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{subscript}H-to-DJ{subscript}H rearrangement of distal, but not proximal V{subscript}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{subscript}H-DJ{subscript}H rearrangements in IkPax5{superscript/+} thymocytes, thus recapitulating the loss-of-function phenotype of Pax5{superscript−/−} pro-B cells. The phenotypic similarities of both cell types include (1) chromatin accessibility of distal V{subscript}H genes in the absence of V{subscript}H-DJ{subscript}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{subscript}H genes from proximal segments in an extended IgH locus. Reconstitution of Pax5 expression in Pax5{superscript−/−} pro-B cells induced large-scale contraction and distal V{subscript}H-DJ{subscript}H rearrangements of the IgH locus. Hence, V{subscript}H-DJ{subscript}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{subscript}H-DJ{subscript}H recombination. Pax5 subsequently activates locus contraction and distal V{subscript}H-DJ{subscript}H rearrangements in collaboration with an unknown factor that is present in pro-B cells, but absent in thymocytes.

[Keywords: Pax5/BSAP; V{subscript}H-DJ{subscript}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 synopsis 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{alpha} and Ig{lambda} light-chain genes in pre-B cells, whereas the TCR{beta} and TCR{alpha} 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 synopsis 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{subscript}H-J{subscript}H rearrangements preceding V{subscript}H-DJ{subscript}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 $D_{\gamma \delta \epsilon}$-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 $D_{\gamma \delta \epsilon}$-rearranged IgH alleles at low frequency [Kurosawa et al. 1981, Born et al. 1988; Corcoran et al. 2003]. Importantly, $V_{\mu}$–$D_{\gamma \delta \epsilon}$ 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 $V_{\mu}$–$D_{\gamma \delta \epsilon}$ 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 [Burorowski et al. 2002].

The IgH locus with its 150–200 $V_{\mu}$ 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 in germ-line configuration [Bain et al. 1994; Lin and Grosschedl 1995]. Both transcription factors appear to control the initial $D_{\gamma \delta \epsilon}$-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 $D_{\gamma \delta \epsilon}$-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; Urbânek et al. 1994; Nult et al. 1997], which still retain a broad lympho-myeloid developmental potential characteristic of uncommitted progenitors [Nult et al. 1999; Rolink et al. 1999]. Restoration of Pax5 expression in Pax5−/− pro-B cells suppresses this multilineage potent-
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}–D_{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}–D_{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}–D_{H} recombination within the proximal domain of the IgH locus. Subsequently, Pax5 activates large-scale contraction and distal V_{H}–D_{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}–I_{H} and V_{H}–D_{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-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}–I_{H} and V_{H}–D_{H} rearrangements [Fig. 1B]. No V_{H}–D_{H} rearrangements and only a low level of D_{H}–I_{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}–I_{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}–I_{H} recombination in thymocytes. More importantly, Ik^{Pax5/−} thymocytes carried V_{H}–D_{H} rearrangements [Fig. 1B], which are normally restricted to the B-lymphoid lineage (Kurosawa et al. 1981). The proximal V_{H}7183 and V_{H}Q52 genes [Fig. 1A] were rearranged in Ik^{Pax5/−} pro-B cells as efficiently as in wild-type pro-B cells, whereas a slightly lower level of proximal V_{H}7183 and V_{H}Q52 gene rearrangements was detected in Ik^{Pax5/−} pro-T cells [Fig. 1B]. Unexpectedly, however, the recombination frequency of the more distal V_{H}Gam3.8, V_{H}3609, and V_{H}558 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}–D_{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}–I_{H} and V_{H}–D_{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}–I_{H} and different V_{H}–D_{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. Three-fold 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 I_{H}1, I_{H}2, and I_{H}3 segments. (C) Quantitation of D_{H}–I_{H} recombination in sorted pro-B and pre-T cells. D_{H}–I_{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 Igk rearrangements in \( I_{\text{K Pax5}+/} \) thymocytes

Sequence analysis of the \( V_{\mu}D_{\mu}J_{\mu} \) PCR fragments revealed that \( V(D)J \) recombination in \( I_{\text{K Pax5}+/} \) pre-T cells involved different members of the \( V_{\mu 7}7183 \) and \( V_{\mu 9}Q52 \) 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 \( V_{\mu r}D_{\mu}I_{\mu H} \) 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* \( I_{\text{K Pax5}+/} \) thymocytes at the expected frequency (Fig. 2A). However, no \( V_{\mu r}–I_{\mu} \) rearrangements could be detected in \( I_{\text{K Pax5}+/} \) pre-T cells, demonstrating that Pax5 is unable to induce rearrangements at the Igk 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 \( I_{\text{K Pax5}+/} \) thymocytes, which is consistent with expression data shown below. Most \( I_{\text{K Pax5}+/} \) thymocytes expressed TCRβ on the cell surface [Fig. 2A], in agreement with the observation that \( D_{\mu 2}–I_{\mu 2} \) and \( V_{\mu 5.1}–D_{\mu 2}I_{\mu 2} \) rearrangements were present at similar frequency in \( I_{\text{K Pax5}+/} \) and control \( I_{\text{K neo}+/} \) pre-T cells [Fig. 2B]. These data further demonstrate that the \( I_{\text{K Pax5}+/} \) thymocytes are of T-lymphoid origin despite ectopic expression of the B-lineage commitment factor Pax5 [Souabni et al. 2002].

Conditional Pax5 activation induces \( V_{\mu r}–D_{\mu}I_{\mu H} \) rearrangements within the T-lymphoid lineage

The expression of \( RAG1 \) and \( RAG2 \) is initiated in the earliest lymphocyte progenitor [ELP], resulting in subsequent \( D_{\mu r}I_{\mu H} \) 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 \( I_{\text{K Pax5}} \) 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 \( I_{\text{K Pax5}+/} \) mice may undergo \( V_{\mu r}–D_{\mu}I_{\mu H} \) rearrangements before migration to the thymus and initiation of T-cell development. Alternatively, the \( V_{\mu r}–D_{\mu}I_{\mu H} \) rearrangements present in \( I_{\text{K Pax5}+/} \) thymocytes may originate within the T-lymphoid lineage. To distinguish between these two possibilities, we took advantage of the conditional \( I_{\text{K neo}} \) 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 \( I_{\text{K neo}+/} \) mouse with a transgenic mouse, which expressed the Cre recombinase under the control of the proximal lck promoter \( \text{lck-cre} \) during pro-T-cell development [Lee et al. 2001; Wolfer et al. 2002]. The \( \text{lck-cre} \) transgene was shown to initiate Cre-mediated inactivation of a floxed \( \text{Notch1} \) 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 \( \text{lck-cre} \) transgene deleted the floxed neomycin gene of the \( I_{\text{K neo}+/} \) allele with lower efficiency, as the activated \( I_{\text{K Pax5}} \) allele was detected in only ~60% of pre-T cells [Fig. 3]. Nevertheless, these thymocytes of \( I_{\text{K neo}+/} \) lck-cre mice were characterized by an increase in \( D_{\mu r}I_{\mu H} \) rearrangements and the presence of \( V_{\mu r}–D_{\mu}I_{\mu H} \) rearranged IgH alleles [Fig. 3]. The \( V_{\mu r}–D_{\mu}I_{\mu H} \) rearrangement frequency was high-
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 \( I^k_{neo} \) mice by depleting Lin\(^{+}\) cells (including B220\(^{+}\) B cells), followed by sorting for Thy1.2\(^{+}\) Lin\(^{-}\) DN thymocytes. Flow cytometric analysis demonstrated that the sorted Thy1.2\(^{+}\) Lin\(^{-}\) pro-T cells were purged to homogeneity [Fig. 4A]. Moreover, RT–PCR analysis failed to detect rearranged Igk and Igk1 mRNAs in these sorted cells [Fig. 4B]. We conclude, therefore, that the sorted \( I^k_{neo} \) 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 (GILT), whereas the abundance of spliced IgH \( (V_H-DJ_H) \) 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 3.** \( V_H-DJ_H \) rearrangements upon conditional Pax5 activation in thymocytes. Pre-T cells were isolated by FACS sorting as DP thymocytes from \( I^k_{neo} \) 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. \( I^k_{neo} \) pro-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 \( I^k_{neo} \) to the \( I^k_{Pax5} \) allele was determined by PCR [bottom row].

**Figure 4.** Activation of germ-line \( V_H \) transcription and B-cell-specific gene expression in \( I^k_{Pax5/−} \) pro-T cells. (A) Flow cytometric reanalysis of sorted \( I^k_{Pax5/−} \) 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 \( I^k_{Pax5/−} \) pro-T cell population. Rearranged Igk \( (V_k-J_C_k) \) and Igk1 \( (V_k-J_C_k1) \) mRNAs could not be detected by RT–PCR in purified \( I^k_{Pax5/−} \) 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 \( I^k_{Pax5/−} \) 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. \( V_H7183-DJ_H \) and \( V_H7183-DJC \) refer to rearranged IgH mRNAs and GILT 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 IkPax5+/+ pro-T cells compared with wild-type pro-B cells. The rearranged mRNAs of the Vα7183 genes were, however, present at similar abundance in all three cell types (Fig. 4C), consistent with efficient \(V_{\alpha}7183-DJ_{\alpha}H\) recombination in these cells [Fig. 1B; Hesselin et al. 2003]. In contrast, the rearranged mRNA of the distal \(V_{\alpha}J558\) gene family was reduced ~100-fold in \(I_{k}Pax5^{+/+}\) pro-T cells and Pax5−/− pro-B cells (Fig. 4C), which undergo \(V_{\alpha}J558-DJ_{\alpha}H\) rearrangements with a similarly low efficiency compared with wild-type pro-B cells (Fig. 1B; Nutt et al. 1997).

Germline \(V_{\alpha}J558\) transcripts were, however, present at a similar abundance in \(I_{k}Pax5^{+/+}\) pro-T cells relative to wild-type and Pax5−/− pro-B cells (Fig. 4C). Germline transcripts of the TCR \(V_{\beta}5\) gene could not be detected in pro-B cells, but were expressed in \(I_{k}Pax5^{+/+}\) 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 \(I_{k}Pax5^{+/+}\) 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 \(I_{\kappa}g\alpha\) (+/−, Nutt et al. 1998) in \(I_{k}Pax5^{+/+}\) pro-T cells (Fig. 4C). More surprisingly, these pro-T cells also expressed the genes \(I_{\kappa}g\beta\) [B29], \(\lambda_{5}\), 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 \(I_{k}Pax5^{+/+}\) 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 \(I_{\kappa}g\beta\) gene [Wang et al. 1998]. Moreover, the efficient expression of all pre-BCR components (\(k_{5}\), VpreB, \(I_{\kappa}g\beta\), and \(I_{\kappa}g\beta\)) strongly suggests that the rearranged Igµ protein is transported as part of the pre-BCR to the cell surface of \(I_{k}Pax5^{+/+}\) 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 \(I_{k}Pax5^{+/+}\) 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). PRD1 conditions, which detect human and mouse Pax5 mRNAs with equal efficiency, revealed that the human Pax5 minigene of the \(I_{k}Pax5^{+/+}\) 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 \(I_{k}Pax5^{+/+}\) pro-T cells.

Relocation of the IgH locus from the nuclear periphery to central positions in \(I_{k}Pax5^{+/+}\) 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 \(I_{k}Pax5^{+/+}\) thymocytes, we next used three-color 3D FISH analysis (Skok et al. 2001) to localize different \(Ig\) gene segments in three-dimensionally (3D) preserved nuclei by confocal laser scanning microscopy [Fig. 5]. The locations of the different \(V_{H}\) and \(C_{y1}\) 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 \(V_{\alpha}J558\) and \(V_{\mu}15\) 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 \(V_{\alpha}7183\) and \(C_{y1}\) 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 \(V_{\mu}\) 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 \(D_{H}-I_{H}\) 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 \(I_{k}Pax5^{+/+}\) 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 \(I_{k}Pax5^{+/+}\) 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 \(V_{\alpha}J558\) and \(V_{\mu}15\) genes were still separated from the proximal \(V_{\alpha}7183\) and \(C_{y1}\) genes in 92%–100% of all \(I_{k}Pax5^{+/+}\) pro-T and pre-T cells [Fig. 5C]. The physical separation of distal \(V_{H}\) genes from the rearranged \(D_{H}\) segment is therefore a likely cause for the low \(V_{H}-DJ_{H}\) recombination efficiency of distal \(V_{H}\) genes in \(I_{k}Pax5^{+/+}\) thymocytes.

The IgH locus is present in an extended chromatin state in Pax5−/− pro-B cells

As the Pax5−/− pro-B cells and \(I_{k}Pax5^{+/+}\) thymocytes exhibit a similar \(V_{H}-DJ_{H}\) recombination phenotype, we
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_{H}\text{J}\text{558} and V_{\text{B}}15 genes were separated from the proximal V_{H}17183 and C_{\text{J}}1 genes in 84%–94% of all Pax5−/− pro-B cells [Fig. 6A,B], similar to the situation observed in IkPax5−/− 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_{\text{H}}\text{I}-DJ\text{H} rearrangements in pro-B cells

To directly demonstrate an involvement of Pax5 in IgH locus contraction and distal V_{\text{H}}\text{I}-DJ\text{H} recombination, we used retroviral infection to restore Pax5 expression 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]. DJ\text{H}−/− and proximal V_{\text{H}}17183-DJ\text{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_{H}\text{J}\text{558}-DJ\text{H} rearrangement above the level seen 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_{H}\text{J}\text{558} and V_{\text{B}}15 genes were separated from the proximal V_{\text{H}}17183 and C_{\text{J}}1 genes in 94%–96% of the Pax5−/− RAG2−/− pro-B cells infected with the parental MiCD2

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 IkPax5−/− pro-T [DN] cells. The different probes with their colors are indicated. The V_{H}\text{J}\text{558} BAC [52E2A21] was directly labeled with Cy5-dUTP [blue]. The V_{H}17183 [167C1] BACs as well as V_{\text{B}}15 [243G9] BAC [not shown in panel B] were labeled with digoxigenin-dUTP and detected with rhodamine- and Texas red-coupled anti-digoxigenin antibodies [red]. The C_{\text{J}}1 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 flt sites in the IkPax5−/− allele [Soubani et al. 2002], were specifically deleted for the three-color FISH analysis by mating IkPax5−/− 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.
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−/− 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\textsubscript{H}–DJ\textsubscript{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\textsubscript{H}–DJ\textsubscript{H} recombination of distal but not proximal V\textsubscript{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\textsubscript{H} genes in pro-T cells. The distal V\textsubscript{H}558 genes are, however, present in accessible chromatin in IK\textsubscript{Pax5+/−} pro-T cells similar to Pax5−/− pro-B cells. Hence, the loss of Pax5 in pro-B cells results in the same V\textsubscript{H}–DJ\textsubscript{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\textsubscript{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\textsubscript{H}–DJ\textsubscript{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\textsubscript{H}–DJ\textsubscript{H} rearrangements (Fig. 7D). According to this hypothesis, the absence of factor X in IK\textsubscript{Pax5+/−} pro-T cells or the loss of Pax5 in Pax5−/− pro-B cells results in equally inefficient recombination of distal V\textsubscript{H} genes. Importantly, the restoration of Pax5 expression in Pax5−/− pro-B cells unequivocally demonstrated that Pax5 promotes distal V\textsubscript{H}–DJ\textsubscript{H} recombination by inducing large-scale contraction of the IgH locus, which leads to juxtaposition of proximal IgH genes next to the proximal DJ\textsubscript{H}–V\textsubscript{H} gene segment.

The IgH locus contains 150–200 V\textsubscript{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\textsubscript{H} genes and proximal DJ\textsubscript{H}–V\textsubscript{H} rearranged gene segment may be ineffective, resulting in a lower efficiency of V\textsubscript{H}–DJ\textsubscript{H} recombination for distal relative to proximal V\textsubscript{H} genes. Indeed, V\textsubscript{H}–DJ\textsubscript{H} recombination exhibits a marked preference for the utilization of the most proximal V\textsubscript{H} genes both in fetal and adult B-lymphopoiesis (Yancopoulos et al. 1984; Malynn et al. 1990). Even within the proximal V\textsubscript{H}7183 family, the more proximally located V\textsubscript{H} genes are preferentially used for V(D)J recombination (Wiliams et al. 2001). This position-dependent bias in V\textsubscript{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\textsubscript{H}–DJ\textsubscript{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 these two separate steps of IgH locus activation. As illustrated by the two-step model in Figure 7D, 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 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 $D_{Hr}$–$J_H$ 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 $V_{Hr}$–$D_{Hr}$ rearrangements as observed in $I_k^{Pax5/−/−}$ 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 $I_k^{Pax5/−/−}$ 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 $V_{Hr}$–$D_{Hr}$ 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 1983], 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 $D_{Hr}$–$I_{Hr}$, and $C_H$ gene segments is first hyperacetylated prior to $V(D)J$ recombination. $D_{Hr}$–$I_{Hr}$ 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-7R\(^{−/−} \) mice (Corcoran et al. 1998). Despite the similar recombination phenotype of IL-7R\(^{−/−} \) 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 \( V_{H} J_{558} \) 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 \( Ik^{nemo^{+}} \) [Souabni et al. 2002], \( Pax5^{−/−} \) [Nutt et al. 1997], \( RAG2^{−/−} \) [Shinkai et al. 1992], and \( ick-cre \) 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), TCRB (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 Ig\( \mu \) protein was performed as described [Thevenin et al. 1998]. Pro-B cells were sorted as B220c-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]. TCRB 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 AOSB [Acouto 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_H558), 243C9 (V_H10), and 167C1 (V_J17;83; Kosak et al. 2002), as well as from the plasmid pRM103 containing a 17-kb genomic insert of the C 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-hCD2t virus was generated and used for infection of Pax5-/- and Pax5+/- pro-B cells as described [Heavey et al. 2003]. Pax5 expression was verified by Western blot analysis of infected pro-B cells.

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