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Expression of *Xenopus tropicalis* HNF6/Onecut-1

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ABSTRACT *Onecut* genes belong to a family of transcription factors that are known to be important in embryonic development. In the present study, we analyzed the pattern of expression of *Onecut-1/HNF6* in *Xenopus tropicalis* using RT-PCR and whole mount *in situ* hybridization. Expression of the *Xenopus tropicalis Onecut-1/HNF6* gene was found to be conserved in the neural tube, the sensory placodes and in the anterior ventral endoderm in a domain consistent with the developing liver primordium.

KEY WORDS: *onecut, HNF6, Xenopus*

Introduction

Onecut genes belong to an ancient superclass of homeobox transcription factors believed to be distantly related to the *Drosophila* Cut protein. Furthermore genome analysis indicates that in *Drosophila* there is a single *Onecut* gene, whilst other animal phyla have several paralogous genes (reviewed in Burglin and Cassata, 2002). In mice, three paralogous genes have been described (Rausa *et al.,* 1997; Jacquemin *et al.,* 1999; 2003a; 2003b; Pierreux *et al.,* 2004) where as in *C-elegans*, there are multiple *Onecut* genes, the majority appearing to have no vertebrate homologue and are believed to result from duplication and diversification within the nematode lineage (Burglin and Cassata, 2002).

In the sea urchin and sea sponge, *Onecut* gene expression is limited to the ciliary band and apical organ containing sensory cilia and neuronal cells (Poustka *et al.,* 2004; Otim *et al.,* 2004; 2005). *Drosophila Onecut* is also expressed exclusively in the nervous system. In lower order animals studied to date, *Onecut* gene appear to be neural specific (Nguyen *et al.,* 2000; Poustka *et al.,* 2004).

The expression of *HNF6* has been described in several vertebrate species (Rausa *et al.,* 1997; Jacquemin *et al.,* 2003a; Matthews *et al.,* 2004; Margagliotti *et al.,* 2007). Consistent with a role in neural development, vertebrate *HNF6* is expressed in the developing ganglia. However, it is also expressed in the ventral foregut endoderm and later in the developing hepatic diverticulum, pancreas and gall bladder (Rausa *et al.,* 1997; Matthews *et al.,* 2004). Disruption of *HNF6* expression results in abnormalities in the development of biliary system, abnormal liver gene expression and hypoplasia of the pancreas (Jacquemin *et al.,* 2003b; Clotman *et al.,* 2002; 2003; Plumb-Rudewiez *et al.,* 2004; Matthews *et al.,* 2004; Pierreux *et al.,* 2006; Margagliotti *et al.,* 2007). Margagliotti *et al.* (2007) have shown that in mice where disruption of both *HNF6* and *Onecut-2* expression occurs, there is delayed migration of hepatoblasts from the liver bud. (The cause is due the failure of breakdown of the basal lamina surrounding the liver bud).

This report describes the expression of *X. tropicalis HNF6* and shows that like other vertebrate homologues, it is expressed in the sensory ganglia and in the ventral endoderm in a region fated to become the liver.

Results

Our inspection of the *X. tropicalis* genomic database identified four *Onecut* family members. Amino acid sequence comparisons and phylogenetic analysis of both *X. tropicalis Onecut* family members and *Onecut* orthologues in other vertebrate species using the www.metazome.net database indicates that *X. tropicalis HNF6* is located on Scaffold 203. Further analysis shows this region of Scaffold 203 is in synteny with human, mouse and zebrafish chromosomes 15, 9 and 18 respectively where *HNF6* in these species is located. This confirms that the *Onecut* orthologue on Scaffold 203 represents *HNF6*. Sequence comparisons, phylogenetic and synteny relationships signify *X. tropicalis Onecut-2* is located on Scaffold 97, *Onecut-3* is located on scaffold 410 and there is a fourth *Onecut* gene on Scaffold 151 which is most closely related to the zebrafish neural *Onecut* (Hong *et al.,* 2002).

Abbreviations used in this paper: HNF, hepatocyte nuclear factor.

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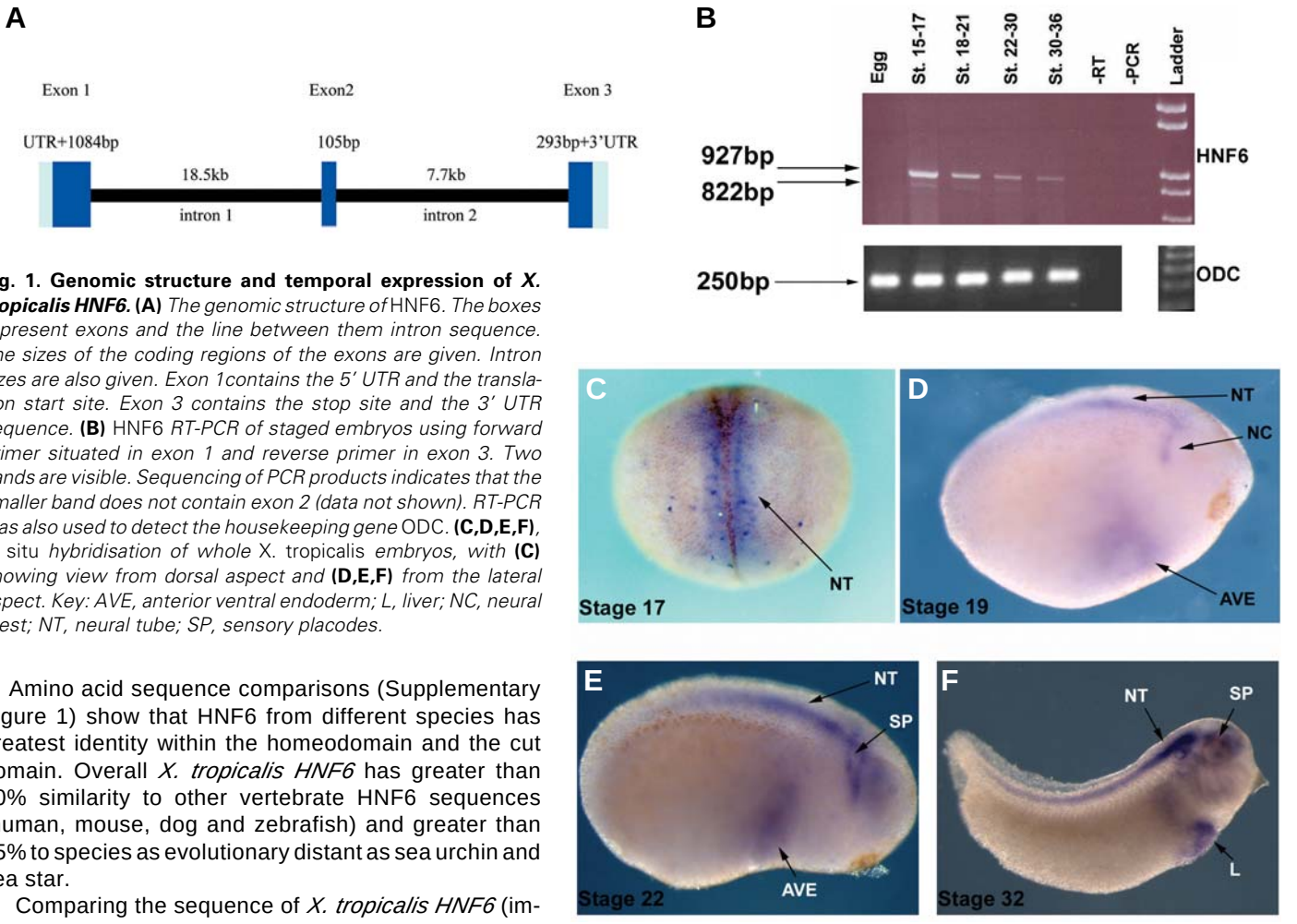

Fig. 1. Genomic structure and temporal expression of *X. tropicalis HNF6*. (A) *The genomic structure of* HNF6. *The boxes represent exons and the line between them intron sequence. The sizes of the coding regions of the exons are given. Intron sizes are also given. Exon 1contains the 5' UTR and the translation start site. Exon 3 contains the stop site and the 3' UTR sequence.* **(B)** HNF6 *RT-PCR of staged embryos using forward primer situated in exon 1 and reverse primer in exon 3. Two bands are visible. Sequencing of PCR products indicates that the smaller band does not contain exon 2 (data not shown). RT-PCR was also used to detect the housekeeping gene* ODC. **(C,D,E,F)**, In situ *hybridisation of whole* X. tropicalis *embryos, with* **(C)** *showing view from dorsal aspect and* **(D,E,F)** *from the lateral aspect. Key: AVE, anterior ventral endoderm; L, liver; NC, neural crest; NT, neural tube; SP, sensory placodes.*

Amino acid sequence comparisons (Supplementary Figure 1) show that HNF6 from different species has greatest identity within the homeodomain and the cut domain. Overall *X. tropicalis HNF6* has greater than 80% similarity to other vertebrate HNF6 sequences (human, mouse, dog and zebrafish) and greater than 45% to species as evolutionary distant as sea urchin and sea star.

Comparing the sequence of *X. tropicalis HNF6* (image clone BC135775, Klein *et al.,* 2002) to the genomic sequence showed *X. tropicalis HNF6* gene, similar to the rat and sea star *HNF6* genes (Rastegar *et al.,* 1998; Otim *et al.,* 2005) is composed of 3 exons (Figure 1A).

The temporal pattern of expression of *HNF6* was analysed using RT-PCR of RNA derived from pooled staged *X. tropicalis* embryos and primers designed within exons 1 and 3. RT-PCR demonstrated *HNF6* is expressed from at least early neurula stages (Figure 1B). Two PCR products (822bp and 927bp) were generated at each age tested and sequencing revealed the smaller product was devoid of exon 2 thus indicating the *HNF6* transcript in the frog is alternatively transcribed to generate predicted open reading frames of 1482bp and 1377bp (data not shown). Alternative transcripts for *HNF6,* lacking exon 2 have also been described in other species (Otim *et al.,* 2005; Rastegar *et al.,* 1998). The spatial expression of staged *X. tropicalis HNF6* was analysed using whole mount *in situ* hybridisation using an RNA probe generated from bases 11-490 of the open reading frame. As this is outside the conserved homeodomain and cut domain, cross hybridisation between different *Onecut* family members would not be expected. Additionally, both alternative transcripts will be detected. Whole mount *in situ* hybridisation was performed on embryos ranging from gastrula to early tailbud stages. No specific staining was observed in embryos at gastrula stages. Whole mount data showed that *HNF6* is expressed in neural tissue and the ventral endoderm (Figure 1C-F).

Neural HNF6 expression

Weak but distinct expression was first observed in early neurula stage embryos in the developing neural tube as shown at st. 17 (Figure 1C), the location of staining was confirmed in cleared and bisected embryos (Figure 2A, 2B). At later stages the expression in the neural tube becomes more elaborate. At st.19, expression is also present in a single narrow stripe of cells perpendicular to the neural tube and caudal to the early developing optic vesicle (Figure 1D). At st. 22-25 expression of *HNF6* continues in the above mentioned domains and additional domains become apparent (Figure 1E; Figure 2C-F). These domains appear to follow the pathway of migrating neural crest cells and expression of *HNF6* becomes more prominent and consistent with the positions of several developing neural placodes and ganglia (Schlosser and Northcutt, 2000; Schlosser, 2002; Schlosser and Aherns, 2004). Expression domains observed at st. 32 (Figure 2G) include trigeminal placode, lens of the eye and the anterodorsal, middle and posterior lateral line placodes. Analysis of sectioned embryos indicates the hybridisation of the *HNF6* probe within the neural tube is non-uniform. Expression appears to occur strongly within the medial part of the neural tube with little expression in either the

dorsal neural tube or the floor plate (Figure 2D-E). Sectioning of st. 24 embryos indicates expression of *HNF6* in the head region is below the level of the surface ectoderm (Figure 2E) and thus suggests the expression is present in neural crest derived mesenchyme cells.

HNF6 is expressed in the anterior endoderm

Weak and diffuse expression of *HNF6* is first observed at st. 19 in the ventral aspect of the embryo at the axial level of the anterior ventral endoderm (Figure 1D). Expression in the ventral part of the embryo continues and becomes more pronounced from this stage until at least st. 32. Sectioning of st. 25 embryo at the level of anterior endoderm (Figure 2H-I) and embryo dissection (Figure 2J) shows this expression corresponds to ventral anterior endoderm. Comparisons of the expression of *HNF6* and *Hex*, another homeobox gene shown to be important in liver development, indicates that the anterior endoderm domain stained by *HNF6 in situ* hybridisation corresponds to the region of the endoderm from which the liver develops (not shown and Zorn and Mason, 2001).

HNF6 in the developing frog embryo appears to be expressed in two distinct types of tissue: neural and endoderm. Our data (Figure 1B) suggests that there are two different transcripts, hence the possibility they might be differentially expressed in these tissues. To test this hypothesis the dorsal neural region of st. 26-29 embryos and the ventral gut region of the embryo were dissected separately. RT-PCR performed on isolated RNA from these tissues indicated both *HNF6* transcripts are expressed in tissues of neural origin and of endoderm origin. Therefore the different transcripts do not appear to have tissue specific expression (data not shown).

Discussion

A search of www.metazome.net database (for genomic sequences) indicates that the *X.tropicalis* genome contains four *Onecut* gene family members. The database indicates that there are also four members in the zebrafish. However, evidence suggests that in the genomes of mammalian species there are only three *Onecut* gene family members. Whether one of the paralogous genes was lost during the evolution of mammalian species or whether another gene was gained in a common ancestor of *Xenopus* and zebrafish is at present unknown.

HNF6/Onecut-1 has been cloned in species ranging from sea Urchins and sea sponges to rats (Otim *et al.,* 2004; 2005; Rastegar *et al.,* 1998). Amino acid comparisons show that the sequence is remarkably well conserved between diverse species perhaps indicating an ancient and evolutionary conserved role for this gene in embryonic development.

We have shown that *X.tropicalis HNF6/Onecut-1* is present in the developing neural tube and ganglia and is comparable with the expression of *HNF6* observed in other vertebrates. *Onecut* in invertebrates *Drosophila*, *C Elegans* and Sea Urchin is similarly expressed in developing neurons (Nguyen *et al.,* 2000; Reece-Hoyles *et al.,* 2007; Poustka *et al.,* 2004). In contrast, endoderm expression of *HNF6/Onecut-1* has only to date been observed in vertebrates suggesting that vertebrates lineage has acquired an additional role for *Onecut* genes in the endoderm.

Materials and Methods

Identification of *Onecut* family members in *X. tropicalis* and other tetrapod species was carried out using the www.metazome.net database. A full length *X. tropicalis HNF6* EST clone was identified from the database (http://www.ensembl.org/Xenopus_tropicalis/index.html) and the intron-exon structure determined by comparison of the coding sequence to the genomic sequence using the database http://

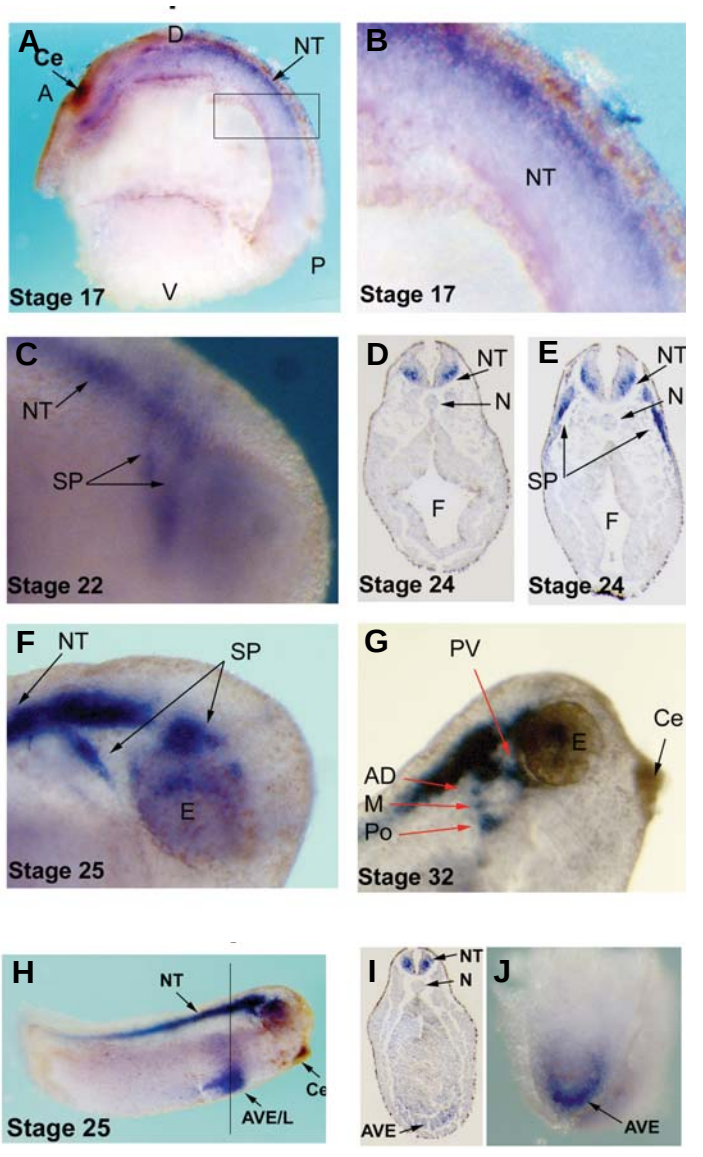

Fig. 2. Spatial expression of *X.tropicalis HNF6*. *Neural expression of HNF6* **(A-G)** *and endoderm expression* **(H,J)**. *Embryos at st. 17* **(A,B)**, *st. 22* **(C)**, *st. 24* **(D,E)**, *st. 25* **(F,H,I,J)**, *st. 32* **(G)** *are shown. The embryo in (A) was bisected along the midline, the anterior region is identified by the presence the pigmented cement gland (Ce). The region highlighted by the rectangular box is shown at higher magnification in (B).* **(C,F,G)** *Expression in the cranial region.* **(D,E)** *Coronal sections.* **(H)** *St. 25 embryo, solid line showing the plane of section of wax section* **(I)** *and bisected embryo* **(J)**. *Abbreviations: A, anterior; AD, anterodorsal lateral line placode; AVE, anterior ventral endoderm; Ce, cement gland; D, dorsal; E, eye; F, foregut; M, middle lateral line placode; N, notochord; NT, neural tube; P, posterior; PV, trigeminal placode; Po, posterior lateral line placode; SP, sensory placodes; V, ventral.*

www.xenbase.org. Comparison of *X. tropicalis HNF6* to homologues in other species and a phylogentic tree constructed using the programme CLC Free Workbench (CLC Bio; http://www.clcbio.com/index.php?id=28).

Preparation of RNA and RT-PCR

X. tropicalis embryos were collected and staged according to morphological criteria as described by Nieuwkoop and Faber (1967). Whole RNA was prepared using Trizol Reagent (Invitrogen) and cDNA prepared using Superscript Polymerase (Invitrogen) and random hexamer primers (Promega). Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed using HNF6 primers F: 5'- TTACAGCCCCTACCACAAGG-3'and R: 5'-CTGCGAGCATTCATGAAAAA-3' to amplify cDNA for temporal expression analysis (PCR product sizes of alternative spliced transcripts, 927bp, 822bp).

Ornithine decarboxylase (ODC) PCR was performed using primers F: AACAAGCAGGCTGCTTCTGG and R: GGCTGGGTTTATCACAGATG genenerating a product of 250bp. The following primers:
F: 5'-AGCTGACTATGGACGCGATT-3' and
R: 5'-CCTTGTGGTAGGGGCTGTAA-3' were used to amplify a fragment of exon 1 for use in *in situ* hybridisation. The PCR product was subcloned into the pGEM T-easy vector (Promega), the inserts were sequenced using the Cardiff University Sequencing Facility (http://watson-bios.grid.cf.ac.uk/seq/), and once verified was linearised using ApaI and digoxigenin (Roche) labelled antisense RNA synthesised using Sp6 polymerase. Transcribed RNA was purified on G50 Sephadex columns as described by Sive *et al.,* (2000). *In situ* hybridisation was performed at high stringency as described previously by Sive *et al.,* (2000). Colour development was performed using the chromogenic reagent BM purple (Roche).

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