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B cell–specific loss of histone 3 lysine 9 methylation in the V_H locus depends on Pax5

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Abstract

Immunoglobulin heavy chain rearrangement (V_H-to-DJ_H) occurs only in B cells, suggesting it is inhibited in other lineages. Here we found that in the mouse V_H locus, methylation of lysine 9 on histone H3 (H3-K9), a mark of inactive chromatin, was present in non-B lineage cells but was absent in B cells. As others have shown that H3-K9 methylation can inhibit V(D)J recombination on engineered substrates, our data support the idea that H3-K9 methylation inhibits endogenous V_H-to-DJ_H recombination. We also show that Pax5, a transcription factor required for B cell commitment, is necessary and sufficient for the removal of H3-K9 methylation in the V_H locus and provide evidence that one function of Pax5 is to remove this inhibitory modification by a mechanism of histone exchange, thus allowing B cell–specific V_H-to-DJ_H recombination.

During hematopoiesis, transcription factors initiate and maintain lineage-specific commitment¹. In B and T lymphocytes, the unique variable (V), diversity (D) and joining (J) (V(D)J) recombination of immunoglobulin and T cell receptor (TCR) gene segments provides critical developmental checkpoints as well as diverse, clonotypic antigen recognition capability². All immunoglobulin and TCR loci use a common recombinase machinery, including proteins encoded by the recombination activating genes *Rag1* and *Rag2* that recognize and cut at common DNA recognition elements called recombination signal sequences (RSSs). However, V(D)J recombination proceeds with strict T lineage–B lineage specificity and developmentally determined order. The mechanisms responsible for these aspects of V(D)J regulation remain poorly understood.

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It is increasingly apparent that V(D)J recombination requires chromatin structure changes to allow the recombinase machinery access to rearranging gene segments. *In vitro*, nucleosomes inhibit V(D)J recombination³⁻⁵, demonstrating that chromatin can act as an obstacle. *In vivo*, multiple hallmarks of accessible chromatin occur at V, D and J gene segments before rearrangement: germline transcription, DNase I sensitivity, demethylation of DNA and histone acetylation⁶. Thus, lineage and developmentally regulated changes in chromatin structure seem to be a prerequisite for V(D)J recombination, although they may not be sufficient⁷.

Methylation of lysine 9 on histone 3 (H3-K9) provides a stable epi-genetic mark that is sufficient to establish repressed chromatin in multiple settings⁸. H3-K9 methylation inhibits V(D)J rearrangement when directed to the promoter of an artificial, stably integrated recombination substrate⁹. However, at present there is no evidence that H3-K9 methylation is involved in V(D)J regulation at endogenous antigen receptor loci in primary lymphocytes.

During early B cell development, the immunoglobulin heavy chain locus (*Igh*) rearranges first. Indeed, D_H-to-J_H recombination can occur in progenitors not fully committed to the B lineage¹⁰. However, V_H-to-DJ_H recombination occurs only in committed B cells, after D_H-to-J_H recombination. This suggests that chromatin in the V_H locus may be subjected to a unique and stringent level of repression that must be overcome during lineage commitment. Irreversible commitment to the B cell lineage requires the transcription factor Pax5 (also known as BSAP)¹¹, and Pax5-deficient bone marrow cells are halted in their development at the stage of V_H-to-DJ_H rearrangement^{12,13}. Specifically, the DJ_H-distal V_H gene segments remain blocked from undergoing V_H-to-DJ_H rearrangement despite the fact that the V_H locus has some hallmarks of accessible chromatin, including germline transcription and histone acetylation^{14,15}. Expression of Pax5 in T cells causes V_H-to-DJ_H recombination for the DJ_H-proximal V_H segments^{15,16}, and Pax5 is involved in the compaction of the V_H locus, which was suggested to promote rearrangement of DJ_H-distal gene segments¹⁵. However, the mechanism by which Pax5 regulates V_H-to-DJ_H recombination is unknown.

In this study we provide evidence that methylation of H3-K9 is present in the V_H locus in hematopoietic cells outside the B lineage and that removal of H3-K9 methylation accompanies Pax5 expression and B cell commitment. Pax5 was both required and sufficient for removal of H3-K9 methylation in the V_H locus. Concomitant accumulation of the histone variant H3.3 at the V_H locus suggests that histone exchange is the likely mechanism by which this heterochromatic mark is lost. These data provide strong evidence for a model in which one important function of Pax5 is removal of H3-K9 methylation in the V_H locus, making V_H genes accessible for V_H-to-DJ_H recombination and allowing further B cell development.

RESULTS

H3-K9 methylation in the V_H locus

Low H3-K9 methylation associated with the V_H locus does not vary between B and T cell lines¹⁷. As our preliminary studies (data not shown) using different cell lines yielded different results from those in this study, we extended our analysis to primary mouse cells. We obtained pro-B cells from *Rag1*^{-/-} bone marrow and cultured them briefly in interleukin 7 (IL-7). We obtained freshly isolated thymocytes from either wild-type or *Rag1*^{-/-} mice. We obtained bone marrow-derived macrophages after 9 d of culture in macrophage colony-stimulating factor. The V_H locus is accessible to RAG-dependent cutting in pro-B cells from *Rag1*^{-/-} mice, as measured by DNase I sensitivity, germline transcription, histone acetylation and the ability to undergo recombination after expression of the RAG proteins^{6,18}. V_H gene segments are inaccessible in cells committed to either the T lineage^{19,20} or the macrophage lineage.

We did chromatin immunoprecipitation assays of *RagI*^{-/-} Abelson virus-transformed pro-B cell lines (6312 and AR2) and a double-negative T cell line (S49; data not shown), as well as on primary pro-B cells, thymocytes and bone marrow-derived macrophages, using an antibody to dimethylated H3-K9 (anti-dimethylated H3-K9; Fig. 1). We designed primers to amplify the promoters or RSS elements of individual V_H gene segments and intergenic sequences with known locations within the V_H locus (Fig. 1a and **Supplementary Table 1** online), as described²¹. The promoter for the gene encoding c-Myc (*Myc*), not associated with H3-K9 methylation²², provided a negative control. Because signals using anti-dimethylated H3-K9 are weak²², only signals fulfilling two criteria were considered to be positive: at least twofold greater than the signal of *Myc* and an appropriate decrease in signal intensity after dilution of the DNA used in PCR. We quantified signals meeting these criteria and plotted the results as percent of input (Fig. 1b).

Methylation of H3-K9 was not associated with V_H promoters, V_H RSSs or V_H intergenic regions in pro-B cells (Fig. 1a) or in *RagI*^{-/-} pro-B cell lines (data not shown). A single exception was the promoter of a V_H gene S107 member, S107b (also known as V11), which was reproducibly associated with methylated H3-K9 in multiple experiments. This gene segment recombines much less frequently than other S107 family members²³. In this and subsequent experiments, we used H3-K9 methylation at the S107b promoter as an internal control to show immunoprecipitation with anti-dimethylated H3-K9 was efficient in B cells.

In the double-negative T cell line S49 (data not shown), primary thymocytes (both *RagI*^{-/-} double-negative and wild-type) and bone marrow-derived macrophages we found H3-K9 methylation associated with most but not all V_H DNA sequences analyzed (9 of 11 sequences in thymocytes and 7 of 11, in bone marrow macrophages). H3-K9 methylation in the V_H locus occurred in promoters, RSSs and intergenic regions and was substantially higher than that of the negative control (*Myc*) and higher than that in pro-B cells (Fig. 1a,b). The H3-K9 methylation pattern was reproducible in T or myeloid lineage cells regardless of whether they were cell lines or primary cells (data not shown) but was different between the two lineages (for example, 7183a, J558b and the intergenic regions in Fig. 1a,b). We found low or undetectable H3-K9 methylation of D_H and J_H regions in both B and T cells, consistent with the fact that some D_H-to-J_H recombination occurs before B lineage-T lineage commitment. Slightly more H3-K9 methylation was associated with D_H and J_H gene segments in myeloid cells.

We next assessed how H3-K9 methylation in the V_H locus compared with H3-K9 methylation in regions where its function had been established before. We analyzed the region 5' of the inactive X-specific transcript gene (*Xist*) on the X chromosome (X3), which is H3-K9 methylated and is thought to act as a nucleation center for Xist RNA-dependent X inactivation²². Consistent with published data, in female mouse embryonic fibroblasts, X3 had low but measurable H3-K9 methylation (1.14% of input DNA); we found slightly higher H3-K9 methylation (1.5% of input) for the V10b RSS in these cells (Fig. 1c). In hematopoietic cells, H3-K9 methylation in the V_H locus was usually higher (5–10% of input; Fig. 1a,b). We conclude that this amount of H3-K9 methylation is in a range consistent with inactive chromatin.

We also studied H3-K4 methylation in the V_H locus, a modification associated with active chromatin⁹. We analyzed *RagI*^{-/-} bone marrow pro-B cells and thymocytes by chromatin immunoprecipitation assay using anti-dimethylated H3-K4. We found reduced H3-K4 methylation associated with the V_H locus in thymocytes compared with that of pro-B cells (**Supplementary Fig. 1** online). Nevertheless, H3-K4 methylation, substantially greater than that found in thymocytes or in the negative control gene (encoding α -amylase 2), was associated with multiple gene segments (7183a, V10b P and V10bRSS) in pro-B cells. For both B and T

cells, H3-K4 methylation was restricted to the gene segments (both promoter and RSS elements) and was not found in intergenic regions, suggesting different mechanisms were responsible for the regulation of H3-K4 and H3-K9 methylation. Thus, we found a general pattern of increased H3-K9 methylation in the V_H locus in non-B cell lineages and absence of H3-K9 methylation from the V_H locus in B cells (Fig. 1). These data provide an inverse correlation between this heterochromatic mark and V_H -to-DJ $_H$ recombination and suggest the possibility that H3-K9 methylation inhibits V_H -to-DJ $_H$ recombination.

H3-K9 and DNA methylation are not associated in the V_H locus

H3-K9 methylation has been linked to methylation of CpG in adjacent DNA⁸. Although varied results have been reported on the function of DNA methylation in regulation of V(D)J recombination⁶, to our knowledge no studies have addressed the DNA methylation status of endogenous V_H gene segments. We sought to determine if H3-K9 methylation of V_H genes in non-B cell lineages was accompanied by increased CpG methylation. We used bisulfite conversion of genomic DNA from the double-negative T cell line S49; the *Rag2*^{-/-} Abelson virus-transformed pro-B cell line 6312; primary pro-B cells; and primary double-negative thymocytes. We analyzed two V_H gene segments that had strong H3-K9 methylation in T cells. We designed primers to amplify coding sequences 5' of each RSS element and to include all (7183a) or a portion (V10b) of the RSS elements (Fig. 2 and **Supplementary Table 1** online). Three of four CpG sites (two in V10b and one in 7183a) in the coding regions were methylated in all clones analyzed, whereas the fourth site (7183a) was partially methylated (about 20% of all clones; Fig. 2). Two CpG sites 3' of the RSS element in the 7183a gene segment were not methylated (Fig. 2). This DNA methylation pattern was not lineage specific, suggesting that H3-K9 methylation and DNA methylation occur independently at the V_H locus. Furthermore, as the V_H gene segments analyzed can rearrange in the pro-B cell line²⁴, these data show that recombination can occur at genes in which DNA methylation occurs outside of the RSS.

H3-K9 methylation is inversely correlated with Pax5 expression

Our data (Fig. 1) suggest that methylation of H3-K9 is involved in repressing V_H -to-DJ $_H$ recombination in non-B cells, indicating involvement of Pax5. Pax5 has the unique function of being required for B cell commitment and of blocking alternate hematopoietic fates, including the T cell and myeloid lineages²⁵. *Pax5*^{-/-} bone marrow cells have normal amounts of D $_H$ -to-J $_H$ recombination but have defective V_H -to-DJ $_H$ joining^{12,13}, specifically for the D $_H$ J $_H$ -distal V_H gene segments^{14,15}. This suggests that the V_H genes are not fully accessible to recombinase, despite having histone acetylation and germline transcription^{14,15}. Although B220⁺ bone marrow cells from *Pax5*^{-/-} mice cultured with IL-7 have hallmarks of the B cell lineage, they cannot develop into B cells. In appropriate conditions, these cells can differentiate into non-B cell lineages, including T cells, macrophages, dendritic cells and osteoclasts^{26,27}. Thus, we reasoned that studying H3-K9 methylation in the V_H locus in *Pax5*^{-/-} bone marrow cells would show the methylation status of V_H H3-K9 in multipotent progenitors and test a requirement for Pax5 in the regulation of V_H H3-K9 methylation.

We did chromatin immunoprecipitation assays of sorted bone marrow pro-B cells isolated from *Pax5*^{-/-} or *Pax5*^{+/+} (wild-type) mice after a short-term culture in IL-7, Fms-like tyrosine kinase 3 ligand and stem cell factor. Because of the potential of *Pax5*^{-/-} bone marrow cells to differentiate into alternate lineages, we periodically used flow cytometry to ensure the cultures were not contaminated with committed myeloid or T cells (data not shown). We analyzed four independent cultures; in each case H3-K9 methylation of the V_H regions was much higher in *Pax5*^{-/-} cells than in wild-type cells (Fig. 3a). D $_H$ and J $_H$ segments could not be analyzed because of prior rearrangement. H3-K9 methylation was associated with most of the V_H gene segments analyzed in the *Pax5*^{-/-} cells (including 7183a, S107b, V10b, J558a and J558b). We conclude that loss of H3-K9 methylation at the V_H locus accompanies commitment to the B

cell fate and that removal of this heterochromatic mark requires the presence of Pax5. Additionally, the data provided evidence that H3-K9 methylation was the 'default state' for the V_H locus in uncommitted hematopoietic progenitors as well as in cells committed to non-B cell lineages.

Removal of H3-K9 methylation in the V_H locus required Pax5 (Fig. 3a), and Pax5 is expressed throughout most of B cell development¹¹. However, V_H -to-DJ H recombination is restricted to pro-B cell development and is inhibited in pre-B cells, in which functional rearrangement of one heavy chain allele inhibits rearrangement of the second allele. Inhibition involves changes in chromatin structure, as demonstrated by decreases in DNase I sensitivity, reduced germline transcription and histone deacetylation^{19,21,28}. To determine whether H3-K9 methylation is involved in allelic exclusion, we analyzed H3-K9 methylation at the V_H locus in pre-B cells of mice expressing a transmembrane form of the human mu protein²⁹. There was no detectable change in H3-K9 methylation associated with the V_H locus (Fig. 3b) in pro-versus pre-B cells. Also, the H3-K4 methylation pattern did not change during the process of allelic exclusion (**Supplementary Fig. 2** online), suggesting that neither H3-K9 methylation nor H3-K4 methylation was involved in regulation of immunoglobulin heavy chain allelic exclusion. Thus, H3-K9 methylation in the V_H locus is a default state in progenitors and its absence correlates with Pax5 expression throughout B cell development.

H3-K9 methylation might be a B cell-specific mechanism for the inhibition of V_H gene recombination or it might be a common mechanism for the inhibition of B and T cell receptor rearrangement in inappropriate lineages. To distinguish these possibilities, we compared TCR V_γ and V_β gene segments in pro-B cells and thymocytes (both *Rag1*^{-/-} double-negative cells and wild-type cells). These loci are inaccessible for V(D)J recombination in B cells²⁰. We found no lineage-specific differences in H3-K9 methylation for five individual TCR V gene segments (two V_γ and three V_β gene segments) in B cells versus T cells (Fig. 3c) or for two V gene segments ($V_\gamma 2$ and $V_\beta 12$) in T versus myeloid cells (data not shown). Thus, lineage specific regulation of H3-K9 methylation of V gene segments is a B cell-specific mechanism, consistent with its dependence on Pax5.

V_H H3-K9 methylation is lost after forced Pax5 expression

Our data establish a requirement for Pax5 in the loss of H3-K9 methylation in the V_H locus but do not distinguish between Pax5 itself or other mechanisms associated with B cell commitment and/or identity. To investigate Pax5 more directly, we took advantage of the B cell lymphoma cell line Myc5 (ref. 30), which was generated by ectopic expression of c-Myc in p53-deficient bone marrow cells³¹. During growth in culture, Myc5 cells reverted to a macrophage phenotype, resulting in loss of B cell surface molecules and the transcription factors Pax5 and early B cell factor. When Pax5 was ectopically expressed in Myc5 cells, macrophage-specific proteins were lost²⁶ but full B cell commitment, determined by surface B220 and CD19 (ref. 30) and transcription factor expression (Fig. 4) did not occur, possibly because of the continued absence of early B cell factor. Thus, Myc5 cells provide a way to separate Pax5 expression from full B cell commitment, thereby allowing us to determine whether Pax5 is sufficient to remove H3-K9 methylation in the V_H locus.

Myc5 cells did demonstrate H3-K9 methylation in the V_H locus (data not shown), consistent with their macrophage phenotype and the absence of Pax5. We infected Myc5 cells with a retrovirus expressing puromycin-resistance gene or a bicistronic virus expressing Pax5 and the puromycin-resistance gene. We selected cells based on puromycin resistance at 2 d after infection and analyzed cells 2 d later. We confirmed Pax5 function by loss of surface CD11b³⁰ (Fig. 4a) and by a reduction in Csf1r mRNA, which encodes the receptor for macrophage colony-stimulating factor³² (Fig. 4b). A substantial loss of H3-K9 methylation resulted at all V_H gene segments tested, except for the promoter of S107b, in Myc5 cells

expressing Pax5 compared with control cells (Fig. 4c). The loss of H3-K9 methylation occurred at both the promoter and RSS elements of V_H gene segments and was not dependent on gene location. These data show that expression of Pax5 is sufficient for removal of H3-K9 methylation in the V_H locus, despite the fact that these cells have not become fully converted to the B cell lineage.

To ensure this finding was not unique to a cell line or type of lineage commitment, we enforced expression of Pax5 in the double-negative T cell line S49 using the same approach. In agreement with published results²⁵, T cell surface markers were not decreased after Pax5 expression (not shown). However, mRNA encoding the T cell-commitment factor Notch1 was reduced in Pax5-expressing cells compared with control cells²⁵ (Fig. 4d). Within 4 d, Pax5 expression resulted in the loss of H3-K9 methylation at the V_H locus in S49 cells (Fig. 4e). Thus, we conclude that expression of Pax5 was sufficient to cause removal of H3-K9 methylation at the V_H locus regardless of cell type or commitment status, demonstrating a previously unknown function for Pax5.

Variant histone H3.3 in the V_H locus after Pax5 expression

The mechanism by which Pax5 expression causes a loss of H3-K9 methylation at the V_H locus (Fig. 4) is not known, and so far no histone demethylases have been described⁸. Although methylated histones can be removed by deposition of new nucleosomes during replication, cell division does not occur after Pax5 expression in pro-B cells before expression of a functionally recombined *Igh* and pre-B cell receptor³³. However, replication-independent histone exchange involving deposition of the H3 variant H3.3 has been linked to the dynamic decondensation of heterochromatin³⁴⁻³⁶. Therefore, we explored the possibility that exchange involving H3.3 might contribute to Pax5-dependent loss of H3-K9 methylation at the V_H locus.

We did chromatin immunoprecipitation assays using polyclonal anti-histone H3.3, focusing on the V_H gene segments. We used S49 cells that had been infected with the Pax5-expressing retrovirus or the control virus expressing the puromycin-resistance gene only, as described above. Because 28S rDNA is associated with the histone variant H3.3 (refs. 34³⁵), we used it as a positive control. As α -amylase 2 is not associated with H3.3, it served as a negative control. There was a Pax5-dependent increase in H3.3 abundance at both the promoter and RSS elements of gene segments V10a and J558a but not in the 7183a RSS (Fig. 5). There was no increase in H3.3 content at the S107b promoter, where H3-K9 methylation remained high (Figs. 4-5). We did not, however, note a change in steady-state mRNA encoding either H3.3 or its chaperone HIRA (histone cell cycle regulation-defective homolog A)³⁷ after Pax5 expression (data not shown). These data provide evidence that Pax5-dependent removal of H3-K9 methylation at the V_H locus may proceed by histone exchange involving H3.3.

DISCUSSION

Our data provide support for the idea that methylation of H3-K9 in the V_H locus is important in enforcement of the strict B lineage specificity of V_H -to-DJ_H recombination. Increasing evidence that H3-K9 methylation is associated with repressed chromatin in centromeric heterochromatin, X chromosome inactivation and gene-specific repression⁸ led us to hypothesize that H3-K9 methylation might be involved in repression of V_H -to-DJ_H recombination in non-B cells. Consistent with this idea, H3-K9 methylation in the V_H locus was absent from B cells for all but one of the sequences analyzed but was found in all non-B cells studied, including hematopoietic progenitors and committed T lymphocytes and myeloid cells. Although a previous study did not find lineage specific H3-K9 methylation associated with the V_H genes¹⁷, this is most likely because of the different populations analyzed (transformed cell lines versus primary cells) and is consistent with the fact that transformed cell lines do not always repress V(D)J recombination properly³⁸.

Targeting of H3-K9 methylation to an engineered, integrated recombination substrate provided direct evidence that H3-K9 methylation is sufficient to inhibit V(D)J recombination⁹. Our data have shown that in primary hematopoietic cells, H3-K9 methylation in the endogenous V_H locus is inversely correlated with B cell commitment and V_H-to-DJ_H recombination. These data provide a compelling argument that the ability of H3-K9 methylation to inhibit V(D)J recombination is physiologically relevant for inhibition of V_H-to-DJ_H recombination in non-B lineage hematopoietic cells, thus providing a mechanism for the strict B cell specificity of this recombination event and the ordered recombination of D_H-to-J_H before V_H-to-DJ_H.

Targeting of the histone methyl transferase G9a to the promoter of a single recombination substrate showed that local H3-K9 methylation can inhibit V(D)J recombination⁹. Our finding that the gene encoding S107b V_H, which rearranges poorly²³, remained associated with methylated H3-K9 in B cells provides evidence that local H3-K9 methylation does inhibit V_H-to-DJ_H recombination of endogenous V_H genes. In non-B lineage cells, we found H3-K9 methylation frequently associated with V_H promoters and RSS elements and, based on the earlier work⁹, propose that V_H-to-DJ_H recombination of these genes is inhibited by local H3-K9 methylation. However, not every V_H gene was associated with H3-K9 methylation, and the pattern of methylated regions varied between T and myeloid lineage cells (Fig. 1). Furthermore, we found H3-K9 methylation of intergenic regions located at least 1 kilobase from any V_H gene segment (Fig. 1). Thus, in non-B lineage cells, H3-K9 methylation in the 2-megabase V_H locus seems to be generalized and not always localized to V_H gene segments. As all V_H genes fail to rearrange in non-B lineage cells, we suggest that generalized H3-K9 methylation in this large chromatin domain may inhibit V(D)J recombination throughout the locus, although additional studies will be necessary to establish this point definitively.

Multiple steps, including acquisition of activating modifications and loss of repressive modifications, are probably required for full accessibility of the V_H locus. Histone acetylation is an early modification that precedes recombination, and its presence in B cells is found at a developmental time when H3-K9 methylation is lost^{21,39}. Methylation of H3K27 seems to affect V(D)J recombination⁴⁰, and localization away from the nuclear periphery and compaction between variable and constant regions^{15,41} have also been linked to regulation of V(D)J recombination. Although our data suggest that H3-K9 methylation inhibits V(D)J recombination, they do not indicate that this modification alone is sufficient in all settings to inhibit recombination. *Pax5*^{-/-} pro-B cells can recombine DJ_H-proximal but not DJ_H-distal V_H genes^{14,15}. Histone H3 acetylation is present throughout the V_H locus in *Pax5*^{-/-} pro-B cells¹⁴; here we have shown that H3-K9 methylation is also present (Fig. 3). Thus, H3-K9 methylation may not block V_H-to-DJ_H rearrangement of DJ_H-proximal V_H genes in this cell environment where factors such as nuclear localization¹⁵ or proximity to regulatory elements may override H3-K9 repression. A differential effect for H3-K9 methylation on DJ_H-proximal and DJ_H-distal V_H genes would be consistent with results of other studies showing that regulation of V_H-to-DJ_H rearrangement varies between DJ_H-proximal and DJ_H-distal regions^{15,19,21,28,39,40,42,43} and that there is preferential rearrangement of DJ_H-proximal V_H genes⁴⁴. Alternatively, if the proportion of *Pax5*^{-/-} pro-B cells that rearrange DJ_H-proximal V_H genes is small, loss of H3-K9 methylation in this subset, if present, might not have been detected in our assay.

DNA methylation is linked with histone methylation in other systems⁸ and in studies using stably integrated V(D)J recombination substrates^{9,45}. DNA demethylation is also important for endogenous V_K-J_K recombination⁴⁶. In our experiments, H3-K9 methylation and DNA methylation did not correlate for the two V_H genes studied. As these two V_H genes recombine *in vivo*²⁴, moderate DNA methylation does not inhibit V_H-to-DJ_H recombination. This is in agreement with studies showing that genes methylated outside the RSS heptamer can recombine⁴⁷ and that inhibitory effects of DNA methylation can be relieved by histone

acetylation^{48,49}. Although more V_H genes must be analyzed, these data suggest that CpG methylation in the V_H locus is not B cell specific or obligatorily linked to H3-K9 methylation.

Pax5 has the unique function in B cell development of inducing genes required for B cells and inhibiting genes required for other lineages¹¹. In the absence of Pax5, bone marrow progenitors retain the ability to develop into T cells, macrophages, dendritic cells and osteoclasts^{26,27}, even though they express B220 and the B cell transcription factors early B cell factor and E2A¹². Our data have identified a previously unknown function for Pax5: it is necessary and sufficient for removal of H3-K9 methylation in the V_H locus. We propose that Pax5-dependent removal of H3-K9 methylation is required for V_H -to-DJ H recombination and further B cell development. This provides an explanation for why lineage-specific V_H -to-DJ H recombination and further B cell development depend on Pax5

Understanding how histone methylation can be altered is advantageous, as no histone demethylase has been described and the half-life of histone methylation is the same as that of the histone protein⁵⁰. There is increasing evidence that histone exchange with the variant H3.3 replacing H3 is involved in the switch from repressed to active chromatin^{34,36}, and the histone chaperone HIRA has been identified as being responsible for replication-independent nucleosome exchange involving H3.3 (ref. 37). Given that pro-B cells do not divide in response to Pax5 expression, we suspected that replication-independent exchange involving H3.3 might mediate the loss of H3-K9 methylation in the V_H region noted after Pax5 expression. Indeed, for four of five V_H regions tested we found increased H3.3 in cells after Pax5 expression (Fig. 5). Although this finding does not rule out the possibility of other mechanisms, it provides an important clue that H3.3 histone exchange is involved in the removal of H3-K9 methylation in the V_H region. We do not understand the lack of H3.3 in the 7183a gene, but it could indicate a different mechanism for removal of H3-K9 methylation. Alternatively, as S49 cells divide in culture, providing the possibility of histone replacement, there may be differential replacement of H3.3 after insertion by histone exchange.

Deposition of H3.3 may occur at areas of active transcription³⁴. Antisense transcription has been reported to precede V_H -to-DJ H recombination at the CD19⁺ stage, when Pax5 is first expressed. The pattern of 'anti-transcription' (transcription of the strand complementary to that transcribed for mRNA encoding immunoglobulin variable regions), in both V_H genes and intergenic sequences, is similar to that of H3-K9 methylation⁵¹. It may be that 'pioneer polymerases', which can transcribe through heterochromatin, might initiate 'anti-transcription' and cause changes in chromatin structure⁵². We speculate that anti-transcription in the V_H region might precede and allow H3.3 histone exchange, which would then remove H3-K9 methylation and prime the locus for V_H -to-DJ H recombination. The known activities of Pax5 provide few clues for how Pax5 might drive this process. Ectopic Pax5 caused removal of H3-K9 methylation within a few days and was independent of full B cell commitment or expression of early B cell factor, 'arguing against' the possibility of a developmental mechanism. We found no evidence that Pax5 induces transcription of H3.3 or its chaperone HIRA. If our speculation regarding H3.3 is correct, Pax5 or a gene product regulated by Pax5 may bind in the V_H locus and recruit pioneer polymerases for anti-transcription. However, many other possibilities remain to be explored. Pax5 expression in T cells was shown to cause rearrangement of DJ H -proximal V_H genes^{15,16}, and Pax5 was found to be required for compaction of the heavy chain chromatin¹⁵. The mechanism of Pax5 action in T cells is also unknown^{15,16} and may not be related to its effects in B cells. Nonetheless, removal of H3-K9 methylation may promote chromatin compaction that juxtaposes V_H and DJ H gene segments and allows their recombination.

METHODS

Cell lines and cell cultures

The 6312 cells (*Rag2*^{-/-} Abelson virus-transformed pro-B cells) and S49 cells (double-negative thymoma; a gift from G. Siu, Amgen) were cultured in RPMI medium plus 10% heat-inactivated FCS (Gemini), 50 μ M β -mercaptoethanol and gentamicin. Myc5 culture conditions have been described³⁰. Bone marrow pro-B cells are derived from *Rag1*^{-/-} mice, and pre-B cells, from mu-transgenic mice on a *Rag1*^{-/-} background. Both B cell types underwent short-term culture in the presence of IL-7 as described²¹. Wild-type thymocytes (which are the majority at the double-positive stage) were obtained from C57BL/6 mice (unsorted) and double-negative thymocytes were pooled from *Rag1*^{-/-} mice, after red blood cell lysis and were used immediately in chromatin immunoprecipitation assays. Primary macrophages were obtained by culture of bone marrow cells in the presence of macrophage colony-stimulating factor for 8 d according to published protocols⁵³. Mature CD3 splenocyte samples were isolated from a wild-type C57BL/6 mouse, subjected to red blood cell lysis and negatively depleted of B220 (Dynabeads) and major histocompatibility complex class II (Miltenyi) as specified by the manufacturer's protocol. All cell populations were checked for purity by flow cytometry (at least 90% pure, using B220-BP1 for pro-B and pre-B cells, CD11b for macrophages and CD4-CD8-Thy-1.2 for thymocytes) and were used immediately in chromatin immunoprecipitation assays. Bone marrow cells were collected from 15-day-old *Pax5*^{-/-} and wild-type control mice and were cultured according to a published protocol⁵⁴, with slight modifications: cells were cultured in X-VIVO-15 medium (Fisher) containing 0.5% endotoxin-free BSA (Stem Cell Technologies), 50 μ M β -mercaptoethanol, 25 ng/ml of Fms-like tyrosine kinase 3 ligand, 20 ng/ml of stem cell factor and 2.5 ng/ml of IL-7 (R&D Systems). Fresh media was added every 3 d. At days 10–14 of culture, pro-B cells were purified by sterile flow cytometry sorting of B220⁺CD43^{hi}GR-1⁻ cells (*Pax5*^{-/-} and wild-type) or CD19⁺BP-1⁺CD11b⁻ cells (wild-type). Cells were then cultured in the X-VIVO media with cytokines as described above and were analyzed by chromatin immunoprecipitation. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Chromatin immunoprecipitation

These assays were done as described²¹ with the following modifications. Each immunoprecipitation used 5×10^6 to 7.5×10^6 cells, with 5 μ l of the following antibodies: polyclonal anti-dimethylhistone H3-K9 (07-212; Upstate Biotechnology), anti-dimethylhistone H3-K4 (07-030; Upstate Biotechnology) and polyclonal anti-histone H3.3 (4263; Abcam). After ethanol precipitation, DNA was resuspended in 100 μ l per 5×10^6 cells and 1–2 μ l was used as a template for each PCR. Serial dilutions of were made at a ratio of 1:5 for 1% input or at a ratio of 1:2 for immunoprecipitated fractions. Comparable dilutions between all primer sets are shown. All PCRs (primers, **Supplementary Table 1** online) used a volume of 50 μ l and the following conditions: 30 s at 94 °C, 30 s at the annealing temperature (**Supplementary Table 1** online) and 30 s at 72 °C. PCR products were separated by 1.5% agarose gel electrophoresis, blotted onto Hybond-N membranes (Amersham Biosciences) and probed with specific oligonucleotides (**Supplementary Table 1** online) that were end-labeled with [γ -³²P]ATP using T4 Polynucleotide Kinase (Amersham Pharmacia). Southern hybridization results were analyzed with a phosphorimager (Molecular Dynamics). The cycle number for each primer set varied from 29 to 37, according to the strength of input signal, and were adjusted so that dilutions fell into the linear range when quantified with the phosphorimager (Molecular Dynamics) after Southern hybridization with an internal probe. No hybridization was required for the H3.3 antibody, as specified by the manufacturer's protocol. In graphs, immunoprecipitated DNA band intensities were expressed relative to the signal of 0.01% input with volume intensity numbers as determined by ImageQuant (Molecular Dynamics) that fell into the linear range, as shown on the vertical axes (**Figs. 1, 4**). The graphed

data are representative of the primary data from a single experiment and represent a trend that was reproduced three to five times from individual preparations. Numbers that were not twofold above background or c-Myc and numbers that did not 'dilute' appropriately (because of low signal and sporadic background) could not be quantified. In some cases, additional dilutions were done (but are not shown) to ensure signals were within the linear range.

RT-PCR

RNA was isolated by the TRIzol method (Gibco-BRL). A total of 1–2 µg RNA was reverse transcribed with random hexamers after DNase (Promega) treatment as described²⁴. Primers and annealing temperatures are listed in **Supplementary Table 1** online.

Retrovirus preparation and infection

Retroviruses were generated as described⁵². Myc5 cells and S49 cells were infected at a multiplicity of infection of 1–2 in the presence of 5 µg of polybrene per ml. After cells had 'recovered' for 2 d after infection, puromycin (9 µg/ml for Myc5 and 4 µg/ml for S49) was added to the media and after 2 d the cells were collected, dead cells were removed with Histopaque-1077 (Sigma) and the remaining cells were used immediately for chromatin immunoprecipitation. RNA was also obtained at this time and flow cytometry was done.

Plasmids

For the generation of retroviruses containing cDNA encoding Pax5, a *NotI* fragment of cDNA from Pax5-pEBB (B. Birshstein, Albert Einstein College of Medicine New York, New York) was cloned into a *NotI* site of the retrovirus vector Vxy-Puro (L. Staudt, National Institutes of Health, Bethesda, Maryland).

Methylation analysis

For methylation analysis, 1.5 µg of genomic DNA was treated for 16 h at 50 °C with sodium bisulfite as described⁵⁵. Bisulfite-treated DNA was subject to nested PCR and was sequenced, with over 99% of all non-CpG cytosine residues being converted to thymidine residues. At least six bisulfite-converted clones were sequenced and multiple bisulfite conversions were done on individual cell types. PCR (primers, **Supplementary Fig. 1** online) used a volume of 100 µl and an annealing temperature of 55 °C.

Flow cytometry

For determination of cell purity and developmental stage, cells were stained on ice for 20 min with 1:100 dilutions of the following antibodies in staining buffer (3% fetal calf serum and 1× PBS): allophycocyanin–anti-CD45R (B220), phycoerythrin–anti-BP-1, phycoerythrin–anti-CD11b (Mac1), fluorescein isothiocyanate–anti-Mac3, phycoerythrin–anti-CD90 (Thy-1.2), phycoerythrin–anti-CD4, fluorescein isothiocyanate–anti-CD8 and phycoerythrin–anti-CD19 (all antibodies were purchased from PharMingen) and were analyzed on a FACSCalibur with CellQuest software (Becton Dickinson).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

H3-K9 methylation associated with the immunoglobulin heavy chain gene segments in *Rag1*^{-/-} bone marrow pro-B cells, thymocytes and bone marrow-derived macrophages. **(a)** Chromatin immunoprecipitation assays, with anti-dimethylated H3-K9, of primary *Rag1*^{-/-} bone marrow pro-B cells after short-term culture in IL-7; primary thymocytes; and bone marrow-derived macrophages. Bottom (c-Myc), the *Myc* promoter serves as a negative control. Wedges indicate 1:5 serial dilutions of 1% input DNA and 1:2 dilutions of H3-K9 immunoprecipitated fraction DNA; each begins (far left) with an undiluted sample. **(b)** Immunoprecipitated (IP) DNA from **a**, quantified with a phosphorimager; the results are presented as percentage of input. Data are from one experiment representative of multiple experiments (four pro-B cell cultures, five thymocyte preparations and three macrophage cultures). BD (below detection), intensities that were not at least twofold greater than that of the *Myc* promoter or background cannot be accurately quantified. – (far right), negative control. Bottom, the V_H locus and relative location of the gene segments. **(c)** Chromatin immunoprecipitation assays, done as described in **a** with primers for Xist (X3), the V_H gene V10b RSS and the *Myc* promoter, of embryonic fibroblasts from female mice (Female MEFs). Input dilutions begin at 1:5. Right, band intensities of immunoprecipitated DNA, presented as percentage of input (vertical axis), representative of three experiments.

Figure 2.

DNA methylation is not linked to lineage-specific H3-K9 methylation of V_H gene segments. DNA sequences of two V_H gene segments, 7183a (81X) and V10b. Portions of each gene were amplified by PCR after bisulfite conversion of genomic DNA from a *Rag2*^{-/-} Abelson virus-transformed pro-B cell line (6312), a double-negative T cell line (S49), *Rag1*^{-/-} primary pro-B cells and *Rag1*^{-/-} double-negative T cells. Black underlining, primers; gray boxed shading, RSS elements. After conversion, PCR products were subcloned and six clones per conversion were sequenced. Two to three bisulfite conversions were done on each cell type. Data were consistent between different cell populations and are representative results of experiments. Partial CpG methylation, about 20% of clones.

Figure 3.

H3-K9 methylation is inversely correlated to Pax5 expression. **(a)** Chromatin immunoprecipitation assay, with anti-dimethylated H3-K9, of sorted primary *Pax5*^{-/-} bone marrow pro-B cells and wild-type (WT) bone marrow pro-B cells after short-term culture in the presence of IL-7, Fms-like tyrosine kinase 3 ligand and stem cell factor. Data are representative of four experiments. Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of immunoprecipitated fractions; input samples begin with a 1:5 dilution. **(b)** Chromatin immunoprecipitation assay, with the anti-dimethylated H3-K9, of primary *Rag1*^{-/-} bone marrow pro-B and *Rag1*^{-/-} pre-B cells that express the transmembrane form of the human mu protein, after short-term culture in the presence of IL-7. Data are representative of three experiments. Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of H3-K9 immunoprecipitated fractions; input samples begin with a 1:5 dilution. **(c)** Chromatin immunoprecipitation assays, with anti-dimethylated H3-K9, of *Rag1*^{-/-} primary pro-B cells and primary thymocytes (also shown in Fig. 1). Data are representative of three experiments. Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of H3-K9 immunoprecipitated fractions; each sample begins with a nondiluted sample.

Figure 4.

Enforced expression of Pax5 in non-B cell lines results in the removal of H3-K9 methylation at the V_H locus. **(a)** Surface expression of the macrophage protein CD11b and the leukocyte marker CD43, analyzed by flow cytometry of Myc5 cells after retroviral infection with a bicistronic retrovirus expressing Pax5 and puromycin resistance (Pax5-Puro) or a control retrovirus expressing only the puromycin-resistance gene (Puro). **(b)** Semiquantitative RT-PCR of total RNA from Myc5 cells after retroviral infection. This analysis includes mRNA encoding factors known to be important in B-cell development (PU.1, E12, E47, early B cell factor (EBF) and Pax5) and macrophage identity (macrophage colony-stimulating factor receptor (M-CSFR)); proteins encoded are included in parentheses along the left margin. Bottom, *Gapd* (glyceraldehyde phosphodehydrogenase) serves as the control. **(c)** Chromatin immunoprecipitation assays, with anti-dimethylated H3-K9, of Myc5 cells after retroviral infection. Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of H3-K9 immunoprecipitated fractions; input samples begin with a 1:5 dilution. Right, immunoprecipitated DNA, representative of one of three experiments, presented as a percentage of input. Hatch marks across bars indicate a disruption in the linear scale on the vertical axis; numbers above bars indicate the final value. **(d)** Semiquantitative RT-PCR of total RNA from the double-negative T cell line S49 after retroviral infection. *Gapd*, control. **(e)** Chromatin immunoprecipitation assays, with anti-dimethylated H3-K9, of S49 cells after retroviral infection. Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of H3-K9 immunoprecipitated fractions; input samples begin with a 1:5 dilution. Right, immunoprecipitated DNA band intensities, representative of one of three experiments, presented as a percentage of input.

Figure 5.

Accumulation of the histone variant H3.3 at the V_H locus correlates with the loss of H3-K9 methylation after Pax5 expression in a double-negative T cell line. Chromatin immunoprecipitation assays, with polyclonal anti-histone H3.3, of S49 cells after infection with a bicistronic retrovirus expressing Pax5 and the puromycin-resistance gene (Pax5-Puro) or the puromycin-resistance gene alone (Puro). Wedges indicate 1:5 serial dilutions of 1% input and 1:2 dilutions of immunoprecipitated fractions; input samples begin with a 1:5 dilution. Top, 28S rDNA serves as a positive control; bottom, α -amylase 2 serves as a negative control. Data are representative of three experiments.