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Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic Development

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Abstract: In zebrafish (*Danio rerio*) pigmentation is initiated during embryogenesis and begins in the retinal epithelium and in the melanophores. The pigment cells develop rapidly, and within hours they constitute a prominent feature of the embryo. In order to improve signal detection by whole mount in situ hybridization, confocal microscopy, or expression of GFP, embryos may be treated with 1-phenyl 2-thiourea (PTU) during embryogenesis. PTU inhibits melanogenesis by blocking all tyrosinase-dependent steps in the melanin pathway but can be toxic at high concentrations. The embryos remain transparent as long as the PTU treatment is continued. However, PTU treatment must be initiated before the initial pigmentation because it does not remove already formed pigment. Here we provide a protocol for generating transparent zebrafish while avoiding the toxic and teratogenic effects of PTU treatment.

Key words: zebrafish, pigmentation, PTU, transparency, embryo development.

INTRODUCTION

In zebrafish pigment formation is initiated during embryogenesis and begins in the retinal epithelium and in the melanophores located dorsolaterally in the skin (Kimmel et al., 1995). This process commences at the prim 5 stage, approximately 24 hours after fertilization. The pigment cells develop rapidly, and within hours they constitute a prominent feature of the embryo. The inhibitory effect of 1-phenyl 2-thiourea (PTU) on formation of melanophores, and in particular its effect on the conversion from tyrosine to melanin, has previously been characterized (Whittaker, 1966; Epping, 1970). The effect on melanogenesis is due to PTU-induced reduction of tyrosinase activity. Tyrosinase is essential for initiation of melanogenesis and acts by a 2-step reaction converting tyrosine into L-dopa and then dopaquinone (Whittaker, 1966). No inhibitory effect on enzymes involved in the synthesis of catecholamines has been reported, and PTU has no known effect on the differentiation of xanthophores and iridophores that normally occurs around hatching (Kelsh et al., 1996).

When working with techniques designed to study gene expression during embryogenesis, especially in teleosts, signal detection may be aggravated by pigmentation. This problem is most prominent when relying on a visually detectable signal such as green fluorescent protein (GFP) expression or whole mount in situ color reagents. Pigmentation can also be a problem in confocal microscopy because

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melanophores can distort signal detection of florescent probes. To reduce these obstacles, different techniques to generate transparency have been developed. These techniques describe production of albino strains through genomic manipulation (Kelsh et al., 1996; Kameyama et al., 1989), postfixative bleaching with hydrogen peroxide (Inohaya et al., 1995), or inhibition of melanin pigmentation by use of compounds such as hydroquinone (Palumbo et al., 1991) or PTU (Whittaker, 1966; Westerfield, 1995). The inhibitory effect of PTU on melanogenesis has been established; however, the available protocols for zebrafish result in a high incidence of embryo mortality, reduced hatching frequency, and teratogenesis. The present study describes an optimized protocol for generating transparent zebrafish using PTU.

Materials and Methods

Chemicals

The 1-phenyl 2-thiourea (PTU) was purchased from Sigma-Aldrich (Sweden AB). All other chemicals were of molecular biology grade.

Experimental Design

Zebrafish eggs were collected, cleaned, and washed with eggwater (Westerfield, 1995) within 1 hour after fertilization. Eggs from different females were pooled and then placed in sterile Falcon 3004 petri dishes containing eggwater and incubated at a temperature of 28.0° to 28.5°C. The experiments were initiated by transferring embryos, at specific developmental stages, to dishes containing different concentrations of PTU. Each solution was prepared fresh in eggwater prior to experimentation. Control embryos were kept in dishes containing eggwater. Each experiment continued for 4 days after initiation of PTU treatment. The dishes were cleaned and embryos were counted twice every day. All photos were taken using a Leica DC-200 CCD camera and a Leica $MZ12_5$ stereomicroscope with a Plan Apo 1.0 × lens. Embryos were illuminated from above and magnification was ×65.

PTU Treatment

Embryo exposure to different PTU concentrations (50, 60, 70, 75, 80, 100, and 200 μM) was initiated at developmental stages between 26 somites and prim 5 and continued for a minimum of 96 hours. The experiments were designed to determine the optimal PTU concentration for inhibition of melanogenesis, the PTU level that resulted in deleterious effects, and the optimal time of treatment initiation. In the first experiment embryos at developmental stage 28 somites were exposed to different PTU concentrations. Four to 10 repeats were performed, and the number of embryos in each treatment group ranged between 35 and 95. In the second experiment embryos at 6 different developmental stages were exposed to 75-μM PTU in order to determine the optimal developmental time for treatment. In this experiment treatment was initiated at the following stages: 25 somites, 26 somites, 27 somites, 28 somites, 29 somites, and prim 5. The experiment was repeated 3 to 6 times, and each group consisted of between 20 and 88 embryos.

Determination of Survival Rate and Hatching Frequency

In order to evaluate the possible embryotoxic effects of PTU, both the concentration-dependent survival rate and the hatching frequency were determined. The numbers of hatched, hatched dead, unhatched dead, and live embryos were counted at 48, 72, 96, and 120 hours postfertilization (hpf). The experiment was terminated at 120 hpf. Live embryos that had not hatched at 120 hpf were counted as being alive and unhatched. Hatching frequency was only determined for embryos remaining alive and early mortality (prior to 48 hpf) was excluded when determining hatching frequency.

Whole Mount In Situ Hybridization

To determine if there was a correlation between PTU treatment and expression of hatching enzyme, in situ hybridization was performed using a zebrafish hatching enzyme-specific antisense RNA probe. Partial complementary DNA encoding zebrafish hatching enzyme (Zh-1), cloned into the *Bam*H1 site of Bluescript II (KS^+), was used to generate a digoxigenin-labeled antisense RNA probe with the DIG RNA Labeling Kit (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Zebrafish embryos, at the 28 somite stage, were grown in 200-μM PTU until long pec stage and then fixed in 4% PFU. Whole mount in situ hybridization and visualization by NBT/BCIP was performed according to Jowett (1999).

Localization of zFF1 expression during long pec stage in PTU-treated zebrafish was performed to exemplify the

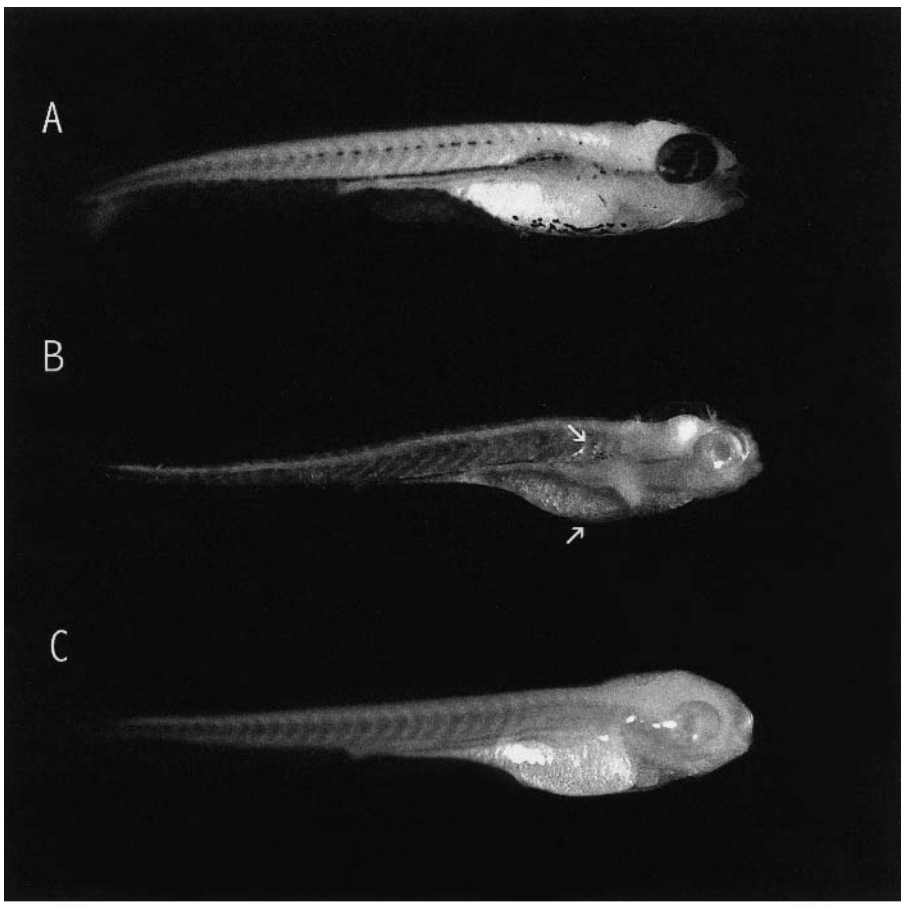
usefulness of depigmentation in whole mount detection of gene expression. Partial cDNA encoding zFF1 was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and cloned into the Srf1 site of pCR-Script amp (Stratagene, La Jolla, Calif.). The identity of the zFF1 sequence was confirmed using an ABI Prism 377 DNA sequencer (PerkinElmer Corp, Upplands Väsby, Sweden). A digoxigenin-labeled antisense RNA probe was generated as described above. Zebrafish embryos, at the 28 somite stage, were grown in 75-μM PTU until long pec stage and were thereafter fixed in 4% PFU. Whole mount in situ hybridization and visualization by NBT/BCIP was performed as described above.

Statistical Analysis

Statistical analysis of data was performed using the 2-tailed heteroscedastic Student *t* test. Statistical significance is indicated at the $P < .05$ level.

Results and Discussion

During recent years the interest in understanding and studying developmental processes in teleost fish has increased. Zebrafish, in particular, has proved to be a suitable model system to work with, and a wide variety of different



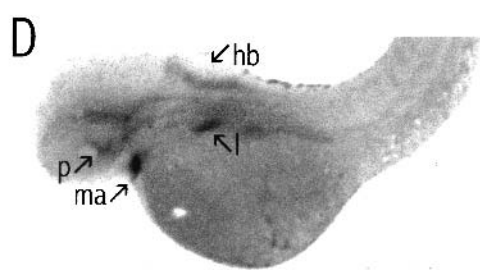


Figure 1. Effects of different PTU concentrations on the level of transparency. **A:** Untreated zebrafish showing normal pigmentation. **B:** Zebrafish treated with 60-μM PTU at the 28 somite stage. Pigmentation remains in head and yolk sac regions as indicated by arrows. **C:** Zebrafish treated with 75-μM PTU at the 28 somite stage. Pigmentation is reduced, rendering the zebrafish transparent. **D:** Whole mount in situ hybridization displaying the expression of zFF1 in a transparent zebrafish treated with 75-μM PTU at the 28 somite stage. Staining was detected in the hypothalamus-pituitary area (p), mandibular arches (ma), hindbrain (hb), and developing liver (1), as indicated by arrows. Detection of the signals in brain and liver was made possible by the PTU treatment and would have been difficult in pigmented zebrafish embryos.

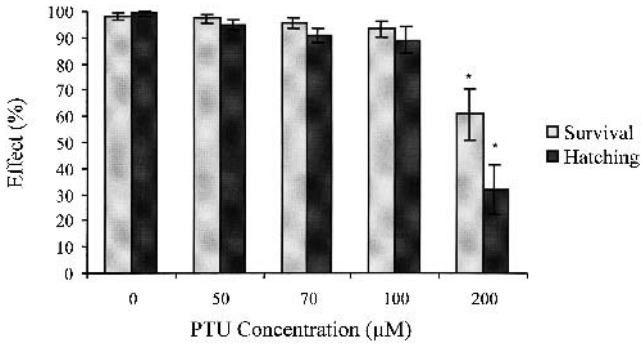


Figure 2. Correlation between PTU concentration, embryo survival, and hatching frequency. Treatment of zebrafish embryos with different concentrations of PTU at the 28 somite stage. Results show reduced embryo survival and hatching frequency when embryos were treated with 200-μM PTU. Rates of hatching and survival were determined at 120 hpf, and the experiment was repeated 4 to 10 times. The number of embryos in each treatment group ranged between 35 and 95. Each bar represents the mean ± SD, and significant differences from the control group are indicated by asterisks ($P < .05$).

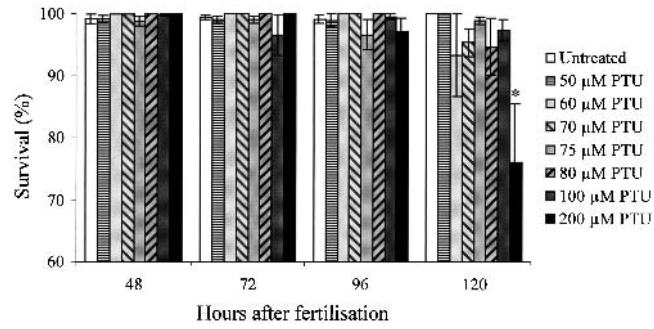


Figure 3. Temporal effects of PTU treatment on embryo survival. The results show a decrease in embryo survival between 96 and 120 hpf when treating embryos with 200-μM PTU at the 28 somite stage. The experiment was repeated 4 to 10 times, and the number of embryos in each treatment group ranged between 35 and 95. Each bar represents the mean ± SD, and significant difference from the control group is indicated by an asterisk ($P < .05$).

strains are commercially available. We have developed a technique, using PTU, to generate transparency in zebrafish without the need of genomic manipulation associated with production of albino strains. The use of PTU to inhibit melanogenesis and formation of melanin pigment has been described previously (Whittaker, 1966; Epping, 1970; Pietzsch-Rohrschneider, 1977; Westerfield, 1995). In contrast to earlier techniques, we have optimized conditions to achieve complete inhibition of pigmentation while avoiding problems with embryo mortality, reduced hatching frequency, and teratogenesis.

Correlation Between PTU Concentration and Level of Transparency

Studies of detection of gene expression during embryogenesis are impaired by the appearance of pigmentation. By using controlled conditions for PTU treatment, it is possible to block pigmentation without affecting normal embryonic development. We observed that inhibition of melanin pigmentation was obtained with 75, 80, 100, and 200-μM PTU, while treatment with 50, 60, and 70 μM was inadequate to block melanin pigmentation (Figure 1). The highest PTU concentration resulted in reduced hatching frequency and embryo mortality (Figure 2). When compared with untreated embryos, the 50-μM to 100-μM PTU concentrations showed no significant effect on hatching or survival while the 200-μM PTU exposure resulted in 67.2% reduction in hatching frequency and 37.3% reduction in survival rate. The main embryo mortality occurred between 96 h and 120 hpf (Figure 3), approximately 24 to 48 hours after all control fish had hatched. Embryo mortality during this time consisted largely of dead unhatched embryos (data not shown).

To determine if there was any correlation between PTU treatment and expression of hatching enzyme during hatch, we performed whole mount in situ hybridization using a zebrafish hatching enzyme-specific antisense RNA probe. There was no reduction in transcriptional levels of hatching enzyme messenger RNA (data not shown). In addition to reduced hatching and embryo survival, treatment with 100-μM and 200-μM PTU induced posterior malformation with a protruding lower snout (Figure 4). This teratogenic phenotype was most prominent with the 200-μM PTU concentration with a 35% incidence. At 100-μM PTU the incidence was 5%. No teratogenic phenotypes were observed among embryos treated with 80-μM PTU concentration or lower.

The toxic and teratogenic effects of high PTU concentrations and the reason for reduced hatching ability have not been fully elucidated. The effects are not likely a consequence of inhibition of tyrosinase activity since albino strains with mutated endogenous tyrosinase genes do not display similar effects. However, observations made during the present study indicate a decreased embryonic motility with increasing concentrations of PTU. The most prominent reasons for the high mortality using 200-μM PTU were that hatching was delayed and the embryos obtained a

Figure 4. Teratogenic phenotype due to treatment with 200-μM PTU. One form of teratogenic effect was frequently observed following treatment with 200-μM PTU at the 28 somite stage. Shown is a posterior malformation with a protruding lower snout. Exposure to 100-μM PTU resulted in occasional malformations.

curved body shape with reduced swimming capacity. Since transparency is obtained with 75-μM PTU without observable toxic or teratogenic effects, we recommend 75-μM PTU to be used when generating transparent zebrafish.

Onset and Duration of PTU Treatment

The inhibitory effect of PTU requires 30 to 60 minutes to take effect following commencement of treatment. It is therefore important to initiate treatment well before the first pigmentation is observed (around the prim 5 stage). To investigate whether embryo mortality and reduced hatching frequency could be correlated to the onset of PTU treatment, we determined the effects of initiating treatment at several different embryonic stages, between 25 somite and prim 5, using 75-μM PTU. Correlation between reduced hatching ability and the onset of PTU treatment was observed at developmental stages 25 to 27 somites, while no significant effect was found on embryo mortality (Figure 5). There was no mortality in the control group. This further indicates that the most appropriate time for initiating treatment with 75-μM PTU is at the 28 somite stage.

Inhibition of pigmentation using PTU is a reversible process (Whittaker, 1966). Embryos depigmented by PTU treatment will again produce pigmentation on discontinuation of PTU treatment. To evaluate if whether PTU treatment affected later developmental stages, we returned embryos to eggwater and monitored their behaviour and development for up to 2 weeks. No difference in mortality or behavior was observed in the PTU-treated embryos as compared with control embryos (data not shown). It was also observed that 2 weeks after removal of PTU the pigmentation had returned to that observed in control fish.

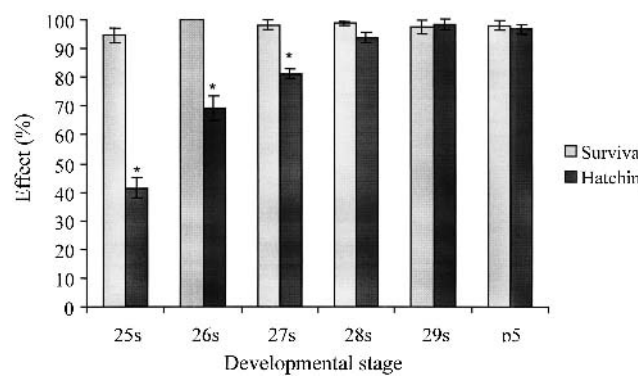


Figure 5. Correlation between onset of PTU treatment, embryo survival, and hatching frequency. The effect of onset of treatment was determined by exposing embryos to 75-μM PTU at 6 different developmental stages. Results show a reduced hatching frequency at stages 25 somites to 27 somites. The experiment was repeated 3 to 6 times, and the number of embryos in each treatment group ranged between 20 and 88. Each bar represents the mean ± SD, and significant differences from the control group are indicated by asterisks ($P < .05$).

The present study presents refined conditions for generation of transparent zebrafish without interfering with their normal embryonic development. The results show that treating zebrafish at the 28 somite stage with 75-μM PTU is optimal for the generation of transparency. This treatment also diminishes the deleterious side effects observed with higher concentrations of PTU or earlier exposure. One of the great advantages of the present protocol is that it can be used on live animals, facilitating the detection of, for instance, GFP signals in genetically manipulated embryos. This method also will be valuable in whole mount detection of gene expression, protein expression, and confocal microscopy screening of intact organisms.

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