

# Extended Duration of DH–JH Rearrangement in Immunoglobulin Heavy Chain Transgenic Mice: Implications for Regulation of Allelic Exclusion

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## Summary

Here we show that suppression of VH–DJH rearrangement in mice bearing a  $\mu$  heavy (H) chain transgene ( $\mu$ -tg mice) is associated with an extended period of DH–JH rearrangement, the first step of Immunoglobulin H chain gene rearrangement. Whereas DH–JH rearrangement is normally initiated and completed at the pro-B cell stage, in  $\mu$ -tg mice it continues beyond this stage and occurs most frequently at the small (late) pre-B stage. Despite ongoing DH–JH rearrangement in late pre-B cells of  $\mu$ -tg mice, VH–DJH rearrangement is not detectable in these cells. We infer that the lack of VH–DJH rearrangement primarily reflects tg-induced acceleration of B cell differentiation past the stage at which rearrangement of VH elements is permissible. In support of this inference, we find that the normal representation of early B lineage subsets is markedly altered in  $\mu$ -tg mice. We suggest that the effect of a productive VH–DJH rearrangement at an endogenous H chain allele may be similar to that of a  $\mu$ -tg; i.e., cells that make a productive VH–DJH rearrangement on the first attempt rapidly progress to a developmental stage that precludes VH–DJH rearrangement at the other allele (allelic exclusion).

Key words: V(D)J rearrangement • immunoglobulin transgenic mice • B cell differentiation • allelic exclusion

In antibody-producing cells, only one of two immunoglobulin (Ig)<sup>1</sup> heavy (H) chain alleles is normally expressed; the other allele is excluded (1, 2). Several models have been proposed to explain allelic exclusion in Ig-producing cells (3–6). The two models of particular interest here are the stochastic and regulatory models. According to the stochastic model (3), allelic exclusion reflects the imprecision of V(D)J rearrangement, the process responsible for rearranging V, D, and J elements to form contiguous VDJ or VJ coding segments for Ig variable regions (reviewed in 7). As this process is error prone, the chance is low that a cell will make in-frame (productive) rearrangements at both alleles of a given locus. Thus, in this model, each allele rearranges independently and has an equal but low chance of being rearranged productively. The regulatory model (4, 5) adds a regulatory rider to the stochastic model and states that rearrangement at one allele may affect rearrangement

at the other allele. An example of apparent nonindependent rearrangement of allelic elements is seen at the H chain locus. B cell plasmacytomas with an incomplete (DJH) rearrangement at one allele generally show a productive, in-frame VDJH (VDJH<sup>+</sup>) rearrangement at the other allele (8). This implies that the product of a VDJH<sup>+</sup> allele is able to prevent further VH–DJH rearrangement (8). In support of this idea, VH–DJH rearrangement is suppressed in  $\mu$  H chain transgenic mice (9–11 and reviewed in 12).

It is still uncertain how the  $\mu$  chain product of a VDJH<sup>+</sup> allele serves to prevent further VH–DJH rearrangement. What is clear is that expression of a  $\mu$  chain, in the form of a pre-B cell receptor (pre-BCR), results in progression of pro-B cells to the pre-B stage and the cessation of VH–DJH rearrangement (reviewed in 13, 14). It is not clear, however, to what extent cessation of VH–DJH rearrangement may reflect (a) rapid differentiation of pro-B cells to a stage (pre-B) at which such rearrangement can no longer occur, or (b) an ability of the pre-BCR to signal direct inhibition of VH–DJH rearrangement in addition to the progression of pro-B to pre-B cells. Similarly, it is not clear to what extent cessation of H and L chain rearrangement after possible premature expression of a BCR may reflect rapid differentiation to the recombinase-inactive B cell stage or direct

<sup>1</sup>Abbreviations used in this paper: BCR, B cell receptor; DSB, double strand break; HSA, heat stable antigen; LM-PCR, ligation-mediated PCR; RAG, recombinase activation gene; tg, transgene.

feedback inhibition. To gain further insight into these issues, we looked at initiation of VDJH rearrangement in scid and scid/+ mice bearing a  $\mu$  transgene (tg) or both a  $\mu$  and  $\kappa$  tg ( $\mu/\kappa$ -tg mice). Scid mice are homozygous for a mutation (*scid*) that severely impairs rearrangement of V, D, and J elements (15). Thus, B cell differentiation in scid mice is arrested at the pro-B cell stage (16), the stage at which H chain gene rearrangement is initiated (17, 18). In  $\mu$ -tg scid mice, however, B cell differentiation proceeds to the late pre-B cell stage before being arrested (19), and in  $\mu/\kappa$ -tg scid mice, differentiation can proceed to the B cell stage (20).

Here we report that tg-induced suppression of VH-DJH rearrangement may primarily reflect accelerated B cell differentiation rather than direct feedback inhibition. In  $\mu$ -tg scid mice, initiation of DH-JH rearrangement was observed to occur predominantly at the late pre-B stage rather than at the pro-B stage, and in  $\mu/\kappa$ -tg scid mice, initiation of DH-JH rearrangement was significantly reduced compared with non-tg scid mice. Similar results were obtained with  $\mu$ -tg and  $\mu/\kappa$ -tg scid/+ mice (heterozygous for the *scid* mutation). We interpret these findings to reflect accelerated development of tg-expressing B lineage cells such that there is insufficient time to initiate or complete DH-JH rearrangement at both alleles in developing pro-B cells. The idea that a  $\mu$  tg might accelerate B cell development has been proposed previously (21). In support of this notion, and consistent with our earlier studies (22), we show that developing pro-B cells in  $\mu$ -tg scid mice appear to bypass the late pro-B stage and progress directly into large (early) pre-B cells, which are known to be deficient in recombinase activation gene (RAG) expression (23). Similarly, in  $\mu/\kappa$ -tg scid mice, we present evidence that developing B lineage cells may transit the pro- and pre-B stages very rapidly during their progression to the RAG-inactive B cell stage. We suggest that the effect of endogenously coded Ig chains may be analogous; i.e., when a cell expresses a  $\mu$  chain, it rapidly progresses to a developmental stage that precludes further rearrangement of VH elements.

## Materials and Methods

**Mice.** Ig transgenic lines of C.B-17 scid mice hemizygous for the H chain tgs, M54 (24), 3H9 (25), or the kappa L chain tg, V $\kappa$ 8 (26), have been described previously (20). M54/V $\kappa$ 8 and 3H9/V $\kappa$ 8 scid mice were obtained by crossing individual tg lines and typing offspring for the presence of the respective tgs. Ig transgenic control mice, heterozygous for the *scid* mutation (denoted as scid/+ or s/+ mice), were obtained by crossing the above transgenic scid lines with C.B-17 wild-type mice. Mice used in this study were between 8 and 12 wk of age.

**Cells.** Bone marrow cells were flushed from femurs with staining medium using a syringe and 22 gauge needle. The cells were then dispersed by gentle pipetting, treated with 0.165 M NH<sub>4</sub>CL, washed, and resuspended in staining medium and passed through a sterile nylon screen. B cell hybridomas were obtained by fusing unstimulated splenic cells from adult M54/V $\kappa$ 8 (or 3H9/V $\kappa$ 8) scid and scid/+ mice in a manner previously described (27) using Ag8.563 (28) as the cell fusion partner.

**Flow Cytometric Analysis.** Bone marrow cell suspensions were

analyzed for the presence of B lineage cells representing different stages of development (17). In brief, cell suspensions were stained with Cy5 (Biological Detection Systems, Inc.) or allophycocyanin (APC; PharMingen) conjugated anti-CD45(B220), FITC-conjugated anti-CD43, or biotinylated anti-IgM. Binding of biotinylated antibodies was revealed by Texas red conjugated Streptavidin (Southern Biotechnology). B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>, B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>, and B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>+</sup> cells were enumerated or sorted by multiparameter flow cytometry using a dual laser FACStar Plus<sup>®</sup> (Becton Dickinson & Co.). Forward and light-angle scatter gates were set to include lymphoid cells only. Dead cells were identified by propidium iodide staining and excluded from analysis. To distinguish early B lineage subsets, B220<sup>+</sup>CD43<sup>+</sup>-gated cells were stained with phycoerythrin-conjugated anti-BP1 (29) and biotinylated anti-heat stable antigen (HSA) (17) (both reagents were provided by R. Hardy, Fox Chase Cancer Center, Philadelphia, PA).

**Analysis of Genomic DNA.** Genomic DNA was prepared from sorted cell subsets ( $0.5\text{--}1.0 \times 10^6$  cells) as described previously (30) and dissolved in water at a concentration corresponding to  $10^5$  cell genome equivalents/ $\mu$ l.

Ligation-mediated PCR (LM-PCR) (31–33) was used to assay DNA samples for double strand breaks (DSBs) resulting from the initiation of H chain gene rearrangement. Initiation of V(D)J rearrangement results in site-specific DSBs at the recombination signal/coding borders of V, D, and J elements: two kinds of broken DNA molecules are generated; covalently closed (hairpin) coding ends and blunt signal ends (34, 35). We assayed for broken molecules with signal ends; specifically, those with JH signal ends and those with 5' or 3' DHfl16.1 (DHfl) signal ends. We also assayed for signal joints (by inverse PCR), completed DH-to-JH rearrangements and unrearranged JH loci as scored by the retention of germline sequence immediately upstream of JH1.

Assays were performed as follows. A double strand linker was ligated to DNA (equivalent to  $\sim 4 \times 10^5$  cell genomes). The linker was constructed according to Roth et al. (32) by annealing two oligonucleotides, DR19 (5'-CACGATCCC-3') and DR20 (5'-GCTATGTACTACCCGGGAATTCGTG-3'). After ligation, different dilutions of the ligation reaction (input DNA) were used to perform PCR amplifications of one or more of the following: (a) linkered JH signal ends using DR20 and an oligonucleotide (MB221) complementary to a sequence immediately 5' of JH1 (5'-TCTCTTGTACAGGTCTCACTATGC-3'); (b) linkered 5' DHfl signal ends using DR20 and an oligonucleotide (MB222) complementary to a sequence 5' of DHfl (5'-GCCTTCACAAGAGGAGAAG-3'); (c) linkered 3' DHfl signal ends using DR20 and an oligonucleotide (MB241) complementary to a sequence 3' of DHfl (5'-TGGGTCAGTGGTCAAGACTCG-3'); (d) signal joints resulting from the joining of JH1, JH2, or JH3 signals to the 3' DHfl signal using MB221 and MB241; (e) DJH coding joints using a DHfl/DHsp primer MB109 (5'-CCGAATTCGTCTCCAGAAACAGACC-3') and a primer (MB 92) complementary to a JH4 sequence (5'-GCCGATCCCTTGACCCAGTAGTCC-3'); (f) retained sequence immediately upstream of JH1 using MB221 and MB92; and (g)  $\alpha$  actin sequence using actin-specific primers (17). The level of amplified  $\alpha$  actin product served as a control for the amount of input DNA.

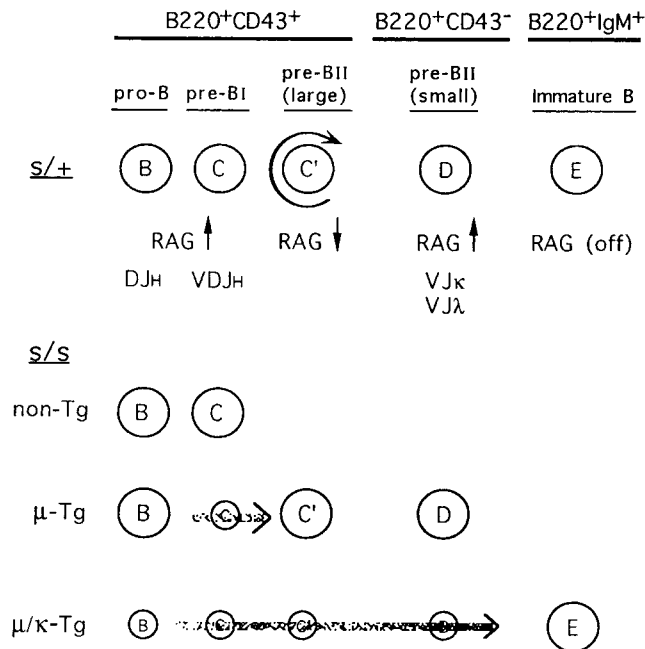
DNA was amplified in a 50- $\mu$ l reaction volume containing each primer at a concentration of 0.5  $\mu$ M, 200  $\mu$ M MgCl<sub>2</sub>, 10 mM Tris-HCL, pH 8.3 at 25°C, 50 mM KCL, 0.001% gelatin, 200  $\mu$ M (each) dTTP, dGTP, dATP, and dCTP (Pharmacia LKB Biotechnology), and 1 U Taq polymerase (Perkin-Elmer Cetus Corp.). The PCR reaction was carried out for 26 cycles of 94°C for 1 min,

60°C for 45 s (or 70°C for 45 s for amplification of JH signal ends), and 72°C for 90 s, followed by a 5-min extension at 72°C. Ligation and PCR amplification with different primers were performed at the same time to minimize experimental variation. Each assay included positive controls and was done several times with independent preparations of DNA. Amplification of PCR products was approximately proportional to the input DNA at several different dilutions. PCR products were separated by electrophoresis and analyzed by Southern blot analysis.

**Probes.** Blots were hybridized with: (a) pJH6.3 (36) to reveal H chain gene rearrangements, DJH coding and signal joints, and unrearranged JH alleles; (b) a genomic fragment corresponding to DHfl and its surrounding region (amplified by PCR using MB222 and MB 241) to reveal LM-PCR-amplified 3' and 5' DHfl signal ends; and (c) pActin (17) to reveal PCR-amplified  $\alpha$  actin. Probes were labeled with  $\alpha$ -[<sup>32</sup>P]dCTP using a Prime-It II kit (Stratagene Inc.).

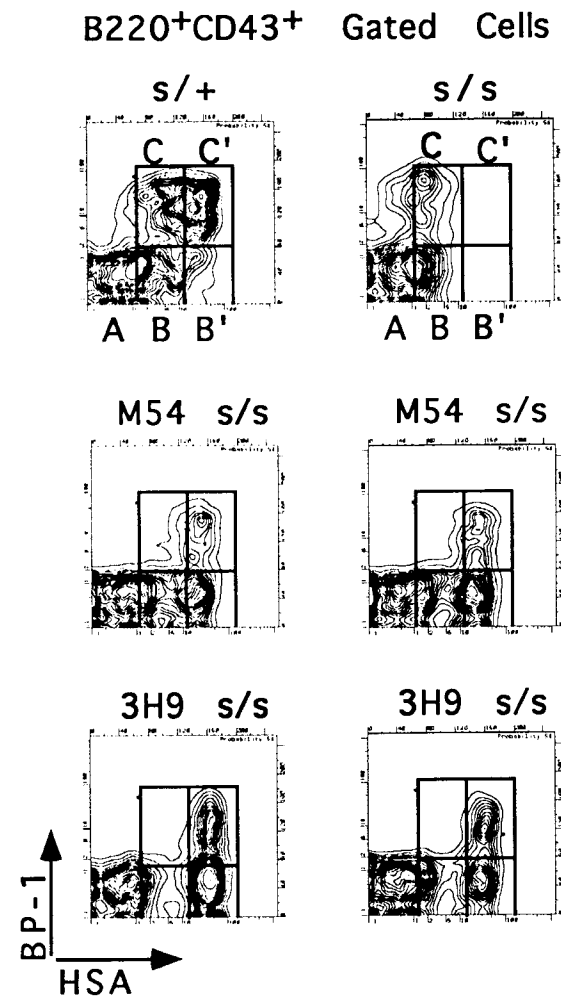
## Results

**Model for B Cell Development in Ig tg scid Mice.** Fig. 1 is a schematic representation of the effects of  $\mu$  and  $\mu/\kappa$  tgs on scid B cell development. The different stages of B cell development are designated with the letter code of Hardy et al. (17); the alternative nomenclature of Rolink and Melchers (37) is shown for comparison. As indicated, B cell develop-



**Figure 1.** Model for B cell differentiation in Ig tg scid mice. Early pro-B, late pro-B (pre-BI), pre-BII (large), pre-BII (small), and immature B cells (37) are designated with the letter code of Hardy et al. (17). Members of subsets B, C, and C' are B220<sup>+</sup>CD43<sup>+</sup> and are distinguished on the basis of their staining for the markers, BP1 and HSA (see Fig. 2); subsets D and E are B220<sup>+</sup>CD43<sup>-</sup> and B220<sup>+</sup>IgM<sup>+</sup>, respectively. The periods at which RAG is normally upregulated and DJH, VH-DJH, and VJL rearrangement are ongoing are indicated. In scid (s/s) and  $\mu$ -tg s/s bone marrow, differentiation is arrested at the C and D stages, respectively. In  $\mu/\kappa$ -tg scid bone marrow, differentiation is not arrested and cells reach the immature B cell stage (E). B cell subsets denoted with a small circle are under represented or missing. The horizontal arrow signifies direct or very rapid differentiation between the stages indicated.

ment in scid bone marrow is blocked at stage C, shortly after B lineage cells initiate H chain gene rearrangement. Relief from this block can be achieved by introduction of a  $\mu$  tg into the scid genome. In  $\mu$ -tg scid mice (22), developing pro-B cells appear to bypass stage C and develop directly into early pre-B cells, denoted as C'. Most cells in subset C' are in cycle (17) and show downregulated RAG expression (23). Cells of subset C' give rise to the D subset. At this late stage of pre-B cell differentiation, RAG expression is again upregulated (23) and L chain gene rearrangement is initiated (17, 18, 38). Differentiation does not proceed beyond stage D in  $\mu$ -tg scid mice, presumably because scid pre-B cells are unable to repair DNA DSBs resulting from the initiation of  $\kappa$  gene rearrangement (22, 30). Complete relief from the scid block can be achieved in double tg scid mice, bearing both a  $\mu$  and  $\kappa$  tg (20). In  $\mu/\kappa$ -tg scid



**Figure 2.** In  $\mu$ -tg scid mice, B cell differentiation from the B to the C' stage appears to bypass the C stage. Flow cytometry was used to analyze bone marrow of scid (s/s), scid heterozygotes (s/+), M54 s/s, and 3H9 s/s mice for the presence of early B lineage subsets. Contour plots show intensity of BP1 versus HSA staining for B220<sup>+</sup>CD43<sup>+</sup>-gated cells. Cells belonging to subset B (BP1<sup>-</sup>HSA<sup>+</sup>), B' (BP1<sup>-</sup>HSA<sup>2+</sup>), C (BP1<sup>+</sup>HSA<sup>+</sup>), and C' (BP1<sup>+</sup>HSA<sup>2+</sup>) are designated in the boxed areas in accordance with the original description of these cell types (17) (B' is an additional designation as explained in the text).

bone marrow, B cell development proceeds to stage E and appears to do so very rapidly, as evidenced by the near-normal percentage of B cells and virtual absence of pro- and pre-B cells.

Data supporting the above model are illustrated in Figs. 2 and 3. Fig. 2 shows the effect of two different  $\mu$  tgs, M54 (24) and 3H9 (25), on scid B cell development before the late pre-B cell stage (stage D). Members of subsets C ( $BP1^+HSA^{dull}$ ) and C' ( $BP1^+HSA^{bright}$ ), both positive for the early B lineage marker BP1 (29), are distinguished by their level of staining for heat stable antigen (17). Note that subset C, which is present in scid mice, appears to be replaced by subset C' in M54 and 3H9 scid mice. Note also that the  $BP1^-HSA^+$  cell fraction, which consists exclusively of  $HSA^{dull}$  cells in scid mice, includes both  $HSA^{dull}$  and  $HSA^{bright}$  cells in M54 and 3H9 scid mice. We designate  $HSA^{dull}$  and  $HSA^{bright}$  cells in the  $BP1^-HSA^+$  fraction as B and B', respectively. The upregulation of HSA when  $\mu$ -tg expressing cells transit from stage B to B' to C' presumably reflects  $\mu$  chain-dependent signaling. The effect of both  $\mu$  and  $\kappa$  chain tgs on scid B cell development is shown in Fig. 3. As indicated, scid mice bearing M54 (or 3H9) and the L chain tg,  $V\kappa 8$  (26), have near-normal percentages of B ( $B220^+IgM^+$ ) cells in their bone marrow, but are severely deficient in early B lineage ( $B220^+CD43^+$  and  $B220^+CD43^-$ ) cells comprising subsets B-D.

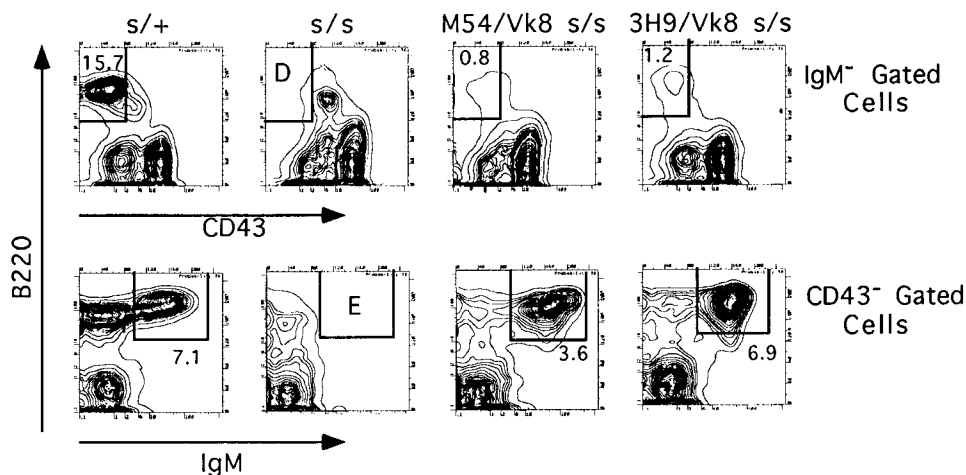
**Initiation of DH-JH Rearrangement Occurs Predominantly at the Late Pre-B Stage in  $\mu$ -tg scid Mice.** Since pro-B cells (subsets B and B') in  $\mu$ -tg scid mice appear to differentiate directly or very rapidly into early pre-B cells deficient in RAG expression (subset C'), we suspected that many developing B lineage cells in these mice might not initiate DH-JH rearrangement until the late pre-B cell stage (D) when RAG expression is again upregulated. To assay for the initiation of DH-JH rearrangement, we tested for DSBs at JH recombination signal/coding borders in FACS<sup>®</sup>-sorted  $B220^+CD43^+$  ( $CD43^+$ ) and  $B220^+CD43^-$  ( $CD43^-$ ) bone marrow cells ( $CD43^+$  cells would include stages B-C' and  $CD43^-$  cells would correspond to stage D). Broken DNA molecules with JH signal ends were detected by LM-PCR (31,

33). We also tested for completed DH-to-JH rearrangements and for retention of JH germline alleles (see Materials and Methods for details).

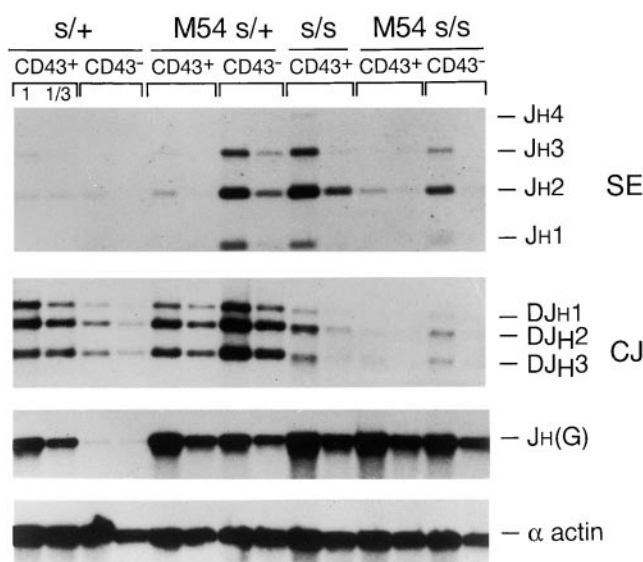
As shown in Fig. 4 and Table I, JH signal ends resulting from the initiation of DH-JH rearrangement in M54 scid mice were much more abundant in the late pre-B ( $CD43^-$ ) cell fraction than in the  $CD43^+$  fraction containing pro-B and early pre-B cells. In M54 scid/+ mice as well, JH signal ends were more abundant in the  $CD43^-$  than  $CD43^+$  cell fraction (Fig. 4 and Table I). We conclude that initiation of DH-JH rearrangement in M54 mice occurs predominantly at the late pre-B cell stage. Despite the relatively low abundance of JH signal ends in the  $CD43^+$  cell fraction of M54 scid/+ mice, alleles with (completed) DH-JH rearrangements were readily detectable in this cell fraction (Fig. 4). This is not surprising as DH-JH rearrangements would be expected to result in DJH complexes that have a much longer half-life than JH signal ends, especially in  $\mu$ -tg-expressing pro-B cells that fail to rearrange their VH elements (20).

Non-tg scid and scid/+ control mice showed widely different levels of JH signal ends; i.e., JH signal ends were more abundant in the  $CD43^+$  cell fraction of scid than scid/+ bone marrow (Fig. 4 and Table I). As discussed later, a possible explanation for this difference is that initiation of DH-JH rearrangement continues unabated in scid mice, whereas in scid/+ mice, DH-JH rearrangement is limited by the onset of VH-DJH rearrangement. JH signal ends were in low abundance in the  $CD43^-$  cell fraction of scid/+ mice, consistent with a low retention of germline JH alleles (Fig. 4) and the completion of H chain gene rearrangement. The high retention of germline JH alleles in the  $CD43^+$  cell fraction of scid/+ (and scid) mice is presumed to reflect in part the presence of early B lineage cells not yet expressing RAG protein and the known contamination of this fraction with non-B lineage cells (39).

**Initiation of VH-DJH Rearrangement Is Not Detectable in Late Pre-B Cells of  $\mu$ -tg Mice, Despite Ongoing DH-JH Rearrangement.** To test for ongoing DH-JH and VH-DJH rearrangement in the  $CD43^+$  and  $CD43^-$  cell fractions of scid, scid/+, and M54 scid/+ mice, we assayed for DSBs at both the 3'



**Figure 3.** In  $\mu/\kappa$ -tg scid mice, B cell development occurs in the absence of significant pre-B cell development. Flow cytometry was used to analyze bone marrow of scid (s/s), scid heterozygotes (s/+), M54/V $\kappa 8$  s/s, and 3H9/V $\kappa 8$  s/s mice for the presence of late pre-B cells (subset D) and immature B cells (subset E) in bone marrow of scid (s/s), scid heterozygotes (s/+), M54/V $\kappa 8$  s/s, and 3H9/V $\kappa 8$  s/s mice. Top row of contour plots shows intensity of B220 versus CD43 staining for IgM<sup>-</sup> gated cells. The percentage of cells in subset D ( $B220^+CD43^-$ ) is indicated within the boxes. Bottom row of contour plots shows intensity of B220 versus IgM staining for  $CD43^-$  gated cells. The percentage of cells in subset E ( $B220^+IgM^+$ ) is indicated outside the boxes.



**Figure 4.** Initiation of DH–JH rearrangement in  $\mu$ -tg mice occurs predominantly at stage D, the late pre-B ( $B220^+CD43^-$ ) cell stage. Genomic DNA from sorted  $B220^+CD43^-$  ( $CD43^-$ ) and  $B220^+CD43^+$  ( $CD43^+$ ) cells of M54 scid ( $s/s$ ) and M54 scid heterozygote ( $s/+$ ) bone marrow was analyzed by LM-PCR for JH signal ends (JH1, JH2, JH3, and JH4) and by PCR for DJH coding joints (DJH1, DJH2, and DJH3) and the retention of JH germline [JH (G)] alleles. Non-tg controls included DNA from sorted  $CD43^+$  cells of  $s/s$  bone marrow and DNA from  $CD43^-$  and  $CD43^-$ -sorted cells of  $s/+$  bone marrow. PCR amplification of the  $\alpha$  actin gene served as an internal control for input DNA. In this and subsequent figures, the first and second lane of PCR products under each bracket proceeding from left to right correspond to undiluted and three-fold-diluted input DNA, respectively. The primers and probes used for amplification and hybridization of PCR products are described in Materials and Methods.

and 5' signals of the DHfl16.1 (DHfl) element. DHfl is the most upstream DH element (40) and is used in  $\geq 50\%$  of DH–JH rearrangements (41–44). Broken DNA molecules with 3' DHfl signal ends signify initiation of DH–JH rearrangement, whereas 5' DHfl signal ends can be taken to reflect initiation of VH–DJH rearrangement (33).

Scid and scid/+ mice showed striking differences in their levels of 3' and 5' DHfl signal ends (Fig. 5). In the  $CD43^+$  cell fraction of scid mice, 3' but not 5' DHfl signal ends were abundant, whereas, in the corresponding cell fraction of scid/+ mice, 5' but not 3' DHfl ends were abundant. Thus, in the  $CD43^+$  cell fraction of scid mice, initiation of DH–JH rearrangement predominates over that of VH–DJH rearrangement, whereas the reverse is true in the  $CD43^+$  cell fraction of scid/+ mice. In the late pre-B ( $CD43^-$ ) cell fraction of scid/+ mice, neither 3' nor 5' DHfl signal ends were detectable, indicating that H chain gene rearrangement is normally completed before this stage, which is in agreement with the results of Fig. 4. In contrast, in the  $CD43^-$  cell fraction of M54 scid/+ mice, DH–JH rearrangement was ongoing, as indicated by the abundance of 3' DHfl signal ends (Fig. 5). Note that 5' DHfl signal ends were not detectable in the  $CD43^-$  (or  $CD43^+$ ) cell fraction of M54 scid/+ mice. Therefore, even though DH–JH rearrangement is ongoing in late pre-B cells of M54 scid/+ mice, initiation of VH–DJH

**Table I.** Relative Abundance of JH2 Signal Ends in  $\mu$ -tg and Non-tg Mice

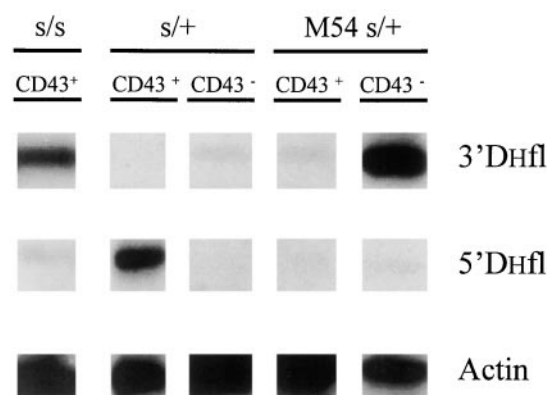
Mouse	Cell subset*	JH2 signal ends <sup>†</sup>
$s/+$	$CD43^+$	0.06
$s/+$	$CD43^-$	0.07
M54 $s/+$	$CD43^+$	0.21
M54 $s/+$	$CD43^-$	1.00
$s/s$	$CD43^+$	0.83
M54 $s/s$	$CD43^+$	0.12
M54 $s/s$	$CD43^-$	0.56

\*Sorted  $B220^+CD43^+$  ( $CD43^+$ ) and  $B220^+CD43^-$  ( $CD43^-$ ) cells were from bone marrow of scid ( $s/s$ ), scid heterozygote ( $s/+$ ), M54  $s/s$ , and M54  $s/+$  mice.

<sup>†</sup>The amount of  $^{32}P$ -labeled probe hybridizing to the JH2 signal end product and the  $\alpha$ -actin internal control was quantitated for the first lane under each bracket in Fig. 4 using a BioImage Analyzer. The values shown correspond to the relative amount of probe hybridizing to each product normalized against the reference control (M54  $s/+$   $CD43^-$  cells). Thus, for example, the value of 0.83 for  $CD43^+$   $s/s$  cells equals the ratio of the amount of probe hybridizing to JH signal/ $\alpha$  actin divided by the amount of probe hybridizing to JH signal/ $\alpha$  actin in the reference control.

rearrangement does not evidently occur in these cells. This apparent inability of the V(D)J recombinase system to target VH elements in late pre-B cells of  $\mu$ -tg mice is consistent with the early findings of Yancoupolus and Alt (45). These investigators found that VH558 transcripts are detectable in  $\mu^-$  but not  $\mu^+$  lines of transformed pre-B cells and concluded that VH elements in  $\mu^+$ -transformed pre-B cells are not accessible to the V(D)J recombinase system.

*Initiation of DH–JH Rearrangement Is Less Frequent in  $\mu/\kappa$ -tg Mice than in non-tg Mice.* To test whether initiation of



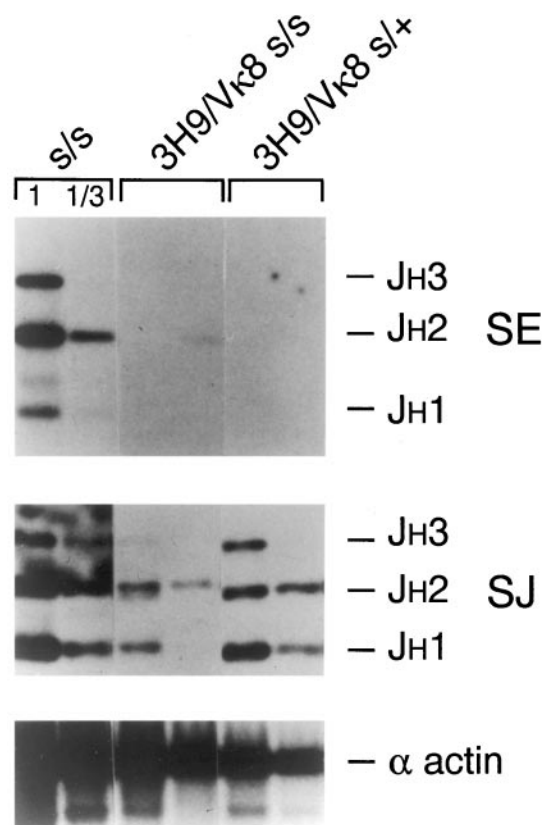
**Figure 5.** Initiation of VH–DJH rearrangement is not detectable in late pre-B ( $B220^+CD43^-$ ) cells of  $\mu$ -tg mice despite ongoing initiation of DH–JH rearrangement. Genomic DNA from sorted  $B220^+CD43^-$  ( $CD43^-$ ) and  $B220^+CD43^+$  ( $CD43^+$ ) cells of scid heterozygotes ( $s/+$ ) and M54  $s/+$  bone marrow were analyzed by LM-PCR for 3' and 5' DHfl signal ends.  $CD43^+$  cells from scid ( $s/s$ ) bone marrow served as a positive control for initiation of DH–JH rearrangement. The primers and probes used for amplification and hybridization of PCR products are described in Materials and Methods.

DH–JH rearrangement occurs at a normal frequency in  $\mu/\kappa$ -tg mice, we sorted B220<sup>+</sup>IgM<sup>-</sup> bone marrow cells from scid, 3H9/V $\kappa$ 8 scid, and 3H9/V $\kappa$ 8 scid/+ mice, and then assayed for the level of JH signal ends. The B220<sup>+</sup> IgM<sup>-</sup> cell population would include B lineage subsets (B–D) before the immature B cell stage (E). We also assayed for circular DNA molecules with signal joints resulting from the joining of the JH1, JH2, or JH3 signals with the 3' DHfl signal (see Materials and Methods). Signal joint formation, in contrast to coding joint formation, is not impaired in scid mice (46, 47). Also, we would expect circular DNA molecules to have a longer half-life than broken molecules with JH signal ends, thus making signal joint formation a sensitive assay for attempted DH–JH rearrangement in scid mice.

As shown in Fig. 6, JH signal ends were more abundant in the B220<sup>+</sup>IgM<sup>-</sup> cell fraction of non-tg scid mice than in the corresponding cell fraction of 3H9/V $\kappa$ 8 scid and 3H9/V $\kappa$ 8 scid/+ mice. Thus, the initiation of DH–JH rearrangement is clearly reduced in the presence of these tgs. This is also apparent from the reduced level of signal joints in 3H9/V $\kappa$ 8 mice compared with control non-tg scid mice (Fig. 6). The level of JH2 signal joints in 3H9/V $\kappa$ 8 scid and 3H9/V $\kappa$ 8 scid/+ mice was estimated to be ~10 and 60%, respectively, the level in non-tg scid mice (see Fig. 6, legend). We suggest later that the lower level of signal joints in 3H9/V $\kappa$ 8 scid than 3H9/V $\kappa$ 8 scid/+ mice may be attributable to premature death of developing scid B cells resulting from persisting DSBs at DH and JH coding elements.

*Cells that Succeed in Reaching the B Cell Stage in  $\mu/\kappa$ -tg scid Mice Do Not Initiate DH–JH Rearrangement.* Given that most developing scid B cells fail to rearrange their D and J elements successfully (15, 48, 49) and die with persisting DSBs (33, 35), the cells most favored to become B cells in  $\mu/\kappa$ -tg scid mice would be those in which DH–JH rearrangement is not attempted. To test this prediction, we generated and cloned B cell hybridomas from the spleen of M54/V $\kappa$ 8 and 3H9/V $\kappa$ 8 scid mice, and then examined these hybridomas for the status of their H chain alleles. Control hybridomas were obtained from M54/V $\kappa$ 8 and 3H9/V $\kappa$ 8 scid/+ mice. Representative results are illustrated in Fig. 7 for 9 3H9/V $\kappa$ 8 scid/+ hybridomas and 10 3H9/V $\kappa$ 8 scid hybridomas. Note that one or two H chain gene rearrangements were clearly evident in all but one of the scid/+ hybridomas. In contrast, none of the scid hybridomas showed a rearranged allele.

44 hybridomas from  $\mu/\kappa$ -tg mice were analyzed and the results are summarized in Table II. 10 scid/+ hybridomas showed one allele to be rearranged with the other allele in germline configuration; 11 (10 of which came from 3H9/V $\kappa$ 8 scid/+ mice) showed both alleles to be rearranged and 5 showed a single rearrangement with the other allele missing or undetectable. Two scid/+ hybridomas showed germline H chain alleles only. As normal B cells and their precursors show H chain rearrangements at both alleles (3, 8, 50), the retention of at least one germline H chain allele in ~40% of the scid/+ hybridomas demonstrates significant transgene-mediated reduction of DH–JH rearrangement.



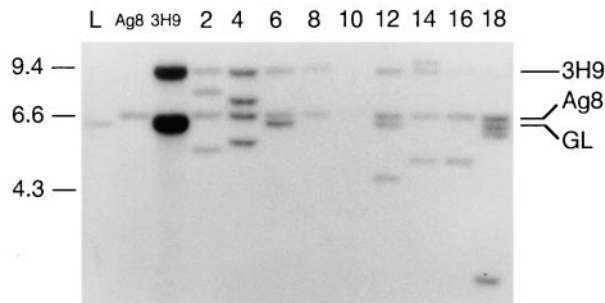
**Figure 6.** Reduced frequency of initiation of DH–JH rearrangement in developing B cells of  $\mu/\kappa$ -tg scid mice. Genomic DNA from sorted B220<sup>+</sup>IgM<sup>-</sup> bone marrow cells of scid (s/s), 3H9/V $\kappa$ 8 s/s, and 3H9/V $\kappa$ 8 s/+ mice was analyzed by LM-PCR for JH signal ends (JH1, JH2, and JH3) and by inverse PCR for signal joints resulting from the joining of JH signal ends with the 3'–DHfl signal. The nonrearranging  $\alpha$  actin gene served as an internal control for the amount of DNA (input DNA). The primers and probes used for amplification and hybridization of PCR products are described in Materials and Methods. To compare the level of signal joints in the three groups of mice, we used a Bio-Image Analyzer to measure the amount of <sup>32</sup>P-labeled probe hybridizing to JH2 signal joint product/ $\alpha$  actin in 3H9/V $\kappa$ 8 s/s (or 3H9/V $\kappa$ 8 s/+) divided by that hybridizing to JH2 signal joint/ $\alpha$  actin in the s/s control. The ratios obtained for 3H9/V $\kappa$ 8 s/s and 3H9/V $\kappa$ 8 s/+ mice were 0.11 and 0.62, respectively.

These results are in agreement with the results of Fig. 6 and with previous reports showing that the frequency of endogenous H chain rearrangement is reduced in B lineage cells of M54 (10, 51) and 3H9/V $\kappa$ 8 (25) wild-type mice. In contrast to the scid/+ results, all M54/V $\kappa$ 8 and 3H9/V $\kappa$ 8 scid hybridomas showed germline H chain alleles only (Table II). The absence of detectable H chain gene rearrangement in the scid hybridomas indicates that cells that succeed in becoming B cells in  $\mu/\kappa$ -tg scid mice do not attempt DH–JH rearrangement.

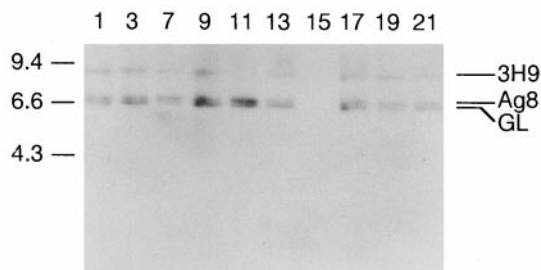
## Discussion

The preceding results show a striking alteration in the representation of B lineage subsets and duration of DH–JH rearrangement in bone marrow of  $\mu$ -tg and  $\mu/\kappa$ -tg scid mice compared with non-tg scid and scid/+ control mice.

A 3H9/Vκ8, s/+



B 3H9/Vκ8, s/s



**Figure 7.** Status of H chain loci in splenic B cell hybridomas from (A) 3H9/Vκ8 scid/+ and (B) 3H9/Vκ8 scid mice. Approximately 10 μg of genomic DNA from each hybridoma (designated with even or odd numbers) was EcoRI digested. Southern blotted, and hybridized to a JH-specific probe. DNA from adult liver (L), the Ag8.563 cell fusion partner (Ag8), and the hybridoma from which the VDJH transgene was derived (3H9) served as controls for the position of the germline (G) H chain fragment, the Ag8.563 H chain allele, and the 3H9 transgene, respectively. The absence of hybridizing fragments in lanes 10 and 15 reflects incomplete DNA digestion.

Specifically, late pro-B cells (subset C) appear to be missing in  $\mu$ -tg scid mice and DH-JH rearrangement occurs predominantly at stage D, the late pre-B cell stage. In  $\mu/\kappa$ -tg scid mice, early B lineage subsets (B-D) are grossly under represented and initiation of DH-JH rearrangement is less frequent than in non-tg control mice. Further, pro-B cells that succeed in reaching the B cell stage in  $\mu/\kappa$ -tg scid mice do not attempt DH-JH rearrangement. Interestingly, in non-tg control mice, we found initiation of DH-JH rearrangement to be greater or more sustained in scid mice than in scid/+ mice. The implications of these findings are discussed below.

**Extended Period of Initiation of DH-JH Rearrangement in  $\mu$ -tg Mice.** In  $\mu$ -tg mice initiation of DH-JH rearrangement was found to occur most frequently at the late pre-B cell stage (stage D). As DH-JH rearrangement is normally completed before this stage (17, 18), initiation of H chain gene rearrangement appears to be somewhat delayed in  $\mu$ -tg mice. To explain this result, we suggest the following model:

**Table II.** Status of the H Chain Alleles in B Cell Hybridomas from M54/Vκ8 and 3H9/Vκ8 Mice

Mouse	Number of hybridomas with indicated status			
	G	R/G	R/R	R/-
M54/Vκ8 s/+	1	5	1	1
M54/Vκ8 s/s	7	0	0	0
3H9/Vκ8 s/+	1	5	10	4
3H9/Vκ8 s/s	9	0	0	0

Germline and rearranged alleles are denoted by G and R, respectively; missing or undetectable alleles are denoted by (-). Hybridomas in the G category would include those with a G/G and G/- genotype as these two categories cannot be distinguished by Southern blot analysis.

$\mu$ -tg- and RAG-expressing pro-B cells (subsets B and B') rapidly differentiate into recombinase-deficient early pre-B cells (subset C'), such that many cells do not have time to initiate or complete DH-JH rearrangement at both alleles until the late pre-B stage (subset D) when RAG expression is again upregulated.<sup>2</sup> Rapid progression of pro-B cells to the C' stage would presumably result from premature expression of a pre-BCR containing a tg-encoded  $\mu$  chain, surrogate light (SL) chain and the signal transducing chains, Ig $\alpha$  and Ig $\beta$  (reviewed in 13, 52-54). Consistent with this model is the known early expression of  $\mu$  tgs (30) and the genes for SL chain (55-57), the apparent absence of subset C in  $\mu$ -tg scid mice (see Fig. 2), the shortened duration of the pro-B stage in  $\mu$ -tg mice (57a), and the finding that the majority of cells corresponding to subset C in non-tg mice contain nonproductive VDJH (VDJH<sup>-</sup>) rearrangements (58). The latter finding has been interpreted to suggest that pro-B cells containing a VDJH<sup>+</sup> rearrangement quickly exit the subset C compartment (58).

Applying the above model to non-tg mice, we suggest that pro-B cells that make a VDJH<sup>+</sup> rearrangement on the first attempt may exclude VH-DJH rearrangement at the other allele by rapidly progressing to the RAG-deficient C' stage, and then to stage D, at which rearrangement of VH elements is no longer permissible. For allelic exclusion to occur in this model, a pre-BCR need only signal developmental progression. This notion is consistent with previous reports showing that exclusion of VH-DJH rearrangement is tightly linked with progression of pro-B cells to the pre-B stage (14, 59-61). Such linkage is even observed in  $\mu$ -tg mice that express a truncated  $\mu$  chain, which results in a pre-BCR complex lacking (specificity) a  $\mu$  variable region and surrogate light chain (62, 63). However, pro- to pre-B progression and VH-DJH rearrangement are both blocked

<sup>2</sup>The extent to which RAG expression is upregulated earlier in subsets B and B' of  $\mu$ -tg mice is not known. If RAG expression is not fully upregulated in these subsets, this could also contribute to the observed lower level of DH-JH rearrangement in the pro-B (B220<sup>+</sup>CD43<sup>+</sup>) versus the late pre-B (B220<sup>+</sup>CD43<sup>-</sup>) cell fraction of  $\mu$ -tg mice (see Fig. 4).

in  $\mu$ -tg mice that express a mutated  $\mu$  chain that precludes assembly of a pre-BCR complex with the signal transducing Ig  $\alpha/\beta$  chains (64–67). Interestingly, the few B lineage cells that reportedly escape the above developmental block show allelic exclusion (67), consistent with our proposed model.

Ongoing initiation of DH–JH (Fig. 4) and  $V\kappa$ – $J\kappa$  rearrangement (30) in late pre-B cells of M54 scid mice could help explain why these mice uniformly lack B cells (20) and appear no more leaky than non-tg scid mice (68). If attempted rearrangement of DH and JH elements in developing M54 scid pre-B cells is initiated before that of  $V\kappa$  and  $J\kappa$  elements, some cells might be expected to succeed in making a DH–JH rearrangement. Indeed, DH–JH rearrangements were recovered from late pre-B cells of M54 scid mice (see Fig. 4). However, the chance of a scid cell surviving attempted rearrangements at both H and L chain loci would seem unlikely, consistent with the absence of detectable  $VJ\kappa$  coding joints in late pre-B cells of M54 scid mice (22, 30).

*Reduced Frequency of DH–JH Rearrangement in  $\mu/\kappa$ -tg Mice.* In  $\mu/\kappa$ -tg mice, we found initiation of DH–JH rearrangement was less frequent than in non-tg scid mice. Signal joints resulting from the initiation of DHfl to JH rearrangement in 3H9/ $V\kappa$ 8 scid and 3H9/ $V\kappa$ 8 scid/+ mice were estimated to be present at ~10 and 60%, respectively, the level observed in non-tg scid mice. Based on the difference in level of recovered signal joints in 3H9/ $V\kappa$ 8 scid/+ and non-tg scid mice, we estimate that initiation of DH–JH is ~40% less frequent in  $\mu/\kappa$ -tg than non-tg mice. This estimate agrees favorably with the observed frequency of germline H chain alleles in B cell hybridomas from 3H9/ $V\kappa$ 8 (6/35 alleles) and M54/ $V\kappa$ 8 (6/14 alleles) scid/+ mice (Table II). The much lower level of signal joints in 3H9/ $V\kappa$ 8 scid than 3H9/ $V\kappa$ 8 scid/+ mice is taken to reflect loss (death) of scid cells that attempt DH–JH rearrangement. This could account for the absence of rearranged H chain alleles in B cell hybridomas recovered from  $\mu/\kappa$ -tg scid mice (Table II).

To explain the reduced level of DH–JH rearrangement in  $\mu/\kappa$ -tg mice, we suggest that expression of a tg-coded BCR in early pro-B cells promotes very rapid progression of these cells to the B cell stage, such that there is little time to initiate DH–JH rearrangement. Consistent with this notion, (a)  $\mu$  and  $V\kappa$ 8 tgs are known to be expressed early in B cell development (30), (b)  $\mu/\kappa$ -tg scid/+ mice contain near-normal percentages of B cells in bone marrow but markedly reduced percentages of pro- and pre-B cells (20), and (c)  $\mu/\kappa$ -tg scid mice show near-normal percentages of B cells in bone marrow but sharply reduced percentages of pro-B cells compared with non-tg scid controls and virtually no pre-B cells ( $\leq 1\%$ ; Fig. 3).

*Basis for the Difference in Level of JH Signal Ends in scid and scid/+ Mice.* In non-tg control mice, we found that early B lineage cells in the CD43<sup>+</sup> cell fraction from scid mice showed a much higher level of JH signal ends than the corresponding cell fraction from scid/+ mice. As scid does not impair the joining of signal ends (46, 47), one cannot attribute the relatively high level of JH signal ends in scid mice to a blockage in signal joint formation. What scid does impair, however, is the processing of coding ends before

their being joined (33, 35). Thus, developing B lineage cells in scid mice do not often succeed in joining DH and JH coding ends (48, 49) and would not be expected to initiate the second step of H chain gene rearrangement (VH–DJH rearrangement). Indeed, 5' DHfl signal ends, signifying the initiation of VH–DJH rearrangement, were not detectable in CD43<sup>+</sup> scid cells (Fig. 5). We suggest that in the absence of VH–DJH rearrangement, initiation of DH–JH rearrangement continues unabated in CD43<sup>+</sup> cells, resulting in a high level of JH signal ends. On the other hand, in the CD43<sup>+</sup> cell fraction of scid/+ mice, initiation of VH–DJH rearrangement was prominent and that of DH–JH rearrangement barely evident (Fig. 5). This implies that initiation of DH–JH rearrangement in scid/+ mice may be limited to the earliest stage of pro-B cell development, consistent with the idea discussed below, that onset of VH–DJH rearrangement may preclude further DH–JH rearrangement.

In wild-type or scid/+ cells, a DH–JH rearrangement may be followed by rearrangement of a VH element to the resulting DJH complex or the complex may be replaced by the joining of an upstream DH element to a downstream JH element (41). The latter event, DJH replacement would seem counterproductive to efficient assembly of VH, DH, and JH elements. Thus, it makes sense, as originally postulated by Alt et al. (69), that after DH–JH rearrangement VH rather than DH elements are preferentially rearranged. How might this happen? Recent evidence suggests that initiation of VH–DJH rearrangement is associated with a shift in the targeting of the V(D)J recombinase activity from the 3' to the 5' side of DH elements (70). Targeting of the recombinase to signals on the 5' side of DH elements would minimize DJH replacement and limit the duration of DH–JH rearrangement to the earliest stage of pro-B cell development. Although DJH complexes can be readily detected in late pro-B cells (subset C) (17, 18, 38), this does not necessarily reflect ongoing DH–JH rearrangement at this stage; the observed DJH complexes could have been formed earlier in cells of subset B.

In sum, DH–JH rearrangement in non-tg mice is normally initiated and completed at the early pro-B stage. In  $\mu$ -tg mice, DH–JH rearrangement may begin at the pro-B stage, but it appears to continue and occur most frequently at the late pre-B stage. Based on the altered representation of pro-B subsets in  $\mu$ -tg scid mice, we suggest that the extended period of DH–JH rearrangement in these mice primarily reflects rapid progression of  $\mu$ -tg-expressing pro-B cells to the recombinase-deficient early pre-B cell stage. Thus, many cells may not have time to initiate DH–JH rearrangement until the late pre-B stage when RAG expression is again upregulated. In addition, ongoing DH–JH rearrangement (including DJH replacement) at the late pre-B stage would not be limited by initiation of VH–DJH rearrangement, as the latter does not apparently occur in late pre-B cells of  $\mu$ -tg mice. Finally, rapid progression of  $\mu/\kappa$ -tg-expressing pro-B cells to the recombinase-inactive B cell stage could explain why in  $\mu/\kappa$ -tg mice we find a reduced initiation of DH–JH rearrangement compared with non-tg mice and a striking deficiency of pro- and pre-B cells despite near-normal numbers of B cells.



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