Activation of V(D)J Recombination at the IgH Chain JH Locus Occurs within a 6-Kilobase Chromatin Domain and Is Associated with Nucleosomal Remodeling

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Activation of V(D)J Recombination at the IgH Chain JH Locus Occurs within a 6-Kilobase Chromatin Domain and Is Associated with Nucleosomal Remodeling

Jérôme Maës, Stéphane Chappaz, Patricia Cavelier, Laura O’Neill, Bryan Turner, François Rougeon, and Michele Goodhardt

IgH genes are assembled during early B cell development by a series of regulated DNA recombination reactions in which DH and JH segments are first joined followed by VH to DJH rearrangement. Recent studies have highlighted the role of chromatin structure in the control of V(D)J recombination. In this study, we show that, in murine pro-B cell precursors, the JH segments are located within a 6-kb DNase I-sensitive chromatin domain containing acetylated histones H3 and H4, which is delimited 5' by the DQ52 promoter element and 3' by the intronic enhancer. Within this domain, the JH segments are covered by phased nucleosomes. High-resolution mapping of nucleosomes reveals that, in pro-B cells, unlike recombination refractory nonlymphoid cells, the recombination signal sequences flanking the four JH segments are located in regions of enhanced micrococcal nuclease and restriction enzyme accessibility, corresponding to either nucleosome-free regions or DNA rendered accessible within a nucleosome. These results support the idea that nucleosome remodeling provides an additional level of control in the regulation of Ig locus accessibility to recombination factors in B cell precursors. The Journal of Immunology, 2006, 176: 5409–5417.

Eukaryotic genomes are contained in densely packed chromatin composed of structural repeating units known as nucleosome core particles, comprised of 147 bp of DNA wrapped around a histone octamer. Beyond their role in DNA compaction, nucleosomes appear to act as the basic regulatory unit of genomic function (1). The assembly of DNA into nucleosomes and higher-order chromatin structures regulates the access of DNA-binding proteins to their target sites and directly influences key cell functions, such as DNA replication, transcription, and recombination. Various strategies have been adopted to overcome the steric and structural constraints imposed by nucleosomal organization. These include posttranslational histone modifications, substitution with histone variants, changes in nucleosome position and conformation, and DNA methylation, which all play an important role in regulating the extent of chromatin condensation. A variety of covalent histone modifications have been documented at transcriptionally active and inactive loci (2). Current hypotheses suggest that successive posttranslational histone modifications constitute a code specifying the recruitment of chromatin-remodeling complexes, which modify nucleosomal organization and chromatin condensation and, in turn, regulate physiological processes (3, 4).

During lymphocyte development, the variable region of Ag receptor (AgR) genes is assembled from individual V, D, and J gene segments through a series of site-specific recombination reactions. V(D)J recombination is catalyzed by the lymphoid-specific proteins, RAG1 and RAG2, that recognize recombination signal sequences (RSS) located immediately 5' or 3' of each V, D, or J gene segment and composed of a conserved heptamer and nonamer motif (5). Despite the fact that recombination at all Ig and TCR loci are mediated by conserved RSS and common recombination factors, V(D)J recombination is regulated in a cell-, locus-, and stage-specific manner (6, 7). Hence, complete Ig gene rearrangement takes place only in B cell precursors and during B cell development; H chain DH-JH then VH-DJH rearrangements precede L chain (IgL) VL-JL recombination.

Given that only a small subset of potential gene substrates undergo V(D)J recombination at any given stage of lymphocyte development, it has been proposed that AgR loci are normally inaccessible to recombination factors and that the recombination machinery gains access to individual loci in a temporal- and development-specific regulated manner (8). Dynamic changes in chromatin structure have now been observed at a number of endogenous AgR loci during lymphocyte differentiation. Many studies have focused on covalent modifications of histones, in particular histone H3 and H4 acetylation, showing that recombination-competent TCR and Ig gene loci are associated with hyperacetylated histones (9–12). Other epigenetic modifications, such as hot spots of histone H3 lysine 4 dimethylation, DNA hypomethylation, and increased general DNase I sensitivity appear as molecular markers of locus accessibility (11, 13, 14), whereas methylation of histone H3 at lysine 9 is observed at recombination incompetent

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5 Abbreviations used in this paper: RSS, recombination signal sequence; MNase, micrococcal nuclease; LM-PCR, ligation-mediated PCR; KAP, kidney androgen-regulated protein; MAR, matrix attachment region.

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NUCLEOSOME STRUCTURE ASSOCIATED WITH IgH RECOMBINATION

Life Technologies) supplemented with 10% FCS, 5
Probes used in nuclease sensitivity and histone acetylation assays

Table I.

DNase I digestion was performed on lysolecithin-permeabilized RAG2
Purification of CD19
Materials and Methods
forms a 6-kb DNase I-sensitive domain associated with acetylated
more, this region of positioned nucleosomes over the JH segments
remodeling provides enhanced accessibility of JH RSSs. Further-
show that in recombination competent pro-B cells, nucleosome
over endogenous RSSs in the more complex chromatin structure
encountered by RAG proteins in vivo has not been investigated in
detail. In this study, we investigated the accessibility of JH RSSs
with respect to nucleosome remodeling and histone modifications
in primary B cell precursors and determined the boundaries of
modified chromatin structure over the JH locus. In this study, we
show that in recombination competent pro-B cells, nucleosome
remodeling provides enhanced accessibility of JH RSSs. Further-
more, this region of positioned nucleosomes over the JH segments
forms a 6-kb DNase I-sensitive domain associated with acetylated
histones H3 and H4, which is flanked by the intronic Eμ enhancer
3' and the DQ52 promoter 5'.

Materials and Methods

Purification of CD19+ pro-B cells and culture conditions

CD19+ B cell precursors were isolated by magnetic separation (Miltenyi
Biotec) from single-cell bone marrow suspensions of 6-wk-old RAG2-
deficient (C57BL/6) mice. Purity was confirmed by simultaneous staining
with anti-B220 (APC-conjugated anti-B220; BD Pharmingen) and anti-
CD43 (FITC-conjugated anti-CD43; BD Pharmingen) Abs.

CD19+ purified cells were cultured in Opti-MEM medium (Invitrogen Life
Technologies) supplemented with 10% FCS, 5 × 10−3 M 2-ME, 100
U/ml penicillin, and 100 U/ml streptomycin in the presence of 125 U/ml
IL-7 and S17 stromal cells irradiated with 1600 rad with a Cesium source,

All animal experiments were done in accordance with the guidelines of
the Pasteur Institute, which are approved by the French Ministry of
Agriculture.

DNase I sensitivity analysis

DNase I digestion was performed on lysolecithin-permeabilized RAG2−/−
CD19+ pro-B cells, as described previously (11).

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as previously described
(11) using affinity-purified Abs to acetylated lys5-histone H4 (R232),
acetylated lys5, lys5-histone H3 (R47), and acetylated lys18-histone H3
(R224) (21). Following chromatin immunoprecipitation, equal amounts of
DNA from the Ab bound, unbound, and input fractions were serially di-
luted and applied to nylon filters (Hybond N+; Amersham Biosciences) by
slot blotting. Specific DNA sequences were detected by hybridization with
radiolabeled probes described in Table I and quantified using a Phosphor-
Imager (Molecular Dynamics).

Micrococcal nuclease (MNase) digestion analysis

RAG2−/− CD19+ pro-B cells were permeabilized with 0.01% lysolecithin,
then incubated with 7.5 U of MNase ( Worthington Biochemicals) at 25°C
as previously described (11). Nuclei were prepared from freshly excised
livers of RAG2−/− mice as described (22). Nuclear suspensions were di-
gested at 25°C with 7.5 U of MNase in 500 μl of RB buffer (60 mM KCl,
15 mM NaCl, 15 mM HEPES (pH 7.5), 2 mM EDTA, 0.15 mM
mercuric, 0.5 mM superoxide, 5 mM CaCl2, and 5 mM MgCl2).

Table 1. Probes used in nucleosome sensitivity and histone acetylation assays

<table>
<thead>
<tr>
<th>Name</th>
<th>Probe</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′DQ52 (A)</td>
<td>850-bp EcoRI-BamH1</td>
<td>5′-TGGAGGCTCGATCTGGTGCG-3′</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
</tr>
<tr>
<td>5′DQ52 (B)</td>
<td>494-bp PCR fragment</td>
<td>5′-GCCCTGCGGAGGAGAAGG-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>JH1</td>
<td>600-bp BamHI-HindIII</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>JH4</td>
<td>470-bp HindIII-MspI</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>Eμ</td>
<td>303-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>Sα</td>
<td>287-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>Cμ</td>
<td>352-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>mμ</td>
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<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
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<tr>
<td>Cδ</td>
<td>270-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
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<tr>
<td>3′ m6</td>
<td>280-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
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<tr>
<td>Cy3</td>
<td>286-bp PCR fragment</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
<tr>
<td>KAP</td>
<td>550-bp PstI-HindIII</td>
<td>5′-AGTCTGCTGGCTTGGGAC-3′</td>
<td>5′-CTCAATCTATTTTGAAGTCG-3′</td>
</tr>
</tbody>
</table>

Restriction endonuclease accessibility analysis

Cell nuclei were prepared from RAG2−/− CD19+ pro-B cell precursors and
S17 stromal cells as described previously (25). For restriction enzyme
digestion, 5 × 106 nuclei were resuspended in 300 μl of appropriate digestion
buffer (New England Biolabs) and incubated with 50 U of restriction en-
zyme at 37°C. Aliquots were taken at 0, 1, 2, 5, 10, and 30 min. Nuclei
were then lysed in extraction buffer (20 mM Tris (pH 7.5), 20 mM NaCl,
20 mM EDTA, 1% SDS) and treated with proteinase K (600 μg/ml) and

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RNase (100 μg/ml) at 56°C for 2 h. An additional overnight RNase treatment (100 μg/ml) was required for S17 stromal cells. DNA was extracted and resuspended in 50 μl of TE. LM-PCR was conducted as described above using either the 3′-JH2A or 3′-JH HA primers. Because HindIII and MaeIII are not blunt-end restriction endonucleases, primer extension was performed before ligation to the unidirectional linker (23). Following the 25 cycles amplification, products were separated on a 2% agarose gel, transferred to a nylon membrane (Positive Membrane; Appligene), and hybridized with either the 3′-JH2B or 3′-JH4B radiolabeled primer. To control for restriction enzyme digestion in CD19+ precursors and liver cells, we performed LM-PCR assays using the β-actin-specific primers A, 5′-GT GGGCCTGGTCCAGAAGGACTCCTGTGG-3′, and B, 5′-CAACCGTG GAAAAGATGACCC-3′, for amplification and hybridization, respectively. Quantification was performed using a PhosphorImager (Molecular Dynamics).

Results

**The DQ52 promoter and Eμ enhancer define the limits of a chromatin domain**

We have previously shown, using Abelson-transformed cell lines, that JH segments are DNase I sensitive at the pro-B cell stage, whereas they are resistant to DNase I in nonlymphoid cells (11). To examine the extent of this DNase I-sensitive domain in primary pro-B cells, we analyzed general DNase I sensitivity of an 80-kb region extending 10 kb upstream and 70 kb downstream of the JH segments in CD19+ bone marrow cells isolated from RAG2−/− mice (Fig. 1). In these experiments, permeabilized cells were incubated with increasing concentrations of DNase I and the genomic DNA then analyzed by Southern blotting using probes hybridizing to different fragments along the IgH locus (Fig. 1A and Table I). Relative sensitivity to DNase I was assessed by measuring the rate of disappearance of individual DNA fragments with increasing DNase I concentration. Because the kinetics of DNase I digestion are dependent on the length of the fragment examined, we analyzed restricted fragments of roughly equivalent lengths to avoid making large corrections for size.

We first analyzed HindIII restriction fragments, because this enzyme cuts at multiple sites along the locus (Fig. 1A). When the results are quantified and normalized for length, a clear transition in general DNase I sensitivity is apparent in the region upstream of the DQ52 segment and downstream of the Eμ enhancer (Fig. 1B). The 2.2-kb HindIII fragments containing either the JH1 (H2) or JH4 (H3) gene segments are sensitive to DNase I. In contrast, the 3-kb HindIII fragment, which is located 2-kb upstream of the DQ52 gene segment (H1) is relatively DNase I resistant. Similarly, a significant drop in the DNase I sensitivity is observed for the 3.7-kb HindIII fragment containing the switch region (H4) and all downstream fragments examined (H5–9). This shows that there is a decrease in general DNase I sensitivity 5′ of the DQ52 promoter and 3′ of the Eμ enhancer.

To extend this analysis, we used BamHI and MspI/HinII digests. We found that fragments containing either the JH segments (M3) or DQ52 sequence and promoter element (B2 and M2) are DNase I sensitive, whereas 5′ fragments are resistant to cleavage, again showing a drop in DNase I sensitivity 2 kb upstream of the DQ52 gene segment. A decrease in DNase I sensitivity is also observed for HinII/MspI fragments containing the SpA switch region and downstream fragments (M4, M5), confirming that the 3′ boundary of the accessibility domain is located between the intronic Eμ enhancer and the SpA region. Therefore, the JH segments reside within a 6-kb DNase I-sensitive domain in pro-B cells, which is delimited 5′ by the DQ52 promoter element and 3′ by the Eμ enhancer.

We next compared the extent of the DNase I-sensitive domain with that of histone H3 and H4 acetylation by chromatin immunoprecipitation experiments, using Abs against the acetylated form of different N-terminal lysine residues (Fig. 2). The kidney androgen-regulated protein (KAP) gene, which is not acetylated in B cell

**FIGURE 1.** Transitions in DNase I sensitivity 5′ of the DQ52 promoter and 3′ of the Eμ enhancer define the boundaries of the JH chromatin domain. A. Schematic representation of the DQ52-JH-CH region. DQ52, JH segments, and CH exons are indicated as black or dark gray boxes; the switch region (SpA) is indicated as a light gray box; and the intronic Eμ enhancer is indicated as a light gray circle. Restriction endonuclease sites HindIII (H), BamHI (B), MspI (M), and HinII (Hc) used for Southern blot analysis of DNase I-treated genomic DNA are shown. Probes used are indicated below the map. A detailed description of probes is given in Table I. B, Quantitative representation of DNase I sensitivity along the IgH locus. Permeabilized RAG2−/− CD19+ pro-B cells were treated with increasing concentrations of DNase I (0, 0.1, 0.2, 0.4, 0.6, 0.8 μg/ml) and extracted DNA digested with either HindIII, BamHI, or MspI/HinII and analyzed by Southern blotting. Relative DNase I sensitivity corresponds to the extent of loss of signal intensities for each band. DNA fragments derived from the KAP gene, which is transcriptionally inactive in B cell precursors are used as DNase I resistant controls. Normalized DNase I sensitivity values (S) were calculated for each DNase I concentration from the following equation (44) and mean values were plotted on a graph: S = log (Ig/K) / [log (K/Kt) × T], where Ig and K are Ig and KAP band intensities for the undigested (U) or digested (D) samples, and T is the size ratio of the KAP to Ig fragments.
studied further. Nuclei isolated from CD19+ pro-B cells and S17 stromal cells were incubated with appropriate enzymes for a limited time course and the extent of cleavage was assessed by LM-PCR. As an internal control for the quantity and degree of digestion of each sample, linker-ligated DNA was amplified in parallel with primers specific for the β-actin gene.

As shown in Fig. 3, restriction sites located in or close to the RSS were more readily cleaved in CD19+ pro-B than in S17 cells. Thus for the JH1 and JH2 segments, RsaI sites R1 and R2 and the MaeIII site M3 were more sensitive to digestion in pro-B than S17 cells (Fig. 3A), as was the HinfI site in the JH4 RSS (H2, Fig. 3B). These results are consistent with RAG-mediated cleavage of Ig genes in isolated nuclei (26) and supports the idea that chromatin modifications in pro-B cells leads to enhanced accessibility at the JH RSS. Furthermore, restriction sites within the RSS were more sensitive to digestion then adjacent sites situated in the JH segments or intervening sequences. Quantification of the Southern blots indicates that even though the locus is globally 2–3 times more sensitive in B cell precursors than in the S17 cells, sites located in the RSS are 5–25 times more sensitive to restriction enzyme digestion in pro-B cells than the equivalent sites in S17 cells or to adjacent restriction sites (Fig. 3C). Hence the M3 site located in the JH2 RSS is 6- and 4-fold more sensitive than the M2 site located in the JH1-JH2 intergenic region and the M1 site in the JH1 coding sequence, respectively. Similarly, H2 site located in the JH4 RSS is 8-fold more accessible than H1 situated at the end of the JH2 coding sequence. Therefore, these results show that the recombination sequences are situated in regions of preferentially accessible chromatin in pro-B cells.

**Nucleosomel organization at the JH locus**

The presence of a nucleosome core particle has been found to inhibit RSS cleavage by the Rag proteins in vitro, suggesting that selective positioning of nucleosomes at endogenous loci may be important in regulating access of V(DJ) recombination factors in vivo. To examine the nucleosomal organization of JH segments in primary cells, CD19+ pro-B and liver cells isolated from RAG2−/− mice were analyzed by MNase digestion of chromatin. This enzyme preferentially cuts DNA in the linker sequences between nucleosomes and in nucleosome-free regions. MNase cleavage sites were mapped with respect to two Mspl sites flanking the JH segments by indirect end-labeling analysis (Fig. 4A, top). In CD19+ pro-B cells, we observed six discrete MNase cleavage sites in the JH1-JH3 region with a 3′ probe consistent with the presence of an ordered nucleosomal structure (Fig. 4A, JH4 probe). Of note, the sizes of the fragments generated indicate that the RSS flanking the three JH segments correspond to MNase cleavage sites. MNase digestion of naked DNA showed that this pattern is not due to the presence of preferential MNase cutting sites (Ref. 11; data not shown). Using the JH1 probe, the position of these sites was confirmed and additional cleavage at the JH4 RSS was observed. These results suggest that, although nucleosomal spacing is not regular throughout the JH region, there is preferential positioning of nucleosomes in pro-B cells resulting in the localization of the four JH RSS in nonnucleosomal DNA.

To extend the analysis to sequences upstream and downstream of the JH segments, the samples were digested with HindIII and hybridized with the JH1 or JH4 probe (Fig. 4, B and C). MNase cleavage was observed between the JH1 and the DQ52 gene segments in CD19+ pro-B cells (Fig. 4, A and B). This pattern was interrupted by two hypersensitive sites associated with the DQ52 gene segment. No visible bands were detected in the 1 kb of sequence examined 5′ of the DQ52 promoter element, indicating that...
this region is most likely not associated with positioned nucleosomes (Fig. 4B). Downstream of the JH4 segment, MNase digestion yielded a regular banding pattern up to the E/H9262 enhancer/MAR element (Fig. 4C). This region is associated with two areas free of MNase cleavage corresponding to the 5'/H11032- and 3'/H11032-MAR and several strong hypersensitive sites, probably due to binding of transcription factors.

A clearly different nucleosomal organization was observed in the liver cells. MNase digestion analysis shows an uninterrupted MNase ladder throughout the DQ52-JH-E/H9262 region. Multiple faint bands are detected over the JH segments, indicating that nucleosomes assembly in a nonrandom fashion in liver cells (Fig. 4A). However, unlike in pro-B cells, MNase cleavage sites appear too closely spaced to correspond to homogeneous positioned nucleosomes. Furthermore, the MNase hypersensitive sites associated with the DQ52 and E/H enhancer/MAR elements are not present, and MNase cleavage sites continue throughout the DQ52 and E/H elements (Fig. 4, B and C). These results show a more homogeneous nucleosomal organization in pro-B than in liver cells. JH segments appear to be covered by positioned nucleosomes in pro-B cells with preferential MNase cleavage occurring immediately 5' of each JH segment. This ordered nucleosomal array is interrupted 5' by the DQ52 promoter and 3' by the E/H enhancer/MAR element.

**High-resolution nucleosome positioning by LM-PCR**

To determine more precisely nucleosome positioning with respect to the JH RSS, we used a LM-PCR technique (23). CD19+/H11001 pro-B cells were subjected to limited MNase digestion and double-stranded breaks visualized after ligation of a unidirectional linker, followed by PCR amplification using a common 5' primer complementary to the linker and JH-specific 3' primers. Cleavage at the β-actin gene was analyzed as an internal control. C. Quantification of restriction enzyme cleavage. Intensity of LM-PCR derived fragments was quantified by PhosphorImager analysis of Southern blots and normalized to the β-actin signal for each time point. Results are means of values for each restriction enzyme in CD19+ cells relative to that in S17 stromal cells. Relative positions of the restriction sites analyzed are indicated on the x-axis. Arrows indicate position of RSS. Results shown are from one experiment, which was repeated two to four times for each restriction enzyme with consistent results.

**FIGURE 3.** Enhanced accessibility of RSS to restriction enzyme cleavage in pro-B cells. Analysis of JH1 and JH2 (A) and JH4 (B) segments. **Top.** Map of JH segments showing location of restriction sites. R, RsaI; M, MaeIII; A, AluI; and H, Hinfl. JH segments are shown as open boxes and RSS are shown as triangles. Relative positions of RSS, JH coding sequences, and restriction sites are shown. Location of the primers used in the LM-PCR assays are shown below the map. **Bottom.** Southern blot analysis of LM-PCR products following restriction enzyme digestion. Nuclei from RAG2−/− CD19+ precursors and S17 stromal cells were treated with 50 U of restriction enzyme for 1, 5, 10, and 30 min at 37°C. Enzyme cleavage at the indicated sites was detected by LM-PCR using 3'JH2 (A) or 3'JH4 (B) primers. Cleavage at the β-actin gene was analyzed as an internal control. C. Quantification of restriction enzyme cleavage. Intensity of LM-PCR derived fragments was quantified by PhosphorImager analysis of Southern blots and normalized to the β-actin signal for each time point. Results are means of values for each restriction enzyme in CD19+ cells relative to that in S17 stromal cells. Relative positions of the restriction sites analyzed are indicated on the x-axis. Arrows indicate position of RSS. Results shown are from one experiment, which was repeated two to four times for each restriction enzyme with consistent results.
segment at nt 1380 to 1290, with new strong cleavage sites detected at the level of the RSS (Fig. 5). These results are consistent with those of the restriction enzyme accessibility assays showing preferential cutting at the RsaI site located in the JH2 RSS. Similarly, strong cleavage sites were observed at the JH4 RSS, whereas sequences 3′/H11032 of JH4 were protected with only two cleavage sites detected at nt 2400 and 2385 (Fig. 5). Most cleavage sites observed with naked DNA were also suppressed at the JH3 segment and 3′/H11032 sequences, but not at the JH3 RSS. Signal intensity was lower with the JH1 primer; however, cleavage sites at early time points occurred always, although not exclusively, in the RSS region. Multiple strong bands over the RSS sequence were also seen when primer extension reactions were conducted with primers hybridizing to the JH segments, indicating that the detection of MNase cleavage at RSS is not related to distance from the primers (data not shown). Together with low-resolution MNase digestion analysis by Southern blot, these data suggest that, in pro-B cells, the RSS flanking the JH gene segments are located in accessible chromatin, perhaps at the boundary of positioned nucleosomes. In liver, MNase cutting is not preferentially localized at the level of the RSS. For the JH4 segment, MNase cutting was observed for nucleotides corresponding to sequences 3′ of the gene segment, whereas the JH4 sequence and 5′-RSS were less digested in liver than in CD19+ pro-B cells (Fig. 5). Even though it is difficult from these data to obtain a precise nucleosome positioning they indicate that nucleosome remodeling leads to enhance accessibility of RSS compared with 3′-JH sequences.

Discussion
Regulation of chromatin structure has been found to be a key component in the control of V(D)J recombination (7, 8). Recombination competent loci are associated with acetylated histones H3 and H4 and changes in histone methylation, indicating that posttranslational modifications of histone tails provides one way of regulating the accessibility of AgR loci to recombination factors. Our results on the nucleosomal structure of the endogenous IgH locus provide support for the idea that nucleosome remodeling is also important in the regulation of V(D)J recombination.
Nucleosomal DNA is generally refractory to sequence-specific DNA binding proteins. This appears to be true for the RAG proteins, because a number of studies have shown that assembly of RSS into mononucleosomes inhibits V(D)J cleavage in vitro (18, 19, 27). The studies of Boyes and colleagues (27) further indicate that the nonamer recombination sequence promotes positioning of nucleosomes over the RSS, suggesting that an active remodeling step is necessary before rearrangement. In this study, we demonstrate that the nucleosomal organization over the JH segments is different in nonlymphoid cells that are refractory to V(D)J recombination and in bone marrow pro-B cells that are poised to undergo D-JH recombination in vivo. In RAG2−/− pro-B cells, the JH segments and immediate 3′ sequences are protected from MNase digestion, while the RSSs are located in regions of enhanced accessibility to both restriction enzymes and MNase, corresponding to either nucleosome free regions or DNA rendered accessible within a nucleosome. This organized structure is restricted to a 2.5-kb region between the DQ52 segment and the Eμ enhancer and may serve to channel the recombination factors to the RSS during D-JH recombination in vivo. In RAG2−/− pro-B cells, the JH segments and immediate 3′ sequences are protected from MNase digestion, while the RSSs are located in regions of enhanced accessibility to both restriction enzymes and MNase, corresponding to either nucleosome free regions or DNA rendered accessible within a nucleosome. This organized structure is restricted to a 2.5-kb region between the DQ52 segment and the Eμ enhancer and may serve to channel the recombination factors to the RSS during D-JH recombination. In contrast, in V(D)J refractory cells, the JH RSS do not exhibit preferential nuclease cleavage, and a more complex nucleosomal structure, not interrupted by the DQ52 and Eμ elements, is observed. These results suggest that onset of IgH recombination is associated with local nucleosome remodeling leading to enhanced JH segment RSS accessibility.

Nucleosomes are remodeled by two families of ATPase-containing complexes, which break histone-DNA interactions and causes sliding or local DNA loops on the histone octamer (28). ATPase subunits belonging to both the SWI/SNF and ISWI families are able to stimulate RAG-mediated cleavage of RSS in vitro (29). Furthermore, BRG1, the catalytic subunit of the SWI/SNF complex, has been shown to be associated with the IgH locus in pro-B cells, suggesting that this complex may be responsible for remodeling of nucleosomes over the JH segments (14).

In pro-B cells, the nucleosomal array covering the JH segments ends 5′ of the DQ52 segment in a region defined as having promoter/enhancer activity by Kohler and colleagues (30) and 3′ at the intronic Eμ enhancer. These two sequences have been shown to correspond to DNase I hypersensitive sites in pro-B cells (12), suggesting that they may act as chromatin boundary elements. Active chromatin domains are classically defined as DNase I-sensitive regions (31). Strikingly, we observed an abrupt decrease in general DNase I sensitivity 5′ of the DQ52 promoter and 3′ of the Eμ enhancer, showing that these two elements delimit a 6-kb domain of decondensed chromatin encompassing the region of nucleosome remodeling in pro-B cells. As previously reported (11, 12, 14), JH segments and the Eμ enhancer are associated with acetylated histones in pro-B cells and the level of acetylation drops off gradually downstream of the Sμ region. However, in this study, we find that sequences situated over 2 kb upstream of the DQ52 segment were associated with hypoacetylated histones H3 and H4 showing that there is also a sharp drop in histone acetylation 5′ of the DQ52 promoter. These results demonstrate that the JH segments are located within a discrete chromatin domain containing the proximal DQ52 segment and suggest the presence of a chromatin boundary element located 5′ of the DQ52 segment. Therefore, DH and JH segments do not all lie within a single chromatin domain as previously suggested (12, 14), but rather are separated...
into at least two distinct domains. Further chromatin analysis will be required to determine whether upstream FL16, SP2, and ST4 DH segments, which are also acetylated in pro-B cells, are contained in one or multiple domains. Apart from the DQ52 promoter and Eμ enhancer, no additional DNase I HS have been described in the 100-kb DH-Cμ region (12). Nevertheless, Morshead et al. (14) have proposed a domain boundary close to the distal DFL16.1 gene segment that is marked by a peak of histone H3 K4 methylation and may correspond to the 5′ limit of the DH chromatin domain.

V(DJ) recombination is a multistep process, with DH-to-JH rearrangement preceding recombination of Vμ to unarranged DH gene segments. This organization may also explain the preferential targeting of the DFL16.1 and DQ52 segments in initial DJH rearrangements (32–34), because these segments would be located at the 5′ extremity of each domain and hence potentially close to cis-acting chromatin regulatory elements. An increased use of DFL16.1 and a decreased frequency of JH3 and JH4 rearrangements were indeed observed in mice deleted for the DQ52 segment (35). Taken together, these results suggest that, during ontogeny, initial rearrangements occur within the DQ52-JH domain, while D-JH rearrangement with DH segments located outside this chromatin domain constitute secondary rearrangements.

Transcriptional promoter and enhancer sequences within the Ig and TCR loci have been shown to act as regulators of V(DJ) recombination. A growing body of evidence indicates that these sequences regulate chromatin structure, probably by recruiting chromatin remodeling factors (9, 10, 36, 37). For example, at the TCRβ locus, the DJβ1 promoter acts in collaboration with the linked Eγ enhancer to recruit histone acetyltransferases and ATP-dependent nucleosomal remodeling factors (37). Our finding that the DQ52 promoter and Eμ enhancer delimit the JH chromatin domain, strongly argues for the functional importance of these elements in the opening of the JH locus in early B cell precursors. Upstream of the DQ52 segment we find a clear transition in DNaseI sensitivity, nucleosomal organization, as well as histone acetylation, suggesting that the DQ52 promoter element acts only on downstream DQ52 and JH segments. This is consistent with the notion that promoter elements have a local, unidirectional effect on chromatin structure, as illustrated by deletion of the DJβ1 promoter and T early α germline promoter at the TCR loci, which only alter the chromatin structure of neighboring downstream J segments (36, 38, 39).

There is a less clear-cut transition in chromatin structure at the 3′ end of the JH domain. Although the DNase I-sensitive region stops at the Eμ enhancer, histone acetylation spreads further downstream. This may be due to the fact that unlike promoters, enhancers appear to have a more wide-ranging effect on chromatin structure (10) and act in a bidirectional manner (40). Targeted gene deletion experiments have shown that the absence of either the Eμ enhancer or DQ52 segment and immediate upstream sequences has only a limited effect on DH-to-JH rearrangement (35, 41, 42). This may indicate that these two elements have redundant or overlapping functions on JH chromatin and analysis of mice carrying both Eμ and DQ52 deletions should provide insight into this question. However, it should be noted that the DQ52 promoter was not entirely deleted in the DQ52−/− mice; only 170 bp of immediate upstream sequence containing enhancer but not promoter activity was removed (30, 43). Moreover, because we find that the 5′ boundary of both the DNase I-sensitive and hyperacetylation domain lies 2 kb upstream of the DQ52 segment, this region could contain additional cis-acting elements involved in locus opening, which have not yet been tested.

In summary, our results indicate that both histone modification and nucleosome remodeling are involved in rendering JH RSSs fully accessible to recombination factors in vivo. These changes occur within a distinct chromatin domain in pro-B cells containing only the proximal DQ52 gene and the JH segments.

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Disclosures

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