

# Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life

(lymphocyte development/antibody genes/molecular evolution)

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**ABSTRACT** The ability to respond to specific antigens develops in a programmed fashion. Although the antibody repertoire in adults is presumably generated by stochastic combinatorial joining of rearranged heavy variable, diversity, and joining ( $V_H$ - $D_H$ - $J_H$ ) and light ( $V_L$ - $J_L$ ) chains, experimental evidence in the mouse has shown nonrandom utilization of variable gene segments during ontogeny and in response to specific antigens. In this study, we have performed sequence analysis of 104-day human fetal liver-derived, randomly isolated constant region  $C_{\mu}^+$  transcripts and demonstrate a consistent preference during fetal life for a small subset of three highly conserved  $V_H3$  family gene segments. In addition, the data show that this preferential gene segment utilization extends to the  $D_HQ52$  and the  $J_H3$  and  $J_H4$  loci. Sequence analysis of two "sterile"  $D_H$ - $J_H$  transcripts suggests that transcriptional activation of the  $J_H$ -proximal  $D_HQ52$  element may precede initiation of  $D_H$ - $J_H$  rearrangement and influence fetal  $D_H$  utilization. Sequence comparisons reveal striking nucleotide polymorphism in allelic gene segments which is poorly reflected in the peptide sequence, implying considerable evolutionary selection pressure. Although vertebrate species utilize a variety of strategies to generate their antibody repertoire, preferential utilization of  $V_H3$  elements is consistently found during early development. These data support the hypothesis that  $V_H3$  gene segments play an essential role in the development of the immune response.

Immunoglobulins are generated by combinatorial joining of rearranged gene segments of the heavy chain variable, diversity, and joining regions ( $V_H$ ,  $D_H$ , and  $J_H$ ) and light chain regions ( $V_L$  and  $J_L$ ) (1). Starting with less than 1000 of these germ-line elements, more than  $10^9$  different antigen binding sites can be generated even in the absence of either junctional diversity or somatic mutation. In mice, only a small fraction of this potential repertoire seems to be expressed as functional antibody (2). Utilization of this repertoire appears to be developmentally regulated in a strain-specific fashion (3–5) with subsequent modification by environmental stimuli (7).

The human neonate is relatively immunodeficient at birth (8). Controlled mobilization of germ-line variable gene segments has been postulated to underlie, in part, the maturation of humoral immunity (5, 9). With these observations in mind, we have concentrated on the use of molecular cloning strategies to dissect the development of the human heavy chain repertoire during fetal life. Because the extent of  $V_H$  region polymorphism in the outbred human population is undefined, we have chosen to analyze individual fetal samples.

Fetal B lymphopoiesis begins in the liver, with pre-B cells first detectable by 8 weeks of gestation (10). We isolated fetal liver mononuclear cells, which are rich in B-cell precursors, and generated oligo(dT)-primed cDNA libraries. We previ-

ously reported evidence of preferential usage of  $V_H$ ,  $D_H$ , and  $J_H$  gene segments by cloning and sequencing 15  $J_H$ -containing constant region  $C_{\mu}^+$  heavy chain transcripts from a 130-day-gestation cDNA library (11). The 14  $V_H$ -containing cDNAs represented only 9 germ-line gene segments, 5 of which belonged to the  $V_H3$  family. Preference was shown for 1 ( $D_HQ52$ ) of more than 20 germ-line  $D_H$  (12, 13) and 2 ( $J_H3$  and  $J_H4$ ) of 6 functional germ-line  $J_H$  elements (14). In this communication, we extend our analysis of heavy chain transcripts present in the 130-day library and also analyze a 104-day library (9) for comparison with our previous results and to determine the extent of restriction during an earlier stage in fetal life.<sup>†</sup>

Striking similarities in the heavy-chain repertoires expressed by two unrelated human fetuses of 4–5 months gestation indicate the existence of a conserved B-cell developmental program of heavy chain variable element expression.

## MATERIALS AND METHODS

Human fetal liver samples were obtained from a karyotypically normal anencephalic abortus at 130 days of gestation, and from a second 104-day abortus with a neural tube abnormality (both gifts of T. Shepard, University of Washington, Seattle). The isolation of mononuclear cells from these tissue samples, purification of poly(A)<sup>+</sup> RNA, generation of oligo(dT)-primed cDNA libraries, and sequencing of  $C_{\mu}^+$  cDNAs have been previously described (9, 11).

## RESULTS

Nineteen  $C_{\mu}^+$  clones were detected in a total of  $8.5 \times 10^5$  recombinants. One-third of the clones contained identical 5' nonvariable sequences with numerous stop codons in all three reading frames. The presence of these "sterile" sequences is common in early lymphoid cells (15). In two clones, reverse transcription terminated in the middle of the  $J_H$  gene segment. Another contained an unusual nontranslated sterile sequence which will be reported elsewhere. The unique sequence at the site of  $V_H$ - $D_H$ - $J_H$  joining in each of the remaining 10 clones demonstrates that each transcript was derived from an independent gene rearrangement event.

Eight of the 10  $V_H$ - $D_H$ - $J_H$ <sup>+</sup> clones include complete  $V_H$  coding sequences (Fig. 1). Of the two incomplete clones, one (M44) ends within the second hypervariable region (CDR II), and the other (M61) contains all but the most amino-terminal portion of framework I. In mouse lymphocytes nonfunctional

Abbreviations: V, variable; D, diversity; J, joining; C, constant; H, heavy; L, light; CDR, complementarity-determining region.

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<sup>†</sup>The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M34020 for clone 70p1, M34021 for clone 74p1, M34022 for clone 83p2, M34023 for clone M26, M34024 for clone M43, M34025 for clone M44, M34026 for clone M49, M34027 for clone M60, M34028 for clone M61, M34029 for clone M71, M34030 for clone M72, M34031 for clone M74, and M34032 for clone M85).

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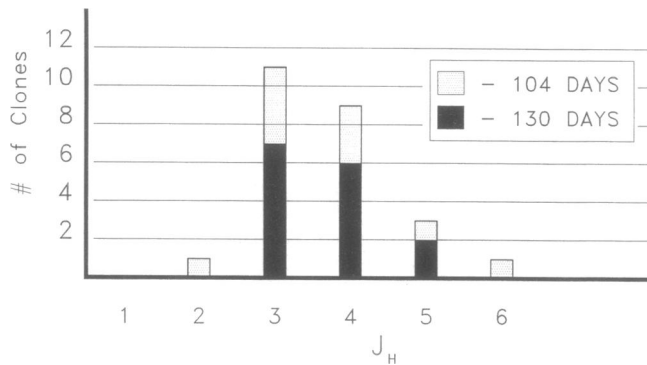


FIG. 3. J<sub>H</sub> utilization in 25 C<sub>μ</sub> clones randomly isolated from 104- and 130-day fetal liver-derived mononuclear cell cDNA libraries. A consistent preference for J<sub>H</sub>3 and J<sub>H</sub>4 was detected.

but in the fetus utilization clearly appears nonrandom.

The third hypervariable region (CDR III) is generated by V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub> joining. Random nucleotide addition (N regions) and junctional flexibility makes assignment of D<sub>H</sub> origin problematic. For example, four clones (M44, M49, M61, and M85) are unassignable (Fig. 4), and transcript M43 shares five bases with D<sub>H</sub>Q52, seven bases with D22/12 (12), and eight bases with DM1 (13). Two CDR III regions appear to contain members of the DN1 family (13): clone M72 shares 14 bases of identity with DN1, and clone (M26) shares 19 bases of interrupted identity. In our 130-day library, 8 of 14 CDR III regions shared between five and nine bases of identity with D<sub>H</sub>Q52 (14). In the current sample of 10 clones, 3 (M60, M71, and M74) share between 8 and 10 base-pair identity with D<sub>H</sub>Q52. The probability that these three transcripts would be drawn from the D<sub>H</sub>Q52 locus at random is <math>3 \times 10^{-5}</math>.

To extend our D<sub>H</sub> locus analysis, we examined 13 C<sub>μ</sub> clones which had been randomly isolated from an additional 3.5 × 10<sup>5</sup> 130-day library recombinants but had not been evaluated (11). Six of the 13 clones did not hybridize to either our sterile specific oligonucleotide [5'-GCCAGACTGT-CATGGCTATCA-3' (11)] or our cocktail of three V<sub>H</sub> complementary oligonucleotides [5'-CGCACAGTAATACACG-GCCGTGTC-3', 5'-ACAGTAATACACGGCTGTGTC-3', and 5'-TGCACAGTAATACACAGCCGTGTC-3' (21)] which identify >95% of known germ-line V<sub>H</sub> gene segments (data not shown). Four of these clones contained further D<sub>H</sub>-J<sub>H</sub> information. The first (70p1) utilized J<sub>H</sub>6 and terminated at the site of D<sub>H</sub>-J<sub>H</sub> joining. The second (83p2) contained a V<sub>H</sub>5 gene segment identical to the V<sub>H</sub>5 element in the 104-day clone M61 (Fig. 1), as well as to the germ-line V<sub>H</sub>5 element 5-1R1 (19). Note that the CDR III region of this V<sub>H</sub>5 transcript shares 8 bases of identity with D<sub>H</sub>Q52 (Fig. 4). The third clone (74p1) was an authentic D<sub>H</sub> transcript joining D<sub>H</sub>Q52 to J<sub>H</sub>2 with an intervening N region sequence (Fig. 5). The fourth clone (84p1) represents a transcript which initiates 5' to the J<sub>H</sub> region; reads through the unrearranged pseudo-J<sub>H</sub>1, D<sub>H</sub>Q52, and J<sub>H</sub>1 gene segments; and then splices appropriately to the 5' terminus of C<sub>μ</sub> (Fig. 5). These transcripts provide further evidence for preferential utilization of D<sub>H</sub>Q52.

DISCUSSION

**Fetal D<sub>H</sub> and J<sub>H</sub> Elements Are Preferentially Rearranged.** Our data indicate a strong preference for D<sub>H</sub>Q52 and J<sub>H</sub>3 and J<sub>H</sub>4 utilization in the second trimester antibody repertoire. Further support for nonrandom D<sub>H</sub>-J<sub>H</sub> rearrangement can be found in analysis of D-J joins in human fetal B-lineage cells at 19 weeks gestation transformed by Epstein-Barr virus (22). A striking gradient of D<sub>H</sub>Q52 rearrangement to J<sub>H</sub>1 > J<sub>H</sub>2, -3, -4 >> J<sub>H</sub>5, 6 is seen. J<sub>H</sub> representation in our two D<sub>H</sub>-J<sub>H</sub> transcripts also reflects D<sub>H</sub>Q52 proximity (Fig. 5).

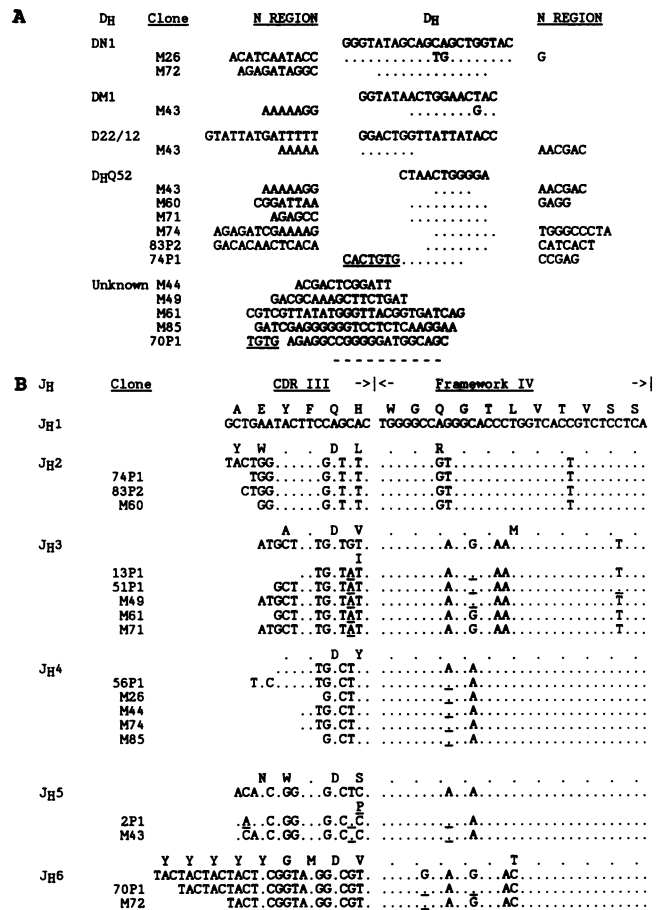


FIG. 4. D<sub>H</sub> and J<sub>H</sub> sequences from human fetal C<sub>μ</sub> cDNA clones. (A) Nucleotide sequences from the D<sub>H</sub>/N region of 13 fetal heavy chain transcripts. Sequences are aligned with their germ-line counterparts where identification is possible: D<sub>H</sub>Q52 (14), DN1 (13), DM1 (13), and D22/12 (12). A dot denotes sequence identity with the given germ-line segment. Clone M43 is shown three times, demonstrating the difficulty of D<sub>H</sub> assignment in a completely rearranged variable element. The heptamer recombination region is underlined in the D<sub>H</sub>-J<sub>H</sub> join 74P1, as well as in the incomplete J<sub>H</sub>-containing transcript 70P1 from the 130-day library (11). (B) Nucleotide sequences from the J<sub>H</sub> region illustrating the rich polymorphism present in the human population. Sequences are grouped by J<sub>H</sub> identity. Dots denote homology to J<sub>H</sub>1 (14). Single base substitutions from previously published sequences (14) are underlined. Clones 13P1, 51P1, 56P1, and 2P1 have been previously reported (11) and are included to illustrate the range of polymorphism seen in this sampling of only five human haplotypes.

The accessibility model propounded by Alt and co-workers (23) posits that a gene can undergo rearrangement only when it is physically accessible to the recombinase. Gene segment accessibility is associated with, and may in fact require, prior transcription of the rearranging element (23). Clone 84p1 (Fig. 5) contains a novel sterile transcript initiating upstream of D<sub>H</sub>Q52, reading through the recombination signals to J<sub>H</sub>1, and then splicing to C<sub>μ</sub>. In keeping with the accessibility model, this transcript may precede initial D<sub>H</sub>-J<sub>H</sub> rearrangement in the pro-pre-B cell and thus could represent the earliest activation event preceding heavy chain rearrangement. Note that few of our transcripts contain J<sub>H</sub>1, whereas V<sub>H</sub>-containing transcripts (e.g., clones 83p2, M74, and M71, Fig. 4) utilize D<sub>H</sub>Q52 in association with J<sub>H</sub>2, J<sub>H</sub>3, and J<sub>H</sub>4, respectively. The relative paucity of J<sub>H</sub>1 and J<sub>H</sub>2 utilization in the expressed V-D-J rearrangements suggests either that additional rounds of D<sub>H</sub>-J<sub>H</sub> rearrangements may precede V<sub>H</sub> splicing or that access to additional D<sub>H</sub> and J<sub>H</sub> gene segments may parallel access to the rest of the V<sub>H</sub> locus.

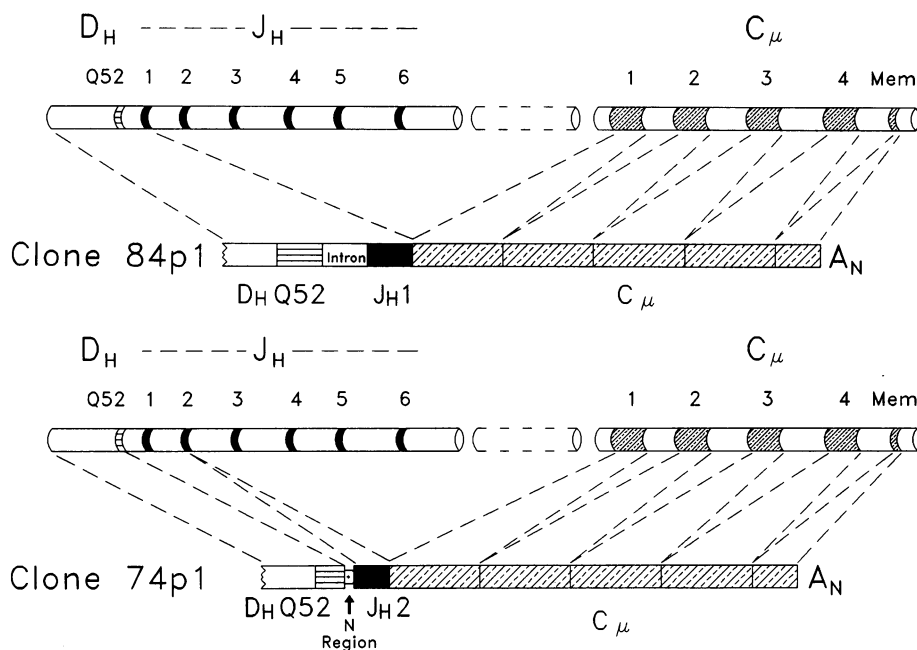


FIG. 5. Schematic map showing the derivation of two D-J transcripts: clones 74p1 and 84p1. Shown are the location of the six functional  $J_H$  gene segments,  $D_H$ Q52, and the  $C_\mu$  exons (14, 31). Mem,  $C_\mu$  membrane exon. Transcription of each  $D_\mu$  cDNA clone begins upstream of  $D_H$ Q52.

Even though use of  $V_H$ ,  $D_H$ , and  $J_H$  elements is selective, junctional flexibility ensures a diverse fetal repertoire. For example,  $D_H$ Q52 is read in all three frameworks and, in concert with N region addition, generates three completely different CDR III regions in M60, M71, and 83P2. This dependence on the third hypervariable region is reminiscent of diversity generation in T-cell receptor rearrangements (24).

**Human Heavy Chain Gene Segments Demonstrate Extensive Nucleotide Polymorphism yet Preserve Peptide Sequence.** The  $J_H$  gene segment codes for the conserved framework IV region (codons 103–113) and can contribute up to nine codons of the highly variable CDR III region (16). Combining the original  $J_H$  region sequence of Ravetch *et al.* (14) with our two samples, five haplotypes have been sampled. Within this limited set of observations, four alleles can be distinguished for  $J_H$ 3, two for  $J_H$ 4, three for  $J_H$ 5, and three for  $J_H$ 6 (Fig. 4). Six of the eight base pair differences are located in framework IV and are silent. Conversely, the three base pair differences in the CDR III region can both result in amino acid substitutions. Note that identification of allelic  $J_H$  segments demonstrates similar heavy-chain preferences in both chromosomes. Similarly, only one of the four nucleotide differences in the two  $V_H$  alleles results in an amino acid change. Hybridization analysis with V-region specific oligonucleotides has also suggested extensive heteromorphism and polymorphism in the human heavy chain locus (25). These data imply that the observed polymorphism may primarily reflect single base pair changes and that the conserved peptide sequences are under significant selection pressure.

**Fetal  $V_H$  Utilization Is Consistently Restricted with Striking Preference for Specific  $V_H$ 3 Elements.** We have identified 24 individual  $V_H$ - $D_H$ - $J_H$  transcripts randomly isolated from two independent cDNA libraries derived from early second-trimester fetal B-lineage cells; 70% of the sequences obtained from the 104-day library are also present in the 130-day set. The human germ-line  $V_H$  repertoire may contain more than 100 segments per haploid genome (19, 20). Hybridization of  $V_H$  probes to 8-week fetal liver-derived mononuclear cell RNA suggests that  $V_H$  utilization is limited to  $V_H$ 5 and  $V_H$ 6 family gene segments (26) when B-lineage development is initiated. Northern analysis of Epstein-Barr virus-transformed cell lines derived from fetal liver and spleen suggested  $V_H$  utilization might be representative of the germ-line repertoire by 19 weeks with 60% of the clones expressing  $V_H$ 3 elements (27). In our sample, however, although 60% of the

fetal cDNAs contain  $V_H$ 3 elements, sequence analysis reveals that these transcripts are drawn from a subset of only 5 individual gene segments out of an estimated total pool of at least 25–30. Note that the  $V_H$ 1 family contains a minimum of 20–25 members:  $V_H$ 2, 5–10;  $V_H$ 4, 6–10;  $V_H$ 5, 2–3; and  $V_H$ 6, 1 member (reviewed in ref. 27). Thus, a small cohort of  $V_H$ 3 elements consistently contributes disproportionately to the fetal antibody repertoire.

The ability to generate antibody responses to specific vaccines follows a predictable course during human infancy. If developmentally controlled restriction of  $V_H$  gene segment utilization regulates, in part, this observed hierarchy of antigen responsiveness, we predict that the expressed antibody repertoire during fetal life would be similar between fetuses and that limitations in the repertoire would continue into early neonatal life. The striking consistency which we have found in these two fetal samples supports the hypothesis that control of variable region utilization contributes to the relative immunodeficiency of the fetus and leaves open the question of whether or not these predictable limitations may extend into neonatal life.

**Preference for  $V_H$ 3-Like Gene Segments Is also Seen in Fetal Mice.**  $V_H$  families arose prior to the mammalian radiation and have since been conserved (28, 29). This conservation appears to reflect selection at the level of protein sequence, and the conserved regions are discretely localized on a solvent-exposed face of the heavy chain, at some distance from the classic antigen-binding site (28). Analysis of hybridomas derived from mouse fetal pre-B cells (5, 30) and Abelson virus-transformed pre-B cell lines have demonstrated a preference for members of the  $V_H$ 7183 family (30), corresponding to less than 10% of the total germ-line  $V_H$  repertoire. It is remarkable that the most highly homologous  $V_H$  gene segments in the mouse and human germ-line repertoires (28, 32) known to us [the  $V_H$ 7183 segment  $V_H$ E415 (30) and the  $V_H$ 3 segment 30p1 (11)] are both favored constituents of the early fetal repertoire.

**$J_H$  Proximity Contributes to Early  $V_H$  Gene Segment Selection but Is Likely Not the Only Factor.** In the mouse, fetal  $V_H \rightarrow D_H J_H$  rearrangements show marked correspondence to the chromosomal order of the gene segments involved (5, 30). In BALB/c, the  $V_H$ 7183 family is positioned at the proximal end of the  $V_H$  locus (30), whereas members of the later-expressed J558 family are more distal. Within the 7183 family, the most  $J_H$  proximal gene segment,  $V_H$ 81X, is preferentially rearranged (30). These data form the basis of

scanning or chromatin potentiation regulatory models postulating initial activation of V<sub>H</sub> gene segments proximal to the D<sub>H</sub>-J<sub>H</sub> locus (5, 30) with subsequent linear access to the more J<sub>H</sub>-distal V<sub>H</sub> elements.

In humans, the two most J<sub>H</sub>-proximal functional V<sub>H</sub> elements are the unique V<sub>H</sub>6 (21, 33) and a V<sub>H</sub>5 (34) gene segment. These elements may represent the favored target for rearrangement at 8 weeks of gestation. However, although present, they are not the most commonly utilized elements in these two second-trimester samples. Provocatively, recent observations in the mouse also suggest that the favored V<sub>H</sub>81X gene segment may not be the most J<sub>H</sub>-proximal V<sub>H</sub> (35). Although a common recombinase is postulated for all antigen receptor rearrangements, we have recently demonstrated striking evolutionary conservation of V<sub>H</sub> family-specific sequence involving the two-turn DNA spacer region between the heptamer and nonamer recombination signals (28). These conserved intervening sequences could influence recombinase activity either directly through differential intermediate complex stability or through associated binding factors. Selective rearrangement has also been reported in some cultured cell lines (36). These observations suggest that control of this developmental program may involve multiple regulatory mechanisms.

**Early Use of V<sub>H</sub>3 Elements Is Common in All Vertebrates, Although the Mechanisms Which Influence the Choice Vary.** Horned sharks contain multiple V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub>-C<sub>H</sub> clusters (37). In the light chain locus in birds, only a single functional V region is found (38) and diversity is generated by gene conversion from upstream pseudogenes. Both birds (39) and rabbits (40, 41) have multiple V<sub>H</sub> gene segments, but all are V<sub>H</sub>3-like. Therefore, in spite of the different mechanisms which are utilized to generate adult diversity in this broad range of species usage of V<sub>H</sub>3-like elements appears to be a common component of the earliest antibody repertoire.

These observations imply that V<sub>H</sub>3-like elements play an important biologic role. One possibility is that these sequences have been selected in response to antigens shared by common pathogens. However, the structural regions which exhibit greatest conservation do not coincide with the classic antigen-binding site (28). Studies in both mice and humans have demonstrated elevated autoreactivity among neonatal antibody repertoires. Two of the three V<sub>H</sub>3 elements we have shown to be most commonly utilized (30p1, 20p1) can form self-reactive antibodies (42, 43). Similarly, early antibody repertoires appear enriched for self-reactivity (6). Studies have shown that perturbation of B cells with anti-idiotypic antibodies during critical windows of development can alter the adult repertoire (6). These observations suggest that V<sub>H</sub>3 elements play a key role in the establishment of the immune system in several classes of vertebrates. If so, alterations in the developmental program of variable element utilization may contribute to autoimmunity, immunodeficiency, or other immune dysfunction.

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