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Xenopus hoxc8 during early development

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Abstract

Vertebrate *hoxc8* homologous genes have been shown to be involved in the formation of lower thoracic/lumbar vertebrae during early embryonic development. We report the isolation of a *Xenopus hoxc8* (*Xhoxc8*), which shows 94% amino acid sequence identity to the mouse counterpart. *Xhoxc8* is initially expressed in a broad region of blastopore lip at gastrular stage; however, at later stages, the region of expression is progressively restricted to the dorsal region caudal to the third somite and to the central trunk region of abdomen. Retinoic acid treatment that caused a severe malformation in antero-posterior axis did not induce any significant change in the spatio-temporal expression pattern of *Xhoxc8* mRNA. Antisense RNA injection into 2- or 4-cell stage embryos resulted in a severe malformation in the abdominal structure leading to embryonic death. The results strongly indicate that *Xhoxc8* expression is critical for the formation of abdominal structure.

Keywords: Homeobox gene; Posterior spinal cord; Development; Abdomen; *hoxc8*; *Xenopus laevis*

Embryonic development is controlled by the expressions of a hierarchy of regulatory genes. In *Drosophila*, critical positions in the developmental hierarchy are occupied by the homeotic gene expression [1]. In vertebrates, *hox* genes are expressed in many types of cells and show dynamic spatial and temporal expression patterns that can be dramatically different even within a single tissue or structure, dependent upon the stage of analysis [2,3]. These genes appear to control the formation of body segment-specific structures by regulating the expression of specific sets of downstream effectors that, in turn, direct the morphogenic events leading to the formation of complex body forms [4], including anterior–posterior axis formation [5,6], muscle patterning [7], and formation of central nervous system [8].

There are four *hox* gene clusters in vertebrates that are located in separate chromosomes, in which, *hox* genes are arranged in the order that the physical order of genes along the chromosome and their expression/function along antero-posterior axis of the embryo are correlated [9,10]. The four copies of the *hox* gene clusters are called as *Hoxa-d* (*HOXA-D*) and each cluster is located on different chromosomes and contains 13 subsets of genes. Studies on function by gene targeting showed that the null mutant mice were born alive, but died within a few days. The mutant mice showed skeletal abnormalities between 7th thoracic vertebra and the 1st lumbar vertebra, which is within the region of *mouse hoxc8* expression [11]. In human, it has been suggested that *hoxc8* gene could be involved in malignant transformation of keratinocytes [12,13]. To have better understanding on the function of *hoxc8* using an easily manipulatable animal system, we have isolated the *Xenopus* parolog of the vertebrate *hoxc8* genes. Here we report the isolation, spatio-temporal expression pattern and function of *Xenopus hoxc8* during early embryonic development.

Materials and methods

Embryos, RNA microinjection, and retinoic acid treatment. *Xenopus laevis* embryos were obtained by standard methods [14] and staged according to Nieuwkoop and Faber [15]. Capped synthetic sense and antisense RNA for *Xhoxc8* was produced from pB*Xhoxc8* plasmid linealized by *Bam*HI and *Hin*dIII, respectively. Four and

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half nanoliters of sense or antisense RNA was injected into each blastomere of 2- or 4-cell stage embryos in 1× MMR plus 5% Ficoll and, at stage 6, the embryos were transferred to 0.1× MMR plus 5% Ficoll for the remainder of the culture period. Total 1 ng of synthetic RNAs diluted in 88 mM NaCl, 5 mM Tris, pH 7.5, was injected into each embryo. Control embryos were injected with 1 ng of sense RNA. To study the effects of retinoic acid on the *Xhoxc8* mRNA expression, embryos were continuously treated with 1 or 10 μM of all-*trans* retinoic acid (Sigma) dissolved in dimethyl sulfoxide (DMSO, Sigma).

cDNA library screenings and 5′-RACE. A 400 bp-long mouse *hoxc8* cDNA (a gift from Dr. Myoung Hee Kim, KIST, Korea) was used as probe to screen a frog neurula stage (stage 17) cDNA library [16]. Four independent positive cDNAs were identified and cloned into the *Eco*RI site of pBluescript vector (Stratagene) yielding pB*Xhoxc8-1* to *-4*. Partial sequencings of all the four independent cDNAs showed that they had originated from the same mRNA. Sequence comparison of the pB*Xhoxc8* clones with that of the mouse *hoxc8* indicated that at least 400 bp 5′ sequence was missing in the *Xenopus* clones. Because repetitive cDNA library screenings failed to isolate the full cDNA sequence, 5′-RACE reaction strategy was applied to isolate the missing 5′-part of the *Xhoxc8* using three internal primers (InP1-3) and two 5′-anchored primers (AnP1-2). Considering the high GC content in the 5′-end sequence of mouse *hoxc8*, reverse transcription was performed at 58 °C using InP1 (5′-caatggcctcacactcagctcc-3′) with AMV reverse transcriptase (Boehringer–Mannheim) in standard buffer. The reverse transcription product was purified, tailed with dATP, and amplified by PCR using AnP1 (5′-gaccacgcgtatcgatgtcgacttttttttttttttttv-3′) and InP2 (5′-ttccttctcca gctccagtgt-3′). The first PCR product was re-amplified with AnP2 (5′-gaccacgcgtatcgatgtcgac-3′) and InP3 (5′-gctgggcaccataaagagatt-3′). The 2nd PCR yielded a 0.6 kb long cDNA and the cDNA was cloned into pGEM-T vector (Promega) and the base sequence was analyzed. Sequence comparison showed that the 5′-RACE product contained the missing part of *Xhoxc8*. By joining the original *Xhoxc8-1* to the 5′-RACE product at their NcoI sites and cloning into pBluescript (pB*Xhoxc8w*), a 2.336 kb full length *Xhoxc8* cDNA was obtained (Fig. 1).

Northern blot, genomic Southern blot, and RT-PCR assay. For Northern blot, 20 μg of stage 20 total RNA was loaded on the 1.2% agarose gel, which was run and transferred to a nylon membrane (Boehringer–Mannheim) in 10× SSC. The blot was pre-hybridized and probed in 50% formamide, 0.02% SDS, 0.1× SSC, 0.1% *N*-laurylsacosine and 2% blocking reagent (Boehringer–Mannheim). RNA probe was prepared by in vitro transcription using 10× DIG RNA labeling mixture (Boehringer–Mannheim) from linealized pBXhoxc8w DNA template. For Southern blot, 10 μg of restriction enzyme digests of genomic DNA were size fractionated on 1% agarose gel in TAE buffer and transferred to nylon membrane. Blots were hybridized at moderate stringency. The same probe used in Northern blot analysis was used for Southern blotting. For RT-PCR assays, one-twentieth of the reverse transcription product was used in each PCR. One-tenth of the PCR product was loaded in each lane and later stained with ethidium bromide for subsequent analyses. Although not shown in all cases, parallel control samples in which reverse transcriptase had been omitted were analyzed in all PCR assays. The sequences of the primers used here are (*Xhoxc9*) 5′ primer, tacttacgggcttggctgga, 3′ primer, agc gtgtaaccagttggctg; (*Xopsin*) 5′ primer, tgcctcacacaattcccatc, 3′ primer, tgggaggtgccttcagacta; (*En2*) 5′ primer, gctgcatttcgcactactgt, 3′ primer, cctagtacatatgcagctg; (*Xotx2*), 5′ primer, ggatggatttgttgcaccagtc, 3′ primer, cactctccgagctcaccttctc; (α-*cardiac actin*), 5′ primer, gctgacag aatgcagaag, 3′ primer, ttgcttggaggagtgtgt; (*Ef1*-α) 5′ primer, cagat tggtgctggatatgc, 3′ primer, actgccttgatgactcctag.

Whole-mount in situ hybridization. Albino or wild type embryos were fixed in MEMFA (0.1 M MOPS, pH 7.5, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 1 h at room temperature and stored in 100% methanol at −20 °C until further processing. In situ hybridization was processed as described in the in situ protocol of Harland with modifications [17]. The same RNA probe used in Northern blot and Southern blot analyses was used for the whole-mount in situ hybridization. For observation, wild type embryos were bleached in 15% hydrogen peroxide in 1× PBS.

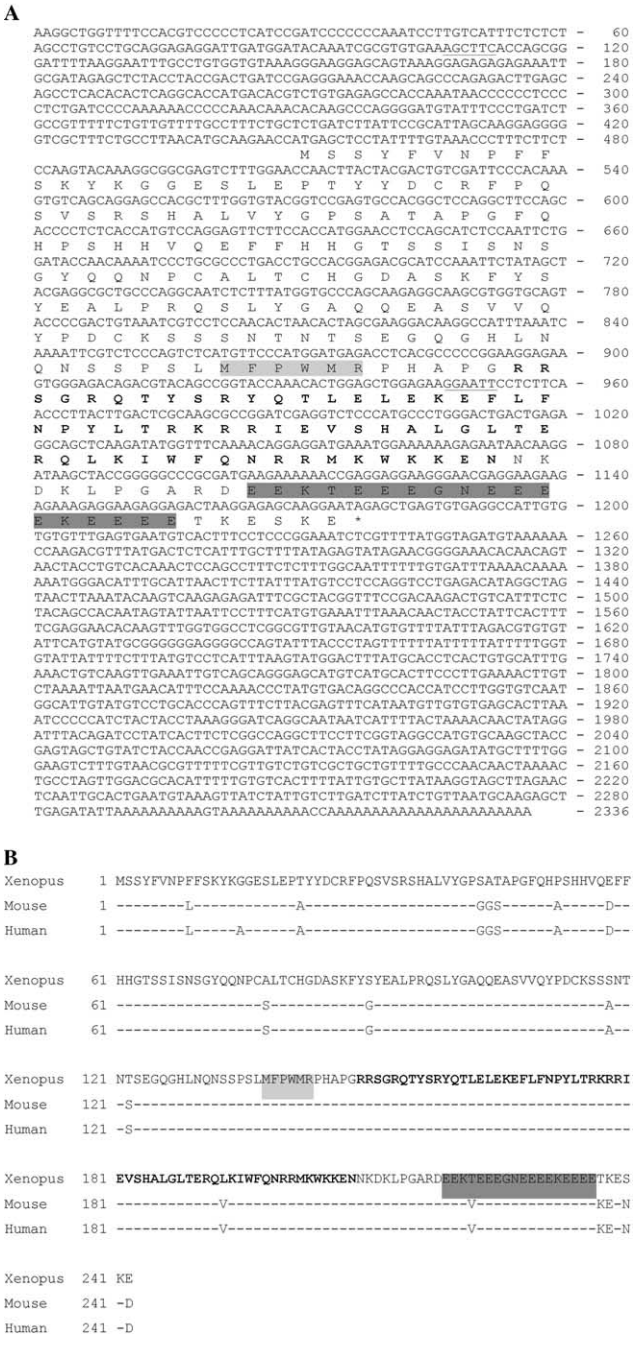

Fig. 1. Nucleotide and deduced amino acid sequences of *Xhoxc8* mRNA. (A) Deduced amino acid sequences are indicated. *Eco*RI (AAGCTT) and *Hin*dIII (GAATTC) sites are underlined. These sequences are deposited in the GenBank database under Accession No. AF001596. (B) Protein sequence comparison among different species. Sequences of mouse [18] and human [19] were compared with *Xenopus* sequence. Dash lines indicate the identical amino acids; bold letters indicate homeobox; hexapeptide region is lightly shaded; polyglutamic acid stretch is heavily shaded.

Results

Isolation of Xhoxc8

A cDNA clone for *Xenopus hoxc8* (*Xhoxc8*) was isolated through the screening of *Xenopus* stage 17 cDNA library with mouses *hoxc8* (*hox-3.1*) cDNA probe and following 5′-RACE reaction. The *Xhoxc8* cDNA contains a single open reading frame (ORF) of 729 nucleotides (nts) flanked by 5′ and 3′ untranslated regions (UTRs) of 449 and 1158 nts, respectively (Fig. 1A). The total 2336 nts length of the *Xhoxc8* cDNA clone is consistent with the size of the single 2.3 kb transcript in Northern blot (Fig. 2A).

The *Xhoxc8* nucleotide sequence predicts a protein of 242 amino acids with a predicted molecular mass of 28 kDa. The mouse and *Xenopus* Hoxc8 proteins are 94% identical in the overall amino acid sequence (Fig. 1B). The homeobox domain of Xhoxc8 is 98% identical to those of mouse [18] and human [19] (Fig. 1B). The sequences of Xhoxc8 and mouse Hoxc8 show high level of similarity in the other two conserved regions—hexapeptide conserved region and polyglutamic acid stretch showing 100% identity and only one amino acid mismatch, respectively (Fig. 1B). Southern blot analysis of genomic DNA identified one fragment in *Xho*I-digested DNA. However, one additional faint band was seen in the *Eco*RI and *Hin*dIII-digested DNAs, respectively (Fig. 2B). Considering the fact that *Xhoxc8* mRNA sequence has *Eco*RI and *Hin*dIII internal sites in it (Fig. 1), the two bands on the *Eco*RI, and *Hin*dIII-digested DNA are supposed to have resulted from the internal digestion of *Xhoxc8* gene by those restriction enzymes. This result indicates that *Xhoxc8* is a single copy gene.

Xhoxc8 mRNA expression during early embryogenesis

The temporal and spatial expression pattern of *Xhoxc8* during embryogenesis was examined by RT-PCR and whole-mount in situ hybridization. Primers used for 5′-RACE (InP3 and 7S) (see Materials and methods) reaction were adopted for the RT-PCR assays. *Xhoxc8* is first expressed at late blastula stage and is continuously expressed at later stages (Fig. 3A), indicating its transcripts are provided by zygotic transcription. To study the regional expression pattern of *Xhoxc8* and to compare its expression pattern with those of other region-specific genes, RT-PCR assay was performed on the five parts of a stage 30 embryo, dissected vertically along the antero-posterior (A-P) axis (Figs. 3B and C). *Xhoxc8* shows region-specific expression pattern with a major expression in the trunk part of embryo. No expression was detected in the head region where *En2* [20], *Xopsin* [21], and *Xotx2* [22] were specifically expressed. Any overlap was not detected between the expression region of *Xhoxc8* and those of head region-specific genes, but the expression pattern of *Xhoxc8* along A-P axis is very similar to the pattern of *Xhoxb9*, a posterior neural marker gene [23]. *Ef1-a* [24] and *a-cardiac actin* [25] were used for internal control.

In order to visualize the anterior and lateral extent of *Xhoxc8* expression, whole-mount in situ hybridization

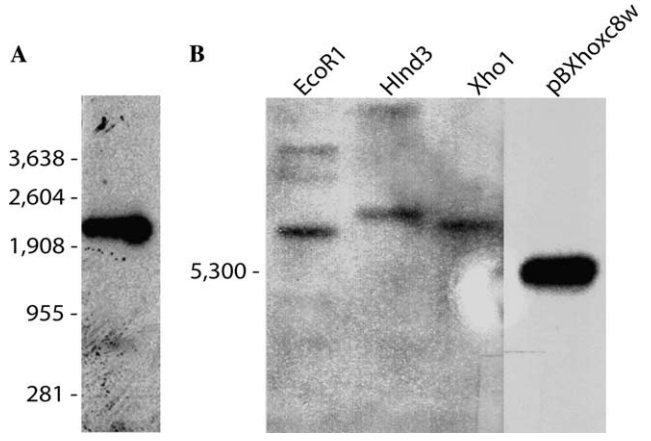

Fig. 2. Northern (A) and Southern blot (B) analyses for the determination of *Xhoxc8* mRNA size and *Xhoxc8* gene-copy number. For Northern blot, 20 μg of stage 20 total RNA was loaded on to the 1.2% agarose gel, which was run and transferred to nylon membrane in 10× SSC. The blot was hybridized with DIG-labeled RNA probe prepared from linealized pB*Xhoxc8w* DNA template. For Southern blot, 10 μg of restriction enzyme digests of genomic DNA was size-fractionated on 1% agarose gel in TAE buffer and transferred to nylon membrane. Blots were hybridized with the same probe used in Northern blot analysis.

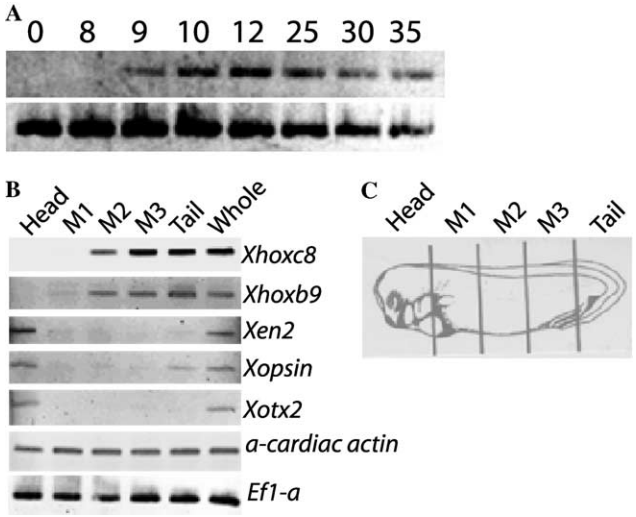

Fig. 3. Expression pattern of *Xhoxc8* analyzed by RT-PCR. (A) One μg of stage 0–35 total RNA was reverse transcribed and PCR amplified using *Xhoxc8* specific primer. (B) Expression of *Xhoxc8* along A-P at stage 30 and comparison of expressions with region-specific marker genes. Total RNAs for analyses were extracted from dissected parts of stage 30 embryos (Head, M1, M2, and M3) or from a whole embryo (Whole). *Ef1-α* and *α-cardiac actin* were used for internal control. (C) Vertical lines indicate the dissected regions.

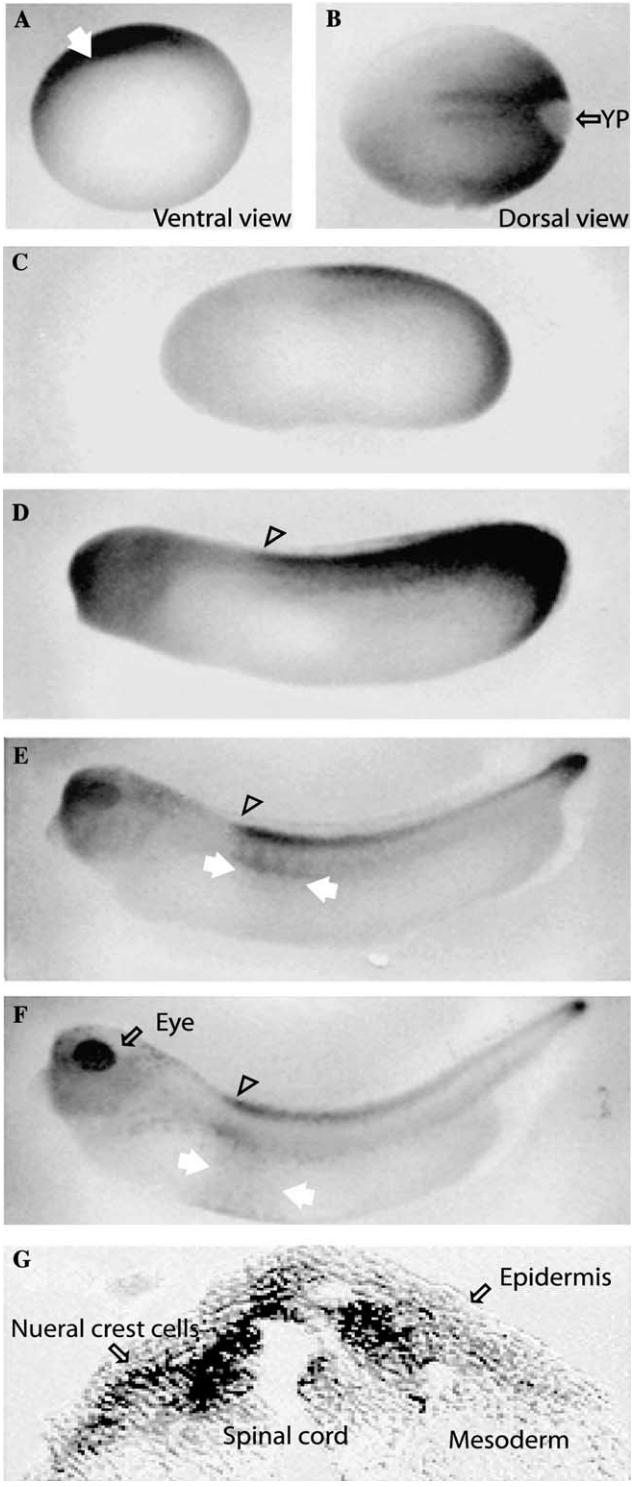

Fig. 4. In vivo *Xhoxc8* expression pattern. Whole-mount in situ hybridization was performed using the same probe used for Northern and Southern blot analyses. (A) Ventral view of stage 10 embryo. Dorsal lip is indicated by arrow. (B) Dorsal view of stage 12 embryo. YP, yolk plug. (C) Lateral view of stage 25 embryo. (D) Lateral view of stage 30 embryo. (E) Lateral view of stage 35 embryo. (F) Lateral view of stage 37 embryo. Open arrowheads indicate the anterior limits of *Xhoc8* mRNA expression. White arrows indicate the anterior and posterior margins of the migrating neural crest cells. (G) Transverse section of stage 30 embryo following in situ hybridization. Notable tissues were indicated.

was performed using the same probe used for Northern blot analysis. At early gastrula stage, it is expressed in a broad region of dorsal blastopore lip (Fig. 4A). Between early gastrula stage and neural plate stage, *Xhoxc8* transcription is detectable in the posterior part of the embryo and the expression becomes progressively restricted spatially and tissue specifically. At neural plate stage, *Xhoxc8* was expressed in the posterior neural plate from the blastopore to about half way along the A-P axis, a position that defines the anterior limit of the future spinal cord [26] (Fig. 4B). At this stage, the major expression is detected in the dorso-lateral region of neural plate. At stage 25, the expression of *Xhoxc8* is restricted to the region caudal to the third somite and this pattern is maintained at later stages (Fig. 4C). After stage 30, although the anterior boundary of expression is not changed, the levels of expression along the A-P axis become position-specific. Compared to the relatively strong expression in the anterior boundary and tail end of the embryo, the level of expression in the middle part of the expression domain became lower at later stages (Figs. 4E and F).

From stage 35, *Xhoxc8* expression is also detected in the lateral side of abdomen and the region of expression is confined to the scope of the highest expression domain of *Xhoxc8* in the spinal cord (Fig. 4G). Vertical section shows that, although *Xhoxc8* is expressed mainly in the spinal cord, a relatively high level of *Xhoxc8* expression is maintained in the adjacent region of spinal cord, through which trunk neural crest cells migrate down to the abdomen (Figs. 4E–G).

Effects of retinoic acid treatments

Almost all homeobox genes are known to be susceptible to retinoic acid treatment. Particularly genes located in the 5′-end of *Hox* gene cluster have been shown to be most sensitive to RA [5]. Hoxc8 gene is located in 3′-end of Hox gene cluster. When the embryos were treated with 1 or 10 μM of RA treatment at stage 5 (16-cell stage) the neural fold closure was significantly inhibited and almost all the head structures were lost. However, the RA-treated embryos showed no significant difference in the expression pattern of *Xhoxc8* (Fig. 5). Even in the head-missing embryos, specific expression pattern along the A-P axis was maintained.

Defects in antisense Xhoxc8 RNA injected embryos

To study the role of *Xhoxc8* in early embryogenesis, synthetic antisense *Xhoxc8* RNA was injected into each blastomere of 2-cell or 4-cell stage embryos and sense *Xhoxc8* RNA was injected for control. Analyzed at early neural fold stage, not only the control but also all the antisense *Xhoxc8* RNA injected embryos displayed a normal development until stage 35 (Figs. 6A and B,

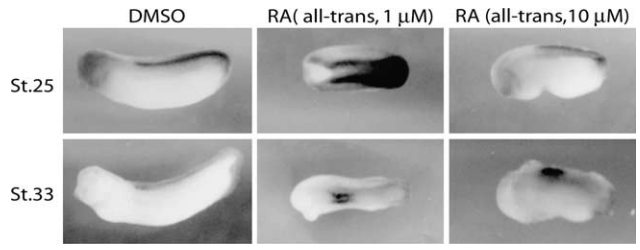

Fig. 5. Effects of retinoic acid treatment on the *Xhoxc8* expression. Embryos were treated with all-*trans* retinoic acid (RA, 1 or 10 μM) or dimethyl sulfoxide (DMSO). Stage 5 embryos were cultured in 0.1× MMR containing DMSO or RA until they reached stage 12. At stage 12, embryos were washed several times with 0.1× MMR and then cultured in the media until they reached stage 25 or 33. The embryos were fixed in MEMFA and the *Xhoxc8* expression level was examined by whole-mount in situ hybridization.

Table 1
Effects of synthetic *Xhoxc8* RNA injection

Injected RNA	Analyzed stages	*n*	Normal (%)	Abnormal (%)
Sense *Xhoxc8*	37/38	40	95 (38/40)	5 (2/40)
	42	38	100 (38/38)	0 (0/38)
Antisense *Xhoxc8*	37/38	40	80 (32/40)	20 (8/40)
	42	32	25 (8/32)	75 (24/32)

Actual numbers of analyzed animals are indicated in parentheses (normal or abnormal embryos/total analyzed animals). At stage 42, only those animals that survived beyond the stage 37/38 were counted.

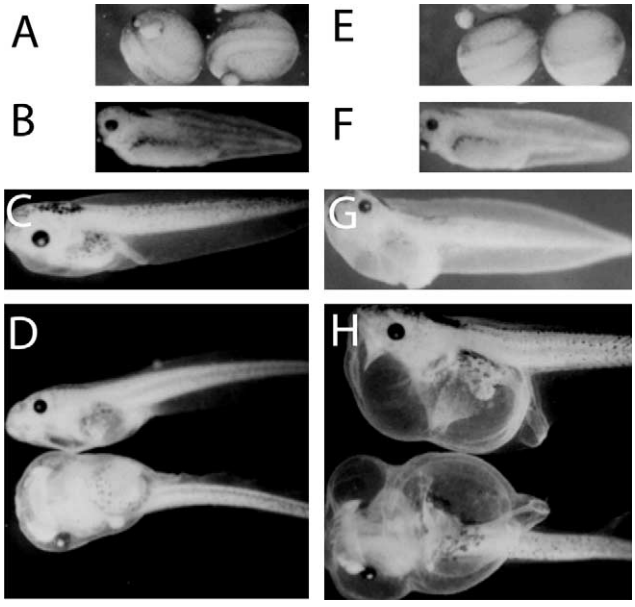

Fig. 6. Effects of *Xhoxc8* antisense RNA injection. Capped synthetic sense and antisense *Xhoxc8* RNAs were produced from linealized pB*Xhoxcw8* plasmid. Total 1 ng of sense (A–D) or antisense *Xhoxc8* (E–H) RNA was injected into each blastomere of 2- or 4-cell stage embryos. The RNA injected animals were analyzed at st. 12 (A,E), 35 (B,F), 41 (C,G), and 48 (D,H).

E and F; Table 1). By stages 37/38, however, 5% of control and 20% of the antisense *Xhoxc8* RNA injected embryos showed a deformed abdominal morphology and died before stage 40 (Table 1). However, the surviving embryos did not show any remarkable morphological disorder until stage 42, from when a significant morphological defect emerged in 75% of antisense *Xhoxc8* RNA injected embryos. They showed a swollen abdomen—severe edema (Fig. 6G). About stage 48, the abdomen lost its golden coloring, the internal organs were smaller than those of normal embryo and they were completely separated from integument (Fig. 6H).

Discussion

Paralogs of Hoxc8 genes

Hox gene products are transcription factors and bind cooperatively to DNA with the assistance of the other class of homeodomain proteins [27]. The hexapeptide conserved region and the polyglutamic acid stretch are involved in the cooperative DNA binding with a subset of Hox proteins and in the modulation of promoter activity [28,29], respectively. The high level of sequence similarity in those regions among the *Hoxc8* parologous genes suggests that these parologs interact with the similar class of homeodomain proteins for the regulation of their target gene transcription. Whole-mount in situ hybridization shows that *Xenopus hoxc8* gene shares the similar spatial expression pattern with the known mammalian *hox* family members [11,30–33]. Like mouse *hoxc8*, *Xhoxc8* mRNA expression is restricted to the spinal cord caudal to the third cervical vertebra, with the highest level of expression just caudal to this vertebra [30,31]. These high levels of sequence homology and pattern of gene expression between *Xhoxc8* and mouse *hoxc8* strongly indicate that the two *hox* genes are highly conserved not only genetically but also functionally in the course of evolution. Also, no remarkable difference in the level and pattern of *Xhoxc8* in the RA treated *Xenoups* (Fig. 5) reveals the general rule that the *hox* genes located in the 3′ end in the *hox* cluster are less sensitive to RA [5].

It is noticeable that no remarkable defect was detected in the spinal structure in the *Xhoxc8* antisense RNA injected embryos, which is the major *Xhoxc8* expressing tissue. Even at later stage, the injected embryos did not show any remarkable defect in the spinal structure. However, severe edematic defects were induced in those embryos (Fig. 6H). It is reminiscent of the similar result observed in the knockout experiment of mouse *Hoxc8*. Rather than showing defects in the spinal structure, *hoxc8*-deficient mice showed a transformation in the rib formation and defects in the

development of internal organs [5,28]. The difference between main expression region and affected region suggests that the *hoxc8* genes in vertebrate may not be involved in the formation of the spinal cord itself. Rather, the *hoxc8* gene likely participates in the determination or differentiation of the precursor cells of the defected tissues. The result that all the antisense *Xhoxc8* RNA injected embryos displayed normal development until stage 35 also adds to the possibility. It has been very well known that many kinds of cells are derived from the trunk neural crest cells [34]. Considering the fact that *Xhoxc8* is strongly expressed in the trunk neural crest and *Xhoxc8* mRNA is expressed in the central trunk region of abdomen (Fig. 4H), it is conceivable to suggest that the blocking of endogenous Xhoxc8 protein expression by the injected antisense RNA inhibit the differentiation or migration of neural crest cells, giving rise to the edematic defects in the abdominal structure.

In summary, we showed that: (1) *Xenopus hoxc8* shares the similar expression pattern with other members of vertebrate *hoxc8* genes; (2) is insensitive to RA treatment; and (3) is involved in abdominal development.

Acknowledgments

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