

Pax5 Activates Immunoglobulin Heavy Chain V to DJ Rearrangement in Transgenic Thymocytes

Lih-Yun Hsu,¹ Hong-Erh Liang,¹ Kristen Johnson,² Chulho Kang,¹ and Mark S. Schlissel¹

¹University of California, Berkeley, Division of Immunology, Department of Molecular and Cell Biology, Berkeley, CA 94720

²Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, NY 10032

Abstract

Mice deficient for the B cell-restricted transcription factor Pax5 show a defect in the V_H to DJ_H rearrangement step of immunoglobulin heavy chain gene assembly even though the expression of the V(D)J recombinase is not diminished in Pax5^{-/-} pro-B cells. To investigate whether Pax5 is limiting for V_H to DJ_H rearrangement, we generated transgenic mice which express Pax5 in developing thymocytes. We show that enforced expression of Pax5 in thymocytes results in a partial block in T cell development due to defective pre-TCR signaling in β -selection. Moreover, our results demonstrate that expression of Pax5 in early thymocytes is sufficient to induce V_H to DJ_H rearrangements in CD4⁺CD8⁺ T cells and lead us to suggest that Pax5 may play a direct role in the lineage-specific regulation of immunoglobulin heavy chain gene rearrangement.

Key words: lymphocyte development • V(D)J recombination • gene expression regulation • transgenesis • immunoglobulin

Introduction

V(D)J recombination is regulated in several ways (1). First, it is lineage specific, with Ig genes completely rearranging only in B cells and TCR genes completely rearranging only in T cells. Second, there is a preferred order to the rearrangement process with Ig heavy chain (IgHC) locus preceding Ig light chain (IgLC) locus rearrangement in B cells and TCR β locus preceding TCR α locus rearrangement in T cells. Also, in both the IgHC and TCR β loci D to J rearrangement precedes V to DJ rearrangement and direct V to D rearrangement occurs rarely if at all. Finally, the gene rearrangement process at most loci is subject to allelic exclusion. That is, an individual B or T cell makes only one productive (in frame) rearrangement at each locus, resulting in its ability to produce only a single Ig or TCR molecule.

Regulated changes in chromatin structure underlie most if not all aspects of substrate choice by the V(D)J recombinase (2). This idea, known as the “accessibility hypothesis,” arose from initial observations which correlated the transcription of unrearranged gene segments with their activation

for rearrangement. It was shown subsequently that mutations which eliminate promoters or enhancers associated with rearranging loci greatly diminish rearrangement of the altered locus (1). Additional work has implicated specific transcription factors in the regulation of recombinase accessibility. For example, overexpression of either E47 or EBF can activate chromosomal Ig gene rearrangement in nonlymphoid cells transfected with RAG1 and RAG2 (3). Furthermore, mice with a null mutation in the Pax5 gene, which encodes the B cell-restricted transcription factor Pax5 (BSAP), show a profound block in B cell development at the pro-B cell stage (4). Most Pax5-null pro-B cells contain DJ_H but not VDJ_H rearrangements at their IgHC loci, leading to the suggestion that Pax5 is necessary to fully activate the V_H to DJ_H step in IgHC gene assembly.

Two other aspects of the regulation of IgHC gene rearrangement focus particular interest on the V to DJ step. First, this is the step which is subject to allelic exclusion. Nearly all developing B cells undergo D to J rearrangement on both HC alleles, but only one of these alleles undergoes

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Address correspondence to Mark S. Schlissel, Div. of Immunology, Dept. of Molecular and Cell Biology, UC Berkeley, 439 LSA (#3200), Berkeley, CA 94720. Phone: (510) 643-2462; Fax: (510) 642-6845; email: mss@uclink4.berkeley.edu

Abbreviations used in this paper: BSAP, B cell-restricted transcription factor Pax5; DN, CD4/CD8 double negative T cells; DP, CD4/CD8 double positive T cells; IgHC, Ig heavy chain; IgLC, Ig light chain; RSS, recombination signal sequence; LM, ligation-mediated.

productive V to DJ rearrangement. Furthermore, IgHC transgenic mice show normal D to J rearrangement but greatly diminished V to DJ rearrangement at their endogenous IgHC loci (1). Finally, this is the step which shows strict lineage specificity. IgHC D to J rearrangement occurs frequently in developing T cells (5), but V to DJ rearrangement is limited to developing B cells.

Given the fact that the state of IgHC rearrangement in *Pax5*-null pro-B cells is similar to that in normal developing T cells and that developing T cells do not express Pax5, we hypothesized that T cells perform D_H to J_H but not V_H to DJ_H rearrangement because Pax5 is absolutely required for the latter step in IgHC gene assembly. To test this idea, we have generated and analyzed Pax5 transgenic mice which express this B lineage transcription factor in developing thymocytes.

Materials and Methods

Antibodies and Flow Cytometry. The following antibodies were purchased from either BD Biosciences or CALTAG Laboratories: FITC-anti-CD3 ϵ (145-2C11), -CD4 (RM4-5), -CD8 α (53-6.7), -Thy1.2 (CD90.2) (53-2.1); PE-anti-CD25 (PC61), -CD4 (RM4-5), -CD19 (1D3); CyChrome-anti-CD44 (IM7); and bi-anti-CD4 (RM4-5), -CD8 α (CT-CD8 α). For analysis, live cells were gated based on forward and side scatter, and over 100,000 events were collected. CD4/CD8 double negative T cell (DN) and CD4/CD8 double positive T cell (DP) thymocytes were purified with an AutoMACS magnetic cell sorter (Miltenyi) as described (6) or using a MoFlo cell sorter (Cytometrix). Routinely, purity of all cell preparations was >95% unless otherwise specified in the text.

Rearrangement PCR and Ligation-mediated-PCR. 100 ng of genomic DNA from DP or pre-B (CD43⁻B220⁺IgM⁻) cells was used to assay for IgHC gene rearrangements. PCR assays for D_H to J_H and V_H to DJ_H rearrangements were performed as described (7). The locus-specific PCR primers and probes used for ligation-mediated (LM)-PCR were described previously (8, 9).

PCR Fragment Length Polymorphism. Total thymocytes were first depleted of DP and SP populations by incubation of cell suspensions with biotinylated antibodies specific for CD4, CD8, and CD3 ϵ followed by negative selection using streptavidin-conjugated microbeads (Miltenyi). DN cells were further stained with PE-conjugated anti-CD25 and CyChrome-conjugated anti-CD44 antibodies. CD44⁻CD25⁺ DN3 cells were purified using a MoFlo cell sorter (Cytometrix). PCR amplification of the TCR β CDR3 region using specific V and J primers was performed essentially as described (10).

Online Supplemental Material. Fig. S1 shows Western blot and RT-PCR analyses of transgene expression. Fig. S2 displays CD19 expression in Pax5 transgenic thymus. Fig. S3 shows RT-PCR analysis of gene expression in Pax5 transgenic thymus. Fig. S4 shows CDR3-length analysis of VDJ β rearrangements in Pax5 transgenic and control thymocytes. Fig. S5 shows VDJ H rearrangement in Pax5 transgenic thymocytes from *Ig β ^{-/-}* mice. In addition, Supplemental Materials and Methods are provided regarding the generation of the transgenic construct, the performance of RT-PCR, and the analysis of transgene expression by Western blot. Figs. S1–S5 and Supplemental Materials and Methods are available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>.

Results and Discussion

Impaired T Cell Development in CD2EP-Pax5 Transgenic Mice. A murine *Pax5* cDNA, cloned in between the human CD2 promoter and its locus control region, was used to generate transgenic mice by pronuclear injection (11). Analysis by Western blot of whole cell thymic extracts from multiple founder lines showed that in two founders the expression level of Pax5 appeared comparable to that in the BM (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>).

In three independent lines of CD2EP-Pax5 transgenic mice, we observed an approximately threefold decrease in thymocyte numbers compared with those of control littermates (Fig. 1 B, data shown from only two founders). Analysis of CD4/CD8 expression revealed a fivefold increase in

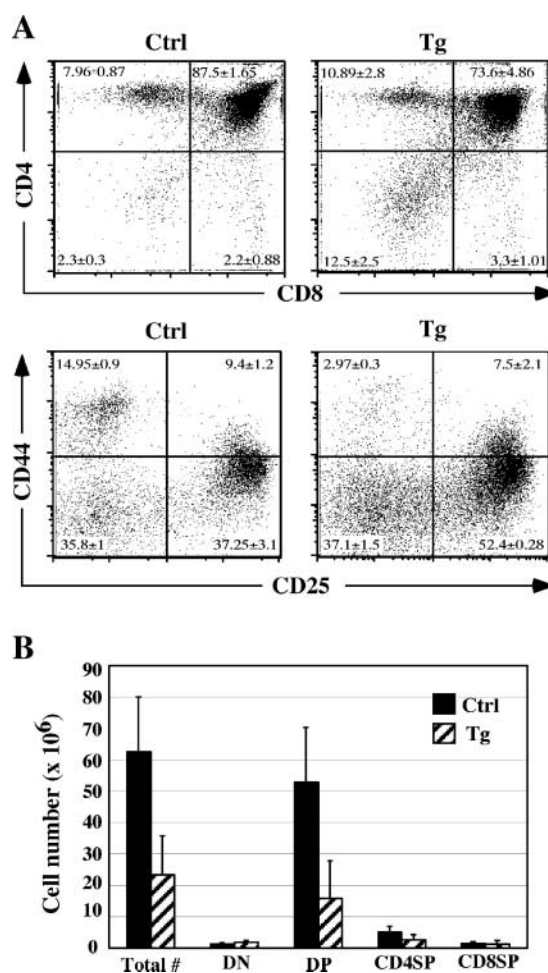


Figure 1. Impaired T cell development in CD2EP-Pax5 transgenic mice. (A) FACS[®] analysis of CD4 and CD8 expression on thymocytes from 4-wk-old littermate control or transgenic mice (top). Electronically gated CD3⁻CD4⁻CD8⁻ DN thymocytes from 2-wk-old control or transgenic mice were stained with anti-CD25 and anti-CD44 antibodies (bottom). Percentages represent the means and SDs of live-gated subpopulations from six mice of each genotype. (B) Absolute cell numbers were calculated for total thymocytes and the thymocyte subsets described in A (top). The error bars represent mean ± SD values for control and transgenic mice ($n = 12$).

the percentage of DN thymocytes and a slight decrease in the percentage of DP thymocytes in the transgenic mice compared with littermate controls (Fig. 1 A, top). Interestingly, the absolute number of DN thymocytes in transgenic mice remained similar to that in control littermates, whereas the number of DP thymocytes was considerably decreased (Fig. 1 B). We went on to examine DN thymocyte populations for CD44 and CD25 expression and observed a small increase in the proportion of CD44⁻CD25⁺ (DN3) cells and a decrease in the proportion of CD44⁺CD25⁻ (DN1) cells in the transgenic mice (Fig. 1 A). The decrease in DN1 cells may be due to expression of Pax5 in a fraction of early thymic progenitors leading to their inefficient entry into the pool of developing thymocytes (12). Collectively, these observations indicate that ectopic expression of Pax5 in early thymocytes leads to a partial developmental block at the DN to DP transition.

Appearance of Rare CD19⁺ Cells in the Thymus of CD2EP-Pax5 Transgenic Mice. Given that ectopic Pax5 expression inhibits T cell development and B cell commitment depends on Pax5 (12), we asked whether overexpression of Pax5 in early thymocyte precursors promotes intrathymic B cell development. We found a small fraction (~1–3%) of CD19⁺ cells in the thymus of transgenic mice but not in control littermates (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>). The majority of the CD19⁺ cells from founder #10 coexpress CD4 and CD8 or Thy1.2 (not depicted), whereas CD19 is not coexpressed with CD4 and CD8 on cells from founder #2. The level of CD19 expression on thymocytes from founder #10 is clearly greater than that expressed on littermate controls but far less than that expressed on wild-type splenic B cells (Fig. S2 C).

To test whether these T cells also express other B lineage markers, we purified DP thymocytes by flow cytometry from either transgenic or control mice and used RT-PCR to assay for expression of early B cell-related molecules. We found that Pax5 transgenic DP T cells express the direct Pax5 targets CD19 and BLNK (13) but do not express other early B cell-specific transcripts such as λ5, VpreB, and Igβ (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>). We conclude from these studies that Pax5 expression in thymocytes activates direct Pax5 targets but does not activate the full genetic program of early B cell development.

These and more detailed analyses failed to reveal a quantitative increase in B cell development in the BM or the induction of significant amounts of B cell development in the thymus (not depicted). These findings are in contrast to those from a recently reported study of knock-in mice in which Pax5 was placed under the control of the *Ikaros* locus (12). In these mice, Pax5 expression in hematopoietic stem cells resulted in an extreme skewing of lymphopoiesis toward the B cell lineage. T cell development was blocked, presumably due to the repression of Notch1 transcription (12). The discrepancy between our results and those of Souabini et al. could be due to a difference in the onset of

ectopic Pax5 expression. It was shown previously that expression of a similar hCD2 promoter/locus control region-driven transgene is first detected at the DN1 stage of T cell development in a heterogeneous fashion but that transgene expression does not become uniform in all thymocytes until the DN3 to DN4 transition (11). Thus, it seems likely that commitment to the T cell lineage takes place before the expression of our CD2 promoter-driven Pax5 transgene. In distinction to the previous study, the relatively later onset of Pax5 expression in our transgenic system allows one to assess the effects of Pax5 expression after the onset of early T cell development.

Ectopic Pax5 Expression in the Thymus Inhibits β-Selection. Transition from the DN to DP stage, often referred to as β-selection, is accompanied by various phenotypic changes, including cell cycle entry and selection for in-frame TCRβ rearrangement (14). To test whether the increased percentage of DN cells in the thymus of transgenic mice was a consequence of faulty β-selection, we first analyzed DN3 (CD25⁺CD44⁻) cells for forward scatter as an indirect marker of cell cycle activation. Strikingly, we found a significant reduction in the percentage of large, presumably cycling cells within the DN3 population of Pax5 transgenic mice (Fig. 2 A). To test whether this defect was due to deficient TCRβ locus rearrangement, we measured recombination reaction intermediates (broken signal ends) in sorted DN3 and DN4 cells from control and transgenic mice (Fig.

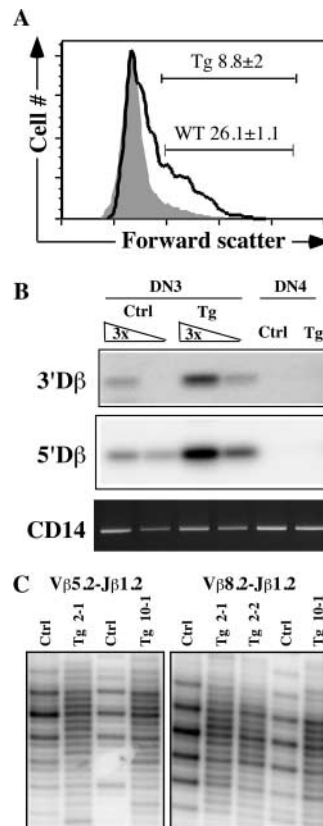


Figure 2. Pax5 expression in the thymus affects β-selection but not TCRβ gene segment rearrangement. (A) Gated DN3 (CD44⁻CD25⁺) cells were analyzed for forward scatter. The results are displayed as overlapping histograms of control (WT, unshaded) and transgenic (Tg, shaded) thymocytes. The percentage of large cycling cells is indicated, including the mean and SD ($n = 3$). (B) Linker-ligated DNAs from control and transgenic DN3 and DN4 T cells were subjected to LM-PCR with primers specific for the detection of double stranded breaks at the RSSs 3' or 5' of Dβ1 as indicated. CD14 is a nonrearranging locus control PCR. (C) PCR fragment length polymorphism analysis of the CDR3 region of fully rearranged TCRβ alleles using template DNA purified from sorted DN3 cells from control (Ctrl) or transgenic (Tg) mice (some assays used two independent samples of founder #2 DNA). Results of analyses focusing on Vβ5.2 or 8.2 to Jβ1.2 rearrangements are shown. Similar results were obtained using a downstream primer specific for Jβ2.1 (not depicted).

2 B). We found that dsDNA breaks at both the 3' and 5' of D β 1 RSSs, which are associated with D β to J β and V β to DJ β rearrangements, respectively, appear slightly increased in DN3 cells from transgenic mice compared with littermate controls. Thus, the lower percentage of large cycling cells in transgenic thymocytes cannot be explained by deficient assembly of genes encoding the TCR β chain.

To determine whether the defect in β -selection might be due to a generalized disruption of the transcriptional program in progenitor T cells associated with the expression of this B lineage transcription factor, we used real-time RT-PCR to compare the levels of *preT α* , *Notch1*, and *LEF-1* mRNA in DN thymocytes from transgenic and control mice (Fig. S3 C). No significant differences were observed, indicating that ectopic Pax5 expression does not affect expression of these critical genes.

Signals derived from the pre-TCR complex result in clonal expansion of thymocytes with functionally rear-

ranged TCR β chains (14). To determine whether ectopic Pax5 expression disrupts this aspect of β -selection, we used a PCR fragment-length polymorphism assay to assess both TCR β CDR3 length heterogeneity and the distribution of in-frame rearrangements in sorted DN3 cells from control and transgenic mice. The imprecise nature of V(D)J joining results in a continuous distribution of CDR3 amplicon fragment lengths. Rapid cell division among cells with a productive TCR β rearrangement results in the enrichment of open reading frames within TCR β . Using primers that amplify across the V(D)J junction within TCR β alleles, PCR products generated using genomic DNA from control DN3 cells showed a length distribution with a periodicity of three nucleotides, which is indicative of enrichment for productive TCR β rearrangements (Fig. 2 C and Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>). In contrast, CDR3 length distribu-

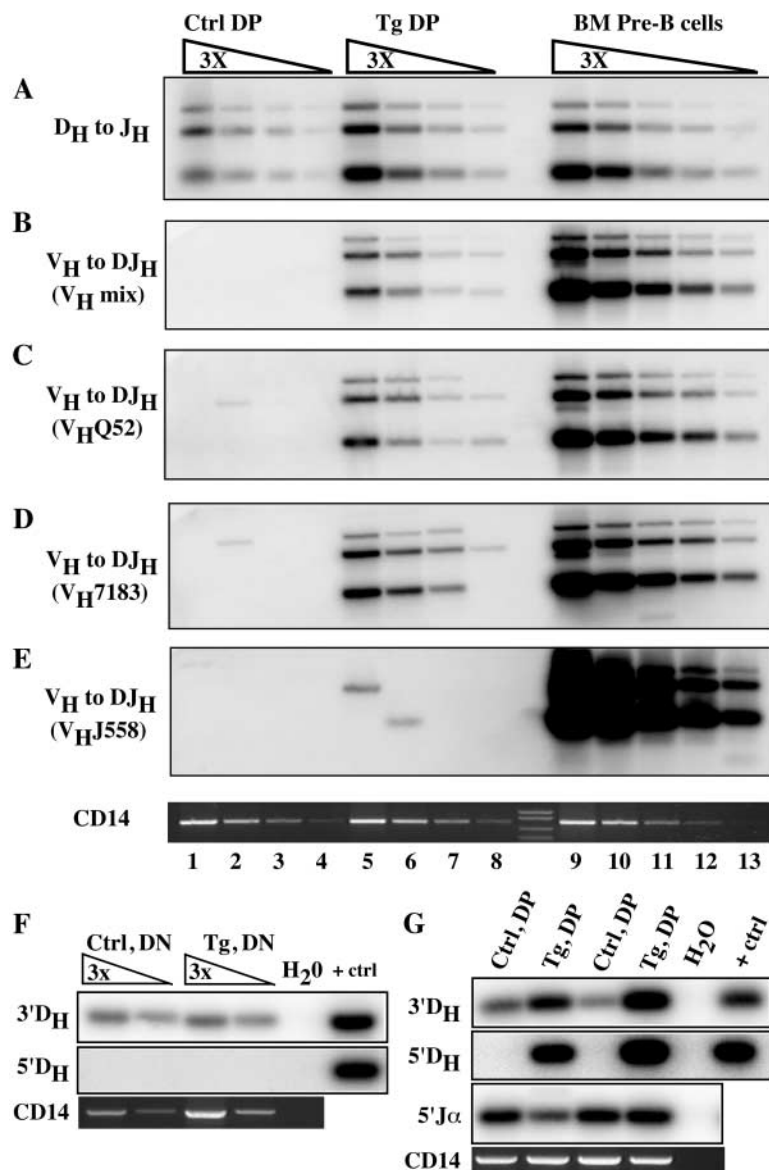


Figure 3. Ectopic Pax5 expression induces V $_H$ to DJ $_H$ rearrangement in transgenic DP thymocytes. Threefold serially diluted genomic DNAs from FACS[®]-sorted control DP thymocytes (lanes 1–4), transgenic DP thymocytes (lanes 5–8), or wild-type BMpre–B cells (B220⁺CD43[–]IgM⁺) (lanes 9–13) were subjected to PCR analysis for D $_H$ to J $_H$ (A) and V $_H$ to DJ $_H$ (B–E) gene rearrangements. An equimolar mixture of degenerate V $_H$ J558, V $_H$ 7183, and V $_H$ Q52 family-specific primers (V $_H$ mix) paired with a unique primer hybridizing 3' of J $_H$ 3 was used in B, whereas each degenerate V $_H$ primer was used in isolation in C–E as indicated. Genomic DNA samples purified from sorted DN (F) and DP (G) thymocytes from two independent samples of control and Pax5 transgenic mice were analyzed by LM-PCR to detect broken signal end recombination intermediates associated with D $_H$ to J $_H$ rearrangement (3'D $_H$, top), V $_H$ to DJ $_H$ rearrangement (5'D $_H$, middle), and V $_\alpha$ to J $_\alpha$ rearrangement (5'J $_\alpha$, bottom). Amplification of a nonrearranging control locus (CD14) was used as a DNA recovery control. H $_2$ O denotes reactions lacking DNA template and + ctrl denotes control assays which contained BM DNA.

tion in DN3 cells from transgenic mice was relatively random, suggesting a defect in β -selection. We hypothesize that defects in pre-TCR signaling in *Pax5* transgenic mice may result from up-regulation of BLNK and CD19 expression in early thymocytes. These components of the B cell signaling machinery may interfere with the function of the T cell-specific adaptors SLP-76 or LAT. Additional work will be required to test this hypothesis.

Pax5 Expression Is Sufficient to Induce V_H to DJ_H Recombination at the IgHC Locus. To test whether *Pax5* plays an important role in controlling the B cell-specific V_H to DJ_H rearrangement step of IgHC gene assembly, we examined the rearrangement status of the IgHC locus in sorted DP thymocytes from transgenic and control mice using a direct PCR assay (7). The purity of these sorted cells was >98%. Consistent with earlier work (5), frequent D_H to J_H rearrangements were readily detectable in both BM pre-B cells and DP thymocytes from either transgenic or control mice (Fig. 3 A). Interestingly, although the DP thymocytes from control mice lacked V_H to DJ_H rearrangements, these rearrangements were readily detectable in DP thymocytes from transgenic mice (Fig. 3 B). By comparing PCR signals between transgenic DP thymocytes and sorted BM pre-B cells from wild-type mice, we estimate the frequency of V_H to DJ_H rearrangements in DP *Pax5* transgenic thymocytes to be ~5–10% that of BM pre-B cells (Fig. 3 B).

This observation was further supported by examination of V(D)J recombination reaction intermediates using a previously described LM-PCR approach. Double strand DNA breaks at RSSs 5' of D_H , which represent intermediates in the V_H to DJ_H rearrangement step, were detected in DNA purified from the DP thymocytes of transgenic but not control mice, whereas DNA breaks associated with TCR α locus rearrangement were detected in all samples (Fig. 3 G). Interestingly, we failed to detect 5' of D_H broken ends in DN thymocytes from either control or transgenic mice (Fig. 3 F), leading us to conclude that *Pax5*-induced V_H to DJ_H rearrangements occur mainly during the DP stage of T cell development. In contrast, 3' of D_H breaks were detected in DN and DP cells from both transgenic and control mice (Fig. 3, F and G). The level of these breaks was greater in DP samples from the transgenic samples, corresponding to greater levels of DJ_H joints observed by direct PCR (Fig. 3 A). We obtained similar results when we crossed the CD2EP-*Pax5* transgene onto a *Ig β ^{-/-}* genetic background where B cell development is blocked at an early stage (15) (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20032249/DC1>). This led us to conclude that contaminating B cells could not account for the V_H to DJ_H rearrangements observed in transgenic thymocytes. In summary, our results indicate that expression of *Pax5* alone in early thymocytes is sufficient to induce V_H to DJ_H rearrangement in T lineage cells.

It was reported recently that pro-B cells from *Pax5*-deficient mice show a far more significant defect in rearrangement of the D_H -distal V_H J558 gene family relative to that of the more D_H -proximal V_H gene families (16). Therefore, we used PCR assays to separately examine the use of

V_H J558, V_H 7183, and V_H Q52 gene segments. As shown in Fig. 3, C and D, the V_H 7183 and V_H Q52 gene families accounted for the vast majority of V_H to DJ_H rearrangements in DP thymocytes from transgenic mice. In striking contrast, rearrangements using the V_H J558 gene family were barely detectable in transgenic thymocytes (Fig. 3 E). In addition, we were able to detect V_H 7183 but not V_H J558 gene segment transcripts in transgenic DP thymocytes (not depicted). Thus, our findings suggest that the extent of V_H to DJ_H rearrangement induced by ectopic *Pax5* expression in the thymus progressively decreases as the distance of a V_H gene family from the D_H cluster increases. However, the results of Hesslein et al. (16) suggest just the opposite—that rearrangement of the distal V_H J558 gene family in pro-B cells is far more dependent on *Pax5* than rearrangement of the more proximal V_H gene families. These differences may be due to differential expression of any of a large number of other transacting factors in B and T cell progenitors.

Given the fact that pro-B cells from *Pax5*^{-/-} mice display amounts of V_H J558 germline transcription and histone acetylation that are indistinguishable from wild-type pro-B cells, these workers (16) concluded that *Pax5* regulates the accessibility of V_H J558 gene segments at a level distinct from germline transcription and histone acetylation. Although histone acetylation appears to correlate well with accessibility of the V(D)J recombinase in many cases (17–19), we were also unable to detect changes in H3 or H4 acetylation which correlated with the *Pax5*-dependent activation of V_H to DJ_H rearrangement in DP transgenic thymocytes (not depicted). Surprisingly, we did find that the distal V_H J558 family of IgHC gene segments was relatively hyperacetylated (although not to the same level as seen in pro-B cells) in both control and *Pax5* transgenic thymus even though they were not found to be accessible to the recombinase in either instance (not depicted). Thus, it appears that variations in histone acetylation may not in a simple fashion explain the selectivity of V_H accessibility. Additional work will be required to determine whether other specific modifications more closely track with V_H gene segment accessibility.

It is possible that the preferential rearrangement of D_H -proximal V_H gene segments observed in the DP transgenic thymocytes is a result of the IL-7 dependence of distal V_H gene segment rearrangement. Several labs have shown that the IL-7 signaling pathway is involved in promoting local accessibility of V_H J558 genes (18, 20). Expression of the IL-7R α chain is low at the DP stage of T cell development (21). Perhaps V_H J558 genes are inactive at the DP stage because low levels of IL-7R α expression make these DP thymocytes poorly responsive to IL-7. Therefore, it is possible that IL-7R signaling and *Pax5* cooperate in the control of distal V_H gene segment recombination. However, given the phenotypic similarity between *IL-7R α ^{-/-}* and *Pax5*^{-/-} mice and the observation that *Pax5* transcript levels are decreased in *IL-7R α ^{-/-}* BM cells (20), we cannot rule out the possibility that *Pax5* acts as a downstream target of IL-7R signaling in developing B cells.

This work shows that Pax5 expression is sufficient to activate V_H to DJ_H rearrangement in T cells and by inference may play a similar role in B cells. Evidence for the involvement of specific transcription factors in modulating the accessibility of specific gene segments to the recombination machinery is scarce. In one study, STAT5 was shown to bind to and promote recombinase accessibility of the TCR γ locus (22). Several studies have implicated E2A proteins (E12 and E47) in the regulation of both Ig and TCR gene segment recombination (3, 23, 24). These effects might depend on the known interaction between transcription factors and chromatin remodeling complexes. However, we have been unable to convincingly demonstrate direct Pax5 interaction with V_H gene segments using a ChIP approach. Thus, further studies on the interactions of various chromatin-modifying complexes with V_H region chromatin in transgenic mice should provide a better understanding of how Pax5 facilitates targeting of recombination machinery to specific V_H gene segments.

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