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Characterization of Chromatin Structure and Enhancer Elements for Murine Recombination Activating Gene-2¹

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Recombination-activating genes (*RAGs*) play a critical role in V(D)J recombination machinery and their expression is specifically regulated during lymphocyte ontogeny. To elucidate the molecular mechanisms regulating murine *RAG-2* expression, we examined a chromatin structure of 25-kb DNA segment adjacent to murine *RAG-2* by analyzing DNase I hypersensitive (HS) sites. In a *RAG-2*-expressing murine pre-B cell line, three lymphoid cell-specific HS sites (HS1, HS2, and HS3) were identified. Among these HS sites, one HS site (HS3) that locates in the *RAG-2* promoter was associated only with *RAG-2*-expressing cell lines. Using the transient enhanced green fluorescence protein reporter gene assays, we identified two enhancer elements in the 5'-upstream region of *RAG-2* that corresponded to HS1 and HS2. One of the enhancer elements (D3) exhibited enhancer activity only in the lymphoid cell lines. Analysis of the transgenic mice carrying the enhanced green fluorescence protein-reporter gene linked with D3 revealed that D3 activated the reporter gene-expression in the primary lymphoid tissues, but not in the secondary lymphoid tissues or nonlymphoid tissues. D3 was active in CD4⁻CD8⁻, but not in CD4⁺CD8⁺ or CD4⁺CD8⁻ thymocytes in the thymus, and also active in B220⁺IgM⁻, but not in B220⁺IgM⁺, cells in the bone marrow. Finally, our data suggested that C/EBP may bind to the D3 enhancer and function as one of the transcription factor(s) responsible for the enhancer activity. These results show that the tissue- and stage-specific expression of murine *RAG-2* is regulated by alteration of the chromatin structure as well as *cis*-regulatory enhancer elements. *The Journal of Immunology*, 2002, 169: 873–881.

During the development of B and T lymphocytes, *Ig* and *TCR* variable region genes are assembled from germline V, (D), J gene segments by a site-specific recombination reaction known as V(D)J recombination (1). The V(D)J recombination is initiated by the products of recombination activating gene (*RAG*)⁵-1 and *RAG-2* (2, 3). They act together to recognize recombination signal sequences that flank V, D, J gene segments and introduce the dsDNA breaks between the recombination signal sequences and the gene segments (4). The expression of *RAG-1* and *RAG-2* is restricted to lymphoid cells undergoing V(D)J gene rearrangement (5–8). In the absence of either the *RAG-1* or *RAG-2* gene products, the development of mature lymphocytes is completely abrogated, and hence results in severe combined immune deficiency in mouse and human (9–11). In humans, missense mutations in either the *RAG-1* or *RAG-2* result in Omenn syndrome, which is characterized by a few T lymphocyte repertoires (12).

The transcription of *RAG* is regulated at different levels. Fuller et al. (13, 14) have reported, and we have also reported, that the DNase I hypersensitive (HS) site was identified in the promoter region of mouse and human *RAG-1* only in *RAG*-expressing lymphocytes, indicating that *RAG*-expression is regulated at the chromatin level. At the promoter level, human *RAG-1* and *RAG-2* promoter regions did not show the lymphocyte-specific expression of the reporter gene in vitro (14–17). On the contrary, it was shown that the mouse *RAG-2* promoter conferred lymphoid specificity in the reporter gene assay and that the distinct transcriptional factors regulate lineage-specific activation of the *RAG-2* promoter in B- and T-lineage cells (18–21). Recently it was shown that *cis*-elements other than the *RAG-2* promoter region were also involved in driving the lymphoid- and differentiation stage-specific *RAG-2*-expression in vivo (22, 23). These indicated the existence of the *cis*-regulatory element, such as enhancer, in the 5'-upstream region of mouse *RAG-2*.

In this study, we analyzed the DNase I HS sites of 25-kb DNA sequences adjacent to the murine *RAG-2* locus and identified three lymphoid-specific DNase I HS sites, one of which corresponded to the promoter region and specifically associated with *RAG-2* expression. We further identified that two enhancer elements in this 25-kb DNA both corresponded to HS sites. We found that one of the enhancer elements (distal enhancer) exhibited enhancer activity only in lymphoid cell lines in transient transfection assays. To test whether the enhancer functions in vivo, we generated and analyzed the transgenic mice carrying enhanced green fluorescence protein (EGFP) reporter gene connected to the distal enhancer element. We show that this enhancer was active in thymus and bone marrow. We further demonstrate that EGFP was expressed only in the CD4⁻CD8⁻ subpopulation in the thymus and B220⁺IgM⁻ cells in the bone marrow. Our data suggest that chromatin structure and the *cis*-regulatory element may regulate the tissue- and stage-specific expression of murine *RAG-2*. We also demonstrate that

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⁵ Abbreviations used in this paper: *RAG*, recombination activating gene; HS, hypersensitive; EGFP, enhanced green fluorescence protein; TK, thymidine kinase; AML, acute myeloid leukemia; Lf, lymphoid transcription factor; LCR, locus control region.

C/EBP may bind to this element and function as one of the activator(s) or coactivator(s) for the enhancer activity.

Materials and Methods

Cell lines and cell culture

18.81 (pre-B cell, *RAG-2*⁺), BAL-17 (B cell, *RAG-2*⁺), WEHI231 (B cell, *RAG-2*⁻), LSB11-1 (T cell, *RAG-2*⁺) (24), LSB11-14 (T cell, *RAG-2*⁺) (24), EL4 (T cell, *RAG-2*⁻), 110TC (T cell, *RAG-2*⁻) (24), WEHI3 (myeloid, *RAG-2*⁻), L (fibroblast, *RAG-2*⁻), and NIH3T3 (fibroblast, *RAG-2*⁻) were grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in 5% CO₂.

Analysis of DNase I HS sites

DNase I HS sites were analyzed as previously described (14). Briefly, 1.5×10^7 nuclei were treated with 0–2 U/ μ l of DNase I (Takara, Kyoto, Japan) at 25°C for 3 min. The reaction was stopped by addition of 3 ml of proteinase K solution (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K). Then the genomic DNA was extracted, digested by appropriate restriction enzymes, electrophoresed on 0.7% agarose gel, blotted onto GeneScreen Plus membrane (NEN, Boston, MA), and hybridized with a ³²P-labeled mouse *RAG-2* fragment.

Construction of plasmids

For the preparation of the EGFP construct with the minimal mouse *RAG-2* promoter (pR2P-EGFP), the -86/+147 region of the murine *RAG-2* promoter (18) was inserted into the *Sall* and *Bam*HI sites of the pEGFP-1 (Clontech Laboratories, Palo Alto, CA). To analyze the 5'-upstream region of murine *RAG-2*, a λ DNA clone containing the 5'-upstream region of murine *RAG-2* was cloned by screening the λ FIX II murine genomic DNA library (Stratagene, La Jolla, CA). An 8-kb *Hind*III-*Hind*III fragment (Fig. 1A) was cut out and subcloned into pBluescript II SK⁺ (Stratagene). The D, E, F, G, H, and P fragments (Fig. 2A), and the D1, D2, D3, P1, P2, and P5 fragments (Fig. 3) were excised from an 8-kb *Hind*III-*Hind*III fragment by restriction enzymes, and subcloned into 5'-upstream of the *RAG-2* promoter in the pR2P-EGFP. The P4 fragment was prepared by PCR using oligonucleotides 1 and 2 as primers. Amplified fragments were inserted into the pR2P-EGFP. The murine *TCRB* 3' enhancer (25) was subcloned into the *Sall* and *Bam*HI sites of the pR2P-EGFP. The HSV thymidine kinase (*TK*) promoter (GenBank accession no., M15234) was subcloned into the *Kpn*I and *Sma*I sites of pEGFP-1 (pTKP-EGFP). pD3-TKP-EGFP was prepared by inserting the D3 fragment into the pTKP-EGFP. For constructing pSVen-TKP-EGFP, the SV40 enhancer (SVen) was excised from the PicaGene Enhancer Vector 2 (Nippon Gene, Tokyo, Japan) and inserted into pTKP-EGFP. The D3 fragment with mutation for the C/EBP binding site (Mut-C/EBP) was prepared by PCR using the cloned genomic DNA as a template and oligonucleotides 3 and 4, as well as oligonucleotides 5 and 6 as primers. All the constructs were verified by restriction enzyme digestion or sequencing analysis.

Transfection and flow cytometry

To introduce EGFP constructs into lymphoid cells, 2.5 μ g of EGFP reporter gene and 2.5 μ g of human CD23 expression vector (pMECD23) (26) were transfected into 2.5×10^6 cells by the DEAE-dextran method as described previously (18). For transfection of EGFP constructs into fibroblasts, 15 μ g of EGFP reporter gene and 15 μ g of pMECD23 were transfected into the cells using the calcium phosphate method as previously described (18). Cells were harvested 22–24 h after transfection, and stained with biotinylated anti-human CD23 mAb (18.6) (26) followed by PE-conjugated streptavidin (BD PharMingen, San Diego, CA). The relative expression of EGFP and CD23 was analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed with CellQuest software (BD Biosciences). Where indicated, the results were analyzed using the Student's *t* test with a *p* value of <0.05 considered significant.

Generation and analysis of transgenic mice

The EGFP reporter gene constructs, pR2P-EGFP and pD3-R2P-EGFP, were digested with *Xho*I and *Afl*II, and the DNA fragments containing the EGFP reporter gene were digested with the murine *RAG-2* promoter/SV40 poly(A) signal, or with the D3 fragment, were purified by a Prep-A-Gene DNA Purification system (Bio-Rad, Hercules, CA). The transgenic mice were generated by Japan SLC (Hamamatsu, Japan). The mice were analyzed for the presence of the transgene by PCR using oligonucleotides 7 and 8, or 9 and 10 for examining D3-R2P-EGFP, and oligonucleotides 8 and 11 to examine R2P-EGFP. The copy number of the integrated DNA

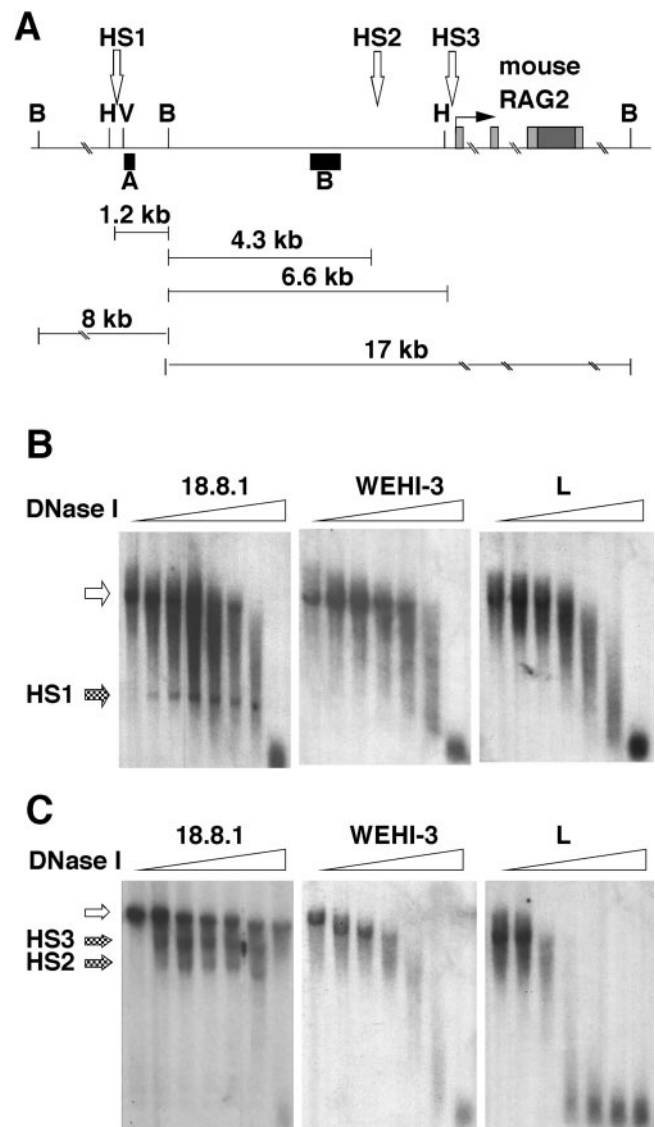


FIGURE 1. DNase I hypersensitive sites 5'-upstream of murine *RAG-2*. A, Schematic representation of the murine *RAG-2* gene and its flanking region. DNase I HS sites are indicated by vertical arrows, and fragments obtained by restriction enzyme digestion with or without DNase I digestion are represented with horizontal lines. Positions of probes are shown by ■. B, *Bam*HI; H, *Hind*III; V, *Eco*RV. C, Southern blot analysis of the DNase I HS site with probe B. DNase I HS sites (HS2 and HS3) are indicated by ⇨⇨ and the 17-kb *Bam*HI-digested DNA fragment is indicated by ⇨⇨.

was determined by Southern blotting. To analyze the expression of the transgene, total RNA was extracted from various tissues and treated with RNase-free DNase I to avoid the contamination of genomic DNA as a template. The transcripts were detected by RT-PCR using oligonucleotides 8 and 9 and by Southern blotting using the *EGFP* gene as a probe as described previously (27).

For analyzing EGFP expression in the thymocyte subpopulation, thymocytes were prepared from the transgenic mice (line 108) and stained with FITC-conjugated anti-CD8 Ab and PE-conjugated anti-CD4 Ab. CD4⁺CD8⁺ and CD4⁺CD8⁻ thymocytes were sorted twice using Epic-Elite (Beckman Coulter, Fullerton, CA). CD4⁻CD8⁻ thymocytes were

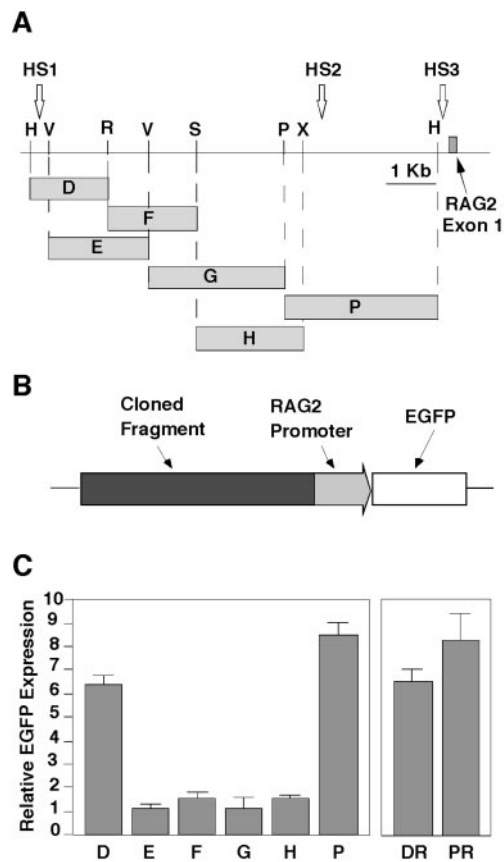


FIGURE 2. Identification of enhancer elements in the 5'-upstream region of RAG-2. *A*, Scheme of the 5'-upstream region of murine RAG-2. The genomic region examined for enhancer activity is shown on the top. Individual fragments (D to P) that were subcloned into reporter constructs are shown as \square . The positions of DNase I HS sites are indicated by vertical arrows. Only restriction enzyme sites used for preparation of each fragment are shown. H, *HindIII*; V, *EcoRV*; R, *EcoRI*; S, *SpeI*; P, *ApaLI*; X, *XbaI*. *B*, Schematic drawing of EGFP reporter constructs. Fragments of the 5'-upstream region of murine RAG-2 were cloned into EGFP reporter gene that is driven by the murine RAG-2 minimal promoter. *C*, Transcriptional activity of reporter constructs in the 18.81 cell line. EGFP reporter genes (5 μ g), together with 5 μ g of pME-CD23 control plasmids, were transfected into 18.81 cells, and EGFP- and CD23-expression was analyzed ~22–24 h later by flow cytometry. Expression of EGFP was normalized by that of CD23. Transcriptional activity of the EGFP construct without the test fragment was given as a value of 1. Error bars indicate deviations of three independent experiments.

prepared by deleting CD4⁺ or CD8⁺ thymocytes using HO2.2 anti-CD8 Ab and RL172 anti-CD4 Ab, and complement, as described previously (24, 28). For analyzing EGFP expression in subpopulations of the bone marrow, bone marrow cells were incubated with biotin-conjugated anti-B220 Ab, followed by streptavidin-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and FITC-conjugated anti-IgM Ab. B220⁺IgM⁺ and B220⁺IgM⁻ bone-marrow cells were sorted using autoMACS (Miltenyi Biotec) and Epics-Elite. The purity of each population was analyzed by FACSCalibur.

EMSA

Nuclear extracts were prepared according to the method described by Schreiber et al. (29). EMSA was performed by incubating nuclear extracts with radio-labeled oligonucleotides and then subjecting them to electrophoresis as previously described (18). The probes of the distal enhancer were prepared by PCR using the primer pairs: oligonucleotides 7 and 14 for probe 1 (-382/-282), 15 and 16 for probe 2 (-281/-198), 17 and 18 for probe 3 (-197/-90), or 19 and 6 for probe 4 (-89/-1). Consensus binding sequences of lymphoid transcription factor (Lyl)-1, acute myeloid leukemia (AML)-1, GATA, E-box, Nkx2.5, NF- κ B, C/EBP, Oct-1, and c-

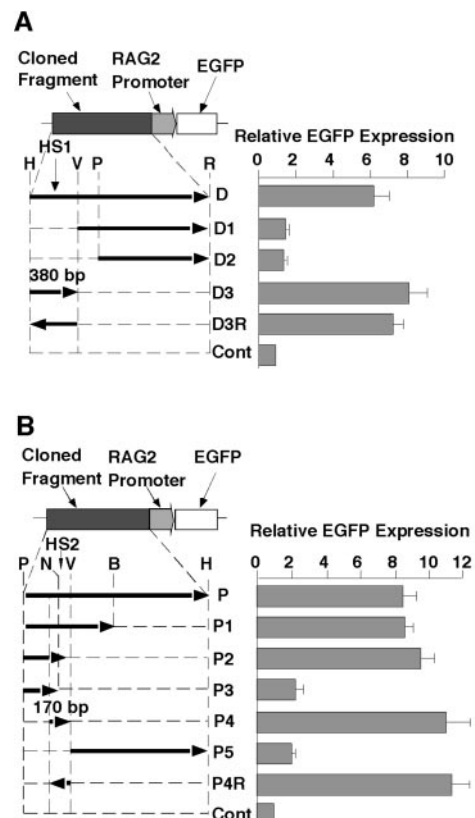


FIGURE 3. Deletion analysis of D and P fragments. *A*, Transcriptional activity of deletion mutants of the D fragment. The indicated deletion mutants of the D fragment were linked to the murine RAG-2 promoter-driven EGFP reporter gene. The 380-bp fragment was subcloned in the reporter construct in both forward (D3) and reverse (D3R) orientations. The reporter construct without test fragment is indicated as Cont. The transcriptional activity of each fragment in 18.81 cells was determined as described in Fig. 2. Position of HS1 is shown with a vertical arrow. H, *HindIII*; V, *EcoRV*; P, *ApaLI*; R, *EcoRI*. Error bars indicate deviations of three independent experiments. *B*, Transcriptional activity of deletion mutants of the P fragment. The indicated deletion mutants of the P fragment were linked to the EGFP reporter gene. The 170-bp fragment was subcloned in the construct in forward (P4) and reverse (P4R) orientations. Transcriptional activity of each fragment was analyzed as described above. P, *ApaLI*; N, *NcoI*; V, *EcoRV*; B, *BstXI*; H, *HindIII*. Position of HS2 is shown with a vertical arrow. Error bars indicate deviations of three independent experiments.

Myb were prepared by annealing oligonucleotides of 20 and 21, 22 and 23, 24 and 25, 26 and 27, 28 and 29, 30 and 31, 32 and 33, 34 and 35, or 36 and 37, respectively.

Oligonucleotides

The primers for PCR were as follows: 1, 5'-AAGCTTTCGCATCCTTT TCTACTTCCGTTG-3'; 2, 5'-GAATTCGGGGATAGGCATGGCTCT TATGA-3'; 3, 5'-AGCTTGCTAGAACAAGAT-T-3'; 4, 5'-GTTGGCTT TAGACTAGTCTAATACACAA-3'; 5, 5'-TTGTGTATTAGACTAGTC TAAAGCCAAC-3'; 6, 5'-ATCACGCTCCACAGGCATTG-3'; 7, 5'-AA GCTTAAAGGCATAAAAATGGCTGT-3'; 8, 5'-CGTCGCCGCTCCAGC TCGACCAG-3'; 9, 5'-CAAGCTGACCCTGAGTTCA-3'; 10, 5'-TCGA TGTTGTGGCGGATCTT-3'; 11, 5'-TTCTGTCTCCCTCAACCATC-3'; 12, 5'-ATGGACGAGCTGTACAAGTA-3'; 13, 5'-ACAAAACCAAC TAGAATGC-A-3'; 14, 5'-ACTTACCATTATGTCCTATGCT-3'; 15, 5'-AAAGGCATAAAAATGGCTGT-3'; 16, 5'-AGAGTGGTCTGTATTTT GTTTCCT-3'; 17, 5'-GCTCTGGCTATCACTGTCAC-3'; 18, 5'-AGGAC TTCTGGGAGCGA-3'; 19, 5'-GTTGTGTATTATTAAGTAAT-3'; 20, 5'-GATCCATTTTGGGAGAAAGT-3'; 21, 5'-ACTTTCCTCCAAAAT GGATC-3'; 22, 5'-AATTCGAGTATTGTGGTAAATACG-3'; 23, 5'-CG TATTAACCAATACTCGAATT-3'; 24, 5'-CACTTGATAACAGAA AGTGATACTCT-3'; 25, 5'-AGAGTTATCACTTTCGTTATCAAGTG-3';

26, 5'-CTTTTACCAGGTGGTCTCTA-3'; 27, 5'-TAGAGACCACCTGG TAAAAAG-3'; 28, 5'-CACTGCCAGTCAAGTGTTCG-3'; 29, 5'-CGA ACATTGACTGGGCAGTG-3'; 30, 5'-GTAGGGGACTTCCGAGCT CG-3'; 31, 5'-CGAGCTCGGAAAGTCCCCTAC-3'; 32 AND 33, 5'-TG CAGATTGCGCAATCTGCA-3'; 34, 5'-ATGAATATGCAAATCAGGT GA-3'; 35, 5'-TCACCTGATTTGCATATTCAT-3'; 36, 5'-TACAGGC ATAACGGTTCGTTAGTGA-3'; 37, 5'-TCACTACGGAACCGTTATG CCTGTA-3'.

Results

Identification of lymphoid-specific DNase I hypersensitive sites 5'-upstream of the murine RAG-2 gene

DNase I HS sites are often associated with *cis*-acting elements, such as enhancers, silencers, or promoters (30–35). To identify the *cis*-acting elements of murine RAG-2, we examined the DNase I HS sites within a 25-kb region encompassing the 5'-upstream and 3'-downstream regions of murine RAG-2 (Fig. 1A). Three DNase I HS sites (HS1, HS2, and HS3) were identified in a RAG-2-expressing pre-B cell line (18.81). HS1, HS2, and HS3 were located at -8.1, -2.6, and -0.2 kb relative to the transcriptional initiation site. These DNase I HS sites were not detected in a myeloid cell line (WEHI3) and a fibroblast cell line (L) (Fig. 1, B and C), suggesting that these HS sites are lymphoid-specific. To confirm this, we examined the DNase I HS sites in T cell lines as well as other B cell lines and nonlymphoid cell lines. As shown in Table I, HS1, HS2, and HS3 existed in both B (18.81 and BAL17) and T cell lines (LSB11-1, LSB11-14) which expressed RAG-2, but not in myeloid (WEHI3) and fibroblast cell lines (L and NIH3T3). HS1 was found not only in RAG-2-expressing cell lines, but also in RAG-2-nonexpressing lymphoid cell lines (WEHI231 and EL4). HS2 was not clearly detected in the RAG-2-nonexpressing EL4 T cell line or the WEHI231 B cell line. HS3, which corresponds to the RAG-2 promoter region, was present only in the cells expressing RAG-2. These DNase I HS sites were also identified using *Stu*I digestion (data not shown).

Effect of DNA fragments containing DNase I HS sites on promoter activities

The presence of the lymphoid-specific DNase I HS sites in the 8-kb 5'-upstream region (Fig. 1A) suggested that this region might be of importance in the lymphoid-specific transcriptional regulation of RAG-2. Therefore, we investigated whether DNA elements in this region could affect the mouse RAG-2 minimal promoter by

Table I. Identification of DNase I hypersensitive sites specifically in lymphoid cells

Cell Line	RAG-2 mRNA ^a	HS1 ^b	HS2	HS3
Lymphoid				
18.81 (pre-B)	+ ^c	+	+	+
BAL17 (B)	+	+	+	+
LSB11-1 (T)	+	+	+	+
LSB11-14 (T)	+	+	+	+
WEHI231 (B)	-	+	+/-	-
EL-4 (T)	-	+	+/-	-
Nonlymphoid				
WEHI3 (myeloid)	-	-	-	-
L (fibroblast)	-	-	-	-
NIH3T3 (fibroblast)	-	-	-	-

^a Expression of RAG-2 mRNA was examined by RT-PCR.

^b DNase I HS sites were assessed as in Fig. 1.

^c +, DNase I-sensitive; +/-, DNase I slightly sensitive; -, DNase I-resistant.

using the EGFP reporter gene (Fig. 2). Constructs containing consecutive or overlapping fragments of the murine RAG-2 5'-upstream region extending from -300 bp to ~-8.3 kb were prepared and assayed for EGFP expression in the transiently transfected 18.81 cells. Two discrete fragments (D and P) were shown to augment EGFP expression ~6- to 8-fold compared with the control EGFP reporter gene (Fig. 2C). The fragment connected in the reverse orientation (DR and PR) to the RAG-2 promoter retained its abilities to stimulate the transcription, indicating that the elements functioned as enhancers (Fig. 2C). D and P fragments coincided with the DNase I HS sites shown in Fig. 1 (HS1 and HS2, respectively).

Identification of the core enhancer regions

To identify the core regulatory elements within P and D fragments, a series of deletion mutants of each fragment was constructed into the EGFP reporter genes (Fig. 3). The EGFP constructs were transfected into 18.81 cells, and the relative EGFP expression was determined. As shown in Fig. 3A, deletion of the 5' portion of the D fragment to the *Eco*RV (D1) or *Pst*I (D2) site completely abolished the enhancer activity. The 380-bp *Hind*III-*Eco*RV fragment (D3)

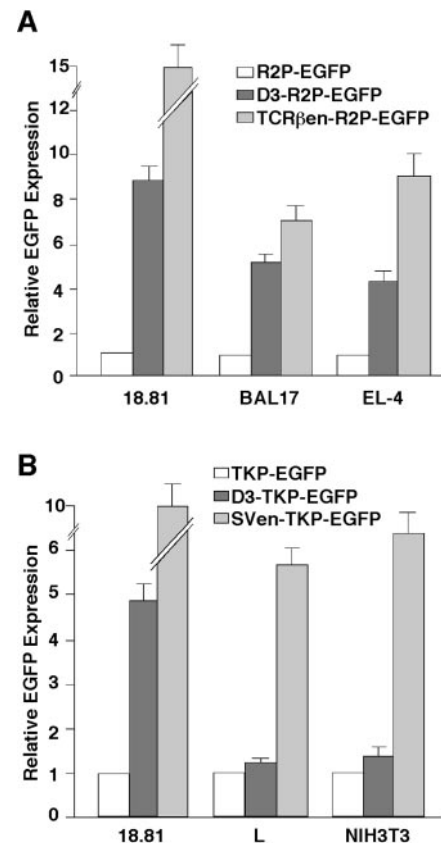
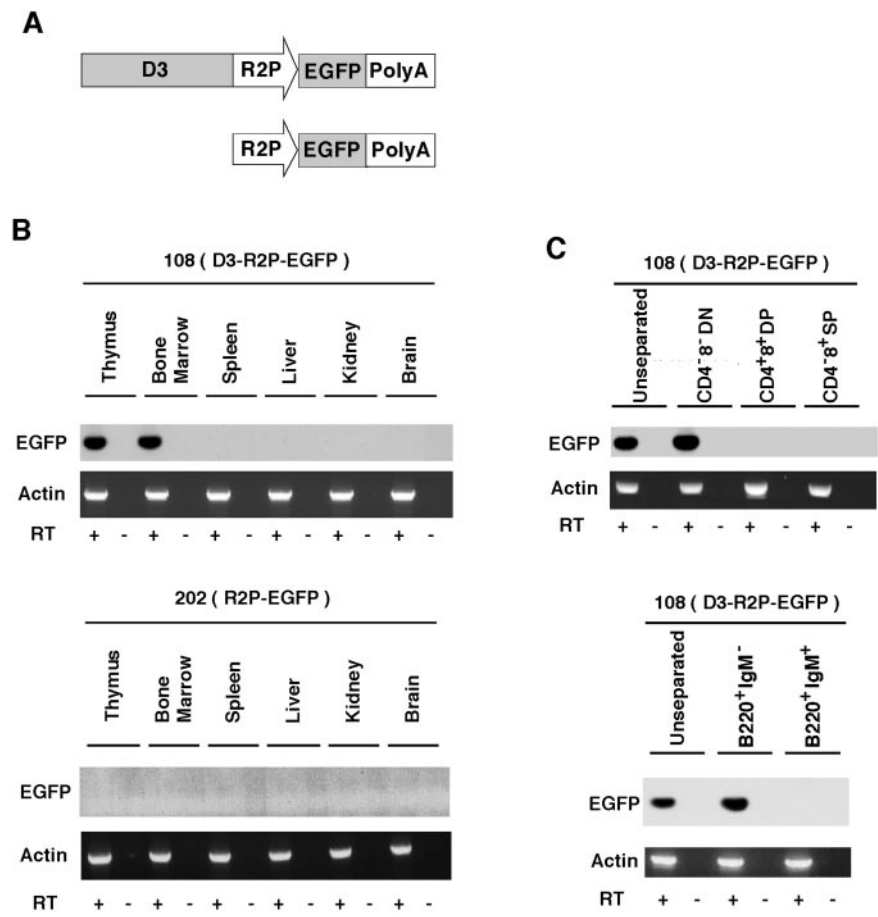


FIGURE 4. Cell lineage-specificity of the distal (D3) enhancer. *A*, Enhancer activity of D3 in lymphoid cells. The EGFP construct with the murine RAG-2 promoter (R2P-EGFP), R2P-EGFP linked with D3 (D3-R2P-EGFP), or that linked with the *TCRβ* enhancer (TCRβen-R2P-EGFP) was transfected into 18.81 (pre-B), BAL17 (B), or EL-4 (T) cells and the transcriptional activity was analyzed as described in Fig. 2. Error bars indicate deviations of three independent experiments. *B*, Enhancer activity of D3 in nonlymphoid cells. The EGFP construct with the TK promoter (TKP-EGFP), TKP-EGFP linked with D3 (D3-TKP-EGFP), or that linked with the SV40 enhancer (SVen-TKP-EGFP) was transfected into 18.81, L, or NIH3T3 cells, and the transcriptional activity was analyzed as described in Fig. 2. Transcriptional activity of TKP-EGFP was given as a reference value of 1. Error bars indicate deviations of three independent experiments.

FIGURE 5. EGFP mRNA expression in various tissues and cells from transgenic mice with D3-R2P-EGFP. *A*, Scheme of the transgenes. R2P, murine *RAG-2* minimal promoter; EGFP, coding sequence of the *EGFP* gene; poly(A), SV 40 polyadenylation signal. *B*, Expression of EGFP mRNA in various tissues of transgenic mice. Total RNA was prepared from thymus, bone marrow, spleen, liver, kidney, and brain of the transgenic mice. RT-PCR for EGFP and β -actin was performed in the presence (+) or absence (-) of reverse transcriptase (RT), and visualized by Southern blot hybridization with a EGFP- or β -actin-specific probe. *Upper panel*, The result of a D3-R2P-EGFP transgenic line (108); *lower panel*, the result of a R2P-EGFP transgenic line (202). *C*, EGFP expression in subpopulations of thymocytes and bone marrow cells. CD4⁻CD8⁻ DN thymocytes, CD4⁺D8⁺ DP thymocytes, or CD4⁺CD8⁻ SP thymocytes and B220⁺IgM⁻ or B220⁺IgM⁺ bone marrow cells were prepared from the transgenic line (108) and transcripts of EGFP and β -actin were analyzed by RT-PCR in the presence (+) or absence (-) of RT.



showed the full enhancer activity, suggesting that the element necessary for maximal enhancer activity is present in D3. Of note is that this 380-bp core enhancer segment coincided with the HS1 site. The fragment D3 constructed in the reverse orientation (D3R) exhibited the corresponding enhancer activity compared with that in forward orientation (D3) (Fig. 3A), showing that the enhancing activity of D3 is orientation-independent. By a similar analysis of series of deletion mutants, the core enhancer element in the P fragment was shown to reside within P4 (a 170-bp fragment) (Fig. 3B). It functioned in an orientation-independent manner and coincided with the HS2 site. Taken together, two enhancer elements, D3 and P4, were identified in the 5'-upstream region of murine *RAG-2* and both of them were located at the lymphocyte-specific DNase I HS sites.

Cell lineage-specific enhancer activity of the distal enhancer element (D3)

To determine the cell specificity of the distal enhancer element (D3), EGFP reporter constructs containing the *RAG-2* promoter with or without D3 (D3-R2P-EGFP and R2P-EGFP) were transiently transfected into 18.81 (pre-B), BAL17 (B), and EL4 (T) cells, and enhancer activity was examined. The EGFP reporter construct containing a *TCRβ* enhancer (*TCRβ*en-R2P-EGFP) was used as a positive control. As shown in Fig. 4A, D3 increased the mouse *RAG-2* promoter activity ~5- to 9-fold in both B (18.81, BAL17) and T cell lines (EL-4). To test the enhancer activity in nonlymphoid cells, the EGFP reporter constructs containing the *TK* promoter with or without D3 (D3-TKP-EGFP and TKP-EGFP) were transfected into the lymphoid cell line (18.81) and nonlymphoid cell lines (L and NIH3T3), and the relative enhancer activity was assessed. The EGFP reporter construct containing the SV40 enhancer (SVen-TKP-EGFP) was used as a positive control. We used the *TK* promoter and the SV40 enhancer in the

EGFP reporter constructs because the *RAG-2* promoter and *TCRβ* enhancer did not function in nonlymphoid cells. As shown in Fig. 4B, the enhancer activity of D3 was detected in 18.81 cells, but not in either L or NIH3T3 cells. These results indicate that D3 functions in a lymphoid-specific manner.

The distal enhancer directs the specific expression of the reporter gene in primary lymphoid organs of the transgenic mice

To verify the results obtained in vitro, we examined the function of the distal enhancer in vivo by generating transgenic mice that carried the EGFP reporter construct containing D3 driven by the murine *RAG-2* minimal promoter (D3-R2P-EGFP). The construct

Table II. Summary of EGFP expression in various tissues from mice with transgene D3-R2P-EGFP

Founder (no. of copies) ^a	Expression of EGFP ^b					
	Thymus	BM	Spleen	Liver	Kidney	Brain
108 (10)	+	+	-	-	-	-
119 (10)	+	+	-	-	-	+
208 (8)	+	+	-	-	-	-
213 (7)	+	+	+	+	+	+
215 (1)	+	+	-	-	-	+
301 (3)	+	+	-	-	-	-
302 (8)	+	+	-	-	-	+
313 (7)	+	+	+	+	+	+
314 (1)	+	+	-	-	-	-
401 (2)	+	+	-	-	-	-
404 (10)	+	+	-	-	-	-

^a Copy number of transgenes was determined by Southern blotting.

^b Expression of EGFP reporter gene in various tissues of the transgenic mice was analyzed as in Fig. 5; BM, bone marrow.

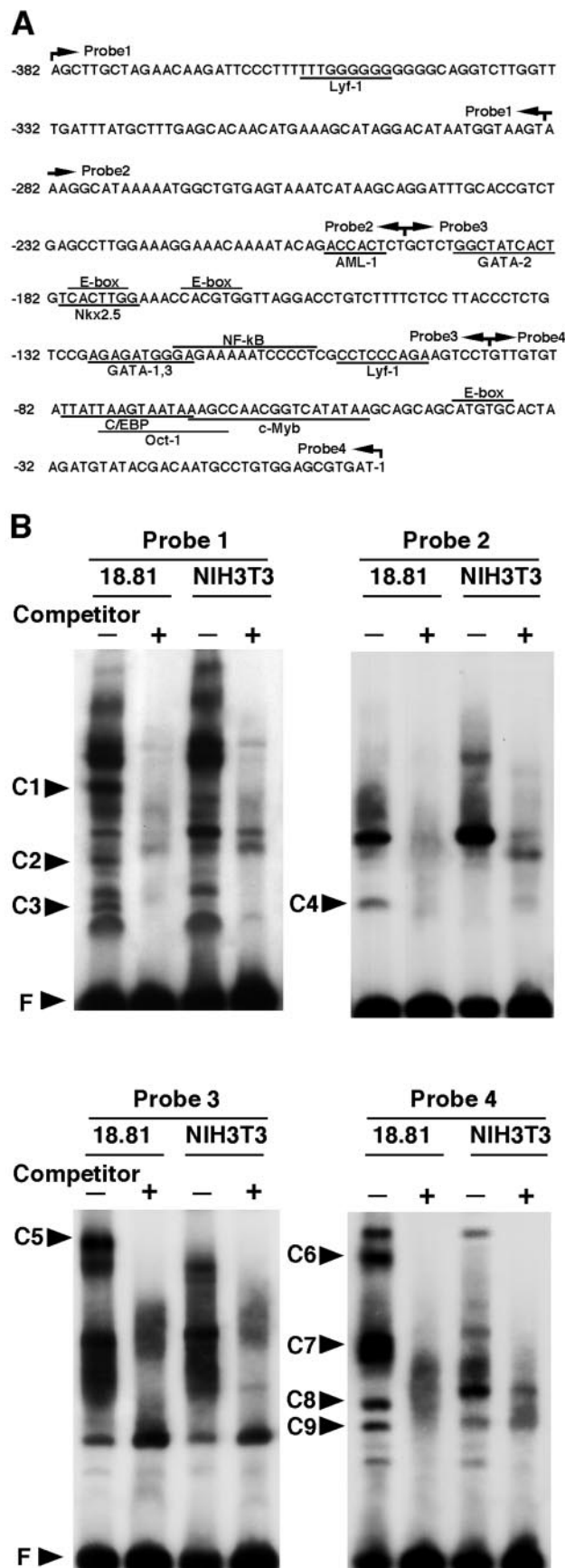


FIGURE 6. Binding of nuclear factors to the distal (D3) enhancer element. *A*, Nucleotide sequence of D3. The nucleotide sequence of 382-bp D3 is shown. The 3'-end is defined as -1. Positions of 5'-end and 3'-end of probes 1, 2, 3, and 4 used in EMSA are indicated by arrows. Some potential recognition sequences for T or B cell-specific transcription factors

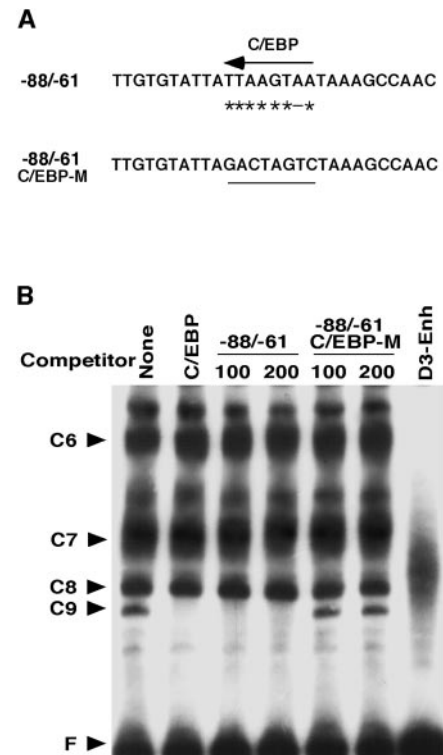


FIGURE 7. C/EBP binds to D3 enhancer and forms the C9 complex. *A*, The oligonucleotide containing the binding site for C/EBP and its mutant (C/EBP-M). The asterisk denotes nucleotides identical with consensus binding sequences for C/EBP; underline indicates mutated sequences. *B*, EMSA competition assay. EMSA was performed as in Fig. 6 using nuclear extracts from 18.81 cells and probe 4. The consensus sequence for C/EBP, the oligonucleotide containing the binding site for C/EBP (-88/-16), its mutant (-88/-16 C/EBP-M), or unlabeled probe 4 (D3-Enh) were added as competitors.

without D3 was used to generate the control mice (R2P-EGFP) (Fig. 5A). The total RNA from thymus, bone marrow, spleen, liver, kidney, and brain of the 11 independent transgenic lines were examined for the expression of the transgene by RT-PCR. Fig. 5B (upper panel) shows a representative result of a transgenic mouse (D3-R2P-EGFP, line 108). D3 directed the expression of the EGFP reporter gene in thymus and bone marrow but not in the spleen, liver, kidney, and brain. In the control transgenic mice (R2P-EGFP), no EGFP expression was detected (Fig. 5B, lower panel). The data of all of the 11 transgenic lines was shown in Table II. Six of 11 lines (108, 208, 301, 314, 401, and 404) expressed EGFP only in thymus and bone marrow, but not in other organs. These data indicate that D3 enhanced mouse *RAG-2* promoter activity in vivo specifically in primary lymphoid organs. Two of the transgenic lines (213 and 313) expressed EGFP in all of the tissues examined, probably due to the integration of the element into the vicinity of other *cis*-acting elements as described by Ellmeier et al.

or other lymphocyte-related transcription factors are indicated with lines under or above the sequences. *B*, Binding of nuclear factors to D3. EMSA was performed using nuclear extracts from lymphoid 18.81 or nonlymphoid NIH3T3 cells with radiolabeled probes 1 to 4. The binding of nuclear factors was competed with a 200-fold excess of each unlabeled probe. Specific complexes (C1 to C9) are indicated by ▶. Free probe is indicated as F.

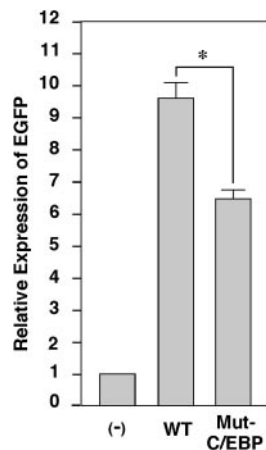


FIGURE 8. Possible involvement of C/EBP in D3 enhancer activity. EGFP constructs were transfected into 18.81 cells and analyzed as described in Fig. 2. Three EGFP constructs were used: R2P-EGFP (-), D3-R2P-EGFP (WT), or D3-R2P-EGFP containing the nucleotide mutation in the binding site for C/EBP (Mut-C/EBP). Error bars indicate deviations of three independent experiments. *, Significant difference ($p < 0.01$).

(36). Interestingly enough, three lines (119, 215, and 302) expressed EGFP not only in primary lymphoid organs, but also in brain, suggesting that D3 is active in some situations in the brain.

To further determine which subpopulations of the thymus or the bone marrow in the transgenic mice express EGFP, various thymocyte subpopulations, CD4⁻CD8⁻ (DN), CD4⁺CD8⁺ (DP), and CD4⁺CD8⁻ (SP), or bone marrow subpopulations, B220⁺IgM⁻ and B220⁺IgM⁺, were prepared from a transgenic line (108) and their EGFP expression was analyzed. As shown in Fig. 5C, EGFP was expressed in the DN thymocytes, but not in the DP or SP thymocytes in the thymus, while it was expressed in B220⁺IgM⁻, but not in B220⁺IgM⁺, cells in bone marrow.

Binding of nuclear factors to the distal enhancer region

To delineate the transcription factors regulating the D3 distal enhancer element, putative binding sites for transcription factors were searched using the Genome Net Database (Bioinformatics Center, Institute for Chemical Research, Kyoto University, Kyoto, Japan). Fig. 6A shows the binding sites for several transcription factors that are thought to function in lymphoid cells such as Lyf-1, AML-1, GATA, E-box, NF- κ B, Nkx2.5, C/EBP, Oct-1, or c-Myb. To try to identify the cell type-specific transcriptional factors that bind to the D3 fragment, an EMSA was performed using probe 1 (-382/-282), probe 2 (-281/-198), probe 3 (-197/-90), and probe 4 (-89/-1) of the D3 region with nuclear extracts from lymphoid 18.81 and nonlymphoid NIH3T3 cells (Fig. 6B). To confirm the specific complex-formation, a 200-fold excess of unlabeled probe was added as a competitor. As shown in Fig. 6B, nine lymphoid-specific complexes (C1-C3 with probe 1; C4 with probe 2; C5 with probe 3; C6-C9 with probe 4) were detected. To determine the binding sites responsible for the complex formation, we then performed a competition assay. We added the excess amount of the oligonucleotides that contained consensus binding sites for each of the possible lymphoid-specific transcription factors in the D3 enhancer, Lyf-1, AML-1, GATAs, E-box, NF- κ B, Nkx2.5, C/EBP, Oct-1, and c-Myb (shown in Fig. 6A), and examined their ability to inhibit the complex formation. Only the addition of the excess oligonucleotide containing the consensus binding site for C/EBP inhibited the complex formation between nuclear extract 18.81 and probe 4 (Fig. 7). This inhibition was specific because the -88/-61-D3 enhancer sequence containing

the mutated binding site for C/EBP did not inhibit the complex formation (Fig. 7). Addition of oligonucleotides containing consensus binding sites for the transcription factors other than C/EBP did not inhibit the complex formation (data not shown). These results show that C/EBP may bind to the D3 enhancer.

To delineate the function of the binding site for C/EBP for D3 enhancer activity, we constructed the D3 enhancer containing the mutated binding site for C/EBP that was linked to R2P-EGFP (Mut-C/EBP). We then transfected it into 18.81 cells, and compared its enhancer activity with that of the wild-type D3-R2P-EGFP (shown in Fig. 4). We found the enhancer activity of Mut-C/EBP decreased significantly compared with that of the wild-type enhancer ($p < 0.01$), although the inhibition was not so remarkable (Fig. 8). These data suggest that C/EBP binds to the D3 enhancer and functions as one of transcription factors or coactivators responsible for the enhancer activity.

Discussion

We and others have shown that the tissue- and stage-specific expression of *RAG-1* and *RAG-2* is regulated by alteration of the chromatin structure of the promoter region (13, 14) and/or transcriptional factors that bind the promoter region (18–21). In the present study, we characterized the chromatin structure of the 25-kb DNA segment adjacent to murine *RAG-2*, and identified three lymphocyte-specific HS sites (Fig. 1). Subsequently, we determined the enhancer element(s) in the 5'-upstream region of *RAG-2* using EGFP reporter assays. Two enhancer elements (D3 and P4) were identified that corresponded to the lymphocyte-specific HS sites (HS1 located at -8.1 kb and HS2 at -2.3 kb 5' upstream of mouse *RAG-2*) (Figs. 2 and 3). One of the enhancer elements, the distal enhancer element (D3), was shown to be specifically active in lymphoid cell lines in *in vitro* transient assays (Fig. 4) and active in the primary lymphoid organs (i.e., thymus and bone marrow) in the D3 minigene transgenic mice (Fig. 5). Our data indicate that the tissue- and stage-specific expression of murine *RAG-2* may be regulated not only by the promoter region, but also by chromatin remodeling and other *cis*-regulatory element(s).

Recently, Yu et al. (22) have reported that approximately the 10-kb 5'-upstream region of murine *RAG-2* is sufficient for the expression of the GFP reporter gene in B lineage cells as well as in a subset of thymocytes *in vivo*. Monroe et al. (23) have also demonstrated that the 9-kb 5'-upstream region of murine *RAG-2* is enough to rescue B cell- and T cell-development using the *RAG-2*^{-/-} blastocyst complementation assay. Their results indicated that there are different regulatory elements in the -2- to -7-kb and the -7- to -9-kb regions, respectively. The proximal element contributed to B cell lineage expression, while the distal element functioned to regulate both B and T cell lineage expression. These studies suggest that at least two lymphoid-specific regulatory elements, such as enhancer(s), may exist in this 5'-upstream region of murine *RAG-2*. With this respect, we identified P4 and D3 elements locating at -2.3- and -8.1-kb 5' upstream of *RAG-2*, respectively, the results corresponding to the putative elements proposed by Monroe et al. (23).

The D3 enhancer was functional *in vivo*. We generated transgenic mice carrying a minigene containing D3, *RAG-2* promoter, and EGFP and analyzed expression of EGFP in various tissues. We showed that 6 of 11 lines expressed EGFP only in the thymus and bone marrow, but not in spleen, liver, kidney and brain, indicating that D3 enhanced mouse *RAG-2* promoter activity *in vivo* specifically in primary lymphoid organs. We further investigated the expression of EGFP in subsets of thymocytes and bone marrow cells in the transgenic mice. We revealed that D3 was active in

CD4⁻CD8⁻ (DN), but not in CD4⁺CD8⁺ (DP) or CD4⁺CD8⁻ (SP), thymocytes in the thymus, and was also active in B220⁺IgM⁻, but not in B220⁺IgM⁺, cells in the bone marrow (Fig. 5C). These results support the previous observation by Yu et al. (22) which found that the 10-kb 5' upstream region of RAG-2 functions for the expression of the GFP reporter gene in CD4⁻CD8⁻ thymocytes, as well as B lineage cells, in vivo.

An interesting finding that emerged during analysis of the transgenic mice is that the transgene was active in the brain in 3 lines of 11 transgenic founders (Table II). Is it an artifact? Because most of the transgenic mice expressed the transgene only in primary lymphoid organs but not in the brain, D3 was not sufficient for the expression of the transgene in the brain. However, those three transgenic lines expressed the transgene in the brain as well as primary lymphoid organs, but not in other organs. This observation indicates that D3 may be able to augment the transcription in the brain in some situations, such as being located near some *cis*-regulatory elements or being in the presence of an additional *cis*-element in RAG locus. With regard to this, Chun et al. (37) have shown that RAG-1, but not RAG-2, is expressed in the nervous system. Yu et al. (22) have also shown that the 5'-upstream region of murine RAG-2 can regulate the expression of mouse RAG-1. Thus, the D3 enhancer could be involved in the expression of murine RAG-1 in the brain.

Although EGFP transcripts were clearly detected by RT-PCR in thymocytes and bone marrow cells in the D3-R2P-EGFP transgenic mice (Fig. 5), we could not detect EGFP proteins by either flow cytometry or Western blotting. There are two possibilities to explain this result. First, it is due to the copy numbers of transgenes in the mice. In fact, the copy numbers in the transgenic lines did not exceed 10 copies (Table II). When we established and analyzed stable D3-R2P-EGFP transfectants of EL-4 cells whose EGFP-proteins were detected by flow cytometry, they contained >50 copies of EGFP-construct (data not shown). Another possibility, which we think is more likely, is that our D3 transgenic mice are missing *cis*-regulatory element(s) such as locus control region (LCR). LCR, which locates near, or sometimes apart from, enhancer element(s), was found to be required for high level, copy number-dependent gene activation in vivo (38, 39). In this respect, Yu et al. (22) generated transgenic mice carrying a bacterial artificial chromosome DNA that spanned a long range of the 5'-upstream to 3'-downstream region of the mouse RAG locus, where the EGFP reporter gene was inserted. It was shown that EGFP proteins were detected by flow cytometry in the thymocytes and bone marrow cells of these transgenic mice. Furthermore, generation of EGFP-knockin mice of the RAG-1 or RAG-2 locus that carry a single copy of EGFP showed that only one copy of EGFP is enough to induce EGFP proteins in the transgenic mice (7, 40). These results strongly suggest that other *cis*-element(s) or LCR are necessary for the D3 enhancer element to induce higher levels of EGFP expression. This element(s) should be determined.

Transcripts of EGFP were detected in bone marrow and thymus, but not detected in spleen, showing that the enhancer activity of distal element is correlated with RAG expression in immature lymphoid cells. The result suggests that the cell-specific transcription of RAG-2 is also regulated by lymphoid-specific *trans*-acting factors. With this regard, a database search revealed the putative binding sites of transcription factors in the 380-bp core distal enhancer element (Fig. 6A). Using EMSA, we demonstrated that C/EBP, one of the transcriptional factors that controls the transcription of genes involved in a broad range of physiological processes, as well as lymphoid cell development (41, 42), may bind to the D3 enhancer (Figs. 6B and 7). We also showed that C/EBP may function as one of the transcription factors or coactivators responsible for

the enhancer activity (Fig. 8). It is necessary to determine other transcription factors and/or associated molecules that are responsible for the full D3 enhancer activity. Identification of these factors may elucidate the transcriptional regulation by the distal enhancer element.

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