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Gene expression, function, and diversity of Nkx2-4 in the rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Nkx2 homeodomain transcription factors are involved in various developmental processes and cell specification: *e.g.* in mammals, NKX2-1 is essential for thyroid-specific gene expression and thyroid morphogenesis. Among Nkx2 proteins, information is still very limited for Nkx2-4. In the present study, we have identified three distinct cDNAs encoding Nkx2-4 isoforms (Nkx2-4a, -b, and -c) from the rainbow trout thyroid tissue, and characterized their transcriptional properties. The trout Nkx2-4 proteins were all predicted to conserve three characteristic domains: the tinman-like amino terminal decapeptide, the NK2 homeodomain, and the NK2-specific domain, and also share 75–89% amino acid similarity. It was shown by dual luciferase assay that Nkx2-4a and Nkx2-4b, but not Nkx2-4c, significantly activated transcription from a cotransfected rat thyroglobulin (TG) promoter. An electrophoretic mobility shift assay indicated that all the Nkx2-4 isoforms could bind to the TG promoter, implying that the faint transcriptional activity of Nkx2-4c might result from some critical amino acid substitution(s) outside the homeodomain. RT-PCR analysis revealed similar tissue distribution patterns for Nkx2-4a and Nkx2-4b mRNAs. Both mRNAs were expressed abundantly in the thyroid, and weakly in the testis. On the other hand, Nkx2-4c mRNA was detected in the ovary as well as in the thyroid. The expression sites of Nkx2-4c mRNA were localized, by *in situ* hybridization histochemistry, to the ovarian granulosa cells and to the thyroid follicular cells. The results suggest that in the rainbow trout, Nkx2-4a and Nkx2-4b might play a major role in TG gene transcription whereas Nkx2-4c might have some functions in the ovary as well as the thyroid.

1. Introduction

The thyroid hormones, thyroxine and triiodothyronine, are produced in all classes of vertebrates, and play crucial roles in the regulation of development, metabolism, homeostasis, and reproduction (Bentley, 1998; Melmed et al., 2011). As to teleost fishes, it is well known that thyroid hormones control flounder metamorphosis (Power et al., 2001) and the parr-smolt transformation of salmonids (Björnsson et al., 2011). Further, these hormones are shown to affect a wide range of other physiological processes such as gonad maturation in the goldfish (Nelson et al., 2010) and changes in the olfactory system in the salmon during an imprinting period (Lema and Nevitt, 2004), suggesting their widespread importance in fish physiology.

Molecular mechanisms for thyroid hormone synthesis and secretion operate in the thyroid follicles, which are characterized by the expression of a specific set of molecules, such as sodium/iodide symporters (NIS), thyroglobulin (TG), thyroid peroxidase (TPO), and thyroid-stimulating hormone (TSH) receptors (TSHRs) (Kovacs and Ojeda, 2012). According to the mammalian scenario, iodide is accumulated in the follicular epithelial cells through NIS, and incorporated into tyrosyl residues within TG by TPO. This enzyme can also catalyze the oxidative coupling of iodotyrosines, followed by storage of the iodinated TG in the colloid. The thyroid hormones are released after endocytosis of the colloid, which is regulated by TSH. This unique phenotype of the thyroid follicular cells is indispensable for thyroid hormone synthesis, and maintained by the fundamental action of transcription factors, including NKX2-1 (also known as TTF1, TITF1, or T/EBP) (Damante

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et al., 2001; De Felice and Di Lauro, 2011; Di Palma et al., 2009; Kimura, 2011).

NKX2-1 is a member of the NK2 family that consists of transcription factors with an unique NK2 homeodomain (Bingle, 1997; Boggaram, 2009). The NK2 family members are widely distributed from invertebrates to vertebrates, and involved in tissue differentiation and organogenesis (Stanfel et al., 2005). In vertebrates, the members of this family are designated as Nkx2 and currently classified into ten types, Nkx2-1 to Nkx2-10, based on the order of discovery (Newman et al., 2000; Price et al., 1992; Stanfel et al., 2005). Among them, Nkx2-1 is of prime importance for the thyroid development and the regulation of thyroid-specific gene expression. The NKX2-1 gene is expressed not only in the thyroid gland, but also in the lung and embryonic diencephalon (Lazzaro et al., 1991). *Nkx2-1* null mice failed to form the thyroid, lung, ventral forebrain, and pituitary, showing the essential role of NKX2-1 in the development of these organs (Kimura et al., 1996; Silberschmidt et al., 2011). NKX2-1 is also shown to function as a key regulator for the maximal transcription of TG (Sinclair et al., 1990), TPO (Francis-Lang et al., 1992), TSHR (Civitareale et al., 1993) and NIS genes (Endo et al., 1997). Additionally, gene expression of NKX2-3 and NKX2-5 is detected in the mouse thyroid during development (Fagman and Nilsson, 2011).

The thyroid glands of most non-mammalian vertebrates are presumed to conserve a similar molecular mechanism for hormone synthesis (Bentley, 1998). However, for these vertebrates, there is limited information on the Nkx2 molecules expressed in the thyroid gland. Because the rainbow trout has been used as a model animal to study the physiological roles of thyroid hormones in fish (Bres et al., 2006; Suliman and Flamarique, 2014), it is of special interest to elucidate the molecular mechanisms operating in the thyroid gland of this species. Hence, in the present study, we have carried out cDNA cloning to clarify which type of Nkx2 is expressed in the trout thyroid, and obtained three distinct cDNAs encoding three isoforms of Nkx2-4. To our knowledge, there is no experimental evidence on transcriptional activity of Nkx2-4 in the thyroid. The functional property of Nkx2-4 isoforms was, therefore, examined by dual luciferase assay. Tissue distribution of the Nkx2-4 mRNAs was also investigated by RT-PCR and *in situ* hybridization, to infer the functions of these proteins.

2. Materials and methods

2.1. Animals and sampling

Rainbow trout, *Oncorhynchus mykiss*, of 1.5-years of age were collected at Fuji Trout Hatchery, Shizuoka, Japan, in January and May. Before sampling, the animals were anesthetized with ethyl m-aminobenzoate methanesulfonate (Nacalai Tesque, Kyoto, Japan). Various organs were carefully removed from the trout, immediately frozen in liquid nitrogen, and stored at −80 °C in a deep freezer. The thyroid was dissected out with the ventral aorta because the thyroid follicles were dispersed along this aorta. The thyroid tissues, including the ventral aortae and thyroid follicles, and ovaries were also fixed in 4% paraformaldehyde at 4 °C overnight for histological analysis. In addition, the liver was removed from a Wistar rat of 6 weeks (Japan SLC, Shizuoka, Japan) anesthetized with ether. All animal experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals of Shizuoka University.

2.2. Preparation of cDNA probe

Poly(A)$^+$ RNA (0.5 μg) from the thyroid tissues were reverse-transcribed in 20 μl buffer containing 80 pmol of dT-adaptor primer (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT), 1 mM dNTP, 20 units RNase inhibitor (Toyobo, Osaka, Japan), and 10 units Rous-associated virus 2 (RAV-2) reverse transcriptase (Takara, Kyoto, Japan), as described previously (Suzuki et al., 1992). Polymerase chain reaction (PCR) was subsequently carried out with Nkx2-1-1 primer (5′-GTKYTMTTCTCBCARGCSCAGGT) and adaptor primer (5′-GACTCGAGTCGACATCGAT) using a Program Temp Control System, PC-701 (Astec, Fukuoka, Japan). The amplification profile for 30 cycles was: dissociation at 94 °C for 1.5 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 2.5 min. The final cycle included polymerization for 8 min. An aliquot of the resultant reaction mixture was further subjected to PCR amplification with Nkx2-1-2 primer (5′-GAAGTACYTGTCSGCGCCGGAG) and adaptor primer. Amplification products were separated by electrophoresis, and a major band was subcloned into pGEM-3Z vector (Promega, Madison, WI, USA). Sequencing reactions were conducted with a thermo sequenase cycle sequencing kit (USB, Cleveland, OH, USA), and nucleotide sequences were analysed using a Li-Cor automated DNA sequencer model 4200L-2G (Li-Cor, Lincoln, NE, USA). A DNA probe was synthesized from the cloned 3′ fragment similar to mammalian NKX2-1 cDNAs, using a digoxigenin (DIG)-high prime kit (Roche Diagnostics, Mannheim, Germany).

2.3. DNA cloning and sequence analysis

A cDNA library from the rainbow trout thyroid was constructed using a ZAP Express cDNA Gigapack III Gold cloning kit (Agilent Technologies, La Jolla, CA, USA). Approximately 54,000 recombinants from the amplified cDNA library were screened by plaque hybridization. Hybridization with the above cDNA probe and post-hybridization washing were performed, basically according to the manufacturer's instructions. Hybridization signals were detected with 25 mM CSPD, a 1,2-dioxetane chemiluminescent enzyme substrate (Tropix, Bedford, MA, USA), on Hyperfilm-ECL film (GE Healthcare, Buckinghamshire, UK) after incubation with alkaline phosphatase-conjugated anti-DIG Fab antibody (Roche). The pBK-CMV phagemid vectors with inserts were excised *in vivo* from the ZAP express vectors of positive recombinants, using the ExAssist helper phage (Agilent Technologies). The nucleotide sequences of these DNAs were determined using a Li-Cor automated DNA sequencer. The sequence data were analyzed using Genetyx, ver. 8 (Genetyx Corporation, Tokyo, Japan) and GenomeNet (2014).

2.4. Phylogenetic tree and synteny analysis

The amino acid sequences of Nkx proteins from the human, mouse, chicken, *Xenopus laevis*, zebrafish, rainbow trout, fugu, and stickleback were aligned using Clustal W (Thompson et al., 1994) after alignment parameters were set according to an instruction manual by Hall (2011). The accession numbers of the sequences are listed in Supplemental Table S1. An optimal unrooted tree was inferred by the neighbor-joining (NJ) method (Saitou and Nei, 1987) in the MEGA program ver. 6.0 (Tamura et al., 2011). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000), and confidence in the NJ tree was assessed with 10,000 bootstrap replications (Felsenstein, 1985). Synteny analysis was carried out using genome data registered in Ensembl (2014).

2.5. Reporter constructs and expression vectors

Genomic DNA was prepared from the rat liver by phenol/chloroform extraction. The 5′-upstream region of rat TG gene was amplified from the genomic DNA by PCR using TG5 primers