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Activation of the chicken Ig-β locus by the collaboration of scattered regulatory regions through changes in chromatin structure

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ABSTRACT

A total of 10 B-lymphocyte-specific DNase I hypersensitive sites located in the chicken Ig-β locus were divided into four regions and combinations of deletions of these regions were carried out. A decrease in transcription of the Ig-β gene to <3% was demonstrated in cells with deletions in all four regions. The Ig-β chromatin was resistant to DNase I digestion in these cells. Thus, the collaboration is shown to convert the Ig-β chromatin from the condensed state to a relaxed state. H3 and H4 acetylation decreased to <8% but H3K4 hypermethylation was observed at the Ig-β promoter and exon 3. The collaboration of four regions had virtually no effect on CG hypomethylation in the region upstream the transcriptional start site. Accordingly, neither the DNase I general sensitive state in the Ig-β chromatin nor hyperacetylation of H3 and H4 histones in the promoter proximal region causes H3K4 di-methylation or CG hypomethylation in the promoter. From these analyses, a chromatin situation was found in which both an active state, such as enhanced H3K4 methylation, or CG hypomethylation, and an inactive state, such as DNase I resistance in the Ig-β chromatin or hypoacetylation of H3 and H4 histones in the Ig-β locus, coexist.

INTRODUCTION

In vertebrate genes that are transcribed cell type specifically, transcriptional regulatory regions are present not only in the promoter proximal region, but also scattered in the upstream region, intron and downstream region (1). However, the role of the scattered regulatory regions for cell type-specific transcription remains obscure. In eukaryotic cells, DNA with genomic information is first stored in nucleosomes as the fundamental units, and then highly folded into chromatin. In the process of differentiation, to switch 'ON' the cell type-specific genes that have not been transcribed so far, it is first necessary to convert the chromatin of a gene and its flanking region from an inactive state to an active state. In vertebrate cells, chromatin of the committed or active gene and its flanking region shows the following features: (i) sensitivity to DNase I (2); (ii) cell type-specific DNase I hypersensitive sites (DHSs) (3,4); (iii) core histone modifications such as acetylation and methylation specific for the active chromatin state, largely at the N- and C-terminal tails (5); and (iv) histone variants such as H3.3 and H2AZ (6). In addition, hypomethylation at the cytosine (C) residues of the cytosine–guanine (CG) dinucleotides is shown in the active promoter (7,8).

Cell type-specific DHSs not only correspond to promoters and enhancers but also are likely to be the regulatory regions participating in the change and maintenance of the chromatin structure from an inactive state to an active state (3,4). In addition to their presence in and close to committed or active genes, cell type-specific DHSs are reported to reside inside the flanking genes (9,10) or between the flanking genes (10–13). Several investigations have been carried out to determine the regions necessary for chromatin changes in the scattered regulatory regions (10,14–18). Deletion of DHS1-6 in the mouse β-globin locus control region (LCR) results in an extraordinary low level of β-globin transcription. However, the acetylation of H3 and H4 histones at the β-globin promoter is the same as in the undeleted control (16) and no change in DNase I sensitivity in the β-globin locus has also been observed (14). Transgenic mice with 0.1 kb deletion of the DHS I region, which constitutes the LCR in the human GH locus, show loss of H3 and H4 acetylation starting from 32 kb upstream of the transcriptional starting site of the GH gene to the GH gene (10). Accordingly, the deleted sequence is essential for the establishment and maintenance of histone acetylation in the GH locus. However, no example has been reported that demonstrates the

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change in chromatin structure from sensitive to resistant to DNase I by deleting DHS.

To determine the *in vivo* role of the scattered DHSs, genetic studies such as gene targeting are effective. Chicken B-lymphocyte-derived DT40 cells are particularly useful for gene targeting because of the high rate of homologous recombination (19,20). Along with membrane immunoglobulin and Ig-α/mb1, Ig-β is a component of the antigen receptor complex (21). Chicken Ig-β gene is specifically expressed in B lymphocytes such as DT40 cells (18) and a project has been planned to elucidate the *in vivo* role of DT40-specific DHSs in B-cell-specific transcription of the Ig-β gene by deleting the DHSs. A total of 13 DT40-specific DHSs were mapped in the 40 kb region spanning from 19 kb upstream to 21 kb downstream from the transcriptional starting site of Ig-β gene (Figure 1) (22). Based on these results, 11 DT40-specific DHSs found in the Ig-β locus were divided into four regions and deleted (18). The deletion of a single region demonstrated no prominent effect on the Ig-β mRNA level. In this study, combinations of deletions of the four DHS regions were carried out. A marked decrease in the level of Ig-β mRNA was observed in cells with deletion of all four regions (All Del cells). Thus, it has been demonstrated that the collaboration of scattered regulatory regions causes a effect on the expression of the Ig-β gene. Examination of Ig-β chromatin in All Del cells showed that the collaboration of the four regions is capable of converting the Ig-β chromatin from a condensed state to a relaxed state and enhancing H3 and H4 acetylation in the Ig-β gene.

MATERIALS AND METHODS

Generation of DHS deletion mutants

DT40/Cre cells (23) were propagated in RPMI 1640 medium (Nissui, Tokyo, Japan)/10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA)/1% chicken serum (Gibco BRL, Grand Island, NY, USA) at 39.5°C. The plasmid pLoxBsr (23) containing the chicken β-actin promoter-driven blasticidin resistance (Bsr) gene with mutant loxP sequences at both ends was used as a targeting vector. For the targeting construct, genomic DNA of ∼2 kb in size was linked on both-sides with the actin promoter-driven Bsr gene. Preparation of the targeting constructs (Figure 1) except for IIS Del was performed as described previously (18). Positions of left and right arms of the IIS Del constructs are as follows (the transcriptional starting site of the Ig-β gene is shown as base number one): IIS-L (−4613 to −2692; 1922 bp) and IIS-R (−612 to +1479; 2091 bp). DT40/Cre cells were electroporated with linearized DNA and Bsr resistance clones were obtained. To determine homologous recombination, PCR was carried out as described previously (18). DT40/Cre clones with homologous recombination were treated with 0.1 mM 4-OH-tamoxifen (Sigma, St Louis, MO, USA) for 19 h and then cloned in 96-well microtiter plates.

DNase I sensitivity analysis and filter hybridization

For DNase I sensitivity analysis, nuclei were prepared and treated with DNase I (Takara Bio), and restriction enzyme digestion and Southern hybridization were carried out as reported previously (18). Probe DNA was labeled by the random-priming methods using [α-^{32}P]dCTP. Preparation of RNA followed by northern hybridization was performed as described previously (18). The intensity of bands on the northern and Southern hybridizations, and the S1 nuclease protection assay was compared using a Typhoon 9210 microarray imager (Amersham Biosciences, Piscataway, NJ, USA) and Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

S1 nuclease protection assays

S1 nuclease protection assays were carried out as described previously (12). Probes were end-labeled with

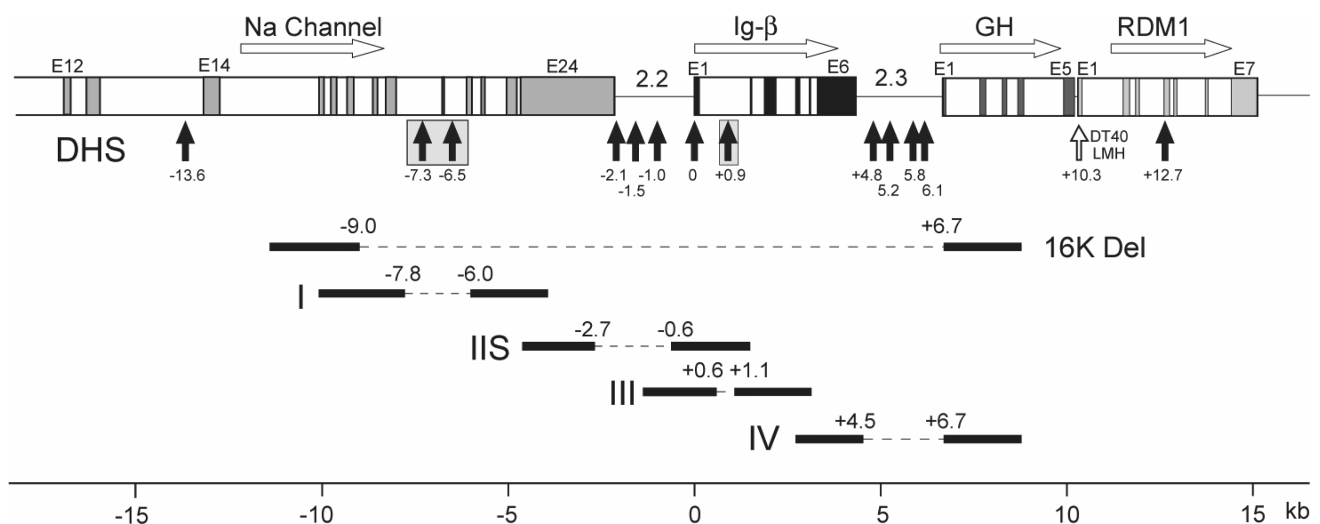

Figure 1. Positions of the DT40-specific DNase I hypersensitive sites (DHSs) and deleted regions in the chicken Ig-β locus. Exons are shown by gray or closed rectangles, introns by open rectangles and intergenic regions by solid lines. Horizontal arrows represent transcriptional orientations. Exon numbers are indicated. Sizes of the intergenic regions are shown in kb. Upward vertical arrows indicate DHSs; distances from the transcriptional starting site of the Ig-β gene in kb are indicated underneath. Closed arrow, DT40-specific; open arrow, common to both DT40 cells and liver-derived LMH cells. DHS arrows with enhancing activity are enclosed by rectangles. Positions of the arm sequences used for the targeting constructs are shown by closed bars and regions deleted are shown by dotted lines. Numbers above the 5′ and 3′ ends of the region deleted are the distances from the transcriptional starting site of the Ig-β gene.

T4 polynucleotide kinase. Total RNA (8 μg) and 50 fmol of end-labeled DNA were hybridized in 30 μl of 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA and 80% formamide for 16 h at 50°C. The hybridization mixture was digested for 30 min at 37°C with 50 U of S1 nuclease (Takara Bio) in 300 μl of 30 mM sodium acetate (pH 4.6), 280 mM NaCl, 1 mM $ZnSO_4$ and 10 μg/ml of sonicated and denatured salmon testis DNA. The protected DNA was phenol-chloroform extracted, precipitated by ethanol with 5 μg of yeast RNA and analyzed on a 6% polyacrylamide gel containing 8M urea.

Histone modification analysis and bisulfite sequencing

The acetylation status of H3 and H4 histones and the di-methylation status of H3K4 were examined by chromatin immunoprecipitation (ChIP) and real-time PCR (RT-PCR) as described previously (24). Anti-acetylated H3 and H4 and anti-di-methylated H3K4 were obtained from Upstate Biotech (Lake Placid, NY). Anti-Pol II was obtained from Santa Cruz Biotech (Santa Cruz, CA). The state of CG methylation in the Ig-β promoter (−409 to +14) was examined by the bisulfite sequencing method (25). Bisulfite-treated DNA was amplified by nested PCR, cloned into pCR2.1-TOPO, and sequenced. The following primers were used for the nested PCR: first round (forward, ATGGGTTGGGG-TTGGATTT; reverse, CTAAAACACCCACAACCTCC), second round (forward, GGATTTGGGGATTTTGGAGG; reverse, AAAATCTCCCATCTTCTCA).

RESULTS

Generation of DT 40 cells with combinations of DHS deletions

The organization of the chicken skeletal muscle sodium channel/Ig-β/growth hormone (GH) locus is the same as that of rat, mouse and human loci, whereas the organization downstream from the GH gene is different between chicken and mammals. The repair-related gene RDM1 (26) with seven exons was found downstream from the GH gene in chicken (Figure 1). A total of 10 DHSs present between −7.3 and +6.1 kb were divided into the following four regions (Figure 1): region I, having transcription-enhancing activity (22) in the sodium channel gene (−7.8 to −6.0 kb; DHSs, −7.3 and −6.5 kb); region IIS, between the sodium channel and Ig-β genes (−2.7 to −0.6 kb; DHSs, −2.1, −1.5 and −1.0 kb); region III, in the first intron of the Ig-β gene (+0.6 to +1.1 kb; DHS, +0.9 kb); and region IV, between the Ig-β and GH genes (+4.5 to +6.7 kb; DHSs, +4.8, +5.2, +5.8 and +6.1 kb). DT40 cells with a 16 kb deletion (16K Del) in one allele of the Ig-β locus was produced and then cells with every combination of deletions were generated. Proper deletions in all combinations were confirmed by genomic Southern hybridization (data not shown) and a representative result is shown in Figure 2.

Level of Ig-β mRNA decreases to <3% in cells with I-IIS-III-IV (All) DHS deletions

The level of Ig-β mRNA in cells with combinations of DHS deletions was examined by northern hybridization (Figure 3). Changes more or less than 30% were observed in cells with a single deleted region, such as I Del (94 and 92%), IIS Del and III Del, whereas a decrease to 47 and 78% was demonstrated in cells with the region IV deletion. The level of Ig-β mRNA decreased to 69 and 75% in I-IV Del cells, to ∼60% in IIS–III Del cells, and to ∼30% in III–IV Del cells, while no prominent change was observed in I–IIS Del and IIS–IV Del cells (Figure 3) and I–III Del (93 and 93%) cells. In deletion cells with a combination of three regions, a marked decrease was observed in two combinations, such as I–III–IV Del (16 and 12%) and IIS–III–IV Del (5 and 10%), whereas a decrease to 71 and 57% was detected in I–IIS–IV Del cells, and no notable change was found in I–IIS–III Del cells (118 and 110%). A decrease was demonstrated in I–IIS–III–IV (All) Del cells in which the level of Ig-β mRNA was <2 and 3% as compared with undeleted 16K Del cells. Thus, it has been demonstrated that the collaboration of scattered regulatory regions causes an effect on the expression of the Ig-β gene in B lymphocytes.

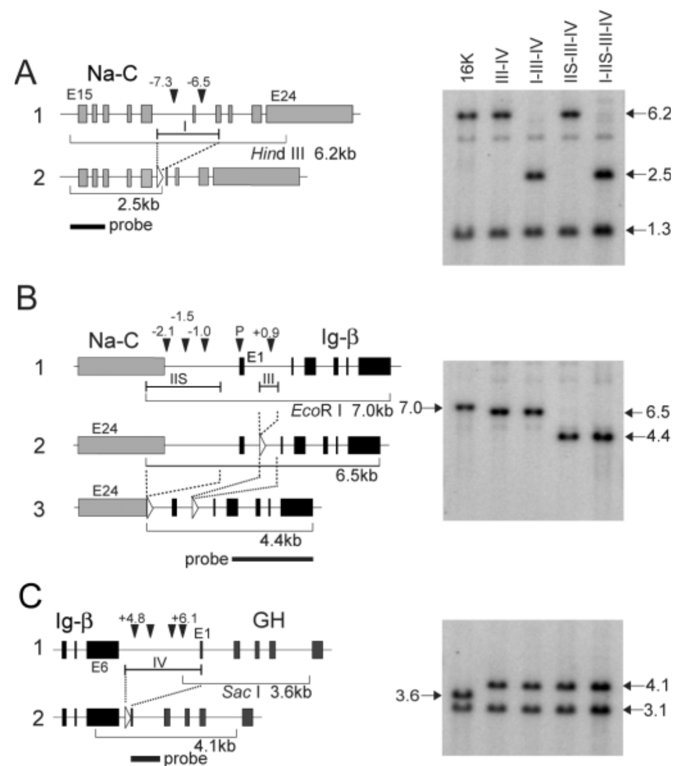

Figure 2. Confirmation of DHS deletions by Southern hybridization. Homologous recombination and excision of the Bsr gene by tamoxifen treatment was first examined by PCR and then confirmed by Southern hybridization. Deleted regions: (**A**) I (−7.8 to −6.0 kb); (**B**) IIS (−2.7 to −0.6 kb), III (+0.6 to +1.1 kb); and (**C**) IV (+4.5 to +6.7 kb). Left, gene organization; right, Typhoon 9210 image of the Southern hybridization. Positions of the probes are indicated by closed bars. Sizes and positions of the restriction fragments hybridized with the probe are shown below the map. Exons are indicated by rectangles and intron and intergenic regions are indicated by solid lines. The exon numbers and the positions of DT40-specific DHSs are shown as in Figure 1. Probes: (A) fragment from the sodium channel gene (−10.4 to −9.4 kb; HindIII/AccI); (B) Ig-β cDNA (nt 312–1542; PmaCI/EcoRI) (in accession no. AB062512); (C) GH gene (+6.6 to +7.4 kb; NcoI/NcoI) (in CHKGHG/D10484). Restriction enzyme digestion: (A) HindIII; (B) EcoRI; (C) SacI. Band sizes are indicated by arrows in the left and right margins of the images. The 1.3 kb bands (A) and 3.1 kb bands (C) are derived from the 16K Del allele.

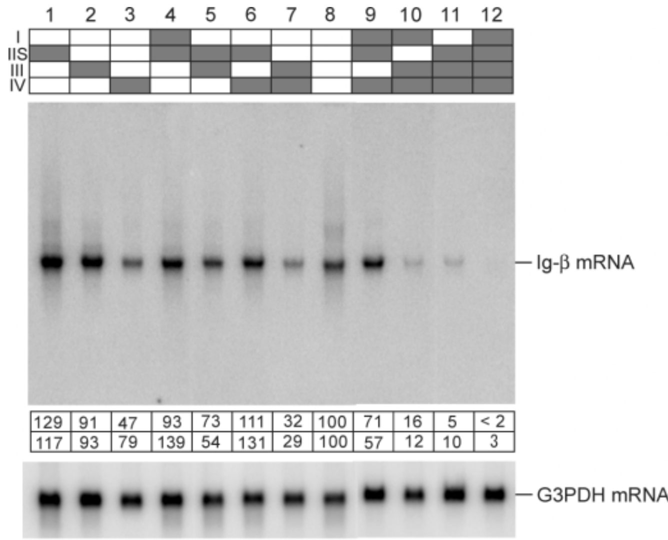

Figure 3. Level of Ig-β mRNA in cells with combinations of deletions in DT40-specific DHSs. Using 3.0 μg total RNA prepared from a deletion, the level of Ig-β mRNA was examined by northern hybridization. Deleted regions are indicated by gray rectangles in the top grid. Northern hybridization with Ig-β cDNA probe is shown underneath the grid and the hybridization with glyceraldehydes-3-phosphate dehydrogenase (G3PDH) cDNA is shown at the bottom. The positions of Ig-β and G3PDH mRNA are indicated in the right margin of the images. The intensity of bands was determined by using a Typhoon 9210 microarray imager and Image Quant software. The values of two clones from each deletion are shown between the Ig-β and G3PDH hybridization images. Ig-β cDNA (nt 312–1542; PmaCI/EcoRI); G3PDH cDNA (nt 325–1020 in CHKGAPDHB/J00849).

Although the level of Ig-β mRNA in cells with the region I deletion was almost the same as that in 16K Del cells, a greater decrease in the Ig-β mRNA level was found in I–III–IV Del cells (lane 10, 16 and 12%) as compared with III–IV Del cells (lane 7, 32 and 29%). Such decrease in the Ig-β mRNA level was again detected in All Del cells (lane 12, <2 and 3%) in comparison with All minus region I Del (IIS–III–IV Del) cells (lane 11, 5 and 10%). Accordingly, the participation of region I in cell type-specific transcription of the Ig-β gene was shown to be present inside the flanking gene, as has been reported in mouse and human β-globin loci and the human GH locus (1).

Decrease in transcription is the reason for low level of the Ig-β mRNA in All Del cells

To investigate whether the decrease in the Ig-β mRNA level in All Del cells is at the transcriptional step, the occupancy of pol II at the Ig-β promoter and exon 3 was examined by ChIP analysis with anti-pol II antibody (Figure 4A). As a positive control, the promoter proximal target of the Ig-J chain gene, which is transcribed equally with Ig-β gene in DT40 cells (data not shown), was used, while the target in the 16 kb condensed chromatin, which is located between the β-globin domain and the folate receptor gene, was used as a negative control because markedly low levels of H3 and H4 acetylation (27) and H3K4 methylation (28) in DT40 cells had been reported at this target. Similar amounts of pol II occupied the Ig-J promoter in 16K Del, III-IV Del and All Del cells. A 13-fold enrichment as compared with the negative control was observed at the Ig-β promoter in 16K Del cells, while the occupancy decreased markedly to 4-fold in III-IV Del cells, and to 1.6-fold in All Del cells where the level is close to that of the negative control. At Ig-β exon 3,

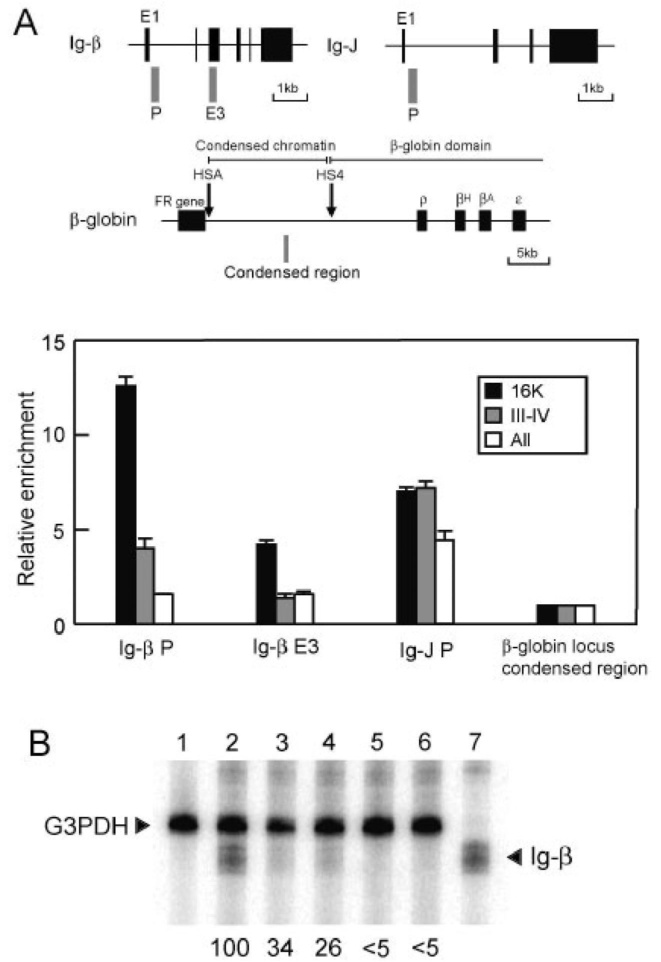

Figure 4. (**A**) ChIP analysis of the occupancy of pol II at the Ig-β promoter and exon 3 in cells with combinations of deletions of DT40-specific DHSs. A target close to the Ig-J chain promoter was used as a positive control and a target in the 16 kb condensed chromatin region located between the β-globin domain and the folate receptor gene (27) as a negative control. Positions of four targets are shown at the top. Primers: Ig-β promoter [forward, 5′-CTGCAGTGAGAAGATGGGAG, reverse, 5′-GAATCCCGTGGTGCACA-AAG; (+)51 to (+)227 in AB062512]; Ig-β exon 3 [forward, 5′-GCTA-CATTGCTGCCAAGAAG, reverse, 5′-TGGAGATGGTGAAGCTGATG; (+)1910 to (+)2086 in AB062512]; Ig-J promoter [forward, 5′-GCGAGA-TGAAGAGCTCTTTG; reverse, 5′-GCAAAACTTCTTGAGCAGGC; (+)21 to (+)220 in AB076374]; β-globin condensed region (forward, 5′-CAGCAGACGCTGTGGTGAA; reverse, 5′-CTTGCAGGATGCAGACT-GGA] (27). Exons and introns are indicated as in Figure 2. Arrows, DHSs. ChIP analysis of the occupancy of pol II is shown at the bottom. Relative enrichment is shown by fold enrichment compared with the negative target (18). Three amplifications were performed for each target. Values are mean SE. 16K Del, closed bars; III–IV Del, gray bars; All Del, open bars. (**B**) Quantitative S1 nuclease protection assays. Total RNA from two clones was hybridized to an excess amount of end-labeled DNA probe and treated with S1 nuclease. RNA: 16K Del (1, 2, 7); III–IV Del (3, 4); All Del (5, 6). Probe: G3PDH (1); G3PDH+Ig-β (2–6); Ig-β (7). The Ig-β S1 probe yields a protected fragment of ∼138 bases (nt 1–138 in the Ig-β cDNA; AB062512); the G3PDH protected fragment is 156 bases (nt 325–480 in the G3PDH cDNA;CHKGAPDHB/J00849). The intensity of bands was quantified by Typhoon and Image Quant, and shown below the image.

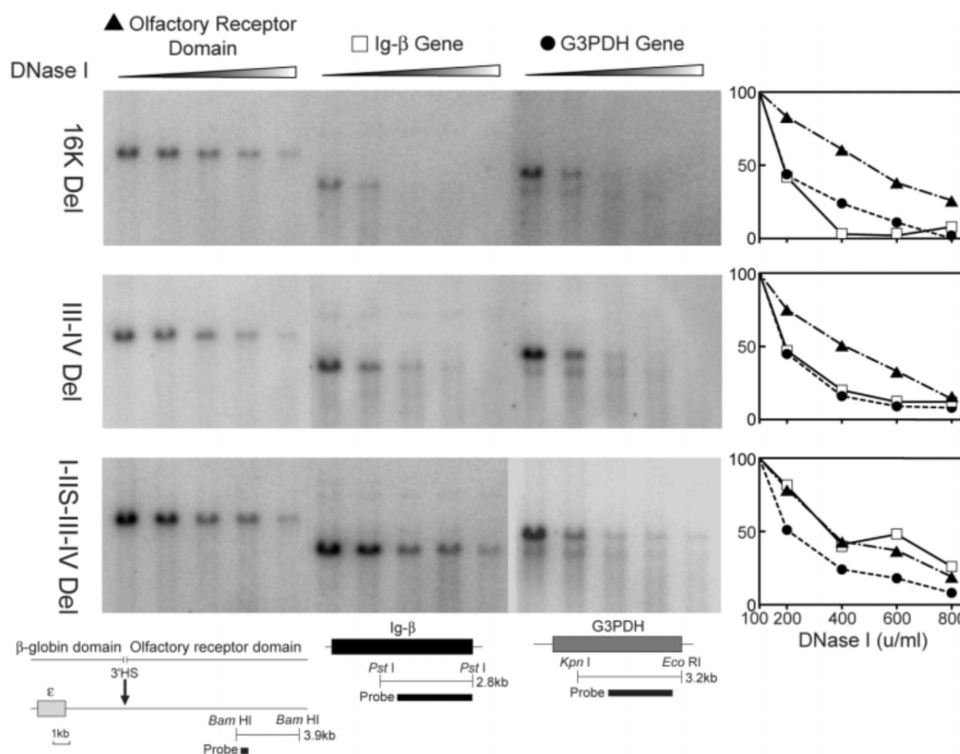

Figure 5. DNase I general sensitivity of the chromatin in cells with combinations of DHS deletions. Isolated nuclei were treated with DNase I for 3 min at 20°C. The concentrations of DNase I for treatment of nuclei from left to right: 100, 200, 400, 600 and 800 U/ml. The DNA was purified from nuclei and digested with BamHI for the olfactory receptor domain, PstI for the Ig-β gene or KpnI /EcoRI for the G3PDH gene. The digests (3.0 μg) were subjected to Southern hybridization with probe P10 (30) for the olfactory receptor domain. The same Ig-β and G3PDH cDNA probes were used as in Figure 3 for the analysis of Ig-β and G3PDH genes. The analyzed region, DHS, and the position of the probe are shown at the bottom. The intensity of bands was determined by using a Typhoon 9210 microarray imager and Image Quant software. Relative values are shown in the right figures, in which the value at 100 U/ml DNase I treatment is plotted as 100%. Closed triangle, olfactory receptor domain; open square, Ig-β gene; closed circle, G3PDH gene.

a decrease in the occupancy was again found in both deletion cells.

Total RNA from 16K Del, III-IV Del and All Del cells was analyzed by quantitative S1 nuclease protection assays with mixed probes for Ig-β and G3PDH mRNAs (Figure 4B). Several protected bands due to multiple transcriptional initiation sites of the TATA box-less Ig-β gene (29) were observed. The band intensity decreased to 34 and 26% in III–IV Del cells and <5% in All Del cells as compared with undeleted 16K Del cells. From these evidences, a decrease in the Ig-β mRNA level in All Del cells is shown to be regulated at the transcription stage.

Chromatin of the Ig-β gene turns out to be DNase I-resistant in All Del cells

DNase I general sensitivity in the chromatin of the Ig-β gene in III–IV Del and All Del cells was examined (Figure 5). Chromatin of the glyceraldehydes-3-phosphate dehydrogenase (G3PDH) gene, which is transcribed in all three kinds of cells (Figure 3) including 16K Del cells, was examined as the positive control, while the chromatin in the olfactory receptor domain, which is located downstream from the β-globin domain, was investigated as the negative control because it had been reported as DNase I-resistant in 15-day-embryo chicken erythrocytes (30). In all cells, the chromatin in the olfactory receptor domain was resistant to DNase I digestion, whereas the G3PDH chromatin was DNase I-sensitive. The chromatin of the Ig-β gene in 16K Del cells was sensitive to DNase I. In contrast, the Ig-β chromatin turned out to be resistant to DNase I digestion in All Del cells. Partial resistance to DNase I was observed in the Ig-β chromatin in III–IV Del cells. These results indicate that the collaboration of I–IIS–III–IV regions is capable of converting the Ig-β chromatin from the condensed state to a relaxed state.

Acetylation and H3K4 methylation at Ig-β promoter and exon 3 in cells with combinations of DHS deletions

The states of H3 and H4 acetylation and H3K4 di-methylation at the Ig-β promoter and exon 3 in cells with combinations of deletions were examined by ChIP analysis followed by real-time PCR quantification (Figure 6). In 16K Del cells, a 67-fold increase at the promoter and a 19-fold increase at exon 3 were found in H3 acetylation as compared with the condensed region flanking the β-globin locus where a hypoacetylation state has been reported in DT40 cells (27), while H3 acetylation decreased to 20% at the promoter and 40% at exon 3 in III-IV Del cells as compared with 16K Del cells. In comparison with III–IV Del cells, a gradual decline in H3 acetylation was observed in

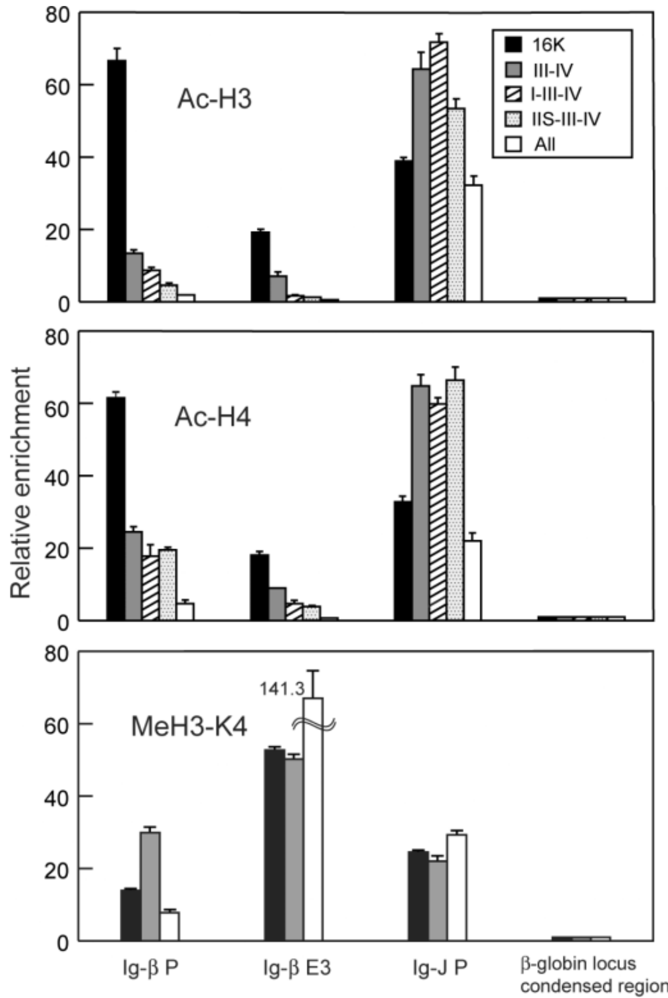

Figure 6. Acetylation and H3K4 methylation in cells with combinations of DHS deletions. The states of acetylation of H3 and H4 and di-methylation of H3K4 at the Ig-β promoter and exon 3 were examined by ChIP analysis. Four targets are shown as in the top of Figure 4. Relative enrichment is shown by fold enrichment compared with the β-globin locus condensed region. Ac-H3, H3 acetylation; Ac-H4, H4 acetylation; MeH3-K4, H3K4 methylation. 16K Del, closed bars; III–IV Del, gray bars; I–III–IV Del, stripped line bars; IIS–III–IV Del, dotted bars; All Del, open bars. Three amplifications were performed for each target. Values are mean ± SE. An overscaled bar is shown by double wavy lines and its mean value is indicated on the left.

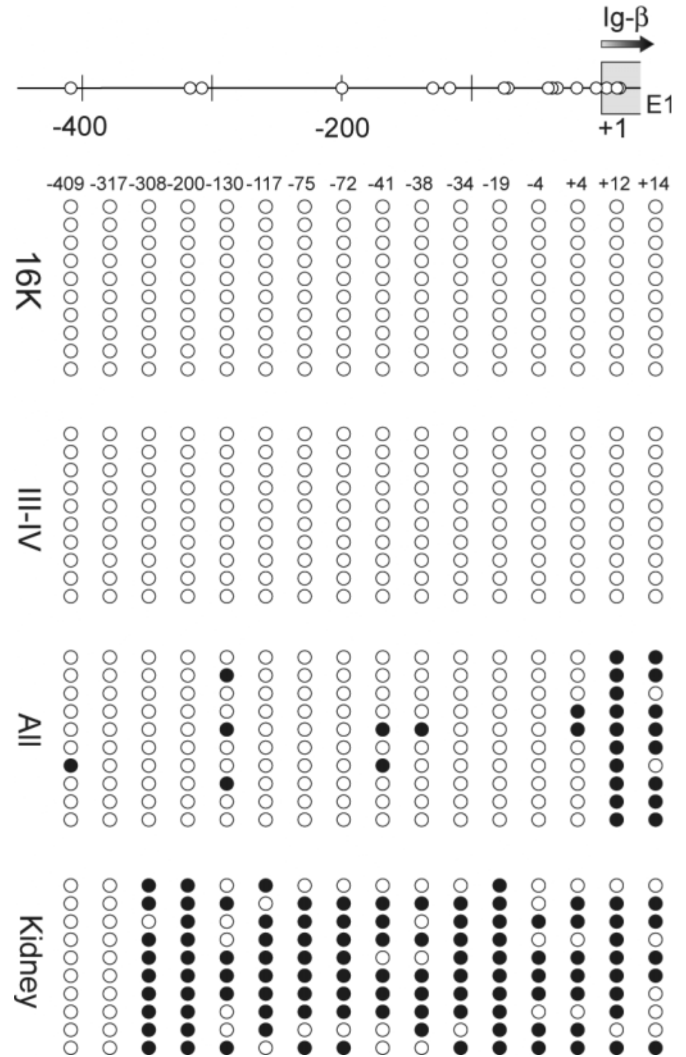

Figure 7. CG methylation in the Ig-β promoter in cells with combinations of DHS deletions. Positions of the CG dinucleotides up to the 0.4 kb upstream region of the Ig-β gene are indicated as open circles in the top map. Below the map are shown the positions of the C in the CG dinucleotides by numbers. A total of 13 are present in the upstream region and 3 in the downstream from the transcriptional initiation site. Bisulfite-treated DNA was amplified by PCR, cloned into pCR2.1-TOPO and sequenced. The methylation status of 10 clones obtained from 16K Del, III–IV Del, All Del and kidney DNA is shown. Open circle, unmethylated; closed circle, methylated.

two triple deletions. In All Del cells, H3 acetylation at the promoter and at exon 3 reached 3% of that in 16K Del-cells, the same level as the Ig-β mRNA reduction. In H4 acetylation, the same profile as H3 acetylation was found. H4 acetylation was 8 and 4% at the promoter and at exon 3, respectively. In all cells, hyperacetylation of H3 and H4 histones was observed at the Ig-J promoter. In contrast to the decrease in H3 and H4 acetylation, there was a decrease to 56% at the Ig-β promoter and 2.7-fold increase at the exon 3 in H3K4 di-methylation in All Del cells. In III–IV Del cells, 2.2-fold increase in H3K4 di-methylation at the Ig-β promoter was observed, while the same di-methylation state as in 16K Del cells was found at the exon 3. Thus, the collaboration of I–IIS–III–IV regions is shown to participate in H3 and H4 acetylation in the promoter proximal region of the Ig-β gene.

CG methylation in the Ig-β promoter in cells with combinations of DHS deletions

The state of CG methylation in the promoter region of the Ig-β gene was examined by the bisulfite sequencing method (25) (Figure 7). All sixteen CG dinucleotides were unmethylated in 16K Del and III–IV Del cells, whereas most CG dinucleotides except at −409 and −317 were methylated in kidney cells, where the Ig-β gene is not transcribed (data not shown). Most CG dinucleotides were unmethylated in All Del cells except for significant methylation at +12 and +14, thus indicating that the collaboration of I–IIS–III–IV regions does not have an effect on hypomethylation in the region situated upstream the transcriptional start site. Although the Ig-β chromatin becomes DNase I-resistant, and

the state of H3 and H4 acetylation in the promoter proximal region decreases in All Del cells, the CG hypomethylation state in the Ig-β promoter is essentially the same as in 16K Del cells except at +12 and +14. This indicates that neither the DNase I general sensitive state in the Ig-β chromatin nor hyperacetylation of H3 and H4 histones in the promoter proximal region causes CG hypomethylation in the region present upstream the transcriptional start site.

DHS formation in further upstream and downstream of the regions I and IV is the same but DNase I sensitivity at Ig-β promoter decreases in All Del cells

In addition to the DHSs deleted in this study, two DT40-specific DHSs are present in further upstream and downstream regions: one at −13.6 kb is positioned in the intron 13 of the upstream sodium channel gene and the other is at +12.7 kb, located close to exon 4 of the downstream RDM1 gene (Figure 1). Examination was carried out to determine whether III–IV Del and All Del deletions have any effect on the formation of these DHSs (Figure 8). In addition, DHSs at the Ig-β promoter and at +10.3 kb, located at the promoter of the RDM1 gene, which is transcribed in DT40 cells and liver-derived LMH cells (data not shown), were analyzed. No difference in DHS formation at −13.6, +10.3 and +12.7 kb was found in 16K Del, III–IV Del and All Del cells. Thus, I–IIS–III–IV regions are shown to be unnecessary for the maintenance of these DHSs. In addition, neither hyperacetylation of H3 and H4 histones in the Ig-β promoter proximal region nor the DNase I general sensitive state in the Ig-β chromatin was found to be required for these DHS formations. In All Del cells, the DHS at the Ig-β promoter was present with a decrease in sensitivity to DNase I. Thus, chromatin parameters such as moderately sensitive DHS, enhanced H3K4 methylation and CG hypomethylation in the promoter were shown to be insufficient for optimal transcription of the Ig-β gene. Based on the analyses of All Del cells, a chromatin situation was found in which both an active state, such as enhanced H3K4 methylation, CG hypomethylation or moderately sensitive DHS in the promoter, and an inactive state, such as DNase I resistance of the Ig-β chromatin or hypoacetylation of H3 and H4 histones in the Ig-β locus, coexist.

DISCUSSION

On the roles of the scattered DHSs in the cell type-specific transcription of vertebrate genes, many questions remain unsolved. To solve these, the deletion effect of the cell type-specific DHSs was investigated in mouse and human β-globin loci and the human GH locus (10,14–17). In any deletion, the change of DNase I general sensitivity had not been obtained so far. In this study, however, combinations of deletions of the scattered DHS regions were shown to convert the chromatin from sensitive to resistant to DNase I digestion. In transgenic mice carrying the 185 kb human β-globin locus, a combination of deletions of HS3 and the β-globin promoter causes changes in chromatin structure not only into a DNase I-resistant state but also into H3 hypoacetylation in the locus (12). However, to convert the chromatin into a DNase I-resistant state with the DHS at the promoter intact, it would be necessary to perform combinations of deletions of the scattered DHSs in human and

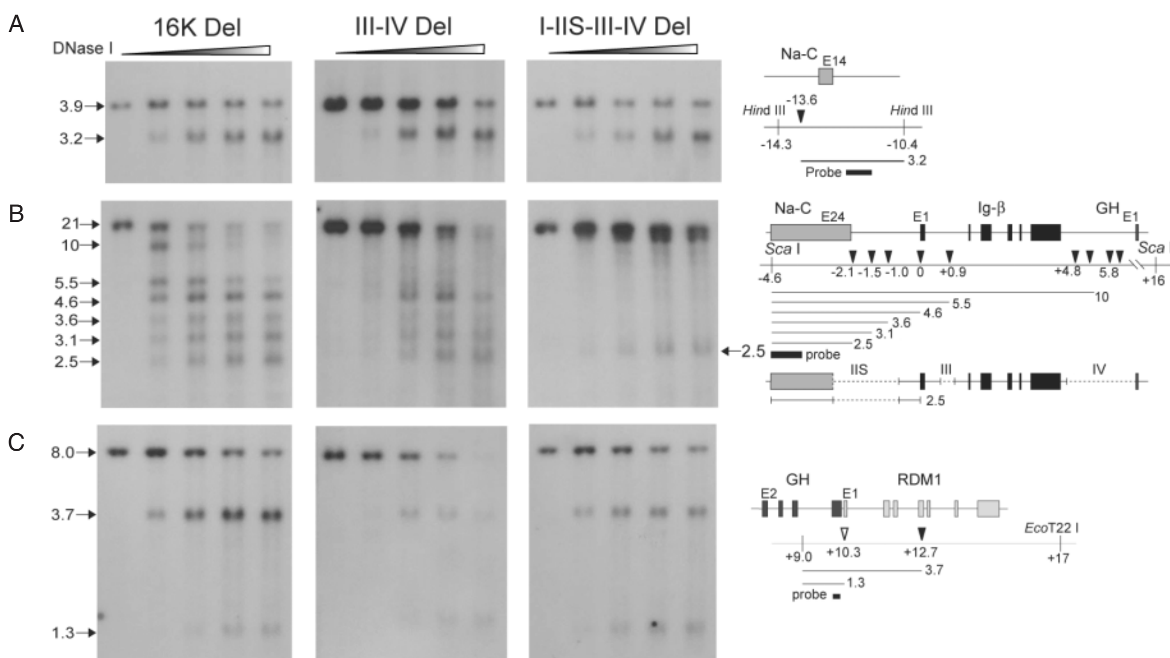

Figure 8. DNase I hypersensitive sites in cells with combinations of DHS deletions. Isolated nuclei were treated with DNase I for 3 min at 20°C. The concentrations of DNase I for treatment of nuclei from left to right: 0, 25, 50, 75 and 100 U/ml. The DNA was purified from nuclei and digested with HindIII (**A**), ScaI (**B**) or EcoT22I (**C**). The digests (3.0 μg) were subjected to Southern hybridization with 0.7 kb SacI/SacI DNA (−12.1 to −11.4 kb) (A), 0.9 kb ScaI/KpnI DNA (−4.6 to −3.7 kb; #849–1751 in AB066568) (B) or 0.2 kb GH exon 5 (#3515–3744 in CHKGHG/D10484) (C). The analyzed region, DHS, and the position of the probe are shown at the right side.

mouse β-globin loci and in the human GH locus (1). As was observed in this study, collaboration of cell type-specific DHSs that are found not only in and around a particular gene but also in the regions situated far upstream or downstream of the gene may participate in cell type-specific transcription in vertebrates through changes in chromatin structure including DNase I general sensitivity. It is unclear how to convert the chromatin by the collaboration of many DHSs. It is reported that scattered DHSs located in more than the 100 kb region assemble to form the active chromatin hub in the transcription of the β-globin gene family in mouse (13) and human (12) β-globin loci. Perhaps the same situation is true in the chicken Ig-β locus. To gain further insight into how the different deleted regions might contribute to Ig-β gene activation, it would be useful to determine whether the lower level of Ig-β mRNA in the deletion mutant such as region IV result from a generalized decrease in all cells, or complete shutdown in some (16).

Among several parameters that determine the active chromatin state, such as (i) DNase I general sensitivity, (ii) active-type histone modifications, (iii) formation of cell type-specific DHSs, (iv) replacement of histone variant and (v) CG hypomethylation, which comes first for chromatin activation? In All Del cells, Ig-β chromatin turned out to be DNase I-resistant and a decrease in H3 and H4 acetylation at the Ig-β promoter and exon 3 was observed, whereas three parameters (H3K4 di-methylation, CG hypomethylation state in the region situated upstream the transcriptional start site, and cell type-specific DHSs in further upstream and downstream regions) were virtually unchanged. In the case of the DHS deletion that causes hypoacetylation of H3 and H4 histones in the wide range of the human GH locus (10), chromatin of the locus still seems to remain sensitive to DNase I, and thus the change in chromatin structure to a DNase I general sensitive state may come earlier than the hyperacetylation of core histones. Di- and tri-methylation of H3K4 is shown to be linked with active transcription in vertebrate genes (31). In this study, a different effect of the DHS deletion on the acetylation and methylation of core histones has been demonstrated. In contrast to the decrease to <8% in H3 and H4 acetylation in All Del cells, H3K4 hypermethylation remained in the Ig-β gene. In murine β-globin (32) and GATA-2 (33) loci, the same situations have been described in which histone acetylation is reduced without a concomitant reduction in H3K4 di-methylation. The hypomethylation state in the Ig-β promoter is virtually unchanged in All Del cells except for significant methylation at +12 and +14. Preventing methylation of these sites might be crucial for proper transcription of the Ig-β gene, or transcription may be necessary to prevent methylation. Intragenic DNA methylation has been reported to repress gene expression and to alter chromatin structure in murine erythroleukemia cells (34), and then it would be interesting to determine the intragenic CG methylation of the Ig-β gene in All Del cells. Based on the data obtained in this study, it is still difficult to determine the changing order of chromatin parameters.

At the Ig-β promoter in III–IV Del cells, H3 and H4 acetylation decreased to 20 and 40%, respectively, whereas H3K4 di-methylation showed a 2.2-fold increase (Figure 6). In place of the GATA-2 protein, the induced GATA-1 protein is reported to bind to the regulatory region present upstream of the GATA-2 gene, which is known to be one of the targets of the GATA-1 transcription factor in mouse proerythroblast-like G1E cells; binding of GATA-1 then blocks the transcription of the GATA-2 gene by disrupting positive autoregulation (33). In this case, the decrease in H3 and H4 acetylation and the increase in H3K4 methylation are demonstrated at the 1G promoter of the GATA-2 gene. To explain this finding, we propose that an unidentified factor(s) increases H3 and H4 acetylation but represses H3K4 methylation at the Ig-β promoter through binding in the III–IV regions, consistent with the decrease in H3 and H4 acetylation and the increase in H3K4 methylation observed in III–IV Del cells. Detailed investigations of the proteins capable of binding to III–IV regions should clarify whether other factor(s) are involved and may facilitate their identification.

For the maintenance of DT40-specific DHSs at −13.6 and +12.7 kb, neither the DNase I-sensitive state in the Ig-β chromatin nor hyperacetylation of H3 and H4 histones is required. The presence of the so-called ‘pioneer transcription factors’ such as HNF3, GATA-4 and MyoD capable of binding to compact chromatin and opening chromatin locally has been reported previously (35–37). Thus, in the first step, the binding of pioneer transcription factors may cause some DHS formation in the inactive chromatin and then active-type change in chromatin structure occurs. The regions required for the maintenance of H3K4 methylation and CG hypomethylation have not been determined in this study; combinations of deletions between the −13.6 and +12.7 kb regions and All Del regions may have an effect on these two parameters, although a single deletion of these upstream and downstream regions showed virtually no effect on the Ig-β mRNA level.

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