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Fluorescence *in situ* hybridization techniques (FISH) to detect changes in *CYP19a* gene expression of Japanese medaka (*Oryzias latipes*)

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ABSTRACT

The aim of this study was to develop a sensitive *in situ* hybridization methodology using fluorescence-labeled riboprobes (FISH) that allows for the evaluation of gene expression profiles simultaneously in multiple target tissues of whole fish sections of Japanese medaka (*Oryzias latipes*). To date FISH methods have been limited in their application due to autofluorescence of tissues, fixatives or other components of the hybridization procedure. An optimized FISH method, based on confocal fluorescence microscopy was developed to reduce the autofluorescence signal. Because of its tissue- and gender-specific expression and relevance in studies of endocrine disruption, gonadal aromatase (*CYP19a*) was used as a model gene. The *in situ* hybridization (ISH) system was validated in a test exposure with the aromatase inhibitor fadrozole. The optimized FISH method revealed tissue-specific expression of the *CYP19a* gene. Furthermore, the assay could differentiate the abundance of *CYP19a* mRNA among cell types. Expression of *CYP19a* was primarily associated with early stage oocytes, and expression gradually decreased with increasing maturation. No expression of *CYP19a* mRNA was observed in other tissues such as brain, liver, or testes. Fadrozole (100 μg/L) caused up-regulation of *CYP19a* expression, a trend that was confirmed by RT-PCR analysis on excised tissues. In a combination approach with gonad histology, it could be shown that the increase in *CYP19a* expression as measured by RT-PCR on a whole tissue basis was due to a combination of both increases in numbers of *CYP19a*-containing cells and an increase in the amount of *CYP19a* mRNA present in the cells.

Introduction

In recent years, an increasing number of genomic and/or proteomic techniques have been developed to identify mechanisms of toxic action in organisms exposed to environmental contaminants. Most of the methods that were developed to meet these objectives such as RT-PCR, Northern blotting, and RNase protection assays rely on relatively high yields of RNA extracted from whole tissues. However, the limitations of these techniques are that they often fail to detect gene expression of low-abundance mRNA in small tissues, or they do not allow localizing changes within certain tissues or cell types. Some genes are only expressed in certain tissues, while others are expressed in specific tissues at only certain times of development (Sanderson et al., 2001). Especially when using small laboratory animal model species, the limited amount of individual tissues available for study and the difficulty in excising them from the organisms has limited the efficacy of these techniques to determine effects during critical windows of time during ontogenesis. While advanced technologies such as laser capture microdissection-based PCR allow identification and quantification of expression of genes in small portions of tissues with great sensitivity, they are labor intensive and typically not feasible for higher throughput multi-tissue studies.

Many of the efforts in endocrine disruptor research have focused on individual endpoints such as receptor-mediated effects (Otsuka, 2002). However, such targeted screening methods may not be sufficient when disruptions are induced through indirect mechanisms. Some chemicals can act as direct agonists or antagonists to certain receptors while others act indirectly by modulating signal transduction, or affecting gene expression or substrate concentrations. For example, the triazine herbicide atrazine does not bind to the estrogen receptor (ER), but *in vitro* in a mammalian cell system,

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atrazine has been found to up-regulate the expression of aromatase (CYP19), the enzyme that transforms testosterone to estradiol. Although atrazine does not act like a typical estrogen via binding the ER, in mammalian cell systems it can, in some situations, at relatively great concentrations result in estrogenic effects by increasing endogenous estradiol production (Sanderson et al., 2000). As a result, it is important not only to develop methodologies that allow for evaluation of chemical-induced effects in multiple target tissues simultaneously, but also to determine subtle effects on multiple endpoints simultaneously within these tissues.

Whole-animal *in situ* hybridization (ISH) is a promising method for determining spatial changes in gene expression (Tompsett et al., in press; Zhang et al., 2008, in press). This methodology allows determination of effects on expression of multiple genes in multiple tissues simultaneously, and it can be used simultaneously with standard histology (Peterson and McCrone, 1993; Lichter, 1997; Hrabovszky et al., 2004; Jezzini et al., 2005; Ijiri et al., 2006). One of the major advantages of ISH is that it allows detection of changes in expression of mRNA for specific genes in organs, tissues, and/or cells of interest in a manner that is consistent with other methods that are used to detect lesions, including histopathology and immunohistochemistry (IHC) (Streit and Stern, 2001). The principle underlying ISH is the hybridization of specifically labeled probes to the complimentary mRNA sequences in tissues or cells. A number of different visualization techniques can be applied to detect an ISH signal including radionucleotides, enzyme linked systems (e.g. biotin, digoxygenin), and fluorophores. Each label type has strengths and weaknesses depending on application. Radiolabeled probes have been widely used to detect specific mRNA sequence in tissues or embryos since the detection of mRNA in invertebrates and vertebrates was originally developed using the radiolabeled probe systems (Simeone, 1999). ISH utilizing radiolabeled probes have been found to be more sensitive and reliable than some other methods such as enzyme linked or fluorophore-based systems. However, radioisotope-based techniques have a number of disadvantages such as a relatively poor resolution, relatively long exposure times for auto-radiographic visualization, and they are expensive and require special certifications in many institutions and extra precautions in the laboratory (Braissant and Wahli, 1998; Simeone, 1999; Pernthaler and Amann, 2004; Tompsett et al., in press). In contrast, enzymatic detection systems such as digoxygenin are very sensitive but tend to be variable. More recently, the application of fluorescent labeling techniques has considerably improved ISH due to the advantage of using different fluorescent tags to simultaneously detect different gene sequences (Wilkinson, 1999). However, application of fluorescent *in situ* hybridization (FISH) methods to detect specific mRNA in tissue sections has not been explored to the same extent as radioisotope or enzyme-based methods due to issues with sensitivity and/or autofluorescence of tissues (Dirks, 1990; Wilkinson, 1999; Andreeff and Pinkel, 1999). Recent improvements in fluorescence labeling techniques render FISH techniques an increasingly useful tool. To effectively utilize FISH, however, a number of technical limitations needed to be overcome. Key issues include probe penetration of sections, autofluorescence of tissues, non-specific binding of probe, type of target tissues and species, and sample preparation (Wilkinson, 1999). Hence, there was a need for the development and optimization of FISH methods to overcome these issues.

The main objective of this study was to develop and optimize an ISH protocol that uses fluorophore-labeled probes to detect specific mRNA sequences in whole animal sections of Japanese medaka (*Oryzias latipes*). Specific goals of this study were: (1) develop and optimize methods to design fluorescent riboprobes for use in ISH; (2) develop and optimize methods to reduce auto- and background fluorescence in fish tissue sections by using a combination of chemical treatment and advanced confocal microscopy techniques; (3) validate the FISH methods developed in this study using Q RT-PCR; and (4) use the optimized FISH methods to examine changes in gonadal *CYP19a* gene expression in Japanese medaka exposed to a competitive pharmaceutical inhibitor of the aromatase enzyme, fadrozole. The physiology, embryology, and genetics of the Japanese medaka have been extensively studied in the past, and more recently, this species has been used as a model in endocrine disrupter research (Wittbrodt et al., 2002). The Japanese medaka has clearly defined sex chromosomes and sex determination (summarized in Wittbrodt et al., 2002). Cytochrome P450 aromatase, encoded by the CYP19 gene, is the key enzyme in estrogen biosynthesis from androgens (Simpson et al., 1994), and it has been extensively used as an endpoint to assess the exposure of endocrine disrupting compounds (EDCs) due to its relation with reproductive processes (Sanderson et al., 2000; Hayes et al., 2002; Rotchell and Ostrander, 2003; Hecker et al., 2006). Fadrozole has been reported to affect *CYP19a* gene expression (Villeneuve et al., 2006) but was also shown to result in other physiological effects in fish including altered plasma estradiol concentrations, gonadal pathologies, and fecundity (Afonso et al., 1999; Ankley et al., 2002; Fenske and Segner, 2004).

Materials and methods

Test chemical

The fadrozole (CGS016949A; MW: 259.74 g) used in this research was provided by Novartis Pharma AG (Basel, CH).

Culture of Japanese medaka

Wild type Japanese medaka were obtained from the aquatic culture facility at the US EPA Mid-Continent Ecology Division (Duluth, MN, USA). Medaka were held in flow-through systems under conditions facilitating breeding (23–24 °C, 16:8 h light/dark). All procedures used during all phases of this study were in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC).

Fadrozole exposure

Prior to initiation of exposure experiments, 12–14 wk old medaka were placed into 10 L tanks with 6 L of carbon filtered tap water and acclimated for 12 d under the same conditions as in the subsequent exposures. One fish died during acclimation. Each treatment group consisted of two replicate tanks, and each tank contained 5 male and 5 female fish. After the acclimation period, fish were exposed to 1, 10, or 100 μg fadrozole/L or carbon-filtered tap water as a control in a 7 d static renewal exposure. Every day one half of the water in each tank (3 L) was replaced with fresh carbon filtered water dosed with the appropriate amount of an aqueous fadrozole stock (5 mg/L). Fish were fed Aquatox flake food (Aquatic Ecosystems, Apopka, FL, USA) *ad libitum* once daily and held at 24 °C with a 16:8 h light/dark cycle. Water quality parameters were measured daily and values were within a normal range for water quality, as follows in all tanks: temperature (24 °C), pH (7.89–8.13), ammonia nitrogen (<0.02–0.04 mg/L), nitrate nitrogen (<0.02–0.3 mg/L), dissolved oxygen (4.3–6.9 mg/L), and hardness (370–480 mg $CaCO_3$/L).

After 7 d of exposure medaka were euthanized in Tricaine S (50 mg/mL) (Western Chemical, Ferndale, WA, USA). Weight and snout length were recorded. Fish were separated into two groups, one group was for ISH and consisted of 2 fish per sex per tank, and a second group that was to be used for Q RT-PCR procedures and included three fish per sex and treatment group. Fish from the ISH group were fixed for ISH and histological investigations as described below. For the Q RT-PCR group, the brain, liver, and gonads were dissected from the fish and weighted individually. The liver somatic