Two distinct immunoglobulin heavy chain isotypes in a primitive, cartilaginous fish, *Raja erinacea*

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ABSTRACT

Immunoglobulin heavy chain genes in *Raja erinacea* (little skate) are organized in clusters consisting of V\(\text{H}\), D\(\text{H}\), J\(\text{H}\) segments and C\(\text{H}\) exons (1). An immunoglobulin heavy chain \(\mu\)-like isotype that exhibits \(61 - 91\%\) nucleotide sequence identity in coding segments to the *Heterodontus francisci* (horned shark) \(\mu\)-type immunoglobulin is described. The overall length of the \(\mu\)-type clusters is \(\sim 16\) kb; transmembrane exons (TM1 and TM2) are located 3 to C\(\text{H}\) exon 4 (C\(\text{H}\)4). In three of four TM-containing genomic clones, a significant deletion is present in TM1. A second isotype of *Raja* immunoglobulin heavy chain genes has been detected by screening a spleen cDNA library with homologous *Raja* V\(\text{H}\)- and C\(\text{H}\)1-specific probes complementing the respective regions of the \(\mu\)-like isotype. Weak hybridization with V\(\text{H}\)-specific probes and no discernable hybridization with C\(\text{H}\)-specific probes were considered presumptive evidence for a second immunoglobulin isotype that nominally is designated as X-type. The V\(\text{X}\) region of the X-type cDNA is \(\sim 60\%\) identical at the nucleotide (nt) level to other *Raja* V\(\text{H}\) segments and thus represents a second V\(\text{H}\) family. Putative D\(\text{X}\) and J\(\text{X}\) sequences also have been identified. The constant region of the X-type immunoglobulin heavy chain gene consists of two characteristic immunoglobulin domains and a cysteine-rich carboxy terminal segment that are only partially homologous with the \(\mu\)-like isotype. Genomic Southern blotting indicates that the V and C segments of both immunoglobulin heavy chain isotypes are encoded by complex multigene families. V\(\text{X}\)- and different C\(\text{X}\)-specific probes hybridize to different length transcripts in northern blot analyses of *Raja* spleen RNA suggesting that the regulation of expression of the X-type genes may involve differential RNA processing.

INTRODUCTION

Multiple classes of immunoglobulin are present in all sarcopterygian species, including the lungfishes, amphibians, reptiles, avians and mammals (2). During the differentiation of B lymphocytes, switch recombination allows the same antigen-binding specificity that is determined by V\(\text{H}\) and V\(\text{L}\) sequences, to be expressed in the context of different secondary biological properties specified by different heavy chain constant region isotypes (3,4). Immunoglobulin class switching depends on the tandem linear order of the genes encoding various constant region isotypes (5) and is influenced by environmental stimuli such as LPS and IL-4 (6,7). Switch recombination occurs via a pathway distinct from that described for immunoglobulin and T cell receptor (TCR) V-(D)-J recombination, although both can lead to the deletion of intervening sequences (8). Unique \(2-10\) kb class switch signal sequences located \(5'\) to each constant region gene (except \(\delta\)) form a basic recognition motif for the recombination of a specific constant region with the rearranged variable region. The biological effects of class switching are apparent; however, the triggering mechanisms of this complex process are not well understood.

The majority of the elasmobranch species studied to date possess only a single immunoglobulin class; prolonged immunization fails to result in the production of additional immunoglobulin classes (9) or in an increase in antibody affinity. In one elasmobranch, *Heterodontus francisci* (horned shark), multiple immunoglobulin heavy chain gene clusters each consisting of a single variable (V\(\text{H}\)), two diversity (D\(\text{H}\)1 and D\(\text{H}\)2), a joining (J\(\text{H}\)) segments and a \(\mu\)-like constant region (C\(\text{H}\)\(\mu\)) have been described (10). Each cluster spans approximately 16 kb and in at least two different individuals, approximately one-half of the clusters exhibit V\(\text{H}\)D\(\text{H}\)\(1\)- or V\(\text{H}\)D\(\text{H}\)\(2\)-J\(\text{H}\)-joining in the germline of non-lymphoid cells (11). Considerable microheterogeneity has been observed in the \(\mu\)-like C\(\text{H}\) exons of *Heterodontus* immunoglobulin genes (12) and rearrangement of V-D\(\text{H}\)1-D\(\text{H}\)2-J may occur only within individual clusters (10) (unpublished).
observations). Due to the high degree of nucleotide similarity between VH segments of Heterodontus, switch recombination between clusters is difficult to discern and cannot be ruled out.

Raja erinacea, a related elasmobranch that is representative of a separate phylogenetic order, possesses μ-like heavy chain constant region (Cμ) genes that exhibit a similar cluster organization (1). Certain members of the Rajiformes (skates and guitarfish) possess two antigenically distinct molecular weight classes of immunoglobulin (13,14). These immunoglobulins share antigenically cross-reactive light chains and possess 70,000 and 45–50,000 Mₗ heavy chains, respectively; there is no information available regarding the primary structures of these molecules. Both the high molecular weight (HMW) and low molecular weight (LMW) forms are present in significant quantities in non-immune serum. The HMW and LMW forms are co-expressed by a significant percentage of Raja spleen cells (15) only during early ontogenetic development. Adult spleen cells, as well as the majority of embryonic cells, are positive for one or the other but never both classes of immunoglobulin, suggesting isotypic exclusion (15). The genetic regulation of differential isotype expression in Raja may be of considerable interest in terms of understanding heavy chain isotype expression in higher vertebrates. In this study, we report the isolation and determination of the complete nucleotide sequences of the heavy chains of two distinct immunoglobulin classes found in Raja.

MATERIALS AND METHODS

Animals

Adult specimens of Raja erinacea were obtained from the Marine Biological Laboratory, Woods Hole, MA. Tissues were processed immediately after the animals were sacrificed.

DNA Libraries

A genomic library was constructed in λDASH™ (Stratagene) from testes high molecular weight DNA that had been partially digested with Sau3A, as described (16). The library was amplified on bacterial host strain P2392, a P2 lysogen of LE392. Approximately 0.9 genome was recovered, based on a genome size of 7 pg/haploid genome (17), an average insert size of 17–19 kb and proportional representation.

RNA Isolation and cDNA Library Construction

Total RNA was isolated from the spleen of an individual specimen of Raja using the guanidium isothiocyanate/CsTFA technique according to the supplier’s specifications (Pharmacia, Piscataway, NJ) and enriched for poly A⁺ RNA by elution from an oligo dT-cellulose column. Poly A⁺-selected RNA was converted to double-stranded, EcoRI adaptor-linked cDNA using a commercial synthesis kit (Pharmacia) that employs a modification of a previously described procedure (18). A cDNA library consisting of 1.3×10⁸ recombinant PFUs was constructed in λgt11. All cDNA library screenings were performed on the unamplified portion of the library in order to facilitate isolation of unique clones.

Filter Hybridization

Nitrocellulose lifts were hybridized in 0.6M NaCl, 0.02M EDTA, 0.2M Tris, 0.5% SDS, 0.1% sodium pyrophosphate (SET) for 12 h at 65°C, washed at 52°C in 1× SSC, 0.1% SDS, 0.05% sodium pyrophosphate and subjected to autoradiography at −70°C for 12 h.

Probes

Heterodontus derived probes utilized in the present study were: TM/5301, containing 84 nts of exon 4, the joined transmembrane (TM) exons and the 3’ untranslated segment of Heterodontus cDNA 5301 (19); SEC, an oligonucleotide 24mer complementing the Heterodontus secretory (SEC) segment (19); Cμ801, a germline exon 1-specific probe derived from genomic clone 801 (12); 6121, a probe derived from a cDNA containing approximately half of exon 1 plus exons 2,3,4 including the entire SEC portion of Cμ4 (19); and 5301/3,4 a 510 nt subinsert containing exons 3 and 4 from cDNA 5301 (19).

All other probes were derived from Raja: 110VH, a ~900 nt XbaI genomic fragment from skate clone Re110 (this is VH-JH specific) (1); 107VH, a 1.5 kb XbaI genomic fragment from Re107 that contains a complete (unrearranged) VH region (1); SkC1, a 1.8 kb EcoRI-SstI fragment from genomic clone Re107 (see below; Fig. 2) containing Cμ1; SkC2, a 1.6 kb SstI-EcoRI fragment from Re107 that adjoins SkC1 and contains Cμ2; 113ex3, a 1.3 kb EcoRI fragment derived from genomic clone Re113 that contains Cμ3. SkTM was isolated from genomic clone (Re27054) that was identified by hybridization with TM/5301 and consists of a 1.5 EcoRI fragment possessing a deletion within the TM coding region (see below; Fig. 3A, 3B). 4bCH consists of the entire Cμ coding region from Raja cDNA clone Re4b and was derived using the polymerase chain reaction (PCR), employing primers that complement the 5’ end of the J region and the 3’ end of SEC. The complete sequence of the constant region of Re4b is illustrated below (Fig. 1).

VX and CX are DNA probes of ~450 and ~750 nts complementing the V and C regions of the X-type isotype, respectively and were derived by PCR from cDNA Re20 using sequence-specific primers. CX1 and CX2-SEC are DNA probes (235 and 347 nts, respectively) that were generated by PCR using primers specific for the predicted first exon and 3’ predicted coding segment of Re20. Insert DNAs were used as templates and all PCR products were gel-purified prior to use.

DNA Labelling

DNA probes were labelled to 10⁸−10⁹ cpm/μg using a modified version of the random hexanucleotide priming method as described (1). Oligonucleotide probes were end-labelled to a specific activity of ~10⁶ cpm/μg by T4 polynucleotide kinase (Bethesda Research Laboratory) in the presence of γ³²P-ATP.

Genomic Southern Analyses

Total high molecular weight DNA was isolated from Raja testes (20). Following standard digestion with various restriction enzymes and electrophoretic separation, the DNA was partially nicked by UV irradiation, denatured and transferred to Zeta-Probe™ (Bio-Rad) in 1M NH₄OAc/20× SSC (1× SSC = 0.15M NaCl, 0.015M NaCitrate) for 20 h. The filters were baked at 80°C under vacuum for 2 h. Hybridization to DNA probes and washing were performed as described (16).

Northern Blot Analyses

Poly A⁺ RNA was separated in a 1.5% agarose/2.2M formaldehyde gel, then transferred to Zeta-Probe™ in 20× SSC. The blots were dried under vacuum for 2 h at 80°C. Hybridization with DNA probes was carried out in SET as described for library lifts.
The relative contributions of D₁, D₂ and potential N-region additions to the Re4b D region cannot be determined in the absence of the germline ‘parent’ cluster. By comparison to Heterodontus C_H sequences, Re4b would represent a full copy length immunoglobulin heavy chain cDNA encompassing all four C_H exons, including the SEC segment of exon 4 and some 3' untranslated (3'UT) sequence.

Table 1. Percent identity of Raja and Heterodontus C_H exons. Alignments were produced by the IFIND program. Gaps in the alignments were scored as a mismatch.

<table>
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<tr>
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<th>CH2</th>
<th>CH3</th>
<th>CH4</th>
<th>SEC</th>
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<th>% Nucleotide Identity</th>
<th>Raja Genomic Exons</th>
<th>3050 CH Exons</th>
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<tr>
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<td>89</td>
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<tr>
<td>64</td>
<td>61</td>
<td>82</td>
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<tr>
<td>% Amino Acid Identity</td>
<td>Raja Genomic Exons</td>
<td>3050 CH Exons</td>
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RESULTS

Constant Region μ Genomic Organization

An unamplified Raja spleen cDNA library was screened with a homologous V_H-J_H-specific probe (110V_H (1)). One V_Ĥ isolate with an insert length of ~1.8 kb was sequenced in its entirety. The complete nucleotide and predicted amino acid sequence of Re4b is shown in Fig. 1.
The location of predicted cysteine and tryptophan residues also is consistent with four typical immunoglobulin \( C_H \) domains. Nucleotide identities between the putative constant region domains of Re4b and *Heterodontus* genomic clone 3050 exons (19) range from 61% for \( C_{H2} \) to 91% for the SEC segment of exon 4 (Table 1). The sequence AATAAC is found 28 nts 3' to the termination codon of the SEC segment; this sequence differs from the consensus polyadenylation signal by one nt (underlined) (22).

The \( C_H \) exon boundaries of cDNA Re4b were assigned by comparison to *Raja* \( C_H \) exon sequences derived from different genomic clones. Nucleotide identities between the cDNA and genomic \( C_{H1} \) and \( C_{H2} \) of Re107 (Fig. 2) are 87 and 89%, respectively (Table 1). This high degree of nucleotide sequence identity between Re4b and the Re107 gene sequences indicates that they represent similar \( C_H \) exons. At the level of predicted amino acid sequence, \( C_{H1} \) and \( C_{H2} \) domains of Re4b and Re107 are 81 and 79% related, respectively. Nucleotide sequence identity between \( C_{H3} \) of Re4b and Re113 (Fig. 2) is ∼84% and the predicted amino acid sequence identity is 70% (Table 1).

In order to facilitate identification of a genomic clone containing all four \( C_H \) exons, a skate genomic library was screened with 5301/TM (19). TM+ clones were isolated and screened with probes for the other \( C_H \) exons. Re27041 hybridizes with SKC1, SKC2, 113ex3, 5301/TM (Fig. 2), but does not hybridize with a homologous \( V_H \) probe. The sequence of \( C_{H4} \) in Re4b is 97% identical at both the nucleotide and predicted amino acid sequence levels to that of Re27041 \( C_{H4} \) (Table 1). The predicted amino acid sequence of the SEC segments of Re4b and Re27041 are identical. Two consensus polyadenylation sites are located 27 nts 3' to SEC and 4 nts downstream of the first AATAAA, as in *Heterodontus* (19).

Nucleotide identities between comparable genomic \( C_H \) exons of *Raja* and *Heterodontus* range from 61% for \( C_{H2} \) to 77% for \( C_{H4} \) and are even higher if the SEC portion of \( C_{H4} \) is included (Table 1). Amino acid identities between genomic \( C_H \) exons of *Raja* and *Heterodontus* are considerably lower, varying from 44% for \( C_{H2} \) to 65% for \( C_{H4} \) and 85% for the SEC segment.

Genomic clone Re27041 contains an intact TM sequence ∼1.8 kb 3' to \( C_{H4} \)/SEC. The coding segments of this sequence are shown in Fig. 3A. TM1 and TM2 are separated by a 130 nt intervening sequence (IVS) (not shown). The presence of separate TM exons is consistent with the assignment of this immunoglobulin locus as \( \mu \)-like. Because the length of the IVS separating \( C_{H1} \) and \( C_{H2} \) is the same in Re107, Re113 and Re27041, and the IVS separating \( V_H \) and \( C_{H1} \) are similar in Re107 and Re113 (Fig. 2), a reasonable estimate of the overall genomic linkage distance (\( V_H - TM \)) of a *Raja* Ig gene cluster is ∼16 kb, similar to that described in *Heterodontus*.

The nucleotide sequence of a 5301/TM+ *EcoRI* fragment from genomic clone Re27054 was determined and is compared to *Heterodontus* (5301/TM) and *Raja* TM-containing sequences (Fig. 3A). Homology of Re27054TM to all other TM+ isolates terminates abruptly in TM1 (Fig. 3A arrow). A splice consensus sequence is not associated with the homology breakpoint. An in-frame stop codon was detected 15 (predicted) amino acids 3' to the homology breakpoint in clone Re27054 (Fig. 3A). TM2-like sequences, typically exhibiting strong phylogenetic conservation, were not detected within 683 nts 3' to this stop codon (data not shown). A consensus polyadenylation site (AATAAA) is located 565 nts 3' to the homology breakpoint.

The *EcoRI* fragment of Re27054 containing this putative TM1-like fragment was used to screen a portion of the unamplified spleen cDNA library. A \( C_{H4}^{+}/TM^{+} \) cDNA (ReTM6) was identified and sequenced. ReTM6 has an intact TM region that is 96% identical at the nucleotide level and 100% identical at the amino acid level to the Re27041TM exons. By comparison to ReTM6 the deletion in Re27054 includes 11 predicted amino acids representing 25% of TM1, all of TM2 and 35 nts of 3' untranslated sequence (not shown).

Therefore, the entire deletion in Re27054 encompasses 33 nts of TM1 coding sequence, the 134 nts IVS (including splice consensus sequences), TM2 and 35 nts of 3' untranslated sequence or 202 nts total (Fig. 3B). This atypical TM-like sequence is exhibited by two genes in addition to 27054; thus
Termination codons are enclosed in boxes. B. Schematic organization of Raja TM + cDNA clones. Genomic clones Re27041 (EMBL accession # M29678) and Re27045 (EMBL accession # M35185) possess an intact and truncated TM regions, respectively. Splice acceptor nucleotides for the C4 splice donor site are in lower case letters. TM1-2 IVS splice consensus sequence (GT/AG) for Re27041 is 130 nts (not shown). The homology breakpoint between Re27041 and all other isolates is indicated by an arrow. CH4 is represented by a shaded box and TM1 and TM2 are shown by cross hatched boxes. IVSs are thin lines and 3' untranslated (3'UT) segments are bold lines.

Figure 3. A. Sequence alignments of Heterodontus (5301/TM, selection probe) and Raja TM segments. ReTM6 (EMBL accession # M29673) and 5301/TM are TM + cDNA clones. Genomic clones Re27041 (EMBL accession # M29678) and Re27045 (EMBL accession # M35185) possess an intact and truncated TM regions, respectively. Splice acceptor nucleotides for the C4 splice donor site are in lower case letters. TM1-2 IVS splice consensus sequence (GT/AG) for Re27041 is 130 nts (not shown). The homology breakpoint between Re27041 and all other isolates is indicated by an arrow. Termination codons are enclosed in boxes. B. Schematic organization of Raja TM exons in cDNA ReTM6 and in Re27041 and Re27045 (genomic) configuration. C4 is represented by a shaded box and TM1 and TM2 are shown by cross hatched boxes. IVSs are thin lines and 3' untranslated (3'UT) segments are bold lines. The distance from TM2 to the putative polyadenylation site (represented by closed circles) is 599 nts for Re TM6. The presence of -150 nts of sequence 3' to the putative polyadenylation site in ReTM6 (not shown) implies that this site was not used to form the end of this mRNA. The predicted second polyadenylation site and poly A+ tract was not found in this clone. The polyadenylation site in Re27041 is 586 nts 3' to TM2 and differs from consensus by one nt. The distance from the end of the 'coding segment of Re27045 to a potential polyadenylation site is 565 nts.

three out of four otherwise unique 5301/TM + genomic isolates (data not shown) are truncated.

A Second Constant Region Isotype is Present in Raja Erinacea

Approximately 1.2 x 10^6 unamplified recombinant cDNA plaques were screened in duplicate using V_H (110V_H (1)) and C_H1-specific (SkC1) probes. Six weakly hybridizing V_H+, C_H1+ plaques were detected. Clone Re20 was found to contain a 1.2 kb insert; inserts of less than 200 bp were identified in the other five clones. The complete nucleotide sequence of Re20 is illustrated (Fig. 4). The 5' (V_H) sequence of Re20 is 60% identical at the nucleotide level to 110V_H, by definition, this represents a unique V_H family. The predicted amino acid sequences of Re110 and Re107 V regions are 46% and 47% related to Re20, respectively (Fig. 5A). This is in contrast to the 78% predicted amino acid homology between the V regions of Re110 and Re107. The V segment (V_X) is separated by 24 nts from a unique but identifiable J region ("J_X") (Fig. 5B). The relative contributions of putative D_X segments to this region cannot be determined without identification and characterization of genomic D_X sequences; however, identity is apparent between this region and known Raja μ-type immunoglobulin D segments (1). It is apparent that the J_X segment of Re107, a μ-type gene, is closely related to the J_X sequence of Re20; however, an extended segment of eight nts at the putative DJ_X boundary also is identical to the sequence of the D_X segment of Re107 reported previously. The most likely explanation is that the X-type gene utilizes a similar D_2 and that in this particular case of D_2 joining, there has been junctional (deletion of nucleotides) and no N-type diversity at the D_2/J_X junction (assuming the general scheme of elasmobranch immunoglobulin gene organization). The more 5' identities between the cDNA and the μ-type genomic sequences are consistent with the utilization of a partially homologous D_1 segment and possible V-D_1/D_2/J_X + D_3/SEC junctional and N diversity. The distributions of cysteine, tryptophan and other critical amino acids in the sequence region 3' to J_X suggests that there at least two immunoglobulin domains (C_X1, C_X2/SEC) (Fig. 4). Re20 constant region domains C_X1 and C_X2 are defined arbitrarily by 100 predicted amino acids. The N terminal 13 amino acids of the putative C_X2 contains five proline residues. The 3' end of the C_X2 segment (44 amino acids) encodes five cysteine residues and terminates in TAA. Based on hydropathicity indices, it is unlikely that this 44 amino acid stretch is involved in membrane anchoring. Multiple hybridizing components were apparent in Southern blots of Raja genomic DNA hybridized with V_X- and C_X-specific probes (Fig. 6), consistent with the designation of the X-type components as members of a multigene family.

Poly A+ selected RNAs from liver and spleen were examined by northern blotting using V_X- and C_X-specific probes. The presence of three different sized messages in spleen (Fig. 7) was noted with both V_X- and C_X-specific probes; no hybridization was evident with liver RNA. In northern blot analyses of Raja spleen poly A+ selected RNA, V_X- and C_X-specific probes hybridize at equivalent intensity to RNA species migrating at gel positions corresponding to 3.3, 1.3 and 1.0 kb (Fig. 7, lanes 1
Nucleotide and predicted amino acid (one letter code) sequence of Raja cDNA clone Re20 (EMBL accession # M29672). Nucleotides are numbered in right margin. Leader, framework (FR), complementarity determining region (CDR), diversity (D) region and joining (J) boundaries were determined by comparison to previously published information (1). D_{2} like sequences are underlined. L: 1 to 57. FR1: 58 to 147. CDR1: 148 to 162. FR2: 163 to 201. CDR2: 202 to 252. FR3: 253 to 339. D region: 340 to 637. J: 638 to 1140. FR1: 1 to 30. CDR1: 31 to 35. FR2: 36-48. CDR2: 49-65. FR3: 66 to 96. Framework (FR) and complementarity determining region (CDR) assignments are based on previously published information (16). L: 1 to 57. FR1: 58 to 147. CDR1: 148 to 162. FR2: 163 to 201. CDR2: 202 to 252. FR3: 253 to 339. D region: 340 to 637. J: 638 to 1140. The assignments of constant region boundaries are based only on considerations of immunoglobulin domain length and are tentative. The predicted location of the carboxy terminal exon is indicated directly. Termination codon is in lower case; otherwise eight additional nts were present 3' to taq in this clone, thus it is not possible to locate a polyadenylation signal sequence.

and 2), whereas a probe specific for C_{x}2 and the putative C_{terminal} segment hybridizes only with the RNA species corresponding to 3.3 and 1.3 kb (Fig. 7, lane 3). These results suggest the significant presence of an RNA species that lacks all or a considerable length of C_{x}2.

**DISCUSSION**

**Constant Region \( \mu \) Genomic Organization**

The skate *Raja erinacea* exhibits a clustered immunoglobulin \( \mu \) genomic organization similar to that described for *Heterodontus*. The entire non-rearranged coding unit from the \( V_{H} \) segments through the TM spans \( \sim 16 \) kb in both species. Multiple copies of the \( V_{H}-C_{H} \) linked clusters are present in the genome. The inter-exon distances between the individual \( C_{H} \) exons, however, are much longer in *Raja* than in *Heterodontus*. The appreciably longer \( J_{H}-C_{H} \) IVS in *Heterodontus* accounts for the overall similar cluster sizes in these two species. It is possible that there is selective pressure to maintain a \( \sim 16 \) kb overall cluster length irrespective of inter-exon distances, although this relationship may be inconsequential.

Another notable difference between *Raja* and *Heterodontus* involves the large percentage of apparently non-functional TM segments. In this study, three out of four genomic clones from *Raja* possess deletions in the 3' end of TM1 and an absence of presumably functional TM2 within the TM1-TM2 linkage distance described for a presumably functional *Raja* immunoglobulin gene clone (Re27041). The genes exhibiting the deletions are presumed to be pseudogenes; however, elements of the TM-deleted clusters may recombine with other genes possessing functional TM...
sequences. Alternatively, a non-functional TM region may essentially regulate the exclusive expression of a secreted form of immunoglobulin, although this would be contrary to our understanding of membrane bound antibodies as primary antigen receptors. Enhanced expression of secretory immunoglobulin through any mechanism would account for the low number of TM+ cDNA clones that have been detected.

A Second Constant Region Isotype

A cDNA representing a second heavy chain isotype (C\text{X}) has been detected in Raja. The open reading frame in Re20 is consistent with three typical immunoglobulin domains ("V\text{X}-C\text{X}1-C\text{X}2") as well as an additional, cysteine-rich region that may serve as the equivalent of a secretory segment. The predicted amino acid sequence of Re20 would encode a 40,000 M\text{r} heavy chain molecule, consistent with the molecular mass of the 'second-class' heavy chain detected in any other rajiform species (14). It is possible that Re20 corresponds to this distinct heavy chain constant region isotype.

Comparisons of Re20 exons to Heterodontus and Raja \mu constant region exons at the nucleotide and predicted amino acid level indicates relatedness between Re20 C\text{X}1 and Heterodontus C\text{H}1 at 21/96 positions. Heterodontus C\text{H} exons lack significant nucleotide or predicted amino acid identity with Re20 C\text{X}2. Re20 C\text{X}2 exhibits a similar level of predicted amino acid sequence identity to both exons 2 and 4 of Re4b (26/106 and 23/106, respectively). As the only significant amino acid and nucleotide identity was between Heterodontus C\text{H} exons, and Raja \mu- and X-type exons, the \mu and X clusters in Raja may have evolved from a primordial \mu-type cluster.

Nucleotide and predicted amino acid identities between Raja C\text{H} exons and most mammalian, amphibian and avian constant region exons exhibit considerably less identity than between the two Raja isotypes, and Raja \mu-type and Heterodontus \mu-type exons. An exception is the human C\text{H}1, which demonstrates 18/106 predicted amino acid identities to Re20 C\text{X}2. The significance of this finding is unclear. As the X-type constant region is a member of a multigene family, the X-type cluster represented by Re20 may exhibit a fortuitous level of predicted amino acid identities to the human C\text{H}1 sequence. Similarly, due to the fact that the X-type constant region exons are members of a multigene family, it is difficult to perform 'molecular clock' analyses on the one nucleotide sequence represented by Re20 and other vertebrate constant region exons.

Due to the lack of predicted amino acid and nucleotide identity, it is unlikely that the X-type cluster is an evolutionary forerunner to the typical immunoglobulin isotypes of higher vertebrates. The X-type gene may be a 'lost' isotype in the evolutionary history of constant regions. An immunoglobulin with an apparent molecular weight consistent with a variable and two constant region domains also is present in at least one avian, lungfish and a reptilian. A two domain constant region (with an additional unrelated sequence segment) may have evolved independently at two points during the radiation of the vertebrate lineage, represented in the skate and these other species.

The V\text{X} sequence is ~60% related to the previously described family of V\text{H} segments in Raja. Although J\text{H} \mu-type segments in Raja vary considerably in predicted amino acid sequence, the J region of Re20 exhibits amino acid motifs that are found in other J segments (1). The D region of Re20 possesses nucleotide sequences similar to those found in germline \mu-type D\text{H} segments. An eight nt sequence which overlaps the first predicted codon of the J\text{X} region is identical to Re107 D\text{X}. Whether this is a coincidence or represents highly conserved D elements between the X- and \mu-type clusters cannot be resolved without identifying the germline segments that gave rise to Re20.

The predicted amino acid sequence of Re20 suggests that two typical constant region domains (segments) are present as well as a shorter carboxy terminal segment. Since a 1.3 kb band is visualized with both V\text{H}+ and C\text{H}-specific probes on northern blots of spleen RNA, it is unlikely that this particular cDNA derives from a potentially degraded mRNA. Without sequence data for genomic clones, it is difficult to predict exon boundaries. However, a proline-rich region of ~13 amino acids is located between the putative ends of C\text{X}1 and the start of C\text{X}2 and may...
correspond to the hinge-region found in higher vertebrate immunoglobulin. Although it is attractive to speculate that the cysteine-rich terminus may be involved in dimer formation, the 320,000 M₆ immunoglobulin dimer found in related rajiform species are not covalently associated (13).

RNA blot transfer analyses with Vₓ and Cₓ-specific probes show the presence of ~3.3, ~1.3 and ~1.0 kb hybridizing components. Whereas the Cₓ₁-specific probe hybridizes to all three RNA classes, a Cₓ₂-specific probe hybridizes only to the 3.3 and 1.3 kb bands, suggesting that the 1.0 kb RNA may lack Cₓ₂ and the unique SEC segment. It is unclear whether the 1.3 and 1.0 kb products arise from alternative splicing of a 3.3 kb (or larger) precursor RNA or whether these are synthesized by different clusters. The 3.3 kb component is distinct from the 2.2 kb µ-type mRNAs that hybridize with 4bCₕ (Fig. 7) as well as with Vₕ (data not shown) probes.

At this time, based on the results described above, it is reasonable to conclude that differential RNA processing of a common µ-X-type RNA and isotype switching (µ-type/X-type) are not likely, since a non-homologous V region (Vₓ) is found in clone Re20 and there is a lack of tandem repetitive DNA structures in the Jₓ to Cₓ₁ IVS of Raja genomic clones (data not shown) such as those mediating immunoglobulin class switching in higher vertebrates (23). Furthermore, no Vₓ or Cₓ sequences are detected by hybridization within 10 kb 3' of the Cₓ TM sequences (unpublished observation). Therefore, although Raja erinacea possess two distinct V region families and two immunoglobulin heavy chain isotypes, these appear to be regulated differently than different isotypes found in the higher vertebrates. The genomic organization and the regulation of expression of the gene encoding the second isotype currently is under study.

CONCLUSIONS

It has been suggested that with respect to immunoglobulin heavy chain gene organization, the elasmobranchs are representative of a primitive form of rearranging gene (24). The multiple clustered organization of elasmbranch immunoglobulin loci presumably reflects the evolutionary decision to duplicate entire V-C linked clusters, whereas the organization of the higher vertebrate immunoglobulin genes is associated with duplication of individual segmental elements (10). The immunoglobulin heavy chain µ-like loci in Raja are similar to those found in Heterodontus; however, the second heavy chain isotype that is associated with a unique V region family suggests an appreciable divergence from the Heterodontus system. Along with previous observations regarding the diversity of Jₓ sequences (1), studies reported here indicate that Raja expands diversity through variation in the sequence of both Vₓ and Cₓ coding regions.

The respective phylogenetic lineages represented by Raja and Heterodontus probably did not diverge until after the putative initial duplication event of V-C linked µ-like clusters. The second immunoglobulin isotype presumably diverged from one (or more) of these clusters. It is possible that the second immunoglobulin isotype present only in Raja represents the evolutionary forerunner of the µ-type cluster (present in both Raja and Heterodontus). Raja is the most phylogenetically primitive species in which the presence of two distinct isotypes has been unequivocally documented. The developmental regulation of these two isotypes and their functional significance in terms of antigen recognition at the cell surface are of considerable interest. Based on these results, an in-depth analysis of the genetic regulation of this system appears to be approachable.

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