The insulin-like growth factor 2 gene and locus in nonmammalian vertebrates: Organizational simplicity with duplication but limited divergence in fish

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Peter Rotwein[1]

From the Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech Health University Health Sciences Center, El Paso, Texas 79905

Edited by Joel Gottesfeld

The small, secreted peptide, insulin-like growth factor 2 (IGF2), is essential for fetal and prenatal growth in humans and other mammals. Human *IGF2* and mouse *Igf2* genes are located within a conserved linkage group and are regulated by parental imprinting, with *IGF2*/*Igf2* being expressed from the paternally derived chromosome, and *H19* from the maternal chromosome. Here, data retrieved from genomic and gene expression repositories were used to examine the *Igf2* gene and locus in 8 terrestrial vertebrates, 11 ray-finned fish, and 1 lobe-finned fish representing >500 million years of evolutionary diversification. The analysis revealed that vertebrate *Igf2* genes are simpler than their mammalian counterparts, having fewer exons and lacking multiple gene promoters. *Igf2* genes are conserved among these species, especially in protein-coding regions, and IGF2 proteins also are conserved, although less so in fish than in terrestrial vertebrates. The *Igf2* locus in terrestrial vertebrates shares additional genes with its mammalian counterparts, including tyrosine hydroxylase (*Th*), insulin (*Ins*), mitochondrial ribosomal protein L23 (*Mrpl23*), and troponin T3, fast skeletal type (*Tnnt3*), and both *Th* and *Mrpl23* are present in the *Igf2* locus in fish. Taken together, these observations support the idea that a recognizable *Igf2* was present in the earliest vertebrate ancestors, but that other features developed and diversified in the gene and locus with speciation, especially in mammals. This study also highlights the need for correcting inaccuracies in genome databases to maximize our ability to accurately assess contributions of individual genes and multigene families toward evolution, physiology, and disease.

The secreted peptide, insulin-like growth factor 2 (IGF2), is produced in many different mammals and nonmammalian vertebrates (1–6) and is part of a small protein family with IGF1 and insulin (5, 7). In mammals, IGF2 plays a central role in fetal development and growth (8) and is involved in a number of other physiological and pathological processes throughout life (9–16). The single-copy gene encoding mammalian *IGF2*/*Igf2* is embedded within a linkage group that includes tyrosine hydroxylase (*TH*/*Th*), *INS* (*Ins2* in mice), *H19*, mitochondrial ribosomal protein L23 (*MRPL23*/*Mrpl23*), and troponin T3, fast skeletal type (*TNNT3*/*Tnnt3*) (17, 18). *IGF2/Igf2* and *H19* gene expression patterns in humans, mice, and likely in other mammals are regulated by parental imprinting, in which *IGF2/Igf2* is selectively active on the paternally derived chromosome and *H19* on the maternal chromosome (19–22). Expression of *IGF2/Igf2* and *H19* on different allelic chromosomes is controlled by DNA sequences within an imprinting control region (ICR). The ICR resides physically between *H19* and *IGF2/Igf2* genes, 5′ to *H19* (23). The regulatory protein, CCTC-binding factor (CTCF)[2] (23–26), can bind to its recognition sequences in DNA within the ICR in maternal chromatin, where the DNA is unmethylated on cytosine residues in CpG dinucleotides (24–26). Under these conditions, DNA-bound CTCF is able to direct distal enhancers to activate the *H19* promoter while simultaneously blocking their access to *IGF2/Igf2* promoters (25–27). In contrast, in paternal chromatin, where ICR DNA is methylated, CTCF binding is blocked, and the enhancers are able to interact selectively with *IGF2/Igf2* promoters (25–27).

Recent advances in genomics and genetics in multiple species now provide unprecedented opportunities for gaining novel insights into comparative physiology, evolution, and even disease predisposition (28–30) through evaluation of information found in public genomic and gene expression databases (31). As an example, examination of the *IGF2/Igf2-H19* locus in different mammals has revealed extensive complexity yet remarkable similarity in individual gene structures, in locus organization, and in gene regulation patterns. Human *IGF2* consists of 10 exons and 5 promoters, as do several other primate *IGF2* genes (3, 18, 21, 22, 32), whereas in the mouse the *Igf2* gene encodes 8 exons and 4 promoters (33–35). *H19* also varies among mammals. Human *H19* has 6 exons and 2 promoters and uses alternative transcription start sites, exon skipping, and differential RNA splicing within exons to generate multiple transcripts (18). Several other primates also have similar regulatory mechanisms for *H19* (18), but these same pro-

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This article contains Fig. S1 and Table S1.

[1] To whom correspondence should be addressed: Dept. of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, 5001 El Paso Dr., El Paso, TX 79905. Tel.: 915-215-4910; Fax: 915-783-5223; E-mail: peter.rotwein@ttuhsc.edu.

[2] The abbreviations used are: CTCF, CCTC-binding factor; UTR, untranslated region; NCBI, National Center for Biotechnology Information; SRA, Sequence Read Archive; ICR, imprinting control region; Myr, million years.

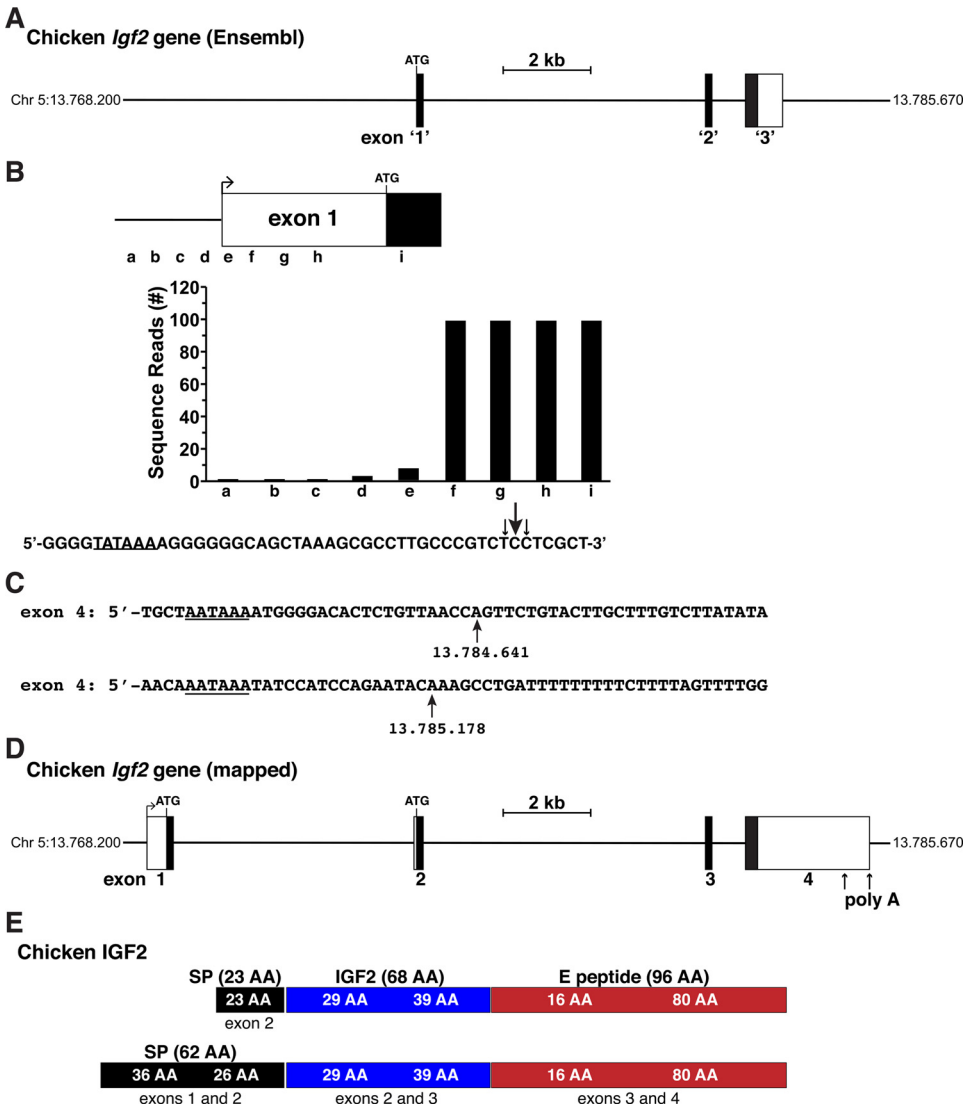


Figure 1. Organization of the chicken *Igf2* gene. *A,* map of the chicken *Igf2* gene as presented in the Ensembl genome database. Chromosomal coordinates are listed; exons appear as *boxes* and are *numbered '1', '2',* and *'3'*, and introns and flanking DNA as *horizontal lines*. A *scale bar* is indicated. *B,* diagram of newly-characterized chicken *Igf2* exon 1 and gene expression data from hepatic RNA-Seq libraries from the SRA NCBI, using 60-bp genomic segments *a–i* as probes. The DNA *sequence below* the *graph* depicts the putative 5′ end of exon 1, with locations of the 5′ ends of the longest RNA-seq clones indicated by *arrows* (the size is proportional to the number of clones identified). A possible TATA box is *underlined*. *C,* DNA sequence of the putative 3′ ends of *Igf2* exon 4. Potential polyadenylation signals are *underlined* and *vertical arrows* denote possible 3′ ends of *Igf2* transcripts. *D,* structure of the chicken *Igf2* gene, after mapping with cDNA XM_015286525 from the NCBI nucleotide data resource and after the other analyses presented in *B* and *C*. Labeling is as in *A*. *E,* diagram of chicken IGF2 protein precursors, illustrating the derivation of each segment from different *Igf2* exons. Mature 68-amino acid (AA) IGF2 is in *blue*; parts of the signal peptide are in *black*, and the E peptide is in *red*.

cesses are not present in other mammalian species, in which just a single promoter has been identified (20, 36). Collectively, these results demonstrate that some common components responsible for controlling *IGF2*/*Igf2* gene expression and IGF2 function appear to have been present in the earliest ancestors of extant mammals, but because there is significant variability in *H19* gene structure, in the ICR, and in transcriptional enhancers, other regulatory elements appear to have developed during species diversification.

The focus of this study is on the *Igf2* gene and locus in non-mammalian vertebrates, where available information is far less extensive than in mammals (37–40). Results based on the combinatorial analysis of public genomic and gene expression databases reveal remarkable conservation of overall locus organization and similarity of *Igf2* exons and IGF2 proteins in >500 Myr of evolutionary diversification, and they support the idea that the *Igf2* gene and its locus are phylogenetically ancient in vertebrates.

Results

Characterizing the chicken Igf2 gene

For this analysis, chicken *Igf2* was selected as the reference gene for terrestrial vertebrates, primarily because it has been more highly studied than any of the other species found in the Ensembl and UCSC Genome browsers. According to a single peer-reviewed publication (37) and information found in both genome databases as of August 2018, the single-copy chicken *Igf2* gene consists of 3 exons and spans 8343 bp of genomic DNA on chromosome 5 (Fig. 1*A*). The 5′ and 3′ ends of the gene

Table 1
Organization of terrestrial vertebrate *Igf2* genes
Length is given in base pairs.

Species	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4	Length
Chicken (*G. gallus*)	568	5601	166	6420	167	740	2840	16,499
Turkey (*M. gallopavo*)	271[a]		166	8484	167	714	2379	11,908
Duck (*A. platyrhynchos*)	464[b]	<5519	166	6127	167	740	2286	15,448
Zebra finch (*T. guttata*)	481	8210	166	6624	167	445	2660	18,660
Flycatcher (*F. albicollis*)	474[b]	7616	511	7500	167	450	2557	19,207
Ch softshell turtle (*P. sinensis*)	126	18,026	163	13425	167	1027	2856	35,783
Anole lizard (*A. carolinensis*)	ND[c]		163	5959	167	1099	3759	11,146
Frog (*X. tropicalis*)	180 or 59	15,283 or 27,386	163	6614	167	969	441	25,263 or 36,772

[a] Data were found in cDNA and could not be mapped to genomic DNA.
[b] This is a poor-quality DNA sequence.
[c] ND indicates no DNA sequence detected.

were not defined in any of these resources, and no promoter was characterized. In fact, in contrast to what is normal eukaryotic gene structure (41, 42), presumptive exon 1 (*exon 1′* in Fig. 1*A*) began with the ATG codon of the IGF2 protein precursor and thus lacked an identified 5′ UTR (Fig. 1*A*). Based on these results, it was clear that the chicken *Igf2* gene was incomplete.

The NCBI nucleotide database contains two different experimentally determined chicken *Igf2* cDNAs. The longer one, XM_015286525, consists of 3369 nucleotides and includes both 5′ and 3′ UTRs, and the shorter one contains primarily coding information. By using the larger cDNA sequence to search the chicken genome, a potential new exon was found within the *Igf2* locus 5′ to the presumptive exon 1 noted above. This new exon contained both coding and noncoding DNA. Mapping with the chicken *Igf2* cDNA also added 9 bp to the 5′ end of Ensembl-defined exon 1, and it resulted in identification of a potential splice donor region being located adjacent to these extra nucleotides (Fig. S1). Subsequent analyses of *Igf2* gene expression by using adjacent 60-bp segments found within the new 5′ exon to query chicken liver RNA-Seq libraries found in the SRA NCBI database identified several potential additional features of the chicken *Igf2* gene. Based on these new results, presumptive exon 1 appeared to be ~568 bp in length and consisted of 108 bp of coding DNA and ~460 bp of 5′ UTR (Fig. 1*B*). Moreover, a potential TATA box, which helps position RNA polymerase II at the start of transcription (43, 44), was identified 29–31 bp 5′ to the ends of the longest *Igf2* transcripts mapped with RNA-Seq libraries (Fig. 1*B*), suggesting that the start of chicken *Igf2* gene transcription may be at this location. However, examination of the region further 5′ using the Promoter 2.0, CNN Promoter, and the UC Berkeley Neural Network Promoter prediction software did not identify many typical components of vertebrate proximal promoters. Thus, although these data extend general understanding of the architecture of the chicken *Igf2* gene, neither the beginning of the gene nor its promoter has been fully established yet.

Genome mapping with the *Igf2* cDNA also extended the 3′ end of the last chicken *Igf2* exon and identified two potential 3′ ends (Fig. 1, *C* and *D*). These two regions, which are separated by ~535 bp, each have typical characteristics of polyadenylation sites, including a "AATAAA" poly(A) recognition sequence and a putative poly(A) addition site 17 or 21 bp 3′ to this element (Fig. 1*C*) (45, 46). Taken together, the results described above indicate that the chicken *Igf2* gene spans at least 16,499 bp on chromosome 5, contains at least 4 exons and 3 introns, and potentially encodes two protein precursors, but a single mature IGF2 (Fig. 1, *D* and *E*, and Table 1).

Characterizing Igf2 genes in terrestrial vertebrates

By using as genomic database queries the four chicken *Igf2* exons and species-homologous cDNAs found in the NCBI nucleotide database, *Igf2* also appeared to be a 4-exon gene in duck, zebra finch, flycatcher, and Chinese softshell turtle, a 3-exon gene in Anole lizard, and a 5-exon gene in frog. It also probably is a 4-exon gene in turkey, although the first exon, which is over 99% identical to the chicken homologue, could not be mapped to the turkey genome, most likely because of poor DNA sequence quality (Fig. 2, Tables 1 and 2). Moreover, in several of the species examined, the annotated genomic data were as incomplete as those for chicken *Igf2* (*e.g.* three exons and no 5′ or 3′ UTRs in turkey and Anole lizard and three exons in flycatcher) or appeared to be unlikely (five proposed exons in duck, including two of 95 and 31 bp separated by a 34-bp intron). When all of the newly identified and mapped information was evaluated, these vertebrate *Igf2* genes appeared to be far simpler than their mammalian homologues, which have up to 10 exons, including several noncoding exons, and up to 5 promoters (human and other primate *IGF2* genes) (18). A possible exception to this lesser complexity is the frog, *Xenopus tropicalis*, which appeared to have two 5′ *Igf2* exons: one, termed exon 1, was located more than 27 kb 5′ to exon 2 and lacks coding potential; the other, termed exon 1a, was ~15 kb from exon 2 and contains an ORF (see Fig. 2, Table 1, and below).

DNA sequence identity with chicken *Igf2* was similar for all four exons in all species examined (88–95% for exon 2, 86–98% for exon 3, 84–95% for exon 4, and 89–100% for exon 1, although in the latter case, there was no match in three species; Table 2). Nucleotide similarity among these vertebrates was more variable in the region located 5′ to chicken *Igf2* exon 1, and it ranged from 87% in turkey to 97% in duck, was minimal in zebra finch and flycatcher, and was not evident in turtle, lizard, or frog. Collectively, these latter observations support other evidence noted above that this DNA segment may not represent the proximal *Igf2* gene promoter in any of these species.

Igf2 gene expression in terrestrial vertebrates

Analysis of information in the SRA NCBI database demonstrated that *Igf2* mRNA was expressed in all species in which

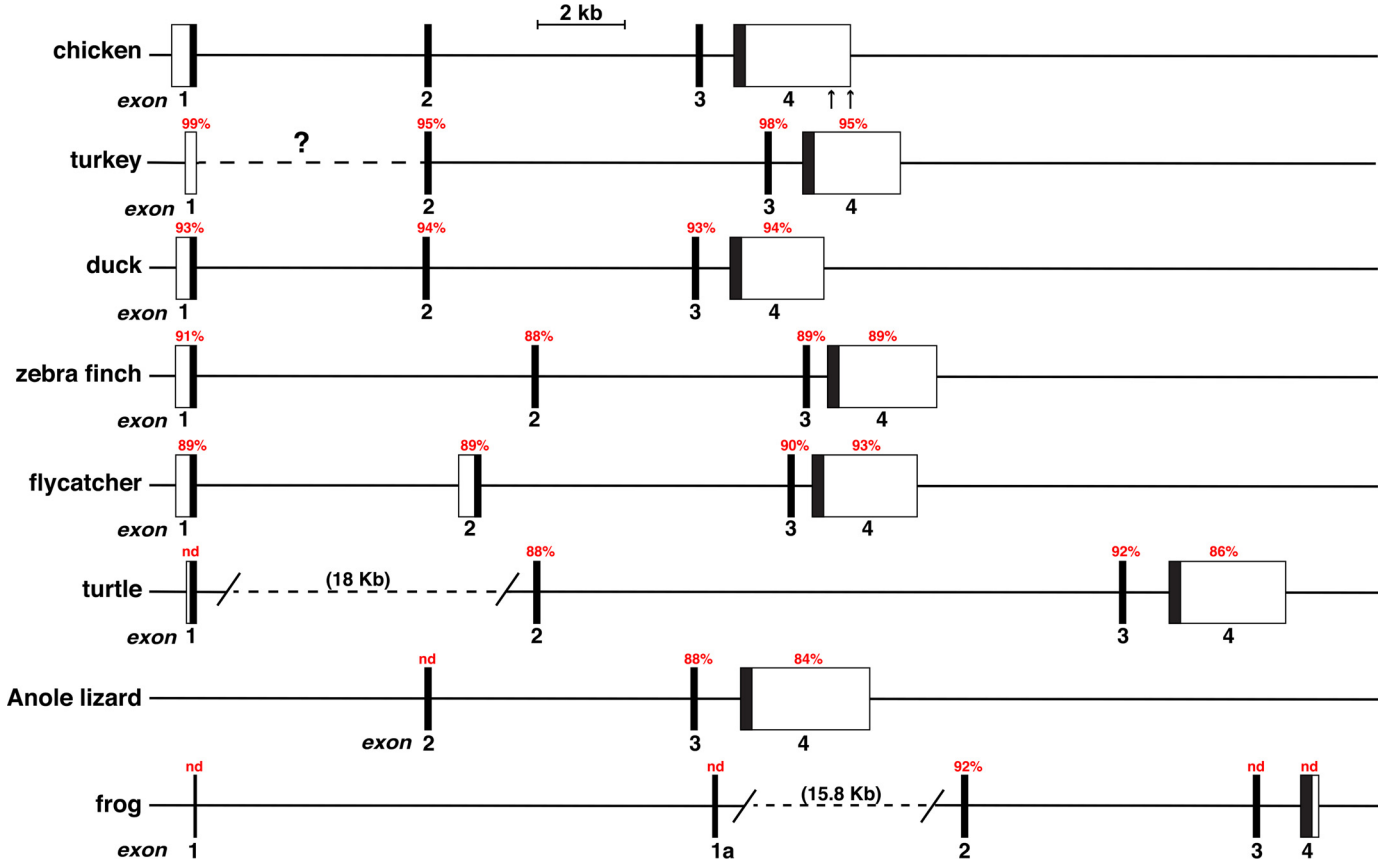


Figure 2. Comparison of terrestrial vertebrate *Igf2* genes. Schematics are shown of chicken *Igf2* and seven other vertebrate *Igf2* genes. Exons are *boxes*, and introns and flanking DNA as depicted as *horizontal lines*. A *scale bar* is indicated, and the two potential 3′ ends of chicken *Igf2* are denoted by *vertical arrows*. The location of turkey *Igf2* exon 1 could not be mapped to the genome, as indicated by a *?*. *Angled parallel lines* indicate discontinuities, with the actual distances spanned in *parentheses*. Percent nucleotide identity with different chicken *Igf2* exons is noted for each gene (*nd,* no identity detected).

Table 2
Nucleotide identity with chicken *Igf2* exons (%)

Species	Exon 1 (568 bp)	Exon 2 (166 bp)	Exon 3 (167 bp)	Exon 4 (2302 and 2840 bp)[a]
Turkey	99[b] (271 bp)	95	98	95 (2320 bp)
Duck	93 (336 bp)	94	93	94 (1824 bp)
Zebra finch	91 (346 bp)	88	89	89 (2167 bp)
Flycatcher	89 (389 bp)	89 (148 bp)	90	93 (1511 bp)
Turtle	No match	88 (120 bp)	92 (89 bp)	86 (1174 bp)
Anole lizard	Not detected	88 (49 bp)	86 (100 bp)	84 (375 bp)
Frog	No match	92 (75 bp)	No match	No match

[a] Two potential poly(A) addition sites are shown.
[b] Data are from cDNA and could not be mapped to genomic DNA.

data were available (Fig. 3). Results from evaluating RNA-Seq experiments for *Igf2* transcripts containing exon 2 are depicted for six species from liver, kidney, spleen, and skeletal muscle (Fig. 3*A*). Further examination of *Igf2* gene expression information for frog revealed marked variability in apparent exon usage. Transcripts containing exon 1a predominated when RNA-Seq libraries were interrogated from either male or female liver, as there were 50–100 times more reads than for exon 1 (Fig. 3*B*). Moreover, no transcripts were identified that contained parts of both exons, and by contrast mRNAs containing exons 1 and 2 or exons 1a and 2 were both found, although the latter predominated (Fig. 3*C*). Mapping studies using the same RNA-Seq libraries from liver also demonstrated that frog exon 1 extended at least 26 nucleotides further in the 5′ direction, and that exon 1a was at least 93 bp longer than recorded in the Ensembl genome browser. Collectively, these results suggest that alternative RNA splicing leads to several classes of frog *Igf2* mRNAs with distinct 5′ ends.

Characterizing Igf2 genes in fish

Zebrafish were selected initially as the index species for studying *Igf2* genes in fish, as it has been more extensively examined than other fish species found in the Ensembl or UCSC Genome Browsers. Based on the information in these databases as of August 2018, the zebrafish genome has two *Igf2* genes, *Igf2a* on chromosome 7, containing four exons within 5984 bp of genomic DNA (Fig. 4*A* and Table 3), and *Igf2b* on chromosome 25, also consisting of four exons and spanning 7506 bp (Fig. 4*B* and Table 3). Examination of these genes and their corresponding cDNAs obtained from the NCBI nucleotide database showed that the 5′ end of *Igf2b* exon 1 matched the 5′ end of the longest cDNA (AF250289), and the 3′ end of exon 4 nearly matched its 3′ end (the last 3 bp are TCA in the gene and GCA in the cDNA). A similarly high degree of DNA sequence identity was found for *Igf2a* and cDNA NM_131433, which differed only within the first four nucleotides at the 5′ end of exon 1. Thus, the annotation of both genes appears to be accurate, unlike the situation with chicken *Igf2* (Fig. 1). Moreover, both zebrafish *Igf2a* and *Igf2b* are structurally similar to chicken *Igf2* (compare Figs. 4, *A* and *B,* with 1*D*). However, even though expression of both genes has been demonstrated during

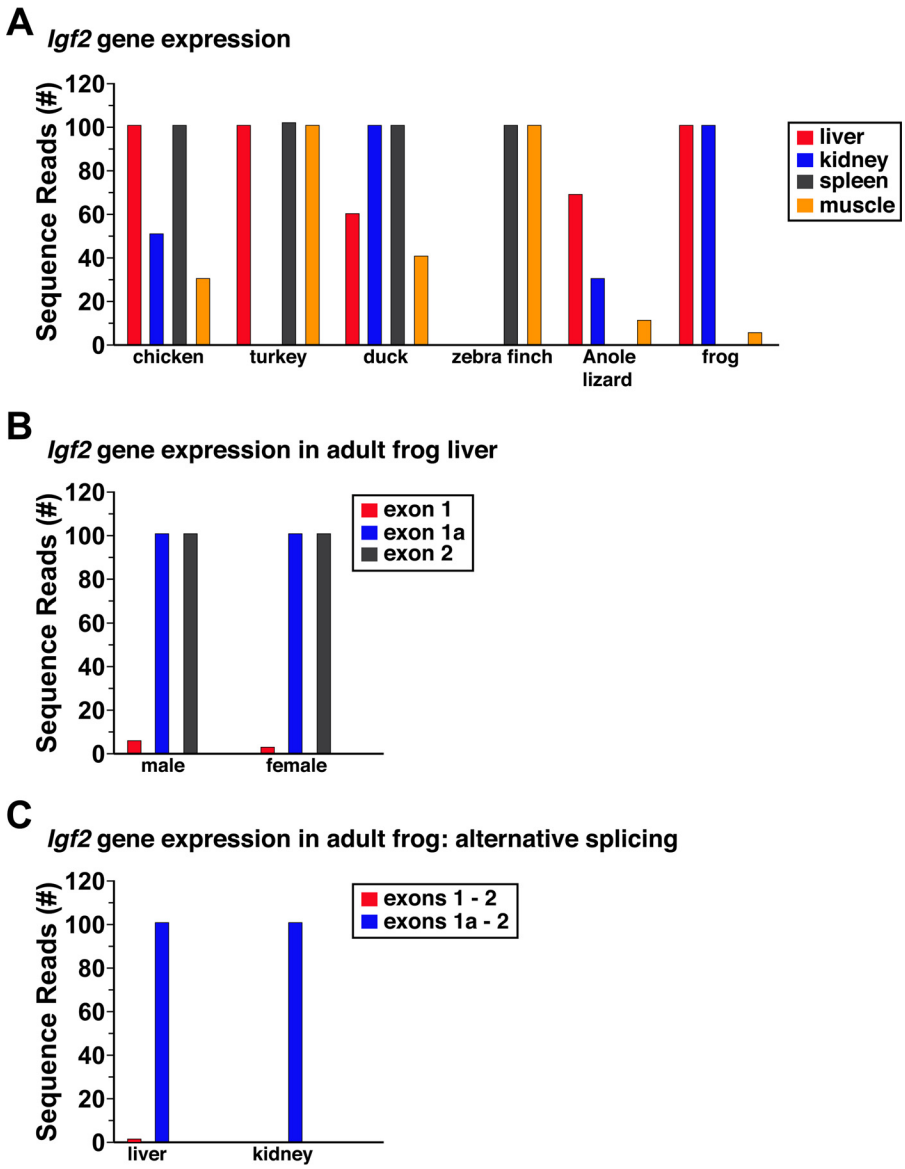


Figure 3. *Igf2* gene expression in terrestrial vertebrates. *A,* levels of *Igf2* transcripts were determined in liver, kidney, spleen, and skeletal muscle by querying RNA-Seq libraries using 60-bp genomic DNA segments from a region equivalent in each species to the same part of chicken *Igf2* exon 2. Results are plotted as the number of sequence reads per species (range = 0–100). Data were obtained from chicken, turkey, duck, zebra finch, Anole lizard, and frog but were not available for flycatcher or Chinese softshell turtle. *B,* comparison of *Igf2* gene expression in hepatic RNA-Seq libraries from adult male and female frogs, using as probes 60-bp fragments of exon 1, exon 1a, and exon 2. *C,* comparison of *Igf2* gene expression in hepatic and kidney RNA-Seq libraries from adult male frog, using as probes 60-bp fragments derived from exons 1 and 2 (3′ 30 bp from exon 1 plus 5′ 30 bp from exon 2) or exons 1a and 2 (3′ 30 bp from exon 1a plus 5′ 30 bp from exon 2). *A–C,* the libraries are listed under "Experimental procedures."

different zebrafish developmental stages, and in adult tissues (47, 48), no gene promoters have been characterized to date, and no studies have been reported on transcriptional control for either *Igf2a* or *Igf2b*.

Searches using *Igf2a* exons as queries revealed just short segments of similarity in only seven other fish genomes. Slightly longer matches were noted with *Igf2b*, although these were found primarily within noncoding portions of exon 4 in 10 species. Searches using chicken *Igf2* exons were similarly uninformative. Of note, a fairly low level of DNA sequence identity with other fish had been observed for the zebrafish *Igf1* gene and prompted using tetraodon *Igf1* exons for mapping this gene in other species (49). The same strategy was subsequently employed here (see below).

In contrast to the two zebrafish *Igf2*s, which are well annotated, the single tetraodon *Igf2* gene has been poorly characterized in Ensembl and in the UCSC Genome Browser. Like zebrafish *Igf2a* and *Igf2b*, tetraodon *Igf2* was reported to consist of four exons, but unlike the former, it appeared to lack identifiable 5′ or 3′ UTRs (Fig. 5*A*). As there were no tetraodon *Igf2* cDNAs in the NCBI nucleotide database, the alternative approach used for chicken *Igf2* was employed to map the beginning and end of the gene. Adjacent 60-bp DNA segments found within and 5′ to presumptive tetraodon exon 1 were used to query the RNA-Seq library, ERX1054374, which was derived from embryo transcripts at 24 h post-fertilization. Results showed that this exon extended for approximately an additional 126 bp in the 5′ direction (Fig. 5*B*). Moreover, as seen for

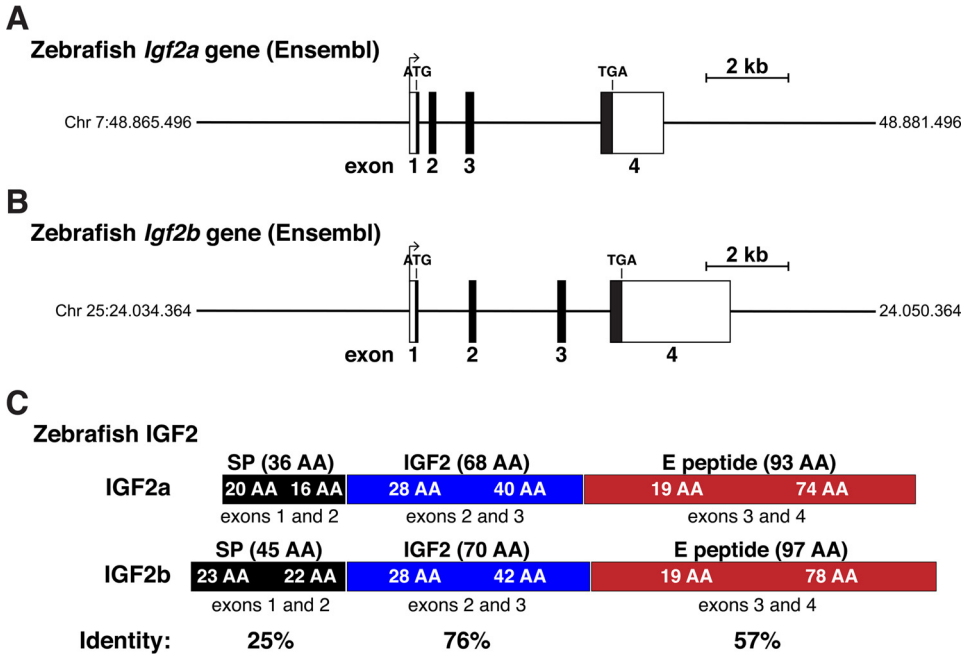


Figure 4. Organization of zebrafish *Igf2* genes. *A,* map of the zebrafish *Igf2a* gene from the Ensembl genome database. *B,* map of the zebrafish *Igf2b* gene from Ensembl genome database. *A* and *B*, chromosomal coordinates are labeled; exons appear as *boxes*; introns and flanking DNA are *horizontal lines*; potential transcription start sites, polyadenylation sites, and locations of ATG and TGA codons are marked; and a *scale bar* is indicated. *C,* diagram of zebrafish IGF2 protein precursors, illustrating the derivation of each segment from different *Igf2a* or *Igf2b* exons. Mature IGF2 is in *blue*; signal peptides are in *black*; and E peptides are in *red*. Percent identities between each part of IGF2a and IGF2b are indicated.

Table 3
Organization of fish *Igf2* genes
Length is given in base pairs.

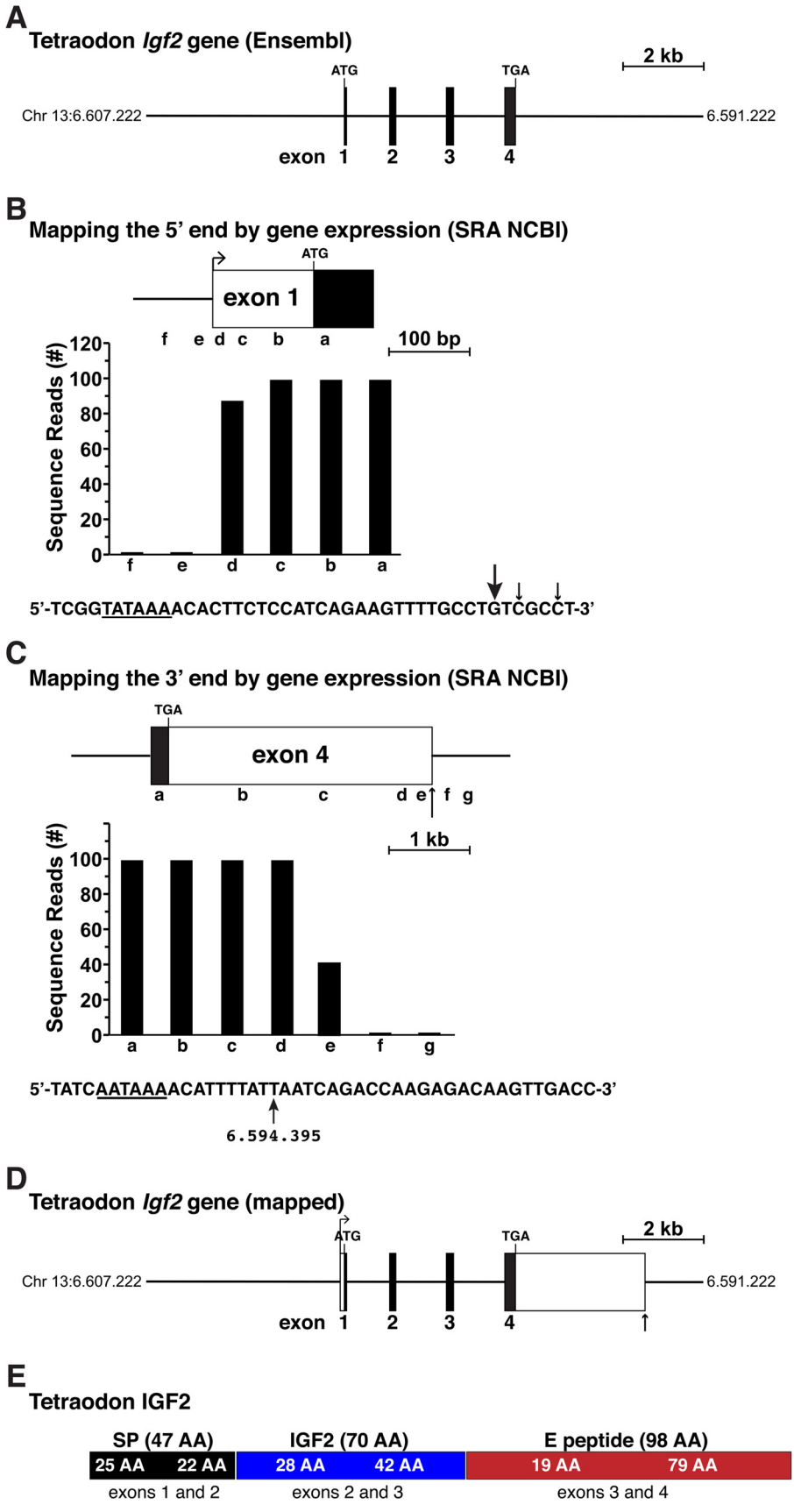
Species	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4	Intron 4	Exon 5	Gene length
Tetraodon (*T. nigroviridis*)	212	1217	151	1352	182	1294	3557			7920
Fugu (*T. rubripes*)	212[a]	899	151	1445	182	1305	3708[a]			8043
Stickleback (*G. aculeatus*)	206[a]	920	151	1473	182	1378	3701[a]			8217
Medaka (*O. latipes*)	306[a]	ND[b]	ND	ND	171	1731	2929[a]			5475
Cod (*G. morhua*)	195[a]	777	151	3156	182	1946	6119[a]			15,139
Cave fish a (*A. mexicanus*)	455	2224	139	4954	176	3341	2028			13,316
Cave fish b (*A. mexicanus*)	401	1035	151	2899	182	958	3530[c]			9168
Tilapia (*O. niloticus*)	195[a]	838	151	2620	182	1291	3548[c]			8986
Amazon molly (*P. formosa*)	813	951	151	2276	184	1150	4264			9789
Platyfish (*X. maculatus*)	135[a]	881	76	257	35	2078	243	1155	3409	7352
Zebrafish a (*D. rerio*)	185	318	133	705	176	2923	1545			5984
Zebrafish b (*D. rerio*)	158	1250	151	1881	182	991	2894			7506
Spotted gar (*L. oculatus*)	195[a]	800	163	2218	176	1741	3934			9226
Coelecanth (*L. chalumnae*)	358	28077	163	18437	167	1126	>213			>48540

[a] Data were defined by 5′ or 3′ end mapping using RNA-sequencing libraries (see Figs. 6 and 7).
[b] ND means not detected.
[c] Data were estimated based on similarity with tetraodon.

chicken *Igf2* (Fig. 1*B*), a potential TATA box, which helps position RNA polymerase II at the start of transcription (43, 44), was identified 26 nucleotides 5′ to the ends of the longest *Igf2* transcript found in this RNA-Seq library (Fig. 5*B*).

An analogous strategy was used to map the 3′ end of presumptive exon 4, and this led to identification of a 3′ UTR of ~3317 bp, and a total exon length of 3557 bp, which included near its 3′ end an "AATAAA" presumptive poly(A) recognition sequence and a putative poly(A) addition site (Fig. 5*C*) (45, 46). Taken together, the results described above, defining both 5′ and 3′ ends of the tetraodon *Igf2* gene, indicate that it spans 7920 bp on chromosome 13 and that it encodes a single protein (Fig. 5, *D* and *E*, and Table 3).

Based on the success of these mapping experiments with chicken and tetraodon *Igf2*, a similar approach was used in seven other fish in which gene annotation was poor: fugu, stickleback, cod, tilapia, platyfish, spotted gar, and medaka, and in which RNA-Seq libraries were available. In six of these fish, the genomic data were nearly as incomplete as those for tetraodon *Igf2* (no 5′ or 3′ UTRs in cod, fugu, and stickleback, and no 5′ UTR in spotted gar, tilapia, and platyfish). Screening of RNA-Seq libraries led to the identification of presumptive beginnings and ends for many of these genes (Figs. 6 and 7). For medaka, in which only two *Igf2* exons had been identified in the genome, most likely because of poor DNA sequence quality, a combination of genomic searches with a medaka *Igf2* cDNA and mapping experiments using a liver-derived RNA-Seq library identified a presumptive exon 1 (but not an exon 2) and extended both exons 1–4 to their presumptive 5′ and 3′ ends, respectively (Figs. 6 and 7).


A
Tetraodon Igf2 gene (Ensembl)
2 kb
ATG
TGA
Chr 13:6.607.222
6.591.222
exon 1 2 3 4
B
Mapping the 5' end by gene expression (SRA NCBI)
ATG
exon 1
f e d c b a
100 bp
Sequence Reads (#)
120
100
80
60
40
20
0
f e d c b a
5'-TCGGTATAAAACACTTCTCCATCAGAAGTTTTGCCTGTCGCCT-3'
C
Mapping the 3' end by gene expression (SRA NCBI)
TGA
exon 4
a b c d e f g
1 kb
Sequence Reads (#)
100
80
60
40
20
0
a b c d e f g
5'-TATCAATAAAACATTTTATTAATCAGACCAAGAGACAAGTTGACC-3'
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Tetraodon Igf2 gene (mapped)
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Chr 13:6.607.222
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exon 1 2 3 4
E
Tetraodon IGF2
SP (47 AA)
IGF2 (70 AA)
E peptide (98 AA)
25 AA
22 AA
28 AA
42 AA
19 AA
79 AA
exons 1 and 2
exons 2 and 3
exons 3 and 4

Additional genomic database searches with tetraodon *Igf2* exons, coupled with information from Ensembl and UCSC Genome browsers, and the mapping data illustrated in Figs. 6 and 7, led to the conclusion that *Igf2* was a 4-exon gene in fugu, cave fish (both *Igf2a* and *Igf2b*), tilapia, Amazon molly, spotted gar, and coelacanth, as well as in zebrafish (*Igf2a* and *Igf2b*), and a 5-exon gene in platyfish (Fig. 8). In stickleback, five *Igf2* exons were predicted in Ensembl, with a 4-nucleotide intron separating the last two exons. As an intron this small is not feasible (50, 51), Ensembl's *Igf2* exons 4 and 5 were combined here into a single *Igf2* exon 4 (Fig. 8 and Table 4). There also was no identifiable *Igf2* gene in lamprey, even though an IGF2 protein has been characterized in this species (see below). When all of this newly characterized information was evaluated, fish *Igf2* genes, like those of terrestrial vertebrates, appeared to be organizationally simpler than their mammalian homologues (Fig. 8) (18). In fact, except for platyfish and frog, with 5 exons, and Anole lizard and possibly medaka, with 3 exons, all the other vertebrate *Igf2* genes in the Ensembl or UCSC Genome Browsers appear to be composed of 4 exons and 3 introns (Figs. 2 and 8).

In addition to structural similarity, DNA sequence identity with tetraodon *Igf2* was relatively high in all fish species examined. This ranged from 84 to 92% for exon 2, 83 to 96% for exon 3, 87 to 94% for exon 4, and 86 to 94% for exon 1, although in three species there was no match for the latter exon (Table 4).

In terrestrial vertebrate *Igf2* genes, an intron divides the exons separating the equivalents of chicken exons 2 and 3 after the first nucleotide of codon 29 of mature IGF2. This is the same codon and codon position and the identical encoded amino acid (serine) found for the intron separating homologous human exons 8 and 9 and mouse exons 6 and 7 (3, 4). The identical exon–intron–exon junctions were observed in all terrestrial vertebrates and in all fish *Igf2* genes, except for medaka, in which no exon 2 could be identified, and platyfish, with an intron interrupted codon 1 of mature IGF2 (threonine) after the first nucleotide.

Igf2 gene expression in fish

Further analysis of RNA-Seq libraries in the SRA NCBI database demonstrated that *Igf2* mRNA accumulated in a variety of different organs, tissues, and developmental stages in different fish (Fig. 9 and data not shown). Results for *Igf2* transcripts containing exon 2 (exon 3 in medaka) are pictured for seven species from liver and for nine from skeletal muscle (Fig. 9). Of note, both *Igf2a* and *Igf2b* genes are expressed in liver and in muscle in cave fish and in zebrafish, and fugu *Igf2* mRNA is detected at different levels in slow *versus* fast twitch skeletal muscle (Fig. 9*B*).

IGF2 protein sequences in nonmammalian vertebrates

The 68-amino acid chicken IGF2 protein resembles the 67-residue human IGF2, as it consists of four domains, termed B, C, A, and D (Fig. 10*A*) (7). This protein appears to be found within two types of precursors with different N-terminal signal peptides, depending on whether mRNA translation begins at the first or second AUG codon (Figs. 1*E* and 10*A*). Among the other species studied here, mature IGF2 was identical to the chicken protein in turkey, duck, and flycatcher; a single amino acid substitution was seen in zebra finch (Ile^{39} to Phe), four changes were found in turtle (Arg^{30} to Ser, Ile^{39} to Phe, Lys^{63} to Arg, and Ser^{64} to Thr), and multiple differences were detected in the other species, including human (Fig. 10*A* and Table 5).

The 70-residue tetraodon IGF2 protein also consists of B, C, A, and D domains (Fig. 10*B*). Among the other fish studied here, only fugu *Igf2* encoded a mature IGF2 identical to the tetraodon protein. In contrast, multiple amino acid substitutions, codon insertions, and/or deletions were found in the other species (range of identity: 58–91%, Fig. 10*B* and Table 6). A phylogenetic comparison demonstrated a greater similarity of mature IGF2 among terrestrial vertebrates and coelacanth than among fish, and it also showed clustering of protein sequences among different groups of fish (*e.g.* cod, stickleback, Amazon molly, tetraodon, fugu, cave fish IGF2b, and zebrafish IGF2b; Fig. 10*C*).

The two potential chicken IGF2 signal peptides either have 23 or 62 residues. The shorter segment starts with the first methionine codon in exon 2. In contrast, the longer signal sequence is encoded by presumptive exons 1 and 2 (36 and 26 codons, respectively; Figs. 1*E* and 11, *A* and *B*, and Table 5). A smaller signal peptide was found in each nonmammalian terrestrial vertebrate analyzed here. It is 23 amino acids in length and varied in all species from the chicken IGF2 signal peptide, with differences ranging from a single amino acid substitution (turkey) to multiple alterations (Fig. 11*A* and Table 5). A longer signal sequence also could be detected in duck, where it is incomplete, and in turtle and frog, but not in other birds (Fig. 11*B* and Table 5). An even longer signal sequence of 80 amino acids is predicted for human IGF2 (17), but its similarity with the chicken signal peptide is negligible (Table 5).

The IGF2 signal peptide in fish is of an intermediate length between short and long chicken signal sequences, ranging from 36 to 53 residues in different species, with amino acid similarity being substantially lower than observed for mature IGF2 (Fig. 11*C* and Table 6). Of note, nearly all of these signal sequences are predicted to have internal in-frame methionine residues

Figure 5. Structure of the tetraodon *Igf2* gene. *A,* map of the tetraodon *Igf2* gene as found in the Ensembl genome database. Chromosomal coordinates are labeled, and exons appear as *boxes* and introns and flanking DNA as *horizontal lines*. Locations of ATG and TGA codons are marked; and a *scale bar* is shown. *B,* mapping the 5′ end of tetraodon *Igf2* with gene expression data from RNA-Seq library, ERX1054374, and 60 bp genomic segments *a–f* as probes. The DNA sequence *below the bar graph* illustrates the putative 5′ end of exon 1, with locations of the 5′ ends of the longest RNA-seq clones indicated by *arrows* (*arrow size* is proportional to the number of clones identified). A possible TATA box is *underlined*. *C,* characterizing the putative 3′ end of tetraodon *Igf2* exon 4 using data from RNA-Seq library, ERX1054374, and 60-bp genomic segments *a–g* as probes. A possible polyadenylation signal is *underlined* in the DNA sequence *below the graph*, and the *vertical arrow* denotes a potential 3′ end of *Igf2* mRNAs with its chromosomal coordinate. *D,* structure of the tetraodon *Igf2* gene based on the analyses shown in *B* and *C*. Labeling is as in *A*. *E,* diagram of the tetraodon IGF2 protein precursor, with the derivation of each segment from different *Igf2* exons indicated. Mature 70-amino acid IGF2 is in *blue*; the signal peptide is in *black*; and the E peptide is in *red*.

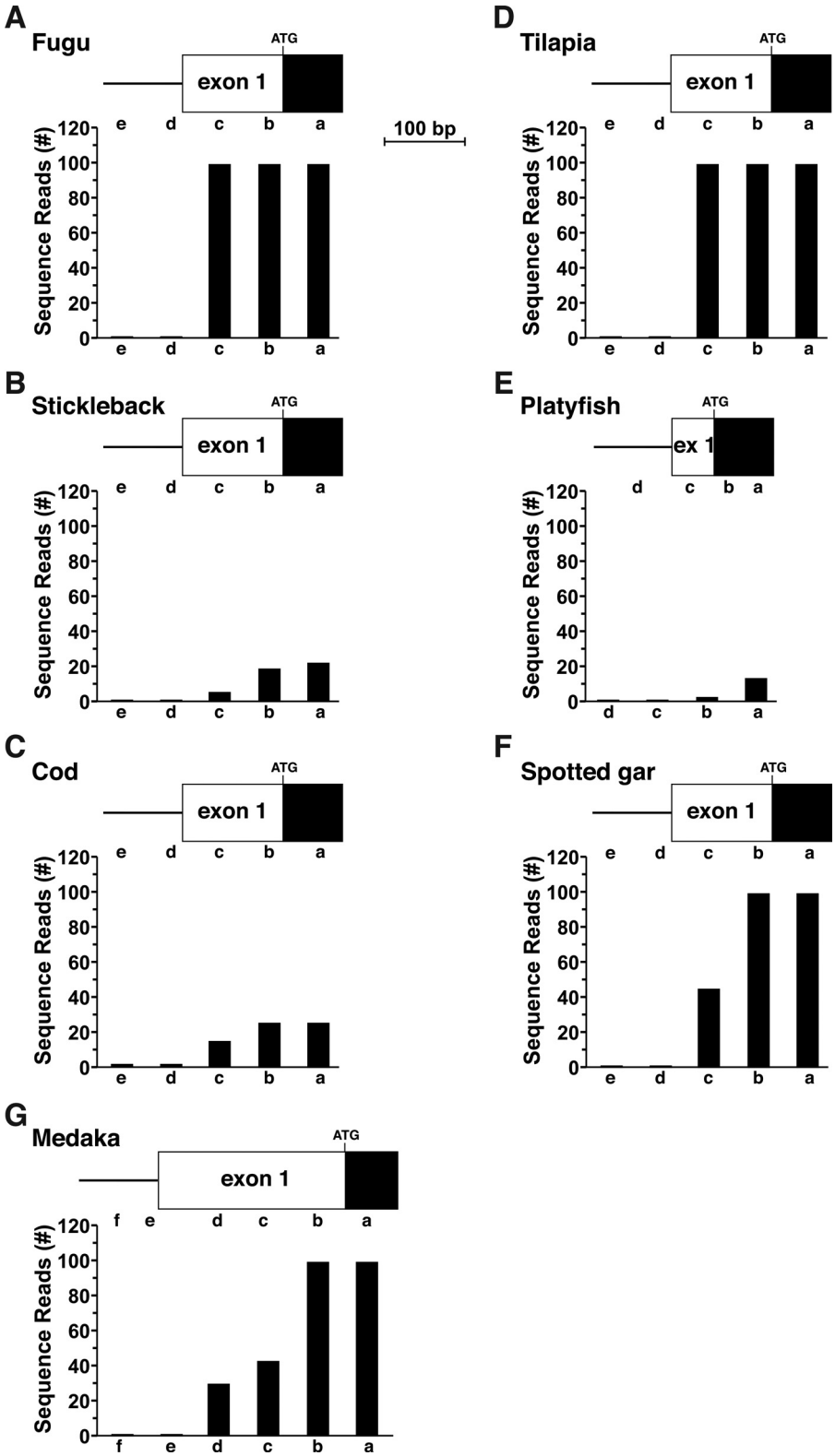

Figure 6. Characterizing 5′ ends of fish *Igf2* genes by analysis of RNA-Seq libraries. *A–G*, mapping putative 5′ ends of fish *Igf2* genes by examination of gene expression data from species-specific RNA-Seq libraries, with 60-bp genomic segments *a–e* or *a–f* as probes. *A*, fugu, library SRX4020085 (liver). *B*, stickleback, library SRX2712198 (liver). *C*, cod, library SRX1044010 (liver). *D*, tilapia, library SRX1257756 (liver). *E*, platyfish, library SRX031881 (whole embryo). *F*, spotted gar, library SRX661023 (whole embryo). *G*, Medaka, library SRX661040 (liver).

(Fig. 11*C*). Because there are no data on the biosynthesis of IGF2 precursors in any nonmammalian vertebrate species, it is not known how effectively mature IGF2 could be generated from a protein precursor with either short or long signal peptides nor which methionine is the initiating residue for protein translation (52, 53).

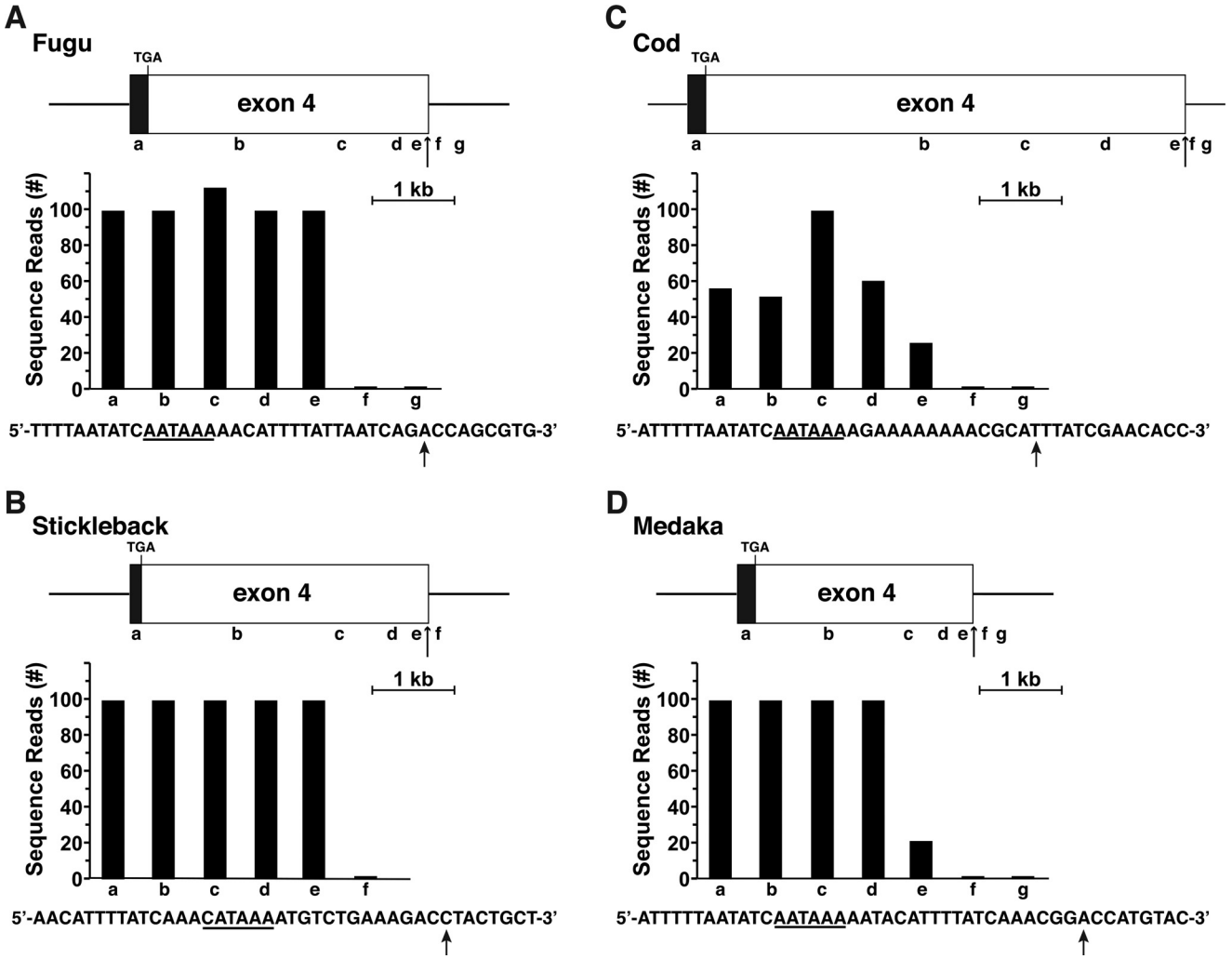

Figure 7. Characterizing 3′ ends of fish *Igf2* genes by analysis of RNA-Seq libraries. *A–D,* mapping putative 3′ ends of fish *Igf2* genes by examination of gene expression data from species-specific RNA-Seq libraries, with 60-bp genomic segments *a–f* or *a–g* as probes. A possible polyadenylation signal is *underlined* in the DNA sequence below each *graph*, and *vertical arrows* denote potential 3′ ends of *Igf2* mRNAs. *A,* fugu, library SRX4020085 (liver). *B,* stickleback, library SRX2712198 (liver). *C,* cod, library SRX1044010 (liver). *D,* Medaka, library SRX661040 (liver).

The E peptide at the C-terminal end of the IGF2 protein progenitor consists of 89 amino acids in human and mouse (4, 17, 32, 54) but is 96 residues in chicken (Fig. 12*A* and Table 5). Except for turkey, in which the IGF2 E region was identical to the chicken segment, it varied in other terrestrial vertebrates in both amino acid sequence and length (*e.g.* flycatcher, 90 amino acids, 85% identity with chicken; Anole lizard, 95 residues, 67% identity; and frog, 94 amino acids, 43% identity; Fig. 12*A* and Table 5). The E peptide comprises 98 residues in tetraodon (Fig. 12*B* and Table 6), and except for fugu, in which there were only 4 amino acid substitutions *versus* tetraodon (96% identity), it was variable in other fish species in both length and amino acid sequence similarity (*e.g.* stickleback, 97 amino acids, 90% identity with tetraodon; platyfish, 103 residues, 59% identity; and spotted gar, 97 residues, 73% identity; Fig. 12*B* and Table 6). The human E peptide shares little similarity with E domains of either terrestrial vertebrates or fish (34% identity with chicken (Table 5) and 24% with tetraodon (Table 6)). The precise functions of this segment of IGF2 have not been established in any species, although it is present in all mammalian and nonmammalian vertebrates that synthesize IGF2 (4, 32, 54).

Igf2 locus organization in nonmammalian vertebrates

Fig. 13 depicts maps of the *Igf2* locus for the terrestrial vertebrates analyzed here. The locus exhibits several similarities in all of these species in the overall topology of the five genes that are present, *Th*, *Ins*, *Igf2*, *Mrpl23,* and *Tnnt3*. In birds, the organization of these genes is congruent, with *Th*, *Ins*, and *Igf2* defining a cluster of three genes in the same transcriptional direction, separated by 213–236 kb from the other two genes, *Mrpl23* and *Tnnt3*, which are in the opposite transcriptional orientation (Fig. 13). In other terrestrial vertebrates, the relative transcriptional orientation is identical with birds, but the distances between individual genes and the two gene clusters are substantially larger, although in frog only 113 kb separates *Igf2* from *Mrpl3*. Of note, the same five genes are found in the same relative orientation within the human *IGF2* locus, although intergenic distances are far shorter than in birds, reptiles, or amphibians (Fig. 13). Also, *H19*, which expresses a long noncoding RNA (20, 55) and is not found here in nonmammalian vertebrates, is present in humans and in other mammals, along with an imprinting control region (ICR), which regulates recip-

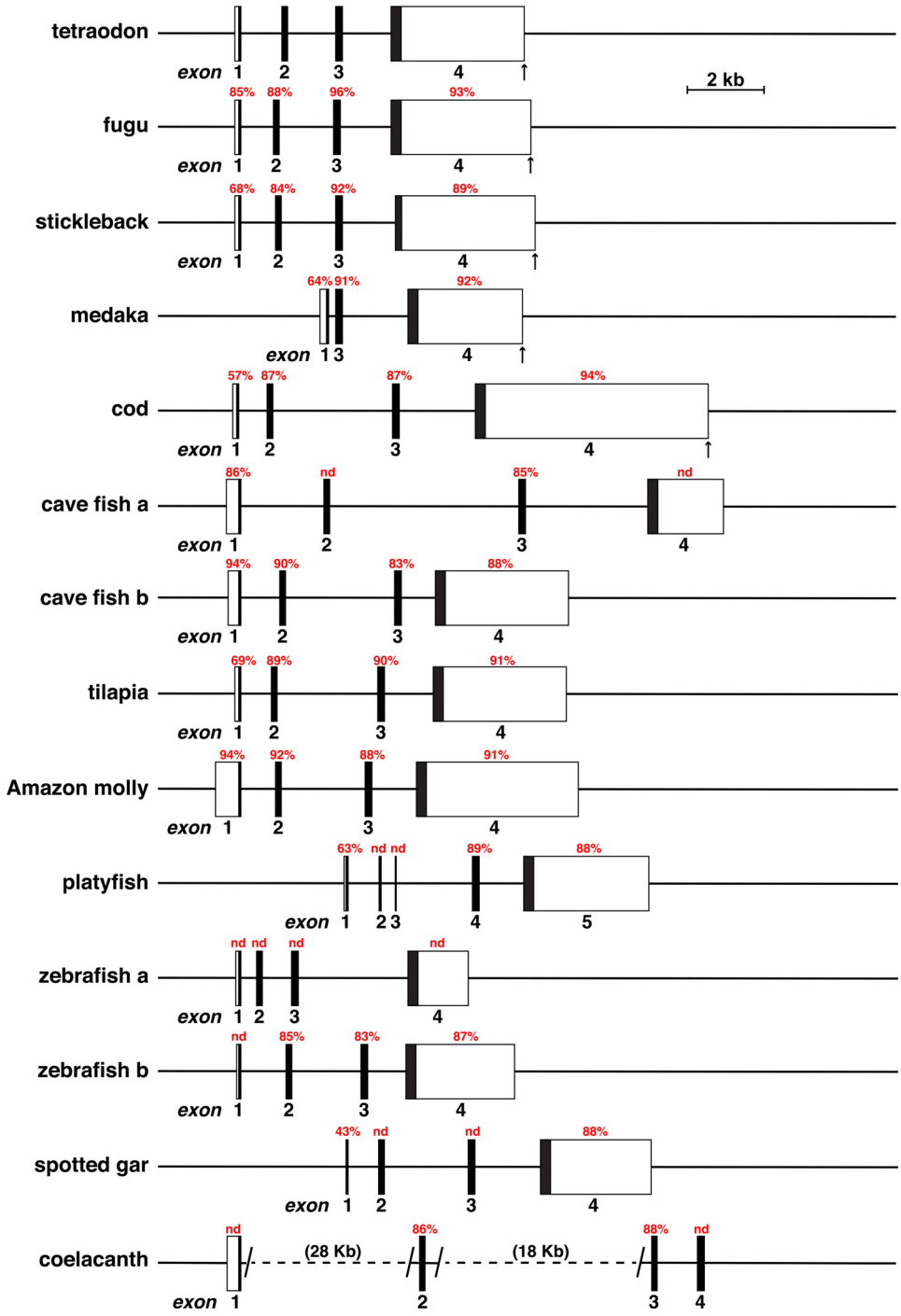

Figure 8. Comparison of fish *Igf2* genes. Diagrams are shown for tetraodon *Igf2*, zebrafish *Igf2a* and *Igfb*, and for *Igf2* genes from 10 other fish species. No *Igf2* gene could be identified in the lamprey genome. Exons are *boxes*, and introns and flanking DNA are shown as *horizontal lines*. A *scale bar* is indicated, and *vertical arrows* denote the 3′ ends of several *Igf2* genes. *Angled parallel lines* and a *horizontal dotted line* indicate a change in scale in coelacanth *Igf2* between exons 1 and 2, and 2 and 3, with the distances spanned in *parentheses*. Percent nucleotide identity with different tetraodon *Igf2* exons is noted for each fish gene (*nd,* no identity detected).

rocal parental chromosome-of-origin–specific expression of *IGF2* and *HI9* in humans and in other mammalian species (23–26, 36). The *Igf2* locus in fish appears to be simpler than in terrestrial vertebrates, as only two other genes, *Th* and *Mrpl23*, are present (Fig. 14). The location and orientation of these genes in the locus are highly similar among the 10 teleost fish studied here but are less so in the nonteleost, spotted gar, in which *Th* is found 5′ to *Mrpl23* (Fig. 14), indicating that an apparent chromosomal rearrangement had occurred after the evolutionary separation of teleosts and nonteleosts. These results also support the idea that *Igf2b* is likely to be the ancestral *Igf2* gene, based on it being embedded in a locus with shared features that also are found in birds and mammals (Figs. 13 and 14), and on the fact that cave fish and zebrafish *Igf2a* loci lack these other genes (data not shown). Moreover, although in medaka and cod *Mrpl23* is apparently not found within this

Table 4
Nucleotide identity with tetraodon *Igf2* exons (%)
Length of DNA sequence similarity is given in parentheses if less than tetraodon exon length.

Species	Exon 1 (212 bp)	Exon 2 (151 bp)	Exon 3 (182 bp)	Exon 4 (3557 bp)
Fugu	85	88	96	93
Stickleback	68	84	92 (172 bp)	89 (1843 bp)
Medaka	64	No exon	91 (120 bp)	92 (1235 bp)
Cod	57	87 (79 bp)	87 (100 bp)	94 (1016 bp)
Cave fish a	86 (80 bp)	No match	85 (68 bp)	No match
Cave fish b	94 (65 bp)	90 (70 bp)	83 (134 bp)	88 (970 bp)
Tilapia	69	89 (75 bp)	90 (172 bp)	91 (1938 bp)
Amazon molly	94 (52 bp)	92 (79 bp)	88 (156 bp)	91 (1600 bp)
Platyfish	63	No match	89 (126 bp)	88 (1760 bp)
Zebrafish a	No match	No match	No match	No match
Zebrafish b	No match	85 (61 bp)	83 (77 bp)	87 (817 bp)
Spotted gar	43	No match	No match	88 (759 bp)
Coelecanth	No match	86 (64 bp)	88 (42 bp)	No match

locus, this could reflect the more incomplete quality of their genome assemblies, because it is present in both species (data not shown). A similar quality control problem may be true for the coelacanth genome, in which *Mrpl23* maps near *Tnnt3* (data not shown), as is observed in both terrestrial vertebrates (Fig. 13) and mammals (18), but could not be localized near *Igf2* (Fig. 14). Taken together, these observations demonstrate that several features of the *Igf2* locus have been retained during more than ~500 Myr of vertebrate and mammalian speciation, and thus they argue that the *Igf2* gene and locus are phylogenetically ancient.

Discussion

Igf2 genes in vertebrates

The goals of the studies presented here were to understand the organization and patterns of expression of *Igf2* genes in nonmammalian vertebrates by mining the resources of public databases and to place these findings in an evolutionary context with mammalian *IGF2*/*Igf2* homologues and the mammalian *IGF2*/*Igf2-H19* locus. In mammals, IGF2 is involved principally in mediating prenatal growth (8), but it also functions in other aspects of physiology and pathophysiology throughout life (9–16). Mammalian *IGF2*/*Igf2* genes are complicated and reside within a complex multigene locus (17, 18, 36, 56). In humans and in mice, multiple gene promoters (5 for human and 4 for mouse) control production of many different types of *IGF2*/*Igf2* mRNAs that are translated and processed into a single mature 67-amino acid IGF2 (4, 32, 54). In both species, *IGF2*/*Igf2* gene promoter activity is regulated by developmental and tissue-specific mechanisms that in turn are controlled by paternal chromosome-of-origin parental imprinting that is reciprocal to the expression of *H19* (25, 26, 57, 58). Similar processes are presumably operative in other mammalian species, although they have not been characterized as fully as in mice and humans (36, 56).

The genomic and gene expression data identified and analyzed here show that *Igf2* genes are far simpler in nonmammalian vertebrates than in mammals (Figs. 2 and 8) and that the locus also is simpler (Figs. 13 and 14). In most of the species described in this paper, the *Igf2* gene is composed of 4 exons and 3 introns and likely has a single gene promoter, although this has not been established

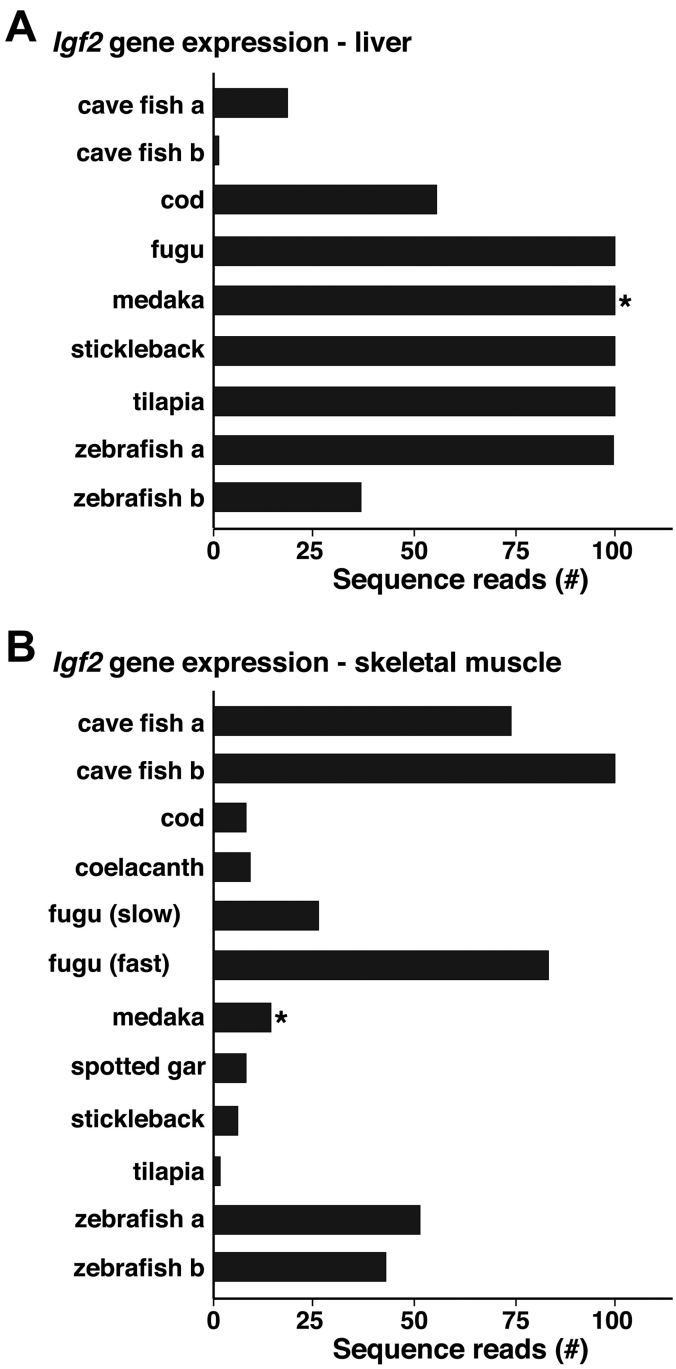

Figure 9. *Igf2* gene expression in fish. *Igf2* transcript levels were identified in liver (*A*) and in skeletal muscle (*B*) by querying RNA-Seq libraries using 60-bp genomic DNA segments from a region equivalent in each species to the same part of tetraodon *Igf2* exon 2 (or * exon 3 in medaka). Results are plotted as the number of sequence reads per species (range = 0–100). Information was not available from either liver or muscle for Amazon molly, platyfish, or tetraodon or for liver for coelacanth. *A* and *B*, libraries searched are listed under "Experimental procedures."

experimentally as yet. Exceptions include frog, in which there are 5 exons and evidence for alternative RNA splicing (Figs. 2 and 3 and Table 1), platyfish, which also has 5 exons (Fig. 8 and Table 3), and possibly Anole lizard and medaka, in which a homologue of exon 1 or exon 2, respectively, could not be identified (Figs. 2 and 8 and Tables 1 and 3). Moreover, in terrestrial vertebrates and in mammals, and in most of the fish species studied here, the *Igf2*/*IGF2*

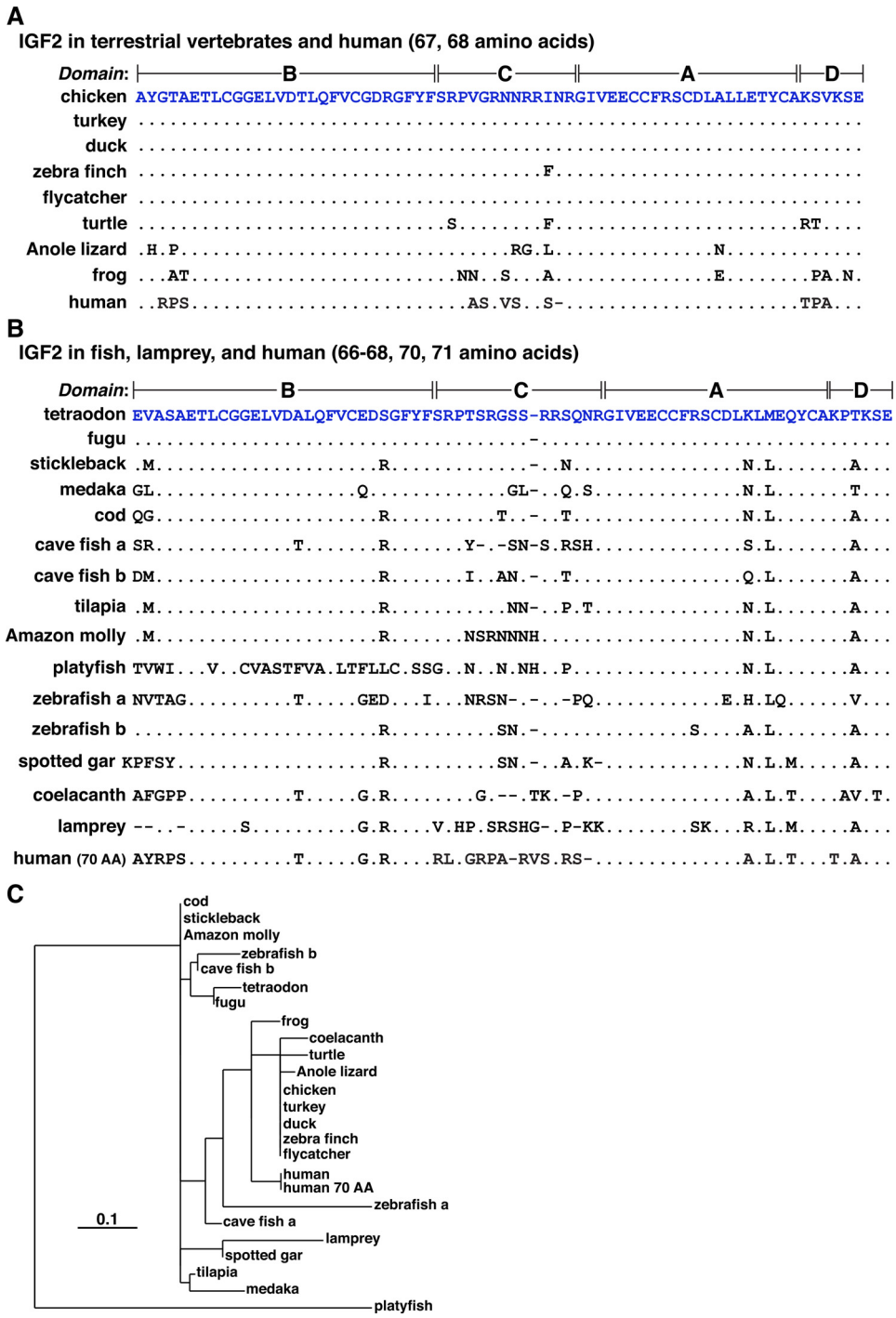

Figure 10. Alignments of vertebrate IGF2 proteins. *A,* amino acid sequences of IGF2 from eight terrestrial vertebrates and humans in *single-letter code*. Differences among species are shown, with identities being depicted by *dots*. *Dashes* indicating no residue have been placed to maximize alignments. *B,* amino acid sequences of IGF2 from 12 fish species, lamprey, and human (70 amino acid variant) in *single-letter code*. Differences among species are shown, with identities being depicted by *dots*. *Dashes* (indicating no residue) have been placed to maximize alignments. *C,* phylogenetic tree of mature IGF2 in vertebrates. The *scale bar* indicates 0.1 substitutions per site, and the length of each branch approximates the evolutionary distance.

gene is present in a single copy in the genome (22). The exceptions are zebrafish and cave fish, in which there are paralogous *Igf2* genes termed *Igf2a* and *Igf2b* (Fig. 8 and Table 3). This latter finding reflects the fact that in a common ancestor of extant ray-finned fish, the entire genome was duplicated ~320–350 Myr ago (59) and that this duplication was followed by re-diploidization in progenitors of many modern teleost lineages (59). However, in some species, such as zebrafish, a substantial fraction of duplicated genes has been retained (60). In both zebrafish and cave fish, the paralogous genes have diverged from one another, as amino acid identities between mature IGF2a and IGF2b are 76 and 84%, respectively, in the two species, and thus are less similar to each other than the corresponding IGF2b proteins are to tetraodon IGF2 (90 and 86%, Table 6). By these criteria, and by other similarities within the locus, it is clear that in both

Table 5
Amino acid identities with chicken IGF2 (%)
AA means amino acids.

Species	Long signal peptide (62 amino acids)	Signal peptide (23 amino acids)	Mature IGF2 (68 amino acids)	E peptide (96 amino acids)
Turkey	None	96	100	100
Duck	[a]	91	100	56 (97 AA)
Zebra finch	None	87	99	88
Flycatcher	None	83	100	85 (90 AA)
Turtle	56 (59 AA)	65	94	71 (95 AA)
Anole lizard	None	61	91	67 (95 AA)
Frog	26 (55 AA)	52	85	43 (94 AA)
Human	<10 (80 AA)	30 (24 AA)	84 (67 AA)	34 (89 AA)

[a] This is a poor-quality DNA sequence.

Table 6
Amino acid identities with tetraodon IGF2 (%)
AA means amino acids.

Species	Signal peptide (47 amino acids)	Mature IGF2 (70 amino acids)	E peptide (98 amino acids)
Fugu	66	100	96
Stickleback	60	91	90 (97 AA)
Medaka	40 (44 AA)	86	79
Cod	55[a] (38 AA)	89	72 (97 AA)
Cave fish a	17 (49 AA)	79 (68 AA)	55 (96 AA)
Cave fish b	6 (53 AA)	86	78 (97 AA)
Tilapia	55	87	88
Amazon molly	17 (51 AA)	83 (71 AA)	85
Platyfish	38 (50 AA)	58 (71 AA)	59 (103 AA)
Zebrafish a	36 (36 AA)	69 (68 AA)	62 (93 AA)
Zebrafish b	38 (45 AA)	90	87 (97 AA)
Spotted gar	32 (50 AA)	80	73 (97 AA)
Coelecanth	17 (53 AA)	74 (68 AA)	42 (86 AA)
Lamprey	15 (53 AA)	70 (66 AA)	ND[b]
Chicken	9 (23 and 62 AA)	75 (68 AA)	42 (96 AA)
Human	2 (24 and 80 AA)	80 (70 AA)	24 (89 AA)

[a] This is a partial sequence.
[b] ND means not detected.

zebrafish and cave fish *Igf2b* represents the descendant of the original *Igf2* locus.

Despite less complexity than in mammals, *Igf2* genes in vertebrates share some common features with mammalian *IGF2*/*Igf2*. In all species studied here, except for platyfish (and medaka, which because of poor genomic sequence quality could not be evaluated), an intron splits the exons that encode the mature IGF2 protein at the identical location (these are the equivalents of exons 2 and 3 in nonmammalian vertebrates, exons 8 and 9 in human, and exons 6 and 7 in mice), interrupting these exons between the first and second nucleotides of serine codon 29 of mature IGF2 (3, 4). Conserved intron positioning also is found in *Igf1* genes from mammals and nonmammalian vertebrates, as in all of these species a large intron interrupts exons encoding the mature IGF1 protein between the first and second nucleotides of codon 26 (49).

The *Igf2* locus in nonmammalian vertebrates also is simpler than in mammals. There is no equivalent of the *H19* gene, and no apparent ICR or distal enhancers, as mapped in mammals (57), although in the absence of a functional promoter, enhancers would be difficult to identify experimentally. However, several of the genes mapped to the locus in mammals also are found in terrestrial nonmammalian vertebrates in the same order and transcriptional orientation, including *Th*, *Ins*, *Mrpl23*, and *Tnnt3* in terrestrial species (Fig. 13), and *Th* and *Mrpl23* in most fish (Fig. 14). Collectively, these results suggest that the *Igf2* locus is phylogenetically old, as it is found in vertebrates separated by over 500 Myr of evolutionary diversification.

Igf2 gene regulation in vertebrates

There is minimal published information on *Igf2* gene expression in nonmammalian vertebrates, with studies being limited to a few analyses of chick embryos (61–64), turkeys (40), ducks (39), zebra finch (38), some observations in zebrafish and medaka embryos (47, 65), and measurements of transcripts in different organs, tissues, and cell types from zebrafish, tilapia (48, 66, 67), and a few other fish species (68, 69). The data presented here using queries of RNA-Seq libraries from the SRA NCBI repository extend previous analyses and show that *Igf2* transcripts are produced in different adult tissues in a number of nonmammalian vertebrates (Figs. 3 and 9). However, mechanisms of gene regulation are unknown, and no *Igf2* gene promoter has been functionally identified to date in any of these species. The situation is potentially different for *Igf1*, in which conserved putative transcription factor-binding sites have been mapped to positions analogous to those characterized experimentally in mammals (49)

In mammals, genetic, epigenetic, and environmental factors all contribute to somatic growth (70, 71), and also influence *IGF2*/*Igf2* gene expression and protein production (9–12). For example, in humans, alterations in levels of IGF2 are associated with genetically determined overgrowth and undergrowth disorders, respectively, termed Beckwith-Wiedemann and Silver-

A

IGF2 Signal Peptide - Terrestrial Vertebrates (23 AA)

```
chicken       MCAARQILL-LLLAFLAYALDSAA
turkey        ......M..-.............
duck          .....RL..-.............
zebra finch   .....RM..-.........M....
flycatcher    ..V..RM..-......G.......
turtle        ...S.R...-.A.T....TV..TL
Anole lizard  ..TS.R...-.A.T....TI..VS
frog          .SVM.HL..-.SIT..V.T....K
```

B

Long IGF2 Signal Peptide - Terrestrial Vertebrates (53, 55, 59, 62 AA)

```
chicken       MASAGAHTDERCRQPAFLPGPPPTEVESGSGSASAKVQRMCAARQILLLLLAFLAYALDSAA
turkey        none
duck          ------------------------------------.........RL...............
zebra finch   none
flycatcher    none
turtle        .SR.ERD..AC.SH....QGCAQ-....S...L--.......S.R....A.T....TV..TL
Anole lizard  none
frog          .EQLSCKHRSSSVD-.EGQLCRQ--A..R.TQLP----..SVM.HL...SIT..V.T....K
```

C

IGF2 Signal Peptide - Fish and Lamprey (36, 45, 47, 49, 50, 51, 53 AA)

```
tetraodon     METQHRPGARSSCHTCRRARSSRMKVKKMSSCSHAVLFALTLALHFA
fugu          ......NA.P.F......TEISI........S...L....A.T.YVV
stickleback   .D......HH.R......TDM....M.....S.R.L.L..ALALYVV
medaka        ..IPQ.H.QQPPR....T.G-.I-..RRR-.PGG.L.L..A.T.CVG
cod           ---------H.V......TER.I......F.S.S.LV...AMT.YIF
cave fish a   ..E.QAGAQH.AAAAAGLC.TC.RSN.QKTTMSSSGGVW.LAVVVALCS
cave fish b   ..E.QQQKKQQQHSYHSVCHTCWRTESARNKVRKMSACSRWLVCALALSLCVL
tilapia       ....QRY.HH.L......TQN.....QR...T.RAL....A.TLYVV
Amazon molly  .PSDMETQQ..GH.SLCHTCRRTESCRMKVKKMCSTSR..LF..ALTLYVV
platyfish     ....Q.S.HH.L......TE.C...IARYKKMCSTSRAL.FALALTLYVV
zebrafish a   .DDY.VF--CA..---.KTEET.TT---.R.LI--.-.V...SMLIS
zebrafish b   ..D.LKHH--.V....S.TD.FVN..I..FWSIRMPICI.F.T.SAF
spotted gar   ..D.QKYSYQAF....LGTENR...MR...TSRQMLV.TIA.TFYIMDVA
coelacanth    ..EYCTHPVVCQIC.KEPED.NSSNF.VSKMSTSRH.LL.SM.IIVYIADVAK
lamprey       ..YKGLASCSLCRF.F.TTRTAAVGCTAARPLTLLAPLL.M.L.GAGNSRPVR
```

Figure 11. Alignments of vertebrate IGF2 signal sequences. *A,* amino acid sequences of IGF2 signal peptides from eight terrestrial vertebrates in *single-letter code*. Differences are shown, and identities are indicated by *dots*. *B,* amino acid sequences of longer IGF2 signal peptides. Differences are indicated, and identities are signified by *dots*. A *dash* indicates no residue. *Bold red text* is identical to the short signal sequence shown in *A*. No longer signal peptides could be detected for turkey, zebra finch, flycatcher, or Anole lizard (= *none*). *C,* amino acid sequences of IGF2 signal peptides from 12 fish species and lamprey in *single-letter code*. Differences are shown, and identities are indicated by *dots*, and a *dash* signifies no residue. *B* and *C, dashes* have been placed to maximize alignments.

Russell syndromes (11, 12). It is not known whether similar growth disorders connected with IGF2 occur in nonmammalian vertebrates. However, DNA polymorphisms have been identified in chickens within the *Igf2* locus that sort with somatic growth and carcass weight (72, 73), and experimental selection for body size in zebrafish has been found to be associated with changes in expression of components of the insulin-like growth factor system, including alterations in levels of *Igf2* transcripts (74).

IGF2 proteins in vertebrates

Mature IGF2 in most mammals is a 67-amino acid single-chain protein consisting of domains termed B, C, A, and D that are related to the analogous parts of IGF1 (4) and also resemble the B and A chains of mature insulin and the C chain of proinsulin (7). In all terrestrial vertebrates examined here, mature IGF2 is 68 residues in length (Table 5), and the proteins are more similar to each other than are IGF2 proteins in fish, where IGF2 ranges from 66 residues (lamprey) to 71 residues (platyfish and Amazon molly), although in the majority of species it is 70 amino acids (Fig. 10*C*, Table 6). In terrestrial vertebrates, the A domains are nearly identical, with only a single amino acid alteration being found in lizard and frog, and B domains also are highly similar (just two differences in lizard and frog), whereas the C region is more divergent in reptiles and amphibians (Fig. 10*A*). In contrast, in fish, there are several amino acid changes in both A and B domains and more in the C region (Fig. 10*B*; exceptions are tetraodon and fugu IGF2, which are identical).

Some mammals, including humans, also express a 70-amino acid form of IGF2 that results from use of an alternative splice acceptor site that adds four codons instead of one to the equivalent

A

IGF2 E peptide - terrestrial vertebrates (89, 90, 94, 95, 96, 97 amino acids)

```
chicken       RDLSATSLAGLPALNK-ESFQKPSHAKYSKYNVWQKKSSQRLQREVPGILRARRYRWQAEGLQAAEEARAMHRPLISLPSQRPPAPRASPEATGPQE
turkey        ................-...............................................................................
duck          ........V.......SPL.ESFQKPSHA..SKYDVWQKKSS..LQREVPGIL.AKKERGEKKKEKSELMLTLNGGGVCIAHL.RIYLAKKHYFYGT
zebra finch   ............S.-........G..............R..DA.N..........V...........L.............A.....TA...K
flycatcher    ............S.-........G.....D.........R..DA.N..Q................K.L.............-A.G.QK
turtle        ....SS..VVF...S.-DG....L.......D...........G..S...............S..TKV-..........K..VVQV.S.TSSTRK
Anole lizard  ....S...VV......-DP...........DI...........G..N.H..HR.........ES..SKI-....MV..T.K.LVVQTTS.TS.S.K
frog          ..V.TAPSTAI.P...QDLYH.H.HT.S...DI..R..IH..R.G..A.V...Q..LLMQQAEES.Q.L-S....TT..IT..LHLQQTS.PSLN
```

B

IGF2 E peptide - fish (86, 93, 96-98, 103 amino acids)

```
tetraodon     RDVSATSLQVVPVMP---ALKQEVPRKQHVTVKYSK--YEVWQRKAAQRLRRGVPAILRAKKFRRQAEKIKAQEQTVF-HRPLISLPSKLPPVLLT-TDSYVNHK
fugu          ..........I....---.V............H..--.....................................-.....................-....I...
stickleback   ..........I.M..---.....................--.....................-......RR....E........A..-.....................A-..DSL...
medaka        ..........I.M..---.Q........PM..R.GD--NKA...T..............S.......G.M....EA..-.....................S.A-..KF....
cod           ..A..SA..GI.M..---P.....QK....NN.L.S--YA..D..........I....K.R........AR.KA.EQT-.....................S-........-
cave fish a   ....G.....I..LQ---T.HKDGT-.KPIN..FT.--.....Q..-.........N....RN...E...AQ...GADI-....MTV.NQR.AM.PP-AH.HAP.N
cave fish b   ..........ISM..---...PD....-.........--.DQ...R........I.....SR..............AN.-.K............WHP-.ED..IP.
tilapia       ..........I....---.......K..........--.....................R.YK.H......K..AI.-.....................-..NF.S..
Amazon molly  ....SSSM.GM....---.....A...P........--.....................................K..AI.-.....................H.-M.NF....
platyfish     ....SSSM.SM....IMPT....AA..P...M..P.SG..L.DKN..R.....................K..AI.-..S..N.........H.-..NF....
zebrafish a   ....S.....F..SQ----..H-----.DTIN.....--.....Q...........S..L.R.....M...QDE...S.-....MT..NRQ.AIVPH-VQISTSR.
zebrafish b   ..........I....---.....-.........--.D.............I..............R......LLH-.....T.......I..P-.EN..S..
spotted gar   ....S....GI.AL.---...-.A...PL-......YD--.............I......R........V.....LL.-.K...T.......AVQSS.EKS.S..
coelacanth    ..LTSS......AVN------KD.F..TSIAR-...--.DW....P.......L.S.A..G.L..L.RARRPLIARPSR.PFTARARPQRY.RRE
lamprey       none
```

Figure 12. Alignments of vertebrate IGF2 E peptides. *A*, amino acid sequences of the IGF2 C-terminal E peptide in eight terrestrial vertebrates in *single-letter code*. Differences are shown, and identities are depicted by *dots*, and a *dash* indicates no residue. *B*, amino acid sequences of the C-terminal E peptide in 12 fish species in *single-letter code*. Differences are shown, and identities are depicted by *dots*, and a *dash* indicates no residue. No E peptide has been defined for lamprey (= *none*). *A* and *B*, *dashes* have been placed to maximize alignments.

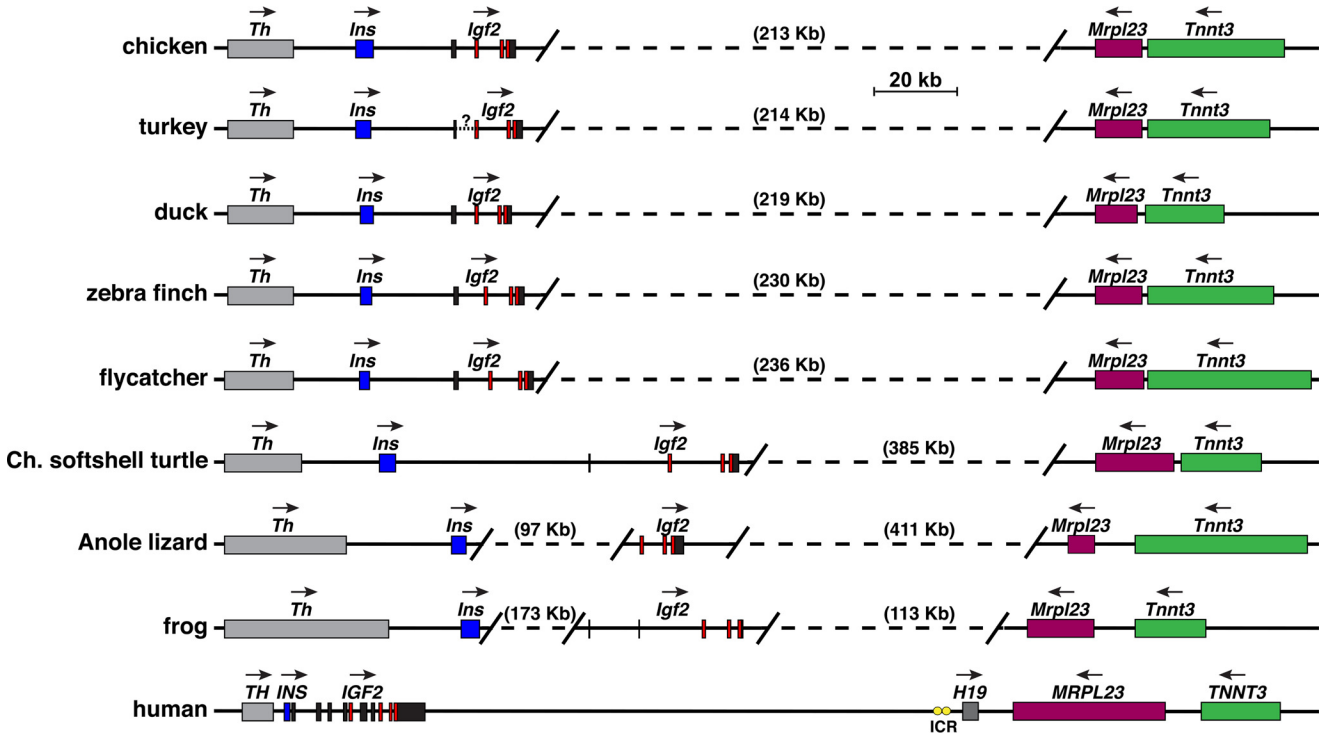

Figure 13. *Igf2* gene and locus in terrestrial vertebrates and human. Diagrams of chicken *Igf2*, seven other terrestrial vertebrate *Igf2* loci, and the human *IGF2–H19* locus are shown. For *Igf2* and *IGF2*, individual exons are depicted as *boxes* (*black* noncoding, *red* coding). Other genes are shown as *single boxes* and include *TH/Th*, *INS/Ins*, *H19*, *MRPL23/Mrlp23*, and *TNNT3/Tnnt3*. A *horizontal arrow* labels the direction of transcription for each gene. *Yellow ovals* depict the ICR located 5′ to human *H19*. *Scale bar* is shown. *Angled parallel lines* indicate discontinuities, with the distances being spanned in *parentheses*.

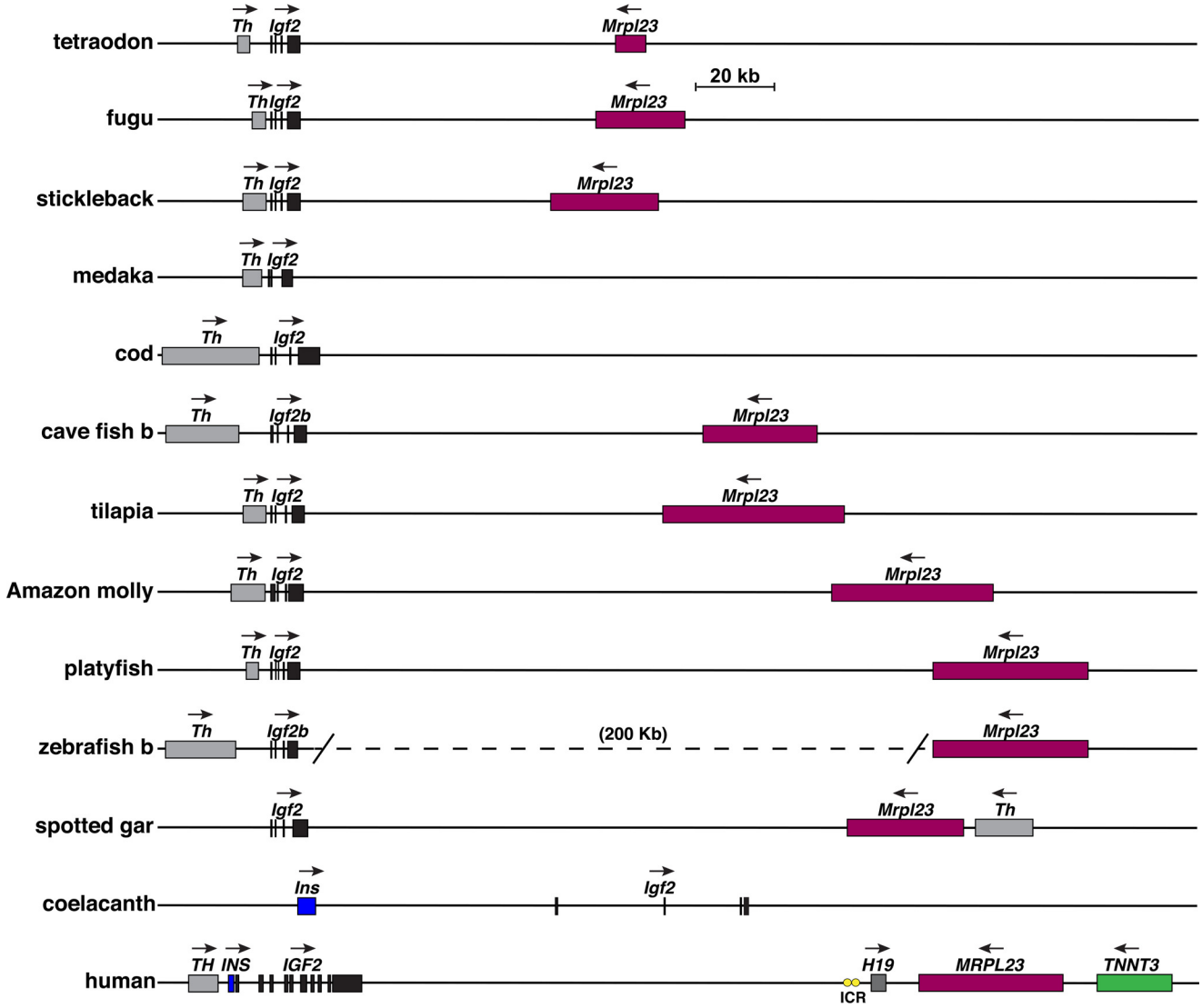

Figure 14. *Igf2* gene and locus in fish and human. Diagrams are shown for the tetraodon *Igf2* locus, cave fish, and zebrafish *Igf2b* loci, nine other fish *Igf2* loci, and the human *IGF2–H19* locus. For *Igf2* and *IGF2*, individual exons are depicted as *boxes*. Other genes are indicated as *single boxes* and include *TH/Th*, *INS/Ins*, *H19*, *MRPL23/Mrlp23*, and *TNNT3/Tnnt3*. A *horizontal arrow* shows the direction of transcription for each gene. *Yellow ovals* depict the ICR 5′ to human *H19*. *Scale bar* is shown.

of the 5′ end of human *IGF2* exon 9, leading to an IGF2 with a longer C domain (15 residues instead of 12 (17)). This longer protein binds to the IGF1 receptor with lower affinity than the 67-residue human IGF2 (76). Although there does not appear to be alternative RNA processing in fish *Igf2* genes, the IGF2 protein also has a 15-residue C domain (Fig. 10*B*). It would be of interest to learn whether a longer C region lowers the affinity of IGF2 for the IGF1 receptor in different fish species and to determine whether the modification of a shorter C domain generally enhances this ligand–receptor interaction in mammals.

Other features of IGF2 protein precursors are similar between nonmammalian vertebrates and mammals. In all species studied, the IGF2 progenitor contains a C-terminal extension or E peptide (Fig. 12) that is cleaved by a post-translational proteolytic processing step (4, 54). IGF1 precursors in both mammals and nonmammalian vertebrates also contain E peptides that are more divergent than other parts of the protein (49, 75), as is seen here with IGF2 (Fig. 12 and Tables 5 and 6). In many mammals (54) and in four of the terrestrial vertebrates analyzed here, *Igf2* mRNAs encode two alternative signal peptides (Fig. 11*C*). One is of a typical length for secreted proteins, 23 amino acids in terrestrial vertebrates and 24 residues in many mammals (52–54), whereas the other is substantially longer, 53–62 amino acids in these four vertebrates (Fig. 11*B* and Table 5), and 80 residues in humans (18). In fish, the lone IGF2 signal sequence is of intermediate length, 36–53 amino acids, and in nearly all species it contains an internal in-frame methionine residue (Fig. 11). As there is no experimental evidence in any nonmammalian vertebrate addressing IGF2 biosynthesis, the methionine or signal peptide responsible for initiating protein translation is unknown.

Improving gene quality in genome databases

Publicly available genomic repositories contain extensive data on different genes from many animal species, yet as shown

here, much of the information for *Igf2* in vertebrates is incompletely or incorrectly annotated. This problem does not appear to be uncommon, as similar deficiencies have been shown for *Igf1* in both mammals and nonmammalian vertebrates (49, 75). It is likely that other genes in these databases also are not described accurately, and it suggests that a concerted effort is needed to improve these data for the general benefit of the scientific community and to accelerate future discoveries. One way to accomplish this goal would be to query different RNA-Seq libraries for transcripts that map to portions of specific genes, as illustrated here for the potential 5′ and 3′ ends of *Igf2* genes from a number of species, and for exon 1 and exon 1a in frog (Figs. 1, 3, and 5–7). A similar strategy also could be used to identify intron–exon and exon–intron junctions and to determine the relative prevalence of alternative RNA splicing (Fig. 3*C*).

Final comments

Conservation of components of the *Igf2* gene and locus and the similarity of IGF2 among mammals and nonmammalian vertebrates suggest that an IGF2 was present in a common vertebrate ancestor (77, 78). Aspects of the biology of IGF2 have been maintained for ~500 Myr of speciation, an idea that with further investigation may lead to new insights into the comparative biology of IGF2 regulation and actions.

Experimental procedures

Database searches and analyses

Vertebrate genomic databases were accessed within the Ensembl Genome Browser (http://www.ensembl.org/) and the UCSC Genome Browser (https://genome.ucsc.edu).[3] *Igf2* cDNA sequences were extracted from the NCBI nucleotide data resource (chicken, XM_015286525; turkey, AY829236; duck, JQ819263; zebra finch, NM_001122966; frog, NM_001113672; cod, HQ263172; medaka, XM_023956176; tilapia, NM_001279643; zebrafish, NM_131433, BC085623; and AF194333 for *Igf2a*, and AF250289 for *Igf2b*). After the chicken *Igf2* gene was fully mapped, genome database queries were performed with chicken *Igf2* exons and adjacent DNA segments for terrestrial vertebrates (*Gallus gallus*, genome assembly Gallus_gallus-5.0), using BlastN under normal sensitivity (maximum e-value of 10; mismatch scores: 1,–3; gap penalties: opening 5, extension, 2; filtered low complexity regions, and repeat sequences masked). Genome assemblies from the following species were examined (Table 1): Anole lizard (*Anolis carolinensis*, AnoCar2.0); chicken (*G. gallus*, Gallus_gallus-5.0); Chinese softshell turtle (*Pelodiscus sinensis*, PelSin_1.0); duck (*Anas platyrhynchos*, BGI_duck_1.0); flycatcher (*Ficedula albicollis*, FicAlb_1.4); frog (*Xenopus tropicalis*, JGI 4.2); turkey (*Meleagris gallopavo*, Turkey_2.0.1); and zebra finch (*Taeniopygia guttata*, taeGut3.2.4). Similarly, for aquatic vertebrates, initial genome database queries were performed with chicken *Igf2* or with zebrafish *Igf2a* and *Igf2b* gene segments (*Danio rerio*, genome assembly GRCz10) and then with tetraodon *Igf2* exons (*Tetraodon nigroviridis*, genome assembly TETRAODON8), as these latter provided more complete recognition of different fish species. Genome assemblies from the following species were examined (Table 3): Amazon molly (*Poecilia formosa*, PoeFor_5.1.2); cave fish (*Astyanax mexicanus*, AstMex102); cod (*Gadus morhua*, gadMor1); coelacanth (*Latimeria chalumnae*, LatCha1); fugu (*Takifugu rubripes*, FUGU 4.0); lamprey (*Petromyzon marinus*, Pmarinus_7.0); medaka (*Oryzias latipes*, HdrR); platyfish (*Xiphophorus maculatus*, Xipmac4.4.2); spotted gar (*Lepisosteus oculatus*, LepOcu1); stickleback (*Gasterosteus aculeatus*, BROAD S1); tetraodon (*T. nigroviridis*, TETRAODON 8.0); tilapia (*Oreochromis niloticus*, Orenil1.0); and zebrafish (*D. rerio*, GRCz10). Data from the human *IGF2* locus were obtained from GRCh38 (*Homo sapiens*). In all species, the highest scoring results mapped to components of the respective *Igf2* gene. Amino acid sequences of proteins were obtained from GENCODE/Ensemble databases, the NCBI Consensus CDS Protein Set (https://www.ncbi.nlm.nih.gov/CCDS/), and the Uniprot browser (http://www.uniprot.org/); when not available, DNA sequences were translated with assistance of SerialCloner1.3 (*e.g.* long signal peptide for Chinese softshell turtle). Potential promoter sequences were examined using Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/) (79), the UC Berkeley Neural Network Promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html) (80), and CNNPromoter.[3] Phylogenetic relationships among IGF2 proteins were defined using the MUSCLE 3.8.31 and PhyML 3.1/3.0 aLRT programs from Phylogeny.fr (http://www.phylogeny.fr/index.cgi) (81).[3] In these analyses, the G-blocks program was employed to remove poorly conserved regions from further consideration. As a result, the first 2–5 amino acids from the N terminus of IGF2 and the C-region were eliminated during curation of the multiprotein alignments prior to construction of the phylogenetic tree (Fig. 10*C*). RNA-Seq information was extracted from the Sequence Read Archive of the National Center for Biotechnology Information (SRA NCBI; www.ncbi.nlm.nih.gov/sra) by querying the following datasets with different 60-bp fragments from the respective *Igf2* genes: chicken, SRX3729588 (female liver), SRX2704299 (male kidney), SRX3566521 (male spleen), and SRX4038245 (male skeletal muscle); turkey: SRX570328 (pooled liver), SRX696650 (male spleen), and SRX696577 (male skeletal muscle from thigh); duck: SRX026110 (liver), SRX3475267 (male kidney), SRX849868 (male spleen), and SRX865197 (male skeletal muscle); zebra finch: SRX2334149 (spleen) and SRX1299467 (skeletal muscle); Anole lizard: SRX3436882 (liver), SRX191161 (kidney), and SRX191163 (skeletal muscle); frog: SRX2704323 (male liver), SRX2704322 (female liver), SRX191166 (kidney), and SRX191168 (skeletal muscle); cave fish: SRX2533243 (liver) and SRX1043997 (skeletal muscle); cod: SRX1044010 (liver) and SRX1044009 (skeletal muscle); coelacanth: DRX001730 (skeletal muscle); fugu: SRX4020085 (liver), SRX2413542 (slow skeletal muscle), and SRX2413433 (fast skeletal muscle); medaka: SRX661040 (liver) and SRX661039 (skeletal muscle); platyfish: SRX031881 (whole embryo); spotted gar: SRX661019 (liver), SRX661018 (skeletal muscle), and SRX661023 (whole embryo); stickleback: SRX2712198 (liver) and ERX1322263 (skeletal muscle); tetraodon: ERX1054374 (whole embryo at 30% epibody); tilapia: SRX1257756 (liver) and SRX790855 (skeletal muscle); and zebrafish: SRX3830285 (liver) and SRX2011208 (skeletal muscle). Results in text, tables, and fig-

[3] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

ures are presented as percent identity over entire query regions, unless otherwise specified.

Experimental strategy

Naming conventions adopted here include the abbreviation "*Igf2*"' for all genes and mRNAs except for human, for which "*IGF2*" is used, and "IGF2" for all proteins. An initial assessment of nonmammalian vertebrate *Igf2* loci, genes, and potential transcripts within Ensembl and UCSC genome browsers revealed that most genes were simpler than human *IGF2* or mouse *Igf2*. However, very few gene assignments appeared to have taken into account available published experimental data. For example, in chicken *Igf2*, the two genomic databases showed three exons, but a comparison with an *Igf2* cDNA from the NCBI nucleotide data resource (XM_015286525) suggested that an additional exon existed. Also, genome databases showed that tetraodon *Igf2* consisted of 4 exons, with the first exon beginning with the ATG codon for the IGF2 precursor and the last exon ending with the TGA translational stop codon, clearly demonstrating that the gene had been incompletely defined. Thus, primary goals were to characterize all genes as completely as possible and then to interpret these more extensive datasets. An iterative process was developed that began with the exon assignments for all vertebrate *Igf2* genes as defined in Ensembl and UCSC browsers. Depending on the species, these assignments were based on the different analytical approaches that had been used to characterize each specific genome (see Table S1). Next, the chicken *Igf2* gene was characterized by a combination of steps that included mapping the gene with its cDNAs and assessing 5′ and 3′ ends by querying RNA-Seq libraries with presumptive exon fragments (Fig. 1). BlastN searches then were conducted against all other terrestrial vertebrate genome assemblies for homologous genomic regions using the chicken *Igf2* gene fragments as queries. These latter results were mapped to each vertebrate *Igf2* locus and were followed by secondary searches relying on cDNAs, gene components from other species, and RNA-Seq libraries. An analogous approach was used for fish *Igf2* genes. BlastN searches were conducted against all 13 genome assemblies for homologous genomic regions using segments of chicken *Igf2* and zebrafish *Igf2a* and *Igf2b* genes as queries. Because limited information was obtained, subsequent BlastN searches were performed using tetraodon *Igf2* exons, after the 5′ and 3′ ends of the gene had been mapped using RNA-Seq files from SRA NCBI. Results of each series of genome searches then were mapped to each fish *Igf2* locus and were followed by secondary searches relying on cDNAs or gene fragments from other fish species and tertiary mapping of potential 5′ and 3′ ends by screening RNA-Seq files from SRA NCBI. Through these steps, all vertebrate *Igf2* genes were defined more completely in most species than had been annotated in either Ensembl or UCSC genome browsers.

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