Early developmental expression of two insulins in zebrafish (Danio rerio)

Madhusudhan R. Papasani,1 Barrie D. Robison,2 Ronald W. Hardy,1 and Rodney A. Hill1

Departments of 1Animal and Veterinary Science and 2Biological Science, University of Idaho, Moscow, Idaho

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Papasani, Madhusudhan R., Barrie D. Robison, Ronald W. Hardy, and Rodney A. Hill. Early developmental expression of two insulins in zebrafish (Danio rerio). Physiol Genomics 27: 79–85, 2006. First published July 18, 2006; doi:10.1152/physiolgenomics.00012.2006.—We have cloned a second insulin gene in zebrafish and studied temporal and spatial expression of two zebrafish insulin genes. Zebrafish insulin-a (insa) and -b (insb) mRNAs are derived from two different DNA contigs on chromosomes 5 and 14, respectively, representing two different insulin genes. Real-time PCR studies suggest that insa is a maternal and also a postzygotic transcript. insa was observed at 1 h postfertilization (hpf) and was rapidly degraded by 6 and 12 hpf but induced at 24 hpf (i.e., after pancreas formation). Expression levels at 24 hpf were ~220-fold higher than at 6 hpf and were significantly different from earlier time points. At 72 hpf (at time of hatching), zebrafish insa mRNA levels tended to be higher than at 24 hpf and were ~727-fold higher compared with 6 hpf. This further increase in insa expression may be one of the many rapid physiological changes associated with hatching. insb expression was observed from 1 hpf and was significantly decreased from 12 hpf onward. Its expression levels at 12 and 24 hpf were approximately twofold and sixfold lower, respectively, compared with expression at 6 hpf. insb expression levels at 48 hpf were significantly lower than at 24 hpf but not different from 72 hpf. Expression levels at 72 hpf were ~61-fold lower than at 6 hpf. In situ hybridization studies showed insb expression in proliferating blastomeres at 3 and 4 hpf. At later time points, insb expression was restricted to the brain and pancreas (24 and 48 hpf). insa expression was observed in the pancreas at 24 and 48 hpf. Expression of insb in blastomeres and head suggests that insb could be acting as a pro-growth, survival, and neurotrophic factor during development. Pancreatic insa and insb may both be involved in regulation of glucose homeostasis as in mammals.

Insulin; gene duplication; polymerase chain reaction

IT IS BECOMING CLEAR that single gene duplication and whole genome duplication have been typical rather than atypical events in the evolutionary history of many species. Two insulin genes have now been identified in rat, mouse, Xenopus laevis, and channel catfish (5, 18, 20, 32). It was hypothesized that the gene duplication events in rodents were different from those occurring in X. laevis and channel catfish (2, 25, 33). The two X. laevis preproinsulins are more similar to each other than the other species and resulted from an insulin gene duplication that occurred during amphibian radiation (33). Recent evidence suggests that a genome duplication event occurred before teleost radiation in ray-finned fish lineage, after divergence from lobe-finned fishes (2, 25). Phylogenetic analysis using fish insulin genes suggests that insulin gene duplication occurred before the divergence of fugu and zebrafish, suggesting that insulin gene duplication occurred as part of a whole genome duplication event that occurred before teleost radiation (15).

Insulin is generally believed to be exclusively expressed by pancreatic β-cells. It has multiple regulatory roles including mediation of glucose homeostasis. However, there is increasing evidence of insulin expression in tissues other than the pancreas. Insulin expression was observed in the central nervous system of adult humans and in embryos of mice, rats, chickens, and X. laevis (8, 9, 22, 35). Insulin-like molecules have been reported in bacteria, protozoa, and fungi and also in invertebrates, and none of these species has pancreatic β-cells (17). These observations suggest that insulin appeared very early in evolution and has extrapancreatic origins. During the course of evolution, insulin might have acquired multiple functions such as regulation of cell survival, cell proliferation, and neuronal development and glucose metabolism.

The metabolic functions of insulin have been well investigated. However, information about its developmental roles is limited. Mice deficient in insulin have shown reduced body size at the time of birth (12), and a similar phenotype was observed in insulin receptor-deficient pups (1, 10). In chickens, insulin and its receptors were shown to be expressed in all stages of early embryonic development, from blastoderm through gastrulation, neurulation, and organ formation (11, 22). Clearly, this is before the development of the circulatory system or pancreas formation. The authors of these papers hypothesized that the insulin system has roles in cell proliferation, differentiation, and cell survival during development and that these actions are probably mediated by an autocrine/paracrine mechanism rather than in an endocrine manner. These observations clearly implicate the insulin-signaling axis in development.

Improved understanding of the insulin-signaling cascade during development may be very important in aiding our understanding of the drivers of the development of insulin resistance in humans in later life. It appears that dysfunctional insulin signaling during development may be a predisposing factor in the development of impaired glucose tolerance and Type 2 diabetes in later stages of life (12).

An early genome duplication event in teleosts appears to have resulted in retention of perhaps 20% of functional genes as co-orthologs of present-day human genes. Knowledge of entire genomes of fishes is now yielding these interesting discoveries. For example, when 74 genes on human chromosome 17 were mapped to putative orthologs in zebrafish, at least 15 (20%) were present as duplicate genes (26). It is thought that pleiotropic functions of a single copy gene in humans may be portioned between these two ancestral co-orthologs (reviewed in Ref. 25). The presence of two co-orthologs makes zebrafish an excellent model. Expression analyses followed by loss-of-function studies provide the potential to discover novel functions of a single copy gene that are masked in humans because of pleiotropy. The presence of
two insulin receptors (40) and also two insulins, plus external development and optical clarity of zebrafish embryos, provides additional tools to investigate the developmental physiology of the insulin system. This may lead to discovery of novel functions of the insulin axis that have not been observed in other model organisms. Toward this goal, we have cloned a second insulin gene in zebrafish and studied temporal and spatial expression of two insulins during early developmental stages.

MATERIALS AND METHODS

Animals

Adult zebrafish were reared on a 14:10-h light-dark photoperiod cycle, at 28.5°C (39). Zebrafish were fed twice daily with either Tetramin flake food or brine shrimp cysts (Inve Aquaculture). The embryos were obtained by natural crosses. The fertilized eggs were collected and grown at 28.5°C in fish tank water.

Identification of Zebrafish Insulin B

Analysis of sequence databases by Irwin (15) revealed the presence of a genomic sequence (GenBank accession no. BX088535) similar to vertebrate insulin sequences. Further in silico analysis identified three expressed sequence tags (ESTs; GenBank accession nos. AL922691, BI885859, and CD599416) containing the putative sequence of zebrafish insulin-b (insb). This information, together with the genomic DNA sequence, was used to identify the intron-exon structure of zebrafish insb (15). On the basis of these published sequences, we designed gene-specific primers to clone and confirm the predicted insb sequence.

RT-PCR and Sequencing

Total RNA was extracted using the Trizol method (Invitrogen) from 6-h postfertilization (hpf) zebrafish. Five micrograms of RNA were reverse transcribed to cDNA using oligo(dT) and superscript III first-strand synthesis system (Invitrogen), following the manufacturer’s protocol. PCR was conducted on cDNA, using gene-specific primers (forward primer 5'-ACTCTCAGACACTTGCTC-3' and reverse primer 5'-TTGCTGGACCTTTACTAC-3') to amplify a partial cDNA using Platinum Taq polymerase (Invitrogen). The PCR profile included initial denaturation at 95°C for 3 min, followed by 39 cycles with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, polymerization at 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were gel purified using an Ultra Free kit (Millipore) and ligated to pGEM-Easy vector (Promega). Top10 cells (Invitrogen) were transformed with ligated PCR products according to the manufacturer’s protocol. The transformed bacteria were cultured, and QIAprep spin minipreps (Qiagen) were used for plasmid extraction. The purified plasmids were sequenced in both directions using SP6 (5'-TAATACGACTCAC-3') and T7 (5'-ATAATACTAC-3') and the putative signal peptide was identified using the search term “zebrafish preproinsulin” to define the locations of zebrafish insulin-a (insa) and insb on the chromosomes.

Whole Mount In Situ Hybridization

Zebrafish insa probe synthesis. A PCR product that was amplified with forward primer 5'-CCATATCCACCATTTCCGCC-3' and reverse primer 5'-CAACGGGAGGACTTAAAGGCCC-3' was cloned and sequenced, and its orientation in the plasmid was determined. The pGEM-T-Easy vector containing insa-cDNA was linearized with either SpeI (antisense) or SacII (sense). The linearized plasmid was purified by phenol-chloroform extraction. Diogoxigenin (DIG)-labeled sense and antisense riboprobes were made with SP6 and T7 polymerase, respectively, using DIG RNA labeling kit (Roche Applied Science), following the manufacturer’s recommendations. Probes were purified with NucAway spin columns (Ambion). The integrity of the probes was tested on 1% agarose gel, and their concentrations were measured using a spectrophotometer.

Zebrafish insb probe synthesis. PCR was conducted on cDNA (forward primer 5'-ACTCTTCAGACACTTGCTC-3' and reverse primer 5'-CATCTTCATACCTTCTTCGAG-3'), the PCR product was cloned and sequenced, and the orientation determined. The pGEM-T-Easy vector (Promega) containing insb-cDNA was linearized with either SphI (antisense) or SpeI (sense). DIG-labeled riboprobes were synthesized from linearized plasmids using either SP6 (antisense) or T7 polymerase (sense) as described above. DIG-labeled probes were purified, quantified, and checked for integrity as described above.

Whole Mount In Situ Hybridization on Zebrafish Embryos

To block melanization, the zebrafish embryos were treated with 0.2 mM phenylthiourea (PTU; Sigma) at 10 hpf. Embryos were collected into 4% paraformaldehyde at 3, 4, 24, and 48 h postfertilization (hpf), using a minimum of 25 embryos/time point, and fixed overnight. The embryos were dechorionated manually after washing twice with phosphate-buffered saline plus 0.1% Tween 20 (PBT20), dehydrated with a series of PBT-methanol solutions (3:1, 1:1, 1:3) at room temperature for 10 min each, and finally stored in 100% methanol overnight at −20°C. The embryos were rehydrated with a series of PBT-methanol solutions (1:3, 1:1, 3:1) and were proteinase K (Fermentas) digested (10 μg/ml proteinase K for 2 min for 3- and 4-hpf, 15 min for 24-hpf, 20 μg/ml for 18 min for 48-hpf embryos). Prehybridization followed by hybridization was conducted with a probe concentration of 1.3 ng/μl at 62°C overnight. After hybridization, the embryos were washed in a series of solutions: four times in 50% formamide-2× 0.15 M NaCl, 0.015 M Na citrate, 0.1% Tween 20 (SSCT), once in 2× SSCT, and twice in 0.2× SSCT, all at 62°C for 20 min each.

The embryos were blocked in a blocking buffer [150 mM malic acid, 100 mM NaCl (pH 7.5), 2% blocking reagent (Roche), and 0.1% Tween 20] overnight at 4°C, followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody (1:3,000 dilution) in blocking solution overnight at 4°C. The embryos were washed with blocking buffer four times (30 min each) and three times in alkaline phosphatase buffer, 100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, and 1 mM levamisol (15 min each). The embryos were allowed to develop color in 0.35 g/ml nitro-blue tetrazolium chloride and 0.175 μg/ml 5-bromo-4-chloro-3-indolyphosphate-p-toluidine salt (Roche) in alkaline phosphatase buffer for 1.5 h. After the embryos developed color, the coloring reaction was stopped by washing twice with PBT and then fixing in 4% paraformaldehyde. The digital pictures were taken on a Nikon FMZ microscope.

Quantitative RT-PCR

Quantitative RT-PCR (QRT-PCR) analysis was done to quantify the developmental expression of insa and insb. For each independent experiment, 40 embryos per developmental stage were pooled for total RNA extraction using the Trizol method. Five micrograms of total RNA at each stage were reverse transcribed using oligo(dT) as described above. A 1:10 dilution of cDNA was used for each stage for PCR, using SYBR Green master mix (MJ Research) and gene-specific primers: insa forward primer 5'-TAAGCACTAACCCAGGCACA-3', reverse primer 5'-GATTAGGAGGAAAGGAAC-3'; insb for-
ward primer 5'-ACTCTTCACAGACTCTGCTC-3', reverse primer 5'-ACAGATGCTGGAGAGGAAA-3'. The housekeeping gene amplicon, elongation factor-1α (forward primer 5'-GTACTCTTCCTGTGATGCC-3', reverse primer 5'-GTACAGTTCCAATACCTCA-3'), was used to normalize the expression of insa and insb at each stage. Each sample was run in triplicate wells on the DNA Engine Option 2 system (MJ Research). No-RT blank controls were run on each plate. The following protocol was used: initial denaturation at 95°C for 3 min followed by the 95°C for 10 s, 72°C for 20 s, repeated for 40 cycles, with one cycle of a finishing program (72°C for 10 min). After amplification, melting curve analysis was done from 60 to 92°C, read every 0.2°C, to determine that there was only one product. insa and insb amplicons were electrophoresed on 1% agarose gels and stained with ethidium bromide. A single band was observed on the gel for each amplicon, and the bands were excised and purified using a Ultrafree kit (Millipore), then ligated to the pGEMT-Easy vector (Promega). TOP10 cells (Invitrogen) were transformed with ligated PCR products according to the manufacturer’s protocol. The transformed bacteria were cultured, and QIAprep spin minipreps (Qiagen) were used for plasmid extraction. The purified plasmids were sequenced in both directions using SP6 and T7 primers. The transformed bacteria were cultured, and QIAprep spin minipreps (Qiagen) were used for plasmid extraction. The purified plasmids were sequenced in both directions using SP6 and T7 as primers. The gene expression data were quantified using the $2^{-\Delta\Delta C_t}$ (Ambion) method relative to the 6-hpf time point.

**Statistical Analyses**

Data were analyzed using a Tukey’s pairwise comparison and ANOVA with the Systat software package. Differences were determined as significant when $P < 0.05$. The original normalized cycle threshold ($\text{Ct}$) values ($\text{Ct}_{\text{interest}} - \text{Ct}_{\text{housekeeping}}$) were used for statistical comparisons. Note (see Fig. 4): fold changes in gene expression are presented as means ± SD. For QRT-PCR of insa and insb, triplicate assays of two and three different biological replicates were run, respectively. In addition, independent studies were performed and reported in Supplemental Fig. S1 (the online version of this article contains the supplemental materials).

**RESULTS**

**Zebrafish insb Sequence**

With the use of gene-specific primers (15), RT-PCR was conducted to obtain a partial cDNA sequence of zebrafish insb (Fig. 1). The partial nucleotide sequence is 498 bp (GenBank accession no. NM_001039064), and it codes for an entire coding sequence of 107 amino acids. This sequence shows a 22-amino acid signal peptide, a 21-amino acid A chain, a 30-amino acid B chain, and a 30-amino acid C-peptide domain (Fig. 2). This sequence has dibasic cleavage sites (KR, RR) as well as six cysteine residues, suggesting endoproteolytic cleavage and protein processing similar to insulin in other species (36, 37). Alignment of the deduced amino acid sequences of insa and insb (Fig. 3) revealed ~51% identity between the two peptides. There are 55 identical residues, 21 conservative substitutions, and 16 partially conservative substitutions between these two peptides. insa and insb show greater conservation across the A-chain and B-chain but less conservation in the C-peptide and signal peptide domains. The comparison of two insulins of zebrafish with human insulin at the amino acid level revealed ~36% identity, and there are 39 identical residues, 14 conservative substitutions, and 13 partially conservative substitutions across these three insulins (Fig. 3).

Greater conservation was observed in the A-chain and B-chain across human and zebrafish insulins (Fig. 3).

**Chromosomal Location of Zebrafish insa and insb**

The Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query) search for zebrafish preproinsulin showed a gene identification (ID; no. 30262) that is identical to insa mRNA (GenBank accession no. NC_071116). This mRNA is encoded by a genomic contig (GenBank accession no. NC_007125) that is present on chromosome 5. On the other hand, gene ID no. 561479 is identical to insb mRNA (GenBank accession no. NC_007125) that is present on chromosome 14. The presence of two insulin genes on two different chromosomes provides evidence for two nonallelic insulin genes in zebrafish that presumably resulted from a genome-wide duplication event that occurred in ancient teleosts.
Temporal Expression of Zebrafish insa and insb

Gene expression levels are reported with reference to 6 hpf. The gene expression analyses using QRT-PCR for insa revealed that it was present at 1 hpf, and its expression level was ~48-fold higher compared with that at 6 hpf. The expression level was greatly decreased at 12 hpf (P = 0.01). We observed induction of insa at 24 hpf; it was ~220-fold higher than its expression at 6 hpf (P < 0.001). The levels were further increased (P < 0.001) at 72 hpf (i.e., at the time of hatching) by ~727-fold compared with 6 hpf. In addition, expression at 72 hpf tended to be greater than at 24 hpf (P = 0.095) (Fig. 4A). Independent biological replicate experiments confirmed these results, the pattern and magnitude of insa expression being closely reflected (Supplemental Fig. S1A).

On the other hand, the highest level of insb expression was observed at 1 hpf. It declined over the following time points, being significantly lower than at 1 hpf from 12 hpf onward. The expression levels of insb at 12 and 24 hpf were approximately two- and sixfold lower, respectively, than at 6 hpf. The levels at 48 and 72 hpf were further reduced compared with those at 24 hpf (P = 0.018 and P < 0.001, respectively). The levels at 48 and 72 hpf were 30- and 61-fold lower, respectively, compared with 6-hpf levels (for both, P < 0.001) (Fig. 4B). Independent biological replicate experiments also confirmed these results, the pattern and magnitude of insb expression being similar (Supplemental Fig. S1B).

Spatial Expression of Zebrafish insa and Zebrafish insb

Tissue-specific expression of insa and insb was examined during early development. Gene expression was detected using DIG-labeled RNA probes and in situ hybridizations on embryo whole mounts. We analyzed insb expression at 3 and 4 hpf and also at 24 and 48 hpf. We observed insb expression in all proliferating blastomeres at 3 and 4 hpf (Fig. 5, Supplemental Fig. S2); however, at both 24 and 48 hpf, insb expression was limited to only the head and pancreas (Fig. 6; see also Fig. 7, C and D, Supplemental Fig. S3B). Whole mount in situ hybridization analyses of insa expression at 24 and 48 hpf showed that, at both time points, expression was restricted to the pancreas (Fig. 7, A and B, Supplemental Fig. S3A).

DISCUSSION

Early developmental expression of two insulins in zebrafish was demonstrated using QRT-PCR and in situ hybridization. insa was detected to be expressed as a maternal transcript, as evident from its expression at 1 hpf. QRT-PCR analyses showed that insa mRNA was rapidly degraded in a time-dependent manner by 6 and 12 hpf, and its expression was induced at the subsequent time point of 24 hpf (Fig. 4A, Supplemental Fig. S1A) and appeared to be expressed in the pancreas. The induction of insa after pancreas formation is in agreement with previous data (4), where insa expression was detected at the 14 somite stage (16 hpf), using in situ hybridization. By 24 hpf, all insulin-expressing cells clustered to form the pancreatic islets (16). In the present study, insa expression was further increased at the time of hatching. These data were corroborated by in situ hybridization analysis, as we observed insa expression in the pancreas at both 24 and 48 hpf, in agreement with existing literature (19).

The temporal expression of insb was highest at 1 hpf, suggesting that it is also present as a maternal transcript. The decreasing gene expression pattern of insb was interesting, and we hypothesize that, as the embryo develops, the expression of insb becomes cell lineage specific and its contribution to the total body mRNA pool decreases. This was supported by in situ hybridization data, where insb was shown to be expressed in all cells at 3 and 4 hpf (Fig. 5, Supplemental Fig. S2), whereas its expression was cell lineage specific in the head and pancreas at both 24 and 48 hpf (Fig. 6, Fig. 7, C and D, and Supplemental Fig. S3B).

The temporal and spatial expression patterns of the two insulin genes were developmentally regulated. Spatial expression patterns of insa and insb were different except that both were expressed in the pancreas. We detected insb mRNA from immediately after fertilization through blastula, gastrula, and neurulation. Our QRT-PCR data, especially the detection of insa and insb mRNAs at 1 hpf, suggest that both insulins are maternal transcripts at this developmental time point. Nevertheless, maternal transcripts are known to regulate early embryonic development (6). This pattern of expression was also observed in X. laevis, as both insulin were expressed in the
mature oocyte, although the roles for the two insulins in embryogenesis were not defined (32).

In the present study, we have observed abundant insb expression at 3 and 4 hpf in proliferating blastomeres, suggesting that it plays a very important role at early developmental stages. insb may act as a cell proliferative and anti-apoptotic factor, as it appears that insulin is involved in the upregulation of cell division and in the rescue of cells from apoptosis (3, 13, 21, 34). We have observed partial differences in the spatial expression of the two insulins after pancreas formation. Both insa and insb were expressed in pancreas, and insb was expressed in the head. The pancreatic insulins are likely to regulate glucose metabolism via endocrine pathways. The expression of insb in the head may represent neuronal expression and thus suggests a role in brain development. There is strong evidence for both neuronal and prepancreatic expression of insulin in other species such as the mouse, rat, chicken, and X. laevis (8, 9, 22, 32). In salmon, immunoreactivity to insulin was observed in the brain, but it was reported that insulin is produced in the pancreas and then transported to the brain via the blood/cerebrospinal fluid system (24). Insulin in fish species is also considered a growth-promoting hormone (23).

Morales et al. (21) have suggested that insulin expression in chicken brain cells is involved in neuronal growth and in protecting cells from undergoing apoptosis. There are also reports suggesting the de novo synthesis of insulin in the rat nervous system. Insulin appears to have a role in increasing axonal growth by triggering the MAPK pathway, suggesting a crucial role in brain development (27–29). Recently, it was shown that insulin and insulin receptors were expressed in the brains of humans (35), and downregulation of the insulin axis was suggested to have a role in the manifestation of Alzheimer’s disease (35). In Alzheimer’s disease, hyperphosphorylated tau protein accumulates in neurons and is implicated in the process that eventually leads to cell death and lesions that correspond to dementia (7). In vitro experiments suggested that insulin and IGF-I regulated tau phosphorylation (14). The dysfunctional insulin axis leads to reduced activity of phosphatidylinositol 3-kinase and Akt, in turn activating glycogen synthase kinase-3β (GSK-3β) (30, 31). Increased activation of GSK-3β leads to hyperphosphorylation of tau (7).

Recently, two insulin receptors were identified and were shown to be expressed in the developing brain of zebrafish during embryogenesis (40). At this point, there appears to be no information about receptor specificity of insa and insb. However, it is likely that insb, which is expressed in brain and pancreas, signals via both receptors to mediate its functions in autocrine/paracrine and endocrine systems. The expression patterns suggest that both insulins have biological functions that are related to those in mammals. The metabolic functions of insulin are very well understood. However, there appears to be much to discover about insulin functions in the central nervous system. During development, insulin may act as a growth factor and increase protein synthesis, neuronal growth, and migration and also cytoskeletal protein synthesis and synapse formation (7). Impairment of the insulin system during development, insulin may act as a growth factor and increase protein synthesis, neuronal growth, and migration and also cytoskeletal protein synthesis and synapse formation (7).
development could lead to defects in growth and also brain deformities including structural changes in the central architecture. Thus further investigation of the roles of insulin in brain development and as an anti-apoptotic factor may provide greater insight into specific roles of this important pleiotropic hormone.

Sequence database searches revealed the presence of two insulins in fugu fish (15). All fish insulin sequences are phylogenetically grouped together (15). This suggests that duplication of the insulin gene occurred in the fish lineage. Fugu insulin-a and insa are more closely related and grouped together with other well-characterized fish insulins, whereas fugu insulin-b and insb are grouped together and are separated from other fish insulins, indicating that insulin gene duplication took place before teleost radiation. There are also two insulins in catfish; however, interestingly, these two peptides have identical C-peptide domains. At the amino acid level, catfish insulin-b differs from catfish insulin-a, showing only three substitutions: A-chain, 14His to 14Gln, B chain, 11Gly to 11Val, and 13Asp to 13Glu (20). However, the zebrafish insulins show considerable divergence at the amino acid level, with the A-chain, B-chain, and C-peptide showing 7, 9, and 23 differences, respectively (Fig. 3). Thus it appears that different selection pressures in these two species have contributed to the differing evolutionary trajectories of their insulin genes. The Wanda database (http://www.evolutionsbiologie.uni-konstanz.de/Wanda/) provides a valuable resource with information on duplicated fish genes and their vertebrate orthologs (38). This information provides a starting point for conducting gene expression and gene knockout/knockdown analyses to understand evolutionary consequences of gene duplication. These analyses are also likely to provide functional information about putative ancestral gene functions masked by pleiotropy in higher vertebrates (25).

N-ethyl-N-nitrosourea (ENU)-induced mutagenesis has revealed novel genes that are implicated in early endocrine pancreatic development. Some phenotypes generated in these mutants exhibited reduced insa expression (16). There may be several explanations for reduced insa expression in these models. It is possible that the process of formation of insulin-expressing cells was disrupted. A second, more direct possibility is that these mutated genes are involved in positive regulation of insulin transcript expression. Further studies that isolate and characterize the affected genes will likely lead to a greater understanding of the regulation and the functional roles of insa and insb.

In the zebrafish, the different temporal and partially different spatial expression patterns of two insulin genes may be attributed to differences in their promoters. Thus characterizing each of the insulin promoters will aid our understanding of insulin regulation and synthesis during development. Promoter analy-
sis, coupled with loss-of-function studies using morpholino oligos, may provide corroborating evidence for the developmental roles of both insa and insb.

GRANTS

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REFERENCES


