Generation of Immunoglobulin Light Chain Gene Diversity in Raja erinacea Is Not Associated with Somatic Rearrangement, an Exception to a Central Paradigm of B Cell Immunity

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Summary

In all vertebrate species examined to date, rearrangement and somatic modification of gene segmental elements that encode portions of the antigen-combining sites of immunoglobulins are integral components of the generation of antibody diversity. In the phylogenetically primitive cartilaginous fishes, gene segments encoding immunoglobulin heavy and light chain loci are arranged in multiple clusters, in which segmental elements are separated by only 300-400 bp. In some cases, segmental elements are joined in the germline of nonlymphoid cells (joined genes). Both genomic library screening and direct amplification of genomic DNA have been used to characterize at least 89 different type I light chain gene clusters in the skate, Raja. Analyses of predicted nucleotide sequences and predicted peptide structures are consistent with the distribution of genes into different sequence groups. Predicted amino acid sequence differences are preferentially distributed in complementarity-determining versus framework regions, and replacement-type substitutions exceed neutral substitutions. When specific germline sequences are related to the sequences of individual cDNAs, it is apparent that the joined genes are expressed and are potentially somatically mutated. No evidence was found for the presence of any type I light chain gene in Raja that is not germline joined. The type I light chain gene clusters in Raja appear to represent a novel gene system in which combinatorial and junctional diversity are absent.

A central paradigm of contemporary molecular immunology is that the generation of antibody and T cell antigen receptor diversity is associated with the rearrangement of segmental elements in individual somatic cells committed to the B or T lineages (1, 2). In mammals, this rearrangement process is associated with combinatorial as well as junctional diversity, which is achieved by deletions and nontemplated additions at the segmental junctions. These somatic changes, along with hypermutation of rearranged variable regions, result in the diversification of the antibody repertoire (3). The relative contributions of these mechanisms to the final immunoglobulin specificity vary among species, as does the total number of individual gene loci. The primary avian model, the chicken, possesses one immunoglobulin heavy chain locus (4) and one immunoglobulin light chain locus (5), whereas cartilaginous fish, such as sharks, have >100 chromosomally dispersed immunoglobulin heavy chain loci and possibly an equal number of immunoglobulin light chain loci (6, 7). Previously, our laboratory demonstrated that the horned shark, Heterodontus francisci (6), and the little skate, Raja erinacea (8), possess two major types of immunoglobulin heavy chain gene organization in which the segmental elements (VH, DH, and JH) are either unjoined or joined in the germline of nonlymphoid cells. On the basis of identities between the predicted coding and flanking region sequences of unjoined and joined genes, it appears that none of the joined heavy chain genes identified to date are pseudogenes. However, the transcription of these genes has not been demonstrated, and their role in adaptive immunity is not understood (9).

Sequence analyses have revealed that there are at least three types of light chain clusters in the cartilaginous fish, some of which are also VJ-J joined in the germline. Type I light chain genes, which were originally isolated using an antibody screening approach (10), are unjoined in Heterodontus (7); however, several joined light chain genes have been identified in Raja (9). Type II light chain genes, which are related to type I light chain genes by ~38% amino acid identity, have been characterized in Heterodontus, Hydrolagus coliei (spotted ratfish), Raja (11), and Carcharhinus plumbeus (sandbar shark) (12), and appear to be joined in each species. Type III light chain genes, which are ~60% related in amino acid sequence to mammalian κ light chain genes in the V
region, have been characterized at the genomic level in *Heterodontus*, in which they are unjoined (11), as well as at the cDNA level in *Ginglymostoma cirratum* (nurse shark) (13). However, we have not been able to detect a type III homologue in either *Raja* or *Hydrolagus* despite extensive attempts using a variety of strategies, including PCR amplification and direct probing (Anderson, M. K., and J. P. Rast, unpublished observations). To establish that germline joining is of functional significance, the distributions of nucleotide sequence differences within a significant portion of the gene family must be characterized and the transcriptional status of the gene must be established. Using several genomic selection strategies combined with analyses of cDNAs, we provide evidence that all type I light chain genes in *R. erinacea* are joined in the germline, diversified, and transcribed, and appear to be somatically mutated.

**Materials and Methods**

**Animals.** Adult specimens of *R. erinacea* were obtained from the Marine Biological Laboratory (Woods Hole, MA). After the animals were sacrificed, tissues were processed immediately.

**DNA Libraries.** A genomic library was constructed from *R. erinacea* nucleated RBCs. RBCs were carefully separated from other cells in whole blood by low speed centrifugation. Cytological staining of such preparations shows these to be devoid of leukocytes. High molecular weight DNA was extracted as previously described (14), treated with RNase, partially digested with Sau3A, and ligated to Lambda DASH® (Stratagene, La Jolla, CA) arms. The unamplified library consisted of ~7.5 × 10^6 PFU, which corresponds to ~2.7 genomic equivalents, assuming 7 pg per haploid genome (15). The library was amplified on the bacterial host P2392, a P2 lysogen of LE392.

A cDNA library was constructed essentially as previously described (16), using *R. erinacea* spleen RNA from the same animal that was used in genomic library construction. RNA was extracted using a commercially available method (RNAzol; Cinna/Biotec Laboratories, Houston, TX), and mRNA was purified using Dynabeads (Dynal, Inc., Oslo, Norway). The mRNA was converted to cDNA using a commercially available cDNA synthesis method (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The cDNA was ligated into λgt11 arms, packaged using Gigapack® Gold (Stratagene) packaging mixes, and plated on *Escherichia coli* strain Y1088. The library was amplified to 4 × 10^9 PFU/ml.

**Probes and PCR Primers.** DNA probes were generated by PCR using primers directed against specific nucleotide sequences of corresponding cDNA or genomic clones. HFL is a 681-bp *H. francisci* type I light chain probe (10) that complements the variable (Vt) and constant (Ct) regions and cross-hybridizes with *Raja* type I light chain genes. HFV1 (288 nucleotides) and HFCt (303 nucleotides) complement the *Heterodontus* type I Vt and Ct regions, respectively. REVt (642 nucleotides) complements the *Raja* type I light chain, and REVc (260 nucleotides) complements the Vt region. PCR-generated probes were labeled by a modification (8) of the random hexanucleotide priming method (17) to a specific activity of 10^9-10^10 cpm/µg.

The primer pair SKVN-X (CCCTCTAGAATTCAGCTCT-GAATCTAA; XbaI linker) and SKCC-S (CCCGGACGCTGTCGCCGCCGGAAGATGTGAG; Stsl linker) was used to amplify by PCR a 1.6-kb fragment, which included the 972-bp J-C intervening sequence (IVS) of genomic light chain clone Λsk102 (the restriction site is indicated in boldface in these and subsequent primer structures). The 3' cDNA primer SKJC (ACAAGCTTGGGATGAGAACGGGTGTC; HindIII linker) was used with the SKVN-X primer to generate Vt-JL regions from genomic isolates. The primer pair RELCFR1 (AAAGCTTCGTCCTCCGGCGACCAGA; HindIII linker) and RELCFR3 (TCTAGACACGTTGCCTAGTGCTCA; XbaI linker) was used to generate the GPLC (genomic PCR-derived light chain; framework region 1 [FR1]-FR3 amplified from genomic DNA) clones (see Fig. 1). The primer pair RELCFR1 and SKLCJ (GAGCTCTTCAGAGTGTGCTCAGG; Stsl linker) was used to generate the FJ (FR1-JL amplified from genomic DNA) series, and the primer pair RELCFR2 (AAAGCTT-TATCGACAGCGTCCGG; HindIII linker) and SKLCJ was used to generate the FR2-JL series (FR2-JL amplified from genomic DNA). The rationale for the use of these various primer pairs is described below.

**DNA and cDNA Library Screening.** To identify several representative type I *Raja* light chain clones, a portion of the *Raja* genomic library (~280,000 PFU) was plated, lifted onto nitrocellolose, hybridized with the radiolabeled probe HFL (*Heterodontus* type I) in SET (0.6 M NaCl, 0.2 M Tris, 0.02 M EDTA, 0.1% SDS, 0.1% sodium pyrophosphate) at 65°C for 16-24 h, and washed in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, 0.05% sodium pyrophosphate at 52°C. The cDNA library (~160,000 PFU) was screened with HFL and REVc, as previously described.

**Genomic PCR.** Genomic DNA was used as a template to potentially amplify portions of the Vt-JL regions of all type I light chains in the germline using the primer pairs previously described. 1 µl of genomic DNA (3 µg/µl) from the same animal used in cDNA and genomic library construction was mixed gently with 9 µl of H2O, heated to 95°C for 7 min, and held at 80°C. PCR (10×) buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3, 15 mM MgCl2, 0.1% gelatin), dNTPs, primers, and 2.5 U of Taq polymerase (0.5 µl of 5 U/µl; Applied Biosystems, Inc., Foster City, CA) were added, followed by 35 cycles at 94°C, 1 min; 55°C, 2 min; 72°C, 4 min. The same conditions were used in parallel analyses in which the template was omitted. This served as a control to rule out potential reagent-based contamination. PCR products were extracted with phenol-chloroform, precipitated with ethanol, digested with appropriate restriction enzymes, ligated into M13 mp18, plated, and selected by absence of LacZ expression and by dot blot hybridization under relaxed stringency to the REVc probe. To obtain the opposite direction strand for sequencing, PCRs were performed using M13-specific primers. The resulting products were digested with appropriate restriction endonucleases and subcloned into M13 mp19.

**DNA Sequencing and Analysis.** DNA sequences were determined by the dideoxynucleotide chain termination method (18) using [³²P]dATP and Sequenase® (United States Biochemical Corp., Cleveland, OH). Oligodeoxynucleotide 18-20-mer primers were used to extend sequences. Analyses of sequences were performed using commercially available software (IntelliGenetics Suite™; IntelliGenetics, Palo Alto, CA), including GEL for managing the sequencing projects and IFIND and GENALIGN for sequence comparisons. All sequences contained herein have been submitted to

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1 Abbreviations used in this paper: CDR, complementarity-determining region; FR, framework region; IVS, intervening sequence; RA G, recombination-activating gene.
GenBank and assigned accession numbers U19001-U19025 for the FJ series, U19045-U19097 and U19203 for the GPLC series, U19187-U19202 for the 27***/28*** series, U19204-19208 for the RRLE series, and U19209 for SK102.

The neighbor-joining tree was constructed using the MEGA (Molecular Evolutionary Genetic Analysis) program kindly provided by Tatsuya Ota and Masatoshi Nei (Pennsylvania State University, University Park, PA; 19). The evolutionary distances between gene pairs over the region examined (codons 19-72, according to Kabat U19187-U19202 for the 27***/28*** series, U19204-19208 for the FJ series, U19045-U19097 and U19203 for the GPLC series, and U19209 for SK102).

Results

Complete Nucleotide Sequence of a Raja Type I Light Chain Gene Cluster. Initially, a Raja genomic library was screened with the heterologous (Heterodontus) type I light chain probe HFL and seven clones that were shown by partial restriction mapping to be unique (λskl02, Λ27101, Λ27102, Λ27103, Λ27104, Λ27105, and Λ27106) were selected. A 2.0-kb EcoRl-Pstl HFVt + fragment and a 0.6-kb EcoRl HFCr + fragment from one of the isolates (λskl02) were subcloned and sequenced. In addition, PCR was performed using Ask102 DNA as a template and the SKVN-X/SKCC-S primer pair (Fig. 1). A 1.6-kb fragment that overlapped the other fragments was identified and subcloned into M13, and the DNA sequence was determined.

The complete genomic sequences of the Vt and Ct segments of λskl02 are shown in Fig. 2. A typical split leader is noted, with the last three codons contiguous with FR1. The Vt, Jr, and Ct segments of λskl02 are in close linkage, exhibiting the cluster-type pattern of gene organization described by us earlier (22). The Raja type I light chain genomic locus as represented by λskl02 is Vt-Jr joined in the germ-line, unlike the Heterodontus type I light chain gene loci, which contain an ~350-bp Vt-Jr IVS (7). Furthermore, λskl02 does not contain the typical light chain regulatory octamer (ATTTGCA) present 134 bp 5' of the putative start codon in the Heterodontus type I light chain gene locus (7). An octamer-like sequence (ATTGGAAT; 7/8 match with consensus) is located 297 bp upstream of the putative start codon. The consensus enhancer elements (TCAATGTG and CAGATG), which are found within the Heterodontus Jr-Cr IVS (7), are not present in the Jr-Cr IVS of λskl02. However, an octamer-like sequence (ATTGCAAG; 7/8 match with consensus) and its reverse complement (TTGCAAAT; 7/8 match with consensus) are located in the Jr-Cr IVS, in positions similar to those of the putative enhancer elements found in Heterodontus. The functional significance of these motifs is uncertain.

Raja Type I Light Chain Gene Clusters Are Vt-Jr Joined in the Germine. Several different approaches were used to determine the nature, extent, and degree of sequence diversity of the type I light chain gene family. PCR analyses were performed on the other six genomic light chain clones that were isolated initially (λ27101, λ27102, λ27103, λ27104, λ27105, and λ27106) using the primer pair SKVN-X/SKCC-S (Fig. 1) to assess whether they are germline joined. An ~1.6-kb product was detected in each case, consistent with the Vt-Cr linkage distance determined by direct cloning and sequencing for λskl02 and thereby with germline joining of Vt and Jr. An additional 32 clones were identified and isolated from a Raja genomic library based on hybridization to HFL under low stringency conditions (14). Similar PCR analyses of each using the SKVN-X/SKCC-S primer pair yielded only ~1.6-kb products. An ~400-bp product was amplified from each of the second group of 32 clones (the SKLCGEN series) using the SKVN-X and SKJC primers. Sequence analyses of the products confirmed that each of these genes is Vt-Jr joined and that 17 of the isolates are unique (i.e., they can be distinguished by at least a one nucleotide sequence difference), as shown in Fig. 3. All of the sequences are 413 bp in length, with the exception of λ28402, λ28423, λ28417,

Figure 1. Approximate locations of PCR priming sites used to obtain the full nucleotide sequence of λskl02 and to generate the SKLCGEN, GPLC, FR2-Jr, and FJ series clones. Schematic representation of PCR primers and products in relation to the genomic type I light chain locus of Raja. The top portion of the figure represents an entire genomic locus. SKVN-X complements the Vt, FR1 region, and SKCC-S complements the end of the constant region, based on the nucleotide sequence of clone λskl02. The Vt region is expanded in the lower portion of the figure to depict the regions complemented by the primers RELCFR1, RELCFR2, RELCFR3, SKLCJ, and SKJC. The GPLC series clones (235 bp) are PCR products from genomic DNA primed with RELCFR1 and RELCFR3. The FR2-Jr series clones (242 bp) are PCR products from genomic DNA primed with RELCFR2 and SKLCJ. The FJ series clones (318 bp) are PCR products from genomic DNA primed with RELCFR1 and SKLCJ. The SKLCGEN series clones (413 bp) are PCR products from genomic library isolates primed with SKVN-X and SKJC.
Figure 2. Nucleotide sequence of XSK102. The predicted amino acid regions. The start codon (ATG), octamer-like sequence, and enhancer-like sequence is shown above the second nucleotide of each codon in the coding region.

and A28406, which possess one fewer codon in the J_1 segment. In addition, A28402 is truncated in the J_1 region. Five of these genes (A28425, A28403, A27104, A28401, and A28409) appear to be pseudogenes, owing to the presence of two shared mutations—one in FR2 and one in FR3, that result in stop codons. A sixth putative pseudogene, A28406, has a stop codon at the 3' end of the constant (C_2) region is denoted by a period.

Previous studies have shown that PCR has a strong bias toward amplification of smaller products, i.e., germline-joined templates are amplified preferentially over unjoined templates even when the overall length of the product varies by <350 bp (reference 23 Anderson, M. K., and G. W. Litman, unpublished observations). In an attempt to circumvent or at least minimize PCR length bias and possibly detect unjoined type I light chain genes, the primers RELCFR2 and SKLCJ were used to amplify the CDR3-J_1 junctions and the surrounding regions from genomic DNA. If a typical (Heterodontus-like) type I light chain gene IVS had been present, the product would have been ~600 bp; however, a single 242-bp fragment was recovered, consistent with uniform germline joining, which subsequently was established by the subcloning and sequencing of four different clones. Notably, each of these was identical in overlapping regions to type I genes selected by different methods, i.e., PCR priming of genomic DNA and λ clones (data not shown).

Genomic DNA was also subjected to PCR amplification using the primers RELCFR1 and RELCFR3 (Fig. 1). This strategy was directed at examining the diversity of germline type I V_1 genes, irrespective of joining status. The high degree of nucleotide identity in the sequences chosen for primer design (Fig. 3) between independently isolated genomic library clones (the initial selection via library screening was based on as little as 60% nucleotide identity) provided a strong basis for potentially priming all genomic type I light chain loci. DNA sequence analyses of the 235-bp PCR product(s) amplified from genomic DNA (the GPLC series) using these
primers resulted in the identification of 54 unique clones in 127 informative cloning events. 14 of these sequences were represented by more than one clone. The GPLC sequences are at least 90% related from FR1 to FR3 to the other longer clones obtained by direct selection from the genomic library (17 unique sequences) or through priming of genomic DNA with RELCFR1 and SKLCJ (25 unique sequences).

As has been emphasized, there is considerable overlap in the three series of isolates, consistent with a representative selection/amplification of an extended gene family. The nucleotide sequence of GPLCL103 matches those of λ27103 and λ28405. GPlCl8 matches FJ36 and λ27101. GPlCl13 matches FJ26 and FJ20. GPLC55 matches FJ33 and FJ17. GPLCl0 matches FJ19 and FJ35, and GPLC82 matches FJ18. Assuming that overlaps between different clones isolated in different ways indicate independent amplification of the same gene, there are 89 type I light chain germline-joined genes that differ by at least 1 bp. Out of these 89 genes, only 7 appear to be pseudogenes (λ28402, λ28406, and GPLC73) because of in-frame stop codons resulting from point mutations.

A neighbor-joining tree constructed from an alignment of 93 FR1–FR3 region nucleotide sequences of representative unique genes from each series (Fig. 6) reveals (a) that since GPLC, FJ, and SKLCGEN clones are intermixed to different degrees in the different clusters, the relatedness of the genes is not a function solely of the method by which they were isolated; and (b) that sequences segregate into four major clusters, the first of which is subdivided into three smaller clusters. One of these consists of only a single representative (GPLC33). The fourth cluster includes five (λ28401, λ28409, λ27104, λ28425, and λ28403) of the seven pseudogenes identified in this study. These putative pseudogenes are identical to FR1–FR3.

Comparison of Genomic and cDNA VL–Jℓ Sequences. To determine whether any of the type I genes are transcribed,
The clones RRLC18, RRLC8, RRLC47, RRLC50, and RRLC36 were selected for analysis. The five cDNAs were compared with the consolidated, 89-member data base of GPLC, Fj, and SKLCGEN sequences (Fig. 7). An exact match was identified between the overlapping portions of cDNAs RRLC8, Fj18, and GPLC82. Similarly, an exact match was identified between the overlapping portions of RRLC47 and GPLC55. RRLC47 exhibits a 1-bp mismatch with Fj7 and with Fj33 in the regions that overlap. RRLC36 differs from GPLC82 by 2 bp and from Fj18 by 3 bp. RRLC50 exhibits a 3-bp mismatch with GPLC10 and a 4-bp mismatch with Fj35. RRLC18 differs from GPLC82 by 1 bp and from Fj18 by 11 bp. These data are consistent with the transcription of at least two germline joined type I light chain genes. The other genes most likely represent somatically mutated forms or could represent allelic or pseudoallelic forms of these genes.

**Discussion**

In mammals, combinatorial diversity results from the different recombination possibilities presented by tandemly arrayed segmental elements. Somatic joining of these elements is associated with the generation of diversity through both nucleotide deletions and nontemplated additions at the joining boundaries. Cartilaginous fish possess multiple clusters of immunoglobulin gene loci that recombine within but not between clusters (23), limiting combinatorial diversity. However, extensive junctional diversity and at least some degree

### Table: Alignment of regions between but not including RELCFR1 and SKLCJ primers of FJ clones, derived from genomic PCR, and SKLCGEN clones, derived from genomic library isolates.

| FR1 | CDR1 | FR2 | CDR2 | FR3 |
|-----|------|-----|------|-----|
| FJ3 | -    | AC  | G    | C   |
| FJ4 | -    | AA  | A    | A   |
| FJ11| -    | AC  | C    | C   |
| FJ19| -    | AC  | A    | A   |
| FJ27| T    | AT  | G    | C   |
| FJ35| -    | AC  | A    | A   |
| FJ4 | -    | GG  | C    | C   |
| FJ5 | -    | TT  | T    | T   |
| FJ6 | -    | TA  | G    | C   |
| FJ12| -    | GG  | C    | C   |
| FJ36| -    | AC  | A    | A   |
| FJ3 | -    | GG  | C    | C   |
| FJ7 | -    | CT  | G    | C   |

**Figure 4.** Alignment of regions between but not including RELCFR1 and SKLCJ primers of FJ clones, derived from genomic PCR, and SKLCGEN clones, derived from genomic library isolates. *Dashes* = nucleotide identity with the consensus sequence; *upper case letters*, nonsynonymous substitutions; *lower case letters*, synonymous substitutions. The FR, CDR, and J regions are denoted at the beginning of each region and are set apart from one another by a space. The predicted amino acid sequence of the consensus nucleotide sequence is shown below the second nucleotide of each codon. Note the abundance of substitutions in the CDRs as compared with the FRs.
of somatic mutation have been demonstrated in rearranged immunoglobulin heavy chain clusters of the most intensively studied cartilaginous fish, H. francisci (23). An apparent second limitation in diversity arises from varying degrees of germline joining of ~50% of the heavy chain loci in this species (6). Similar preliminary findings of germline joining of type I light chain genes in Heterodontus (11), Carcharhinus (12), and Raja (11) suggest that the phenomenon is widespread and functionally significant. We provide unequivocal evidence that at least 41 of the 89 unique type I immunoglobulin light chain genes examined in Raja are germline VL-JL joined, in contrast to the absence of joining in all 63 type I VL+ gene clusters that have been characterized in Heterodontus (7). Other experiments directed at amplifying potential V1-JL intervening sequences in Raja failed to identify unjoined genes. Finally, we provide evidence that is consistent with transcription of at least some germline-joined genes, suggesting some physiological relevance. Immunoglobulin light chain clusters in Raja potentially represent an immunoglobulin gene system in which extensive germline diversification compensates for the loss of combinatorial and junctional diversity.

Although these studies did not result in the cloning and sequencing of all type I VL genes, it is likely that a representative sampling of type I light chain genes has been achieved. Specifically, 17 individual clones were identified and isolated from a genomic library using an approach that is capable of identifying genes that are only 60% related to the probe sequence. The ~90% relatedness between the overall sequences of these 17 clones indicates an absence of family divergence in VL regions, a finding entirely consistent with previous reported studies of Heterodontus VL genes, which are ~90% related. In addition, PCR primers designed on the basis of conserved regions of these 17 clones were used to amplify portions of VL-JL genes directly from genomic DNA to identify genes that had not been incorporated in the library. Significantly, all of the genes amplified by the various primer pairs are related closely in nucleotide sequence to each other and to the genomic library isolates. Although all of the PCR products spanning the VL to JL regions are germline joined, it is possible that unusually long VL-JL IVSs exist that would not be identified using these PCR strategies. However, the FR2-JL primers, which should have been able to amplify a Heterodontus-like (unjoined) type I light chain, because of the relatively short IVS characteristic of this type of gene, yielded germline-joined amplification products. If any unjoined type I clusters exist, they either must be present in very low abundance (relative to the joined type), possess extremely long VL-JL IVSs, or differ appreciably from the joined genes in the primer regions. However, the identity between germline genes and cDNAs described in the next discussion is inconsistent with the last possibility.

The germline-joined state of the type I light chain genes in Raja results in a loss of junctional diversity, in addition to the lack of combinatorial diversity, because of the clustering of the type organization of the gene loci. However, there are several lines of evidence which suggest that junctional diversity is not as important in the generation of light chain diversity as it is in generating heavy chain diversity, both in mammals and in the more phylogenetically distant vertebrates. For example, significant limitations in both nontemplated (N) additions and deletions at the VL-JL coding joints have been demonstrated in mammalian V\(_{\kappa}\) (24) and V\(_{\lambda}\) (25) genes. In addition, CDR3 length and variability are more limited in both murine and human light chains as compared with heavy chains (26). Other systems, including the avian (4) and ovine (27) V\(_{\kappa}\) genes, are diversified primarily by gene conversion and somatic point mutation, respectively. Perhaps the high level of somatic diversification associated with the presence of the D segment(s) in heavy chains provides a strong selective advantage for recombination of these genes. In the absence of D segments, the adaptive advantage of gene rearrangement in the generation of light chain diversity would be markedly reduced. There are no described cases of an entirely germline-joined heavy chain isotype, whereas this and
previous studies (11, 12) provide strong evidence that two types of light chain genes are entirely germline joined, at least within a species. It is therefore conceivable that the level of germline heterogeneity in the *Raja* type I V\(_r\)-J\(_r\) segments, especially in combination with somatic mutation mechanisms, compensates for the lack of both combinatorial and junctional diversifying mechanisms in this system.

The degree of germline diversity of the type I V\(_r\)-J\(_r\) segments is a function of the number of unique genes, the amount of nucleotide identity between these genes, and the regional distribution of the differences. Although the 89 unique type I light chain V\(_r\) (FR1–FR3) genes described in this study indicate a high level of germline-encoded heterogeneity (i.e., there are only 32 potentially functional human V\(_r\) genes [28]), many of these genes differ from each other by only 1–2 bp. Although these 1–2-bp mismatches may indicate Taq polymerase or Sequenase misincorporation, previous studies (28), including a large study from our laboratory (29), have indicated that such errors are negligible (~1/1,800) over relatively short nucleotide sequences and are inconsistent with repeated instances of recovery of identical sequences using different isolation and detection methods. These minor differences also could potentially represent allelic or pseudoallelic forms, although there is no a priori reason to assume that such differences would be associated with small versus large differences. Furthermore, these differences predominate in the CDRs and favor replacement substitutions, both of which are functionally significant.

By analogy to higher vertebrate immunoglobulin, the clustering of nucleotide sequence differences in CDRs would maximize variability in antigen-binding regions (30). Such nonsomatic variation would be subject to direct evolutionary selection, unlike the rearranged immunoglobulin genes of higher vertebrates, which are selected indirectly at a somatic level. The higher levels of variability at all three CDRs indicates that positive Darwinian selection may be acting specifically at these regions to maintain heterogeneity, which has been shown to occur also in mammalian CDR1 and CDR2 regions (31). Many of the substitutions in both the FRs and the CDRs occur in adjacent positions as blocks, similar to those observed in human \(\kappa\) genes (28). Such blocks are typically indicative of genes that were derived from duplication and modification of an ancestral gene. However, the distribution of these blocks among the different regions of different genes indicates that they have not arisen solely by the process of evolutionary duplication and diversification. It also is unlikely that this distribution is a result of recombination of multiple templates during the PCR amplification since the sequences do not represent simple hybrids of known genes and some of these genes are represented independently by closely related cDNA sequences. Furthermore, the extension time of 4 min should be long enough to limit recombinants to <2% (32). In addition, one of the clones identified in the direct genomic library screening (λ27101) exhibits this type of distribution; i.e., in the FR1, CDR1, and CDR3 of λ27101, blocks of nucleotide sequence are shared with λ28402, λ28423, λ28417, and λ28406, whereas in CDR2, λ27101 does not share blocks with these clones, but instead shares blocks with λ28429, λ28412, λ28405, and λ27103. It seems more likely
that these genes are templates for multiple germline gene conversion events.

Although both sharks and skates exhibit ~50% germline joining in the IgM-type heavy chain gene clusters, it has not been possible to demonstrate that these genes are expressed. The identical sequences shared by two cDNAs (out of five) are indicative of alleles or pseudoalleles, as indicated previously. The existence of multiple closely related gene loci; (b) the absence of approaches for obtaining purified lymphoid cell preparations or cell lines; and (c) the absence of a method for distinguishing joined genes from lymphoid versus nonlymphoid sources. Currently, the most reliable way to distinguish these events in lymphoid cells involves comparison of genes from a nonlymphoid source with cDNAs, which should be representative of genes from lymphoid sources. Comparisons between the cDNAs and the germline-joined genes in and around the mismatched nucleotides do not provide evidence for somatic gene conversion events in the Raja type I light chain gene loci, but rather are consistent with point mutation, although the lack of exact matches between some cDNAs and the germline-joined genes defined in this study also could be explained by the presence of additional germline-joined genes that were not amplified by PCR or integrated into the genomic library. The absence of an octamer in the region 100–150 bp 5' of the putative start codon distinguishes the Raja gene from both the Heterodontus type I light chain genes (7), which are not joined, and the Heterodontus and Raja type II light chain genes (11), which are germline joined. This difference suggests that these genes are under a type of control other than that normally associated with vertebrate light chains. Examination of additional 5' and 3' sequences will help clarify this issue.

The available evidence suggests that germline-joined genes represent a derived rather than a primordial arrangement of segmental elements. The existence of unjoined TCR and immunoglobulin heavy chain genes in cartilaginous fish (6, 33)

| Figure 7. Alignment of the Raja type I light chain cDNAs and the type I V<sub>λ</sub> germline-joined genes with which they share the greatest nucleotide sequence identity. Each cDNA is aligned with the genomic sequences from each group (i.e., SKLCGEN or FJ, and GPLC) with which it has the least number of mismatches. The top lines of each set are genomic sequences, the second lines represent the cDNA sequences, and the bottom lines are other closely matching genomic sequences. Dashes indicate nucleotide identity; FR, CDR, and JL regions are indicated at the beginning of each sequence identity. Each cDNA is aligned with the genomic sequences from each group (i.e., SKLCGEN or PJ, and GPLC) with which it has the least number of mismatches. The top lines of each set are genomic sequences, the second lines represent the cDNA sequences, and the bottom lines are other closely matching genomic sequences. Dashes indicate nucleotide identity; FR, CDR, and JL regions are indicated at the beginning of each sequence identity. Each cDNA is aligned with the genomic sequences from each group (i.e., SKLCGEN or PJ, and GPLC) with which it has the least number of mismatches. The top lines of each set are genomic sequences, the second lines represent the cDNA sequences, and the bottom lines are other closely matching genomic sequences. Dashes indicate nucleotide identity; FR, CDR, and JL regions are indicated at the beginning of each sequence identity. Each cDNA is aligned with the genomic sequences from each group (i.e., SKLCGEN or PJ, and GPLC) with which it has the least number of mismatches. The top lines of each set are genomic sequences, the second lines represent the cDNA sequences, and the bottom lines are other closely matching genomic sequences. Dashes indicate nucleotide identity; FR, CDR, and JL regions are indicated at the beginning of each |
Heterodontus more, the presence of unjoined type I light chain genes in heavy chain, and light chain genes was unjoined. Furthermore, the presence of unjoined type I light chain genes in Heterodontus indicates that the type I light chain gene in the hypothetical common cartilaginous fish ancestor was unjoined, whereas all type II light chain genes appear to be joined. Therefore, it seems likely that two independent events occurred during evolution, possibly involving in part the activation of a recombination-activating gene (RAG) homologue in germline cells, resulting in the joining of type II light chain genes at one early time point (affecting all cartilaginous fish), and the joining of type I light chain genes at another time after the divergence of the sharks and skates. A RAG homologue has recently been identified in a cartilaginous fish (34). The hypothesis that the joining of type I and II light chain genes represents independent events is supported by the finding of variability in length in CDR3 of type II, but not type I, light chain genes. Specifically, the type II light chain genes of Garcharhinus (12), Raja, and Hydrolagus (ratfish) (11) exhibit CDR3 segments of varying lengths within each species (5–12 predicted amino acids); all of the type I light chain genes examined in this study possess CDR3 segments of 8 predicted amino acids. Moreover, this difference may reflect variation in the lengths of the original recombining segmental elements, or it may reflect specific types of joining processes, one of which (type II) resembles somatic rearrangement in the addition and/or deletion of nucleotides at the junctional boundaries. It has been suggested that the restrictions in CDR3 length observed in α/β TCR genes are caused by functional constraints in their recognition of MHC–antigen complexes, whereas the immunoglobulin genes and the γ/δ TCR genes are less restricted, since their specificities are not MHC restricted (26, 35). Therefore, it is possible that the difference in CDR3 length variability between type I and II light chain genes reflects varying affinities and specificities for distinct epitope types. Precommitted specificities presumably conferred a selective advantage to the germline-joined genes, resulting in the eventual loss of unjoined light chain genes.

It would be very informative to relate the Raja sequences described here to serum light chain proteins to determine the usage of type I versus type II light chains, particularly since both type I light chains described in this study and type II light chains described previously (11) may exist only in the germline-joined form. These two light chain types are ~36% related at the predicted peptide level and appear to be transcribed at similar levels in the spleen (11); i.e., equivalent numbers of type I and II genes are identified in library screening. Although unequivocal distinction of type I and II light chain genes is difficult, owing to intrinsic peptide heterogeneity, preliminary peptide analyses of serum immunoglobulin (pooled) light chains suggest the expression of both type I and type II light chain genes (Anderson, M. K., unpublished observations).

Weighing the relative contributions of various diversifying mechanisms in the context of overall immune function in lower vertebrate species is complicated. Although at first consideration the phenomenon described here appears to limit diversity, it needs to be recognized that (a) out-of-frame joinings, which could eliminate two out of three recombinations, do not occur; (b) potentially deleterious self-reactive antibodies would be selected against; (c) these genes would be ideally suited for a situation in which selection mechanisms, particularly complex cellular interactions associated with antigen recognition and expansion of antibody-producing clones, may not be present or may be inefficient; and (d) there are far larger numbers of gene loci compared with mammalian systems. In addition, the amount of somatic mutation operating in this system is still unknown and may provide a significant amount of additional diversity. In this regard, further examination of how antigen receptors are expressed on immunocytes of cartilaginous fish is critical to understanding the net functional consequences and selective advantages of the unique form of germline joining described here and the independent multicluster form of gene organization that encodes other types of antigen receptors in these species.

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