

BIASED EXPRESSION OF J_H-PROXIMAL V_H GENES OCCURS
IN THE NEWLY GENERATED REPERTOIRE OF
NEONATAL AND ADULT MICE

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The large diversity of antibody molecules produced by B lymphocytes is accomplished in part by the somatic assembly of multiple germline gene segments that encode the variable region of the molecule. Variable region genes are assembled during the antigen-independent phase of B cell differentiation, which in mammals occurs in primary B cell differentiation organs (fetal and neonatal liver and bone marrow) (reviewed in reference 1). Precursor B (pre-B) lymphocytes assemble and express heavy and light chain genes to become surface Ig⁺ B cells. The set of variable genes expressed by the population of newly formed B lymphocytes is the "newly generated" antibody repertoire, which is unselected by external antigens (reviewed in reference 2). B lymphocytes migrate to peripheral lymphoid organs, such as the spleen and lymph nodes, where they may mature into Ig-secreting cells (plasma cells) if appropriately stimulated (1).

The heavy chain variable region is assembled from three germline elements: variable (V_H), diversity (D), and joining (J_H) segments (reviewed in reference 3). The estimated 100–1,000 (4, 5) murine V_H gene segments have been grouped into 11 families of related genes based on nucleotide sequence homology (4, 6–8). Murine V_H gene families are generally clustered on the chromosome, but there is extensive interspersed of some families (9–13). The relative chromosomal positions of V_H gene families have been determined to be as follows: (V_HJ558-V_H3609)-V_HJ606-(V_HGAM3.8-V_H36-60-V_HX24-V_HS107)-(V_HQ52-V_H7183)-DJ_H (11–13); families with significant interspersed of members are indicated in parentheses (9–13). Analysis of Abelson murine leukemia virus-transformed pre-B cell lines (10, 14, 15) and fetal liver hybridomas (14, 16) suggested that selection of V_H gene segments for V_H to DJ_H rearrangements in pre-B cells was biased; V_H segments from the most J_H-proximal V_H gene families were preferentially used. More recently, we demonstrated that the V_H repertoire expressed in neonatal liver is also biased with dis-

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proportionately high expression of J_H proximal gene segments (17). Together, the data from the cell lines and neonatal liver suggest that developing pre-B cells generated a biased V_H repertoire as a result of mechanistic constraints on the Ig gene assembly process that leads to preferential rearrangement and expression of J_H-proximal V_H segments.

The pattern of V_H gene family expression in the adult spleen differs dramatically from that observed in the neonatal liver (17). Several different analyses have shown that the V_H repertoire in adult spleen is "randomized" such that expression of V_H gene families correlates approximately with the size (i.e., number of individual members) of the family encoded in the genome, suggesting that individual V_H gene segments are represented at equal frequencies (17-20). Although strain-dependent variations that may not be due to the size of the V_H gene family have been observed (17, 20-22), for simplicity we will refer to the shift away from the position-related V_H repertoire of the neonatal liver to the distribution observed in adult spleen as the "randomization" of the repertoire. Randomization could be effected by several, not mutually exclusive, mechanisms. One possibility is that programmed changes in recombinational mechanisms may alter the frequency with which different V_H segments are used during development; according to such a model the rearrangement mechanism operating in fetal pre-B cells would preferentially use proximal V_H gene segments, while that operating later in ontogeny randomly use all V_H gene segments (14, 16, 17, 23). Alternatively (and not mutually exclusively), rearrangement frequencies of V_H gene segments in pre-B cells could be the same throughout ontogeny but cellular selective interactions acting subsequent to V gene rearrangement would normalize the representation of the various V_H gene families in mature B cell populations. To elucidate mechanisms that operate to shift the V_H repertoire from the biased pattern observed in the neonatal liver to the more random pattern observed in the adult spleen, we assayed the patterns of V_H expression in primary and peripheral lymphoid organs at various times in ontogeny. We find that the biased V_H repertoire is apparent early in fetal ontogeny and is constant throughout fetal and neonatal development until 5-7 d after birth. Furthermore, we find that the primary repertoire generated in adult bone marrow is also biased with respect to expression of J_H-proximal V_H gene segments. Together, these findings suggest that selection, rather than changes in rearrangement frequency, is the major contributor to randomization of the V_H repertoire in adult splenic B cells.

Materials and Methods

Mice. BALB/cByJ and C57BL/6J mice of specified age were purchased from The Jackson Laboratory (Bar Harbor, ME). Fetal and newborn samples were derived from timed-pregnant matings purchased from The Jackson Laboratory. C.B-17 and C.B-17*scid* mice (hereafter designated scid mice) were maintained in specific pathogen-free animal facilities at the Institute for Cancer Research (Philadelphia, PA) and were provided by Dr. M. Bosma and G. Bosma (Fox Chase Cancer Center, Philadelphia, PA). The scid mutation occurred in the C.B-17 inbred strain, a congenic partner of BALB/cAnICR that has the Igh^b haplotype from C57BL/Ka. Unless otherwise indicated, spleen and bone marrow samples were pooled from two to three mice and fetal and neonatal liver samples were pooled from three to five litters. Adult samples were removed at 5-6 wk of age; otherwise samples were removed at the age indicated in the text.

Preparation of Tissue Samples. Splens and livers were dissected and RNA isolated without

further manipulation. Bone marrow cell suspensions were prepared from femurs and tibias by perfusion with media containing FCS and washed once in PBS.

V_H and C μ Probes. The V_H-specific probes representative of each of the V_H gene families and the C μ probe (μ_2) have been described in detail previously (17). The V_H81X and V_H.B4 (17) probes were used to identify the V_H7183 and V_HJ558 families, respectively. The μ_m probe is the 1.4-kb Pst I fragment isolated from the genomic DNA clone μ_5 (24) which encodes the second exon and 3' untranslated portion of the membrane form of μ mRNA.

Preparation of RNA and Northern Blotting. Total and poly(A)⁺ RNA were prepared as previously described (25). Northern blotting, probe preparation, and hybridization procedures have previously been described (25).

V_H Utilization Assay. The assay to measure utilization of V_H gene families in tissue samples has been described in detail previously (17). Briefly, total RNA from spleen or bone marrow or poly(A)⁺ RNA from liver samples were titered for the level of C μ expression and amounts of RNA that gave approximately equal signals were assayed for hybridization to V_H-specific probes. The amounts of RNA differed for the various tissue samples approximately as follows: 10–20 μ g poly(A)⁺ RNA from fetal and neonatal livers; 15–20 μ g total RNA from spleens day 1 to day 7; 5 μ g from spleens at 2–3 wk; 1–2 μ g total RNA from adult spleen; 5–10 μ g total RNA from adult bone marrow. Replicate Northern blots were hybridized to each of the V_H or μ probes and signal intensities were quantitated. The V_H-to-C μ ratio was calculated for each sample. Preference values are a comparison of the relative expression of a particular V_H gene family in a primary lymphoid organ (that is, fetal liver, neonatal liver, or adult bone marrow) to that expressed in normal, adult spleen. Thus a preference value of 1 indicates that a V_H family comprises an equivalent proportion of the μ mRNA in the experimental sample as in the adult spleen; a value >1 indicates the proportion to which that family is over-represented compared with adult spleen; and a value <1 indicates the proportion to which that family is under-represented compared with adult spleen. Within each experiment, all samples were compared with the same adult samples so that values between experimental samples can be directly compared. The percent expression of V_H gene families was calculated by comparison of the V_H to C μ ratio in the tissue samples to the ratio obtained in reference cell lines, as previously described (17).

Densitometry. Densitometry was performed on multiple exposures of the autoradiograms using the Joyce Loebel Chromoscan 3.

Results

Expression of V_H Gene Families Is Constant Throughout Fetal and Neonatal Development. To determine if the relative expression of V_H gene families changes during fetal development, we analyzed V_H expression in fetal livers at different times during gestation. The predominant C μ -hybridizing mRNA species in fetal liver between days 15 and 17 fail to hybridize to V_H gene probes (26) and most likely correspond to germline C μ transcripts (data not shown, 26–29). Because of the difficulty in resolving some germline transcripts from those mRNAs derived from fully assembled V_HDJ_H-C μ Ig heavy chain genes (27–29), it was not possible to accurately standardize the expression level of V_H-containing C μ mRNA transcripts for fetal liver samples of different ages. Therefore, replicate Northern blots containing equivalent amounts of poly(A)⁺-enriched RNA from fetal samples at different times in gestation were hybridized to V_H probes specific for the V_H7183 and V_HJ558 families. The signal intensities were determined by densitometry and the ratio of the signals obtained with the V_H7183 and V_HJ558 probes for each of the fetal liver samples was compared with that of newborn liver, which was arbitrarily set at 1. Although the absolute signal intensities with the V_H probes increased between day 15 and day 18 fetal liver (probably reflecting the increased frequency of pre-B and virgin B cells)

(1, 30), the relative expression of V_H7183 and V_HJ558 was similar throughout fetal development until birth (Fig. 1).

To determine the expression levels of other V_H gene families in fetal development, fetal liver samples from day 18 of gestation were examined in more detail. In both BALB/c and C57BL/6 mice, the pattern of expression in day 18 fetal liver was similar to that observed in newborn liver for six V_H gene families examined (Fig. 2, compare lanes 1 and 2, 8 and 9). To facilitate comparison of V_H usage patterns in the different tissue samples, the relative expression of each V_H family for a standardized level of μ mRNA was compared with that observed in adult spleen (Fig. 2, lanes 7 and 14) and preference values were calculated (Table I). A marked preference for expression of J_H-proximal V_H gene families was observed in fetal liver relative to adult spleen (Table I). The only change in expression between day 18 fetal liver and newborn liver is a two- to fourfold increase in expression of V_HJ558 (Fig. 2 and Table I). Biased usage of proximal V_H gene families is still apparent in liver samples taken 3 and 7 d postnatally in both BALB/c (Fig. 2, lanes 3 and 4) and C57BL/6 (Fig. 2, lanes 10 and 11) mice. Thus, the V_H repertoire expressed in differentiating B cells in the liver is relatively constant throughout fetal development and the first days of postnatal life, suggesting that V_H segments continue to be rearranged and expressed at a rate that reflects chromosomal position.

Normalization of V_H7183 Expression Occurs between 1 and 2 wk of Age. To elucidate further the shift between fetal and adult V_H repertoires, we assayed the relative

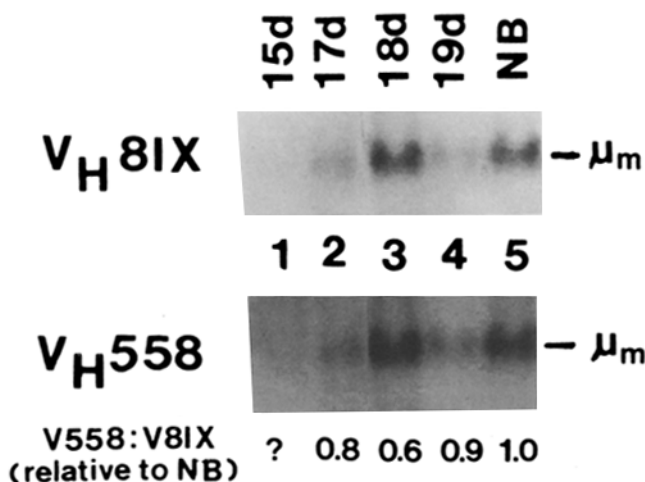


FIGURE 1. Expression of the V_H7183 and V_HJ558 gene families during fetal development of BALB/c mice. 5–10 μ g of poly(A)⁺ RNA prepared from pooled BALB/c livers removed at the indicated day of fetal development or the first day after birth (newborn, NB) were fractionated on agarose-formaldehyde gels and transferred to nitrocellulose filters. Duplicate filters were hybridized to either the V_H81X probe or the V_H.B4 probe. Signal intensities were determined by densitometry, and were used to determine the V_HJ558/V_H81X ratio for each of the samples; these ratios are expressed relative to the ratio in the NB liver sample which was arbitrarily set at 1. The signal intensities of V_H hybridization to day 15 fetal liver (lanes 1) were too weak for accurate densitometric scanning; however, the V_HJ558/V_H81X ratio as assessed by visual examination of the original autoradiograms was similar to the liver samples taken later in ontogeny.

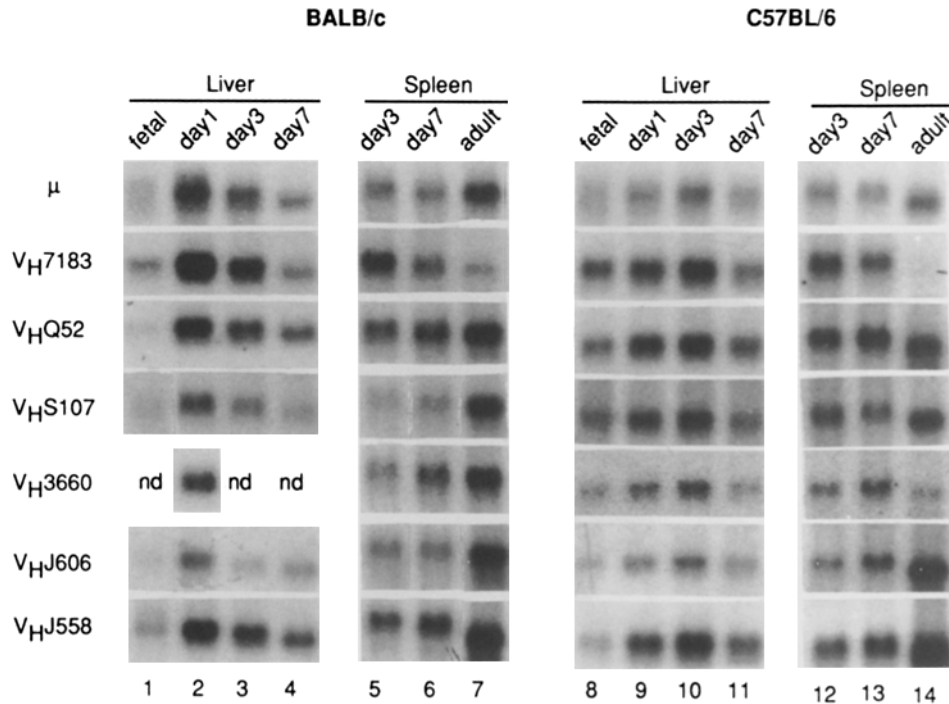


FIGURE 2. Expression of V_H gene families during neonatal development of BALB/c and C57BL/6 mice. Pooled samples of 18 day fetal livers (lanes 1 and 8), neonatal livers (lanes 2-4 and 9-11), and neonatal spleens (lanes 5-7 and 12-14) were isolated from BALB/c (lanes 1-7) and C57BL/6 (lanes 8-14) mice. Total RNA from the spleen samples and poly(A)⁺ RNA from the liver samples was standardized for μ mRNA levels and amounts that gave approximately equal signals were fractionated. Replicate Northern blots were assayed for hybridization to V_H -specific probes as indicated. Densitometric analyses of multiple autoradiographic exposures of these Northern blots are summarized in Table I.

representation of each V_H gene family in the μ mRNA expressed in neonatal splenic B cells. The initial repertoire expressed in the neonatal spleen is biased for expression of J_H -proximal V_H families; relative expression of the six V_H families in the day 3 spleen is almost identical to that in newborn liver in both BALB/c (Fig. 2, compare lanes 2 and 5) and C57BL/6 (Fig. 2, compare lanes 9 and 12) strains. Changes in expression are observed in day 7 spleen: although still biased toward proximal families, relative expression of V_H7183 is decreased in BALB/c spleen (Fig. 2, lane 4) and expression of V_HJ606 and V_HJ558 are slightly increased in both BALB/c (Fig. 2, lane 4) and C57BL/6 (Fig. 2, lane 13) day 7 spleens.

To analyze when in ontogeny the V_H repertoire is normalized to adult levels, the absolute utilization of the V_H7183 and V_HJ558 gene families was determined in BALB/c splenic B cells at various times from neonate to adult. The level of V_H7183 expression remained constant until 5 d after birth; subsequently, the level decreased steadily and reached approximately adult levels by 2 wk after birth (Fig. 3, A and B). Although there is some variation in the absolute expression of the V_HJ558 family in the first week after birth, adult levels were reached by 2 wk of age.

TABLE I
V_H Usage During Development in BALB/c and C57BL/6 Mice

Mice		Liver				Spleen		Adult
		Fetal	Day 1	Day 3	Day 7	Day 3	Day 7	
BALB/c	V _H 7183	4-5	4-8	4-5	3-4	5-6	2-3	1.0
	V _H Q52	0.5-2	0.8-0.8	0.7-0.8	0.7-1	0.9-1	1.0-1	1.0
	V _H S107	0.1-0.2	0.4-0.5	0.3-0.4	0.3-0.3	0.2-0.3	0.4-0.5	1.0
	V _H 36-60	ND	0.6	ND	ND	0.6	1	1.0
	V _H J606	0.1-0.2	0.2-0.3	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	1.0
	V _H J558	0.2	0.5	0.5	0.5	0.5	0.6	1.0
C57BL/6	V _H 7183	15-20	7-10	10-20	9-20	10-25	10-20	1.0
	V _H Q52	0.6-1.0	0.8-1	0.8-1	1-2	0.9-2	2-3	1.0
	V _H S107	0.5-1	0.8-2	0.6-1	0.7-2	0.5-2	0.7-2	1.0
	V _H 36-60	2-3	1-2	2-3	2-3	2-3	3-5	1.0
	V _H J606	0.1-0.1	0.2-0.3	0.2-0.4	0.3-0.4	0.2-0.6	0.6-1	1.0
	V _H J558	0.1-0.2	0.5-0.6	0.5-0.6	0.7-0.9	0.4-0.5	0.9-1	1.0

Northern blots as described in Fig. 2 were analyzed by densitometry. Signal intensities were determined and used to derive the preference values listed in the table. Preference values were calculated according to the following equation: Preference (V_H) = [I_{NB}(V_H)/I_{sp}(V_H)]/[I_{NB}(C_μ)/I_{sp}(C_μ)]; where I_{NB} and I_{sp} denote the intensities of hybridization of a given V_H probe and the C_μ probe to the indicated tissue samples. The range of values given for each comparison encompasses all the values obtained after densitometric analysis of multiple (2-4) autoradiographic exposures of each Northern blotting experiment (except for hybridization of the V_H36-60 and V_HJ558 probes to the BALB/c samples for which only one exposure suitable for densitometric scanning was obtained); numbers are rounded off to a single significant digit. Preference values are further described in Materials and Methods.

Alternative splicing of primary V_HDJ_H-C_μ transcripts results in two species of μ mRNA that contain different 3' exons: processed μ mRNAs of 2.7 and 2.4 kb encode the membrane-bound (μ_m) and the secreted (μ_s) forms of μ heavy chain proteins, respectively (24, 31, 32). B-lineage cells at different developmental stages produce different steady-state amounts of the two species of μ mRNA: pre-B cells and mature B cells produce equal or higher levels of μ_m mRNA than μ_s mRNA, whereas plasma cells produce predominantly μ_s mRNA (28, 29). Thus, the relative expression of μ_m versus μ_s mRNA in a given tissue is in part related to the maturation state of the cells expressing the predominant level of μ mRNA sequences in the tissue. To determine whether the shift in V_H7183/V_HJ558 expression was correlated with a shift in the pattern of μ mRNA produced, we quantitated more precisely the apparent shift in μ mRNA expression suggested from the V_H hybridizations observed in day 1 and day 3 spleens (Fig. 3A). For this analysis, the relative intensity to which BALB/c splenic RNA samples hybridized to a probe specific for the μ_m exons was compared with that obtained with a probe (μ₂) that detects all μ mRNAs. When the amount of RNA electrophoresed for each sample is adjusted to give a relatively constant signal of total μ mRNA, the μ_m-specific hybridization steadily decreased after 3 d of age (Fig. 3A). An estimate of the relative expression of μ_m mRNA was quantitated by comparing the ratio of the signal intensities for the two probes (μ_m/C_μ) obtained for each spleen sample to that obtained for an Abelson murine leukemia virus (A-MuLV)-transformed pre-B cell line (Fig. 3B). These data clearly demonstrated that the decrease in V_H7183 expression correlates with a gradual shift

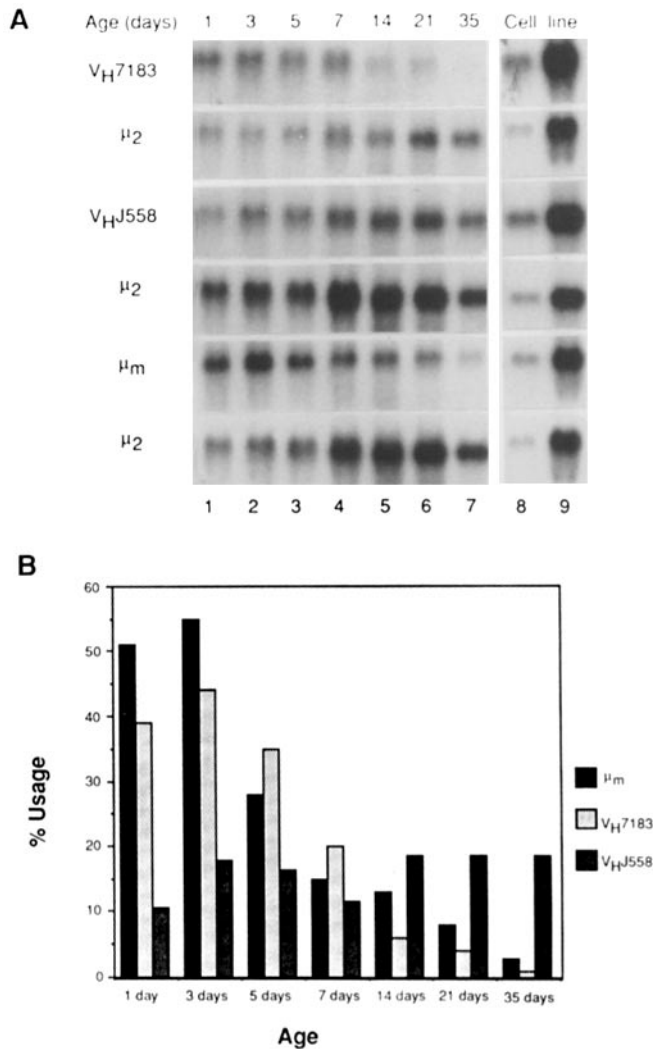


FIGURE 3. Splenic V_H gene expression during ontogeny of BALB/c mice. (A) Total RNA was prepared from BALB/c spleens isolated at different days after birth (lanes 1–7) and from reference cell lines (lanes 8 and 9). The day 1 sample was a pool of spleens derived from four litters of newborn mice; day 3, 5, and 7 samples were pooled spleens from 12 mice each; day 14 and 21 samples were pooled spleens from 6 mice each; and the 5-wk sample was pooled spleens from 3 mice. RNA amounts were standardized to contain approximately equal levels of μ mRNA; a fivefold lower amount of RNA from the reference cell lines (lane 8) were also examined to allow easier comparison and quantitation of the relatively high levels of expression of a single V_H family in the cell lines with the lower levels found in spleen samples. The reference cell line for the V_H81X and μ_m probes was 22D6-G2; the reference cell line for the V_H.B4 probe was MOPC104E. Replicate Northern blots were hybridized to the V_H probes or a probe specific for the membrane form of μ mRNA, as indicated. Blots were subsequently hybridized to a probe (μ₂) that detects all μ mRNAs. (B) Signal intensities of the bands depicted in A were determined by densitometry and used to determine the V_H/C_μ ratio or μ_m/C_μ ratio for each sample. These ratios are expressed as percentages of the ratios in the reference cell lines, which were set at 100%; the percent usage was plotted against the age of the mice from which the spleen samples were removed.

in the expression of μ mRNA from predominantly μ_m to predominantly μ_s in the adult spleen (Fig. 3 B).

V_H Gene Utilization in Differentiating Pre-B Cells in the Adult Is Also Biased to J_H-proximal Families. To determine if a repertoire biased toward utilization of J_H-proximal V_H segments continues to be generated in differentiating B cells in the adult, we analyzed three independent pools of total bone marrow cells for V_H gene expression.

Migration of B cells from the bone marrow to peripheral lymphoid organs is not unidirectional; B cells stimulated by antigen in the periphery may recirculate into the bone marrow and mature into μ - and γ -secreting plasma cells (33-35). Plasma cells produce as much as 100-fold or more μ mRNA, predominantly μ_s , than B-lineage cells of earlier stages (36). Thus, even a small number of contaminating plasma cells would prevent detection of mRNA contributed by the developing B cells. The number of plasma cells in the bone marrow accumulates with age (33). To minimize contamination of bone marrow with recirculating cells from the periphery, bone marrow samples were derived from young adult (4-5 wk old) BALB/c mice and only those samples that had a high proportion of μ_m mRNA (indicative of pre-B and B cells) were tested. The relative representation of each V_H gene family in the μ mRNA of bone marrow cells was compared with that in the μ mRNA of adult spleen. For comparison, newborn liver samples were assayed in the same experiments. The pattern of V_H expression in adult bone marrow differs from that of spleen and is similar to but not identical to that of newborn liver (Fig. 4 and Table II). In particular, the V_H7183 family is preferentially expressed in adult bone marrow relative to adult spleen; however, the relative V_H7183 utilization in adult bone marrow is clearly less than that of newborn liver. The biased expression of proximal V_H gene segments in adult bone marrow is consistent with the notion that these V_H gene segments are also preferentially rearranged in differentiating pre-B cells in the adult.

A curious feature of V_H expression in adult bone marrow was the high expression of the V_HS107 family, which was higher than that expressed in both adult spleen and newborn liver (Fig. 4 and Table II). However, the bulk of V_HS107-containing μ mRNA was the size of the μ_s species of mRNA (2.4 kb), suggesting bone marrow contains a disproportionate number of plasma cells or secreting B cells producing antibodies with heavy chains encoded by V_H segments from this family. This result also is consistent with the observation that adult mice naturally produce a high titer of antibodies bearing the T15 idiotype (37), which is encoded by a member of V_HS107 family (38).

Biased V_H Gene Expression in Adult Spleens of scid(μ^+) Mice. As another approach to studying the V_H repertoire generated in adult mice, we analyzed expression in the mutant mouse strain that is homozygous for the *scid* mutation (scid mice; 39, 40). In general, scid mice lack T cells and B cells due to a mutation that does not allow developing B and T lineage cells to functionally rearrange their respective antigen receptor loci (41). However, a certain percentage of adult scid mice, termed "leaky" scid, produce significant amounts of serum Ig (42). To analyze the potential of this immunodeficient mouse strain to express V_H genes, pooled or individual scid spleens were screened for μ mRNA expression and V_H usage analyzed in those samples that had detectable μ expression. The majority of scid spleens had extremely low levels of μ mRNA (>1,000-fold lower levels than those of normal C.B-17 of comparable age) (data not shown); however, 1 of 3 pooled scid spleen samples and 2 of 12 individual scid spleens had detectable levels of μ mRNA (10-100-fold lower than that of normal C.B-17) (Fig. 5). These spleen samples also expressed complete κ mRNA transcripts in proportions similar to the level of μ mRNA (data not shown), suggesting that expression is due to mature B cells and not to pre-B cells that may be present in spleens of some of these defective mice (43).

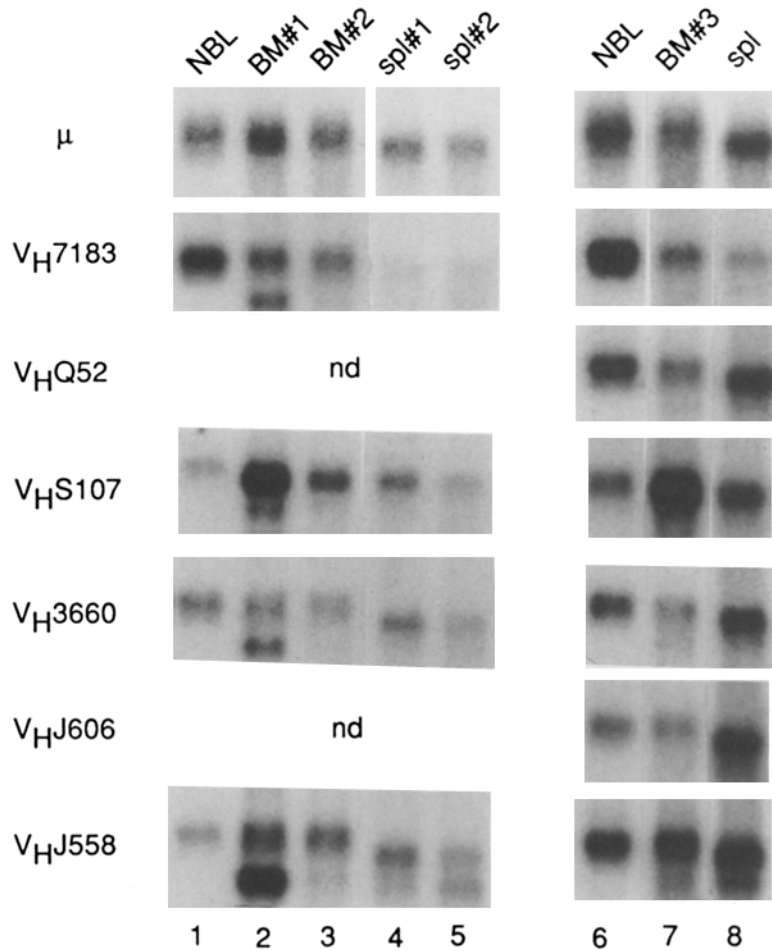


FIGURE 4. V_H gene expression in bone marrow from adult BALB/c mice. Expression of V_H gene families in total RNA prepared from pooled adult BALB/c bone marrow (BM) samples (lanes 2, 3, and 7) was compared with that observed in RNA from adult spleen (*spl*; lanes 4, 5, and 8) and poly(A)⁺-RNA from day 1 newborn liver (*NBL*, lanes 1 and 6) as described in Fig. 2. The lower V_H-hybridizing bands observed in some samples (e.g., lanes 2 and 5) correspond to γ_s mRNA and were not included in the densitometric analyses summarized in Table II. Lanes 1-5 represent Exp. 1 and lanes 6-8 represent Exp. 2. In Exp. 2, the *NBL* and adult spleen samples are the same as those depicted in Fig. 2.

The V_H expression in the selected scid(μ^+) spleens differed significantly from that of normal C.B-17 spleens (Fig. 5 and Table III). To facilitate comparison between normal and scid spleens, a ratio of the relative utilization of each V_H gene family for a standardized amount of μ mRNA was derived by dividing the relative (V_H/C _{μ}) expression in the scid spleens by the relative expression in normal spleens (Table III). The V_H7183 gene family was highly preferentially expressed in two of three scid spleens (Fig. 5 and Table III). The third scid spleen sample had substantially

TABLE II
V_H Gene Expression in Adult BALB/c Bone Marrow

	Exp. 1			Exp. 2	
	NBL	BM1	BM2	NBL	BM3
V _H 81X	10-20	4-5	4-6	4-8	2-2
V _H Q52	ND	ND	ND	0.8-0.8	0.3-0.5
V _H S107	0.2-0.4	2-4	1-2	0.4-0.5	2-3
V _H 36-60	0.8-1	0.3-0.4	0.4-0.5	0.6	0.5
V _H J606	ND	ND	ND	0.2-0.3	0.2-0.3
V _H J558	0.3-0.7	0.5-1.0	0.7-1.5	0.5	0.6

The Northern blots described in Fig. 4 were analyzed by densitometry and preference values were calculated as described in Table I and Materials and Methods. The intensities of hybridization of the given V_H probe and the C_μ probe to the newborn liver (NBL) or adult bone marrow (BM) sample were compared with each adult spleen. The range of values for each comparison represents all the values obtained from densitometric analysis of multiple autoradiographic exposures; numbers are rounded off to a single significant digit.

lower levels of μ mRNA, making quantitation of V_H family expression difficult. However, the relative expression of the two V_H families that were detectable (V_HQ52 and V_HJ558) was biased towards the J_H-proximal family (V_HQ52). Thus, the general pattern of V_H gene expression in adult scid(μ^+) spleens is biased in a manner reminiscent of that observed in pre-B cell lines derived from normal C.B-17 bone marrow (44) and in neonatal livers and neonatal spleens from normal BALB/c and C57BL/6 mice.

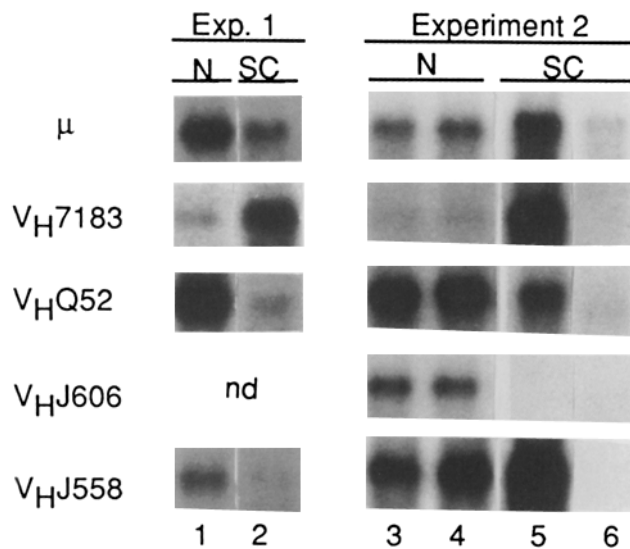


FIGURE 5. V_H utilization in spleens from adult scid(μ^+) mice. 20 μ g of total RNA prepared from adult scid spleens were screened for expression of detectable levels of μ mRNA. One of 3 pools of scid spleen samples (lane 2) and 2 of 12 individual spleen samples (mouse no. 8203, lane 5; and mouse no. 8186, lane 6) were subsequently analyzed for V_H gene expression as indicated and compared with expression observed in normal C.B-17 adult spleens (2 μ g total RNA) (pooled sample, lane 1; individual samples, lanes 3 and 4). The signal intensities were determined and the results are summarized in Table III. Expression of V_HJ558 in the scid spleen shown in lane 5 was re-examined in other Northern blots containing levels of μ RNA more comparable to those in the normal samples so that a better comparison could be made (data not shown); the quantitation of this data is given in Table III.

TABLE III
V_H Usage in Adult Spleens from Scid Mice

	Exp. 1	Exp. 2	
	Pooled spleen	Mouse 8203	Mouse 8186
V _H 81X	10-20	7-10	Not detected
V _H Q52	0.2-0.3	0.3-0.5	0.2-0.3
V _H J606	Not done	<0.1	Not detected
V _H J558	0.3-0.4	1-2	<0.1

The Northern blots described in Fig. 5 were analyzed by densitometry. Values represent a comparison of the expression of V_H gene families in scid(μ^+) spleens relative to that observed in normal C.B-17 spleen for a given amount of μ mRNA. Values were calculated as follows: $[I_{SC}(V_H)/I_{SC}(C_\mu)]$ was divided by $[I_N(V_H)/I_N(C_\mu)]$; where I_{SC} and I_N denote the intensities of hybridization to a given probe in scid and normal spleens, respectively.

Discussion

Expression of the V_H Gene Repertoire in Fetal and Neonatal Development. We have demonstrated that the position-dependent bias of V_H gene expression previously observed in neonatal liver (17) is also evident in earlier stages of fetal development. The relative expression of the V_H7183 and V_HJ558 gene families is constant from day 15 of fetal life through birth. Six V_H gene families that are representative of regions throughout the V_H locus (11-13) were expressed at the same relative level through fetal and neonatal liver development. These findings suggest that the rate at which particular V_H segments are rearranged and expressed remains constant during early ontogeny and that this rate is influenced by chromosomal position.

An ontogenic analysis of V_H gene expression in spleen revealed that the neonatal splenic repertoire was similar to that of neonatal liver. The shift away from the position-dependent V_H repertoire was first apparent at ~ 1 wk of age. Although there is a transient appearance of pre-B cells in the spleen early in ontogeny, the total number of pre-B (cytoplasmic μ^+ , surface Ig⁻) cells plateaus at birth and is only a fraction of the number of B (surface Ig⁺) cells at 3 d postnatally (30, 45). Because B cells produce equal or greater amounts of steady-state μ mRNA than pre-B cells (28), B cells most likely contribute the predominant μ mRNA detected in the postnatal spleen. Thus the primary B cell repertoire expressed in the neonatal spleen reflects the biased repertoire generated by the position-dependent rearrangement of V_H gene segments that occurred in the differentiating pre-B cells, suggesting that the mechanisms that "normalize" the V_H repertoire to the random representation of V_H genes in the adult act subsequent to expression of surface Ig. These data agree with other studies that found an over-representation of J_H-proximal V_H gene families in hybridomas derived from unstimulated neonatal spleens (46, 47), antigen-selected hybridomas (48), and in polyclonally activated B cells assayed by in situ hybridization (49). In the latter study, the representation of V_H gene families reached approximately adult levels by 7 d after birth (49).

B cell responsiveness to some antigens is acquired in a temporally ordered fashion during ontogeny (50-59). Our finding that the utilization of V_H gene families by differentiating pre-B cells remains constant during fetal and adult B cell differentiation implies that it is unlikely that the ordered appearance of antibody specificities

can be explained solely on the basis of the absence of rearrangements of particular V_H gene segments. However, it is possible that the relative bias in the representation of certain V_H gene segments in the newly generated versus normalized repertoires might account for the usage of different antibody specificities in response to particular antigens in neonatal versus adult mice. For example, in neonatal antibody responses a more frequently rearranged V gene of lower affinity may predominate at the expense of a less commonly encountered V gene of higher affinity. Recently, it has been demonstrated that the representation of κ light chain families differs in B cell colonies derived from neonatal versus adult spleen (60). Thus, it is possible that biased rearrangements at light chain loci or combinatorial associations (e.g., 61) of particular heavy and light chains might contribute to the ordered acquisition of particular antibody specificities.

The gradual shift in V_H gene expression in the spleen during the first 2 wk of life suggests that during this time in development other factors act on the initially biased repertoire of newly formed B lymphocytes to diversify the expressed V_H repertoire. These mechanisms may include positive or negative regulatory forces, such as tolerance induction, immunoregulatory networks, and antigen selection. For example, hybridomas derived from neonatal B cells have been found to have reactivity to self antigens (46, 47, 62), including reactivity to variable regions of Ig molecules (i.e., antiidiotypes) (46, 62). The potential modulatory effects of these antibodies during neonatal development may play a role in the generation of a randomized adult repertoire (46, 62). Differential V_H gene expression by distinct B cell subsets may also contribute to the developmental pattern of V_H gene expression. For example, the Ly-1 B cell subset makes up a larger proportion of splenocytes in the neonate than in the adult (63); thus the shift away from expression of J_H-proximal V_H families may correlate with the decrease in proportion of Ly-1 B cells in the spleen. However, available evidence (64, 65) suggests that the V_H repertoire expressed by the Ly-1 B cell subset does not significantly differ from the conventional B cell population (64, 65; and our unpublished data).

Generation of the B Cell Repertoire in Adult Mice. Unlike pre-B cell differentiation early in ontogeny, pre-B cell differentiation in adult mice occurs exclusively in the bone marrow. It has been suggested that there is a difference in the repertoire generated by pre-B cells derived from adult bone marrow versus fetal or newborn liver that leads to the developmental shift in V_H gene expression. We found increased representation of V_H7183-related gene segments in the adult bone marrow relative to adult spleen but less than fetal or newborn liver. The bone marrow is a heterogeneous population of B-lineage cells that includes about equal numbers of pre-B and newly formed B cells (30, 35, 45), as well as memory B and plasma cells that have recirculated from peripheral lymphoid organs (33–35). Although μ -secreting plasma cells that have recirculated from the periphery may contribute to the μ mRNA signal and thus skew the V_H repertoire measured, the fact that preferential expression of the V_H7183 family is observed suggests that the V_H repertoire generated in differentiating B cells in the adult has a position-dependent bias similar to that early in ontogeny. This conclusion is consistent with the nonrandom usage of V_H genes in rearrangements of pre-B cell lines derived from adult bone marrow (10, 14) or hybridomas

from long-term bone marrow cultures (66), as well as in nonproductive rearrangements of adult B lineage cells (15).

In contrast to these findings, another study found that polyclonally activated adult bone marrow cells analyzed by in situ hybridization displayed a pattern of V_H usage similar to adult spleen (49). The different results in that study (49) versus the analysis presented here may be explained by differences in the cell populations assayed. The former study (49) examined functional B cells stimulated with LPS, whereas in our assay V_H expression is contributed by pre-B and newly formed, unstimulated B cells. It is possible that regulatory mechanisms that influence the antibody repertoire in mature cells have already acted on the LPS-responsive cells in the adult bone marrow. Examination of purified populations of pre-B cells and unstimulated B cells from the bone marrow may clarify these apparent discrepancies.

Biased V_H Expression in Spleens of Leaky $scid(\mu^+)$ Mice. The *scid* mutation is an autosomal, recessive mutation that arose spontaneously in an inbred strain of mice (39). Mice homozygous for this mutation are deficient in B and T lymphocytes; other hematopoietic cell types (erythrocytes, monocytes, granulocytes, and megakaryocytes) are normal (39, 40). The *scid* mutation appears to affect the ability of T and B lymphocytes to functionally rearrange their respective antigen receptor loci (41). The majority of *scid* mice lack detectable serum Ig due to the deficiency of functional lymphocytes. However, serological screening of *scid* mice have revealed circulating Ig in $\sim 15\%$ of the mice (42); mice with detectable serum Ig were denoted $scid(Ig^+)$. Analyses of circulating Ig in $scid(Ig^+)$ mice revealed a restricted κ light chain heterogeneity in most mice; however, some mice showed more complex serum Ig patterns, suggesting variable degree of leakiness among individual mice (42). In this study, we screened *scid* spleens for the presence of μ mRNA; a small percentage of mice had low, but detectable, levels of μ mRNA and are denoted $scid(\mu^+)$. The μ mRNA detected was predominantly of the secreted type and κ mRNA was detected in proportion to the level of μ mRNA (data not shown). These observations suggest that the μ mRNA found in $scid(\mu^+)$ spleens was produced by plasma cells and was not due to the presence of nonactivated B cells or pre-B cells, which can sometimes be recovered from *scid* spleens by transformation with A-MuLV (43). This conclusion is consistent with the lack of detectable B220⁺ cells in the spleens of $scid(Ig^+)$ mice (42).

The analysis of V_H gene families in $scid(\mu^+)$ spleens revealed that multiple V_H gene families were expressed in individual spleen samples, suggesting that multiple clones of B cells were present. However, the pattern of V_H expression in these $scid(\mu^+)$ spleens was strikingly different from that observed in their normal counterparts. The higher expression of J_H -proximal V_H gene families in $scid(\mu^+)$ adult spleens relative to normal adult spleens is similar to that observed in livers and spleen of normal neonatal mice. We suggest that the biased V_H gene expression in the adult spleens of leaky *scid* mice reflects the position-dependent rearrangements occurring in pre-B cells of bone marrow. Potentially, a lack of immunoregulation due to the deficiency of normal numbers of B and T lymphocytes in *scid* mice may result in an unselected repertoire that becomes activated in the spleen.

Together our findings strongly indicate that the position-dependent V_H repertoire

generated in differentiating B cells reflects constraints imposed by the Ig gene assembly process and that this process is unchanged during development. Regulatory and selection mechanisms acting subsequent to the rearrangement events appear to be the major pathway leading to randomization of the V_H repertoire to adult levels.

Summary

We have previously demonstrated a dramatic preference for utilization of the most J_H-proximal V_H gene segments in the newborn liver versus adult spleen. We now examine in detail the relative expression of different V_H gene families throughout ontogeny and in immunodeficient mice to gain insight into factors that cause the shift in V_H usage. We find that the relative expression of V_H gene families remains constant and biased throughout fetal and neonatal liver development. In addition, the primary V_H repertoire expressed in neonatal spleen displays a similarly biased, position-dependent V_H repertoire. The pattern of V_H gene expression begins to change at 5–7 d postnatally and reaches the adult randomized pattern at ~2 wk of age. We also find biased expression of J_H-proximal V_H gene families in adult bone marrow and in spleens of adult leaky scid mice, suggesting that the spontaneously generated repertoire of adult mice is similar to that observed in neonates. Together, these data suggest that a position-dependent repertoire is generated in differentiating pre-B cells at all stages of ontogeny, at least in part, as a result of preferential rearrangement of proximal V_H gene segments. Therefore, mechanisms subsequent to V gene rearrangement, such as regulatory interactions and antigen selection, must play a major role in normalizing the repertoire.

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