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Antisense Intergenic Transcription Precedes *Igh* D-to-J Recombination and Is Controlled by the Intronic Enhancer E_μ ▽‡

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V(D)J recombination is believed to be regulated by alterations in chromatin accessibility to the recombinase machinery, but the mechanisms responsible remain unclear. We previously proposed that antisense intergenic transcription, activated throughout the mouse *Igh* V_H region in pro-B cells, remodels chromatin for V_H-to-DJ_H recombination. Using RNA fluorescence in situ hybridization, we now show that antisense intergenic transcription occurs throughout the *Igh* D_HJ_H region before D-to-J recombination, indicating that this is a widespread process in V(D)J recombination. Transcription initiates near the *Igh* intronic enhancer E_μ and is abrogated in mice lacking this enhancer, indicating that E_μ regulates D_H antisense transcription. E_μ was recently demonstrated to regulate D_H-to-J_H recombination of the *Igh* locus. Together, these data suggest that E_μ controls D_H-to-J_H recombination by activating this form of germ line *Igh* transcription, thus providing a long-range, processive mechanism by which E_μ can regulate chromatin accessibility throughout the D_H region. In contrast, E_μ deletion has no effect on V_H antisense intergenic transcription, which is rarely associated with D_H antisense transcription, suggesting differential regulation and separate roles for these processes at sequential stages of V(D)J recombination. These results support a directive role for antisense intergenic transcription in enabling access to the recombination machinery.

In order to generate the primary repertoire of immunoglobulin (Ig) and T-cell receptor (TCR) molecules, antigen receptor loci undergo variable, diversity, and joining [V(D)J] recombination in B and T lymphocytes. Recombination is catalyzed by a recombinase complex containing the protein products of the recombinase-activating genes *Rag1* and *Rag2* (28). Within precursor lymphocytes, this process is strictly lineage specific, with heavy (*Igh*) and light (*Ig*κ and *Ig*λ) immunoglobulin loci fully recombining only in B lymphocytes and T-cell receptor loci (*Tcra*, *Tcrb*, *Tcrg*, and *Tcrd*) recombining only in T cells. Further, within lineages, loci are recombined in a precise order. Recombination of the *Igh* locus is the earliest step in the generation of the mature antibody repertoire in B lymphocytes. The *Igh* locus of the C57BL/6 mouse spans 3 Mb and comprises 195 V_H genes spanning 2.5 Mb, 10 D_H genes (~60 kb), 4 J_H genes (2 kb), and 8 constant (C_H) genes (200 kb) (31, 68). D_H-to-J_H recombination occurs on both *Igh* alleles before V_H-to-DJ_H recombination takes place (16).

Lineage and stage specificity of V(D)J recombination are regulated by differential chromatin accessibility to the RAG proteins. Several mechanisms may contribute, but their relative importance is still unclear. The first process discovered was germ line transcription, which occurs in all antigen receptor loci across gene segments competent for recombination (34). This transcription was termed "sterile" or "germ line" to distinguish it from coding V(D)J transcription. In the *Igh* locus, the earliest germ line transcripts detected occur before D_H-to-J_H recombination and initiate from two regions: the intronic enhancer E_μ (I_μ transcript) (35) and a promoter, PDQ52, immediately upstream of the most 3′ D_H gene segment, DQ52 ($_\mu$0 transcript) (60). Following D_H-to-J_H recombination, the DJ_H gene segment produces D_μ transcripts (51) and sense germ line transcription initiates over the V_H genes (17, 67). The discovery of V_H gene germ line transcription formed the basis of the accessibility hypothesis, which proposed that lineage and stage specificity of recombination are regulated by differential chromatin accessibility of antigen receptor gene segments to the recombinase machinery, with germ line transcription associated with open chromatin (58, 67). However, a function for V_H germ line transcription has not been demonstrated, and it has been argued that it may be a secondary effect of the V_H gene promoters becoming accessible for V_H-to-DJ_H recombination.

We have recently provided evidence in support of a functional role for germ line transcription with the discovery that an additional process, intergenic antisense transcription, not controlled by V_H gene promoters, occurs throughout the V_H region before V_H-to-DJ_H recombination. We proposed that this large-scale transcription remodels the V_H region to facilitate accessibility for V_H-to-DJ_H recombination (8).

Intergenic transcription has been proposed to drive through repressive chromatin, recruiting remodeling factors, thus facilitating further chromatin opening over genes (12). In several loci, intergenic transcription delineates domains of modified chromatin that surround active genes and their regulatory el-

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ements (7, 21, 25). RNA polymerase II is associated with, or recruits, a wide range of chromatin-remodeling and histone-modifying factors (14, 65, 66). Furthermore, transcription triggers histone turnover and the deposition of variant histone H3.3, enriched with active modifications (54). Collectively, these activities suggest several mechanisms by which the processing activity of the elongating RNA polymerase II complex can achieve chromatin accessibility (42, 47).

The process of intergenic transcription may therefore direct the changes in chromatin structure that precede V(D)J recombination. In the IgH V region, these include acetylation of histones H3 and H4, markers of accessible chromatin (15, 29, 38); histone H3.3 exchange (30); and methylation of H3 lysine 27 (H3-K27) (59). Indeed, it was recently demonstrated that disruption of intergenic transcription in the Jα domain of the mouse TCRα locus results in failure both to methylate H3 lysine 4 and to recombine several corresponding Jα genes (1), demonstrating a requirement for intergenic transcription to effect V(D)J recombination. In addition to localized changes, several large-scale changes in nuclear configuration occur, including repositioning of the *Igh* loci away from the nuclear periphery (32) and Pax-5-dependent contraction of the V_H region, via higher-order looping of chromatin (23, 32, 53).

In the D region, increased DNase I sensitivity (15, 37), enrichment of acetylated histones H3 and H4, H3K4 methylation, and nucleosome remodeling over D_H and J_H genes occurs in pro-B cells poised for D_H-to-J_H recombination (15, 37, 43). Overall, while it is evident that several processes play critical roles in the regulation of V(D)J recombination, the precise role of each remains unclear.

It also remains to be clarified which regulatory elements direct V(D)J recombination of the *Igh* locus. It was originally proposed that the intronic enhancer E_μ regulated V_H-to-DJ_H recombination (52, 55). However, more recent studies by ourselves and others have shown that targeted deletion of E_μ causes a defect in D_H-to-J_H recombination, indicating that E_μ regulates this process primarily (2, 49). Thus, previously observed defects in V_H-to-DJ_H recombination are likely to be secondary to this earlier defect, and a V region-specific recombination control element has not yet been found. Although the function of E_μ in V(D)J recombination is now known, its mechanism of action remains unclear, and much remains to be understood about the chromatin regulation of the D_H region before D_H-to-J_H recombination in vivo.

Our discovery of intergenic antisense transcription over the *Igh* V region before V-to-DJ recombination raised the question of whether similar transcriptional processes precede other V(D)J recombination events. Here we ask whether germ line transcription occurs over the D_H region and whether this precedes and is functionally associated with D_H-to-J_H recombination.

MATERIALS AND METHODS

Mice. *Rag1*$^{-/-}$ mice (57), backcrossed to a C57BL/10 background, were kindly provided by M. Turner. C57BL/6 wild-type (WT) mice and timed matings were from the Small Animal Barrier Unit, Babraham Institute. PDQ52 and E_μ gene-targeted mice were as described previously (2). Animal work was performed under project license PPL 80/1644, in compliance with Home Office guidelines.

Sorting of B-lymphocyte populations. $CD19^+$ B lymphocytes from *Rag1*$^{-/-}$ bone marrow (BM) were sorted for RNA by positive selection using anti-CD19 magnetic affinity cell sorting (MACS) magnetic beads (Miltenyi Biotech; purity, >80% $CD19^+$). $B220^-$ interleukin 7R (IL-7R)$^+$ cells (common lymphoid progenitors [CLPs]) were fluorescence-activated cell sorter (FACS) sorted directly from WT BM without prior enrichment. We sorted $B220^+CD19^+$ *Rag1*$^{-/-}$ BM B cells for RNA fluorescence in situ hybridization (RNA FISH) directly by FACS. The following antibodies, obtained from BD PharMingen, were used: phycoerythrin-labeled, allophycocyanin (APC)-labeled, or biotinylated anti-B220 (RA3-6B2); fluorescein isothiocyanate (FITC)-, APC-, or peridinin chlorophyll-cy5.5-labeled or biotinylated anti-CD19 (1D3); and biotinylated anti-IL-7R (B12-1). The purity of each cell fraction was always >90%. Cells were washed twice in phosphate-buffered saline and then applied to poly-L-lysine-coated slides (Sigma) and fixed with 3.7% formaldehyde (Merck).

Fetal liver B cells were isolated by negative MACS selection using a biotinylated anti-Ter119 antibody (BD Pharmingen). $B220^+CD19^+$ cells were then sorted from the negative fraction by FACS using the same antibodies described above. These cells were then used to make either RNA or slides for RNA FISH as described above. $B220^+$ cells were sorted from BM of *Rag*-deficient mice harboring either WT, PDQ52$^{-/-}$E$^{+/+}$, or P-E$^-$ alleles, using biotinylated anti-B220, followed by streptavidin-conjugated MACS beads.

Reverse transcription-PCR analysis. Total RNA was extracted from BM cell populations and cell lines using an RNeasy kit (QIAGEN) according to the manufacturer's protocol, except for total RNA from *Rag*-deficient mice harboring WT, PDQ52$^{-/-}$E$^{+/+}$, or P-E$^-$ alleles, which was isolated using TRIzol (Invitrogen Life Technologies). The RNA was treated with RNase-free DNase I (1 unit per μg of total RNA; Promega) for 1 h at 37°C followed by repurification using a QIAGEN RNeasy kit according to the manufacturer's RNA cleanup protocol. Between 0.1 and 0.5 μg of total RNA was reverse transcribed for 2 h using Superscript III (Invitrogen) at either 50°C, with 100 ng of random hexamers (Amersham Pharmacia), or 55°C, with 2 pmol of specific reverse transcription primer (see Table S1 in the supplemental material). Reverse transcription reactions were assembled at 55°C to reduce endogenous priming. Following reverse transcription, PCR was performed with the primers listed in Table S1 in the supplemental material, using the following conditions: 30 to 40 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 30 s at 72°C. Annealing temperatures varied according to the melting temperatures of the primer pairs (see Table S1 in the supplemental material). Inverted gel images were sufficient to visualize products, with the exception of 81X (see Fig. 3), which was detected by Southern blotting using a ^{32}P-labeled genomic DNA PCR product probe. DNA bands were excised, gel purified using a QIAquick gel extraction kit (QIAGEN), and cloned into the pGEM-T Easy vector (Promega) for sequencing. Real-time PCR was performed in triplicate on cDNA reversed transcribed with random hexamers as previously described, except 1 μg of total RNA was used. PCRs were detected with SYBR green fluorogenic dye (Applied Biosystems) in an ABI PRISM 7000 sequence detection system (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60 to 64°C (depending upon primer pair) for 1 min. The PCR primers used are listed in Table S1 in the supplemental material. Relative quantification was performed after construction of standard curves using serial dilutions of genomic DNA, and values were then normalized to J558.

RNA FISH. Sorted cells fixed on slides were analyzed by two-color RNA FISH essentially as described previously (24), with the modifications listed by Gribnau et al. (25). For three-color FISH, biotinylated probes were generated and detected with streptavidin Alexa 350 (Molecular Probes), followed by a biotinylated goat antistreptavidin antibody (Cambio), followed by an additional layer of streptavidin Alexa 350. When Alexa 350 was used, DAPI (4′,6′-diamidino-2-phenylindole) was omitted from the mounting media. Probes for FISH were generated by PCR (primers are listed in Table S1 in the supplemental material), cloned into pGEM-T Easy vector (Promega), and verified by sequencing. Single-stranded cDNA probes for detection of either sense or antisense transcripts were generated by in vitro transcription using a MAXIscript kit (Ambion) followed by reverse transcription with Superscript II (Invitrogen) including either digoxigenic-11-dUTP (Roche), 2,4-dinitrophenol-11-dUTP (Perkin Elmer), or biotin-11-dUTP (Roche) in the reaction (11). In hybridizations, antisense probes detected complementary sense transcripts, while sense probes detected antisense transcripts. Signals were visualized and captured using Olympus BX40 and BX41 microscopes. Experiments were performed two to three times, and at least 100 nuclei (two colors) or 100 alleles (three colors) were counted each time. Total numbers of cells counted are shown in Table S2 in the supplemental material.

RESULTS

Germ line antisense and intergenic transcription occurs throughout the D_H region. We assembled in silico the entire

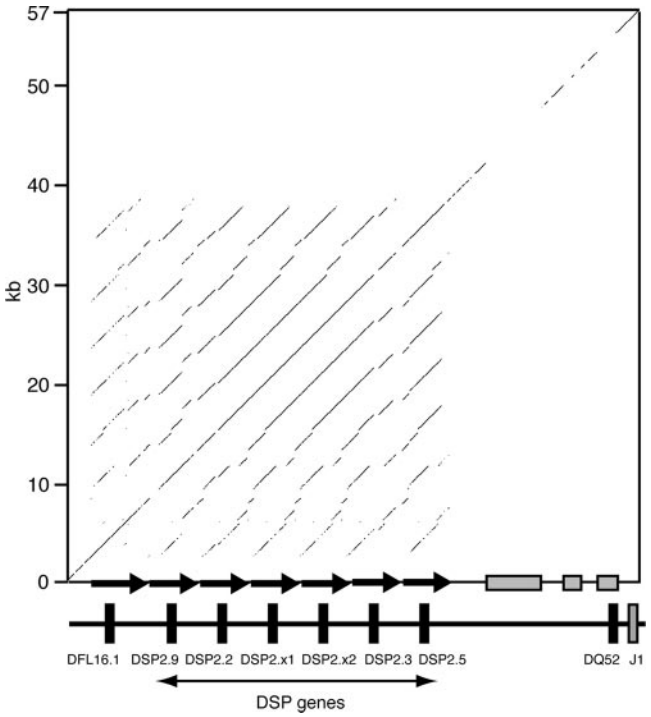

FIG. 1. DFL and DSP genes are located in a tandem array of ~4.7-kb repeats. The sequence of the D_H region of C57BL/6 was aligned against itself using the DotPlot program from the Omiga package (Accelrys). The interspersed repeats within the sequence on the vertical axis were masked with X's using RepeatMasker (www.repeatmasker.org) to exclude these from the alignment, resulting in a discontinuous diagonal line, in particular in the DSP2.5-DQ52 intergenic region, which contains several interspersed repeats (gray boxes on *x* axis). Black arrows depict the ~4.7-kb direct repeats containing the DSP and DFL16.1 genes. A sliding window of 30 bp was used; lines indicate ≥90% identity. D_H genes are labeled in accordance with Ye (68). For clarity, the DST4 and P2-P6 genes are not depicted.

D_H region of the C57BL/6 mouse *Igh* locus from available genomic sequences. Subsequently, the assembly of this region was reported elsewhere (68). Within this sequence, all D_H gene segments are oriented in the same (sense) direction relative to the constant region genes. The C57BL/6 D_H region is organized as a tandem array of seven 4.7-kb repeats, each repeat containing a DFL16.1 or DSP gene (Fig. 1). The DSP repeats are almost identical to each other (95 to 99%), while the DFL16.1 repeat is ~85% identical to each of the DSP repeats. The most 3′ D gene, DQ52, is separated from the DFL/DSP tandem array by ~20 kb of unique sequence and has very different flanking sequences, including the PDQ52 promoter/enhancer (33).

We first investigated germ line transcription of the DFL16.1/DSP region, since this constitutes most of the D_H region and the vast majority of D_H gene usage in V(D)J recombination in C57BL/6 mice (20, 68). We designed reverse transcription and nested PCR primers to detect all the DSP genes equally in a single reaction and a set to detect the DFL16.1 gene separately. Reverse transcription and PCR primers were located downstream of the D_H gene 3′ recombination signal sequence (RSS) (Fig. 2A). As the 3′ RSS is lost due to D_H-to-J_H recombination, only transcripts originating from unrearranged D_H segments will be detected in WT mice. We first analyzed ex vivo $CD19^+$ BM cells from *Rag1*$^{-/-}$ mice (57) using a sensitive strand-specific reverse transcription-PCR assay (8). As these mice cannot perform V(D)J recombination, any D_H transcripts detected would be exclusively germ line in origin.

We reverse transcribed total RNA from *Rag1*$^{-/-}$ $CD19^+$ BM cells using random hexamers or gene-specific primers and performed PCR on the cDNA using nested primers. In both random and antisense-specific reverse transcription-PCRs, cDNA products of the same size as those from genomic DNA were amplified for both DSP and DFL, while no sense-specific products were amplified (Fig. 2C, i and ii). We did not detect any sense D_H germ line transcription using two further sets of reverse transcription and nested PCR primers, confirming previous findings (3). However, as previously described (8), we detected sense, but not antisense, transcription over the I_μ intron and C_μ exons 2 to 3 (Fig. 2B and C). We conclude that antisense, but not sense, transcription occurs over the germ line D genes of the *Igh* locus.

We next determined whether D_H antisense transcription was exclusively associated with the genes or extended into intergenic regions. As the intergenic sequences between the DSP genes are 95 to 99% identical, we were able to design one set of nested primers that would amplify interspersed repeat-free regions between each of the six DSP genes with equal efficiency (Fig. 2B, iii). Strand-specific reverse transcription-PCR showed an unspliced antisense band of the expected size, indicating that antisense transcription also occurred in intergenic regions (Fig. 2C, iii). We cloned and sequenced both the genic and intergenic reverse transcription-PCR products to find out whether the whole DSP array was transcribed or if the observed transcription was occurring across only certain regions. All but one DSP gene and one intergenic region were represented, indicating that transcription occurs across the entire DSP region (Fig. 2D).

We next investigated if the antisense transcription extended outside the DFL/DSP array using reverse transcription and nested PCR primers located between the most 3′ DSP gene, DSP2.5, and the most J-proximal D gene, DQ52 (Fig. 2B, iv). A strong antisense-specific band, but no sense band, was detected, indicating that the DSP-DQ52 intergenic region was also transcribed in the antisense orientation (Fig. 2C, iv). Taken together, the data indicate that antisense intergenic transcription extends throughout the D_H region.

To gain insight into how abundant these transcripts are and how their amounts compared to antisense transcripts we have previously detected in the V region, we performed quantitative real-time reverse transcription-PCR. We compared transcription from the unique DF16 gene with two unique intergenic regions, the first within the J558 gene family which comprises the 5′ half of the V region and the second from the J606 family in the middle of the V region. The reverse transcription reaction was performed with random hexamers so that the subsequent PCRs could be performed on the same sample. In all three cases, transcription has previously been detected only from the antisense strand. DFL16 transcript amounts were intermediate between J558 and J606, and variation was no greater than 2.5-fold among all three regions (Fig. 2E), indicating that steady-state amounts of antisense transcription over the D region are comparable to the V region in *Rag1*$^{-/-}$

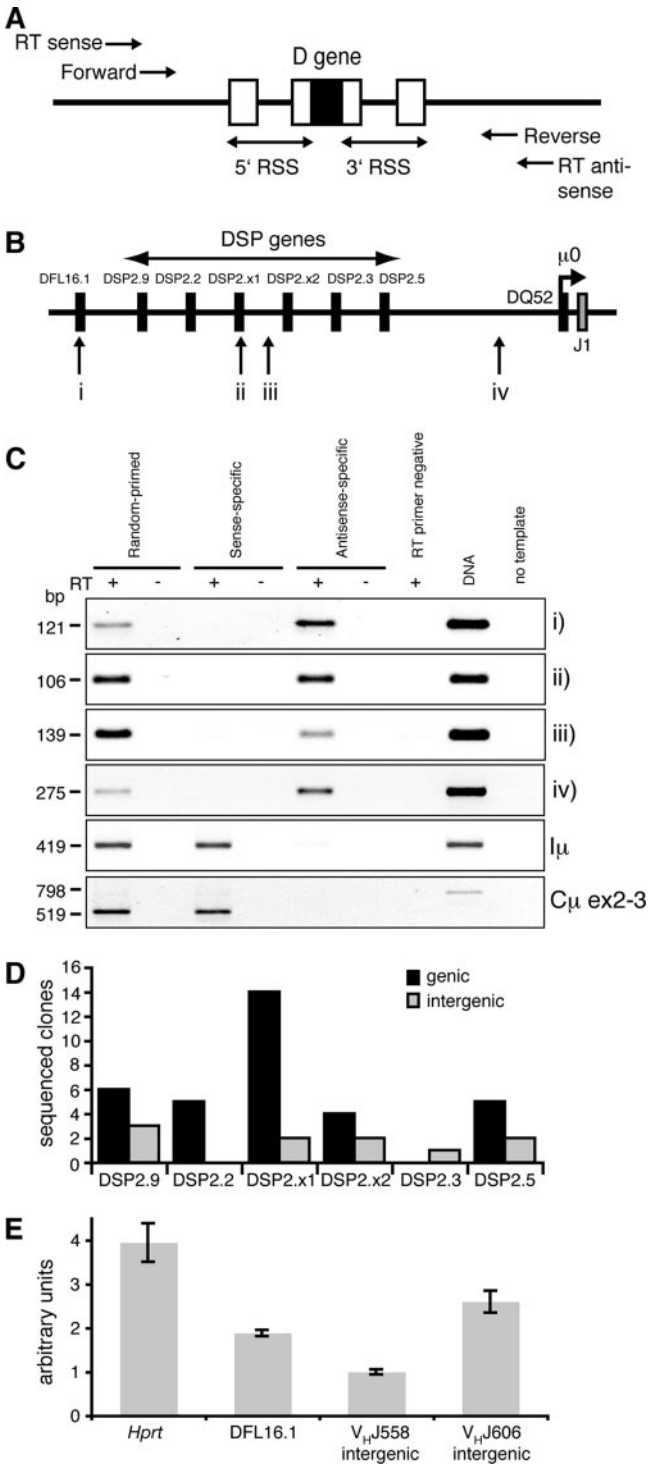

FIG. 2. Detection of germ line transcription across the *Igh* D region. (A) Design of the D_H region germ line transcription reverse transcription-PCR assay. Reverse transcription antisense (to detect sense transcription) and sense (to detect antisense transcription) primers were designed 5′ and 3′ of the D_H gene RSS sequences. Nested primers were used for PCR. Similar schemes were used for intergenic regions. (B) Schematic of the DFL16.1-J1 sequence showing regions analyzed by reverse transcription-PCR. Not to scale. (C) Reverse transcription-PCR of *Rag1*$^{-/-}$ CD19$^+$ total RNA. Above lanes, with (+) or without (−) reverse transcriptase (RT). Right margin, regions amplified (shown in panel B); left margin, molecular sizes. DNA, genomic DNA positive control. (D) Sequencing of DSP genic and intergenic reverse transcription-PCR products. The number of reverse transcription-PCR products cloned and sequenced is shown for each D_H gene and intergenic region. (E) Real-time quantitative reverse transcription-PCR of *Rag1*$^{-/-}$ CD19$^+$ random-primed cDNA showing relative transcription levels for *Hprt*, DFL16.1, V_HJ558 intergenic, and V_HJ606 intergenic regions. The level of V_HJ558 intergenic transcription was arbitrarily set to 1.

CD19$^+$ BM cells. Furthermore, amounts were within fourfold of the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), indicating that these transcripts are relatively abundant.

Developmental regulation of D_H antisense transcription. We next sought to investigate the stage specificity of D_H antisense transcription during B-lymphocyte development and V(D)J recombination. B lymphopoiesis initiates in the fetal liver with B-cell progenitors appearing by day 12.5 (18). D_H-to-J_H recombination initiates on day 12 and increases rapidly on days 13 and 14 (19). Thus, fetal liver on day 14 and day 15 includes B cells that have initiated and those that have completed D_H-to-J_H recombination (26). Consequently, initiation of V_H-to-DJ_H recombination is similarly asynchronous, and this is reflected by the reported expression of V_H germ line sense gene transcripts from day 14 until day 16 (36). Thus, in fetal liver, D_H-to-J_H recombination and V_H-to-DJ_H recombination occur in partially overlapping temporal windows.

We first investigated steady-state transcript levels in BM progenitor B lymphocytes from *Rag1*$^{-/-}$ (CD19$^+$) and day 14.5, 15.5, and 16.5 WT fetal liver B cells. Further, to obtain a more detailed picture of when D_H antisense transcription initiates, we also examined BM CLPs (IL-7R$^+$ B220$^-$), which undergo a low level of D_H-to-J_H recombination (4). We analyzed thymus to study the association of D_H antisense transcription specifically with D_H-to-J_H recombination, since up to 50% of thymocytes undergo D_H-to-J_H but are blocked from completing V_H-to-DJ_H recombination (9). We used brain as a negative control. By semiquantitative reverse transcription-PCR, we first analyzed expression of V region germ line transcripts. Random-primed reverse transcription followed by PCR to detect the largest J558 gene family in the D-distal end of the V region yielded two transcript bands, as previously shown for BM early B cells. The lower band corresponds to the spliced sense transcript, while the upper band includes both antisense and unspliced sense primary transcripts (8). Both bands were present in fetal liver B cells, with little variation between days 14.5 and 16.5 (Fig. 3), confirming previous Northern analysis (36). We found a similar developmental distribution of germ line transcripts from the D-proximal 7183 V gene family, albeit transcript levels were lower, due in part to PCR detection of fewer genes (Fig. 3). Neither transcript was present in the thymus, consistent with the V region's being inaccessible in thymocytes (Fig. 3). Steady-state D_H antisense transcription levels were similar to those in *Rag1*$^{-/-}$ CD19$^+$ cells in fetal liver B-cell populations, with little change between days 14.5, 15.5, and 16.5 (Fig. 3). D_H antisense transcripts are absent from brain (not shown) and low in CLPs but are readily detectable in thymocytes (Fig. 3). Taken together, these results

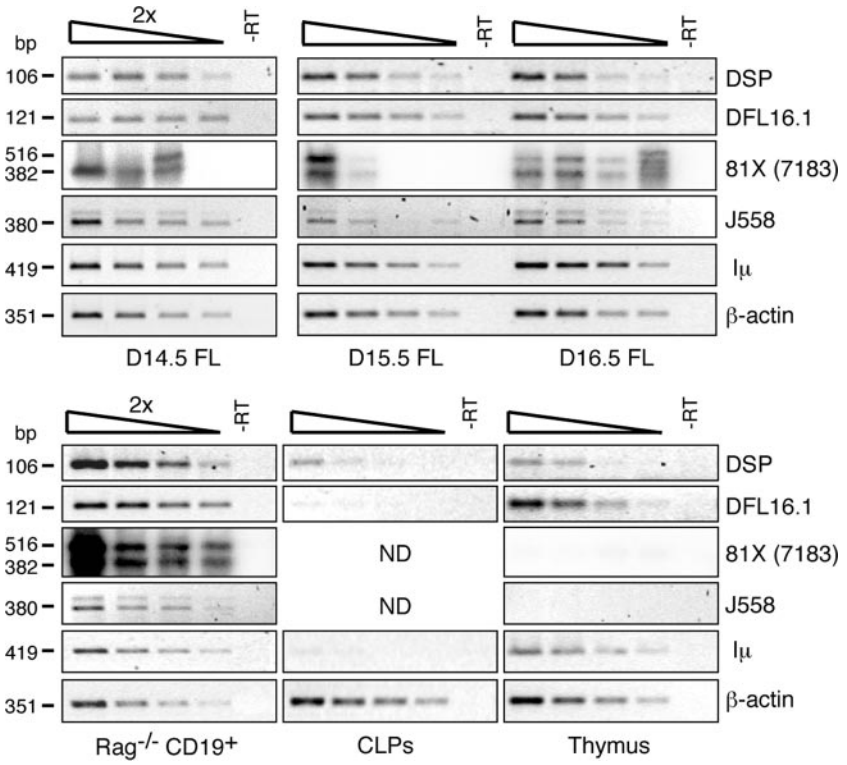

FIG. 3. Semiquantitative reverse transcription-PCR analysis of D_H antisense and V_H genic germ line transcription through development. Top panel: Day (D)14.5, D15.5, and D16.5 fetal liver B lymphocytes. Bottom panel: *Rag1*$^{-/-}$ CD19$^+$ BM B lymphocytes, CLPs, and ex vivo thymocytes. Right margin, regions amplified; left margin, molecular sizes. −RT, without reverse transcriptase. β-actin was used as a loading control. DSP and DFL16.1 antisense transcripts were detected as in Fig. 2. 81X and J558 were amplified by random-primed reverse transcription-PCR. The PCR primers were designed to amplify one (81X) or nine (J558) V genes. 81X products were subjected to blotting Southern and probed with a genomic DNA PCR product to aid visualization.

suggest that D_H antisense transcription initiates on *Igh* alleles poised for D_H-to-J_H recombination.

Reverse transcription-PCR was used to assess steady-state transcript levels in bulk populations. However, we sought to analyze D_H antisense primary transcription on individual *Igh* alleles in single cells to determine its association with the different steps of V(D)J recombination of the locus and thus provide clues to its function. To do this, we used RNA FISH. We designed 3.5-kb DFL16.1 and 2.8-kb DSP2.x1 RNA FISH probes containing each gene and respective flanking region (Fig. 4A). As the DSP repeats are >95% identical, it is likely that the DSP2.x1 probe detects transcription of all DSP genes. As the DFL and DSP segments are highly homologous, it was also probable that the probes would cross-hybridize. Therefore, for optimal detection efficiency, we combined the probes in all cases.

To first determine if we could detect D antisense intergenic transcription by RNA FISH, we analyzed CD19$^+$ pro-B cells from *Rag1*$^{-/-}$ mice. Sense or antisense D_H probes were cohybridized with a probe for I_μ, described previously (8). We used I_μ as a positive control, as it is transcribed from a high proportion of alleles (8) (Fig. 4A). We detected D_H antisense signals in 37% of *Rag1*$^{-/-}$ CD19$^+$ pro-B cells with I_μ signals. We did not detect any D_H sense signals, in agreement with the reverse transcription-PCR results. D_H antisense signals were closely associated with or overlapping the I_μ signals, as expected, given that the DSP/DFL region is located just ~24 kb from E_μ (Fig. 4B). We detected biallelic D_H antisense transcription in ~10% of cells, indicative of the stochastic output of a biallelically expressed transcription unit transcribing at a low level (61). In conclusion, we were able to detect D_H antisense transcription by RNA FISH in a high proportion of *Rag1*$^{-/-}$ pro-B cells.

We next investigated D_H antisense intergenic transcription in day 14.5, 15.5, 16.5, and 17.5 fetal liver B220$^+$CD19$^+$ B-lymphocyte populations (Fig. 4C). I_μ signals were detected in approximately 60% of cells in each of these samples, indicating that a large proportion of *Igh* alleles are active in fetal liver B lymphocytes. In day 14.5 and 15.5 populations, we detected D_H antisense signals in comparably high proportions of cells (30% and 32% of cells with I_μ signals, respectively). By day 16.5, the number of B cells with D_H antisense signals was reduced to ~18%, and this number was further reduced to 7% by day 17.5 (Fig. 4F). Thus, D_H antisense transcription occurs over an extended time window during fetal liver B-lymphocyte development but is progressively lost, probably as a result of ongoing V(D)J recombination (see below).

To further clarify how D_H antisense transcription relates to V(D)J recombination in fetal liver, we analyzed the appearance of sense transcripts from VDJ_H-rearranged alleles. V_H sense germ line transcripts are undetectable by our RNA FISH technique (8); thus, all V_H sense transcription signals detected

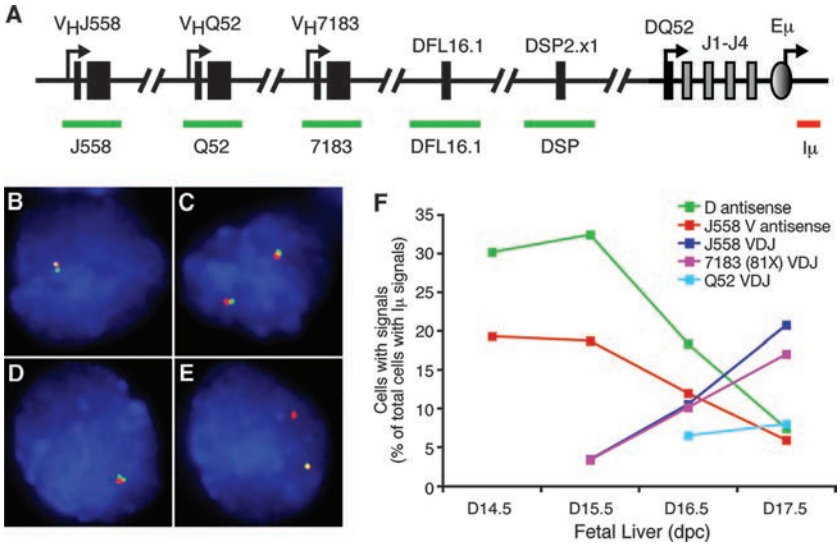

FIG. 4. Developmental regulation of D_H antisense transcription in fetal liver B lymphocytes. (A) Locations of RNA FISH probes: red, detected with Texas Red; green, detected with FITC. RNA FISH detection of D_H antisense intergenic and I_μ sense transcription in (B) $CD19^+$ *Rag1*$^{-/-}$ BM and (C) WT E15.5 fetal liver B lymphocytes. (D) RNA FISH detection of V_HJ558 antisense transcription in day 15.5 fetal liver B lymphocytes. (E) RNA FISH detection of V_HJ558 VDJ-rearranged (VDJ_H) transcription in day (D)16.5 fetal liver B lymphocytes. Similar signals were obtained for V_H7183 and V_HQ52. In all cases, nuclei were counterstained with DAPI (blue). (F) Quantitation of D_H antisense, V_HJ558 antisense, J558 VDJ_H, 7183 (81X) VDJ_H, and Q52 VDJ_H RNA FISH signals in $CD19^+$ B lymphocytes from D14.5, D15.5, D16.5, and D17.5 fetal liver.

originate from VDJ_H-rearranged alleles. We used probes to detect VDJ_H-rearranged transcription for the 3′ V_H7183 (81X) and V_HQ52 families and the large 5′ V_HJ558 family (8). In day 15.5 fetal liver B lymphocytes, we detected VDJ_H signals for both V_H7183 and V_HJ558 in similarly low numbers of cells (~3% of cells with I_μ) (Fig. 4E and F). By day 16.5, signals for both families had increased to 10%, while at day 17.5, V_H7183 signals were found in 17% and V_HJ558 signals in 21% of cells with I_μ signals. This distribution is as expected, given the greater number of J558 genes (31), tempered by a mild bias toward V_H7183 recombination in C57BL/6 fetal liver (5). These results correlated well with a previous report of Northern blot detection of J558 VDJ_H transcripts from day 16.5 of development (36). Signals from the V_HQ52 family were found in 6.5% and 8% of day 16.5 and 17.5 B lymphocytes, respectively (Fig. 4F). Thus, V_H-to-DJ_H recombination also occurs asynchronously over an extended temporal window. Together, these findings show that by the time VDJ_H signals are seen in appreciable numbers (day 16.5), the number of D_H antisense signals is greatly reduced, consistent with D_H antisense transcription's being lost as a result of ongoing V(D)J recombination.

Due to the overlapping time windows of D_H-to-J_H and V_H-to-DJ_H recombination, we had not yet determined the extent to which D_H antisense intergenic transcription was associated with each of these processes. To place it in context relative to the accessibility of the V_H region, we next analyzed V_H antisense transcription in fetal liver. Using a probe to the V_HJ558 antisense transcripts from the 5′ half of the V region (8), we discovered that V_H region antisense transcription is present in a relatively high number of cells by day 14.5 (19% of cells with I_μ signals) (Fig. 4D and F). This proportion remains similar on day 15.5 (18.5%) but is reduced by day 16.5 (12%) and further reduced by day 17.5 (6%). Thus, V_H and D_H antisense transcription processes also overlap significantly within the same time window, albeit transcripts may occur on different alleles or indeed in different cells.

The sharp reduction in both D and V antisense primary transcripts between day 15.5 and day 16.5 and thereafter (Fig. 4F) contrasted with steady-state transcript levels, which showed little variation between days 14.5 and 16.5 (Fig. 3). RNA FISH detects a window of primary transcript generation and thus is exquisitely dependent on the presence of an actively transcribing locus and appropriate target sequence, which is lost due to ongoing V(D)J recombination. In contrast, steady-state levels reflect the balance between generation and degradation of transcripts, and the relatively stable levels observed suggest low transcript turnover.

D_H antisense transcription initiates on germ line alleles and before V_H antisense transcription. To distinguish between these possibilities, we first determined if D_H and V_H antisense transcription occurred on germ line alleles in day 15.5 fetal liver B lymphocytes using three-color RNA FISH. We cohybridized probes to detect I_μ, $_\mu0$, and either D_H (DSP and DFL) or V_H (J558) antisense transcription (Fig. 5A). The $_\mu0$ probe is located between DQ52 and J1; since this region is removed by D_H-to-J_H recombination, this probe is a marker of unrearranged alleles. We detected I_μ in blue, $_\mu0$ in red, and D_H or V_H antisense transcription in green. We found that D_H antisense signals colocalized with $_\mu0$ and I_μ in 36% of total alleles counted in day 15.5 fetal liver B lymphocytes, while D_H antisense signals occurred without $_\mu0$ on 17% of total alleles (Fig. 5A and C). Thus, 70% of D_H antisense transcripts were associated with $_\mu0$, indicating that D_H antisense transcription initiates on germ line alleles in fetal liver. A total of 30% of alleles expressed D_H antisense transcripts without $_\mu0$, suggesting that D_H antisense transcription continues on DJ_H-rearranged alleles.

In contrast, V_H antisense, $_\mu0$, and I_μ colocalized in only 9%

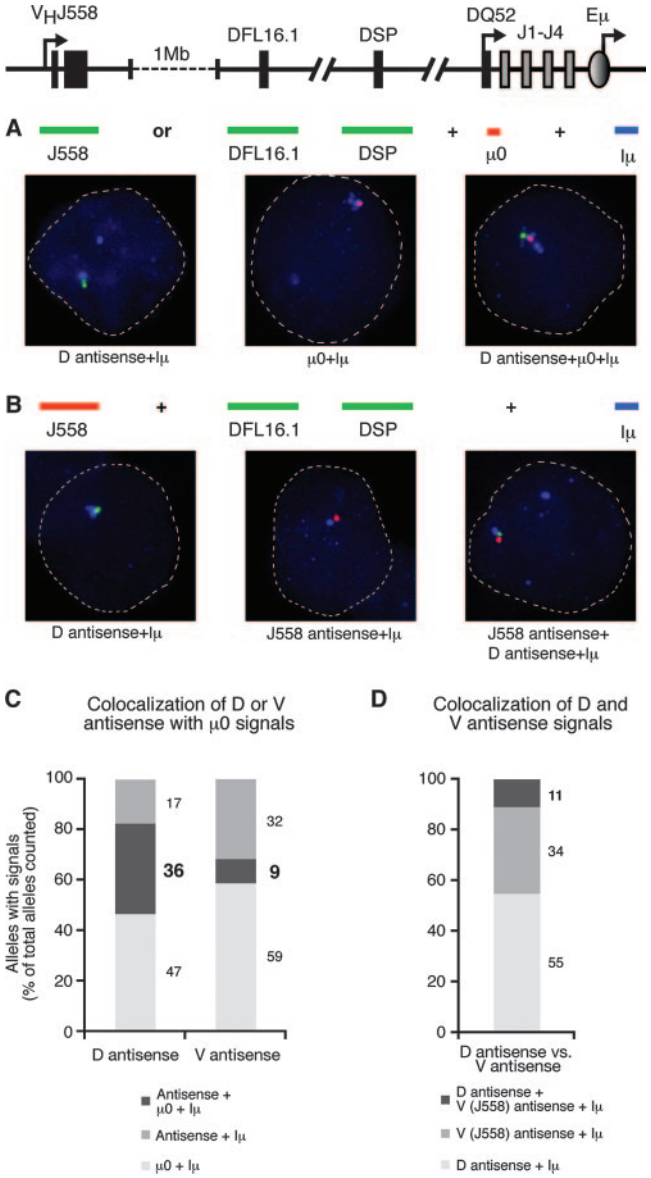

FIG. 5. Analysis of colocalization of D_H and V_H antisense signals with $_\mu0$ and with each other. (A) Locations of RNA FISH probes: red, detected with Texas Red; green, detected with FITC; blue, detected with Alexa 350. Three-color RNA FISH detection of D_H antisense or V_H antisense, $_\mu0$, and I_μ transcription. (B) Locations of RNA FISH probes: red, detected with Texas Red; green, detected with FITC; blue, detected with Alexa 350. Three-color RNA FISH detection of D_H antisense, V_H antisense, and I_μ sense transcription. All experiments were performed with day (D)15.5 fetal liver B lymphocytes; representative examples of the different signals observed are given in each case. (C) Analysis of colocalization frequencies of D_H antisense or V_H antisense with $_\mu0$ and I_μ. (D) Analysis of colocalization frequencies of D_H antisense, V_H antisense, and I_μ.

of total alleles, while V_H antisense transcripts were detected without $_\mu0$ on 32% of total alleles (Fig. 5C). Thus, only 20% of V_H antisense signals were associated with $_\mu0$. Together, these results suggest that D_H antisense transcription precedes D_H-to-J_H recombination of the *Igh* locus in fetal liver, and that V_H antisense transcription can initiate before D_H-to-J_H recombination on a small number of alleles, but usually occurs concomitant with or following D_H-to-J_H recombination, as previously shown in BM (8).

Given the differing associations with germ line alleles, we hypothesized that D_H and V_H antisense transcription would rarely be found on the same allele. To test this, we cohybridized probes to I_μ, D_H (DSP and DFL), and V_H (J558) antisense transcripts in day 15.5 fetal liver B lymphocytes (Fig. 5B). We detected I_μ in blue, D_H antisense in green, and V_H antisense in red (Fig. 5B). We found that D_H and V_H antisense signals colocalized in 11% of alleles (Fig. 5D). Thus, while D_H and V_H antisense transcription can occur simultaneously on a low number of alleles, the majority (89%; 55% D_H, 34% V_H) occurs separately. These data demonstrate a developmental shift from D_H antisense transcription to V_H antisense transcription concomitant with D_H-to-J_H recombination in fetal liver.

The *Igh* intronic enhancer E_μ regulates D antisense intergenic transcription. The data thus far provided strong correlative evidence in support of a role for D_H antisense intergenic transcription in chromatin accessibility for D_H-to-J_H recombination. To test this model functionally, we assessed the role of the known regulatory elements PDQ52 and the intronic enhancer E_μ in regulating D_H antisense transcription.

The PDQ52 promoter region initiates $_\mu0$ transcription in the sense direction through the J-Cμ region and is also thought to have enhancer function (33). We first asked if, similar to one other immunoglobulin promoter (45), PDQ52 was bidirectional and thus also the promoter of the D_H antisense transcription. To test this, we designed reverse transcription and nested PCR primers to amplify regions upstream and downstream of PDQ52 and did reverse transcription-PCR on $CD19^+$ *Rag1*$^{-/-}$ BM RNA (Fig. 6). Antisense-specific products of similar band intensities were amplified for both of these regions, indicating that antisense transcription occurs both upstream and downstream of PDQ52 and, hence, PDQ52 is unlikely to be the promoter of, or to regulate, the antisense transcription. This suggested that the downstream enhancer E_μ might regulate D_H antisense transcription. This was an attractive hypothesis, as we had previously shown that E_μ was required for D-to-J recombination (2).

To investigate this hypothesis, we analyzed D_H antisense transcription in two gene-targeted mouse models we have previously generated (2). The first contains a 300-bp deletion of the region upstream of DQ52 containing the $_\mu0$ promoter PDQ52 and has no effect on V(D)J recombination of the *Igh* locus. The second contains a deletion of both PDQ52 and a 700-bp region spanning the core E_μ and its 5′ matrix attachment region (MAR). These mice have a dramatic defect in D_H-to-J_H recombination, which, in the absence of a phenotype with the PDQ52 deletion alone, indicates that this process is regulated by E_μ alone (2, 49).

We examined D_H region sense and antisense transcription by reverse transcription-PCR in $B220^+$ BM B lymphocytes from mice harboring these deletions on a *Rag*-deficient background, which ensured no interference from DJ_H- or VDJ_H-rearranged transcripts. D_H antisense transcripts were detected in PDQ52$^{-/-}$ mice at levels similar to WT (Fig. 7A), indicating that this deletion has no effect on D_H antisense transcription and supporting our reverse transcription-PCR data (Fig. 6). In

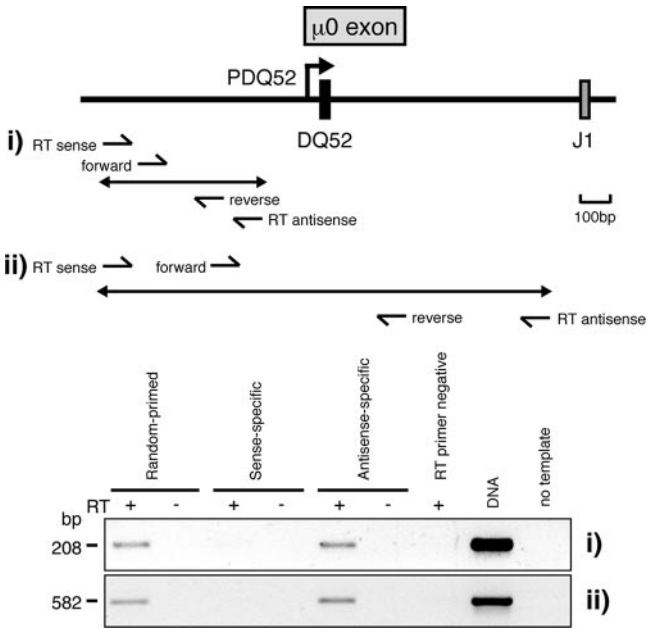

FIG. 6. Analysis of transcription surrounding PDQ52 in *Igh* WT *Rag1*$^{-/-}$ mice. Reverse transcription sense and antisense primers were designed in regions 5′ and 3′ of PDQ52. Nested primers were then used in PCR. Sense transcripts corresponding to $_{\mu}0$ were not detected due to placement of the 5′ PCR primer upstream of the $_{\mu}0$ transcription start site.

striking contrast, the additional absence of E_{μ} caused a dramatic reduction in antisense transcription over both DFL and DSP gene segments (Fig. 7A and B). These data indicate that D_H antisense transcription is regulated, either directly or indirectly, by E_{μ}, which also regulates D-to-J recombination. In contrast, deletion of E_{μ} did not inhibit V_H antisense transcription, demonstrating that this process is not regulated by E_{μ} and correlating with the lack of effect of E_{μ} on V-to-DJ recombination (Fig. 7B).

D_H antisense transcription initiates either within or immediately upstream of the E_{μ} core element. As we have previously shown that antisense transcription does not occur in the region immediately downstream of E_{μ} (8), we reasoned that the promoter of D_H antisense transcription was located within E_{μ} or its immediate upstream flanking sequences and thus sought to map transcription further and identify putative promoters in the region between DQ52 and E_{μ}. Bioinformatic analyses using Genomatix Promoter Inspector (10) did not identify any canonical promoter elements. We attempted strand-specific reverse transcription-PCR across this interval using RNA from *Igh*-WT *Rag*-deficient mice. However, this proved impossible due to detection of the high-level $_{\mu}0$ sense transcription in the antisense-specific and primer-negative controls due to endogenous priming in the reverse transcription step (not shown). We considered the possibility that $_{\mu}0$ primary transcription, and hence endogenous priming, might be significantly reduced in the PDQ52$^{-/-}$ *Rag*-deficient mice and that these mice could thereby be used to map the antisense transcription within this region. We have previously shown that levels of mature, spliced $_{\mu}0$ mRNA are little changed when PDQ52 is deleted (2) but had not assessed $_{\mu}0$ primary transcription in these mice. Semi-

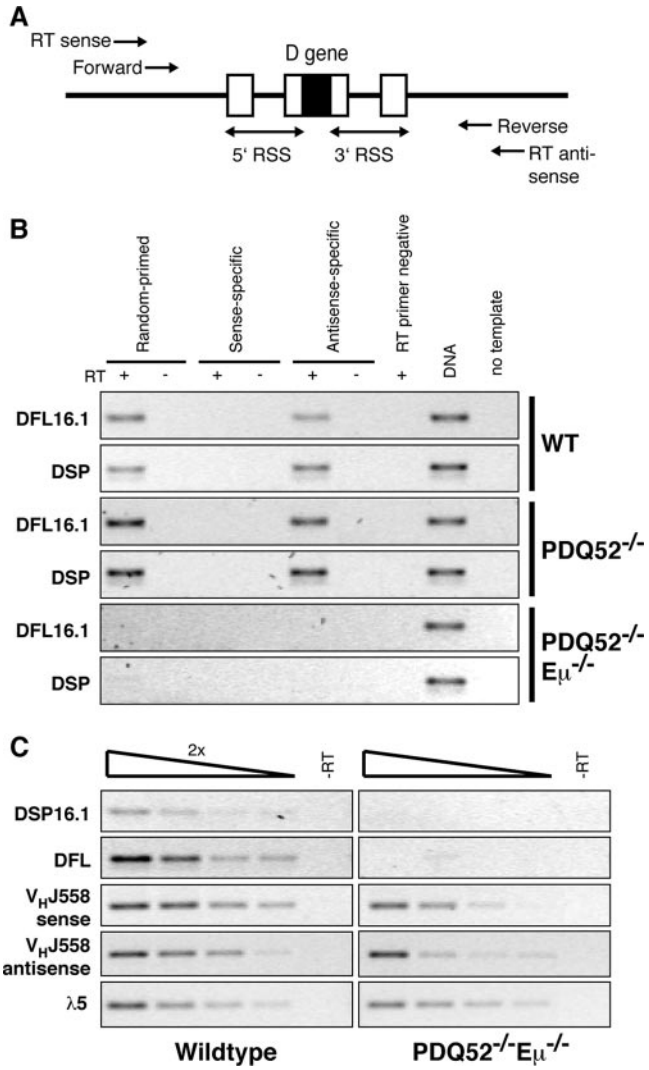

FIG. 7. Reverse transcription-PCR analysis of D and V region germ line transcription in *Igh* WT, PDQ52$^{-/-}$, and PDQ52$^{-/-}$E$_{\mu}^{-/-}$ *Rag1*$^{-/-}$ mice. (A) Design of the D_H gene germ line transcription reverse transcription-PCR assay. (B) D_H gene strand-specific reverse transcription-PCR of total RNA from B220$^+$ BM B lymphocytes from *Igh* WT, PDQ52$^{-/-}$, and PDQ52$^{-/-}$E$_{\mu}^{-/-}$ *Rag1*$^{-/-}$ mice. (C) Semiquantitative reverse transcription-PCR of D_H and V_H (V_HJ558) germ line transcription in WT and PDQ52$^{-/-}$E$_{\mu}^{-/-}$ *Rag1*$^{-/-}$ mice. λ5 was used as a loading control.

quantitative reverse transcription-PCR using primers in the $_{\mu}0$ intron demonstrated that $_{\mu}0$ primary transcription is six- to eightfold reduced when PDQ52 is deleted (data not shown).

As it seemed likely that the antisense transcription was initiating either within or immediately upstream of E_{μ}, we analyzed transcription between J4 and the 222-bp E_{μ} core (22) in PDQ52$^{-/-}$ mice. We did strand-specific reverse transcription-PCR using reverse transcription primers located immediately 3′ of J4 (antisense specific) and at the 3′ margin of the E_{μ} core (sense specific), followed by PCR using three sets of primers in the intervening region (Fig. 8). Antisense-specific products were amplified for the two 5′ regions but not the 3′ region spanning the E_{μ} core, demonstrating that transcription ini-

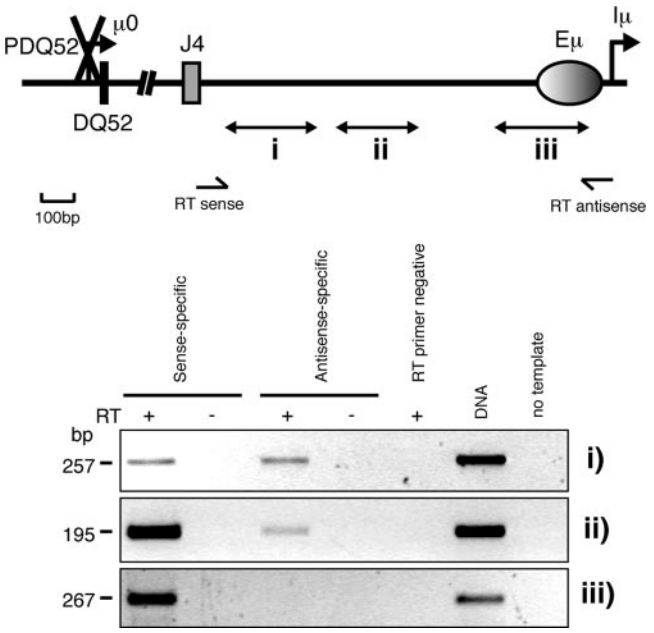

FIG. 8. Mapping of an antisense transcriptional start site to the 5′ margin of the E_μ core. Reverse transcription primers were designed immediately 3′ of J4 and at the 3′ boundary of the E_μ core to detect antisense and sense transcription, respectively, across this interval. Three sets of PCR primers in the intervening region were then used to map transcription across this region. Above lanes, with (+) or without (−) reverse transcriptase (RT). DNA, genomic DNA positive control.

tiates either within the E_μ core or in the 235-bp region immediately upstream.

DISCUSSION

We report the discovery of antisense intergenic transcription throughout the D_H and J_H regions of the mouse *Igh* locus in pro-B cells and propose that this process remodels the D_H cluster for D_H-to-J_H recombination. In support of this model, we show that D antisense transcription is activated before D_H-to-J_H recombination and is regulated by the intronic enhancer E_μ. E_μ was recently demonstrated to regulate V(D)J recombination of the *Igh* locus at the earliest D_H-to-J_H recombination step (2). Taken together, these data suggest that E_μ controls D_H-to-J_H recombination by activating this form of germ line *Igh* transcription and that the processivity of this transcription renders the D region accessible for D_H-to-J_H recombination. To our knowledge, this is the first report for an endogenous locus of a regulatory element (E_μ) regulating both intergenic transcription and a process that requires chromatin accessibility of the transcribed region, in this case, D_H-to-J_H recombination. This model is supported by a recent report that intergenic transcription regulates Jα gene recombination in the TCRα locus (1).

In contrast to transcription over the D region, we show that deletion of E_μ has no effect on V_H antisense transcription, suggesting that accessibility of the V_H region is not regulated by E_μ, in agreement with E_μ's not having a major role in V_H-to-DJ_H recombination (2, 49). Furthermore, we show that D_H antisense transcripts initiate on germ line alleles and that D_H and V_H antisense transcripts are rarely associated on individual alleles. Thus, we demonstrate a stepwise progression of antisense intergenic transcription, first over the DJ region, under the control of E_μ, then over the V region, under the control of a separate, unknown, regulatory element. In both cases, transcription precedes the corresponding V(D)J recombination event. This pattern is strikingly similar to the observed stepwise progression of active histone marks during *Igh* V(D)J recombination (15). Our results raise an important question: what regulates V region intergenic transcription? We have ruled out the PDQ52 promoter/enhancer, since V antisense transcription is normal in PDQ52 knockout mice. Interestingly, a novel pro-B cell-specific hypersensitive site has recently been identified 5′ of the V region, although its function is not yet known (48).

We have discovered that a major transcription start site for D region antisense transcription lies either within the E_μ core or in the upstream J-proximal 235-bp region including the 5′ MAR (Fig. 8). Since the enhancer core and upstream region are both deleted in the E_μ knockout mice we analyzed here, we are currently unable to distinguish between a putative antisense promoter and the known enhancer functions of the E_μ core. However, we have compared these mice with mice that lack the E_μ core but have the upstream region intact (49), and both have a similar defect in *Igh* D-to-J recombination (2). This suggests a dominant role for the E_μ core in this process, either as a bona fide enhancer, an as-yet-unclassified promoter, or both.

We propose that antisense intergenic transcription over D_H facilitates the establishment of an active chromatin domain for D_H-to-J_H recombination, either by histone exchange or recruitment of chromatin modifiers. Our model is supported by several observations. First, intergenic transcription is concomitant with a decrease in repressive histone marks, including dimethyl histone H3K9, and an increase in active histone marks, including H3 acetylation, and chromatin-remodeling enzymes over the DJ_H region prior to D_H-to-J_H recombination (15, 37, 43). Histone acetylation is highest over the J region but is also widespread throughout the D region (15). This is consistent with histone acetylation's being propagated by intergenic transcription initiating near E_μ and decreasing with increasing distance from the start site. However, it is not yet clear whether there is a continuous antisense transcript starting close to E_μ or multiple smaller transcripts initiating throughout the D locus. Since this is a large region (60 kb), it is also possible that E_μ controls transcription in cooperation with more than one antisense promoter. This model is in agreement with a recent suggestion that the region encompassing DQ52 and the four J genes and E_μ forms a separate chromatin domain from the rest of the D region (37).

Conversely, this study provides substantial evidence opposing a role for antisense transcription in generation of double-stranded RNA (dsRNA) and heterochromatin formation in the *Igh* locus. Strikingly, the J region, which displays the highest histone acetylation before D-to-J recombination, forms part of the transcription overlap between the $_\mu$0 sense transcript and antisense transcription originating near E_μ, suggesting that the transcription units are not antagonistic. Indeed, they are coordinately upregulated by E_μ. Furthermore, over DQ52, the single D gene that expresses sense transcripts, D_H

antisense and $_{\mu}0$ (sense) are cotranscribed on the same allele, yet DQ52 is rather more acetylated than the rest of the D genes (15, 43) and is preferentially used in early D-to-J recombination (6). Additionally, our finding that there is no sense germ line transcription in the remainder of the D_H region indicates that there is no possibility for dsRNA formation. Intergenic transcription has recently been shown to be required for V(D)J recombination at the TCRα locus, but in this case, it originates from the sense strand (1). This suggests that the strand origin is not important, which supports our model that the processing activity is the key function of this transcription.

Why is a large-scale chromatin remodeling process necessary? The D_H genes are small (11 to 16 bp) relative to the large intergenic sequences between them (4 kb). However, relocation of the *Igh* locus from the nuclear periphery to the interior precedes D-to-J recombination and requires chromatin unfolding (32). Furthermore, *Igh* must be made accessible to the large RAG recombinase complex. It is unlikely that either could be achieved through remodeling of the genes alone. Instead, intergenic transcription may alter the chromatin structure throughout the large DJ region (60 kb) in a similar manner to other large developmentally regulated loci (40), which undergo transcription-dependent (39, 44), higher-order chromatin remodeling and looping out of their chromosome territories (13, 62). Furthermore, we speculate that E_{μ} may promote nuclear relocation by recruiting the D region to a transcription factory in the nuclear interior to facilitate intergenic transcription, in a similar manner to the β-globin locus control region (50).

Recombination of most antigen receptor loci (e.g., TCRα, TCRβ, and Igκ loci) requires the cooperative activity of germ line promoters and enhancers and is characterized by transcription from germ line promoters (34). Of particular interest are the promoters upstream of each Dβ gene cluster in the TCRβ locus (PDβ1 and PDβ2) (56) and both upstream of and within the TCRα J cluster (27), which are required for efficient Dβ-Jβ (63, 64) and Vα-Jα (27) joining, respectively. The transcribed regions are associated with active histone marks such as acetylation (41, 64). These promoters require the presence of their respective intronic enhancer elements (E_{β} and E_{α}) for transcriptional activity (34). We have recently shown that E_{β} has two distinct roles prior to V(D)J recombination of the TCRβ locus: an intrinsic function that directs general chromatin opening over most of the DβJβ cluster and a cooperative function that facilitates the assembly of a promoter/enhancer holocomplex with PDβ1 (46). Here we show that the D_H cluster differs from this and other antigen receptor loci in that transcription is undetectable from most D_H germ line promoters. This raises the possibility that interaction between D promoters and E_{μ} may not occur and may not be necessary for D_H-to-J_H recombination. Further, deletion of the only promoter from which germ line transcription was detected, DQ52, had no effect on antisense intergenic transcription, shown here, or D-to-J recombination (2), although in this case arguably a promoter-enhancer interaction might not be necessary due to proximity of DQ52 to E_{μ}. Thus, it is possible that E_{μ} enhancer-driven antisense intergenic transcription may be the major force driving accessibility over the D_H region prior to DJ recombination. However, absence of transcription does not preclude interaction of the germ line promoters with E_{μ}, and such an interaction may occur before recombination and expression of the D_{μ} transcript. It will be interesting to distinguish between these possibilities by direct analysis of the interaction of these elements.

In summary, we have discovered that, in addition to the V_H region, antisense intergenic transcription occurs throughout the *Igh* D region and is thus a widespread process during V(D)J recombination. D_H antisense intergenic transcription precedes D_H-to-J_H recombination and initiates near to and is regulated by E_{μ}. Since E_{μ} also regulates D_H-to-J_H recombination, antisense intergenic transcription may provide a long-distance, processive mechanism by which E_{μ} is able to regulate accessibility across the D_H region.

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