DEVELOPMENTALLY REGULATED AND STRAIN-SPECIFIC EXPRESSION OF MURINE V_H GENE FAMILIES

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The genes that encode the antigen-binding portions (variable regions) of mammalian Ig heavy (H) and light (L) chains are assembled from multiple germline DNA elements (1). Precursor B (pre-B) lymphocytes in "primary" B cell differentiation organs (the fetal liver and adult marrow) assemble and express first H and subsequently L chain genes in an ordered process that culminates in the generation of primary B-lymphocytes that express complete Ig molecules on their surface (2). Primary B lymphocytes then migrate to "peripheral" lymphoid organs, such as the spleen and lymph nodes, where they mature into Ig-secreting cells (plasma cells) after interaction with cognate antigens or nonspecific activators (3). Different populations of B-lineage cells may express distinct sets of variable regions (variable region "repertoires") (4). Newly generated B cells express a primary repertoire; this primary repertoire may reflect constraints of the Ig gene assembly process, and presumably has not yet been perturbed by external selective forces. The repertoire of peripheral B-lineage cells, on the other hand, may be molded by positive or negative selective forces (4).

The H chain variable region gene (V_HDJ_H) is assembled from three germline DNA elements denoted V_H for variable, D for diversity, and J_H for joining (reviewed in Reference 1). 12 D segments lie within the 80 kb immediately upstream of the J_H cluster and 100–1000 or more V_H segments lie upstream of the D and J_H clusters; in BALB/c mice the most proximal V_H segments are found within 200 kb of the D locus (Morrow, M., and F. Alt, manuscript in preparation). Murine V_H segments have been divided into nine families based on amino acid or nucleotide sequence homology (5–8), with the size of these families varying from a few (eg. Reference 9) to as many as hundreds of members (10, 11; see Fig. 1 *A*). Individual members of a V_H family are usually grouped together on the chromosome (5, 12); the relative positions of the V_H families were initially determined by deletion and recombinant inbred strain analyses (5, 12; see Fig. 1 *A*). Modifications of this order, including some interspersion of the V_H families, have been suggested (13, 14, 15).

The assembly of V_H, D and J_H segments follows an ordered two-step process in

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which V_H segments are joined to pre-existing DJ_H complexes (2). Analyses of Abelson murine leukemia virus (A-MuLV)[1]-transformed pre-B lines, which actively performed V_H to DJ_H joining in culture, provided an opportunity to examine V_H utilization in the absence of in vivo antigenic or immunoregulatory forces (16). Such lines from BALB/c mice (or mice with a similar V_H organization) (B. Malynn et al., manuscript in preparation) preferentially utilized V_H segments from their most J_H-proximal family (V_H7183); in particular, the most J_H-proximal segment (V_H81X) was used at very high frequency (16). Significantly, an A-MuLV transformant derived from a mouse strain that had a different J_H-proximal V_H family (V_HQ52) than BALB/c mice used V_H segments from that family most frequently (13). Similar conclusions regarding position-dependent V_H gene utilization were reached from studies of fetal liver hybridomas and A-MuLV-transformed pre-B lines that had formed V_HDJ_H rearrangements at the time of isolation (16, 17). Together, these observations suggested that the chromosomal position of V_H segments is a major determinant of their rearrangement frequency, resulting in expression of a "non-random" repertoire in pre-B cells, which is biased towards utilization of J_H-proximal V_H segments (16, 17).

The adult spleen consists of a major population of resting B cells and a minor population of plasma cells and other activated cells (3). Recent studies examined V_H utilization patterns in hybridomas (18) or B cell colonies (19, 20) derived from bacterial LPS-activated spleen cells of adult BALB/c or C57BL/6 mice. Two of these analyses revealed "random" utilization of V_H families; thus, family representation occurred approximately in proportion to family size and displayed no bias towards J_H-proximal families (18, 20). One report used these findings to support a model that suggests that the rearrangement process stochastically utilizes all V_H segments (20). An alternative interpretation consistent with the finding of biased V_H utilization in permanent pre-B lines is that an initially biased repertoire generated in differentiating pre-B cells is normalized during ontogeny or in the transition from primary to peripheral tissues (16, 17). However, one of the colony assays did not find significant differences between V_H utilization in the fetal liver and adult spleen (19). To elucidate the primary V_H repertoire in vivo and how it relates to the V_H repertoire of peripheral B cell populations, we devised a simple assay that provides an instantaneous representation of V_H family usage in primary and peripheral lymphoid tissues. This assay lacks complex manipulations out of the animal and thus minimizes the risk of in vitro artifacts. We have used the assay for comparative analyses of V_H utilization patterns in neonatal liver and in unstimulated or polyclonally-activated spleen cells of adult BALB/c and C57BL/6 mice. These studies provide support for a model in which the rearrangement process generates a position-dependent repertoire in early development which is modified in a strain-specific manner in peripheral B-lineage cells.

Materials and Methods

Cells and Tissues. Derivation, growth, and characteristics (including description of the expressed V_H gene segment) of the pre-B cell lines used in the following studies have previ-

[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; μ_m, membrane-bound form of μ; μ_s, secreted form of μ.

ously been described (16, 21), as have the reference cell lines 22D6-G2 (16), A23-10 (22), UN42-10 (22), MRL5-51 (23), and MOPC104E (24, 25). BALB/cByJ (BALB/c) and C57BL/6J (C57BL/6) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. The adult spleen and newborn liver RNA samples described were prepared from pools of these tissues in all cases. At least two litters were pooled for each newborn liver sample presented. In all cases, four spleens, obtained from 4–8-wk-old mice, were pooled for each adult spleen sample presented. For LPS treatment, spleen cells were plated at 10^6 cells/ml (in RPMI 1640 with 10% FCS and 50-μmol 2-ME) and grown in the presence of 10–40 μg/ml of *Salmonella typhimurium* LPS (Difco Laboratories Inc., Detroit, MI) for 4–5 d.

Preparation of RNA and Northern Blotting. Preparation of total and poly(A)$^+$ RNA from cells and tissues was performed as previously described (25). Northern blotting, probe preparation, and hybridization procedures have previously been described (25).

Preparation of V_H and Cμ Probes. V_H-specific probes were prepared from genomic DNA clones and cDNA clones described in Fig. 1 *B*; detailed characterization and restriction mapping analysis of the DNA fragments used as the V_H probes are available to interested readers upon request. The Cμ probe was prepared from a cDNA clone described in Reference 26.

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

Results

Expression Assay to Determine V_H Gene Utilization Frequency: Theoretical Considerations. To examine the development of the expressed V_H repertoire in vivo, we determined either the relative or absolute contribution of a given V_H gene family to the total steady state level of μ H chain mRNA produced in various murine lymphoid tissues at different stages of development. The V_HDJ_H complex is assembled just upstream from the first constant region gene (Cμ) expressed during B cell differentiation; appropriate transcription and RNA processing mechanisms result in production of μ mRNAs that encode both variable and constant portions of the H chain (1). Alternative splicing of primary V_HDJ_H-Cμ-containing transcripts results in μ mRNAs of 2.7 and 2.4 kb that encode membrane-bound (μ_m) and secreted (μ_s) forms of the protein, respectively (27–29; see Fig. 2). Pre-B cells usually produce higher levels of μ_m mRNA than μ_s mRNA, cells of the mature B cell stage express similar levels of both, and cells of the plasma cell stage (Ig secreting) produce predominantly μ_s mRNA (30). In addition, Ig-secreting plasma cells express as much as 1,000-fold greater levels of μ mRNA as their pre-B or B cell precursors (31). However, the absolute level of μ mRNA expression appears relatively consistent among cells of the same differentiation stage and usually does not depend on the particular V_H segment utilized in the expressed V_HDJ_H gene (30, unpublished observations; see below). Thus, in a population of B-lineage cells at the same differentiation stage but which contain heterogeneous rearrangements, the relative level of μ mRNA transcripts containing a particular V_H segment should roughly reflect the proportion of the expressed V_HDJ_H joins within that population which utilize that particular V_H segment (expressed V_HDJ_H joins can include both "productive" and "nonproductive" V_HDJ_H rearrangements; see legend to Fig. 2).

To assay for developmentally specific patterns of V_H segment expression, we derived probes specific for six separate V_H gene families and a probe from the V_H81X segment (Fig. 1 *B*). The probe for a given V_H family identified all or most of the members of that V_H family but not members of other V_H families under the hybridization conditions we used (not shown). Multiple independent samples of total or poly(A)$^+$ RNA from various sources was assayed by standard electropho-

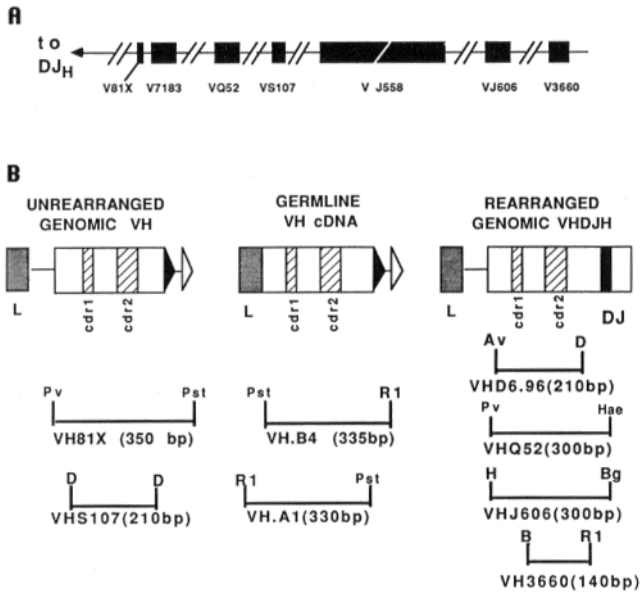

FIGURE 1. (*A*) Relative location and size of the V_H families, adapted from Reference 5; the V_H3660 and V_HJ606 families were not mapped relative to each other, although both families were mapped J_H-distal to the V_HJ558 family (5). Relative size of the V_H families is reflected by length of box representing the V_H family; the break in the box representing the V_HJ558 family reflects the controversy concerning its size (reviewed in Reference 40). The most J_H-proximal V_H segment, V_H81X, is indicated (16). Recent modifications of this original V_H locus map have been reported and described in the text (14, 15). (*B*) Derivation of V_H probes. DNA fragments used as probes were derived from unrearranged genomic V_H segments, germline V_H cDNA clones, and genomic V_HDJ_H rearrangements, as indicated. The fragments used as probes did not contain any D, J_H, or Cμ hybridizing regions. V_H81X (which is relatively more specific for the most J_H-proximal V_H segment but also hybridizes to other closely related members of the V_H7183 family; see Reference 16) and V_HD6.96 probes (representing V_H7183 family) were derived from clones described in Reference 16. The V_HQ52 probe was derived from the VQbDJ$_H$3 rearrangement described in Reference 13. The V_H.B4 and V_H.A1 probes (representing V_HJ558 family) were described in Reference 25. The V_HS107 probe was derived from the V_H107 clone described in Reference 55, the V_HJ606 probe from a genomic clone containing rearranged heavy chain variable region gene of the HGAC9 hybridoma (56), and the V_H3660 probe from a clone (K. Kruger, G. Yancopoulos, and F. Alt, unpublished data) representing the V_H81Y rearrangement partly characterized in Reference 30; the sequence of this V_H3660 segment is 98% homologous to previously published V_H3660 genes (57). The portions of the variable region genes that encode the leader (*L*), complementarity determining regions (*cdr1* and *cdr2*), heptameric (*closed triangle*) and nonameric (*open triangle*) recombination recognition sequences, and D and J_H regions are indicated. Restriction sites: *Av* = Ava2; *B* = BamH1; *Bg* = Bg12; *D* = DdeI; *Pst* = PstI; *Pv* = PvuII; *R1* = EcoR1; *Hae* = Hae3.

resis/Northern blotting procedures for hybridization to the V_H probes and to a Cμ probe; hybridization signal intensities were determined by densitometric analyses. The total amount of μ mRNA among the different samples was standardized by relative hybridization intensity to the Cμ probe. Although pre-B cells produce detectable levels of several Cμ-hybridizing transcripts that do not contain V_H sequences, these transcripts can be distinguished from μ mRNA species on the basis of size and are usually expressed at lower steady state levels (30, 32–34). Thus, the presence of such "Cμ-transcripts" did not significantly affect our estimates of the amount of μ mRNA in a given sample. Comparison of the relative hybridization intensities of a standardized quantity of μ mRNA when assayed with the different V_H probes was used to estimate the relative contribution of the given V_H gene families to the total μ mRNA in the various samples tested. Utilization of RNA samples from reference cell lines, in which 100% of the μ mRNA utilized V_H segments from a single V_H family, allowed estimation of the absolute contribution of each V_H gene family to the μ mRNA in a given tissue sample (see below).

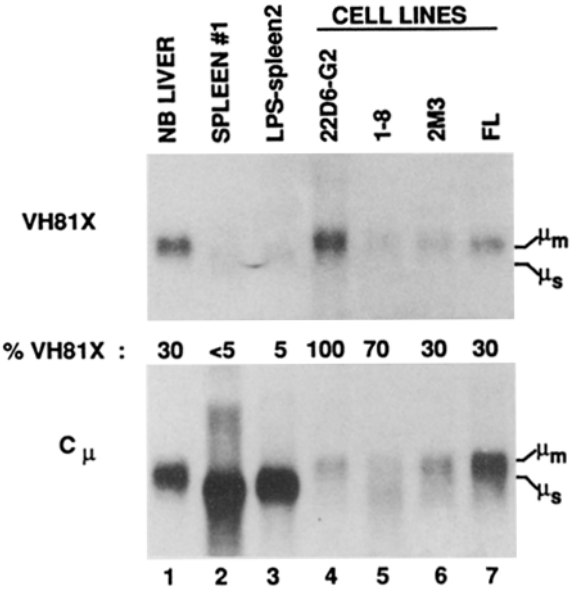

FIGURE 2. Preferential V_H7183 usage early in BALB/c development. 5 μg of poly(A)$^+$ RNA prepared from pooled BALB/c newborn (*NB*) livers, 3 μg of total RNA prepared from pooled BALB/c adult spleens, 0.5 μg of total RNA prepared from LPS-stimulated BALB/c splenocytes, and 0.5 μg of poly(A)$^+$ RNA from each of the pre-B cell lines (lanes *4–8*) were fractionated on agarose-formaldehyde gels and transferred to nitrocellulose filters; duplicate filters were probed with either the V_H81X probe or the Cμ probe. The 22D6-G2 line is described in the text. The pre-B cell lines *1-8, 2M3,* and *FL* have previously been shown to contain a single V_H81X-DJ_H rearrangement (16). Signal intensities for the appropriate bands were determined by densitometry, and used to determine the V_H81X:Cμ ratio for each of the samples; these ratios are expressed as percentages of the ratio in the line *22D6-G2*, which was set at 100% because all of the μ mRNA in this line should hybridize to the V_H81X probe (see text).

As described in the text, the percentage of μ mRNA (expressed by a population of B-lineage cells in the same differentiation stage but with heterogeneous rearrangements) comprised by a given V_H family should roughly reflect the proportion of the expressed V_HDJ_H joins within that population that utilize that particular V_H family. In this regard, murine pre-B cells generally express relatively equal levels of productive and non-productive V_HDJ_H rearrangements (i.e., those that can or cannot encode μ protein), while productive V_HDJ_H rearrangements dominate expression in plasma cells (reviewed in Reference 2). Therefore, V_H expression in pre-B cells should reflect V_H utilization in all rearrangements, whereas V_H expression in both unprimed and LPS-stimulated spleen samples should reflect V_H utilization only on productive rearrangements.

Membrane (μ_m) and secreted (μ_s) forms of μ mRNA are indicated.

Preferential V_H7183 Utilization Occurs Early in Normal Development. To accurately examine utilization of V_H81X and other members of the most J_H-proximal V_H family (V_H7183) at various developmental stages in the BALB/c mouse, we compared the relative intensity with which a V_H81X probe hybridized to a standardized amount of μ mRNA derived from various sources. These sources included the neonatal liver, adult spleen, and pre-B cell lines that had rearrangements that utilized V_H81X or a closely related V_H7183 segment on one or both alleles. The 22D6-G2 line has V_HDJ_H rearrangements on both alleles; one utilizes a V_H81X segment and the other a closely related V_H7183 segment (16). Therefore, all of the μ mRNA sequences in 22D6-G2 should hybridize to the V_H81X probe; thus, the ratio of V_H81X to Cμ hybridization for μ RNA from this reference cell line was set at 100% (Fig. 2, lane *4*). Correspondingly, several pre-B lines that utilize a V_H7183 gene on only one of their two J_H rearrangements had V_H81X to Cμ hybridization ratio of ~30–70% that of 22D6-G2 (Fig. 2, lanes *5–7*), as expected for roughly equal expression of each rearranged chromosome at the RNA level in pre-B cells.

As in the pre-B cell lines, mostly μ_m mRNA is detected in samples from neonatal liver (Fig. 2). Thus, as noted previously (35), pre-B and B cells contribute most of

the μ mRNA expressed in the neonatal liver. Strikingly, μ mRNA isolated from BALB/c neonatal liver has a V_H81X to Cμ hybridization ratio that is ~30% of the ratio in the 22D6-G2 line (Fig. 2, lane *1*); this ratio is comparable to the ratio observed in cell lines with V_H81X rearrangements on one of their two alleles. This result demonstrates that a high proportion of the μ mRNA produced in BALB/c neonatal liver contains V_H81X or a closely related gene segment; by extension, a major proportion of the V_HDJ_H rearrangements in the immature B cells of this primary differentiation organ have utilized these gene segments.

Most of the μ mRNA detected in unprimed spleen cells was of the μ_s form that predominates in plasma cells (Fig. 2, lane *2*), indicating that the bulk of the μ mRNA expressed in the unprimed adult spleen is apparently contributed by the high level expression from the relatively small population of activated plasma cells normally located there. Significantly, the ratio of V_H81X to Cμ hybridization in μ mRNA from unstimulated BALB/c adult spleen is <5% that observed for 22D6-G2 μ mRNA (Fig. 2, lane *2*). These results demonstrate that only a small fraction of the expressed V_H repertoire in the unprimed adult spleen is comprised of V_H7183 segments. The minor population of plasma cells that dominate μ mRNA expression in the unprimed spleen probably arose via activation by endogenous and incidental antigens; because they presumably represent an antigen-selected population, their V_H repertoire might not reflect that of the resting splenic B lymphocytes that comprise the major B-lineage population in the unprimed spleen. LPS can polyclonally activate a large fraction of the B cells in the spleen, regardless of antigen specificity, to divide and differentiate into Ig-secreting cells (36). V_H7183 expression in the μ_s mRNA of the LPS-stimulated spleen should therefore reflect V_H7183 usage in the expressed V_HDJ_H joins of the major B-lineage cell population in the adult spleen. Notably, the ratio of V_H81X to Cμ hybridization in μ mRNA prepared from LPS-stimulated BALB/c splenocytes is only slightly higher than the ratio observed in μ mRNA from the unstimulated BALB/c spleen (Fig. 2, lanes *2* and *3*). Thus, in BALB/c mice, the V_H7183 family comprises a major portion of the V_H repertoire expressed in the immature B lineage cells of the neonatal liver but only a small proportion of the V_H repertoire expressed in the peripheral B-lineage cells of the adult spleen.

Position-dependent V_H Utilization across the Entire V_H Locus. To further define developmentally regulated differences in V_H utilization during early B cell development, we compared the relative levels with which the various V_H family probes hybridized with μ mRNA sequences derived from neonatal liver and unprimed or LPS-stimulated adult spleens of BALB/c and C57BL/6 mice (Fig. 3). These analyses demonstrated that μ mRNA transcripts containing V_H segments from each of the V_H families were detectable in the neonatal liver, but revealed significant differences in the relative representation of different V_H families in the neonate compared with the adult spleen samples. For example, although all of the samples analyzed were standardized to contain approximately equal amounts of μ mRNA (Fig. 3, *top panel*), the V_H81X probe hybridized with much greater intensity to neonatal liver μ mRNA than to adult spleen μ mRNA, while the V_H558 probe hybridized with greater intensity to adult spleen μ mRNA than to neonatal liver μ mRNA (Fig. 3; note that the lower V_H-hybridizing band seen in some spleen samples corresponds to γ_s-mRNA and was not considered in the following calculations involving V_H utilization in μ mRNA).

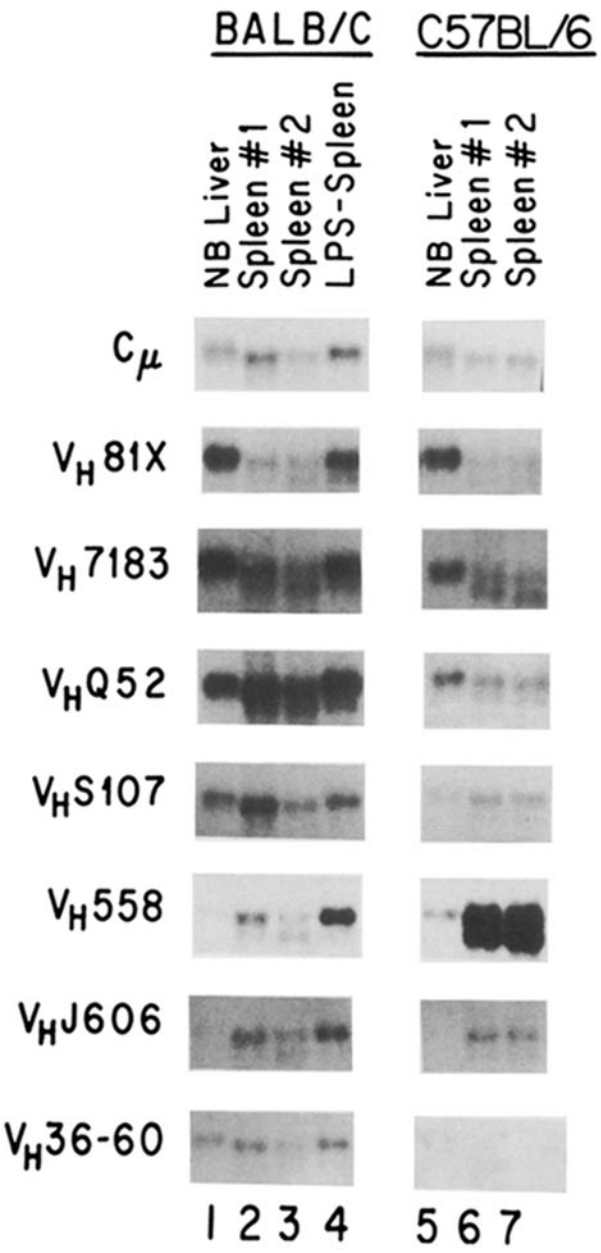

FIGURE 3. Position-dependent V_H utilization across the entire V_H locus occurs in the neonate. RNA was prepared from pooled samples (see Materials and Methods) of newborn (*NB*) livers, adult spleens, or LPS-stimulated spleen cells isolated from BALB/c or C57BL/6 mice, as indicated. The RNA was standardized for μ mRNA levels, and RNA amounts were assayed for each sample that contained approximately equal levels of μ mRNA; this amounted to ~5.0 μg of poly(A)$^+$ RNA from the newborn liver samples, 2.5 μg of total RNA from the spleen samples, and 1.0 μg of total RNA from the LPS-stimulated spleen sample. Duplicate RNA blots, prepared as described in Fig. 2, were probed with the Cμ probe and the series of V_H-specific probes as indicated.

To facilitate comparison of V_H utilization between the neonatal liver and adult spleen, a simple formula was devised that assigns a preference value (derived by dividing relative utilization in neonatal liver by relative utilization in adult spleen; see legend to Table I) for the expression of each V_H family; a preference value of 1 indicates that a V_H family comprises an equivalent proportion of the μ mRNA in neonatal liver and adult spleen samples, a value >1 indicates that the family is

TABLE I
V_H FAMILY PREFERENCE VALUES

	Preference values in BALB/c			Values in C57BL/6	
	NB liver #1/ spleen #1 and 2	NB liver #2/ spleen #2	NB liver #1/ LPS-spleen #1	NB liver #1/ spleen #1 and 2	NB liver #2/ spleen #1
V_H7183 (VH81X)	4–10	5–6	3–4	4–8	5–10
V_H7183 (VHD6.96)	2–3	ND	1–2	2	ND
V_HQ52	0.6–0.7	0.9	0.6–0.9	1–2	1–2
V_HS107	0.6–0.7	0.5	1	0.4–0.6	0.7–0.9
V_H J558 (A.1)	0.3–0.5	ND	0.2–0.3	0.2–0.3	ND
V_H J558 (B.4)	0.2–0.4	0.5	0.2	0.1–0.2	0.1–0.3
V_H J606	0.1–0.2	0.3	0.2	0.1–0.2	0.2–0.3
V_H3660	0.6–0.9	ND	0.8	ND	2.0–2.5

Duplicate Northern blots containing RNA from the various tissue-derived sources were probed with the Cμ probe and the series of V_H-family probes, as described for Fig. 3 (if we have described multiple probes for a given family, the actual probe used is indicated in parenthesis). Signal intensities for the appropriate bands were determined by densitometry and used to obtain the preference values listed in this table; preference values were calculated according to the following equation: Preference (V_H) = $[I_{NB}(V_H)/I_S(V_H)]/[I_{NB}(C\mu)/I_S(C\mu)]$; where I_{NB} and I_S denote the intensities of hybridization of the given VH probe and the Cμ probe to the indicated newborn liver or adult spleen samples (with or without LPS stimulation, as indicated). The range of values given for each newborn liver to spleen comparison encompasses all the values obtained after densitometric analysis of multiple autoradiographic exposures of at least two independent Northern blotting experiments for each comparison (except for the values in the last column, which derive from multiple exposures of a single experiment); numbers are rounded off to a single significant digit. Preference values are further described in the text.

relatively over-represented in neonatal liver compared to adult spleen, and a value <1 indicates relative under-representation in the neonate. Strikingly, the preference value for each V_H family (with the possible exception of the V_H3660 family, see below) was roughly related to its reported J_H-proximity (Table I; Fig. 4 *D*); the highest preference value was obtained with the V_H81X probe, which is particularly specific for the most J_H-proximal V_H segment. The position dependence of preference values was evident whether the newborn repertoire was compared with unprimed or LPS-stimulated adult splenocytes (Fig. 3, Table I). Preference values were similar for both murine strains analyzed (Table I; Fig. 4 *D*).

Strain-specific Differences in V_H Utilization in the Adult Spleen. To examine absolute utilization of the different V_H families in peripheral B-lineage cells, RNA from the spleens of unprimed 4–8-wk-old BALB/c or C57BL/6 mice, from LPS-stimulated BALB/c spleen cells, and from the reference cell lines was assayed for hybridization to the Cμ and V_H probes as described above. Roughly equal levels of μ mRNA from each source (e.g., examine panels probed with Cμ probe in Fig. 5) were compared for hybridization with each of the V_H family probes (Fig. 5); a sixfold lower amount of RNA from each of the reference cell lines (in lanes denoted by * in Fig. 5) was also examined to allow easy comparison of the relatively high level expression of a single V_H family in these samples with the lower levels generally found in spleen samples. The assays were repeated on multiple replicate samples; all gave consistent

Figure 4

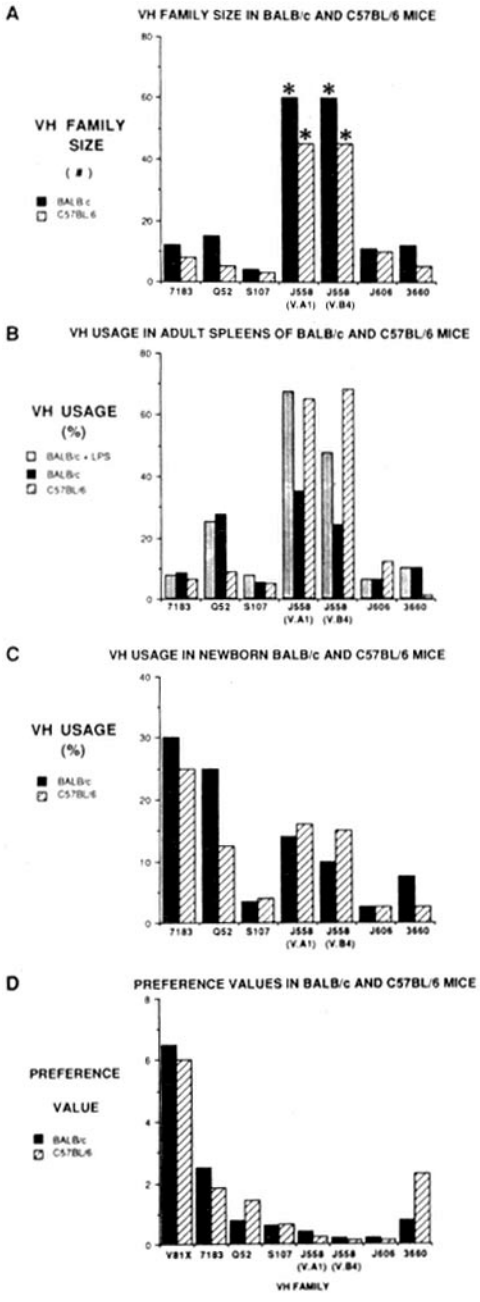

FIGURE 4. (*A*) Estimated V_H family size in BALB/c and C57BL/6 mice, as adapted from Reference 15. V_HJ558 family size is presented twice, for comparison with the usage data obtained with the two different V_HJ558 probes; asterisks denote controversy concerning the size of the V_HJ558 family (reviewed in Reference 40), and the minimum V_HJ558 family size estimates from Reference 15 are provided. Recent evidence indicates that the V_HJ558 family is larger in BALB/c than in C57BL/6 (39). (*B*) V_H utilization in the adult spleens of BALB/c and C57BL/6 mice; usage in LPS-stimulated BALB/c splenocytes and unprimed BALB/c and C57BL/6 spleens is indicated. (*C*) V_H utilization in the newborn livers of BALB/c and C57BL/6 mice. (*D*) Preference values derived by comparing V_H usage in the newborn livers with that in unprimed adult spleens (see text). The values used in *B–D* are derived from the values presented in Tables I and II; in cases where ranges and/or multiple samples were provided in the tables, the midpoint of these values is used in *B–D*. In *D*, preference values are reported separately for V_H81X and the other V_H7183 probe (as reported in Table I); V_H7183 data presented in *B* and *C* were derived using the V_H81X probe (as reported in Table II). Because the absolute level of V_H3660 usage could not be determined (see Fig. 4), its level in LPS-stimulated spleen in panel B was set at 10% for comparison purposes; V_H3660 usage levels in other samples were varied accordingly in *B* and *C*.

results (Table II). Typical results are shown in Fig. 5 and calculated utilization values are compared in Fig. 4 *B*.

We find that V_H family utilization in the unprimed adult spleen roughly correlates with family size and not with chromosomal position (Fig. 4, *A* and *B*). Examination of the utilization of the V_H81X, V_HS107, and V_HJ606 families among the var-

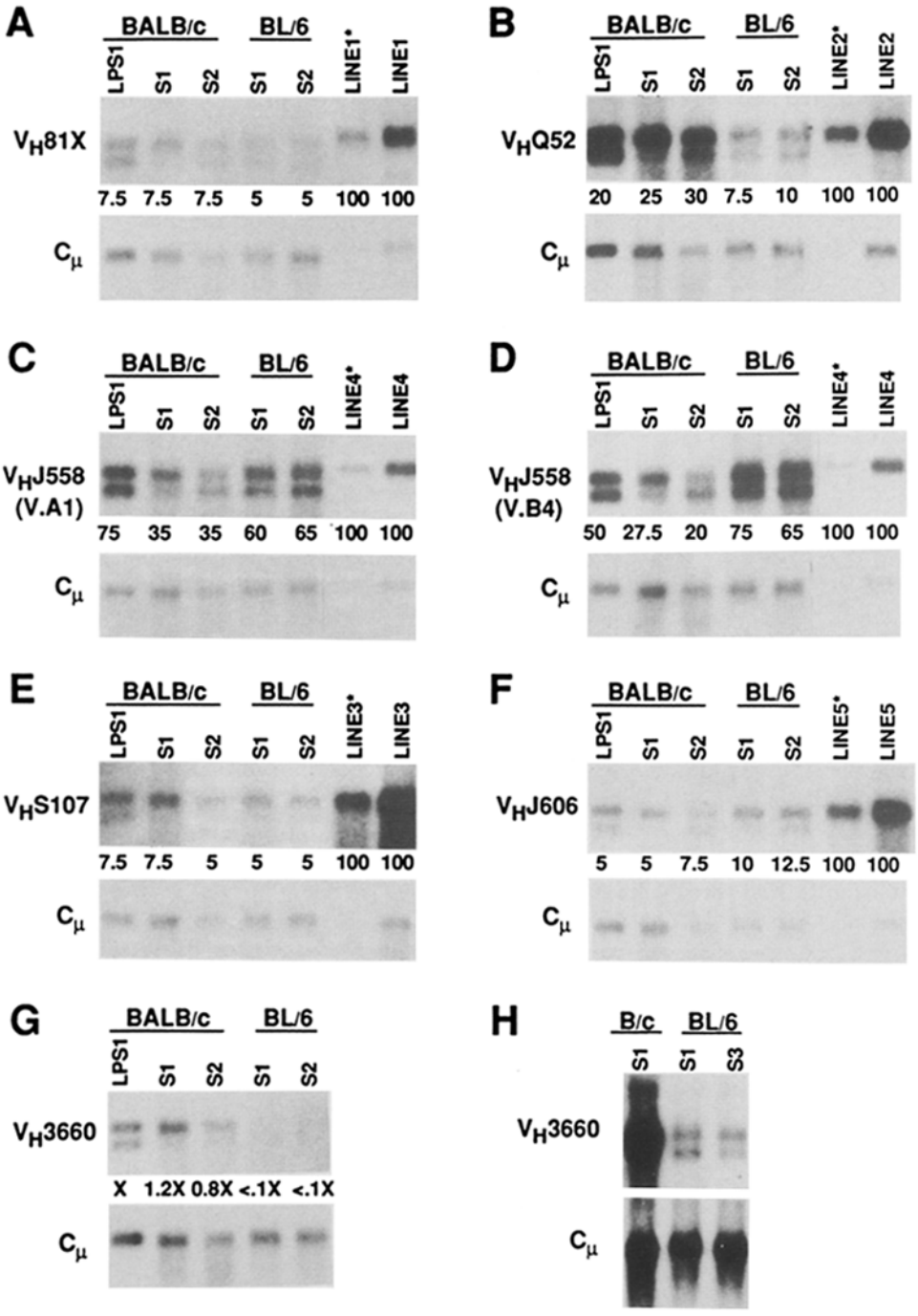

FIGURE 5. Utilization of the V_H gene families in the adult spleens of BALB/c and C57BL/6 mice. RNA was prepared from a series of reference cell lines (*LINE1* through *LINE5*) or from independent pools of unprimed adult spleens (S1, S2, or S3) or LPS-stimulated adult spleens (*LPS1*) isolated from BALB/c or C57BL/6 mice, as indicated. RNA amounts were standardized to contain approximately equal levels of μ mRNA from all analyzed samples; a sixfold lower amount of RNA from the reference cell lines (lanes marked by asterisk) was also examined for reasons described in the text. Duplicate Northern blots, prepared as described in Figs. 2 and

ious spleen samples and appropriate reference lines indicated that the V_H families represented by these probes comprised roughly similar proportions of the splenic μ mRNA in both BALB/c and C57BL/6 mice; these V_H families were utilized in only a small fraction (<5–10% each) of the μ mRNA in the spleen samples (Fig. 5, *A, E,* and *F*; Table II; Fig. 4 *B*). In contrast, the relative hybridization of the V_HQ52 and V_HJ558 probes demonstrated striking (and compensatory) strain-specific utilization differences, with the V_HQ52 family displaying higher utilization in BALB/c spleens and the V_HJ558 family displaying higher utilization in C57BL/6 spleens (Fig. 5 *B, C,* and *D*; Table II; Fig. 4 *B*). V_HJ558 expression was examined with two different V_HJ558 probes: the V.A1 and V.B4 probes identify overlapping subsets of the very large V_HJ558 family, with the V.B4 probe more specific for the V_H segments utilized in response to the NP hapten by C57BL/6 mice but not BALB/c mice (25). Notably, the strain-specific differences in V_HJ558 expression are more pronounced with the V.B4 probe (Fig. 5, *C* and *D*; Table II; Figure 4 *B*); thus V_HJ558 segments closely related to the V.B4 segment are preferentially utilized in spleens from unprimed C57BL/6 mice. Although we did not have a reference line to allow estimation of the absolute utilization of the V_H3660 family in the tissue samples, it is clear that V_H segments detected by the V_H3660 probe are utilized at a much higher relative level in BALB/c spleens than in C57BL/6 spleens (Fig. 5 *G*; Table II; Fig. 4 *B*); in fact, we could only detect V_H3660 expression in the adult spleens of C57BL/6 mice when we analyzed much higher levels of μ mRNA from these samples (Fig. 5 *H*).

Both samples of LPS-stimulated BALB/c splenocytes utilized most V_H families at approximately the same levels as they were utilized in unprimed BALB/c spleens (Fig. 5; Table II; Fig. 4 *B*; but see discussion). These results are, in general, consistent with previous studies of B cell colonies (20) or hybridomas (18), which indicated that V_H utilization in LPS-stimulated splenocytes correlated with family size. Thus, sufficiently widespread activation of splenic lymphocytes to the plasma cell stage occurs in unprimed mice to allow the actively expressed V_H repertoire in the spleens of such mice to reflect the extensively and presumably randomly activated V_H repertoire resulting from LPS treatment.

Discussion

Comparison Between Our Assay and B Cell Colony Assays. We have defined strain-specific variations in the utilization of certain V_H families in unprimed adult spleens. Furthermore, we have found striking differences in the relative expression of different V_H families in neonatal liver vs. adult spleen within a given strain (Fig. 3, Table I). Analyses of V_H expression in B cell colonies derived from adult spleen or fetal liver yielded certain results that apparently are contradictory to these. In particular,

3, were probed with the Cμ probe and a V_H probe as indicated. Signal intensities for the appropriate bands (in the autoradiographic exposures depicted) were determined by densitometry, and used to determine the V_H:Cμ ratio for each sample in *A–F*; these ratios are expressed as percentages of the ratios in the reference cell lines, which were set at 100% as described for Fig. 2 and in the text. No reference cell line was available for the V_H3660 family; thus, the V_H3660:Cμ ratio in LPS-stimulated spleen was arbitrarily set at *X*, and ratios in other samples are reported relative to *X*. Tenfold higher amounts of mRNA (i.e., 25 μg of total RNA), from the indicated samples, were assayed in *H. LINE1* = 22D6-G2; *LINE2* = MRL5-51; *LINE3* = A23-10; *LINE4* = MOPC104E; *LINE5* = UN42-10.

TABLE II

Absolute Utilization (%) of V_H families in BALB/c and C57BL/6 Mice

	V_H usage in BALB/c mice						V_H usage in C57BL/6 mice			
	Newborn liver #1	Newborn liver #2	Adult spleen #1	Adult spleen #2	LPS-spleen #1	LPS-spleen #2	Newborn liver #1	Newborn liver #2	Adult spleen #1	Adult spleen #2
V_H7183 (VH81X)	25–35	25–35	2.5–7.5	2.5–10	5–7.5	5–10	20–30	20–30	5–7.5	5
V_HQ52	15–20	22.5–30	20–30	25–35	20–30	25–30	7.5–15	10–15	7.5	10
V_HS107	2.5–5	2.5–5	5–7.5	5	2.5–5	7.5–10	2.5	2.5–5	5	5
V_H J558 (A.1)	10–20	ND	32.5–37.5	30–35	50–70	65–80	12.5–20	ND	55–65	60–70
V_H J558 (B.4)	5–10	10–15	25–30	20–25	30–55	45–55	5–15	10–20	70–80	55–65
V_H J606	0–2.5	2.5	5	5–7.5	2.5–10	5–7.5	0–2.5	2.5	7.5–10	12.5–15
V_H3660	0.5–1X	ND	1.2X	0.8X	ND	1X	ND	0.1–0.3X	<0.1X	<0.1X

Values represent the percent of μ mRNA, in each of the indicated tissue-derived samples, which is comprised by the V_H families identified by the listed V_H probes (if we have described multiple probes for a given family, the actual probe used is indicated in parentheses). Values were obtained as described for Figs. 2 and 5 in the text; absolute utilization levels in the spleen samples were directly obtained by comparison to the reference cell lines (as illustrated in Fig. 5) whereas absolute utilization levels in the newborn liver samples were obtained by multiplying the absolute utilization levels for the adult spleen by the preference values for that family. The range for each sample encompasses all the values obtained after densitometric analysis of multiple autoradiographic exposures of at least two, and usually four or more, independent Northern blotting experiments using each sample. As described in the text, the absolute utilization of the V_H3660 family could not be determined because no relevant reference cell line was available; thus the V_H3660 level in LPS-stimulated spleen was arbitrarily set at "X", and all other values for V_H3660 are given relative to this value.

one study found no strain-specific differences in V_H utilization in splenic colonies (20); the other found strain-specific differences in splenic colonies somewhat similar to those we report, but detected no differences in V_H utilization between colonies isolated from fetal liver and adult spleen (19). Differences between the results of the two colony assays and our assay must reflect inherent differences in the assays. We have examined the instantaneous representation of V_H families within a minimally manipulated and heterogeneous cell population (often within an unmanipulated tissue sample); the differences we define reflect those of the predominant cell population responsible for H chain expression within a given sample. Conceivably, B cell colony assays, in which only a small percentage of the input cells give rise to an assayable colony, select for a sub-population of "clonable" cells with the V_H usage patterns described. Further elucidation of the factors that lead to the different results among the different assays may yield information relevant to normal repertoire development.

Position-dependent V_H Rearrangement. Multiplication of absolute utilization values for the various V_H families in adult spleen by the neonatal liver/adult spleen preference value for that family allowed estimation of absolute V_H utilization levels in the neonate (Table II; Fig. 4 *C*). These absolute expression levels of particular V_H gene families in newborn liver do not correlate with either family-size or chromosomal position; however, the absolute expression of different V_H gene families in the adult spleen is clearly correlated with family size (Fig. 4, *A–C*). On the other hand, preference values (the ratio of the relative expression of a given V_H gene family in newborn liver compared with adult spleen) are directly related to chromosomal position (Fig. 4 *D*). Based on our analyses of V_H to DJ_H rearrangements and their subsequent expression in pre-B cell lines (16), it seems likely that V_H family expression levels in newborn liver should reflect V_H family rearrangement frequency. A direct relationship between preference number and J_H-proximity of a given V_H family would result if the absolute rearrangement frequency of a family (the sum of the individual rearrangement frequencies of all family members) depended both on proximity to the J_H locus and on the total family size. Thus, the observed position-dependence of the preference numbers would result from dividing the frequency with which a given V_H family is rearranged (a product of J_H proximity and size: the proposed basis for representation in newborn liver) by its total number of members (size: the basis for representation in adult spleen). In this regard, preference numbers should reflect the absolute probability of rearranging an individual member of a given V_H family. For example, the probability of rearranging an individual member of the V_HJ558 family would be low due to its J_H-distal position (resulting in a low preference number; Fig. 4 *D*); however, the absolute utilization of this family in the newborn liver is relatively high due to the large number of members (Fig. 4 *C*).

The position-dependent preference of V_H family utilization in neonatal liver confirms that the preferential V_H gene rearrangement frequency observed in permanent pre-B cell lines reflects processes operative in normal pre-B cells. Such position-dependent rearrangement makes it unlikely that segments are joined exclusively by mechanisms that rely on collisions during three-dimensional diffusion (dissociative joining), supporting the idea that the recombination machinery works by a one-dimensional "tracking" mechanism during V_H to DJ_H joining (associative joining) (16, 37). In this regard, only the V_H3660 family displays a preference number significantly higher than would have been expected from its originally reported J_H-

distal location (5); V_H3660 segments are also preferentially utilized in certain neonatal, but not adult, immune responses (38). Although specific mechanisms may act to increase the frequency of cells expressing V_H3660 rearrangements early in development, recent findings suggest that at least some members of the V_H3660 family are located more J_H-proximally than previously thought in both the C57BL/6 and BALB/c strains (14, 15), in a position more compatible with the V_H3660 preference value.

Preference values do not clearly distinguish between each of the V_H families, in particular, those of the more J_H-distal V_HJ558 and V_HJ606 families. Several factors could complicate the simple relations that we have proposed to explain relative V_H utilization. For example, predominate dissociative, as opposed to associative, rearrangement of J_H-distal V_H families could increase their overall utilization and minimize position-dependent utilization differences among these families; such cooperation may have evolved to ensure rearrangement of V_H segments across the entire V_H locus. Interspersion of V_H family members would also negate family position-dependent differences in preference values (13–15). Finally, factors that modify the size-dependence of V_H utilization in adult spleen (see below) would also affect the position-dependence of preference numbers.

V_H Expression in the Adult Spleen. The naturally activated V_H repertoire expressed in the unprimed adult spleen has not been measured by any other method. We find reproducible strain-specific differences in this repertoire between BALB/c and C57BL/6 mice. The V_HQ52 and V_H3660 families represent a higher percentage of splenic expression in BALB/c relative to C57BL/6, while the V_HJ558 family (and to a lesser extent the V_HJ606 family) represents a relatively higher percentage in C57BL/6 (Fig. 4 *B*). Although the strain-specific differences in V_HQ52 and V_H3660 expression correlate with strain-specific variations in the size of these families, V_HJ558 utilization differences do not clearly correlate with variations in the size of this family in the two strains (Fig. 4, *A* and *B*). Such strain-specific differences could result from mechanisms encoded by loci outside of the V_H locus, which select for or against expression of particular V_H segments. However, such differences also could readily be explained with respect to the relative content and organization of the V_H locus in the two strains. Although V_H558 family size (i.e., number of V_H segments detected by hybridization to family-specific probes) may be larger in BALB/c than in C57BL/6, this does not necessarily reflect the relationship between V_HJ558 family "complexity" (i.e., number of V_H segments in a family available to encode distinct functional H chains) in the two strains. For example, the BALB/c strain may have undergone a recent duplication in its V_H558 locus, resulting in a large number of identical V_HJ558 segments (39); BALB/c may also lack a number of functional V_HJ558 segments found in the C57BL/6 strain (26). In the same context, the occurence of large numbers of non-functional V_H gene segments (pseudogenes) could also obscure the relationship between family size and family complexity. If selective mechanisms which result in repertoire randomization depended on functional family size (complexity) and not actual family size, the rough correlation between family size and expression in the adult spleens could reflect a precise correlation between family complexity and expression in the two strains examined. In support of this possibility, strain-specific differences in V_HJ558 expression were less obvious when expression was compared between either LPS-stimulated BALB/c splenocytes and total C57BL/6

spleen or between neonatal liver samples from the two strains (Fig. 5, *B* and *C*); LPS-stimulated splenocytes and neonatal liver presumably represent B-lineage cells, which, as a population, have undergone less selection than the naturally-activated cells responsible for V_H expression in the unprimed adult spleen.

Functional Significance of V_H Rearrangement Patterns. Randomization (to be more representative of family size) of the position-dependent V_H repertoire must occur at a point between the B cell developmental stages represented in newborn liver and adult spleen. Several mechanisms (reviewed in Reference 40), not mutually exclusive, could be involved in the normalization process. Programmed changes in recombinational mechanisms involved in V_HDJ_H assembly could generate distinct primary repertoires at different developmental stages (41). Such programmed changes would be consistent with, and could provide a basis for, the programmed appearance of particular antibody specificities during early development (38, 42–49); however, we have noted expression of all V_H families in the newborn, including those with members that are used to encode late responses. In addition, evidence from adult marrow-derived pre-B cell lines indicates that the adult primary repertoire may remain position-dependent (13, 16), while other evidence suggests that the V_H segments over-represented in early development may also be over-represented in nonproductive (and, therefore, non-selected) rearrangements in adult B lineage cells (50). Thus, cellular selection mechanisms operating subsequent to the rearrangement process could play a major role in normalization of primary repertoires that remain relatively constant throughout development; such selection mechanisms could be related to those resulting in strain-specific V_H expression (reviewed in Reference 40).

Autoreactive antibodies, anti-idiotypic antibodies that are expressed early in development, and anomalous antibodies produced in transgenic mice may all preferentially utilize J_H-proximal V_H segments (23, 51–53). These findings may reflect important binding specificities uniquely encoded by J_H-proximal segments; preferential rearrangement of these V_H segments early in murine development may have evolved to ensure their appropriate expression. In this regard, related V_H segments also may be utilized preferentially in early human development (54). Alternatively, preferential V_H rearrangement may merely reflect a necessary by-product of the V_HDJ_H assembly mechanism that must be overcome by normalization processes to result in a maximally diverse peripheral repertoire. In the latter case, the association of J_H-proximal V_H segments with autoreactive or other anomolous responses may be related to a breakdown in the selection forces that result in normalization, simply reflecting the incidentally frequent expression of these segments in the primary B cells or particular B cell subsets that generate these antibodies.

Summary

We have devised a simple assay that provides an instantaneous representation of V_H family usage in primary and peripheral lymphoid tissues. This assay lacks complex manipulations out of the animal and thus minimizes the risk of in vitro artifacts. We have used this assay to demonstrate a dramatic preference for utilization of the most J_H-proximal V_H segments in the newborn liver of BALB/c and C57BL/6 mice. Furthermore, we find that V_H segments from across the entire V_H locus are utilized early in development, but at frequencies directly related to their J_H proximity. A major shift away from the position-dependent V_H repertoire of the neo-

nate is seen in unprimed or polyclonally-activated adult spleen cells, in which relative utilization of the various V_H families is related to family size. We also report consistent strain-specific differences in the expression of certain V_H families.

Our data indicate that a position-dependent V_H repertoire is generated in differentiating pre-B lymphocytes (probably reflecting constraints imposed by the immunoglobulin gene assembly process), and that mechanisms that operate subsequent to rearrangement then randomize this position-dependent repertoire in a strain-specific manner.

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