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Genomic characterization and expression analysis of the first nonmammalian renin genes from zebrafish and pufferfish

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Liang, Ping, Craig A. Jones, Brent W. Bisgrove, Lei Song, Sean T. Glenn, H. Joseph Yost, and Kenneth W. Gross. Genomic characterization and expression analysis of the first nonmammalian renin genes from zebrafish and pufferfish. *Physiol Genomics* 16: 314–322, 2004. First published November 25, 2003; 10.1152/physiolgenomics.00012.2003.—Renin is a key enzyme in the renin-angiotensin system (RAS), a pathway which plays an important physiological role in blood pressure and electrolyte homeostasis. The origin of the RAS is believed to have accompanied early evolution of vertebrates. However, renin genes have so far only been unequivocally identified in mammals. Whether or not a bona fide renin gene exists in nonmammalian vertebrates has been an intriguing question of physiological and evolutionary interest. Using a genomic analytical approach, we identified renin genes in two nonmammalian vertebrates, zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*). Phylogenetic analysis demonstrates that the predicted fish renins cluster together with mammalian renins to form a distinct subclass of vertebrate aspartyl proteases. RT-PCR results confirm generation of the predicted zebrafish mRNA and its expression in association with the opisthonephric kidney of adult zebrafish. Comparative in situ hybridization analysis of wild-type and developmental mutants indicates that renin expression is first detected bilaterally in cells of the interrenal primordia at 24 h postfertilization, which subsequently migrate to lie adjacent to, but distinct from, the glomerulus of the developing pronephric kidney. Our report provides the first molecular evidence for the existence of renin genes in lower vertebrates. The observation that the earliest renin-expressing cells, arising during ontogeny of this teleost vertebrate, are of adrenocortical lineage raises an interesting hypothesis as regards the origin of renin-expressing cells in the metanephric kidney of higher vertebrates.

renin-angiotensin system; pronephros; adrenocortical; interrenal gland

THE RENIN-ANGIOTENSIN SYSTEM (RAS), has long been recognized to play a critical role in blood pressure homeostasis and electrolyte balance (44). More recently, it has become evident that a functional RAS is required for normal mammalian renal development (19, 41, 54). Most studies have focused on the metanephros, the functional definitive kidney of adult mammals, where renin expression is observed throughout the branching vascular tree during renal organogenesis, ultimately resolving to the classic juxtaglomerular cell upon maturation (20, 29). Various lines of evidence, however, suggest that the RAS is a phylogenetically ancient pathway, existing in all vertebrates and associated with all three nephric structures, pronephros, mesonephros, and metanephros, that are observed to form from intermediate mesoderm ontogenetically and evolutionarily (23, 38, 51). Nevertheless, much of the supporting documentation for RAS components in the kidneys of more primitive vertebrates is indirect, consisting of morphological and physiological evidence (36, 38). As of this writing, no renin gene has been identified in any nonmammalian vertebrate, raising the question of whether true orthologs of mammalian renin genes exist in nonmammalian vertebrates or whether a closely related aspartyl protease performs renin's function in these species. Here we report the first identification, via an in silico approach, of renin gene sequences in two fish genomes and the confirmation of renin gene expression in association with zebrafish pronephric and opisthonephric kidneys. We also provide evidence that the earliest expression of renin is found in adrenocortical derivatives, which as components of the interrenal gland are intimately associated with the developing pronephric kidney.

MATERIAL AND METHODS

Identification of fish renin gene sequences by genomic sequence analysis. In our search for renin gene sequences from nonmammalian vertebrates, we adapted in silico strategies making the use of available genomic sequences for zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*). For zebrafish, trace sequences with open reading frames (ORFs) sharing higher similarity to renin relative to other aspartyl proteases were collected by downloading the trace data from the ongoing genome sequencing project (**http://wwww.sanger.ac.uk/Projects/D_rerio/**) and querying with mammalian renin protein sequences using a locally installed BLAST package (2). More traces overlapping with the above traces were collected and assembled to build small contigs, which were then extended to a maximal length by repeating this procedure and were connected to each other to form a scaffold. In parallel, the genomic sequence for a potential renin gene in fugu fish was identified by searching the genome sequences provided by the International Fugu Genome Consortium (4). Complete gene structure predictions for both renin sequences were made by first identifying the approximate location and the correct reading frame of each exon using TBLASTN. Then the exact intron/exon boundaries were determined based on GenScan results (9) and fine-tuned by manual adjustment. Several exons missed by GenScan were added manually based on the protein-DNA alignment.

Phylogenetic analysis. All aspartyl protease protein sequences that share sequence similarity with renin for a selected list of species, including human, mouse, rat, sheep, chicken, South African clawed toad, zebrafish, pufferfish, fruit fly, and *Caenorhabditis elegans*, were

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identified and retrieved from GenBank. A multiple sequence alignment was performed using a locally installed Clustal X (27) with the output saved in PHYLIP format. A distance matrix for all protein sequences was generated using Poisson correction, and a phylogenetic tree was inferred by the NJ method. The latter two steps, as well as tree displaying, were done with TreeCon for Windows (50). To validate the tree, the same sequence set was analyzed with tools in the PHYLIP package (17), using PROTDIST followed by FITCH or NEIGHBOR programs.

Identification and sequencing of zebrafish renin genomic DNA. Arrayed filter sets for the RPCI-71 zebrafish genomic bacterial artificial chromosome (BAC) library were obtained from BACPAC Resources (Oakland, CA). Overgo probes (see Supplemental Material for sequences, available at the *Physiological Genomics* web site)[1] for library screening were designed based on the assembled sequences and used to screen the BAC library using a standard procedure (45). Positive BAC clones were purchased from BACPAC Resources for PCR and directed sequencing. To obtain the complete genomic sequence, primers were designed in various regions of the assembled scaffold to fill small gaps. Additionally, some chromosomal walking was done to close bigger gaps, as well as to validate the assembled sequences. The 24 kb of zebrafish renin genomic sequence was covered with at least one pass of directed sequencing for the areas that have high-quality assembled sequence, whereas regions with conflicting sequence between the assembled traces and our in-house sequences were sequenced more than once to resolve the conflicts.

Preparation of zebrafish RNA, RT-PCR, real-time PCR, and identification of 5′-untranslated regions (5′-UTRs). To prepare RNA, 1.5- to 2.5-cm adult zebrafish were euthanized by immersion in 0.25 μg/ml tricaine. Kidney tissue was identified as saddle-like in shape and surrounded by connective tissues containing numerous melanophores, giving it a spotted appearance (16, 25). The indicated tissues were diced, snap frozen, and stored at −70°C until RNA extraction with TRIzol (Invitrogen).

Total RNA was treated with RNase-free DNase I (Promega, 0.125 U/μg RNA) before being converted to cDNA with TaqMan reverse transcription reagents (ABI kit no. N808-0234) using either poly(dT) or random primers. Overlapping renin primer sets for PCR (6) covering the entire cDNA were designed from cDNA sequences predicted from the genomic sequence. These same primers were used to forward and reverse prime PCR products for sequencing.

RNA samples from additional adult zebrafish tissues, including gill, liver, spleen, heart, bladder, brain, gonad, skeletal muscle, eye, and gut, were prepared and used to make cDNA as described above. Real-time PCR reactions were set up using 1.5 μl of cDNA, 12.5 μl 2× SYBR Green Master Mix (Applied Biosystems, ABI), and 0.1 μg of each primer in a total volume of 25 μl. The real-time PCR was run for 30 cycles on the ABI Prism 7700 Sequence Detection System (ABI) according to ABI protocol. A threshold cycle (C_t) value was determined, and the relative expression level of a specific gene is expressed as ΔC_t (the C_t value of the gene of interest minus the C_t value of β-actin). Primers for each gene were designed to generate a product around ∼100 bp in size, and their efficiency was validated by graphical representation of slope and fluorescence intensity/cycle number (see Supplemental Material for primer sequences).

Determination of transcription initiation site. To determine the transcription initiation sites, both RNase protection and RNase primer extension assays following standard protocols (18, 31) were used. Briefly, for primer extension, a 32-mer consisting of 5′-TTATGCT-TCAATGCTCTTATACCCACTTGTTC-3′ was end labeled by T4 polynucleotide kinase (New England Biolabs) with [α-^{32}P]ATP and hybridized to 50 μg of zebrafish kidney total RNA. This hybrid was extended with AMV reverse transcriptase (ABI), digested with RNase A (Sigma), and loaded on an 8% sequencing gel with Decade size markers (Ambion). Gels were dried and visualized by exposure on PhosphorImager screens (Molecular Diagnostics). For RNase protection assays, a 200-bp protecting fragment was generated by PCR with 5′-GAGGACAGCGAAAGCTAAGGGT-3′ as the forward primer and 5′-TCTCACTGATGGATCTATTCAC-3′ as the reverse primer and cloned into a transcription vector to generate antisense RNA labeled with [α-^{32}P]CTP (Amersham). This was gel purified, hybridized to 10 μg of sample, and digested with RNase A (Sigma). The protected RNA fragment was resolved on an 8% sequencing gel and visualized as described above.

Analysis of renin gene expression in zebrafish embryo by in situ hybridization. Zebrafish embryos were collected from natural spawnings, cultured for 24 h, then transferred to water containing 0.003% 1-phenyl-2-thiourea to inhibit melanin synthesis (52). For in situ hybridization (ISHs), embryos staged by developmental time and morphological criteria were fixed in 4% paraformaldehyde in sucrose buffer (52), rinsed in PBS, dehydrated into absolute methanol and stored at −20°C. Riboprobes were synthesized from linearized DNA templates using T3 or T7 polymerases and digoxigenin labeling mixes (Roche Applied Science). ISHs were carried out as previously described (7). Probes used include: renin (this report), *pax2.1* [*pax(zf-b)*, Ref. 32], *ff1b* (11), *wt1* (15), and *flk-1* (33). Embryos were cleared in 70% glycerol/PBS and photographed with a Leica MZ12 microscope. Images were captured with a Nikon Coolpix 995 digital camera and processed using Adobe Photoshop.

RESULTS

Identification and characterization of renin genes from fugu fish and zebrafish genomes. The search for a renin gene in zebrafish genomic sequence was initiated before the pufferfish draft genome data became available. Trace sequences representing exons 2, 3, 5, and 7–9 were first identified with the data generated from the early stage of zebrafish genome project, while those for exons 4 and 6 were identified later using the predicted pufferfish renin peptide sequence. Because of the very low degree of sequence conservation among exon 1 of renin genes, we were unable to identify the first exon based on known renin sequences. GenScan also fails to predict any initial exon in this region. Two possible exon 1 regions were proposed by manually examining the 4-kb genomic region upstream of exon 2, and one of them was confirmed by RT-PCR amplification and sequencing.

A putative fugu fish renin gene was identified within the scaffold 285 (emb|CAAB01000285) in the published version of the genome data (4). GenScan predicts a complete gene in the region similar to mammalian renins. Some adjustments were made to the GenScan prediction based on the peptide sequence alignment with known renins. For example, GenScan predicts a longer exon 2 and misses exon 6, which is detectable by BLAST search.

With all trace sequences available from the zebrafish genome project, we assembled a 24-kb scaffold covering all exons plus a 2-kb promoter region. Meanwhile, BAC clones containing the identified renin sequences were obtained, and one of the clones, RPCI-71-25K18, was chosen for directed sequencing using primers located at various positions on the assembled scaffold. The sequencing results confirmed the sequence assembly and completed the genomic sequence of

[1]The Supplementary Material for this article (sequences for overgo probes used in BAC filter screening; primer sequences used in SYBR Green real-time RT-PCR; accession nos. for sequences used in the phylogenetic tree; and Fig. S1, showing sequences of predicted renin genes from fugu fish and zebrafish) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00012.2003/DC1**.

A

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CATD_HUMAN    1 ----MQPSSLLPLALCLLAAPA------SALVRIPLHKFTSIRRTMSEVGGSVEDLIAKG
CATD_MOUSE    1 ----MKTPGVLLLILGLLASSS------FAIIRIPLRKFTSIRRTMTEVGGSVEDLILKG
CATD_ZFISH    1 ----MR--IRFCCSLLPFSAR-------RRDCRIPLKKFRTLRRTLSDSGRSLEELVSSS
CATD_FUGU     1 ----MK--LLILCVFAALALTN------DALVRIPLKKFRSIRRELTDSGRKIEELLADR
REN_MOUSE     1 MD-RRRMPLW-ALLLLWSPCTFSLPTRTATFERIPLKKMPSVREILEERGVDMTRLSAEW
REN_HUMAN     1 MDGWRRMPRWGLLLLLWGSCTFGLPTDTTTFKRIFLKRMPSIRESLKERGVDMARLGPEW
REN_FUGU      1 --MALQLSHWMSLAALSLALTS-----SQALRRITLHKMPSIRETLGEMGVSVEQVLSEM
REN_ZFISH     1 ----MKI-HCLTLLILSLSAIS-----TKALWRVKLKKMPSIRETLKEMSVTPAQVLSEI

CATD_HUMAN   51 PVSKYSQAVPAVTEGPIPEVLKNYMDAQYYGEIGIGTPPQCFTVVFDTGSSNLWVPSIHC
CATD_MOUSE   51 PITKYSMQSSPKTTEPVSELLKNYLDAQYYGDIGIGTPPQCFTVVFDTGSSNLWVPSIHC
CATD_ZFISH   48 NSLKYNLGFP-ASNDPTPETLKNYLDAQYYGEIGLGTPVQTFTVVFDTGSSNLWVPSVHC
CATD_FUGU    49 RINKYNYGFP-TAGAPTPETLKNYLDAQYYGEIGLGTPPQPFTVVFDTGSSNLWVPSVHC
REN_MOUSE    59 G---VFTKRPSLTNLTSPVVLTNYLNTQYYGEIGIGTPPQTFKVIFDTGSANLWVPSTKC
REN_HUMAN    61 S---QPMKRLTLGNTTSSVILTNYMDTQYYGEIGIGTPPQTFKVVFDTGSSNVWVPSSKC
REN_FUGU     54 A---EKSAG-DVFNKTVPTPLTNYLDTQYFGEISIGSPAQMFNVVFDTGSANLWVPSQSC
REN_ZFISH    51 M---PKYQEPSPTNGTAPTPLINYLDTQYFGEISIGSPAQMFNVVFDTGSANLWVPSHSC

CATD_HUMAN  111 KLLDIACWIHHKYNSDKSSTYVKNGTSFDIHYGSGSLSGYLSQDTVSVPCQSASSASALG
CATD_MOUSE  111 KILDIACWVHHKYNSDKSSTYVKNGTSFDIHYGSGSLSGYLSQDTVSVPCK--SDQSKAR
CATD_ZFISH  107 SLTDIACLLHHKYNGGKSSTYVKNGTQFAIQYGSGSLSGYLSQDTC-----------TIG
CATD_FUGU   108 SLLDIACLLHHKYNSAKSSSYVKNGTAFAIRYGSGSLSGYLSQDTC-----------TLG
REN_MOUSE   116 SRLYLACGIHSLYESSDSSSYMENGSDFTIHYGSGRVKGFLSQDSV-----------TVG
REN_HUMAN   118 SRLYTACVYHKLFDASDSSSYKHNGTELTLRYSTGTVSGFLSQDII-----------TVG
REN_FUGU    110 SPFSTACFTHNRYDASKSQTHVENGTGFSIQYASGNVRGFLSEDVV-----------VVG
REN_ZFISH   108 SPLYTACFTHNRYDASKSLTHIFNGTGFSIQYASGNVRGFLSEDVV-----------VVG

CATD_HUMAN  171 GVKVERQVFGEATKQPGITFIAAKFDGILGMAYPRISVNNVLPVFDNLMQQKLVDQNIFS
CATD_MOUSE  169 GIKVEKQIFGEATKQPGIVFVAAKFDGILGMGYPHISVNNVLPVFDNLMQQKLVDKNIFS
CATD_ZFISH  156 DIAVEKQIFGEAIKQPGVAFIAAKFDGILGMAYPRISVDGVPPVFDMMMSQKKVEKNVFS
CATD_FUGU   157 DLAVEKQLFGEAIKQPGIAFIAAKFDGILGMAYPRISVDGVTPVFDNIMSQKKVEKNVFS
REN_MOUSE   165 GITVT-QTFGEVTELPLIPFMLAKFDGVLGMGFPAQAVGGVTPVFDHILSQGVLKEEVFS
REN_HUMAN   167 GITVT-QMFGEVTEMPALPFMLAEFDGVVGMGFIEQAIGRVTPIFDNIISQGVLKEDVFS
REN_FUGU    159 GIPVI-QVFAEATSLSAMPFVFAKFDGVLGMGYPNMAIDGITPVFDRIMSQHVLKEEVFS
REN_ZFISH   157 GIPVV-QVFAEATALPAIPFIFAKFDGVLGMGYPDVAIDGITPVFDRIMSQHVLKENVFS

CATD_HUMAN  231 FYLSRD--PDAQPGGELMLGGTDSKYYKGSLSYLNVTRKAYWQVHLDQVEVASGLTLCKE
CATD_MOUSE  229 FYLNRD--PEGQPGGELMLGGTDSKYYHGELSYLNVTRKAYWQVHMDQLEVGNELTLCKG
CATD_ZFISH  216 FYLNRN--PDTQPGGELLLGGTDPKYYTGDFNYVDISRQAYWQIHMDGMSIGSGLSLCKG
CATD_FUGU   217 FYLNRN--PDTQPGGELLLGGTDPKYYTGDFDYVNVTRQAYWQIHMDGMSVGSQLSLCKS
REN_MOUSE   224 VYYNR---GSHLLGGEVVLGGSDPQHYQGNFHYVSISKTDSWQITMKGVSVGSSTLLCEE
REN_HUMAN   226 FYYNRDSENSQSLGGQIVLGGSDPQHYEGNFHYINLIKTGVWQIQMKGVSVGSSTLLCED
REN_FUGU    218 IYYSRD--PKHSPGGELVLGGTDPNYYTGSFNYMGTRETGKWEITMKGVSVGMEMMFCTE
REN_ZFISH   216 VYYSRD--PTHIPGGELVLGGTDPNYHTGPFHYINTKEQGKWEVIMKGVSVGADILFCKD

CATD_HUMAN  289 GCEAIVDTGTSLMVGPVDEVRELQKAIGAVPLIQGEYMIPCEKVSTLPAITLKLGGKGYK
CATD_MOUSE  287 GCEAIVDTGTSLLVGPVEEVKELQKAIGAVPLIQGEYMIPCEKVSSLPTVYLKLGGKNYE
CATD_ZFISH  274 GCEAIVDTGTSLITGPAAEVKALQKAIGAIPLMQGEYMVDCKKVPTLPTISFSLGGKVYS
CATD_FUGU   275 GCEAIVDTGTSLLTGPSEEVKALQKAIGAMPLIQGQYMVSCDKIPSLPVITFNIGGKPFS
REN_MOUSE   281 GCAVVVDTGSSFISAPTSSLKLIMQALGAKEKRIEEYVVNCSQVPTLPDISFDLGGRAYT
REN_HUMAN   286 GCLALVDTGASYISGSTSSIEKLMEALGAK-KRLFDYVVKCNEGPTLPDISFHLGGKEYT
REN_FUGU    276 GCTAVIDTGSSYITGPASSVSLLMKTIGA-QLDESGYKVNCDAVKTLPSVTFHLGGQEYP
REN_ZFISH   274 GCTAVIDTGSSYITGPASSISILMKTIGAVELAEGGYTVSCNVVRLLPTVAFHLGGQEYS

CATD_HUMAN  349 LSPEDYTLKVSQAGKTLCLSGFMGMDIPPPSGPLWILGDVFIGRYYTVFDRDNNRVGFAEAARL
CATD_MOUSE  347 LHPDKYILKVSQGGKTICLSGFMGMDIPPPSGPLWILGDVFIGSYYTVFDRDNNRVGFANAVVL
CATD_ZFISH  334 LTGEQYILKESQGGHDICLSGFMGLDIPPPAGPLWILGDVFIGQYYTVFDRENNRVGFAKAKSV
CATD_FUGU   335 LSGDQYVLKVSQAGKTICLSGFMALDIPAPAGPLWILGDVFIGQYYTVFDRDNNRVGFAKAK--
REN_MOUSE   341 LSSTDYVLQYPNRRDKLCTLALHAMDIPPPTGPVWVLGATFIRKFYTEFDRHNNRIGFALAR--
REN_HUMAN   345 LTSADYVFQESYSSKKLCTLAIHAMDIPPPTGPTWALGATFIRKFYTEFDRRNNRIGFALAR--
REN_FUGU    335 LTQEDYILWQSQIEGDVCIVTFRGLDIPPPVGPIWILGANFIARYYTEFDRHNNRIGFATAV--
REN_ZFISH   334 LTDEDYILWQSEFGEDICTVTFKALDVPPPTGPVWILGANFIARYYTEFDRGNNRIGFARAV--
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B

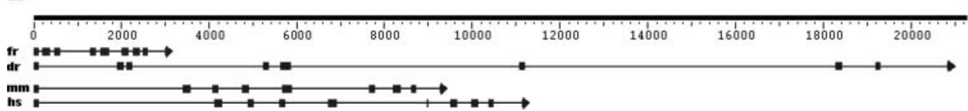


Fig. 1. Comparative analysis of protein sequences and gene structures. *A*: multiple sequence alignment of renin and cathepsin D peptides from human, mouse, zebrafish, and fugu fish. Dark-shaded residues are identical; light-shaded residues are similar; unshaded residues differ. Carat symbol (ˆ) indicates the residue above is unique to renin peptides; pound symbol (#) indicates the residue above is unique to fish renin peptides; exclamation mark (!) indicates residue below is unique to cathepsin D peptides. *B*: alignment of the exon-intron structure of renin genes from human, mouse, zebrafish, and fugu fish. Exons are represented by thick solid bars, while introns are indicated by thin lines. The lengths of exons and introns are approximately proportional to their actual lengths. Arrowhead indicates the last exon. The scale bar at the *top* indicates the approximate base position starting from the "ATG" start codon. Species abbreviations are *hs* (*Homo sapiens*), *mm* (*Mus musculus*), *fr* (*Takifugu rubripes*), and *dr* (*Danio rerio*).

zebrafish renin gene. Supplemental Figure S1 shows the prediction of gene structure and conceptual translations for the two renin genes. The complete genomic sequences and annotation for the two genes have been deposited into GenBank as BK000648 (fugu) and AY216499 (zebrafish).

Fish renin genes are similar in sequence and gene structure to mammalian renin genes. The two newly identified renin sequences differ from any previously reported aspartyl protease sequences from the two fish genomes (16, 42, 43), and they show higher similarity to mammalian renin sequences than to any other aspartyl protease members. Figure 1*A* shows the protein sequence comparison of the two new sequences with mammalian renins and cathepsin D, the closest paralog of renin, from human, mouse, and the two fish species. At the peptide level, exclusive of exon 1 sequences which are normally found only in pro-renin, zebrafish renin is 53% identical to human renin compared with its 46% identity to human cathepsin D, whereas there is 66% identity between cathepsin D from zebrafish and human. Specific sequence properties distinctive and conservative for all known renins are found. For examples, there are 89 (~25%) residues that are identical among all cathepsin D and renins. Nevertheless, the two fish renins share 26 additional identical residues with mammalian renins, but not with cathepsin D sequences. They also share 56 residues that are different from cathepsin D and mammalian renin, possibly suggesting that physical features required for optimal physiological enzymatic activity and substrate recognition may not exactly correspond to those of mammalian renins.

As shown by the phylogenetic tree (Fig. 2), the two new sequences cluster with all mammalian renin sequences to form a distinct renin cluster in the aspartyl protease superfamily while retaining a close relationship to cathepsin D. This data provides strong support for our conclusion that the two newly identified sequences correspond to bona fide renin genes in these two fish genomes.

In addition to undertaking an examination of sequence similarity with known renin genes, a preliminary comparison of overall gene structure reveals general conservation of gene structure over several hundred million years. Despite the huge differences in the intron sizes, the two fish renin genes share the same exon structure, both having nine exons using the exactly same exon boundaries as the mouse genes in relation to the translated proteins (Fig. 1*B*). The human renin gene shares the same gene structure except that it has a 9-base extra exon after exon 5 (named exon 5a) (22, 35). The fugu fish renin gene is roughly half of the size of human renin gene, due to the small size of constituent introns, a phenomenon consistent with its compact genome (8). In contrast, the zebrafish renin gene is unexpectedly large, more than double of the size of mouse and human renin genes, with introns 5 and 6 being particularly long (5 and 7 kb, respectively, vs. 1.8 and 0.4 kb, respectively, for the corresponding introns in mouse renin gene). Nevertheless, introns 2 and 4 are comparable in size to the corresponding introns of the fugu fish renin gene.

Expression of renin in adult zebrafish tissues. To confirm the renin gene prediction and document expression of the predicted transcript, RNA was extracted from adult zebrafish kidney and RT-PCR using primers spanning the complete coding sequence (CDS). Weak bands corresponding to the predicted fragment size were consistently obtained as shown in Fig. 3. Sequencing of the PCR fragments confirmed the predicted CDS based on the assembled genomic DNA. The transcription initiation site was determined using primer extension and RNase protection assays, both of which predict a similar initiation site when allowance is made for AT-rich "breathing" at the 5′ end (Fig. 4, *A* and *B*). This site is 25 bases downstream of a typical TATA box (Fig. 4*C*).

The expression level of renin appears to be lower than the expression levels of β-actin and cathepsin D, as the renin amplicons required a second run of amplification to obtain a robust PCR yield. To find out whether the gene is expressed in

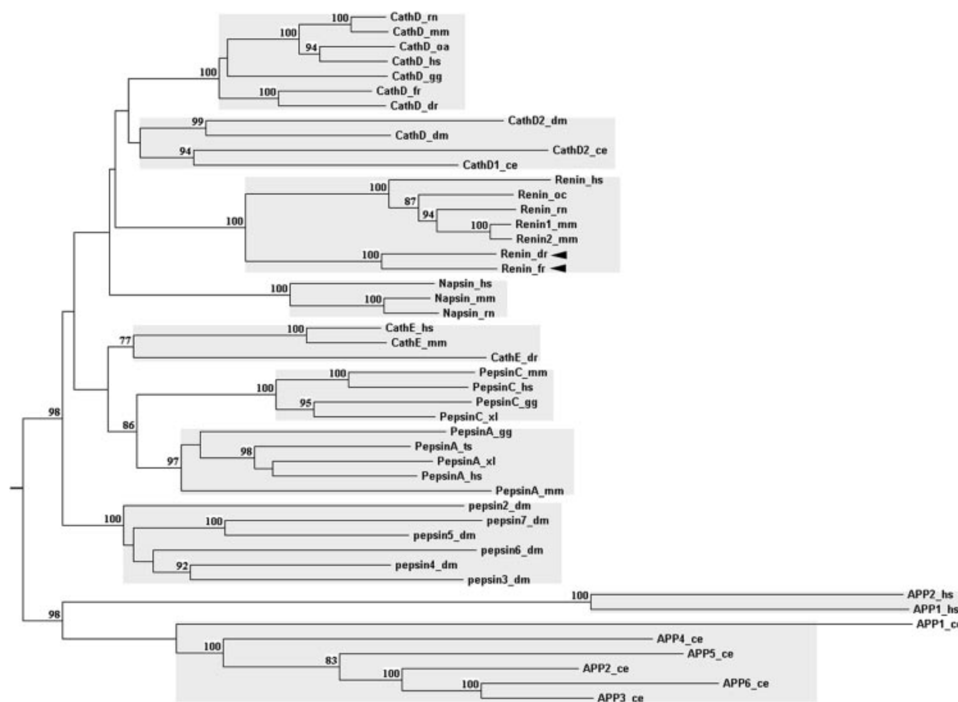


Fig. 2. Phylogenetic tree for aspartyl proteases from a selected list of animal species. Species abbreviations in addition to those used in Fig. 1 are *rn* (*Rattus norvegicus*), *ts* (*Testudines sp.*), *oc* (*Oryctolagus cuniculus*), *xl* (*Xenopus laevis*), *dm* (*Drosophila melanogaster*), *gg* (*Gallus gallus*), and *ce* (*Caenorhabditis elegans*). Each shaded box represents a distinct cluster on the tree. Bootstrap values above 75 for 100 samples are shown for each node, and the tree was rooted with sequences from a group of *C. elegans* aspartyl proteases as the outgroup. The accession numbers of all used sequences are available in the Supplemental Materials, at the *Physiological Genomics* web site. The two fish renin sequences are indicated by arrowheads beside the sequence IDs.

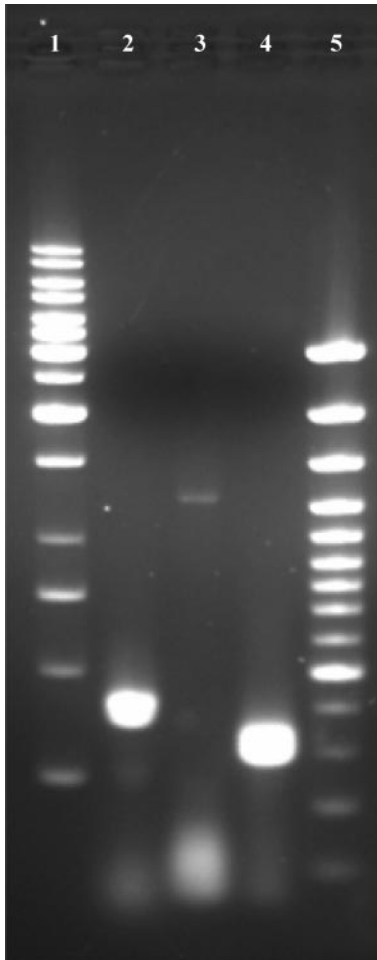


Fig. 3. Detection of renin expression in adult zebrafish kidney by RT-PCR. *Lanes 1* and *5* contain size ladders, *lane 2* contains a 371-bp amplification product of cathepsin D, *lane 3* contains the ~1,180-bp amplification product of renin, and *lane 4* contains the 300-bp amplification product of β-actin.

other tissues, a semiquantitative real-time PCR was performed for various adult zebrafish tissues. As shown by the data in Table 1, among the tissues examined, kidney shows the smallest ΔC_t, indicating that renin gene has the highest expression in kidney tissue ($P < 0.05$). In comparison, cathepsin D, a renin paralog known to be expressed ubiquitously in tissues, shows approximately equivalent expression among the tissues ($P > 0.05$). The greater ΔC_t values of renin compared with cathepsin D suggest that renin expression is lower than that of cathepsin D, as also suggested by the RT-PCR results shown in Fig. 3. This data is consistent with the fact that we have been unable to identify any renin EST sequence from available zebrafish EST databases despite the fact that there are kidney cDNA libraries among those used to generate these EST databases. Taken together, these observations suggest that the renin gene is either expressed at low levels and/or it is only expressed in a small fraction of the cell population associated with kidney.

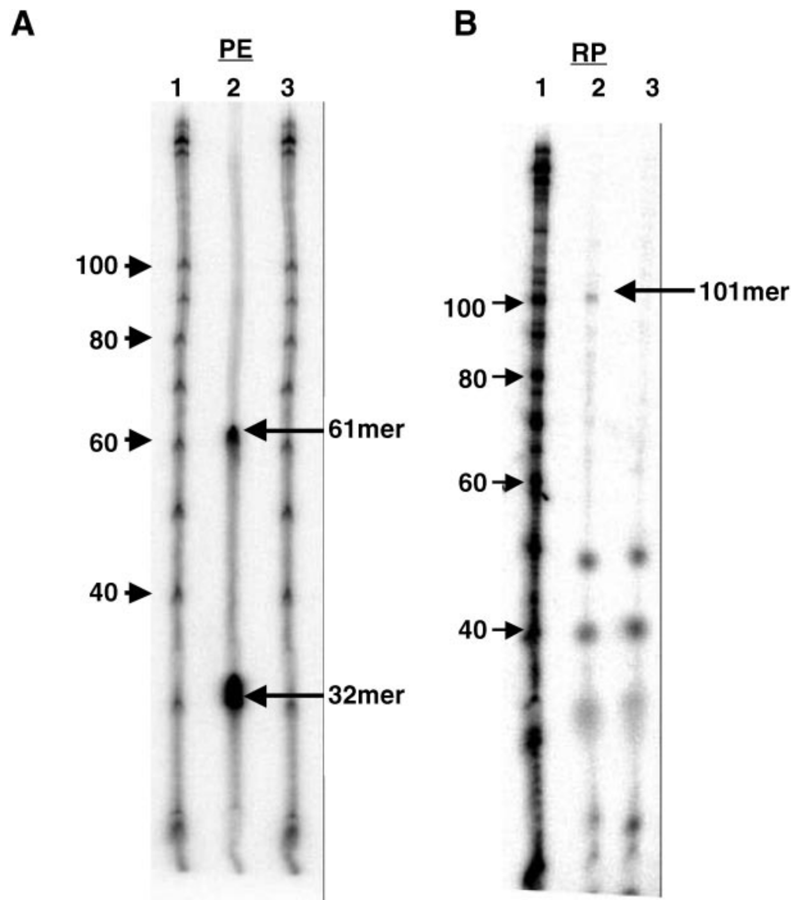
Localization of renin mRNA during early zebrafish development. As shown by ISH, localized renin expression is first detected at 24 h postfertilization (hpf) in two bilateral clusters of 1–3 cells that lie in the intermediate mesoderm lateral to the notochord at the level of the third somite (Fig. 5*A*). By 30 hpf the clusters of cells have migrated medially (Fig. 5*B*) to a position immediately ventral to the notochord where they subsequently fuse into a single group of cells that lies slightly to the right of the midline. Renin expression remains restricted to a small group of cells in the region of the developing pronephros through at least the first 5 days of development (Fig. 5*C*). To determine whether the morphogenetic movement of the renin-expressing cells toward the midline is dependent on signals from the midline, we examined renin expression in two mutants defective in midline development. Both mutants affect the Nodal signaling pathway. The "one-eyed pinhead" (*oep*^{m134}) mutants result from a loss of function in an EGF-CFC coreceptor protein and have defects in floorplate and midline mesoderm and endoderm formation (46, 56). The "casanova" (*cas*^{ta56}) mutants result from loss of function of a novel Sox-related transcription factor and lack a gut tube and other endoderm derivatives and develop cardia bifida (1, 13, 30). In both *oep* and *cas* mutants the clusters of renin-expressing cells fail to fuse at the midline and remain lateral to the notochord through at least 48 hpf (Fig. 5*D*, and not shown), indicating that the migration of these cells is dependant on midline nodal signaling and/or proper endoderm formation.

To examine whether development of the renin-expressing cells was altered in zebrafish mutants with abnormal pronephric development, we examined renin expression in embryos mutant for *locke* (*lok*^{to237b}), a presently unidentified gene, which develop pronephric cysts (13). In these mutants a fused cluster of renin-expressing cells indistinguishable from that seen in wild-type embryos develops at the midline at 48 hpf (Fig. 5*E*), suggesting that migration and development of the renin-expressing primordia is independent of development of epithelial components of the pronephros.

To definitively identify the structure that expresses renin, we performed double ISHs with renin and other markers that localize to specific components of the developing pronephric and interrenal primordia. The small cluster of renin-expressing cells is located medial and posterior to the pronephric tubules which are identified by expression of *pax2.1* (Fig. 5*F*) (32). The *wt1* expression identifies two groups of cells, the pronephric primordia that lie medial to the pronephric tubules (Fig. 5*G*). The two pronephric primordia will subsequently fuse and develop into the glomeruli of the pronephric kidney (47). The renin-expressing cells lie immediately posterior to the right-side expression domain of *wt1* in an identical location to cells that express *ff1b* (10), a marker of the interrenal primordium (26), the progenitor of the zebrafish homolog of the adrenal cortex (compare Fig. 5, *H* and *I*). The zebrafish interrenal gland has recently been proposed to function in osmoregulation (11). The renin-expressing cells of the interrenal primordium are adjacent to, but appear to be separate from, the vascular system of the developing glomerulus as identified by *flk-1* expression at 72 hpf (Fig. 5*J*).

DISCUSSION

A considerable body of evidence points to emergence of the RAS at an early stage of vertebrate evolution. Biochemical and pharmacological evidence suggest the presence of renin substrate, "renin-like" enzyme, ANG I, ANG II, ACE, and receptors for ANG II in bony fish and in more advanced nonmammalian vertebrates (23, 36, 38, 39, 53). DNA and protein sequences of angiotensinogens have been available in the



C DNA sequence in 5' UTR and surrounding regions

Base 61 by PE
Base 108 by RP
Base 101 by RP

```
gaggacagagaaagctaagggtttgtgtcaaggtcaaactgagcatatataagagaaata
gtataaaaacacagaaaggaggaaagtctcttaaataaagcgcagtaaaagagaagttta
cgaacaagtgggtataagagcattgaagcataaaacgtgtggccggagtaactggacagt
gaatagatccatcagtgagaATGAAAATCCACTGCTTGACTCTTCTTATTCTGTCTCTCT
```

Fig. 4. Determination of zebrafish renin gene transcriptional initiation site. *A*: *lane 2* shows a typical primer extension gel using 50 μg of total RNA from adult zebrafish kidney as detected by phosphor imaging. Numbered arrows represent fragment size in bases. *Lanes 1* and *3* are size markers. *B*: *lane 2* shows a typical RNase protection gel using 10 μg of adult zebrafish kidney total RNA, while *lane 3* is 10 μg of a yeast tRNA-negative control. *Lane 1* contains size markers. *C*: double-underlined sequences represent the primers used for RNase protection (RP), whereas the underlined sequence indicates the primer used in primer extension (PE). The bold "a" (indicated by an arrow) represents the 61st base from the start of the primer used for primer extension and the 108th base for RNase protection. The sequence in grayed background represents a typical TATA box. The black box indicates the predicted start codon.

sequence database for zebrafish (gb|AY049731) and pufferfish (TPA|BK001021), and the peptide sequences for ANG I for many other fish species have been reported (3, 23, 48, 49). However, attempts to clone renin from zebrafish using traditional gene cloning approaches have not been successful (16). The failure to clone a bona fide renin gene from any nonmammalian vertebrate has prompted the question of whether true renin orthologs exist in these species and the hypothesis that other aspartyl proteases, such as cathepsin D, may substitute as the angiotensin-generating enzyme in primitive vertebrates (12, 16). Counterposed to the above hypothesis is a considerable body of evidence suggesting the existence of "renin-like" enzymes in these species that function optimally at neutral pH, as found for mammalian renins. For example, a "renin-like" activity detected in teleost fish exhibits a neutral pH optimum and a substrate specificity that is distinguishable from cathepsin D (40). In addition, plasma renin activity has also been measured in several other primitive vertebrate species (24, 37).

Table 1. *Detection of renin gene expression in pronephric kidney by real-time PCR*

Tissue	Kidney	Gill	Liver	Spleen	Heart	Air Bladder	Brain	Gonad	Skeletal Muscle	Eye	Gut
β-Actin C_t	18.1±0.1	19.6±0.3	22.1±0.1	18.5±0.2	20.4±0.3	18.2±0.3	19.9±0.2	17.3±0.3	20.0±0.1	20.3±0.3	21.3±0.3
Renin C_t	25.9±0.1	30.5±0.3	30.6±0.1	30.5±0.3	32.2±1.0	29.7±0.8	29.8±0.8	29.7±0.5	28.3±0.4	31.1±0.5	31.5±0.8
Renin ΔC_t	7.8±0.1	10.9±0.2	8.6±0.1	12.0±0.3	11.8±0.8	11.5±0.7	9.8±0.7	12.4±0.2	8.3±0.3	10.9±0.6	10.2±0.9
Cathepsin D C_t	21±0.1	22.6±0.2	23.2±0.3	20.7±0.3	24.2±0.4	22.1±0.4	22.4±0.3	20.2±0.4	22.6±0.4	22.9±0.5	24.6±0.4
Cathepsin D ΔC_t	2.9±0.1	3.1±0.4	1.2±0.2	2.2±0.2	3.8±0.2	3.9±0.2	2.5±0.2	2.9±0.2	2.6±0.3	2.6±0.3	3.4±0.2

Values are means ± SE. C_t, threshold cycle value. Standard error values are based on measurements from 4 repeats.

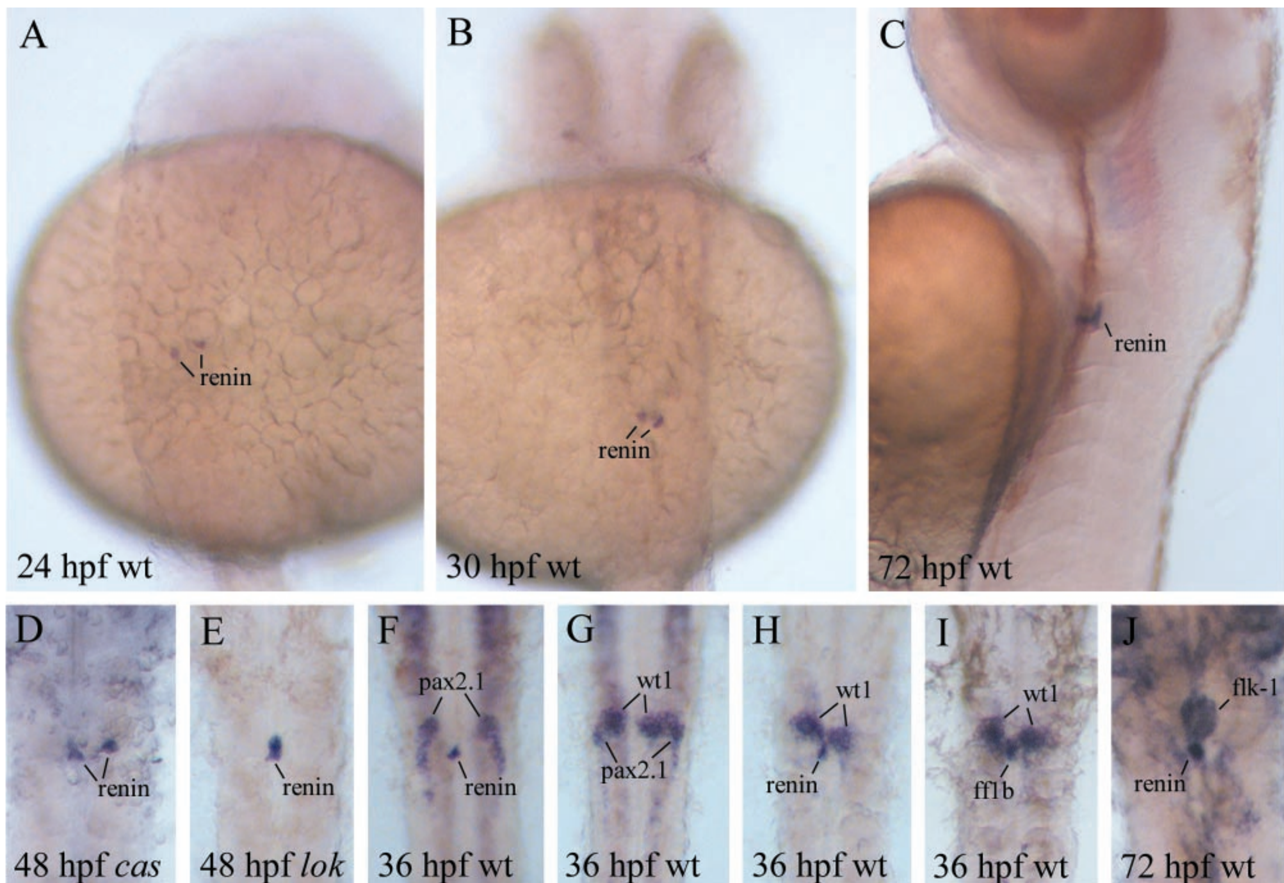


Fig. 5. Expression and localization of renin and other markers of early kidney and adrenal development in wild-type (wt) and mutant zebrafish embryos. *A–E*: localization of renin mRNA by antisense RNA in situ hybridization of in 24 hpf (*A*), 30 hpf (*B*), and 72 hpf (*C*) wild-type embryos and 48 hpf *cas* (*D*) and *lok* (*E*) mutant embryos. *F–I*: double in situ localization of gene markers of pronephric and interrenal primordia in 36 hpf wild-type embryos: renin and *pax2.1* (*F*); *wt1* and *pax2.1* (*G*); renin and *wt1* (*H*); *ff1b* and *wt1* (*I*). *J*: localization of renin and the vascular marker *flk-1* in 72 hpf wild-type embryos. *A* and *B* are dorsal views; *C* is a lateral view; *D–J* are ventral views; anterior to top in all; hpf, hours postfertilization.

Over the evolutionary time spans under consideration, there may be low sequence conservation at the DNA level among renin orthologs. Therefore, we have used a bioinformatics approach, "hybridization in silico," where the "stringency" of sequence recognition may be exquisitely controlled. Searches performed at the level of protein sequences, which preserves conservation much more sensitively than the DNA sequence, were integral to success. In fact, almost no sequence conservation at the DNA level can be detected between renin genes from zebrafish and mouse.

Our report provides the first molecular evidence for the existence of renin genes in lower vertebrates. This, in turn, implies that renin genes should also exist in other higher nonmammalian vertebrates, such as amphibians, reptiles, and birds, and therefore supports the suggestion that the RAS exists in all vertebrates. Notably, as seen in Fig. 2, both *C. elegans* and *Drosophila* have two copies of cathepsin D-like proteins, whereas all vertebrates examined, including three species (human, mouse, and fugu fish) for which close-to-complete genome sequence is available, appear to have only one copy of the cathepsin D gene. Renin and cathepsin D sequences from vertebrates share a similar phylogenetic tree topology, suggesting that renin and cathepsin D evolved in parallel during the evolution of vertebrates. It is possible that two copies of cathepsin D-like genes were present in their common ancestor and that one evolved as renin in the vertebrate lineage to have a more specific or restricted function while the other remained and/or evolved as the current vertebrate cathepsin D.

Renal tissue, as isolated from adult teleosts, consists of opisthonephric tissue lying caudal to pronephric nephrons that form during earlier development (25). Moreover, adrenocortical-like and catecholamine-producing chromaffin tissues are distributed within the renal tissue, intimately associated with the portal circulation of the posterior cardinal vein, and correspondingly termed the interrenal gland (14). We have therefore sought to assess renin expression during the earliest stages of pronephric kidney development. A particularly intriguing finding of the current studies is that renin is first detected in adrenocortical primordia that morphogenetically migrate to lie in juxtaposition with the developing pronephros. Studies addressing the origin of mammalian adrenocortical tissue have suggested that it arises by the coalescence of degenerating pro and mesonephric derivatives at the cephalic end of the metanephric kidney (55). Certainly, there is considerable plasticity evident phylogenetically for adrenal structure within vertebrates (5, 21). In mammals, pronephric renal units are generally not thought to achieve a functional state, while the more caudal and later forming mesonephric renal units have been

characterized to exhibit variable functional utility according to species and placental efficiency (51). Interestingly, screens for renin expression during ontogeny in mice suggest that the earliest and most abundant expression of renin is evident at 13–14 days gestation as the adrenocortical tissue coalesces with medullary tissue of neural crest origin to form the composite adrenal gland (28, 29). The identification of renin expression within adrenal steroidigenic tissue, in association with pronephric renal tissue in teleosts, raises interesting questions about the origin and nature of the renin-expressing cells of mesonephric and metanephric kidney of higher vertebrates. This is made all the more intriguing by the observation that a renin-expressing cell of testes, the Leydig cell, has also been proposed to arise from derivatives of degenerating mesonephric tissue during development (34).

Isolation of the first nonmammalian renin from zebrafish will permit future studies directed at examination of its biochemical activity using angiotensinogen of fish origin, cellular localization of renin by immunofluorescence or ISH, and expression profiling of renin throughout the organogenesis of the pro- and opisthonephric kidneys. Moreover, the availability of zebrafish renin gene sequences should allow us to take advantage of the unique attributes of the zebrafish system, as regards accessible organogenesis, powerful genetics, and open important new avenues for addressing the roles of the RAS in vertebrate development through evolution.

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