Molecular Characterization of Transgene-induced Immunodeficiency in B-less Mice Using a Novel Quantitative Limiting Dilution Polymerase Chain Reaction Method

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Summary

B-less mice express a human immunoglobulin (Ig)λ transgene that induces a severe deficiency of both immature pre-B and mature B lymphocytes. To understand this perturbation in B lymphopoiesis, we have devised a sensitive limiting dilution polymerase chain reaction assay that quantitates specific Ig rearrangements and thus quantitates B lineage cells at various stages of differentiation within unfractionated bone marrow. We find that there are significantly reduced frequencies of both Vn-to-DJn andVk-to-JK rearrangements in the transgenic strain, whereas the frequency of D-to-Ja rearrangements approximates that of wild type. Since Ig gene rearrangements occur in a stepwise fashion in which D-to-Ja joining precedes that of Vn-to-DJn andVk-to-JK, these results indicate that the major block of B lymphocyte development in the B-less strain occurs after D-to-Ja rearrangement. Interestingly, sequence analysis of residual VnDJn junctions from transgenic pre-B lymphocytes reveals that an abnormally high proportion of these are out of frame and therefore nonproductive. Taken together, these data suggest that early expression of the transgenic λ protein specifically prevents the development of a normal-sized population of precursor B lymphocytes coexpressing functional IgH. The transgene-induced immunodeficiency appears to arise by a precocious maturation process in which precursors bypass a developmental stage associated with cellular expansion.

The protein products of the Ig loci are inextricably involved in the regulation of several aspects of early B lymphocyte development. This is prominently illustrated by the related phenomenon of allelic and isotypic exclusion, in which expression of one productive Ig allele inhibits functional production of the remaining allele (1). Extensive analyses of transgenic mice bearing IgH and/or IgK genes (2) together with more recent studies of Igλ-bearing transgenics (3-5) indicate that all three Ig chains participate in this process. Apparently critical to this form of regulation is the signaling function provided by the H chain of IgM (μ). Specifically, it is the membrane-bound version (μm), and not the secreted form (μs), that is required (6-8). In what may be a related process, μm appears to stimulate rearrangements of the IgL chain genes (9), although μm is clearly not essential in this regard since IgL rearrangement proceeds in the absence of μ protein (8, 10).

Recently, the analysis of genetically modified mice that lack the μm gene has demonstrated that its function is essential for the development of the B lineage (11). As such, μm-deficient mice fail to generate mature B lymphocytes due to a developmental arrest that occurs early in B lymphopoiesis (11). Consistent phenotypes are also exhibited by RAG-deficient (RAG-1 [12] or RAG-2 [13]) and homozygous scid mice (14), each of which lack functional Ig recombinase activity and are therefore unable to assemble functional Ig proteins. Presumably, each of these recombinase-deficient strains exhibit similar early blocks in B lymphocyte differentiation as a consequence of the failure to produce functional Ig proteins. This is supported by the observation that the introduction of Ig transgenes can partially overcome the arrest of B lymphopoiesis in homozygous scid mice (15).

B lymphopoiesis is also regulated by the product of incompletely (DJn) rearranged alleles of IgH (referred to as Dμ proteins) (16). In early pre-B cells establishment of the DJn junctional reading frame bias has been shown to be dependent upon the membrane-bound version of Dμ (16). During this early phase of B lymphopoiesis, additional proteins that are known to physically associate with μ (and Dμ) are also likely to be essential for μm regulatory function (reviewed in reference 17). This hypothesis was recently tested using mice deficient for one such gene, λs, that encodes a surrogate light chain protein (18). Consistent with expectations, these mice fail to establish a DJn junctional reading frame...
bias and, furthermore, exhibit an arrest in B lymphocyte development similar to what is observed in mice that lack μm (18).

Relevant to understanding the regulation of B lymphocyte development by Ig, we have recently described the B-less line of transgenic mice, which contains a rearranged human λ gene fused to the murine IgH enhancer (19). Mice that carry this transgene exhibit a severe deficiency in both immature pre-B and mature B lymphocytes. This defect is entirely restricted to the B lymphocyte lineage and results in a development block that occurs early in this differentiation pathway. B-less mice contain markedly fewer bone marrow cells bearing the B lymphocyte-specific surface Ag CD45R(B220) (20), reflecting reductions of both mature B (CD45R(B220)-positive; surface IgM-positive) and pre-B (CD45R(B220)-positive; surface IgM-negative) populations. The existence of an early block in B lymphocyte development in B-less mice is supported by a dramatic reduction of fetal liver-derived, surface IgM-negative populations. The existence of this B-lineage block in B-less mice is likely to reflect an interaction of the transgenic λ (λtransgenic) gene with regulatory mechanisms controlling early B lymphocyte differentiation.

To increase our understanding of the process by which the λtransgenic interferes with B lymphocyte development, we have undertaken experiments to define precisely the point at which this process is blocked. We use the fact that the genes encoding both IgH and IgL undergo an ordered process of somatic recombination during B lymphocyte differentiation (1). The first rearrangements occur at IgH and join together sequences of the Vn, Dn, and Jn segments. This assembly occurs in a two-step process in which D-to-Jn joining occurs first, followed by Vn-to-Dn joining. Subsequently, the V regions of IgL (K or λ) undergo assembly in a process similar to that when VL-to-JL joints are formed. We reasoned that given a sufficiently quantitative assay, the block in B lymphocyte differentiation in B-less mice could be defined in terms of this established order of Ig gene rearrangement.

To pursue this approach, we developed a quantitative PCr-based assay that accurately determines the relative frequencies of specific Ig rearrangements in genomic DNA prepared from unfractionated bone marrow cells. A brief account of a similar, independently devised assay has recently been described by Sykes et al. (21). With this technique we show that B-less and wild-type bone marrow samples contain essentially equivalent numbers of D-to-Jn rearrangements, whereas the subsequent Vn-to-Dn and Vk-to-Jk rearrangements are significantly reduced in B-less mice. These results indicate that B lymphopoiesis is arrested after D-to-Jn rearrangement in these mice. Furthermore, sequence analysis of unrearranged Vn-Dn junctions present in transgenic animals reveals that they are largely out of translational frame and thus non-functional. Taken together, these findings indicate that the human λ protein acts to specifically inhibit the development of the subpopulation of precursor B lymphocytes that express functional IgH. We interpret these results to reflect a process by which the expression of the transgenic λ (λtransgenic) drives B cell precursors to precociously mature, thereby circumventing a stage of B lymphopoiesis that is associated with dramatic cellular expansion.

Materials and Methods

Cultured Cells. The WEHI 231 cell line was purchased from American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 4.5 g/liter glucose, 0.05 mM 2-ME, 50 U/ml penicillin, 50 mg/ml streptomycin, and 4 mM L-glutamine. FvB/N tail fibroblasts (a gift of Dr. Tom Vogt) were obtained at passage 30 and DNA was extracted without further culture.

Bone Marrow Cells. The B-less strain of mice has been described (19). Mice were maintained under specific pathogen-free conditions until the point of bone marrow harvest. Two pairs of littermates were used in the limiting dilution (LiD) PCR analysis. The littermate pairs of nos. 3334/3338 and nos. 4173/4175 were aged 6 wk and 8 mo at the time of death, respectively. Mice were killed by cervical dislocation. Intact femurs and tibias were removed from freshly killed mice and flushed with cold PBS. Cells harvested from the bones of a single mouse were combined in PBS and pelleted by centrifugation. Purified bone marrow pre-B lymphocytes were obtained from three independent sets of age-matched mice that ranged from 4 to 12 wk of age.

Genomic DNA Preparation. Cells were resuspended in proteinase K solution (17 mM Tris [pH 7.6], 167 mM NaCl, 17 mM EDTA, 0.8% SDS, and 0.17 mg/ml proteinase K [Boehringer, Mannheim, Germany]) and incubated at 50°C until completely solubilized (at least 4 h). After solubilization, additional water was added to any samples that were excessively viscous. The aqueous solution containing DNA was phenol-chloroform extracted, precipitated in ethanol, and centrifuged to pellet the DNA. The pellets were dried and resuspended in 0.1× TE. The concentration of DNA solutions was determined by OD260. Thereafter, samples were stored at or below -20°C.

LiD PCR Dilutions and Amplification Reactions. Twofold serial DNA dilutions were made by transferring 150 μl of concentrated DNA into a fresh microcentrifuge tube containing 150 μl of diluent (1 ng/μl pBR322 plasmid [Boehringer] in water). The new dilution was vortexed extensively and pulse centrifuged. 150 μl of the diluted sample was transferred to a fresh tube containing an equal volume of diluent for the next dilution. This process was repeated until the desired range of DNA concentrations was attained. All dilutions were maintained at 4°C throughout the procedure. Each individual dilution served as a DNA source for 12 separate PCR reactions. An individual PCR run consisted of 96 individual reactions that included 12 independent reactions for each of eight serial dilutions. Two to three separate runs were performed to generate each data point shown in this paper (i.e., 24–36 independent PCR reactions were performed to generate each data point). PCR reactions contained 10 μl of diluted genomic DNA solution, 4 μl 10×

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1 Abbreviations used in this paper: A-MuLV, Abelson murine leukemia virus; λtransgenic, transgenic λ; LiD, limiting dilution.
MgCl₂, 0.1% [wt/vol] gelatin), 0.4 µl 100x DNA polymerase mix (20 mM dNTP; Pharmacia Fine Chemicals, Piscataway, NJ), 1 µmol of each primer, and 0.1 µl (0.5 U) AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 40 µl. PCR reactions were carried out on a GeneAmp PCR 9600 thermal cycler with the MicroAmp 96-well tube/tray assembly (Perkin Elmer Cetus). Each sample was initially denatured at 94°C for 30 s and then subjected to 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. At the end of the final cycle, the samples were incubated at 72°C for 10 min. Thereafter, samples were stored at 4°C.

Hybridization Analysis of PCR Reactions. Dot blot analyses were performed as follows: 10 µl of each PCR reaction was denatured by mixing with 4 µl 1 N NaOH and subsequently with 60 µl neutralization buffer (0.6 M Tris [pH 7.6], 1.5 M NaCl). The neutralized samples were transferred to nitrocellulose in a 96-well dot blot manifold (Schleicher & Schuell, Inc., Keene, NH). All membranes were hybridized to a 32p-labeled randomly primed probe at 5 x 10⁵ cpm/ml at 42°C for at least 12 h and washed in 0.2x SSC, 0.1% SDS at 70°C for 30 min.

Mathematical Analysis of Data. The numbers of responding and nonresponding amplification reactions for each dilution were tabulated and plotted using the KaleidaGraph™ (Synergy Software [PCS Inc.], Reading, PA) data analysis and graphics software. Curve-fitting was accomplished using a least squares algorithm described by the equation J(x) = e^(-x), where J(x) is the frequency of negative reactions, x is the frequency of effective amplification templates within the DNA, and a is the amount of genomic DNA per reaction. The value, a, was determined by using the linear analysis feature with Quasi-Newton minimization of the Systat™ statistical software package (Systat, Inc., Evanston, IL). a was determined for each curve by considering the series of four adjacent data points corresponding to the highest J(x) excluding points for which the number of positive responses was <2.

Primers and Probes. The sequences of PCR primers used in this report are as follows: c-myc sequences were amplified with c-myc2916 5'-GAT GAA GGT CTC GTC GTC AGG-3' and c-myc2561 5'-CCA ACA GGA ACT ATG ACC TC-3'. Primers used for amplification of Ig sequences (except oligo J4.2) were adapted from those previously published (10, 22). Ig sequences were amplified with primers Vx0-16) and J4.2. IgH rearrangements that form D-to-J and V-to-DJ joints are amplified with the primer pairs D.J and Va7183/J4.2, respectively. V4-to-J4 and J4.2 primers. Coding sequences of c-myc exon 2 are amplified with the c-myc2916 and c-myc2561 primers. Probes used in detection of amplified products are represented as black bars underneath the appropriate sequence. Figures are not drawn according to scale.

Figure 1. Strategy for PCR amplification and detection of genomic sequences. (A) IgH, (B) IgK, and (C) c-myc loci. IgH rearrangements that form D-to-J and V4-to-DJ joints are amplified with the primer pairs D.J and Va7183/J4.2, respectively. V4-to-J4 rearrangements are amplified with the Vx0-16) and J4.2 primers. Coding sequences of c-myc exon 2 are amplified with the c-myc2916 and c-myc2561 primers. Probes used in detection of amplified products are represented as black bars underneath the appropriate sequence. Figures are not drawn according to scale.
The remaining D elements are clustered within a 60-kb region that is located ~20 kb 5' of J,1 (25). Rearrangement of the Ig genes results in the juxtaposition of coding elements and the removal (or displacement) of noncoding sequences. The large distances separating the coding elements in the germline configuration prohibit efficient replication so that PCR amplification will only occur if a rearrangement has taken place.

Two classes of recombination products, D-to-J, and Vn-to-DJn, can be amplified at the IgH locus (Fig. 1 A). First, recombinants that have joined any of the D elements encoded by the DFl16 or Dcp2 families to any of the four J, elements are amplified using the D4n and Jma primers because the D4n primer is degenerate and hybridizes to upstream sequences flanking each of the identified D elements (excluding the DQ5 element that is just 5' of the J, region cluster and is not amplified with these primers). The nondegenerate Jma primer hybridizes specifically to a sequence 3' of the fourth J, element. Second, rearrangements that join V segments of B). This primer pair will amplify only those rearrangements in the deletion of the Jra primer recognition sequence. In our assay, the mouse c-myc gene is included as a nonrecombinant endogenous control that provides for the normalization of DNA sample quality. Amplification of the c-myc gene is performed with the cmyc2841 and cmyc2915 primers that are complimentary to sequences within exon 2 (Fig. 1 C). All classes of amplification reactions are detected by blot hybridization to the appropriately radiolabeled probes and generate bands of the expected molecular weight upon examination by Southern blot analysis (data not shown). Additional confirmation of appropriate c-myc and VJK amplification was obtained by restriction digestion of the amplified products (data not shown).

Quantitation of Ig Gene Recombination Frequencies by LiD PCR. The ability to detect Ig rearrangements as described above provides the basis for an assay to detect, but not accurately quantitate, Ig recombination in samples of genomic DNA. To overcome this limitation, we devised an assay that we refer to as LiD PCR, outlined in Fig. 2 A. This assay relies on the ability to amplify specific DNA molecules even when those molecules are diluted to exceedingly low concentrations. At such concentrations, individual templates distribute small, serially diluted sampling aliquots in accordance with the Poisson probability equation, which relates the number of aliquots receiving templates to the concentration of the template in the original sample solution. In practice the frequency of a particular Ig rearrangement within a sample of genomic DNA is detected by serially diluting the stock solution of bone marrow DNA over a broad range of concentrations. Multiple aliquots of equal volume are taken from each dilution and amplified by PCR with an individual primer pair. After amplification, each reaction is analyzed by blot hybridization to the appropriate radiolabeled probe and scored as either positive (i.e., those reactions containing one or more

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**Figure 2.** Description and theoretical considerations of the LiD PCR assay. (A) Basic design. Genomic DNA to be tested is twofold serially diluted to produce a set of at least eight subdilutions. Equal volumes of each subdilution are aliquoted to individual wells of a 96-position tray and amplified. After amplification, reactions are blotted to nitrocellulose and scored as either positive or negative for hybridization to a radiolabeled probe. (B) Generation of LiD PCR curves and determination of effective template frequency. LiD analysis should generate a curve in which the frequency of negative PCR reactions, f0(1), is a decreasing function of the amount of DNA per reaction (plotted here as percent negative reactions). This relationship is described by the Poisson probability equation, which states that the probability that a given PCR reaction containing an average amount of genomic DNA, X, will contain exactly c templates as 

\[ P(c|X) = \frac{e^{-\lambda} \lambda^c}{c!} \]

where \( \phi \) is the frequency of effective templates within the DNA sample being tested, \( c \) is the exact number of templates per reaction, and \( e \) is the base of the natural log (derived from reference 45). Considering the case when \( c = 0 \), the equation reduces to 

\[ P(c=0) = e^{-\lambda} \]

Therefore, for an average template concentration of one per reaction, that is when \( \lambda = 1 \), \( P(c=1) = e^{-1} = 0.37 \). Therefore, the frequency of negative reactions, \( f_{0}(1) \), will approximate 37%, given a sufficiently large number of reactions. A best-fit curve (least square), described by this equation, is fitted to the data (see Materials and Methods). The determination of the effective template concentration, \( \phi \), is facilitated by the use of a statistical software package (see Materials and Methods). Visually, the effective template frequency may be estimated by extrapolation to the best-fit curve corresponding to the \( f_{0}(1) = 37\% \) (dotted lines).
Ig recombinant templates) or negative (i.e., those reactions that did not receive a recombinant template). Thus, instead of evaluating the quantity of DNA amplified, only the presence or absence of DNA need be scored. Initially, all PCR reactions were assayed by Southern blot analysis. However, after processing hundreds of amplification reactions in this manner, it was apparent that only products of the appropriate sizes were being produced. We therefore substituted a simpler dot blot hybridization strategy that enabled us to process larger numbers of samples.

A theoretical treatment of the LiD PCR procedure is illustrated in Fig. 2B. The Poisson probability equation describes the curve that is expected to be generated by this type of LiD assay (for review, see reference 26). Using this equation, it is possible to relate the frequency of nonresponding PCR reactions, $f(0)$, to the number of effective templates per reaction. In this context, $f(0)$ is equal to 37% at an effective template frequency of one per PCR reaction (see legend to Fig. 2 and Materials and Methods). Given that each of the amplification reactions received the same volume of DNA solution, the frequency of effective templates per volume can be determined (i.e., the effective concentration of templates). In the theoretical curve shown in Fig. 2B, there is approximately one template per 3 pg genomic DNA (3 pg is the approximate theoretical mass of one haploid murine genome).

**LiD PCR Analysis of Genomic DNA.** Two considerations must be met to generate informative results with LiD PCR: (a) individual DNA sequences must segregate independently under conditions of limiting-dilution; and (b) a single template must be reliably amplified and unambiguously detected. To test whether the LiD PCR assay conforms to these expectations, genomic DNA from the WEHI 231 cell line was analyzed for the frequencies of both c-myc templates and $V_k$-to-Jk rearrangements. WEHI 231 is an immature B cell line that carries a $V_k$-to-Jk1 rearrangement (10). The result of that analysis is shown in Fig. 3. The shapes of the resultant curves conform to that predicted by the Poisson distribution, suggesting that genomic DNA templates randomly distribute within the indicated range of DNA dilutions (see Materials and Methods for details on statistical treatment). These results indicate a frequency of approximately one effective c-myc template per 5.5 pg of genomic DNA, which is close to the theoretical value of 3 pg for a single-copy sequence of haploid murine genomic DNA. The value determined for the $V_k$-to-Jk recombinant template was 14 pg. It was found that even at the highest dilutions of DNA, individual reactions could be unambiguously scored as either positive or negative (data not shown).

To generate informative LiD PCR curves, DNA dilutions must be used that span the concentrations at which the PCR template sequences are limiting. Therefore, the amount of genomic DNA required for amplification is inversely proportional to the frequency at which the amplification template exists within a given sample of DNA. We were concerned that amplification efficiency could be affected by using greatly differing amounts of genomic DNA. Furthermore, the accuracy of the assay needed to be determined. An experiment to evaluate these considerations is shown in Fig. 4. $V_k$-to-Jk dilution curves were generated from DNA mixtures containing predetermined proportions of DNA from WEHI 231 (a source of $V_k$-to-Jk1 recombinant templates) and cultured fibroblasts. Fibroblast DNA did not contain any detectable Ig recombination (Fig. 4, 100% FvB/N fibroblast DNA) and,

![Figure 3. LiD PCR analysis of the WEHI 231 cell line. Frequency determinations of both c-myc (open circles) and $V_k$-to-Jk (filled circles) templates in genomic DNA from the B cell line WEHI 231. c-myc templates are found at a frequency of one per 5.5 pg and $V_k$-to-Jk templates at one per 14 pg genomic DNA with 95% confidence intervals of 3.9-9.2 and 11-18 pg, respectively. See Materials and Methods for curve-fitting procedures.](image)

![Figure 4. LiD PCR analysis of DNA stock solutions containing different concentrations of effective templates. Stock mixtures containing WEHI 231 and fibroblast DNAs in predetermined proportions were analyzed by LiD PCR. 100% WEHI 231 DNA (circles) (same curve as in Fig. 3); 10% WEHI 231 DNA/90% fibroblast DNA (squares); 1% WEHI 231 DNA/99% fibroblast DNA (diamonds); and 100% fibroblasts DNA (triangles).](image)
Table 1. Summary of Ig Recombination Frequencies in Bone Marrow DNA from Wild-Type and B-less Mice

| Amplification template | Wild-type (no. 3334) | 95% C.I.  | B-less (no. 3338) | 95% C.I.  | Wild-type (no. 4173) | 95% C.I.  | B-less (no. 4175) | 95% C.I.  |
|------------------------|----------------------|-----------|-------------------|-----------|----------------------|-----------|-------------------|-----------|
| c-myc                  | 7.7*                 | (4.4-29)  | 3.9               | (2.2-15)  | 2.6                  | (2.3-2.8) | 3.2               | (2.7-4.0) |
| D-to-Jh                | 180                  | (140-260) | 290               | (240-390) | 170                  | (130-250) | 250               | (210-300) |
| Vh-to-DJh              | 1,700                | (1,500-1,900) | 8,100      | (6,700-10,000) | 660         | (470-1,100) | 7,700            | (5,800-11,000) |
| Vh-to-Jh               | 330                  | (230-590) | 5,100             | (3,800-7,900) | 150                  | (110-210) | 3,700             | (2,500-7,500) |

* Picograms DNA per single effective template (1/φ).
† Values corresponding to the lower and upper 95% confidence interval (C.I.) are shown in parentheses.
are separated by a line. D element reading frames were assigned according to the convention of reference 27. Frame designations for multiple D regions are separated by a slash and are listed in the order, 5' to 3', in which the D sequence homologies reside. Productive (P) coding joints and nonproductive (NP) rearrangements are determined on the basis of translational reading frame. Nonproductive rearrangements that contain stop codons are indicated (NP). Nucleotides that could not be unambiguously identified are listed (N). Clones are derived from each of three independent sets of age-matched mice as follows: set 1 (clones 3-11 and 17-47), set 2 (clones 140-150 and 109-156), and set 3 (clones 303-344 and 202-240).
to contain essentially identical frequencies of D-to-J rearrangement while the other pair exhibited a threefold effect. The results of c-myc amplification shown in Fig. 5, D and H, indicate that all DNA samples contained similar frequencies of effective c-myc templates. Therefore, it is unlikely that any of the genomic DNA samples were either significantly degraded or contaminated with "amplification inhibitors." Finally, because the two littermate pairs differed significantly with respect to age (6 wk vs. 8 mo), the observed recombination frequencies do not appear to be age sensitive.

Sequence Analysis of V_{7183}DJ rearrangements from Pre-B Lymphocytes. Given that functional Igs play a role in regulating B cell development, we considered the possibility that arrest of B lymphocyte differentiation in B-less mice might involve production of functional Igs. This possibility was examined by sequencing cloned V_{7183}DJ junctions from purified pre-B lymphocytes of both wild-type and B-less mice. As shown in Fig. 6 A, cells harvested from wild-type animals carry 79% functional V_{7183}DJ junctions as ascertained by the preservation of reading frame and the absence of stop codons. In contrast, only 21% of the sequenced V_{7183}DJ junctions from transgenic pre-B lymphocytes were found to be functional (Fig. 6 B). These results strongly suggest that the transgene specifically impedes the development of a subpopulation of pre-B lymphocytes that express functional IgH chains.

Before the expression of complete IgH proteins, nascent H chains (D\mu proteins) are thought to serve in a signaling function; that is, they might be in part responsible for the establishment of a D\mu reading frame bias (16, 27). Although D\nu-to-J\nu recombination should generate equal frequencies of all three possible D\nu reading frames, in practice, reading frame 2 usage is rare. This might be due to a counter-selection against cells that express D\mu proteins generated by D\nu-to-J\nu rearrangements that place D\nu in reading frame 2 (16). If the transgene interferes with this D\mu-mediated signaling function, then it might be expected that such a reading frame bias fails to become established in transgenic pre-B lymphocytes. However, in Fig. 6 we see that the D\nu reading frame bias is maintained regardless of genotype; only 6% of wild-type clones and 14% of B-less clones are found to contain D elements in reading frame 2. These findings suggest that the \lambda protein does not interfere with the putative signaling function of D\mu proteins required for the establishment of D segment reading frame bias.

Discussion

In B-less mice, the expression of the human Ig \lambda transgene induces a stage-specific perturbation of B lymphocyte development. We have defined the stage at which B lymphopoiesis is affected in these mice by comparing the relative frequencies of D-to-J\nu, V\nu-to-DJ\nu, and V\kappa-to-J\kappa Ig rearrangements in genomic DNA harvested from unfractionated bone marrows of B-less and wild-type animals. To accurately assess the relative frequencies of these Ig rearrangements, we developed a quantitative PCR-based assay, LiD PCR, that has significant advantages over conventional quantitative PCR-based protocols. Significantly, the data generated by this method are in binary form inasmuch as only those reactions that receive one or more effective templates generate product. This avoids the potential inaccuracies associated with conventional methodologies due to nonlinear aspects of conventional PCR amplification (e.g., plateau effect), and the potential of significant variation due to the exponential nature of the amplification reaction. The data in this report demonstrate that the theoretical underpinnings of this approach are sound and

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**Figure 7.** The block in B lymphocyte differentiation can be defined in terms of Ig rearrangements. Schematic representation of B lineage precursors defined according to the rearrangement configuration of Ig genes. Cell types A-G reflect an ordered progression of Ig rearrangements (described in reference 28). A block in B lymphocyte differentiation within region I (top left) results in failure to develop DJ\nu precursor cells (A) and their descendants (B-G). A block that occurs in region II (top right) could result in loss of any, or all, of the cell types indicated in this region (see text). The data presented in this paper are consistent with a model in which all cell types that express functional IgH chains are depleted (i.e., cell types D-G). *Alleles that are either productive or non-productive.

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that LiD PCR can be used to accurately detect small differences in the relative frequencies of DNA templates reliably.

**Defining the Stage of Altered B Lymphopoiesis in B-less Mice.** The data from these analyses can be contrasted with predictions based on the ordered pattern of Ig rearrangements that normally occurs during B lymphocyte differentiation. As is shown in Fig. 7, the D-to-J_{H} recombination step can occur on both chromosomes and is the first in a series of Ig gene rearrangements that take place during early B lymphocyte differentiation in bone marrow (1). A block in B lymphocyte differentiation that occurs before or coincident with the onset of Ig rearrangement (under region I in Fig. 7) would result in the failure to produce such precursors (Fig. 7, cell type A), and therefore preclude the generation of all subsequent stages of B lymphocyte differentiation (cell types B–G). Thus, a decrease in the frequency of each class of Ig rearrangement (D-to-J_{H}, V_{H}-to-DJ_{H}, V_{K}-to-J_{K}, and V_{\lambda}-to-J_{\lambda}) should be observed in B-less mice relative to wild-type mice. A block in B lymphocyte development that occurs after the D-to-J_{H} rearrangement step (under region II in Fig. 7) would lead to a different outcome. In this case, the predicted effect on Ig rearrangement frequencies depends on the relative proportion of precursor cells that carry the following three allelic configurations: V_{H}DJ_{H}/V_{H}DJ_{H} (both IgH alleles completely rearranged) (Fig. 7, cell types C and D), V_{H}DJ_{H}/DJ_{H} (one IgH allele completely rearranged, the other partially rearranged) (cell types B and E), and DJ_{H}/DJ_{H} (both alleles partially rearranged) (cell type A). If a large percentage of bone marrow B cell precursors carry a V_{H}DJ_{H}/DJ_{H} allelic configuration, then the elimination of this population would result in net loss of bone marrow D-to-J_{H} intermediates in addition to reductions of V_{H}-to-DJ_{H} and V_{K}-to-J_{K} rearrangements.

The results of the analyses of two matched littermate pairs are qualitatively similar and show that the bone marrow from B-less mice contain wild-type (or very marginally reduced) frequencies of D-to-J_{H} recombination, but are markedly deficient for V_{H}-to-DJ_{H} and V_{K}-to-J_{K} rearrangements. The finding that B-less mice contain essentially normal frequencies of D-to-J_{H} rearrangements (Fig. 5, C and G, and Table 1) is informative and indicates that B lymphocyte development is perturbed after the D-to-J_{H} recombination stage of differentiation in B-less mice. In contrast, V_{K}-to-J_{K} joining, which represents the most distal step of recombination assayed, is sharply reduced in B-less mice. A similar, although not as severe, reduction is also observed for V_{H}-to-DJ_{H} rearrangements in B-less bone marrow. These frequency reductions were not unexpected because B-less bone marrow is essentially devoid of later-stage B lymphocytes (19).

There is conflicting evidence on the relative proportion of bone marrow cells containing both V_{H}DJ_{H} and DJ_{H} alleles. Most A-MuLV-transformed bone marrow cell lines are V_{H}DJ_{H}/V_{H}DJ_{H} (28). However, a recent report indicates that a large fraction of CD45R(B220)-positive bone marrow cells bear both the V_{H}DJ_{H} and DJ_{H} alleles (29). If this is correct, then a block in B lymphocyte development that eliminates cell types of this rearrangement configuration (Fig. 7, cell types B and E) should result in a measurable reduction of bone marrow D-to-J_{H} recombinant alleles. However, in spite of the deficiency of CD45R(B220)-bearing bone marrow precursors in B-less mice, D-to-J_{H} rearrangements persist at approximately wild-type frequencies. Thus, our results suggest that the population of bone marrow cells bearing both V_{H}DJ_{H} and DJ_{H} alleles is relatively small compared with the population bearing only DJ_{H} rearrangements. We have investigated the possibility that the D-to-J_{H} junctions we de-

![Figure 8. Precocious maturation as a model of transgene-induced immunodeficiency in B-less mice.](image-url)
tect may be contributed by non-B lineage cells (namely T cells) present in the bone marrow (16, 30–33) by amplifying rearrangements from purified CD45R(B220)-negative bone marrow cells. We find that this fraction does not contain a significant frequency of D-to-Jα sequences (data not shown).

Precursors Bearing Functional IgH Rearrangements Are Underrepresented in B-less Mice. The stage at which B lymphopoi-

esis is disrupted resides within region II (Fig. 7), which in-

cludes cells of several stages of differentiation. We have been able to further resolve this location of B cell precursors by sequencing VαDJa junctions from purified populations of pre-B lymphocytes. Accordingly, we find that the pre-B lymphocyte population of B-less mice contains a disproportionately high fraction of nonproductive VαDJa rearrangements (Fig. 6), which strongly suggests that the subset of pre-B lymphocytes that normally express functional IgH are profoundly deficient in these mice (Fig. 7, cell types D–G). Consistent with this finding is the observation that B-less bone marrow contains only one-fourth as many CD45R(B220)+; surface (s)Ig- pre-B lymphocytes as wild-type mice (data not shown).

Precocious Maturation as a Mechanism for Transgene-induced Immunodeficiency. Murine B cell precursors carrying functional VαDJa alleles (pre-B cells) are thought to undergo several mitoses (at least five or six) before differentiating into nondividing sIgM-bearing B lymphocytes (34, 35). The expression of a μm/λαg complex may drive B lymphocyte precursors to mature precociously and therefore bypass this stage of differentiation required for cellular expansion. This could potentially cause as much as a 64-fold reduction in the number of B lymphocytes formed. This point is illustrated in Fig. 8. In wild-type mice the rate at which functional IgH/IgL complexes are formed presumably depends on the independent rates at which functional rearrangements are generated at these loci. This contrasts with the situation for B-less mice in which the functional λαg protein is expressed throughout all stages of differentiation. This would effectively reduce the rate at which μm/λαg complexes are formed to that at which functional VαDJa rearrangements are expressed. A maturation signal provided by a chimeric μm/λαg Ig could cause mitotically active pre-B cells to prematurely cease dividing and exit the bone marrow. In accordance with this model, precursors that carry only DJα or nonfunctional VαDJa rearrangements should be refractory to the λαg effect (Fig. 8 B, open circles). Our present results are clearly consistent with this hypothesis. That is, functional VαDJa alleles are depleted in B-less mice and DJα intermediate alleles are maintained at wild-type levels. Once formed, mature B cells bearing chimeric IgH/λαg Ig receptors apparently fail to significantly expand in the periphery. However, examination of every B-less mouse to date has revealed a very small population of splenic B lymphocytes (19).

The finding that B-less mice are severely depleted for A-MuLV transformation-sensitive targets (19) is also consistent with our hypothesis since pre-B lymphocytes would be forced to differentiate into nontransformation-sensitive (i.e., mature) B cells. The reduction of transformation-sensitive targets is an interesting departure from several other strains of mice in which B lymphocyte differentiation is apparently arrested (discussed below). An alternative hypothesis is that the λαg induces apoptosis of the B lineage cells in which it is expressed. We believe that this is unlikely, however, since another of our strains expresses high levels of λαg on the surface of apparently healthy splenic B lymphocytes (data not shown).

Other Ig Transgenic Strains Exhibiting Altered B Lymphocyte Populations. To date several strains of Ig-bearing transgenic mice have been reported to exhibit unexpected perturbations in B lymphocyte development. Although the B-less deficiency is the most severe among reported strains of λαg mice (3–5), a strain bearing a murine λαg transgene was found to contain only 30% wild-type numbers of B lymphocytes (5). We have also generated several other lines with the B-less construct that also display reduced B lymphocyte populations. Two of these lines, tg.ET and tg.EH, exhibit two- and fourfold reductions, respectively (36, and data not shown). Among our lines (five in all) the extent of the B lymphocyte deficiency is clearly correlated with the level of transgene expression in bone marrow (data not shown). Other strains of Ig-transgenic mice (bearing either IgH or both IgH and IgK) also exhibit significant reductions in B lymphocyte populations (for example, see reference 37). At least three strains, M54 (carrying a rearranged μ transgene; 38), Tμm (also carrying a μ transgene; 39), and TμmK38 (a double-expressing IgH/IgK strain; 39), exhibit severe deficiencies of both mature and pre-B cell compartments. An additional strain that expresses a truncated human μ chain (lacking the entire V region) has also been reported to exhibit large reductions of both bone marrow and splenic B lymphocyte populations (40). In general, most Ig transgenic lines reported have at least a measurable reduction in B lymphocyte populations. The spectrum of phenotypes exhibited by these reported strains presumably reflects both the intrinsic properties of the particular transgene as well as strain-specific patterns of transgene expression. The precocious maturation model of Ig transgene-induced immunodeficiency implies that the developmental onset of transgene expression is expected to profoundly influence the size of the precursor population. We suspect that positional influences could affect this parameter of transgene expression and thereby cause significant strain-dependent variation in phenotype.

Comparison of the B-less Phenotype to Other Genetically Modified B Lymphocyte–deficient Strains. Recently, mice that lack μm have been shown to exhibit a profound B lymphocyte deficiency due to a stage-specific arrest in the differentiation of B cell precursors (11). The developmental block in these mice corresponds to the transition at which large cycling pre-B lymphocytes give rise to smaller, noncycling descendants (11). Early blocks in B lymphocyte differentiation are also seen in mice that are genetically deficient for either the RAG-1 (12) or RAG-2 genes (13), neither of which are capable of producing functional μm chains. Detailed analysis of the bone marrow CD45R(B220)-positive cell population of RAG-2-deficient
mice indicates that B lymphocyte differentiation is arrested at a point that corresponds to the onset of Ig rearrangement in wild-type mice (12). That \( \mu_m \) normally functions to direct precursors to mature at this stage of B lymphocyte differentiation is also supported by recent experiments with B lymphocyte precursors bearing Ig transgenes (41). Precursors that express \( \mu_m \) transgenes are skewed to a later stage of differentiation (based on growth signal requirements) than cells that do not express the transgene. An analogous finding was recently reported for T lymphocytes in which TCR transgenes do not express the transgene. An \( \alpha \) transgene also appears to drive maturation of thymocytes in RAG-1-deficient mice (42). The finding that \( B \)-less mice have dramatically reduced numbers of A-MuLV transformation-sensitive targets contrasts with the lymphocyte-deficient mutant strains scid, RAG-1, and RAG-2, all of which appear to have approximately normal-sized populations of such targets (12, 13, 43). Because A-MuLV-sensitive targets generally appear to represent early B lymphocyte precursors that carry IgH, but not IgL, rearrangements (reviewed in reference 44), it is likely that the arrest of B lymphopoiesis in each of these recombination-deficient strains occurs after the expansion of precursors corresponding to the A-MuLV transformation-sensitive stage of differentiation. Taken together, the aforementioned strains appear to exhibit deficiencies resulting from arrested maturation, whereas the \( B \)-less deficiency appears to reflect accelerated maturation.

Potential Mechanism Underlying the \( \lambda_k \) Effect. Due to extensive homology between the \( \lambda_k \) and \( \lambda_5 \), a potential biochemical mechanism may be that \( \mu_m/\lambda_k \) complexes compete with endogenous \( \mu_m/\lambda_5 \) complexes that normally regulate maturation of B lymphocyte precursors (19). Recently, mice deficient for the \( \lambda_5 \) gene have been shown to exhibit a B lymphocyte deficiency that may also result from arrested B lymphocyte maturation (18). A potential functional interaction of the \( \lambda_k \) and \( \lambda_5 \) proteins could be investigated by comparing the phenotypes of the \( \lambda_k \)-deficient and \( B \)-less strains, particularly in relation to our finding that most \( V_k/\text{DJ}_{\text{int}} \) rearrangements in residual pre-B lymphocytes are nonfunctional. Interestingly, it has been recently reported that \( \lambda_5 \) function is required to establish the DJ frame reading bias (17). Our results show quite clearly that a reading frame bias is established normally in \( B \)-less mice. Thus, it appears that \( \lambda_k \) does not act to preclude this specific function of \( \lambda_5 \). It remains possible, however, that the \( \lambda_k \) may functionally substitute for \( \lambda_5 \), thereby providing excess \( \lambda_5 \) function.

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