

EVOLUTION OF ANTIGEN BINDING RECEPTORS

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KEY WORDS: vertebrate phylogeny, immunoglobulin, T cell antigen receptors, adaptive immunity, gene rearrangement

ABSTRACT

This review addresses issues related to the evolution of the complex multigene families of antigen binding receptors that function in adaptive immunity. Advances in molecular genetic technology now permit the study of immunoglobulin (Ig) and T cell receptor (TCR) genes in many species that are not commonly studied yet represent critical branch points in vertebrate phylogeny. Both Ig and TCR genes have been defined in most of the major lineages of jawed vertebrates, including the cartilaginous fishes, which represent the most phylogenetically divergent jawed vertebrate group relative to the mammals. Ig genes in cartilaginous fish are encoded by multiple individual loci that each contain rearranging segmental elements and constant regions. In some loci, segmental elements are joined in the germline, i.e. they do not undergo genetic rearrangement. Other major differences in Ig gene organization and the mechanisms of somatic diversification have occurred throughout vertebrate evolution. However, relating these changes to adaptive immune function in lower vertebrates is challenging. TCR genes exhibit greater sequence diversity in individual segmental elements than is found in Ig genes but have undergone fewer changes in gene organization, isotype diversity, and mechanisms of diversification. As of yet, homologous forms of antigen binding receptors have not been identified in jawless vertebrates; however, acquisition of large amounts of structural data for the antigen binding receptors that are found in a variety of jawed vertebrates has defined shared characteristics that provide unique insight into the distant origins of the

rearranging gene systems and their relationships to both adaptive and innate recognition processes.

INTRODUCTION

The adaptive immune system is enormously complex. Its primary effector molecules are the antigen binding receptors [immunoglobulin (Ig) and T cell receptors (TCR)], which are members of the Ig gene superfamily that encompasses large numbers of related gene families serving diverse functions in separate cell lineages (1). The evolutionary acquisition of the complex set of diversification mechanisms found in the antigen binding receptors of present day mammals can be traced in part by comparisons to homologous genes of progressively more divergent vertebrate species. Superimposition of characters related to both sequence and genomic organization of Ig and TCR on the relatively well-established vertebrate phylogeny has proven critical in achieving our current understanding of their evolution. From both structural and functional perspectives, the evolution of Ig and TCR, which appear to be confined to the jawed vertebrates (Figure 1*a*), is unique and remarkably complex, as it is associated with particularly large families of diverse genes. Furthermore, the somatic diversification of the antigen binding regions of Ig and TCR, which occurs in all jawed vertebrates, is typically associated with a unique form of genetic rearrangement. In higher vertebrates, a second, unrelated form of somatic gene rearrangement takes place during Ig heavy-chain class switching and is associated with additional somatic diversification of germline genes. Antigen binding receptors also share signaling pathways with the nonrearranging mediators of innate immunity (2, 3). Thus, a broad interpretation of the evolution of antigen binding receptors needs to be framed within the boundaries of a highly diversified system in which effector molecules are selected on the basis of both templated germline diversity and untemplated somatic differences.

Although comparisons between Ig-type receptors in widely divergent groups have revealed extraordinary diversity in recombining systems, they simultaneously have defined common features that possibly represent constraining elements in the immune system. Broadscale comparisons can be used to generate hypotheses regarding the evolution of this diversity; however, only comparisons of more closely related species can be used to test their validity. As becomes apparent, the degree of plasticity in Ig gene organization and the mechanisms of diversification represent confounding aspects of a pure sequence comparison approach to the evolution of antigen binding receptors. This variation could not have been predicted (reconstructed in an evolutionary sense) by examining structure (and function) in the mammals alone. Although relatively

few phylogenetic groups have been examined, within-group variation can be extreme. Every effort has been made in this review to emphasize common features in the Ig and TCR of diverse vertebrates and to relate differences in gene structure and organization that influence function.

TWO MAJOR ANTIGEN BINDING RECEPTOR CLASSES ARE ENCODED BY REARRANGING GENES

In addition to Ig and TCR, various members of the Ig superfamily have defined roles in the adaptive immune response, including major histocompatibility complex (MHC) I and MHC II, which recognize and bind portions of antigen and share C1 domain types with Ig and TCR (1). All four classes of genes are present in jawed vertebrates (Figures 1*a* and 2). The structurally related but functionally distinct Igs and TCRs are considered to be the principal antigen binding receptors (4–6) and are the principal focus of this review. Ig and TCR are both structurally and organizationally related, function at the surface of cells, transduce signals along with coreceptors, and are the products of developmentally programmed rearrangement of segmental elements. Although somatic rearrangement and diversification are distinguishing features of Ig and TCR, even genetic rearrangement is not characteristic of all Ig loci (7, 8). In some species, specialized genetic processes exist for diversifying more limited numbers of recombining elements, whereas other species possess extremely large numbers of recombining elements. Rearranged Ig loci undergo extensive somatic hypermutation, whereas TCR genes tend to be mutated only in specialized cellular microenvironments (9, 10). The overall extent of mutation, of forces driving selection of mutations (i.e. antigen dependence versus independence), and of temporal dependence of the mutation process likewise exhibit considerable interspecies variation (10, 11). Considering the relatively few species that have been characterized, the number of different gene diversification themes that have been identified is extraordinary.

IMMUNOGLOBULIN GENES IN CARTILAGINOUS FISH

Heavy-Chain Genes

The living cartilaginous fish, comprised of the two major radiations, 1. sharks, skates, and rays and 2. chimaeras and ratfish, are the most phylogenetically distant vertebrate group relative to mammals in which Ig genes have been identified (12–14) (Figures 1*a* and 1*b*). The cartilaginous fish diverged from a common

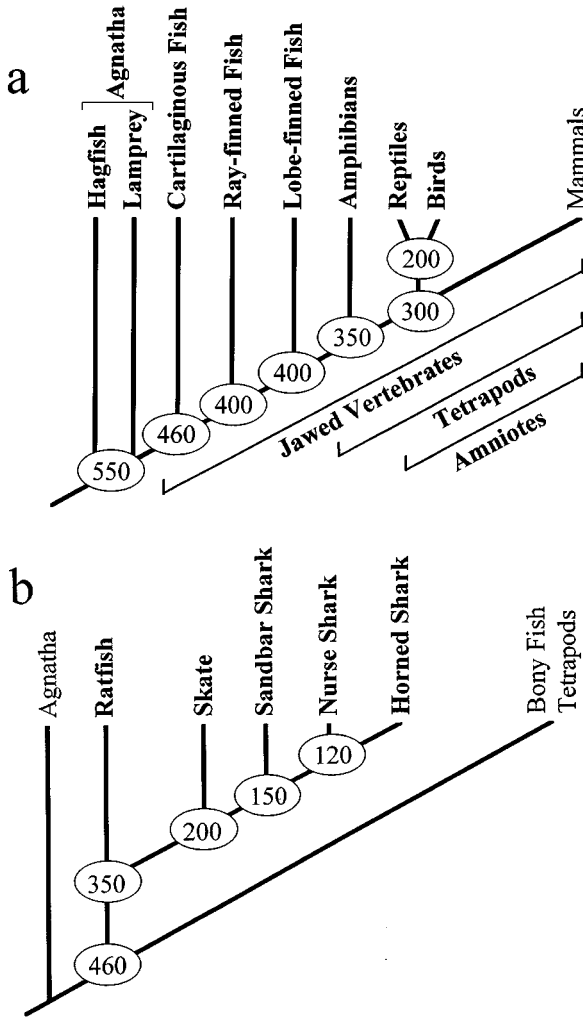


Figure 1 Phylogenetic relationships among the vertebrates. (a) Relationships among major vertebrate taxa; (b) relationships among the cartilaginous fishes (class Chondrichthyes); (c) relationships among the bony fishes: the ray-finned fishes (Actinopterygii) and the lobe-finned fishes (Sarcopterygii); and (d) relationships among the tetrapods. (Ovals) Approximate divergence times, in millions of years ago, based on paleontologic evidence (15) or molecular comparisons (16); the accuracy of these estimates vary.

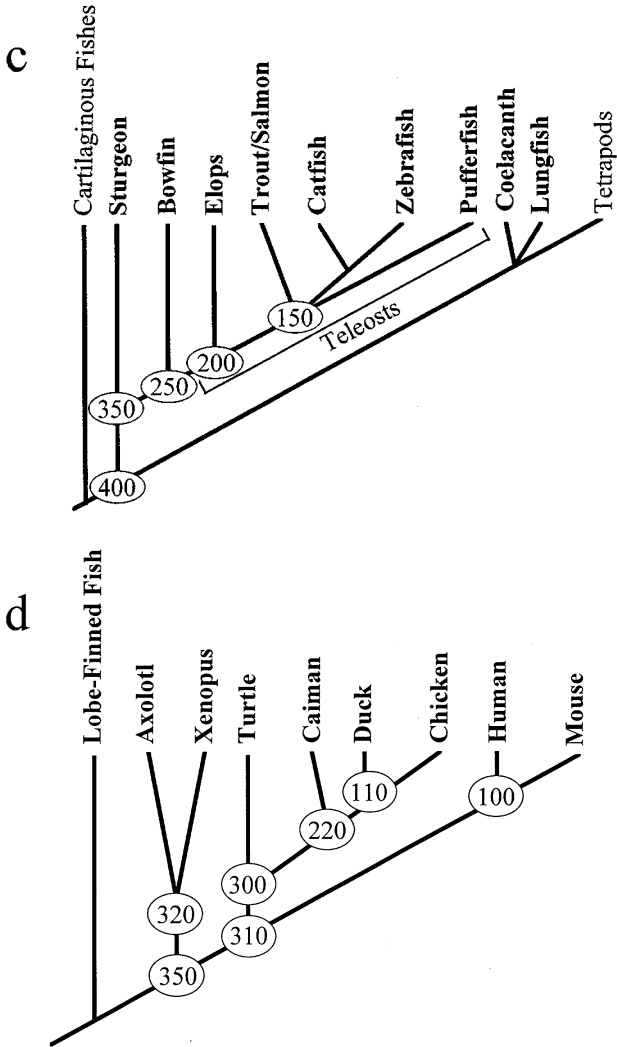


Figure 1 (Continued)

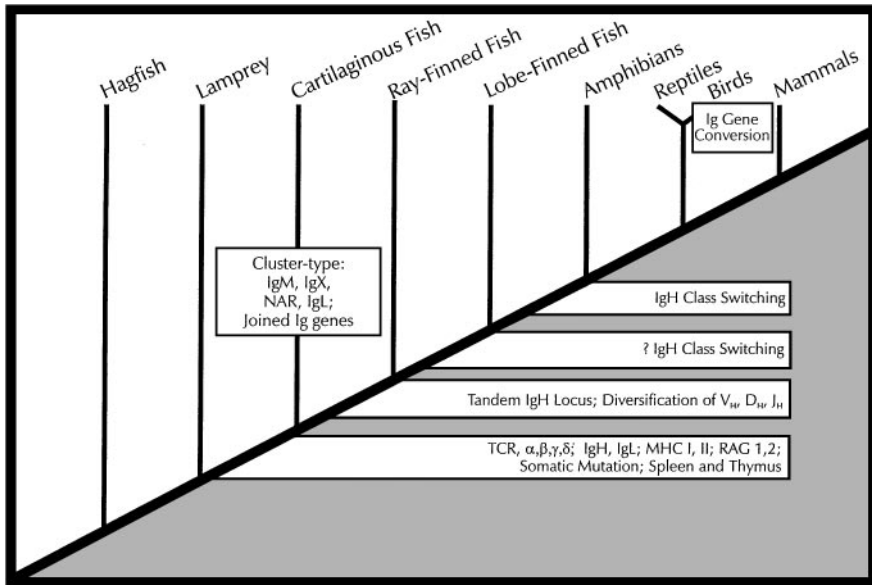


Figure 2 Major events in the evolution of rearranging antigen binding receptors superimposed on a phylogenetic tree of the major vertebrate groups. The placement of events relative to major vertebrate group divergence points is inferred from the data described in this review. Ig, immunoglobulin; IgH, Ig heavy chain; IgL, Ig light chain; NAR, novel antigen receptor; D_H, Ig heavy chain diversity region; J_H, Ig heavy chain joining region; V_H, Ig heavy chain variable region; RAG, recombination activating gene; TCR, T cell receptor; MHC, major histocompatibility complex.

ancestor with the other living jawed vertebrates between 450 and 575 million years ago (MYA) (15, 16). In addition to IgM-type genes (hereafter referred to as IgM), other classes of Ig genes have been identified in these species. Certain features of IgM gene organization, structure, and function have been shown to be shared with higher vertebrate Ig, including the following: segmental rearrangement of V, D, and J elements (12); amino acid identity with the corresponding rearranging elements found in higher vertebrates (7, 17, 18); separate Ig domains in single C region exons; differential processing of secretory and transmembrane (TM) forms (14, 19); and somatic mutation of rearranged genes (18) (see below). However, major differences are evident: (a) Multiplicity of loci, i.e. Ig heavy-chain genes in horned shark (*Heterodontus francisci*) are encoded in as many as 100 independently functional clusters in the genomic forms V-D₁-D₂-J-C-TM (20); (b) there is restricted intracluster rearrangement (18); (c) short (typically <350 bp) intersegmental distances exist between rearranging elements (7, 13, 21–23); (d) an additional D segment exists within clusters

that increases the potential for junctional diversification (7, 18); (e) there is germline joining of segmental elements in significant numbers of clusters (7, 8, 13) (see below); and (f) there is an absence of octamer binding sites and a presence of TCR β -type CRE elements (24). Furthermore, although the V_H genes of tandemly organized loci, i.e. mammals, appear to evolve in a birth-and-death turnover process (25), it is unclear whether cluster-type Ig genes are subject to the same diversifying process.

A single IgM V_H family (V_H1) in the horned shark in which the members are ~90% related at the peptide level accounts for all but one gene cluster (7, 18). The monotypic V_HII gene cluster, which is ~60% identical to V_HI at the predicted peptide level, presumably diverged from a V_HI-type gene by a process in which the intervening sequence (IVS) between D₁ and D₂ was excised and recombined in an inverted orientation, equivalent to a hybrid signal join. This results in a different utilization pattern for D₁ and D₂, and possible relief from any gene correction processes, thus allowing greater divergence. Analyses of the rearrangements of specific Ig gene clusters in horned shark have revealed typical patterns of junctional diversity and ruled out intercluster recombination of segmental elements. Junctional diversity presumably preceded combinatorial diversity in the evolution of mechanisms that diversify Ig genes (18).

The phenomenon of germline joining is unique to the cartilaginous fish. Germline-joined horned shark IgM heavy-chain genes (VD-J-C; VDJ-C), which comprise 50% of the clusters analyzed, resemble their unjoined counterparts in terms of overall exon organization and sequence relatedness (7, 18). Both VD-J-C- and VD-DJ-C-joined germline genes also are found in the IgM genes in little skate (*Raja erinacea*), which similarly possess nonjoined forms (13). Various lines of evidence indicate that these genes are derived from typical unrearranged ancestral genes (7, 8, 26).

IgM gene clusters also have been identified in a separate lineage of cartilaginous fish represented by the ratfish (14). These are found in unjoined and joined forms and are related to the shark and skate IgMs. A variant IgM-type cluster recovered from a ratfish cosmid library possesses a first constant region exon that lacks significant sequence identity with C_H1 from the IgM genes found in this species. Duplication of a C_H2 exon and loss of a typical C_H1 exon accounts for this difference (14). The IgM variant lacks the cysteine residue in C_H1 that participates in interchain disulfide bonding with light chain, which suggests that it could function either as a single-folded chain or as a homodimer. This presumably derived character also has been reported in the nurse shark (*Ginglymostoma cirratum*) novel antigen receptor (NAR) (22, 27) and in camel IgG (28, 29). A completely germline-joined IgM, which has been shown to be expressed in both secretory and TM forms, and an extensive family of pseudogenes, comprising ~90% of all V_H hybridizing segments, also are present in this species (14).

IgX, a second distinct class of cluster-type Ig heavy-chain gene, has been described from skates. V_X is $\sim 60\%$ identical at the nucleotide and amino acid levels to the V_H of the cartilaginous fish IgM genes (30). IgX clusters contain two D and one J segment as well as two C_X exons followed by a single cysteine-rich exon lacking a TM character (and by inference designated secretory) (21, 30). Multiple IgX (as well as IgM) clusters are present in skate (30). In situ hybridization studies with interphase nuclei demonstrate that both IgM and IgX clusters are found on multiple chromosomes and do not appear to be linked (21). The overall regulation, including possible allelic exclusion, of multiple cluster-type Ig loci, is not understood (24, 26); however, other multigene families encoding diverse specificities are also organized in multiple potentially independently functional loci (31). The complete genomic nucleotide sequence of an IgX locus up to the secretory exon has been reported (21). Although the structures of short regulatory motifs in the J-C IVS of both shark IgM and skate IgX are related closely to those important in the transcriptional regulation of mammalian Ig expression, their relative positions are not conserved (32). The functional significance of these motifs is not yet established (G Warr, D Ross, M Anderson, unpublished observations).

Longer IgX-related transcripts have been identified (30) and shown to consist of V_X , C_{X1} , and C_{X2} as well as four additional Ig domains (21). IgW described in sandbar shark (*Carcharhinus plumbeus*) (33) and NARC (new antigen receptor from cartilaginous fish) described in nurse shark (34) consist of IgX-like V , C_1 , and C_2 regions as well as four additional C terminal regions (C_3 – C_6) (34), which are homologous to the last four exons of the long IgX transcript and exhibit significant identity with the corresponding regions of the nurse shark NAR (22) (see below). The relationship of IgX to IgW, NARC, and NAR in these three phylogenetically disparate species was examined further by characterizing both short and long forms of IgX in the skate; the short form has not been identified in any shark species at the molecular genetic level. The coding regions of the long and short forms of IgX share $\sim 90\%$ nucleotide identity, with differences concentrated in complementarity determining regions (CDRs) (23). Differential processing of the same locus most likely accounts for the extraordinary identities, which extend to include IVSs between D_X elements (23).

NAR, a third class of Ig in cartilaginous fish, was identified in the nurse shark using polymerase chain reaction (PCR) that targeted shared sequence motifs in C_1 -type Ig domains (22). NAR possesses an unusual V region, which is not related closely to those of any particular Ig or TCR V regions, a truncated C_1 domain, and four C-terminal Ig domains. Furthermore, NAR V regions, unlike those of conventional Ig and TCR, do not form dimers but presumably function as independent domains (22, 27). NAR possesses the same cluster-type

genomic organization as IgM and IgX but each cluster contains three versus two D elements. Only four NAR loci have been identified in nurse shark, of which two are expressed and two presumably are pseudogenes. Two of the three D genes are germline-joined in one of the pseudogenes. Two V types are defined on the basis of differences in noncanonical cysteine residues that may stabilize the functional V monomer against variation (35). Cysteine residues in CDR3 are encoded typically by preferred reading frames of rearranged D segments (27). NAR has been identified in another species of shark and in sting ray (M Flajnik, personal communication). NAR undergoes extensive hypermutation (see below).

Several, but not all, of these features of NAR, i.e. its function as a single chain and the intermediate TCR-Ig V-like sequences are potential properties of a primordial antigen binding receptor (22, 27); however, the three D segments and extensive hypermutation may be derived characters. Phylogenetic analyses indicate that the last four C-region domains of NAR diverged from an IgX/IgW/NARC/NAR common ancestor after the IgM/IgX divergence but prior to the divergence of the sharks and skates. Presumably, NAR would have existed in a common ancestor of both sharks and rays/skates (23). Alternatively, NAR could represent a chimeric structure, reflecting C terminal elements of IgX and a C₁-type domain as well as a V region of divergent origin.

Light-Chain Genes

Light-chain genes have been identified in both major radiations of cartilaginous fish. Type I light-chain genes in horned shark and skate cannot be classified as either κ or λ chains (36). In horned shark, light-chain genes exist in multiple, independently functioning clusters consisting of closely linked single V_L and J_L segments, and a C_L exon (37). The type I light-chain genes in horned shark possess a typical regulatory octamer (37), and germline joining has not been detected. In contrast, a multisite PCR priming strategy and analyses of genomic clones in the little skate failed to identify any unjoined type I light-chain genes. Several of these germline-joined genes are expressed and appear to undergo somatic mutation (8, 38).

Type II light-chain genes have been characterized in sandbar shark (39–41), two different skate species (42), and ratfish (14). Genomic cloning and PCR analyses suggest that type II light-chain genes in the sandbar shark are germline-joined (40) and are encoded in clusters that can be classified in two sequence groups (41). All type II light-chain genes in skate are also germline-joined (42). Type II light-chain genes have been shown at the protein level to associate with IgM heavy chains in clearnose skate (*Raja eglanteria*) (M Anderson, unpublished observations). Unlike the heavy-chain genes in the cartilaginous fish, light-chain genes of a given type within a species are either

completely joined or unjoined. Although type I genes can be joined (skate) or unjoined (shark), type II genes are joined in sharks, skates, and ratfish, confounding definition of any simple pattern for the acquisition of the joined character. However, the observations are consistent overall with germline joining being a derived characteristic.

Attempts to classify type I and II light-chain genes relative to κ - or λ -type are complicated by the large phylogenetic distances between the cartilaginous fish and the higher vertebrate prototypes, as well as the relatively rapid rates of evolution of antigen binding receptors. However, the type I genes certainly are unlike those of higher vertebrates. The classification of a third type of light chain found in the nurse shark (43) and horned shark (42) as κ -like is well supported by phylogenetic analyses (43, 44). The κ -like genes are not germline-joined. The distinctive character of the three families of Ig light-chain genes will facilitate studies of potential isotypic exclusion.

IMMUNOGLOBULIN GENES IN RAY-FINNED FISH

Heavy-Chain Genes

Unlike the organization of heavy-chain genes in the cartilaginous fish, an IgM heavy-chain gene is encoded at a single mammalian-like locus in representative species of the chondrosteian, holostean, and teleost radiations of the ray-finned fish (Figure 1c). The IgM loci in teleosts such as ladyfish (*Elops saurus*), channel catfish (*Ictalurus punctatus*), Southern pufferfish (*Spheroides nephelus*), and rainbow trout (*Oncorhynchus mykiss*) consist of multiple V_H , D_H , and J_H elements linked to IgM C_H exons that are linked within ~ 100 kb. Shorter intersegmental (V_H - D_H) distances are found in teleost fish Igs than are found in mammalian Igs (45, 46).

The constant regions of IgM heavy chains in teleosts are encoded by four C_H and two TM exons that are related to IgM heavy-chain genes in higher vertebrates. However, differences have been noted in cysteine distributions and potential glycosylation patterns (47, 48) as well as in the relative hydrophobicity of the C_{H3} domain(s) relative to C_{H3} in IgM heavy chains of other vertebrates (47). TM exons have been shown to be spliced directly into C_{H3} , producing a shorter membrane-bound form of heavy chain (48, 49). The phenomenon is tissue independent and may be generalized in the teleost fish (50). Both the C_{H3} -to-TM mode of differential processing found in teleost fish and the C_{H4} -to-TM pattern found in sharks, amphibians, and mammals (14) are found in the bowfin (*Amia calva*), a representative of the holostean lineage that diverged prior to the lineage that included the latest common ancestors of the teleosts (Figure 1c) (51). Ig accessory molecules, which possibly relate to signal transduction molecules found in higher vertebrates, are associated with the membrane form

of Ig in catfish (52). Studies of enhancer function in catfish B cell lines have revealed that the Ig heavy-chain enhancer lies downstream of C_{H4} and not in the J-C intron. It is possible that enhancer function had to arise in the J-C intron as a precondition to the emergence of class switching in order to avoid deletion of the enhancer during the switch recombination event (53).

The potential for generating V_H gene diversity by combinatorial joining in ray-finned fish is considerable. In catfish, seven different V_H families, multiple D segments, and at least nine J_H segments, which are linked within 1.9 kb to C_{H1} , have been described (54). Trout encode at least 11 V_H gene families and 10–20 D_H segments (55, 56); rearrangement joins in CDR3 are relatively short by comparison with mammals. Although the reduced length complicates assessment of D_H contributions, there is no direct evidence that the short CDR3 lengths compromise antibody diversity (57).

A second class of Ig heavy-chain gene, which encodes an IgM C_{H1} domain plus seven additional C_H exons, is contiguous with the IgM heavy-chain gene in catfish. A similar situation has been observed in the pufferfish; however, the second isotype contains only six additional C_H exons (T Ota, C Amemiya, personal communication). The extent of amino acid sequence identity between the second fish isotype and corresponding mouse and human IgD exons is quite low; however, identity between some C_H exons in mouse and human IgD is also very low (58). The chromosomal location of the IgM and IgD-like exons, the predicted alternative splicing, the separate secretory and TM domains, and the coexpression with IgM (μ -type) heavy chains in some B cells support a relationship of the catfish gene to IgD but a hinge region, such as that found in IgD, is not present. The difference in gene organization between the cartilaginous and ray-finned fish is extreme. Although subsequent radiations of vertebrates preserved the overall mammalian-like organization of the Ig heavy-chain gene locus seen in the ray-finned fish, changes have taken place in its form and function that underscore the unusually fluid character of Ig gene evolution.

Light-Chain Genes

The chondrosteian radiation, represented by the sturgeon (*Acipenser*), is the most distant lineage of the ray-finned fish relative to the teleosts (Figure 1c). In this species, multiple V_L genes are encoded upstream of J_L sequences and a single (or relatively few) C_L region(s), a pattern that is distinct from the organization patterns described in the teleosts (59) and reminiscent of the κ light-chain gene locus in mammals. In contrast, Southern blot analyses suggest that one form of Ig light-chain (IgLC1) gene in two teleost species, trout and Atlantic cod (*Gadus morhua*), is organized in a cluster-type organization, similar to the cluster-type I (unjoined) light-chain genes in horned shark (60); however, in teleost fish, the individual clusters are closely linked (60, 61). A second

light-chain isotype in trout (IgLC2), which is only distantly related to IgLC1, also exhibits cluster-type organization. Two repeat sequences that are found upstream of mammalian J_{κ} and may represent transcription factor binding sites were identified in sterile IgLC2 transcripts (62). In catfish, two classes of light chains designated G and F, which share 52% and 34% amino acid identity in the V and C regions, respectively, have been identified. The V_F region is present in multiple families and is homologous to higher vertebrate V_{κ} , and V_G is closely related to IgLC1. C_G and C_F domains cannot be unequivocally assigned to other vertebrate light-chain classes (60, 61, 63). Southern blot analyses are consistent with at least 15 G clusters and more than 50 F clusters. The G loci consist of at least two V_G elements upstream of two J_G elements, one of which is likely to be a pseudogene, and a single C_G exon. F loci resemble basic V_L - J_L - C_L cartilaginous fish-like light-chain clusters. As in the G cluster, the V_F and J_F segments are in opposite transcriptional polarity, indicating rearrangement by inversion rather than by deletion (61, 63). Neither type of cluster is associated with germline joining. The close linkage of the individual clusters could permit intercluster recombination, although this has not been demonstrated. The relationships among light-chain gene organization patterns in the cartilaginous fish, the sturgeon, and several different teleost lineages cannot be reconciled in a simple model (59).

IMMUNOGLOBULIN GENES IN THE LOBE-FINNED FISH

Heavy-Chain Genes in a Living Fossil

Different forms of organization of Ig heavy-chain genes are associated with different mechanisms of somatic diversification and regulation of gene expression. Specifically, combinatorial rearrangement of segmental elements does not appear to be used to diversify gene clusters in cartilaginous fish (18). Furthermore, unlike Ig gene loci in higher vertebrates, gene rearrangement per se is not an obligate requirement (7, 8, 39, 40). The living coelacanth (*Latimeria chalumnae*) is a surviving member of an ancient superorder that was long thought to be extinct (Figure 1c). V_H and D_H segments in coelacanth Ig heavy-chain genes are linked closely (~ 190 bp), a similar character to that described in the cartilaginous fish (64). The V-D pairs themselves are in close tandem linkage (3–4 kb), presumably upstream of J_H and C_H . The putative coding regions of D_H contain recombination signal sequences (RSSs) with 12-bp spacers and are related to a “join” of shark-type D_{H1} and D_{H2} (7). The apparent absence of additional D_H segments (and RSSs) downstream of V-D is consistent with precommitted joining of V-D pairs. Southern blotting of coelacanth DNA using a V_H -specific

probe reveals a large number of hybridizing elements. All of the genomic V_H genes thus far sequenced are members of a single family; cDNA analyses will be required to further assess V_H family divergence and to characterize J_H and C_H contributions.

Heavy-Chain Gene Isotype Diversity

Modern lungfish are a critical group in terms of understanding possible distinctions between Ig genes in ray-finned fish and in representatives of the phylogenetic radiation that includes the ancestors of the amphibian and reptiles (Figure 1*d*). Three distinct heavy-chain cDNAs have been identified in African lungfish (*Protopterus aethiopicus*): (a) an IgM gene encoding four C_H region domains, (b) a second type encoding seven C_H domains, and (c) a third type encoding two C_H domains and a 3' cysteine-rich domain, similar to that in an avian IgY gene (see below). The seven C_H domain-containing genes resemble the IgD-like gene in the ray-finned fish discussed above; however, C_{H1} is not IgM-like. Six distinct V_H families have been identified in lungfish (65) (T Ota, C Amemiya, personal communication). By analogy to avians and through general phylogenetic inference, it is possible that the heavy-chain gene class switch arose prior to the divergence of the lungfish and tetrapods. Light-chain gene structures have not been described for either the coelacanth or lungfish.

IMMUNOGLOBULIN GENES IN AMPHIBIANS

Heavy-Chain Genes

In addition to their phylogenetic position, amphibians are of particular significance as an immunological model system that undergoes metamorphosis (66). The Ig heavy-chain gene locus in the African clawed frog (*Xenopus laevis*), an anuran amphibian, encodes three types of C regions: IgM, IgX, and IgY. The TM regions of IgX and IgY are more closely related to the TM regions of *Xenopus* IgM than to mammalian forms, supporting a hypothesis that IgM duplicated to give rise to IgY and IgX (67), which is not related to IgX in cartilaginous fish but rather is considered to be an IgA functional equivalent (68). No evidence has been found, in either developing or mature lymphocytes, for IgD-like genes or for alternative splicing of C_H regions. Amphibians are the most divergent species relative to the mammals (Figure 1*a*), in which a mammalian-type heavy-chain class-switching mechanism has been documented, although this characteristic could be present in lobe-finned fish (Figure 2). The μ -type switch region (S) in *Xenopus* is ~ 5 kb and consists of 23 repeats of ~ 150 bp; each repeat consists of shorter internal repeats and palindromic sequences. Class switching occurs after gene rearrangement and is greatly accelerated in the secondary immune response. In IgX-expressing B

cells, the μ -type heavy-chain gene is deleted and $S\mu$ and Sx , which are not related at the sequence level, are joined. As in mammals, switching recombination occurs at microsites formed through repetitive palindromes and is associated with tissue-specific gene expression (69).

Eleven different V_H gene families, extensively diversified D_H genes, and a high level of J_H diversity are found in *Xenopus* (70–72). Certain V_H families have multiple members and all but the V_{HIII} and V_{HVII} families have been found to be interspersed (73). Multiple 5' Ig regulatory octamer sequences and octamer-like motifs are found upstream of *Xenopus* V_H genes, which suggests that their regulation could have unique features (70, 73). Restricted utilization of D_H sequences and limited N region diversity are associated with early development in *Xenopus* (74).

Axolotl (*Ambystoma mexicanum*) is a neotenic amphibian, i.e. the mature form of the animal retains larval features. Both IgM and IgY have been identified in axolotl. In axolotl IgY, additional cysteines, as have been found in *Xenopus* C_v , avian C_v , and human C_e , are found in the C_{v1} and C_{v2} regions. The IgY molecule in axolotl is secretory (75).

Three axolotl Ig V_H families as well as four D_H and six J_H elements have been identified (75, 76). Analyses of spleen Ig cDNAs reveal greater numbers of out-of-frame sequences in early embryos than at later developmental stages. A slight increase in the length of CDR3 between subadult and adult axolotls has been reported (77). The CDR3 loop of axolotl V_H genes has been interpreted to be less somatically diversified than CDR3 in any developmental stage of mouse or *Xenopus*. The discovery that V_H regions in axolotl have both shorter junctional sequences and extensive conservation of the germline J_H sequences possibly relates to the decreased affinities in antibody responses in this species compared with *Xenopus* and teleost fish (78).

Light-Chain Genes

Three classes of light-chain genes have been identified in *Xenopus* (79–81). The ρ locus contains at least four V_ρ and five J_ρ elements as well as a single C_ρ gene and is classified as κ -like on the basis of both V_ρ sequences and organizational features of the ρ locus (44, 80). The σ -type light chain consists of multiple $V_\sigma 1$ and $V_\sigma 2$ as well as $C_\sigma 1$ and $C_\sigma 2$ segments (79); both types of genes rearrange to their respective J_σ types. The σ and ρ genes are encoded at separate loci. Expressed V_σ sequences are highly conserved and diversity of σ and ρ light-chain genes in *Xenopus* may be markedly restricted, as opposed to the heavy-chain genes, which are as diversified, if not more so, than their mammalian counterparts (72).

A third light-chain type in *Xenopus* represents a higher vertebrate λ light-chain homolog. Six distinct V_λ families, two J_λ segments, and two distinct C_λ

exons have been characterized. Southern blotting of DNA as well as sequence analysis from individual animals indicates that the V_λ and C_λ sequences are diverse and polymorphic (81). The complex diversity in the λ -type light-chain genes argues against diversity restriction in the overall light-chain repertoire in *Xenopus*.

IMMUNOGLOBULIN GENES IN REPTILES

Heavy-Chain Genes

Southern blotting of reptilian [caiman (*Caiman crocodylus*) and turtle (*Chelydra serpentina*)] DNAs indicates large numbers of V_H genes that cross hybridize with murine and species-specific V_H probes (82) (G Litman, unpublished observation). Screening of a single genomic equivalent of a turtle genomic λ DNA library using homologous V_H probes resulted in hybridization to >500 individual clones. At the stringency used, the hybridizing elements could be as little as 60% related to the probe used for screening. Each λ genomic clone typically consists of multiple, linked V_H genes; however, many of the hybridizing bands may represent pseudogenes. Overrepresentation of individual clones does not account for the large number of positives. In situ chromosomal hybridization mapped turtle genes to four separate linkage groups (26) (C Amemiya, G Litman, unpublished observation). More recent studies in another species of turtle (*Pseudemys scripta*) defined four families of V_H genes, which are encoded at a single μ -type locus (83). Northern blot analyses using a C_{H4} probe identified two μ -type transcripts and at least two non- μ -type transcripts. Analyses of rearranged Ig heavy-chain gene transcripts from a single animal suggest large numbers of J_H segments and/or extensive somatic mutation in FR4. In terms of the generation of antibody diversity, it is apparent that reptiles contain particularly large numbers of V_H genes. Although avians technically are members of the reptilian radiation of vertebrates, their Ig genes represent a marked departure from the typical reptilian organization, as defined in caiman and turtles.

IMMUNOGLOBULIN GENES IN AVIANS

Heavy-Chain genes

The chicken (*Gallus domesticus*) is the most comprehensively characterized nonmammalian immunological model system (84–86). Three classes of heavy-chain genes are present in this species. Class switching occurs from IgM to either IgY or IgA; IgM to IgY switching occurs during the secondary antibody response and is associated with increased antibody affinity (see below). In addition to IgM and IgA (87), two forms of IgY, possessing nearly identical C_{H1}

and C_H2 domains, are present in the duck *Anas platyrhynchos* (88). The shorter transcript encodes C_H1 and C_H2 plus a unique short exon encoded between C_H2 and C_H3. The longer transcript encodes C_H1–C_H4 in either secretory or TM forms. On the basis of inferred intrachain disulfides and the number of secretory exons, the full-length form of IgY resembles IgE; however, TM sequences and similar RNA processing are more characteristic of IgG, which suggests that IgG and IgE diverged from a common ancestral molecule resembling IgY (89, 90).

Chickens possess a >60–80 kb heavy-chain gene locus consisting of a single functional V_H gene, 16 relatively similar D_H segments, and a single J_H element, which are arrayed upstream of the C_H region genes (84, 91). Only limited combinatorial diversity and little junctional diversity is achieved through differential rearrangement of D_H segments. The principal means of diversification is achieved through gene conversion that utilizes a pool of 80–100 5'-truncated upstream V_HΨ (pseudogenes), which lack RSSs and are in different relative transcriptional polarities. Certain V_HΨ possess D_H-like and J_H-like sequences at the 3' ends, and gene conversion can extend into these regions; preferred V_HΨ exchanges have also been described (92). Furthermore, a high incidence of D_H-D_H joining and P nucleotide addition also diversifies the V_H locus (92). Given the phylogenetic positions of avians as having diverged from a common ancestor with the reptiles after the divergence of the mammals, the avian Ig heavy-chain gene presumably is a derived evolutionary character. Gene conversion also has been documented unequivocally in one mammal (93) and presumably occurs in others (94–96).

Light-Chain Genes

The somatic hyperconversion process described above was characterized initially for the single λ-like light-chain locus of the chicken (97, 98). Twenty-six V_LΨ are upstream of single, closely linked V_L and J_L segments (97, 99). A variant of this arrangement, in which the pseudogenes are upstream of two functional V_L segments, has been described in another avian species (100). The frequency of V_LΨ use depends on the proximity of the pseudogene and target genes as well as the extent of identity and their relative orientation. Homology in the 5' pseudogene and target is most essential and evidence for polarity in the gene conversion mechanism(s) has been presented (101). In addition to gene conversion, a number of mechanisms, including imprecise joining, somatic point mutation, single nonrandom nucleotide additions (102), and V gene replacement (99), further diversify light-chain genes.

The diversification mechanisms found in avians are of particular interest from an evolutionary standpoint in terms of the stability and maintenance of VΨ (donors). Comparisons of V_LΨ and functional alleles between three inbred lines of chicken identified multiple interstrain polymorphisms as well as

polymorphisms that could arise by meiotic gene conversion. The incidence of mutations within the $V_L\Psi$ is lower than in the corresponding flanking regions, consistent with the hypothesis that the $V_L\Psi$ cluster behaves as a functional multigene family under selective pressure for its role in the diversification of the antibody repertoire (103). The similarities between the organization of the Ig heavy- and light-chain loci in both avian and cartilaginous fish models suggests parallel evolution of both form (organization) and function (mechanisms of diversification) in antigen binding receptors (37).

PHYLOGENETIC ORIGINS OF SOMATIC HYPERMUTATION

Targeted somatic hypermutation has been demonstrated in all vertebrate Ig gene systems thus far examined (11) and is undoubtedly an ancient mechanism for the diversification of the antigen-binding sites of Igs, regardless of their genomic organization (8). The degree of somatic hypermutation varies by approximately an order of magnitude among vertebrate groups (11). Somatic hypermutation in mammals is associated with affinity maturation (10), which can be several orders of magnitude greater than in anuran amphibians and teleost fish. Temporal increases in antibody affinity have not been observed in cartilaginous fish (18, 104). The discrepancy between the presence of somatic hypermutation and the absence of affinity maturation in the cartilaginous fish is difficult to reconcile. One explanation is that the absence of germinal centers in cartilaginous fish may result in a lower efficiency of cellular selection of high-affinity mutants (10, 18, 105). However, this supposition raises the question of why targeted somatic mutation would have evolved prior to the development of a selection system. One possibility is that somatic mutation could diversify the preimmune repertoire prior to selection, as occurs in sheep Peyer's patches (106). However, recent studies indicate that NAR mutations are accumulated after exposure to antigen and are not used to generate the preimmune repertoire (35), which argues against this theory. Although the basis for mutation of the shark NAR gene could be different from the other cluster-type genes, this observation is consistent with NAR mutations being a shared character with mammalian somatic mutation and, thus, ancestral. It is possible that rather than repeated rounds of mutation and expansion in response to antigen receptor signaling, as occurs in mammalian germinal centers (107), lymphocytes in lower vertebrate groups are biased toward terminal differentiation. It is also possible that high-affinity mutants reside in other (unknown) tissue(s) in these animals. Further study is required to relate differences in antigen binding receptor structures in various vertebrate groups to the differences observed in immune function assays.

B CELL MEMORY AND THE SECONDARY IMMUNE RESPONSE

The evolution of antigen binding receptors involves organizational and mechanistic change and occurs within the context of highly specialized systems that potentiate function of effector molecules. B and T cell memory, defined in terms of the ability to respond more rapidly and effectively to secondary challenge with a specific antigen, are found in all jawed vertebrates, with the possible exception of the cartilaginous fish (66). Specifically, upon secondary antigenic challenge, horned sharks do not show a dramatic rise in levels of antibody. Rather, the increase requires repeated monthly immunizations and occurs over a much longer time interval than is seen in mammals (108, 109).

Both teleost fish and amphibians demonstrate a secondary rise in antibody levels that is greater and more rapid than the primary response, but less vigorous than that seen in mammals. The secondary response is generally 10- to 20-fold higher than the primary response, without a measurable increase in affinity. It has been shown that a low priming dose is required to achieve optimal memory development in carp (*Cyprinus carpio*) (110). Whether IgM memory in mammals exists is debatable, and thus the role of isotype switching in triggering changes characteristic of the memory response is unclear. The modest secondary response consisting primarily of IgM antibody, which is seen in teleost fish, indicates that a limited form of memory exists in the absence of class switching.

Anuran amphibians (such as *Xenopus*) exhibit a peak in the primary response at 4 weeks. The secondary response peak occurs at 2 weeks postimmunization and is accompanied by class switching from IgM primarily to IgY. The secondary response is associated with 10–40 times greater antibody production (111). However, in urodele amphibians [such as the newt (*Notophthalmus viridescens*)] class switching does not appear to occur. The peak level of antibody in response to T-dependent antigens does not increase in the secondary response, although the rise in antibody occurs more rapidly (112).

Current views on mammalian memory emphasize the importance of the affinity of antigen receptor–antigen interactions in controlling which signaling pathways are activated and, thus, how the functional response is modulated (113). A strong-affinity interaction results in a full activation of all possible signaling pathways, leading to activation of the effector function gene expression program. However, a low-affinity interaction results in partial activation, which sensitizes the cell to further stimulation without inducing proliferation or effector function and allows entry into the memory compartment. Studies of memory B cells (114) support a model in which clonal expansion occurs primarily in cells that are undergoing the effector program response, not in cells that are

fated to become memory cells. The observation that a low priming is needed to achieve memory development in carp whereas high-priming doses result in poor memory responses suggests a similar mechanism in the development of memory in teleost fish (110). Two key differences between cartilaginous fish and higher vertebrate immunity are the low-affinity Ig receptors that do not mature, and the apparent lack of sensitization leading to a memory response. It is difficult to understand how the shark lymphocytes, which bear low-affinity antigen receptors, become activated to a full effector response unless there are major differences in the way their antigen receptors interact with the signaling apparatus, or unless there is a significant change in the signaling threshold.

THE IDENTIFICATION OF T CELL ANTIGEN RECEPTOR HOMOLOGS IN NONMAMMALIAN VERTEBRATES

T cell-mediated immunity was thought to exist in all the phylogenetic groups in which rearranging Ig genes were known to exist. However, with the exception of chicken TCR β (115), nonmammalian TCRs have proven refractory to isolation by DNA cross hybridization strategies that were used successfully to identify Ig genes in lower vertebrates. The primary basis for this technical disparity most likely relates to the higher rates of sequence divergence among TCRs. High rates of divergence are characteristic of immune genes relative to their nonimmune counterparts (116). The forces driving this rapid divergence are unclear but could stem from a generally lower level of constraint on immune proteins. Alternatively, this rate could result from sequence divergence in response to interactions with molecules—such as the TCRs themselves and MHC proteins—that are under heavy positive selection to vary (117). Paradoxically, as more data are acquired, it appears that although TCR genes are generally diverged in primary sequence, TCRs may be more conserved than Igs in terms of gene organization, diversification mechanisms, and subclass heterogeneity.

T CELL ANTIGEN RECEPTOR GENES IN CARTILAGINOUS FISH

The particular interest in TCR genes in cartilaginous fish relates not only to their phylogenetic position but also to functionally unique aspects of immunity in these species, e.g. apparent absence of affinity maturation and chronic allograft rejection (118). A PCR strategy based on shared 3–4 amino acid motifs in FR2 and FR3 of higher vertebrate TCR and V_L genes of higher vertebrates (119) was used to amplify candidate gene segments using horned shark cDNA as template.

Sequence analyses of cDNAs selected with the candidate segments identified clones with V, J, and C regions that were most TCR β -like. Comparisons of cDNA clones reveal (a) a D β region core element and (b) that combinatorial joining plays a role TCR β diversification in shark. Genomic V β and J β region sequences were shown to have typical RSS elements separated by 23-bp (V) and 12-bp (J) spacers, respectively (119). Southern blotting indicates that V β and C β gene elements are present in multiple copies yet are organized (unlike the shark Ig heavy-chain genes) in several tandem arrays consisting of multiple V β and J β elements, similar to mammalian TCR genes. The differences in restriction enzyme digestion patterns of genomic lambda clones containing C β Ig domain exons and cDNA analyses are consistent with multiple C β region genes (120). However, this multiplicity may not be the rule for TCR genes in cartilaginous fish, as the four classes of TCR genes that have been identified thus far from the skate (see below) are encoded in one or at most two copies (121).

Additional information about V β diversity in horned shark has been obtained by sequencing large numbers of spleen TCR β cDNAs. Seven V β families, 18 different J β sequences, and a putative D β core sequence (GGGACAAC) were identified (120). Phylogenetic analyses comparing the shark V β sequences to those of other vertebrates suggest that at least some V β family divergence occurred prior to the divergence of the cartilaginous fish and the lineage leading to mammals.

Two TCR δ -like genes have been identified in horned shark (122). Initially it was unclear whether these genes represented a TCR δ ortholog; however, the later identification of homologs of TCR α and TCR δ in the skate (121) and the relationship of these genes to the horned shark genes suggest that the α/δ divergence occurred prior to or early in jawed vertebrate evolution. The two related but divergent TCR δ homologs as well as the multiple TCR β loci may relate to genomic polyploidy in horned shark (122).

Determination of orthology among mammalian and cartilaginous fish TCR genes is problematic given the divergent nature of TCR genes. A more comprehensive, though by no means exhaustive, approach based on short primer PCR was used to amplify TCR genes from the clearnose skate (121), a well-defined developmental model (123). cDNAs were identified that are similar to the four mammalian TCR gene types in terms of V and C region sequence, absence or presence (inferred) of D regions, and other junctional characteristics. TM regions associated with each of the four TCR types conform to conserved antigen receptor TM motifs described in higher vertebrates (124). Sequencing of representatives of the different V families for each putative gene type is completely consistent with TCR α , β , γ , and δ orthology. Specifically, four skate V α and six distinct J α region families were identified. TCR α

junctional sequences are short, consistent with direct V-J joining. Six skate $V\beta$ region families, two potential $D\beta$ core elements, and four $J\beta$ region families are associated with the $C\beta$ homolog. Although each of the $D\beta$ s is G-rich, neither is otherwise similar to the putative $D\beta$ region identified from horned shark TCR β . Five $V\gamma$ and two $J\gamma$ families were identified and direct $V\gamma J\gamma$ joinings were observed, although some cDNAs possess more complex junctions. Five $V\delta$ and two $J\delta$ families have been identified. Three potential $D\delta$ core elements are present in TCR δ VJ junctions. The long junctional lengths are characteristic of mammalian TCR δ genes. Pulsed field gel analyses suggest that $C\alpha$ and $C\delta$ are linked, although the distance between the elements is probably considerably greater than in mammals.

T CELL ANTIGEN RECEPTOR GENES IN RAY-FINNED FISH

TCR genes have been described from four representative species of ray-finned fish. A TCR β gene from the rainbow trout was cloned using a degenerate PCR amplification strategy with primers that targeted conserved V sequence motifs (125). The sequences of amplified products were used to generate specific primers, and an anchor PCR strategy employing thymocyte cDNA as template was used to clone a corresponding $C\beta$ region. Three trout $V\beta$ families, exhibiting between 30% and 38% pairwise amino acid identity, and 10 different $J\beta$ sequences were described (126). The $J\beta$ segments possess canonical 5' 7-mer elements, and although a number of these lack typical 9-mers, they are utilized frequently in TCR rearrangements (127). A core $D\beta$ -like sequence (GGACAGGG) in trout is identical to a corresponding sequence in axolotl, chicken, and mouse $D\beta$ regions. Genomic analyses show that the $D\beta$ segment in trout is flanked by typical RSS elements. Although Southern blotting analyses potentially indicate multiple $C\beta$ -hybridizing elements, only a single $C\beta$ sequence was identified. Junctional lengths of the in-frame trout TCR β CDR3 regions are somewhat shorter than those observed in mice and chickens and can be accounted for by the relatively short (eight nucleotide) $D\beta$. Approximately 40% of trout $D\beta$ - $J\beta$ junctions lack N-region nucleotide additions compared with 26% in the mouse.

The TCR β VDJ junctions, along with a portion of $C\beta$, have been recovered from Atlantic salmon (*Salmo salar*) using reverse transcription-PCR and leukocyte cDNA as template (128). Only one $C\beta$ region, closely related to trout $C\beta$, has been fully characterized. Minor sequence differences in salmon $C\beta$ were attributed to allelic variation or *Taq* error; interpretation of these data is complicated by a recent ancestral tetraploidization event, which is expected to result in two TCR β loci. Eight $J\beta$ region sequences were isolated, of which

five can be paired readily with trout $J\beta$. A single $D\beta$ core sequence, which is identical to that of trout, has been inferred, along with junctional diversity.

A rainbow trout $TCR\alpha$ homolog has been isolated using anchored PCR and a thymocyte RNA template (129). $C\alpha$ -specific primers were used to amplify $V\alpha$ - $J\alpha$ regions in a 5'-RACE PCR analysis. Of 40 cDNA sequences analyzed, 6 different $V\alpha$ families and 32 different $J\alpha$ segments were identified. The high level of J diversity is characteristic of $TCR\alpha$. Sequence comparisons of the trout $C\alpha$ reveal a close match with a Southern pufferfish putative $TCR\alpha$ sequence (122).

The catfish, in which lymphocytes and immunocyte cell lines are well characterized, has proven to be an excellent model system for studying lower vertebrate immune function (130). $TCR\alpha$ and β have been identified by V region-directed PCR amplification with degenerate primers from peripheral blood leukocyte cDNA. Three $V\alpha$ families and a single $C\alpha$ gene as well as five $V\beta$ and two $C\beta$ families were identified. Southern blotting using $V\alpha$ and $V\beta$ family-specific probes are consistent with additional genes. Seven different $J\alpha$ and seven $J\beta$ regions have been identified, as have two potential $D\beta$ regions, one of which matches corresponding sequences found in a variety of vertebrate $TCR\beta$ genes. Analyses of the transcription patterns of these genes in phenotypically characterized catfish T and B cell lines is consistent with their assignments.

T CELL ANTIGEN RECEPTOR GENES IN AMPHIBIANS

The $TCR\beta$ genes of *Xenopus* were isolated by an anchored PCR strategy employing a DNA template from a spleen/thymus cDNA library (131). The single $TCR\beta$ -like C region that was identified shares TM region features (124) as well as predicted peptide identity (31%) with axolotl $TCR\beta$. Ten different, highly divergent $V\beta$ gene families and 10 different $J\beta$ segments have been identified. Two putative $D\beta$ contributions, one of which matches the trout, axolotl, chicken, and human $D\beta$ sequences, have been inferred along with a possible third $D\beta$ sequence, similar to that identified in horned shark. N-region diversification most likely occurs in the rearranged junctional regions. RSS sequences for $V\beta$, 5'- $D\beta$, and $J\beta$ were identified in partially rearranged transcripts and are similar to those found in other TCR genes. Notably, the *X. laevis* $TCR\beta$ probes do not cross hybridize with DNA from members of the *Xenopus tropicalis* species group (131), which diverged ~120 MYA from a common ancestor with the *X. laevis* group. This lack of sequence similarity is unusual among genes of the *Xenopus* immune system (Ig, MHC, etc) which typically cross hybridize between these species. The finding is an example of the higher rate of sequence divergence for TCR than Ig genes. Although the genomic

sequence of a putative TCR α V region from *Xenopus* has been reported (122), a corresponding cDNA has not been described.

The first TCR gene from a nonamniote was identified in the axolotol using an anchored PCR strategy employing degenerate primers complementing a conserved region in TCR β and Ig light-chain C regions (132). Two closely related C β and two additional C β sequences were identified. Nine different V β families were described, of which the V β 7.1 gene is utilized most frequently (133). The junctional regions of 189 V β 7.1 cDNAs from animals at 2.5, 10, and 24 months were compared and equivalent numbers of C β 1 and C β 2 positive transcripts were identified along with nine different J β elements. Three J β 1 elements were associated with C β 1 and six J β 2 elements were associated with C β 2. Two different D β elements were identified; D β 1, J β 1, and C β 1 elements associate preferentially, as do D β 2, J β 2, and C β 2. D β 2-J β 1-C β 1 and D β 1-J β 2-C β 2 transcripts that could arise by trans- or inversional rearrangements, as well as by rearrangements that skip J β and C β elements, respectively, were also identified. Several V β -J β rearrangements were identified that appear to arise either from direct V β -J β joining or from extensive exonucleolytic trimming. N region addition increases from 40% of junctions in the 2.5-month-old animals, to 73% in the 10- and 24-month-old animals.

A PCR strategy employing 3' V α - and C α -TM specific primers was used to identify a TCR α homolog in the axolotol. Five different V α families and 14 different J α elements have been identified. As with TCR β from this species (132), a high fraction of rearrangements are out-of-frame (134).

T CELL ANTIGEN RECEPTOR GENES IN AVIANS

As indicated above, chicken TCR β genes were the first nonmammalian TCR genes to be described (115). Two V β gene families, which have mammalian V β counterparts, were identified that appear to define the chicken TCR2 and TCR3 cell lineages (135, 136). A total of six V β 1 and four V β 2 genes encode the chicken V β repertoire. All V β genes rearrange to a single 14 nucleotide D β element. Four J β segments and a single C β element were identified. The extent of N-region addition increases during development and is consistent with ontogenetic up-regulation of terminal deoxynucleotide transferase (TdT) in the thymus (137). The chicken D β region encodes a glycine residue in all three reading frames that presumably participates in the formation of a CDR3-encoded loop structure (137).

The chicken TCR α genes were isolated by a coprecipitation-peptide sequencing strategy employing antibodies to chicken CD3 (138). Multiple V α and J α elements are linked to a single C α region. Two V α gene families, which exhibit ~24% amino acid identity, account for all V α expression by T cell lines

(136). Southern blotting is consistent with more than 25 $V\alpha$ elements as well as multiple $J\alpha$ elements. Typical RSS configurations are associated with chicken genomic $V\alpha$ and $J\alpha$ elements.

In mammalian $TCR\alpha/\delta$ rearrangements, $V\alpha$ elements associate with $D\delta$ - $J\delta$ - $C\delta$ at a low but significant frequency. cDNA library screening with $V\alpha$ and $C\alpha$ probes identified $V\alpha^+/C\alpha^-$ clones. Sequencing revealed a putative $C\delta$ gene exhibiting 33% amino acid identity with human and mouse $C\delta$. Northern blot analyses showed that this gene is transcribed in thymus and spleen but is absent in chicken $\alpha\beta$ T cell lines. Two $V\delta$ families have been identified, and $C\delta$ associates with both chicken $V\alpha$ families (136).

$TCR\gamma$ genes in chicken were isolated by a short primer degenerate PCR strategy using chicken genomic DNA as template, followed by screening of a chicken spleen cDNA library with a candidate $TCR\gamma$ -like amplified product (122). Two different $V\gamma$ families and a putative $C\gamma$ region were identified. The extracellular Ig domain of putative chicken $C\gamma$ exhibits ~31% identity with mouse $C\gamma$. A third $V\gamma$ family was identified and each of the three families consists of eight to ten members (139). Three $J\gamma$ segments and a single $C\gamma$ region gene have been described. Transcripts that hybridize with a $C\gamma$ probe are prevalent in a $\gamma\delta$ cell line and are absent in $\alpha\beta$ T cell and B cell lines. No evidence was found for the early waves of invariant rearrangement of $TCR\gamma\delta$ T cells seen for mouse and human (139). Notably, chicken—like sheep, cattle, and pigs—possess a high frequency of $\gamma\delta$ T cells and also express a more complex repertoire of γ and δ rearrangements. Thus, the $TCR\gamma\delta$ characteristic described for the mouse and human may not be typical of vertebrates as a whole.

In the initial stages of the comparison of complex genetic systems, such as TCRs, similarities are likely to be recognized before differences are defined. With this bias in mind, the emerging consensus is that the broad-scale features of mammalian TCR genes can be found throughout the living representatives of the jawed vertebrates. This putative conservation of structure may result from an increased level of functional constraint that possibly represents an outcome of the more central role these receptors play in adaptive immunity. However, it is clear that this constraint does not extend to the TCR primary sequence, which exhibits extraordinary divergence relative to the Igs. It is likely that additional interpretations will emerge as TCR gene structures from an even wider variety of vertebrate taxa are elucidated and more extensive genomic studies are reported.

T CELL MEMORY IN LOWER VERTEBRATES

The recent description of TCR α , β , γ , and δ genes in diverse vertebrate groups raises questions about conservation of T cell function throughout vertebrate phylogeny. T cell function typically has not been examined directly in

nonmammalian species, but accelerated alloimmune reactions to secondary skin grafts have been used as a measure of T cell memory in many lower vertebrate species. Although alloantigens are probably poor indicators of the T cell response (140), these studies generally showed mildly accelerated responses to second-set grafts (141); however, the specificity of these results is unclear because of a lack of a third-party, unrelated donor control group. Given these caveats, two major types of allograft rejection patterns are observed. Mammals, avians, anuran amphibians, and teleost fish exhibit acute primary allograft rejection followed by accelerated secondary rejection, whereas urodele amphibians, reptiles, cartilaginous fish, and lamprey respond more slowly to primary allograft and show a gradual stepwise increase in reactivity to repeated allografts (141–143). The basis for the differences in allograft rejection patterns in these animal groups remains unknown, but it is clear that there is no simple phylogenetic pattern that implies stepwise acquisition of this cell-mediated function.

Strong evidence for cell-mediated immune memory in teleost fish is suggested by a study that used recombinant protein fragments from viral haemorrhagic septicemia rhabdovirus (VHSV) to stimulate trout leukocytes from survivors of VHSV infection or leukocytes from uninfected trout (144). Cell-mediated immunity was detected by lymphocyte proliferation assays to detect increased sensitivity to activation. The persistence of sensitized, antigen-specific cells that proliferated in response to subsequent exposure to the primary stimulus is indicative of memory. Furthermore, the recombinant proteins were able to provide partial protection to trout after secondary challenge with the virus. The findings that chondrichthyan TCR and MHC genes are as diverse as their mammalian homologs essentially negates early speculation that restricted diversity in these molecules could relate to differences seen in allograft rejection in cartilaginous fish (4, 66, 121). The recent definition of molecular markers for different lymphocyte populations (22, 30, 42, 121, 145, 146), along with progress in elasmobranch culture systems (147) (C Luer, C Walsh, personal communication), and a better understanding of the molecular mechanisms of mammalian memory should facilitate efforts to measure similar parameters in the cartilaginous fish.

JAWLESS VERTEBRATE IMMUNITY

The jawless vertebrates (Agnatha) are comprised of two extant groups (hagfishes and lampreys), which are the surviving remnants of a major evolutionary radiation and represent the most divergent extant vertebrate groups relative to the species that have been described in this review (Figure 1a). This divergence is reflected in significant anatomic, biochemical, and physiological differences relative to jawed vertebrates. The current consensus view places

each of these two agnathan groups in lineages derived from separate common ancestors (polyphyly) with the jawed vertebrates (148), although evidence also exists for a single common ancestor (monophyly) (149). Although the mode of divergence of the hagfish and lamprey from common ancestor(s) with the higher vertebrates has important implications as to how to interpret immunity in these species, there is no doubt that study of them will offer a unique phylogenetic view into the immune system of the jawed vertebrates.

The agnathans lie across a phylogenetic boundary with regard to our current knowledge of vertebrate antigen binding receptors. Agnathans lack both a spleen and a thymus, which are present in all jawed vertebrate groups. Furthermore, the inducible immune response in lamprey is associated with a highly specific molecule(s) of a lectin-like character (24, 26). Although no fully systematic descriptions have been reported, a variety of approaches for identifying homologs of Ig, TCR, MHC I and II, and RAG genes have been unsuccessful with hagfish (*Eptatretus stoutii*) and lamprey (*Petromyzon marinus*), underscoring the major phylogenetic break in adaptive immune function between the jawed and jawless vertebrates (Figure 2). This failure could relate to the absence of such a receptor or to either qualitative or quantitative complications, e.g. low levels of expression, transient expression at different stages of development or expression in "atypical" tissues. Alternatively, antigen binding receptor gene homologs in these species may be present but have diverged appreciably. The successes in recovering Ig, MHC, and TCR in jawed vertebrates using PCR-based techniques has to some degree depended on the statistical advantages afforded by multiplicity of target motifs as well as inferred knowledge of their anatomic sites of expression. If the primary evolutionary event(s), presumably a transposition (5) (see below), which led to the rearrangement of segmental elements, was followed (under radically new selective pressure) by an expansion of gene number, then nonrearranging homologs in agnathans are possibly present in single- or low-copy number. Recent observations of extreme interspecies sequence divergence among orthologous novel immune-type receptor genes underscores how limited variation can impede the identification of homologous structures, even among different lineages of teleost fish (J Yoder, unpublished observation). A universal genetic strategy directed at the identification of the homologs of Ig and TCR in jawless vertebrates may not be feasible. Although it is plausible that homologs of relatively unconserved cell surface proteins may be difficult to identify in lamprey and hagfish, it should be possible to isolate highly conserved genes such as RAG (see below) from these species if homologs are present, i.e. failure to identify them is not likely to represent a technical artifact. It should be noted that the proportional difference in divergence time between the chondrichthyans and other jawed vertebrates (~500 MYA) is not greatly different from that of the Agnathans and jawed vertebrates (<600 MYA),

which suggests that more than mere clock-like divergence is responsible for the failure to isolate homologs in these species. Although presently based on entirely negative data, the findings from agnathan representatives are entirely consistent with the emergence of the jawed vertebrate adaptive immune system by a transposition event that took place after their divergence.

An important consideration of this real or perceived difference as regards antigen binding receptors in agnathans relates to the developmental biology of these species. Specifically, lampreys undergo metamorphosis from ammocoete larvae to the adult form. Characterization of gut-associated tissue (typhlosole) and the opisthonephros in the larval form of lampreys indicates the presence of lymphocytic/plasmocytic lineages. The typhlosole involutes prior to the first internal signs of metamorphosis and the opisthonephros involutes during metamorphosis. Metamorphosis is associated with a gain in lymphohemopoietic character of the protovertebral arch (supraneural body) (150; A Miracle, unpublished observation). It is possible that the adult (parasitic phase) of the lamprey life cycle is associated with a markedly different immune system than that seen in the larval form.

Even if jawless vertebrates lack readily recognizable homologs of MHC I, MHC II, TCR, and Ig, viewing the emergence of these molecules in the jawed vertebrates as a sudden event relative to the origins of jawed vertebrates needs to be reconsidered, given the significant time period separating these vertebrate radiations, a period of time that clearly was of sufficient length to allow for drastic morphological change. Finally, it remains possible that species that diversified prior to the jawless vertebrates, e.g. protochordates, could retain homologs of antigen binding receptor genes that are more related to their higher vertebrate counterparts.

GENES AFFECTING DIFFERENTIATION OF LYMPHOCYTES AND THE GENERATION OF IMMUNOLOGICAL DIVERSITY

The commitment of specific hematopoietic lineages to the expression of specific antigen binding receptor genes is significant both in terms of defining the anatomical sites of synthesis of immune receptors and in the potential for identifying related lineages in species that diverged prior to the jawed vertebrates. T, B, and NK cell lineage differentiation is dependent on the expression of the genes related to the *Ikaros* transcription factor. The *Ikaros* gene undergoes differential processing to produce a variety of different zinc-finger DNA binding transcription factors (151, 152). *Ikaros* is expressed in chickens from day 2 onward, prior to the transfer of the first precursor cells to the lymphoid primordia, thymus and bursa (153), as well as in *Xenopus* (154). In trout, *Ikaros*,

a single copy gene, is expressed in six different spliced isoforms also found in mammals and in two novel forms (154). The expression of *Ikaros* in trout and *Xenopus* is primarily lymphoid and occurs early in development (154). Unique differentially processed forms of *Ikaros* as well as related family members [*Helios*, *Aiolos* (152)] have been identified in skate (R Haire, A Miracle, unpublished observation). Regardless of the function of these genes, they are potentially valuable markers of immune cells in divergent species.

Identification and characterization of various other transcription factors, which in general are more conserved than cell surface markers, in different vertebrate species could prove informative in understanding similarities and differences in B cell development and function. Specifically, studies in higher vertebrates have identified transcription factors that are critical to both the initial formation of germinal centers and the differentiation pathways toward either the plasma cell or the memory cell phenotype, e.g. Blimp-1 and BSAP (Pax-5) promote plasma cell or germinal center formation, respectively. These factors also influence germinal center B cells, promoting either plasma cell or memory cell formation following affinity maturation (155).

The evolutionary stability of RSSs (5, 32, 156, 157) and the requisite involvement of RAG1 and 2 in Ig and TCR gene rearrangement represent other potential approaches for examining the immune systems of species found below the phylogenetic level of the jawed vertebrates. Specifically, RAG1 has been identified in mammals, avians, amphibians, ray-finned fish, and cartilaginous fish (5). Shared regions of RAG1 in sandbar shark and zebrafish (*Danio rerio*) as well as in other higher vertebrates reveal high degrees of amino acid identity (158, 159). RAG2 is somewhat less conserved phylogenetically than is RAG1 (160), but RAG1 and RAG2 are found in the same relative close proximity in lower vertebrates as in mammals (161). In mammals, avians, and amphibians, RAG1 and 2 are encoded by single exons, in opposite transcriptional orientation. However, in zebrafish the coding region of RAG1 is interrupted by two introns (162), and in trout, the coding region is interrupted by a single intron (163). These intronic relationships need to be considered in the design of strategies to identify RAG homologs in other species. The conserved close physical linkage of RAG1 and 2 in trout, zebrafish, and *Xenopus* (160–162) and higher vertebrates suggests that the identification of a homolog of one of these genes would lead to the isolation of the second. The observations of RAG2 gene expression in oocytes in *Xenopus* (160) and early in development in trout (161) are of strategic significance in terms of potential PCR template sources in other species. Although identification of RAG in a jawless vertebrate species would be of great significance, there is a distinct possibility that its presence in jawed vertebrates is the result of a horizontal gene transfer event and thus a homolog would not necessarily be present in jawless vertebrates or protochordates.

TdT functions in generating junctional diversity, which has been documented in species as phylogenetically distant from mammals as the cartilaginous fish (18, 121). TdT expression has been characterized in species as phylogenetically distant from man as amphibians (164) and ray-finned fish (165). Bruton's tyrosine kinase (BTK), a nonreceptor protein tyrosine kinase, is essential for normal B lymphocyte development but is not expressed in T cells; a *Btk* homolog has been identified in skate (145). CD45, a TM tyrosine phosphatase that is required for antigen-induced signal transduction in mammalian lymphocytes, is expressed in horned shark (146). Investigations into the concordance of expression of antigen receptors, lymphocyte-specific enzymes such as RAG and TdT, transcription factors, and other genes implicated in lymphoid development have the potential for providing additional insight into the sites of hematopoiesis and the molecular events governing lymphocyte development and activation in diverse vertebrate groups. However, it also must be recognized that the transcription factors are often utilized in a variety of unrelated systems.

ORIGINS OF ANTIGEN BINDING RECEPTORS

The phylogenetic approach that has been at the core of this review has provided a wealth of information regarding the evolution of rearranging gene families; however, it offers little in the way of defining the early evolutionary origins of antigen binding receptors, other than to constrain the time of origin and diversification of all six rearranging gene classes (TCR α , β , γ , δ , and Ig light-chain and Ig heavy-chain genes) to a period prior to the divergence of the cartilaginous and ray-finned fishes, ~460–550 MYA. Inferring characteristics of primitive receptor forms through phylogenetic analysis of existing rearranging genes is complicated by the highly derived nature of the antigen binding receptors. Nevertheless, some relationships are evident, e.g. TCR β is more related to TCR γ , and TCR α is more related to TCR δ at the sequence level than either is between these two groups. The D regions appear to have evolved or were lost more than once during the evolution of TCRs. Additional sources of information may prove useful in cases where the limited data from primary sequence is inadequate, e.g. the syntenic organization of genes encoded in the chromosomal vicinity of the rearranging receptors could be useful in relating them to one another. Also, as more information regarding the coreceptors and transcriptional regulation of antigen binding receptor genes becomes available, a more complete picture of the early evolutionary history of these proteins may emerge from affinities in regulation and function.

Nonrearranging genes that descended from the prerearranging ancestral antigen binding receptor may still exist in invertebrates and protochordates as well

as in jawed vertebrates. A number of potential nonrearranging ancestral-like candidate genes are known that contain TCR/Ig-like V regions, including CD8 β and VpreB. Notably, both of these genes are encoded very near rearranging genes in mice and have functions that are consistent with an ancient association with these antigen recognition systems. A particularly interesting set of non-rearranging genes termed novel immune-type receptors (NITR) was isolated initially from the Southern pufferfish using a V region degenerate PCR strategy that was employed to isolate TCR genes (122). The prototypic NITR gene (Sn193) consists of a V domain and a C2 domain, as well as TM and cytoplasmic (Cyt) regions. The V region domain exhibits remarkable similarity to TCR and Ig V regions beyond that which is displayed by other nonrearranging V region-containing Ig domain proteins; a J-like region is located 3' of V. P1 artificial chromosomes were isolated using Sn193 as well as several related genes as probes, and a large number of related genes were identified in tandem linkage over a >100-kb region. The NITR genes exhibit extensive TCR-like V region diversification, and the majority characterized thus far contain ITIM motifs in their cytoplasmic regions (S Strong, G Mueller, R Litman, N Hawke, R Haire, A Miracle, J Rast, C Amemiya, G Litman, unpublished observation). NITR genes may represent a link between conventional V-type recognition, innate function, and signaling pathways known to function in recognition molecules such as certain NK receptors that are members of a newly defined superfamily of ITIM-containing genes, the inhibitory receptor superfamily (166).

A number of additional V region domain-containing cell surface molecules, including CD8 β , OX-2, CTX (167), and ChT1 (168, 169), have varying degrees of Ig/TCR-like character. The two *Xenopus* CTX genes contain a V and C2 Ig domain, both of which are split by introns. A J-like region is present, including a glycine bulge motif C terminal to the V region (167, 170). One of the *Xenopus* genes is encoded within the MHC region. The chicken ChT1 protein has a domain and intron/exon structure similar to that of CTX but is not linked to the chicken MHC (168; K Katevuo, personal communication). Both these genes are expressed in thymic T cells and possibly derive from an early TCR ancestral molecule. Recently, mammalian homologs of CTX and ChT1 were identified; however, these genes are not expressed in thymus (169). It is of great interest to researchers to determine if CTX/ChT1 genes can be detected in ray-finned and/or cartilaginous fish. All vertebrate rearranging receptors, along with class I and II MHC proteins, contain Ig C1-type domains. If the MHC genes are primitively nonrearranging, then the initial hypothesized transposition event that led to rearranging receptors is likely to have interrupted a V-C1-TM-Cyt-type molecule. The V-C2-TM-Cyt general structure, found in a variety of vertebrate genes along with the pufferfish NITR, may reflect the antigen receptor predecessor of IgC1-containing, nonrearranging receptor.

Further clarification of the origins and interrelationships of these putative receptors will come through phylogenetic comparisons between rearranging and nonrearranging antigen binding receptors found in jawed vertebrates. Such analyses will also be significant in terms of designing strategies for identification of related systems in agnathans, lower chordates, hemichordates, and echinoderms. The recent discovery that RAG1 and RAG2 proteins together constitute a transposase, capable of excising a piece of DNA containing recombination signals from a donor site and inserting into a target DNA molecule, has enormous bearing on mechanisms whereby nonrearranging receptors diverged into the rearranging antigen binding receptor genes (171).

CONCLUSIONS

The origins of Ig and TCR, as inferred from studies of contemporary species, can be traced to a distant period in the phylogenetic development of the jawed vertebrates. Available information suggests that the character of TCR genes has been conserved throughout vertebrates, whereas Ig genes have undergone a series of changes, of which some segregate to major phylogenetic radiations and others are specific to less inclusive taxonomic groups. A series of genetic divergences can be recognized throughout the jawed vertebrates that reflect a process of continued evolutionary variation of potential mechanisms of diversification. One such step separates species in which heavy- and light-chain genes are encoded at a single locus from those in which large numbers of individual loci are present. In the latter case, which is found in all cartilaginous fish (e.g. sharks), certain gene clusters are joined in the germline, whereas others hypermutate extensively. It is likely that the regulation of expression of single and multicluster Ig gene loci differs markedly. Other variations in Ig structure and regulation are seen throughout the radiations of ray- and fleshy-finned fish as well as the tetrapods, including the following: the use of alternative processing mechanisms, partial precommitment of rearranging segment elements, nonassociation of light chains with heavy chains, and the use of both gene conversion and antigen-independent somatic hyperconversion as a primary basis for varying receptor structure. Some of the exceptional phenomena typically are associated with specialized lymphocyte compartments and corresponding unique microenvironments. The basis for the remarkable plasticity in form and function of both Ig and TCR is not understood. Studies to date in jawless vertebrates have failed to provide evidence for antigen binding receptors resembling those found in jawed vertebrates, nor have homologous structures been identified in more phylogenetically distant forms. It remains open to question whether other heretofore unrecognized genetic systems related to Ig and TCR also function in antigen binding. Several nonrearranging genes that have been characterized

in jawed vertebrates exhibit varying levels of sequence identity with the rearranging antigen binding receptors and could represent either modern forms of progenitor genes or derived products of Ig and TCR loci. Such gene products, which would function in an innate capacity, could offer new information as to how the highly complex diversified process of antigen recognition has evolved.

ACKNOWLEDGMENTS

We would like to thank Chris Amemiya, Louis Du Pasquier, Martin Flajnik, Robert Haire, Noel Hawke, Kasia Katevuo, Carl Luer, Ann Miracle, Tatsuya Ota, David Schatz, Scott Strong, Gregory Warr, and Jeffrey Yoder for sharing their unpublished findings and their valuable comments. We would also like to thank Michael Sexton for his assistance with the figures and Barbara Pryor for editorial assistance. This work was supported by Grant R37 AI23338 to GWL from the National Institutes of Health. MKA is supported by the Stowers Institute for Medical Research. JPR is supported by an NRSA Grant GM 18478.

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