

T CELL RECEPTOR β GENE HAS TWO
DOWNSTREAM DNase I HYPERSENSITIVE REGIONS
Possible Mechanisms of Tissue- and Stage-specific Gene Regulation

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TCR molecules recognize a variety of foreign antigens in the context of self MHC antigens. The genes encoding TCR- α , - β , - γ and - δ chains have been isolated and characterized (1-10). The overall structure of the TCR genes reveals many similarities with the Ig genes. Both the TCR and Ig genes consist of variable (V), joining (J), and constant (C) regions. These segments, which are separated in germline DNA, are combined during differentiation to generate functional and diverse receptor genes that encode the heterodimeric Ig or TCR molecules (11).

Changes in local chromatin structure often reflect developmental regulation of genes expressed in a tissue-specific manner. Neither the mechanisms that control the physical changes in chromatin structure nor the physiological significance of nuclease hypersensitive regions are clearly understood. However, a number of studies have demonstrated that hypersensitive regions are within or near DNA regulatory regions (12-15). Stimulation of the pre-B cell line 70Z/3 with bacterial LPS induces a DNase I-hypersensitive site in the enhancer region of the J-C intron of the Ig κ gene, Ig κ transcription, and activation of the enhancer-binding protein NF- κ B (12, 15). The human Ig heavy chain (IgH) gene has a DNase I-hypersensitive site in the putative enhancer region in a human B cell (16). These observations strongly suggest a correlation between regulatory regions and sites of nuclease hypersensitivity.

The TCR- β gene has a tissue-specific nuclease-hypersensitive region in the J-C intron (17), although the function of this region remains unclear. Another regulatory region in the TCR- β gene was found downstream of the C- β -2 region (18, 19). This region shows enhancer activity, as detected by transient CAT assay, and the DNA sequence of this region has several regions homologous with characteristic enhancer sequences. However an enhancer activity was detected in B cells as well as T cells (18), so that the mechanism of tissue-specific regulation by this region remains unclear.

This paper reports the detection of two DNA-hypersensitive regions located 3' of the C- β -2 region. One hypersensitive region was found in all T cell lines examined but not in cell lines from other tissues. This region coincides with the regulatory site thought to be an enhancer element. DNA sequences of this region show several identical or homologous DNA sequences with regulatory elements. The other hyper-

This work was supported by a W. W. Smith Award, and by grant AR-38187 from the National Institutes of Health, the Lucille Markey Charitable Trust, and by the Leukemia Society of America.

sensitive site, located between the C- β -2 and the enhancer regions, was detected in T cell lines expressing the TCR- β gene, but not in T cell lines that do not express the transcript or in other cell lines from other tissues. The nucleotide sequences of the second hypersensitive region also has DNA sequences homologous with typical regulatory elements. Thus, there appear to be two regulatory regions downstream of the TCR- β gene, and tissue-specific regulation by these two regions appears to be controlled, at least in part, by changes in chromatin structure during T cell development.

Materials and Methods

Cell Lines. Table I lists the cell lines used in this study and the mouse strain from which they were derived. Clone L is a Friend leukemia virus-transformed mouse cell line (20). A20 cells were derived from a spontaneous reticulum cell neoplasm (21). The KKE, NCKA, KgV cell lines are Gross leukemia virus-transformed mouse cell lines (Hashimoto, Y., and K. Blank, manuscript submitted for publication). YAC-1 is a Molony murine leukemia virus-transformed mouse cell line (17). RAD-T is a radiation-induced T cell line (17). AKRB is an AKR strain derived from a spontaneous thymoma. S49.1 cells were cloned from cell line S49, established from a lymphoma induced by bacteriophage and oil induction (22).

Detection and Mapping of the DNase I-hypersensitive Region. Nuclei were prepared and digested with DNase I essentially by the method of Parslow and Granner (15). Briefly, nuclei were prepared from washed cells (2×10^8) by gentle homogenization in a solution containing 0.25 mM sucrose, 10 mM Tris, pH 8.0, 10 mM MgCl₂ and 1% (wt/vol) Triton X-100, then washed three times in the same solution without detergent, and suspended to a DNA concentration of 1 mg/ml in ice-cold 0.25 mM sucrose 10 mM Tris, pH 8.0, 10 mM MgCl₂. Aliquots (0.2 ml) of the nuclear suspension were preincubated at 37°C for 1 min. DNase I was added to a final concentration of 0–20 U/ml and incubated for 2 min. Digestion was stopped by adding

TABLE I
T Cell Lines Used in this Study and Pattern of TCR- β Gene Expression

Cell line	Source strain	TCR- β gene expression	RNA	Lineage
Clone L	BALB.B	D β 1-J β 1C β 1-D β 2-J β 2C β 2 D β 1-J β 1C β 1-D β 2-J β 2C β 2	None	non-B, non-T
A20	BALB.C	D β 1-J β 1C β 1-D β 2-J β 2C β 2 D β 1-J β 1C β 1-D β 2-J β 2C β 2	None	B
KKE	BALB.K	VDJ β 2C β 2 D β 1J β 2C β 2	None	Pre-T (?)
NCKA	B10.K	D β 1-J β 1C β 1-D β 2-J β 2C β 2 D β 1J β 2C β 2	None	Pre-T
KgV	BALB.K	VD β 1J β 1C β 1-D β 2J β 2C β 2 VD β J β 2C β 2	1.3, 1.0	↑
YAC-1	A/Sn	XJ β 2C β 2 XJ β 2C β 2	+ ?	
RAD-T	B10A(3R)	XJ β 2C β 2 XJ β 2C β 2	+ ?	↓
S49.1	BALB.C	XJ β 2C β 2	+	
AKRB	AKR	VDJ β 2C β 2 VDJ β 2C β 2	1.3 ?	

V implies the presence of a V β gene segment, while X indicates an unidentified segment. The sizes of expressed RNAs are given where known. (+) Indicates the presence of β RNA.

0.1 vol each of 10% SDS and 2 mg/ml proteinase K per milliliter. After incubation at 37°C for 2 h, the reaction mixture was extracted twice with anhydrous ether and adjusted to 0.5 M NaCl. Nucleic acids were precipitated by adding of 2.5 vol ethanol. The precipitate was resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA, and digested successively with 0.1 mg ribonuclease A per milliliter and 0.2 mg proteinase K per milliliter for 2 h each at 37°C. Samples were extracted twice with phenol/chloroform (1:1) and twice with anhydrous ether, then adjusted to 0.2 M ammonium acetate and precipitated with ethanol. After digestion with selected restriction enzymes, aliquots of the purified DNA were electrophoresed on an agarose gel and transferred to nitrocellulose. Nitrocellulose filters were hybridized with ³²P-labeled probe and exposed to x-ray film.

DNA Sequences of the Hypersensitive Region. A genomic clone was obtained from the BALB/c liver library previously described (23). A Bgl II fragment (~2.3 kb) containing the tissue- and stage-specific hypersensitive region was subcloned into the pGEM7Z (+) vector (Promega Biotec, Madison, WI), and sequenced by the dideoxy chain termination method (24) with both T7 and Sp6 promoter-specific primers on double-stranded plasmid DNA. All sequences reported were determined from both strands. To facilitate sequencing, deletional subclones were generated by Exo III and S1 nuclease treatment.

Results

Downstream DNase I-hypersensitive Regions in T Cells. Nuclei from the T cell line S49.1 were isolated and digested with various amounts of DNase I. The purified DNA was then digested with two restriction endonucleases (Bam HI and Bgl I), electrophoresed, blotted, and hybridized to a 0.6-kb fragment derived from the most 5' region of the Bam HI-Bgl I fragment as probe (Fig. 1 A). The Bam HI and Bgl I-digested DNA without DNase I digestion gives a band of ~6.8-kb (Fig. 1 B). With increasing amounts of DNase I, the major 6.8-kb bands grow weaker and minor faint bands appear and become more intense (Fig. 1 B). This pattern is thought to reflect the resulting fragments of partial digestion by DNase I; thus, it contains at least two DNase I-hypersensitive regions in the Bam HI and Bgl I fragment. (DHD 1 and DHD 2 thick and thin arrows, respectively). Analysis of nuclei from other T cell lines for the presence of these same hypersensitive sites revealed two faint bands in all samples after DNase I treatment, corresponding to DHD 1 and -2 (Fig. 2, C and D). In most of the T cell samples, subbands generated by the DHD 1 region were more intense and broader, possibly representing more than one band, than those resolved from the DHD 2 region. These results indicate that DNase I-hypersensitive region in the DHD 1 region is larger and more sensitive to DNase I than that in the DHD 2 region.

Developmental Specificity of TCR-β Downstream Hypersensitive Region. Changes in chromatin structure often reflect the developmental regulation of genes expressed in a tissue-specific manner. To analyze tissue specificity of DNase I-hypersensitivity in the DHD 1 and DHD 2 regions, nuclei from a B cell line (A20) and a non-T, non-B cell line (clone L) were prepared and analyzed by the same restriction enzymes and probe as described above (Fig. 2 A). Unlike the T cell lines previously examined, no subbands were detected at any DNase I concentrations used. Thus, nuclease hypersensitive sites are not present in the DHD 1 and DHD 2 regions in the TCR gene of A20 and clone L cells, but are present in the T cell lines examined, suggesting that both the DHD 1 and DHD 2 regions are generated in a tissue-specific manner and that these regions are related to tissue-specific gene regulation. To further dissect the developmental regulation of these hypersensitive regions, DNase I hypersensitivity of these regions was examined in T cells representing earlier develop-

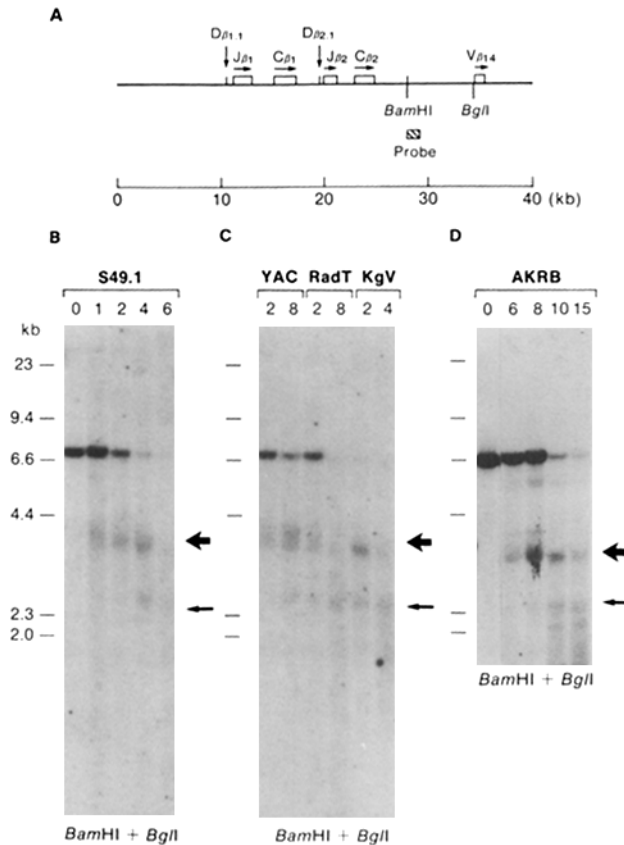


FIGURE 1. (A) Map of the mouse TCR- β gene. The region examined for DNase I hypersensitivity is downstream of the C- β -2 region between two indicated Bam HI and Bgl I restriction enzyme sites. The probe used for all Southern blots described here is indicated under the map. (B) DNase I hypersensitivity of the TCR- β Bam HI-Bgl I region in the S49.1 T cell line. DNase I-treated DNA was double-digested with Bam HI and Bgl I restriction enzymes and analyzed by Southern blotting (details described in Materials and Methods). Concentrations (units) of DNase I for nuclei digestion are indicated on the top of each lane. Hind III-digested λ phage DNA fragments (*left*) were used as size markers and electrophoresed in parallel. (C, D) DNase I hypersensitivity in a panel of T cell lines: YAC-1 (YAC), a radiation-induced T cell line (RadT), KgV, and AKRB cell lines. The thick and thin arrows indicate the positions of subbands generated from the DHD 1 and DHD 2 regions, respectively. DNase I concentration is indicated at top of each lane.

mental stages, i. e., NCKA cells in which V β regions are not rearranged on either chromosome, and KKE cells in which one allele(s) has a rearranged VDJ β and the other chromosome, only a rearranged DJ β gene. TCR- β gene expression was not detected in either cell line. Southern hybridization analysis of DNase I-treated nuclei from both cell lines revealed one subband from the DHD 1 region but not from the DHD 2 region (Fig. 2 B). Although subbands generated from the DHD 2 region were consistently weaker so that very faint subbands might have been missed, repeated experiments with a wider range of DNase I concentrations for nuclei digestion did not reveal any detectable subbands from the DHD 2 region. Thus, hypersensitivity in the DHD 1 region appears to be regulated in a tissue-specific manner, whereas that in the DHD 2 region appears to be both tissue- and stage-specific.

Location of the DNase I-hypersensitive Regions. The probe for these experiments was obtained from a Bam HI-Sau3 AI fragment of ~ 0.6 kb and located in the 5' region of the Bam HI-Bgl I restriction fragment (Fig. 3). Therefore, the size of bands detected by this probe reflects the distance from the Bam HI site to a region downstream where the particular restriction enzyme or DNase I cleaves. The subbands generated by DNase I (as measured from the center of each band) were ~ 3.5 kb

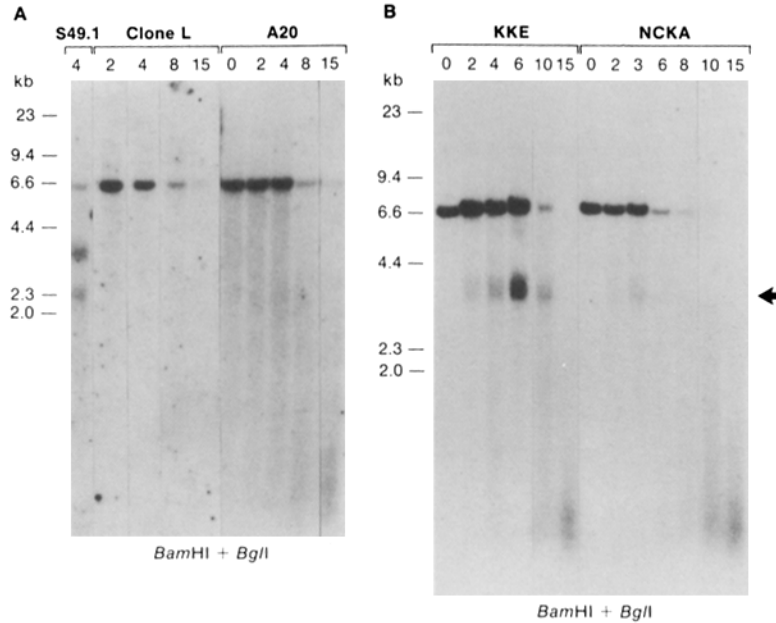
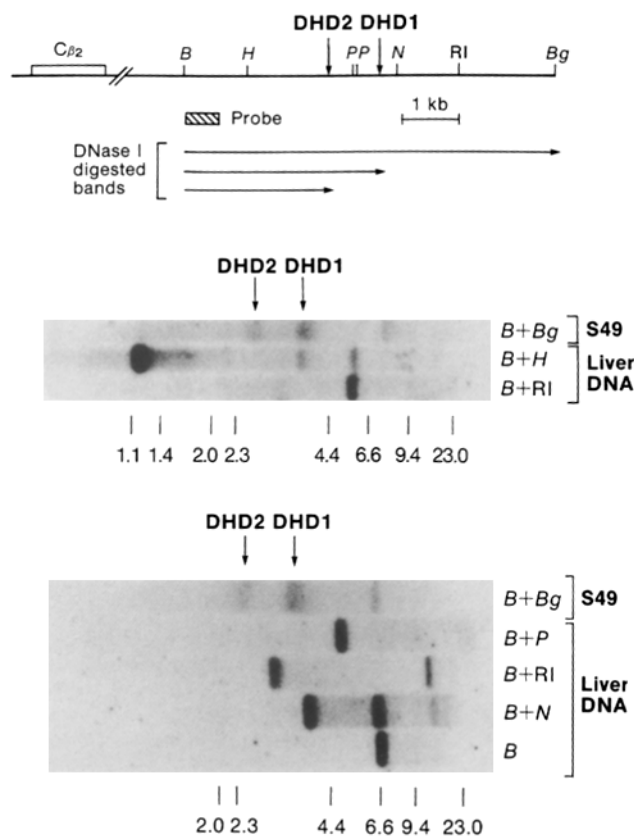


FIGURE 2. (A) DNase I hypersensitivity in non-T cell lines. DNase I-treated DNA from clone L and A20 cell lines was analyzed as described in Fig. 1. DNase I-treated DNA from the S49.1 cell line was analyzed in parallel as a positive control. (B) DNase I hypersensitivity in T cell lines that do not express TCR- β gene. DNase I-treated DNA from the KKE and NCKA cell lines was analyzed as described in Fig. 1 and Materials and Methods.

(DHD 1) and 2.3 kb (DHD 2) in size (Figs. 1 and 3). Thus, the DNase I-hypersensitive regions are located 3.5 kb and 2.3 kb downstream of the Bam HI sites. To further localize these regions, DNase I-treated nuclei were analyzed together with DNA digested with different combinations of restriction enzymes but not with DNase I. The band generated from the DHD 1 region migrated between two bands, representing the Bam HI plus Pst I digest and the Bam HI plus Nco I digest, respectively. Our analysis of the surrounding restriction enzyme sites suggests that the two highest molecular weight bands in the B + H lane (Fig. 3, *top gel*) and the highest molecular weight band in the B + P lane and the two highest molecular weight bands in the B + N lane (Fig. 3, *bottom gel*) are products of partial digestion. The band seen in the Bam HI plus Pst I digest lane is derived mainly from the 5' end of the Pst I and the Bam HI cleavage site, because the probe is located upstream of the two Pst I sites, between the Bam HI and the 5' end of the Pst I site (see Fig. 3). The band generated by the DHD 2 region is ~ 400 bp shorter than the band from the Bam HI plus Pst I digestion. Thus, the DHD 1 region is located between the 5' Pst I site and the Nco I site, and the DHD 2 region is located ~ 400 bp upstream of the 5' Pst I site. Recently a regulatory region with enhancer activity was described and mapped between the Bgl II and Nco I sites (18, 19). Our mapping shows that the DHD 1 region is that regulatory region.

DNA Sequences of the Tissue- and Stage-specific DNase I-hypersensitive Region. The bio-



side corresponds with the order of restriction enzyme sites from the Bam HI site. Restriction enzyme sites used for this study are shown in the map. The subbands observed in DNase I-treated S49.1 DNA are shown schematically below the restriction enzyme map.

logical function and the DNA sequences of the DHD 1 regions have been determined previously (18, 19); an enhancer activity and oligonucleotide sequences with characteristic enhancer motifs were identified. To analyze the DHD 2 region, DNA sequences of this and the flanking region were determined. Several oligonucleotide sequences homologous with characteristic regulatory DNA elements were found within ~150 bp upstream and downstream from the center of the DHD 2 hypersensitive region (Fig. 4). Three oligonucleotides show significant homology with the enhancer core sequence of SV40 or polyoma virus (25, 26). Several DNA sequences homologous to other characteristic regulatory sequences were found in this region: (a) sequences similar to the octamer sequence ATGCAAAT present in the promoters of IgH and κ chain genes and in the IgH enhancer (27-29); (b) sequences similar to the binding site of the transcription-activating protein AP-1 (30); and (c) sequences similar to the protein binding site detected by chemical modification using dimethyl-sulfate (DMS-modified IgH enhancer sequences) in the IgH enhancer (31, 32).

Two stretches of sequences homologous to the SV40 enhancer core sequences and sequences homologous to the octamer and AP-1 binding sequences are located close

FIGURE 3. Mapping the DNase I-hypersensitive regions in the S49.1 cell line. The top of the gel is on the right side. Four units of DNase I-treated DNA obtained from the S49.1 cell line was digested with Bam HI and Bgl I restriction enzymes and analyzed by Southern blotting (indicated as B + Bg/S49). BALB/c mouse-derived liver DNA digested with Bam HI plus Hind III (B + H) or Bam HI + Eco RI (B + RI) restriction enzymes were analyzed in parallel (*top gel*). In a separate experiment, BALB/c mouse-derived liver DNA was digested with Bam HI plus Eco RI (B + RI), Bam HI plus Pst I (B + P), Bam HI plus Nco I (B + N) restriction enzymes or Bam HI restriction enzyme alone (B) and compared with DNase I-treated S49.1 cell DNA digested with Bam HI and Bgl I (B + Bg/S49) restriction enzymes (*bottom gel*). Since the DNA fragment for the probe is located 5' of the Bam HI and Bgl I sites (shown under the map), the sizes of bands are identical with the distance from Bam HI to a downstream site where the restriction enzyme or DNase I cleaves; the order of the bands from the left

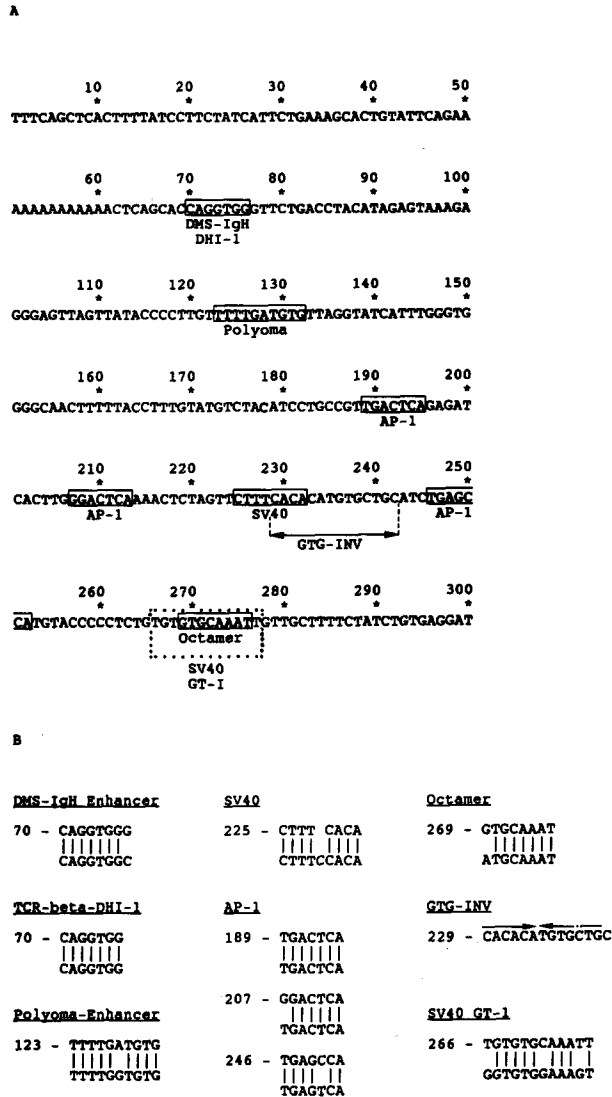


FIGURE 4. (A) DNA sequence of the DHD 2 region. The nucleotide stretches that show significant homology with the characteristic DNA sequences within the regulatory elements are shown in B. (B) DHD 2 region sequences (*top sequences*) are compared with consensus sequences (*bottom sequences*); the location of each DNA element within the DHD 2 region is indicated on the left. The GTG-containing inverted repeat is shown as GTG-INV, with arrows indicating an inverted repeat. Note that the DNA elements starting at positions 189 and 207 are aligned with the AP-1 binding site consensus sequence, while the DNA element at position 246 is aligned with the opposite strand of the AP-1 consensus sequence.

together in a region downstream of the center of the DHD 2 region. Interestingly, GTG symmetrical DNA sequences were also found in this cluster. A similar symmetrical motif was shown to be a nuclear protein binding region in the well-defined prokaryotic and eukaryotic regulatory regions (33-35), and is detected in the promoter of the TCR- β gene (33, 36, 37). Thus, this downstream 100-bp fragment appears to be a core region of the DHD 2 segment.

Discussion

Two DNase I-hypersensitive regions, DHD 1 and DHD 2, were found downstream of the C- β -2 region of the TCR- β gene. Hypersensitivity in the DHD 1 and DHD

2 regions was expressed in a tissue-specific and a tissue- and stage-specific manner, respectively. Mapping studies of DHD 1 and DHD 2 showed that a previously described regulatory region is identical with the DHD 1 region. Other investigators have demonstrated an enhancer activity in this regulatory region by transient CAT assay, and activity of the enhancer element was observed in both T and B cells (18). Our data indicating tissue specificity of DNase I hypersensitivity at the DHD 1 region strongly suggest that tissue-specific regulation of the TCR- β gene by the enhancer region is determined, at least in part, by tissue-specific changes of the local chromatin structure.

The biological function of the DHD 2 hypersensitive region is not clear. The strong enhancer activity observed in the DHD 1 region was not detected in the DHD 2 region (18, 19). DNA sequencing of the DHD 2 region revealed several stretches homologous with characteristic regulatory motifs. One of the sequences in the DHD 2 region is homologous with the sequences of the protein binding site in the IgH enhancer detected by chemical modification (31, 32). A sequence similar to that of the DMS-modified IgH enhancer sequence has also been identified in the DNase I-hypersensitive region of the TCR- β J-C2 intron (designated DHI-1) (Hashimoto, Y., M. I. Greene, and A. Maxam, manuscript submitted for publication). The first seven nucleotides (GTCCACC) of the DHD 2, DHI 1, and the DMS-modified IgH enhancer region are identical, differing only in the last nucleotide. It is possible that the conserved nucleotide sequence is sufficient in itself for recognition by specific T cell factors. Three stretches of nucleotides similar to the binding site of transcription-activating protein AP-1 were found in the DHD 2 region. Similar sequences to the AP-1 binding region were also found in the promoter and enhancer regions of the TCR- β gene (18, 19, 37); these motifs were located within the known regulatory regions of this gene. Thus, these AP-1 binding region-like sequences may have a critical role in T cell-specific gene expression. Studies by other investigators using TCR- β gene transgenic mice revealed that the TCR- β gene is expressed only when both DHD 1 and DHD 2 regions are present (18). Still other experiments with TCR- β gene transgenic mice showed that the presence of DHD 1 is sufficient for low level expression of the TCR- β (Selsing, E., Brandeis University, Waltham, MA, personal communication). These observations, together with our data indicating tissue- and stage-specific DNase I hypersensitivity at the DHD 2 region, suggest that the DHD 2 is a regulatory region for tissue-specific gene expression.

Recently, several examples of a downstream enhancer element were described (38-40). In addition to indicating the presence of an enhancer element, our data strongly suggest the presence of another regulatory element located downstream of the C- β -2 region in the TCR- β gene. Moreover, our demonstrating the tissue and stage specificity of DNase I hypersensitivity suggests that changes in chromatin structure control tissue-specific gene expression.

Summary

Two DNase I-hypersensitive regions were identified downstream of the TCR gene constant region. One of these regions is located at the site of a putative enhancer element and was observed only in T cell lines and not in cell lines derived from other tissues. The other DNase-hypersensitive region was also detected only in T cell lines but only in those expressing TCR- β RNA. Thus, the first region is probably tissue

specific, while the second region is probably tissue and stage specific. The DNA sequence of the second DNase I-hypersensitive region revealed several stretches of nucleotides that are characteristic of consensus sequences for regulatory elements. These results, together with the observations in transgenic mice that indicate a requirement for two distinct regions for optimal TCR gene expression, suggest the presence of at least two regulatory regions downstream of the C- β -2 region; one is an enhancer region and the other is a transcriptionally related regulatory region. The tissue/stage specificity of these DNase I-hypersensitive regions supports the notion that changes in chromatin structure control tissue-specific gene expression.

We thank Mr. Eric Asel for technical assistance. We also thank Dr. Susan Faas and Ms. Marina Hoffman for critical reading of the manuscript and Ms. Mayumi Hashimoto for preparation of the manuscript.

Received for publication 8 December 1988 and in revised form 7 March 1989.

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