Zebrafish ae2.2 encodes a second slc4a2 anion exchanger

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Shmukler BE, Clark JS, Hsu A, Vandorpe DH, Stewart AK, Kurschat CE, Choe S-K, Zhou Y, Amigo J, Paw BH, Alper SL. Zebrafish ae2.2 encodes a second slc4a2 anion exchanger. Am J Physiol Renal Physiol 289: F835–F849, 2005, now called ae2.1. We now report the structural and functional characterization of Ae2.2, the product of the second zebrafish slc4a2 gene, ae2.2. The ae2.2 gene of zebrafish linkage group 24 encodes a polypeptide of 1,232 aa in length, sharing 70% amino acid identity with zebrafish Ae2.1 and 67% identity with mouse AE2a. Zebrafish Ae2.2 expressed in Xenopus oocytes encodes a 135-kDa polypeptide that mediates bidirectional, DIDS-sensitive Cl−/HCO3− exchange and Cl−/HCO3− exchange. Ae2.2-mediated Cl−/HCO3− exchange is cation independent, voltage insensitive, and electroneutral. Acute regulation of anion exchange mediated by Ae2.2 includes activation by NH4+ and independent inhibition by acidic intracellular pH and by acidic extracellular pH. In situ hybridization reveals low-level expression of Ae2.2 mRNA in zebrafish embryo, most notably in posterior tectum, eye, pharynx, epidermal cells, and axial vascular structures, without notable expression in the Ae2.2-expressing pronephric duct. Knockdown of Ae2.2 mRNA, of Ae2.1 mRNA, or of both with nontoxic or minimally toxic levels of N-morpholino oligomers produced no grossly detectable morphological phenotype, and preserved normal structure of the head and the pronephric duct at 24 h postfertilization.

chloride/bicarbonate exchanger; Xenopus oocyte; isotopic flux; in situ hybridization; two-electrode voltage clamp; N-morpholino oligomer


THE SLC4 GENE FAMILY IN MAMMALS INCLUDES AMONG THE Na+-INDEPENDENT CI−/HCO3− EXCHANGER MEMBERS AT LEAST THREE GENES DIFFERING IN TISSUE DISTRIBUTION AND REGULATORY PROPERTIES (11, 17). WE THEREFORE SOUGHT ADDITIONAL AE-RELATED SLC4 GENES IN THE ZEBRAFISH. WE REPORT HERE THAT THE ZEBRAFISH GENOME ENCODES A SECOND SLC4A2 GENE, AE2.2, AND SO RENAME THE ORIGINALLY REPORTED ZEBRAFISH AE2 GENE AS AE2.1. THE AE2.2 GENE, THOUGH LOCATED ON A DIFFERENT CHROMOSOME, SHARES A COMMON INTRON-EXON GENOMIC ORGANIZATION WITH THE HOMOLOGOUS AE2.1 GENE. THE AE2.2 POLYPEPTIDE ENCODES A Na+- AND VOLTAGE-INDEPENDENT, ELECTRONEUTRAL ANION EXCHANGER INHIBITED BY NH4+ AND EXTRACELLULAR pH (pHo), AND IS ACTIVATED BY NH4+. AE2.2 mRNA EXPRESSION APPEARS ABSENT FROM THE PRONEPHRIC DUCT OR HEMATOPOIETIC CELLS, BUT IS EXPRESSED AT LOW LEVELS IN BRAIN, EYE, AND ADDITIONAL STRUCTURES. N-MORPHOLINO OLIGO-MEDIATED INDIVIDUAL KNOCKDOWN OF THE CDNAS ENCODING AE2.2 OR AE2.1, OR COMBINED KNOCKDOWN OF BOTH, PRODUCED NO DETECTABLE GROSS ALTERATION OF EARLY EMBRYONIC DEVELOPMENT, INCLUDING APPARENT PRESERVATION OF NORMAL STRUCTURE OF THE HEAD AND PRONEPHRIC DUCT.

METHODS

MOLECULAR CLONING OF ZEBRAFISH AE2.2 CDNA. AN ARRAYED ZEBRAFISH PHAGEMID CDNA LIBRARY IN pBK-CMV (STRATEGANE, LA JOLLA, CA) SCREENED AT LOW STRINGENCY WITH A 32P-Labeled ZEBRAFISH SLCA41/AE1 CDNA (10) YIELDED 11 CLONES ENCODING AE2.1 (DESCRIBED IN REF. 14 AS ZAE2), AND ONE CLONE (CG385) WITH TERMINAL SEQUENCES IDENTICAL TO ZEBRAFISH INFORMATION NETWORK (ZFIN) CLONE CB330 (19). ALIGNMENT OF THE COMPLETELY SEQUENCED CLONE CG385 WITH AE2 SEQUENCES REVEALED THREE DELETIONS UNRELATED TO CONSENSUS INTRON-EXON JUNCTIONS, AS WELL AS AN INTERNAL TRANSLATION OF SEQUENCE FROM THE 5′-CODING REGION TO THE MIDDLE OF THE CDNA. INDEPENDENT PCR AMPLIFICATION PRODUCTS FROM THE SAME LIBRARY WERE SEQUENCED AS POOLS TO IDENTIFY SEQUENCES BRIDGING THE DELETIONS. 5′- AND 3′-RACE PRODUCTS GENERATED FROM THE SAME CDNA LIBRARY WERE SEQUENCED AS POOLS OR AS INDIVIDUAL CLONES IN pCRII (INVITROGEN) TO EXTEND THE CDNA SEQUENCE

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into 5'- and 3'-untranslated regions (utr). These procedures restored most of the ae2.1-like open reading frame, with the exception of a
into 5' - and 3' -untranslated regions (utr). These procedures restored

R1082
Ac2.2. A SECOND ZEBRAFISH Slc4a2 ANION EXCHANGER

H11032
H11003

Ae2.2, A SECOND ZEBRAFISH Slc4a2 ANION EXCHANGER

CAGTGAGCACTTTGAGG-3
(Hot start with 36 cycles of 94°C denaturing

DANIO RERIO

The genomic location of the

two-electrode voltage clamp of X. laevis oocytes. Two-electrode
voltage clamp was performed as previously described, with modifications (2, 14). Oocytes injected 2–3 days previously with water or with 10 ng

H11001
H11002
H11350
H9262

immunofluorescence detection of zebrafish ae2.2 polypeptide in transiently transfected HEK-293 cells. Zebrafish Ae2.2 cDNA (plasmid 15-3) subcloned into pcDNA3 was transiently transfected into subconfluent HEK-293 cells on glass coverslips using lipofectamine (Invitrogen). After 48 to 72 h, cells were fixed with 2% paraformaldehyde, quenched, and immunostained with affinity-purified anti-

Immunofluorescence detection of zebrafish ae2.2 polypeptide in transiently transfected HEK-293 cells. Zebrafish Ae2.2 cDNA (plasmid 15-3) subcloned into pcDNA3 was transiently transfected into subconfluent HEK-293 cells on glass coverslips using lipofectamine (Invitrogen). After 48 to 72 h, cells were fixed with 2% paraformaldehyde, quenched, and immunostained with affinity-purified anti-

Antisense N-morpholinolo-oligo knockdown studies. N-morpholino-
oligos (MO) of a design verified with GeneTools software were synthesized by GeneTools (Philomath, OR; see Supplemental Table 3). Ae2.2 oligos MO-1 (spanning the intron 4-exon 15 junction of the ae2.2 gene) and MO-2 (spanning the exon 15-intron 15 junction of the ae2.2 gene) were designed to interrupt the Ae2.2 open reading frame by deletion of exon 15 from Ae2.2 mRNA. Ae2.1 oligos MO-5 (spanning the intron 5-exon 6 junction) and MO-6 (spanning the exon 6-intron 6 junction) were designed to interrupt the Ae2.1 open reading frame by deletion of exon 6 from Ae2.1 mRNA. One-cell embryos were injected with 1 nl volumes containing pairs of MOs at individual concentrations of 0.2 to 1.5 mM (total injected load 3.3–25 ng). Knockdown efficacy was estimated from the relative abundance of RT-PCR products generated from wild-type mRNA and from the

Xenopus

Ae3

AE2

H11003

AJP-Regul Integr Comp Physiol • VOL 294 • MARCH 2008 • www.ajpregu.org

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intended knockdown mRNA product, as detected by agarose gel electrophoresis. The oligonucleotide sequences used for these diagnostic RT-PCR amplifications are presented in Supplemental Table 3. MO-3 and MO-4 were found to be ineffective as reagents for Ae2.1 knockdown.

RESULTS

cDNA cloning. Among the 14 cDNA clones selected from the zebrafish adult kidney cDNA library by low-stringency screen with an Slc4a1/Ae1 probe, only three of the isolated cDNAs did not encode Ae2.1 (14). One of these three (the Ae2.1-related CG385) had terminal sequence identical to that of a previously sequenced clone CB330 (ZDB-GENE-030429-14), represented by GenBank partial cDNA sequences CB417274 (5'-end) and CB417275 (3'-end). Complete sequencing of CG385 revealed three internal deletions and one internal rearrangement. After Ae2.2 sequences were extended at both ends by 5'- and 3' RACE, cDNAs encoding a full-length Ae2.2 open reading frame were amplified from zebrafish whole embryo cDNA prepared from pooled embryos.

Two allelic full-length coding sequences of Ae2.2 cDNA were determined. Supplemental Fig. 1 indicates sites at which sequences of alleles 1 and 2 differ. The two nonsynonymous coding polymorphisms (so named because they alter amino acid sequence) and 19 additional synonymous polymorphisms (leaving amino acid sequence unaltered) that distinguish the two alleles are summarized in Supplemental Table 1. Three additional nonsynonymous coding polymorphisms and eight more synonymous polymorphisms contributing to neither allele 1 nor allele 2 were also found in two or more Ae2.2 cDNA clones isolated in early stages of the study from independent pools of embryos (Supplemental Table 1). Thus, the ae2.2 gene is highly polymorphic.

Mapping of the zebrafish ae2.2 gene. The ae2.2 gene was assigned to linkage group 24 (LG24) by RH mapping on the Goodfellow TH51 RH panel. The LG24 markers unpi191, fj04c06.x1, and zfish44908-1385h06 at 29 cR exhibited linkage to the ae2.2 gene with logarithmic odds (LOD) scores of 7.5. LG24 markers z4921 at 31 cR, fb61h03.x1 at 35 cR, and z42003 were linked to ae2.2 with respective LOD scores of 6.7, 5.9, and 5.1. The RH localization of the ae2.2 gene to LG24 was confirmed by physical linkage of LG24 marker fi03f09.x1 to a portion of the ae2.2 gene in fosmid CH1073-406K10 from the double-haploid library (M. Caccamo, Wellcome Trust Sanger Institute, personal communication from Zv7 genomic assembly). Both ae2.2 on LG24 and ae2.1 on LG2 are syntenic with the human SLC4A2 locus at chromosome 7q36 (Table 1). Although most of the surrounding linked zebrafish genes have a duplicated homolog elsewhere in the genome, the homologous cul1b and cul1a genes of zebrafish have each remained linked to ae2.2 and to ae2.1, respectively (Table 1). None of the human genes adjacent to the human AE3 locus on chromosome 2q35 are syntenic with the zebrafish ae2.1 or ae2.2 genes.

Exon-intron organization of the zebrafish ae2.2 gene. The exon-intron organization of the zebrafish ae2.2 gene (Table 2) was revealed by nucleotide sequence alignment of the ae2.2 cDNA sequence with ae2.2 gene fragments (Supplemental Fig. 1). Twelve of the 23 ae2.2 exons are identical in length to the corresponding exons of the ae2.1 gene. All 23 introns of the ae2.2 gene are bound by consensus donor and acceptor splice

Table 1. Zebrafish genes ae2.2 and ae2.1 are both syntenic with human SLC4A2

<table>
<thead>
<tr>
<th>Human 7q36</th>
<th>Position, nt</th>
<th>Danio rerio LG24</th>
<th>Danio rerio LG2</th>
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<tr>
<td>CUL1</td>
<td>@148M</td>
<td>cul1b</td>
<td>cul1a</td>
</tr>
<tr>
<td>PDA4</td>
<td>@148.3M</td>
<td>pda4</td>
<td></td>
</tr>
<tr>
<td>ABCB8</td>
<td>@150.3M</td>
<td>abcb8</td>
<td></td>
</tr>
<tr>
<td>ACCN3</td>
<td>@150.3M</td>
<td>accn2(acco3)</td>
<td></td>
</tr>
<tr>
<td>CDK5</td>
<td>@150.3M</td>
<td>N/Aadir</td>
<td>N/A$</td>
</tr>
<tr>
<td>SLC4A2</td>
<td>@150.39M</td>
<td>ae2.2</td>
<td>ae2.1</td>
</tr>
<tr>
<td>FASTK</td>
<td>@150.4M</td>
<td>Faskt2</td>
<td></td>
</tr>
<tr>
<td>TMUB1</td>
<td>@150.4M</td>
<td>tmub13</td>
<td></td>
</tr>
<tr>
<td>CENTG3</td>
<td>@150.4M</td>
<td>N/Aadir</td>
<td>N/A$</td>
</tr>
<tr>
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<td>@150.5M</td>
<td>cbx1</td>
<td>abc2</td>
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<tr>
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<td>@150.5M</td>
<td>abc2f2</td>
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<td>@154.9M</td>
<td>eng2b*</td>
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<td>@155.3M</td>
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</tr>
</tbody>
</table>

Linkage groups (LG) are genetically linked genes that together reside on a single chromosome. *The gene duplication products for these genes are on LG7. †The gene duplication product shha is on LG15. §Orthologous coding sequences identified in whole genome shotgun sequences were later mapped on Zv6 by BLAT on the UCSC genome browser. §No orthologs identified among current Danio rerio genomic and cDNA databases. Nearby human genes to SLC4A2 with yet unmapped zebrafish orthologs include EZH2, KCNH2 (EAG2), and ABCB8 (M-ABC-1). Those telomeric to SLC4A2 include RHEB, ACTR3B, and CRYGN (duplicated in zebrafish as crygn1 and crygn2).

sites, as is also true for zebrafish ae2.1 and mouse Ae2 genes. The 22 exon-exon junctions of the ae2.2 gene include 20 with locations precisely aligned with those of the zebrafish ae2.1 gene. Sixteen of the 21 exon-exon junctions within the ae2.2 gene protein coding region exhibit amino acid sequences across the splice junctions that are conserved in the Ae2.1 polypeptide (14). The exon 13-exon 14 junction in all three genes falls after codon position +1, but the ae2.2 gene has a single codon insertion (E654) at that site (Fig. 1). The exon 10-exon 11 junction is located identically in the zebrafish ae2.2 and 2.1 genes, but is shifted in the mouse gene. The exon 5-exon 6 junction in all three genes falls after coding position +2, despite the low degree of amino acid sequence conservation in a wide region surrounding this splice junction.

Deduced amino acid sequence of the zebrafish ae2.2 polypeptide. The 1232 aa zebrafish Ae2.2 polypeptide is 70% identical to its homologous zebrafish Ae2.1 polypeptide (Fig. 1). Kyte-Doolittle hydrophathy analysis predicts for Ae2.2 a hydrophilic NH2-terminal cytoplasmic domain of ~701 aa, followed by a hydrophobic polytopic transmembrane (TM) domain of ~494 aa comprising 12–14 predominantly o-helical TM spans and a COOH-terminal hydrophilic cytoplasmic domain of ~37 aa. Three consensus N-glycosylation sites are located in the nominally extracellular loop between putative TM5 and TM6. The putative covalent binding site for stilbene disulfonate inhibitors of anion transport is also conserved at the extracellular end of TM5 at K838 of Ae2.2. The putative electrostatic binding site for carbonic anhydrase 2 (aa 1207–11) is identical to that in human AE1 and mouse Ae1. Pairwise alignments of the Ae2.2 aa sequence with those of other SLC4/AE family members (Supplemental Table 3) revealed greater identity with zebrafish Ae2.1 and with AE2 of other species than with AE1 or AE3 polypeptides. This held true also in comparisons of individ-
Ae2.2 intron sizes are compared with those of zebrafish ae2.1 (zae2.1) and mouse Ae2 (M) genes. Introns 2, 7 and 13 remain of unknown length. In bold are exon sizes that are identical in ae2.2 and ae2.1 genes, and splice junction amino acid residues that are identical in Ae2.2 and Ae2.1. Period at end of the intron 13 5'-junction sequence marks the genomic clone terminus. Zebrafish ae2.2 genomic DNA was found as the following partially overlapping genomic scaffold contigs: Exons 1 and 2 in 20788 bp Zv6_scaffold741.5.32 (GenBank accession no. CAAK03012650); Exons 3-5 in 13152 bp Zv6_scaffold741.5.33 (GenBank accession no. CAAK03012651); Exons 5-7 in 8678 bp Zv6_scaffold741.5.33 (GenBank accession no. CAAK03063217); Exon 7 in 3598 bp Zv6_scaffold3510.1.4 (GenBank accession no. CAAK03063217); Exons 8-13 in 8909 bp Zv6_scaffold3510.1.3 (GenBank accession no. CAAK03063216); Exons 14-23 in 14747 bp Zv6_scaffold3510.1.2 (GenBank accession no. CAAK03063215); Exon 23 in 48956 bp Zv4_NA13401 (GenBank accession no. CAAK01015987.1).

### Table 2. Exon-intron boundaries of the zebrafish ae2.2 gene

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<th>Exon</th>
<th>5’ Junction</th>
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<td>3</td>
<td>gttagtcag</td>
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<td>2</td>
<td>gtaagtcag</td>
<td>4</td>
<td>tctcttcag</td>
<td>6</td>
<td>ttgtagcag</td>
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<td>21</td>
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Ac2.2 intron sizes are compared with those of zebrafish ae2.1 (zae2.1) and mouse Ae2 (M) genes. Introns 2, 7 and 13 remain of unknown length. In bold are exon sizes that are identical in ae2.2 and ae2.1 genes, and splice junction amino acid residues that are identical in Ac2.2 and Ac2.1. Period at end of the intron 13 5'-junction sequence marks the genomic clone terminus. Zebrafish ae2.2 genomic DNA was found as the following partially overlapping genomic scaffold contigs: Exons 1 and 2 in 20788 bp Zv6_scaffold741.5.32 (GenBank accession no. CAAK03012650); Exons 3-5 in 13152 bp Zv6_scaffold741.5.33 (GenBank accession no. CAAK03012651); Exons 5-7 in 8678 bp Zv6_scaffold741.5.33 (GenBank accession no. CAAK03012652); Exon 7 in 3598 bp Zv6_scaffold3510.1.4 (GenBank accession no. CAAK03063217); Exons 8-13 in 8909 bp Zv6_scaffold3510.1.3 (GenBank accession no. CAAK03063216); Exons 14-23 in 14747 bp Zv6_scaffold3510.1.2 (GenBank accession no. CAAK03063215); Exon 23 in 48956 bp Zv4_NA13401 (GenBank accession no. CAAK01015987.1).
Zebrafish Ae2.2 is a cation-independent, electroneutral Cl⁻/H¹¹0₂Cl⁻/H¹¹0₂ exchange. Ae2.2 polypeptide was abundantly expressed in Xenopus oocytes (Fig. 2A), and its expression was associated with concentration-dependent ³⁶Cl⁻/H¹¹0₂ influx at rates comparable to those exhibited by zebrafish Ae2.1 (Fig. 2B). The Cl⁻ transport was confirmed as Cl⁻/H¹¹0₂Cl⁻/H¹¹0₂ exchange by demonstration of trans-Cl⁻/H¹¹0₂ dependence of ³⁶Cl⁻/H¹¹0₂ efflux (Fig. 2, C and D). Both Cl⁻/H¹¹0₂Cl⁻/H¹¹0₂ and Cl⁻/H¹¹0₂/H¹⁰CO₃⁻/H¹¹0₂ exchanges were sensitive to the stilbene disulfonate inhibitor, DIDS (Figs. 2, C and D and 3). zAe2.2-mediated Cl⁻/H¹¹0₂Cl⁻/H¹¹0₂ exchange was cation-independent, insofar as bath Na¹¹⁰/H¹¹0¹ substitution with N-methyl-D-glucamine (Fig. 4A) or with K⁺ (Fig. 4B) diminished neither ³⁶Cl⁻/H¹¹0₂ efflux rate constants nor sensitivity to inhibition by DIDS.

Zebrafish Ae2.2 is subject to acute regulation. Mouse Ae2 and zebrafish Ae2.1 are acutely and independently regulated by intracellular and pH₀, and by NH₄⁺/H¹¹0₈ (14). We therefore examined zebrafish Ae2.2 for similar types of acute regulation. Fig. 6 shows that the rate of ³⁶Cl⁻/H¹¹0₂Cl⁻/H¹¹0₂ exchange by Ae2.2 is nearly doubled by bath addition of NH₄⁺/H¹¹0₈. As shown in Fig. 7, A and B, removal of butyrate following a period of butyrate-induced intracellular acidification accelerates the rate of ³⁶Cl⁻/H¹¹0₂ exchange by Ae2.2 nearly threefold. However, the relative inhibition of Ae2.2 by 40 mM butyrate may not be so great as for Ae2.1 (Fig. 7 and Ref. 14). Fig. 7C shows that acidic pH₀ inhibits Ae2.2 with a pH₀(50) value of 7.33 ± 0.16 (n = 10).
Although this value is slightly alkaline-shifted compared with that of Ae2.1 (6.94 ± 0.19, n = 9; Ref. 14), the shift is not statistically significant (P = 0.14).

Localization of ae2.2 mRNA expression. Recombinant Ae2.2 polypeptide and Ae2.1 polypeptide were detected equally well by antibody to the mouse AE2 COOH-terminal peptide (Fig. 7D), such that this antibody could not be used for unambiguous localization of Ae2.2 polypeptide. Therefore, Ae2.2 mRNA was localized in embryos by whole mount in situ hybridization (Fig. 8). Staining was faint at the 5, 10, and 17 somite stages (not shown). At 24 h postfertilization, low-abundance mRNA was evident in eye (likely retina) and tectum, and in a faint axial pattern suggestive of axial vasculature. The observed pattern reproduced that reported for in situ hybridization with a probe generated from the multiple deletion clone CB330 (19). Ae2.2 expression was not detected in pronephric duct, the major site of Ae2.1 expression. Ae2.2 localization was not altered in hematopoiesis-defective cloche mutants at the same developmental stages (not shown).

As shown in Fig. 7D by immunofluorescence in transiently transfected HEK-293 cells, and in Fig. 2A by immunoblot in oocytes, Ae2.2 and Ae2.1 are both detected by antibody to the highly conserved mouse AE2a COOH-terminal aa 1124–1237. Similar cross-reactivity was noted for antibody-to-mouse AE2a aa 330–352 (not shown). Therefore, neither antibody reagent is appropriate for immunolocalization of Ae2.2 polypeptide expression in zebrafish.

N-morpholino oligo knockdown of Ae2.2 and of Ae2.1 mRNAs. Injection of pairs of MOs at concentrations of 1.5 mM, 0.75 mM, and 0.4 mM each led to dose-dependent hemorrhagic necrosis in brain and eye accompanied by developmental delay, constriction of yolk sac extension, and retarded axial extension with enhanced body curvature (not shown). Injection of individual MO pairs at the concentration of 0.2 mM did not produce grossly evident toxicity (Supplemental Fig. 2A). At these concentrations, knockdown of Ae2.1 mRNA was nearly complete, as evidenced by generation of the intended exon 6 deletion product leading to termination of the open reading frame (Supplemental Fig. 2C). Knockdown of Ae2.2 mRNA was also of high efficiency. However, in addition to the intended exon 15 deletion product terminating after Ae2.2 TM span 1 (Supplemental Fig. 2B), the MO1/MO-2 pair also generated two alternate deletion products with in-frame deletions of 24 or 48 nt at the 3′ end of exon 15.
documented by DNA sequencing, these deletion products encoded Ae2.2 polypeptides lacking 8 (aa 761–768) or 16 amino acids (aa 761–776) from putative TM span 3 of Ae2.2, both predicted to be nonfunctional and/or unstable.

Knockdown of either Ae2.2 or Ae2.1 using MO concentrations of 0.4 mM or greater led to dose-dependent hemorrhagic necrosis. Individual knockdown of Ae2.2 or Ae2.1 using MO concentrations of 0.2 mM produced little if any necrosis, and produced mild developmental delay without evident specific developmental phenotype (Fig. 9). However, even with MO concentrations by 10.220.33.6 on April 20, 2017 http://ajpregu.physiology.org/ Downloaded from

**DISCUSSION**

Low stringency screening for Ae1 homologs of the zebrafish uncovered two zebrafish Ae2 genes, ae2.1 (14) and ae2.2 (present study). The two genes are each syntenic with human AE2, exhibit highly conserved intron-exon structures and encode multiple polymorphisms. The ae2.1 and ae2.2 genes encode polypeptides that share 70% amino acid identity, and each mediates electroneutral, cation-independent, anion exchange with similar properties of acute regulation. Whereas Ae2.1 is expressed at moderate levels in embryonic development and is predominantly localized at later stages in the anterior pronephric duct, Ae2.2 is expressed at low levels in head structures and in a diffuse pattern suggestive of axial vasculature. MO knockdown of Ae2.2 and Ae2.1 or both resulted in no apparent specific defect in early development. MOs at higher concentrations produced a nonspecific phenotype of developmental retardation, and inhibition of axial extension with curvature.

**Ae2 gene duplication in zebrafish.** The highly conserved genomic structure and the conserved sequence encoding a
conserved domain arrangement with conserved motifs all suggest that the \( ae2.2 \) and \( ae2.1 \) genes arose by gene duplication, a remnant of the whole genome duplication in ray-finned fishes believed to have occurred between 250M and 400M years ago (13). The 70% amino acid sequence identity is consistent with this remote gene duplication. Among the three of 21 exon-exon junctions of the \( ae2.2 \) gene coding region, which do not share superimposable locations with the zebrafish \( ae2.1 \) gene and the mouse \( Ae2 \) gene, the exon 10–11 junction is identical in the \( ae2.2 \) and \( ae2.1 \) genes, but differs in the mouse \( Ae2 \) gene, consistent with the occurrence of this gene duplication after the evolutionary divergence of fish from other vertebrates (13). The 5–6 junction which differs between the \( ae2.2 \) and \( ae2.1 \) genes is the site of alternative promoter usage in the mouse gene generating the functionally distinct \( Ae2c1 \) polypeptide (7). The \( ae2.1 \) exon 13–14 junction is conserved in mouse \( Ae2 \) gene but not in zebrafish \( ae2.2 \). This junction lies adjacent to a predicted proteolytic cleavage site, which, in the homologous \( Ae1 \) genes, defines the topological boundary separating most of the NH2-terminal cytoplasmic domain from the TM domain.

The hypothesis of \( Ae2 \) gene duplication is substantiated by shared synteny of both zebrafish \( Ae2 \) genes, \( ae2.1 \) on LG2 and \( ae2.2 \) on LG24, with apparently nonoverlapping regions of human chromosome 7q36 closely adjacent to the human \( AE2 \) gene. Such dual LG24 and LG2 synteny with human 7q36 is shared by only one linked zebrafish gene pair shown in Table 1, \( cul1b \) and \( cul1a \). Additional genes pairs preserve synteny for only one of the duplicated genes. Thus \( abcf2, smarcd3, \) and \( eng2b \) reside on LG2, but their duplicated homologs are found en bloc on LG7, suggestive of a postduplication intrachromosomal recombination. The duplicated homolog of LG2 gene \( shhb, shha, \) is found on LG15. Additional examples of preserved gene duplications in the zebrafish genome include insulin-like growth factor 1 receptors (12), voltage-gated Na+ Fig. 5. \( zAe2.2 \) does not mediate an anion conductance. A: current-voltage relationships of oocytes previously injected with water (n = 4), measured sequentially in ND-96 (white circles), in Na gluconate (black circles), and with subsequent addition of 200 \( \mu \)M DIDS (white triangles). B: current-voltage relationships of \( zAe2.2 \)-expressing oocytes (n = 5), measured sequentially in Na gluconate (black circles), ND-96 (white circles), and with subsequent addition of 200 \( \mu \)M DIDS. The small currents are not altered by expression of \( zAe2.2 \).

![Fig. 5](https://example.com/f5.png)

Fig. 6. \( zAe2.2 \)-mediated \(^{36}\text{Cl}^-/\text{Cl}^- \) exchange is stimulated by \( \text{NH}_4^+ \). A: \(^{36}\text{Cl}^- \) efflux traces from representative individual oocytes previously injected with water or with \( zAe2.2 \) cRNA and exposed sequentially to ND-96, ND-96 in which 20 mM NaCl was replaced with NH4Cl, and to 200 \( \mu \)M DIDS. B: summary of \( \text{NH}_4^+ \) stimulated, DIDS-sensitive \(^{36}\text{Cl}^- \) efflux from oocytes expressing \( zAe2.2 \) or \( zAe2.1 \). Number of oocytes tested from (n) frogs is shown above bars.

![Fig. 6](https://example.com/f6.png)
channels (9), and two of the 29 muscular dystrophy gene homologs (15). Genes encoding cKit receptors and cKit ligands are both duplicated, and have evolved paralogous receptor-ligand specificity (6).

However, many neighboring genes of human chromosome 7 immediately adjacent to the AE2 gene have only one known homolog in the zebrafish genome, a situation reflecting most of the zebrafish genome. This is consistent with zebrafish diploidy, and reflects the evolutionary loss of one of the duplicated genes (13). Fish genes preserved in duplicated form are hypothesized to have been maintained under selective pressure. Most fish gene coding regions have evolved faster than their mammalian orthologs (1), as perhaps reflected in the high degree of polymorphism detected in the ae2.2 gene (Supplemental Table 1; note, however, that 10 nonsynonymous and 15 synonymous cSNPs have been reported in the human AE2 gene as of Sept. 2007). Many fish gene pairs also exhibit asymmetrically accelerated evolution of the paralogs (1). However, this generalization appears not to apply to zebrafish ae2.2 and ae2.1 coding regions, which are equidistant in sequence similarity from the most closely related mammalian SLC4 polypeptides. Thus, the preservation of two ae2 genes evolving at apparently equivalent rates supports the hypothesis that both gene products play important functions in the zebrafish, despite the absence of identifiable specific phenotype arising from mRNA knockdown during early development.

**Functional characterization of ae2.2 polypeptide.** Maintenance of two related genes through evolution might reflect acquisition of distinct functional activities or distinct patterns of acute regulation. However, the Ae2.2 polypeptide did not differ from the Ae2.1 polypeptide in any tested functional index.

**Fig. 7.** zAe2.2-mediated $^{36}$Cl$^{-}$/H$^+$ exchange is stimulated by intracellular and extracellular alkalinization. 

A: $^{36}$Cl$^{-}$ efflux traces from representative individual oocytes previously injected with water or with zAe2.2 cRNA and exposed sequentially to ND-96 in the presence and subsequent absence of 40 mM sodium butyrate, followed by 200 μM DIDS. B: summary of butyrate removal-stimulated, DIDS-sensitive $^{36}$Cl$^{-}$ efflux from oocytes expressing zAe2.2 or zAe2.1. Number of oocytes tested from (n) frogs is shown above bars. C: $^{36}$Cl$^{-}$ efflux rate constants were measured sequentially at each extracellular pH (pHo) in each individual oocyte and normalized to each oocyte’s value at pHo 8.0. Results are from 10 oocytes from 2 frogs. D: Ae2.2 is recognized by anti-mouse AE2 aa 1224-1237. HEK-293 cells on coverslips were transiently transfected with cDNA encoding zAe2.2 (a and b) or zAe2.1 (c and d). At 48 h later, cells were fixed, permeabilized, and immunostained with anti-mAE2 COOH-terminal aa 1224-1237 in the presence of 24 μg/ml irrelevant peptide (a and c) or peptide antigen (b and d).
Ae2.2, a second zebrafish Slc4a2 anion exchanger

was DIDS-sensitive, cation-independent, and electroneutral, all properties shared with Ae2.1. Ae2.2-mediated Cl⁻/H⁺ exchange was also acutely activated by NH₄⁺, acutely inhibited by acidic pHᵢ, and acutely inhibited by acidic pHₒ, again properties in common with Ae2.1. The pHₒ(50) value of Ae2.2 was not significantly different from that of Ae2.1. Thus, as judged by the assays tested to date, distinct anion transport function does not explain preservation of the duplicated Ae2 genes in evolution.

It is curious that three of four cSNPs of the NH₂-terminal cytoplasmic domain of Ae2.1 (P147R, I149T, E152D) reside within a region, which, when mutated in mouse Ae2a or when absent from mouse Ae2c1, lead to an alkaline-shifted pHₒ(50) (7). The single TM domain cSNP (V882M) is located at the COOH-terminal end of the ecto-loop connecting TM5 and TM6 (“Z loop”) immediately adjacent to two residues, which, when mutated in mouse Ae2, acid-shifts or alkali-shifts pHₒ(50), respectively, (16) (only the latter is conserved in Ae2.1 as K884).

Localization of Ae2.2 mRNA in zebrafish embryo. Ae2.2 and Ae2.1 mRNAs do differ in localization pattern of expression. In contrast to the moderately high level of Ae2.1 expression in pronephric duct (14), Ae2.2 is absent from pronephric duct and expressed at low level in eye (likely retina) and tectum, and in axial vasculature. Ae2.2 is expressed at lower levels than Ae2.1 during early somite stages of development. The low expression level is evident also in the proportional representation of cDNAs encoding Ae2.1 (51 hits, most from kidney, some from testis, few from heart) and Ae2.2 (4 hits, all from embryo) in the Expressed Sequence Tag (EST) database (Sept 15, 2007).

Role of the two ae2 genes in embryonic development. Preservation of both ae2 genes through evolution suggests a selective advantage or selective pressure at work. Distinct sites of expression constitute the best evidence for nonoverlapping functions of Ae2.2 and Ae2.1. However, these nonoverlapping expression patterns do not reveal whether in each expression site Ae2 function is essential or redundant.

Combined knockdown of both Ae2.2 and Ae2.1 (at effective MO concentrations, which, as individual MO pairs, were apparently nontoxic) produced minimal generalized developmental delay and minimally impaired axial elongation (Fig. 9).
and Supplemental Fig. 2A). In all surviving embryos (the large majority of those injected) this developmental delay did not lead to any abnormality grossly evident at later developmental stages.

**Perspectives and Significance**

We conclude that neither Ae2.1 nor Ae2.2 are essential for normal early embryonic development. This conclusion is consistent with the developmental role of the single mouse Ae2 gene, whose knockout allows early embryonic development and birth, but is manifest as peri- and postnatal growth retardation and periweaning death (4). Ae2.2 may contribute to regulation of cell pH, cell volume, and cell [Cl\(^{-}\)] in embryonic head structures, as Ae2.1 likely does in the anterior segment of the pronephric duct. However, the knockdown results suggest that the zebrafish genome expresses redundant functions that compensate for loss of Ae2.2 and Ae2.1 expression during early development. These redundant functions may be encoded by additional SLC4 genes, by SLC26 genes, or by yet other genes. Knockdown embryos might exhibit a phenotype under imposed stress conditions indicating conditional requirement for one or both Ae2 polypeptides. Ae2.2 and/or Ae2.1 might alternatively play more important physiological roles in the more mature or adult fish at times beyond the efficacy of MO-mediated mRNA knockdown.

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