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Zebrafish *dax1* Is Required for Development of the Interrenal Organ, the Adrenal Cortex Equivalent

Y. Zhao, Z. Yang, J. K. Phelan, D. A. Wheeler, S. Lin, and E. R. B. McCabe

Department of Human Genetics (Y.Z., E.R.B.M) and Department of Pediatrics (J.K.P., E.R.B.M.), David Geffen School of Medicine at UCLA; Department of Molecular, Cell and Developmental Biology (Z.Y. S.L.), UCLA; Mattel Children's Hospital at UCLA (E.R.B.M.); and UCLA Molecular Biology Institute, Los Angeles, California 90095; and Human Genome Sequencing Center (D.A.W.), Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

Mutations in the human nuclear receptor, *DAX1*, cause X-linked adrenal hypoplasia congenita (AHC). We report the isolation and characterization of a *DAX1* homolog, *dax1*, in zebrafish. The *dax1* cDNA encodes a protein of 264 amino acids, including the conserved carboxy-terminal ligand binding-like motif; but the amino-terminal region lacks the unusual repeats of the DNA binding-like domain in mammals. Genomic sequence analysis indicates that the *dax1* gene structure is conserved also. Whole-mount *in situ* hybridization revealed the onset of *dax1* expression in the developing hypothalamus at approximately 26 h post fertilization (hpf). Later, at about 28 hpf, a novel expression domain for *dax1* appeared in the trunk. This bilateral *dax1*-expressing structure was located immediately above the yolk sac, between the otic vesicle and the pronephros. Interestingly, weak and transient expression of *dax1* was observed in the interrenal glands (adrenal cortical equivalents) at approximately 31 hpf. This gene was also expressed in the liver after 3 dpf in the zebrafish larvae. Disruption of dax1 function by morpholino oligonucleotides (MO) down-regulated expression of steroidogenic genes, *cyp11a* and *star*, and led to severe phenotypes similar to *ff1b* (*SF1*) MO-injected embryos. Injection of *dax1* MO did not affect *ff1b* expression, whereas *ff1b* MO abolished *dax1* expression in the interrenal organ. Based on these results, we propose that dax1 is the mammalian DAX1 ortholog, functions downstream of ff1b in the regulatory cascades, and is required for normal development and function of the zebrafish interrenal organ. (*Molecular Endocrinology* 20: 2630–2640, 2006)

DOSAGE-SENSITIVE SEX reversal, adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1 (DAX1), encoded by the gene *NR0B1*, is an unusual member of the orphan nuclear receptor family of transcription factors. DAX1 owes its name to its dual role in human pathology. Duplications of the 160-kb dosage-sensitive sex-reversal region in Xp21, containing the *NR0B1* gene, result in male-to-female phenotypic sex reversal (1, 2). However, mutations in human *NR0B1* gene cause X-linked AHC, a hereditary disorder of the adrenal cortex commonly manifested by early-onset adrenal insufficiency (3–6). *DAX1* is expressed in the hypothalamic-pituitary-adrenal-gonadal (HPAG) axis, *i.e.* ventromedial hypothalamic nucleus, pituitary gonadotropes, adrenal cortex, testis, and ovary (7–11). This pattern of expression reflects the phenotypic features of DAX1 deficiency, specifically AHC and hypogonadal hypogonadism.

DAX1 is conserved in gene structure and orthologs are present throughout vertebrates (3, 12–18). The *DAX1* gene has a very simple genomic structure with two exons separated by a single intron, which is located in the same relative position across species (19). Structurally, the DAX1 protein is a member of the nuclear receptor superfamily (20). The C-terminal region of this orphan nuclear receptor contains the ligand binding-like (LBL) domain common to other members of the nuclear receptor superfamily. However, DAX1 lacks the conventional DNA-binding domain; instead, its N terminus is composed of 3.5 alanine/glycine rich repeats of a 65- to 70-amino acid motif that has no known homology to any other proteins except SHP (short heterodimer partner, *NR0B2*), the only other nuclear receptor NR0B family member present in mammals (19). The 3.5 N-terminal repeat structure with an unusual DNA binding-like (DBL) domain has been conserved among mammalian DAX1s. However, in nonmammalian vertebrates, including chick (16), alligator (15), frog (17), and tilapia (18), cloned DAX1 orthologs lack these repeats.

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Abbreviations: AHC, Adrenal hypoplasia congenital; BAC, bacterial artificial chromosome; DAX, dosage-sensitive sex reversal, AHC critical region on the X chromosome; DBL, DNA binding-like; DIG, digoxigenin; dpf, days post fertilization; EST, expressed sequence tag; HPAG, hypothalamic-pituitary-adrenal-gonadal; hpf, hours post fertilization; ISH, *in situ* hybridization; LBL, ligand binding-like; mMO, mismatch MO; MO, morpholino oligonucleotides; SF1, steroidogenic factor 1; SHP, short heterodimeric partner.

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DAX1 is a transcriptional repressor of a number of other nuclear receptors expressed in the steroidogenic axis, particularly steroidogenic factor 1 (SF1) (21). DAX1 is a negative regulator of SF1-induced transactivation of many genes and inhibits the synergistic transcriptional activation of SF1 interacting with other SF1 heterodimeric partners like WT1 (21, 22). However, the exact mechanisms of DAX1 action at the different levels of the HPAG axis during normal development and adulthood are not fully understood (21).

One of the reasons for the lack of understanding of DAX1 function is the difficulty in studying mammalian embryogenesis. To better understand the roles of this gene in normal HPAG axis development, and adrenal development in particular, we investigated the zebrafish, *Danio rerio*, as a model organism, because zebrafish embryos are amenable to molecular manipulation and genetic dissection (23, 24). The adrenal cortex homolog in teleosts is called the interrenal gland, because together with chromaffin cells (counterpart of adrenal medulla), it is embedded inside the anterior part of the kidney, commonly referred to as the head kidney (25). The interrenal gland is the major site of steroid synthesis in most teleosts (26), as is the adrenal cortex in mammals (27, 28). Interrenal and adrenocortical cells both express genes encoding steroidogenic proteins, such as *cyp11a*, 3*βhsd*, and *star* (28–31).

In this study, we isolated and characterized the zebrafish *dax1* by expressed sequence tag (EST) database searching and molecular cloning. Using whole-mount *in situ* hybridization (ISH), the spatial and temporal expression patterns of the *dax1* gene throughout zebrafish embryogenesis were determined. Disruption of *in vivo* dax1 function by morpholino (MO) led to larval phenotypes that were suggestive of impaired interrenal function and very similar to *ff1b* (zebrafish *SF1* ortholog) MO-injected embryos. Injection of *dax1* MO also down-regulated the expression of steroidogenic genes, *cyp11a* and *star*. These lines of evidence strongly support the direct involvement of dax1 in zebrafish interrenal development. In addition, our gene knockdown experiments showed that *dax1* MO did not affect the expression of *ff1b*, whereas MO knockdown of *ff1b* activity abolished the interrenal expression of *dax1*, suggesting the function of dax1 downstream of ff1b in the regulatory cascades underlying normal interrenal development in zebrafish.

RESULTS

A DAX1 Homolog, *dax1*, Is Identified in Zebrafish

The C-terminal consensus sequence of known DAX1 proteins was used as a probe to search zebrafish cDNA and EST databases for homologous sequences. A single EST clone (CK029520) was identified that encoded amino acids with significant homology to the DAX1 consensus sequence. Subsequent sequencing showed that this clone contained the polyadenylation signal and poly(A) tail (Fig. 1). This putative *dax1* cDNA included the open reading frame encoding a predicted 264-amino acid protein, and a 69-bp 5′-untranslated region. The *dax1* genomic sequence containing the complete coding sequence and a 1487-bp intron was also obtained by bacterial artificial chromosome (BAC) library screening and subsequent sequencing. The position of the intron was exactly the same as that of known *DAX1* genes (19). The zebrafish *dax1* gene was composed of two exons separated by a single intron between the first and second nucleotides of the codon 185 for the highly conserved amino acid aspartate (D185) in the LBL domain (Figs. 1 and 2). We compared the amino acid sequences of the putative zebrafish dax1 with DAX1 orthologs in tilapia, frog, alligator, chick, mouse, rat, and human (Fig. 2). The predicted zebrafish dax1 protein contained the conserved 3′-LBL motif; but the 5′-region lacked the unusual repeat motif of the DBL domain in mammals. As a consequence of the truncated DBL, zebrafish dax1 contained only the third and fourth of four LXXLL motifs seen in mammals; this feature is typical of other nonmammalian DAX1 proteins (Fig. 2). Based on the alignment results, we constructed a phylogenetic tree of DAX1 proteins in vertebrates (Fig. 3). Not unexpectedly, the zebrafish dax1 is most closely related to tilapia DAX1 protein.

The similarities between the predicted zebrafish dax1 and other reported DAX1 proteins are listed in Table 1. The zebrafish dax1 shows the highest levels of identity and similarity to tilapia DAX1 (65.5% identity and 79.5% similarity) over the full-length protein sequence. It also shows more than 48% and 43% amino acid identity to other nonmammalian (frog, alligator, and chick) and mammalian (mouse, rat, human, and pig) DAX1s, respectively.

To determine whether a *dax1* isoform analogous to human *DAX1a* (32, 33) exists in zebrafish, the 1487-bp intron sequence of the zebrafish *dax1* gene was analyzed. The sequence comparison of the zebrafish *dax1* and the human DAX1 introns did not reveal conserved regions. The zebrafish *dax1* intron sequence was further searched for potential splice sites. Combined with polyA-addition signal analysis, we identified three potential splice sites, which had the potential to result in an exon 2a. RT-PCRs were then performed to amplify the original exon 2 and each potential exon 2a, respectively. RT-PCR detected the original *dax1* gene, but not any of the potential *dax1a* isoforms (data not shown). In addition, we searched the zebrafish EST database with the exon 1 sequence. No EST indicating alternative splicing was found.

dax1 Is Expressed during Zebrafish Embryogenesis

In the central nervous system, expression of *dax1* was first detected at around 26 h post fertilization (hpf) in two strips of cells extending from the midline of the rostral basal forebrain (Fig. 4, A and B). By 31 hpf, *dax1*

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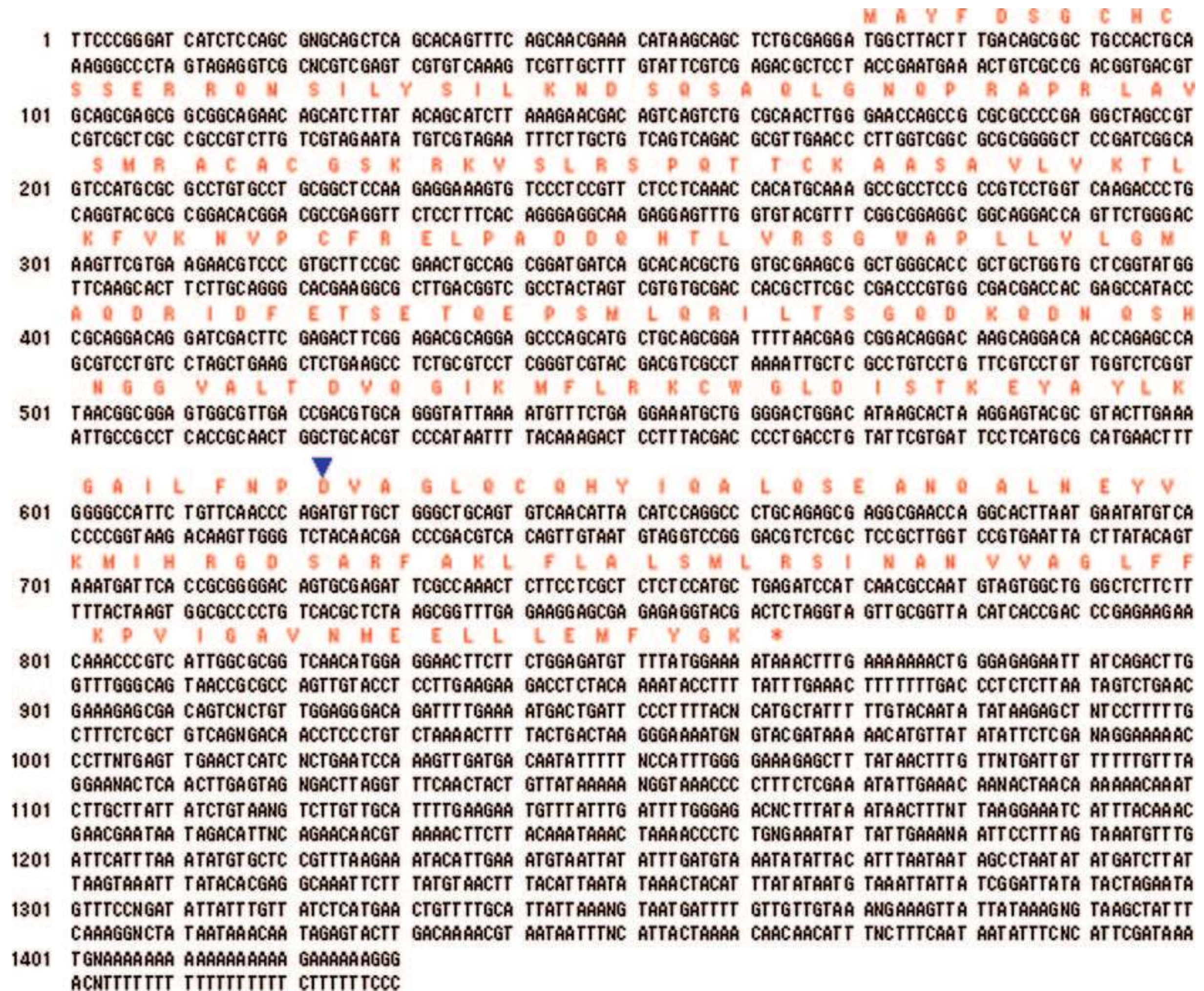

Fig. 1. Nucleotide and Amino Acid Sequences of Zebrafish dax1

The translated amino acid sequence is shown in standard *one-letter code above* the nucleotide sequence. The *arrowhead* indicates the position of the intron.

was strongly expressed in the rostral diencephalon between the eye fields (Fig. 4, C and D), immediately anterior to the pituitary, shown by double ISH with the pituitary marker *pomc* (34) (Fig. 4, E and F). Subsequent to 36 hpf, *dax1* expression in the brain was down-regulated and by 4 days post fertilization (dpf), no specific *dax1* expression could be observed in this region (Fig. 4, Q and R).

From 28 hpf, a second *dax1*-expressing domain appeared in the trunk, emerging as two clusters of cells symmetric to the embryo body midline (Fig. 4G). Expression of *dax1* mRNA in this bilateral structure peaked at around 32 hpf (Fig. 4H) and disappeared by 48 hpf. To characterize further this novel *dax1*-expressing domain, we performed double ISH of *dax1* with the interrenal marker *ff1b* (35–37) (Fig. 4, I and J), otic vesicle marker *otx1* (38, 39) (Fig. 4, K and L), pectoral fin bud marker *tbx5* (40) (Fig. 4, M and N), pronephric tubule marker *pax 2.1* (41) (Fig. 4O), and tooth germ marker *pitx2a* (42) (Fig. 4P). The bilateral *dax1*-expressing structure was clearly distinct from all five organs/tissues investigated. It was located immediately above the yolk sac, ventral to both the otic vesicle and the pectoral fin buds, caudal to the otic vesicle, cephalic to the pectoral fin buds and head kidney, and laterally adjacent to the tooth germ (Fig. 4P).

Weak and transient expression of *dax1* was observed in the interrenal organ at around 31–32 hpf (Fig. 4H, *arrow*), determined by its colocalization with the interrenal marker *ff1b* (Fig. 4J). Another intriguing result for *dax1* ISH is that this gene was expressed in the liver after 3 dpf in the zebrafish larvae (Fig. 4, Q and R).

MO-Mediated Knockdown of *dax1* Function Down-Regulates Expression of the Steroidogenic Genes *cyp11a* and *star*

To study the potential role of dax1 in interrenal development, MO was used to block its translation during zebrafish embryogenesis. We injected either *dax1* MO or *dax1* mismatch MO (mMO), the mismatch control,

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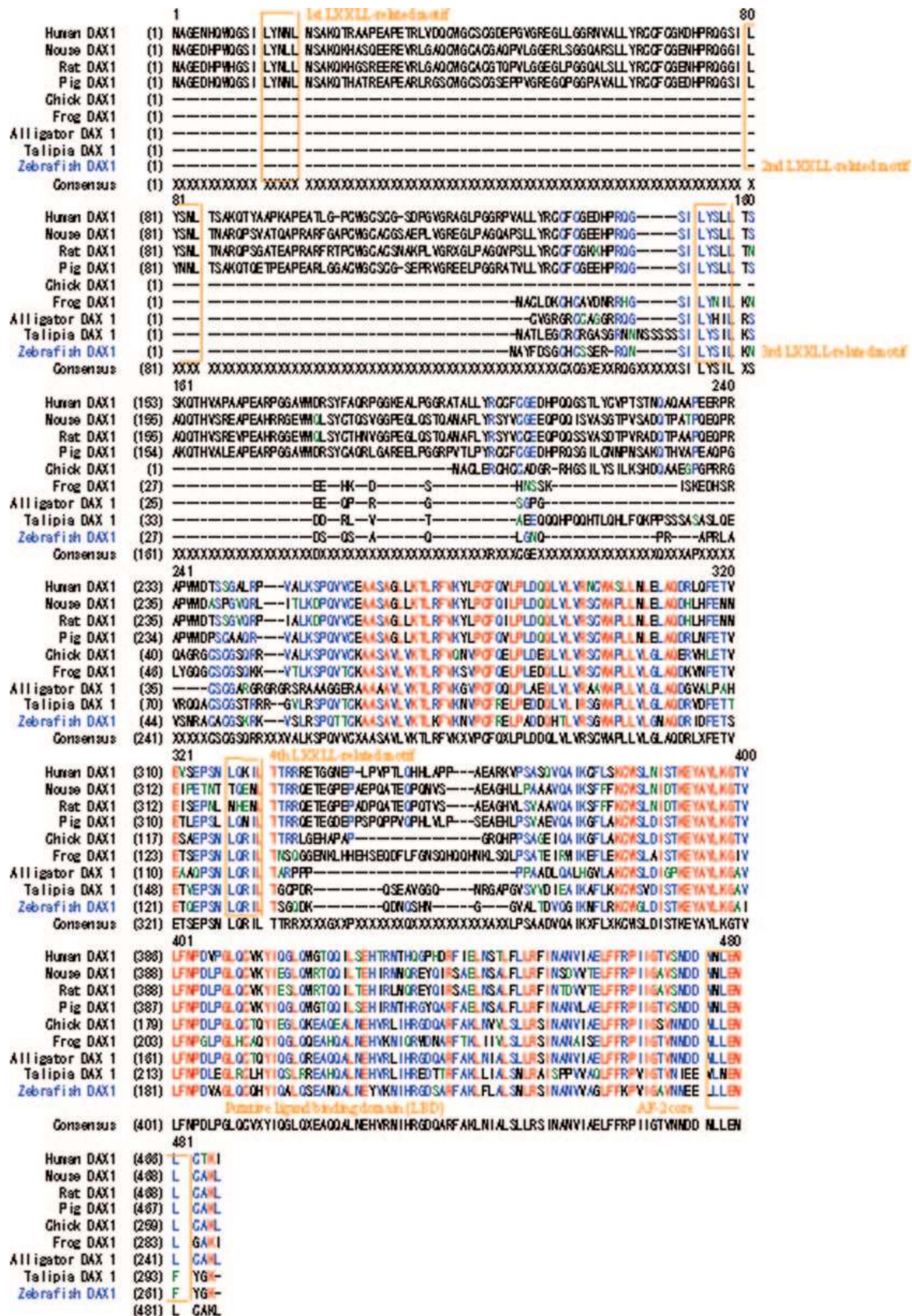

Fig. 2. Alignment of the Amino Acid Sequences of Zebrafish dax1 with Those of Other DAX1s

GenBank accession numbers of DAX1 orthologs used for the analysis are: tilapia (AY135397), frog (AB079550), American alligator (AF180295), chick (AF202991), rat (NM_053317), mouse (U41568), pig (U82466), and human (NM_000475).

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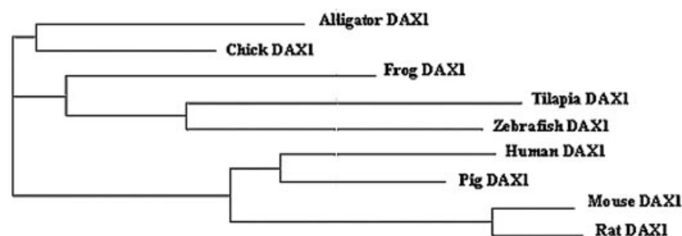

Fig. 3. Phylogenetic Analysis of Zebrafish dax1 and Other Known DAX1s in Vertebrates
GenBank accession numbers are given in the legend of Fig. 2.

into one- to two-cell embryos and subsequently examined the effects of MO injection on the expression of the steroidogenic genes, *cyp11a* and *star*, both of which were shown to be expressed in the developing zebrafish interrenal organ (29, 31), by ISH. Embryos injected with 9 ng of *dax1* MO and *dax1* mMO were collected at 36 hpf for ISH with *cyp11a.* Injection of *dax1* MO significantly down-regulated *cyp11a* expression in 77.1% (81/105) of embryos, whereas at the same dosage, only 5.4% (5/93) of *dax1* mMO-injected embryos showed the same degree of down-regulation of *cyp11a* (Fig. 5, A and B). Similarly, knockdown of *dax1* function by MO significantly reduced the number of embryos showing *star* expression in the interrenal organ (Fig. 5, C and D). Of *dax1* MO-injected embryos 80.2% (77/96) were *star* negative at 40 hpf. However, 96.6% (85/88) of embryos injected with *dax1* mMO showed positive *star* staining, therefore establishing the specificity of *dax1* MO effects.

Morpholino Knockdown of *dax1* Function Leads to Impaired Osmoregulation

Embryos injected with 9 ng of *dax*1 MO and *dax1* mMO were allowed to develop to larval stages. Injection of *dax1* MO led to severe phenotypes similar to *ff1b* (SF1) MO-injected embryos (37). These phenotypes could be grouped into three different classes as previously described by Chai *et al*. (37). The percentages of larvae exhibiting different classes of phenotypes are presented in Table 2. Morphological changes had begun to appear by 4 dpf. Some *dax1* MO-injected larvae showed signs of fluid accumulation in their body cavities, predominantly in the abdomen. By 7 dpf, varying degrees of sc edema were observed, consistent with *ff1b* morphants. Edema of the optic sacs led to protrusion of the optic cup. Edema of the pericardial sac interfered with cardiac pumping, leading to slowing or even cessation of heartbeat. As a result, blood circulation slowed or ceased, and blood islands formed in the ventral tail. About 8.7% of control embryos injected with *dax1* mMO displayed phenotypes ranging from classes A to C.

We also investigated the effects of *dax1* MO at the histological level. Either *dax1* MO or mismatch control MO-injected embryos were collected at 36 hpf and 48 hpf, respectively. These embryos were stained by *ff1b* ISH and randomly sampled for cross-sectioning. Examination of the sections revealed that *dax1* MO injection did not cause any obvious structural difference in the interrenal tissues, labeled by *ff1b* staining (data not shown). In addition, the apoptosis profiles of *dax1* morphants and controls were examined using acridine orange staining and terminal deoxynucleotide transferase-mediated dUTP nick end labeling assay. *dax1* morphants presented similar numbers of apoptotic cells in the interrenal region compared with those in control embryos (data not shown).

dax1 May Function Downstream of ff1b during Zebrafish Interrenal Development

As previously described, the disruption of dax1 activity by *dax1* MO led to severe phenotypes that are similar to those reported in *ff1b* morphants; MO knockdown of either protein was sufficient to interrupt the expression of steroidogenic enzymes. To explore the relationship of these two genes in the regulatory network involved in the interrenal development and function, we first examined the effects of *dax1* MO injection on the expression of *ff1b* in the interrenal organ. Embryos injected with 9 ng of *dax1* MO and *dax1* mMO were collected at 36 hpf for ISH with *ff1b.* Similar proportions, 91.3% (73/80) of embryos injected with *dax1* MO and 93.1% (67/72) embryos injected with *dax1* mMO, showed positive *ff1b* staining in the interrenal organ (Fig. 6, A and B). These results indicated that the disruption of *dax1* function did not affect the normal expression of *ff1b*.

TABLE 1. Amino Acid Sequence Identities and Similarities between Zebrafish dax1 and Other Vertebrate DAX1s

Zebrafish dax1	Tilapia DAX1	Chicken DAX1	Frog DAX1	Alligator DAX1	Porcine DAX1	Human DAX1	Rat DAX1	Mouse DAX1
Identity	65.5	58.4	56.4	48.2	46.4	45.9	44.0	43.3
Similarity	79.5	72.7	70.4	58.4	61.6	58.7	60.3	57.1

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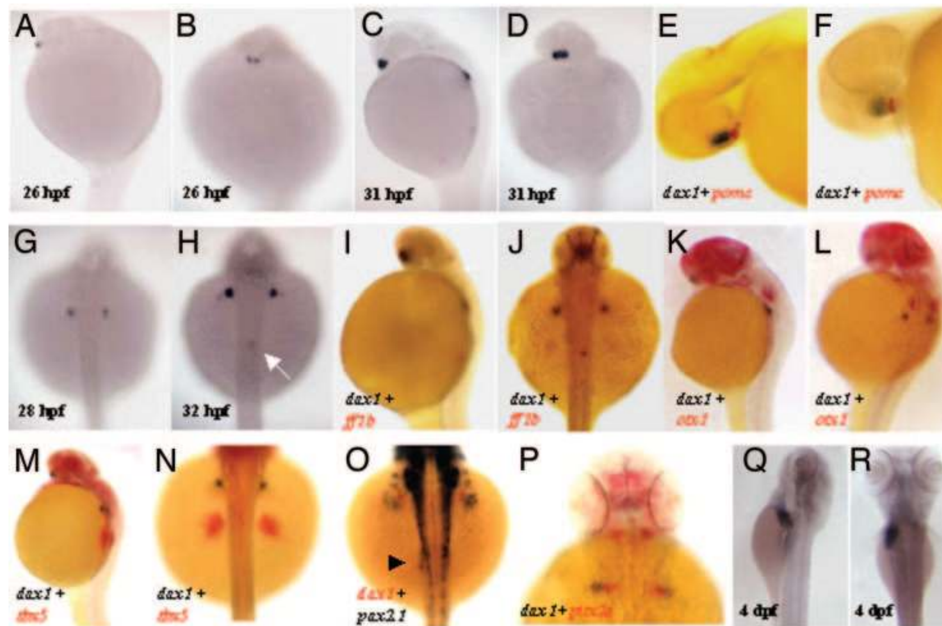

Fig. 4. Expression Analysis of *dax1*

A–D, G, H, Q, and R, ISH for dax1 was performed with DIG-labeled *dax1* riboprobe and visualized with *purple.* E, F, I–N, and P, Two-color ISH was performed with fluorescein-labeled *dax1* riboprobe and DIG-labeled marker gene riboprobes and were visualized with *purple* and *Fast Red* staining, respectively. O, Two-color ISH was performed with fluorescein-labeled *pax2.1* riboprobe (*purple*) and DIG-labeled *dax1* riboprobe (*Fast Red*). Lateral views of *dax1* expression at 26 hpf (A), 31 hpf (C), and 4 dpf (Q). Ventral views of *dax1* expression at 26 hpf (B) and 31 hpf (D). Dorsal views of *dax1* expression at 28 hpf (G), 32 hpf (H), and 4 dpf (R). *Arrow* in panel H indicates the expression of *dax1* in the interrenal gland. Lateral (E) and lateral-ventral (F) views of 32 hpf embryos stained for *dax1* and pituitary marker *pomc*. Lateral (I) and dorsal (J) views of 32 hpf embryos stained for *dax1* and interrenal marker *ff1b*. Lateral (K) and lateral-dorsal (L) views of 32 hpf embryos stained for *dax1* and ear marker *otx1*. Lateral (M) and dorsal (N) views of 32 hpf embryos stained for *dax1* and pectoral fin bud marker *tbx5*. Dorsal view (O) of 32 hpf embryo stained for *dax1* and pronephric tubule marker *pax2.1*. *Arrowhead* indicates stained pronephric tubules. Dorsal view (P) of 38 hpf embryo stained for *dax1* and tooth germ marker *pitx2a*.

Furthermore, we investigated the interrenal expression of *dax1* in *ff1b* morphants. An antisense morpholino and a mutated antisense morpholino were synthesized and injected as previously described by Chai *et al*. (37). Because *dax1* expression in the interrenal organ is weak and transient at approximately 31–32 hpf, we collected both *ff1b* MO and *ff1b* mMO-injected embryos at 30–33 hpf for ISH with *dax1*. We observed that 16.4% (29/177) of *ff1b* mMO (control)-injected embryos displayed weak expression of *dax1* in the interrenal organ. In contrast, only 4.8% (9/186) of embryos injected with *ff1b* MO retained comparable *dax1* staining (Fig. 6, C and D). Thus, the injection of *ff1b* MO significantly down-regulated the interrenal expression of *dax1* ($P < 0.001$).

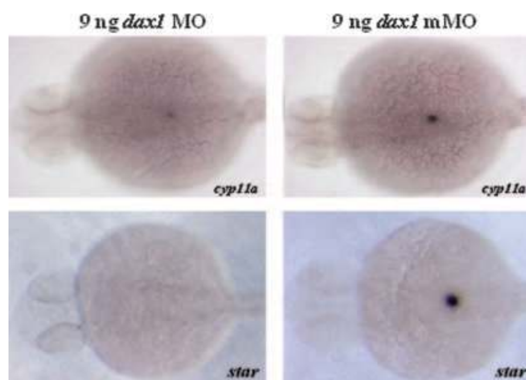

Fig. 5. Effects of *dax1* MO Injection on the Expression of *cyp11a* and *star*

A and B, ISH for *cyp11a* on 36 hpf embryos injected with 9 ng per embryo of *dax1* MO (panel A) and *dax*1 mMO control (panel B). C and D, ISH for *star* in *dax1* MO (panel C) and *dax1* mMO control (panel D)-injected embryos (40 hpf). Embryos were oriented with anterior to the *left*.

To characterize further the relationship of dax1 and ff1b during zebrafish early development, we determined whether there is the classical SF1 response element sequence in the *dax1* promoter region. We sequenced the 5′-flanking region of the *dax1* gene and aligned the putative promoter sequences of human, mouse, rat, and zebrafish. No consensus SF1 response element was identified in the zebrafish *dax1* gene, unlike the human, mouse, and rat *DAX1*s (Fig. 7). We did find, however, conserved blocks of sequence, all relatively short, in the promoter regions for these four species, *e.g*. the region shown in Fig. 7. This

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TABLE 2. Classification of *dax1* Morphant Phenotypes at 7 dpf

Phenotype	Class A	Class B	Class C
Optic sac	Normal	Slight edema	Severe edema
Pericardial sac	Edema	Edema	Edema
Heart beat	Slow	Slow	Very slow
Blood flow	Slow	Very slow	None
Swim bladder inflation	Normal	None	None
Mouth	Protruding	Protruding	Protruding
Percentage of larvae	28.4	23.8	18.7

Total number of larvae scored = 689.

conservation suggests the possibility that regulatory strategies may have been maintained from fish to human.

DISCUSSION

In the present study, we cloned and characterized a *DAX1* homolog, *dax1*, in zebrafish embryos and larvae. We provide several lines of evidence to support a role for *dax1* in the development of the interrenal organ in the zebrafish. The *dax1* gene encodes a protein of 264 amino acid residues, including the conserved 3′-LBL motif, and the 5′-region resembles other nonmammalian DAX1 proteins and lacks the unusual repeat motif of the DBL domain in mammals. The genomic structure of the *dax1* gene is highly conserved when compared with those of known *DAX1*s. Whole-mount ISH revealed that *dax1* shares a similar expression pattern with other *DAX1* orthologs as well. Knockdown of dax1 activity by MO led to severe phenotypes that are consistent with impaired osmoregulation and down-regulated the expression of steroidogenic genes. The fact that the injection of *ff1b* MO abolished the interrenal expression of *dax1* whereas knockdown of *dax1* did not significantly affect *ff1b* expression suggests that dax1 is downstream of ff1b in transcriptional regulation during zebrafish interrenal development.

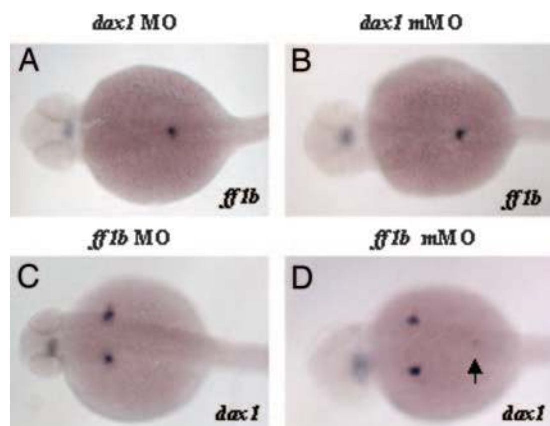

Fig. 6. Effects of *ff1b* MO Injection on the Expression of *dax1*

A and B, ISH for *ff1b* on 36 hpf embryos injected with *dax1* MO (panel A) and *dax*1 mMO control (panel B). C and D, ISH for *dax1* on approximately 31–32 hpf embryos injected with *ff1b* MO (panel C) and *ff1b* mMO control (panel D). *Arrow* indicates the expression of *dax1* in the interrenal organ.

dax1 Is Required for the Development of the Steroidogenic Component of the Zebrafish Interrenal Organ

Microinjection of MOs into one- to four-cell zebrafish embryos has proven to be an effective and specific means to inhibit the translation of a target gene. The inhibitory effects can persist in all cells throughout the first 72 h of development, allowing many early developmental pathways to be analyzed (43).

A role for zebrafish *dax1* in interrenal organ development and function is demonstrated by MO-mediated knockdown of dax1 activity *in vivo*. Injection of the *dax1* MO clearly abolishes the expression of the steroidogenic protein genes *cyp11a* and *star* in the interrenal organ. Detailed histological analysis revealed that *dax1* MO injection did not cause any obvious structural difference in the interrenal tissues. In addition, no abnormality in the pattern of apoptosis was observed in those *dax1* morphants. These results

Fig. 7. Alignment of the Promoter Sequences of Zebrafish *dax1* with Mammalian *DAX1s*

A small portion of the alignment, including the SF1 response element (SF1-RE), is shown. The nucleotides are numbered based on their positions relative to the start codon ATG in the *dax1* sequences of human (*top*) and zebrafish (*bottom*). The mammalian SF1 consensus response element does not appear to be present in this teleost. Blocks of sequence are conserved, however, in the 5′-untranslated regions of these four species, as, for example the 5′-CATGG-3′ at position −157 to −161, suggesting conservation of certain aspects of *dax1* expression throughout vertebrates.

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suggest that the zebrafish dax1 is required not for the structural or the organizational development of the interrenal organ, but for some other functional characteristics of this organ, *e.g*. perhaps the acquisition of the steroidogenic identity for the interrenal cells. This hypothesis is also consistent with the expression of *dax1* after *ff1b* and after the coalescence of the paired interrenal structures in the midline.

The phenotypes of larvae caused by the knockdown of *dax1* expression are consistent with impaired osmoregulation. In teleosts, the interrenal organ plays a critical role in osmoregulation by producing corticosteroids, principally cortisol, which is essential for hydromineral control in both seawater and fresh-water fish (44–46). Fresh-water fish produce highly diluted urine, which is hypotonic, to excrete excess water. Therefore, the edema phenotype observed in *dax1* morphants is likely due to reduced ability to produce hypotonic urine, as the result of the disruption of interrenal cortisol production.

dax1 Functions Downstream of ff1b in Regulation of Interrenal Organ Development

SF1 (NR5A1), an Ftz-F1 member of the nuclear receptor superfamily, is a key transcriptional factor critical for adrenal development in mammals (47). *SF1* shares a similar tissue expression profile with *DAX1* and could be involved in a common developmental pathway with *DAX1* (21).

ff1b, an *SF1* homolog, was identified in zebrafish embryos and was specifically expressed in the hypothalamus and the interrenal organ (35). Knockdown of *ff1b* gene function led to the loss of structural component of the interrenal organ (37). To date, *ff1b* is the earliest known molecular marker for teleost interrenal development (35–37). In zebrafish, the primordial interrenal cells first appear as bilateral clusters expressing *ff1b* ventral to the third somite by 22 hpf (Zhao, Y., and E.R.B. McCabe, unpublished results). These cells then migrate toward the axial midline, coalesce at around 30 hpf, and subsequently begin to acquire a steroidogenic identity (36, 37). However, *dax1* expression in the interrenal region is first observed at around 31 hpf, right after the primordial interrenal paired cell clusters fuse together and before these cells develop their steroidogenic identity. These data suggest that dax1 acts downstream of ff1b in interrenal gland development.

In this report, we present MO knockdown data for both *dax1* and *ff1b* that suggest the relative hierarchical roles of these two factors in regulation of normal interrenal development and function. Whereas *dax1* MO did not alter the *ff1b* gene expression in the interrenal organ, knockdown of *ff1b* activity abolished the interrenal expression of *dax1*. These observations are consistent with the temporal expression patterns, strongly suggesting that ff1b acts upstream of dax1 in regulation of interrenal gland development.

Previous investigations have shown that mammalian SF1 directly regulates transcription of the *DAX1* gene (21). In the present study we did not find any evidence of the consensus SF1 response element in the zebrafish *dax1* gene, unlike the mammalian *DAX1*s. To determine the mechanistic relationship of dax1 and ff1b, a series of transfection analyses in appropriate zebrafish cell lines will be necessary in future investigations.

dax1 May Have Novel Functions outside the HPAG Axis

In this report, we describe the zebrafish *dax1* gene expression not only in the hypothalamus and adrenal, where other known *DAX1*s are specifically expressed, but also in novel bilateral structures and in the liver at a later larval stage, suggesting novel functions for dax1 outside of the HPAG axis.

The later expression of *dax1* in the liver is particularly intriguing, because liver is one of the major expression sites for another orphan nuclear receptor SHP (48). SHP and DAX1 belong to the same nuclear receptor family. SHP functions as a transcriptional repressor, as does DAX1 (49–51). The mammalian SHPs possess the equivalent of one of the DAX1 repeats in the DBL domain and show high homology to nonmammalian DAX1s (18, 48). Moreover, phylogenetic analysis of DAX1s, SHPs, and homologous EST fragments indicates a paralogous relationship between DAX1 and SHP with origin from duplication of a common ancestral gene (18).

Our search for an SHP ortholog in zebrafish yielded an EST that shares significant sequence similarity to tilapia and mammalian *SHP*s. Subsequently we obtained the potential zebrafish *shp* full-length cDNA and studied its expression pattern by ISH. Surprisingly, this potential *shp* gene is expressed neither in the liver, where mammalian SHPs are expressed, nor in any internal organs. Instead, it is expressed diffusely in the superficial cell layers of the yolk sac, the yolk sac extension, and part of the trunk (data not shown). We are now in the process of further characterizing the function of this zebrafish *shp* gene and the fine structure of those tissues that express this gene.

The lack of *shp* expression and the atypical expression of *dax1* in the liver raise the possibility that zebrafish dax1 may carry out certain functions that are usually executed by SHPs in mammals. To evaluate the potential function of the relatively late expression of *dax1* in the liver, we examined the expression of liver markers in both the *dax1* morphants and the control-injected embryos. We did not observe any significant difference between the two panels, possibly due to the limitations of the morpholino technique beyond 72 h of development (data not shown). Future analysis of the relationship between zebrafish dax1 and the classical target genes of SHP may help characterize the potential involvement of dax1 in zebrafish liver development,

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and eventually offer new insights into the relationship of these two unusual nuclear receptors.

Summary

We propose that zebrafish is an appropriate vertebrate model in which to explore the roles of DAX1 and other regulatory factors in the development and normal function of the HPAG axis, and, in particular, the adrenal cortex.

MATERIALS AND METHODS

Zebrafish Stock and Embryo Collection

Wild-type zebrafish were maintained at 28.5 C. Embryos were obtained by natural spawning and cultured in embryo medium following standard procedures. Staging of embryos was carried out according to Kimmel *et al.* (52). Embryos used for expression analysis were cultured in 0.03% phenylthiourea (Sigma Chemical Co., St. Louis, MO) solution from 10 h post fertilization (hpf) to inhibit pigment formation.

Cloning of *dax1* cDNA and Phylogenetic Analysis

One *DAX1*-like EST clone was identified from the zebrafish genome databases (http://www.ensembl.org/Danio_rerio/blastview and http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml) by BLAST with the consensus sequence of the tilapia DAX1 and other known DAX1 proteins, and was purchased from Open Biosystems (Huntsville, AL). After sequencing and multiple alignments with known *DAX1* homologs, the complete coding sequence of the putative zebrafish *DAX1* gene was determined from this EST. Homology analyses of nucleotide sequences and deduced protein alignments and the subsequent phylogenetic analysis were performed with Vector NT1 software (Infomax, North Bethesda, MD). Sequences used for alignments other than those reported here were extracted from the NCBI UniGene and Nucleotide databases. GenBank accession numbers of DAX1 orthologs used for the analysis are listed below: tilapia (AY135397), frog (AB079550), American alligator (AF180295), chick (AF202991), rat (NM_053317), mouse (U41568), pig (U82466), and human (NM_000475).

Cloning of *dax1* Genomic Sequence

The zebrafish genome database from Sanger (http://www.sanger.ac.uk/Projects/D_rerio/) was screened by BLAST search with the putative zebrafish *DAX1* cDNA and a single BAC clone from the zebrafish CHORI-211 BAC library was identified that contained the putative exon 1 sequence. Subsequent sequencing of this BAC clone confirmed that it actually contained the complete coding sequence and the intronic sequence of the putative zebrafish *DAX1* gene.

Analysis of the *dax1* Promoter Region

The sequence of the *dax1* promoter region was obtained by sequencing the BAC clone that contained this gene. The mRNA sequences of the human, mouse, and rat *DAX1*s were used to search the NCBI genome databases to identify the contigs that contained the complete gene sequences. Subsequently the promoter regions were determined by comparing the genomic sequences and the corresponding mRNAs. Multiple alignments were performed with Vector NT1 software.

Whole-Mount ISH

Digoxigenin (DIG)- or fluorescein-labeled *dax1* riboprobes were synthesized from *Avr*II linearized *dax1* cDNA using T7 RNA polymerase. DIG-labeled riboprobes were synthesized from plasmids containing zebrafish cDNAs for *ff1b, pomc, tbx5, otx1, pax2.1*, *cyp11a*, and *StAR,* respectively. Plasmids for *pomc*, *tbx5, otx1, pitx2a*, and *cyp11a* were digested with *Not*I and transcribed with *T7* RNA polymerase. *ff1b* and *star* plasmids were linearized with *Nco*I and *Bam*HI, respectively, and transcribed with *Sp6* polymerase. *pax2.1* plasmid was linearized with *Bam*HI and transcribed with *T7* polymerase. Single-color ISH for *dax1* was performed on zebrafish embryos at 10 different developmental stages with DIG-labeled *dax1* riboprobe as previously described (53). For two-color ISH, embryos at around 32 hpf were hybridized with both fluorescein-labeled *dax1* and DIG-labeled *pomc, ff1b*, *tbx5*, *otx1*, or *pax2.1,* respectively, as previously described (54); and double ISH of *dax1* and *pitx2a* was performed on embryos at around 38 hpf. DIG-labeled probes were detected with alkaline phosphatase (AP)-conjugated anti-DIG antibody and visualized with BM Purple AP substrate (Roche Applied Sciences, Indianapolis, IN), whereas fluorescein-labeled probes were detected with AP-conjugated antifluorescein antibody and stained with Fast Red (Roche). Fully stained embryos were washed three times for 10 min in PBS/Tween and postfixed in 4% paraformaldehyde, followed by tissue clarification in gradient concentrations of glycerol (in PBS/Tween). Specimens were then mounted on glass slides and photographed under Normaski optics on a Zeiss Axio microscope system (Carl Zeiss, Thornwood, NY).

Morpholino Injection

MOs were synthesized at Gene Tools (Corvallis, OR). A stock solution of 10 μg/μl was prepared by dissolving the lyophilized powder in doubly distilled water. The stock solution was diluted to a working concentration of 2 μg/μl in 1× Danieau solution (58 mM NaCl; 0.7 mM KCl; 0.4 mM $MgSO_4$; 0.6 mM $Ca(NO3)_2$; 5 mM HEPES, pH 7.6).

Freshly laid embryos were collected and placed into the embryo medium (55) and transferred onto an agrose platform. MO solution (1–6 nl) was injected into the yolk of one- to two-cell embryos from the vegetal pole. Microinjection was performed using a Nanojet injector.

The morpholino sequences were as follows

dax1 MO: 5′-CAGAGCTGCTTATGTTTCGTTGCTG-3′

dax1 mismatch control, *dax1* mMO: 5′-CAcAGgTGCTTA-TcTTTCcTTGgTG-3′

ff1b MO: 5′-AATCCTCATCTGCTCTGAAGTC-3′

ff1b control, *ff1b* mMO: 5′-AATC-TCATC-GCTC-GAAG-TCat-3′

Both the *ff1b* MO and the *ff1b* control were synthesized with the sequence previously described by Chai *et al*. (37).

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Address all correspondence and requests for reprints to: Edward R. B. McCabe, Department of Pediatrics, David Geffen School of Medicine at UCLA, 10833 LeConte Avenue, Room 22-412 MDCC, Los Angeles, California 90095-1752, E-mail: EMcCabe@mednet.ucla.edu.

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REFERENCES

1. Bardoni B, Zanaria E, Guioli S, Floridia G, Worley KC, Tonini G, Ferrante E, G. Chiumello G, McCabe ER, Fraccaro M, Zuffardi O, Camerino G 1994 A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. Nat Genet 7:497–501
2. McCabe ER 2001 Adrenal hypoplasias and aplasias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. Vol 3, 8th ed. New York: McGraw-Hill Medical Publishing Division; 4263–4274
3. Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Moser C, Walker AP, McCabe ER, Meitinger T, Monaco AP, Sassone-Corsi P, Camerino G 1994 An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 372:635–641
4. Muscatelli F, Strom TM, Walker AP, Zanaria E, Recan D, Meindl A, Bardoni B, Guioli S Zehetner G, Rabl W, Schwarz HP, Kaplan JC, Camerino G, Meitinger T, Monaco AP 1994 Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. Nature 372:672–676
5. Guo W, Mason JS, Stone CG, Morgan SA, Madu SI, Baldini A, Lindsay EA, Biesecker LG, Copeland KC, Horlick MN, 1995 Diagnosis of X-linked adrenal hypoplasia congenita by mutation analysis of the DAX1 gene. JAMA 274:324–330
6. Phelan JK, McCabe ER 2001 Mutations in NR0B1 (DAX1) and NR5A1 (SF1) responsible for adrenal hypoplasia congenita. Hum Mutat 18:472–487
7. Guo W, Burris TP, McCabe ER 1995 Expression of DAX-1, the gene responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism, in the hypothalamic-pituitary-adrenal/gonadal axis. Biochem Mol Med 56:8–13
8. Swain A, Zanaria E, Hacker A, Lovell-Badge R, Camerino G 1996 Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. Nat Genet 12:404–409
9. Ikeda Y, Swain A, Weber TJ, Hentges KE, Zanaria E, Lalli E, Tamai KT, Sassone-Corsi P, Lovell-Badge R, Camerino G Parker KL 1996 Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages: potential links in endocrine development. Mol Endocrinol 10:1261–1272
10. Tamai KT, Monaco L, Alastalo TP, Lalli E, Parvinen M, Sassone-Corsi P 1996 Hormonal and developmental regulation of DAX-1 expression in Sertoli cells. Mol Endocrinol 10:1561–1569
11. Ikeda Y, Takeda Y, Shikayama T, Mukai T, Hisano S, Morohashi KI 2001 Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. Dev Dyn 220:363–376
12. Guo W, Lovell RS, Zhang YH, Huang BL, Burris TP, Craigen WJ, McCabe ER 1996 Ahch, the mouse homologue of DAX1: cloning, characterization and synteny with GyK, the glycerol kinase locus. Gene 178:31–34
13. Bae DS, Schaefer ML, Partan BW, Muglia L 1996 Characterization of the mouse DAX-1 gene reveals evolutionary conservation of a unique amino-terminal motif and widespread expression in mouse tissue. Endocrinology 137:3921–3927
14. Parma P, Pailhoux E, Puissant C, Cotinot C 1997 Porcine Dax-1 gene: isolation and expression during gonadal development. Mol Cell Endocrinol 135:49–58
15. Western PS, Harry JL, Marshall Graves JA, Sinclair AH 2000 Temperature-dependent sex determination in the American alligator: expression of SF1, WT1 and DAX1 during gonadogenesis. Gene 241:223–232
16. Smith CA, Clifford V, Western PS, Wilcox SA, Bell KS, Sinclair AH 2000 Cloning and expression of a DAX1 homologue in the chicken embryo. J Mol Endocrinol 24:23–32
17. Sugita J, Takase M, Nakamura M 2001 Expression of Dax-1 during gonadal development of the frog. Gene 280:67–74
18. Wang DS, Kobayashi T, Senthilkumaran B, Sakai F, Sudhakumari CC, Suzuki T, Yoshikuni M, Matsuda M, Morohashi K, Nagahama Y 2002 Molecular cloning of DAX1 and SHP cDNAs and their expression patterns in the Nile tilapia, *Oreochromis niloticus*. Biochem Biophys Res Commun 297:632–640
19. Zhang Z, Burch PE, Cooney AJ, Lanz RB, Pereira FA, Wu J, Gibbs RA, Weinstock G, Wheeler DA 2004 Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. Genome Res 14:580–590
20. Burris TP, Guo W, McCabe ER 1996 The gene responsible for adrenal hypoplasia congenita, DAX-1, encodes a nuclear hormone receptor that defines a new class within the superfamily. Recent Prog Horm Res 51: 241–259
21. Iyer AK, McCabe ER 2004 Molecular mechanisms of DAX1 action. Mol Genet Metab 83:60–73
22. Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA 1998 Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93:445–454
23. Briggs JP 2002 The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol 282:R3–R9
24. Penberthy WT, Shafizadeh E, Lin S 2002 The zebrafish as a model for human disease. Front Biosci 7:d1439–d1453
25. Chester-Jones I, Mosley W 1980 The interrenal gland in Pisces. In: Chester-Jones I, Henderson IW, eds. General, comparative and clinical endocrinology of the adrenal cortex. Vol 3. London and New York: Academic Press; 395–523
26. Grassi Milano E, Basari F, Chimenti C 1997 Adrenocortical and adrenomedullary homologs in eight species of adult and developing teleosts: morphology, histology, and immunohistochemistry. Gen Comp Endocrinol 108: 483–496
27. Hanke W, Kloas W 1995 Comparative aspects of regulations and function of the adrenal complex in different groups of vertebrates. Horm Metab Res 27:389–397
28. Keegan CE, Hammer GD 2002 Recent insights into organogenesis of the adrenal cortex. Trends Endocrinol Metab 13:200–208
29. Hsu HJ, Hsiao PH, Kuo MW, Chung BC 2002 Expression of zebrafish cyp11a1 as a maternal transcript and in yolk syncytial layer. Gene Expr Patterns 2:219–222
30. Lai WW, Hsiao PH, Guiguen Y, Chunag BC 1998 Cloning of zebrafish cDNA for 3β-hydroxysteroid dehydrogenase and P450scc. Endocr Res 24:927–931
31. Bauer MP, Bridgham JT, Langenau DM, Johnson AL, Goetz FW 2000 Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol Cell Endocrinol 168:119–125
32. Hossain A, Li C, Saunders GF 2004 Generation of two distinct functional isoforms of dosage-sensitive sex reversal-adrenal hypoplasia congenita-critical region on the X chromosome gene 1 (DAX-1) by alternative splicing. Mol Endocrinol 18:1428–1437
33. Ho J, Zhang YH, Huang BL, McCabe 2004 NR0B1A: an alternatively spliced form of NR0B1. Mol Genet Metab 83:330–336
34. Hansen IA, To TT, Wortmann S, Burmester T, Winkler C, Meyer SR, Neuner C, Fassnacht M, Allolio B 2003 The pro-opiomelanocortin gene of the zebrafish (*Danio rerio*). Biochem Biophys Res Commun 303:1121–1128

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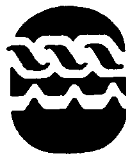
35. Chai C, Chan WK 2000 Development expression of a novel Ftz-F1 homologue, ff1b (NR5A4), in the zebrafish *Danio rerio.* Mech Dev 91:421–426
36. Hsu HJ, Lin G., Chung BC 2003 Parallel early development of zebrafish interrenal glands and pronephros: differential control by wt1 and ff1b. Development 130: 2107–2116
37. Chai C, Liu YW, Chan WK 2003 Ff1b is required for the development of steroidogenic component of the zebrafish interrenal organ. Dev Biol 260:226–244
38. Mori H, Miyazaki Y, Morita T, Nitta H, Mishina M 1994 Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. Brain Res Mol Brain Res 27:221–231
39. Leger S, Brand M 2002 Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. Mech Dev 119:91–108
40. Ahn DG, Kourakis MJ, Rohde LA, Silver LM, Ho RK 2002 T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417:754–758
41. Majumdar A, Lun K, Brand M, Drummond IA 2000 Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. Development 127:2089–2098
42. Jackman WR, Draper BW, Stock DW 2004 Fgf signaling is required for zebrafish tooth development. Dev Biol 274:139–157
43. Ekker SC 2000 Morphants: a new systematic vertebrate functional genomics approach. Yeast 17:302–306
44. Wendelaar Bonga SE 1997 The stress response in fish. Physiol Rev 77:591–625
45. Evans, DH 1993 Osmotic and ionic regulation. In: Evans DH, ed. The physiology of fishes. Boca Raton, LA: CRC Press; 315–341
46. McCormick SD 2001 Endocrine control of osmoregulation in teleost fish. Am Zool 41:781–794
47. Parker KL, Rice DA, Lala DS, Ikeda Y, Luo X, Wong M, Bakke M, Zhao L, Frigeri C, Hanley NA, Stallings N, Schimmer BP 2002 Steroidogenic factor 1: an essential mediator of endocrine development. Recent Prog Horm Res 57:19–36
48. Lee HK, Lee YK, Park SH, Kim YS, Lee JW, Kwon HB, Soh J, Moore DD, Choi HS 1998 Structure and expression of the orphan nuclear receptor SHP gene. J Biol Chem 273:14398–14402
49. Seol W, Chung M, Moore DD 1997 Novel receptor interaction and repression domains in the orphan receptor SHP. Mol Cell Biol 17:7126–7131
50. Seol W, Hanstein B, Brown M, Moore DD 1998 Inhibition of estrogen receptor action by the orphan receptor SHP (short heterodimer partner). Mol Endocrinol 12: 1551–1557
51. Bavner A, Johansson L, Thomsen JS, Gustafsson JA, Treuter E 2002 A transcriptional inhibitor targeted by the atypical orphan nuclear receptor SHP. EMBO Rep 3:478–484
52. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF 1995 Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310
53. Jowett A 1999 Analysis of protein and gene expression. Methods Cell Biol 59:63–85
54. Dheen T, Sleptsova-Friedrich I, Xu Y, Clark M, Lehrach H, Gong Z, Korzh V 1999 Zebrafish tbx-c functions during formation of midline structures. Development 126: 2703–2713
55. Westerfield M 2000 The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Eugene, OR: University of Oregon Press



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