

Pax5 and Linker Histone H1 Coordinate DNA Methylation and Histone Modifications in the 3' Regulatory Region of the Immunoglobulin Heavy Chain Locus^{∇†}

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The 3' regulatory region (3' RR) of the murine immunoglobulin heavy chain (IgH) locus contains multiple DNase I-hypersensitive (hs) sites. Proximal sites hs3A, hs1.2, and hs3B are located in an extensive palindromic region and together with hs4 are associated with enhancers involved in the expression and class switch recombination of IgH genes. Distal hs5, -6, and -7 sites located downstream of hs4 comprise a potential insulator for the IgH locus. In pro-B cells, hs4 to -7 are associated with marks of active chromatin, while hs3A, hs1.2, and hs3B are not. Our analysis of DNA methylation-sensitive restriction sites of the 3' RR has revealed a similar modular pattern in pro-B cells; hs4 to -7 sites are unmethylated, while the palindromic region is methylated. This modular pattern of DNA methylation and histone modifications appears to be determined by at least two factors: the B-cell-specific transcription factor Pax5 and linker histone H1. In pre-B cells, a region beginning downstream of hs4 and extending into hs5 showed evidence of allele-specific demethylation associated with the expressed heavy chain allele. Palindromic enhancers become demethylated later in B-cell differentiation, in B and plasma cells.

The immunoglobulin heavy chain (IgH) gene locus undergoes a panoply of regulated DNA events during B-cell development, including VDJ joining to construct variable-region genes; class switch recombination (CSR) to achieve the production of non-IgM classes, such as IgG, IgE, and IgA; and somatic hypermutation (reviewed in reference 23). The identification of the specific *cis* regulatory regions that regulate these processes is of considerable interest. Candidates include promoters that are locally associated with V and D segments and also with I regions upstream of each constant-region gene, except for C δ . In addition, regulators capable of acting at considerable distances from their target sequences flank the C H genes (Fig. 1A). The first of these to be identified was the ~1-kb intronic enhancer E μ , which is situated in the intron

between J H and C μ . Targeted deletion studies with mice have revealed that E μ is important for VDJ joining (1, 29, 32, 36).

A 3' regulatory region (3' RR) extends ~35 kb downstream of the C α gene and includes multiple DNase I-hypersensitive (hs) sites. The murine 3' RR contains modules (Fig. 1A) that are developmentally regulated, as assessed by DNase I hypersensitivity, reporter assays, and engagement with active chromatin marks. The three most proximal sites, i.e., hs3A, hs1.2, and hs3B, form a 25-kb palindromic region by virtue of almost identical hs3A and hs3B sequences in inverted orientation at either end of this segment and by intervening families of locally repetitive sequences flanking the central hs1.2 enhancer (4, 34). hs3A, hs1.2, and hs3B are DNase I hypersensitive at later stages of B-cell development, i.e., in plasma cells (14, 20). A second unit contains hs4, which appears to be active beginning in pro-B cells and continuing throughout B-cell development (14, 20, 24). A third 3' RR module located downstream of hs4 is marked by DNase I-hypersensitive sites hs5, -6, and -7 and binding sites for CTCF, a protein associated with mammalian insulators, and displays *in vivo* insulator activity (13). DNase I hypersensitivity of the hs5 to -7 sites, like that of hs4, is detected at all stages of B-cell development. Knockout studies with mice have revealed that a region in the 3' RR in which enhancers hs3B and hs4 are located is critical for CSR (30). The contribution of hs5 to -7 to IgH locus function is not yet known.

In accord with acquisition of hypersensitive sites, chromatin immunoprecipitation (ChIP) assays showed that during B-cell development, marks of active chromatin were progressively acquired (3' to 5') by modular units of the 3' RR (13). In pro-B

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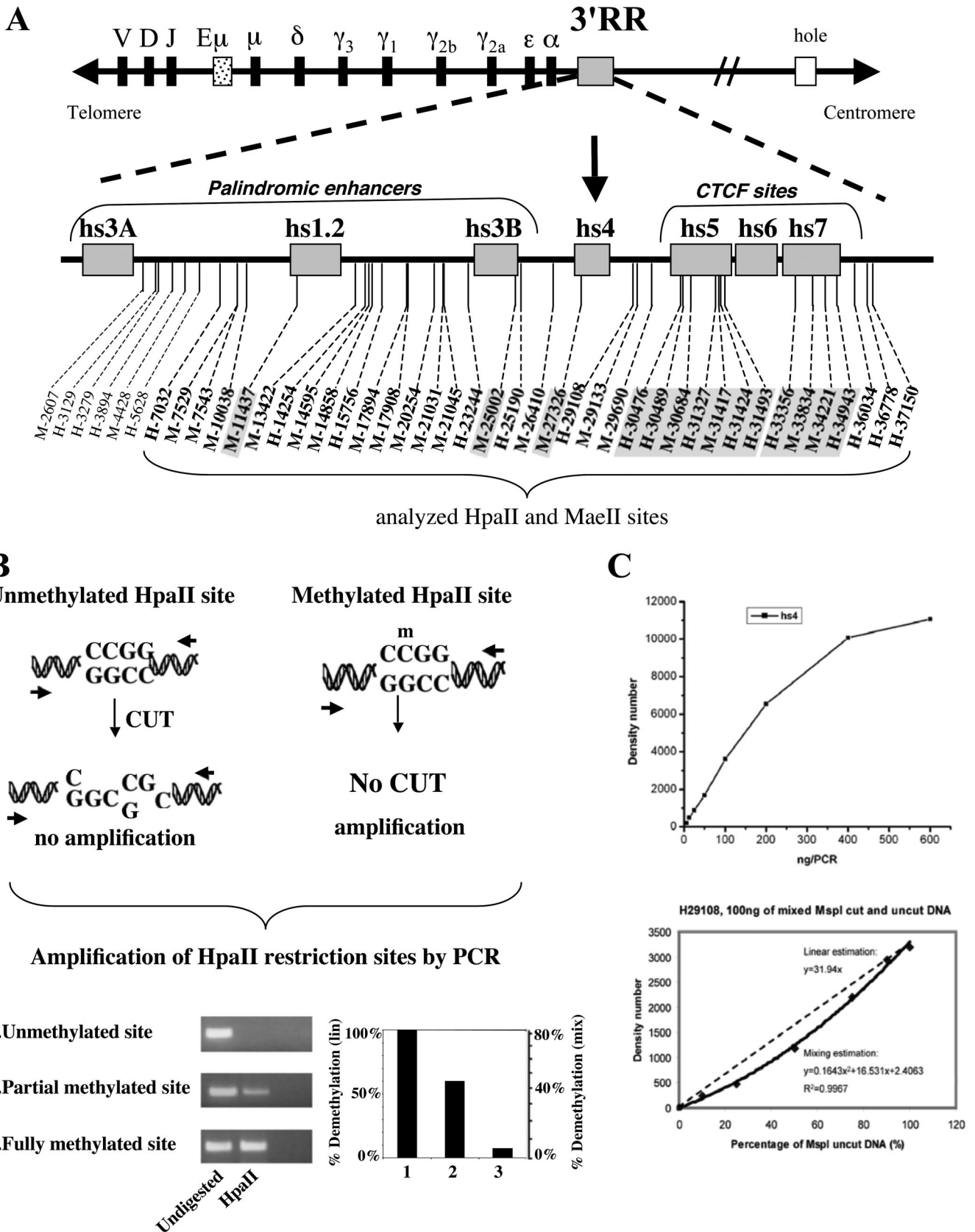
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cells, the most downstream regulators hs4 and hs5 to -7 are both associated with acetylated histone H4 (AcH4), and hs5 to -7 are also associated with AcH3. In pre-B cells, hs4 acquires association with AcH3. Enhancers hs3A, hs1.2, and hs3B become associated with AcH3 and AcH4 in B cells. No additional changes in these histone marks have been detected in plasma cell lines.

Analysis of the DNA methylation status of the 3' RR was first attempted in 1993 using genomic Southern analysis with the methylation-sensitive HpaII restriction enzyme and its methylation-insensitive isoschizomer MspI (14). However, this was limited by the relatively cursory knowledge of the structure of this region. Only sites hs3B and hs1.2 were known with confidence, and the region in the vicinity of not-yet-identified downstream hs sites had not yet been sequenced. Southern analysis depended on a probe from the palindromic region, which detected multiple members of families of locally repetitive sequences. Overall, these experiments showed that much of the 3' RR is hypermethylated at the pre-B-cell stage and undergoes progressive demethylation during B-cell development (14). A constitutively demethylated region (14) was identified, which appears to be located in the vicinity of now-mapped CTCF binding sites in hs7 (13). The complete sequence of the 3' RR has been determined (43), providing the foundation for the localization of additional hs sites, i.e., hs4, -5, -6, and -7, and making DNA methylation and histone analysis of this entire region feasible. Here, we tested the prediction that during B-cell development, the 3' RR is coordinately regulated by DNA methylation and histone modifications.

In these studies, we have examined DNA methylation at 37 specific CpG sites in the murine 3' RR in non-B and B cells, using PCR amplification at individual HpaII and HpyCH4IV (an isoschizomer of MaeII) methylation-sensitive restriction sites. Our experiments have shown that in B cells, DNA demethylation begins in hs4 and hs5 to -7 and occurs progressively during B-cell development in parallel with acquisition of marks of active chromatin. An allelic demethylation mark detected in pre-B cells correlates with allelic heavy chain gene expression. The region containing the palindromic enhancers, hs3A, hs1.2, and hs3B, constitutes a separately regulated module, and palindromic enhancers are demethylated only later in development. Non-B-cell sources also show extensive demethylation of the 3' RR, in concert with a variety of chromatin profiles generally associated with inactive DNA regions.

We have tested transacting factors that may coordinate DNA demethylation and acquisition of active chromatin marks in the 3' RR in B cells. One candidate is Pax5 (reviewed in reference 5), which regulates 3' RR enhancers (37; reviewed in reference 19) and multiple other genes (6, 31, 35) as both an

activator and a repressor. Pax5 expression is critical for generating and maintaining B-cell identity, beginning in pro-B cells and extending throughout the mature B-cell stage. Pax5's role with Ets1 in *mb1* activation is affected by DNA methylation (21). In addition, Pax5 expression has been shown to have an impact on histone marks associated with the IgH locus: when reexpressed in Pax5-deficient cells, Pax5 reduced the association of V_H genes with di-meK9H3 (17). Furthermore, Pax5 has been shown to interact with both histone acetylases and chromatin-remodeling complexes (2). A second candidate is the linker histone H1, which plays an important role in chromatin folding. Significant reduction in H1 levels through targeted deletions resulted in decreased global nucleosome spacing, reduced local chromatin compaction, and decreases in certain core histone modifications (10). Interestingly, the expression of only a small number of genes was affected, some of which, e.g., the IGFR/H19 locus, are imprinted and are normally regulated by CTCF binding and DNA methylation. In our experiments, we used cells depleted of Pax5 or H1 to analyze the effects of these factors on DNA methylation of the 3' RR. We found that both histone H1 and Pax5 contribute to regulating both DNA methylation and histone modifications of the 3' RR.

MATERIALS AND METHODS

Cell lines and murine primary B cells. Mouse cell lines (AH7 pro-B, 18-81 pre-B, W231 B, MPC 11 plasma cells, Bac1.2F5 macrophage [25], BW5147 T cells, and MEL mouse erythroleukemic cells) were maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin, and β -mercaptoethanol. Various additional sources of pro-B cells were analyzed. ChIP analysis was done on bone marrow cells isolated from wild-type and Pax5^{-/-} mice, which were cultured with interleukin-7 (IL-7) to generate pro-B cells as previously described (17). DNA methylation was assayed in pro-B cells that were isolated from the bone marrow of Rag1^{-/-} mice as previously described (13) and in two cultured Pax5^{-/-} pro-B-cell sources generously provided by Meinrad Busslinger. One was a pro-B-cell clone (P5N5) derived from the bone marrow of a Pax5^{-/-} mouse of 129/C57Bl6 background. A second source (B1.1) was derived from a heterogeneous population of pro-B cells extracted from the bone marrow of an 8-day-old Pax5^{-/-} mouse (129/C57Bl6 background). B1.1 cells were transduced with the retroviral vector MSCV Pax5ER-IRES-GFP (ER, estrogen receptor) (27). Green fluorescent protein (GFP)-positive cells were fractionated by fluorescence-activated cell sorter (FACS), after which Pax5ER was induced with 4-hydroxytamoxifen for 24 h. CD19⁺ cells increased from 1.4% before induction to 47% after induction. DNA was extracted from the total population of cells (CD19⁺ and CD19⁻).

Normal pre-B cells [fraction D; CD19⁺ IgM⁻ CD43(S7)⁻ CD93(AA4)⁺] were purified from mouse bone marrow by flow cytometry using a BD FACS-VantageSE/DiVa cell sorter (16). B cells were obtained from spleens of 6- to 8-week-old wild-type female C57BL/6 mice. Spleens were depleted of red blood cells by lysis (Puregene), after which CD43⁺ cells were removed with anti-CD43 (Ly-48) MACS microbeads (Miltenyi Biotec, Auburn, CA). Negative selection of resting B cells resulted in a purity of 95.3%, as evaluated by FACS analysis of CD43⁻

sites for CTCF, a protein associated with mammalian insulator regions (13), and have been hypothesized to serve as an insulator of the IgH locus. Each CpG site is identified by H or M, referring to HpaII and MaeII, respectively, and by a number identifying its location in Bac199M11 (GenBank accession number AF450245). Gray bars and shaded numbers identify CpGs associated with hypersensitive sites. hs3A and hs6 were excluded from DNA methylation analysis because they lack HpaII and MaeII sites. Primer pairs detected DNA products in the linear range with templates ranging from 6.25 to 200 ng, as shown for hs4 in panel C and for other primer pairs in Fig. S1 in the supplemental material. (B) As illustrated, methylation-sensitive restriction enzymes, such as HpaII (or MaeII), cut only target sites that are unmethylated at cytosine residues; hence, a PCR product generated by primers flanking the restriction site represents only methylated (or uncut) DNA. (C) The proportion of methylated DNA in a sample was estimated by two approaches: a linear estimation (lin) and a mixing experiment (mix). Additional details are shown in the supplemental material.

B220⁺ CD3ε⁻ cells. Mouse primary B cells (4.5 × 10⁶ cells in 3 ml RPMI) were induced to undergo CSR *in vitro* by addition of lipopolysaccharide (LPS) (50 μg/ml) (*Escherichia coli* 055:B5) (catalog no. 437625; Calbiochem). Germ line transcripts were monitored by reverse transcription-PCR (26), using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a control as previously described (13) (data not shown). T cells from spleen and lymph nodes were isolated with the MACS pan-T-cell isolation kit (catalog no. 130-090-861), plated in 2 ml of complete RPMI containing 20 to 40 units/ml of recombinant human IL-2 in a well coated with anti-CD3ε, and incubated for 96 h. Livers were isolated and disrupted by pressing with glass slides; cells were collected by centrifugation and used to prepare genomic DNA. Peritoneal cells were isolated by flushing the peritoneal cavity with 5 to 10 ml medium followed by plating on petri dishes overnight in the incubator. Adherent cells were subjected to genomic DNA isolation. Wild-type and triple-H1 null embryonic stem (ES) cells were previously described (10).

DNA extraction from cell lines and primary cells. Cells (10⁷) were centrifuged at 1,800 rpm for 5 min at 4 to 10°C (Beckman). The pellet was resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% sodium dodecyl sulfate, 0.2 M NaCl) and 10 μl of 20-mg/ml proteinase K. The solution was incubated at 65°C for 1 h. One volume of chloroform was added, and the sample was mixed and centrifuged at full speed for 15 min. DNA was precipitated from the upper phase by addition of 1 volume of isopropanol and resuspended in distilled water. The DNA concentration was determined with a nano-drop instrument (NanoDrop Technologies, Wilmington, DE).

Analysis of extent of DNA methylation of CpGs in HpaII and MaeII sites by PCR. The sequence of the 3' RR from 129Sv (extending from position 1 to ~40,000 in BAC199M11; GenBank accession number AF450245) provides the template to locate individual CpG dinucleotides (Fig. 1A). Genomic DNA was digested initially with EcoRI and then with methylation-sensitive restriction enzymes HpaII (New England BioLabs) (target site, CCGG) or HpyCH4IV (a Mae II isoschizomer; target site, ACGT) (New England BioLabs) or with MspI, the methylation-insensitive isoschizomer of HpaII, at a concentration of 10 U/μg. The percentage of methylation at these sites (prefixed H for HpaII sites and M for MaeII isoschizomer sites) was assayed by PCR amplification before and after enzyme digestion (primer pairs are shown in Table S1 in the supplemental material). The reaction conditions were as follows: 1 unit of *Z. Taq* (generously provided by P. Scherer, Albert Einstein College of Medicine) or *Taq* DNA polymerase (Roche) in the buffer (1×) Platinum High Fidelity (200 mM Tris-HCl [pH 8.4], 500 mM KCl), deoxynucleoside triphosphates (0.2 mM), MgCl₂ (1.5 mM), primers (15 pmol), and water to give a final volume of 50 μl. The reaction was done at 94°C for 2 min, followed by 27 cycles at 94°C for 30 s, 60°C (or as indicated in Table S1 in the supplemental material) for 30 s, and 72°C for 30 s and one final extension at 72°C for 7 min. PCR products were analyzed on agarose gels (1.2%) stained with ethidium bromide. Unmethylated DNA in segments containing HpaII or MaeII sites is sensitive to restriction enzyme cleavage, precluding amplification by primers flanking the cut site. Hence, PCR products represent methylated DNA (Fig. 1B). Because most analyzed CpG sites are initially methylated at early stages of B-cell development, we plotted the percent demethylation (100% - percent methylated) to more clearly identify the dynamic demethylation that occurs during B-cell development. The intensity of bands was measured by Chemimager V 5.5 software. Each PCR signal was normalized with an internal control obtained by amplification of the hs4 fragment (see Table S1 in the supplemental material) that contains neither HpaII nor MaeII restriction sites.

Standard curves were generated using the hs4 product (Fig. 1C) and four other CpG sites (see Fig. S1 in the supplemental material) with eight different DNA amounts ranging from 6.25 ng to 600 ng. Each point of the standard curves was the average from two independent reactions. While the efficiency of individual primer pairs differed, the linear range for each of the primer pairs was similar and extended to ~200 ng. The percent methylation at a HpaII or MaeII site was calculated by two approaches, as presented in Fig. 1B and C and described in detail in the supplemental material. One approach presumed a linear approximation between the signal and the percent methylation. A second approach analyzed various ratios of cut and uncut DNA. This mixing experiment showed a curvilinear relationship between PCR signal and methylated DNA amounts and indicated that methylated DNA can be detected only when present at a ratio of >1:10. Nonetheless, there is a general correlation between the two approaches, and graphs of the data show the linear approximation on the left axis and the mixing estimation on the right axis.

ChIP. ChIP was carried out as described previously (39) except for a few modifications. In summary, 10⁸ cells were cross-linked for 10 min at room temperature in 30 ml of medium by the addition of formaldehyde to the fixation solution at a final concentration of 1.1%. Cross-linking was terminated with

glycine. Cells were washed twice with cold phosphate-buffered saline, and nuclei were prepared as described. Chromatin fragments were generated by sonication using a Bioruptor (Diagenode model UCD 200). For immunoprecipitations, in addition to the previously described antibodies, the following antibodies were used: normal rabbit IgG (Santa Cruz catalog no. 2027), rabbit polyclonal anti-H1 (Upstate pan-H1 [AE-4] antibody), and affinity-purified rabbit anti-Pax5. Rabbits were immunized with Pax5 from which the paired domain had been deleted. Serum containing anti-Pax5 was affinity purified using an *N*-hydroxysuccinamide-activated Sepharose column (Amersham) to which full-length Pax5 protein had been coupled. Anti-Pax5 specificity was confirmed by Western blots and by immunostaining. After immunoprecipitation, cross-links were reversed and DNA was isolated with a Qiagen QIAquick PCR purification kit (catalog no. 28106). Samples were analyzed quantitatively by real-time PCR using the method and primers described previously (13).

RESULTS

Modular regulation of DNA methylation of the 3' RR during B-cell development. Previous studies have shown progressive acquisition of marks of active chromatin by the 3' RR enhancers and insulator fragments during B-cell development (13). In pro-B cells, both hs4 and hs5 to -7 are associated with AchH4 and di-meK4H3, but only hs5 to -7 is also associated with AchH3. In pre-B cells, hs4 acquires association with AchH3. In B and plasma cells, the palindromic enhancers hs3A, hs1.2, and hs3B become associated with all three marks of active chromatin, i.e., AchH3, AchH4, and di-meK4H3.

We determined whether progressive changes in DNA methylation in the 3' RR accompanied marks of active chromatin during B-cell development by studying the state of DNA methylation of 37 CpGs of the 3' RR with methylation-sensitive endonucleases HpaII and HpyCH4IV (an isoschizomer of MaeII) (see Materials and Methods) (Fig. 1). These sites represent ~20% of the total number of CpG sites in this region. Hs3A and Hs6 do not contain HpaII or MaeII sites. The horizontal axis in each graph locates the position of analyzed CpGs in the 3' RR (with position 1 beginning at position 1 of BAC199M11, GenBank accession no. AF450245), and the vertical axis plots the corresponding percentage of "unmethylation" (Fig. 1 and 2), as assessed by a linear approximation (left axis) and by a mixing experiment (right axis) (see Materials and Methods and the supplemental material for fuller descriptions). Sites that are fully methylated are 0% unmethylated. Data were represented in this fashion in order to more easily focus on the demethylation that occurs during B-cell development.

Studies of the AH7 pro-B-cell line (Fig. 2) revealed that several sites in the hs4 to -7 region were subject to demethylation. The only site that was completely unmethylated was associated with hs4. In contrast, except for a site in hs3B, the palindromic region in which hs3A, 1.2, and 3B are located was essentially fully methylated. To determine whether the modules of differential DNA methylation in the 3' RR detected in AH7 cells were apparent *in vivo*, we examined pro-B cells directly isolated from the bone marrow of RAG1^{-/-} mice. These cells had a DNA methylation profile similar to that detected in AH7 cells (Fig. 2). Analysis of the 18-81 pre-B-cell showed a DNA methylation profile similar to that of pro-B-cell sources, except for a segment of ca. five CpGs located between hs4 and hs5 that were ~50% demethylated. When normal pre-B cells were examined for the methylation profile in the same vicinity, four of the five sites showed a similar degree of

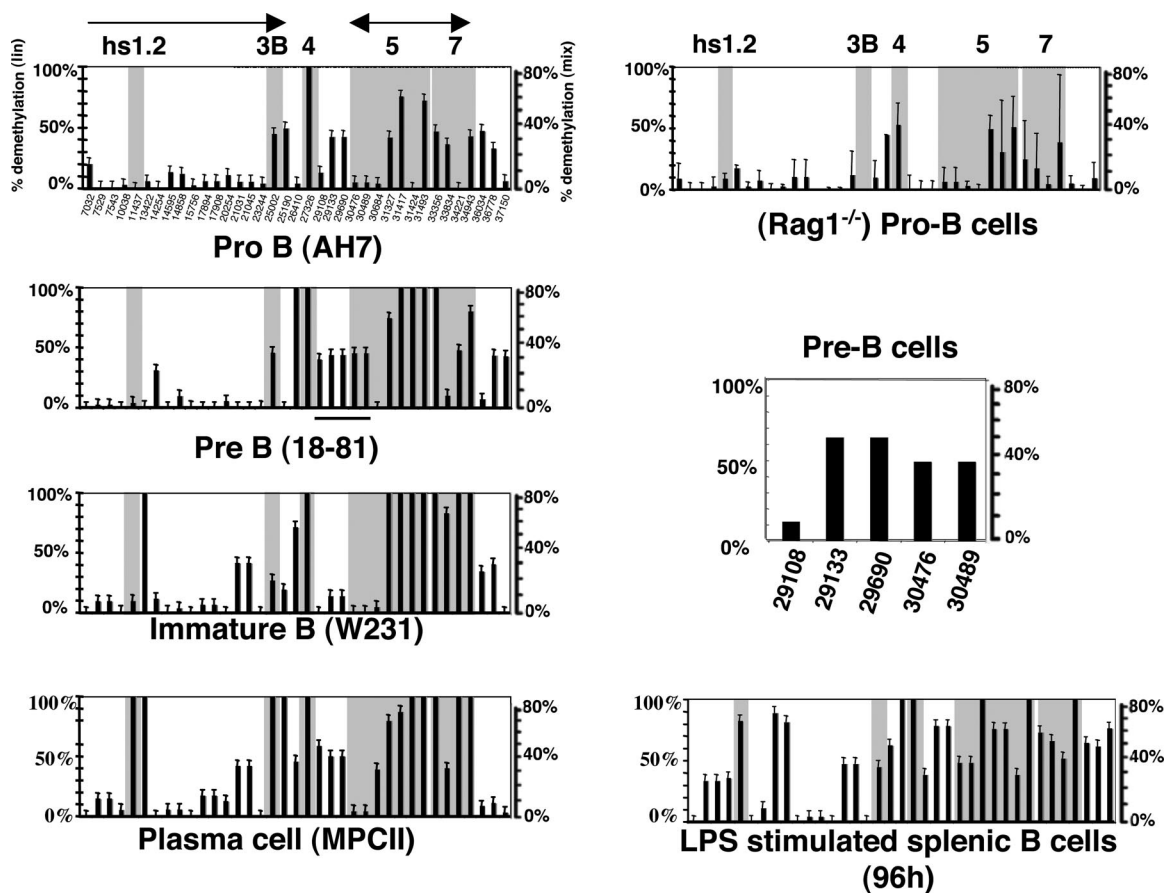


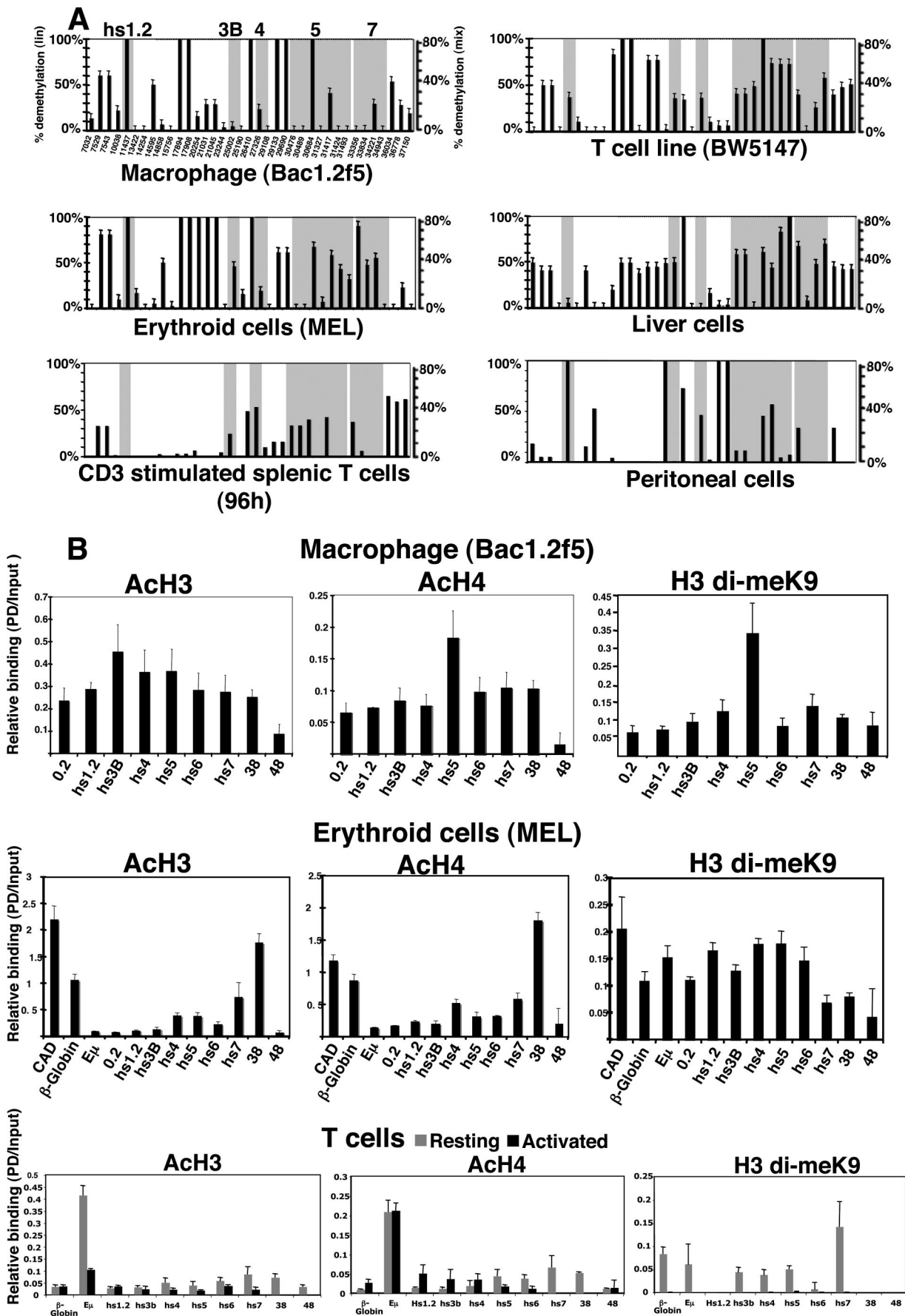
FIG. 2. Progressive DNA demethylation of the 3' RR during B-cell development. The percentage of demethylation (vertical axis; linear estimation on left and mixing estimation on right) at 37 CpGs (horizontal axis) was measured by PCR amplification after digestion with methylation-sensitive HpaII or HpyCH4IV (MaeII). CpGs are located in the context of BAC199M1 (see Materials and Methods). Shaded boxes indicate DNase I-hypersensitive sites of the murine 3' RR. hs3A and hs6 were excluded from analysis because they lack HpaII or MaeII sites. Pro-B cells were represented by the AH7 cell line and by bone marrow-derived cells from RAG^{-/-} mice. Pre-B cells were represented by 18-81 and by pre-B (fraction D) cells isolated from normal mice (16). The bar between hs4 and hs5 identifies a region of potential allele-specific methylation, which was assessed in normal pre-B cells, and is discussed further below. The W231 B-cell line and MPC11 plasma cells represented later stages of B-cell development. Normal mature activated B cells were represented by splenic B cells stimulated by LPS for 96 h. The hs4 site is demethylated in all examined B-cell lines. Sites in the hs5 to -7 region are extensively unmethylated, especially prominently in pre-B, B, and plasma cells. Sites related to hs1.2 and hs3B are fully demethylated only in a plasma cell line (MPC11), where sites flanking hs1.2 are extensively methylated.

demethylation. These data were potentially indicative of an allelic mark and will be discussed further below. Study of the W231 and MPC11 cell lines, representing later stages of B-cell development, provides evidence about activation of the palindromic enhancers (Fig. 2). Scattered demethylation of the palindromic region is evident in W231 immature B cells. However, only in MPC11 plasma cells is hs1.2 itself fully demethylated, and this occurs on a background of extensively methylated flanking sequences. The pattern in LPS-stimulated normal splenic B cells is much like those in W231 and MPC11, showing demethylation of the hs1.2 enhancer and at hs1.2 flanking sites and more extensive demethylation throughout the hs4 to -7 region. This stepwise demethylation of 3' RR modules parallels acquisition of marks of active chromatin by 3' hs sites during B-cell development (13).

To determine whether there was a B-cell-specific 3' RR methylation signature associated with IgH activity, we examined various non-B hematopoietic cells and normal liver cells for DNA methylation of the 3' RR (Fig. 3A). Except for T

cells, where DJ joining has been shown to occur, the IgH locus is not known to be active in non-B cells. Of the six cell sources studied, four showed extensive DNA demethylation throughout the 3' RR, while stimulated splenic T cells and adherent peritoneal cells showed proportionately less demethylation in the palindromic region, a feature shared with most B-cell sources (Fig. 2). Comparison of non-B cells (Fig. 3A) and B cells (Fig. 2) showed that complete demethylation of hs4 and virtually complete demethylation of sites in hs5 to -7 were detected only in B cells. The demethylation in the palindromic region that was evident in four of the non-B-cell sources was also observed, although less extensively, only in splenic B cells stimulated with LPS for 96 h. These observations suggested that while demethylation of the 3' RR can occur in multiple non-B and B-cell sources, complete demethylation of hs4 and of the hs5 to -7 region, and general methylation of the palindromic region may be under B-cell-specific regulation (see below).

To assess the mode of chromatin regulation of the 3' RR in



non-B cells, we carried out ChIP for AcH3 and AcH4 and for di-meK9H3 in Bac1.2f5 macrophage cells, MEL erythroleukemic cells, and resting and activated T cells (Fig. 3B). ChIP showed that different epigenetic patterns were associated with the inactive 3' RR in these cells. In macrophages, the 3' RR had considerable association with AcH3 and AcH4 and modest levels of association with di-meK9H3, with a spike of interaction with hs5. In MEL cells, 3' regulators were associated with low levels of AcH3 and AcH4 and high levels of di-meK9H3, the inverse of the patterns detected in B cells. Resting and activated T cells were similar to MEL cells in having low levels of association with AcH3 and AcH4, and resting but not activated T cells showed interaction with di-meK9H3. These observations suggest that the DNA demethylation of the 3' RR in non-B cells can occur independently of specific histone modifications.

Allelic differences in DNA methylation of the 3' RR in pre-B cells. An allelic mark provided by DNA methylation in the 3' RR would be evidenced by ~50% demethylation at specific sites. The 18-81 BALB/c mouse-derived pre-B-cell line contained a region starting downstream of hs4 and extending into hs5, spanning five CpGs located in methylation-sensitive restriction sites, each of which was found to be ~50% demethylated (Fig. 2 and 4). To determine whether this entire segment was regulated by DNA demethylation in an allele-specific manner, we turned to study of 70Z/3, a heterozygous pre-B-cell line, in which the expressed allele is derived from C57BL/6 while the unexpressed allele, which is blocked at DJ recombination, is derived from the DBA mouse (33). DNA sequence analysis of this region (primers 15A and 17B [see Table S1 in the supplemental material], position 30089 to 30923 [accession no. AF450245]) showed that the two alleles in 70Z/3 could be distinguished by a 17-bp segment located between hs4 and hs5, which is repeated 5 times in the DBA allele and 35 times in the C57BL/6 allele (and 12 times in the BALB/c allele) (Fig. 4B and C). All other sequences were identical. Using primers that amplified the polymorphic repeated region and flanking sequences, we could individually analyze five CpGs that were candidates for allelic regulation. Our data (Fig. 4D) demonstrate that an ~800-bp segment containing M29690, H30474, and H30488 is extensively demethylated on the expressed C57BL/6 allele and, reciprocally, is highly methylated on the unexpressed DBA allele. Two CpG sites located within 500 bp upstream, i.e., H29108 and M29133, showed a similar trend. These data show that DNA demethylation in a region from hs4 to the 5' end of hs5 occurs preferentially on the single expressed allele in 70Z/3 pre-B cells. Like 18-81 and 70Z/3 cells, normal pre-B cells (Fig. 2) contained a similar region in which adjacent sites showed ~50% demethylation. The demethylated region in normal pre-B cells appeared to be shifted slightly 3' of the region identified in pre-B-cell lines (Fig. 2). These stud-

ies are consistent with the prediction that in the transition from pro- to pre-B cells during which heavy chain allelic exclusion is established, demethylation in the hs4 to -5 region of the 3' RR occurs specifically on the expressed heavy chain allele.

Pax5 expression coordinates DNA methylation and histone modifications in pro-B cells. IL-7-dependent cell lines from Pax5-deficient mice provided a source of pro-B cells and the vehicles to test the hypothesis that Pax5 has an impact on the 3' RR through epigenetic regulation. Pax5^{-/-} cells have many pro-B-cell-specific features, including evidence of DJ joining, but are blocked in further B-cell differentiation (28). Intriguingly, Pax5^{-/-} pro-B cells showed an increase in 3' RR DNA demethylation (Fig. 5) compared to Rag1^{-/-} pro-B cells and the AH7 pro-B-cell line (Fig. 2), which was especially evident at several sites in the palindromic region (Fig. 5A and B). In addition, the hs5 to -7 region was more extensively demethylated, albeit with a similar profile of affected sites. To assess a role for Pax5 in promoting DNA methylation of the 3' RR, we exploited an independent source of Pax5^{-/-} pro-B cells, which contained a vector directing the nuclear localization of Pax5 on exposure to tamoxifen via its coupled estrogen receptor (Fig. 5B). After 24 h of tamoxifen treatment, there was a general reestablishment of 3' RR methylation patterns, as detected in AH7 and wild-type pro-B cells, including complete methylation at sites associated with hs1.2 and hs3B (Fig. 5C). These data imply that in pro-B cells, Pax5 expression affects the extent of DNA methylation of sites throughout the 3' RR, including the complete methylation of the palindromic hs3A-1.2-3B region.

Previous experiments had revealed that in Pax5^{-/-} cells, but not in wild-type pro-B cells, V_H genes were associated with di-meK9H3 (17). To determine whether the differences in 3' RR DNA methylation observed in Pax5^{-/-} and wild-type cells were accompanied by differences in chromatin modifications, we carried out ChIP analysis of di-meK9H3 with the 3' RR. By using bone marrow cells from wild-type and Pax5^{-/-} mice cultured in IL-7 as the source of pro-B cells, we obtained sufficient numbers of cells for ChIP analysis. ChIP experiments (Fig. 5D) showed that palindromic enhancers of the 3' RR, like V_H genes, are enriched with di-meK9H3 in Pax5^{-/-} cells but not in wild-type pro-B cells. Hence, Pax5 expression in pro-B cells was correlated not only with promotion of increased DNA methylation of the 3' RR but also with loss of the associated di-meK9H3 mark.

To determine whether Pax5's effects on DNA methylation and histone modifications of the 3' RR could result from direct protein-DNA interaction, we carried out ChIP with anti-Pax5 antibody. We observed that Pax5 bound preferentially to hs1.2 in wild-type pro-B cells (Fig. 5E). These experiments suggest that binding of Pax5 to hs1.2 may help initiate both DNA

FIG. 3. DNA methylation (A) and chromatin modifications (B) of the 3' RR in non-B cells. (A) The percentage of demethylation was assessed as described for Fig. 2. All sources show demethylation of the hs5 to -7 region at reduced levels compared to in B cells. hs4 is substantially methylated. hs1.2 flanking sequences show partial to complete demethylation. (B) ChIP analysis of association of AcH3, AcH4, and di-meK9H3 with the 3' RR. Inactivity of the IgH locus in each cell source is associated with various patterns of histone marks. CAD, β -globin, E μ , and "48" were controls for histone modifications, as described previously (13). Positions 38 and 48 are located 38 and 48 kb downstream of position 1 of BAC199, respectively. Experiments with splenic T cells and peritoneal cells were carried out once, precluding calculation of standard deviations.

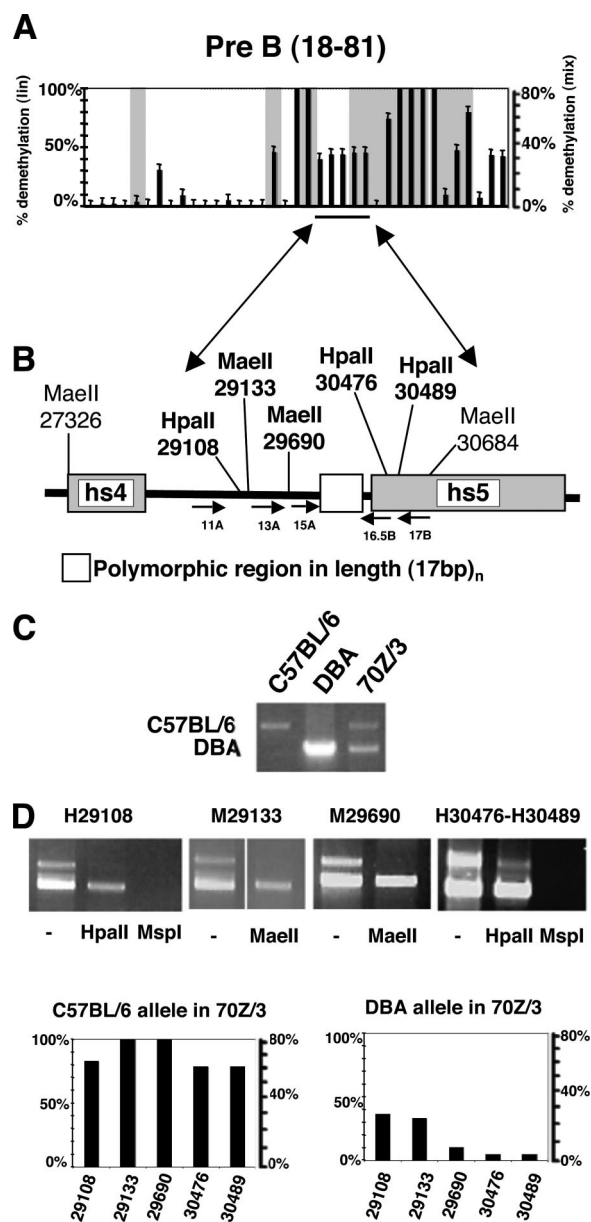


FIG. 4. Demethylation of a 3' RR segment located downstream of hs4 and extending into hs5 is associated with the expressed heavy chain allele in pre-B cells. (A) Methylation profile of the 3' RR in 18-81 pre-B cells (as also shown in Fig. 2) detects a region of ~50% demethylation (underscored bar) that was similarly unmethylated in bone marrow pre-B cells (Fig. 2). (B) Schematic map of the polymorphic region in the 3' RR. A 17-bp sequence, repeated 5 times in the DBA allele and 35 times in the C57BL/6 allele, is located between hs4 and hs5 (open box). Primers flanking the polymorphic region (see Table S2 in the supplemental material) are indicated by arrows. The polymorphic 17-bp repeat region is an end point of an extensive deletion of the 3' RR from hs3A to hs4 in the LP1.2 cell line (15, 24). (C and D) Analysis of allelic methylation in 70Z/3. (C) Amplification of homozygous genomic DNA from DBA and C57BL/6 mice and of heterozygous DBA/C57BL/6 genomic DNA from 70Z/3 pre-B cells. The PCR amplification shows the differences in length between the DBA and C57BL/6 alleles. (D) Analysis of allelic methylation in the 70Z/3 pre-B-cell line. PCR amplification after HpyCH4IV (MaeII) and HpaII digestions shows that the unexpressed DBA allele is highly methylated while the expressed C57BL/6 allele is extensively demethylated, as graphed.

methylation and histone demethylation of the palindromic region.

Contribution of linker histone H1 to B-cell-specific demethylation of the 3' RR. Recent studies have shown that the H1 linker histone promotes DNA methylation of some imprinting control regions that when unmethylated are bound by CTCF (10). The hs5 to -7 region of the 3' RR contains a number of CTCF binding sites and the observations we report here show regulation of the 3' RR by DNA methylation, both features that are shared with imprinted genes affected by H1. We therefore sought to analyze H1's association with the 3' RR in pro-B cells. ChIP assays in pro-B cells (Fig. 6A) showed that H1 was associated with all the enhancers of the 3' RR except for an especially low association with hs4 and hs5. For the most part, the patterns of association were similar in wild-type and Pax5 deficient cells, implying that association of di-meK9H3 with the 3' RR was regulated independently of H1.

Published studies have reported that those genes whose methylation status and subsequent expression were particularly sensitive to H1 dosage in ES cells were associated, in wild-type situations, with substoichiometric association of H1 (10). This inference suggested the possibility that DNA methylation of the 3' RR might also be influenced by histone H1, with sites hs4 and hs5 being of particular interest in this regard. We compared 3' RR methylation patterns in wild-type and triple-H1 null ES cells (Fig. 6B). In two independently derived wild-type ES lines, the 3' RR was essentially fully methylated at all analyzed HpaII and MaeII sites; the percent of "demethylation" did not exceed 20% for any analyzed CpGs (Fig. 6B). However, a number of 3' RR sites were demethylated in two triple-H1 null ES cell lines, including several in the interval beginning downstream of hs4 and punctuating the hs5 to -7 region (Fig. 6B). These experiments show that methylation of specific 3' RRs in ES cells depends on normal levels of linker histone H1.

DISCUSSION

Our experiments reveal that during B-cell development, stepwise demethylation of the 3' RR modules parallels acquisition of marks of active chromatin. Previous studies (13) had shown that marks of active chromatin, i.e., acetylated histones and di-meK4H3, are progressively associated with modules of the 3' RR, beginning with hs4 and hs5 to -7 in pro-B cells and extending in B and plasma cells to the palindromic region in which hs3A, hs1.2, and hs3B are located. Here we show that in all B-cell populations, hs4 is extensively demethylated, reaching demethylation levels of ~100% in several cell lines. The hs5 to -7 region has sites of substantial demethylation. The palindromic region, on the other hand, appears to be generally methylated even in plasma cells, where the hs1.2 enhancer is demethylated, although this region also acquires some demethylation in B cells stimulated by LPS for 96 h. Hence, during B-cell development, there is coordination between two epigenetic marks associated with the 3' RR, i.e., acquisition of marks of active chromatin and reduction of DNA methylation.

Non-B cells also showed extensive demethylation of the 3' RR, but only in B-cell sources did we observe complete demethylation of hs4. By comparing B and non-B cells, we infer that B-cell-specific regulation of 3' RR DNA methylation is

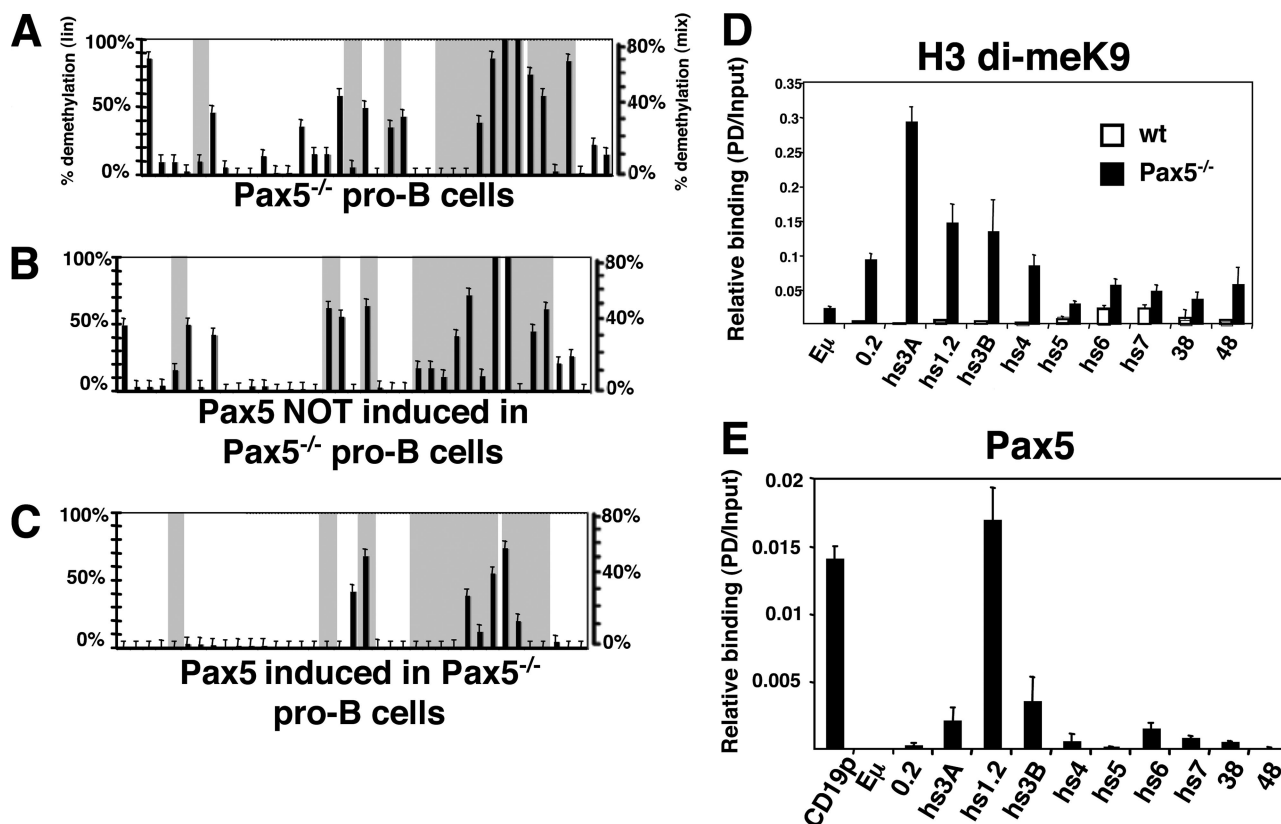


FIG. 5. Pax5 coordinates DNA methylation and histone modifications of the 3' RR. (A to C) Pax5 expression promotes DNA methylation in the palindromic region of the 3' RR in pro-B cells. Two Pax5^{-/-} pro-B-cell sources (A and B) were analyzed, one of which (B) contained a tamoxifen-dependent Pax5 expression vector. (C) The expression of Pax5 in Pax5^{-/-} pro-B cells promotes DNA methylation of the 3' RR after 24 h. Data are the averages from two independent experiments. (D) ChIP analysis for di-meK9H3 in wild-type (wt) and Pax5^{-/-} pro-B cells. Pro-B cells were isolated from normal or Pax5^{-/-} mice and cultured with IL-7. A di-meK9H3 mark on palindromic enhancers is detected in Pax5^{-/-} cells but not in wild-type cells. (E) ChIP detects Pax5 association with hs1.2 of the 3' RR in bone marrow-derived pro-B cells.

evidenced by demethylation of hs4 and hs5 to -7 and by methylation of the palindromic region. In various non-B cells, the 3' RR was maintained in an apparently inactive state by reduced association with "active" chromatin (i.e., AcH3 and/or AcH4), by increased association with "inactive" chromatin (di-meK9H3), or by both kinds of marks. Hence, in contrast to B cells, in non-B cells demethylation of 3' RR sequences does not parallel active histone modifications.

A similar lack of coordination between DNA methylation and histone modifications was apparent in Pax5^{-/-} pro-B cells. In Pax5^{-/-} pro-B cells, the 3' RR palindromic region is associated both with di-meK9H3, a repressive mark, and with a general increase in DNA demethylation, a mark ordinarily associated with activation of this region at later stages of B-cell development. Direct binding of Pax5 to hs1.2 in pro-B cells as revealed by ChIP is associated with an exchange of "repressive marks" of this region, i.e., loss of interaction with di-meK9H3 and gain of DNA methylation on a background of only modest interactions with AcH3 and AcH4 (13). We speculate that Pax5 binding to hs1.2 as detected in pro-B cells initiates active spreading of DNA methylation and loss of di-meK9H3 via mechanisms associated with structural features of this palindromic region, e.g., families of locally repetitive sequences that are dispersed in direct and inverted repeats on both flanks of

hs1.2 (34). Hence, Pax5 is a candidate for initiating the coordination of histone modifications and DNA methylation of the 3' RR in B cells, reinforcing the variety of roles that Pax5 plays in B cells, including its activity as a central regulator of both gene activation and repression, potentially mediated by its interaction with both histone acetylases and chromatin-remodeling complexes (2) and with known repressors (7, 9).

Like the palindromic region of the 3' RR, V_H genes are similarly rendered free of di-meK9H3 upon Pax5 expression in Pax5^{-/-} cells (17). This appears to be associated with a direct role for Pax5 in IgH locus contraction (12) and the utilization of distal V_H genes in VDJ joining (42). That Pax5 expression results in a similar reduction in the di-meK9H3 mark in V_H and 3' RR sequences is provocative in considering whether this involves physical interaction between these two distally located sequences. Evidence for this long-range interaction in mature B and plasma cells has been reported (18, 40), and analysis of an E μ ^{-/-} mouse has led to consideration of a potential role for the 3' RR in early steps of VDJ joining (29).

B-cell-specific epigenetic regulation of the 3' RR appears to interface with linker histone H1 and is especially evident in the hs4/5 region. Like the H19 gene, which is activated in the triple-linker histone H1 null ES cells (10), the hs4/5 segment is, in wild-type pro-B cells, associated with very low levels of H1

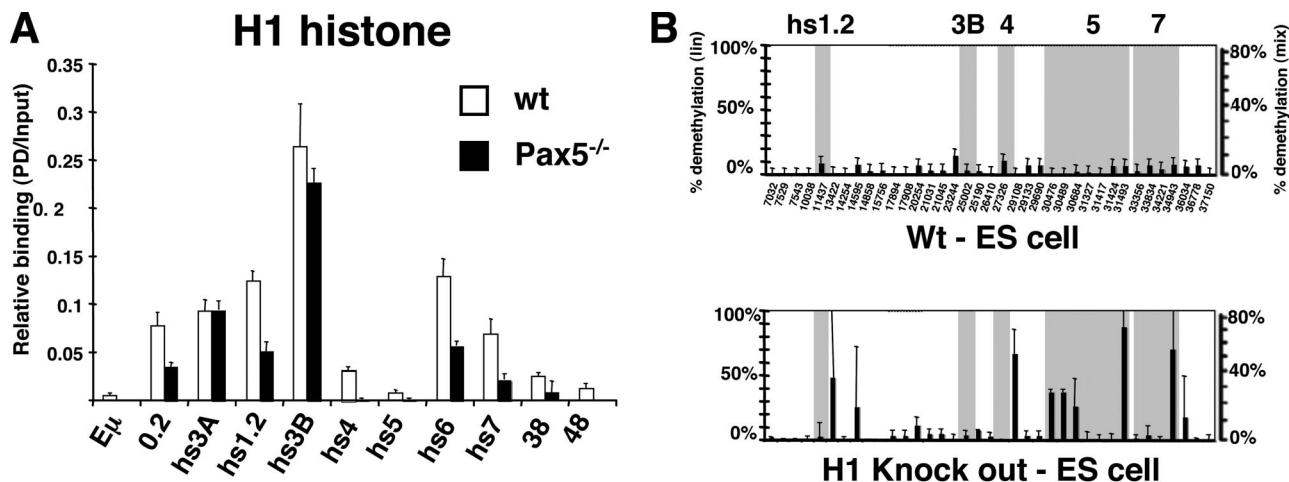


FIG. 6. Epigenetic influence of H1 histone on the 3' RR. (A) ChIP analysis of H1 association with 3' RR hs sites in wild-type (wt) and Pax5^{-/-} pro-B cells. H1 is associated with palindromic enhancers, but there is substoichiometric association of H1 with hs4 and hs5, identifying these hs sites as potentially affected in extent of DNA methylation by further reduction in H1 levels. Positions 38 and 48 serves as a control DNA segment that is independent of 3' RR regulation. (B) DNA methylation in wild-type and triple-H1 null ES cells shows that a reduction in H1 levels results in demethylation at multiple sites in the 3' RR, especially in the hs4/h5 region.

histone. It has been suggested that when H1 levels are reduced, those genes ordinarily regulated by reduced H1 more easily exhibit a phenotypic threshold (10). Also similar to activation of the H19 gene by DNA demethylation, the hs4/5 region appears to be a target for regulated demethylation in triple-H1 null ES lines and during B-cell development. These experiments predict that hs4 and hs5 have unique associations with chromatin and are particularly sensitive to interactions with H1 linker histone.

This same hs4/h5 region is the focus of an ~50% demethylation mark, which was evident in normal pre-B cells and in two pre-B-cell lines, 18-81 and 70Z/3. A similar level of demethylation was detected for two sites in the same region in the AH7 pro-B-cell line but was not evident in other sources of pro-B cells. Hence, we hypothesized that the ~50% demethylation status of five linked methylation-sensitive CpG sites was predictive of allelic regulation in pre-B cells. In fact, analysis of the F₁ pre-B-cell line 70Z/3 showed that demethylation of the hs4/h5 region was specific to the expressed (C57BL/6-derived) allele. The 70Z/3 cell line is distinctive in that it contains a deletion on the expressed allele that extends from membrane sequences to a position between hs1.2 and h3B; enhancers hs3A and hs1.2 are absent (33). No phenotype has been associated with this deletion (33), in accord with the absence of any detected phenotype from independent targeted deletions of hs3A and hs1.2 in mice (22). We therefore posit that in normal pre-B cells, as in the 70Z/3 cell line, the heavy chain allele becomes demethylated in the hs4/5 region congruent with allelic expression. What confers pre-B-cell-specific demethylation, whether it may occur prior to or after heavy chain expression, and the potential consequences of this mark are not known. Other studies have shown allelic regulation of demethylation associated with Ig genes (3, 11), as well as a general association of demethylation with heavy chain gene expression (38, 41).

It would be of interest to identify which enzymes are in-

involved in the methylation and demethylation of the 3' RR. It is noteworthy that a rare immunodeficiency affecting Ig expression (immunodeficiency, centromeric region instability, and facial anomalies [ICF]) has been associated with mutations in the de novo DNA methyltransferase DNMT3B. The phenotype is associated with normal B-cell numbers, a reduction in serum Ig levels, and changes in expression of several B-cell-specific genes (8). Patients are uniquely susceptible to infections and are successfully treated with intravenous Ig. DNMT3B targets that are directly associated with these phenotypes have not been identified.

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