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Gene expression pattern

Inner ear and lateral line expression of a zebrafish *Nkx5-1* gene and its downregulation in the ears of FGF8 mutant, ace

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Abstract

An orthologue of the mouse homeobox gene Nkx5-1 was cloned and characterized in the zebrafish. As in the mouse and chick, the zebrafish Nkx5-1 gene is expressed in the ear placode and vesicle and in cells forming the vestibulo-acoustic ganglion. In addition, a novel expression domain, the lateral line, appears in the zebrafish, supporting a common precursor hypothesis for these two organs. In the FGF8 zebrafish mutant ace, expression of Nkx5-1 in the otic structures is diminished. The most significant reduction of zfNkx5-1 expression was observed in cells of the vestibulo-acoustic ganglion. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Epidermal placodes give rise to several sensory organs (nose, eye, lateral line) and some cranial ganglia and possess a 'common placodal potential' at early developmental stages. With ongoing development placodes become continuously determined to their individual fates (Jacobson, 1963; Torres and Giraldez, 1998). During vesicle stages, when the inner ear anlagen consist of simple, single cell layered epithelial sacs, patterning processes are already well advanced and become apparent through specific, regionally restricted gene expression patterns. For example, the dorso-lateral wall of the otic vesicle, which gives rise to vestibular structures, expresses Nkx5-1, whereas the ventro-medial wall expresses Pax2 and later forms the cochlea (Rinkwitz-Brandt et al., 1996; Hadrys et al., 1998; Torres et al., 1996). Such specific gene expression patterns are initially established in the otic placode by external signals (Herbrand et al., 1998; Fekete, 1996; Giraldez, 1998).

Comparisons between different vertebrate species revealed a high degree of homology in inner ear development. Several genes, for example kreisler, pax2 and msh, show similar expression patterns in otocysts of fish and higher vertebrates and in many cases might fulfil similar functions as was already documented for the kreisler gene (Moens et al., 1996). A recently described, inactivating mutation in the zebrafish FGF8 gene, ace, causes defects in the mid-hindbrain development and thus might also affect the inner ear (Reifers et al., 1998; Léger and Brand, 2000).

Besides the accessibility of valuable mutations, the fish presents an interesting model for investigation of evolutionary interrelationships between individual epidermal placodes. In particular, the origin and molecular control of inner ear and lateral line placodes can be studied in the fish system. According to Jorgensen (1989) the inner ear and the lateral line are derived from a common ancestor organ, although the common origin for these placodes has been questioned by others (Northcutt, 1986). To date, no molecular markers have been identified that would support one or the other hypothesis at the molecular level. In this report we present the isolation of a zebrafish homologue of the mouse and chick Nkx5-1 gene. Nkx5-1 is specifically expressed in otic placodes and vesicles in mouse and chick (Rinkwitz-Brandt et al., 1995; Herbrand et al., 1998). This expression pattern is conserved in fish otocysts. Interestingly, in the zebrafish Nkx5-1 is also expressed in the lateral line primordia. In the zebrafish FGF8 mutant, ace, Nkx5-1 expression is

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significantly diminished in otic structures. In contrast, Nkx5-1 expression in the lateral line placode does not depend on the FGF8 signal.

2. Results

2.1. Isolation of zebrafish Nkx5-1 cDNA

To isolate a zebrafish homologue of the Nkx5-1 gene a genomic fragment encoding 77 amino acids (aa) from the carboxyterminus, including most of the homeodomain, was amplified (Fig. 1). Nkx5-1 cDNA was subsequently isolated from a stage 15–19 h (28°C) embryonic cDNA library, kindly provided by B. Appel (Vanderbilt). The full-length zebrafish Nkx5-1 protein sequence deduced from the cloned cDNA is presented in Fig. 1. The entire aminoterminal part upstream of the homeodomain is highly conserved in all vertebrate species investigated so far. However, the mouse sequence contains one alanine-rich stretch at the N-terminus (aa 59–74) and another 17 aa long sequence stretch (aa 193–210), that are both absent in chick and fish. In addition, there is one short proline-rich sequence at the N-terminal end (aa 15–26), which is partly conserved in the chick but not in the zebrafish. If those two deletions are not taken into account, the proteins in the mouse and zebrafish are 69% identical. Comparison to partial human and rat sequences, which were obtained in our laboratory (see Section 3), revealed that the additional sequence stretch observed in the mouse Nkx5-1 protein (aa 193–210) is also present in rat and in man (Fig. 1).

2.2. Nkx5-1 is expressed in the ear and the lateral line and in specific regions of the brain of zebrafish embryo

zfNkx5-1 expression starts at the four somite stage (11.3 h of development) in lateral line placodes. At the 14 somite stage (16 h), an additional expression domain, the otic placode, appears. Later (28 h) Nkx5-1 expression starts in distinct domains of the developing brain. The spatial and temporal distribution of Nkx5-1 transcripts in representative zebrafish embryos is illustrated in Fig. 2.

As in mouse and chick (Hadrys et al., 1998; Herbrand et al., 1998) zfNkx5-1 is first expressed in the rostral aspect of the otic placode. Just after vesicle formation the Nkx5-1 expression domain is confined to its antero-medial aspect (see Figs. 2C and 3A,B), whereas at the late vesicle stage the Nkx5-1 domain translocates to the lateral wall (see cross-

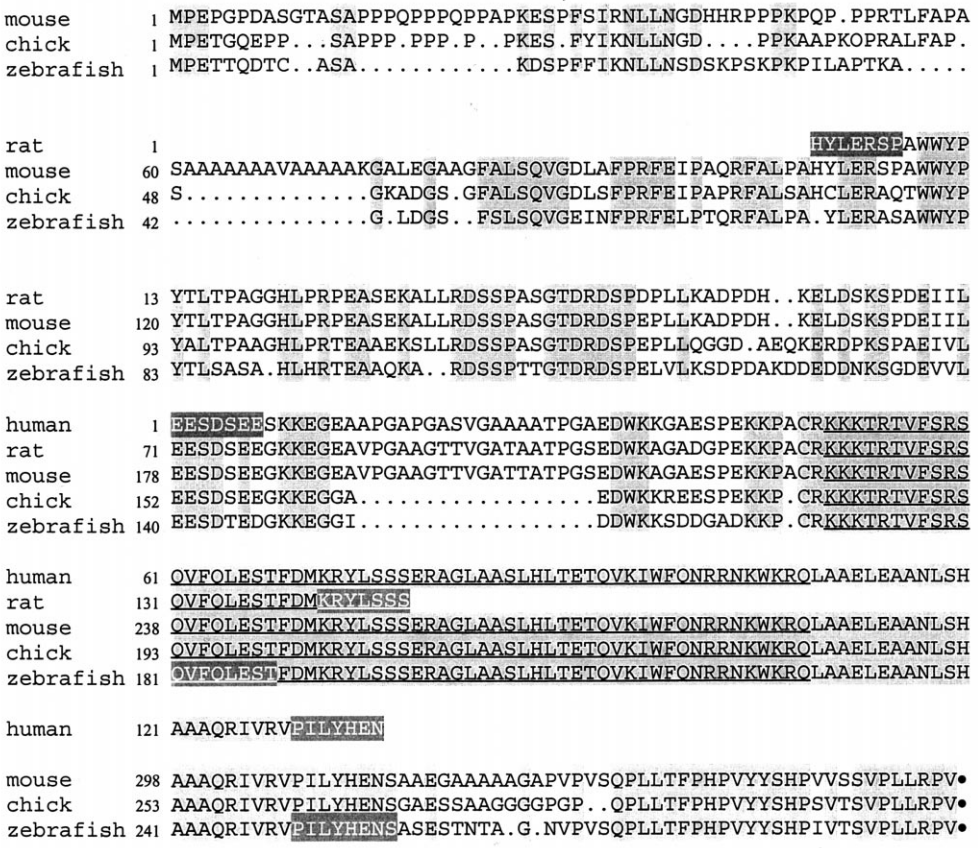

Fig. 1. Nkx5-1 amino acid sequences are strongly conserved in different vertebrates. Amino acid Nkx5-1 sequences of man, rat (this report), mouse (Mennerich et al., 1999), chicken (Herbrand et al., 1998), and zebrafish (this report) are aligned. Gaps were introduced to some of the presented sequences (marked by dots) to allow detection of all homologous regions. Amino acids identical in all analyzed species are shaded in grey. Primers used for PCR amplification of rat, human, and zebrafish sequences (see Section 3) are written in white and shaded. Homeodomains are underlined and stop codons are indicated by thick dots.

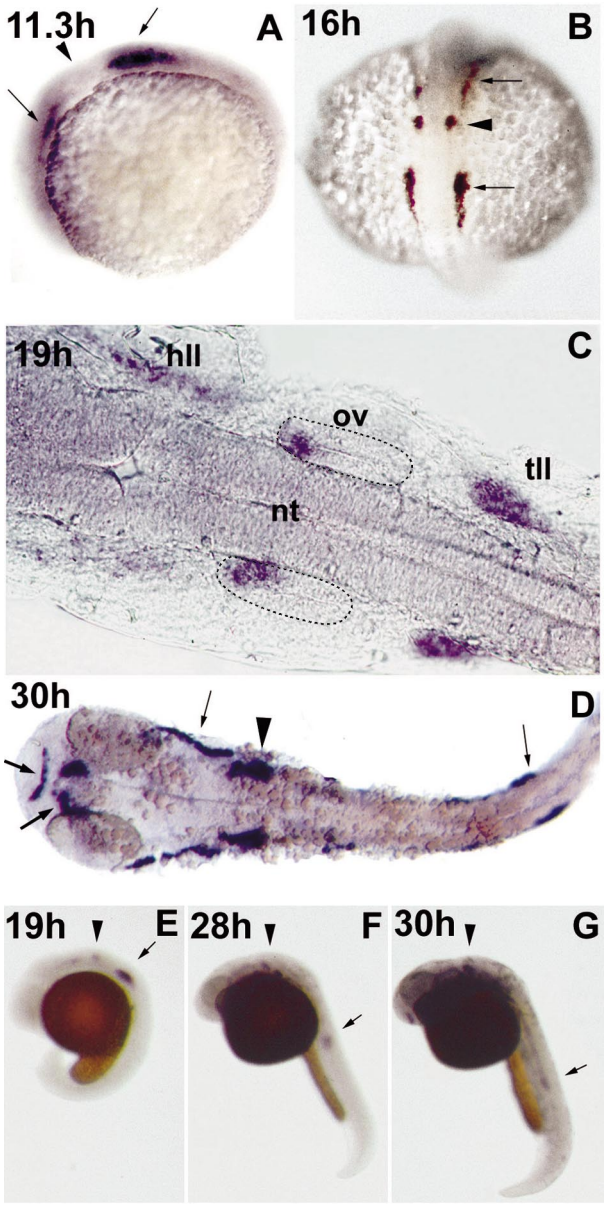

Fig. 2. The zebrafish Nkx5-1 gene is specifically expressed in the otic vesicle, lateral line primordium and distinct brain regions. Nkx5-1 in situ hybridization signals are first detected at 11.3 h of development (four somites) in the lateral line placodes indicated by arrows in (A). No Nkx5-1 activity can be detected in the otic placode at this stage (arrowhead in A). At 16 h (14 somites) Nkx5-1 expression is restricted to otic (arrowhead) and lateral line (arrows) placodes (B). (C) Higher magnification of a 19-h-old embryo with Nkx5-1 expression domains in head and trunk lateral lines (hll and tll, respectively) and in the otic vesicle (ov); note that Nkx5-1 expression is confined to the rostral part of the otic vesicle (see the dotted outline) and is adjacent to the neural tube (nt). (D) An older embryonic stage (30 h). In this stage distinct brain domains (thick arrows) express Nkx5-1. Lateral line (thin arrows) and otic vesicles (arrowheads) continue to express Nkx5-1. (E–G) Nkx5-1 expression in the primordium of the trunk lateral line (arrow) correlates with its caudal migration during ongoing development (19, 28 and 30 h, respectively). In contrast, the otic vesicle (arrowhead) stays at its original position, lateral to the hindbrain.

section in Fig. 3D), the transcripts still confined to the rostral part of the vesicle (Fig. 3C). This dynamic distribution seems to be conserved in all vertebrates despite the different modes of vesicle formation (cavitation in fish and invagination in birds and mammals). Interestingly, in the zebrafish one additional expression domain appeared that was not present in birds or rodents. This new expression area is confined to the lateral line placodes in the head and trunk. It starts at the four somite stage when the placodes form (11.3 h, see Fig. 2A). The Nkx5-1 gene continues to be expressed in what appears to be the migrating placodal primordium, as illustrated in Fig. 2E–G (views on whole embryos of 19, 28 and 30 h of age in the lowest row). The position, timing of appearance, and migrational behaviour strongly corresponded to morphological characteristics of the lateral line placodes and their primordia in zebrafish (Metcalfe, 1985). Therefore, we concluded that Nkx5-1 marks early lateral line structures and thus represents the first specific molecular marker for the ear and lateral line placodes. Nkx5-1 is not expressed in any other epidermal placodes such as the lens or cranial ganglia.

2.3. Nkx5-1 expression in the ear but not in the lateral line and the brain depends on FGF8 signalling

As reported recently the zebrafish mutation ace inactivates the FGF8 gene and results in severe defects of the midbrain and hindbrain (Reifers et al., 1998). A general diminishment of the otic placode as indicated by changes in Pax2.1 expression in this region was also observed (Reifers et al., 1998). As shown in Fig. 3, Nkx5-1 expression in the otic placode (19 h of development, arrowheads) is significantly reduced in the ace mutant (Fig. 3E,F) as compared to wild-type fish (Fig. 3A,B). In contrast, Nkx5-1 expression in the lateral line seems to be completely unaffected at 19 h and at later stages of development (arrows in Fig. 3A,B,E,F). The later appearing expression domains in the brain remained unchanged in the ace mutant (data not shown).

Fig. 3C,D and G,H illustrate another aspect of Nkx5-1 expression in the otic vesicle derived structure, namely the vestibulo-acoustic ganglion in the wild-type and ace mutant, respectively. In wild-type zebrafish embryos cells that delaminate from the otic epithelium and subsequently build the ganglion show abundant Nkx5-1 expression at 24 h of development (Fig. 3C, asterisk). At the same developmental stage almost no expression is seen in this region of the ace mutants although ganglion cells are still morphologically discernible (asterisk in Fig. 3G). At the same time Nkx5-1 expression in the otocyst is still present.

3. Experimental procedures

3.1. PCR amplification of the conserved Nkx5-1 gene fragments

All PCR amplifications were initiated for 4 min at 95°C followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and

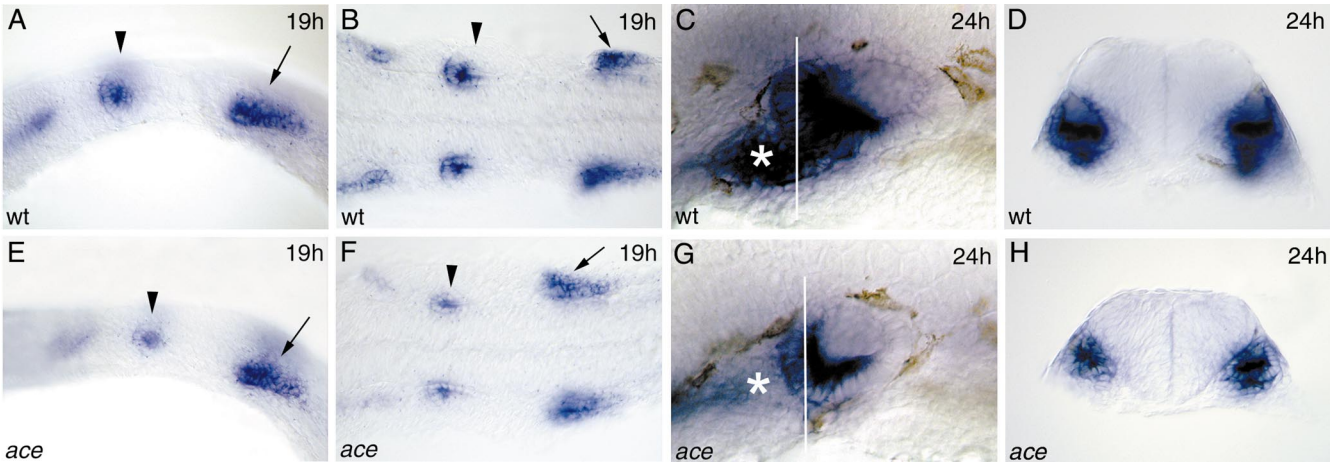

Fig. 3. Nkx5-1 expression is specifically affected in the ears of the FGF8 zebrafish mutant, ace. All pictures show embryos after whole-mount in situ hybridization with an Nkx5-1 antisense RNA (the anterior is to the left). (A,E) Side views of the ear region of wild-type and ace embryos, respectively. It is apparent that all Nkx5-1 expression domains are preserved in the ace mutant. The level of Nkx5-1 expression in the otic vesicle (arrowhead) is, however, significantly reduced in the ace mutant as compared to the wild-type embryo. (B,F) Dorsal views of the otic and trunk lateral line areas of embryos presented in (A,E), respectively; note the reduction of Nkx5-1 expression in the ears of the ace mutant (arrowhead in F) as compared to the wild-type (arrowhead in B), whereas the level of Nkx5-1 expression in the trunk lateral line is generally unchanged in the ace mutant (compare structures marked with arrows in B and F). (C,G) Lateral views at the ear region of whole-mount embryos, illustrating a lack of Nkx5-1 expression in the developing vestibulo-acoustic ganglion (marked by asterisk) of the ace mutant (G). The ear expression, although diminished, can be observed in the ace mutant. (D,H) Cross-sections of the ears of embryos on (C,G), respectively (levels of sections are indicated by white lines).

72°C for 1 min. DNA was synthesized by ExTaq polymerase (Takara). Fragment (230 bp) of zebrafish Nkx5-1 homologue was amplified using genomic DNA and degenerated primers from conserved regions of mouse and chicken Nkx5-1 (5′-CAGGTCTTCCAGCTNGAGTCCAC-3′ and 5′-AGTTCTCGTGGTANAGGATGGG-3′). The 408 bp fragment of the human Nkx5-1 gene was amplified using primers 5′-GAAGAGAGCGACTCGGAGG-3′ and 5′-AGTTCTCGTGGTAGAGGATG-3′ and the genomic DNA. For amplification of the 447 bp rat fragment primers 5′-GCACTACCTGGAGCGCTCC-3′ and 5′-TCCGAGCTGCTCAGGTAGCG-3′ and cDNA from neuroectodermal rat cell line PC12 were used.

3.2. Isolation of the zebrafish Nkx5-1 cDNA

The cDNA library constructed from poly(A)$^+$ embryonic zebrafish RNA (15–19 h, 28°C), kindly provided by Dr Appel (Vanderbilt), was screened using the 230 bp amplified zfNkx5-1 fragment as a probe. A single positive cDNA clone was identified which contained a complete coding zebrafish Nkx5-1 cDNA sequence.

3.3. Whole-mount in situ hybridization

In situ hybridizations were performed using a standard protocol (Thisse et al., 1993) and an antisense RNA probe encompassing the full length zfNkx5-1 cDNA.

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