ELEVEN DISTINCT V_H GENE FAMILIES AND ADDITIONAL PATTERNS OF SEQUENCE VARIATION SUGGEST A HIGH DEGREE OF IMMUNOGLOBULIN GENE COMPLEXITY IN A LOWER VERTEBRATE, *XENOPUS LAEVIS*

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The antibody response of higher vertebrate species typically is heterogeneous and increases in affinity upon antigenic restimulation. By contrast, during the humoral immune response of lower vertebrates, antibody affinity fails to increase, even after multiple reimmunizations (1–5) and in many cases antibody is relatively homogeneous (5). To date, immunoglobulin genes have been well characterized in only two lower vertebrate species. In an avian, *Gallus domesticus* (chicken), a single functional light chain variable (V_L) gene (6) and single functional heavy chain variable (V_H) gene (7) are targets for gene correction by flanking pseudogenes. By contrast, in *Heterodontus francisci* (horned shark), a large number of independent gene clusters consisting of V_H, diversity (D_H), joining (J_H), and constant (C_H) segments have been described (8, 9). A similar cluster-type gene organization also has been found for the *Heterodontus* light chain gene family (10). The close genetic relatedness between all *Heterodontus* V_H genes, including those selected using homologous C_H- (11), J_H- (Hinds, K., and G. Litman, unpublished data) as well as V_H-specific probes (9), is consistent with the classification of these genes in a single family that extends to include V_H genes found in a species belonging to another distant phylogenetic order, last sharing a common ancestor with *Heterodontus* some 200 million years ago (12). Thus, antibody diversity in these two lower vertebrate species may be limited, relative to mammalian antibodies, by gene families that are less extensive and/or do not use combinatorial joining to generate diversity.

Both the humoral and cellular immune responses of *Xenopus* have been described in considerable detail (13). The spectrotypes of hapten-specific antibody are not as complex as those found in higher vertebrates and are shared by different isogeneic animals (14, 15). Furthermore, sharing of antibody idiotypes by isogeneic *Xenopus* is consistent with a restricted repertoire; however, no sharing of idiotypic specificity was detected among anti-DNP antibodies from individual outbred frogs (16). The

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basis for this restriction is unclear and it has been suggested that it may arise from a relatively small number of progenitor lymphocytes and/or a narrow temporal window of lymphocyte differentiation (13). To address the molecular genetic basis for the possible restriction, we have characterized the complexity of the V_H genes expressed in the adult form of *Xenopus laevis* (XL).[1]

Materials and Methods

Animals. Outbred frogs (obtained from *Xenopus* I, Ann Arbor, MI) were anesthetized in 5 g/liter tricaine methanesulfonate before they were killed and splenectomy was performed.

cDNA Library Construction and Screening. Hybond mAP™ paper (Amersham Corp., Arlington Heights, IL) was used to isolate poly(A)$^+$ RNA. A cDNA kit (Pharmacia Fine Chemicals, Piscataway, NJ) was used to produce 500 ng of Eco RI-linkered cDNA from 5 μg of poly(A)$^+$ RNA. The cDNA was packaged into λgt11 vector with Gigapak Gold packaging mix (Stratagene, La Jolla, CA). A total of 10^6 recombinants were obtained, and $\sim 3 \times 10^5$ recombinants (unamplified) were screened under conditions of moderate stringency (17) using J_H-, C_μ- (18), and C_x-specific (19) probes. The C_μ- and C_x-specific probes were hybridized under conditions of moderate hybridization/wash stringency (17); the J_H-specific 33-mer (−) mixed oligonucleotide (see below) was hybridized in 6× SSC at 52°C, and washed in 6× SSC at 42°C. With J_H-selected clones, the purified DNA was digested with Eco RI. cDNA inserts that were C_μ^- (IgM) and ~2 kb were classified tentatively as C_x (IgX) (20); inserts that were <2 kb and contained internal Eco RI fragments were classified tentatively as C_υ (IgY) (21). At subsequent stages of analysis, the assignment of C_H isotype was confirmed by hybridization with gene-specific probes. 190 positive clones were recovered from three library platings (see below); based on the length of the cDNA inserts, 180 were judged to be full to near full copy length.

The initial cDNA library (replica) plating, 80,000 recombinants, was screened with V_HI-, V_HII-, and a C_μ (74mer oligodeoxyribonucleotide)-specific probes (see below). The C_μ^+, $V_H I^-$, $V_H II^-$ recombinants were cored, plaque purified, and the insert size was established. Several different ~2-kb insert regions were subcloned into M13 and sequenced. A new probe (V_HIII) was derived from one of these and the remaining unclassified recombinant phage were screened. The process of negative selection and sequencing was repeated until all C_μ clones were characterized with respect to V_H family. From this screening, V_HIII, VI, and VII were identified. A second library screening with J_H-selected recombinants resulted in the identification of V_HIV, V, VIII, IX, and X. A third library plating was designed to identify recombinants containing rare V_H family genes. The initial screening was done with a mixture of C_μ and C_x probes and V_HI-III and V_HVI-IX (not all V_H probes were available at that time). The V_H coding regions were amplified from the C_μ^+, C_x^+, V_H^- cores using polymerase chain reaction (PCR) technology in conjunction with C_H-specific primers and 18-mer probes complementing sequences in the LacZ gene that flanked either side of the λgt11 Eco RI cloning site. The amplified (V_H) DNA was then Southern blotted and screened with all available V_H probes and negative cores were plaque purified and analyzed, further leading to the identification of V_HXI. All clones in the study could be classified in 11 V_H families with the exception of nine clones that were C_H^+ and/or J_H^+ but did not contain a V_H region. These latter clones could result from truncated cDNA production or some may represent sterile mRNA transcripts as described (22).

DNA Sequence Analysis. Insert segments were excised from positive λgt11 clones by digestion with Eco RI and subcloned into the replicative forms of M13 mp11. Isolated plaques were screened with J_H 33-mer oligodeoxyribonucleotide probes complementing the (+) and (−) strands (see below). DNA sequences were determined in both directions by the dideoxynucleotide chain termination method (23) with the use of α-[^{32}S]dATP and T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). Primers specific for exon 1 of C_μ (18), C_x (19), and C_υ (21) were used to determine V(D)J sequences in one direction and the universal M13

[1] *Abbreviations used in this paper:* PCR, polymerase chain reaction; XL, *Xenopus laevis*.

primer was used to obtain the sequence of the opposite strand. DNA sequence comparisons were made using the GENALIGN routine (Bionet). Family relatedness for mammalian V_H genes was estimated using the same criteria that were applied in comparisons of XL sequences. Murine V_H genes, typically representing the prototypic member of a respective family, were selected: V186-1 (24), V_H36-60 (25), 81X (26), 23.9 (27), T15V1 (28), VH441 (29), 22.1 (30), PJ14 (31), DB3 (32), B5-3 (33), JV9 (34).

Genomic Southern Blot Analyses. Genomic Southern blots of Eco RI-digested XL DNA (10 μg/lane) derived from an individual animal were transferred to nylon membrane then hybridized with 10 ng of probe (plasmid or PCR derived) DNA (specific activity ≈ 2×10^9 cpm/μg) for 18 h at 65°C in 0.6 M NaCl, 0.2 M Tris, 0.02 M EDTA, 0.5% SDS, 0.1% $Na_4P_2O_7$, pH 8.5. Blots were washed at 65°C for 2.5 h in 1× SSC wash (0.15 M NaCl, 0.015 M sodium citrate, 0.1% SDS, 0.05% $Na_4P_2O_7$). Autoradiogram exposures varied from 2 to 6 d.

Probes. An XL V_HI (35) containing plasmid was obtained from Professor T. Honjo (Kyoto University, Kyoto, Japan). A V_HI probe prepared from the genomic segment consisted of 500 nucleotides 5′ of the leader through FR1 (nucleotide position 41; Fig. 1 *A*). Another V_H clone (8741) was identified by genomic library screening under conditions of moderate hybridization/wash stringency using the murine S107V probe essentially as described (17). The sequence of the V_H segment of this clone did not crosshybridize with the V_HI probe and its nucleotide sequence was <60% related. The hybridizing component was subcloned in pUC13 and used as a homologous V_HII probe consisting of 269 nucleotides from position (-72), (in the leader intervening sequence) through CDR2 (equivalent to position 197, Fig. 1 *A*).

Probes complementing V_HIII-V_HXI were obtained by standard subcloning techniques or by PCR from isolated single phage using internal 15–18-mer probes complementing sequences in FR1 and FR3 (see also Fig. 3 legend). The classification of the V_HIII probe is based on reference 36, and the V_HIV and V_HV probes have been assigned the same designation used in reference 37. Probes for V_HIII and V_HV were prepared from cDNAs closely related to those shown in Fig. 1 *A*, corresponding to positions 1–251 of the V_HIII and 5–268 of the V_HV (prototype) sequences. The remaining probes were derived from the sequences shown: V_HIV 19–261, V_HVI 1–294, V_HVII 1–223, V_HVIII 7–258, V_HIX 1–155, V_HX 114–263, V_HXI 57–264 (referring to sequences in Fig. 1 *A*).

An XL C_μ-specific probe was generated from two opposite polarity oligodeoxyribonucleotide 42 mers (C_μA and C_μB) that were complementary over 10 bp at the 3′ end of the A oligonucleotide and at the 5′ end of the B oligonucleotide. The annealed, parial duplex was copied (filled in) by a standard reaction using dNTPs and the Klenow fragment of *Escherichia coli* DNA polymerase. The complete duplex was phosphorylated by addition of ATP and T4 polynucleotide kinase. The 74-mer (complementing part of codon 16 through codon 40 [18]) was concatamerized by addition of T4 DNA ligase. A C_x-specific probe was derived by digesting a C_x clone (19) with Eco RI and Xba I. An ~0.6-kb fragment consisting of exons 3, 4, and a portion of 3′ untranslated sequence was subcloned in pUC19. An IgY-specific probe was derived from a V_H^+, C_μ^-, C_x^- cDNA (21) by digestion with Eco RI; a ~0.7 kb C_υ fragment was subcloned in Bluescript (Stratagene, La Jolla, CA). Two oligodeoxyribonucleotide 33 mers complementing both the (+) and (−) orientations of XL J_H2 and J_H7 (36) were synthesized:

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5′-TGGGGACAAGGTACCATGGTCACCGTCACCTCA-3′  (+)
                  G                   T

TGAGGTGACGGTGACCATAGTTCCTTGTCCCCA      (−)
  A                       G  C
                             A
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All probes were labeled using the (N^6) random hexamer method as described (38), except that the concentration of the primer is reduced to 10^{-1}, yielding larger transcripts that hybridize more effectively (lower background) with distant members of a related family (unpublished data).

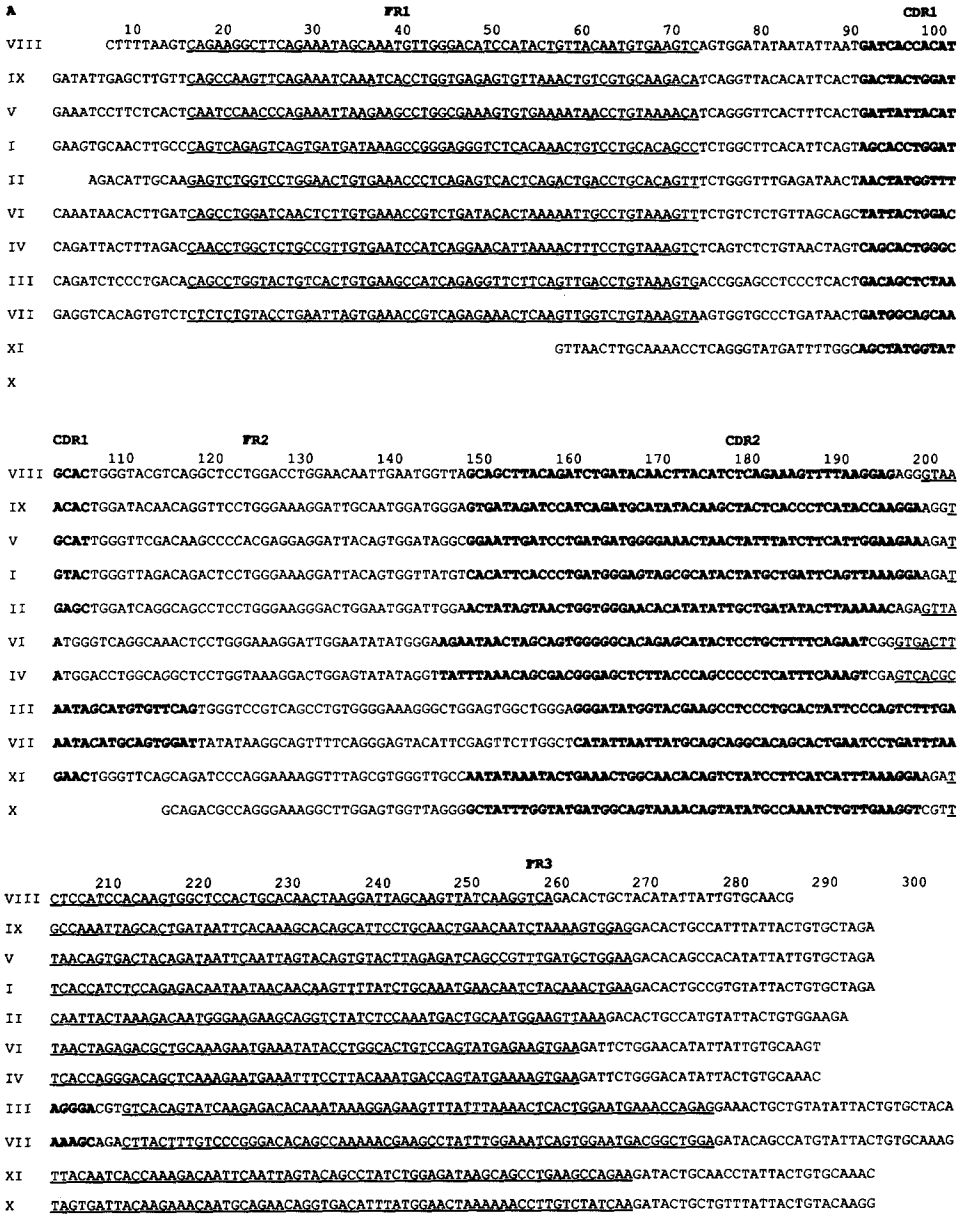
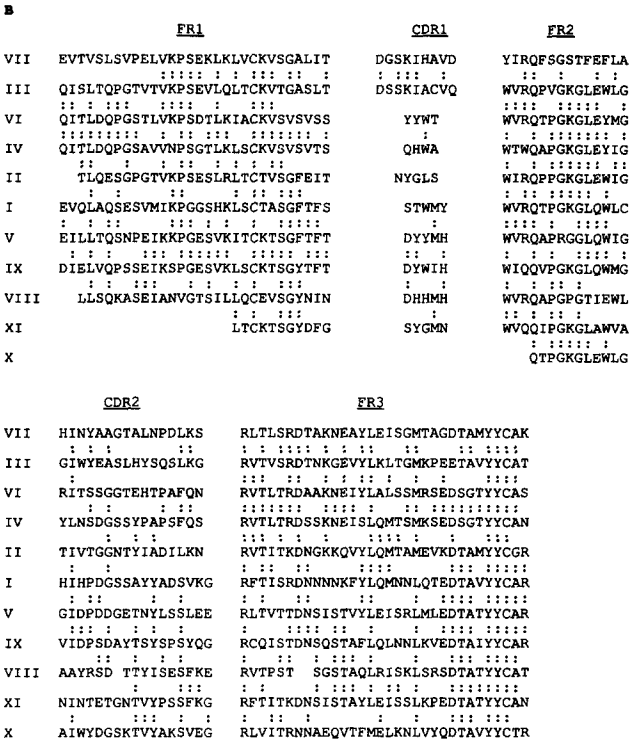


FIGURE 1. (*A*) Nucleotide sequences of XL V_HI-V_HIX and partial sequences of truncated V_HX-XI cDNA clones. V_HVIII and II clones presumably lack the first six and four nucleotides, respectively. CDRs are in bold. The sequences are shown through the conserved Tyr, Tyr, Cys, and two additional codons. The actual length of some FR3 regions may be one to four nucleotides longer than shown. V_HI, II, and III examples, longer than those depicted, are known (7), and some V_HVIIIs appear to have a longer FR3 as well (our unpublished observation). The sequences are ordered by GENALIGN in terms of relatedness to the first sequence. The sequences of the family specific regions are shown by underlining. GenBank accession numbers for the nucleotide sequences are: V_HI/M24673, V_HII/M24674, V_HIII/M24675, V_HIV/M24680, V_HV/M24681, V_HVI/M24678, V_HVII/M24679, V_HVIII/M24676, V_HIX/M24677, V_HX/M27254, V_HXI/M27244. (*B*) Predicted amino acid translations of cDNA sequences from *A*. Functional



boundaries are defined by analogy to mammalian genes (45). The conserved Cys in FR1 is assigned codon 22. All full-length FR1 sequences begin with Gln, Asp or Glu. Note that FR3 may be longer for some V_H families (see legend to *A*). The sequences are ordered by GENALIGN in terms of relatedness to the first (*top*) sequence and this order differs from that shown in *A*. Identities between adjacent sequence pairs are shown (:).

Results

Iterative Screening of an XL Spleen cDNA Library. RNA was prepared (39) from the combined, equivalent size spleens of six adult XL, poly(A)$^+$ RNA was isolated, and a cDNA library was prepared. The rationale for combining the spleens of several animals was based on the assumption that certain V_H families may not be expressed in all normal individuals in any given time frame. The rationale for using unimmunized animals was to prevent possible selection bias arising from antigen-selective expression of certain V_H families. The unamplified library was screened with various probes complementing the V_H (18), J_H2 and J_H7 (36), C_μ (36), or C_χ (19) regions of XL heavy chain genes (see Materials and Methods). Initially, the J_H^+ and C_H^+ clones were screened using XL V_HI-(35) and V_HII-specific probes. The V_HII probe was recovered from an XL genomic DNA library by heterologous screening with the murine S107V probe (40). Clones bearing full or near-full copy length cDNAs were screened at moderate stringency with the XL V_HI and V_HII probes; $V_H I^-$, $V_H II^-$ clones were selected and a few were sequenced. A region corresponding only to the V_H coding portion was subcloned or PCR-amplified (41), and a new probe, i.e., V_HIII, was used to rescreen all other $V_H I^-$, $V_H II^-$ clones under the same con-

ditions. This process, which is based on negative hybridization with respect to new families, has been repeated eight times; more than 180 individual Ig clones have been recovered and characterized by family-specific hybridization and/or selective sequencing (see below). In the course of these studies, the complete V_H-D_H-J_H sequences of 40 unique cDNA clones have been determined; and in this group of clones, the same cDNA has not been encountered twice. Based on the selection method used and nucleotide relatedness of $\leqslant 70\%$, the level of sequence identity ordinarily applied in distinguishing V_H families (42), at least 11 gene families are expressed in the spleen of adult XL. The distributions of V_H families relative to the probe used in the initial selections are summarized in Table I. The 54 J_H selected clones also were hybridized to the three C_H probes and classified by isotype to assess the apparent frequency of expression of C_μ (38%), C_υ (49%), and C_x (13%).

DNA Sequences of V_H Genes Belonging to Different Families. The nucleotide and predicted amino acid sequences of V_H genes belonging to 11 different families are shown in Fig. 1, *A* and *B*. These sequences shown are in most cases the first member of each family detected in the screening procedure, with no other selection criteria imposed. All $V_H{}^+C_H{}^+$ cDNAs that have been sequenced are members of one of the families shown, with >85% overall nucleotide similarity to the family prototype. Recent analysis of all available nucleotide sequences of human and mouse V_H sequences has revealed the utility of using characteristic family-specific identification regions for the classification of V_H gene families (43). The nucleotide sequences corresponding to codons 6–24 of the first framework region (FR1) and codons 67 through 85 (FR3 codons 2–23) (Fig. 1 *A*) are both highly conserved between members of a V_H family and widely divergent between different V_H families in those species; an alignment matrix for the XL FR1 data is shown in Fig. 2 *A* and for the FR3 data in Fig. 2 *B*. This analysis method also has been useful in delineating putative evolutionary interfamily relatedness (43). The results of the comparisons based on family-specific regions are similar to those obtained with full-length sequence comparisons

TABLE I

Initial Selection Method

V_H	$J_H{}^+$ *	$C_\mu{}^+$ ‡	$C_\mu{}^+ + C_X{}^+$ § V_HI-III, VI-IX(−)
I or II	21	39	
III	15	12	
IV	7	1	4
V	1		3
VI		1	1
VII	1	1	
VIII	3		
IX	5		
X	1		
XI			1

The Ig containing clones identified in three screenings are classified by V_H family.

* J_H AND ‡C_μ probes initially were used to find V_H families I through X. The third screening §$C_\mu{}^+ + C_x{}^+$ was designed to find rare V_H families. Thus, all V_H genes that were positive for mixed V_H probe (I, II, III, VI, VII, VIII, IX) were not analyzed further and do not appear in the table.

A	II	III	VIII	IX	VI	VII	IV	V
I	60	53	40	58	51	49	54	54
II		68	35	46	63	65	61	47
III			40	44	61	63	65	49
VIII				37	39	39	42	40
IX					47	42	44	65
VI						65	74	51
VII							60	51
IV								53

B	II	III	VIII	IX	VI	VII	IV	V	X	XI
I	59	55	22	56	55	50	62	45	48	59
II		53	22	45	59	53	56	50	45	55
III			20	39	53	61	58	50	35	50
VIII				26	22	26	26	20	28	20
IX					44	36	48	53	47	56
VI						55	71	52	45	52
VII							55	48	39	56
IV								55	41	55
V									36	73
X										47

FIGURE 2. (*A*) Family specific region nucleotide sequences of XL V_H FR1 (corresponding to codons 6 through 24, see Fig. 1 *A*) are compared using GENALIGN. Sequences representing V_H families I–IX were aligned and ordered according to relatedness. The values given in the scoring matrix are percent nucleotide similarity. Families X and XI are not included in the analysis. (*B*) Family-specific region of FR3 (corresponding to codons 2 through 23 of FR3, see Fig. 1 *A*) compared as in *A*. Scoring matrix percentages are as in *A*. Percentages for comparisons with V_HVIII are calculated on 60-nucleotide length; all others are 66 nucleotides.

because inclusion in the analysis of FR2, which exhibits somewhat less variation between families, as well as the complementarity determining regions (CDR1 and CDR2), which exhibit more variation than the family-characteristic regions, does not alter assignment of family status of a given cDNA using the 70% overall similarity level criterion (42, 44) (except as noted immediately below). The most closely related sequence pair is V_HIV and V_HVI, which exhibit 74% and 71% nucleotide identity in the family characteristic FR1 and FR3 regions, respectively; however, these V_H genes have been assigned separate family status because of the selection method and overall nucleotide and amino acid sequence identity of 70% and 68%, respectively. In most cases where three or more members of a family have been sequenced (e.g., V_HI, II, III, and IX), nucleotide identities in the family specific regions are ≥90%. An additional V_HIV gene has been sequenced (not shown) and exhibits 86% overall nucleotide similarity (82% and 94% in the family-specific segments of FR1 and FR3) to the family prototype gene but only 71% to the V_HVI prototype, supporting the classification of V_HIV and VI as separate families. The FR3 family-specific segments of V_HV and V_HXI share 73% similarity but the cDNAs are only 65% similar overall. The V_HX and V_HXI families are represented by truncated cDNAs and only FR3 is included in the comparison matrix shown in Fig. 2 *B*. Over a comparable region, the V_HX gene has only 60% nucleotide identity to V_HI, its most similar counterpart, and exhibits blot hybridization characteristics (as well as behavior in the selection assay) consistent with this assignment (see below). Furthermore, a genomic clone containing a full-length V_HX gene has been isolated and sequenced

(our unpublished observation). This gene is highly related to and may code for the cDNA shown in Fig. 1.

The overall nucleotide (FR1-FR3) relatedness of the XL V_H cDNAs belonging to different families ranges from 35 to 70%. The sequence differences in the XL V_H families exceed those calculated in parallel comparisons using randomly selected members of each of the 11 murine Ig gene families. In a comparison matrix (not shown), the nucleotide similarity of a single gene to a member of each other family in turn yields a score. The mean score for that gene to all other families represents a measure of the intraspecies V_H family diversity. For selected mouse V_H genes (see Materials and Methods, *DNA Sequence Analysis*), mean scores range from 103 to 184 for comparisons of full-length V_H sequences (average length of 293 ± 3 nucleotides). For XL V_H genes, exclusive of comparisons involving V_HVIII, V_HX, and V_HXI which introduce penalties for differences in sequence length and gaps, mean comparison scores are lower (indicating higher diversity) and range from 66 to 126 for sequences (average length of 294 ± 6 nucleotides). Inclusion of XL V_H VIII, V_HX, and V_HXI results in even greater V_H family diversity, i.e., lower comparison scores. At the amino acid sequence level, the overall identity between XL V_H genes range from 68% to only 21% (Fig. 1 *B*, comparison matrix not shown).

The only region of extended sequence identity between all 11 families involves the phylogenetically hyperconserved sequence Tyr-Tyr-Cys (17) in FR3. Furthermore, the amino acid sequences of some of the XL cDNAs is noteworthy. In contrast to human, mouse (45), shark (17), caiman (46), chicken (7) and *Elops* (47), a teleost, Ig where four key V_H FR1 and FR2 residues are "invariant," i.e., occur in ~99% of known murine or human V_H sequences or in all known V_H genes of the other species listed, some V_H families of XL contain alternate amino acids at one or more of these positions. At FR1 position four ($FR1^4$), Leu is replaced by Val in V_HVII as in many rabbit and a few mouse Igs (45). $FR1^{26}$ is invariantly Gly, but in XLV_HIV and VI Val occurs in our cDNAs. In $FR2^1$ the invariant Trp is replaced by Tyr in V_HVII. Two representative cDNAs of the V_HVII gene family have been sequenced and found to be highly related in FR1, FR2, and CDR1 regions with different CDR2s and a few FR3 substitutions but are the only XL cDNAs that do not have Trp at this position. There is no other reason to assume that these represent pseudogene transcripts. Finally, at $FR2^{10}$ the expected Leu is Phe in V_HVII and Thr in V_HVIII (all these substitutions are confirmed in sequences of genomic clones, our unpublished observations). Thus, diversity of XL V_H structure expands the presumed limits placed on functionally allowable amino acid sequences (assuming productive translation).

Genomic DNA-Southern Blot Analyses Using V_H-specific Probes. Probes complementing the predicted coding (mature) segments of V_H genes were hybridized to individual tracks of Southern blotted XL genomic DNA obtained from a single animal (Fig. 3). Variation in the number of hybridizing components is apparent; furthermore, there appears to be little similarity in the patterns of hybridization associated with each of the family-specific probes. While some of this complexity potentially is allelic (see below), each V_H family, with the possible exception of V_HX, most likely consists of several members and a total of 150 unique bands can be identified. Many of these bands presumably represent multiple gene copies, thus underestimating complexity; however, the known presence of pseudogenes in XL (48) would overesti-

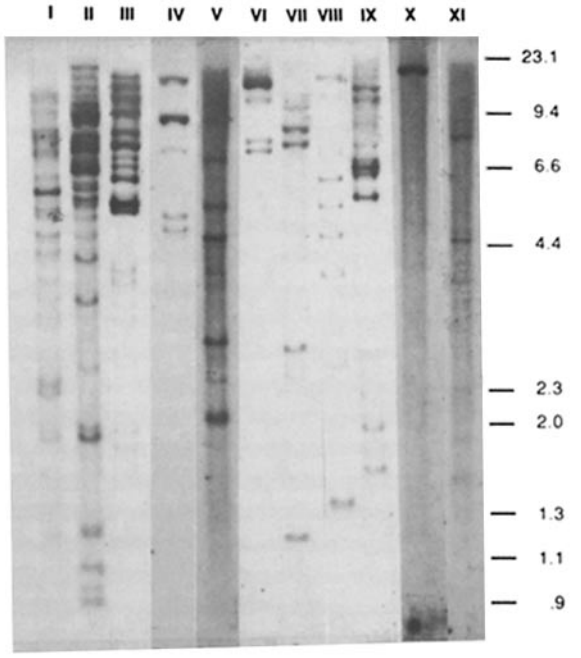


FIGURE 3. Genomic Southern blots of Eco RI-digested XL DNA prepared from a single individual. Probes are derived from (*1*) V_HI, a plasmid containing leader and part of FR1, as well as noncoding sequence 700 nucleotides (35). (*2*) V_HII, FR1-FR3 coding sequence from a genomic clone; (*3*) V_HIII, FR1-FR3 cDNA sequence; (*4*) V_HIV, a PCR-amplified probe complementing FR1-CDR2; (*5*) V_HV, a probe complementing FR1-FR3, derived from a cDNA; (*6*) V_HVI, FR1-FR3 coding sequence; (*7*) V_HVII, leader, FR1-CDR2 cDNA sequence; (*8*) V_HVIII, FR1-CDR3 cDNA sequence; (*9*) V_HIX, FR1-CDR2 cDNA sequence; (*10*) V_HX, a PCR-derived probe complementing FR2-FR3 and (*11*) V_HXI, a PCR-derived probe complementing CDR2-FR3. Precise boundaries of probe sequences (relative to Fig. 1 *A*) are indicated in Materials and Methods. Standards are λ digested with Hind III and øX174 digested with Hae III and indicated in kilobases at right.

mate V_H complexity. The absence of similarity between the different V_H patterns and genomic DNA sequence information recently obtained for each of the additional V_H families described herein (our unpublished observations), suggests that the cDNAs recovered from the library and used subsequently to generate probes, do not arise from intergenic recombination involving extended sequence segments, although the effects of regionally limited gene conversion would not be possible to discern. Hybridization of the family-specific probes to a series of phage DNA clones representing V_HI-V_HXI results in little or no crosshybridization (data not shown).

Sequence Comparisons of CDR Segments. Additional diversity within the same V_H gene family is evident from sequence comparisons of CDR1 and CDR2 (Fig. 4). The level of substitution within XL CDR2 appears to be comparable to that observed in murine intra-family comparisons (28, 49–51). On average, the CDR2 regions of different murine genes vary by 5-10 nucleotide changes in 51-57 nucleotides. The corresponding average rate of substitution in the XL V_HIII CDR2s is 8 changes in 48 nucleotides, assigning clone 26055 as the "prototype." In the CDR1 of XL, more changes are evident than in the corresponding segment of the murine genes even when the extra length of XLV_HIII CDR1 is taken into account. However, two V_HIII genes, 26510 and 26947, share an identical CDR1 sequence that varies only by a single nucleotide from the shared CDR1 segments of XL V_HIII genes reported in another study (48). Different mammalian V_H genes belonging to the same family also share CDR1 sequences (24). In interpreting these data, it is essential to note that XL cDNAs are being compared with genomic sequences and that nucleotide differences may reflect somatic mutation or limited gene conversion. However, doing the same analysis on recently published V_H genomic sequences from isogeneic XL (48) yields a nearly identical substitution rate (7–8) to that found for our cDNAs.

In another study (37) comparing XL cDNAs, five members of the V_HII family show CDR2 substitution rates of 11 changes per gene, comparable to the substitution rate demonstrated in V_HII germline genes (48). The XL CDRs shown in Fig. 4 appear to be no more highly related than are their murine counterparts although human V_H gene families (52) may be more highly variable in CDR regions than are isogeneic or wild-type XL and inbred mouse strains.

Comparison of D Segments. Further evidence for variation in the V_H gene repertoire of XL comes from the comparison of associated D segments (Fig. 5), which are defined operationally as the sequence occurring between the 3′ of the V_H coding segments, i.e., following codon position 32 of FR3 (see Fig. 1) and extending to the 5′ of J_H as inferred from genomic sequences (36; Suzuki, D., C. Amemiya, R. Haire, and G. W. Litman, unpublished data). The lengths of the deduced D regions vary from 4 to 29 nucleotides that would encode 1–10 amino acids. Presumably the extended regions of nucleotide identity between different cDNAs correspond to germline D_H sequences, although fortuitous sequence identity could occur through a template-independent mechanism (53). Based on patterns of conserved nucleotides, 23 of 36 sequences in this comparison of randomly selected sequences can be categorized in four primary families, i.e., having four or more members. The extended length of some D regions (e.g., 26035 and 26510) suggests that two D segments may contribute to a single rearrangement event as can occur in *Heterodontus* (9) (Hinds, K., and G. Litman, unpublished data) and possibly in an avian (54). Joining of two D segments also may occur in XL (37). In addition, 13 D segments are unassigned with regard to the primary families. By analogy to equivalent studies of other vertebrate V_H genes, including *Heterodontus* (9), it is presumed that both junctional and N-type diversity (53) account for a major portion of the sequence variation in this segment.

Comparison of J_H Segments. In addition to the variation in D segments, five different J_H sequences (Fig. 6), that vary by more than three nucleotide changes,

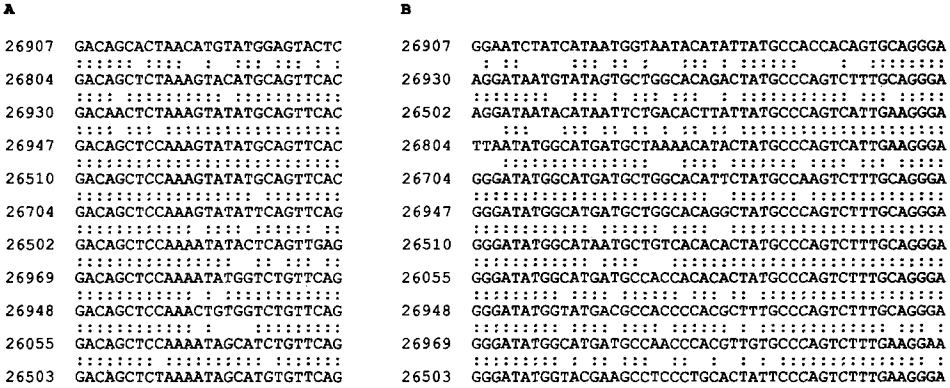


FIGURE 4. Comparison using GENALIGN of CDR1 (*A*) and CDR2 (*B*) sequences of 11 V_HIII clones. Designation at left refers to clone number. Note that clones were not selected by hybridization with a V_HIII probe but were selected initially on the basis of being negative for V_HI and V_HII and plaque purified on that basis, thus avoiding bias in the sequence distributions. Clone 26907 is a transcript of a V-D-J joined to an intervening sequence segment with no C_H. The relatedness order of the clones is different for CDR1 and CDR2.

	D	CLONE	V_H
1	TGGGGTGGAGCTGGG	26606	I
	TCACTGGGGTGGAGCTC	26031	XI
	TGGGGTGGGAGAAT	26945	IV
	GAGCTCACTGGGGTGGGA	26602	I
2	GAAGGAG	26704	III
	GGAG	26304	II
	GGAGTACAGCG	26503	III
	GATCCGGAGTGGCATTA	26083	I
	GACTGGAGTGGCC	26965	IX
	TGGACTGGAGTGGGAGCT	26105	II
3	GATCGGGGGGAGTGGGAGC	26510	III
	TATCGGGGGGCTAGTGGTTACAGGGGGTA	26035	IV
4	GAAGCTTGGGCTAGCGGGTACCTCA	26804	III
	GACGCTAGCGGGTACAG	26943	I
	CATGGGGCTAGCGGGTACAG	26051	V
	GAACAGCTGGGTACGCTAGCGGGTACA	26505	III
	AGAGGATTAGCGGGTACGGG	26929	IX
	GAAAGCGGTTACAG	26401	II
5	GGTACGGGGGTGG	26918	X
	TACGGGGGTGGCAGC	26910	I
	CGGGGGTGGCAAT	26959	I
	AATACGGGGG	26962	VIII
	CCTACGGGTGACAGCTGTC	26502	III
6	GAAGAAA	26948	III
	GAAGGAGG	26704	III
7	CAAAAT	26907	III
	CAAAC	26963	V
	TGGGGGGCATTTG	26609	I
	CAGG	26607	VII
	ATGGGGCCCGAC	26601	VI
	GTAGGCCT	26055	III
	TACGG	26926	IV
	GCACC	26947	III
	GAAA	26969	III
	AGATCAGGATCTCCTTCTGGAATGGG	26972	IX
	ACAGACGTCCGTGGATCCG	26920	I

FIGURE 5. Representation of putative D segments (presumably reflecting junctional and N diversity) deduced from several XL cDNAs. Sequences considered to be prototypic for a given D family are shown in bold, other relationships within and in some cases between D families are shown by underlining or by aligning sequences relative to one another in order to emphasize regions of absolute sequence identity. Distinct D families are arbitrarily assigned as consisting of at least two members with at least four identical bases. In some cases, such as between type 3 and 4, extended regions of sequence identity are present; however, these are considered as being separate families since there is some discontinuity in the overall pattern of sequence identity. These assignments are tentative and would require complete analysis of genomic V_H and D sequences in order to access the contribution of junctional and N diversity as well as other somatic changes. Recently, the available genomic sequences of XL V_HI, II, III (48), and V_HVIII (our unpublished observations) indicate that FR3 may be longer than shown here. This may require the deletion of putative D nucleotides and their inclusion in V_H sequences (see Fig. 1 *A*, legend). The V_H family designation of the cDNA is indicated at right. The phage clone identification number also is given. Elements similar to the shared core sequences of groups 1, 4, and 5 are described in (37, 48).

including representatives of four known genomic J_H sequences (36) have been noted in these cDNAs. In addition a sequence comparable to J8, known from a cDNA (37), has been detected. Five additional J_H sequences varying from each other by 1–3 nucleotides have been observed. Genes belonging to different V_H families are associated with the same J_H segments and the same V_H family is associated with different J_H segments (data not shown).

Discussion

Evidence presented to date suggests that the Ig heavy chain locus in XL is arranged similarly to the V_H locus in mammals and exhibits combinatorial diversity between elements, in contrast to the "cluster" or single gene-multiple pseudogene organization patterns of elasmobranchs (9) and avians (55), respectively. The studies reported here reveal both an exceptional level of complexity in Ig gene families and the presence of multiple individual members within a single family. Specifically, we have detected 11 distinct V_H families, four (or five) primary and 13 additional D_H sequences (families) and at least 10 different J_H sequences. It is likely that at least some of the sequence segments in the various categories are allelic variants and/or reflect the effects of somatic mutation. Further evidence that the V_H genes repre-

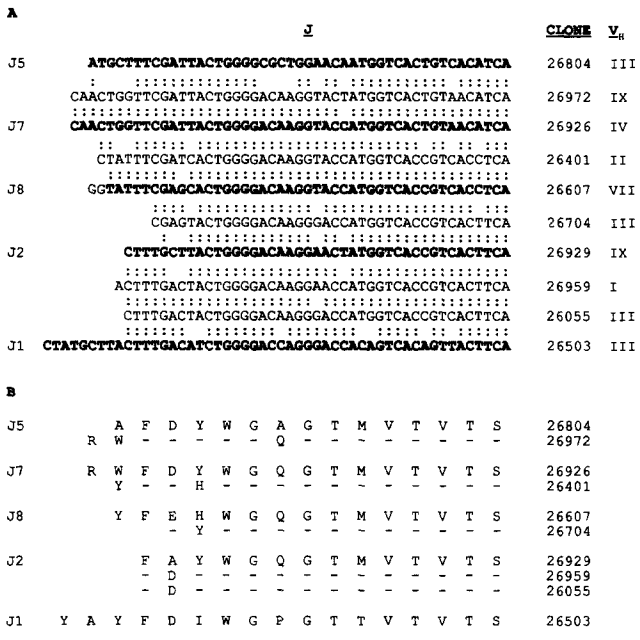


FIGURE 6. J region sequences of XL cDNAs. (*A*) Sequences shown in bold correspond over their length to genomic sequences J1, 2, 5, and 7 (36) and to J8 observed in a cDNA (37). Clone identification number and associated V_H family for a cDNA containing the representative J_H sequence shown are indicated at right. Variability in sequence at 5′ end is presumably a result of junctional deletion of genomic J_H sequence. Some of the sequences shown occur in two or more clones but with minor differences in 5′ nucleotide sequence. (*B*) Amino acid translations of J_H sequences. The first sequence for each J_H type is shown with observed substitutions shown below. No change is indicated by (-), and a space indicates no residue present due to 5′ truncation (see *A*). J_H family designations are from reference 37.

sent individual families comes from the unique genomic Southern blot patterns associated with each of the 11 V_H probes. The absence of common hybridization patterns and other preliminary observations suggest that the cDNAs are not the products of intergenic recombination or gene conversion events that lead to introductions (exchange) of significant portions of one sequence, e.g., V_Ha into another gene, V_Hb, that would yield a $V_H$ab product. This conclusion is supported further by the absence of sequence relatedness between the cDNAs representing individual families. It is unlikely that V_H family diversity originates from genetic exchanges among a limited number of germline components.

From possibly as few as one (V_HX) to as many as 40 germline components are present in each V_H family and the absence of internal Eco RI sites in the known cDNA sequences suggests that the individual bands do not arise through internal cleavage. The relative numbers of hybridizing components are consistent with observations made in two other studies with five XL V_H families (36, 37) and indicate that the V_H gene complexity in XL is as extensive as, if not more than, that found in mammals. Thus, use of an outbred animal model cannot be identified as the source of a significant degree of Southern blot band complexity; although the use of animals selectively bred for the maintenance of a homozygous recessive trait (albino) has been shown to reduce the complexity of some hybridizing elements (37). The presence of D segments and presumably junctional/N-type diversity is consistent with these mechanisms of diversification. Furthermore, the relative variation within CDR sequences is at least as great as that observed between members of different V_H gene families in higher vertebrates. This conclusion extends to the predicted amino acid sequence level.

Recently the nucleotide sequences of 22 genomic V_H genes belonging to three

families have been determined and various sequence patterns have been interpreted to be consistent with restricted antibody diversity in this species (48). Specifically, three V_HI clones were found to have the same CDR1 sequence as were two V_HIII clones; one of the V_HI clones shares a CDR2 sequence with a fourth V_HI clone. Sequence comparisons of other pairs of clones show patterns of variation ranging from single differences to nonidentity in CDR segments. The V_HI clones that exhibit the highest degree of absolute sequence sharing in CDR1 and CDR2 have identical restriction maps and probably are allelic, whereas the pair of V_HIII genes that share identical CDR1s do not appear to be allelic. In the studies reported here, which include 40 informative comparisons, three V_HIII genes and a V_HIX gene pair share CDR1s and no cDNAs share CDR2.

Sizeable numbers of pseudogenes and limitations in V_H family complexity also have been proposed to account for restrictions in antibody diversity; however, the numbers of pseudogenes detected in XL (48) are equivalent to or somewhat less than are found in mammalian systems (56). As reported here, the number of V_H gene families is equivalent to that reported in mouse (42–44) and considerably greater than that found in humans (43, 52). It is essential to note that assessment of V_H complexity on the basis of genomic sequences alone is inadequate. Furthermore, estimating the total number of V_H genes from Southern blot patterns is not reliable unless parallel gene titrations are carried out, and even then, determining whether pseudogenes are recombined functionally can be complex. Only a single functional germline component is present in both the V_H and V_L loci of an avian; however, these genes are converted, giving rise (at the RNA level) to highly complex V genes (7, 55). The number of germline genes per se cannot be used as a reliable means for estimating diversity unless these have been isolated and characterized completely in both structural and functional terms, and even then the preferred usage of relatively few genes within a family of potentially functional sequences can occur (57). Ig gene diversity is estimated best by sequence comparisons of expressed gene products even though the inability to establish productive translation of such sequences introduces some uncertainty.

The Ig V_H gene system of XL represents the most complex antibody gene system described to date for a lower vertebrate. V_H gene families found in this species are highly divergent and actually may reflect more extreme evolutionary diversification than is seen in contemporary vertebrates. Some XL V_H families contain amino acid substitutions at positions previously thought to be universal by invariance over a broad evolutionary spectrum. In this regard, it is interesting to note that Igs of the V_HVII type have incorporated alternative amino acids at three positions previously thought to be "invariant." Unless XL V_H genes do not undergo somatic mutation (preliminary studies suggest that they do) and/or gene conversion, or otherwise have unique constraints placed on the potential patterns of rearrangement, it is unlikely that the restricted diversity reported in this species arises from differences in gene structure, organization, or complexity. Reconciling earlier observations on spectrotype (14, 15) and idiotype (16) sharing with molecular genetic data is difficult; however, changes may occur that do not influence charge and antigenic properties of V regions as dramatically as those occurring in mammalian antibodies. It also is possible that IgY, the class of functional antibody visualized in the spectrotype analyses, exhibits less variation than IgM or IgX, which are undetectable in this assay

(37). IgY also is known to possess only a subset of light chains and thus may exhibit restricted heterogeneity (37, 58). Alternatively, the antigen-combining sites found in each XL V_H family may differ extensively, limiting the potential for crossreactivity between different families relative to that seen in mammals. Thus spectrotypes of hapten-specific antibody would be less complex, i.e., reflect a limited number of families, while antibody heterogeneity, as measured by differences in CDR sequence, junctional and N diversity, and perhaps even somatic mutation, may be as extensive as that observed in higher vertebrates.

Summary

Lower vertebrate species, including *Xenopus laevis*, exhibit restricted antibody diversity relative to higher vertebrates. We have analyzed more than 180 V_H gene-containing recombinant clones from an unamplified spleen cDNA library by selective sequencing of J_H and C_H positive clones following iterative hybridization screening with family-specific V_H probes. 11 unique families of V_H genes, each associated with a unique genomic Southern blot hybridization pattern, are described and compared. Considerable variation in the number of hybridizing components detected by each probe is evident. The nucleotide sequence difference between V_H families is as great as, if not more than, that reported in other systems, including representatives of the mammalian, avian, and elasmobranch lineages. Some *Xenopus* Ig gene families encode alternative amino acids at positions that are otherwise invariant or very rarely substituted in known Igs. Furthermore, variations in complementarity determining region sequences among members of the same gene family and high degrees of D_H and J_H region complexity are described, suggesting that in at least this lower vertebrate species, the diversity of expressed Ig V_H genes is not restricted.

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