Changes in Histone Acetylation Are Associated with Differences in Accessibility of V_H Gene Segments to V-DJ Recombination during B-Cell Ontogeny and Development

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Although V(D)J recombination is thought to be regulated by changes in the accessibility of chromatin to the recombinase machinery, the mechanisms responsible for establishing “open” chromatin are poorly understood. We performed a detailed study of the acetylation status of histones associated with 11 V_H gene segments, their flanking regions, and various intergenic elements during B-cell development and ontogeny, when V(D)J recombination is highly regulated. Histone H4 shows higher and more-regulated acetylation than does histone H3 in the V_H locus. In adult pro-B cells, V_H gene segments are acetylated prior to V(D)J rearrangement, with higher acetylation associated with J_H -distal V_H gene segments. While large regions of the V_H locus have similar patterns of histone acetylation, acetylation is narrowly confined to the gene segments, their flanking promoters, and recombinase signal sequence elements. Thus, histone acetylation in the V_H locus is both locally and globally regulated. Increased histone acetylation accompanies preferential recombination of J_H -proximal V_H gene segments in early B-cell ontogeny, and decreased histone acetylation accompanies inhibition of V-DJ recombination in a transgenic model of immunoglobulin heavy-chain allelic exclusion. Thus, changes in histone acetylation appear to be important for both promotion and inhibition of V-DJ rearrangement during B-cell ontogeny and development.

V(D)J recombination of immunoglobulin (Ig) and T-cell receptor (TCR) genes depends on lymphocyte-specific recombinase-activating gene (RAG) proteins and ubiquitous double-strand break repair enzymes (for a review, see reference 4). Since double-strand breaks jeopardize the integrity of the genome, V(D)J recombination must be tightly controlled. However, conserved recombinase signal sequences (RSSs) and common enzymatic machinery are employed for V(D)J recombination of Ig and TCR loci, leaving important aspects of V(D)J regulation unexplained, including (i) B- and T-cell-specific rearrangement of Ig and TCR loci, respectively, (ii) developmentally ordered rearrangement, (iii) preferential rearrangement of gene segments, and (iv) inhibition of rearrangement after successful rearrangement of one allele.

The chromatin accessibility hypothesis, which posits that V(D)J rearrangement must be preceded by acquisition of an accessible chromatin structure to allow RAG proteins to recognize and cleave RSSs (41), provides an explanation for these aspects of V(D)J regulation. Consistent with this hypothesis, various indicators of chromatin structure change prior to V(D)J recombination in Ig and TCR loci, including nuclease sensitivity, germ line transcription, and DNA hypomethylation (for a review, see reference 26). The mechanism(s) that determines altered chromatin accessibility in Ig and TCR loci is not known, but there is increasing evidence that covalent modifi-

cations of histone tails may play an important role in establishing active or inactive chromatin (34). Acetylated histones are associated with TCR and Ig loci prior to V(D)J recombination (1, 7, 17, 20, 43). In B cells, histone acetylation is associated with the V_H region after interleukin-7 (IL-7) stimulation and DJ rearrangement but prior to V-DJ rearrangement (7) and is accompanied by increased nuclease sensitivity and reorganization of nucleosome structure (17). However, the pattern of histone acetylation in the Ig heavy-chain (IgH) locus and whether and how it correlates with regulation of V(D)J recombination during ontogeny and later development are not known.

We studied histone acetylation in the murine V_H locus, which occupies >2 Mb on mouse chromosome 12 and contains >130 V_H gene segments (6). Although D_H - J_H recombination occurs in T cells, V_H - D_H - J_H recombination is strictly B-cell specific. Early in B-cell ontogeny, there is a bias toward recombination of J_H -proximal gene segments in the fetal liver (5, 19, 25, 41) which cannot be explained by the V_H promoters or RSSs (16, 40). Also, when V(D)J recombination at one IgH allele creates a functional gene that expresses the mu heavy chain, development proceeds and recombination of the unrearranged IgH allele is inhibited (13, 22). This inhibition establishes IgH allelic exclusion and ensures that each B-cell clone expresses a single antibody molecule.

Our data show that histone acetylation in the V_H locus is regulated both locally and regionally and that it changes during B-cell ontogeny and B-cell development in ways that correlate with V_H -DJ recombination. Acetylation of histones H3 and H4 is specific to pro-B cells, but H4 is particularly regulated in the V_H locus. Early in B-cell ontogeny, pro-B cells have H4 acetylation associated with J_H -proximal V_H gene segments, corre-

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TABLE 1. Primers and probes used in ChIP assay

Primer	Sequence (5'-3')	Internal probe	Annealing temp (°C)
Promoters			
7183a up	CTTCTCTGTGCCCCATGAA	ACTTATCCTGCAGCTCTG	50 ^a
7183a down	CAAAGAGTGTCTGGTCAGA		
S107a up	TAGGCAGCCTTAGATATT	AGTTGGAAGGTGAAC	55 ^a
S107a down	GGACAGGATCCACTGCTA		
S107b up	CAAGGACAGGGTATTCTT	TTGCTAGTAGCAAGG	50 ^a
S107b down	AGGGCTAGGTTTCATACGC		
VGAMa up	AATGAGCACTGAGGGCTGT	GCTAAGATAAAGGCACAGG	55 ^a
VGAMa down	CTAACTGGTCACTCCCTT		
V10a up	GGACAAATACAAATTAAGGCT	CTCTCAGTTGTGCCATTT	50 ^a
V10a down	ATTGTCAGTGTGAACACTA		
V10b up	CTCAGACAGTTGTGCCAT	ATTGCTGTCCCAAAGGCT	50 ^a
V10b down	GTCACCTCTCTCTATGAC		
J558a up	TAGCCTCAACACAAGGTT	GAATGGGATGCATTCTCT	50
J558a down	ATGAGGATGATACAGCTC		
J558b up	CTTCCTCAGAGAGGGCTTT	CTTGACCTGAGAATTCT	50 ^a
J558b down	GCAGTTACTGACAGGAGA		
J558c up	AAGTGGAGCAGCAGACTC	TCCCTAGTTCTTCTCCAG	50
J558c down	GGAGAAGACAGGAGTCAT		
HPRT up	ATTTGAGATCCAGCCGCG	GGTAGCTGGGCATAAAAGCC	50
HPRT down	CTGGGTAGGTCGAATATG		
amylase up	CAGCTGTGCACATCATTG	GGCGCTAGAGAGAAAGAA	55
amylase down	TTCCTTGGCAATATCAACC		
RAG1 up	CACCCTGAATGTTTCTGC	GAGTGTTAACAACCAAGC	55 ^a
Rag1 down	GCCAGAGTGCTAGAATGT		
RAG2 up	ACTGGTATCTCGGGACTT	CTGTCAGTAATGAAAAGG	55 ^a
Rag2 down	CCAGAGGGGCTGCTTATC		
lambda5 up	CTGCAGAGACTCTTGTTT	AGGCAGCTGTGAGTGAAA	55 ^a
lambda5 down	TGCAGGTGGATCTGTGTA		
VpreB up	ACTCTTGTCCCTTTACC	GCTTCCATTAGACCATT	55 ^a
VpreB down	ACTCTCCTCCCTGCTTTG		
RSS			
7183a up	ACGAATTCCCTTCCCATG	AAGAGGCTGGAGTGGGTC	55 ^a
7183a down	CTGCTGGTCTTAGATGCT		
S107a up	AAGAGACTGGAGTGGATT	GCAAGTAGAAACAAAGCTAATG	55 ^a
S107a down	TGAAGAGGTCATACAGTC		
S107b up	GAAGGGTCGGTTCACCAT	TTACTTAACATCTGAAAATT	55 ^a
S107b down	TTGGAGATAAAGCCTGGT		
VGAMa up	GAAACAGGCTCCAGGAAA	GAAGCAACCAGAGGAAAC	50 ^a
VGAMa down	CCAAACACCAAGGAGAGA		
V10a up	CTCCAGAGATGATTACAGA	ATTGCTATGTCTGGGCTC	55 ^a
V10a down	TGTAAAGATGACCCCTT		
V10b up	GGTTTGGAATGGGTTGCT	GGGTGCTTAGTACACACA	50 ^a
V10b down	CTGAGCTGATCTTGCTC		
J558d up	TGAGCAGAGCCATGGAAA	GGCTGCTTGACAGATTTTC	50 ^a
J558d down	CACACTGTTCAAATGATTAC		
3609a up	CAGGGAAGGGTCTAGAGT	CAGCTCTGTTCTTCTTA	55
3609a down	GGAAACCACAGAGACCAG		
J558e up	GGCGCTTCAGTGAAGATA	TGGAGGTGCAGCAAGCTC	55 ^a
J558e down	GTCTTGTCAGTTCTAAGG		
J558a up	AAGCAGAGGCCTGGACAA	GTGGAGACTCAGAGAAGT	50
J558a down	TGAGCAAGTCTACAAGCA		
J558b up	CTGGAGGTGGTTATACTA	CGAATGTCCATGTATTCC	50 ^a
J558b down	CTGTGAGGAGGAAGGAAT		
J558c up	CCTGGAGATGGAGATACT	ATCTTTAGACTTGCTCAG	55 ^a
J558c down	TTCTCCAAACAGCATCAC		
Intergenic			
I-a up	CAGTTCTTCAACTGTTCC	CCCATATCCAAGAGAATG	50 ^a
I-a down	ATCTCTCTGAGTCTCACA		
I-b up	GTCTCTATAGGCGGCATC	CCAATACCCGTTACTGC	50
I-b down	GAAGATTCCGGTTTGCTG		
I-c up	GGCTAGAACACCATTTAG	TGGAAAGAGAATCTCGTG	48 ^a
I-c down	CTTCATGTGCTTCTTCT		
I-d up	CACTGTCCAGTCTTCAGAAA	GGTATTCCAGGAGAGCTT	50 ^a

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TABLE 1—Continued

Primer	Sequence (5'-3')	Internal probe	Annealing temp (°C)
I-d down	CTTGATTAACATACAGGCTC		
V10b p +200 up	GCCAATGTAGGACCAGTA	CTCAGACAGTTGTGCCAT	55 ^a
V10b p +200 down	TCAGGGCTGTGATGGTTT		
V10b RSS +200 up	GGACACAGCCATGTATTA	CTGAGCTGATCTTGTCTC	50 ^a
V10b RSS +200 down	GAATATGATTGCCCTTCC		
J558a p +400 up	CATGTTCAAACGCAGTGA	TTGGTCATTTGGGTGATT	55 ^a
J558a p +400 down	ATTAGAAGTCACAGCCTC		
J558a p +200 up	TTGGTCATTTGGGTGATT	GAATGGGATGCATTCTCT	55 ^a
J558a p +200 down	TCAAGAATCAGGATGCTG		
J558a RSS +200 up	AGTGTCCAAAACCTTGGA	TGAGCAAGTCTACAAGCA	55 ^a
J558a RSS +200 down	TACCATGCCTAATCAGAG		
J558b p +600 up	GATCATAGCATATTTTCATAGAT	GCATACATAGTTGTCTAC	50 ^a
J558b p +600 down	CCATATTAATTAAGACAATCTC		
J558b p +400 up	GCATACATAGTTGTCTAC	ACCTGTGGGAAAGGAAATA	50 ^a
J558b p +400 down	GATGATGATGATGATTTTGA		
J558b RSS +200 up	GATTAATCATTAGAATTGCTC	CGAATGTCCATGTATTCC	55 ^a
J558b RSS +200 down	CCCAAAAATTTCAAGAAATTC		

^a Addition of 1μM MgCl₂ is required.

lating with their preferential rearrangement. In adult bone marrow, there is a sharp bias in histone H4 acetylation in the J_H-distal V_H gene segments. Within this region, histone acetylation is localized to each V_H gene segment, including the promoter and RSS, but does not extend into intergenic regions. Along with developmental progression to pre-B cells and heavy-chain allelic exclusion, expression of transmembrane mu causes a significant decrease in histone H4 acetylation in J_H-distal V_H gene segments.

MATERIALS AND METHODS

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (45), with the following modifications. Approximately 10×10^6 cells were used for each immunoprecipitation. Immunoprecipitations were done using one of the following polyclonal antibodies: acetylated histone H3 (catalog no. 06-599; Upstate Biotechnology), acetylated histone H4 (catalog no. 06-866; Upstate Biotechnology), and cdk4 (sc-260; Santa Cruz). Following ethanol precipitation, DNA was resuspended in 200 μl/10⁶ cells and 2 to 5 μl was used as the template for each PCR. Input samples represent 1% of total DNA and were diluted 1:5, and IP fractions were diluted 1:2. PCR conditions were designed to amplify single products and were therefore specific to each primer set. Gene family-specific primers and PCR conditions have been described previously (2), as have kappa primers (27). The gene-specific primer sequences and conditions are listed in Table 1. The general conditions for PCR were as follows: 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and 72°C for 10 min for 34 cycles. All PCRs were amplified in 100-μl mixtures containing 1× PCR buffer, 100 nM concentrations of each primer, 200 μM deoxynucleoside triphosphate, and 1 U of DNA *Taq* polymerase (Sigma). Some primer sets also contained an additional 1 μM concentration of MgCl₂ as specified in Table 1. The number of cycles also varied depending on the primer set, being adjusted so that the input dilutions fell into the linear range. PCR samples were separated on 1.7% agarose gels, transferred to a nylon membrane, hybridized with internal probes, and quantified by phosphorimager. In the figures, band intensities are expressed relative to the signal of 0.1% of input and are presented as relative intensity on the y axis of the graphs. All calculations were done by comparing input and IP dilutions that fell into the linear range. The primary data in each figure show equivalent dilutions between different gene sets and/or preparations so that the differences are visually apparent and comparable, but in many cases further dilutions were required for the IP fractions to fall into the linear range. These numbers were then used for the graphs. In those cases where the numbers were expressed relative to the negative control amylase gene, the results were first normalized to input.

Cell lines and cell cultures. Culture of the Abelson murine leukemia virus-transformed RAG-deficient pro-B-cell lines was described previously (2). The NIH 3T3 cell line was grown in Dulbecco's modified Eagle medium supple-

mented with 10% fetal calf serum and gentamicin. Short-term bone marrow and fetal-liver cells were established according to the methods described by Whitlock and Witte, with slight modifications (39). Briefly, bone marrow cells from 6- to 12-week-old RAG1^{-/-} mice (Jackson Laboratory) were harvested and directly placed at a concentration of 1×10^6 to 2×10^6 cells/ml in T-25 flasks in 8 ml of RPMI medium supplemented with 5% heat-inactivated fetal calf serum, 50 μM β-mercaptoethanol, and gentamicin. One-half of the medium was replaced every 3 to 4 days, and after 7 to 10 days, cultures were given recombinant IL-7 (R&D) to achieve a final concentration of 12.5 ng/ml. Cultures were harvested 3 to 4 days later and assayed for purity and developmental stage by monitoring B220⁺/BP-1 expression by fluorescence-activated cell sorter analysis. Enriched B-cell populations were used directly in the ChIP assay. As described previously (33), slightly different culture conditions were required for culturing μ transgenic bone marrow cells, including plating of the cells at a higher density (3×10^6 to 5×10^6 cells/ml) and the addition of IL-7 (final concentration of 12.5 ng/ml) at the start of the culture. In this case, the RAG-deficient bone marrow cells without the μ transgene were cultured by using the same conditions. The results obtained by using the two culture conditions were similar. To ensure that the μ transgene was enforcing allelic exclusion and pushing the cells forward in development as previously reported (22, 33, 44), cell surface markers (BP-1/B220) and the level of J558 germ line transcripts were monitored and compared with those of littermate controls at the end of the culture. The μ transgenic mice were kindly provided by M. C. Nussenzweig (22) and were crossed into the RAG1^{-/-} background. Short-term fetal-liver cells were cultured in a manner similar to that for culturing the μ transgenic bone marrow cells. Fetal-liver cells were obtained from 15- to 16-day-old RAG1^{-/-} embryos and plated at 2×10^6 /ml in the presence of IL-7 at a final concentration of 12.5 ng/ml. After 10 days, cultures of enriched B-cell populations (90 to 99% B220⁺ purity) were used directly in the ChIP assay.

RESULTS

Acetylated histones are specifically associated with the V_H locus. ChIP assays using a polyclonal antibody to acetylated histone H3 were performed on Abelson virus-transformed RAG^{-/-} pro-B-cell lines and one fibroblast line. The chromatin structure of the V_H locus in the pro-B-cell lines is accessible, as measured by DNase I sensitivity, the presence of germ line transcripts (2, 17, 27), and V(D)J recombination upon expression of RAG protein; the fibroblast line has an inaccessible V_H locus and cannot undergo recombination (30). Primers unique for V_H families (2), for an active gene (hypoxanthine phosphoribosyltransferase gene), and for an inactive gene (amylase gene) were used. The data (Fig. 1) show that

FIG. 1. H3 acetylation is associated with V_H gene families in pro-B cells. ChIP assays were performed on Abelson virus-transformed pro-B lines (AH7 and AR2) and a fibroblast line (3T3) by use of a polyclonal anti-acetylated H3 antibody. Shown are serial dilutions (1:2) of IP fractions comparing acetylations associated with the housekeeping gene (hypoxanthine phosphoribosyltransferase gene) and the pancreatic specific gene amylase with those of V_H gene families (SM7 and 3609). Serial dilutions of a nonspecific antibody are also shown.

only the pro-B cells have histone H3 acetylation associated with V_H families 3609 and SM7. Thus, histones in the V_H locus are acetylated in pro-B cells prior to the rearrangement of the locus.

Histone H3 and H4 acetylation is biased toward the J_H-distal half of the V_H locus in bone marrow pro-B cells. To thoroughly characterize histone acetylation in the V_H locus, we studied histones H3 and H4 and analyzed eight individual V_H gene segments. Although the sequence of the V_H locus is not complete, V_H gene segments located within particular bacterial artificial chromosome (BAC) clones were identified. J_H-proximal, intermediate, and J_H-distal gene segments were studied (Fig. 2A). Primers were designed to span the promoter and RSS elements for each gene segment (Fig. 2B).

Histone acetylation of most V_H promoter and RSS elements was higher than what was observed with the negative control (Fig. 3). However, acetylation levels, calculated as the band intensity relative to 0.1% input, varied significantly between individual gene segments (Fig. 3B). Histone acetylation showed a clear bias toward the J_H-distal (telomeric) portion of

the locus, especially for histone H4 acetylation associated with the RSS. For example, histone H3 is barely detectable for several of the J_H-proximal gene segments analyzed, including both the promoter and the RSS elements of S107a (V1) and S107b (V11) gene segments. In contrast, the distal gene segments J558a and J558b have 5- to 10-fold higher levels of histone H3 acetylation in the promoter and RSS elements, respectively, compared with proximal gene segments. These differences are even more pronounced for histone H4, where there is a 45-fold increase in histone H4 acetylation when the RSS elements of the same distal and proximal gene segments are compared. However, one exception is J558c, the most telomere-proximal V_H gene segment, which has moderate levels of histone H4 acetylation with relatively little H3 acetylation. Although the absolute percentages in immunoprecipitated fractions varied in different experiments, the pattern of high acetylation in the J_H-distal portion of the locus was highly reproducible and the J_H-proximal gene segments consistently had acetylation levels comparable to that of the negative control gene, amylase gene.

FIG. 2. Diagrams of the V_H locus and individual V_H gene segments. (A) Schematic representation of the V_H locus. The relative locations of the V_H gene segments analyzed in this study were determined based on DNA sequence and contig information provided by R. Riblet (personal communication). The locus is shown with the centromere to the left and the telomere to the right. The J_H segments are located to the left as shown. (B) Diagram of a typical V_H gene segment, including the promoter and RSS, with the location of gene-specific primers used for PCR indicated. All primers are ~18 bp, and the products are ~300 bp.

FIG. 3. A detailed analysis of the acetylation pattern of the V_H locus in bone marrow pro-B cells from adult $RAG^{-/-}$ mice. (A) ChIP assays were performed on bone marrow pro-B cells from $RAG^{-/-}$ mice following short-term culture in the presence of IL-7 by using antibodies to acetylated H3 and acetylated H4. Primers were designed to span the promoter and RSS elements of specific V_H gene segments whose relative location in the locus is known (Fig. 2). Serial dilutions (1:5) of 1% of input and serial dilutions (1:2) of IP fractions for individual gene segment promoters (P) were prepared. RSS elements and intergenic segments (I-a to I-d) are shown. Amylase gene was used as a control for an unexpressed gene. Representative data, showing comparable dilutions between gene segments, from one of three experiments are shown. (B) Graphs depicting data in panel A quantified by phosphorimager and showing the relative intensity of IP fractions relative to 0.1% input. Levels of H3 and H4 acetylation are graphed separately, as are different portions of the amplified DNA. Only dilutions which fell into the linear range were used for calculations. The relative intensity of amylase is incorporated into each graph and is used as a reference to determine significant levels of histone acetylation. (C) Schematic showing the portion of the V_H locus where additional gene segments are located and presentation of ChIP data and graphs of the relative intensity of H4 acetylation associated with the RSS elements of additional gene segments. Asterisks signify gene segments that have been used previously. The order and spacing of the V_H gene segments are based on sequence information from R. Riblet (personal communication).

Several lines of evidence suggest that the accessibilities of the V_H locus may be different between J_H -proximal and J_H -distal subregions (7, 9, 11, 23). Therefore, we suspected that the degree of histone acetylation could be determined by the location of V_H gene segments within the locus. However, an alternative possibility was that sequences unique to different V_H families determined different histone acetylation patterns. To investigate this question further, we expanded the number of gene segments analyzed, focusing on the portion of the locus where a transition between low and high levels of histone acetylation was observed. We analyzed acetylation of histone H4 at the RSS elements of three additional V_H gene segments in the region between V10b (J_H proximal, low acetylation) and J558a (J_H distal, high acetylation) (Fig. 3B). The most J_H -

proximal gene segment, J558d, had low H4 acetylation; two gene segments that were more J_H -distal, J558e and 3609a, had high H4 acetylation (Fig. 3C). Interestingly, J558d and J558e are located approximately 96 kb apart (based on DNA sequence information from R. Riblet, personal communication). Together, the data in Fig. 3B and C show that J_H -proximal V_H gene segments from multiple families, including 7183, S107, VGAM, V10, and J558, all have low H4 acetylation associated with their RSS regions, while more- J_H -distal V_H gene segments from both J558 and 3609 families have high H4 acetylation in their RSS regions. Thus, the degree of H4 acetylation does not correlate with V_H gene family but does correlate with location within the V_H region.

Histone H4 acetylation is narrowly localized to V_H gene

FIG. 3—Continued.

segments. We wished to determine whether nucleosomes associated with DNA throughout the V_H locus, including regions between V_H gene segments, were acetylated to the same degree as those associated with V_H promoters and RSSs. Four primer sets, separated by at least 1 kb from a V_H gene segment, were designed to span intergenic sequences within BACs which contained V_H gene segments previously analyzed (Fig. 2B). H4 acetylation for all primer sets was less or nearly equivalent to what was seen with amylase gene (Fig. 3A and B). Histone H3 acetylation for intergenic regions was also low in most areas.

To define more precisely the boundaries of elevated histone H4 acetylation, overlapping primer sets that extended from 5' of the promoter to 3' of the RSS elements for three V_H gene segments were designed (Fig. 4A). A similar pattern was observed for all three V_H gene segments. Histone H4 acetylation decreased dramatically within 200 bp 3' of the RSSs (Fig. 4B). Upstream of the promoters, H4 acetylation also decreased, somewhat more gradually within a distance of 200 to 400 bp, as shown for gene segments V10b and J558a (Fig. 4B). H4 acetylation 5' of J558b remained high for ~1.5 kb, as it is very close to another J558 gene segment. Thus, histone H4 acetylation is tightly restricted to V_H gene segments and does not extend into intergenic regions, suggesting local, gene-specific control of H4 acetylation.

Histone H4 acetylation changes within the J_H-proximal V_H locus during B-cell ontogeny. Although V_H gene segments appear to recombine randomly in adult bone marrow, during early B-cell ontogeny in the fetal liver there is preferential recombination of J_H-proximal V_H gene segments (5, 19, 25, 42). We therefore investigated the pattern of histone acetylation in V_H gene segments in fetal liver to determine whether there was a correlation between preferential recombination and acetylation status. ChIP assays were performed on RAG-deficient pro-B cells from fetal-liver cells isolated from 15- and 16-day-old embryos. These cells were subjected to a short-term culture in the presence of IL-7 and obtained a purity of 90 to 99% as assayed by B220⁺ staining in fluorescence-activated cell sorter analysis (data not shown). Examination of histone H3 and H4 acetylation associated with RSS elements from V_H gene segments throughout the V_H locus revealed a very different pattern of histone H4 acetylation compared with that observed in adult bone marrow (Fig. 5A). Histone H4 acetylation was not biased towards the J_H-distal portion of the locus. In fact, the RSS elements of J_H-proximal gene segments S107b (V11) and VGAMa have acetylation similar to that of three J_H distal gene segments, J558a, J558b, and J558c. This is in sharp contrast to what was observed with the bone marrow, where the same distal gene segments have 5- to 10-fold more acetylation than do the same two proximal gene segments (Fig. 5B). No differences were observed in histone H3 acetylation between fetal and adult pro-B cells (Fig. 5A).

Histone H4 acetylation in V_H gene promoter and RSS regions was determined in each population and compared with histone H4 acetylation of the amylase gene, following normalization to input, to eliminate experiment-to-experiment variation in absolute acetylation levels (Fig. 5C and D). The purities and developmental stages of the fetal and adult pro-B-cell populations were similar as determined by flow cytometry using antibodies to B220 and BP-1 (data not shown). The results show that H4 acetylation of J_H-proximal V_H gene segments is increased by at least sevenfold in fetal liver compared to adult bone marrow for RSS elements (Fig. 5D) and by threefold for promoter regions (data not shown). In addition, H4 acetylation in the J_H-distal V_H gene segments did not decrease (compare Fig. 5A with Fig. 3A) in fetal liver but remained approximately comparable to that in adult pro-B cells. H4 acetylation remained predominantly limited to regions surrounding the V_H gene segments and was not found in intergenic regions (data not shown). Thus, we conclude that histone H4 acetylation of the J_H-proximal V_H gene segments is elevated in fetal versus adult pro-B cells, correlating with the preferential recombination of these gene segments in fetal liver and providing a possible explanation for differential V_H gene recombination during ontogeny.

Transmembrane mu expression decreases H4 acetylation in the V_H locus and increases acetylation in the kappa locus in bone marrow cells. IgH allelic exclusion occurs as a result of mu protein expression, causing an inhibition in V-DJ recombination at the V_H locus. To analyze histone acetylation in the V_H locus during IgH allelic exclusion, we studied mice expressing a transmembrane form of the human mu protein from a transgene (22). Endogenous V_H gene segments are not rearranged in these mice, and enforced mu expression drives development to the pre-B stage (data not shown) (33, 44).

FIG. 4. Histone acetylation associated with intergenic sequences within the V_H locus. (A) Schematic diagram showing the primers used to analyze regions directly flanking an individual V_H gene segment. Primers were designed to amplify overlapping regions on both the 3' and 5' sides of individual gene segments. Each amplified region was ~ 300 bp long and overlapped its neighbors by ~ 100 bp. (B) ChIP assays were performed using antibodies to acetylated H3 and H4 on pro-B cells from bone marrow of $RAG^{-/-}$ mice following a short culture in IL-7. Shown are 1:5 serial dilutions of 1% input and 1:2 serial dilutions of IP fractions. The amylose promoter is used as a negative control. The data are representative and were repeated in separate preparations. The relative intensity of amylose is also shown. (C) H4 acetylation levels from panel B are graphed as relative intensity of 0.1% input after being quantified by phosphorimager.

Histone acetylations of V_H gene segments in pro- and pre-B cells from adult bone marrow were compared in $RAG1^{-/-}$ transgene⁺ mice and $RAG1^{-/-}$ littermate controls (Fig. 6). The most striking change was a 50 to 80% reduction in the levels of H4 acetylation in the J_H -distal portion of the locus (Fig. 6B). Reductions were observed at both the promoter and RSS elements. Thus, expression of membrane μ causes a decrease in histone H4 acetylation of the J_H -distal V_H gene segments, consistent with the notion that H4 acetylation status in this region helps determine lack of accessibility to the recombinase machinery during allelic exclusion.

In pre-B cells the transmembrane form of the μ protein not only causes allelic exclusion at the heavy-chain locus but also activates recombination of light-chain loci (29). Using primers which recognize conserved sequences of V_κ gene segments (27), we observed an increase of at least 3-fold for H4 acetylation and 13-fold for H3 acetylation following μ expression. Thus, the histone acetylation statuses of both V_H and V_κ loci correlate with chromatin accessibility and V(D)J recombination following expression of transmembrane μ .

Transmembrane μ causes decreased histone acetylation at promoters of pro-B-cell-specific genes. Transmembrane μ permits pro-B cells to become pre-B cells (33, 44). Decreased expression of specific genes accompanies this developmental change (15). Some genes, such as *RAG1* and *RAG2*, are transiently repressed, while others, such as $\lambda 5$ and V_{preB} , are permanently repressed. Therefore, primers which span the promoters of *RAG1*, *RAG2*, $\lambda 5$, and V_{preB} were designed. A 6- to 12-fold reduction in the level of H3 acetylation and a less dramatic reduction in H4 acetylation occurred upon expression of μ (Fig. 7). We conclude that, in addition to Ig genes, transmembrane μ affects the histone acetylation status of other genes involved in B-cell development, including genes that are repressed both transiently and permanently.

DISCUSSION

A detailed analysis of the acetylation status of histones associated with coding and intergenic regions located throughout the murine immunoglobulin V_H locus during B-cell ontogeny and development has revealed several important facts. (i) Acetylation precedes V-DJ recombination and is higher and more regulated for histone H4 than for histone H3, suggesting a unique role for histone H4 in determining V_H chromatin. (ii) In adult pro-B cells, V_H gene segments in the J_H -distal part of the locus are associated with highly acetylated H4 while those in the J_H -proximal part of the locus have lower amounts of acetylation, revealing a global pattern of regulation. (iii) Histone H4 acetylation is narrowly confined to V_H promoter, coding, and RSS regions, with a particularly sharp boundary occurring just 3' of RSS elements, demonstrating local, gene-specific regulation within the context of global regulation. (iv) Pro-B cells formed early in ontogeny have high H4 histone acetylation in J_H -proximal V_H gene segments, which correlates with their preferential rearrangement. (v) Enforced expression of transmembrane μ in a transgenic model of IgH allelic exclusion decreases histone acetylation of V_H gene segments, consistent with their inaccessibility to RAG recombinases. In addition, μ expression increases acetylation in the Ig κ locus and decreases acetylation of promoters for several genes, in-

cluding *RAG1*, *RAG2*, $\lambda 5$, and V_{preB} . In sum, our data provide strong evidence that chromatin structure determined by histone acetylation is important for both activation and inhibition of V_H -DJ recombination and is subject to both global and local regulation.

Histone acetylation in the V_H locus suggests B-cell-specific, global regulation. Histone acetylation is not an innate property of the V_H locus because histones associated with V_H genes are not acetylated in pluripotent stem cells or cells outside the B-cell lineage (data not shown) (Fig. 1). Consistent with the results of previous reports (7, 17), we show that histone acetylation in the V_H locus is elevated in adult bone marrow pro-B cells (Fig. 1, 3, and 5) when the locus is accessible as indicated by DNase I sensitivity (17), V_H germ line transcription (11, 27, 38, 41), and RAG-dependent double-strand breaks (8). Introduction of RAG genes into $RAG^{-/-}$ pro-B cells leads to DJ and V-DJ recombination, demonstrating that the IgH locus is accessible in these cells (2, 32).

Elevated histone acetylation in adult bone marrow cells was primarily found in the J_H -distal portion of the V_H locus (Fig. 3). Our data confirm and extend a previous report that IL-7 causes increased H3 acetylation in the J_H -distal part of the V_H locus (7). To obtain sufficient cell numbers, the bone marrow cells used in this analysis were subjected to short-term culture in IL-7 and therefore may not exactly reflect in vivo pro-B cells. Additionally, transformation with the Abelson virus, used to establish the cell lines analyzed, mimics IL-7 signaling (3). However, it seems likely that our primary cultures in IL-7 mimic the natural opening in the V_H locus because in IL-7R-deficient mice, V-DJ rearrangement is blocked specifically for J_H -distal V_H gene segments (9), consistent with our observation of increased histone acetylation of V_H gene segments in this location following culture in IL-7 (Fig. 3) in bone marrow pro-B cells.

Our data show that histone acetylation of V_H gene segments corresponds with their location in the locus. It may be that histone acetylation of V_H gene segments is regulated in a global manner, perhaps by an element like a locus control region or an insulator, which could regulate acetylation within large J_H -proximal and J_H -distal regions. A precedence for such a boundary element correlating with sharp changes in the acetylation profile is found at the beta globin locus (10). Interestingly, the boundary between V_H gene segments with low versus high H4 acetylation appears to occur within a region of ~96 kb between J558d and J558e (Fig. 3C). Further analysis of this region might reveal a boundary element.

In spite of differential histone acetylations, V_H gene segments in the J_H -proximal and J_H -distal regions rearrange with similar frequencies (18). However, the amount of histone acetylation required for V-DJ recombination is not known and low levels of histone acetylation may be sufficient for rearrangement of the J_H -proximal V_H gene segments. Alternatively, acetylation of J_H -proximal V_H gene segments earlier in ontogeny (Fig. 5) or proximity to enhancers in the DJ region (7) may be sufficient to permit their recombination.

Acetylation of histone H4 in V_H gene segments was higher and more regulated than acetylation of histone H3 (Fig. 3, 4, and 6). Increased H4 versus H3 acetylation may be unique to Ig loci in B cells since the TCR β locus has a reversed situation in T cells (36). Although H3 acetylation of Ig κ changed signif-

FIG. 5. Analysis of histone acetylation in the V_H locus in pro-B cells from $RAG^{-/-}$ fetal liver. (A) ChIP assays using anti-acetylated H3 and H4 antibodies were performed on pro-B cells from $RAG^{-/-}$ fetal-liver cells isolated from day 15 and 16 embryos following a short-term culture in IL-7. The histone acetylation status associated with RSS elements from individual gene segments located throughout the locus is shown, and the amylase gene was used as a negative control. The 1% input fractions were diluted 1:5, and the IP fractions were diluted 1:2. Shown are representative data from three separate experiments performed using pooled fetal-liver cells. All data shown were obtained from use of comparable dilutions between gene segments within each preparation. (B) H4 acetylation levels from panel A represented as relative intensity of 0.1% input after being quantified by phosphorimager. Graphs make use of samples which fell into the linear range; therefore, additional dilutions were performed in analysis of the many gene segments. An equivalent graph of the bone marrow cells (primary data shown in Fig. 6) is shown for comparison. (C) Shown are the results of ChIP assays analyzing both the promoter (P) and RSS elements of proximal gene segments and using both anti-acetylated H3 and H4 antibodies on embryonic day 15 and 16 fetal-liver pro-B cells following a short culture in the presence of IL-7. Data are representative of three experiments. Also shown is a schematic representation which highlights the portion of the locus for which data are presented. (D) Data from panel C were graphed as increases (n -fold) above the results with amylase, after normalizing to input, in order to adjust for background histone acetylation levels between experiments. The increases (n -fold) are compared for pro-B cells from bone marrow and fetal liver.

FIG. 5—Continued.

icantly following μ expression (Fig. 6), substantial acetylation of H4 was associated with the kappa locus during the pro-B stage (Fig. 6), which could account for the kappa rearrangement that occurs prior to or concomitant with heavy-chain rearrangement (28).

Histone acetylation in the V_H locus is confined to V_H genes, suggesting local regulation. Even within the highly acetylated J_H -distal region, only histones associated with V_H -coding regions and the promoter and RSS elements associated with them were acetylated (Fig. 3 and 4). This highly focused pattern of V_H histone acetylation suggests the possibility that a sequence in the promoter or RSS elements of each gene segment might be important for local recruitment of proteins with histone acetyltransferase (HAT) activity. It may be that once large portions of chromatin are "opened," other proteins with HAT activity are recruited to local regions. Deletion of enhancers and lineage-specific proteins in both B and T cells has provided examples of single factors that are able to regulate

recombination of large portions of DNA (for a review, see reference 4). The marked decrease in histone acetylation 3' of the RSSs also suggests that RSSs could be nucleosome free. Consistent with this idea, nucleosomes inhibit RAG-dependent recombination and Swi/SNF-dependent remodeling increases RAG-dependent recombination in vitro (14).

Histone acetylation in the V_H locus accompanies preferential V_H rearrangement during B-cell ontogeny. H4 acetylation was not skewed to the J_H -distal region in pro-B cells from fetal liver (Fig. 5), and H4 acetylation in the J_H -proximal V_H gene segments was significantly higher in pro-B cells from fetal liver compared with those from adult bone marrow (Fig. 5C and D). Thus, histone acetylation in the V_H locus is differentially regulated during B-cell ontogeny. Other differences in pro-B cells during ontogeny have been noted (15, 37). Interestingly, absence of Pax5 causes a complete loss of V_H -DJ joining in the fetal liver but only a loss of recombination of J_H -distal V_H gene segments in adult bone marrow (24), suggesting that Pax5

FIG. 6. Comparison of the histone acetylations associated with the V_H and Ig kappa loci following mu expression in B cells from $RAG^{-/-}$ mice. (A) ChIP assays using anti-acetylated H3 and H4 antibodies were performed on pre-B cells from $RAG^{-/-}$ mice expressing the human mu protein as a transgene (TG+), and the results were compared to those with pro-B cells from littermate controls (TG-) following a short culture in the presence of IL-7. Data are representative of results with three to five mice for each genotype. Individual V_H gene segments are shown, and the amylose gene was used as a negative control. The kappa primers recognize conserved portions of the V_K gene segments. Shown are 1:5 serial dilutions of 1% input and 1:2 serial dilutions of IP fractions for each animal. The increases (n -fold) of H3 and H4 acetylation relative to that for TG- animals are shown for kappa genes. (B) Graphic representation, using samples which fell into the linear range, of H4 acetylation data from panel A expressed as relative intensity of 0.1% input. The relative intensity of the pancreatic specific gene amylose represents nonspecific levels of histone acetylation.

FIG. 7. The effects of enforced mu expression on the histone acetylation associated with promoters for transiently expressed pro-B-cell genes. (A) ChIP assays using anti-acetylated H3 and H4 antibodies were performed on pre-B cells from RAG^{-/-} mice expressing the human mu protein as a transgene (TG+), and the results were compared to those with pro-B cells from littermate controls (TG-) following a short culture in the presence of IL-7 (as in Fig. 6). Primers were designed to span a ~300-bp fragment of the RAG1, RAG2, VpreB and lambda5 promoters. Shown are 1:5 serial dilutions of 1% input and 1:2 serial dilutions of IP fractions for each animal. The amyase gene served as a negative control. The relative intensity of amyase is also shown. (B) A graphic representation of the data from panel A comparing the levels of H3 acetylation when expressed over the inactive amyase gene, after being normalized to input.

could be important for determining H4 acetylation in the V_H locus at both developmental stages.

Neither differential promoter activity nor RSS strength can explain the biased rearrangement of J_H -proximal V_H gene segments in the fetal liver (16, 40). Our data show a direct correlation between H4 acetylation and preferential recombination. Interestingly, our data also show that J_H -proximal gene segments are associated with high levels of histone acetylation prior to DJ rearrangement, which is contrary to the requirement of DJ rearrangement for high levels of histone acetylation associated with these gene segments found by Chowdhury et al. in bone marrow pro-B cells. Although these two findings are not mutually exclusive, our data suggest that the proximal portion of the V_H locus is regulated in a unique fashion early during ontogeny in a way that is more permissive for recombination to occur. In the context of a growing body of data showing that histone acetylation is permissive for V(D)J recombination in TCR α/δ (20) and TCR γ (1, 43) loci, our results suggest that increased H4 acetylation is important for prefer-

ential recombination of the J_H -proximal V_H gene segments early in B-cell ontogeny.

Histone acetylation decreases upon inhibition of V-DJ recombination during IgH allelic exclusion. The mu heavy chain provides a checkpoint in early B-cell development and is required for progression from the pro-B to pre-B stage (33, 44). Mice expressing high levels of a transmembrane mu from a transgene allelically exclude rearrangement of the endogenous V_H locus and proceed with kappa gene rearrangement (22). Changes in gene expression also occur during the transition from pro-B to pre-B cells that accompanies mu expression (33, 44). We found that mu heavy-chain expression caused a significant decrease in histone acetylation in the J_H -distal portion of the V_H locus, consistent with inhibition of V-DJ recombination (Fig. 6). Concomitantly, histone acetylation associated with the Ig κ locus increased. Similar results have been obtained independently by Sen and colleagues (personal communication) for the V_H locus and by the Krangel group for the TCR β locus (36). Thus, histone acetylation is not only associated with establishment of accessibility for recombination in the V_H and Ig κ loci in pro-B cells but is also associated with inhibition of further recombination in the V_H locus during IgH allelic exclusion in pre-B cells.

The degree of histone deacetylation sufficient to inhibit accessibility to RAG proteins is not clear. Complete inhibition was not observed in the V_H locus (Fig. 6) or the TCR β locus (36). The most dramatic decreases occurred in the J_H -distal portion of the V_H locus. This region was more highly acetylated in the absence of mu, possibly providing more contrast. Alternatively, H4 acetylation may be more critical for establishing accessibility of this region. It is also possible that IgH allelic exclusion operates primarily or exclusively in the J_H -distal region. Another study has shown that only J_H -distal J558 transcripts were downregulated in pre-B cells, suggesting that only this portion of the V_H locus is inhibited during allelic exclusion (11).

At present, we do not know the signaling pathways responsible for mu-dependent changes in histone acetylation. Allelic exclusion may be independent of the pre-B-cell receptor since mice lacking surrogate light chains maintain allelic exclusion (21, 31, 35). Also, various covalent modifications of histone tails, especially acetylation and methylation, are interdependent, and other changes may precede acetylation (12). However, since histone acetylation is more dynamic and sensitive to signaling pathways than is methylation, it is likely that local recruitment of proteins with HAT or histone deacetylase activity is an early event in mu-dependent developmental changes.

Overall, our data show that in the V_H locus, histone acetylation, especially histone H4 acetylation, is regulated both globally and locally during B-cell ontogeny and development. This provides strong evidence that these changes are important for regulating V-DJ recombination.

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