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Stepwise activation of the immunoglobulin μ heavy chain gene locus

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The immunoglobulin heavy chain (IgH) gene locus spans several megabases. We show that IgH activation during B-cell differentiation, as measured by histone acetylation, occurs in discrete, independently regulated domains. Initially, a 120 kb domain of germline DNA is hyperacetylated, that extends from $D_{FL16.1}$, the 5′-most D_H gene segment, to the intergenic region between Cμ and Cδ. Germline V_H genes were not hyperacetylated at this stage, which accounts for D_H to J_H recombination occurring first during B-cell development. Subsequent activation of the V_H locus happens in at least three differentially regulated domains: an interleukin-7-regulated domain consisting of the 5′ J558 family, an intermediate domain and the 3′ V_H genes, which are hyperacetylated in response to DJ_H recombination. These observations lead to mechanisms for two well-documented phenomena in B-cell ontogeny: the sequential rearrangement of D_H followed by V_H gene segments, and the preferential recombination of D_H-proximal V_H genes in pro-B cells. We suggest that stepwise activation may be a general mechanism by which large segments of the genome are prepared for expression.

Keywords: B cells/gene expression/hyperacetylation/immunoglobulin heavy chain

Introduction

The genome of higher eukaryotes is packaged into chromatin (Kornberg and Lorch, 1999; Wolffe and Guschin, 2000). The resulting compaction of DNA is not only essential to fit it into the cell nucleus, but also serves to suppress gene expression. Against this backdrop of primarily inactive genes, a restricted number of cell- and tissue-specific genes are activated in appropriate cells during differentiation. Regions of chromatin that contain active genes differ in several ways from those that contain inactive genes. For example, active genes are readily cleaved by DNase or micrococcal nuclease (MNase) (Gross and Garrard, 1988), and are usually hypomethylated at CpG dinucleotides (Siegfried and Cedar, 1997; Ng and Bird, 1999). Covalent modification of core histones recently has gained prominence as an important marker of chromatin domains. The N-termini of histones H3 and H4 associated with active genes are acetylated at several lysine residues (Workman and Kingston, 1998; Strahl and Allis, 2000). Conversely, inactive genes are associated with hypoacetylated histones. A mechanistic link between methylated DNA and hypoacetylated histones, both of which correlate with gene inactivity, was provided recently by the observation that methylcytosine-binding proteins interact with histone deacetylases (Jones *et al*., 1998; Nan *et al*., 1998). These parameters have been analyzed comprehensively in the β-globin locus with regard to differential gene expression (Hebbes *et al*., 1988, 1994; Litt *et al*., 2001).

Genes encoding B- and T-lymphocyte antigen receptors are assembled by DNA recombination events. The accessibility model of Alt and colleagues was proposed to explain the observation that the same machinery recombines all six antigen receptor loci, but that it does so in different cell types, and at different stages of differentiation (Sleckman *et al*., 1996, 1998). The crux of the accessibility model is that the recombinase machinery gains access to individual loci in a regulated manner. For example, when the immunoglobulin heavy chain (IgH) locus is rearranged in pro-B cells, the recombinase can only access IgH sequences, but not recombination signal sequences (RSSs) associated with the other five antigen receptor loci. Regulated changes in chromatin structure are believed to be the basis of gene-specific recombinase accessibility (Stanhope-Baker *et al*., 1996).

Locus-specific accessibility of the recombinase to antigen receptor genes is governed by *cis*-acting regulatory elements, including transcriptional enhancers. At the IgH locus, the μ heavy chain gene enhancer (μE) is one such element which activates recombination in stably transfected, or transgenic, substrates (Ferrier *et al*., 1990; Oltz *et al*., 1993; Fernex *et al*., 1994). Conversely, deletion of this enhancer from the endogenous locus abolishes V_H to DJ_H recombination without significantly affecting D_H to J_H recombination (Chen *et al*., 1993; Serwe and Sablitzky, 1993; Sakai *et al*., 1999). Similarly, enhancers and sterile promoters associated with the T-cell receptor (TCR) loci also serve as recombination activators (Capone *et al*., 1993; Lauzurica and Krangel, 1994; McMurry *et al*., 1997; Sleckman *et al*., 1997; Whitehurst *et al*., 1999; Tripathi *et al*., 2000). Recent studies show a striking correlation between histone acetylation and V(D)J recombination at TCR loci (Mathieu *et al*., 2000; McBlane and Boyes, 2000; McMurry and Krangel, 2000). Recombinationally competent TCR loci were associated with hyperacetylated histones; deletion of TCRα or TCRβ enhancers that abolished recombination also diminished histone acetylation. In addition, deletion of the Dβ1 sterile promoter resulted in reduced recombination and enhanced methylation at proximal sites (Whitehurst *et al*., 2000).

The murine IgH locus comprises several hundred V_H gene segments, 16 D_H gene segments and four J_H gene segments spread over 3 Mb (Figure 1A). During B-cell differentiation, two ordered DNA recombination events

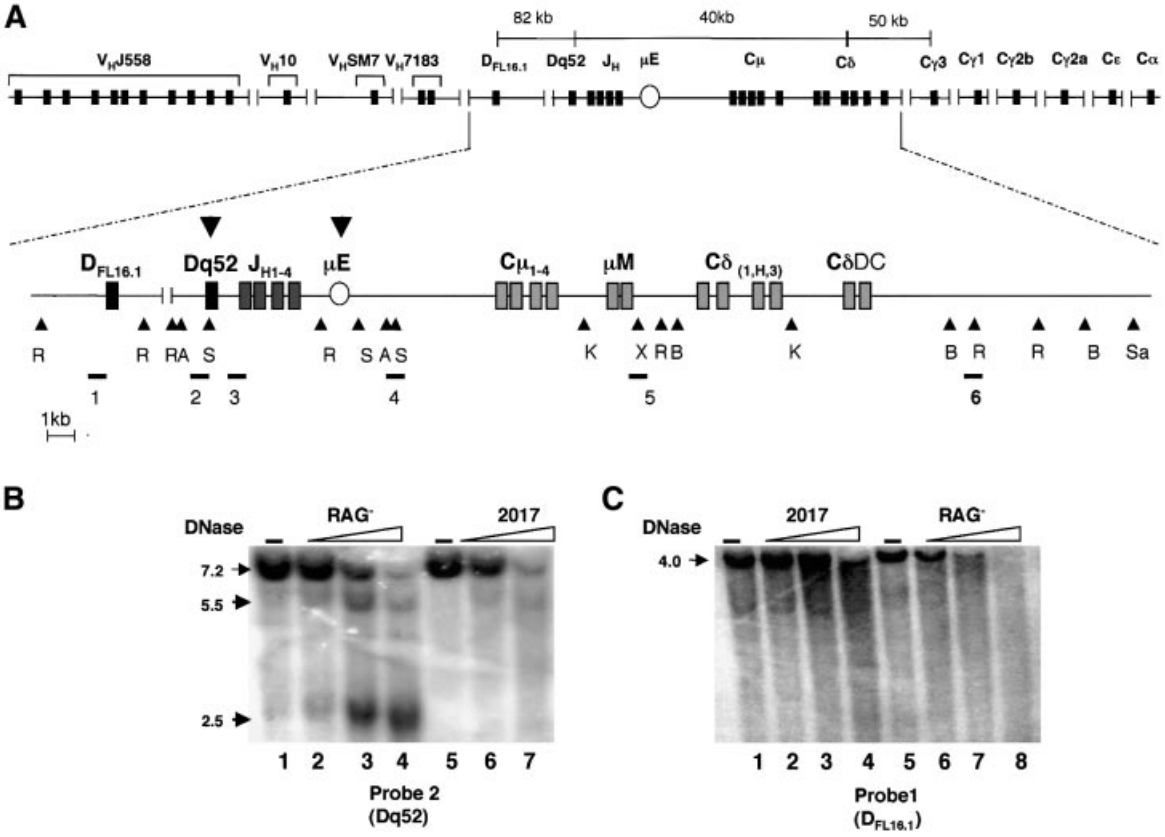

Fig. 1. DNase I-hypersensitive site analysis of the murine IgH gene locus. (**A**) Schematic representation of the IgH locus with DNase I-hypersensitive sites. The complete locus including V_H gene segments and all heavy chain isotypes is shown on the top line. The second line is a detailed schematic of part of the locus starting from the 5′ $D_{FL16.1}$ gene segment to 7 kb downstream of the Cδ membrane exon. The restriction endonuclease sites *Eco*RI (R), *Ase*I (A), *Sac*I (S), *Kpn*I (K), *Sal*I (Sa), *Xho*I (X) and *Bgl*II (B) were used for Southern blot analysis of DNase I-treated genomic DNA. Probes used in the study are labeled 1–6, and are shown below their corresponding hybridizing fragments. The position of the intronic μ enhancer is marked with an oval μE. The nuclease-hypersenstive sites identified are indicated by the bold arrowheads. (**B** and **C**) DNase I sensitivity assays. Nuclei from RAG^- (pro-B) cells or 2017 (pro-T) cells were treated with increasing concentrations of DNase I as indicated, and purified genomic DNA analyzed by Southern blotting. DNA from untreated nuclei is shown in lanes 1 and 5. Restriction enzymes and probes used were as follows: (B) *Ase*I, probe 2; (C) *Eco*RI, probe 1. DNase-hypersensitive sites are indicated by the bold arrows. The bands at 2.5 and 5.5 kb correspond to hypersensitive sites at the Dq52 region and the μE, respectively. Data shown are from one out of four independent experiments.

juxtapose a V_H, D_H and J_H gene segment to generate functional IgH genes. Hardy *et al*. (1991) have classified B-cell precursors into six subsets, A2–F, using cell surface marker expression. In this nomenclature, A2 cells are the earliest B lineage-committed cells which have no gene rearrangements at either IgH or Ig light chain loci. IgH rearrangements are first evident in the B fraction which contains cells that have undergone D_H to J_H recombination to create DJ_H joins. V_H gene recombination follows in fraction C to generate fully rearranged VDJ alleles. Because recombination is error prone, only a small proportion of cells that contain VDJ alleles can make IgH protein. These are selected to differentiate further to fraction D, which are the cells in which the bulk of light chain gene recombination occurs. Successful light chain recombination results in immature B cells that express immunoglobulin on the surface (fraction E).

Several aspects of the regulation of IgH gene recombination remain poorly understood. For example, despite appropriate RSSs flanking V_H and D_H gene segments, V_H to D_H recombination occurs only after D_H to J_H rearrangements. Secondly, V_H genes that lie close to the D_H cluster, such as the V_H7183 family, have been shown to rearrange preferentially during B-cell ontogeny (Yancopoulos *et al*., 1984; Jeong and Teale, 1989; Malynn *et al*., 1990; ten Boekel *et al*., 1997). Therefore, these genes are over-represented at early stages of differentiation compared with the more distal (and more numerous) V_HJ558 family. Moreover, in interleukin-7 receptor (IL-7R)-deficient mice, rearrangement of the J558 family is impaired relative to that of the D_H-proximal V gene families (Corcoran *et al*., 1998). Finally, it is unclear why deletion of the μE located in the J_H-Cμ intron affects V_H to DJ_H recombination, rather than the more proximal D_H to J_H recombination.

Here we used histone hyperacetylation to assay activation of the IgH locus during B-cell differentiation. We show that the several megabase IgH locus is activated in discrete steps. An ~120 kb domain, that includes the D_H gene sements and extends till the Cμ exons, is hyperacetylated first prior to initiation of recombination. D_H to J_H recombination presumably occurs within this domain. The V_H locus was inactive at this stage of differentiation, providing a simple explanation for the observation that D_H

to J_H rearrangements precede V_H to D_H rearrangements. The V_H locus itself contained at least three independently regulated domains; genes closest to D_H/Cμ were associated with hyperacetylated histones only in cells that contained DJ_H recombined alleles, suggesting that they may be activated as a consequence of the first recombination event. The distal V_H genes were IL-7 responsive, and genes within an intervening domain were activated by the v-*abl* tyrosine kinase. Independent regulation of segments of the IgH locus suggests a mechanism for preferential rearrangement of proximal V_H genes, and provides a model for activation of large segments of the genome.

Results

Nuclease mapping

The observation that genetic deletion of the IgH μE (Figure 1A) does not affect D_H to J_H recombination hints at other recombination-activating regulatory sequences at this end of the locus. To search for additional regulatory sequences, we examined the chromatin structure of the locus prior to rearrangement using DNase I and MNase (see Figure 1A lower panel for probes and restriction sites). In these studies, we compared two cell lines whose characteristics resembled pre-rearrangement cells obtained from primary lymphoid tissue. RAG$^-$ is an Abelson virus-transformed cell line derived from the fetal liver of RAG2$^-$ mice and represents early B-lymphoid cells. 2017 cells were derived by intra-thymic injection of Moloney virus and represent early T-lymphoid cells (Spolski *et al*., 1988). DNase I digestion revealed a strong hypersensitive site in the vicinity of the μE in RAG$^-$ (pro-B) cells, but not in 2017 cells (pro-T) (Figure 1B, and summarized in Figure 1A, lower level, bold arrow). This site was evident using either *Ase*I- or *Sac*I-digested DNA and probes 2 or 3, respectively. In addition, pro-B-specific hypersensitivity was detected near Dq52, which probably corresponds to the Dq52 promoter where μo transcripts are initiated (Alessandrini and Desiderio, 1991; Kottmann *et al*., 1992, 1994). No other DNase I- or MNase-hypersensitive sites were detected in the region extending to ~5 kb 3′ of the Cδ transmembrane exons (data not shown).

Furthemore, no additional hypersensitive sites were detected in ~10 kb surrounding $D_{FL16.1}$, the most 5′ D_H gene segment (Figure 1C), and the adjacent $D_{SP2.2}$. That Dq52 contained a hypersensitive site but $D_{FL16.1}$ and $D_{SP2.2}$ did not indicated that the chromatin structure surrounding D_H gene segments was not identical. We conclude that in a 40 kb region that encompasses Dq52, Cμ and Cδ, or in a 10 kb region encompassing $D_{FL16.1}$ and $D_{SP2.2}$, there are only two nuclease-hypersensitive sites which are likely to determine the structure of this part of the IgH locus.

Histone acetylation at the IgH locus

Association of genes with acetylated histones has been implicated in the regulation of gene expression and V(D)J recombination. To determine the extent of the IgH locus that was associated with acetylated histones prior to rearrangements, chromatin immunoprecipitations were carried out using anti-acetylated H3 or H4 antibodies followed by analysis of the co-precipitated DNA by PCR. As with the studies described above, we compared the acetylation status of the IgH locus in RAG$^-$ (pro-B) and 2017 (pro-T) cells. PCR primers that span the D_H–Cδ regions are indicated in Figure 2A. The region close to the two identified nuclease-hypersensitive sites (Dq52 and μE), as well as the intervening region that contains the four J_H gene segments, co-precipitated efficiently with anti-acetylated histone antibodies from RAG$^-$ cells where the IgH locus is accessible to recombinase (Figure 2B and C). Histone acetylation of the IgH locus was markedly diminished in 2017 (pro-T) cells. Comparable immunoprecipitation of β_2-microglobulin (β2m) sequences from both cell lines served as a positive control.

We examined the state of $D_{FL16.1}$ and found that it was also associated with acetylated histones in RAG$^-$ (pro-B) cells, but not in 2017 (pro-T) cells (Figure 2B). Thus, gene segments at either extremity of the D_H gene cluster were in an activated state as assessed by histone acetylation. To probe the middle of the D_H gene cluster, we used primers specific for $D_{SP2.4}$ and $D_{SP2.8}$ which lie at similar distances from the 5′ and 3′ ends of the D_H cluster, respectively (Figure 2A). Both regions co-precipitated with acetylated histones from RAG$^-$ cells, but not from 2017 cells (Figure 2B). These observations suggest that the entire D_H cluster is activated simultaneously at the earliest stages of B-cell differentiation. It is interesting to note that $D_{FL16.1}$ is associated with acetylated histones, though it is not marked by a nuclease-hypersensitive site like Dq52. The 5′ end of the acetylated domain has not been defined in these studies; however, the lower levels of $D_{FL16.1}$ DNA detected in the immunoprecipitates suggest that the domain does not extend much further upstream.

To identify the 3′ end of the domain of hyperacetylation, we used primer sets that hybridized within and beyond the Cμ exons (Figure 2A). Histone acetylation was evident at Cμ, as well as in the intergenic region between Cμ and Cδ (Cμ–Cδ) exons (Figure 2B). Quantitation of the proportion of input DNA that immunoprecipitated with acetylated histone antibodies indicated that the level of acetylation between Cμ and Cδ was significantly reduced compared with the J_H region (Figure 2C). Cδ sequences located only 3 kb 3′ of the intergenic Cμ–Cδ region did not co-precipitate with acetylated histones, and neither did downstream Cγ2b sequences. As expected, none of these regions were associated with acetylated histones in 2017 cells.

To confirm these observations, CD19$^+$ cells were purified from the bone marrow of RAG2$^{-/-}$ mice (Figure 2B, third column) and used for chromatin immunoprecipitation assays. Most of the results paralleled those in cell lines. $D_{FL16.1}$, $D_{SP2.4}$, $D_{SP2.8}$ and Dq52 regions were associated with acetylated histones, as were the J_H cluster, the μE and the Cμ exons (Figure 2B). The main difference between the two populations of pro-B cells was that the Cμ–Cδ intergenic region did not precipitate from primary cells as had been seen in the RAG$^-$ cell line. As before, the Cδ and Cγ2b regions were not associated with acetylated histones. We conclude that the 3′ end of the activated IgH locus in pro-B cells terminates abruptly in the 5 kb region between Cμ and Cδ. This domain of hyperacetylation of the germline IgH locus spans ~120 kb and includes all D_H and J_H gene segments, and the Cμ exons. Our observations suggest that V(D)J recombination at the IgH locus is initiated within this domain.

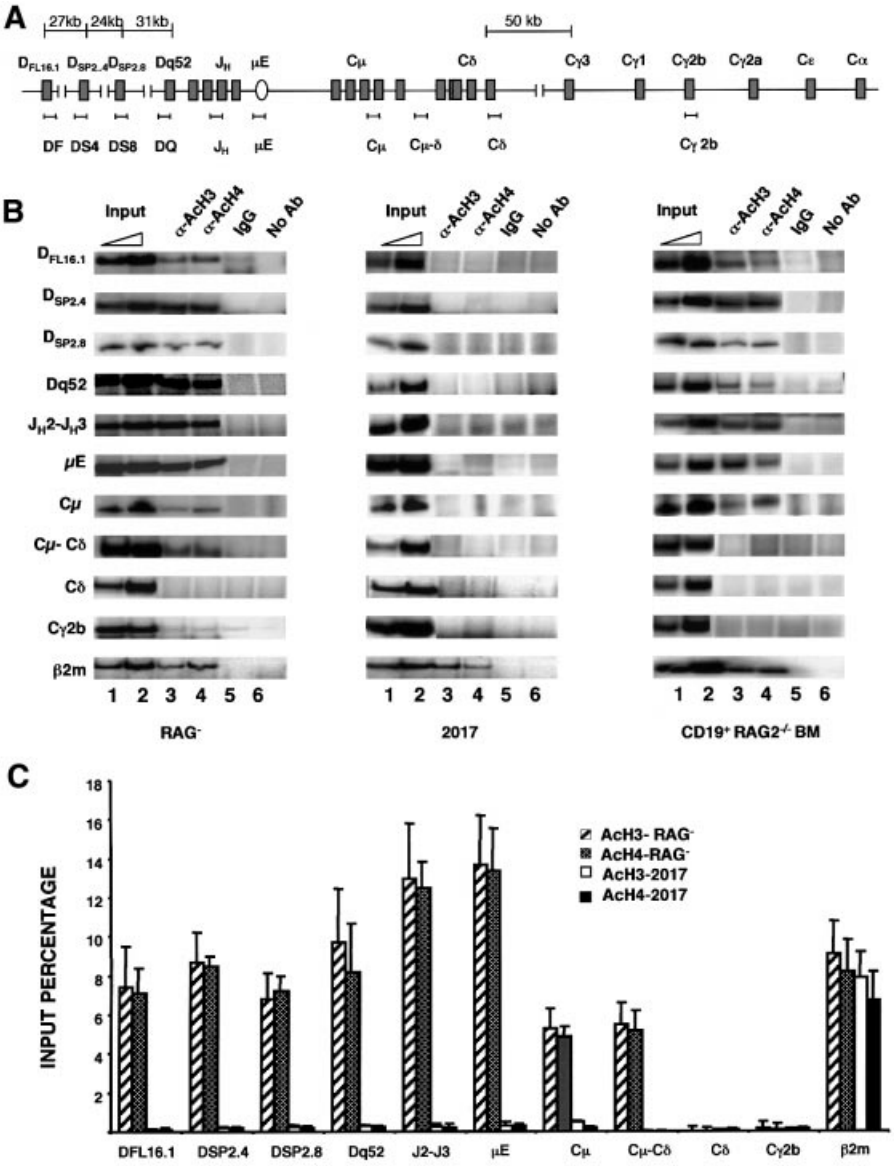

Fig. 2. Histone acetylation in the IgH locus. (**A**) Schematic representation of the D_H–Cμ locus with approximate locations of the primer sets used in chromatin immunoprecipitation assays. Sequences of the primers are provided in Table I. (**B**) Chromatin immunoprecipitation assays using pro-B (RAG^-) and pro-T (2017) cell lines, and primary pro-B cells from RAG2-deficient bone marrow. Formaldehyde-cross-linked chromatin prepared from cells as described in Materials and methods was incubated with anti-acetylated histone H3 (α-AcH3) or anti-acetylated histone H4 (α-AcH4) antibodies. Control immunoprecipitations were carried out using a 2-fold excess of non-specific rabbit IgG (lane 5) or no antibody (lane 6). Antibody-bound DNA was collected by adsorption to protein A–agarose, uncross-linked and amplified by PCR with the primer sets indicated in (A). The 3′ primer was radiolabeled for quantitation, and the products were visualized after fractionation through 6% polyacrylamide gels. Phosphoimager analysis was used to detect and quantitate reaction products. Lanes marked Input (lanes 1 and 2) correspond to DNA purified from chromatin before immunoprecipitation. The amount of input DNA used as the template in the PCR was one-tenth (lane 1) or one-fifth (lane 2) that used for immunoprecipitations (lanes 3–6). Primers hybridizing to the β_2-microglobulin gene were used as a positive control. Results shown are representative of one out of three independent experiments. (**C**) Data in (B) of the RAG^- and 2017 cell lines were quantitated by phosphoimager analysis and are represented graphically. Results shown are an average of three independent experiments, with the error bars representing the standard deviation. The input percentage was calculated taking the subsaturating PCR product of the input DNA (lane1) and also taking into account that the input had 10-fold less DNA template per PCR.

Distinction between the D_H-Cμ and V_H locus activation

The most μ-proximal V_H gene family, V_H7183, lies ~40 kb upstream from $D_{FL16.1}$ (Figure 1A). The remaining several hundred V_H gene segments are spread over >2 Mb, with the largest V_HJ558 family occupying almost half of this region (Haines and Brodeur, 1998). Two facets of the regulation of V_H gene recombination are well known. First, V_H gene recombination in Hardy fraction C follows D_H to J_H recombination (in fraction B) during B-cell development. Secondly, μ-proximal V_H genes, particularly those of the V_H7183 family, are over-represented in fraction C compared with the larger numbers of V_HJ558 genes. These observations have led to the idea that V_H7183 genes rearrange preferentially during B-cell ontogeny. The molecular mechanisms of neither phenomenon are clear. Wu and colleagues have proposed that part of the selectivity of V_H gene rearrangements resides in the

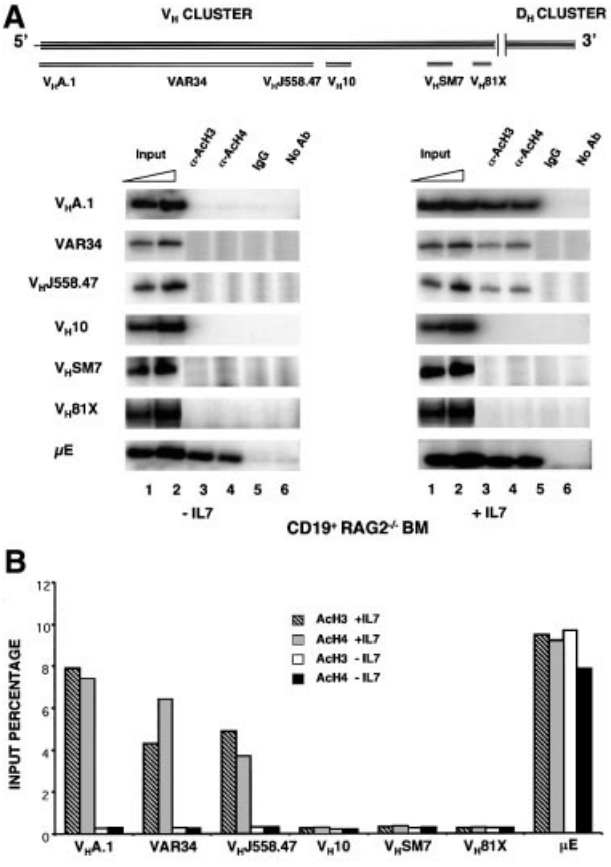

Fig. 3. IL-7-dependent histone acetylation of V_HJ558 gene segments. (**A**) Schematic representation of the murine V_H gene locus showing the approximate location of gene segments analyzed in this study (adapted from Haines and Brodeur, 1998). CD19$^+$ pro-B bone marrow cells from RAG2$^{-/-}$ mice were cultured for 4 days with or without IL-7 (20 ng/ml). Chromatin immunoprecipitation assays were done as described using primers from the V_H region as shown in the top panel. The μ enhancer region (μE) was used as a positive control. Representative data from one of two experiments is shown. (**B**) The results in (A) were quantified by phosphoimager analysis and are represented as a proportion of the input DNA that was immunoprecipitated.

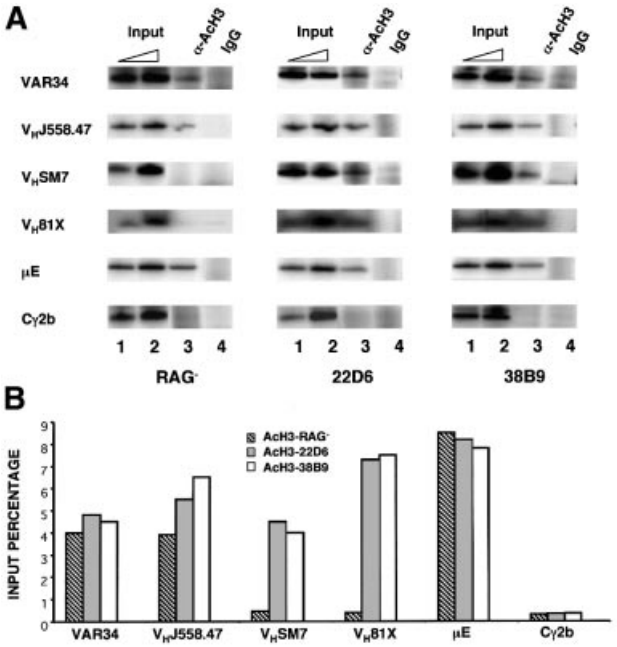

Fig. 4. Acetylation of the D_H-proximal V_H genes in pro-B- and pre-B-cell lines. (**A**) Chromatin immunoprecipitation assays were done using Abelson murine leukemia virus-transformed pro-B (RAG$^-$) and pre-B cell lines (22D6 and 38B9). The IgH locus is in germline configuration in RAG$^-$ cells, whereas 22D6 and 38B9 cells contain DJ_H recombination on both alleles. Representative data from one of two experiments are shown. (**B**) Phosphoimager analysis and graphical representation of the data in (A).

sequences of the genes and their flanks (Yu *et al*., 1998). Furthermore, a comprehensive analysis of the chromatin structure of the V_H locus by DNase I sensitivity also did not reveal significant differences amongst family members, or stages of differentiation (Haines and Brodeur, 1998) that could explain V_H gene regulation.

We assayed V_H gene acetylation in CD19$^+$ bone marrow cells from RAG2-deficient mice using representative examples of the V_H genes from the 5′ end, the 3′ end and the middle of the V_H cluster. The 5′ V_H J558 family was represented by three genes: V_HA.1, which is considered to be one of the 5′-most V_H genes (Haines and Brodeur, 1998); V_HJ558.47 which is one of the most 3′ J558 genes; and VAR34 which maps between A.1 and J558.47. D_H/Cμ-proximal (3′) V_H genes were represented by V_H81X and V_HSM7, and the middle of the V_H cluster was represented by V_H10.1A. An approximate map of these genes is shown in Figure 3A (upper panel). None of the V_H genes that we examined co-precipitated with acetylated histones from CD19$^+$ RAG2$^-$ bone marrow cells (Figure 3A, left panel) though the D_H–Cμ locus was activated in these cells (Figure 2). The efficient precipitation of μE DNA in these assays served as a positive control. We conclude that the domain of hyperacetylation that reflects the earliest opening of the IgH locus includes the D_H cluster, the J_H gene segments and the Cμ exons, but excludes all V_H gene segments. These observations suggest that the order of rearrangements at the IgH locus may be determined by the ordered activation of rearrangeable gene segments, i.e. D_H to J_H rearrangements occur first because these segments are available first to the recombinase machinery.

Activation of distal V_Hs

IL-7 and IL-7R interaction affects B lymphopoiesis by providing proliferative, survival and differentiative signals to pro-B cells. The earliest developmental defect in IL-7Rα-deficient (IL-7R$^{-/-}$) mice is a 5- to 10-fold reduction in the cell numbers of pro-B cells, in which IgH gene rearrangements take place (Maraskovsky *et al*., 1998). In addition, V_H genes at the 5′ end of the locus are significantly under-represented in VDJ recombined alleles in IL-7R$^{-/-}$ mice (Corcoran *et al*., 1998). To determine whether the effects of IL-7 on recombination were due to a direct effect on V_H gene chromatin structure, CD19$^+$ bone marrow cells from RAG2-deficient mice were cultured *in vitro* in the presence or absence of IL-7. After 4 days in culture, viable cells were recovered and used for chromatin immunoprecipitation assays with anti-acetylated histone antibodies. In response to IL-7, all three V_H genes of the J558 family co-precipitated efficiently with acetylated histones (Figure 3A, right panel). However, V_H10 located in the middle of the V_H cluster, as well as

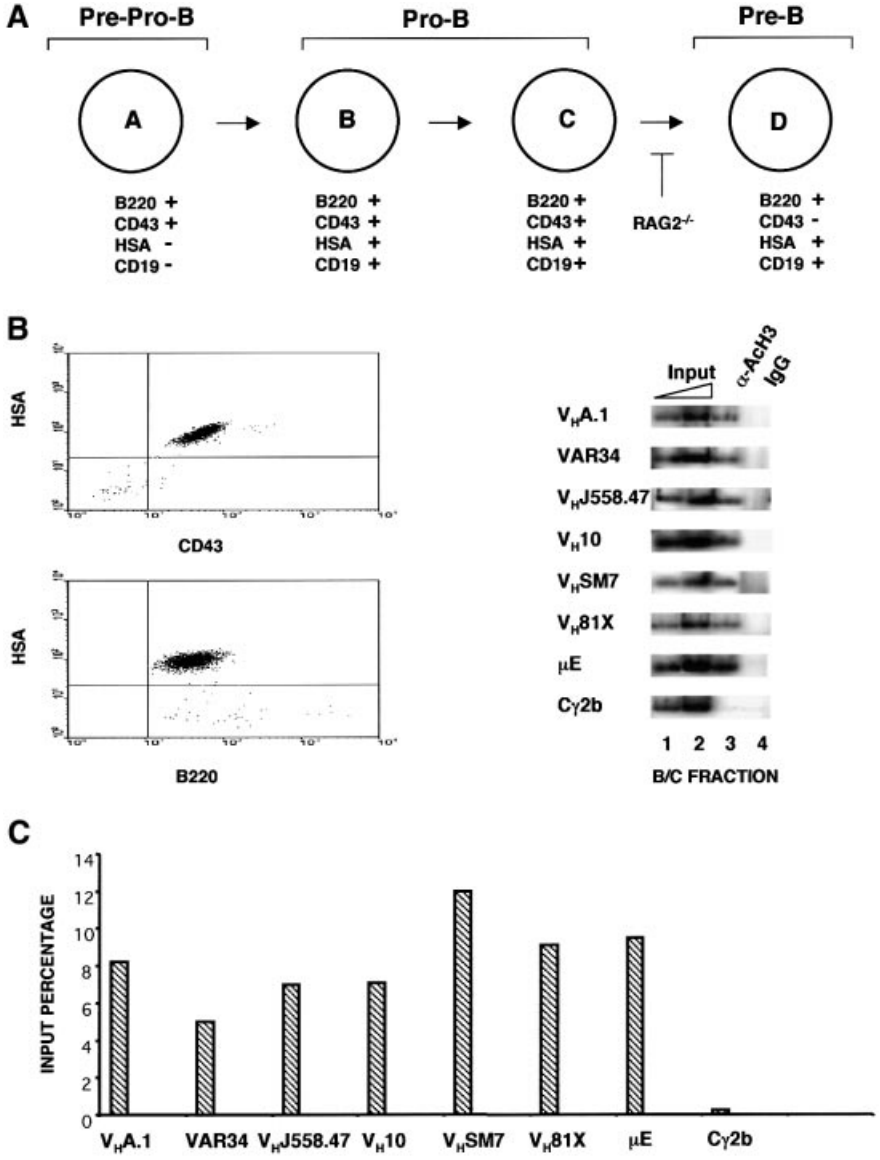

Fig. 5. Histone acetylation of V_H genes in primary bone marrow pro-B cells. (**A**) Hardy classification of early B-cell development listing some of the cell surface markers used to distinguish the different cell populations (Hardy *et al*., 1991). DJ_H recombination is seen in fraction B cells and VDJ recombination is seen in fraction C cells. (**B** and **C**) The B/C fraction pro-B cells were purified from bone marrow of wild-type BALB/c mice by magnetic depletion of erythroid, myeloid and T cells followed by sorting for the $B220^+$ $CD43^+$ HSA^+ cells. Chromatin immunoprecipitation assays were carried out using the primers shown in Figure 3A. Phosphoimager quantitation was done as described in the legend to Figure 2. Representative data from one of two experiments using two preparation of bone marrow cells are shown.

V_HSM7 and V_H81X representing the 3′ end of the V_H cluster, remained hypoacetylated under these conditions. Thus IL-7 induces histone hyperacetylation of a domain that encompasses the J558 genes and thereby may activate these genes for recombination. These results provide a plausible explanation for reduced V_HJ558 gene rearrangements in the bone marrow of the IL7Rα-deficient mice.

Activation of proximal V_Hs

We found that the D_H-proximal V_H genes were inactive in $RAG2^-$ bone marrow cells and could not be activated by IL-7. Analysis of V_H gene acetylation in an Abelson virus-transformed RAG-deficient cell line showed that V_H J558 genes were acetylated, but the 3′ V_HSM7 and V_H81X genes were not (Figure 4, left panel). We attributed constitutive acetylation of the J558 genes to an earlier observation that the v-*abl* oncogene mimics aspects of IL-7R signaling (Banerjee and Rothman, 1998). However, lack of acetylation of the D_H-proximal V_H genes was surprising because these genes rearrange preferentially in early B cells, a phenomenon that was first described in Abelson transformants, and more recently reproduced after reconstitution of recombinase in RAG-deficient Abelson cell lines (Angelin-Duclos and Calame, 1998). We tested the possibility that the rearrangement state of the IgH locus played a role in activation of the proximal genes by comparing V_H gene acetylation in three Abelson transformants: $RAG2^-$, 22D6 and 38B9. The latter two cell lines contain DJ_H rearrangements on both alleles and undergo low frequency V_H to DJ_H recombination in

culture (Yancopoulos *et al*., 1984). In contrast to the observation in RAG$^-$ cells, proximal V_H81X and V_HSM7 genes co-precipitated with acetylated histone H3 in the two new cell lines (Figure 4A, middle and right columns). Efficient co-precipitation of the μE sequences served as a positive control, and Cγ2b sequences were unacetylated in all three lines. We suggest that DJ_H recombination may activate the proximal V_H genes.

To confirm and extend these observations, we assayed the state of V_H gene activation in Hardy fractions B/C (Figure 5A) purified from BALB/c bone marrow. These cells have been shown previously to contain DJ_H rearrangements on both alleles and are undergoing V_H to DJ_H rearrangement (Ehlich *et al*., 1994). Total bone marrow depleted of myeloid, erythroid and T cells was sorted to obtain B220$^+$HSA$^+$CD43$^+$ pro-B cells (Figure 5B). Proximal V_H gene sequences were amplified easily in anti-acetylated histone immunoprecipitates from these cells (Figure 5B). Note that the 3′ primer used for amplification of the V_H81X region is from germline 3′ non-coding sequences downstream of the RSS; therefore, the V_H81X signal being detected arises only from the unrearranged gene. 5′ V_H genes were also hyperacetylated in these cells, consistent with this cell population being the one in which the bulk of V_H to DJ_H recombination takes place. The significant difference in the pattern of V_H gene activation in B/C fraction cells from normal compared with RAG2$^-$ mice (Figures 3 and 5) strengthens the idea that DJ_H recombination is a prerequisite for activation of proximal V_H gene segments.

Discussion

General features of the IgH locus

We used histone hyperacetylation as a measure of genome activation to examine the state of the IgH locus during B-cell differentiation. A domain of ~120 kb, that included all D_H gene segments, J_H gene segments and Cμ exons, was activated first by this criterion. Given the reported correlation between acetylation and V(D)J recombination (Mathieu *et al*., 2000; McMurry and Krangel, 2000), it is likely that IgH rearrangements are initiated within this domain. The region containing the four J_H gene segments precipitated more efficiently with anti-acetylated histone antibodies than other parts of the 120 kb domain. This microdomain may be the consequence of being flanked by two DNase I-hypersensitive sites corresponding to the Dq52 promoter and the μE. We speculate that the J_H microdomain may help to initiate V(D)J recombination by targeting the recombinase to the J_H gene segments. Microdomains of higher histone acetylation have also been detected in the murine β-globin locus (Litt *et al*., 2001) where they coincide with the expressed β-like gene.

Two other features of the D_H–Cμ hyperacetylated domain are noteworthy. First, the domain terminates abruptly in the 5 kb region between the Cμ and Cδ exons. This intergenic region did not contain a nuclease-hypersensitive site that could indicate the presence of a classical insulator element. Presumably, the 3′ end of the domain is established by a different mechanism. Secondly, the chromatin structure surrounding D_H gene segments was not identical. Whereas the level of histone acetylation of the five D_H gene segments we examined was comparable, only the Dq52 region was marked by a closely associated DNase I-hypersensitive site. We conclude that a closely located hypersensitive site is not required for recombinase accessibility or histone acetylation.

Implications for V_H gene rearrangements

DJ_H before V_HDJ_H rearrangements. The 12/23 rule specifies that V(D)J recombination occurs only between gene segments whose RSSs contain 12 and 23 nucleotide spacers. In the IgH locus, the RSSs of murine J_H and V_H gene segments are associated with 22 bp spacers, while the D_H gene segments are flanked by RSSs with 12 bp spacers. Therefore, V_H can rearrange only to D_H, but not J_H gene segments. However, D_H to J_H rearrangements invariably occur before recombination of V_H to D_H gene segments. Our observations provide a plausible explanation for the order of rearrangements at the IgH locus. We found that V_H genes were not associated with hyperacetylated histones in bone marrow pro-B cells, whereas D_H and Cμ regions were. These observations suggest that the V_H gene segments are not accessible to recombinase at the time that cells are undergoing D_H to J_H recombination. The later activation of V_H ensures that V_H rearrangement only occurs after DJ_H recombination.

V_H subdomains. We find that the V_H gene locus contains at least three independently regulated subdomains. The 5′-most genes were IL-7 responsive, genes lying in the middle of the cluster (represented by V_H10) were activated by v-*abl* (unpublished data) and the 3′ most genes were only acetylated in cells that contained DJ_H recombinants. The close correlation between the DJ_H rearranged status of the IgH locus and proximal V_H gene acetylation suggests that DJ_H recombination may be a prerequisite for activation of these genes. In this model, the state of DJ_H rearrangements must be sensed by proximal V_H genes. An obvious possibility is that D_H to J_H rearrangement brings these genes closer to Cμ. By doing so, perhaps they come under the influence of Cμ-associated recombination enhancers, such as the μE. Obviously, how close they get depends on which D_H segment rearranges on a particular allele. With the exception of Dq52, however, rearrangement of any other D_H gene segment deletes, or inverts, at least 19 kb of genomic DNA. This is because the D_H closest to Dq52, $D_{SP2.10}$, is 19 kb away; every other D_H gene segment is separated by a further 5–10 kb. Therefore, proximity changes as a result of D_H to J_H recombination can be significant, particularly in light of our observations that acetylation boundaries can be affected by even a few kilobases of DNA (e.g. between Cμ and Cδ).

Dysregulation of V gene recombination at the IgH and TCR γ chain loci has been noted in IL-7Rα-deficient mice (Corcoran *et al*., 1998; Schlissel *et al*., 2000). Lack of Vγ recombination in these animals has been attributed to reduced accessibility of the TCR-γ locus (Schlissel *et al*., 2000), but the mechanisms that operate at the IgH locus have not been clarified. IL-7-induced hyperacetylation of J558 genes indicates that impaired rearrangement of upstream V_Hs in IL-7R$^{-/-}$ pro-B cells (Corcoran *et al*., 1998) is likely to be a direct consequence of the control of chromatin structure of these V_Hs by IL-7, rather than the indirect result of the IL-7–IL-7R pathway in promoting proliferation, or survival, of early lymphoid precursors.

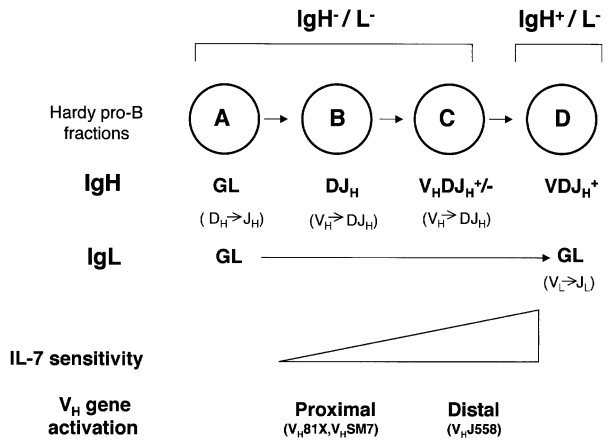

Fig. 6. Mechanism of ordered V_H recombination. Pro-B cell subsets as defined by Hardy are shown in the circles. Genomic structures of the Ig loci are shown in bold below each circle (GL = germline). Presumed ongoing rearrangements in each subset are indicated in parentheses below the genomic structure. Recombination at the IgH locus is initiated in late A fraction cells; our observations suggest that only the D_H–Cμ part of the locus is accessible to recombinase at this stage, resulting in D_H to J_H recombination. Proximal V_H gene families are activated on DJ_H recombined alleles as outlined in the text. We propose that distal V_H genes, that are activated by IL-7, lag behind the proximal V_Hs because IL-7 responsiveness is gained gradually during B-cell development based on Wei *et al.* (2000) (indicated by a triangle). Preferential rearrangement of proximal V_H genes is therefore a consequence of the late activation of the 5′ V_H genes via the IL-7 receptor.

We suggest that the genomic regions containing the upstream V_Hs never get hyperacetylated in IL-7R$^{-/-}$ mice and are therefore not accessible to the recombinase machinery. This direct effect on VDJ recombination in pro-B cells also explains why B lymphopoiesis cannot be rescued in IL-7R$^{-/-}$ mice by expression of a transgenic *bcl-2* gene (Akashi *et al*., 1998; Maraskovsky *et al*., 1998).

Order of V_H gene rearrangements. IL-7 responsiveness of the 5′ V_H genes also suggests a model for the preferential rearrangement of D_H-proximal V_H genes in pro-B cells, a characteristic feature of IgH gene assembly. The model couples our results with the observation that developing pro-B cells become increasingly responsive to IL-7. This is manifest at the level of both receptor expression and receptor responsiveness. Though IL-7Rα is expressed at the earliest stages of B-cell development (Kim *et al*., 2000; Wei *et al*., 2000), highest levels are reached only in Hardy fractions C and D (Figure 6). However, high surface expression of the receptor is not sufficient for enhanced IL-7 responsiveness, as shown most clearly by the requirement for high concentrations of IL-7 to induce proliferation of RAG$^-$ pro-B cells (Marshall *et al*., 1998). It has been proposed that the threshold for IL-7 responsiveness may be regulated by the pre-B-cell receptor. We propose the following model for V_H gene activation during B-cell ontogeny.

Activation of the 120 kb D_H–Cμ region takes place first; V_H genes are inactive at this stage because low levels of IL-7R expression makes these cells non-responsive to IL-7 and D_H to J_H recombination has not yet occurred. This is presumably the state in late A fraction cells and allows initiation of D_H to J_H recombination (Figure 6). DJ_H recombination on both alleles (fraction B, Figure 6) primes the proximal V_H genes to become hyperacetylated, and consequently recombinogenic, as described above. Though these cells steadily gain IL-7 responsiveness, the lag between DJ_H rearrangement and IL-7 signaling delays activation of the 5′ V_H gene families. By default, the proximal V_H gene families become accessible earlier, and therefore undergo preferential rearrangement in pro-B cells. Thus, preferential rearrangement of the V_H 7183 family is the result of independent activation of different parts of the V_H locus and the complex pattern of IL-7 responsiveness of developing pro-B cells. Our model also accounts for the observation that the frequency of V_H81X utilization drops even in the absence of selection mediated by IgH (Marshall *et al*., 1996).

Table I. Primer sets used in chromatin immunoprecipitation assays

Primer	Sequence
VHA1-5′	GGACCTGAGCATCCTGTTGC
VHA1-3′	CGAGAGCACACTGATCATAGGTAGG
VAR34-5′	CCTGGGATGTCACTGATATACACTCTG
VAR34-3′	GTAGTAGCCAGTAAATGAGTAACCAGAAGC
VHJ558.47-5′	GCAAGGCTTCTGGATACACATTCAC
VHJ558.47-3′	CCTTGCCTTTGAACTTCTCATTGTACTTAG
VH10-5′	CTCTCTGCCAATGTAGGACCAG
VH10-3′	GCCTGAATTTCAGGGTCAGGG
VHSM7-5′	CGTTATCCTCATTGCTACTACCACC
VHSM7-3′	CCAAGTCCTAACTCTGTCTGAAGAAC
VH81X-5′	GAGATGAGATTCTGTCTGTTGTATGCAC
VH81X-3′	CTGCAAACAAAGAGTGCTGGTCAG
VH81X(G)-5′	CTCCAGAGACAATACCAAGAAGACCC
VH81X(G)-3′	CCCCCTGCTGGTCCTAGATG
DF-5′	GGGCAGGCATGTCTCAAAGCACAATGC
DF-3′	GGAATGGTCTGTTTCTGGGGGACTTCCTC
DS4-5′	GGCAGGGATTTTTGTCAAGGGATC
DS4-3′	GGGTTTTTGCTGGATATATCACTGTGG
DS8-5′	GTTACCTTACTTGGCAGGGATTTTTGTC
DS8-3′	GTCTGCTGGGCATAATGGGTTTTTG
DQ-5′	CCCCACAGGCTCGAGAACTTTAGCGACTG
DQ-3′	CAGTCAGAGACCACAGGGACTCTGAGGC
JH-5′	GCTGATGCAGACAGACATCCTCAGCTCC
JH-3′	GGGCTCCAGGATTATCTCAGATGGAGGCC
EN-5′	GGAATGGGAGTGAGGCTCTCTC
EN-3′	GCTGCAGGTGTTCCGGTTCTGATCGGCC
Cδ-5′	GGATGGCCTCCTACCACCTC
Cδ-3′	CGTGGAGCTACATAGGGCCC
Cμ-5′	GGTAGGTATCCCCCCTTCCC
Cμ-3′	GAAGACAGTAGTGAGGATAGGGTGG
Cμ-δ-5′	CAGCCCACCATCTTGGGCTGGTG
Cμ-δ-3′	CCCTAGGGCTTGTCATGTTGTGGGAGAG
Cγ-2b-5′	GGGAGGAGGGAATCACCAGAGTTGTAGGC
Cγ-2b-3′	CCCTGGTATGGGCTTAGTCCAGGATGATCC
β_2-microglobulin-5′	GCGGTCCCAGGCTGAACGACCAG
β_2-microglobulin-3′	GAGAGACCAGCTAGGGCGCGCG

Materials and methods

DNaseI and MNase assays

The DNase hypersensitive assay was done as described (Landry *et al*., 1993), using 10^8 RAG$^-$ and 2017 cells, respectively. The MNase digestion was done similarly, with the addition of 2 mM $CaCl_2$ in the RSB buffer (Landry *et al*., 1993). An 8–10 μg aliquot of DNase- or MNase-treated DNA was digested with appropriate restriction endonucleases, fractionated by agarose gel electrophoresis and analyzed by Southern blotting. Probes indicated in Figure1 were labeled by random priming in the presence of [α-^{32}P]dCTP.

Chromatin immunoprecipitations

The method of Parekh and Maniatis (1999) was adapted as follows. RAG$^-$ (10^8), 2017 cells (10^8), 22D6 cells (5×10^6), 38B9 cells (5×10^6), B/C fraction pro-B bone marrow cells and CD19$^+$ bone marrow cells (5×10^6) were cross-linked by addition of 1% formaldehyde to the medium for 5 min. Cells were lysed in hypotonic lysis buffer and nuclei resuspended in sonication buffer (Parekh and Maniatis, 1999). The nuclear suspension was sonicated to reduce DNA length to between 400 and 1000 bp, and debris removed by centrifugation. The chromatin solution was diluted 2-fold in immunoprecipitation buffer (Parekh and Maniatis, 1999) pre-cleared with non-specific IgG and protein A beads for 3 h at 4°C. The supernatant was incubated with 0.5 µg of α-acetylated H3 or H4 antibodies for 2 h at 4°C. The supernatant from B/C fraction cells was incubated with 5 µg of α-acetylated H3 antibody. Immune complexes were collected with protein A beads pre-absorbed with sonicated single-stranded DNA. Following washes and elution (Braunstein *et al*., 1993; Parekh and Maniatis, 1999), cross-links were reversed by heating at 65°C for 6 h; DNA was recovered after proteinase K treatment, two phenol extractions and ethanol precipitation. Specific sequences in the immunoprecipitates were detected by PCR under conditions in which product yield was dependent on input DNA dose. The sequences of the primers used are given in Table I. The reactions also contained 10 ng of ^{32}P-radiolabeled 3′ primer as a tracer. The PCR products were fractionated through 6% polyacrylamide gels and quantified using a phosphoimager.

Purification of CD19$^+$ pro-B cells

Bone marrow cells were recovered from 6-week-old RAG2-deficient (BALB/c) mice by flushing the femur and tibia with 10% calf serum in phosphate-buffered saline (PBS). The cell suspension was filtered through nylon mesh to remove debris, and the cells collected by centrifugation. From six mice, we obtained 2.5×10^8 cells. The cells were resuspended in 2.25 ml of magnetic cell sorting (MACS) buffer (PBS pH 7.2, 0.5% bovine serum albumin) and incubated with 250 µl of anti-mouse CD19 antibody-coated paramagnetic microbeads (Miltenyi Biotech.) at 4°C for 15 min. The cells were washed with 10–20 vols of MACS buffer. The resuspended cells were loaded onto positive selection (RS+) minimacs columns (Miltenyi Biotech.) and attached to a magnet at 4°C. Columns were washed twice with MACS buffer, and cells were collected from the flow through. These fractions typically contained <10% CD19$^+$ cells. The column was removed from the magnet, and the cells were washed free with MACs buffer. A small aliquot of cells was removed and restained with phycoerythrin (PE)-conjugated anti-CD19 antibody (Pharmingen) to monitor the purity of the cells by flow cytometry. More than 90% of the cells were CD19$^+$. Purity was also confirmed by simultaneous staining of independent aliquots of cells with anti-B220 antibodies (APC-conjugated anti-B220; Pharmingen). Recovery of CD19$^+$ cells was ~50%.

Purification of B/C fraction pro-B cells from wild-type mouse

Bone marrow cells were recovered from 6-week-old wild-type (BALB/c) mice as described above. From 10 mice we obtained 5×10^8 cells. The cells were incubated with biotinylated antibodies (Pharmingen, San Diego, CA) against Ter119, Mac1, Gr1, Thy1 and CD3, respectively. After repeated washing, the labeled cells were then incubated with streptavidin-coated paramagnetic microbeads (Miltenyi Biotech, Auburn, CA). Again after repeated washing, the cell suspension was loaded on to a depletion (CS+) column (Miltenyi Biotech). Approximately 10% of the cells were recovered and these were then sorted in a MoFlo cell sorter for CD43$^+$B220$^+$HSA$^+$ cells. We obtained 8×10^6 cells with >95% purity.

Culturing CD19$^+$ pro-B cells with IL-7

CD19$^+$ pro-B cells were purified as described above with the following modification. The cell suspension recovered from the bone marrow was layered on lympholyte, and centrifuged at room temperature at 1800 r.p.m. for 20 min. Live cells were aspirated from the interphase, leaving the dead cells and red blood cells in the pellet. The live cells were then washed in several volumes of the MACS buffer and the CD19$^+$ cells were purified. Cells were cultured in RPMI medium (Gibco) supplemented with 20% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 100 U/ml streptomycin at a concentration of 10^6 cells/ml. Purified recombinant mouse IL-7 (Endogen, Woburn, MA) was added at a concentration of 20 ng/ml and replenished to the same level after 3 days. Cells were cultured for 4 days and then harvested for chromatin immunoprecipitation assays.

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