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Immunology

Isolation of a shark immunoglobulin light chain cDNA clone encoding a protein resembling mammalian κ light chains: Implications for the evolution of light chains

(cartilaginous fish/λ light chains/phylogenetic trees)

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ABSTRACT **The time of emergence of immunoglobulin κ and λ light (L) chains in evolution is unknown. An L chain cDNA clone was isolated from the nurse shark (*Ginglymostoma cirratum*), a cartilaginous fish, whose predicted variable (V) region amino acid sequence has up to 60% sequence identity to mammalian $V_κ$ domains. Genomic analyses suggest a cluster-type gene organization for this L chain locus, similar to the shark λ-like immunoglobulin L chain loci rather than mammalian κ loci. We propose that divergence of the ancestral L chain into isotypes likely occurred before the emergence of elasmobranchs 400–450 million years ago. Similarities in gene organization between the two isotypes in sharks may reflect the gene organization utilized by the ancestral L chain.**

Immunoglobulins are the central effector proteins in the humoral adaptive immune system and are composed of two heavy (H) and two light (L) chains. In mammals, immunoglobulin L chains are designated as κ or λ, which are believed, on the basis of sequence similarities, identical domain structures, and a common function, to have arisen from an ancestral L chain gene (1). However, the timing in evolution of divergence into κ and λ isotypes is unknown, since L chains in nonmammalian vertebrates have not been studied extensively. Chickens have L chains apparently only of the λ isotype (2). Two L chains have been characterized in the amphibian *Xenopus laevis*; one appears κ-like (3, 4), and the other is not more related to mammalian κ or λ (5). The L chain constant (C) domain of another amphibian, *Rana catesbeiana*, cannot be clearly classified as $C_κ$ or $C_λ$ (6). λ-like L chains are present in two species of sharks (7–9).

κ rearranging gene segments in mammals are arranged in clusters of variable (V) and joining (J) segments upstream of a single C (reviewed in ref. 10), while λ loci in BALB/c mice and humans have either multiple or single V segments upstream of J-C clusters (11–14). However, not all L chain loci of nonmammalian vertebrates are organized as are those of mammals. For example, chicken B cells have single V and J segments, with diversity achieved by gene conversion events employing V pseudogenes upstream of the functional V (15, 16). Shark λ-like L chain loci are organized in clusters of V-J-C, with the rearranging segments linked tightly within each cluster (8, 17).

The finding of only λ-like L chains in sharks led to the hypothesis that only one L chain isotype would be found in representatives of primitive vertebrate classes (7). Furthermore, the presence of λ-like L chains in a chondricthyian led to the suggestion that λ may have been the first isotype to emerge in evolution (9). However, the presence of other isotypes in sharks was not ruled out (9, 18), and phylogenetic trees of all described κ and λ suggested that the ancestor of sharks and all other vertebrates possessed both isotypes (4). We report here the isolation of an L chain cDNA clone from the nurse shark, *Ginglymostoma cirratum*.¶ The predicted V region amino acid sequence is similar to mammalian $V_κ$ (60% identity). This L chain differs from all other κ described in that it has a cluster-type gene organization similar to the shark H (19) and λ-like loci (8, 17).

MATERIALS AND METHODS

Oligonucleotides and PCR. The following three oligonucleotides were used for amplification of nurse shark cDNA: the immunoglobulin-superfamily-specific CB2 primer (described in *Results*; 5′-GCGAATTCAARGCNACNCTBGTNTG-3′, where N represents A, C, G, or T, R is A or G, and B is G, T, or C), an adapter primer (5′-GACTCGAGTCGACATCG-3′) (20), and a $(dT)_{17}$ adapter primer (5′-GACTCGAGTCGA-CATCGATTTTTTTTTTTTTTTT-3′) (20). The first eight bases of CB2 contain an *Eco*RI recognition sequence. Preparation of poly(A)$^+$ RNA and its reverse transcription with the $(dT)_{17}$ adapter primer were done as previously described (21). The PCR mixture (50 μl) contained 3 μl of cDNA, 200 μM dNTPs, 0.5 μM CB2 primer, 0.5 μM adapter primer, 5 μl of 10× reaction buffer (Stratagene), and 2.5 units of *Taq* DNA polymerase (Stratagene). The amplification protocol used was three cycles of 1 min at 94°C, 2 min at 37°C, and 3 min at 72°C with a ramp time of 4 sec/°C as suggested in ref. 22; followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with a final extension of 10 min at 72°C. Second-round PCR was performed on the original PCR product, and bands which reamplified with both CB2 and adapter primers were gel purified and then treated with Geneclean (Bio 101) and subcloned in the *Eco*RV site of pBluescript II SK (+) (Stratagene). V (nt 106–342 in Fig. 1) and C region-specific (nt 418–732) probes were also generated by PCR, and oligonucleotides present in the V segment (nt 106–123) and complementary to the J segment (nt 379–396) were used to amplify the fragment separating V and J segments in the genomic clones.

Southern Blotting and cDNA Library Screening. For Southern blot analyses (23), 10 μg of genomic DNA, isolated from erythrocytes according to a standard protocol (ref. 24, pp. 9.16–9.19), was subjected to restriction enzyme digestion (Boehringer Mannheim) and electrophoresed on a 0.8% agarose gel. Prehybridization and hybridization were performed at 42°C in the same solution [50% (vol/vol) formamide/6× SSC/5× Denhardt's solution/0.3% SDS, and denatured salmon sperm

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Abbreviations: V, variable; C, constant; J, joining; L, light; H, heavy; PIR, Protein Identification Resource.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L16765).

DNA at 100 μg/ml (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)], with hybridization carried out for at least 16 hr. The membrane was washed in 2× SSC/0.1% SDS for 5 min twice at room temperature and then in 0.2× SSC/0.1% SDS for 20 min twice at 65°C. Twenty-five nanograms of the C region probe was labeled with [α-^{32}P]dCTP by using a Random-Primed labeling kit (Boehringer Mannheim). The V region probe was labeled by using a V-specific oligonucleotide (nt 106–123) as a primer instead of random hexamers to increase the specific activity (this probe labels poorly when random hexamers are used, probably because of its small size) (25). The hybridization conditions used for screening a nurse shark spleen cDNA library were the same as above, and the filters were washed three times with 0.2× SSC/0.1% SDS for 20 min at 65°C before overnight autoradiography.

Preparation and Screening of Genomic Library. Erythrocyte genomic DNA was partially digested with *Sau*3AI, and the 17- to 22-kb fragments were ligated to the vector arms of LambdaGem-11 according to the supplier's instructions (Promega). Amplification of the genomic library was performed by using a standard protocol (ref. 24, p. 9.30), and approximately 900,000 plaques were screened with the insert of clone C16 (Fig. 1), using the conditions described above.

DNA Sequencing. Double-stranded DNA was sequenced by the dideoxynucleotide termination method (26), using Sequenase (United States Biochemical).

DNA Sequence Analysis and Phylogenetic Tree Construction. The Protein Identification Resource (PIR) data base (release 33.0) was searched by using the FASTDB program of IntelliGenetics. The construction of phylogenetic trees has been described previously (4, 27, 28). The amino acid sequences of C and V (not including J) regions of nurse shark L chain (Fig. 1) were aligned by the "progressive alignment procedure" (29) with the set of C and V sequences, respectively, used in refs. 4 and 28. The alignments were adjusted to position gaps between segments of β-sheets (3, 4, 28). The "parsimony after progressive alignment" procedure (30) was used to generate phylogenetic trees based on the aligned sequences. The DRAWTREE program (within PHYLIP 3.4) used to print the phylogenetic tree was supplied by J. Felsenstein (Univ. Washington, Seattle).

RESULTS

Isolation of Nurse Shark Immunoglobulin L Chain cDNA Clones. The amino acids preceding the first cysteine of immunoglobulin superfamily C1-SET members [T-cell receptor, immunoglobulin, and major histocompatibility complex (MHC) class I and II molecules] are well conserved (31). Therefore, we reasoned that oligonucleotides encoding this motif (underlined in Fig. 1) and ending in the second base of the cysteinyl codon would preferentially amplify immunoglobulin superfamily molecules of the C1-SET. Nurse shark spleen mRNA was reverse-transcribed with the $(dT)_{17}$ adapter primer and used for anchored PCR (20) with CB2 and adapter primers. An 850-bp product encoded a protein similar to L chain C domains when used in FASTDB searches of the PIR data base, and the PCR product was used to screen a spleen cDNA library (32). Approximately 200 positive

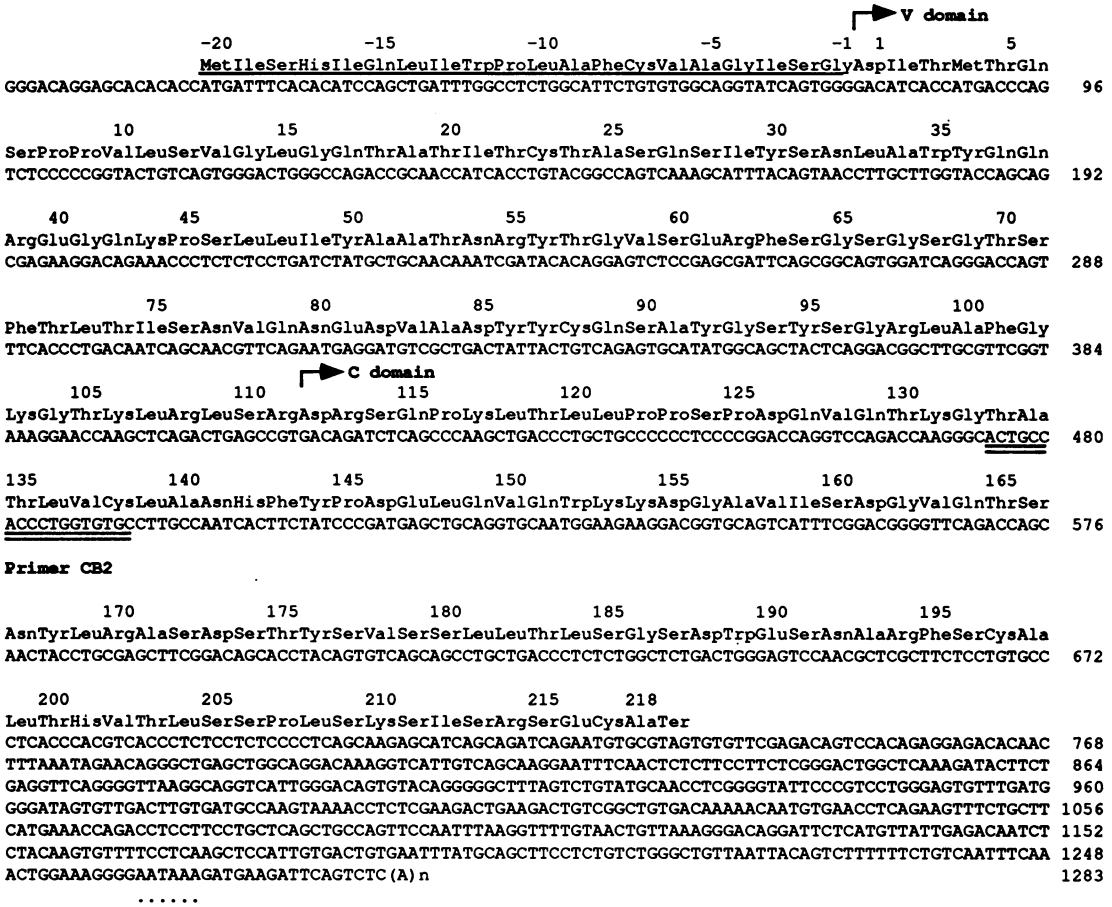

FIG. 1. Nucleotide sequence of clone C16. The deduced amino acid sequence is shown above the nucleotide sequence. The putative signal peptide is numbered from −20 to −1 and is shown underlined. The location of the primer used for PCR is shown as a double line, and a potential polyadenylylation signal (AATAAA) is dotted. (A)n, poly(A) tail.

plaques were obtained per 100,000 plaque-forming units, and 30 randomly picked positives were plaque purified.

The sequence of one of these clones, C16, is shown in Fig. 1. The size of this clone is 1283 bp [excluding the poly(A) tail] and is consistent with the transcript size determined by Northern blot analysis (data not shown). A polypeptide of 238 amino acid residues was predicted from the sequence; the first 20 amino acids probably constitute the signal peptide, reasoning on the basis of earlier N-terminal sequencing of nurse shark L chains (33). A methionine located at nucleotides 19–21 is most likely the translational start site, since it is embedded in Kozak's consensus sequence (34).

Amino Acid Comparisons. Previous phylogenetic analyses of immunoglobulin L chains revealed that V and C domains evolve independently (4, 28). The V domain (residues 1–111) was used for searches of the PIR data base; the top 200 scores were mammalian V_κ (10.06–14.02 SD) and were 57–60% identical. An alignment of the nurse shark V region with several L chain V sequences is shown in Fig. 2*A*. Amino acid identities among L chain V sequences were found mainly in the β strands (Fig. 2*A*). The nurse shark V region contains a phenylalanine at position 71, similar to that found in other κ chains (4, 37, 38). The nurse shark V region was only 47–50% identical to the 5 top scoring mammalian V_λ regions.

The C domain (residues 112–217) was used to search the PIR data bank, and the top scores were the C domain of the sandbar shark *Carcharhinus plumbeus* L chain (7) as well as C_κ and C_λ from several species. The C domain was not as similar to C_κ as the V domain was to V_κ (39–47% vs. 57–60%). An alignment of the C domain with the C domains of several L chains is seen in Fig. 2*B*. We tentatively concluded from these analyses that the nurse shark L chain cDNA clone is more related to κ than to λ, but the V domain shows greater similarity to κ than does the C domain. That the V domain is a more reliable indicator of isotype (or family) classification has been described previously for mammalian (39) and nonmammalian vertebrates (28, 40).

Phylogenetic Tree Construction. The relationship of nurse shark V and C regions to these regions of L chains in a variety of species was evaluated by constructing phylogenetic trees (Fig. 3). As shown in Fig. 3*A*, the mammalian C_κ and C_λ each form clusters, with the chicken C_λ on a branch near the branch containing the mammalian C_λ. The C_κ group is divided into two subclusters, the rabbit sequences lying in one and the human/mouse/rat sequences in the other. The C regions of the lower vertebrate species lie between the κ and λ branches. The three shark sequences form a cluster, and this cluster and the branch containing *Xenopus* L2 emerge near the center of the tree. It is not clear how this tree should be rooted and we cannot place the time of C_κ/C_λ divergence in relation to the time of divergence of the lower vertebrate species from the lineage that led to birds and mammals.

An unrooted phylogenetic tree depicting relationships among V sequences is shown in Fig. 3*B*. A striking feature of this tree is that the mammalian V_κ sequences form a distinct cluster, whereas the mammalian V_λ are much more diverse. As previously shown, V_λ appear to lie in three clusters, designated V_λI, V_λII, and V_λIII (4); the sequences in each V_λ cluster are as different from those in the two other V_λ clusters as they are from those in the V_κ cluster. It is clear from Fig. 3*B* that the nurse shark V region clusters with the mammalian V_κ. A similar clustering of the nurse shark V region sequence

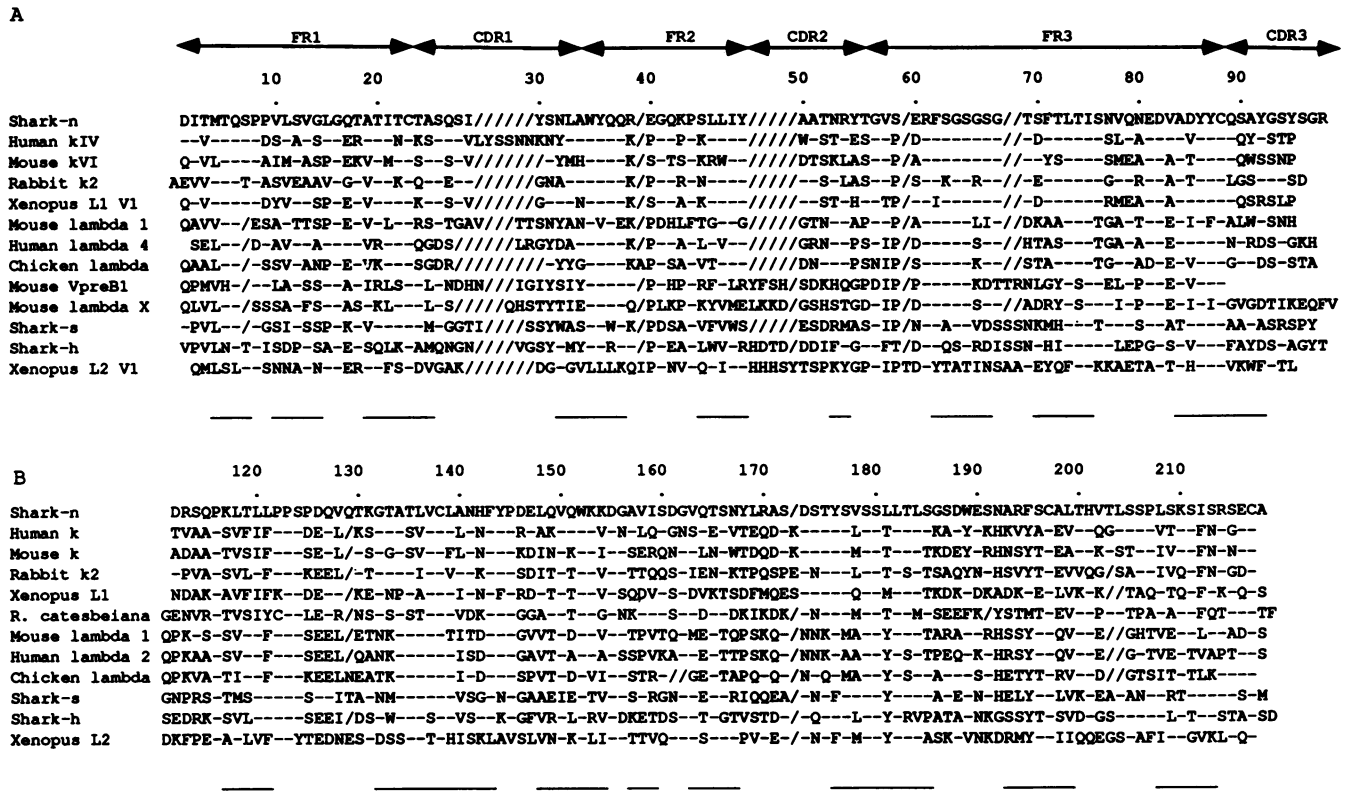

FIG. 2. Amino acid sequence comparisons between the nurse shark L chain and representative L chains from other species. (*A*) Alignment of the V regions. (*B*) Alignment of C domains. Alignments were performed as described in *Materials and Methods*. Gaps in the sequence generated by the alignment are shown as /, and amino acids identical with the nurse shark sequence are shown as –. The position of β-strands is indicated as solid lines below the alignments and is based on the crystal structure of J539 (35, 36). The boundaries of the framework (FR) and complementarity-determining regions (CDR) for the nurse shark L chain are shown above the V domain alignment (37). Shark-n, nurse shark; Shark-s, sandbar shark; Shark-h, horned shark. The source of the sequences can be found in ref. 37 (with the page and entry numbers given in refs. 4 and 28); sequences not given in ref. 37 are sandbar shark λ (7, 8), horned shark λ (9), *Xenopus* L2 V1 and L2 (5), and *R. catesbeiana* (6).

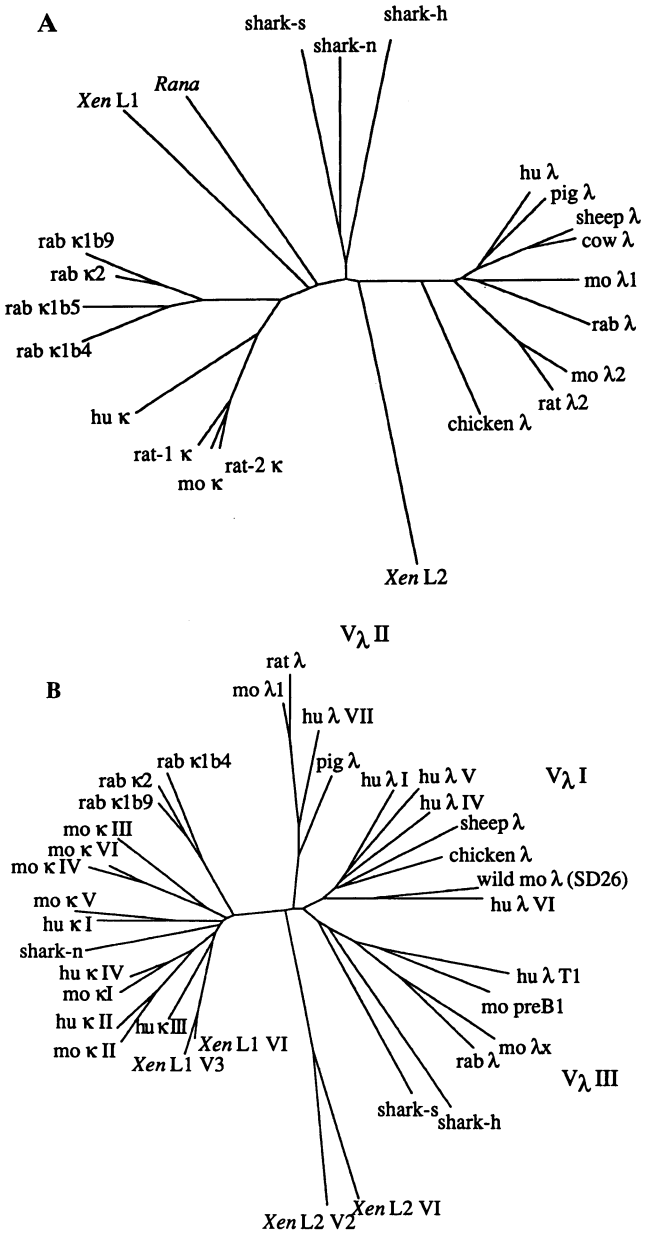

FIG. 3. Unrooted phylogenetic trees of L chain C (*A*) and V (*B*) sequences. The sequence alignments on which these trees are based are from refs. 4 and 28, with the addition of the nurse shark V and C sequences from this paper. The lengths of the branches are a measure of sequence divergence; the angles between branches are arbitrary. The branch order was modified to optimize the least-squares fit of distances along branches to the distance scores of pairs of aligned sequences. mo, Mouse; hu, human; Xen, *Xenopus laevis*; rab, rabbit; shark-h, horned shark; shark-n, nurse shark; shark-s, sandbar shark; Rana, *R. catesbeiana*. The source of the sequences not given in Fig. 2 is ref. 37 (with the page and entry numbers given in refs. 4 and 28) except for Xen L1 V1 and V3 (4), Xen L2 V2 (5), Xen L1 (4), Xen L2 (5), wild mouse λ (SD26) (27), and human λ T1 (41).

with V_κ sequences was observed (unpublished results) by using Nei's neighbor joining method with nonsynonomous substitutions (42, 43).

Gene Organization of the Nurse Shark L Chain. To determine the number of recombining gene segments, Southern blot analyses were performed with V and C region-specific probes. Many bands were detected with both probes (Fig. 4) for several restriction endonucleases. The existence of multiple bands hybridizing with a C probe suggested a clusterlike gene organization for this L chain; to confirm this, several V^+

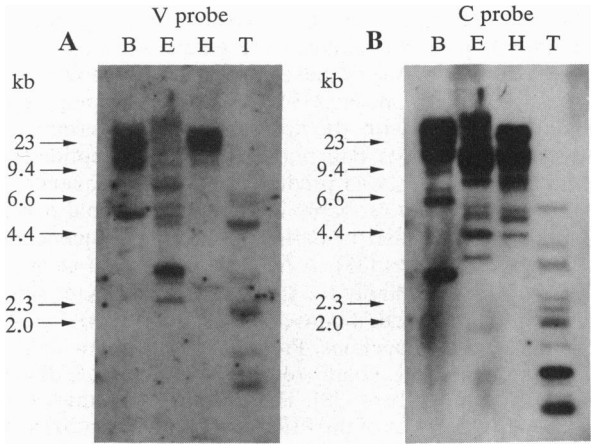

FIG. 4. Southern blot analyses using V and C probes. B, E, H, and T refer to digestion of genomic DNA with *Bam*HI, *Eco*RI, *Hin*dIII, and *Taq* I, respectively. The same membrane was sequentially hybridized with probes to either the V (*A*) or C (*B*) regions. Probe specific activities were 9.0×10^5 dpm/ng and 3.1×10^6 dpm/ng for the V and C probes, respectively. Size markers were generated by digestion of bacteriophage λ DNA with *Hin*dIII.

genomic clones with different restriction endonuclease patterns were isolated. PCR analysis with V- and J-specific oligonucleotides (see *Materials and Methods*) demonstrated that all of the V^+ clones contained J segments. In 8/9 clones, an amplified V-J fragment of 800 bp was observed (data not shown), implying that the V and J segments are only 500 bp apart; one clone gave an amplified product of 300 bp, which may represent a germ-line joined fragment. One V^+ C^+ hybridizing genomic clone was isolated out of 29 clones initially surveyed; therefore, the distance between the J and C segments may be greater than that described for the shark λ-like L chains (8, 17). These data suggest a $(V\text{-}J\text{-}C)_n$ cluster gene organization for this L chain, similar to that of shark λ-like L chains (8, 17). The orientation of the recombination signal sequences was determined by sequencing the region 5′ of the J segment for one of the genomic clones. The J segment is flanked by a heptamer-nonamer unit separated by 23 nucleotides (data not shown), similar to that described for other κ sequences (4, 10).

There are more bands that hybridize with the C than with the V probe (Fig. 4), and a greater number of V^- C^+ than V^+ C^- hybridizing genomic clones were isolated, suggesting that there may be V_κ gene families as in the mouse (44). However, under lowered stringency conditions (30% formamide), the V probe did not hybridize to the C^+ genomic clones or detect any additional bands by Southern blotting; we also have not identified cDNA clones with different V regions associated with the C region in clone C16 (data not shown). Thus, we favor the idea that there is only one nurse shark V_κ-like family but that there are some orphan C_κ gene segments not linked to V.

DISCUSSION

We describe the isolation of an L chain cDNA clone from the nurse shark that is similar to mammalian κ. The deduced amino acid sequence is nearly identical to the previously reported N-terminal sequence obtained with pooled nurse shark L chains (33); therefore, this L chain may be the predominant L chain in the nurse shark. The isolation of a κ-like L chain cDNA clone from sharks suggests that the divergence of the ancestral L chain into isotypes preceded the emergence of elasmobranchs some 400–450 million years ago (4, 28). Further studies of L chains in agnathans [if immunoglobulin exists in these vertebrates (45)] are needed to determine the nature of primordial L chain isotypes. Since

κ-like L chains are present in frogs and sharks, the failure to find κ in chickens is probably due to its loss or inactivation. Nonproductive rearrangements are not observed on the nonexpressed allele in the chicken λ locus (46), suggesting that the unusual manner of generating diversity has exerted a strong pressure on immunoglobulin loci to generate viable joins. Such a system over evolutionary time would likely render a second L chain locus unnecessary.

We have also recently isolated two additional nurse shark L chain cDNA clones; one is 80% identical at the amino acid level to the L chain described from the sandbar shark (8), and the other is 80% identical at the amino acid level to that described in the horned shark (17) (A.S.G. and M.F.F., unpublished data). The λ-like L chains characterized from the horned and sandbar sharks are only 40% identical to one another; it was originally believed that these L chains were unique to these two shark species and that the low sequence similarity reflected divergence from a common ancestral λ (7). In contrast to this idea, our data from the nurse shark suggest that all sharks will have the κ described here, as well as the two λ-like L chains. Furthermore, additional L chain classes may exist in elasmobranchs which are neither κ-like nor λ-like. We conclude that L chain class diversity is more extensive in representatives of primitive vertebrate classes than previously realized (7).

The gene organization of the nurse shark L chain is different from mammalian κ, yet the recombination signal sequences are similar to those described for mammalian κ rather than mammalian λ. That the cluster-type organization is seen for all immunoglobulin H and L chain classes in elasmobranchs indicates that the primordial rearranging receptor may have been clusterlike in its gene organization; in the absence of knowledge of the organization of T-cell receptor genes of primitive vertebrates, however, one cannot make firm conclusions. Our results also suggest that gene organizational pattern alone is not an accurate measure in predicting whether L chain isotypes are more κ- or λ-like.

The existence of at least one κ and two λ L chain classes in sharks raises interesting questions about whether there are hierarchies of rearrangement within the L chain loci (47). In mammals, κ deleting elements (48, 49) are thought to promote rearrangement of λ genes if κ loci have undergone nonproductive rearrangements. Considering the clusterlike genomic organization of all shark L chain genes, it is difficult to envisage how the same mechanism could be involved in regulating rearrangement. Indeed, since there seems to be no affinity maturation of the specific antibody response in sharks (50), it may be that there is no isotypic (and allelic) exclusion of shark L chain loci; i.e., each shark B lymphocyte may be capable of producing multiple L chains and thus may not be subjected to stringent clonal selection. Antisera specific for shark L chain isotypes should allow us to test this hypothesis.

Our data also shed light on the question of why different L chain isotypes arose in evolution. If shark H chain loci are reflective of the ancestral rearranging immunoglobulin receptor, then the presence of only one V_H family (51) may have selected for the emergence of more than one L chain isotype to increase diversity by H–L chain combinatorial pairing. Some of these L chain genes then evolved into canonical κ and λ.

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