

Histone Acetylation Determines the Developmentally Regulated Accessibility for T Cell Receptor γ Gene Recombination

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Abstract

Variable/diversity/joining (V(D)J) recombination of the T cell receptor (TCR) and immunoglobulin (Ig) genes is regulated by chromatin accessibility of the target locus to the recombinase in a lineage- and stage-specific manner. Histone acetylation has recently been proposed as a molecular mechanism underlying the accessibility control. Here, we investigate the role for histone acetylation in the developmentally regulated rearrangements of the mouse TCR- γ gene, wherein predominant rearrangement is switched from V γ 3 to V γ 2 gene during the fetal to adult thymocyte development. Our results indicate that histone acetylation correlates with accessibility, as histone acetylation at the fetal-type V γ 3 gene in accord with germline transcription is relatively high in fetal thymocytes, but specifically becomes low in adult thymocytes within the entirely hyperacetylated locus. Furthermore, inhibition of histone deacetylation during the development of adult bone marrow-derived thymocytes by a specific histone deacetylase inhibitor, trichostatin A, leads to elevated histone acetylation, germline transcription, cleavage, and rearrangement of the V γ 3 gene. These data demonstrate that histone acetylation functionally determines the chromatin accessibility for V(D)J recombination in vivo and that an epigenetic modification of chromatin plays a direct role in executing a developmental switch in cell fate determination.

Key words: V(D)J recombination • germline transcript • ligation-mediated PCR • chromatin immunoprecipitation • histone deacetylase

Introduction

Lymphocyte development is regulated by V(D)J recombination that assembles V, D, and J gene segments in the TCR and Ig loci. V(D)J recombination reaction is catalyzed by the common enzymatic machinery that includes recombination activating gene (RAG)-1 and RAG-2 proteins (1, 2). RAG-1 and RAG-2 recognize recombination signal sequences (RSSs) that flank all the coding gene segments and then introduce cleavages of DNA at RSSs. Lymphocyte-specific recombination is primarily ensured by the restriction of RAG-1 and RAG-2 expression to defined stages of T and B lymphoid development. However, lineage- and stage-specificity of the rearrangements is regulated by the accessibility of RSSs within chromatin to the recombinase (3–5). Recent studies revealed that

the cis-acting elements within TCR and Ig loci including enhancers and promoters play crucial roles in recombination by establishing the locus- and gene segment-specific accessibility (3–5). However, the molecular nature of chromatin structural modifications responsible for accessibility has long been unknown. Acetylation of the NH₂-terminal tails of histones, the most extensively characterized modification of chromatin, has been implicated for transcriptional regulation through the alteration of chromatin structure (6, 7). Recently, acetylation of histone H3 was shown to tightly correlate with locus-wide accessibility for V(D)J recombination directed by the enhancer in vivo (8), suggesting that histone acetylation might be functionally relevant to the accessibility control.

In this report, we investigate the role for histone acetylation in the accessibility control during the physiological thymocyte development. We focused on the mouse TCR- γ gene (9, 10), wherein rearrangement of V γ 3 gene is re-

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stricted to fetal stages, whereas V γ 2 recombination predominates in the adult thymus (11–13). Our data demonstrate that targeted histone deacetylation is responsible for the inhibition of V γ 3 rearrangements in adult thymocytes.

Materials and Methods

Cell Preparation, Fetal Thymic Organ Culture, and Flow Cytometry. Fetal and adult thymocytes were prepared from embryonic day 14 (E14) and E16 C57BL/6 fetuses and 4-wk-old C57BL/6 mice. CD3⁺CD4⁺CD8⁺CD19⁺TER119⁺ thymocytes were isolated by MACS (Miltenyi Biotec) and used as CD3⁺CD4⁺CD8⁺ triple negative (TN) thymocytes. We routinely obtained the population of >95% purity. Fetal thymic organ culture (FTOC) was performed as described (14, 15). Fetal liver cells of E14 C57BL/6 fetuses and adult bone marrow cells of 8-wk-old C57BL/6 mice (2 × 10⁵ cells/lobe) were transferred into 2'-deoxyguanosine-treated E15 fetal thymic lobes from ICR mice in a hanging drop for 24 h, and then organ cultured on a floating Nucleopore polycarbonate filter (Corning) with trichostatin A (TSA; 3 ng/ml; WAKO) or equal volume of DMSO as a vehicle control. Flow cytometric analysis was performed as described (14, 15). The following monoclonal antibodies were used: FITC-conjugated anti-CD3 ϵ (145-2C11) and anti-V γ 2 (UC3-10A6) antibodies; and PE-conjugated anti-CD3 ϵ (145-2C11) and anti-V γ 3 (536) antibodies (BD Pharmingen). Viable cells were analyzed by a FACS-Calibur™ with CELLQuest™ software v3.1 (Becton Dickinson).

PCR Analysis. Genomic DNA was prepared by the standard proteinase K method (16). Fivefold serial dilutions (50, 10, and 2 ng) of fetal and adult thymocyte DNAs were prepared in 25 μ g/ml sonicated pBSKS. Diluted DNA and organ-cultured thymocyte DNA (equivalent to 10³ cells) were analyzed by PCR in 10 μ l reaction with 0.25 mM dNTP, 0.5 μ M each primer, 5 μ g/ml sonicated pBSKS, and 0.5 U of rTaq DNA polymerase (Takara). PCR was performed for 24, 28, or 32 cycles (CD3 ϵ , V γ coding joints, V γ signal joints, respectively) of 94°C, 45 s, 55°C, 120 s, and 72°C, 60 s with the following primers: CD3 G1/2 F141, 5'-TCAAAGGCCAGGACGGCTAC-3'; CD3 G1/2 R636, 5'-GCCATAGTAGGATGAAGGAG-3'; for V γ coding joints, L2, L3 (13), and STP100 (J γ 1 [17]); for V γ signal joints, V2S-R1, 5'-GGAGTGATTCAGGGGATTCA-3', V3S-R1, 5'-TGACATCCTTGAACCGAGTC-3', and J1P-F1, 5'-ACAGTTTCAACACGAGTGAG-3'. PCR products were run on a 2% agarose gel, transferred to HybondN⁺ membrane (Amersham Pharmacia Biotech), and hybridized with ³²P-end-labeled oligonucleotide probe as follows: CD3 G1/2 F443, 5'-GGC-CGGATTCCTCAGTTA-3'; for V γ coding joints, STS1008 (J γ 1 [17]); for V γ signal joints, V2-3'b and V3-3'b (13). All the Southern blots were analyzed and quantitated using a Bio-image Analyzer (Fujix BAS5000).

Ligation-mediated PCR. The same set of the genomic DNAs was analyzed by ligation-mediated PCR as described (18). Fetal and adult thymocyte DNA (10 ng) and organ-cultured thymocyte DNA (equivalent to 2 × 10³ cells) were ligated to annealed BW linker. Ligated DNA was amplified for 12 cycles with the BW-1H primer and the following distal locus-specific primer: 3'V γ 2 3'-1, 5'-CATTCAGCAGGAAACAGTGCTAACCAG-3'; V γ 3EXT (19). The first PCR product was then amplified for 30 cycles with the following linker- and proximal locus-specific primers: for V γ 2, BW-1H5, 5'-ACCCGGGAGATCTGAAT-TCCACAGCA-3', 3'V γ 2 3'-2, 5'-TGATAGCAAGATAGTC-CCTGAGGCT-3'; for V γ 3, BW-1H3, 5'-ACCCGGGAGAT-

CTGAATTCCACAGT-3', V γ 3INT (19). PCR products were analyzed by Southern blotting using the V2-3'b and V3-3'b oligonucleotides as probes.

Reverse Transcription PCR. Total RNA was prepared using TRIzol (GIBCO BRL). Random hexamer-primed cDNA was synthesized from RNA equivalent to 2–6 × 10⁴ cells using Moloney murine leukemia virus (MMLV) reverse-transcriptase Superscript II (GIBCO BRL) according to the manufacturer's instructions. Fivefold serial dilutions of cDNA (equivalent to 5, 1, and 0.2 × 10³ cells) were amplified as in genomic PCR with the following primers: for V γ 2 germline transcript (GLT), LV2, 5'-TCTTATGCCTCTTGACATTTGG-3', V2-3'a (13); for V γ 3 GLT, LV3, 5'-TGTCTTTGACCTGTGTTTATGG-3', V3-3'a (13); for J γ 1 GLT, 5'J γ 1 5'-2, 5'-TAACTCCAGGGAGAA-CAGTG-3', C γ 1 3'-3, 5'-GACAAAGGTATGTCCCAGTC-3'; for J κ 1 GLT, 5'J κ 1 5'-4, 5'-CAGCAGTTCTGTGTCAGAGA-3', C κ 3'-1, 5'-GATGGTGGGAAGATGGATAC-3'; mMyoD 10691-F1, 5'-TCTACGCACCTGGACCGCTGC-3'; mMyoD 10692-R1, 5'-CAGGAGTGCCTACGGTGGTGC-3'; CD3 RT F503, 5'-AAGCCTGTGACCCGAGGAAC-3'; CD3 RT R1111, 5'-GTGTAACAGTCGGAGGATGG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-S1, 5'-CCATCACCATCTTCCAGGAG-3'; GAPDH-AS1, 5'-CCT-GCTTACCACCTTCTTG-3'. Numbers of PCR cycle were as follows: 32 (V γ 2 and V γ 3 GLTs), 26 (J γ 1 GLT), 30 (J κ 1 GLT), 25 (MyoD), 23 (CD3 ϵ), and 22 (GAPDH). To specifically amplify the spliced forms of cDNA, each LV2 and LV3 primer was designed to span the intron between the leader and following exons of each V γ 2 and V γ 3 gene. PCR products were analyzed by Southern blotting using the following oligonucleotide as probe: V2-3'b; V3-3'b; STS1008 (J γ 1); 5'J κ 1 5'-3, 5'-CTT-TCGCCTACCCACTGCTCTG-3'; MyoD RT-R2, 5'-CGG-GGCTGTCTGTGGAGATGC-3'; CD3 RT-F543, 5'-GAGG-GCAAAACAAGGAGCGGC-3'; GAPDH-S2, 5'-GGAGCCA-AACGGGTCATCATC-3'.

Chromatin Immunoprecipitation. Soluble chromatin was prepared from 1–2 × 10⁶ cells fixed with formaldehyde as described (20, 21). The chromatin solution (2 ml) was precleared with 120 μ l of 50% protein G-Sepharose slurry preadsorbed with 100 μ g/ml sonicated salmon sperm DNA (ssDNA), then aliquoted and incubated with 4 μ g of acetylated histone H3 antibody, 1 μ l of acetylated histone H4 antiserum (Upstate Biotechnology), or 4 μ g of normal rabbit IgG (Santa Cruz Biotechnology, Inc.) overnight. Immunoprecipitates were recovered with 20 μ l of 50% protein G-Sepharose/ssDNA for 2 h, washed with the buffers as described (20), and resuspended in 200 μ l of 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.3 M NaCl, and 0.5% SDS. The beads and an input fraction saved before preclear were incubated at 65°C for at least 6 h. DNA was purified and serial dilutions of DNA were prepared in 25 μ g/ml sonicated pBSKS and amplified as in genomic PCR for 28 cycles of 94°C, 20 s, 55°C, 60 s, and 72°C, 60 s with 2 s increment per each cycle using the following primers: V2P-F1, 5'-GCTCAGAACAGCTACTC-TTC-3'; V2P-R1, 5'-GGATTCTCATCTGCCAGGTT-3'; V2S-F1, 5'-TTGGAGGAAGAAGACGAAGC-3'; V2S-R1, 5'-GGAGTATTACGGGATTCA-3'; V3P-F1, 5'-AACAGC-TCAGAAATAAGTCT-3'; V3P-R1, 5'-CTCAGGACACAAAATAACG-3'; V3S-F2, 5'-CCACGTACTACTGTGCC-TGC-3'; V3S-R1, 5'-TGACATCCTTGAACCGAGTC-3'; J γ 1 5'-2, 5'-TAGCTCAGGTTTTTACAAGG-3'; 3'J γ 1 3'-1, 5'-TCTGGAGGGAAGAAGCAATC-3'; 5'J κ 1 5'-4, 5'-CAG-CAGTTCTCTGTGAGAGA-3'; 3'J κ 1 3'-1, 5'-ACAGACAT-AGACAACGGAAG-3'; MyoD-F1, 5'-CGCCCTACTACAC-

TCCTATTG-3'; MyoD-R1, 5'-AAGGTTCTGTGGGTT-GGAATG-3'; CD3 G1/2 F443, 5'-GGCCCGGATTTCTC-CAGTTA-3'; CD3 G1/2 R636, 5'-GCCATAGTAGGAT-GAAGGAG-3'. PCR products were analyzed by Southern blotting using the following oligonucleotide as probe: V2P-F2, 5'-TGGAAGGCGGGAGGATGAGGA-3'; V2-3'b (for V2S); V3P-F2, 5'-CTTCCTCCCCTGGCCCTTTC-3'; V3-3'b (for V3S); 3'J γ 1 3'-3, 5'-GACTCAGGCTAAGAATATTGT-AGT-3'; 5'J κ 1 5'-3, 5'-CTTTCGCCTACCCACTGCTCTG-3'; MyoD-F621, 5'-GCCAGGACGCCCCAGGACACG-3'; CD3-F557, 5'-CCTGCCTCCCCTGTCTGCTTC-3'. Southern blots were quantitated using a Bio-image Analyzer. Quantitative analysis was confirmed by comparing the dilution series. To calculate the relative histone acetylation levels, a mean value of the serial dilutions was calculated for the input (I) and immunoprecipitated (P) fractions after adjusting by the dilution factors. The P/I ratio of each locus was then compared with that of the positive control CD3 ϵ locus.

Results and Discussion

Histone Acetylation at V γ Genes Correlates with Accessibility during Fetal to Adult Thymocyte Development. To investigate the accessibility control of the TCR- γ gene rearrangement during the thymocyte development, we first assessed

the accessibility of unrearranged V γ 2 and V γ 3 genes by measuring their germline transcripts in fetal and adult thymocytes by reverse transcription (RT)-PCR (Fig. 1, A and B). For adult thymocytes, we analyzed CD3⁻CD4⁻CD8⁻TN thymocytes, in which TCR- γ gene rearrangements take place. E14 thymocytes expressed both germline transcripts at high levels. In E16 thymocytes, V γ 3 germline transcripts began to decline by threefold compared with E14, whereas V γ 2 germline transcripts remained comparable. In contrast, germline transcripts as well as rearrangements of V γ 3 dramatically decreased in adult TN thymocytes, whereas those of V γ 2 were still predominant (Figs. 1 B and 2 B). These results confirm the previous findings that chromatin at V γ 3 is inaccessible and V γ 3 rearrangements are consequently inhibited in adult thymocytes (13). Unrearranged J γ 1 gene and CD3 ϵ gene were vigorously transcribed in both fetal and adult thymocytes, whereas J κ 1 and MyoD were not (Fig. 1 B).

We next examined whether histone acetylation at V γ genes correlates with accessibility. Histone acetylation was measured by chromatin immunoprecipitation (ChIP) assay (20, 21) using antiacetylated histone H3 and H4 antibodies. Immunoprecipitated DNA was analyzed by quantitative PCR to assess the association of specific DNA regions with

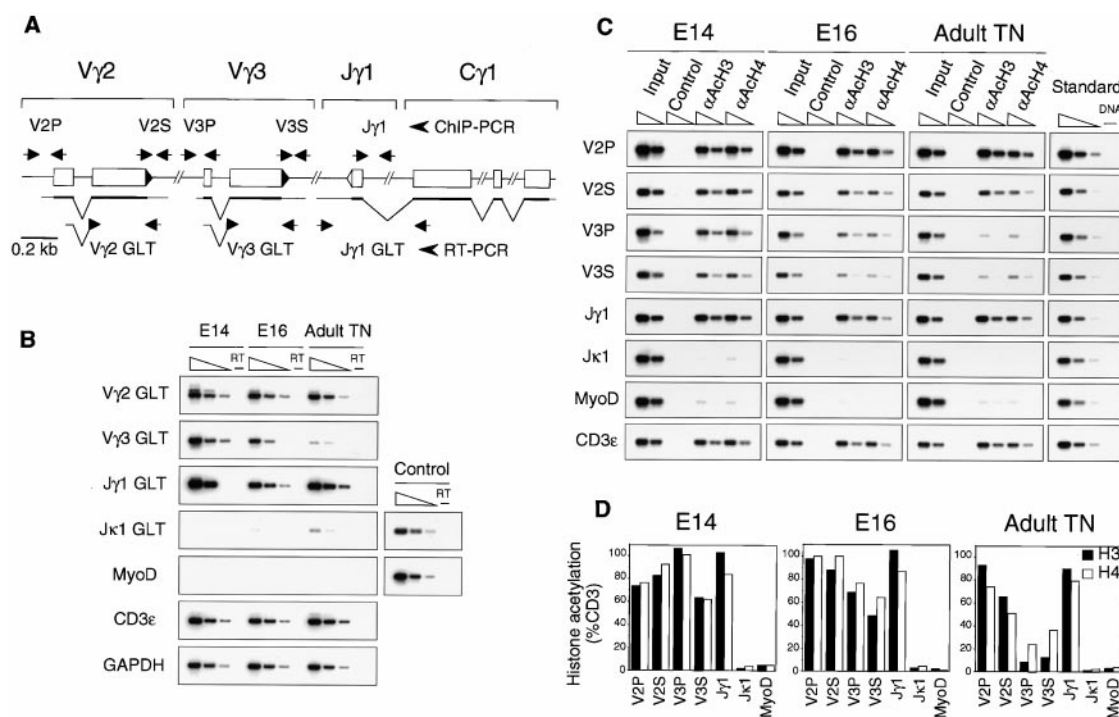


Figure 1. Histone acetylation at V γ genes correlates with accessibility during fetal to adult thymocyte development. (A) Schematic diagram of the TCR- γ locus and location of PCR primers used in RT-PCR and ChIP assays. The promoter (P) and RSS (S) regions of each V γ 2 and V γ 3 gene were analyzed in ChIP assays. GLTs of V γ 2, V γ 3, and J γ 1 genes were measured by RT-PCR. The V γ RSSs with 23-bp spacer and J γ 1 RSS with 12-bp spacer are indicated by filled and open triangles, respectively. (B) RT-PCR analysis of E14 and E16 total, and adult CD3⁻CD4⁻CD8⁻TN thymocyte RNA. Fivefold serial dilutions of cDNAs and reaction without reverse transcriptase (RT⁻) were amplified for indicated transcripts. For J κ 1 GLT and MyoD transcripts, cDNAs from adult bone marrow and C2C12 cells were used, respectively (Control). (C) Histone acetylation measured by ChIP assay in E14 and E16 total, and adult TN thymocytes. Threefold serial dilutions of input DNA and DNAs immunoprecipitated with antiacetylated histone H3 (α AcH3) and H4 (α AcH4) antibodies or with normal rabbit IgG (Control) were analyzed by PCR. Fivefold serial dilutions of input DNA from Ba/F3 cells (Standard) and no DNA control (DNA⁻) were also subjected to PCR. (D) Histone acetylation levels measured in C are shown as relative histone H3 (black bars) and H4 (white bars) acetylation levels to that of the CD3 ϵ locus.

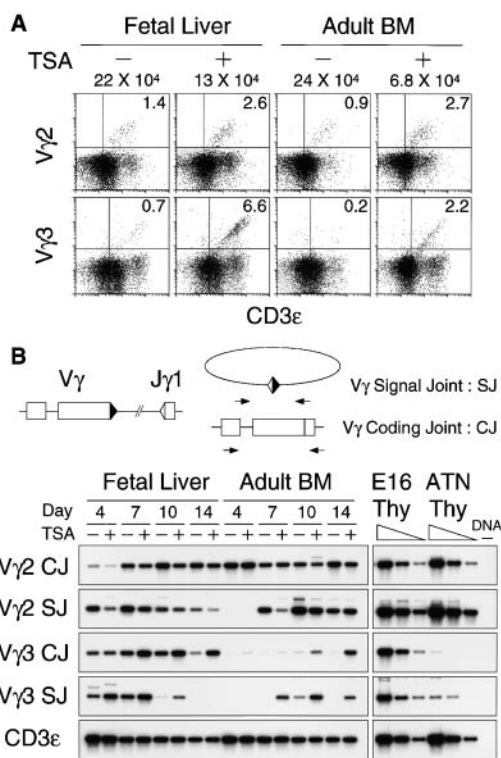


Figure 2. TSA induces V γ 3 rearrangements in adult-derived thymocytes. (A) Flow cytometric analysis of organ-cultured thymocytes in fetal thymic lobes repopulated with E14 fetal liver and adult bone marrow (BM) cells. Thymocytes cultured for 14 d with (+) or without (-) TSA were stained with FITC-V γ 2 and PE-CD3 ϵ , or PE-V γ 3 and FITC-CD3 ϵ antibodies. Average cell numbers per lobe from four lobes and percentages of cells for a given phenotype are shown above and inside each panel, respectively. (B) PCR analysis of organ-cultured thymocyte DNA to measure coding joints (CJ) and signal joints (SJ) of V γ 2-J γ 1 or V γ 3-J γ 1, and CD3 ϵ gene for a control. Fivefold serial dilutions of E16 and adult TN (ATN) thymocyte (Thy) DNA and no DNA control (DNA-) were also subjected to PCR.

acetylated histones. We examined two regions corresponding to the promoter and RSS for each V γ gene (Fig. 1 A). Histone acetylation at the promoters may reflect transcription on productively rearranged alleles. On the other hand, PCR products of RSS regions that are deleted by recombination reflect the acetylation state before recombination. To compensate deletions by recombination, histone acetylation levels were normalized with input DNA, and then compared with that of the positive control CD3 ϵ locus. Consistent with abundant germline transcripts, we detected high levels of histone H3 and H4 acetylation at V γ 2, V γ 3, and J γ 1 in E14 thymocytes, whereas acetylation levels were extremely low at J κ 1 and MyoD (Fig. 1, C and D). We reproducibly observed that acetylation at V γ 3 promoter was slightly higher than that of V γ 2 promoter (Fig. 1 D). In E16 thymocytes, the acetylation pattern at promoters became opposite, whereas that of RSSs was unchanged (Fig. 1 D), indicating that histone acetylation at V γ 3 promoter correlates well with germline transcription during fetal stages. Nevertheless, general acetylation levels in the promoter and RSS regions are relatively high at both V γ 2 and

V γ 3. In contrast, acetylation levels at V γ 3 promoter and RSS specifically decreased in adult TN thymocytes, whereas those at V γ 2 and J γ 1 remained relatively high (Fig. 1 D). It might be argued that the decreased levels of histone acetylation and germline transcription at V γ 3 in adult TN thymocytes are due to excision out of V γ 3 gene by the rearrangements of V γ 2 (or V γ 4) to J γ 1. However, we confirmed by Southern blotting that extrachromosomal species were estimated to be less than one-third of V γ 3 gene in adult TN thymocytes (data not shown). Thus, reduced histone acetylation and germline transcription at V γ 3 that we detected were not solely attributed to the excision. Furthermore, similar reduction was also found in adult thymocytes of RAG-2 knockout mouse (data not shown). These results indicate that histone acetylation is tightly correlated with germline transcription and hence accessibility on the chromosomal V γ 3 gene during fetal to adult thymocyte development. These data led us to the hypothesis that histone deacetylation at V γ 3 may play a role in establishment and maintenance of the inaccessible chromatin structure of V γ 3 gene in adult thymocytes.

Inhibition of Histone Deacetylase Induces V γ 3 Rearrangements in Adult-derived Thymocytes. To address this hypothesis, we tested whether inhibition of histone deacetylation by TSA (22) affects development of V γ 3⁺ T cells in FTOC (Fig. 2 A). E14 fetal liver and adult bone marrow cells were transferred into 2'-deoxyguanosine-treated fetal thymic lobes and then cultured with or without TSA. After 14 d, thymocytes were prepared from the cultured lobes and analyzed by flow cytometry. Both fetal liver and adult bone marrow cells gave rise to V γ 2⁺ T cells irrespective of TSA treatment. In contrast, a small but discrete V γ 3⁺ T cell population was detected in fetal- but not adult-derived thymocytes without TSA, confirming our previous finding that the potential to differentiate into V γ 3⁺ T cells is determined at the stem cell level (14). However, in the presence of TSA both fetal liver and adult bone marrow cells gave rise to distinct V γ 3⁺ populations. This is not due to relative enrichment because the absolute numbers of V γ 3⁺ cells, but not of V γ 2⁺ cells, were significantly increased by TSA. These data indicate that TSA treatment promotes development of fetal-type V γ 3⁺ T cells. We also verified the donor origin of V γ 3⁺ cells by allotypic marker (CD45.1 and CD45.2) staining (data not shown).

To determine whether V γ 3 rearrangements are also promoted by TSA, we next analyzed organ-cultured thymocyte DNA to quantitate coding joints between V γ and J γ 1 exons by PCR (Fig. 2 B). V γ 2 coding joints were readily detected in both fetal- and adult-derived thymocytes. V γ 3 coding joints were also detected in fetal-derived cells, but barely detectable in adult-derived cells without TSA (Fig. 2 B, TSA⁻ lanes). By TSA treatment, however, V γ 3 but not V γ 2 coding joints were significantly increased in adult-derived cells (5- and 17-fold on day 10 and 14, respectively, compare TSA⁺ to - lanes in Fig. 2 B). In fetal-derived cells, TSA also increased V γ 3 coding joints sevenfold on day 14, but did not significantly affect them until day 10. Consistent with the data in the flow cytometric analysis, these results

suggest that inhibition of histone deacetylation by TSA leads either to specific induction of V γ 3 recombination or to selective expansion of V γ 3-rearranged cells.

To discriminate these two possibilities, we examined reciprocal V γ -J γ 1 signal joints that are recombination by-products generated by joining between V γ and J γ 1 RSSs (Fig. 2 B). The amount of signal joints usually reflects de novo recombination events, because they are present on circular DNAs that do not replicate in most cases. V γ 3 but not V γ 2 signal joints were specifically reduced in adult TN thymocytes as well as adult-derived thymocytes without TSA (Fig. 2 B, TSA⁻ lanes). However, TSA strongly increased V γ 3 signal joints in adult-derived cells (Fig. 2 B, compare TSA + to - lanes). A small amount of V γ 3 signal joints was detected on day 10 without TSA, but was increased sevenfold by TSA. These results demonstrate that de novo V γ 3-J γ 1 recombination is induced by TSA in adult-derived thymocytes. In addition, TSA-induced recombination apparently included productive V γ 3-J γ 1 recombination, as revealed by the flow cytometric analysis (Fig. 2 A). However, it should be noted that expansion of V γ 3-rearranged cells partially contributed to the increased V γ 3 coding joints from day 10 to 14 in adult-derived cells cultured with TSA because V γ 3 signal joints reciprocally decreased (Fig. 2 B). In fetal-derived thymocytes, high levels of V γ 3 coding and signal joints were detected until day 7 irrespective of TSA treatment, and then signal joints were rapidly decreased and coding joints were also gradually reduced without TSA. However, TSA treatment maintained high levels of V γ 3 coding joints up to day 14 despite the rapid loss of signal joints, indicating that TSA prolonged the growth of V γ 3-rearranged cells. This explains the apparent increase of V γ 3⁺ population in fetal-derived thymocytes detected by the flow cytometric analysis (Fig. 2 A).

Accessibility at V γ 3 Is Increased by TSA in Adult-derived Thymocytes. VDJ recombination is initiated by cleavages at RSSs. We thus assessed whether TSA induces cleavages at V γ 3 RSS in adult-derived thymocytes. To this end, we analyzed V γ signal broken ends (SBEs) in organ-cultured thymocytes by ligation-mediated PCR (18, 23; Fig. 3 A). SBEs are recombination intermediates produced on cleavage at RSSs by the RAG proteins, which directly reflect the accessibility of RSSs (1, 2). Both V γ 2 and V γ 3 SBEs were detected in E16 and fetal liver-derived thymocytes. In contrast, V γ 3 SBEs were hardly detectable in adult TN thymocytes and adult-derived thymocytes without TSA, whereas V γ 2 SBEs were readily detected. By TSA treatment, however, V γ 3 SBEs were strongly induced in adult-derived cells. This is not due to accumulation by inhibiting joining process because the signal joints were concomitantly increased (Fig. 2 B). These results demonstrate that TSA treatment induces initiation of the recombination event by increasing the accessibility at V γ 3 RSS in adult-derived thymocytes.

To further confirm the TSA-induced accessibility of V γ 3 chromatin, we also analyzed germline transcripts of V γ genes in organ-cultured thymocytes by RT-PCR (Fig. 3 B). V γ 3 but not V γ 2 germline transcripts were specifically

reduced in adult-derived cells cultured for 7 d without TSA by threefold compared with fetal-derived cells. TSA treatment augmented V γ 3 germline transcript level three- to fourfold. Kinetic analysis clearly showed three- to fourfold enhancement of V γ 3 germline transcription by TSA from day 7 to 14 in adult-derived cells (Fig. 3 C). This is in good agreement with the kinetics of V γ 3 SBEs (Fig. 3 A). V γ 2 germline transcripts also increased in parallel with SBEs from day 7 to 10 irrespective of TSA treatment, although TSA slightly enhanced germline transcription (Fig. 3 C). In striking contrast to adult-derived cells, fetal-derived thymocytes exhibited prominent germline transcripts and SBEs of both V γ genes in the early phase of culture (Fig. 3, A and C), indicating that chromatin is accessible at both V γ genes in fetal-derived cells. These data demonstrate that TSA specifically alters V γ 3 chromatin from inaccessible to accessible state in adult-derived thymocytes.

Histone Acetylation at V γ 3 Is Increased by TSA in Adult-derived Thymocytes. If TSA induces accessibility by directly increasing histone acetylation, the acetylation level at V γ 3 should be elevated by TSA treatment. To confirm this, we

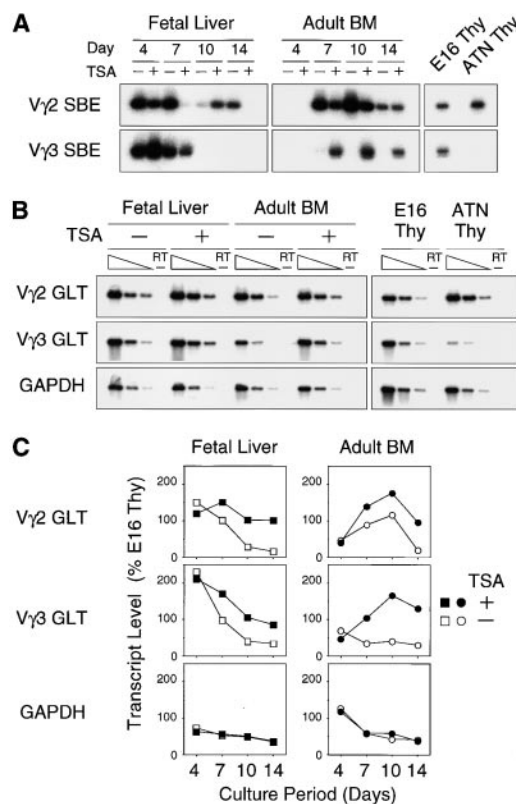


Figure 3. Accessibility at V γ 3 is increased by TSA in adult-derived thymocytes. (A) Ligation-mediated PCR analysis of organ-cultured thymocyte DNA used in Fig. 2 B to measure SBEs at V γ 2 and V γ 3. (B) RT-PCR analysis of RNA prepared from fetal liver- and adult bone marrow (BM)-derived thymocytes cultured for 7 d with (+) or without (-) TSA, and from E16 and adult TN (ATN) thymocytes (Thy). Fivefold serial dilutions of cDNAs and reaction without reverse transcriptase (RT⁻) were amplified for V γ 2, V γ 3 GLTs, and GAPDH transcripts. (C) Kinetic analysis of transcript levels in organ-cultured thymocytes. Transcript levels measured as in B are shown as the ratio to those in E16 thymocytes.

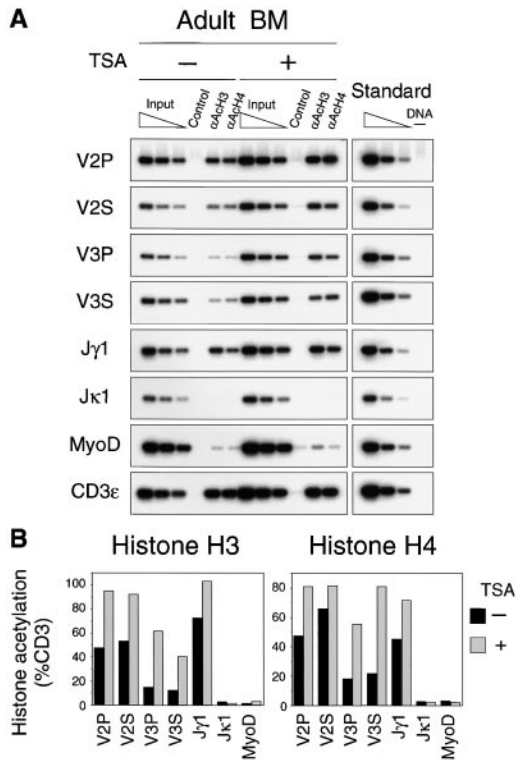


Figure 4. Histone acetylation at V γ 3 is increased by TSA in adult-derived thymocytes. (A) Histone acetylation measured by ChIP assay in adult bone marrow (BM)-derived thymocytes cultured for 15 days with (+) or without (-) TSA. ChIP and PCR analysis were performed as in the legend to Fig. 1 except that twofold serial dilutions of input DNA were amplified. (B) Histone acetylation levels in thymocytes cultured with (shaded bars) or without (black bars) TSA measured in A are shown as relative histone acetylation levels to that of the CD3 ϵ locus.

assessed histone acetylation of V γ 3 chromatin in organ-cultured thymocytes by ChIP assay (Fig. 4). Adult bone marrow-derived thymocytes cultured without TSA showed low levels of histone H3 and H4 acetylation at V γ 3, whereas modest acetylation could be detected at V γ 2 and J γ 1. TSA increased acetylation of both histone H3 and H4 at V γ 3 by three- to fourfold. Acetylation levels at V γ 2 and J γ 1 were slightly elevated, suggesting that TSA affects histone acetylation along the entire TCR- γ locus. Nevertheless, the most striking effect was observed at V γ 3, consistent with the specific enhancement of cleavage and germline transcription at V γ 3. Although elevated acetylation at V γ 3 promoter may simply reflect the increased V γ 3 rearrangements, similar enhancement was observed for acetylation at V γ 3 RSS as well as V γ 3 germline transcription that were derived from unrearranged alleles. These data collectively indicate that TSA indeed increases histone acetylation and accessibility of V γ 3 chromatin, thereby inducing V γ 3 rearrangements in adult bone marrow-derived thymocytes.

The accessibility model for V(D)J recombination predicts that specific modifications of chromatin at RSSs regulate cleavage by RAG proteins in a lineage- and stage-specific manner (3–5). Recent biochemical studies revealed

that packaging of RSSs into mononucleosomes severely inhibited cleavage in vitro (24, 25) and that histone acetylation did not stimulate cleavage (25) despite the functional involvement of histone acetylation was suggested in the accessibility control in vivo (8). Thus, it has been postulated that histone acetylation may regulate accessibility in vivo depending on the higher order chromatin structures or chromatin remodeling activities that are not reproduced in vitro (8). We demonstrate here that inhibition of histone deacetylation leads to elevated histone acetylation, accessibility, and rearrangement of the V γ 3 gene. Our data thus provide evidence that histone acetylation is the chromatin modification that triggers all the in vivo processes that induce accessibility, possibly including chromatin remodeling. During the preparation of our manuscript, several studies supporting our conclusion have been reported. Histone deacetylase inhibition by TSA also stimulated V(D)J recombination in cultured cell line (26) and thymocytes of the TCR- β enhancer-deleted mice (27). Moreover, hSWI/SNF-mediated chromatin remodeling was shown to act in concert with histone acetylation to stimulate cleavage on mononucleosomal DNA (28).

What is the mechanism underlying differential acetylation and accessibility in the V γ regions? The entire TCR- γ locus is hyperacetylated and accessible in fetal thymocytes, whereas V γ 3 chromatin specifically becomes hypoacetylated and inaccessible in adult thymocytes. Inhibition of histone deacetylation by TSA increased transcription and accessibility at V γ 3, despite the fact that TSA does not cause the widespread gene activation (29). These data can not be attributed to the selective loss of factors inducing accessibility at V γ 3 in adult thymocytes. Rather, they strongly suggest that an active mechanism must exist to reduce the localized accessibility at V γ 3. This idea is consistent with the previous report that the V γ 3 upstream sequence, when swapped with the V γ 2 upstream sequence in transgenes, reduced rearrangements of the V γ 2 gene in adult thymocytes (30). A specific factor may bind to the V γ 3 upstream sequence and recruit a histone deacetylase activity, which reduces histone acetylation and accessibility at V γ 3 chromatin. Once established, such inaccessible chromatin probably plays a dominant role over the factors inducing accessibility. Thus, our data imply that establishment of inaccessible chromatin by targeted histone deacetylation may also serve as an active and dynamic mechanism for other developmentally regulated processes such as allelic exclusion in the Ig heavy chain and TCR- β loci.

We thank Drs. T. Kina, K. Kuroda, H. Kurooka, K. Maki, E. Matsuda, C. Murre, W.J. Romanow, and Y. Yokota for materials and discussion; and Ms. S. Hirano, M. Tanaka, T. Taniuchi, Y. Tabuchi, T. Toyoshima, and S. Nomura for their technical assistance.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

Submitted: 6 December 2000

Revised: 16 January 2001

Accepted: 24 January 2001

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