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_{H}D_{JH}) 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
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-

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\(^1\) Abbreviations used in this paper: A-MuLV, Abelson murine leukemia virus; \( \mu_m \), membrane-bound form of \( \mu \); \( \mu_s \), secreted form of \( \mu \).
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 C\_\mu Probes.** V\_H-specific probes were prepared from genomic DNA clones and cDNA clones described in Fig. 1B; detailed characterization and restriction mapping analysis of the DNA fragments used as the V\_H probes are available to interested readers upon request. The C\_\mu 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 \( \mu H \) chain mRNA produced in various murine lymphoid tissues at different stages of development. The V\_H DJ\_\mu complex is assembled just upstream from the first constant region gene (C\_\mu) expressed during B cell differentiation; appropriate transcription and RNA processing mechanisms result in production of \( \mu \) mRNAs that encode both variable and constant portions of the H chain (1). Alternative splicing of primary V\_H DJ\_\mu-C\_\mu-containing transcripts results in \( \mu \) mRNAs of 2.7 and 2.4 kb that encode membrane-bound (\( \mu m \)) and secreted (\( \mu s \)) forms of the protein, respectively (27–29; see Fig. 2). Pre-B cells usually produce higher levels of \( \mu m \) mRNA than \( \mu s \) mRNA, cells of the mature B cell stage express similar levels of both, and cells of the plasma cell stage (Ig secreting) produce predominantly \( \mu s \) mRNA (30). In addition, Ig-secreting plasma cells express as much as 1,000-fold greater levels of \( \mu \) mRNA as their pre-B or B cell precursors (31). However, the absolute level of \( \mu \) 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\_H DJ\_\mu 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 \( \mu \) mRNA transcripts containing a particular V\_H segment should roughly reflect the proportion of the expressed V\_H DJ\_\mu joins within that population which utilize that particular V\_H segment (expressed V\_H DJ\_\mu joins can include both “productive” and “non-productive” V\_H DJ\_\mu 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\_H 81X segment (Fig. 1B). 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-
EXPRESSION OF THE MURINE V\_\(\mu\) GENE FAMILIES

FIGURE 1. (A) Relative location and size of the V\(\mu\) families, adapted from Reference 5; the V\(\mu\)J606 and V\(\mu\)J606 families were not mapped relative to each other, although both families were mapped J\(\mu\)-distal to the V\(\mu\)J558 family (5). Relative size of the V\(\mu\) families is reflected by length of box representing the V\(\mu\) family; the break in the box representing the V\(\mu\)J558 family reflects the controversy concerning its size (reviewed in Reference 40). The most J\(\mu\)-proximal V\(\mu\) segment, V\(\mu\)J1X, is indicated (16). Recent modifications of this original V\(\mu\) locus map have been reported and described in the text (14, 15). (B) Derivation of V\(\mu\) probes. DNA fragments used as probes were derived from unrearranged genomic V\(\mu\) segments, germline V\(\mu\) cDNA clones, and genomic V\(\mu\)DJ\(\mu\) rearrangements, as indicated. The fragments used as probes did not contain any D, J\(\mu\), or C\(\mu\) hybridizing regions. V\(\mu\)J6X (which is relatively more specific for the most J\(\mu\)-proximal V\(\mu\) segment but also hybridizes to other closely related members of the V\(\mu\)183 family; see Reference 16) and V\(\mu\)D6.96 probes (representing V\(\mu\)J783 family) were derived from clones described in Reference 16. The V\(\mu\)Q52 probe was derived from the VQbDJ\(\mu\)3 rearrangement described in Reference 13. The V\(\mu\)B4 and V\(\mu\)A1 probes (representing V\(\mu\)J558 family) were described in Reference 25. The V\(\mu\)J107 probe was derived from the V\(\mu\)107 clone described in Reference 55, the V\(\mu\)J606 probe from a genomic clone containing rearranged heavy chain variable region gene of the HGAC9 hybridoma (56), and the V\(\mu\)J606 probe from a clone (K. Kruger, G. Yancopoulos, and F. Alt, unpublished data) representing the V\(\mu\)J606 rearrangement partly characterized in Reference 30; the sequence of this V\(\mu\)J606 segment is 98% homologous to previously published V\(\mu\)J606 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\(\mu\) regions are indicated. Restriction sites: A\(v\) = AvaI; B = BamHI; Bg = BglII; D = Ddel; Pst = PstI; Pvu = PvuII; R1 = EcoRI; Hae = HaeIII.

resis/Northern blotting procedures for hybridization to the V\(\mu\) probes and to a C\(\mu\) probe; hybridization signal intensities were determined by densitometric analyses. The total amount of \(\mu\) mRNA among the different samples was standardized by relative hybridization intensity to the C\(\mu\) probe. Although pre-B cells produce detectable levels of several C\(\mu\)-hybridizing transcripts that do not contain V\(\mu\) sequences, these transcripts can be distinguished from \(\mu\) mRNA species on the basis of size and are usually expressed at lower steady state levels (30, 32–34). Thus, the presence of such “C\(\mu\)-transcripts” did not significantly affect our estimates of the amount of \(\mu\) mRNA in a given sample. Comparison of the relative hybridization intensities of a standardized quantity of \(\mu\) mRNA when assayed with the different V\(\mu\) probes was used to estimate the relative contribution of the given V\(\mu\) gene families to the total \(\mu\) mRNA in the various samples tested. Utilization of RNA samples from reference cell lines, in which 100\% of the \(\mu\) mRNA utilized V\(\mu\) segments from a single V\(\mu\) family, allowed estimation of the absolute contribution of each V\(\mu\) gene family to the \(\mu\) mRNA in a given tissue sample (see below).
Preferential V\textsubscript{n}7183 Utilization Occurs Early in Normal Development. To accurately examine utilization of V\textsubscript{n}81X and other members of the most J\textsubscript{n}-proximal V\textsubscript{n} family (V\textsubscript{n}7183) at various developmental stages in the BALB/c mouse, we compared the relative intensity with which a V\textsubscript{n}81X probe hybridized to a standardized amount of \( \mu \) mRNA derived from various sources. These sources included the neonatal liver, adult spleen, and pre-B cell lines that had rearrangements that utilized V\textsubscript{n}81X or a closely related V\textsubscript{n}7183 segment on one or both alleles. The 22D6-G2 line has V\textsubscript{n}DJ\textsubscript{n} rearrangements on both alleles; one utilizes a V\textsubscript{n}81X segment and the other a closely related V\textsubscript{n}7183 segment (16). Therefore, all of the \( \mu \) mRNA sequences in 22D6-G2 should hybridize to the V\textsubscript{n}81X probe; thus, the ratio of V\textsubscript{n}81X to C\textsubscript{\mu} hybridization for \( \mu \) RNA from this reference cell line was set at 100\% (Fig. 2, lane 4). Correspondingly, several pre-B lines that utilize a V\textsubscript{n}7183 gene on only one of their two J\textsubscript{n} rearrangements had V\textsubscript{n}81X to C\textsubscript{\mu} hybridization ratio of \( \sim 30-70\% \) 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 \( \mu \)\textsubscript{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<sub>μ</sub>81X to C<sub>μ</sub> hybridization ratio that is ~30% of the
ratio in the 22D6-G2 line (Fig. 2, lane 1); this ratio is comparable to the ratio ob-
erved in cell lines with V<sub>μ</sub>81X rearrangements on one of their two alleles. This
result demonstrates that a high proportion of the μ mRNA produced in BALB/c neo-
natal liver contains V<sub>μ</sub>81X or a closely related gene segment; by extension, a major
proportion of the V<sub>μ</sub>DJ<sub>μ</sub> rearrangements in the immature B cellsof this primary
differentiation organ have utilized these gene segments.

Most of the μ mRNA detected in unprimed spleen cells was of the μ<sub>s</sub> 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<sub>μ</sub>81X to C<sub>μ</sub> 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<sub>μ</sub>
reertoire in the unprimed adult spleen is comprised of V<sub>μ</sub>7183 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<sub>μ</sub> 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<sub>μ</sub>7183 expression in the μ mRNA of the
LPS-stimulated spleen should therefore reflect V<sub>μ</sub>7183 usage in the expressed V<sub>μ</sub>DJ<sub>μ</sub>
joints of the major B-lineage cell population in the adult spleen. Notably, the ratio
of V<sub>μ</sub>81X to C<sub>μ</sub> 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<sub>μ</sub>7183 family comprises a major portion of the V<sub>μ</sub> repertoire expressed in the
immature B lineage cells of the neonatal liver but only a small proportion of the V<sub>μ</sub>
repertoire expressed in the peripheral B lineage cells of the adult spleen.

Position-dependent V<sub>μ</sub> Utilization across the Entire V<sub>μ</sub> Locus. To further define develop-
mentally regulated differences in V<sub>μ</sub> utilization during early B cell development,
we compared the relative levels with which the various V<sub>μ</sub> family probes hybridized
with μ mRNA sequences derived from neonatal liver and unprimed or LPS-stim-
ulated adult spleens of BALB/c and C57BL/6 mice (Fig. 3). These analyses demon-
strated that μ mRNA transcripts containing V<sub>μ</sub> segments from each of the V<sub>μ</sub>
families were detectable in the neonatal liver, but revealed significant differences in the
relative representation of different V<sub>μ</sub> families in the neonate compared with the
adult spleen samples. For example, although all of the samples analyzed were stan-
dardized to contain approximately equal amounts of μ mRNA (Fig. 3, top panel),
the V<sub>μ</sub>81X probe hybridized with much greater intensity to neonatal liver μ mRNA
than to adult spleen μ mRNA, while the V<sub>μ</sub>558 probe hybridized with greater in-
tensity to adult spleen μ mRNA than to neonatal liver μ mRNA (Fig. 3; note that
the lower V<sub>μ</sub>-hybridizing band seen in some spleen samples corresponds to γ-
mRNA and was not considered in the following calculations involving V<sub>μ</sub> utiliza-
tion in μ mRNA).
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 $\mu$ mRNA in neonatal liver and adult spleen samples, a value >1 indicates that the family is
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 \( V_n \) family (with the possible exception of the \( V_n3660 \) family, see below) was roughly related to its reported \( J_n \)-proximity (Table I; Fig. 4D); the highest preference value was obtained with the \( V_n81X \) probe, which is particularly specific for the most \( J_n \)-proximal \( V_n \) 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. 4D).

**Strain-specific Differences in \( V_n \) Utilization in the Adult Spleen.** To examine absolute utilization of the different \( V_n \) 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 \( C_H \) and \( V_n \) probes as described above. Roughly equal levels of \( \mu \) mRNA from each source (e.g., examine panels probed with \( C_H \) probe in Fig. 5) were compared for hybridization with each of the \( V_n \) 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 \( V_n \) 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. (A) Estimated V_{\mu} family size in BALB/c and C57BL/6 mice, as adapted from Reference 15. V_{\mu}J558 family size is presented twice, for comparison with the usage data obtained with the two different V_{\mu}J558 probes; asterisks denote controversy concerning the size of the V_{\mu}J558 family (reviewed in Reference 40), and the minimum V_{\mu}J558 family size estimates from Reference 15 are provided. Recent evidence indicates that the V_{\mu}J558 family is larger in BALB/c than in C57BL/6 (39). (B) V_{\mu} 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_{\mu} utilization in the newborn livers of BALB/c and C57BL/6 mice. (D) Preference values derived by comparing V_{\mu} 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_{\mu}S1X and the other V_{\mu}J606 probe (as reported in Table I); V_{\mu}J606 data presented in B and C were derived using the V_{\mu}S1X probe (as reported in Table II). Because the absolute level of V_{\mu}S660 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_{\mu}S660 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_{\mu} 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_{\mu}S1X, V_{\mu}S107, and V_{\mu}J606 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 $\mu$ 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 \( V_n \) families represented by these probes comprised roughly similar proportions of the splenic \( \mu \) mRNA in both BALB/c and C57BL/6 mice; these \( V_n \) families were utilized in only a small fraction (<5-10% each) of the mRNA in the spleen samples (Fig. 5, A, E, and F; Table II; Fig. 4 B). In contrast, the relative hybridization of the \( V_nQ52 \) and \( V_nJ558 \) probes demonstrated striking (and compensatory) strain-specific utilization differences, with the \( V_nQ52 \) family displaying higher utilization in BALB/c spleens and the \( V_nJ558 \) family displaying higher utilization in C57BL/6 spleens (Fig. 5 B, C, and D; Table II; Fig. 4 B). \( V_nJ558 \) expression was examined with two different \( V_nJ558 \) probes: the \( VA1 \) and \( VB4 \) probes identify overlapping subsets of the very large \( V_nJ558 \) family, with the \( VB4 \) probe more specific for the \( V_n \) segments utilized in response to the NP hapten by C57BL/6 mice but not BALB/c mice (25). Notably, the strain-specific differences in \( V_nJ558 \) expression are more pronounced with the \( VB4 \) probe (Fig. 5, C and D; Table II; Figure 4 B); thus \( V_nJ558 \) segments closely related to the \( VB4 \) 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 \( V_n3660 \) family in the tissue samples, it is clear that \( V_n \) segments detected by the \( V_n3660 \) 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 \( V_n3660 \) expression in the adult spleens of C57BL/6 mice when we analyzed much higher levels of \( \mu \) mRNA from these samples (Fig. 5 H).

Both samples of LPS-stimulated BALB/c splenocytes utilized most \( V_n \) 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 \( V_n \) 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 \( V_n \) repertoire in the spleens of such mice to reflect the extensively and presumably randomly activated \( V_n \) 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 \( V_n \) families in unprimed adult spleens. Furthermore, we have found striking differences in the relative expression of different \( V_n \) families in neonatal liver vs. adult spleen within a given strain (Fig. 3, Table I). Analyses of \( V_n \) expression in B cell colonies derived from adult spleen or fetal liver yielded certain results that apparently are contradictory to these. In particular,
|          | V_{n} usage in BALB/c mice |          | V_{n} usage in C57BL/6 mice |
|----------|----------------------------|----------|-----------------------------|
|          | Newborn liver #1 | Newborn liver #2 | Adult spleen #1 | Adult spleen #2 | LPS-spleen #1 |  | Newborn liver #1 | Newborn liver #2 | Adult spleen #1 | Adult spleen #2 |
| V_{n} 7183 | 25-35 | 25-35 | 2.5-7.5 | 2.5-10 | 5-7.5 | 5-10 |  | 20-30 | 20-30 | 5-7.5 | 5 |
| (VHB1X)  |  |  |  |  |  |  |  |  |  |  |  |
| V_{n} 052 | 13-20 | 22.5-30 | 20-30 | 25-35 | 20-30 | 25-30 |  | 7.5-15 | 10-15 | 7.5 | 10 |
| V_{n} S107 | 2.5-5 | 2.5-5 | 5-7.5 | 5 | 2.5-5 | 7.5-10 |  | 2.5 | 2.5-5 | 5 | 5 |
| V_{n} 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_{n} J558 (B.4) | 5-10 | 10-15 | 25-30 | 20-25 | 30-55 | 45-55 |  | 5-15 | 10-20 | 70-80 | 55-65 |
| V_{n} 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_{n} 3660 | 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_{n} families identified by the listed V_{n} 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_{n}3660 family could not be determined because no relevant reference cell line was available; thus the V_{n}3660 level in LPS-stimulated spleen was arbitrarily set at "X", and all other values for V_{n}3660 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 \( V_H\)J558 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 \( V_H\)3660 family displays a preference number significantly higher than would have been expected from its originally reported J\( _H \)-
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distal location (5); V<sub>H</sub>3660 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<sub>H</sub>3660 rearrangements early in development, recent findings suggest that at least some members of the V<sub>H</sub>3660 family are located more J<sub>H</sub>-proximally than previously thought in both the C57BL/6 and BALB/c strains (14, 15), in a position more compatible with the V<sub>H</sub>3660 preference value.

Preference values do not clearly distinguish between each of the V<sub>H</sub> families, in particular, those of the more J<sub>H</sub>-distal V<sub>H</sub>J558 and V<sub>H</sub>J606 families. Several factors could complicate the simple relations that we have proposed to explain relative V<sub>H</sub> utilization. For example, predominate dissociative, as opposed to associative, rearrangement of J<sub>H</sub>-distal V<sub>H</sub> 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<sub>H</sub> segments across the entire V<sub>H</sub> locus. Interspersion of V<sub>H</sub> family members would also negate family position-dependent differences in preference values (13-15). Finally, factors that modify the size-dependence of V<sub>H</sub> utilization in adult spleen (see below) would also affect the position-dependence of preference numbers.

V<sub>H</sub> Expression in the Adult Spleen. The naturally activated V<sub>H</sub> 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<sub>H</sub>Q52 and V<sub>H</sub>3660 families represent a higher percentage of splenic expression in BALB/c relative to C57BL/6, while the V<sub>H</sub>J558 family (and to a lesser extent the V<sub>H</sub>J606 family) represents a relatively higher percentage in C57BL/6 (Fig. 4 B). Although the strain-specific differences in V<sub>H</sub>Q52 and V<sub>H</sub>3660 expression correlate with strain-specific variations in the size of these families, V<sub>H</sub>J558 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<sub>H</sub> locus, which select for or against expression of particular V<sub>H</sub> segments. However, such differences also could readily be explained with respect to the relative content and organization of the V<sub>H</sub> locus in the two strains. Although V<sub>H</sub>558 family size (i.e., number of V<sub>H</sub> 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<sub>H</sub>J558 family “complexity” (i.e., number of V<sub>H</sub> 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<sub>H</sub>558 locus, resulting in a large number of identical V<sub>H</sub>J558 segments (39); BALB/c may also lack a number of functional V<sub>H</sub>J558 segments found in the C57BL/6 strain (26). In the same context, the occurrence of large numbers of non-functional V<sub>H</sub> 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<sub>H</sub>J558 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<sub>H</sub> expression in the unprimed adult spleen.

**Functional Significance of V<sub>H</sub> Rearrangement Patterns.** Randomization (to be more representative of family size) of the position-dependent V<sub>H</sub> 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<sub>H</sub>D<sub>Ja</sub> 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<sub>H</sub> 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<sub>H</sub> segments over-represented in early development may also be over-represented in non-productive (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<sub>H</sub> 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<sub>a</sub>-proximal V<sub>H</sub> segments (23, 51–53). These findings may reflect important binding specificities uniquely encoded by J<sub>a</sub>-proximal segments; preferential rearrangement of these V<sub>H</sub> segments early in murine development may have evolved to ensure their appropriate expression. In this regard, related V<sub>H</sub> segments also may be utilized preferentially in early human development (54). Alternatively, preferential V<sub>H</sub> rearrangement may merely reflect a necessary by-product of the V<sub>H</sub>D<sub>Ja</sub> 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<sub>a</sub>-proximal V<sub>H</sub> segments with autoreactive or other anomalous 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<sub>H</sub> 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<sub>a</sub>-proximal V<sub>H</sub> segments in the newborn liver of BALB/c and C57BL/6 mice. Furthermore, we find that V<sub>H</sub> segments from across the entire V<sub>H</sub> locus are utilized early in development, but at frequencies directly related to their J<sub>a</sub> proximity. A major shift away from the position-dependent V<sub>H</sub> repertoire of the neo-
nate is seen in unprimed or polyclonally-activated adult spleen cells, in which relative utilization of the various \( V_n \) families is related to family size. We also report consistent strain-specific differences in the expression of certain \( V_n \) families.

Our data indicate that a position-dependent \( V_n \) 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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