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Evolutionarily Conserved TCR Binding Sites, Identification of T Cells in Primary Lymphoid Tissues, and Surprising Trans-Rearrangements in Nurse Shark

Michael F. Criscitiello,[1] Yuko Ohta, Mark Saltis, E. Churchill McKinney, and Martin F. Flajnik

Cartilaginous fish are the oldest animals that generate RAG-based Ag receptor diversity. We have analyzed the genes and expressed transcripts of the four TCR chains for the first time in a cartilaginous fish, the nurse shark (*Ginglymostoma cirratum*). Northern blotting found TCR mRNA expression predominantly in lymphoid and mucosal tissues. Southern blotting suggested translocon-type loci encoding all four chains. Based on diversity of V and J segments, the expressed combinatorial diversity for γ is similar to that of human, α and β may be slightly lower, and δ diversity is the highest of any organism studied to date. Nurse shark TCRδ have long CDR3 loops compared with the other three chains, creating binding site topologies comparable to those of mammalian TCR in basic paratope structure; additionally, nurse shark TCRδ CDR3 are more similar to IgH CDR3 in length and heterogeneity than to other TCR chains. Most interestingly, several cDNAs were isolated that contained IgM or IgW V segments rearranged to other gene segments of TCRδ and α. Finally, in situ hybridization experiments demonstrate a conservation of both α/β and γ/δ T cell localization in the thymus across 450 million years of vertebrate evolution, with γ/δ TCR expression especially high in the subcapsular region. Collectively, these data make the first cellular identification of TCR-expressing lymphocytes in a cartilaginous fish. ***The Journal of Immunology*****, 2010, 184: 6950–6960.**

T cell receptors, Igs, and MHC genes are present in jawed cartilaginous fish (Chondrichthyes: sharks, skates, rays, and chimaeras), but not in more ancestral vertebrates (e.g., lamprey and hagfish) (1). Thus, sharks represent the oldest living vertebrates with the basic components of the adaptive immune system in mammals. Their study provides a window into the natural history of the genes critical to the system as well as the most fundamental aspects of its physiology.

Ig genes of cartilaginous fish are not arranged in a single large translocon organization common to other vertebrates. Instead, sharks employ many clusters or mini-loci to generate a diverse Ab repertoire (2). Such a system in an ancient vertebrate has confounding implications for the origins of allelic and isotypic exclusion, problems that are only beginning to be addressed (3, 4). In contrast to Ig, the organization of shark and skate TCR loci was suggested to parallel that of other vertebrates, with evidence for a single translocon locus encoding each chain (5–7). The many Ig loci in the shark could increase the possibility of Ig-TCR trans-rearrangement between juxtaposed or even distant Ag receptor loci normally thought to provide receptors on distinct B and T cells. The translocon TCR organization yet multiple cluster Ig arrangement was consistent with two distinct trends emerging among vertebrate Ag receptors: a plasticity of isotypes, primary lymphoid organs, and diversification mechanisms for the BCR, whereas TCRs showed much more evolutionary conservation (perhaps a result of MHC restriction, at least for the TCR α- and β-chains) (8). However, interesting variants also have been found in vertebrate TCRs, including wide variation in number of gene segments (9), bizarre CDR1 and -2 lengths in pathogen-specific Vs (10), and both allelic polymorphism (11, 12) and multiple, distinct C region loci (13, 14). Sandbar shark was shown to use (B cell-like) somatic hypermutation at the TCRγ locus (7). In the nurse shark, we previously reported the first doubly rearranging Ag receptor, the new Ag receptor (NAR)-TCR δ-chain (15). This longer (three-domain) form of TCRδ uses an N-terminal V domain generated by VDJ rearrangement that is very similar to IgNAR, a shark Ig H chain isotype that does not associate with L chains (16). Subsequently, a fifth TCR chain (μ) expressed in a marsupial was identified that also was predicted to contain two V domains that are IgVH-like. TCRμ is related to TCRδ, but it is not orthologous to NAR-TCR despite the fact that both Ag receptors have three domains (17). These recent findings demonstrate that like Ig, the TCR, especially γδ TCR, is more evolutionarily plastic than previously appreciated. TCR employing IgH domains in disparate vertebrates via convergent evolution prompted a comprehensive study of the TCR gene products and their tissue localization in the shark.

Like the molecular hardware of adaptive immunity, the required primary (thymus) and secondary lymphoid tissues (spleen) are also found in jawed vertebrates but not in agnathans. Cartilaginous fish possess a thymus derived from the first pharyngeal pouches that is composed of lobes with a distinct cortex and medulla (18) and has

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The online version of this article contains supplemental material.

Abbreviations used in this paper: APAR, agnathan paired receptors resembling Ag receptors; BAC, bacterial artificial chromosome; c, cortex; IgSF, Ig superfamily; m, medulla; NAR, new Ag receptor; RSS, recombination signal sequence; t, trabeculae.

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high expression of TCR genes (19). In mammals, T cell progenitors enter the thymus through the corticomedullary blood vessels and accumulate in the subcapsular region of the cortex. These cells proliferate and migrate deeper into the cortex as they mature into αβ or γδ cells. In the cortex, the αβ thymocytes that do not bind self-MHC with high enough affinity fail positive selection and die by apoptosis. As the thymocytes pass from the cortex into the medulla, to generate self-tolerance, they scan a complex set of organ-specific Ags under the control of the AIRE gene expressed by medullary epithelial cells. The thymus has been characterized anatomically and cytochemically in several elasmobranchs (20), yet gene expression and development of T lymphocytes in the most ancient organisms with a thymus has not been studied in detail. Analysis of nurse shark secondary lymphoid tissue (spleen) has been performed for B cells and APCs, but in fact, T cells have never been identified by TCR expression in cartilaginous fish (21).

In this work, we have studied the repertoire of nurse shark TCR chains and complemented the molecular work with TCR expression analysis by in situ hybridization. We have discovered unexpected trans-rearrangements between Ig and TCR loci. Whether these transcripts are by-products of the multiple Ig clusters in shark or if the Ig V segments are functional on T cells is not addressed, but the adaptive repertoires of sharks (7, 15, 16), noneutherian mammals (22), and now higher poikilothermic vertebrates (Z.E. Parra, Y. Ohta, M.F. Criscitiello, M.F. Flajnik, and R.D. Miller, submitted for publication) have blurred the boundary between B and T cell Ag receptors. Thus, these findings of genetic and mechanistic plasticity may be redefining the fundamental boundaries of Ag receptor repertoires.

Materials and Methods

Library screening and radioprobe labeling

cDNA libraries were constructed from nurse shark spleen, pancreas, and WBC RNA as previously described (23) in the Gateway system using the pDONR222 vector with Cloneminer (Invitrogen, Carlsbad, CA). Low-stringency hybridization conditions were 30% formamide washing to 2× SSC/0.1% SDS at 55°C, and high-stringency hybridization conditions were 50% formamide washing to 0.2× SSC/0.1% SDS at 65°C (24). PCR was used to label all probes with [^{32}P] 2′-deoxycytidine 5′-triphosphate as previously described (25). Probes routinely labeled to 3×10^7 dpm/μl. Positive clones were isolated and converted into plasmid vectors. Plasmid

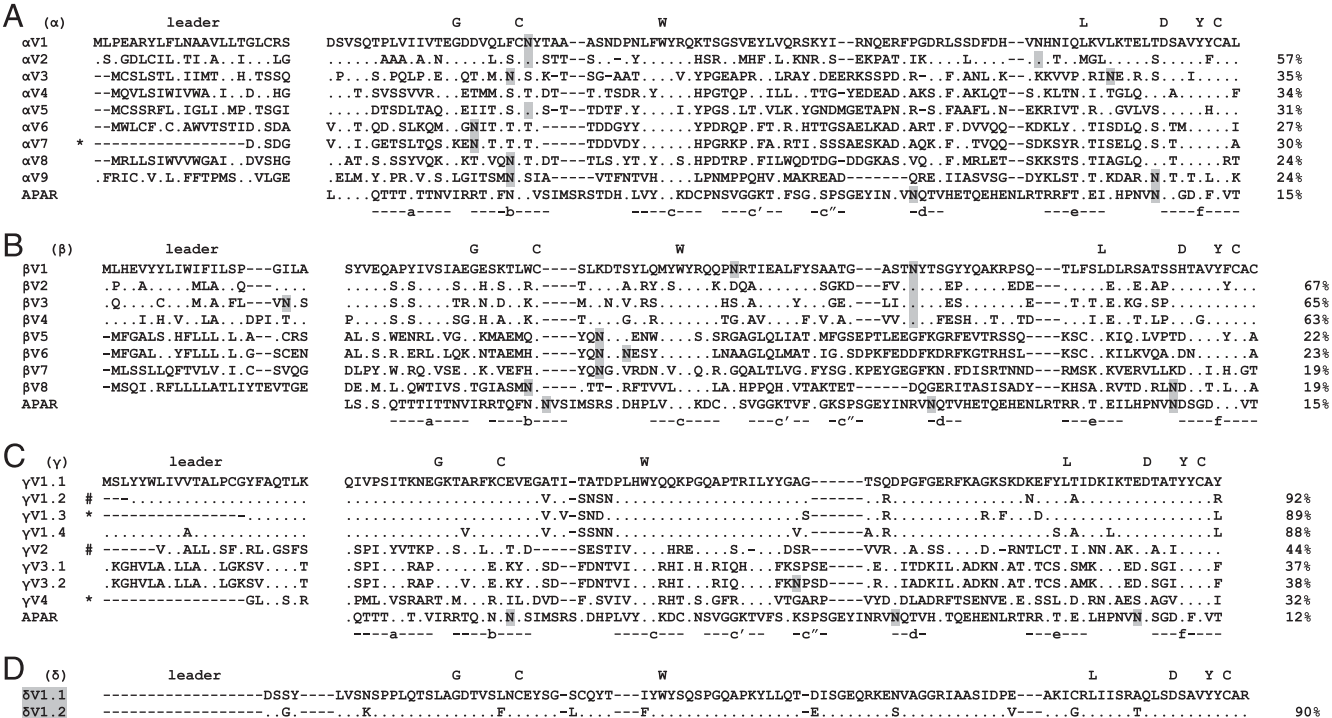

FIGURE 1. Nurse shark TCR V gene segment amino acid alignments. TCRα (*A*), TCRβ (*B*), TCRγ (*C*), and TCRδ (*D*). Conserved residues of Ag receptor variable domains are marked above the first sequence of each set (36). Gray highlighting marks sites of potential N-linked glycosylation (NxS/T, where x is any residue other than proline). Dashes indicate gaps in alignment, and dots mark identity to the first sequence. Percent amino acid identity compared with the first sequence is shown on the right. Predicted β strands are indicated below each alignment (37–39). Highlighted δVs are found only supporting N-terminal NAR-TCRV and therefore do not have leader sequences immediately preceding (15). Four spaces separate predicted leader peptide from mature protein. V sequences preceded by an asterisk (*) were only isolated with absent (unspliced) leader peptide exons, whereas those preceded by a number sign (#) are known only from incomplete 5′ cDNA clones. For contrast, the hagfish APAR-A sequence is shown aligned at the bottom (accession number BAD90578, www.ncbi.nlm.nih.gov/sites/entrez).

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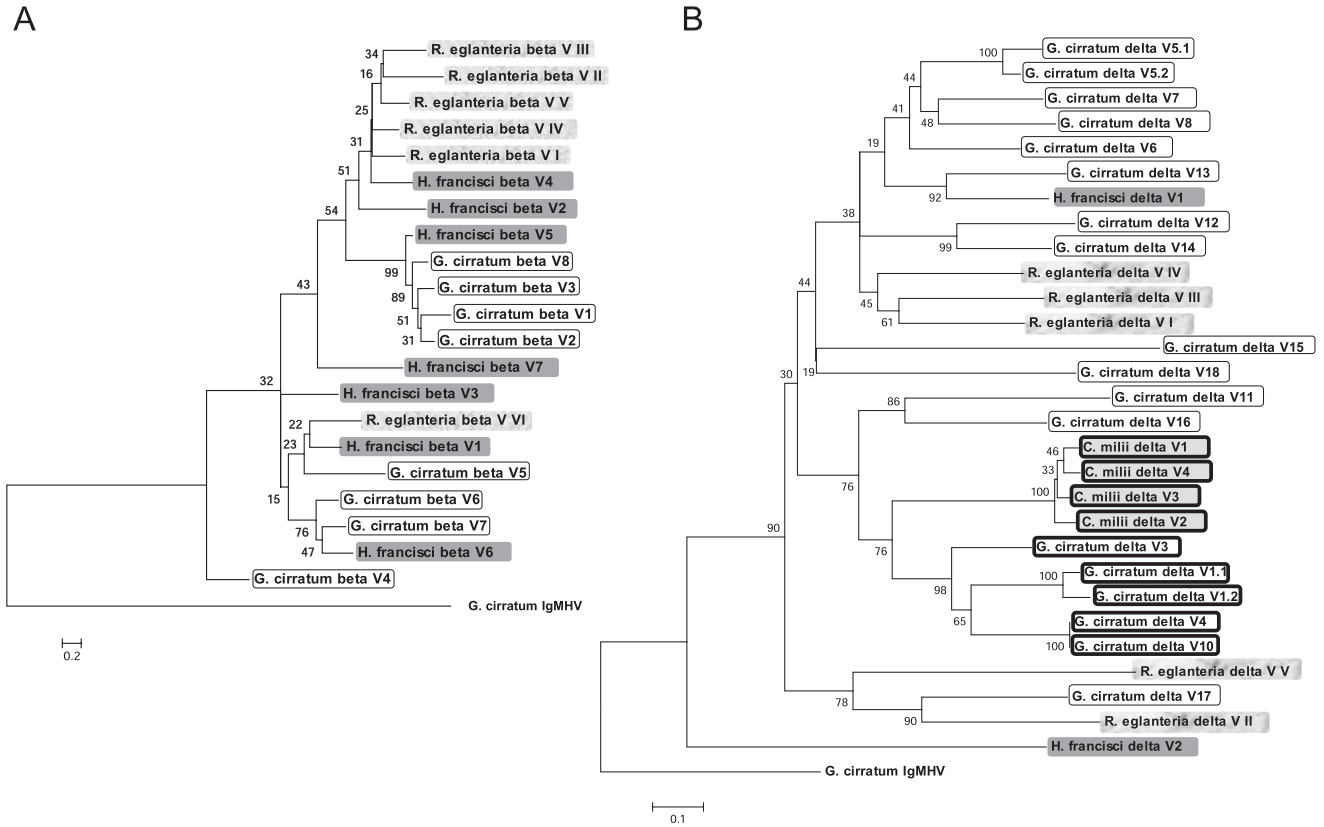

FIGURE 2. Phylogenetic analysis of cartilaginous fish TCR βV (*A*) and δV gene (*B*) families. Neighbor-joining tree with genetic distance scale bar shown. Numbers at nodes represent percentage support from 1000 bootstrap replications. NAR-TCR supporting δV are outlined in bold. Species: *H. francisci* (horn shark), *Ginglymostoma cirratum* (nurse shark), *R. eglanteria* (clearnose skate), and elephant shark (*C. milii*) (46).

preparations were purified using Qiaprep (Qiagen, Valencia, CA) and readied for sequencing by the University of Maryland Biopolymer/Genetics Core facility (Baltimore, MD).

Reverse transcription and PCR amplification

Oligo(dT)-primed cDNA was made from 5 μg total RNA from various tissues and used as a template for standard PCR amplification as described (26). 5′ and 3′ RACE products were amplified using the SMART RACE system (BD Biosciences, San Jose, CA) (15). Primers are listed in Supplemental Table I. PCR products were resolved by agarose gel electrophoresis, purified with Geneclean (Bio101, La Jolla, CA), and cloned into pCRII vector with the TA Cloning Kit (Invitrogen), and sequences were analyzed.

DNA sequence analysis

DNA sequence data were managed in DNAstar (Madison, WI) and Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html) and submitted to the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/sites/entrez). Signal peptides were predicted with SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/). Amino acid sequences were aligned with ClustalW using default parameters and then manually adjusted. The evolutionary histories were inferred using the neighbor-joining method (27). Phylogenetic analyses were conducted in MEGA4 (28). The bootstrap consensus trees inferred from 1000 replicates are taken to represent the evolutionary history of the genes analyzed (29). Phylogenetic trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. The evolutionary distances were computed using the Dayhoff matrix-based method (30) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

Southern and northern blotting

Southern blot was performed on genomic DNA from erythrocytes of three unrelated nurse sharks digested with BamHI, EcoRI, HindIII, PstI, and SacI (Roche, Basel, Switzerland) as previously described (31). Total RNA was prepared for northern blotting as previously described (24), and 10 μg was loaded in each lane. The nurse shark nucleotide diphosphate kinase (32) probe used as a loading control was amplified with primers listed in Supplemental Table I and radiolabeled as above.

Table I. *CDR3 lengths in amino acids*

	Shark				Human[a]				Mouse[a]			
	α	β	γ	δ	α	β	γ	δ	α	β	γ	δ
Maximum	12	19	12	27	12	12	12	21	12	13	11	19
Minimum	3	0	6	4	6	6	1	8	6	4	4	6
Range	9	19	6	23	6	6	11	13	6	9	7	13
Median	8	9	9.5	15	9	9	7	14	8	9	9	13
Mean	8.04	9.58	9.05	14.8	9.2	9.5	7.2	14.5	8.5	8.9	8.8	12.7
Variance	3.5	13.3	3	25.7	2.8	4	5.5	11	1.6	2	1.8	6.4

[a]See Ref. 51.

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In situ hybridization

Nurse shark thymus tissue was harvested and fixed in 4% paraformaldehyde/3% sucrose and washed in sucrose to 30% gradually over 2 d at 4˚C. Fixed tissues were then frozen in OCT media using liquid nitrogen-chilled isopentane bath. Sections were cut to a thickness of 8 μm and adhered to microscope slides. RNA probes were transcribed and labeled with digoxigenin from linearized probe template DNA using the DIG RNA Labeling Mix (Roche). Cryosections were prefixed in 4% paraformaldehyde in shark PBS and digested in proteinase K (Sigma-Aldrich, St. Louis, MO). After a wash in shark PBS, sections were acetylated for 10 min in 0.25% acetic anhydride. A total of 60 ng probe per slide was hybridized in Hybridization Solution (Sigma-Aldrich) with 50% formamide and baker's yeast tRNA (Sigma-Aldrich) under the coverslip overnight at 67˚C in a humidifying chamber. Slides were washed twice for 30 min in 0.2× SSC at 72˚C, then 5 min in 0.2× SSC at room temperature. Blocking was performed with 10% heat-inactivated horse serum in 0.1 M Tris/0.15 M NaCl (pH 7.6) for at least 1 h at room temperature before binding to antidigoxigenin alkaline phosphatase Fab fragments at 4˚C overnight. Sections were washed four times for 5 min in 0.1 M Tris/0.15 M NaCl (pH 7.6), then 10 min in alkaline phosphate buffer (100 mM Tris [pH 9.5]/50 nM $MgCl_2$/100 nM NaCl), then developed in NBT/BCIP substrate solution (Roche) until the desired stain is obtained. Final washes in water (three times for 5 min), a fix in 4% paraformaldehyde (5 min), and again in water (three times for 5 min) were completed before allowing the slides to air dry (33). Images were obtained on a Nikon Eclipse microscope (Nikon, Melville, NY) with Diagnostic Instruments camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI).

Results

Cloning of genes of the four nurse shark TCR chains

Isolation of the genes for each of the four TCR chains was achieved in a different way. Nurse shark TCRβ was cloned from a spleen cDNA library with a horn shark (*Heterodontus francisci*) TCRβ C probe (34). A probe to nurse shark IgW (an equivalent of mammalian IgD that has been very plastic in evolution) (35) isolated the TCRδ C domain gene. This surprising clone was from a nurse shark WBC cDNA library, hybridized to the IgWV domain, and was one of the unexpected Ig/TCR trans-rearranged products described below. TCRγ appeared in an in-house expressed sequence tag library generated from nurse shark spleen and pancreas to identify shark immune genes. A portion of TCRαV segment was isolated with minimally degenerate primers designed to the conserved motifs in the framework 2 and framework 3 regions of TCR and Ig L chains (6). Once a partial clone was isolated for each chain, cDNA library screening and RACE PCR were used to complete full-length sequences and analyze the rearrangement repertoire for each.

TCR V gene segments

The diversity of nurse shark V gene families cloned from cDNA to date is shown in Fig. 1 (36–39). Cloning techniques based on C domain sequence but not requiring V domain knowledge (5′ RACE PCR with C domain primer and cDNA library screening with C domain probe) maximized the discovery of repertoire components. V gene segments were assigned to families based on 75% or greater identity at the predicted amino acid level (Supplemental Table II) (40). Almost all of the V genes contain the tryptophan and two canonical cysteines common to Ig superfamily (IgSF) domains and thought to be critical for the β-sandwich tertiary structure. Only δV16 and δV18 do not contain the first cysteine needed for the intradomain disulfide bond, and δV16 has a potential compensatory cysteine two positions carboxyl-terminal in β strand b. In addition to lacking the N-terminal conserved cysteine, δV18 is also peculiar in the absence of the tryptophan of the WYRQ motif.

Sequence divergence among V families is great for each of the four nurse shark TCR chains; for each chain, there are families that differ by as much as 71% at the amino acid level (Fig. 1, Supplemental Table II) (36–39). Similar high levels of amino acid sequence diversity among V families has been seen in other vertebrate groups; for example, the horned shark βV1 and βV5 share only 20% identity (41), cod αV2 and αV3 share 32% (42), axolotl αV1 and αV5 share 25% (43), and cow γV1 and γV3 share only 18% predicted peptide identity (44). BLASTP of each of the 43 translated V genes in Fig. 1 (36–39) resulted in lowest E scores to TCR V domains of other jawed vertebrates. Agnathan paired receptors resembling Ag receptors (APAR), a multigene family encoding receptors with a single V-type domain that pair similarly to B and TCRs but do not rearrange, is one receptor employed by leukocytes of jawless vertebrates with features most expected of a primordial lymphocyte Ag receptor (45). Although TCR V domains from other TCR chains and IgL V domains were found by BLASTP, APAR were not retrieved in this manner using any of the nurse shark TCR V domains as bait. APAR does not show high identity with the V families of the four TCR chains of nurse shark and therefore may not be closely related to the ancestral Ag receptor or TCR.

Evolution of V genes

Predicted peptides of published TCRV genes from other cartilaginous fish were aligned with these new nurse shark sequences, and theoretical phylogenies were constructed (Fig. 2, Supplemental Fig. 1) (46). The interleaved pattern with V genes from three different elasmobranchs often clustering together demonstrates *trans*-species maintenance of V gene families over large periods of evolutionary time. Like the *trans*-species maintenance of MHC alleles (47), it has been observed that TCRV gene families in mammals are preserved across successive speciation events (48, 49). Fig. 2 (for TCRβ and δ) and Supplemental Fig. 1 (for TCRα and γ) show trans-species maintenance of V families for all four

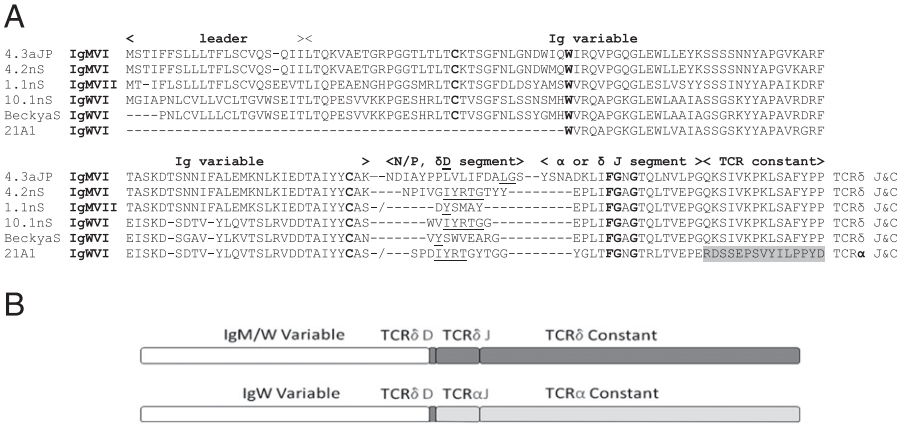

FIGURE 3. *Trans* rearrangements between Ig V domains and TCRα and δ gene components. *A*, Amino acid alignment of clones; slashes indicate frame shifts in CDR3. Gene segments contributing are listed at the top of alignment. Gray highlighted C sequence is from TCRα, and C genes from other five clones are TCRδ. *B*, Drawing showing derivation of gene segments in these six chimeric transcripts. *Top panel*, First five sequences in *A*. *Bottom panel*, TCRα clone 21A1.

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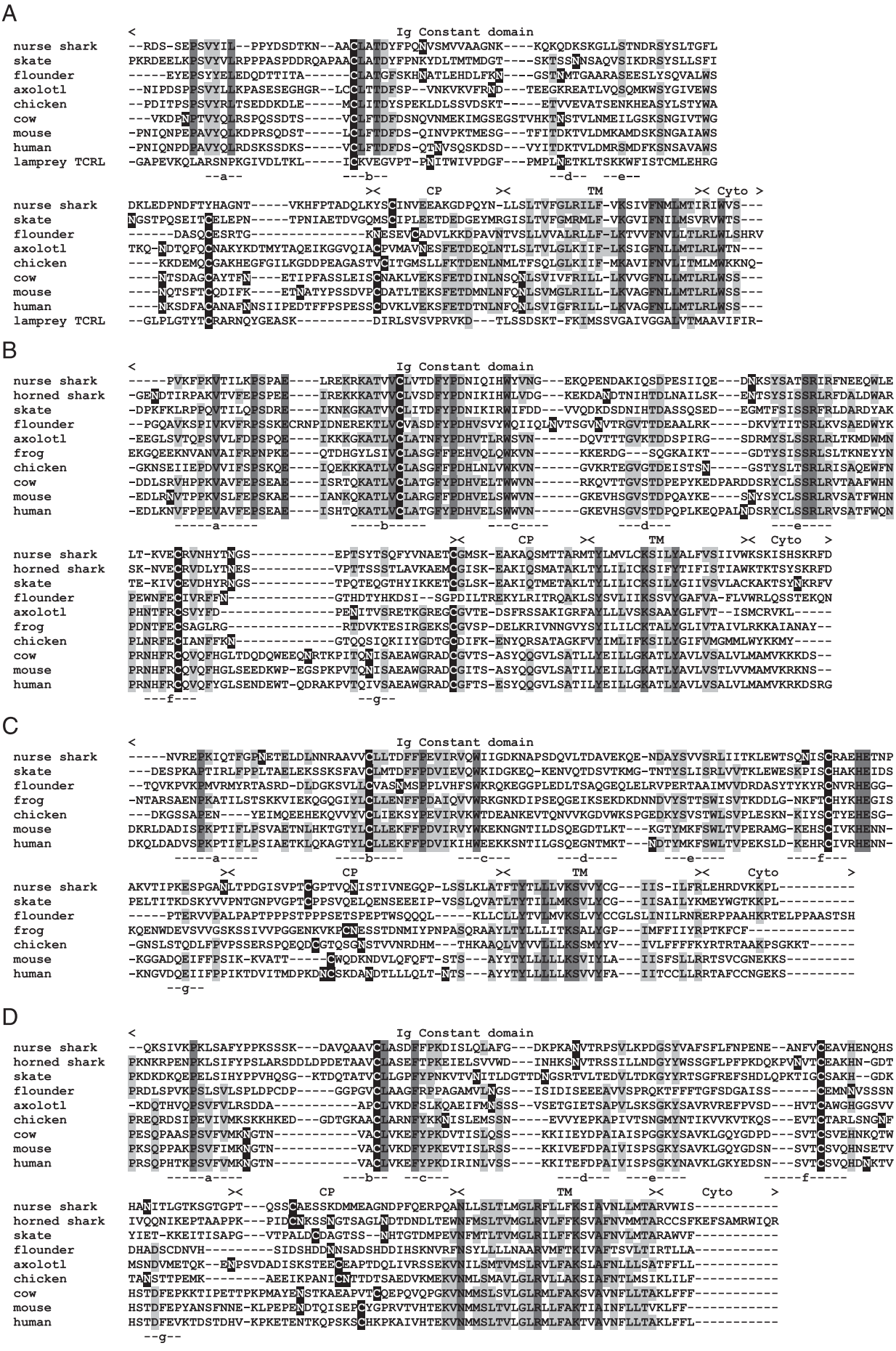

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chains, as sequences from nurse shark, horned shark, and clearnose skate (*Raja eglanteria*) interleave rather than clustering solely by species (as would be predicted if the Vs had expanded only after the divergence of the shark and ray lineages and the split of the orectolobiform from the heterodontiform sharks) (50). The trees show both trans-species maintenance and possibly some expansion of clusters in particular lineages (e.g., nurse shark δV5.2, δV5.2, δV6, δV7, and δV8 and the four Vs from the holocephali elephant shark, *Callorhinchus milii*). Only elephant shark δVs (all NAR-TCRV supporting) cluster with the nurse shark V domains that support the NAR-TCRV domain, suggesting that such orthologs have not yet been cloned in the modern elasmobranchs, despite the fact that they were shown to be present in all cartilaginous fish by genomic Southern blotting (15).

Combinatorial diversity

Junctional regions from CDR3 of all of the TCR chains were aligned and analyzed for J segment use, overall length, and heterogeneity (Supplemental Fig. 2). CDR3 length in amino acids was calculated for each and compiled (Table I) (51). TCRδ CDR3 is longer than the other chains and has the largest range of lengths and the most variance. This range and variance of TCRδ CDR3 length is unusually high compared with other vertebrates (51), although the mean is similar to that seen in humans. CDR3 nucleotide alignments of TCRα and TCRγ show no evidence of D segment contribution (as expected based on TCR L chains from other vertebrates), with the one notable α clone exception discussed below. Nucleotide alignments of TCRβ and TCRδ junctional regions allowed discernment of one D in each of these TCR H chains: TCRβD1 and TCRδD1 (Supplemental Fig. 3). Thus, the mean lengths of CDR3s of nurse shark TCRs show conservation of the binding paratopes used in higher vertebrates.

The frequency of V gene families and J genes employed in nurse shark TCR mRNA was compared with those of other vertebrates (Supplemental Table III). Only the mammals in the table have had both comprehensive genomic and cDNA analysis, so it is expected that the other vertebrate numbers could be higher than these initial assessments. What stands out is that the nurse shark has much greater TCRδ junctional diversity than other vertebrates. The 18 TCRδV sequences are at least three times the number of dedicated TCRδV described in other species. The TCRδV are not found in TCRα rearrangements, suggesting that the TCRδ locus may not be nested within the TCRα locus in sharks as it is in mammals (52). Unlike its presumed heterodimerization partner δ, nurse shark TCRγ displays combinatorial diversity more typical of other vertebrates, including sandbar shark (7). This initial assessment of TCRα and β from the nurse shark shows lower than average combinatorial diversity, as might be anticipated for those TCR chains expected to recognize MHC-peptide complexes. Diversity at CDR3, however, is still great (particularly after TdT action), supporting the primordial significance of these loops in Ag receptor-binding sites in general and MHC restricted αβ TCR in particular (53).

Trans-rearrangements

Adding to this exceptional complexity of nurse shark TCRδ transcripts are chimeric *trans* rearrangements of IgHV segments with D, J, and C genes of TCR (Fig. 3). (As mentioned, the nurse shark TCRδC gene was initially isolated with a probe for IgWV.) Four TCRδ clones from neonatal nurse shark spleen and one from peripheral blood leukocytes of an adult nurse shark are rearrangements of Ig V genes with D-J-C of TCRδ (Fig. 3). Furthermore, one TCRα clone from adult spleen is derived from a transcript of IgWV with the C gene of TCRα (54). This chimeric TCRα clone joins the TCRδ D to what appears to be a TCRα J (it has not been isolated in any other α or δ clone; Supplemental Fig. 2) and employs an IgW V, spliced to the TCRα C domain gene. This IgW-TCRα clone and one IgM-TCRδ clone contain junctional frame shifts that could not encode a functional protein, but the other four trans-rearranging clones are in frame. Two different IgMV families and one IgWV family are represented in the TCR chimeras.

C domains

Only one C domain gene sequence was found for each of the four TCR chains. When the amino acid sequences of these genes were compared with those of other vertebrates, conservation of basic domains and key residues is evident (Fig. 4). C domain genes of the β-, γ-, and δ-chain encode typical IgSF C domains, connecting peptides, transmembrane regions, and short cytoplasmic tails. TCRα contains an Ig-related domain before the connecting peptide, transmembrane, and similarly short cytoplasmic tail. The nurse shark TCR C domains contain the two hallmark cysteines used in intradomain disulfide bonding in each chain except TCRα, which lacks the second (carboxyl-terminal) of the pair. TCRαC from higher vertebrates has been recognized for adopting a less compact and possibly more flexible β-strand structure than other C domains, in which β strands c, f, and g do not form the canonical top β sheet of an IgSF fold (55). This unorthodox C domain articulates with the CD3 complex, and its structural flexibility may be important in signal transduction (56, 57). However, in TCRαC, the hallmark cysteines are usually present. Connecting peptides of the elasmobranchs, like those of tetrapods, typically contain acidic amino acids as well as cysteines for interchain disulfide bonds that are absent in most teleost TCR αβ (58, 59) and some vertebrate γδ (60).

Genomic analysis

Southern blotting of nurse shark genomic DNA usually produced one band when probed for the TCR α, β, γ, or δ C gene (Fig. 5*A*). The TCRγC probe contains an internal HindIII site, yielding the weak band at 4 kb. This suggests that only one copy of each gene is present in the nurse shark, like skate but unlike the horned shark (5, 6). With the five enzyme digestions used with genomic DNA, no clearly coincidental hybridization was found among the C domains, suggesting that these TCR loci are not in very close proximity to one another. Preliminary analysis with bacterial artificial chromosome (BAC) clones (with average insert size of 150 kb; data not shown) demonstrated no close linkage of TCR loci.

FIGURE 4. Amino acid alignment of TCR C domains of representative vertebrates. TCRα (*A*), TCRβ (*B*), TCRγ (*C*), and TCRδ (*D*). The predicted IgSF domain, transmembrane region, and cytoplasmic tail are indicated above the alignments and the β strands beneath. Dashes mark gaps introduced into alignment. Canonical cysteines that make intradomain and interchain disulfide bonds are highlighted in black, as are putative N-linked glycosylation sites. More or less conserved residues are indicated by dark and light gray highlighting, respectively. Accession numbers for other species are as follows: horned shark: β AAA61563, δ AAA87016; skate: α AAB51495, β AAB51496, γ AAB51498, δ AAB51497; flounder: α BAC65457, β BAC65459, γ BAC65461, δ BAC65464; axolotl: α AAA98473, β AAA48534, δ AY029365; lamprey: TCR-like AAU09668; frog: β BAC67174, γ AAM21541; chicken: α AAC60277, β AB092341, γ AAA87009, δ AAD51740; cow: α AAO42514, β BAA14168, δ BC104586 (predicted from nucleotide); mouse: α AAB47020, β DQ340294, γ CAA25294, δ AAA51274; and human: α AAO72258, β AAO72258, γ M16768, δ A31326.

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Therefore, if TCRα and δ (or β and γ) (61) are linked, the distance may be >85 kb seen in mammals between human TCRα and δ.

Expression

Northern blotting with C domain probes (Fig. 5*B*) was used to assess the expression levels of the four TCR chains in adult nurse shark tissues. Strong hybridization of mRNA was noted in thymus and spleen for all four nurse shark TCR loci. Although high expression was previously seen for only TCRα and β in the skate (19), relatively lower expression was indicated in spiral valve (shark intestine), gill, and peripheral blood leukocytes. TCRγ and δ were not only expressed highly in the spleen and thymus, but also in the spiral valve, gill, and pancreas. Though these data are not quantitative, this trend is consistent with an early origin of γδ T cell defense of epithelial and mucosal tissues (high levels of IgW have also been found in shark pancreas) (62). The strong TCRδC expression reported in skate liver was not seen in the nurse shark (19).

T cells in thymus

To definitively identify T cells in cartilaginous fish, we performed in situ hybridization studies. Thymi are situated dorsomedial to the gills (20), but we have found variance among individual sharks regarding the encasement of the thymus in the crevasse between the epaxial and brachial constrictor muscle bundles. Rather than attempting to excise thymic tissue cleanly, reproducibly excellent sections have been obtained by including the musculature and connective tissue enveloping the thymus. Nurse shark thymus shows highly symmetrical petal-shaped lobules of cortex around a central medulla (Fig. 6). We used some non-TCR probes to further identify specific regions of the thymus (21, 63, 64).

RAG1 and TdT were expressed in the cortex, with highest expression in the subcapsular region. This pattern is consistent with the expression of these genes in mammalian CD4/CD8 double-negative 2 and 3 cells in which the TCR β, γ, and δ genes rearrange. Both MHC class I and II were expressed in the medulla, whereas in the cortex, more punctate individual MHC-expressing cells were seen for both classes, presumably due to staining of epithelium and accessory cells. As in most vertebrates, MHC class I is highly expressed by medullary thymocytes (presumably in addition to the epithelium and APC), whereas class II is expressed only on cells with dendritic morphology (and presumably epithelium), consistent with previous studies showing mature nurse shark T cells to be class II^- (21). The TCRα and β probes hybridized strongest in the central cortex and weakly in the medulla and subcapsular region. TCRδ and γ expression was also detected in isolated central cortical cells, but they were most highly expressed in cells in the subcapsular region of cortex. TCRδ stained the medulla more clearly than the other three TCR chains. Thus, the differential expression of RAG, MHC, and TCRs in this primary lymphoid architecture is generally conserved between shark and mammalian thymus, but we believe there are a greater number of γ/δ T cells in nurse shark as compared with most other species.

Discussion

Nurse shark thymic lymphocytes

The in situ hybridization studies on nurse shark thymus have shown the organ and its lymphocyte traffic to be similar to that of higher vertebrates. The cortex and medulla are clearly defined, with the cortex being divided into evenly proportioned lobes by trabeculae and containing densely packed cells with lymphocyte morphology. Hassall's corpuscles are absent in the shark and seem to be confined to warm-blooded vertebrates (65). Cells just arriving in the thymus express RAG and TdT as they rearrange TCR loci in the subcapsular zones of the cortex, and RAG expression persists into the central cortex, probably to perpetuate TCRα re-rearrangements during positive selection. TCRβ and α expression is detected presumably when the new thymic immigrants in the subcapsular region have proliferated and become the shark equivalent of double-positive cells (T cell coreceptors have yet to be defined in elasmobranchs) in the central cortex. TCRγ and δ are expressed earlier than TCRβ and α in the subcapsular regions of the cortex and then in the medulla, perhaps because T cells committing to this lineage quickly navigate the central cortex. mRNA for the γ- and δ-chains appears to be expressed by more cells in both the cortex and medulla and more distinctly than in mouse, human, and other nonmammalian vertebrates (66).

TCR evolution: C domains

A lack of the second canonical cysteine in the nurse shark TCRαC domain is more curious than canonical cysteines missing in Vs and suggests that this domain's tertiary structure may be even more divergent than that of other vertebrate TCRαCs. We found that, like in other vertebrates, nurse shark αC likely lacks the c, f, and g strands, as evidenced by the difficulty in aligning those residues (Fig. 4*A*). That this unconventional structure of αC arose early in the evolution of adaptive Ag receptors supports the significance of the lost β-sheet to TCRαβ Ag recognition or signal transduction.

C domains in nurse shark TCR at least are truly constant, unlike the situation of allelic polymorphism in some teleost fish (67). Shark TCR C domains are rich in potential sites of N-linked glycosylation, as are those from other vertebrates, but to varying degrees. A recent study showed that removal of glycosylation sites enhances the avidity of αβ TCR of various specificities in mouse and man (68). The TCRαCs of cartilaginous and bony fish lack the α-chain connecting peptide motif (FETDxNLN) that is conserved in tetrapods and has been linked to signal transduction and CD8 coreceptor function (69, 70). When both extracellular and proximal intracellular signaling components are understood in shark lymphocyte activation, a major step will be made in understanding lymphocyte evolution.

C domain amino acid alignments in Fig. 3 were used to make phylogenetic trees (Supplemental Fig. 4). Using the nurse shark IgMC1 domain as an outgroup, the cartilaginous fish (horned shark, nurse shark, and skate) TCR sequences always cluster together, as do those included from mammals (cow, mouse, and human). TCR C sequences from teleost fish, amphibians, and birds do not always behave in the dendrograms as predicted by their natural history, probably due to the particular sequences included. This C domain analysis does, however, concur with the previous assertion that the four chains in mammals (α, β, γ, and δ) arose very early near the genesis of the adaptive immune system (6). The membrane proximal domain of lamprey TCR-like is included in the TCRC analyses because it was originally identified by its similarity with porcine TCRα (71). Using several tree-building methods, the agnathan TCR-like C domain clusters with teleost sequences between the TCRαC of Chondrichthyes and tetrapods, and in the case of TCRβ with elasmobranchs, supporting its purported descent from early, nonrearranging IgSF receptors. The alignment in Fig. 4*A* shows that this molecule lacks many of the conserved residues in vertebrate TCRαC.

V genes and families

Nurse shark TCR V genes showed remarkable evidence for *trans*-species maintenance when analyzed with those from other elasmobranchs (Fig. 2, Supplemental Fig. 1). The instances in which V

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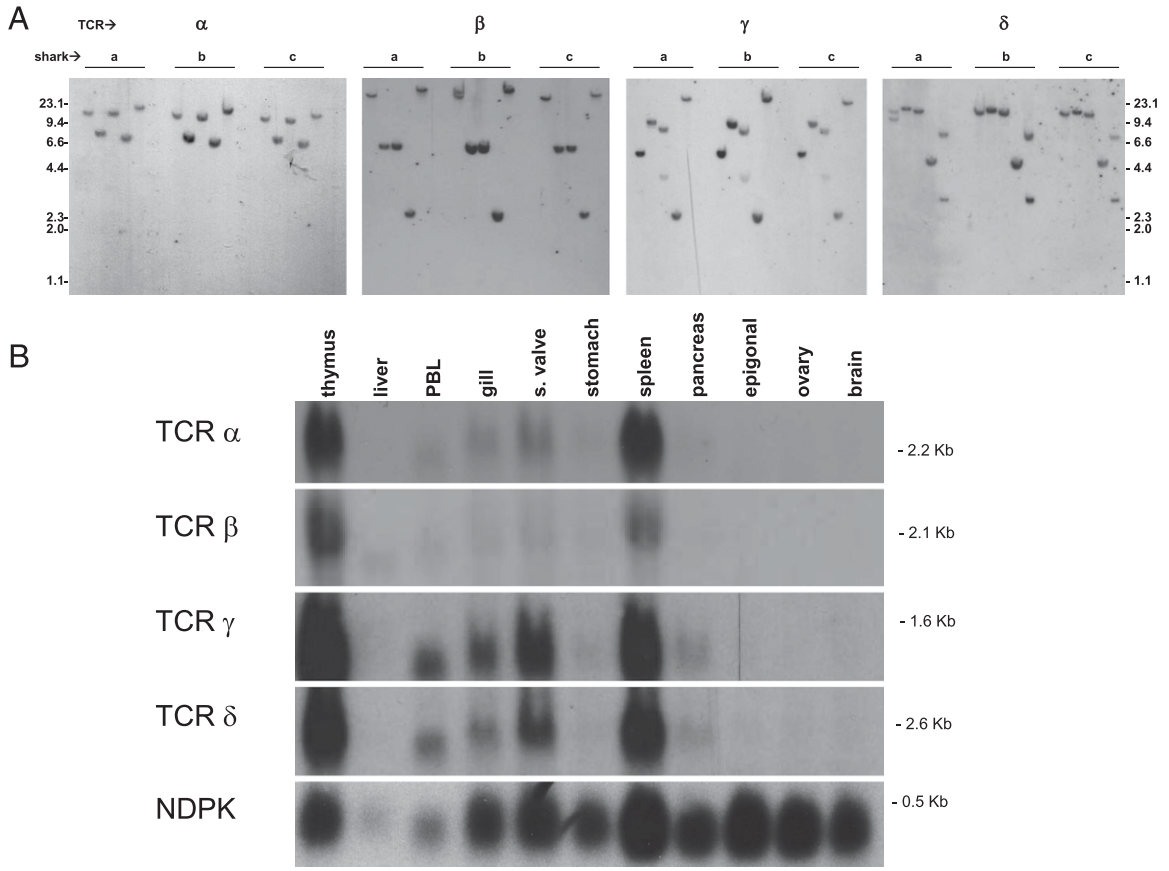

FIGURE 5. Genomic Southern and Northern blotting. Probes to the C domain Ig regions of each TCR chain were used to probe blotted nucleic acid agarose gels. *A*, Genomic DNA of three individual nurse sharks (*a*, *b*, *c*) digested (from *left* to *right*) with BamHI, EcoRI, HindIII, PstI, and Sac I. *B*, RNA from various nurse shark tissues. Size of migration markers is shown in kilobases.

families from the same species grouped with robust statistical support (i.e., nurse shark αV4, αV6, αV7, and αV8 or skate γVII and γVIII) may eventually be supported as trans-species maintained groups by V families annotated from more species in the future or may be expansive diversification within a smaller taxonomic clade. After all, the organisms in this analysis diverged 350 (holocephaloid elephant shark and elasmobranchs), 220 (sharks and skates), and 120 (horned and nurse sharks) million years ago and would not show more recent trans-species maintenance of V families (1).

Somatic hypermutation has been recently identified at the TCRγ locus of the sandbar shark (*Carcharhinus plumbeus*), where the entire germline locus was sequenced, and only five V genes were revealed (7). We think it likely that this nurse shark cDNA dataset contains similar evidence of somatic hypermutation at γ and other loci. Future complete analyses of germline loci will be required before we can distinguish genuine somatic hypermutation from addition of V genes or PCR/cloning-induced errors.

Combinatorial diversity and CDR3

Supplemental Table III shows the potential combinatorial diversity at nurse shark TCR genes and how that of nurse shark TCRδ is greater compared with other vertebrates. The length of the loop generated by V(D)J recombination has been used as a metric in assessing the mode and possible range of binding paratopes made by an Ag receptor. CDR3 length distribution has been analyzed in mammalian Ag receptors (51), and some of these data are reproduced for contrast with the shark analysis in Table I. The CDR3 loops of TCRα and β in humans are only 6–12 aa (averaging 9), presumably limited by the recognition of peptide/MHC. TCRγ CDR3 averages only 7 aas yet ranges wider, from 1–12. However, human TCRδ is much longer and shows greater variance, ranging from 8–21 aa with a mean of 14.5. These data provide further evidence that γδ T cells recognize Ag in a manner akin to Ig, because Ig H and L chains have CDR3 length characteristics similar to the δ- and γ-chains, respectively (53). RAG-mediated recombination and TdT-catalyzed addition of nontemplate nucleotides are ancient methods of CDR3 diversification (72, 73) and in situ hybridization confirms their activity in nurse shark thymus (Fig. 6). So in the nurse shark, it is clear that the chain-specific pattern in CDR3 length was an original characteristic of the system (Table I). This is supported by similar findings in the CDR3 lengths of flounder (a teleost fish) and frog TCRs, in which α, β, and γ appear to be under more constraint (74, 75).

T cell evolution: αβ versus γδ T cell usage

Analyses of V segment (both TCR and Ig) diversity in endothermic vertebrates have found correlations between higher V segment diversity and a lower percentage of γδ T cells in the periphery (49, 76). Hence, mouse and human (~5% γδ in periphery) have higher potential combinatorial diversity in their V gene segments than chicken, rabbit, sheep, and cow (~20–30% γδ in peripheral blood). It is still not clear whether sharks belong to the γδ high, γδ low, or neither designated group recognized in higher vertebrates (77). Equivalent library screening for TCRβC and TCRγC yielded more β clones from multiple tissues, and the combinatorial diversity of shark TCRδ is extreme, yet Northern blotting suggests TCRγ and δ expression is as high as TCRα and β in all tissues sampled. It is possible that the γδ high/low dichotomy may not be very useful beyond warm-blooded vertebrates, as it is also often linked to B cell diversification in GALT-associated structures (γδ high species) versus bone marrow (γδ low species), and the nurse

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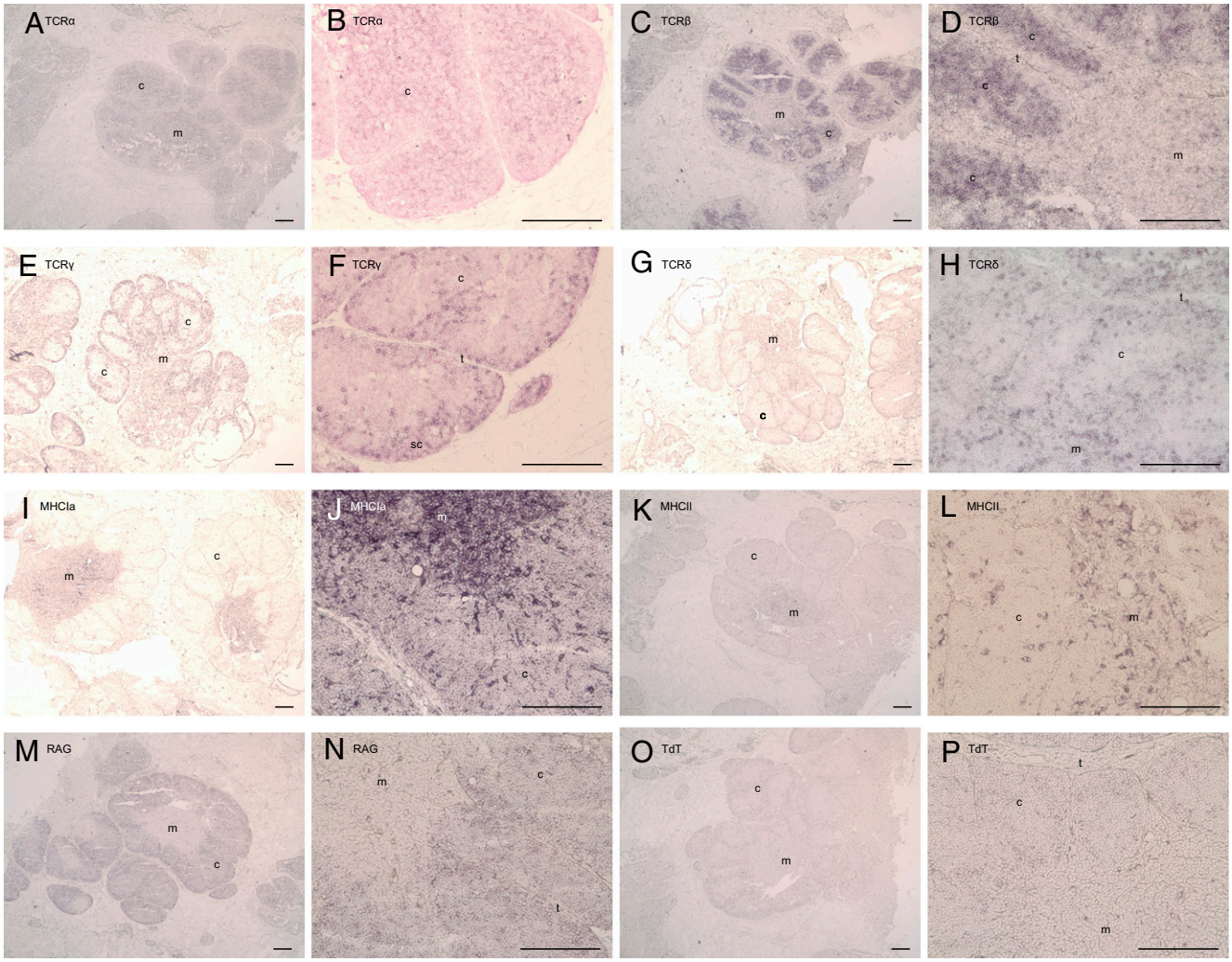

FIGURE 6. In situ hybridization of shark thymus. Positive hybridization is purple. TCRαC probe (*A*, *B*), TCRβC probe (*C*, *D*), TCRγC probe (*E*, *F*), TCRδC probe (*G*, *H*), MHC class Ia probe (*I*, *J*), MHC class IIb probe (*K*, *L*), RAG1 probe (*M*, *N*), and TdT probe (*O*, *P*). Scale bar, 100 μM; original magnification ×10 and ×20. Negative controls with sense probes (not shown) showed no staining. c, cortex; m, medulla; t, trabeculae.

shark epigonal organ is neither. It is clear, however, that nurse shark γδ T cells appear to have a much richer repertoire than the γδ low primates and rodents (and in fact all vertebrates) without the relative paucity of γδ T cells in the periphery seen in those animals. We provisionally would suggest that the nurse shark is a γδ high species but without the accompanying repertoire restriction that has been reported in some tetrapods (49).

Trans *rearrangements*

The cDNA clones showing *trans* rearrangements between Ig and TCR loci are remarkable for several reasons. First, they confirm that at least some shark Ig loci are in a rearrangement-permissive state, whereas thymocytes are rearranging TCR loci (3). Secondly, they mandate genomic mapping to determine whether multiple Ag receptor loci are clustered in a larger mega-locus, because the clones reported in this study suggest that TCRδ, NAR-TCRδ, TCRα, IgM, and IgW may all be encoded by genes in close enough proximity to accommodate RAG recombination. Third, we must re-examine the constraints on the V domain and CDR3 of receptors in light of the possibility of IgV (from IgM or IgW) or a V with B/T dual capabilities (IgNARV/NARTCRV) functioning on TCRδ (and maybe α), as well as the rearrangement of an α-chain employing the D segment usually reserved for Ag receptor H chains. These initial data should not be used to extrapolate the possible ramifications to the T cell repertoire, as reprobing the blot in Fig. 5*B* with an IgMV probe gives a weak signal of the appropriate size in the thymus. Future work must better characterize these rearrangements, the extent that these chimeric transcripts are translated, and what role they have in the shark's immune system.

Shortly after the initial identification of mammalian Ag receptor loci, it was recognized that *trans* rearrangements between Ig and TCR loci could be associated with lymphomas (78, 79). *Trans* rearrangements are known between human TCR loci and can contribute to chimeric TCR protein chains on the cell surface (80). Such rearrangements are studied more for their predictive value of genomic instability and neoplastic transformation than for their contribution to repertoire diversity (81). The TCR α/δ locus can draw from a shared pool of V gene segments for use on both the δ- and α-chains, and alternative splicing can even place VDJδ with Cα occasionally in wild-type mice (but more frequently in TCRδ-deficient mice) (82). The TCRδ genes are not particularly known for *trans* rearrangements, as TCRγ-TCRβ *trans* rearrangements are the most studied. The common theme in the existing literature of Ag receptor *trans* rearrangement is that this is a phenomenon of cancer cells, not of normal physiology. However, the multicluster organization of the Ig loci in cartilaginous fish could permit interlocus rearrangements without chromosomal translocation.

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Data from incompletely rearranged clones and genomic PCR sequencing so far have confirmed the conservation of recombination signal sequence (RSS) orientations that are shown in Supplemental Fig. 5. Assuming that the 12/23 rule is an ancient property of the RAG recombinase, then the nucleotide spacing between the heptamer and nonamer motifs that are the enzymes' substrate are in a permissive orientation for all of the Ig/TCR products cloned so far. For example, IgWV(23)-(12)TCRδD(23)-(12)TCRαJ obeys the rule of 12-spaced RSS only rearranging to 23-spaced RSS. This is not only important for these rearrangements in shark, but also as a basis for understanding the birth of all rearranging Ag receptor loci. Most recently, TCRμ was postulated to have evolved in early mammals by just such a germline rearrangement between IgH and TCR loci (17).

The Ig/TCR chimeric transcripts may be mere by-products of multiple Ig clusters in shark, and it does seem doubtful if the IgWV-TCRδD-TCRJ-TCRαC product would ever be expressed at the cell surface. But the shark adaptive repertoire now has a history of disregarding the distinctions between B and T cell tools (7, 15, 16), and experiments must determine the functional significance of these rearrangements that can appear in primary and secondary lymphoid tissues at levels comparable to canonical TCRV-TCRC transcripts. The permissive mechanisms of the DNA rearrangements that created the transcripts likely depend upon epigenetic markings, nuclear localization, and chromatin topography, tiers of control that are only beginning to be understood in the Ag receptor loci of more experimentally tractable mammals (83). Examining these rearrangements should help us understand the evolution of the extant adaptive receptor loci and may even open new design space in Ag receptor engineering.

The adaptive immune system depends on T lymphocytes for regulation and execution of cellular immunity and essential help for most humoral immunity. αβ T cells must develop through checkpoints for recognition of self-MHC without high affinity for self-peptide, which special architecture in a primary lymphoid organ has evolved to provide. We found that the thymus evolved this structural design early in the history of gnathostomes as that primary T lymphoid organ and that T cells have been testing combinations of α-, β-, γ-, and δ-chain rearrangements in it since the common ancestor of shark and man. Our repertoire analysis shows still more surprises from the shark TCR δ-chain's diverse bag of rearrangement tricks. Future work will determine whether the chimeric rearrangements are attributable to receptor locus proximity, locus accessibility in thymocytes, RAG promiscuity, or new V domain functional plasticity in this oldest vertebrate adaptive immune system.

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Disclosures

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