Brief Definitive Report

Regulation of T Cell Receptor δ Gene Rearrangement by c-Myb

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Summary

Developmental activation of VDJ recombination at the T cell receptor (TCR) δ locus is controlled by an intronic transcriptional enhancer (E_δ). Transcriptional activation by E_δ is dependent on c-Myb. To determine whether c-Myb plays a role in the activation of TCR-δ gene rearrangement, we compared VDJ recombination in transgenic mice carrying two versions of a human TCR-δ gene minilocus recombination substrate. One includes a wild-type E_δ, whereas the other carries an E_δ with a mutation that abrogates c-Myb binding. We demonstrate that an intact Myb binding site is necessary for efficient rearrangement of the minilocus substrate, suggesting that c-Myb plays a crucial role in activating VDJ recombination at the endogenous TCR-δ locus.

The protein c-Myb plays important roles in the differentiation and proliferation of hematopoietic cells (1, 2). Gene-targeted c-*myb*–homozygous mutant mice die in utero and display severe anemia and other defects in hematopoiesis by fetal day 14.5 (3). Further, T cell–specific overexpression of dominant-negative *myb* alleles in transgenic mice results in impaired T cell proliferation and differentiation (4). However, because inhibition of Myb function has pleiotropic effects, specific roles for Myb proteins in vivo have remained elusive. Myb proteins activate transcription by specific binding to the nucleotide sequence PyAAC$^{T}/_{G}$G (1, 2). Yet only a few genes that are specifically expressed in hematopoietic cells are known to be Myb targets (5–10). We have previously shown in transient transfection experiments that TCR-δ gene transcription is regulated by the T lineage–specific TCR-δ enhancer (E_δ)[1] (11), and that the binding of c-Myb to the δE3 element of E_δ is essential for enhancer activity (7).

The regulated activation of VDJ recombination at TCR and Ig gene loci is an essential feature of lymphoid cell development (12, 13). VDJ recombination has been correlated with germline transcription, suggesting a link between the two processes (14–18). Direct evidence that transcriptional promoters and enhancers can regulate VDJ recombination in developing lymphocytes has been obtained from studies of transgenic mice that carry recombination substrates (19–24) and from studies that use homologous recombination to eliminate regulatory elements from endogenous loci (25–27).

We have previously studied VDJ recombination in transgenic mice carrying an unrearranged human TCR-δ gene minilocus (22, 23). This construct includes germline $V_\delta1$, $V_\delta2$, $D_\delta3$, $J_\delta1$, $J_\delta3$, and C_δ segments, as well as the TCR-δ enhancer (E_δ) within the $J_\delta3$-C_δ intron. Frameshift mutations in the V segments prevent the expression of functional TCR chains such that the transgene serves as a neutral reporter of VDJ recombination. We found that transgene rearrangement is T cell specific and occurs stepwise, first V to D and then VD to J. Notably, in most lines of transgenic mice carrying a minilocus with E_δ deleted, the initial V to D step still occurs, but the subsequent VD-to-J step is dramatically inhibited. This result suggests that E_δ is required for J segment accessibility, but is not required for V or D segment accessibility. This system therefore offers the opportunity to test the role of specific *cis*-acting enhancer sequences in regulating VDJ recombination. In this study, we specifically address the role of Myb proteins in the recombinational enhancer activity of E_δ.

Materials and Methods

Production of Transgenic Mice. E_δ with a Myb–binding site mutation (E_δmMyb) was generated by PCR using as a template the 1.4-kb wild-type E_δ subcloned into the XbaI site of pBluescript KS+ (1.4E_δBS). Mutagenic oligonucleotides MybPCRup and MybPCRdn that include a 2-bp change in the δE3 element and generate a HindIII site were described (7). Linearized plasmid was subjected to PCR in two different reactions including either oligonucleotides MybPCRup and E_δPCR (AAGGTTTAATTC-

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; E_δ, TCR-δ enhancer; E_δmMyb, E_δ with a TCR-δ enhancer with a Myb–binding site mutation; 1.4E_δBS, 1.4-kb wild-type E_δ subcloned into the XbaI site of pBluescript KS+; E^-, enhancerless.

AGTCAGAC) or MybPCRdn and the reverse primer. The 5′ 550-bp fragment was digested with HindIII and PflMI, the 3′ 290-bp fragment was digested with HindIII and BamHI, and the two were ligated together into PflMI- and BamHI-digested 1.4E_δBS. The insert was sequenced to confirm its structure, after which the 1.4-kb E_δmMyb was excised with XbaI and cloned into XbaI-digested, phosphatase-treated pBluescript carrying the enhancerless minilocus (22). Minilocus DNA was purified and microinjected into fertilized C57BL/6 × SJL F2 eggs as described previously (22). Transgenes were maintained on a mixed C57BL/6 × SJL background.

PCR. The preparation of genomic DNA, conditions for PCR, probes for Southern blot analysis, and quantification of PCR signals were as described (22). The amount of template DNA used for PCR reactions was 12 ng for single-copy integrants. Template quantity was reduced for multicopy integrants to account for copy number and to keep all PCR signals in the linear range. Reported VD and VDJ rearrangement signals were normalized to the C_δ signal for each DNA sample.

Transfection and Chloramphenicol Acetyl Transferase (CAT) Assays. Enhancers were cloned upstream of the $V_\delta1$ promoter in XbaI-digested and phosphatase-treated $V_\delta1$-CAT (11). The human T cell line Jurkat was transfected, and CAT assays were performed as described (7, 11).

Results

A 2-bp mutation within the δE3 element of a 370-bp E_δ fragment eliminates in vitro binding of c-Myb and abrogates transcriptional activation (7). The same 2-bp mutation was introduced into the 1.4-kb E_δ fragment previously shown to regulate transgene rearrangement to generate E_δmMyb (Fig. 1 *A*). We first examined the effect of the Myb binding site mutation on E_δ transcriptional activity by subcloning E_δ and E_δmMyb into the enhancer-dependent test construct $V_\delta1$-CAT, and assaying CAT activity after transient transfection of the constructs into Jurkat cells (Fig. 2). E_δ was active in both orientations (38.7- and 34.8-fold induction), whereas E_δmMyb was completely inactive (0.5- and 0.2-fold induction). Thus, an intact Myb binding site within δE3 is essential for the transcriptional activity of the 1.4-kb E_δ as assayed by transient transfection.

E_δmMyb was then substituted for the wild-type E_δ within the transgenic minilocus (Fig. 1 *A*), and three independent lines of transgenic mice carrying the mutant enhancer were established. Minilocus rearrangements in three transgenic lines carrying wild-type E_δ (A, B, and C) (22) were compared with those in the three transgenic lines carrying E_δmMyb (P, Q, and R). E_δ lines A, B, and C each carry a single copy of the minilocus (22). Among the E_δmMyb lines, P carries three copies, Q carries four copies, and R carries 15 copies. To assess transgene rearrangement, thymocyte genomic DNA templates were analyzed by quantitative PCR, and specific PCR products were identified by hybridization with radiolabeled $V_\delta1$ and $V_\delta2$ cDNA probes (Fig. 1 *B*). The primer combinations $V_\delta1$-$J_\delta1$ and $V_\delta2$-$J_\delta1$ can amplify a product of 0.3 kb that reflects VDJ rearrangement and one of 1.2 kb that reflects VD rearrangement (22). The primer combinations $V_\delta1$-$J_\delta3$ and

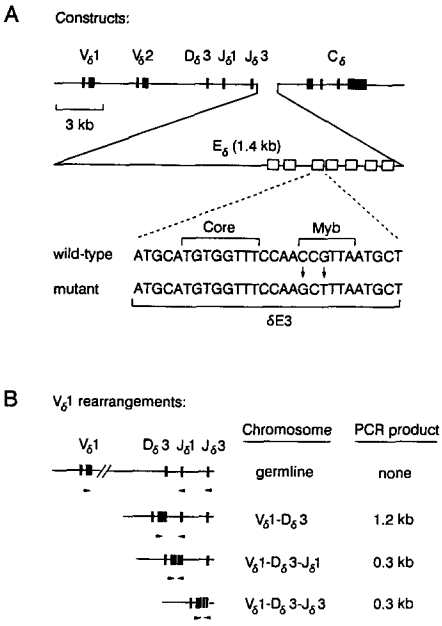

Figure 1. Human TCR-δ gene minilocus. (*A*) Filled boxes represent exons, open boxes represent protein-binding sites within E_δ, and sequences of wild-type and mutant δE3 elements are shown. (*B*) $V_\delta1$ rearrangement products generated using $V_\delta1$, $J_\delta1$, and $J_\delta3$ primers are depicted. Arrows denote PCR primers. A similar set of $V_\delta2$ rearrangement products are generated using $V_\delta2$, $J_\delta1$, and $J_\delta3$ primers. No products are amplified from unrearranged templates.

$V_\delta2$-$J_\delta3$ amplify only a 0.3-kb product that reflects VDJ rearrangement (Fig. 1 *B*). Amplification was also performed using a pair of C_δ primers as an internal control. Of note, E_δ line C carries a truncated minilocus that lacks the $V_\delta1$ gene segment, limiting the analysis to $V_\delta2$ rearrangements in this line (22).

Consistent with previous experiments (22), VDJ rearranged products were readily detectable with all four primer combinations in E_δ lines A and B, and with $V_\delta2$ and J_δ primers in E_δ line C (Fig. 3 *A*). Furthermore, as assessed using the primer combinations $V_\delta1$-$J_\delta1$ and $V_\delta2$-$J_\delta1$, VDJ-

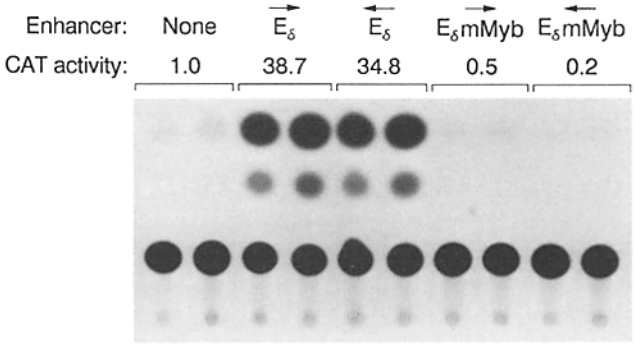

Figure 2. Transcriptional activation by wild-type E_δ and E_δmMyb. The indicated enhancers were tested in both orientations (*arrows*) upstream of the $V_\delta1$ promoter in the enhancer-dependent test construct $V_\delta1$-CAT. Jurkat cells were transfected in duplicate, and values for percent chloramphenicol acetylation were averaged and then normalized to the activity of the E^- $V_\delta1$-CAT construct.

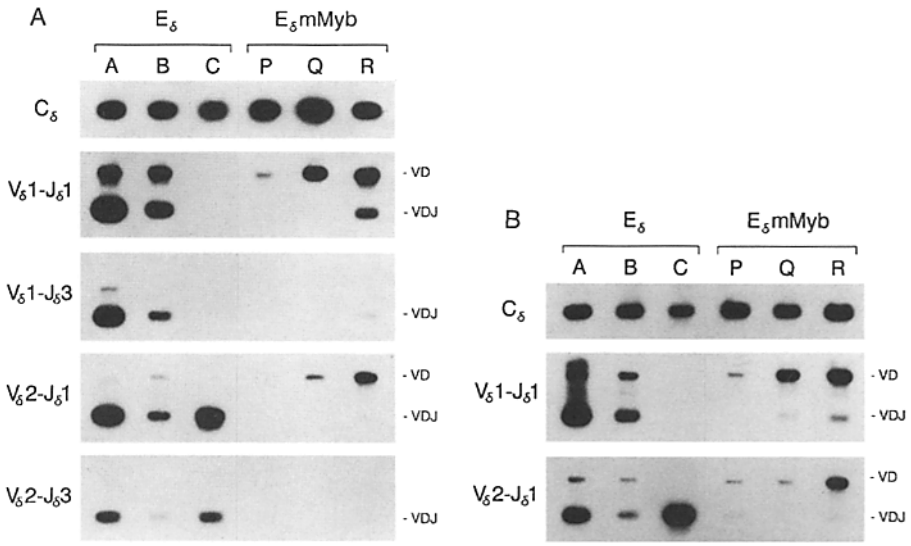

Figure 3. Minilocus rearrangement analyzed by PCR. (*A*) Genomic DNA from thymi of wild-type E_δ mice from lines A, B, and C, and from E_δmMyb mice from lines P, Q, and R were amplified by quantitative PCR using the indicated primers, and Southern blots were developed using radiolabeled $V_\delta1$ or $V_\delta2$ cDNA probes. A pair of C_δ primers and a radiolabeled C_δ probe served as internal controls. The mice analyzed were A584 (3 wk old), B31 (8 wk old), C114 (4 wk old), P13 (4 wk old), Q29 (2 wk old), and R18 (4 wk old). (*B*) Analysis of additional E_δmMyb mice P34 (8 wk old), Q60 (5 wk old), and R41 (6 wk old). E_δ mice were identical to those in *A*.

rearranged products were more abundant than VD-rearranged products in each case, indicating that the enhancer-dependent VD-to-J step of transgene rearrangement is highly efficient. Remarkably, the E_δmMyb lines displayed a very different phenotype. In all three lines and with every primer combination, the absolute amount of VDJ rearrangement was dramatically inhibited. Relative to E_δ line A, the levels of $V_\delta1$-$D_\delta3$-$J_\delta1$ rearrangement in the E_δmMyb lines were 0.1% in P (i.e., [0.036/25.618] × 100), 0.2% in Q, and 5.4% in R, and the levels of $V_\delta1$-$D_\delta3$-$J_\delta3$ rearrangement were 0.1% in line P, 0.1% in Q, and 1.5% in R (Table 1). Levels of $V_\delta2$ rearrangements were similarly diminished. Furthermore, in all three lines, VD-rearranged products were more abundant than VDJ-rearranged products, indicating that the VD-to-J step of transgene rearrangement was preferentially inhibited. Some inhibition of the V-to-D step of rearrangement was nevertheless apparent, most notably in line P. Similar conclusions were drawn from analysis of a second individual in each of the E_δmMyb-transgenic lines (Fig. 3 *B*).

Table 1. *Quantification of Minilocus Rearrangement*

	E_δ			E_δmMyb		
	A	B	C	P	Q	R
$V_\delta1$-$D_\delta3$	4.491	4.423	nd	0.322	2.391	6.194
$V_\delta1$-$D_\delta3$-$J_\delta1$	25.618	5.255	nd	0.036	0.059	1.388
$V_\delta1$-$D_\delta3$-$J_\delta3$	14.110	1.452	nd	0.015	0.011	0.215
$V_\delta2$-$D_\delta3$	0.051	0.060	0.012	nd	0.045	0.427
$V_\delta2$-$D_\delta3$-$J_\delta1$	2.297	0.261	1.219	nd	0.007	0.026
$V_\delta2$-$D_\delta3$-$J_\delta3$	0.419	0.064	0.260	nd	nd	0.016

Hybridization signals from the PCR experiment presented in Fig. 3 *A* were quantified using a Betascope (Betagen, Waltham, MA). The reported VD and VDJ rearrangement signals were normalized to the C_δ signal for each DNA sample. *nd*, not detectable.

We also analyzed minilocus rearrangement directly by genomic Southern blot (Fig. 4). Unlike the PCR experiments, in which DNA samples were adjusted to normalize for copy number before analysis, in this experiment similar quantities of genomic DNA were analyzed. In accord with the PCR data, thymus DNA from E_δ line A displayed a low level of $V_\delta1$-$D_\delta3$ rearrangement (0.9 kb), and higher levels of $V_\delta1$-$D_\delta3$-$J_\delta1$ and $V_\delta1$-$D_\delta3$-$J_\delta3$ rearrangement (1.7 and 3.2 kb, respectively). This rearrangement profile was dramatically perturbed in E_δmMyb lines P, Q, and R. Rearrangement was undetectable in line P. Line Q displayed a high level of VD rearrangement but no VDJ rearrangement, whereas line R displayed a high level of VD rearrangement and a very low level of VDJ rearrangement. Notably, VDJ rearrangement in line R was just barely detected by genomic Southern blot despite a 15-fold increase in transgene copy number relative to E_δ line A. The South-

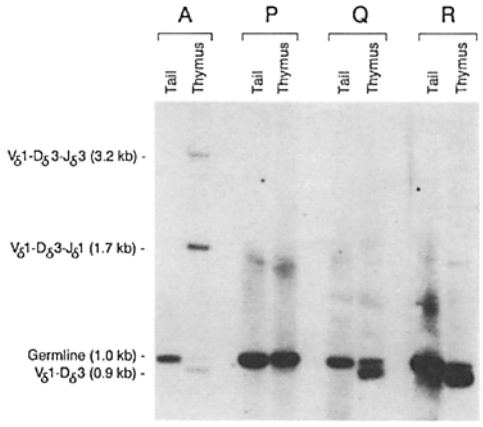

Figure 4. Minilocus $V_\delta1$ rearrangement analyzed by genomic Southern blot. PstI plus EcoRI-digested tail (germline control) and thymus DNA samples (7 μg) from lines A, P, Q, and R were electrophoresed through 1% agarose and blotted onto a nylon membrane. The blot was probed with a radiolabeled 1.0-kb $V_\delta1$ genomic PstI fragment (22). Mice A584, P13, Q29, and R18 are analyzed.

ern blot data are therefore highly consistent with those of PCR.

The quantitative differences among E_δmMyb lines could result from differences in the properties of transgene integration sites, differences in transgene copy number, or both. Importantly, the range of phenotypes displayed by the E_δmMyb-transgenic lines is very similar to the range of phenotypes displayed by lines of transgenic mice carrying distinct integrations of an enhancerless (E^-) minilocus (22). We conclude that the E_δmMyb minilocus is functionally equivalent to an E^- minilocus, and that disruption of the δE3 Myb-binding site essentially eliminates the ability of E_δ to activate VDJ recombination.

Discussion

c-Myb clearly has pleiotropic effects on cell proliferation and differentiation within multiple hematopoietic lineages. It has therefore been difficult to obtain information from either gene-targeting or dominant negative approaches that would implicate c-Myb in the regulation of specific molecular events. As an alternative, we chose to disrupt a functional Myb-binding site within a phenotypically neutral VDJ recombination reporter construct in transgenic mice. This approach has the clear advantage over the gene-targeting and dominant-negative approaches in that any defect in VDJ recombination must be a direct effect of the mutation, rather than an indirect effect that is secondary to a developmental perturbation. Using this strategy, we found that efficient VDJ recombination of a TCR-δ gene reporter construct requires an intact Myb-binding site within the δE3 element of E_δ.

Our data argue strongly that a member of the Myb family plays a crucial role in activating TCR-δ gene rearrangement. However, the disruption of a *cis*-acting element cannot by itself formally implicate c-Myb. Nevertheless, we have previously shown that c-Myb can bind to the δE3 site in vitro and can transactivate gene expression in vivo (7). Furthermore, the activation of TCR-δ gene rearrangement correlates closely with the apparent onset of c-Myb expression in developing hematopoietic cells. c-Myb is known to be expressed at highest levels in immature thymocytes (28), and the defect in hematopoiesis in c-Myb knockout mice is first apparent at day 14.5 of fetal development (3). In accord with this, VDJ recombination at the endogenous murine TCR-δ locus occurs in the immature $CD3^-CD4^-CD8^-$ population of postnatal thymocytes and is initiated at day 14.5 of fetal development (29, 30), and the enhancer-dependent VD-to-J step of transgenic minilocus rearrangement is activated in the same population of postnatal thymocytes and at the same stage of fetal thymocyte development (23). Given all of the available data, we conclude that c-Myb plays a direct role in the developmental activation of VDJ recombination at the TCR-δ locus.

It is generally accepted that enhancers regulate VDJ recombination by modulating the accessibility of chromosomal substrates to the recombinase (12). However, the mechanism by which accessibility is regulated is poorly understood. Although VDJ recombination correlates with germline transcription (14–18), a causal relationship has not been established (24, 31, 32). Our results implicate a transcription factor that is known to be important for TCR-δ gene transcription as an important regulator of VDJ recombination. Although consistent with the correlation between transcription and rearrangement, our results do not necessarily argue that transcription is causal in activating VDJ recombination. Enhancers can affect local chromatin accessibility, even in the absence of detectable transcription (33, 34). Thus, the binding of c-Myb to E_δ could play a direct role in modulating TCR-δ locus accessibility to the recombinase that is at least in part distinct from its role in transcription. Additional studies are clearly necessary to resolve this issue.

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Note added in proof: Recent data indicates that an additional E_δmMyb transgenic line (O) displays a phenotype that is consistent with those of lines P, Q, and R.

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