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Identification of distal enhancers for *Six2* expression in pronephros

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ABSTRACT The embryonic nephric mesenchyme contains pluripotent progenitor cells. *Six2,* a homeodomain transcription factor, is expressed in a subset of the nephric mesenchyme, and it functions to maintain a progenitor state by suppressing nephrogenesis. Despite the functional significance of Six2 in nephric development, its regulatory mechanisms remain unclear. To identify the *cis*-regulatory elements for *Six2,* we focused on the evolutionarily conserved sequences known as conserved noncoding sequences (CNSs) associated with the *Six2* locus. Transgenic experiments using *Xenopus laevis* embryos revealed that three of the eight CNSs located within a 317-kb segment of the *Six2* genomic locus were nephric enhancers. Motif analysis of transcription factors combined with phylogenetic footprinting revealed the enrichment of putative T-cell factor (Tcf)-, Hox-, and SWI/SNF complex helicase-like transcription factor (Hltf)- and AT-rich interactive domain 3A (Arid3a)-binding motif sequences in these enhancers.

KEY WORDS: *Six2, conserved noncoding sequence, nephric enhancer, Xenopus laevis, transgenic system*

Introduction

Six2 is a vertebrate homolog of the *Drosophila* homeobox gene *sine oculis* and is expressed in developing neural tissues, such as the eyes, otic placodes, and cranial ganglia, and in non-neural tissues, such as the head mesenchyme, somite, muscle anlagen, and mesenchyme associated with the nephron in *Xenopus* embryos (Ghanbari *et al*., 2001). Targeted disruption of Six2 in mouse embryos results in ectopic premature epithelialization of the nephric mesenchyme (Self *et al*., 2006). Moreover, Six2 was recently found to control both the self-renewal of nephron progenitors and the commitment to nephric lineages with Wnt-signaling components, β-catenin and lymphoid enhancer factor (Lef)/T-cell factor (Tcf) in mouse embryos (Park *et al*., 2012). Although these studies have illustrated the essential roles of Six2 in the early steps of nephrogenesis, its regulatory mechanisms remain unclear.

C*is*-regulatory analysis of a key regulatory gene is a straightforward approach for understanding the mechanisms of tissue induction and development. However, because some enhancers are located distantly from their target genes, identification via traditional genome walking was a slow and painstaking process. This situation has recently been changed by the development of comparative genomics approaches, which discovered a number of evolutionarily conserved noncoding sequences (CNSs) associated with developmental genes (Woolfe *et al*., 2005). Transgenic analyses using mice, zebrafish, and frogs have revealed that some CNSs are actually important enhancers and/or silencers for gene expression (Woolfe *et al*., 2005)(Ochi *et al*., 2012).

In this study, we identified eight CNSs that are conserved in tetrapods and are located within a 317-kb segment of the *Xenopus tropicalis Six2* genomic locus. A transgenic reporter assay in *Xenopus* embryos revealed that three of these CNSs function as nephric enhancers. Phylogenetic footprinting analysis of these enhancers followed by motif searches identified putative Tcf-, Hox-, helicase-like transcription factor (Hltf)-, and AT-rich interactive domain 3A (Arid3a)-binding sequences as the enriched motifs. These results suggest that the combination of input transcription factors, Tcf, Hox, and SWI/SNF complex on the distal enhancers drive *Six2* expression in pronephros.

Abbreviations used in this paper: CNS, conserved noncoding sequence; GFP, green fluorescent protein.

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Results and Discussion

Conserved noncoding sequences in the Six2 *locus*

The homeodomain transcription factor Six2 expressed within the kidney mesenchyme suppresses nephrogenesis to maintain progenitor renewal (Ghanbari *et al.*, 2001)(Self *et al.*, 2006). An enhancer assay using cultured kidney cells revealed that the Hox–Eya–Pax complex regulates *Six2* expression through a 450-bp genomic region upstream of the *Six2* translational start site (Gong *et al.*, 2007). A transgenic enhancer assay using mice also illustrated that a 1-kb segment upstream of *Six2* containing the Hox–Eya–Pax complex-binding site drove reporter expression in kidney cells (Yallowitz *et al.*, 2009). Although the *cis*-regulatory elements controlling developmental gene expression are often associated within the proximal region of the gene, some *cis*-regulatory elements are known to have the ability to function over distances of 100 kb or longer (Kleinjan *et al.*, 2006). To date, no distal enhancers for Six2 were reported, since the identification of such distal enhancer using traditional genome walking were very costly and laborious. The evolutionarily CNSs represent landmarks for identifying such distal enhancers (Woolfe *et al.*, 2005)(Ochi *et al.*, 2012). To explore the *cis*-regulatory candidates for the distal enhancers of *Six2*, we initially compared the genomic sequence of a 364-kb segment encompassing human *SIX2* with the orthologous regions in the mouse, opossum, and frog (*X. tropicalis*) genomes using the MulitPipMaker alignment tool (Schwartz, 2000). This analysis identified eight CNSs (CNS1–CNS8) as candidate *Six2* *cis*-regulatory elements (Fig. 1A).

Identification of Six2 *nephric enhancers using a* Xenopus *transgenic reporter assay*

The *X. laevis* transgenic system is known as an efficient transgenesis technique for a non-mosaic founder assay and as a useful system to screen for *cis*-regulatory elements (Ogino and Ochi, 2009). *Six2* from *X. laevis*, a tetraploid frog, is expressed in the region of the developing pronephric kidneys, resembling mouse *Six2* expression (Ghanbari *et al.*, 2001). A diploid frog *X. tropicalis* was recently developed as a model animal, and its genomic sequences are available. Hence, this genomic resource is suitable for enhancer screening using the *X. laevis* transgenic system. The expression of endogenous *Six2* in *X. tropicalis* embryo was first examined by *in situ* hybridization, and the results confirmed that its expression in pronephric kidneys closely resembled that of *X.laevis* (Fig. 1B–D)(Ghanbari *et al.*, 2001). Each *X. tropicalis* *Six2* CNS was cloned into a GFP reporter plasmid carrying a β-actin basal promoter. Each construct was then used to generate transgenic embryos, and their reporter expression was examined once the resulting embryos reached stage 35/36, at which point endogenous *Six2* is expressed in the proximal tubule region (Fig. 1 C,D)(Ghanbari *et al.*, 2001). The reporter constructs without a CNS displayed non-significant GFP expression (Table 1). Of the eight *Six2* CNSs tested, three CNSs, CNS2, CNS3, and CNS5, drove strong and reproducible pronephric expression (Fig. 2 B,C, and E; Table 1). CNS6 was immediately adjacent to *Six2*, for which the corresponding region previously identified as the nephric enhancer in mice displayed reporter expression in the pronephros, although the frequency of pronephric positive embryos was less than that of CNS2, CNS3, and CNS5 expression (Fig. 2F; Table 1)(Gong *et al.*, 2007) (Yallowitz *et al.*, 2009). CNS4 was also expressed in the pronephros, and its frequency of expression was less than those of CNS2, CNS3, and CNS5 (Fig. 2D; Table 1). CNS7, located downstream of *Six2*, drove GFP reporter expression in otic vesicles, in which endogenous *Six2* is expressed (Fig. 2G; Table 1). CNS8, which was located near *Six3*, was active in the eye tissues (Fig. 2H; Table 1). Transverse sections indicated that CNS2 and CNS3 drove strong GFP reporter expression both in the pronephric tubules and in the non-glomus mesenchyme, while CNS5 was mostly expressed in the pronephric tubules (Fig. 2. B'',C'',E''). Thus, we identified CNS2, CNS3, and CNS5 as nephric enhancers of *Six2*. These enhancers are located 82, 75, and 40 kb upstream of the *Six2* transcription start site in the *X. tropicalis* genome (Fig. 1A). Shadow enhancers drive a pattern of transcription that resembles the drive pattern of previously identified primary enhancers (Barolo, 2012). According to this category, CNS6, immediately adjacent to *Six2*, is categorized as the primary enhancer, and CNS2, CNS3, and CNS5 are considered shadow enhancers (Barolo, 2012). Because shadow enhancers modulate pheno-

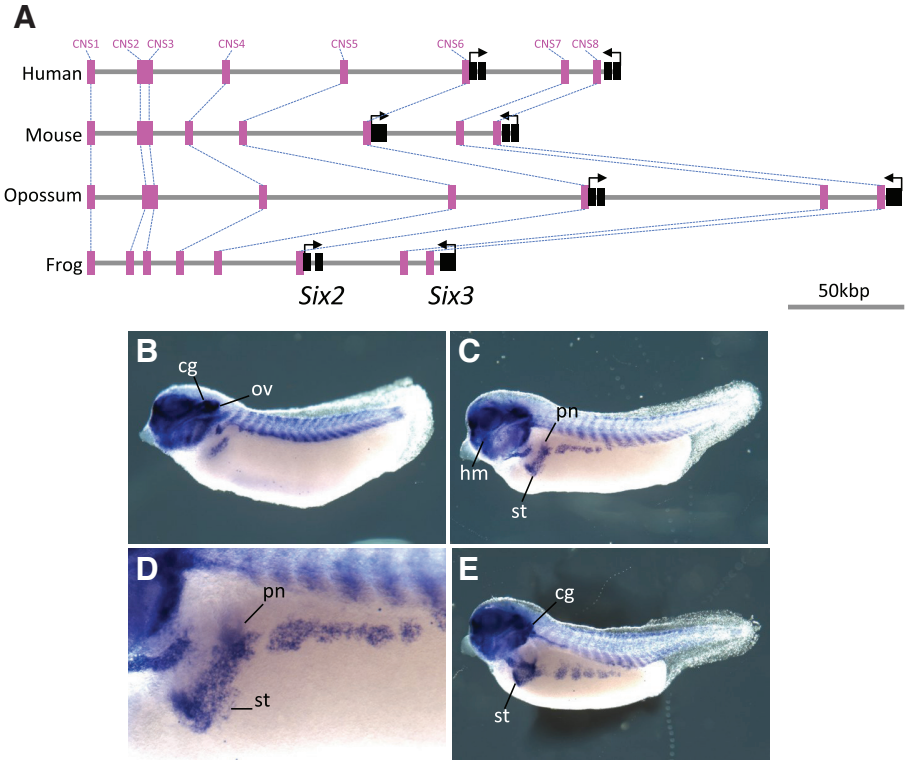

Fig. 1. Conservation of noncoding sequences across the *Six2* locus in tetrapods. **(A)** *Schematic overview of the* Six2 *locus. Magenta and black boxes indicate conserved noncoding sequences and exons, respectively.* **(B–D)** In situ *hybridization analysis illustrating the expression of* Six2 *in* Xenopus tropicalis *at the stages 26, 35/36, and 43* **(B,C,E)**. *Higher magnification image of stages 35/36 embryos* **(D)**. *Pn, pronephros; st, stomach; hm, head mesenchyme; ov, otic vesicle; cg, cranial ganglia.*

TABLE 1

SCORING RESULTS FROM THE *XENOPUS* TRANSGENIC REPORTER ASSAY

Construct	Analysed embryos (n)	Number of GFP positive (n)	Pronephrons	MHB	Neural tube	Eye	Otic Vesicle	Pharyngealarch	Somite	% of pronephric activity
β-GFP	82	2				+			+	0%
		1				+		+	+	
Six2-CNS1-β-GFP	103	5						+	+	3%
		5				+				
		3							+	
		2	+						+	
		1	+		+				+	
		1			+			+		
		1		+				+	+	
Six2-CNS2-β-GFP	106	10	+					+		18%
		5	+	+				+		
		3	+		+			+		
		3						+		
		2		+				+		
		1	+	+				+		
		1						+	+	
		1		+				+		
Six2-CNS3-β-GFP	92	18	+					+	+	19%
		1		+						
		1			+					
		1				+				
Six2-CNS4-β-GFP	104	4	+					+		6%
		3				+		+	+	
		3							+	
		2	+							
		2				+			+	
Six2-CNS5-β-GFP	142	9	+					+	+	22%
		6	+			+		+	+	
		5						+	+	
		3	+							
		2	+							
		1	+						+	
		1	+		+				+	
		1	+			+	+		+	
		1	+				+		+	
		1							+	
		1				+				
Six2-CNS6-β-GFP	89	4	+					+	+	8%
		2	+		+					
		1	+					+		
Six2-CNS7-β-GFP	74	3					+			5%
		2	+			+		+	+	
		2		+			+	+		
		1	+	+		+	+	+		
		1	+			+		+		
		1				+		+		
Six2-CNS8-β-GFP	133	13				+				6%
		9				+		+	+	
		4	+			+		+		
		3	+			+			+	
		2				+		+		
		1				+			+	
		1	+			+				

Green fluorescent protein (GFP) expression patterns were examined at stage 35/36. The expression domains for each construct are indicated by +. For each construct, the reproducible expression patterns were consistent with representative examples shown in Figure 2. The percentage of pronephric activity indicates the total number of GFP-expressing embryos in the pronephros among all examined embryos. MHB, midbrain-hindbrain boundary

typic robustness, *Six2* distal nephric enhancers may function as redundant enhancers (Barolo, 2012).

Characterization of the candidate transcription inputs

To dissect the transcriptional inputs for the *Six2* nephric enhancers, the open-access database JASPAR ver. 5 was used to define potential transcription factor-binding sites (Mathelier *et al.*, 2014). The candidate transcription factors were narrowed on the basis of their nephric expressions using the Expression Atlas (the baseline atlas)(Petryszak *et al.*, 2014). Finally, candidate transcription factors were narrowed by phylogenetic footprinting. Hox, Eya, and Pax are known regulatory factors for *Six2* expression (Gong *et al.*, 2007). The secreted molecule Wnt9b and its downstream transcription factor TCF/Lef1 is also known to regulate kidney development (Karner *et al.*, 2011). Transcriptional input motif analysis uncovered Hox motifs in CNS2 and CNS5 and Tcf motifs in CNS3 and CNS5 (Fig. 3 A–C). Pax2, the aberrant expression of which is sufficient to induce ectopic nephric structures, was present in CNS5 (Bouchard *et al.*, 2002). Other motifs, such as Sox9, a regulator of nephric duct branching, and early B-cell factor (Erb1), a regulator of podocyte differentiation and glomerular maturation, were found in CNS5 (Fig. 3C)(Reginensi *et al.*, 2011)(Fretz *et al.*, 2014). Motifs for the components of the SWI/SNF complex, Arid3a and Hltf, were found in CNS2 and CNS5.

CNSs were further analyzed using the motif-finding program MEME to identify the consensus motifs in *Six2* nephric enhancers, and two consensus motifs were identified (Bailey *et al.*, 2009) (Fig. 3D). Motif 1 is partially overlapped with the putative Hoxa5-binding motif derived from JASPAR in CNS2 and with Tcf3 in CNS3 (Fig. 3). Motif 2 is partially overlapped with Tcf3 and basic helix-loop-helix family, member e40-binding motif in CNE5 (Fig. 3).

The consensus motifs in *Six2* nephric enhancers driven by the

TABLE 2

PRIMER SEQUENCES FOR CLONING *SIX2* CONSERVED NONCODING SEQUENCES (CNSS)

Target	Location in genome assembly	Primer Sequences
X. tropicalis Sxi2-CNS1	xenTro3,GL172661:1,324,307-1,325,472	tctagaTTGCCAAATCATTCCAAACCGCTAAAAGTC ggatccGGGAAAGTATTATAAACAGAACAGATGGGTGC
X. tropicalis Sxi2-CNS2	xenTro3,GL172661:1,345,329-1,345,807	tctagaTCTAGA GAGTCTCTATGCCTTCACTACAAGC ggatccCAAACGTACATGTTTTTGCTGAAT
X. tropicalis Sxi2-CNS3	xenTro3,GL172661:1,352,848-1,352,986	tctagaTTTGTATTCTGCCTCGGTTACGTTCC ggatccCTTCTATTATATACAAAAGGATTAAATACAGATGTTTCCC
X. tropicalis Sxi2-CNS4	xenTro3,GL172661:1,367,624-1,367,928	tctagaGAAGAGGCCCAAACAACCCCTGATTAATTA ggatccAGATGAATATACGAACCTTGAGTTTTAGTGCTAAAAGC
X. tropicalis Sxi2-CNS5	xenTro3,GL172661:1,386,790-1,387,226	tctagaCTGCTTCCTTCTAAATTCGTTCAGCGTG ggatccCCCTGACTGTCACTTTGGAAATAAAACTGAG
X. tropicalis Sxi2-CNS6	xenTro3,GL172661:1,427,350-1,427,819	ggatccGGAGCAATCTGTCAAGCCAGCCCGG ctgcagATCCTGGCTGGGGCCCCCTG
X. tropicalis Sxi2-CNS7	xenTro3,GL172661:1,475,679-1,475,909	tctagaGCAGGTATCACTGGTGAGGGGG ggatccTTGTTGAATGTTTTACTCCATCTCCCCACAG
X. tropicalis Sxi2-CNS8	xenTro3,GL172661:1,487,514-1,487,667	tctagaGTCAGGGCCAAATGTATCACCGAG ggatccGTTGGTGCCCTGTTAGTGCTCTG

Evolutionarily CNSs were extracted using MultiPipMaker. Primers for CNS1, CNS3, CNS4, CNS5, CNS6, and CNS8 targeted the ends of the CNS. Primers for CNS2 targeted a region outside the CNS because we failed to amplify the target sequences using primers targeting the end of the CNS. The locations of the *cis*-regulatory elements in genome assemblies were identified using the UCSC genome browser. Primer linker sequences are shown in lowercase letters.

MEME motif-finding program illustrated that they are partially overlapped with putative Hoxa5- and Tcf3-binding motifs, whereas the transcription factors that bind to the consensus motifs are unclear. The Hox–Eya–Pax complex and Wnt signaling component Tcf/Lef1 are known to regulate *Six2* expression (Gong *et al.*, 2007) (Park *et al.*, 2012). Therefore, the input by Tcf3, Pax2, and Hoxa5 with unidentified transcription factors that bind to the consensus motifs 1 and 2 may be common features among the *Six2* distal enhances. Although the functional roles of the SWI/SNF complex Arid3a and Hltf with regard to *Six2* expression are unknown, they are expressed in cells of nephric linage (Petryszak *et al.*, 2014). Hence, Arid3a and Hltf are possibly involved in nephric development. Moreover, although further analysis is required, candidate transcription inputs with motifs that are evolutionarily conserved between humans and frogs may contribute to *Six2* expression in both amphibians and mammals during nephrogenesis.

Materials and Methods

Ethics statement

All animal care and experiments conformed to the Guidelines of Animal Experiments of Yamagata University, and the experimental protocols were approved by the Animal Research Committee of Yamagata University (26-075).

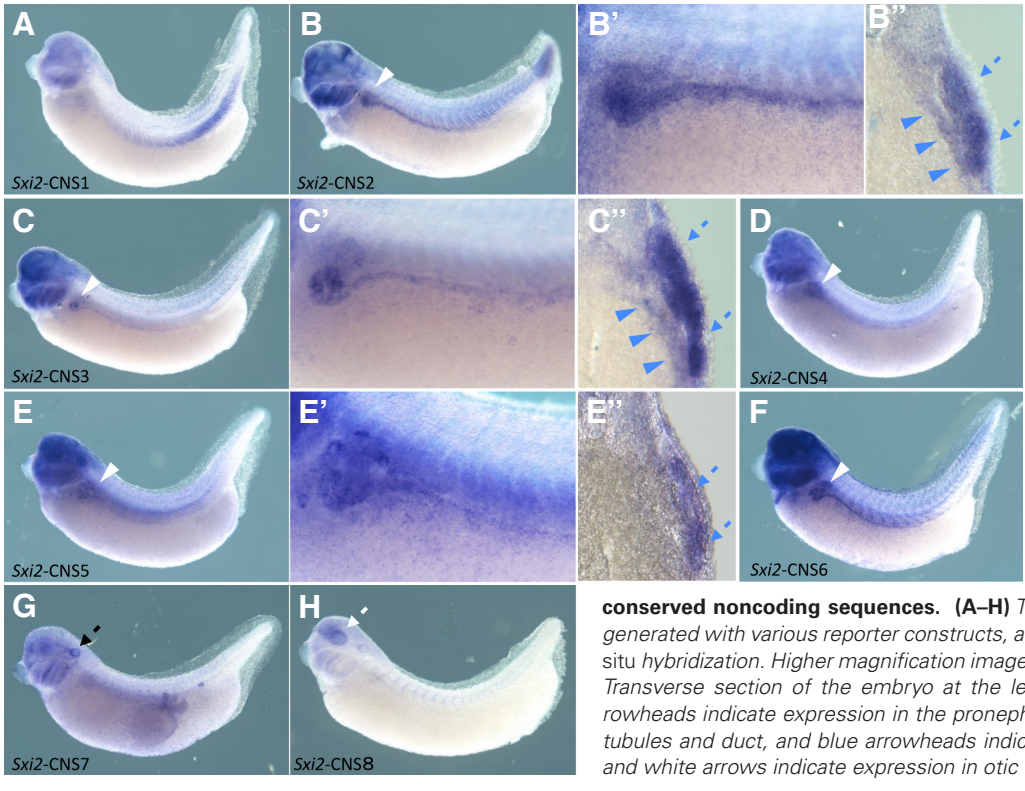

Fig. 2. Green fluorescent protein expression pattern driven by *Six2*-conserved noncoding sequences. (A–H) *Transgenic* Xenopus laevis *embryos were generated with various reporter constructs, and their expression was analyzed using* in situ *hybridization. Higher magnification image of pronephric positive embryos* **(B′,C′,E′)**. *Transverse section of the embryo at the level the pronephros* **(B″,C″,E″)**. *White arrowheads indicate expression in the pronephros. Blue arrows indicate the pronephric tubules and duct, and blue arrowheads indicate the non-glomus mesenchyme. Black and white arrows indicate expression in otic vesicle and the eye tissues, respectively.*

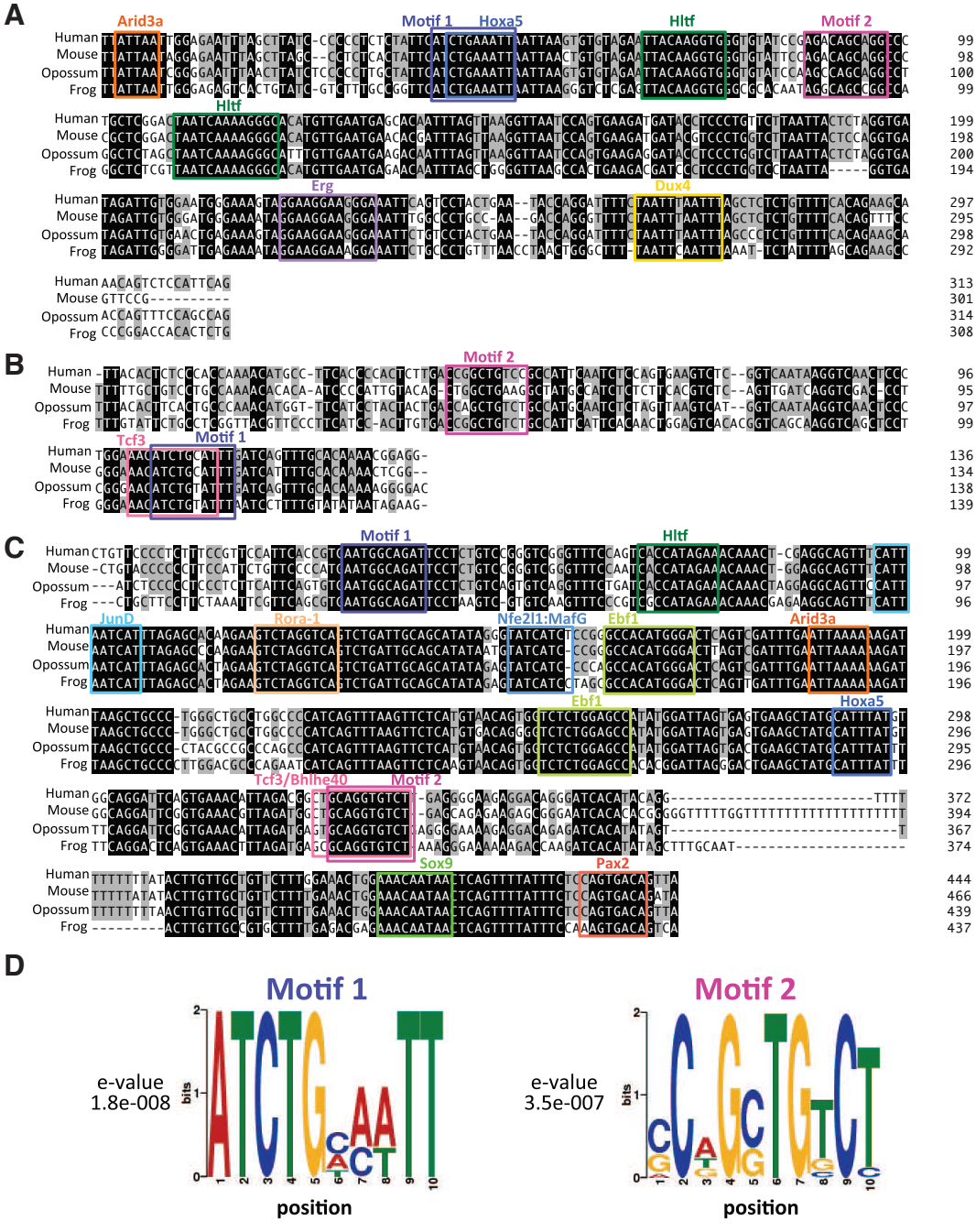

Fig. 3. Phylogenetic footprinting analysis for *Six2* nephric enhancers. **(A–C)** *Alignment of* Six2 *with conserved noncoding sequence 2 (CNS2)* **(A)**, *CNS3* **(B)**, *and CNS5* **(C)**. *Potential transcription factor-binding motifs in nephric enhancers were extracted using JASPAR ver. 5. Among them, candidates expressed in nephric tissues were selected and further analyzed by phylogenetic footprinting.* **(D)** *Sequence logo representation of the consensus motifs in all nephric enhancers. The size of the characters represents the relative frequency of the corresponding position. Sequence of motif 1 in CNS5 (C) and motif 2 in CNS2 (A) indicate reverse complement motifs. The colored boxes denote the evolutionarily conserved putative transcription factor-binding motifs.*

Identification of conserved non-coding sequences

The 364-kb genomic sequence of the human *SIX2* locus (hg19-chr2_45112852-45477594) and its orthologous sequences in mice (mm10 chr17-84875598-85861124), opossums (monDom5 chr1-652674101-653951640), and *X. tropicalis* (Xenbase 7.1, scaffold_5_108443461-108867602) were downloaded from the UCSC Genome Browser and Xenbase Genome Browser. These sequences were aligned using MultiPipMaker (Schwartz, 2000).

Construction of reporter plasmids

The green fluorescent protein (GFP) reporter plasmid carrying a chicken β-actin basal promoter (–55 to +53) was previously described as β-GFP (Ogino and Ochi, 2009). The CNSs were amplified from *X. tropicalis* genomic DNA by PCR and cloned into β-GFP reporter vectors. The primer sequences used in this study are summarized in Supplementary Table 2.

***Cloning of* X. tropicalis Six2**

A cDNA fragment of *Six2* was amplified from a cDNA pool of *X. tropicalis* tailbud embryos (stage 26) using the following primers: Xl_Six2_F: ATGTCGATGCTTCCGACTTTTGGC, Xl_Six2_R: TTAAGAGCCAAGGTCTACCAAGTTGGATG. The product was introduced into the *Xho*I and *Xba*I sites of pCS2+MT plasmid, and correct cloning was verified by sequencing analysis. The resulting plasmid was linearized by *Nco*I and used as the template for RNA probe synthesis.

Transgenic reporter assay

Transgenic *Xenopus laevis* embryos were generated by the sperm nuclear transplantation method with oocyte extracts (Kroll and Amaya,

1996). The manipulated embryos were cultured until stages 35-36, and all normally developed embryos were subjected to *in situ* hybridization to examine their GFP expression with maximum sensitivity. All CNS-carrying reporters were tested at least three times. The frequency of GFP expression varied depending on the constructs, but all constructs exhibited a reproducible expression pattern. The CNSs that drove nephric expression in more than 15% of the examined embryos were defined as nephric enhancers.

Motif analysis for transcription factor-binding sites

The open-access database JASPAR ver. 5 was used to search for potential transcription factor-binding sites in nephric enhancers (Mathelier *et al*., 2014). The candidate transcription factors were narrowed on the basis of their expression using the Expression Atlas (Petryszak *et al*., 2014). CNSs were aligned using ClustalW, and conserved sequences for the candidate transcription factor-binding sites were further analyzed by phylogenetic footprinting (Blanchette and Tompa, 2002). The MEME suite was used to discover the consensus motifs in *Six2* nephric enhancers (Bailey *et al*., 2009).

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