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EVOLUTIONARY CONSERVATION AND MOLECULAR CLONING OF THE RECOMBINASE ACTIVATING GENE 1++

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A 700-bp fragment of the recombinase activating gene 1 (RAG-1) was cloned from several evolutionarily distant (sandbar shark, paddlefish, goldfish, axolotl and pig) species using PCR. The nucleotide and deduced amino acid sequences revealed a highly conserved region that has remained essentially unaltered during 400 million years of evolution; e.g, shark and human sequences were 75% identical at the nucleic acid level and 87% as protein. The RAG-1 mRNA levels in the shark were analyzed using semi-quantitative PCR to reveal expression patterns contrary to normal mammalian expression. These results establish that the genetic mechisms for Ig gene rearrangement are present in all extant gnathanstomes.

The characterization of immune recognition molecules from cartilaginous fishes, such as sharks and from other phylogenetically ancient species indicates that most elements of the immune system arose very early in vertebrate evolution [1]. Antibodies occur in all jawed vertebrate species and genes specifying T-cell receptors (TCRs) have been identified in amphibians [2], and birds [3]. Antibody and TCR genes are homologous structures composed of different genetic elements that are spatially separated in the genome, sometimes by extremely large distances. In order for a functional gene to be formed, an ordered process of gene rearrangement and recombination must occur. This process is not well understood, but the products of two genes termed recombination activating proteins RAG-1 and RAG-2 have been shown to be essential [4]. Inactivation of either the RAG-1 or RAG-2 genes in mice completely suppresses recombination

++Sequence data from this article have been deposited with the GenBank Data Libraries under Accession Nos. U15610, U15611, U15612, U15613, and U15614.

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activity and the formation of functional antibody and TCR genes and consequently, prevents the development of cellular and humoral immunity. Recent characterization of RAG genes from mammals [5], the chicken [6], and an anurian amphibian [7] suggest that these molecules have been highly conserved in evolution. The genetic elements of shark immunoglobulin genes are very similar to their mammalian counterparts, though their arrangement in the genome is quite different [8]. However, with some notable exceptions [9], rearrangement of these genetic elements in the genome is still needed to form functional genes. Here, we utilize PCR primers based upon homologous regions of characterized RAG-1 genes to identify and partially characterize the RAG-1 gene in sharks and several other phylogenetically ancient vertebrates including a chondrostrian fish (the paddle fish, *Polydon spatula*), a teleost fish (the goldfish, *Crassius auretus*), and a urodel amphibian, (the axolotl *Ambystoma mexicana)*. Our results confirm that the RAG-1 gene has been highly conserved in evolution. We also show that the RAG-1 gene is expressed in the thymus as well as the spleen of sharks.

MATERIALS AND METHODS

Cloning of the RAG-1 gene.

Shark and goldfish genomic DNA was isolated from the respective spleens after Maniatis *et al.*, [10]. Axolotl and paddlefish genomic DNA was isolated from the respective PBLs also after Maniatis *et al.*, [10]. A pig cDNA library was the kind gift of Dr. J. Butler(University of Iowa, Department of Microbiology). Oligonucleotide primers for PCR cloning from shark, paddlefish, goldfish and axolotl were based on Steiner *et al.*, [7]. Primer sequence: sense, CA(C/T) Tg (C/T) AT(A/C/T) gg(g/A/T/C) AA(C/T)gC, antisense, TT(A/g) Tg(g/A/T/C) gC(A/g) TT CAT(A/g) AA(C/T) TT(C/T) Tg. Primers used for cloning from the pig were designed on the consensus alignment of the shark, paddlefish, goldfish, and the published sequences of the mouse, human, chicken, and clawed toad (Xenopus). Primer sequence: sense, TA(T/C) gA(A/g) gg(g/A) AT(A/T/C) AC(g/A/T/C) AA(C/T)TA, antisense, gT(A/g) TA(g/A/T/C/) A(A/g) CCA(g/A) Tg(g/A) Tg(T/C)TT. All primers were synthesized at the University of Arizona Biotechnology Facility. An aliquot of the PCR mixture was electrophoresized on a 3% nuseive gel (FMC Bioproducts, Rockland ME) and the band corresponding to the expected size was removed and purified using Quiagen glass beads (Quiagen Inc., Chatsworth, CA). The fragment was then ligated into T-vector (Promega, Madison, WI) and subjected to DNA sequence analysis using the Sequenase protocol (United States Biochemical, Cleveland OH). Sequences were analyzed with the BLASTX, BLASTN, PILEUP, GAP and BESTFIT programs (NCBI, gcg: Genetics Computer Group Inc. Madison WI).

Semi-quantitative PCR analysis.

Primers for the semi-quantitative PCR to monitor expression levels in the shark spleen and thymus were designed with the Oligo program (National Bioscience

Inc. Plymouth, MN) from the shark sequence obtained above. Primer sequence: sense, AgA CTg Tgg AAg CCT gTg AAC TgA, antisense, TTg CCC gAT TCA TTg CCT TCA CTT. Total shark RNA was isolated from shark spleen or thymus using Trizol (Gibco-BRL, Gaithersburg, MD). cDNA was then synthesized using a first strand synthesis kit (Invitrogen, San Diego, CA). The semi-quantitative PCR was cycled for 30 cycles at 60 degree annealing temperatures for specificity. An aliquot of the PCR mixture was electrophorsized as above.

RESULTS

Cloning of RAG-1 genes

Alignment and comparison of protein sequences of RAG-1 from human, mouse, chicken, and xenopus show that there are extensive regions of complete sequence identity. Fully degenerate primers based on conserved peptide segments [7], were used to perform homology probing by PCR. Except for the pig for which a cDNA library was used, genomic DNA was used to amplify RAG-1 products. PCR fragments of the expected size were cloned and sequenced. Similarity with known sequences allowed the unequivocal identification of RAG-1 gene products. The segment cloned corresponds to the carboxyl terminal end of the protein from residues 808 to 1005 by comparison to the 1045 residue long Xenopus sequence. Alignment of the deduced amino acid sequence of the amplified fragments and known RAG-1 genes is shown in Figure 1. Identities with the shark sequence are highlighted since the shark represents the most phylogenetically ancient species. Homologies range from 75% identity with goldfish, 82% identity with xenopus and 87% identity with the human sequence. If substitutions of similar amino acids are taken into account, the score goes as high as 91% with human. The last 60 residue segment is 95% identical for all sequences.

Pattern of expression of RAG-1 in sharks

Using specific primers derived from the amplified product, the expression of the RAG-1 gene in shark thymus and spleen was assessed by PCR. Owing to the difficulty of obtaining tissue samples, the thymus and spleen tissue were obtained from two very closely related carcharhine species, the bull shark (*Carcharhinus leucas*) and a sandbar shark respectively. Both tissue samples were obtained from young adult sharks. As shown in Figure 2, there is a high level of RAG-1 expression in the thymus. RAG-1 is also expressed in the spleen, albeit at lower levels.

DISCUSSION

Our results extend and confirm the observation that RAG-1 has been highly conserved during evolution. For example, the fragment isolated from the shark is 87% identical with human RAG-1. Recombination of immunoglobulin gene segments to

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Shark      TEFYRIFQDEIGEVYKNSNSSKEERKRWQSMLDKHLRKKMNLKPIMRMNGNFARKLMTKETVEAVCELIP
Paddlefish QEFYKIFQDEIGEVYQNYNPTHEERKRWQSALDKQLRKKMNLRPVMRMNGNFAVKLMTKETVEAVCELVP
Axolotl          QLEIGEVFKNPDASKEERKRWQSTLDKHLRKKMNLKPIMRMDGNFARKLIGQETVEAVCELVH
Goldfish         FQDEIGEVYQKSNPSREERRRWASTLDKHLRKNLKLKPVMRMNGNYARRLMTREAVEVVCELVL
Rabbit     AEFYKIFQLEIGEVYKNPSASKEERKRWQATLDKHLRKKMNLKPIMRMNGNFARKLMTIETVEAVCELIP
Human      AEFYKIFQLEIGEVYKNPNASKEERKRWQATLDKHLRKKMNLKPIMRMNGNFARKLMTKETVDAVCELIP
Mouse      AEFYKIFQLEIGEVYKHPNASKEERKRWQATLDKHLRKRMNLKPIMRMNGNFARKLMTQETVDAVCELIP
Chicken    TEFYRIFQMEIGEVYKNPDATKEERKRWQLTLDKHLRKKMKLKPMMRMSGNFARKLMSKETVEAVCELIK
Xenopus    AEFYRIFQLEIGELYKNLSATKEEKKRWQATLDNHIRKRMNLKPIMRMNGNFARKLMSKETVEAVCELVP

Shark      SEERREILRELMHLYLLMKPVWRSTFPTTECPDLLCQYSFNSQRFAELLHTEFSHRYEGKITNYLHKTLA
Paddlefish SQERREALRELVHLYLQMKPVWRVNCPAKECPDLLCRYSFNSQRFAELLSTTFKYRYDGKITNYLHKTFA
Axolotl    SEERRTALRELMDLYLKMKPVWRSSCPAKECPELLCQYSFNSQRFAELLSTKFKYRYEGKITNYFHKTLA
Goldfish   SEATREALRELMDLYLQMIACVASTRPSQDCPVQLCQYSYNSQQFADLLSTTFKYRYDGKITNYLHKTLA
Pig                                                                  YFHKTLA
Rabbit     SKERHEALRELMELYLKMKPVWRSSCPAKECPESLCQYSFNSQRFAELLSTKFKYRYEGKITNYFHKTLA
Human      SEERHEALRELMDLYLKMKPVWRSSCPAKECPESLCQYSFNSQRFAELLSTKFKYRYEGKITNYFHKTLA
Mouse      SEERHEALRELMDLYLKMKPVWRSSCPAKECPESLCQYSFNSQRFAELLSTKFKYRYEGKITNYFHKTLA
Chicken    CEERHEALKELMDLYLKMKPVWRSSCPAKECPELLCQYSYNSQRFAELLSTKFKYRYEGKITNYFHKTLA
Xenopus    CEERQAALTELMDLYLKMKPVWRSSCPAKECPELLCQYSFHSQRFAELLSTKFKYRYEGKITNYFHKTLA

Shark      HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKSYELEDILKHHWLYTSKYL
Paddlefish HVPEIIEREGSIGAWASEGNESGNKLFRRFRKMNARQSKCYELEDILKHHWLYTSKYL
Axolotl    HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKFYEMEDVLKHHWLYTSKHL
Goldfish   HVPEIVERDGSIGAWASEGNESGNKLFRRFRKMNARQSKTFELEDVLKHHWLYTSKYL
Pig        HVPEITERDGSIGAWASEGNESGNKLFRRFRKMNARQSKFYEMEDVL
Rabbit     HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKCYEMEDVLKHHWLYTSKYL
Human      HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKCYEMEDVLKHHWLYTSKYL
Mouse      HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKCYEMEDVLKHHWLYTSKYL
Chicken    HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKFYEMEDVLKHHWLYTSKYL
Xenopus    HVPEIIERDGSIGAWASEGNESGNKLFRRFRKMNARQSKFYEMEDVLKHHWLYTSKYL
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Figure 1. Alignments of deduced amino acid sequence of all known RAG-1 gene products corresponding to residues 808-1005 of the clawed toad (Xenopus) sequences. The sequence for the shark, paddlefish, axolotl, goldfish and pig are from this paper. Sequence from the rabbit, human, mouse, chicken and clawed toad (Xenopus) were taken from GENBANK. Shared residues correspond to identities with the shark, the most phylogenetically ancient species depicted.

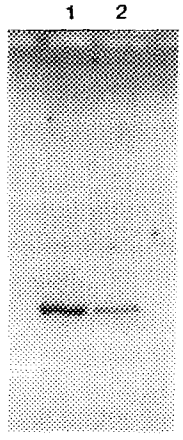

Figure 2. Semi-quantitative for results showing the levels of RAG-1 mRNA in a shark thymus (Lane 1) and spleen (Lane 2). 800 ng of total RNA of each tissue was used for the first strand cDNA synthesis. Reaction: 1 μl of cDNA was used from each reaction for the PCR transplantation. Controls in which no template was added produced no product. (Data not shown).

form a functional gene is a critical mechanism for the generation of diversity and for the regulation of gene expression and lymphocyte development in higher vertebrates [4]. The RAG-1 gene product is an indispensable element in this process [11]. The finding of RAG-1 in the shark indicates that the components necessary to mediate recombination arose concurrently with the development of the primordial immune system.

Although the exact function of the RAG-1 product has yet to be elucidated, speculation derived from various gene homologies abounds. A cysteine rich N-terminal segment of the molecule has drawn comparisons to the zinc-finger family of proteins suggesting a DNA transcription mode of action [12]. However, recent experiments deleting this N-terminal portion of the protein have shown that it is not essential for recombination *in vitro* [11]. Subsequently, the N-terminal region upstream of the zinc-finger motif has been shown to bind to the human homolog of yeast SRP1-1. Since SRP1-1 binds to the nuclear membrane [13], this region may serve as a localizer of the RAG-1 gene product. The carboxyl-terminal half of the molecule shows similarity to the yeast HPR1 product, which in turn, has homology to yeast topoisomerase [14]. This suggests that RAG-1 is a direct component of the recombination machinery since, like recombinases, topoisomerases catalyze transesterification reactions [11]. Inclusion of the sequences reported here in comparison significantly increases the similarity to yeast HPR1 (Data not shown). The extraordinary conservation of the C-terminal region suggests that this is a crucial portion of the RAG-1 protein for its recombination activity.

Additionally, since, thymus is traditionally a site for T-cell development, and differentiation, the high levels of RAG-1 gene expression are consistent for this function in the thymus of the shark. Although the spleen can be a site for lymphocyte development during fetal ontogeny, this is very rarely the case in adults of other species. The significant level of RAG-1 gene expression in a spleen of a young adult suggests that the spleen may continue in sharks to be a site for lymphocyte development contrary to accepted vertebrate models.

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