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Patterns of Receptor Revision in the Immunoglobulin Heavy Chains of a Teleost Fish 1,2

Miles D. Lange,* Geoffrey C. Waldbieser,† and Craig J. Lobb3*

H chain cDNA libraries were constructed from the RNA derived from seven different organs and tissues from the same individual catfish. Sequence analysis of > 300 randomly selected clones identified clonal set members within the same or different tissues, and some of these represented mosaic or hybrid sequences. These hybrids expressed VH members of the same or different VH families within different regions of the same clone. Within some clonal sets multiple hybrids were identified, and some of these represented the products of sequential VH replacement events. Different experimental methods confirmed that hybrid clones identified in the cDNA library from one tissue could be reisolated in the cDNA pool or from the total RNA derived from the same or a different tissue, indicating that these hybrids likely represented the products of in vivo receptor revision events. Murine statistical recombination models were used to evaluate cryptic recombination signal sequences (cRSS), and significant cRSS pairs in the predicted hybrid joint formation. The heptamers of the cRSS pairs were located at different locations within the coding region, and different events resulted in the replacement of one or both CDR as well as events that replaced the upstream untranslated region and the leader region. These studies provide phylogenetic evidence that receptor revision may occur in clonally expanded B cell lineages, which supports the hypothesis that additional levels of somatic H chain diversification may exist. The Journal of Immunology, 2009, 182: 5605–5622.

The structural diversity of the VH, D, and JH gene segments that undergo somatic recombination to form the rearranged VDJ provides the inherent structural diversity of Ig H chains (1). Recombination requires the presence of the recombination signal sequence (RSS) located at the ends of these segments. Each RSS is composed of a heptamer(e.g., CA CAGT G), a 12- or 23-bp spacer (denoted 12-RSS or 23-RSS, respectively), and an A-rich or T-rich nonamer (e.g., ACAAAAACA or TGGTTTTTG). Recombination is mediated by RAG1 and RAG2 and occurs more efficiently when one segment has a 12-RSS and the other has a 23-RSS (the 12/23 rule). The combinatorial diversity provided by the recombination of these different segments is extended by the processes of junctional diversity and N-region nucleotide additions at the coding region junctions (2–4). The potential to further increase the diversity of H chains post-VDJ rearrangement may occur by the activation-induced cytidine deaminase (AID)-dependent processes of somatic mutation, class switch recombination, and gene conversion (5–11).

There is an additional level of postrearrangement Ig diversification that has been termed receptor replacement (12–16). Receptor replacement is divided into two major forms. The first is termed receptor editing. These events appear to principally occur in naive B cells that present with an Ig receptor that reacts with self-Ags. Studies have shown that the RSS at the usual 3'-end of an upstream VH segment is partnered in a RAG-dependent manner with a cryptic RSS (cRSS) located near the end of the framework region (FR) 3-encoded region of the VDJ rearrangement. This secondary rearrangement replaces most of the original VH segment, usually resulting in a lengthened CDR3 and altered receptor reactivity (15–20).

The second type of receptor replacement is termed receptor revision (21–29). In contrast to receptor editing, receptor revision appears to occur in B cells that may have undergone clonal expansion as the result of Ag stimulation (26–29). In addition, the B cells in these populations may have been the targets of somatic mutation. Revision events also differ from those known in receptor editing because the cRSS within both the VH donor and the targeted VDJ recipient appear to be used. In these cases an unusual situation is presented. These alternative cRSS must be located in nearly identical positions in homologous regions within both the donor and recipient coding regions if Ig function is to be preserved. If not, nucleotides essential to coding region structure and function may be deleted during the replacement rearrangement. These requirements have led to the hypothesis that if receptor revision is RAG dependent, then the resulting replacement is dependent upon the formation of a hybrid joint (26–28). As opposed to the standard recombination event that joins the coding ends from two rearranging gene segments, a hybrid joint recombines the signal end containing the RSS from one segment with the coding end from the other segment (30). As suggested by earlier studies, if the heptamers in both the donor cRSS and the recipient cRSS are in the same orientation and the same relative coding region locations, then a
RAG-mediated recombination event that results in a hybrid joint could yield a functional and apparently seamless recombination product (26–28). Alternatively, it has been postulated that recombination might result from an AID-dependent, gene conversion-type event (12, 27, 28, 31). The mechanisms, the frequency, and the location where the recombination may occur present major challenges in understanding the potential biological significance of recombination in mouse cells.

In early phylogeny at the level of the bony fish, there was an explosion of structural diversity that occurred in the VH, DJH, and JH gene segments that undergo rearrangement to encode the Ig H chain V region. In our studies with the channel catfish, different VH H families representing ~200 VH segments have been defined, and each of these 15 VH families have been shown to be expressed in the splenic repertoire of an individual animal. The genomic sequences of representative members from these families have been characterized, and the sequences of at least five functional DJH and nine functional JH segments located upstream from Cμ have been determined (32–38). We have recently focused our studies upon the utilization patterns of the H chain gene segments in the adult repertoire as we seek to better understand the relationships between the systemic and mucosal immune systems. Toward this goal, separate Ig H chain cDNA libraries were constructed from different organs and tissues derived from an individual adult channel catfish. The analyses on these cDNA libraries have led to this report in which we observe that some members of clonal sets represent hybrids that expressed VH members of the same or different family within different regions of the same clone. The experimental design of these studies allowed the results to be confirmed in cDNA libraries, cDNA pools, or total RNA derived from the same or different tissues. These results, coupled with structural analyses of the donor and recipient partners, provide new insight into VH replacement events.

Materials and Methods

Construction of H chain cDNA libraries, hybridization, and sequence analysis

An individual adult outbred channel catfish (Ictalurus punctatus) was examined, and the following tissues were excised: anterior kidney (AK); gill lamellae (GL); spleen (SP); skin (SK); ~2 cm2 from the dorsolateral surface; and intestine (~30 cm). The mesenteries and blood vessels were removed from the intestine, and the organ was thoroughly washed in 90% RPMI 1640 (diluted with sterile water). The intestine was cut into three equal sections (each was ~10 cm), and 2-cm samples were taken from the middle of each section. These sections were designated as intestine (I) 1 (I1; from the proximal third), I2 (from the middle third), and I3 (from the distal third). The PBL were isolated as described (39). These experiments were conducted under an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center (Jackson, MS).

Total RNA was extracted from each tissue using TRIzol (Invitrogen), and first-strand cDNA synthesis was initiated with a CpA2 domain primer as previously described (35). The products were treated with poly(C) at the 5′-end. Following RNase digestion and spin column purification, the products were amplified using a CpA1 domain primer and the abridged adapter primer that is supplied with the 5′-RACE kit (Invitrogen). PCR amplification was conducted with Taq polymerase (Invitrogen) using the following parameters: 1 min at 94°C, 45 s at 52°C, and 45 s at 72°C for 30 cycles with the terminal cycle given a 7-min final extension time. PCR products between 450 and 550 bp in length were gel excised (subsequently termed the cDNA pool), aliquots were ligated into cloning vector pCR2.1, and TOP10F’ competent cells were transformed (Invitrogen). Five hundred fourteen to 534 H chain cDNA colonies derived from each of these tissues except for I1 were arrayed onto Luria-Bertani agar plates. Replica lifts of the colonies were made using Nytran membranes (Schleicher & Schüll) that were separately hybridized with radiolabeled probes specific for the VH1, VH2, VH6, or VH7 families under high stringency conditions that did not permit these probes to cross-hybridize (35), a confirmation also confirmed in this study by sequencing of hybridization positive clones. The cDNA libraries were also hybridized with γ-32P-labeled oligonucleotides designed to be specific for the CDR3-encoded region in various hybrid sequences defined in this study. Filters were prehybridized for at least 2 h at 65°C in 3 × SSC, 0.1% SDS, 5 × Denhardt’s solution, and 0.1% sodium pyrophosphate. Hybridizations were conducted overnight at a temperature dependent upon the Tm of the oligonucleotide in 3 × SSC, 0.2% SDS, 1 × Denhardt’s solution, and 0.1% sodium pyrophosphate. The membranes were washed once in 3 × SSC for 5 min at the same wash temperature as that used for hybridization, with the last two 5-min washes in 0.5 × SSC at a probe-specific temperature. The respective hybridization and final wash temperatures used were 50°C and 54°C for the VH2CSICDR3 primer, 39°C and 42°C for the VH4CSICDR3Rev primer, 50°C and 54°C for the VH1CS2VHRev primer, and 47°C and 50°C for the VH2CS2CDR3Rev primers. The sequences of these primers are shown in Figs. 6A and 7, respectively. Plasticized filters were hybridized on an ABI PRISM BigDye Terminators chemistry on an ABI 3100 DNA analyzer (Applied Biosystems) at the U.S. Department of Agriculture, Agricultural Research Services, Mid-South Area Genomic Laboratory in Stoneville, MS. H chain FR and CDR were assigned using the nomenclature of IMGT, the international ImMunoGeneTics information system (40). The sequences of 308 clones from these cDNA libraries and 13 confirmatory clones directly derived from the cDNA pools (see below) have been submitted to GenBank under the accession numbers EU492547-EU492867 (www.ncbi.nlm.nih.gov/GenBank). Nucleotide substitutions are designated using IMGT nomenclature (ImMunoGeneTics), e.g., A322-G denotes that the A at position 322 is substituted by a G. The nomenclature for catfish germline Dμ and I segments is from earlier studies (36–37). Database searches were conducted using the basic local alignment search tool (BLAST; Ref. 41). The accession numbers for additional sequences in this article are NG22 (M58670), 3D09AVH2 (DQ230574), 1A11AVH5 (DQ230591), 3B12AVH5 (DQ230597), and the germline segments VH2-6, VH3-47, VH7-12, and VH11-2 (DQ400445; Ref. 38).

Direct amplification of hybrid sequences from H chain cDNA pools derived from different tissues

Experiments were done to confirm the presence of hybrid clones in the H chain cDNA pools from the different tissues. Forward primers were designed to be restricted to the VH member expressed in the hybrid, whereas the reverse primers corresponded to nucleotides in the CDR3-encoded region. PCR amplifications were done in a volume of 50 μl containing 1 μl of the H chain cDNA pool diluted 1/25 (the same cDNA pools as used to construct the cDNA libraries), 20 mM Tris-HCl (pH 8.4), 1 mM MgCl2, 10 pmol of each primer, and 1 U of Taq polymerase (Invitrogen). The amplification parameters used an initial denaturation of 5 min at 94°C followed by cycles of 30 s at 94°C, 30 s at an annealing temperature dependent on the Tm of the primer pair, 30 s at 72°C, and a final extension of 7 min at 72°C. The primer pairs (primer sequences are shown in the figures), annealing temperature, number of cycles, and tissue source of the H chain cDNA were as follows: VH7FFor and VH7CS1CDR3Rev, 58°C for 32 cycles using I2 and I3 cDNA; VH1AFor and Cμ1Rev, 58°C for 30 cycles using I3 cDNA; VH1IFor and VH1CS2VHRev, 60°C for 35 cycles using I3 cDNA; VH6BFFor and VH2CS2CDR3Rev, 65°C for 30 cycles with SP and AK cDNA; and VH6BFFor and VH2CS2CDR3Rev, 65°C for 30 cycles with AK cDNA. After 30 cycles, faint bands of the correct size were present for the VH6B and VH6M products. These gel-excised products were reamplified for an additional 30 cycles using the same primers before cloning. Our earlier analyses had determined that under similar amplification conditions the resultant Taq polymerase error rate was 0.30 × 10–3 mutations per base pair per cycle. Therefore, the likelihood that any sequence within the VH-encoded region of the clones from the cDNA libraries in this report would be expected to be no more than 0.32–0.42 mutations per sequence (42). The confirmatory PCR clones from 30 or 60 rounds of direct amplification from the cDNA pools would be expected to be no more than 0.26–0.34 or 0.44–0.67 errors per sequence, respectively. Therefore, it is unlikely that any one sequence has a mutation resulting from amplification error.

Additional experiments to confirm hybrid products can be amplified from RNA pools derived from different tissues

Additional experiments were conducted to determine whether the hybrid products identified in the cDNA libraries and cDNA pools could also be directly amplified from the total RNA derived from these tissues. The experiments focused upon four hybrids: 18E02SK (Fig. 1B); 19E0212 (Fig. 4C); 21F01AK (Fig. 6B); and 19E10SP (Fig. 6C). For these experiments, Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017
the various total RNA pools derived from the tissues of the animal were used, and first-strand cDNA was synthesized using a primer designed to be specific for the CDR3-encoded region. PCR then used sense and nested antisense primers designed to specifically amplify the hybrid sequence identified in the cDNA libraries. The sequences of these primers are shown in the figures unless otherwise listed. To confirm 18E02SK, VH2CS3Rev3 (5'-GTAATCGAGAAGCTCTGTAG-3') was used for cDNA synthesis with 5 µL of GL or SK RNA, and the VH6For and VH2CS2Rev primer pair was used for amplification (60°C for 30 cycles with one-tenth of the reaction product reamplified for an additional 30 cycles). To confirm 19E0212, VH1CS2SDRev (5'-TCCCATGATGCTAAGAATGCCTGA-3') was used for cDNA synthesis using 5 µL of GL or I3 RNA, and the VH1EFor and VH1CS2VHDRev primer pair was used for amplification (60°C for 30 cycles with one-tenth of the reaction product reamplified for an additional 30 cycles). To confirm 21F01AK, VH2CS2DRev3 (5'-AAAGCCCCGCTAGATCC-3') was used for cDNA synthesis using 5 µL of AK RNA, and the VH2f2FR1For and VH2CS2DRev3 primer pair was used for amplification (65°C for 30 cycles with one-fifth of the reaction product reamplified for an additional 30 cycles). To confirm 19E10SP, VH2CS2DRev3 was also used for cDNA synthesis using 5 µL of AK or SP RNA, and the VH66For and the VH2CS2VHDRev primer pair was used for amplification (67°C for 30 cycles with one-tenth of the reaction product reamplified for an additional 30 cycles). Products were gel excised, cloned, and sequenced as described above.

cRSS analysis in recipient and donor partners

To determine the presence of cRSS in likely donor and recipient VH sequences, the statistical models for defining murine RSS developed by Cowell et al. were used (43). These models, which have been made available online at www.dulci.org/rsc, provide scores for the recombination information content (RIC) of potential cRSS at 28- or 39-bp nucleotide intervals (i.e., 12-bp RSS and 23-bp RSS, respectively). In these models, the CA dinucleotide at positions 1 and 2 of the heptamer is required for scoring by RIC analysis. The applicability of these models was evaluated using the functional catfish germine VH, DJ, and JH sequences (see Results).

Results

Hybrid H chain cDNA clones express members of different VH families

RNA was derived from eight different tissues from the same individual adult channel catfish, and seven of these were used to construct different H chain cDNA libraries. These seven tissues were anterior kidney (a major hematopoietic organ in bony fish; denoted AK throughout), PBL, spleen (denoted SP throughout), gill lamellae (denoted GL throughout), skin (denoted SK throughout), and two nonadjacent regions of the intestine designated I2 and I3. Probes specific for the catfish VH families VH1, VH2, VH6, and VH7 were separately hybridized with each library, representative positive clones were sequenced, and sequence alignments were constructed. From these analyses clones were identified and assigned to clonal sets (CS). These related sequences, some of which were found in different cDNA libraries, expressed the same VDJ and a characteristic CDR3 likely specific to that rearrangement. Within a CS, the pattern of the somatic mutations identified in the VHstr, DJstr, and/or JHstr-encoded regions allowed likely genealogies of clonal lineages to be determined.

During these analyses, hybrid clones were identified that expressed members of different VH families in different regions of the same sequence. The sequences of six hybrid clones are shown in Fig. 1. These clones were isolated from five different cDNA libraries, and each encoded an open reading frame. This suggested that a donor segment from a different VH family had replaced the upstream coding region in a recipient rearrangement. Hence, we sought to determine whether likely recipients were also present in the H chain libraries. These recipients would be expected to share the same CDR3/FR4 sequence as the hybrid but express the full-length sequence of the VH member used in the initial rearrangement.

The I2 hybrid 21D0212 expressed members of the VH3 and VH11 families with the junctional boundary located in the CDR1/FR2-encoded region (Fig. 1A). Another CS member, clone 15A10VH4GL from the GL library, was not a hybrid because it expressed the full-length VH1 segment. This sequence from FR2 through CDR3/FR4 was identical, except at one position, to that in the hybrid. BLAST analyses (41) indicated that the VH3 donor was similar to germline segment VH3-47, which differed from the hybrid by 2 nt in the region extending from the 5’-untranslated region (UT) downstream through CDR1.

Hybrid SK clone 18E02SK expressed members of the VH6 and VH2 families with the junctional boundary in FR2 (Fig. 1B). Clone 18E03VH2SK, also from the SK library, expressed the full-length VH2 but was a member of the same CS, as its sequence from near the beginning of FR2 through Cµ was almost identical to that of the hybrid (2-nt differences). The predicted donor was a VH6 member similar to that expressed in 18H12VH6L (Fig. 1B). This VH6 sequence, extending from the 5’-UT through FR2, differed by 2 nt from that in the hybrid.

This hybrid (18E02SK) was also of interest because VH2 and VH6 represented two of the four families chosen for library hybridization and sequencing. If this hybrid resulted from PCR error due to template switching or bridging during cDNA library construction (44–47), then the probability of its detection would be expected to be related to the proportion of template partners available for amplification. Hybridization with a VH6 family-specific probe showed that only 16 of the 514 clones in the SK library were VH6 positive (3.1%), and none of the nine clones that were sequenced used the VH6 member expressed in the hybrid. The clonally related nonhybrid clone (18E03VH2SK) was one of only three VH2-positive rearrangements in this library (0.2% of the total library). VH6 primers were not used during cDNA construction, which also made it less likely that these specific VH6 and VH2 cDNA templates underwent PCR bridging.

Hybrid clones 16A08GL and 20F032 were derived from two different libraries (GL and I2, respectively), and both are members of the same CS because they shared the identical CDR3/FR4. The upstream and downstream regions are encoded by segments from the VH2 and VH5 families, with the junctional boundary located in the CDR1/FR2 region (Fig. 1C). These hybrids differ by 9 nt, indicating that somatic mutation had occurred. BLAST results indicated that the VH5 recipient was similar to that expressed in 18E03VH5 (Fig. 1C). Another CS member, clone 15A10VH4GL, was not a hybrid because it expressed the full-length VH1 member used in the initial rearrangement. In these hybrids the junctional boundary occurred near or within the FR2 region.

Hybrid cDNA clones express different members of the same VH family

The above results suggested that hybrid formation may result from VH replacement events involving donors from VH families different from those expressed in the original VDJ rearrangement. These
observations were pursued to determine whether \( V_H \) members of the same family might also serve as sequence donors. Sequence alignments indicated that the \( V_H \)-encoded regions could be assigned to different groups. Each group shared between 95 and 100\% nucleotide identity and exhibited coding region similarities likely restricted to a specific germline gene. Nucleotide differences were principally single positions, and these infrequent differences were usually located at different sites within the encoded region. These results allowed \( V_H \) consensus sequences to be constructed that were generally derived from three or more independent rearrangements. The consensus sequence shared 98\% or greater nucleotide identity to the \( V_H \)-encoded region of the clones assigned to that group. A summary of the consensus sequences pertinent to this study is shown in Table I.

**FIGURE 1.** Hybrid Ig H chain sequences express members of different \( V_H \) families in different regions of the same clone. Ig H chain cDNA libraries were separately constructed from seven different tissues derived from the same individual adult catfish. The hybrids shown in each panel (designated as revisions) were identified by random sequencing following library hybridization with probes specific for the \( V_H \)1, \( V_H \)2, \( V_H \)6, or \( V_H \)7 family. Tissue origin is designated by the last two letters of the clone name: AK, GL, I2 (from the middle third of the intestine), I3 (from the distal third of the intestine), PBL, SK, and SP. The arrows demarcate regions within the hybrid that were encoded by members of different \( V_H \) families, with the upstream and downstream regions assigned to sequences most similar to the potential donor and recipient respectively. The sequence shared in common by the hybrid, recipient, and donor lies between the arrows. The sequences are demarcated into FR and CDR regions. In A and B, the hybrid and recipient were clonally related because each encoded the identical CDR3/FR4 regions. In B the clone 25A09GL (designated as ConfmRNA) was derived by PCR from the GL total RNA pool using experimental methods and primer pairs different from those used in H chain cDNA construction (see Materials and Methods). Other PCR clones derived from GL or SK total RNA pools were either identical to 25A09GL or differed by one or two nucleotides (positions of difference are shown by an asterisk; \( ^* \); see Results). The underlined recipients in C–E were not clonally related to the hybrid but were identified in BLAST searches as sequences with the highest similarity to the downstream \( V_H \)-encoded region in the hybrid. Dots indicate sequence identity and dashes indicate gaps introduced to maximize nucleotide similarity. The heptamer (7-mer) and nonamer (9-mer) of the cRSS predicted to be recombinogenic when evaluated using the statistical models of Cowell et al. (43) are boxed in A and B (see also Table III).
When known germline and consensus sequences were aligned with the library sequences, some CS members appeared to represent hybrids. The hybrids shared the same CDR3/FR4 and sequence identity with other CS members continued upstream toward the leader region (LDR) until an unexpected change in sequence occurred. At that point the sequence matched the sequence of a different VH member. These observations suggested three different possibilities: 1) these hybrids were PCR artifacts; 2) the nucleotide differences were due to multiple somatic mutations resulting in substitutions identical to the nucleotides in potential donors; or 3) the hybrids represented bona fide in vivo VH replacement events. These hypotheses were pursued to determine the most likely explanation.

Clonal lineage VH2-CS1 likely resulted from rearrangement of VH2 member VH2D with germline segments DH2 and JH2 (Fig. 3). Each clonal member carried the sequence of a different VH member. These observations suggested three different possibilities: 1) these hybrids were PCR artifacts; 2) the nucleotide differences were due to multiple somatic mutations resulting in substitutions identical to the nucleotides in potential donors; or 3) the hybrids represented bona fide in vivo VH replacement events. These hypotheses were pursued to determine the most likely explanation.

Table I. Summary of catfish VH consensus sequences expressed in H chain cDNA libraries

<table>
<thead>
<tr>
<th>VH Consensus Sequence</th>
<th>Number of Independent VDJ Rearrangements</th>
<th>Number of cDNA Clones in Clonal Sets Expressing the Consensus VH Sequence</th>
<th>Total Number of cDNA Clones Expressing the VH Consensus Sequence</th>
<th>Mean ± SD, and Range of the Percentage of Nucleotide Identities in cDNA Clones when Aligned with the VH Consensus Sequence</th>
<th>Representative cDNA Clone Expressing the VH Consensus Sequence (accession no.)</th>
<th>Total Number of Nucleotide Differences Between Representative cDNA Clone and the VH Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1A (400 nt)</td>
<td>7</td>
<td>31 (CS1)* 2 (CS6)</td>
<td>38</td>
<td>98.9 ± 0.4 (97.8 –99.7)</td>
<td>20A06V15SK_CS1 (EU492754)</td>
<td>1</td>
</tr>
<tr>
<td>VH1B (398 nt)</td>
<td>10</td>
<td>10 (CS2)</td>
<td>19</td>
<td>99.6 ± 0.5 (97.7 –100)</td>
<td>15C07V1PBL1PBL (EU492557)</td>
<td>0</td>
</tr>
<tr>
<td>VH1E (400 nt)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>99.2 ± 0.7 (98.5 –100)</td>
<td>15E06V1ISP (EU492641)</td>
<td>0</td>
</tr>
<tr>
<td>VH1B2 (369 nt)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>98.8 ± 0.2 (98.6 –98.9)</td>
<td>16C02V2PBL (EU492565)</td>
<td>4</td>
</tr>
<tr>
<td>VH2D (369 nt)</td>
<td>4</td>
<td>4 (CS1)</td>
<td>7</td>
<td>99.7 ± 0.2 (99.4 –100)</td>
<td>16H3V1213_CS1 (EU492821)</td>
<td>0</td>
</tr>
<tr>
<td>VH2H (350 nt)</td>
<td>3</td>
<td>3 (CS7)</td>
<td>5</td>
<td>98.8 ± 0.4 (98.2 –99.1)</td>
<td>16E08V2ISP (EU492651)</td>
<td>3</td>
</tr>
<tr>
<td>VH2N (369 nt)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>98.7 ± 1.0 (97.5 –99.5)</td>
<td>21H05V2213 (EU492846)</td>
<td>2</td>
</tr>
<tr>
<td>VH2O (369 nt)</td>
<td>2</td>
<td>2</td>
<td>2d</td>
<td>99.5 ± 0.4 (99.2 –99.7)</td>
<td>16C03V2PBL (EU492566)</td>
<td>1</td>
</tr>
<tr>
<td>VH6B (366 nt)</td>
<td>11</td>
<td>2 (CS5)</td>
<td>12</td>
<td>99.3 ± 0.5 (98.6 –100)</td>
<td>17B09V6GL (EU492717)</td>
<td>0</td>
</tr>
<tr>
<td>VH6M (366 nt)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>99.5 ± 0.7 (98.4 –100)</td>
<td>17C11V6PBL (EU492572)</td>
<td>0</td>
</tr>
<tr>
<td>VH7F (399 nt)</td>
<td>4</td>
<td>2 (CS2)</td>
<td>5</td>
<td>99.5 ± 0.4 (99.0 –99.7)</td>
<td>18A02V7CS2 (EU492727)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Clonal sets expressed the identical VDJ rearrangements. The number of clones analyzed in these sets precedes the CS designation.

The VH2H-encoded region in clones 15E09V11SP (EU492643), 19C10V113_CS2 (EU492834), and 21D06V113 (EU492842) was also identical to the VH2B consensus sequence.

The VH2M consensus sequence was derived by including one clone from a previous study (M58670; Ref. 33).

The VH2O consensus sequence was derived by including one clone from a previous study (DQ230574; Ref. 42).

The VH16-encoded region in clones 18B05V166SP (EU492664) and 18F08V16SP_CS5 (EU492675) was also identical to the VH6B consensus sequence.

had the same T249→C and A322→G mutations that were present in the hybrid (16H0713), which supports a common ancestry (VH2-CS1H1 in Fig. 2A).

A different CS (VH7-CS1) was initially identified in I2, and the cDNA libraries were hybridized with a CDR3 probe to determine the locations of other CS members. These results showed that 40 of the 42 VH7-positive I2 clones as well as one of two VH7 PBL clones were members of VH7-CS1. A PBL clone and 34 of the I2 clones were sequenced and multiple alignments were constructed. VH7-CS1 likely resulted from the recombination of germline segments VH7-12, DH1, and JH6 (Fig. 3). Each clonal member carried two VH mutations, G25C and G25A (differences that might also represent polymorphisms). VH7-CS1 had undergone expansion and somatic mutation because clonal members differed at one or two additional positions (Fig. 3A). I2 clonal member 20C07T22, however, represented a hybrid in which a VH7 member similar to VH7F had replaced the 5′-UT downstream through the end of FR2 (Fig. 3B). Each of the 24 nt differences in this region were identical to nucleotides in VH7F. This event maintained the open reading frame but replaced the upstream point mutations that distinguished various clonal members (e.g., C-37→T, T-27→C, G25→A, etc.). Thus, it could not be determined which sublineage was the likely recipient. Because it was unlikely this hybrid would be represented in other library clones, PCR approaches were used to obtain confirmatory evidence.

The I2 and I3 H chain cDNA pools were separately amplified with a VH7F-restricted FR1 forward primer and the VH7-CS1 CDR3 reverse primer. Products of the expected size were cloned, and three clones from each product were sequenced. Although none of the six matched the 20C07T22, a different hybrid was identified. A VH7 member similar to VH7F appeared to have replaced VH7-12 from the 5′-UT downstream through the beginning of the FR3 region. The hybrid sequence was identical in clones 22A02I2 from I2 and 22A04I3 from I3 (Fig. 3C). Sequences of additional clones indicated that somatic mutation had occurred in this hybrid lineage (Fig. 3A).
Hybrids may result from sequential VH donor replacements

These results indicated that different hybrids may be present in the same CS and that a hybrid identified in the H chain cDNA population from one tissue may be present in a different tissue. Additional studies to support these results were pursued during the analysis of VH1-CS2 (initially identified in I3). VH1-CS2 resulted from the recombination of member V H1B with germline segments DH1 and JH2. The libraries were hybridized with a CDR3 CS-specific probe, and multiple I3 clones, an I2 clone, and a PBL clone were sequenced. The I3 and PBL clones differed by 1–4 mutations (Fig. 4A). However, clone 19E02I2 from the I2 library represented a hybrid with 23-nucleotide differences in the 5′/H11032-UT through FR2 regions when compared with V H1B (Fig. 4C). When aligned with other VH1 members, these differences grouped into two subregions, with each assigned to a different VH1 member. Eleven nucleotide differences (located
between the 5' end of the LDR) were identical to those in member VH1A (Fig. 4C). Nine other differences (between the end of CDR1 and the end of FR2) were identical to nucleotides in member VH1E. The remaining three nucleotide differences were located near the end of FR1, and two of these were present in both VH1A and VH1E (positions 60 and 78; G68T was a likely mutation; Fig. 4C).

These results suggested that 19E02I2 might be the product of sequential replacements wherein the upstream region was initially replaced by a segment similar to VH1A, and this intermediate hybrid was secondarily replaced with a member similar to VH1E. We reasoned that if this hypothesis was to receive further consideration, then the hypothetical intermediate as well as the secondary hybrid would have to be identified in the cDNA population from a different tissue. The I3 cDNA pool was chosen for these experiments and different primer pairs were used for amplification. A CDR1 primer designed to amplify member VH1A, but not VH1B or VH1E (VH1Afor; Fig. 4B), was used in conjunction with a Cµ1 reverse primer. A product of the expected size was amplified and two clones were sequenced. These results showed that both clones (21E01I3 and 21E07I3) had the same CDR3/FR4 as the other clonal members, and the sequences matched that of the predicted hybrid intermediate (Fig. 4B). Two nucleotide differences between these clones indicated that this intermediate lineage had undergone somatic mutation.

Another primer pair was used to determine whether the sequential hybrid could be identified in the I3 H chain cDNA pool. A primer designed to amplify VH1E, but not VH1A or VH1B (VH1Efor; Fig. 4C), was used with the CDR3 primer, and a product of the expected size was cloned and sequenced. This I3 product, 22A07I3, was identical except for two nucleotide mutations to the hybrid clone 19E02I2 from I2. Thus, these results confirmed the presence of this hybrid in a different tissue and supported the hypothesis that sequential VH replacements may occur.
FIGURE 4. Genealogical relationships between clonally related cDNA sequences in clonal set VH1-CS2. A, The dashed circle indicates the common progenitor VH1B/DJ, which likely resulted from the recombination of VH1 member VH1B with the germline segments DH1 and JH2. The sequences of the predicted common progenitor and the I3 library clone 19C10I3 were identical. The solid circles represent cDNA library clones designated by their names and tissue source (see legend to Fig. 1). The dotted circle represents the hypothetical clonal intermediate VH1A/B which was the initial hybrid product (subsequently retargeted with donor member VH1E). The double circles represent PCR clones amplified from I3 H chain cDNA pool using the underlined primers shown in B and C. The triple circles represent PCR clones derived from the total RNA pool derived from either I1 or I3 using different experimental methods and primers than those used for H chain cDNA construction (see Materials and Methods). The mutations that occurred during clonal expansion are shown beside the arrows (see Fig. 2 legend).

B Revision 1: 21E01 I3

C Revision 2: 19E02 I2, 22A07 I3 and 25A03 I1
Different hybrids in the same CS may result from independent and sequential events and may be isolated from different tissues.

The last CS examined was VH2-CS2, which arose by recombination of a VH2 family member with the germline DH2 and JH1 segments (Fig. 5). The clonal precursor was designated as clone 16C12AK, with identical clones identified in the PBL and SP libraries. Each clone had the A328 mutation in the DH2-encoded region, and this was the only example of this mutation in the >20 independent rearrangements from this fish that expressed DH2. Hybridization of the libraries with a CS-specific CDR3 probe (VH2CS2DJRev; Fig. 6A) identified seven other clones; two AK clones differed from the clonal precursor by only 1 or 2 nt. However, the other five clones (two from SP and one each from the AK, I2, and PBL libraries) represented different hybrids, two of which expressed members of different VH families (Figs. 5 and 6).

**FIGURE 5.** Genealogical relationships between clonally related cDNA sequences in clonal set VH2-CS2. A, The dashed circle indicates the common progenitor VH2/DJ-CS2, which resulted from recombination of a VH1 member with germline DJ2 and JH2 gene segments. The solid circles represent H chain cDNA library clones designated by their names and tissue source (see legend to Fig. 1), and the dotted circles represent hypothetical clonal intermediates. Clonal intermediate VH2H/VH2 represents a hybrid that was sequentially targeted for VH replacement by donor member VH6M of the VH6 family. The double circles represent PCR clones amplified from the H chain cDNA pool of the indicated tissues (see the legend to Fig. 1) using the underlined primers shown in Fig. 6. The triple circles represent PCR clones derived from the total RNA pool of the indicated tissues using experimental methods and primer pairs different from those used during H chain cDNA construction (see Materials and Methods). The mutations that occurred during clonal expansion are shown beside the arrows (see Fig. 2 legend). B, The predicted amino acid sequences of different hybrids within VH2-CS2 aligned with the clonal progenitor 16C12AK. The coding region is demarcated into the LDR, FR, CDR and partial H region. Dots indicate sequence identity and dashes indicate gaps that were introduced to maximize sequence similarity.
FIGURE 6. Sequence alignments of the hybrid clones in clonal set VH2-CS2 (as depicted in Fig. 5). In all five panels (A–E), the sequences of hybrid clones (REV) with arrows demarcating regions within the hybrids that were assigned to different donor (DNR) and recipient (REC) sequences are shown. The consensus sequence for VH members VH6B, VH2H, VH6M, VH2B, or germline VH2-6 are respectively shown in A–E as the donor (see also Table I). VH6B and VH6M are members of the VH6 family, whereas the others are members of VH2. The hybrids are demarcated into the 5′-H11032-UT, LDR, FR, CDR, and partial C′/H9262 regions. The 5′- and 3′-ends of some library clones as well as the 5′-ends of some VH consensus sequences are not shown because their full-length sequence is not necessary to indicate the derived donor or recipient regions. The utilized regions of the germline DH2 and JH1 segments are shown in A. Nucleotides designated as V/N were either derived from the germline VH2 segment or from N region additions. Dots indicate sequence identity and dashes indicate gaps introduced to maximize nucleotide similarity. The heptamer (7-mer) and nonamer (9-mer) of cRSS predicted to be recombinogenic (43) are boxed (see also Table III). The sequences of the primers used to derive the PCR clones from the H chain cDNA pools or the total RNA pools (shown as double or triple circles, respectively, in Fig. 5) are underlined (see also Materials and Methods).
The number of potential recipients in the H chain cDNA library where the hybrid was identified (designated by the last two letters of the clone name) was determined by hybridization with CS-specific CDR3 probes. The estimated percentage of recipients was calculated by dividing the number of potential recipients by the total number of clones in the cDNA library (all cDNA libraries were composed of 534 clones except SK, which was composed of 514 clones). Similarly, the number of clones in the VH family of the donor member (as shown in the designated figures) was determined by library hybridization with VH family-specific probes. Various clones were sequenced and the number of clones that potentially expressed the donor VH member was determined. Clones that expressed the VH<sub>I</sub> family but were not sequenced were assumed to be potential donors (an improbable assumption). The estimated percentage of potential donors was determined by dividing the number of potential donors by the total number of H chain clones in the respective cDNA library. The number of potential recipients shown for hybrid clone 18E02SK was determined by directly sequencing the VH<sub>I</sub>2 positive SK clones.

The number of potential donors in the VH family of the donor member (as shown in the designated figures) was determined by library hybridization with VH family-specific probes. Various clones were sequenced and the number of clones that potentially expressed the donor VH member was determined. Clones that expressed the VH<sub>I</sub>2 family but were not sequenced were assumed to be potential donors (an improbable assumption). The estimated percentage of potential donors was determined by dividing the number of potential donors by the total number of H chain clones in the respective cDNA library. The number of potential recipients shown for hybrid clone 18E02SK was determined by directly sequencing the VH<sub>I</sub>2 positive SK clones.

A VH<sub>H</sub>6 member similar to VH<sub>H</sub>6B was the likely replacement donor expressed in hybrid clone 19E06AK. The 5'-UT downstream through the beginning of FR2 differed extensively from CS precursor 16C12AK, yet differed by only 1 nt when aligned with VH<sub>H</sub>6B. Downstream from the FR2 boundary the sequence was identical to 16C12AK. To determine whether this hybrid was present in other libraries, the AK and SP H chain cDNA pools were used in separate PCR with a FR2/CDR3 primer designed to be restricted to VH<sub>H</sub>6B and the CDR3 primer (Fig. 6A). Products of the expected size were obtained from both reactions, and the products were cloned and sequenced. The sequences of two AK clones and the sequence of a SP clone were either identical or differed at two positions. The primary intermediate by separately amplifying AK and SP cDNA with a VH<sub>H</sub>2 member-restricted LDR/FR1 primer and a CDR3 primer designed to not encompass the hallmark D<sub>H</sub>2 A328→C mutation (Fig. 6B). A product of the expected size was amplified from both tissues. The SP and AK sequences (21F01AK, and 21E08SP) matched that predicted for the intermediate hybrid, and A328→C was present (Figs. 5A and 6B). Studies were then done to determine whether the secondary hybrid product could be amplified from a different tissue. The AK cDNA pool was amplified using the VH<sub>H</sub>6M LDR/FR1 primer and the CDR3 primer. The sequence of this product (22C03AK) was identical to the hybrid SP library clone 19E10SP (Fig. 6C). Thus, these experiments also supported the hypothesis of sequential donor replacements.

The fourth hybrid in VH<sub>H</sub>2-CS2 was represented by clones from the PBL and I2 cDNA libraries, 20C02PBL and 16G06I2. These two clones shared the hallmark CDR3 features of the CS, yet the 5'-UT downstream through CDR1 had 6 or 10 nt differences, respectively, from the clonal precursor. Five or seven of these differences were identical to nucleotides in member VH<sub>H</sub>2B (Fig. 6D), which suggested that this regional difference was the result of an additional replacement event resulting in a sublineage that had undergone somatic mutation.

Lastly, the third hybrid in VH<sub>H</sub>2-CS2 was represented by SP library clone 20C04SP (Fig. 5). This clone differed from clonal precursor 16C12AK at nine positions in the region extending from the 5'-UT downstream through the beginning of FR1. Three of these nine nucleotides represented a deletion within the 5'-UT (Fig. 6E). Yet, when aligned with other VH<sub>H</sub>2 members, eight of these nine positions were identical to those in germline VH<sub>H</sub>2 member VH<sub>H</sub>2-6. Thus, 20C04SP likely represented the product of another event. Thus, these combined results indicated that VH<sub>H</sub>2-CS2 was the likely target of multiple VH<sub>H</sub> replacements, each resulting in an open reading frame that preserved the CDR3 coding region of the original rearrangement and served to modestly or extensively diversify the CDR1 and/or CDR2 combining sites (Fig. 5A).

### Table II. Estimated percentage of potential recipients and donors in cDNA libraries where hybrid clones were identified

<table>
<thead>
<tr>
<th>Clonal Set Name</th>
<th>Figure Shown</th>
<th>Hybrid cDNA Clone</th>
<th>Estimated Percentage of Potential Recipients in cDNA Library (%)</th>
<th>Estimated Percentage of Potential Donors in cDNA Library (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH&lt;sub&gt;H&lt;/sub&gt;2-CS3</td>
<td>B</td>
<td>18E02SK</td>
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<td>B</td>
<td>16H07I3</td>
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<td>0.7</td>
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<td>VH&lt;sub&gt;H&lt;/sub&gt;2-CS1</td>
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<td>20E01SP</td>
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<td>13.5</td>
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<td>7.3</td>
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<td>19E02I2 (primary)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>VH&lt;sub&gt;H&lt;/sub&gt;1-CS2</td>
<td>C</td>
<td>19E02I2 (secondary)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>19E06I6AK</td>
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<td>19E10I6SP (primary)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>C</td>
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</tbody>
</table>

<sup>a</sup> The number of potential recipients in the H chain cDNA library where the hybrid was identified (designated by the last two letters of the clone name) was determined by hybridization with CS-specific CDR3 probes. The estimated percentage of recipients was calculated by dividing the number of potential recipients by the total number of clones in the cDNA library (all cDNA libraries were composed of 534 clones except SK, which was composed of 514 clones). Similarly, the number of clones in the VH family of the donor member (as shown in the designated figures) was determined by library hybridization with VH family-specific probes. Various clones were sequenced and the number of clones that potentially expressed the donor VH member was determined. Clones that expressed the VH<sub>I</sub> family but were not sequenced were assumed to be potential donors (an improbable assumption). The estimated percentage of potential donors was determined by dividing the number of potential donors by the total number of H chain clones in the respective cDNA library. The number of potential recipients shown for hybrid clone 18E02SK was determined by directly sequencing the VH<sub>I</sub>2 positive SK clones.

<sup>c</sup> A potential recipient was not identified. The estimated frequency was calculated as described above by assuming that there was one additional clone in the cDNA library that represented this hypothetical recipient.

<sup>c</sup> Clones 19E02I2 and 19E10I6SP represented hybrids that appeared to be the products of sequential VH replacement events. These events are designated as primary and secondary (see Results).
Table III. Comparison of the distribution of mutational hotspots with the distribution, sequence, and estimated recombinatorial efficacy of cryptic recombination signals (eRSS) in recipient and donor VH sequences identified in chimeric hybrid clones.

<table>
<thead>
<tr>
<th>Hybrid clone</th>
<th>Figure shown</th>
<th>VH member</th>
<th>Length of sequence region used to construct the recipient and donor chimeras</th>
<th>Number of mutated VH CDR3 cores in the entire VH region of the recipient and donor (in frequency)</th>
<th>Number of VH CDR3-containing motifs within the entire VH region of the recipient and donor (in frequency)</th>
<th>Total number of scoring eRSS pairs in the entire VH region of the recipient and donor (in frequency)</th>
<th>Number of high scoring eRSS pairs in the entire VH region of the recipient and donor (in frequency)</th>
<th>Values and orientations for highest scoring eRSS pairs within the region shared in common by the recipient and donor (K-D Orientation)</th>
<th>Values and orientations for highest scoring eRSS pairs within the region shared in common by the recipient and donor (K-D Orientation)</th>
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</thead>
<tbody>
<tr>
<td>21020R2</td>
<td>1A</td>
<td>VH1</td>
<td>12</td>
<td>5 (9)</td>
<td>9</td>
<td>1 (12-25)</td>
<td>29</td>
<td>9</td>
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<tr>
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<td>1B</td>
<td>VHB</td>
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<td>2 (19)</td>
<td>16</td>
<td>3 (21-23)</td>
<td>46</td>
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<tr>
<td>3100202</td>
<td>4C</td>
<td>VHH</td>
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<td>2 (2-12)</td>
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<tr>
<td>30055AK</td>
<td>6A</td>
<td>HC12AK</td>
<td>32</td>
<td>6 (57)</td>
<td>1 (13)</td>
<td>19</td>
<td>3 (1-25-25)</td>
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<td>19</td>
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<td>3 (1-25-25)</td>
<td>3 (1-25-25)</td>
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</tbody>
</table>

*Refers to the number of nucleotides in the indicated hybrid clone that are located in between the spurious sequence assigned to the donor and the downstream sequence assigned to the recipient. These boundaries delimit the region where the likely recombination occurred and these boundaries are shown in the indicated figures (See Figures 1A-3). The number of high scoring eRSS pairs located within the entire VH region of the recipient and donor are also shown. The eRSS scores for physiological mouse 12RSS ranged from 0-10 (predicted to be highly efficient) to 48.50 (very low efficiency). For the values for 21RSS ranged from 56.80 to 69.90 (35). The upper limit for a marginal 12RSS was defined as 56.60 with the marginal upper limit for a 21RSS defined as 79.90. The number of high scoring eRSS pairs located within the region shared in common by the recipient and donor are also shown. The eRSS scores for physiological mouse 12RSS ranged from 0-10 (predicted to be highly efficient) to 48.50 (very low efficiency). For the values for 21RSS ranged from 56.80 to 69.90 (35). The upper limit for a marginal 12RSS was defined as 56.60 with the marginal upper limit for a 21RSS defined as 79.90.

*The "orientation" of the eRSS indicates that the nomenclature is consistent with the orientation of the eRSS as defined by the direction of the DNA strand. The number 23 refers to the number of nucleotides in the spacer region between the regions marked by the eRSS and the donor.

*The values shown represent the highest scoring eRSS pairs and their orientations in the recipient and donor. For these values to be listed, the eRSS score for the recipient and donor must be within the above limits defined (cf Table footnotes). Only one high scoring eRSS pair was defined within the region shared in common by the recipient and donor for hybrid clones 210202, 38620K, 30030, and 30055AK. The sequence of the eRSS and orientation in the recipient and donor are shown in the 3' direction of the donor DNA strand. Hybrid joint formation requires that the eRSS from either the recipient or donor be present in the hybrid clone (30). The eRSS and orientation from the recipient or donor are underlined if the eRSS was present in the hybrid clone. If the hybrid and donor are not aligned in both the recipient and donor, then the potential origin of the hybrid joint within the hybrid could not be assigned by sequence alone. In these cases, the hybrid and donor are not underlined. Hybrid joint formation would predict that eRSS pairs in the "○" orientation would use the signal end of the recipient whereas those in the "○" orientation would use the signal end of the donor.

*No high scoring eRSS pairs were defined.
than the number of potential recipients, as these values were calculated by assuming that any nonsequenced clone expressed the same VH member as that assigned to the replacement donor (an improbable assumption). The donor values ranged from 0.4 to 24.2% in the various libraries. These percentages of potential recipients and donors within the libraries indicate that it was unlikely that these specific templates underwent PCR bridging to result in hybrid formation.

**Hybrids identified in H chain cDNA libraries can be amplified from total RNA derived from these different tissues**

An additional series of experiments were done to determine whether the hybrids from the H chain cDNA libraries were the result of in vivo VH replacement events. For these experiments an aliquot of the original total RNA pool derived from these various tissues was used. Rather than initiating cDNA synthesis with a Cµ primer (as was done in the previous phase of this study), first-strand cDNA synthesis was initiated with a CDR3/FR4 primer designed to be specific to the rearrangement expressed in the hybrid. A sense primer corresponding to the VH member used in the replacement was then paired with an internally nested CDR3/FR4 primer to determine whether the hybrid sequence could be amplified (see Materials and Methods).

These experiments focused upon four hybrids: 18E02SK (Fig. 1B), 19E02I2 (Fig. 4C), 21F01AK (Fig. 6B), and 19E10SP (Fig. 6C). Hybrid clone 18E02SK from the SK H chain cDNA library expressed members of the V46 and V82 families. The PCR experiments amplified a band of the expected size from both GL and SK total RNA pools. The excised products were cloned, and four clones from GL and three from SK were sequenced. Two of the GL clones and two of the SK clones were identical to each other as well as identical to 18E02SK (representative clone 25A09GL is shown in Fig. 1B). When compared with 18E02SK, the other two GL clones had either one (C146\(\rightarrow\)T) or two (T147\(\rightarrow\)C and T116\(\rightarrow\)G) nucleotide differences. The other SK clone differed by one nucleotide (G188\(\rightarrow\)A).

Hybrid clone 19E02I2 from the I2 H chain library appeared to result from sequential replacements (initially by member V441A and secondarily by member V441E). Following first-strand synthesis, a V441E member-restricted primer was used in PCR and bands of the expected size were amplified from the total RNA pools derived from the other two intestinal regions, I1 and I3. The products were gel excised, and four I1 clones and four I3 clones were sequenced. Three of the I1 clones and three of the I3 clones were identical in sequence to 19E02I2 (representative sequence 25A03I1 is shown; Fig. 4C). The other I1 and I3 clones had one (A152\(\rightarrow\)C) or two (C59\(\rightarrow\)T and T116\(\rightarrow\)C) nucleotide differences, respectively.

The last two experiments focused on hybrid clone 19E10SP, which also appeared to be the result of sequential events in which V42H had initially replaced the V42E member expressed in the original rearrangement and, in turn, the upstream region of V42H had been replaced by a member of a different VH family, V46M. For these experiments, first-strand cDNA was synthesized with a CDR3/FR4 primer and PCR subsequently used either a V42H or a V46M member-restricted primer with an internally nested CDR3 primer. In both experiments, products of the expected size were recovered from AK RNA and the V46M product was also recovered from SP RNA. Three AK clones corresponding to the intermediate V42H product were sequenced, and two were identical to the intermediate hybrid amplified earlier from the H chain cDNA pool (representative clone 25B07AK is shown aligned with 21F01AK in Fig. 6B). The other AK clone had one mismatch (T204\(\rightarrow\)C). Four AK clones and three SP clones corresponding to the 19E10SP sequential product were also sequenced. All four of the AK clones and one of the SP clones were identical in sequence to 19E10SP (representative clone 25C01AK is shown in Fig. 6C). The other two SP clones had two (A17\(\rightarrow\)G and A240\(\rightarrow\)G) or one (A152\(\rightarrow\)G) nucleotide difference. Thus, the hybrids studied in these experiments could be independently amplified from the total RNA derived from one or more different tissues.

**Presence of cryptic recombination signals within functional boundaries**

As noted in the Introduction, mammalian VH revisions occur in a region where the recipient and the donor share a common sequence. The occurrence of cRSS within this common region has suggested that receptor revision may be RAG mediated and occurs via hybrid joint formation (26–28). The hybrid clones identified in this study were examined to evaluate this possibility.

Cowell and colleagues (43) developed statistical models to compute RIC values that estimated the recombinogenic potential of murine RSS. These models were also used to define potential cRSS within their genomic contexts (cRSS are also composed of a heptamer, a 12- or 23-bp spacer, and a nonamer). To evaluate whether these models could identify catfish cRSS, RIC values for the 23-RSS located at the conventional positions of 34 previously characterized functional germline catfish VH and JH segments were determined (34–36, 38). These RIC scores ranged from −34.3 to −64.5, and each was below the −69.69 value defined for functional murine 23-RSS (43). Similarly, RIC scores for the conventional 12-RSS that flank the functional catfish D\(\gamma\) segments (37–38) ranged from −16.0 to −31.0, and each was below the −48.16 RIC value defined for functional murine 12-RSS (43). These results on the conventional catfish RSS indicated that RIC murine models should provide a reasonable approach to evaluate whether potentially functional cRSS may be present.

The formation of a functional revision via hybrid joint formation requires the heptamers of the cRSS in the recipient and the cRSS in the donor to exist in the same orientation and coding region location in order for a seamless revision to occur and thus preserve H chain functionality (26–28). Revisions can theoretically occur with recipient/donor cRSS pairs in any of four possible spacer region combinations, i.e., 12-RSS in both, 23-RSS in both, 12-RSS/23-RSS in recipient and donor, or 23-RSS/12-RSS in recipient and donor. Thus, eight possible cRSS pair combinations might lead to receptor revision: four combinations of spacers in one of two orientations (the “positive” (+) orientation where the 9-mer is downstream of the 7-mer and the “negative” (−) orientation where the 9-mer is upstream of the 7-mer). There were 13 hybrids in this study in which both recipient and donor partners could be assigned. RIC analyses predicted that the total number of spatially correct cRSS pairs that had recombinogenic potential in the VH\(\gamma\) encoded region of these partners ranged from a low of nine to a high of 37 (Table III). Potential cRSS pairs were also identified where the recipient and donor were members of different families.

The number of possible cRSS pairs located only in the sequence region shared in common by the predicted recipient and donor used in hybrid formation were then determined. In 12 of the 13 hybrids, potential recipient and donor cRSS pairs were identified. The number of potential cRSS recombinogenic pairs generally increased as the length of the common sequence region increased (Table III). The 7-mer and 9-mer sequences that reflect those cRSS pairs with the highest RIC scores in Table III are boxed in Figs. 1–4, and Fig. 6. Thus, cRSS pairs that may be functional in recombination events appear to be present in both the recipient and the donor in the required orientations and locations.
Presence of mutational targets within junctional boundaries

Earlier studies determined the mutability indexes of mononucleotides, dinucleotides, and trinucleotides and defined AGCT and AGCA as the significant RGYW tetranucleotide targets for somatic mutation in catfish H chains (42). The high concentration of somatic mutations in these few RGYW motifs indicated that the motifs likely targeted by AID are restricted in the catfish compared with those known in mammals. These results allowed us to pursue the alternative hypothesis that hybrid formation was mediated by AID (12, 27–28, 31). If revision occurred via an initiating AID-induced DNA lesion, the targeted site would be expected to be within the junctional boundary where the recipient and donor shared the same sequence. The frequency of these targets in the recipient was determined and compared with the frequency of these targets within the complete V_H coding region. These results showed that these mutational targets were not within the junctional boundary of five of the 13 recipients (Table III). In the eight recipients that contained these targets within the junctional boundary, the frequency of these targets was generally higher than that within the total V_H coding region. Thus, these results do not uniformly support the hypothesis that receptor revision is mediated by AID (see Discussion).

Discussion

These studies have sought to provide insight into the patterns and potential mechanisms of V_H replacements that may serve to diversify Ig H chains at the phylogenetic level of the bony fish. In the experimental design of this study, RNA was isolated from eight different organs and tissues from the same individual adult catfish and seven of these were used to construct C_H chain cDNA libraries. These libraries were screened with probes specific for four V_H families and >300 clones were randomly sequenced. These analyses identified clonally related sequences that expressed the same VDJ and encoded a characteristic CDR3, including deletions and N regions likely specific to that clonal rearrangement. Different clonal members were present in the same as well as in different tissues, and differences between some members appeared to extend beyond somatic mutation to include the formation of hybrid sequences. If hybrid sequences had been rare, an occasional clone might have been discarded with its origin attributed to PCR amplification error (e.g., PCR splicing by overlapping extension or bridging; Refs. 44–47). However, the observation that multiple hybrid sequences had been identified in these cDNA libraries, which were constructed from RNA isolated from eight different organs and tissues, differed from those expressed in the original rearrangement. Overall, nine of the 140 sequenced clones from the six different clonal sets hybridized with CS-specific CDR3 probes represented hybrids. The percentage of hybrids varied within a CS, and not all clonal sets contained hybrids. The largest clonal set sequenced in this study (V_H1-CS1, not shown) was represented by 60 clonal members, and none represented a hybrid. In contrast, five of the 10 different V_H2-CS2 clonal members (Figs. 5 and 6) represented four different hybrids, and these were identified in the H chain cDNA libraries derived from four different tissues. The structural features of the H chain sequences that resulted from sequential events were also distinct from those that resulted from a single event. In a primary replacement event the 5'-end of the recipient was replaced with the sequence from the incoming donor resulting in two distinct regions: the 1) upstream region extending into the 5'-UT/LDR encoded by the donor; and 2) the downstream region, including the CDR3/FR4 region encoded by the recipient. In the sequential replacements there were three regions, with each region encoded by a different V_H member. The upstream region (including the 5'-UT/LDR region) was encoded by the donor member used in the secondary event, a middle region was encoded by the V_H member used in the primary event, and the downstream region was encoded by the V_H member used in the original rearrangement.

The supporting series of experiments to confirm the presence of the hybrids in the H chain cDNA libraries focused upon four library clones, two of which appeared to represent sequential V_H replacements. The approach taken was 2-fold. First, the same H chain cDNA pools used for library construction were directly analyzed by PCR with primers designed to specifically amplify the hybrids identified in the libraries. Secondly, the original total RNA pools used to construct H chain cDNA were directly analyzed by RT-PCR, with first-strand cDNA synthesized using primers designed to be CS specific. PCR then used sense and nested antisense primers designed to specifically amplify the hybrids identified in the libraries. The RNA or H chain cDNA pools that were used in these experiments were derived from either the same or a different tissue than that from which the library hybrid was initially identified. The results showed that hybrid library clone 18E02SK (Fig. 1B) was confirmed to be present in the total RNA pools from both GL and SK. Hybrid 20C0712 (Fig. 3B) was not confirmed, but another hybrid within this same CS that used the same V_H7F donor member was confirmed in the H chain cDNA pools from both I2 and I3 (Fig. 3C). Separate experiments were done to confirm the intermediate and sequential V_H events that likely resulted in hybrid library clone 19E0212 from the I2 library (Fig. 4). The predicted intermediate hybrid (V_H1A/V_H1B) was confirmed to be present in the cDNA H chain pool derived from I3, and the sequential hybrid corresponding to 19E0212 was identified in the I3 H chain cDNA pool as well as in both the I1 and the I3 total RNA pool. It is noted that this sequential hybrid was thus identified in each of the three
nonadjacent sections of the intestine that were analyzed in these experiments (see Materials and Methods). Library hybrid 19E06AK (Figs. 5A and 6A) was confirmed to be present in the H chain cDNA pool from both SP and AK. Lastly, separate experiments were done to confirm the intermediate and sequential events that led to the library hybrid 19E10SP (Figs. 5A and 6, B and C). The predicted intermediate hybrid product was identified in both the AK and the SP H chain cDNA pool as well as the AK total RNA pool. The sequential hybrid product resulting from replacement events involving members of different VH families was confirmed in the AK H chain cDNA pool and in both the AK and the SP total RNA pool. Thus, these combined experiments support the hypothesis that in vivo VH receptor revision occurred.

The donor sequences in the hybrids were assigned to 13 VH members representing five different VH families, whereas the recipients were assigned to eight VH members from five different families. Although the size and number of VH genes in the catfish IgH locus have been estimated, only ~25% of the germline VH genes have been sequenced (34–35, 38, 49). Thus, the location of donor segments within the locus relative to the VH recipients used in the primary rearrangement cannot be presently addressed. It is noted, however, that hybrid germline VH genes similar to those known in pigs (50) have not been identified in any of the germline families. Although the size and number of VH genes in the catfish IGH locus have been estimated, only ~25% of the germline VH genes have been sequenced (34–35, 38, 49). Thus, the location of donor segments within the locus relative to the VH recipients used in the primary rearrangement cannot be presently addressed. It is noted, however, that hybrid germline VH genes similar to those known in pigs (50) have not been identified in any of the germline analyses conducted in catfish, or to our knowledge, in the VH genes identified in any other bony fish. But it is known that the germline members of the different catfish VH families are extensively interspersed and closely linked (34–35, 49). Thus, hybrids that result using donors from families different from that of the recipient would not be inconsistent with the organization of the IgH locus. It was also generally evident that nonhybrid clonal members remained present within the B cell population isolated from these tissues. This is one of the potential features that serves to distinguish receptor revision from receptor editing, as the latter mechanism would be expected to replace the original and potentially autoreactive rearrangement before clonal expansion (14–20).

It is noted, however, that editing has been found to occur in the absence of self-reactivity (51–53). Different hybrids resulting from different replacement events were also identified in the same CS, suggesting that replacement events may be a targeted occurrence. Multiple VH replacement events within the same clonal set had also been identified in the analysis of revised clonal H chains derived from human B cells (27–29). The consecutive progression of replacement events targeted toward a specific and preceding VDJ rearrangement had also been observed during receptor editing, where it was referred to as sequential (54) or serial replacement (18). In the primary event, the embedded cryptic FR3 heptamer of the recipient (which partially encodes Cys104) was recombined with the RSS located at the conventional 3′-end of the donor VH segment. This resulted in replacement of the entire upstream V region, leaving only a few of the original V/N nucleotides upstream of those assigned to DJ. When the primary event was sequentially targeted, the above steps again occurred, leaving V/N nucleotides from the primary revision adjacent to those few nucleotides from the original V/N junction. CDR3 lengthening occurred, and a characteristic footprint was defined (18). However, as shown in the studies of Koralov et al. (52), nucleotide deletion and N region insertion at the site of replacement events are not necessarily observed, indicating to these authors that there is strong selection for replacement joints without N region additions. They further postulate that this may be due to sequence homology in the involved spacer sequences, a conclusion further supported by functional analyses (55). Receptor editing is known to be RAG mediated and dependent upon a recombination signal, and it is estimated to be responsible for as many as 25% of the VDJ rearrangements in mammalian B cells (56).

The seamless replacement products observed by Koralov et al. (52) were also observed in the revisions identified in earlier studies (27–29). These events likely occurred in mature B cells, which suggested that RAG reactivation led to these seamless revision products via hybrid joint formation. A hybrid joint is a composite of signal and coding joints, such that the signal end from one recombinant is located adjacent to the coding end of the other. Hybrid joint formation requires that a pair of RSS participate in the recombination event (30, 57) and that certain criteria are met. The pair of RSS (one RSS from the donor and the other from the recipient) can be in one of two relative orientations. If the RSS of the pair are in opposite orientations (relative to each other), deletion of the intervening DNA is the usual outcome. This is observed in receptor editing where the FR3 heptamer of the recipient undergoes recombination (see above). In contrast, hybrid joint formation requires that the RSS in the donor and the recipient reside in the same orientation. Thus, the RSS heptamers in the recipient and the donor must both be either upstream (the positive (+) orientation) or downstream (the negative (−) orientation) of their respective nonamers (30).

The analyses of the catfish hybrid sequences allowed us to define boundaries that delineated the regions assigned to the donor and the recipient. The common sequence region (located in between recipient- and donor-assigned regions) was then evaluated to determine whether a heptamer might be present. It had been shown that the first three nucleotides of the heptamer (CAC) are required for efficient recombination (58–60). Inspection showed that CA (GT) motifs were present, but our initial efforts to define criteria necessary to identify a cRSS were problematic. This same difficulty led Cowell and colleagues (43, 61) to conclude that consensus RSS sequences may not provide sufficient insight into recombination efficiencies (62). This led to their development of the RIC algorithm which scans for 28- or 39-bp sequences within their genomic context (12-RSS or 23-RSS, respectively). The recombinogenic potential of these motifs is then correlated to known functional murine RSS and values are assigned (43). This theoretical model, when used to evaluate catfish RSS in their conventional locations within functional V, D, and J segments, defined RIC values within the threshold limits defined for functional RSS in murine systems. It is noted that RIC scoring of human VH segments was lower than that demonstrated for mouse segments (48, 53, 55, 61); thus, the predictive value of RIC scores when applied to a new and untested system such as catfish VH genes will need to be confirmed experimentally.

These results, however, laid the foundation for determining whether potentially functional cRSS may be present within the sequence region shared in common by the recipient, the donor, and the hybrid. The analyses on the 13 hybrid clones where both donor and recipient partners could be defined are summarized in Table III. Although many of these cRSS pairs would likely have low recombinogenic potential if tested experimentally, these results indicated that these cRSS pairs have the potential to undergo recombination via hybrid joint formation and form seamless revision products. These cRSS pairs were distributed over multiple coding region locations and ranged in number from a low of nine to a high of 37 (Table III). In 12 of the 13 hybrids a cRSS pair was identified within the region shared in common by the recipient and the donor. RIC significant cRSS pairs were in both orientations in five of the hybrids. The positive orientation was the only orientation identified in two of the hybrids, and the negative orientation was the only orientation found in five of the hybrids.
Davila et al. (53) evaluated high RIC scoring cRSS in the coding regions of murine V<sub>H</sub> germline segments. Their analyses of 12-cRSS in the negative orientation identified conserved sites designated as site I through site V. Sites I and II essentially flank the upstream and downstream borders of CDR1, whereas sites III and IV flank and extend through CDR2. Site V encompasses the FR3 Cys<sup>106</sup> cryptic heptamer that is usually associated with RAG-dependent receptor editing in B cell precursors as discussed above (12–14, 18). The authors subsequently evaluated four of these predicted cRSS sites and showed that each was active in recombination assays, leading the authors to postulate that cRSS sites adjacent to CDR1 and CDR2 might be phylogenetically conserved to provide somatic CDR reassortment. The present study supports this general hypothesis. Of the hybrids in this study where a high RIC scoring cRSS heptamer was identified within the region shared between the recipient and donor (Table III), four had undergone replacement of both CDR1 and CDR2, five had undergone replacement of CDR1 only, and three resulted in the replacement of the 5′-UT/LDR. The other four (those where a high scoring RIC cRSS could not be assigned because the recipient or donors partners were either unassigned (Fig. 1, C–E) or the nearest RIC-significant cRSS pair was outside the common boundary (Fig. 3B)) each resulted in the replacement of CDR1. Thus, the hypothesis that revision provides for somatic CDR reassortment is supported. However, as discussed below, some of the hybrid lineages also appear to have undergone somatic mutation. Thus, receptor revision may function beyond repertoire diversification to include the rescue of B cells from lethal mutations or their rescue from replacement mutations that promote autoreactivity. It may even provide a CDR-independent mechanism that replaces the 5′-UT, thus altering V<sub>H</sub> promoter-regulated Ig expression (53, 63–65).

The cumulative results of this study indicate that hybrid formation is occurring within expanded, presumably Ag-stimulated, clonal B cells. It was also observed that in CS where these hybrids were identified, unrevised clonal members had undergone somatic mutation (Figs. 2A, 3A, 4A, and 5A). It cannot be concluded from sequence information alone whether somatic mutation preceded hybrid formation, because mutations within the distal 5′-end of a targeted recipient would have been replaced by the donor. However, there is evidence to indicate that hybrid lineages occurred after a clonal precursor had undergone somatic mutation. Examples of this are the CDR3 A322→G mutation found in all clonal members of V<sub>H</sub>2-CS1 (Fig. 2) or the CDR3 A328→C mutation within all clonal members of V<sub>H</sub>2-CS2 (Figs. 5 and 6), where mutations occurred within the germline encoded region of D<sub>H</sub>2 (see Results). There was additional evidence that somatic mutation had occurred after revision, as many of the confirmatory clones derived from either H chain cDNA pools or the RNA from these different tissues had different mutational patterns. Somatic mutation had also been observed to occur both before and after receptor editing (66). This interwoven relationship between somatic mutation and replacement events had suggested that receptor revision might be AID mediated (13, 27–28, 31, 63).

Earlier studies had shown that AGCT and AGCA were the expected V(D)J recombinase targets for somatic mutation in catfish (42). If AID was involved in hybrid formation, these targets would be expected to be present within the region shared in common by the donor and the recipient. The presence of these targets within this region was determined and compared with their frequency within the entire V<sub>H</sub> coding region. These results showed that these motifs were not present in almost 40% of the hybrids (five of 13; Table III). In the other eight hybrids the frequency of these targets within the region shared in common was equal to or greater than that found in the entire V<sub>H</sub> region. Thus, the hypothesis that receptor revision is due to AID-dependent, gene conversion-like mechanisms becomes less likely (although it cannot be discarded because less favored nucleotide motifs are also the targets of somatic mutations; Ref. 42). However, these results do not preclude the possibility that an AID-induced, perhaps crippling somatic mutation occurred during clonal expansion that led to RAG reactivation (see below).

Receptor editing in immature B cells is dependent upon RAG (18, 67), and studies indicate that RAG may also be reactivated in mature B cells. RT-PCR approaches showed that RAG transcripts were induced in splenic B cells from immunized mice and that RAG<sup>−/−</sup> lymphocytes were present in germinal centers from spleen as well as regional lymph nodes (21–22). In addition, RAG-dependent, double-stranded RSS signal breaks have been identified in peritoneal B cells and splenic B cells from immunized animals. They have also been shown to be present in splenic, PBL, and tonsillar B cells following appropriate in vitro stimulation (23–24, 68–70). GFP marker studies with the RAG enzymes, however, have found little evidence to support RAG reactivation in mature B cells (26, 63, 71, 72) suggesting that immature B cells that expressed RAG may have migrated to germinal centers or that RAG reactivation occurred below the level of detection used in these assays. Recently, however, Wang and Diamond, studying a murine lupus-like disease, found that RAG was reinduced during an autoimmune response in Ag-activated, early memory, or preplasma B cells. They also showed that L chain receptor revision had occurred (73).

Our studies would not have identified the number of hybrids defined if it were not for the fact that multiple tissues were examined. This suggests possibilities that extend beyond repertoire diversification. B cells, upon migration to a different tissue, might be subjected to adverse conditions that promote RAG reactivation. Migrating B cells might encounter “new” autoantigens that were previously either not expressed or expressed below activation thresholds. RAG reactivation and receptor revision may occur in these B cells to escape clonal deletion, similar to that known in B cells that undergo receptor editing to achieve peripheral tolerance (14, 74, 75). Alternatively, the Ag or the required cellular interactions that led to clonal expansion in the primary tissue may be absent from the secondary tissue. If mature B cells require tonic signals to maintain homeostasis (76, 77), the lack of or diminished signaling (perhaps due to somatic mutation) in these new environments may result in RAG reactivation, leading to altered specificities and potential autoreactivity. Experimental analyses are now required to determine the developmental B cell stage and anatomical locations where H chain receptor revision may occur.

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