikely to be another MAR, as MAR activity was not detected in other segments that comprise the transgene (Fig. 1*A*, and data not shown). One possibility is that the compensating element is the core Eδ itself. It may be the case that two or more copies of the core Eδ, even though in different transgene copies, may obviate the need for MARs because they can synergize with each other to establish an extended region of accessible chromatin. Alternatively, it could be the case that the core Eδ, in multiple copies, displays true functional redundancy with the MARs. Because transcription factors may be matrix-associated rather than free in the nucleus, enhancer occupancy by transcription factors may recruit the enhancer to the nuclear matrix (10). Core Eδ occupancy could be intrinsically unstable and incapable of such recruitment by itself, therefore requiring flanking MARs to bring the enhancer into an environment that is rich in transcription factors and that promotes stable occupancy. However, two or more copies of the core Eδ might function cooperatively to convert the intrinsically unstable occupancy and association with the nuclear matrix into a more stable association, which would further stabilize enhancer occupancy. Although the mechanisms for enhancer-MAR synergy and multicopy compensation are uncertain, our experimental system may prove useful for further exploration of these issues in future studies.

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