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 V_H-to-DJ_H and V_K-to-J_K rearrangements in the transgenic strain, whereas the frequency of D-to-J_H rearrangements approximates that of wild type. Since Ig gene rearrangements occur in a stepwise fashion in which D-to-J_H joining precedes that of V_H-to-DJ_H and V_K-to-J_K, these results indicate that the major block of B lymphocyte development in the *B-less* strain occurs after D-to-J_H rearrangement. Interestingly, sequence analysis of residual V_HDJ_H 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 (μ_m), and not the secreted form (μ_s), that is required (6–8). In what may be a related process, μ_m appears to stimulate rearrangements of the IgL chain genes (9), although μ_m 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 μ_m gene has demonstrated that its function is essential for the development of the B lineage (11). As such, μ_m-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 (DJ_H) rearranged alleles of IgH (referred to as Dμ proteins) (16). In early pre-B cells establishment of the DJ_H 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 μ_m regulatory function (reviewed in reference 17). This hypothesis was recently tested using mice deficient for one such gene, λ_5, that encodes a surrogate light chain protein (18). Consistent with expectations, these mice fail to establish a DJ_H junctional reading frame

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bias and, furthermore, exhibit an arrest in B lymphocyte development similar to what is observed in mice that lack μ_m (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, Abelson murine leukemia virus (A-MuLV)[1] transformation-sensitive target cells and by the inability to derive pre-B cell cultures from either fetal liver or bone marrow cells (19). This deficiency is likely to be a direct consequence of λ transgene expression because two other lines, generated with the identical construct, also display significant alterations in B lymphocyte development (see Discussion). The mechanism by which the λ transgene perturbs B lymphocyte development in *B-less* mice is likely to reflect an interaction of the transgenic protein with regulatory mechanisms controlling early B lymphocyte differentiation.

To increase our understanding of the process by which the λ_{tg} 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 V_H, D, and J_H segments. This assembly occurs in a two-step process in which D-to-J_H joining occurs first, followed by V_H-to-DJ_H joining. Subsequently, the V regions of IgL (K or λ) undergo assembly in a process similar to that when V_L-to-J_L 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-J_H rearrangements, whereas the subsequent V_H-to-DJ_H and V_K-to-J_K rearrangements are significantly reduced in *B-less* mice. These results indicate that B lymphopoiesis is arrested after D-to-J_H rearrangement in these mice. Furthermore, sequence analysis of rare V_HDJ_H junctions present in transgenic animals reveals that they are largely out of translational frame and thus nonfunctional. 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 (λ_{tg}) 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 DME 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.

Mice and 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 OD_{260}. 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×

[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; λ_{tg}, transgenic λ; LiD, limiting dilution.

PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin), 0.4 μl 100× DNA polymerization 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 ^{32}P-labeled randomly primed probe at 5×10^5 cpm/ml at 42°C for at least 12 h and washed in 0.2× 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 defined by the equation $f_{(0)} = e^{-(\phi x)}$, where $f_{(0)}$ is the frequency of negative reactions, ϕ is the frequency of effective amplification templates within the DNA, and x is the amount of genomic DNA per reaction. The value, ϕ, was determined by using the linear analysis feature with Quasi-Newton minimization of the Systat™ statistical software package (Systat, Inc., Evanston, IL), ϕ was determined for each curve by considering the series of four adjacent data points corresponding to the highest $f_{(0)}$ 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 $J_{H4.2}$) were adapted from those previously published (10, 22), Ig_K sequences were amplified with primers $V_{K(1-16)}$ 5′-GGC TGC AG(C/G) TTC AGT GGC AGT GG(A/G) TC(T/A) GG(A/G) AC-3′ and J_{K2} 5′-CCA AGC TTT CCA GCT TGG TCC CCC CTC CGA A-3′. IgH sequences were amplified with D_{HL} 5′-GGA ATT CG(A/C) TTT TTG T(G/C)A AGG GAT CTA CTA CTG TG-3′, J_4-5′-TCC CTC AAA TGA GCC TCC AAA GTC C-3′, and V_{H7183} 5′-CGG TAC CAA GAA (G/C)A(A/C) CCT GT(A/T) CCT GCA AAT GA(G/C) C-3′. The $J_{4.2}$ oligo, 5′-GCG CTC GAG GAG ACG GTG ACT GAG GTT-3′, is complimentary to the J_{H4} coding sequences and is upstream of oligo J_4 (described in reference 23). c-*myc* sequences were detected by hybridization to a 3-kb XbaI/HindIII fragment containing exons 2 and 3 of the murine c-*myc* genomic DNA. Both D-to-J_H and V_H-to-DJ_H rearrangements were detected with a 1.5-kb PstI-PstI fragment containing the murine J_H region sequences. Rearranged V_K-to-J_K sequences were detected with a 2.7-kb HindIII-HindIII fragment containing the genomic sequences of the murine J_K region cluster.

Cloning and Sequencing of $V_{H7183}DJ_{H4}$ Junctions from Pre-B Lymphocytes. Bone marrow cells were harvested as described above except that cells were flushed into HBSS (Whittaker, Inc., Walkersville, MD) containing 20% FCS. The following Abs were used: PE-labeled CD45R(B220) (PharMingen Inc., San Diego, CA), FITC-labeled goat anti-mouse IgM and FITC-labeled goat anti-mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). The latter Ab contains balanced activity against mouse IgM, IgA, and IgG. Labeled cells were sorted on a Cytofluorograf IIS (Ortho Diagnostic Systems, Inc., Westwood, MA). Pre-B lymphocytes were collected as cells that are CD45R(B220) positive and either IgM or Ig negative. Purified populations were found to be >99% free of double-positive cells upon reanalysis. Genomic DNA was prepared from purified cells as described and amplified in a two-step, hemi-nested strategy as follows: 200 ng of genomic DNA was amplified with V_{H7183} and J_4 primers as previously described. 5% of the first reaction was reamplified with the V_{H7183} and $J_{H4.2}$ oligos by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C of 40 s. Bands of the expected sizes were cloned into pBluescript (Stratagene, Inc., La Jolla, CA). Clones containing $V_{H7183}DJ_{H4}$ junctions were sequenced using a sequenase kit (United States Biochem. Corp., Cleveland, OH).

Results

Detection of Specific Templates in Genomic DNA. A sensitive PCR method has been developed (10, 22) to detect recombination at both IgH and IgK, and this method provides the basis for our quantitative assay. In brief, primer pairs are used that amplify Ig coding sequences in the recombined state but not in the germline configuration. The overall strategy for the amplification and detection of these rearranged sequences is shown in Fig. 1. In the germline configuration, the Ig coding segments, and thus the sequences recognized by the PCR primer pair, are separated by long stretches of noncoding DNA. For example, the murine V_H sequences lie ~100 kb 5′ of the J_H sequences (reviewed in reference 24), and although a single Q52-type D element lies ~700 bp 5′ of the

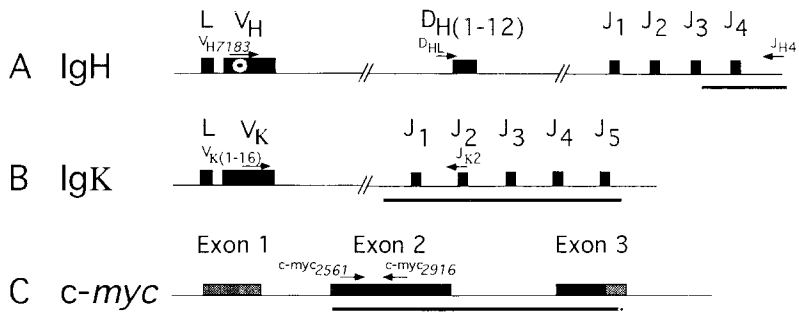

Figure 1. Strategy for PCR amplification and detection of genomic sequences. (*A*) IgH, (*B*) IgK, and (*C*) c-*myc* loci. IgH rearrangements that form D-to-J_H and V_H-to-DJ_H joints are amplified with the primer pairs D_{HL}/J_{H4} and V_{H7183}/J_{H4}, respectively. V_K-to-J_K rearrangements are amplified with the $V_{K(1-16)}$ and J_{K2} primers. Coding sequences of c-*myc* exon 2 are amplified with the $c\text{-}myc_{2561}$ and $c\text{-}myc_{2916}$ primers. Probes used in detection of amplified products are represented as black bars underneath the appropriate sequence. Figures are not drawn according to scale.

J_H cluster, the remaining D elements are clustered within a 60-kb region that is located ~20 kb 5′ of J_{H1} (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_H and V_H-to-DJ_H, can be amplified at the IgH locus (Fig. 1 *A*). First, recombinants that have joined any of the D elements encoded by the D_{FL16} or D_{SP2} families to any of the four J_H elements are amplified using the D_{HL} and J_{H4} primers because the D_{HL} primer is degenerate and hybridizes to upstream sequences flanking each of the identified D elements (excluding the D_{Q52} element that is just 5′ of the J_H region cluster and is not amplified with these primers). The nondegenerate J_{H4} primer hybridizes specifically to a sequence 3′ of the fourth J_H element. Second, rearrangements that join V segments of the 7183 V_H subfamily (V_{H7183}) to DJ_H intermediates are amplified with the V_{H7183} and J_{H4} primers. The amplification of D-to-J_H recombination intermediates cannot occur from genes that have undergone complete V_H-to-DJ_H recombination due to the deletion of the D_{HL} primer recognition sequence upon V_H-to-DJ_H joining. IgK rearrangements are amplified with a degenerate V_K region primer ($V_{K[1-16]}$) together with a nondegenerate J_K region primer (J_{K2}) (Fig. 1 *B*). This primer pair will amplify only those rearrangements in which a V_K segment is joined to either J_{K1} or J_{K2}, since the joining of a V_K segment to either J_{K3}, J_{K4}, or J_{K5} results in the deletion of the J_{K2} 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 c-myc_{2561} and c-myc_{2916} 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 VJ_K 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

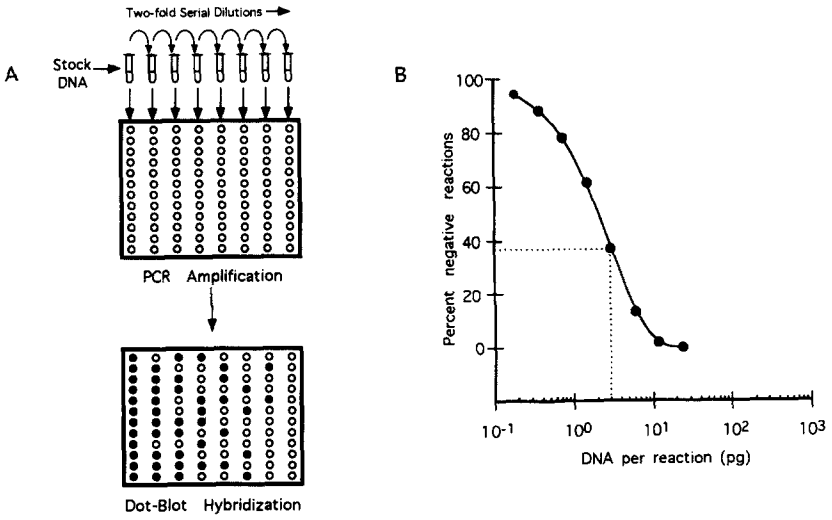

Figure 2. Description and theoretical considerations of the LiD PCR assay. (*A*) Basic design. Genomic DNA to be tested is twofold serially diluted to produce a set of at least eight subdilutions. Equal volumes of each subdilution are aliquoted to individual wells of a 96-position tray and amplified. 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, $f_{(0)}$, 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 $P(_c)X = (\phi x)e^{-\phi x}/c!$; where ϕ 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 $P_{(0)} = e^{-\phi x}$. Therefore, for an average template concentration of one per reaction, that is when $\phi x = 1$, $P_{(0)} = e^{-1} = 0.37$. Therefore, the frequency of negative reactions, $f_{(0)}$, will approximate 37%, given a sufficiently large number of reactions. A best-fit curve (least squares), described by this equation, is fitted to the data (see Materials and Methods). The determination of the effective template concentration, ϕ, 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 $f_{(0)} = 37\%$ (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. 2 *B*. 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. 2 *B*, 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-J_K rearrangements. WEHI 231 is an immature B cell line that carries a V_K-to-J_{K1} 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-J_K 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-J_K dilution curves were generated from DNA mixtures containing predetermined proportions of DNA from WEHI 231 (a source of V_K-to-J_K recombinant templates) and cultured fibroblasts. Fibroblast DNA did not contain any detectable Ig recombination (Fig. 4, 100% FvB/N fibroblast DNA) and,

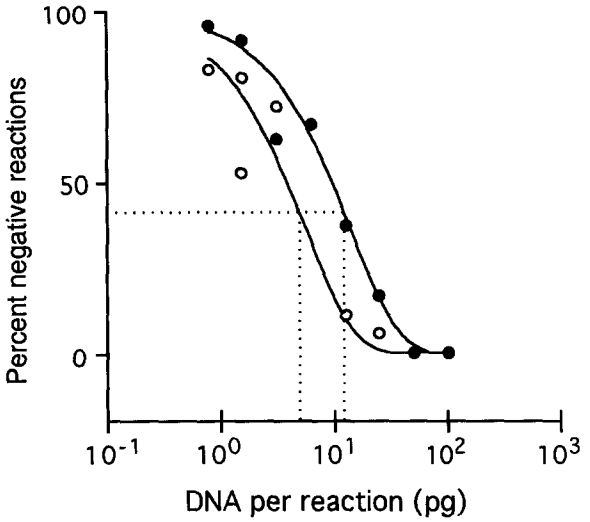

Figure 3. LiD PCR analysis of the WEHI 231 cell line. Frequency determinations of both c-*myc* (*open circles*) and V_K-to-J_K (*filled circles*) templates in genomic DNA from the B cell line WEHI 231. c-*myc* templates are found at a frequency of one per 5.5 pg and V_K-to-J_K templates at one per 14 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.

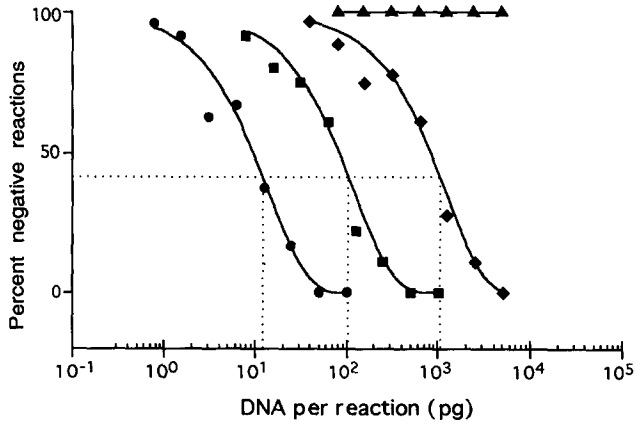

Figure 4. LiD PCR analysis of DNA stock solutions containing different concentrations of effective templates. Stock mixtures containing WEHI 231 and fibroblast DNAs in predetermined proportions were analyzed by LiD PCR. 100% WEHI 231 DNA (*circles*) (same curve as in Fig. 3); 10% WEHI 231 DNA/90% fibroblast DNA (*squares*); 1% WEHI 231 DNA/99% fibroblast DNA (*diamonds*); and 100% fibroblasts DNA (*triangles*). The frequency (and 95% confidence interval) of V_K-to-J_K recombinant templates in mixtures containing 100, 10, and 1% WEHI 231 DNA are 1 per 14 (11–18) pg (same curve as in Fig. 3), 120 (92–170) pg, and 1,200 (800–2,400) pg, respectively. No rearrangements were detected in pure fibroblast DNA.

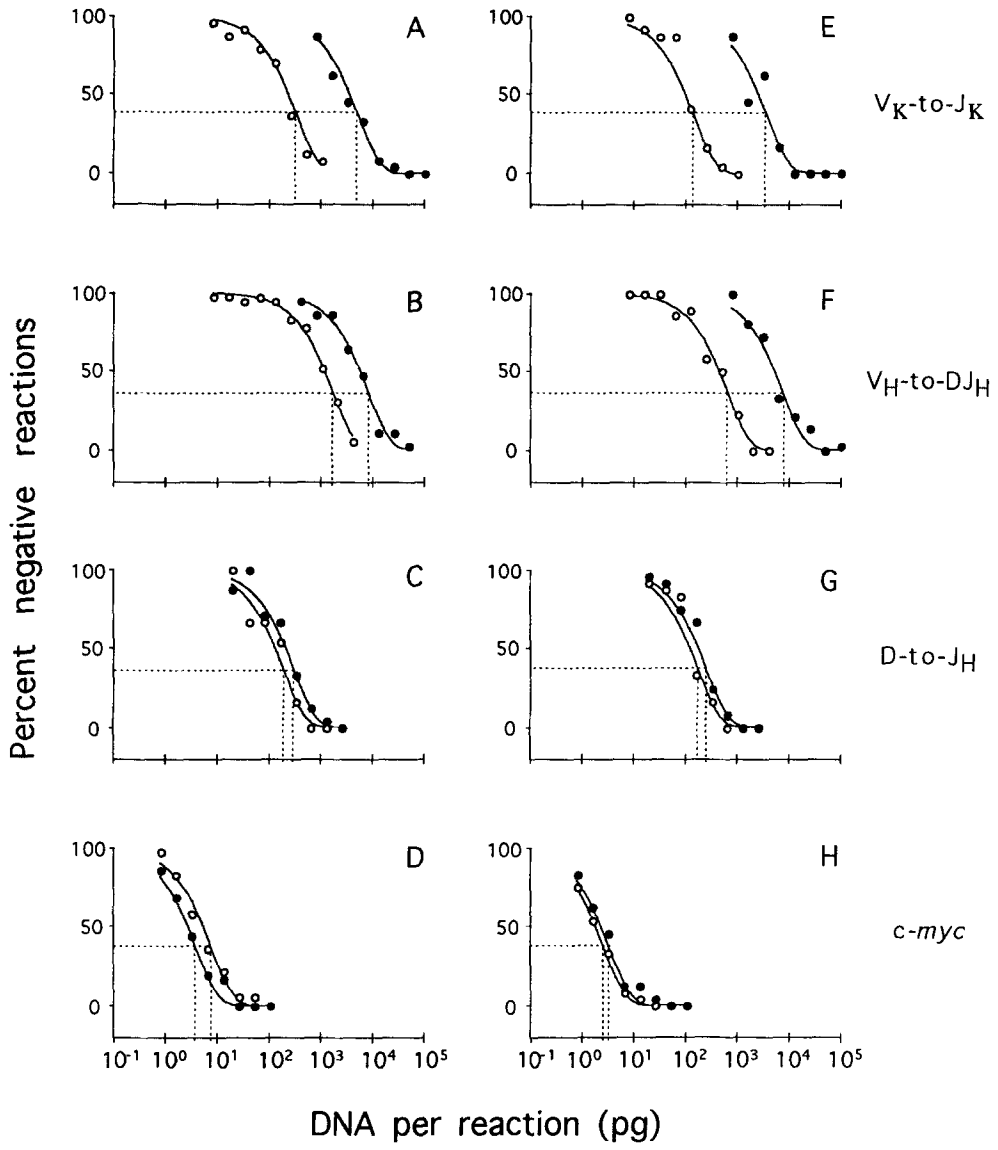

Figure 5. LiD PCR analysis of genomic DNA harvested from bone marrow cells of two independent littermate pairs. 6-wk-old animals (pair no. 1) are shown in *A–D*, and 8-mo-old animals (pair no. 2) in *E–H*. Specific amplification reactions are shown as follows: *A* and *E*, V_K-to-J_K; *B* and *F*, V_H-to-DJ_H; *C* and *G*, D-to-J_H; *D* and *H*, c-*myc*. Open and filled circles indicates data from wild-type and *B-less* animals, respectively.

Table 1. *Summary of Ig Recombination Frequencies in Bone Marrow DNA from Wild-Type and B-less Mice*

	Pair no. 1				Pair no. 2			
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-J_H	180	(140–260)	290	(240–390)	170	(130–250)	250	(210–300)
V_H-to-DJ_H	1,700	(1,500–1,900)	8,100	(6,700–10,000)	660	(470–1,100)	7,700	(5,800–11,000)
V_K-to-J_K	330	(230–590)	5,100	(3,800–7,900)	150	(110–210)	3,700	(2,500–7,500)

* Picograms DNA per single effective template ($1/\phi$).
‡ Values corresponding to the lower and upper 95% confidence interval (C.I.) are shown in parentheses.

therefore, served as a source of nonrecombinant competitor DNA. The relative frequencies of V_K-to-J_K recombination were experimentally determined for each of the stock solutions. Again, all resulting curves closely conformed to a Poisson distribution, indicating that the presence of different concentrations of nonamplified DNA did not impart a significant effect on either amplification efficiency or template dilution. More importantly, the experimentally determined values of recombinant template frequency were in close agreement with the known values, demonstrating the accuracy of the assay within the range of DNA concentrations tested. As mentioned, WEHI 231 genomic DNA was determined to contain one effective V_K-to-J_K template per 14 pg DNA (same curve as in Fig. 3). Mixtures containing 10 or 1% of this DNA in fibroblast DNA were determined to have one template per 120 and 1,200 pg, respectively, as would have been expected from these dilutions.

The Relative Frequencies of Ig Recombination in Bone Marrow DNA from B-less and Wild-Type Mice. As noted above, the purpose of designing the LiD PCR assay was to allow us to use recombinant Ig genes as surrogates for B cells at various stages of development and thus specify the block in B lymphopoiesis in *B-less* mice. To determine the relative frequencies of Ig rearrangements, bone marrow cells from both *B-less* and wild-type littermates were assayed using this technique. The results of the analysis for two pairs of littermates are shown in Fig. 5 and Table 1. Comparison of recombination frequencies at the IgK locus demonstrate that *B-less* mice were severely deficient for V_K-to-J_K recombination relative to their wild-type littermates (Fig. 5, *A* and *E*). After normalization for c-*myc* amplification, *B-less* mice were determined to have a frequency of V_K-to-J_K rearrangements of ~3–5% that of wild-type. *B-less* bone marrow was also deficient for V_H-to-DJ_H rearrangements (Fig. 5, *B* and *F*) as compared with wild-type mice (~11% of wild-type levels). In contrast, the analysis of D-to-J_H templates demonstrates that this class of rearrangements is virtually unaffected (Fig. 5, *C* and *G*, and Table 1). One littermate pair was found

A Wild-type clones:

CLONE	V_{H7183}	N-D-N	J_{H4}	PROD.	D REGION FAMILY	READING FRAME
3	TGT GCA AGA C	TGGGGGCAGCTCGGGCCTT	T TAC TAT GCT ATG GAC AAC	P	-	-
4	TGT GCA AGA C	TGAGGTTAGGGG	AT GCT ATG GAC TAC	P	DSP2.3,6,9	3
5	TGT GCA AGA C	GGGACTACGATGGTAGCTACG	AT GCT ATG GAC TAC	P	DFL16.1,.2|DFL16.1E	1/1
6	TGT GCA AGA CA	GCTCGGGCCCC	CT ATG GAC TAC	P	-	-
8	TGT GCA AGA C	TCCCGTCCGGA	NCT ATG GAC TAC	P	-	-
9	TGT GCA AGA CA	TGAGGGGGGTTACTACGAGGG	G GAC TAC	P	DFL16.1,2	1
10	TGT GCA AGA C	GGTATGGTAACTACGG	C TAT GCT ATG GTC TAC	P	DSP2.8	1
11	TGT GCA	GGAAGGGGGACCT	C TAT GCT ATG GAC TAC	P	DQ52	1
18	TGT GCA AGA C	TTAGGGTCTTTAGTAACT	AT TAC TAT GCT ATG GAC TAC	P	DSP2.X	2
140	TGT GCA AGA CA	AAGCTACGATGGTAGCCCAC	AC TAT GCT ATG GAC TAC	P	DFL16.1|DFL16.1E	1/1
141	TGT GCA AGA	GATATGATTCTCCGCCGATGGCTCTAT	CT ATG GAC TAC	NP	DSP2.2|DSP2.9|DSP2.9	1/2/3
142	TGT GCA AGA	GGATCCCC	CT ATG GAC TAC	NP	-	-
143	TGT GCA AGA CA	TGCCCC	T GCT ATG GAC TAC	P	-	-
144	TGT GCA AGA C	CCCTACGTC	AT TAC TAT GCT ATG GAC TAC	P	DSP2.6,7,11,X	3
149	TGT GCA AGA C	TAGAGCTACGATGGTAGCTACGGG	GCT ATG GAC TAC	NP	DFL16.1,1E,.2|DFL16.1E	1/1
150	TGT GCA AGA	GAAAGATNTNGTGACTAC	GCT ATG GAC TAC	P	DSP2.1,5,7,8,9,X,DFL16.1,.2	1
303	TGT GCA AGA C	GGCTCCGCT	AT TAC TAT GCT ATG GAC TAC	P	DFL16.2	1
304	TGT GCA AGA C	GGGGCTCCGACT	AT TAC TAT GCT ATG GAC TAC	P	DFL16.2	1
306	TGT GCA A	AAGATTGTTACTACGGGGGGG	AT GCT ATG GAC TAC	P	DSP2.9	1
307	TGT ATG AGA	TCACTATAGTAACTC	TAC TAT GCT ATG GAC TAC	NP	DSP2.X	3
310	TGT GCA AG	GGGGGTCCACCGC	GCT ATG GAC TAC	P	DFL16.2	1
311	TGT GCA AG	CTCAGGGACCTACT	AT TAC TAT GCT ATG GAC TAC	P	DSP2.6,7,11,X	1
313	TGT GCA AG	CTC	GCT ATG GAC TAC	NP	-	-
324	TGT GCA AGA	GTTGGTAACTCCT	AT TAC TAT GCT ATG GAC TAC	P	DSP2.1,5,7,8	1
327	TGT GCA AG	GCCCCATCGATGGTTACTCC	AT TAC TAT GCT ATG GAC TAC	P	DSP2.9	3
328	TGT GCA AG	GCGCTATAGAGAGG	AT GCT ATG GAC TAC	P	DSP2.10,11,X	1
331	TGT GCA AGA	GGGGGTTACTTT	TAT GCT ATG GAC TAC	P	DSP2.9	1
333	TGT GCA AGA CA	TGAGATCTATGATGGTTACT	AT TAC TAT GCT ATG GAC TAC	P	DSP2.9	1
335	TGT GCA AGA	GTTGGTAACTCCT	AT TAC TAT GCT ATG GAC TAC	P	DSP2.1,5,7,8	1
336	TGT ATG AGA	TAGCATAATACGTTATTACTACGATGGTAGCTACCG	T TAC TAT GCT ATG GAC TAC	NP	DFL16.1|DFL16.1E	1/1
337	TGT GCA AGA C	TAAATGCTAGCCAT	AT TAC TAT GCT ATG GAC TAC	P	DFL16.1E	1
341	TGT GCA AGA CA	GGGTAGCTACAGA	GCT ATG GAC TAC	P	DFL16.1E	1
344	TGT	ATAGTTACTATAGTTTACGAGGAAG	T TAC TAT GCT ATG GAC TAC	NP	DSP2.10,11,X|DSP2.2,3,6	3/3

PRODUCTIVE REARRANGEMENTS: 26/33 (79%)

B *B-less* clones:

CLONE	V_{H7183}	N-D-N	J_{H4}	PROD.	D REGION FAMILY	READING FRAME
17	TGT GCA AGA C	GGACTATGGTTACTATT	T TAC TAT GCT ATG GAC TAC	NP	DFL16.1E|DSP2.1-7,10,11,X	3/3
18	TGT GCA AGA C	CGACCACTGTTTAACGTA	TAT GCT ATG GAC TAC	NP	-	-
22	TGT GCA AGA C	GAAAGTAACCAATA	TAC TAT GCT ATG GAC TAC	NP	DSP 2.X	1
30	TGT GCA AGA C	TGGGGTATACTATAGTAACTAGCTATCATGGG	C TAC	NP	DSP2.X	3
32	TGT GCA AGA C	GACAACTGTTTAACGTA	TAT GCT ATG GAC TAC	NP	DQ5 2	2
33	TGT GCA AGA CA	TG	GCT ATG GAC TAC	P	-	-
38	TGT ATG AGA	TGAGATGTTGTATTGTTACTATGTATA	TAC TAT GCT ATG GAC TAC	NP	DSP2.8|DSP2.1-7	3/3
40	TGT GCA AGA C	CTACTATAGTAACTC	C TAT GCT ATG GAC TAC	NP	DSP2.X	1
41	TGT GTA AGA CA	AGACCTCTATATC|ACGACGTGGGG	AC TAT GCT ATG GAC TAC	NP	DSP2.9|DSP2.2,3,6,10,11	3/3
43	TGT GCA AGA C	TAAACTATAGTAACTAGGCGG	C TAT GCT ATG GAC TAC	P	DSP2.X	1
47	TGT GCA AGA C	CCCGCTTGTATAGGTAGAG	GCT ATG GAC TAC	P	DSP2.10,11	1
53	TGT GCA AG	GTATAGTTTACTATAGTTTACGACGGAACTGGGACGAGG	GCT ATG GAC TAC	P	DSP2.X|DSP2.X|DSP2.2,3,6,10,11|DQ52	1/1/1/2
109	TGT ATG AG	CCCCCATAACCACGAGGGAA	AC TAT GCT ATG GAC TAC	NP	-	-
118	TGT ATG AGA	GCNNNNNGTTACTATGGTGACC	T TCT ATG GAC TAC	NP	DFL16.1E	3
120	TGT ATG AG	CCCCCAGTAACCACGAGGGAA	AC TAT GCT ATG GAC TAC	NP	DSP2.X	1
151	TGT GCA AGA CA	CTACTATAGTAATC	TAC TAT GCT ATG GAC TAC	NP	DSP2.X	2
155	TGT GTA AGA C	GTGATTACTACGATGGTAGCTACGAGATT	AC AT GCT ATG GAC TAC	NP	DFL16.1,2|DFL16.1E|DSP2.2	2/2/3
156	TGT GCA AGA C	GGGTNCCTATTACTACGG|ATGGTAGAC	C TAT GCT ATG GAC TAC	NP	DFL16.1|DSP2.1,5,7,8	3/3
202	TGT GTA AGA CA	AGACTCCTATGGTAACTAC|GATGG	C TAT GCT ATG GAC TAC	P	DSP2.1,5,7|DSP2.9	1/1
209	TGT GCA AGA C	GGGG	T TAC TAT GCT ATG GAC TAC	P	-	-
212	TGT GCA AGG C	CTACTATCCC	CT ATG GAC TAC	NP	DSP2.1-7,10,11,X,	3
213	TGT GCA AGA	GATAGTTACTATAGCCCTCATA	TAC TAT GCT ATG GAC TAC	NP	DSP2.10,11,X	2
218	TGT GCA GGA C	TACCGGGT	T TAC TAT GCT ATG GAC TAC	NP	-	-
224	TGT GCA AGA C	GGTCCCATATAGAGGGTATGG	T ATG GAC TAC	NP	DSP2.10,11,X|DSP2.8	1/1
226	TGT GCA AGA CA	TGGGCTATGGTAACTACGC	T TAC TAT GCT ATG GAC TAC	NP	DSP2.1,5,7	1
228	TGT GCA AGA C	TCTCTATGGG|ATGGTTACTACGT	C TAT GCT ATG GAC TAC	NP	DSP2.1,3-7|DSP2.9	3/1
230	TGT GCA AGA C	GAGGACTGGGAGT	AT TAC TAT GCT ATG GAC TAC	NP	DQ52	-
235	TGT GCA AGA C	TGAGGGCTACTATAGTAACCCCGA	TAT GCT ATG GAC TAC	NP	DSP2.X	3
240	TGT GCA AGA C	TACCTACTATAGTTTACTATAGTTTACGAGGGAGGGAGGGG	TAC TAT GCT ATG GAC TAC	NP	DSP2.X|DSP2.X|DSP2.2,3,6,10,11	1/1/1

PRODUCTIVE REARRANGEMENTS: 6/29 (21%)

Figure 6. Sequence analysis of $V_{H7183}DJ_{H4}$ junctions obtained from purified populations of bone marrow pre-B lymphocytes. Pre-B lymphocytes are obtained from wild-type (*A*) and *B-less* (*B*) mice. Precursor B lymphocytes were purified from total bone marrow by two-color FACS® (Becton Dickinson & Co., Mountain View, CA) and identified as cells that are $CD45R(B220)^+/IgM^-$ (clones 3–156) or $CD45R(B220)^+/Ig^-$ (i.e., IgM^-, IgG^-, and IgA^-) (clones 202–344). Clone sequences are aligned to those compiled for V_{H7183} on the left and J_{H4} on the right (46). Whenever possible, junctional nucleotides are designated as belonging to either V_{H7183} or J_{H4} sequences. N-D-N sequences are defined as junctional nucleotides that fail to match either V_{H7183} or J_{H4} elements and may be partly composed of D elements, N nucleotides (47, 48), and P nucleotides (49). D region homologies are underlined and are recognized as junctional sequences with a minimum homology of 5 bp with published D region sequences (compiled in references 50 and 46). Only those published D segments that match the longest stretch of identified sequence homology are listed. Adjacent D element sequence homologies and corresponding designations 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_H 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 $V_{H7183}DJ_{H4}$ 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 $V_{H7183}DJ_{H4}$ 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 $V_{H7183}DJ_{H4}$ junctions as ascertained by the preservation of reading frame and the absence of stop codons. In contrast, only 21% of the sequenced $V_{H7183}DJ_{H4}$ 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_H reading frame bias (16, 27). Although D_H-to-J_H recombination should generate equal frequencies of all three possible D_H 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_H-to-J_H rearrangements that place D_H 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_H 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 λ_{tg} 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 λ 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_H, V_H-to-DJ_H, and V_K-to-J_K 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

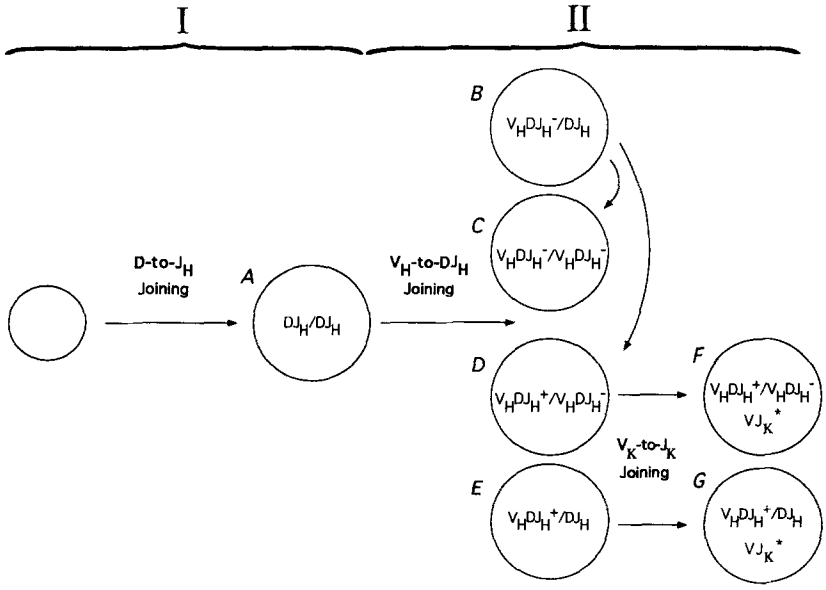

Figure 7. The block in B lymphocyte differentiation can be defined in terms of Ig rearrangements. Schematic representation of B lineage precursors defined according to the rearrangement configuration of Ig genes. Cell types *A*–*G* reflect an ordered progression of Ig rearrangements (described in reference 28). A block in B lymphocyte differentiation within region *I* (*top left*) results in failure to develop DJ_H/DJ_H 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 nonproductive.

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 recombination (D-to-J_H, V_H-to-DJ_H, V_K-to-J_K, and V_λ-to-J_λ) 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_HDJ_H/V_HDJ_H (both IgH alleles completely rearranged) (Fig. 7, cell types *C* and *D*), V_HDJ_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_HDJ_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, though 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_HDJ_H and DJ_H alleles. Most A-MuLV-transformed bone marrow cell lines are V_HDJ_H/V_HDJ_H (28). However, a recent report indicates that a large fraction of CD45R(B220)-positive bone marrow cells bear both the V_HDJ_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_HDJ_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-

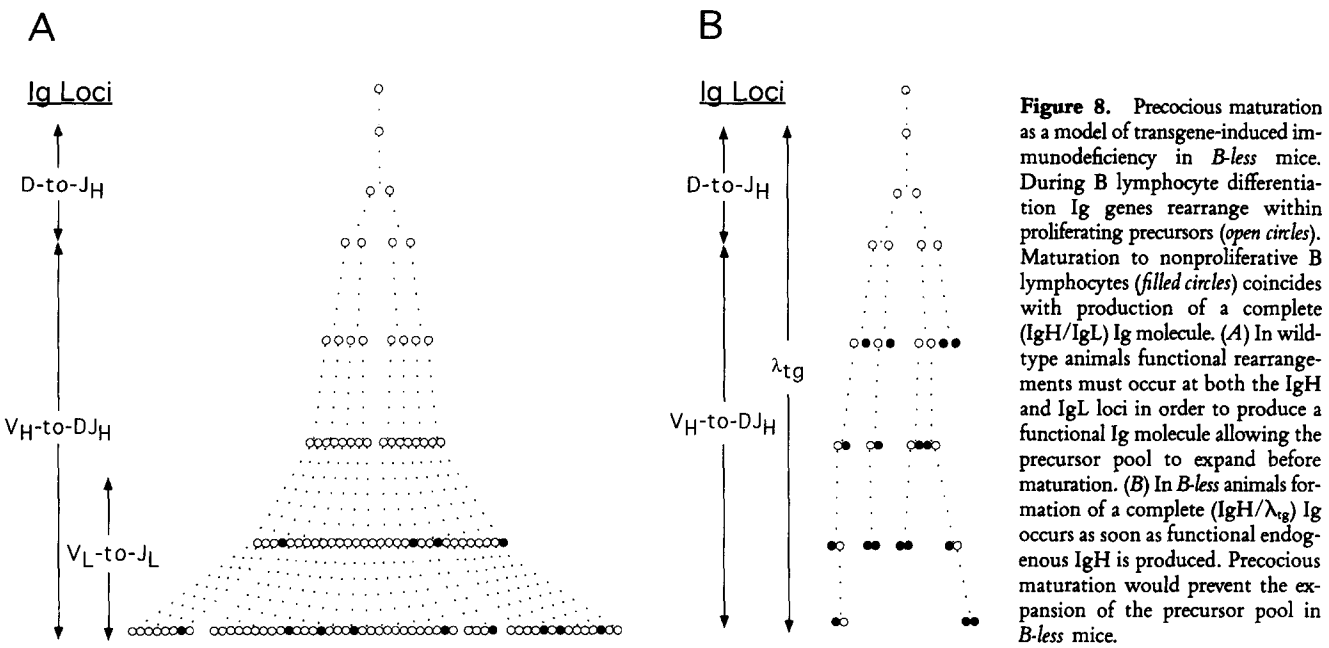

Figure 8. Precocious maturation as a model of transgene-induced immunodeficiency in *B-less* mice. During B lymphocyte differentiation Ig genes rearrange within proliferating precursors (*open circles*). Maturation to nonproliferative B lymphocytes (*filled circles*) coincides with production of a complete (IgH/IgL) Ig molecule. (*A*) In wild-type animals functional rearrangements must occur at both the IgH and IgL loci in order to produce a functional Ig molecule allowing the precursor pool to expand before maturation. (*B*) In *B-less* animals formation of a complete (IgH/λ_{tg}) Ig occurs as soon as functional endogenous IgH is produced. Precocious maturation would prevent the expansion of the precursor pool in *B-less* mice.

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-J_H recombinant sequences (data not shown).

Precursors Bearing Functional IgH Rearrangements Are Underrepresented in B-less Mice. The stage at which B lymphopoiesis is disrupted resides within region II (Fig. 7), which includes cells of several stages of differentiation. We have been able to further resolve the affected subpopulation of B cell precursors by sequencing V_HDJ_H 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 $V_{H7183}DJ_{H4}$ 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 is the finding 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 V_HDJ_H 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 μ_m/λ_{tg} 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 λ_{tg} protein is expressed throughout all stages of differentiation. This would effectively reduce the rate at which μ_m/λ_{tg} complexes are formed to that at which functional V_HDJ_H rearrangements are expressed. A maturation signal provided by a chimeric μ_m/λ_{tg} 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 DJ_H or nonfunctional V_HDJ_H rearrangements should be refractory to the λ_{tg} effect (Fig. 8 *B*, *open circles*). Our present results are clearly consistent with this hypothesis. That is, functional V_HDJ_H alleles are depleted in *B-less* mice and DJ_H intermediate alleles are maintained at wild-type levels. Once formed, mature B cells bearing chimeric IgH/λ_{tg} 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 λ_{tg} 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 λ_{tg} 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 λ_{tg} mice (3–5), a strain bearing a murine λ_2 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 four-fold 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 μ_m 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 μ_m 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 μ_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 μ_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 appear 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 recombinase-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 λ_{tg} Effect. Due to extensive homology between the λ_{tg} and λ_5, a potential biochemical mechanism may be that μ_m/λ_{tg} complexes compete with endogenous μ_m/λ_5 complexes that normally regulate maturation of B lymphocyte precursors (19). Recently, mice deficient for the λ_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 λ_{tg} and λ_5 proteins could be investigated by comparing the phenotypes of the λ_5-deficient and *B-less* strains, particularly in relation to our finding that most $V_{H7183}DJ_{H4}$ rearrangements in residual pre-B lymphocytes are nonfunctional. Interestingly, it has been recently reported that λ_5 function is required to establish the DJ_H junctional reading frame bias (17). Our results show quite clearly that a reading frame bias is established normally in *B-less* mice. Thus, it appears that λ_{tg} does not act to preclude this specific function of λ_5. It remains possible, however, that the λ_{tg} may functionally substitute for λ_5, thereby providing excess λ_5 function.

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