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# Complete sequence of a cDNA clone specifying sandbar shark immunoglobulin light chain: Gene organization and implications for the evolution of light chains

(λ chain/phylogeny/*Carcharhinus plumbeus*)

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**ABSTRACT** **A full-length cDNA clone specifying sandbar shark (*Carcharhinus plumbeus*) immunoglobulin light chain has been isolated and sequenced. By alignment with human λ chains, the leader, framework, complementarity-determining, joining, and constant regions are clearly identified in the shark light chain. Approximately 40–50% identity is shared between the human and shark sequences in the variable and constant regions. We have performed sequence comparisons of the individual segments and constructed phylogenetic trees for the variable region. These studies identify the shark protein as a λ chain. In addition, the sandbar shark light chain is only distantly related to that of horned shark (*Heterodontus francisci*) [Shamblott, M. J. & Litman, G. W. (1989) *Proc. Natl. Acad. Sci. USA* 86, 4684–4688], demonstrating that the long evolutionary time of divergence among shark species has led to the generation of substantial differences in sequence. The positions of the variable, joining, and constant gene segments in 14 genomic clones have been mapped. The segments are linked in individual clusters (variable, joining, constant) occupying 3–7 kilobases. Cluster arrangement can be grouped into two patterns based upon spacing between the genes in the individual clones. This arrangement is fundamentally different from that observed in higher vertebrates.**

Serum immunoglobulins of primitive vertebrate species are polydisperse in sequence and no monoclonal gammopathies have been found. Consequently, it has been necessary to use recombinant DNA technology to isolate and characterize clones specifying individual polypeptide chains (1, 2). A knowledge of the structure and arrangement of shark immunoglobulin genes is of considerable importance to our understanding of the evolution of antibodies because ancestral elasmobranchs arose early in vertebrate evolution, dating back more than 400 million years (3).

Here we report the complete cDNA sequence of an immunoglobulin light-chain gene* of a carcharhine shark, the sandbar shark *Carcharhinus plumbeus*. Carcharhinformes are the dominant order of living sharks, comprising 55% of all shark species (4). They are an ancient group of vertebrates, with fossil carchinoids dating back to the Jurassic period (3). We use the primary data obtained here to construct a phylogenetic tree for the evolution of immunoglobulin light chains among vertebrate groups ranging from elasmobranchs to man and also to make a comparison with light chains of a distantly related shark, the horned shark *Heterodontus francisci* (2, 5). Comparison of these shark species allows us to make comparisons of homologous sequences of species whose ancestors must have diverged approximately 200 million years ago. Our data show that fundamental features of immunoglobulin light-chain sequence have been conserved in vertebrate evolution but also that major differences, particularly in the arrangement of the genetic elements, occur within major taxonomic groups of vertebrates, including the single class of elasmobranchs.

## MATERIALS AND METHODS

**Library Construction and Screening.** The cDNA library was constructed from spleen mRNA isolated from four sandbar sharks in the expression vector λgt11, as has been described (1). Genomic DNA was prepared from liver tissue of a single sandbar shark by standard methods (6). The genomic library was made commercially in the replacement cloning vector λFIX II by Stratagene.

Approximately $10^6$ plaques of the cDNA library were screened using the constant (C)-region probe Shlc3 (1). Hybridization was overnight at 65°C in 5× standard saline citrate (SSC)/5× Denhardt's solution (6)/0.5% SDS. Final washes in 0.1× SSC/0.1% SDS at 55°C represent moderate stringency conditions. One clone of sufficient size to be full length, Shlc5.1, was sequenced. After λ DNA purification (6, 7), the *Eco*RI insert was purified by HPLC on a Waters Gen-Pak FAX column and subcloned in pBluescript (Stratagene). DNA sequences were determined in both directions by the dideoxy method using $^{35}S$ label and Sequenase (United States Biochemical). A series of deletion subclones for sequencing covering the entire length of the insert were obtained using a Pharmacia double-stranded nested deletion kit.

The genomic library was screened at a density of approximately 50,000 plaque-forming units per plate using the C-region probe and two different 3′-untranslated-region probes (probes Untrans-1 and Untrans-4). Screening was performed using the Genius nonradioactive detection kit (Boehringer Mannheim) essentially as described by the manufacturer. High-stringency wash conditions (0.1× SSC/0.1% SDS at 68°C) were used.

**Synthesis of Probes.** Probes specific for shark light-chain variable (V) region, C region, and untranslated regions were prepared from cDNA clones previously described (Shlc1, Shlc3, Shlc4) (1) or from the cDNA clone described in this paper (Shlc5.1). The probes were prepared as follows: probe Shlc3, *Eco*RI fragment of clone Shlc3; V region, 5′ fragment of *Eco*RI/*Nhe* I-digested clone Shlc5.1; C region, 5′ fragment of *Eco*RI/*Nsi* I-digested clone Shlc4; Untrans-1 probe, 3′ fragment of *Eco*RI/*Bgl* II-digested clone Shlc1 corresponding to the 3′ untranslated region; Untrans-4 probe, 3′ fragment of *Eco*RI/*Nsi* I-digested clone Shlc4 corresponding to the 3′ untranslated region. The probes were purified by HPLC using a Gen-Pak FAX column. The joining (J)-region

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Abbreviations: V, variable; J, joining; C, constant; FR, framework region; CDR, complementarity-determining region.
*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M81314).

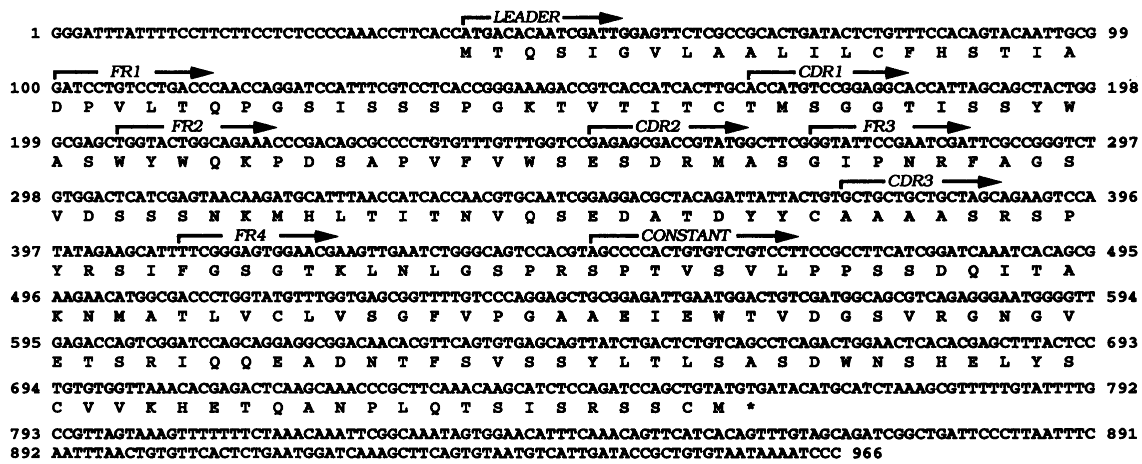


FIG. 1. DNA and corresponding amino acid sequence of full-length sandbar shark light-chain clone Shlc5.1. Leader, leader peptide sequence; FR1–FR4, framework regions 1–4; CDR1–CDR3, complementarity-determining regions 1–3; Constant, C-domain coding sequences.

probe was a mixed synthetic oligonucleotide (synthesized by Charlene Alford, Medical University of South Carolina, Charleston). Its sequence, 5′-AA(C/G)AAG(Y)TGRRYC-3′, was a consensus derived from the J-region sequences of clones Shlc1, Shlc3, and Shlc5.1.

Shlc3 was labeled using [$\alpha$-$^{32}$P]dCTP and a random-priming labeling kit (Boehringer Mannheim). Untrans-1, Untrans-4, C-region, and V-region probes were labeled using the Genius nonradioactive labeling kit. The J-region probe was labeled with dioxigenin-11-dUTP by using a tailing kit (Boehringer Mannheim) as recommended by the manufacturer.

**Southern Blots.** DNA was transferred to Immobilon-N poly(vinylidene difluoride) transfer membranes (Millipore) by a Posiblot pressure blotter (Stratagene) under alkaline conditions (0.4 M NaOH/0.6 M NaCl) and fixed to the membrane by baking at 80°C for 1 hr. Hybridization with DNA probes and development procedures were the same as those described for genomic library screenings. Blots screened with oligonucleotide probes were hybridized in 6× SSC/5× Denhardt's solution/0.5% SDS and washed in 6× SSC/0.5% SDS.

**Restriction Mapping of Genomic Clones.** Genomic clones were mapped for *Eco*RI, *Hin*dIII, and *Bam*HI restriction sites by using a nonradioactive gene-mapping kit (Stratagene) as recommended by the manufacturer.

**Identification of V, J, and C Gene Arrangements.** Fourteen mapped genomic clones were digested with *Eco*RI, *Bam*HI, and/or *Hin*dIII and analyzed by Southern blot hybridization with V, J, and C probes to identify the locations of these genes.

**Computer Analysis.** DNA sequence data were organized and analyzed using programs of the Genetics Computer Group (University of Wisconsin, Madison) (8) and the Protein Identification Resource (National Biomedical Research Foundation, Georgetown University Medical Center, Washington) (9). Programs for phylogenetic analysis by the progressive sequence alignment approach (10) were obtained from Russell F. Doolittle (University of California, San Diego).

## RESULTS AND DISCUSSION

**Isolation of a Full-Length Light-Chain cDNA Clone.** A full-length cDNA clone, sandbar shark light-chain clone 5.1 (Shlc5.1), was isolated from the λgt11 library by using a probe specific for the light-chain C region (probe Shlc3). Fig. 1 shows the complete cDNA sequence of clone Shlc5.1 as well as the derived amino acid sequence. The identity with some mammalian light chains at the protein level is 40–50%, and sequence alignment (Fig. 2) with these proteins allows the leader, V (including FRs and CDRs), J, and C regions of the shark light chain to be clearly delineated. The 3′ untranslated region is 197 nucleotides long with a single polyadenylylation signal sequence at its end. A second possible polyadenylylation signal sequence present in all other identified clones (1) is absent from this clone due to a T → C base change at position 817. A tryptic peptide obtained in high yield from purified polyclonal light chains (12) with sequence DPVLTQPGSISSSPGK is identical to a FR1 V-region segment predicted from Shlc5.1 gene sequence (Fig. 1, residues 100–148), demonstrating that this gene specifies a major serum V-region sequence. This FR1 sequence must therefore represent a segment highly conserved among sandbar shark light chains.

The C region of clone Shlc5.1 is extremely similar to previously characterized clones (1). Approximately 95% nucleic acid sequence identity is shared between all clones in the C region. A similar degree of identity is seen in the 3′ untranslated region of clones Shlc5.1, Shlc4, and Shlc6, but there is less than 60% identity in this region between these clones and clone Shlc1. Since the sequence differences are at

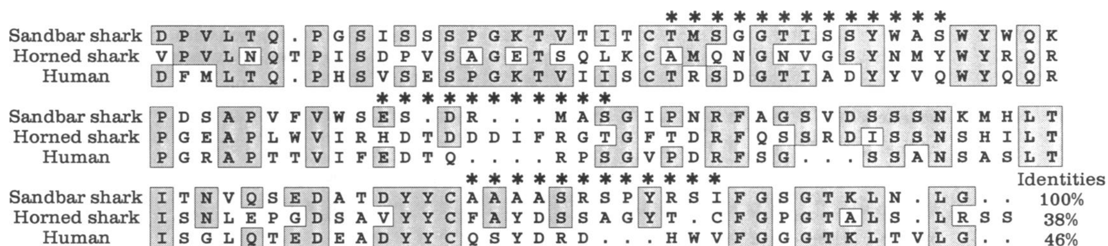


FIG. 2. Comparison of VJ sequences of sandbar shark $V_\lambda$, horned shark $V_\lambda$, and human $V_\lambda$VI. Horned shark sequence is from Shamblott and Litman (5) and human $V_\lambda$VI is from Kabat *et al.* (11). Asterisks, CDR; dots, gaps introduced to maximize alignment.

least 5%, it is apparent that these clones represent separate genes and not merely allelic differences.

**Phylogeny of Immunoglobulin Light Chains.** As we have previously discussed (1, 13), the shark light-chain C regions are most related to λ chains. This follows from a consideration of the greater overall sequence identities with λ chains (≈40%) than with κ chains (≈30%), the conservation of certain signature sequences (e.g., the sequence ATLVCL around Cys-134 is found only in λ chains), and an analysis of the phylogenetic relationships of light-chain C regions (13).

Similar arguments lead to the conclusion that shark V regions are also λ-chain V regions. Thus, the 20-amino acid leader sequence is most similar to other λ leader sequences, with its best match being to that of human $V_{\lambda}1$ followed by chicken light chain. As shown in Fig. 3, leader sequences of κ chains demonstrate lower homology to the shark leader sequence as compared with λ chains.

In data base searches, the best matches with shark V region are obtained with λ chains. Fig. 2 compares the VJ sequences of sandbar shark, human $V_{\lambda}VI$ (which gave the best data base match), and horned shark. The horned shark is a distantly related shark and is the only other elasmobranch for which light-chain sequence is available. In this alignment, human $V_{\lambda}VI$ and sandbar shark share 46% identity, although greater than 50% identity results when the two sequences are aligned directly. Significantly, our sandbar shark sequence has greater homology with the human chain than with the horned shark sequence. There is only 36% identity between the sandbar and horned shark V regions, and the C regions are only 42% identical. This result illustrates the overall conservation of immunoglobulin structure. It also documents that the long evolutionary time of divergence of individual shark species has led to the generation of substantial differences in light-chain sequences within a single class of vertebrates.

The J-region sequences of Shlc5.1 and two previously sequenced clones, Shlc1 and Shlc3 (1), are very similar. The J segments share greater identity with $J_{\lambda}$ than with $J_{\kappa}$, especially in the carboxyl-terminal region (13). These residues further identify clone Shlc5.1 as a cDNA encoding a λ-like light chain.

To further ascertain the relationship of the shark V-region genes with other known V regions, we constructed a phylogenetic tree by the progressive alignment method of Feng and Doolittle (10, 14). Sequence alignments were assessed from similarity scores derived using the minimum mutation matrix of Dayoff (15). This approach seems to be effective in establishing phylogenetic relationships (15, 16). In the tree, horizontal length is proportional to the distance scores generated by the computer program. Although this analysis reveals relationships and phylogenetic histories, actual evolutionary time is difficult to infer, especially for multigene families such as the immunoglobulin family. As expected, the λ and κ chains form two groups of the tree (Fig. 4). Consistent with the long phylogenetic history of sharks, the two shark light chains are near the root of the tree but clearly segregate as λ chains. Our and other (41) analyses indicate that λ chains can be divided into at least two classifications. One is composed of the "classical" λ chains and the other appears to comprise more "primitive" sequences characterized by the "VpreB" proteins expressed on pre-B cells (38, 39). Indeed, in our analysis this group is suggested as the evolutionary precursor of classical λ and κ chains. Interestingly, the horned shark sequence segregates with the primitive

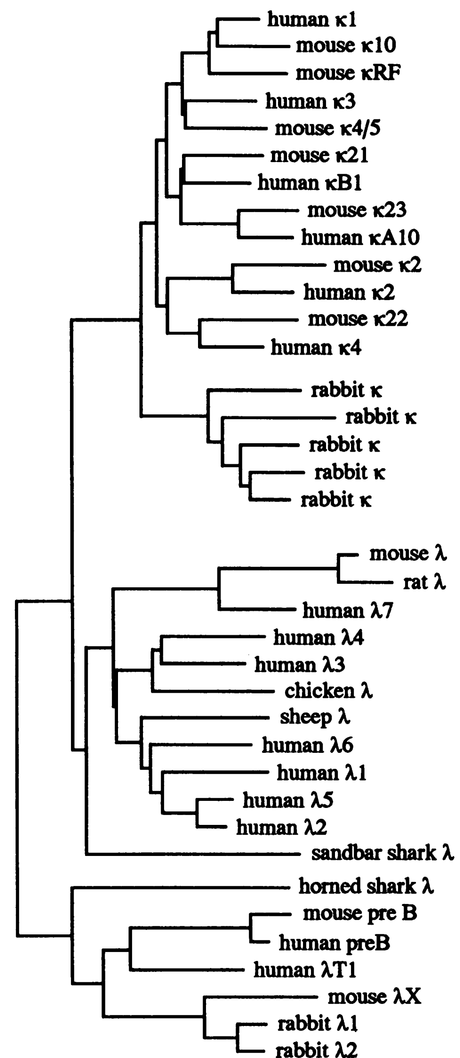


FIG. 4. Phylogenetic tree for immunoglobulin V regions. A preliminary tree was constructed using 64 sequences. This was simplified to 37 sequences to show the overall topology by removing sequences internal to the same branch. Mouse $V_{\kappa}$ subgroup classification is from Strohal *et al.* (17), while all other subgroup classifications are from Kabat *et al.* (11) except human κA10 (18), human κB1 (19), human λT1 (20), human λ4A (21), and mouse λx (22). Sequence references are as follows: human κ1 and κ2 (11), human λ1–λ6 (11), mouse κ10 (23), mouse κRF (24), mouse κ4/5 and κ2 (25), mouse κ21 (26), mouse κ23 (27), mouse κ22 (28), rabbit κ sequences (29–33), human κB1 (19), mouse λ1 (34), rat λ (35), chicken (36), sheep λ (37), mouse preB (38), human preB (39), and rabbit λ (40).

| | | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | Matches with sandbar shark |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sandbar shark | M | T | Q | S | I | G | V | L | A | A | L | I | L | C | F | H | S | T | . | I | A | . | | 20 |
| human Vλ1 | M | T | C | S | P | L | L | L | T | L | L | I | H | C | T | G | S | W | . | . | A | . | | 9 |
| mouse λ (104E) | M | A | W | I | S | L | I | L | S | L | L | A | L | S | S | G | A | S | . | I | S | . | | 5 |
| chicken | M | A | W | A | P | L | L | L | A | V | L | A | H | T | S | G | S | L | V | Q | A | . | | 6 |
| human κ | M | D | M | R | V | L | A | Q | L | L | G | L | L | L | L | C | F | P | . | G | A | R | C | 3 |
| mouse κ (21B) | M | E | S | D | T | L | L | L | W | V | L | L | L | V | W | P | G | S | T | G | | | | 4 |

FIG. 3. Comparison of leader sequences of sandbar shark light chain with those of vertebrate λ and κ chains. Sequences are from Kabat *et al.* (11).

group whereas the sandbar shark light-chain clusters with the rest of the λ chains. The evolutionary significance of this result is unclear, but it again points out that the two shark sequences are only distantly related.

**Analysis and Mapping of Genomic Light-Chain Genes.** A consideration of cDNA sequence and Southern blot data (1) led us to conclude that multiple copies of light-chain genes are present in the genome and that these can be classified into at least two families based on 3′-untranslated-sequence homologies. We have extended these findings using several approaches. A shark liver genomic library was screened with a C-region-specific probe, and since the individual constant regions are greater than 90% identical most, if not all, of the genomic light-chain genes should have been detected by this procedure. The C-region-positive clones were tested for reactivity with the different untranslated-region probes in a "plaque dot blot assay." Of the 24 clones analyzed, 3 hybridized specifically with the Untrans-1 probe, 15 with the Untrans-4 probe, and 6 with neither probe. Restriction maps of the unreactive clones showed that they possessed untranslated regions and so must therefore represent a third family (based on 3′-untranslated-sequence homologies) of genomic genes. A majority of the genomic clones belong to the Untrans-4 family, and this is reflected in the transcribed genes since 75% of the cDNA clones also belong to this family.

Fourteen genomic clones comprising examples from each of the groups described above were selected for detailed mapping using probes specific for the V region, J region, and C region. Clones ranged from 11 to 19 kilobases (kb) in length. The most striking result from these studies is that each clone contains a single copy of V, J, and C gene segments. The genes do not appear to be rearranged. Two patterns for the arrangement of the V, J, and C exon clusters could be discerned. In about half of the clones (Fig. 5, pattern A), the genes can be arranged so that the entire V–C region spans 3 kb or less. This type of arrangement is also the pattern exhibited by horned shark light-chain gene clusters (2). The other clones (Fig. 5, pattern B) exhibit a different type of arrangement in that the distance between the V and C genes appears to be slightly larger, between 3.0 and 3.5 kb. In all the clones the V and J genes appear to be separated by 1 kb or less. The maximum distance the clones could span on the genome is 5 kb. Two of the clones failed to hybridize with the V and J probes. In these clones the C region mapped at the end of the insert, demonstrating that individual clusters must be separated by at least 12 kb.

Intriguingly, there appears to be a correlation between the Untrans groups and the arrangement pattern. Clones hybridizing to the Untrans-1 probe conform to pattern A. Conversely, the clones that hybridize to the Untrans-4 probe conform to pattern B.

**Conclusions.** The shark light chains are clearly homologous to corresponding mammalian immunoglobulin light chains. Based upon the analysis of the leader, V, J, and C elements

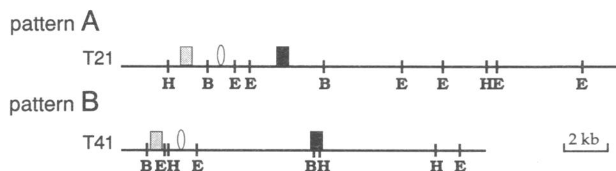


FIG. 5. Partial restriction maps of light-chain clones demonstrating two patterns of cluster arrangement. V (stippled box), J (oval), and C (solid box) assignments are based on the sizes of hybridizing bands in Southern blots. Restriction enzyme sites are *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B). Pattern A: V and C genes possibly separated by 3 kb or less. Pattern B: V and C genes separated by 3.0–3.5 kb.

using several criteria, we conclude that sandbar shark light chains are λ chains.

The arrangement of shark light-chain genes, in which there are multiple clusters comprising closely spaced V, J, and C elements, is radically different from that of other vertebrates. There appear to be many of these clusters. Approximately 0.03% of the unamplified genomic library hybridized with the C-region probe, and most of these clones probably represent different clusters since all of the 14 mapped clones were unique. With this arrangement, it is challenging to predict how clonal selection, if it exists at all, could be regulated in sharks and it remains possible that immunoglobulin regulation in sharks is also radically different from that in higher vertebrates. The functional significance of the different arrangement patterns and sequence polymorphisms we have observed is unclear at this time, but the mapping and sequence analysis of the immunoglobulin genes should provide us with the necessary experimental foundation for resolving these questions. It seems likely that continued study of the sharks should provide us many interesting results and perspectives concerning the regulation and evolution of the immunoglobulins.

**Note Added in Proof.** Since this manuscript was submitted, we have obtained sequence data on two genomic clones representing pattern A. In these clones, the V and J segments were fused in the germline.

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