Pax5 induces *V*-to-*DJ* rearrangements and locus contraction of the *immunoglobulin heavy-chain* gene

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The subnuclear location and chromatin state of the *immunoglobulin heavy-chain* (*IgH*) locus have been implicated in the control of *V(D)J* recombination. V_H-to-DJ_H rearrangement of distal, but not proximal V_H genes, furthermore, depends on the B-lineage commitment factor Pax5 (BSAP). Here we demonstrate that ectopic *Pax5* expression from the *Ikaros* promoter induces proximal rather than distal V_H–DJ_H rearrangements in $Ik^{Pax5/+}$ thymocytes, thus recapitulating the loss-of-function phenotype of $Pax5^{-/-}$ pro-B cells. The phenotypic similarities of both cell types include (1) chromatin accessibility of distal V_H genes in the absence of V_H–DJ_H rearrangements, (2) expression of the B-cell-specific regulator EBF, (3) central location of *IgH* alleles within the nucleus, and (4) physical separation of distal V_H genes from proximal segments in an extended *IgH* locus. Reconstitution of *Pax5* expression in $Pax5^{-/-}$ pro-B cells induced large-scale contraction and distal V_H–DJ_H rearrangements of the *IgH* locus. Hence, V_H–DJ_H recombination is regulated in two steps during early B-lymphopoiesis. The *IgH* locus is first repositioned from its default location at the nuclear periphery toward the center of the nucleus, which facilitates proximal V_H–DJ_H recombination. Pax5 subsequently activates locus contraction and distal V_H–DJ_H rearrangements in collaboration with an unknown factor that is present in pro-B cells, but absent in thymocytes.

[*Keywords*: Pax5/BSAP; V_H–DJ_H recombination; immunoglobulin heavy-chain; locus contraction; subnuclear location; T cells]

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V(D)J recombination is of fundamental importance for the generation of diverse antigen receptor repertoires, as this process assembles the variable regions of immunoglobulin (*Ig*) and T-cell receptor (*TCR*) genes from discontinuous variable (*V*), diversity (*D*), and joining (*J*) gene segments during B- and T-cell development (Tonegawa 1983; Hesslein and Schatz 2001). All of these gene segments are flanked by conserved recombination signal sequences (RSSs) that constitute recognition sites for the V(D)J recombinase proteins RAG1 and RAG2 (Hesslein and Schatz 2001; Bassing et al. 2002). Upon binding and synapsis of two compatible RSS sites, the RAG1/2 complex introduces double-strand DNA breaks between the RSSs and flanking gene segments. Subsequently, the RAG proteins and repair factors of the nonhomologous end-joining machinery complete the recombination reaction by processing and religating the DNA ends (Hesslein and Schatz 2001; Bassing et al. 2002).

V(D)J recombination takes place only in lymphocytes, where it is tightly controlled in a lineage- and stage-specific manner. Within the B-lymphoid lineage, the *immunoglobulin heavy-chain* (*IgH*) locus is rearranged in pro-B cells prior to recombination of the *Ig*κ and *Ig*λ *light-chain* genes in pre-B cells, whereas the *TCR*β and *TCR*α genes are rearranged in pro-T and pre-T cells, respectively (Hesslein and Schatz 2001; Bassing et al. 2002). As the *RAG1* and *RAG2* genes are expressed in all lymphoid progenitors (Igarashi et al. 2002) and immature T cells and B cells, *V(D)J* recombination is primarily regulated by limiting the accessibility of RSS sites within chromatin (Yancopoulos and Alt 1985; Stanhope-Baker et al. 1996; Krangel 2003). As a consequence, only the sites of particular gene segments are available for RAG1/2-mediated synapsis and DNA cleavage in different cell types and developmental stages.

V(D)J recombination of the *IgH* gene occurs in a defined temporal order with D_H–J_H rearrangements preceding V_H–DJ_H recombination. The earliest lymphocyte

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progenitor (ELP; Igarashi et al. 2002) and later common lymphoid progenitor (CLP; Allman et al. 2003) already initiate D_H–J_H rearrangements, which are completed during development to the early pro-B-cell stage (fraction B; Li et al. 1993). As the ELP and CLP also give rise to T, NK, and dendritic cells (Kondo et al. 1997; Traver et al. 2000; Igarashi et al. 2002), it may not be surprising that these cell types carry D_H–J_H-rearranged *IgH* alleles at low frequency (Kurosawa et al. 1981; Born et al. 1988; Corcoran et al. 2003). Importantly, V_H–DJ_H rearrangements could never be observed in thymocytes and dendritic cells (Kurosawa et al. 1981; Corcoran et al. 2003), as the second *IgH* rearrangement step takes place only in committed pro-B cells (fractions B and C; Li et al. 1993). Successful V_H–DJ_H recombination leads to expression of the Igμ protein as part of the pre-B-cell receptor (pre-BCR), which acts as an important checkpoint to control the transition from the pro-B- to the pre-B-cell stage (Burrows et al. 2002).

The *IgH* locus with its 150–200 V_H genes spans a large chromosomal region of ~3 Mb pairs (Chevillard et al. 2002), which is likely to be an impediment for efficient synapse formation and *V(D)J* recombination of distantly separated *IgH* gene segments. A recent fluorescent in situ hybridization (FISH) analysis revealed that *V(D)J* recombination correlates with changes in the subnuclear location and chromatin state of the *IgH* locus (Kosak et al. 2002). Both *IgH* alleles are present in an extended chromatin configuration at the nuclear periphery of non-B-lymphoid cells, whereas they are relocated to central positions of the nucleus and undergo large-scale contraction in committed pro-B cells (Kosak et al. 2002). Subnuclear compartmentalization was thus proposed as a novel mechanism for regulating *IgH* transcription and recombination during B-cell development (Kosak et al. 2002), particularly because the nuclear periphery in higher eukaryotes may function as a repressive compartment for transcriptional silencing (Baxter et al. 2002) in analogy to yeast (Hediger and Gasser 2002).

Differentiation of the CLP to committed pro-B cells critically depends on the three transcription factors E2A, EBF, and Pax5 (BSAP; Schebesta et al. 2002a). All three factors are also essential for *V(D)J* recombination of the *IgH* gene during early B-cell development. The absence of E2A or EBF arrests B-cell development at the stage of $B220^+$ $CD43^+$ progenitor cells (fraction A) that contain the *IgH* locus still in germ-line configuration (Bain et al. 1994; Lin and Grosschedl 1995). Both transcription factors appear to control the initial D_H–J_H rearrangement step by activating the expression of *RAG1* and *RAG2* (Bain et al. 1994; Lin and Grosschedl 1995; O'Riordan and Grosschedl 1999) and by promoting the accessibility of the D_H–J_H region to the V(D)J recombinase (Romanow et al. 2000). In the absence of Pax5, B-lymphopoiesis proceeds in the bone marrow to early pro-B cells (fraction B; Urbánek et al. 1994; Nutt et al. 1997), which still retain a broad lympho-myeloid developmental potential characteristic of uncommitted progenitors (Nutt et al. 1999; Rolink et al. 1999). Restoration of *Pax5* expression in $Pax5^{-/-}$ pro-B cells suppresses this multilineage potential and rescues development to mature B cells, thus identifying Pax5 as the critical B-lineage commitment factor that restricts the developmental options of lymphoid progenitors to the B-cell pathway (Nutt et al. 1999). In addition, Pax5 controls the second, B-cell-restricted step of V_H–DJ_H recombination, as rearrangements of the distal (5′) V_HJ558 genes are ~50-fold reduced in $Pax5^{-/-}$ pro-B cells, although D_H–J_H recombination occurs at normal frequency (Nutt et al. 1997). Despite this defect, the distal V_H genes are present in accessible chromatin, as indicated by their germ-line transcription and histone H3 acetylation in $Pax5^{-/-}$ pro-B cells (Hesslein et al. 2003). Interestingly, the V_H–DJ_H recombination efficiency of $Pax5^{-/-}$ pro-B cells progressively increases with decreasing distance of the V_H gene to the proximal D_H–J_H region (Hesslein et al. 2003). Hence, Pax5 is essential for the rearrangement of distal but not proximal V_H genes.

Pax5 is expressed within the hematopoietic system exclusively from the pro-B- to the mature B-cell stage, consistent with its role as B-lineage commitment factor (Adams et al. 1992). We have recently complemented our Pax5 loss-of-function analyses with a gain-of-function approach by expressing a human *Pax5* minigene under the control of the endogenous *Ikaros* (*Ik*) locus in all blood cell types (Souabni et al. 2002). Pan-hematopoietic expression of the Ik^{Pax5} allele efficiently promotes B-cell development at the expense of T-lymphopoiesis. Whereas ectopic *Pax5* expression completely blocks T-cell development in chimeric mice reconstituted with wild-type and $Ik^{Pax5/+}$ bone marrow, thymocyte differentiation is reduced and abnormal under noncompetitive conditions in $Ik^{Pax5/+}$ mice (Souabni et al. 2002). At the molecular level, Pax5 down-regulates transcription of the T-cell specification gene *Notch1* and activates expression of the B-lymphoid gene *CD19* in thymocytes (Souabni et al. 2002).

By analyzing the rearrangement status of the *IgH* locus in $Ik^{Pax5/+}$ thymocytes, we now demonstrate that ectopic *Pax5* expression is sufficient to increase D_H–J_H recombination and to initiate V_H–DJ_H rearrangements in immature cells of the T-lymphoid lineage. Unexpectedly, the V_H–DJ_H recombination phenotype of Pax5-expressing thymocytes resembles that of Pax5-deficient pro-B cells. The distal V_HJ558 and V_H3609 genes are rearranged with very low efficiency in contrast to the proximal V_HQ52 and V_H7183 genes, although germ-line transcripts of the distal V_HJ558 genes could be readily detected in $Ik^{Pax5/+}$ pro-T cells as in $Pax5^{-/-}$ pro-B cells. Importantly, the expression of the B-cell-specific transcription factor EBF was induced to the same level in $Ik^{Pax5/+}$ pro-T cells as in $Pax5^{-/-}$ pro-B cells. FISH analysis of three-dimensionally preserved nuclei further extended the phenotypic similarity between $Ik^{Pax5/+}$ pro-T and $Pax5^{-/-}$ pro-B cells. The *IgH* alleles were centrally located in the nuclei of both cell types and were present in an extended chromatin state that physically separates the distal from the proximal V_H genes. In contrast, the *IgH* loci were localized at the nuclear periphery in wild-type pro-T cells. In Pax5-expressing pro-B cells, the *IgH*

loci were found in central nuclear positions and underwent large-scale contraction, as published (Kosak et al. 2002). Moreover, Pax5 induced *IgH* locus contraction and distal V_H–DJ_H rearrangements in retrovirally reconstituted $Pax5^{-/-}$ pro-B cells. Based on these data, we propose a two-step model for the transcriptional activation of V_H–DJ_H recombination in early B-cell development. The *IgH* locus is first relocated, possibly under the control of EBF, from the periphery to the center of the nucleus, thus facilitating V_H–DJ_H recombination within the proximal domain of the *IgH* locus. Subsequently, Pax5 activates large-scale contraction and distal V_H–DJ_H rearrangements of the *IgH* locus in collaboration with an unknown factor that is present in pro-B cells, but absent in thymocytes.

Results

Pax5 induces D_H–J_H *and* V_H–DJ_H *rearrangements at the* IgH *locus in* $Ik^{Pax5/+}$ *thymocytes*

To investigate whether ectopic *Pax5* expression is able to induce *IgH* rearrangements in T cells, we isolated $CD4^-$ $CD8^-$ double-negative (DN) pro-T cells and $CD4^+$ $CD8^+$ double-positive (DP) pre-T cells from the thymus of 2-week-old $Ik^{Pax5/+}$ and control $Ik^{neo/+}$ mice (Souabni et al. 2002) by FACS sorting after depletion of $B220^+$ B-lymphocytes. As positive control, we sorted $c\text{-}Kit^+$ $B220^+$ pro-B cells from the bone marrow of wild-type and $Pax5^{-/-}$ mice (Urbánek et al. 1994). DNA was isolated from the different cell types and normalized by PCR amplification of a DNA fragment from the *IgH Cμ* region prior to quantitative PCR analysis of D_H–J_H and V_H–DJ_H rearrangements (Fig. 1B). No V_H–DJ_H rearrangements and only a low level of D_H–J_H rearrangements were detected in control $Ik^{neo/+}$ thymocytes (Fig. 1B), as previously described for wild-type T cells (Kurosawa et al. 1981; Born et al. 1988). The frequency of D_H–J_H recombination was increased fivefold in $Ik^{Pax5/+}$ thymocytes, reaching half the level observed in wild-type and $Pax5^{-/-}$ pro-B cells (Fig. 1B,C). Hence, ectopic *Pax5* expression promotes further D_H–J_H recombination in thymocytes. More importantly, $Ik^{Pax5/+}$ thymocytes carried V_H–DJ_H rearrangements (Fig. 1B), which are normally restricted to the B-lymphoid lineage (Kurosawa et al. 1981). The proximal V_H7183 and V_HQ52 genes (Fig. 1A) were rearranged in $Ik^{Pax5/+}$ pre-T cells as efficiently as in wild-type pro-B cells, whereas a slightly lower level of proximal V_H7183 and V_HQ52 gene rearrangements was detected in $Ik^{Pax5/+}$ pro-T cells (Fig. 1B). Unexpectedly however, the recombination frequency of the more distal $V_HGam3.8$, V_H3609, and V_HJ558 genes (Fig. 1A) was reduced 30- to 100-fold in $Ik^{Pax5/+}$ pro-T and pre-T cells in contrast to wild-type pro-B cells (Fig. 1B). A similar position-dependent decrease of V_H gene recombination was observed in $Pax5^{-/-}$ pro-B cells (Fig. 1B), in agreement with recently published data (Hesslein et al. 2003). Hence, ectopic expression of *Pax5* leads to the same V_H–DJ_H recombination phenotype in thymocytes as does the loss of Pax5 in pro-B cells. In summary, these data indicate that Pax5 is sufficient to induce *V(D)J* recombination within the proximal *IgH* domain in thymocytes. However, efficient rearrangement of the distal V_H genes appears to require the cooperation of Pax5 with an unidentified second factor that is absent in Pax5-overexpressing T cells.

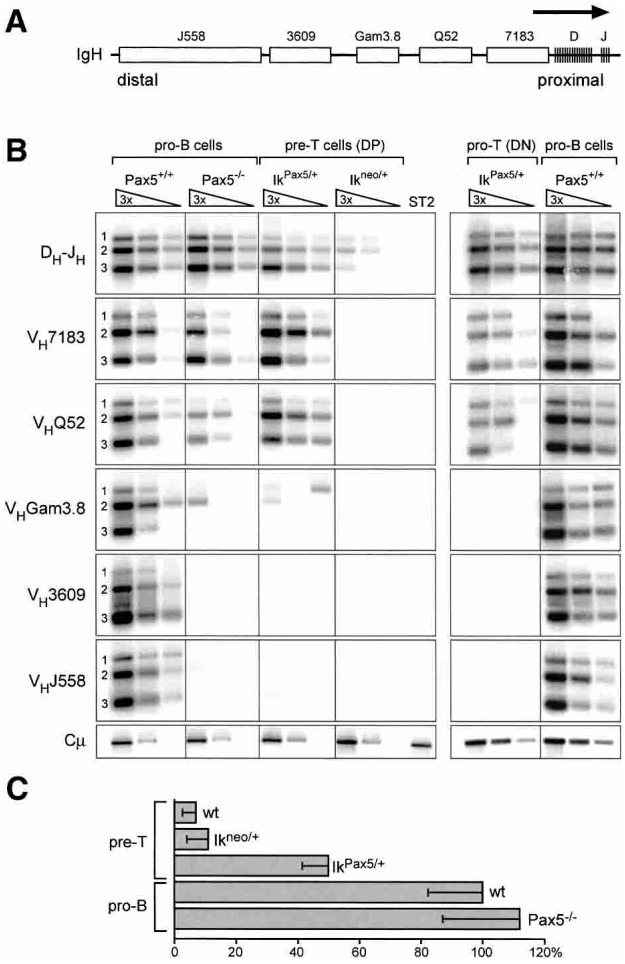

Figure 1. Pax5 induces D_H–J_H and V_H–DJ_H rearrangements of the *IgH* locus in thymocytes. (*A*) Schematic diagram of the V_H gene cluster of the *IgH* locus. Only the V_H gene families analyzed are shown together with their transcriptional direction (arrow) and distal or proximal position within the V_H gene cluster. (*B*) PCR detection of D_H–J_H and different V_H–DJ_H rearrangements in $Ik^{Pax5/+}$ and $Ik^{neo/+}$ thymocytes (pro-T [DN] and pre-T [DP] cells) as well as in bone marrow $Pax5^{+/+}$ and $Pax5^{-/-}$ pro-B cells, which were directly sorted from 2-week-old mice. Threefold serial DNA dilutions were analyzed by PCR. Input DNA was normalized by amplification of a PCR fragment from the *IgH Cμ* regions, and DNA of the stromal ST2 cells was used as negative control. Numbers to the *left* indicate rearrangements to the J_H1, J_H2, and J_H3 segments. (*C*) Quantitation of D_H–J_H recombination in sorted pro-B and pre-T cells. D_H–J_H rearrangements were analyzed by PCR in three independent preparations of sorted cells of the indicated genotypes. The average recombination frequency with its standard deviation is shown as relative percentage of the rearrangements detected in wild-type (wt) pro-B cells.

Surface Igμ expression in the absence of Igκ *rearrangements in* $Ik^{Pax5/+}$ *thymocytes*

Sequence analysis of the V_HDJ_H2 PCR fragments revealed that *V(D)J* recombination in $Ik^{Pax5/+}$ pre-T cells involved different members of the V_H7183 and V_HQ52 gene families, although with a strong bias for more proximally located genes within each family (data not shown). Moreover, 13% of the sequenced PCR fragments contained a functional in-frame V_H–DJ_H rearrangement (data not shown). This finding was confirmed by flow cytometric analysis demonstrating that functionally rearranged Igμ chains were expressed not only in the cytoplasm, but also on the cell surface of Thy1.2$^+$ $Ik^{Pax5/+}$ thymocytes at the expected frequency (Fig. 2A). However, no V_κ–J_κ rearrangements could be detected in $Ik^{Pax5/+}$ pre-T cells, demonstrating that Pax5 is unable to induce rearrangements at the *Igκ light-chain* locus (Fig. 2B). As a consequence, the productively rearranged Igμ protein is likely to be expressed as part of the pre-BCR on the cell surface of $Ik^{Pax5/+}$ thymocytes, which is consistent with expression data shown below. Most $Ik^{Pax5/+}$ thymocytes expressed TCRβ on the cell surface (Fig. 2A), in agreement with the observation that $D_\beta2$–$J_\beta2$ and $V_\beta5.1$–$DJ_\beta2$ rearrangements were present at similar frequency in $Ik^{Pax5/+}$ and control $Ik^{neo/+}$ pre-T cells (Fig. 2B). These data further demonstrate that the $Ik^{Pax5/+}$ thymocytes are of T-lymphoid origin despite ectopic expression of the B-lineage commitment factor Pax5 (Souabni et al. 2002).

Conditional Pax5 *activation induces* V_H–DJ_H *rearrangements within the T-lymphoid lineage*

The expression of *RAG1* and *RAG2* is initiated in the earliest lymphocyte progenitor (ELP), resulting in subsequent D_H–J_H recombination (Igarashi et al. 2002). Hence, the *V(D)J* rearrangement machinery is already active in lymphoid progenitors of the bone marrow prior to B- and T-lineage commitment. On the other hand, expression of the Ik^{Pax5} allele is initiated in the hematopoietic stem cell and is maintained in the progenitors and differentiating cells of all major hematopoietic lineages (Souabni et al. 2002). It is therefore conceivable that the Pax5-expressing lymphoid progenitors of $Ik^{Pax5/+}$ mice may undergo V_H–DJ_H rearrangements before migration to the thymus and initiation of T-cell development. Alternatively, the V_H–DJ_H rearrangements present in $Ik^{Pax5/+}$ thymocytes may originate within the T-lymphoid lineage. To distinguish between these two possibilities, we took advantage of the conditional Ik^{neo} allele, which contains a floxed *neomycin* stop cassette upstream of the *Pax5* minigene in the *Ikaros* locus (Souabni et al. 2002). *Pax5* expression from this allele is only activated upon Cre recombinase-mediated deletion of the *neomycin* gene (Souabni et al. 2002). To this end, we crossed the $Ik^{neo/+}$ mouse with a transgenic mouse, which expressed the Cre recombinase under the control of the proximal *lck* promoter (*lck-cre*) during pro-T-cell development (Lee et al. 2001; Wolfer et al. 2002). The *lck-cre* transgene

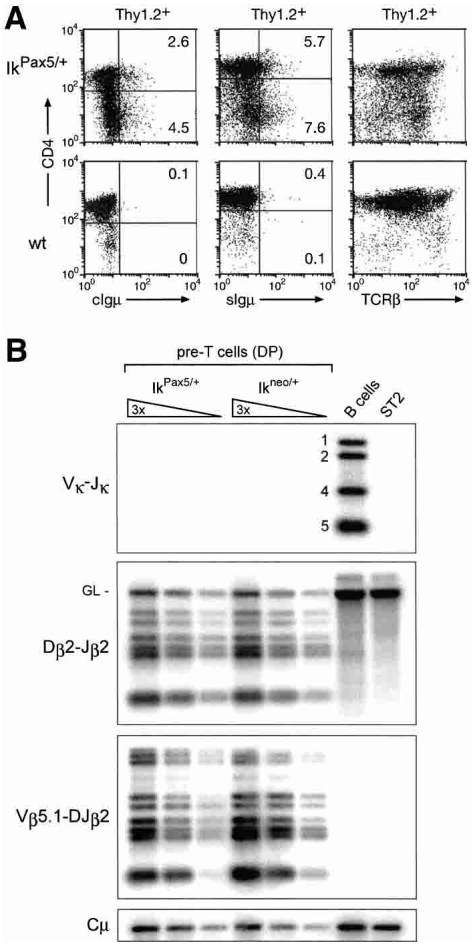

Figure 2. Surface Igμ expression in the absence of *Igκ* rearrangements in $Ik^{Pax5/+}$ thymocytes. (*A*) Flow cytometric analysis. Cytoplasmic (c) and cell surface (s) expression of functionally rearranged Igμ protein and cell surface TCRβ expression were analyzed in wild-type (wt) and $Ik^{Pax5/+}$ thymocytes (gated as Thy1.2$^+$ cells). The percentage of Igμ$^+$ cells is indicated. (*B*) Normal *TCRβ* recombination and absence of V_K–J_K rearrangements in $Ik^{Pax5/+}$ thymocytes. The same sorted $Ik^{Pax5/+}$ and $Ik^{neo/+}$ pre-T cells, which were used for the experiment shown in Figure 1, were analyzed by PCR for V_K–J_K rearrangements at the *Igκ* locus and for $D_\beta2$–$J_\beta2$ and $V_\beta5.1$–$DJ_\beta2$ rearrangements at the *TCRβ* locus. Splenic B220$^+$ B cells and stromal ST2 cells served as positive and negative controls, respectively. The numbers next to the B-cell lane indicate rearrangements to the J_K1, J_K2, J_K4, and J_K5 segments. GL denotes the position of the germline PCR product.

was shown to initiate Cre-mediated inactivation of a floxed *Notch1* allele in CD44$^+$ CD25$^+$ (DN2) pro-T cells and to complete gene deletion in CD44$^-$ CD25$^+$ (DN3) pro-T cells (Wolfer et al. 2002). However, the same *lck-cre* transgene deleted the floxed *neomycin* gene of the Ik^{neo} allele with lower efficiency, as the activated Ik^{Pax5} allele was detected in only ~60% of pre-T cells (Fig. 3). Nevertheless, these thymocytes of $Ik^{neo/+}$ *lck-cre* mice were characterized by an increase in D_H–J_H rearrangements and the presence of V_H–DJ_H-rearranged *IgH* alleles (Fig. 3). The V_H–DJ_H rearrangement frequency was high-

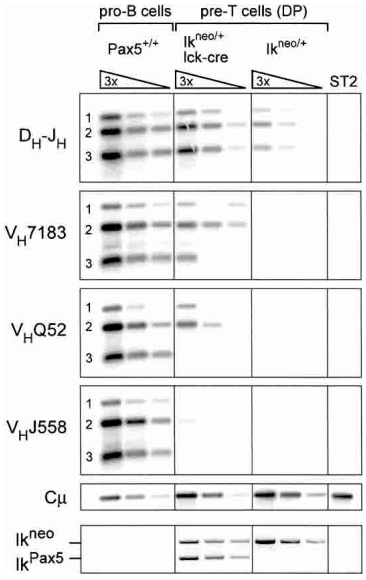

Figure 3. V_H–DJ_H rearrangements upon conditional *Pax5* activation in thymocytes. Pre-T cells were isolated by FACS sorting as DP thymocytes from $Ik^{neo/+}$ mice carrying the *lck-cre* transgene followed by PCR quantification of D_H–J_H and V_H–DJ_H rearrangements as described in Figure 1B. $Ik^{neo/+}$ pre-T cells and stromal ST2 cells served as negative controls and $Pax5^{+/+}$ pro-B cells as positive control for the detection of V_H–DJ_H rearrangements. Cre-mediated conversion of the Ik^{neo} to the Ik^{Pax5} allele was determined by PCR (*bottom* row).

est for the proximal V_H7183 genes and lowest for the distal V_HJ558 gene family (Fig. 3), thus recapitulating the *V(D)J* recombination phenotype of $Ik^{Pax5/+}$ thymocytes (Fig. 1B). The rearrangement frequency was, however, lower in $Ik^{neo/+}$ *lck-cre* pre-T cells, possibly because of the inefficient activation of the Ik^{neo} allele during T-cell development. Together these data unequivocally demonstrate that Pax5 is able to induce V_H–DJ_H rearrangements within the T-lymphoid lineage.

Pax5 activates germ-line V_H *transcription and multiple B-lymphoid genes in pro-T cells*

To study the extent of B-cell-specific gene activation by Pax5 in thymocytes, we purified pro-T cells from $Ik^{Pax5/+}$ mice by depleting Lin^+ cells (including $B220^+$ B cells), followed by sorting for $Thy1.2^+$ Lin^- DN thymocytes. Flow cytometric reanalysis demonstrated that the sorted $Thy1.2^+$ Lin^- pro-T cells were purified to homogeneity (Fig. 4A). Moreover, RT–PCR analysis failed to detect rearranged *Igκ* and *Igλ1* mRNAs in these sorted cells (Fig. 4B). We conclude, therefore, that the sorted $Ik^{Pax5/+}$ pro-T cells were free of contaminating B cells. In addition, we sorted wild-type pro-T and pro-B cells as well as $Pax5^{-/-}$ pro-B cells. cDNA was prepared from all four cell types and normalized for equal expression of the control *HPRT* gene prior to semiquantitative RT–PCR analysis of B-cell-specific transcripts (Fig. 4C).

The accessibility of a particular V_H gene in active chromatin can be monitored by expression of its germ-line transcript (GLT), whereas the abundance of spliced *Igμ* (V_H–*DJCμ*) mRNA is a direct measure of the V_H–DJ_H recombination frequency in progenitor cells (Yancopoulos and Alt 1985). The germ-line transcripts of the proximal V_H7183 gene family were expressed at a fivefold

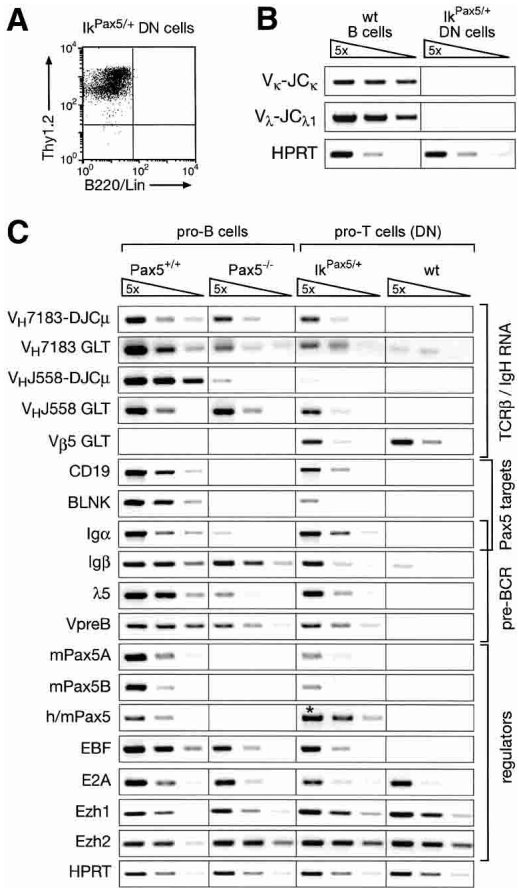

Figure 4. Activation of germ-line V_H transcription and B-cell-specific gene expression in $Ik^{Pax5/+}$ pro-T cells. (*A*) Flow cytometric reanalysis of sorted $Ik^{Pax5/+}$ DN thymocytes. DN pro-T cells of 2-week-old mice were sorted as $Thy1.2^+$ Lin^- cells after depletion of Lin^+ cells, which were stained with antibodies recognizing B220, CD4, CD8, DX5, CD11c, Mac-1, Gr-1, and Ter119. (*B*) Absence of B cells in the sorted $Ik^{Pax5/+}$ pro-T cell population. Rearranged *Igκ* (V_K–JC_K) and *Igλ1* (V_λ–$JC_{\lambda 1}$) mRNAs could not be detected by RT–PCR in purified $Ik^{Pax5/+}$ pro-T cells in contrast to IgM^+ IgD^+ B cells isolated from wild-type (wt) spleen. The *hypoxanthine phosphoribosyltransferase* (*HPRT*) gene was equally expressed in both cell types. (*C*) B-cell-specific gene expression in $Ik^{Pax5/+}$ pro-T cells. Transcripts of the indicated genes were analyzed by semiquantitative RT–PCR of fivefold serial dilutions of cDNA that was prepared from ex vivo sorted pro-B cells and pro-T cells of the indicated genotypes. The cDNA input was normalized according to the expression of the control *HPRT* gene. V_HJ558-*DJCμ* and V_H7183–*DJCμ* refer to rearranged *Igμ* mRNAs and GLT to the corresponding germ-line transcripts. Transgenic human (h) and endogenous mouse (m) *Pax5* transcripts were amplified with conserved primers, giving rise to the same PCR fragment (denoted by an asterisk) for both transcripts.

lower level in $Pax5^{-/-}$ pro-B cells and $Ik^{Pax5/+}$ pro-T cells compared with wild-type pro-B cells. The rearranged mRNAs of the V_H7183 genes were, however, present at similar abundance in all three cell types (Fig. 4C), consistent with efficient V_H7183–DJ_H recombination in these cells (Fig. 1B; Hesslein et al. 2003). In contrast, the rearranged mRNA of the distal V_HJ558 gene family was reduced ~100-fold in $Ik^{Pax5/+}$ pro-T cells and $Pax5^{-/-}$ pro-B cells (Fig. 4C), which undergo V_HJ558–DJ_H rearrangements with a similarly low efficiency compared with wild-type pro-B cells (Fig. 1B; Nutt et al. 1997). Germ-line V_HJ558 transcripts were, however, present at a similar abundance in $Ik^{Pax5/+}$ pro-T cells relative to wild-type and $Pax5^{-/-}$ pro-B cells (Fig. 4C). Germ-line transcripts of the *TCR* $V_\beta5$ gene could not be detected in pro-B cells, but were expressed in $Ik^{Pax5/+}$ pro-T cells, further confirming the T-lymphoid origin of these cells (Fig. 4C). These data indicate, therefore, that ectopic expression of *Pax5* establishes an accessible chromatin state at both the proximal and distal V_H genes in $Ik^{Pax5/+}$ pro-T cells, although only the proximal V_H genes undergo efficient V_H–DJ_H recombination.

Ectopic *Pax5* expression activated the Pax5 target genes *CD19* (Nutt et al. 1998), *BLNK* (Schebesta et al. 2002b), and *Igα* (*mb-1*; Nutt et al. 1998) in $Ik^{Pax5/+}$ pro-T cells (Fig. 4C). More surprisingly, these pro-T cells also expressed the genes *Igβ* (*B29*), λ*5*, and *VpreB* (Fig. 4C), which are known to be cooperatively regulated by the transcription factors EBF and E2A (Sigvardsson et al. 1997, 2002; O'Riordan and Grosschedl 1999). Consistent with this finding, Pax5 induced expression of the B-cell-specific *EBF* gene in $Ik^{Pax5/+}$ pro-T cells to a level that is normally seen in wild-type and $Pax5^{-/-}$ pro-B cells. In contrast, the expression of *E2A* was similar and independent of Pax5 in pro-T cells as in pro-B cells (Fig. 4C). None of the B-cell-specific transcripts analyzed could be detected in wild-type pro-T cells except for low-level expression of the *Igβ* gene (Wang et al. 1998). Moreover, the efficient expression of all pre-BCR components (λ5, VpreB, Igα, and Igβ) strongly suggests that the rearranged Igμ protein is transported as part of the pre-BCR to the cell surface of $Ik^{Pax5/+}$ thymocytes (Fig. 2A).

The *Pax5* gene gives rise to two distinct mRNAs by alternative promoter usage and splicing of the different 5′-exons onto common coding sequences (Busslinger et al. 1996). The presence of the mouse *Pax5A* and *Pax5B* transcripts in $Ik^{Pax5/+}$ pro-T cells demonstrated that the ectopically expressed human Pax5 protein is able to induce transcription of the endogenous *Pax5* gene in thymocytes (Fig. 4C). This *Pax5* activation is likely to be an indirect effect of EBF expression, which is known to regulate the *Pax5* gene in pro-B cells (O'Riordan and Grosschedl 1999). PCR conditions, which detect human and mouse *Pax5* mRNAs with equal efficiency, revealed that the human *Pax5* minigene of the Ik^{Pax5} allele is expressed at a fivefold higher level in pro-T cells compared with the endogenous *Pax5* gene in wild-type pro-B cells (Fig. 4C). In conclusion, moderate overexpression of *Pax5* activates its own gene as well as many other B-cell-specific genes in $Ik^{Pax5/+}$ pro-T cells.

Relocation of the IgH *locus from the nuclear periphery to central positions in* $Ik^{Pax5/+}$ *thymocytes*

A recently published FISH analysis indicated that the subnuclear location and chromatin state of the *IgH* locus may regulate *V(D)J* recombination in non-B versus pro-B cells (Kosak et al. 2002). In particular, the 3-Mb-long *IgH* locus with its 150–200 V_H genes was shown to be present in an extended chromatin state at the nuclear periphery of thymocytes (Kosak et al. 2002). To further investigate the discrepancy between distal and proximal V_H gene recombination in $Ik^{Pax5/+}$ thymocytes, we next used three-color 3D FISH analysis (Skok et al. 2001) to localize different *IgH* gene segments in three-dimensionally (3D) preserved nuclei by confocal laser scanning microscopy (Fig. 5). The locations of the different V_H and *Cγ1* gene probes used are shown in Figure 5A together with the minimal number of base pairs separating these probes in the *IgH* locus.

As illustrated by the confocal images of Figure 5B, the distal V_HJ558 and V_H15 genes were colocalized at the nuclear periphery in 92%–95% of all wild-type pro-T and pre-T cells (Fig. 5D), in agreement with published data (Kosak et al. 2002). The signals of the distal V_H genes were separated in only 2% of all wild-type thymocytes (Fig. 5C). In marked contrast, the distal V_H genes were separated from the proximal V_H7183 and *Cγ1* genes by a distance of 0.3–1.5 μm in 89%–98% of the wild-type nuclei (Fig. 5C). In addition, the proximal *IgH* domain was positioned away from the nuclear periphery toward the center in 96% of wild-type pro-T and pre-T cells (Fig. 5B; data not shown). Hence, the *IgH* locus is anchored via the distal V_H genes at the nuclear periphery and is oriented in its extended chromatin state toward the center, which may facilitate access of the V(D)J recombinase to the proximal *IgH* domain, thus accounting for the D_H–J_H rearrangements observed in wild-type thymocytes.

Ectopic *Pax5* expression resulted in subnuclear repositioning of the *IgH* locus in pro-T and pre-T cells, as the majority (62%) of *IgH* loci were located at central positions in the nuclei of $Ik^{Pax5/+}$ thymocytes (Fig. 5B,D). A similar percentage of centrally located *IgH* alleles was observed in $RAG2^{-/-}$ and $Pax5^{-/-}$ $RAG2^{-/-}$ pro-B cells (Figs. 5D, 6A), indicating that the relocation of the *IgH* locus is as efficient in $Ik^{Pax5/+}$ thymocytes as in pro-B cells. Importantly, the *IgH* locus remained in an extended chromatin state even in its more central location, as the distal V_HJ558 and V_H15 genes were still separated from the proximal V_H7183 and *Cγ1* genes in 92%–100% of all $Ik^{Pax5/+}$ pro-T and pre-T cells (Fig. 5C). The physical separation of distal V_H genes from the rearranged DJ_H segment is therefore a likely cause for the low V_H–DJ_H recombination efficiency of distal V_H genes in $Ik^{Pax5/+}$ thymocytes.

The IgH *locus is present in an extended chromatin state in* $Pax5^{-/-}$ *pro-B cells*

As the $Pax5^{-/-}$ pro-B cells and $Ik^{Pax5/+}$ thymocytes exhibit a similar V_H–DJ_H recombination phenotype, we

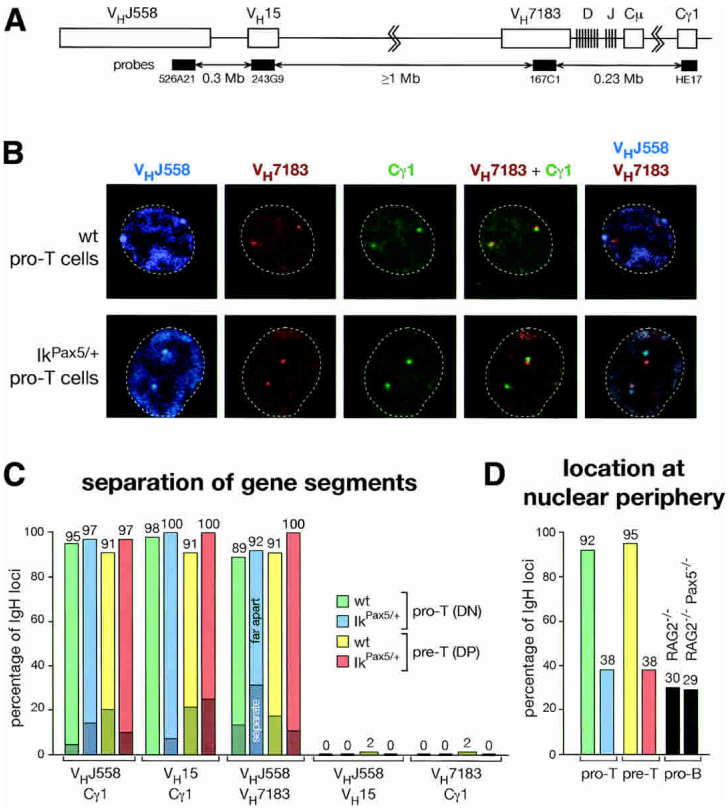

Figure 5. Subnuclear compartmentalization and extended chromatin state of V_H genes in thymocyte nuclei. (*A*) Schematic diagram indicating the positions of the DNA probes in the *IgH* locus (not drawn to scale). The minimal number of megabase pairs (Mb) separating the probes was estimated according to the mouse genome database (Ensembl release of July 1, 2003). (*B*) Three-color 3D DNA-FISH analysis of the *IgH* locus. Confocal laser scanning microscopy identified the subnuclear locations of different gene segments of the two *IgH* alleles, which are shown on representative single optical sections through the nucleus of sorted wild-type (wt) and $Ik^{Pax5/+}$ pro-T (DN) cells. The different probes with their colors are indicated. The V_HJ558 BAC (526A21) was directly labeled with Cy5-dUTP (blue). The V_H7183 (167C1) BACs as well as V_H15 (243G9) BAC (not shown in panel *B*) were labeled with digoxygenin-dUTP and detected with rhodamine- and Texas red-coupled anti-digoxygenin antibodies (red). The *Cγ1* probe (HE17) was labeled with biotin-dUTP and detected with FITC-avidin and biotinylated FITC-coupled anti-avidin antibodies (green). The *IRES-GFP* sequences, which were flanked by *frt* sites in the Ik^{Pax5} allele (Souabni et al. 2002), were specifically deleted for the three-color FISH analysis by mating $Ik^{Pax5/+}$ mice with the transgenic *ACTB:FLPe* deleter line (Rodriguez et al. 2000). The contour of the cell is indicated by a broken line. (*C*) Statistical analysis of the distance between proximal and distal V_H genes in thymocyte nuclei of the indicated genotypes. Signals of the different *IgH* gene probes, which were separated in the nucleus by 0.3–0.5 μm (separate; dark color) or 0.5–1.5 μm (far apart; light color), were scored as percentage of all signals analyzed. The actual numbers and sample sizes are shown in Supplementary Table 1A. (*D*) Statistical analysis of the peripheral location of distal V_H genes in thymocytes and pro-B cells of the indicated genotypes.

next studied the nuclear location and chromatin state of *IgH* loci in wild-type and $Pax5^{-/-}$ pro-B cells. The *IgH* alleles were centrally located in ~70% of the pro-B cell nuclei regardless of the presence or absence of Pax5 (Figs. 5D, 6A). Interestingly, the distal V_HJ558 and V_H15 genes were separated from the proximal V_H7183 and *Cγ1* genes in 84%–94% of all $Pax5^{-/-}$ pro-B cells (Fig. 6A,B), similar to the situation observed in $Ik^{Pax5/+}$ pro-T cells (Fig. 5B,C). In contrast, the distal and proximal *IgH* gene segments were colocalized in 76%–86% of wild-type pro-B cells, whereas they were separated by only a short distance (0.3–0.5 μm) in the remaining 14%–24% of pro-B cells (Fig. 6B). These data therefore point to an essential role for Pax5 in regulating large-scale contraction of the *IgH* locus. To rule out the possibility that this Pax5 function depends on *V(D)J* recombination, we analyzed $Pax5^{+/+}$ and $Pax5^{-/-}$ pro-B cells on a *RAG2* mutant background, which prevents immunoglobulin gene rearrangements (Shinkai et al. 1992). The *IgH* locus was still in an extended state in $Pax5^{-/-}$ $RAG2^{-/-}$ pro-B cells and in a contracted state in $Pax5^{+/+}$ $RAG2^{-/-}$ pro-B cells (Fig. 6A,B), indicating that Pax5-mediated contraction of the *IgH* locus can precede *V(D)J* recombination in pro-B cells.

Pax5 induces IgH *locus contraction and distal* V_H–DJ_H *rearrangements in pro-B cells*

To directly demonstrate an involvement of Pax5 in *IgH* locus contraction and distal V_H–DJ_H recombination, we used retroviral infection to restore *Pax5* expression in in vitro cultured $Pax5^{-/-}$ pro-B cells. The retrovirus M-Pax5-iCD2 as well as the parental virus MiCD2 (Heavey et al. 2003) express a human CD2 indicator protein, which facilitated FACS sorting of the infected cells prior to PCR quantification of *IgH* rearrangements (Fig. 7A). D_H–J_H and proximal V_H7183–DJ_H rearrangements were present at similar levels in $Pax5^{-/-}$ pro-B cells regardless of whether these cells were infected with the parental or Pax5-expressing virus (Fig. 7A). In contrast, retroviral *Pax5* expression led to a 10-fold increase of distal V_HJ558–DJ_H recombination above the level seen in in vitro cultured $Pax5^{-/-}$ pro-B cells (Fig. 7A). We next infected double-mutant $Pax5^{-/-}$ $RAG2^{-/-}$ pro-B cells to study the effect of *Pax5* on *IgH* locus contraction in the absence of *V(D)J* recombination. As expected, the distal V_HJ558 and V_H15 genes were separated from the proximal V_H7183 and *Cγ1* genes in 94%–96% of the $Pax5^{-/-}$ $RAG2^{-/-}$ pro-B cells infected with the parental MiCD2

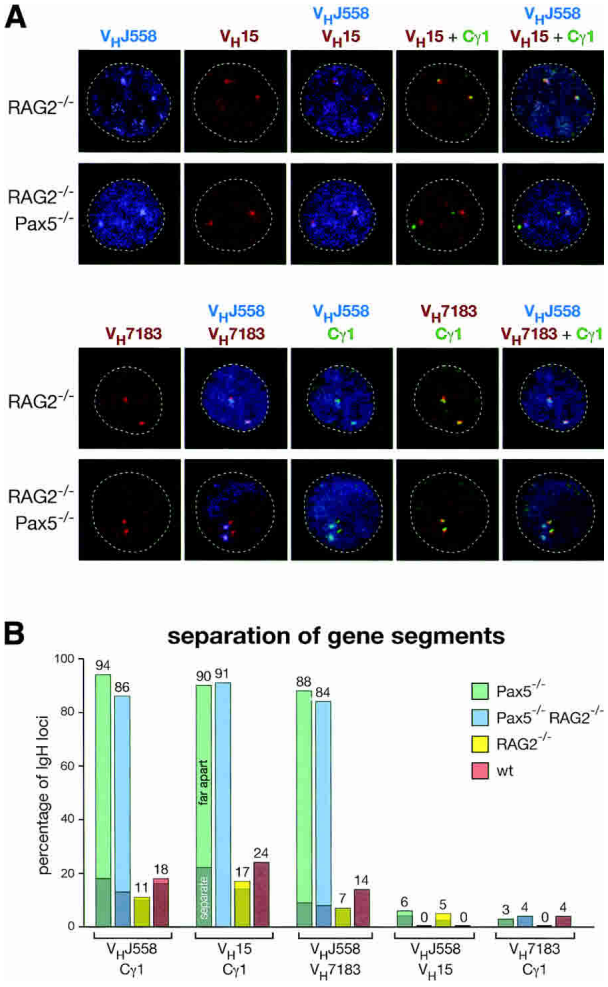

Figure 6. Absence of *IgH* locus contraction in the nucleus of *Pax5*$^{-/-}$ pro-B cells. (*A*) Three-color 3D DNA-FISH analysis of the *IgH* locus. In vitro cultured *RAG2*$^{-/-}$ and *Pax5*$^{-/-}$ *RAG2*$^{-/-}$ pro-B cells were analyzed by FISH and confocal microscopy as described in the legend for Figure 5B. (*B*) Statistical analysis of the distance between proximal and distal V_H genes in pro-B cells of the indicated genotypes. A distance of 0.3–0.5 µm (separate) or 0.5–1.5 µm (far apart) between the signals is indicated by dark or light color, respectively. See Supplementary Table 1B for further information.

virus (Fig. 7B,C). In contrast, both distal and proximal gene segments were colocalized and thus present in a contracted state in 69%–75% of the Pax5-reconstituted *Pax5*$^{-/-}$ *RAG2*$^{-/-}$ pro-B cells, which compares favorably with the corresponding frequency (83%–93%) observed in *Pax5*$^{+/+}$ *RAG2*$^{-/-}$ pro-B cells (Fig. 7B,C). We conclude therefore that Pax5 facilitates distal V_H–DJ_H rearrangements by inducing *IgH* locus contraction in pro-B cells.

Discussion

Previous loss-of-function analyses identified an essential role for Pax5 in controlling V_H–DJ_H recombination of distal but not proximal V_H genes in pro-B cells (Nutt et al. 1997; Hesslein et al. 2003). Here we have reported the unexpected finding that ectopic expression of Pax5 promotes efficient rearrangement of proximal rather than distal V_H genes in pro-T cells. The distal V_HJ558 genes are, however, present in accessible chromatin in *Ik*$^{Pax5/+}$ pro-T cells similar to *Pax5*$^{-/-}$ pro-B cells. Hence, the loss of Pax5 in pro-B cells results in the same V_H–DJ_H recombination phenotype as expression of Pax5 in pro-T cells. This phenotypic similarity was further extended by FISH analyses demonstrating that the *IgH* loci in both cell types are located at central positions of the nucleus and are present in an extended chromatin state (Fig. 7D). As a consequence, the distal V_H genes are separated from the proximal *IgH* domain by a large distance, which is likely to prevent efficient synapse formation between distal and proximal gene segments, thus resulting in a dramatic reduction of distal V_H–DJ_H recombination in both cell types (Fig. 7D). The seemingly conflicting data of the Pax5 gain- and loss-of-function analyses may be explained by the existence of an unknown factor X that is expressed in B cells but not in T cells and that cooperates with Pax5 in the control of locus contraction and distal V_H–DJ_H rearrangements (Fig. 7D). According to this hypothesis, the absence of factor X in *Ik*$^{Pax5/+}$ pro-T cells or the loss of Pax5 in *Pax5*$^{-/-}$ pro-B cells results in equally inefficient recombination of distal V_H genes. Importantly, the restoration of *Pax5* expression in *Pax5*$^{-/-}$ pro-B cells unequivocally demonstrated that Pax5 promotes distal V_H–DJ_H recombination by inducing large-scale contraction of the *IgH* locus, which leads to juxtaposition of distal V_H genes next to the proximal D_HJ_H-rearranged gene segment.

The *IgH* locus contains 150–200 V_H genes, which are positioned over a chromosomal region of ~3 Mb (Chevillard et al. 2002). The large size of the *IgH* locus is likely to constitute a mechanistic constraint for *V(D)J* recombination, as synapse formation between the distal V_H genes and proximal D_HJ_H-rearranged gene segment may be ineffective, resulting in a lower efficiency of V_H–DJ_H recombination for distal relative to proximal V_H genes. Indeed, V_H–DJ_H recombination exhibits a marked preference for the utilization of the most proximal V_H genes both in fetal and adult B-lymphopoiesis (Yancopoulos et al. 1984; Malynn et al. 1990). Even within the proximal V_H7183 family, the more proximally located V_H genes are preferentially used for *V(D)J* recombination (Williams et al. 2001). This position-dependent bias in V_H gene rearrangements is observed already in wild-type pro-B cells, but is dramatically increased in the absence of Pax5, as *Pax5*$^{-/-}$ pro-B cells essentially fail to undergo distal V_H–DJ_H recombination (Nutt et al. 1997). The recently published FISH analysis of Kosak et al. (2002) provided the first evidence that *V(D)J* recombination may be controlled by the subnuclear location and contraction state of the *IgH* locus. Our detailed *V(D)J* recombination and 3D FISH analyses have now considerably extended these published data by implicating the two B-cell-specific transcription factors EBF and Pax5 in controlling two separate steps of *IgH* locus activation. As illustrated by the two-step model in Figure 7D, the *IgH* locus is

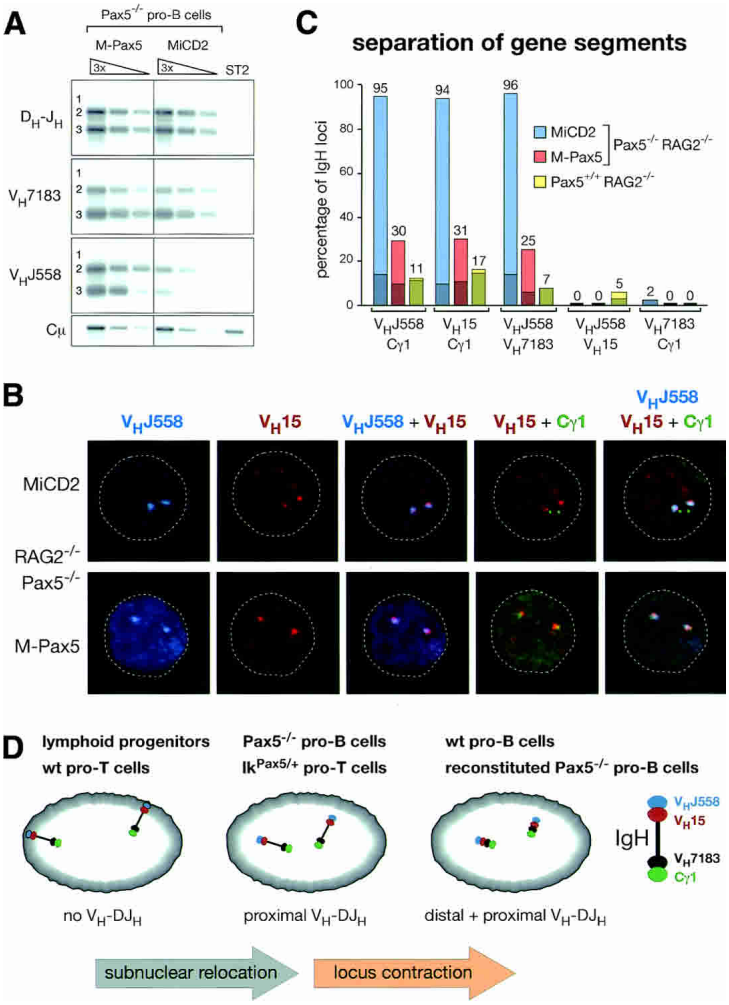

Figure 7. Distal V_H gene rearrangements and *IgH* locus contraction upon reconstitution of *Pax5* expression in $Pax5^{-/-}$ pro-B cells. (*A*) Induction of distal V_H gene rearrangements by retroviral *Pax5* expression in $Pax5^{-/-}$ pro-B cells. In vitro cultured $Pax5^{-/-}$ pro-B cells were infected with the M-Pax5-iCD2 or parental MiCD2 retrovirus followed by PCR analysis of D_H–J_H and V_H–DJ_H rearrangements. Note that the starting $Pax5^{-/-}$ pro-B cell culture contained only two prominent D_H–J_H rearrangements (D_H–J_H2 and D_H–J_H3). (*B*) *IgH* locus contraction after retroviral reconstitution of *Pax5* expression in $Pax5^{-/-}$ $RAG2^{-/-}$ pro-B cells. Double-mutant pro-B cells were infected with the M-Pax5-iCD2 or control MiCD2 virus prior to three-color 3D FISH analysis as described in the legend for Figure 5B. (*C*) Statistical analysis of the distance between proximal and distal V_H genes in pro-B cells of the indicated genotypes. For further information, see Supplementary Table 1B. (*D*) Two-step model for *IgH* locus activation in early lymphopoiesis. A schematic diagram of the nucleus illustrates the location and contraction of proximal and distal V_H genes in lymphoid progenitors, wild-type (wt), and $Ik^{Pax5/+}$ pro-T cells as well as in $Pax5^{-/-}$, retrovirally reconstituted $Pax5^{-/-}$ and wild-type pro-B cells. In a first step, the *IgH* locus is repositioned, possibly under the control of EBF, from the periphery to the center of the nucleus, resulting in proximal V_H–DJ_H rearrangements. Pax5 subsequently activates large-scale contraction of the *IgH* locus, leading to distal V_H–DJ_H rearrangements. Gray shading highlights the perinuclear compartment.

anchored via the distal V_H genes at the nuclear periphery and is oriented in its extended chromatin state toward the center of the nucleus in all non-B cells such as pro-T cells. In this default configuration of the *IgH* locus, the distal V_H genes are likely to be silenced, as the nuclear periphery may function as a repressive compartment in higher eukaryotes in analogy to yeast (Baxter et al. 2002; Hediger and Gasser 2002). The more centrally located, proximal *IgH* domain may, however, be accessible for the V(D)J recombinase, which could account for the low level of D_H–J_H rearrangements observed in thymocytes and dendritic cells (Kurosawa et al. 1981; Corcoran et al. 2003). The first step of *IgH* locus activation consists of relocation of the *IgH* locus from the nuclear periphery to a central position within the nucleus (Fig. 7D). In the absence of locus contraction, this subnuclear repositioning in concert with chromatin changes leads to proximal V_H–DJ_H rearrangements as observed in $Ik^{Pax5/+}$ pro-T cells and $Pax5^{-/-}$ pro-B cells. Interestingly, the subnuclear relocation of the *IgH* locus correlates with expression of the B-cell-specific transcription factor EBF in both cell types. EBF is known to function upstream of *Pax5* in early B-lymphopoiesis, as it is normally expressed in a Pax5-independent manner in $Pax5^{-/-}$ pro-B cells (Nutt et al. 1997). Unexpectedly, ectopic expression of Pax5 activates *EBF* in $Ik^{Pax5/+}$ pro-T cells, thus identifying a novel cross-regulatory interaction between these two transcription factors. Based on the correlation between *EBF* expression and *IgH* relocation, we hypothesize that EBF may be involved in controlling the first step of *IgH* locus activation. In a second step, Pax5 together with factor X induces *IgH* locus contraction and distal V_H–DJ_H rearrangements in wild-type pro-B cells (Fig. 7D).

Another important regulatory step in *V(D)J* recombination controls the accessibility of the *IgH* locus (Yancopoulos and Alt 1985), which is determined by the local chromatin structure of the different gene segments (Stanhope-Baker et al. 1996; Maes et al. 2001). Histone acetylation, which is a characteristic feature of open chromatin, plays an important role in determining the chromatin accessibility of *Ig* and *TCR* loci (Chowdhury and Sen 2001; Krangel 2003). Analysis of the histone acetylation state revealed a stepwise activation of discrete chromatin domains in the *IgH* locus (Chowdhury and Sen 2001). A 120-kb genomic region encompassing the D_H, J_H, and *Cμ* gene segments is first hyperacetylated prior to *V(D)J* recombination. D_H–J_H rearrangements

subsequently induce histone acetylation and rearrangements of the proximal V_H genes (Chowdhury and Sen 2001). Finally, the distal 2-Mb domain containing the majority of V_H genes is activated by IL-7 signaling (Chowdhury and Sen 2001) consistent with the observation that V_H–DJ_H recombination of the distal, but not proximal V_H genes is severely impaired in B-cell progenitors of *IL-7Rα*$^{-/-}$ mice (Corcoran et al. 1998). Despite the similar recombination phenotype of *IL-7Rα*$^{-/-}$ pro-B cells and *Ik*$^{Pax5/+}$ thymocytes, we do not regard IL-7 signaling to be the missing component in T cells that normally cooperates with Pax5 in the control of distal V_H–DJ_H rearrangements for the following reasons. First, IL-7 signaling is active during early T-cell development, where it plays an essential role in regulating cell proliferation and survival (Fry and Mackall 2002). Second, the expression of germ-line V_HJ558 transcripts in *Ik*$^{Pax5/+}$ pro-T cells indicates that the distal *IgH* domain is present in an accessible chromatin state (Fig. 4C), which is likely established under the influence of IL-7 signaling (Chowdhury and Sen 2001). Finally, the incubation of sorted *Ik*$^{Pax5/+}$ pro-T cells in IL-7 medium for 2 d failed to promote distal V_H–DJ_H rearrangements (M. Fuxa and M. Busslinger, unpubl.). Based on these considerations, we hypothesize that the recombination of distal V_H genes is controlled by two independent pathways. IL-7 signaling is essential for the establishment of an accessible chromatin state in the distal *IgH* domain, whereas a second Pax5-dependent pathway is responsible for contraction of the activated V_H locus, thus leading to distal V_H–DJ_H rearrangements.

It is important to note that the Pax5-dependent contraction of the *IgH* locus occurs under conditions that do not affect the accessible chromatin state of the V_H genes. Hence, locus contraction cannot be caused by chromatin condensation of the entire *IgH* locus. Interestingly, histone acetylation and thus chromatin accessibility are narrowly confined to individual V_H gene segments, their promoters and RSS sites (Johnson et al. 2003). It is, therefore, conceivable that the intergenic regions between V_H genes contain regulatory elements that control locus contraction. An interesting paradigm for such regulatory sequences are the Polycomb response elements (PREs), which have the potential to form clusters with each other through the binding of Polycomb group (PcG) proteins (Pirrotta 1998; Francis and Kingston 2001; Orlando 2003). In apparent conflict with a possible role in locus contraction, the PcG proteins are generally thought to function in gene repression by maintaining the transcriptionally silent state (Pirrotta 1998; Francis and Kingston 2001; Orlando 2003). Interestingly however, the histone methyltransferase Ezh2 is not only a member of the PcG protein family, but is also an essential regulator of distal V_H–DJ_H rearrangements (Su et al. 2003). *Ezh2* and its related gene *Ezh1* are similarly expressed at early stages of B- and T-cell development, as demonstrated by RT–PCR analysis of pro-B cells and immature thymocytes (Fig. 4C). Hence, Ezh1 and Ezh2 are unlikely candidates for factor X, which could, however, be another PcG protein that is expressed in pro-B cells, but not in thymocytes. We are presently testing the hypothesis that Pax5 cooperates with the PcG system in controlling *IgH* locus contraction and distal V_H–DJ_H rearrangements. It is, however, also conceivable that ectopic *Pax5* expression in *Ik*$^{Pax5/+}$ thymocytes fails to properly activate a Pax5 target gene, which codes for a chromatin regulator mediating *IgH* locus contraction.

Materials and methods

Mice

Ik$^{Pax5/+}$ and *Ik*$^{neo/+}$ (Souabni et al. 2002), *Pax5*$^{-/-}$ (Nutt et al. 1997), *RAG2*$^{-/-}$ (Shinkai et al. 1992), and *lck-cre* mice (Lee et al. 2001) were maintained and genotyped as described.

FACS sorting and analysis

The following phycoerythrin (PE)- or allophycocyanin (APC)-coupled antibodies were used for flow cytometry: anti-B220 (RA3-6B2), CD4 (L3T4), CD8 (53-6.7), CD11c (HL3), CD19 (1D3), DX5 (DX5), Gr-1 (RB6-8C5), c-Kit (2B8), IgM (M41.42), Mac-1 (M1/70), TCRβ (H57-597), Ter119 (TER-119), and Thy1.2 (30-H12) antibodies. Unspecific antibody binding was suppressed by preincubation of cells with CD16/CD32 Fc-block solution (PharMingen). Intracellular staining of cytoplasmic Igμ protein was performed as described (Thévenin et al. 1998). Pro-B cells were sorted as B220$^+$ c-Kit$^+$ cells after enrichment of c-Kit$^+$ bone marrow cells by magnetic cell sorting (MACS). DP thymocytes were sorted as CD4$^+$ CD8$^+$ cells after depletion of B220$^+$ cells. DN thymocytes were sorted as Thy1.2$^+$ Lin$^-$ cells following depletion of Lin$^+$ cells (stained with PE-anti-B220, CD4, CD8, DX5, CD11c, Mac-1, Gr-1, and Ter119 antibodies) with anti-PE MACS beads (Miltenyi Biotec).

V(D)J *recombination analysis*

Sorted cells from 2-week-old mice were digested with proteinase K, and DNA was isolated by phenol extraction and ethanol precipitation. PCR analyses of immunoglobulin genes were performed with published primers (Supplementary Table 2) as described (Schlissel et al. 1991; Angelin-Duclos and Calame 1998). *TCR*β rearrangements were analyzed as described (Wolfer et al. 2002). PCR cycle numbers were adjusted to be in the linear range, based on the analysis of serially diluted DNA. PCR products were separated on agarose gels, transferred to a porablot NYamp membrane and analyzed by Southern blotting using published oligonucleotide probes (Supplementary Table 2; Schlissel et al. 1991).

RT–PCR analysis

RNA was prepared from sorted cells, using the Trizol Reagent (GIBCO-BRL). Reverse transcription (with random hexamers) and semiquantitative PCR were performed as described (Horcher et al. 2001), using the primers shown in Supplementary Table 3. PCR products were separated on agarose gels and visualized by ethidium bromide.

3D DNA-FISH and confocal analysis

Three-color 3D FISH experiments were carried out with sorted pro-T and pre-T cells as well as with in vitro cultured pro-B cells as previously described in detail (Skok et al. 2001). The fixation

conditions used were designed to preserve nuclear integrity (Skok et al. 2001). Cells were analyzed by confocal microscopy on a Leica SP2 AOBS (Acousto Optical Beam Splitter) system. Optical Z-sections were collected at 0.3-µm steps through individual nuclei. Only cells containing signals of both *IgH* loci were evaluated. DNA probes were prepared from the BACs 526A21 (*V_HJ558*), 243G9 (*V_H10*), and 167C1 (*V_H7183*; Kosak et al. 2002), as well as from the plasmid pγ1/HE17 containing a 17-kb genomic insert of the *Cγ1* region (Skok et al. 2001). The distance between the signals of the different *IgH* gene probes in the nucleus was measured on individual confocal images. A distance of 0.3–0.5 µm was evaluated as "separate," whereas "far apart" referred to a distance of 0.5–1.5 µm (Supplementary Table 1; Figs. 5–7).

Retroviral infection of pro-B cells

A human *Pax5* cDNA was inserted upstream of the IRES-hCD2t gene of the retroviral vector MiCD2, which results in expression of a C-terminally truncated (t) human (h) CD2 indicator protein (Heavey et al. 2003). The M-Pax5-iCD2 virus was generated and used for infection of *Pax5*$^{-/-}$ and *Pax5*$^{-/-}$ *RAG2*$^{-/-}$ pro-B cells as described (Heavey et al. 2003). Pax5 expression was verified by Western blot analysis of infected pro-B cells.

Acknowledgments

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