The toxic effects of cadmium and other heavy metals have been well established, and many of these and other environmental pollutants are known to be embryotoxic or teratogenic. However, it has proven difficult to identify individual cells that respond to toxicants among the wide range of cell populations in an intact animal, particularly during early development when cells are continually changing their molecular and physiologic characteristics as they differentiate. Here we report the establishment of an in vivo system that uses hsp70 gene activation as a measure of cadmium toxicity in living early larvae of transgenic zebrafish carrying a stably integrated hsp70-enhanced green fluorescent protein (eGFP) reporter gene. We demonstrate that eGFP expression in this strain of fish acts as an accurate and reproducible indicator of cell-specific induction of hsp70 gene expression. Furthermore, the transgene responds in a dose-dependent manner at concentrations similar to those observed for morphologic indicators of early-life-stage toxicity and is sensitive enough to detect cadmium at doses below the median combined adverse effect concentration and the median lethal concentration. The stable nature of this trangenic line should allow for extremely rapid and reproducible toxicologic profiling of embryos and larvae throughout development. Key words: cadmium, green fluorescent protein, hsp70 promoter, stable transgenic zebrafish, toxicology, zebrafish.

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using standard procedures (14,18). Fish were originally obtained from wild-type stocks and maintained in the laboratory for multiple generations. A maximum of 25 embryos were reared in 25 mL petri dishes with system water/treatment solutions changed twice per day.

**Cadmium exposures.** To determine median lethal concentration (LC50) and median combined adverse effect concentration (EC50) and to select relevant Cd exposure doses for the gene expression treatments, we first performed acute toxicity tests. As part of the acute toxicity tests, posthatch larvae were continuously exposed to a range of Cd doses (0–125 µM) for 96 hr beginning at 72 hr after fertilization. Three replicate treatments (3 × 25 larvae) were exposed to each dose. Observations for morphologic effects were made, and solutions were changed twice daily; any dead larvae were counted and removed. The average proportion of larvae responding for a given end point was calculated for each dose. LC50 and EC50 were calculated from a linear regression of probit transformations of the dose–response data (19).

Using the Cd acute toxicity dose–response relationship, doses for the hsp70 expression experiments were selected to represent low doses [no observed adverse effect concentration (NOAEC)], middle doses (near the EC50), and high doses (≥ LC50). Exposures for hsp70 expression were conducted similarly to those for the acute toxicity test on both wild-type (n = 35–45 per dose) and transgenic (n = 12–16 per dose) larvae. Exposures began at 80 hr of development for a duration of 3 hr. In some experiments, exposure periods were followed by a chase period in clean water as indicated.

**hsp70 detection.** Wild-type larvae were fixed in 4% paraformaldehyde after Cd exposure, and expression of hsp70 mRNA was detected using in situ hybridization. A digoxigenin-labeled antisense riboprobe was synthesized from the inducible zebrafish hsp70 polymerase chain reaction fragment previously cloned by Lele et al. (16). In situ hybridization was performed according to Jowett and Yan (20), with modifications. After hybridization, larvae were incubated for 4 hr in alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:4,000; Roche Diagnostics, Quebec, Canada). Larvae were then washed overnight in phosphate-buffered saline. The blue-purple color reaction was performed using the alkaline phosphatase substrates 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Photomicroscopy of hybridization product was conducted on whole larvae cleared in a solution of 2:1 benzyl benzoate/benzyl alcohol and also on serial sections of methacrylate-mounted larvae.

Expression of the hsp70 reporter gene in living transgenic larvae was assessed at 0, 8, 16, and 24 hr after Cd exposure. eGFP fluorescence was detected using a Nikon Y-FL epifluorescence attachment on a Nikon E-600 microscope, and photos of both wild-type and transgenic larvae were captured with a Nikon Coolpix digital camera (Nikon, Tokyo, Japan).

**Statistical analysis.** We calculated the proportion of larvae expressing hsp70 mRNA or eGFP for a given tissue at each dose. Differences between controls and treatment doses were tested for significance using Fischer’s exact test (19,21).

**Results**

**Acute toxicity of cadmium.** Zebrafish embryos in their first day of development after fertilization have been reported to be more resistant to Cd exposure than are hatched larvae (10,22), a result that is in agreement with our own preliminary studies (data not shown). We therefore performed a 96-hr acute toxicity test for Cd on larvae at 3–7 days of age to establish a dose range for the hsp70 expression experiments and to determine acute toxicity of Cd in early-larval-stage zebrafish. The predominant effects observed were mortality, edema, and trunk abnormalities such as spinal kyphosis, scoliosis, and lordosis (Figure 1), with not all effects necessarily occurring in any one individual. Edema was first observed in the pericardial region and, in some individuals, developed into more widespread subcutaneous edema and ascites, as shown in Figure 1. The LC50 and EC50 and their 95% confidence intervals

![Figure 1](image-url)
were calculated to be 18.8 µM (4.8–73.8 µM) and 1.7 µM (0.7–4.1 µM), respectively. The dose–response toxicity data allowed for three doses to be chosen for the hsp70 expression studies: 0.2 µM (low dose) was the NOAEC over the 96-hr exposure, 5 µM (middle dose) was near the EC50, and 125 µM (high dose) caused 100% mortality during the acute test.

Expression of the endogenous hsp70 gene in wild-type larvae. To determine the response of the wild-type hsp70 gene to Cd at the cell and tissue level, we used whole-mount in situ hybridization analysis using a digoxigenin-labeled antisense hsp70 RNA probe. Whole-mount photos of wild-type larvae revealed a dose-dependent increase in hsp70 mRNA-expressing cells within the developing gill and head region (Figure 2). Larvae given the 125-µM dose exhibit hsp70 mRNA throughout the head and trunk region (Figure 2G), which is particularly evident in the anterior region of the head (Figure 2H). At 5 µM Cd, this expression is reduced to the gill region in the lateral view (Figure 2E) and has become more localized in the anterior head (Figure 2F). At the NOAEC dose for morphologic indicators of 0.2 µM, hsp70 transcripts are still observed in the gill region but no longer in the anterior head (Figure 2C and Figure 2D, respectively). Serial sections of larvae from the same Cd treatment doses were made in order to determine the specific tissues expressing hsp70. The proportions of larvae per treatment dose expressing the gene in a given tissue were calculated from counts of the serial sections (Table 1). The 125-µM dose resulted in expression in epithelial cells of the developing skin, gill, olfactory organ, digestive tract, liver, pronephric ducts of the pronephros, and in the lateral line neuromasts (Figure 3). The 5-µM dose revealed hsp70 expression in the olfactory tissue, gill, and skin, and only cells of the gill and skin exhibited any detectable mRNA signal at 0.2 µM Cd (sections not shown for 0.2 and 5 µM doses). The proportions positive for tissue expression were significantly different from controls in all treatments (p < 0.001) unless otherwise stated.

hsp70-eGFP reporter expression in transgenic larvae. Our laboratories have previously described the development of a stable line of transgenic zebrafish containing the eGFP reporter gene driven by the 1.5-kb promoter of the inducible zebrafish hsp70 gene (23). This promoter is from the same gene that has been used as a probe for our previous in situ hybridization studies (16,17) and those presented in Figures 2 and 3. The strain of fish has now been stably maintained for multiple generations over 4 years in the laboratory and passes the transgene to offspring in typical Mendelian ratios. Furthermore, eGFP expression in these fish faithfully mimics that of the endogenous gene during both normal

| Gene type/treatment (µM Cd) | Skin | Gill | Olfactory system | Digestive tract | Liver | Pronephros |
|----------------------------|------|------|------------------|----------------|-------|------------|
| hsp70                      | 0 (n = 45) | 0 | 0 | 0 | 0 | 0 |
| 0.2 (n = 41) | 100 | 97.8 | 80 | 0 | 0 | 0 |
| 5 (n = 35) | 97.1 | 91.4 | 8.6*  | 0 | 0 | 0 |
| 125 (n = 45) | 100 | 100 | 100 | 100 | 97.8 | 77.8 |
| hsp70-eGFP reporter | 0 (n = 10) | 0 | 0 | N/A | 0 | 0 |
| 0.2 (n = 12) | 100 | 100 | 100 | N/A | 0 | 0 |
| 5 (n = 15) | 100 | 100 | 100 | N/A | 13.3* | 0 |
| 125 (n = 15) | 100 | 100 | 100 | N/A | 75 | 62.5 |

NA, not applicable. Tissue counts for endogenous hsp70 were made from sections of fixed larvae, whereas tissue-specific hsp70-eGFP expression was observed in living transgenic larvae. Expression data for the digestive tract could not be obtained in transgenic larvae because of yolk fluorescence in the optical path. All values are significantly different from controls for both hsp70 (p < 0.0001) and hsp70-eGFP (p < 0.0025), except for values indicated (*).
development under nonstress conditions (lens-specific expression) and after heat shock (ubiquitous up-regulation) (17,23). The constitutive burst of expression of endogenous hsp70 gene expression in the developing lens occurs between 26 and 42 hr of age but is no longer detectable at later stages and therefore is not seen in the in situ hybridizations shown in Figures 2 and 3. Although the hsp70-eGFP reporter gene mimics this lens-specific constitutive expression pattern, the stable eGFP product remains in the lens until later stages and is therefore still detectable in the transgenic larvae shown in Figures 4 and 5.

Cd-induced expression of the hsp70-eGFP reporter gene in live larvae was assessed 24 hr after a 3-hr Cd exposure to allow for maximal eGFP accumulation. eGFP fluorescence followed a dose-dependent pattern in terms of the number and types of different tissues expressing (Figure 4) and is clearly similar to that observed for endogenous hsp70 in wild-type larvae. The 125-µM dose caused eGFP fluorescence in the developing olfactory organ, gill, skin, liver, and pronephros. Weak expression was occasionally observed in the digestive tube, but the signal was obscured by interference with other fluorescence in the optical path. Olfactory, gill, and skin expression was also seen at 5 µM, whereas cells in only the gill and skin exhibited detectable fluorescence at 0.2 µM Cd. All proportions expressing in a given tissue were significantly different from the control (p < 0.003) unless otherwise stated (Table 1).

Figure 3. Serial transverse sections of in situ hybridized wild-type larvae demonstrating hsp70 expression after 3 hr-exposure to 125 µM Cd (B,D,F,H) relative to untreated controls (A,C,E,G). Location of transverse sections 1 (A,B), 2 (C,D), 3 (E,F), and 4 (G,H) are shown (I). Abbreviations: de, digestive tract epithelium; ep, skin epithelium; gi, gill; gl, glomerulus; gt, gut; le, lens; li, liver; nc, notochord; oe, olfactory epithelium; pd, pronephric ducts. Pigmented cells of the retinal epidermis and pronephric tubule are reddish brown in C–H. Magnification: 30× (A,C,E,G), 45× (B,D,F,H), and 160× (I).

Figure 4. Exposure of hsp70-eGFP transgenic larvae to Cd results in a dose-dependent increase in reporter gene expression (eGFP fluorescence) (C–I) relative to the control treatment (A,B). Abbreviations: ep, skin epithelium; gi, gill; le, lens; li, liver; oe, olfactory epithelium; pd, pronephric ducts. Fluorescence in the lens of all larvae represents residual eGFP from constitutive lens expression earlier during development. Controls did not show any Cd-induced hsp70-eGFP expression, whereas background autofluorescence in the yolk region is evident at all doses. Magnification: 30× (A,C,E,G), 45× (B,D,F,H), and 160× (I).
We were also interested in monitoring the appearance of eGFP activity in real time after Cd exposure (Figure 5). No induced fluorescence is observable immediately after the 3-hr dose period, likely because of the lag between the initiation of hsp70-eGFP transcription and accumulation of enough eGFP protein to generate a signal (Figure 5A). The gills, skin, and olfactory tissues are the first to exhibit fluorescence 8 hr after being returned to clean water (Figure 5B). After 16 hr of recovery (Figure 5C), liver expression is evident, and by 24 hr (Figure 5D) expression is also detectable in the pronephric ducts. Fluorescence in neuroectoderm of the head lateral line can also be seen (Figure 5C). Similar expression is sometimes seen in the trunk lateral line, as is expression of the endogenous gene (data not shown).

Discussion

The use of GFP reporter gene constructs has become increasingly popular for the analysis of gene expression in developmental systems. Motoike et al. (24) monitored vascular development in real time using stable transgenic zebrafish expressing GFP under the control of a promoter expressed in vascular endothelial cells, and Perz-Edwards et al. (25) used a retinoic acid response element/GFP line of zebrafish to examine retinoic acid-induced gene expression in early embryos. In toxicologic analysis, Carvan et al. (26) have suggested using transgenic adult zebrafish carrying GFP under control of aryl hydrocarbon response elements (AHREs), electrophile response elements, and metal response elements as reliable sentinels for aquatic pollution but had been unable to maintain transgenes beyond the F₂ generation. At the embryonic level, Mattingly et al. (12) recently reported 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced GFP activity in zebrafish embryos transiently microinjected with an AHRE-regulated GFP construct. However, only a small number of cells that are capable of expressing any given reporter gene construct will actually express it in transient microinjection assays, and they do so in a mosaic pattern when individual embryos are compared (27,28). This mosaicism was also apparent in the GFP-positive embryos of the AHRE/GFP study, and 57% of all injected embryos exhibited no fluorescence at all (12). These difficulties limit the extent to which transient injection assays can be reproducibly used in toxicology but are overcome with the stable line of transgenic zebrafish described in our study.

The in vivo reporter gene model presented here addresses many of the shortcomings of previously used vertebrate hsp70-based toxicity assessment models, particularly as they relate to early life stages. First, a significant amount of work has already characterized the normal patterns of hsp70 gene expression in developing zebrafish embryos and larvae, and additional information can be easily obtained using whole-mount in situ hybridization analysis with existing probes. Because the eGFP reporter gene mimics expression of the endogenous hsp70 gene, more meaningful interpretation of eGFP expression patterns after toxin exposure can be made. The dose dependency of tissue-specific eGFP expression also occurred in a similar manner to that observed for the endogenous hsp70 gene, and for severity of classical end points based on mortality and morphology. Finally, the eGFP expression assay is extremely rapid and simple to perform; it can be carried out in living embryos, and because this eGFP model uses a stable line of transgenic fish, it is also highly reproducible.

The hsp70 gene promoter used to create the stable line of transgenic zebrafish contains several putative heat shock elements (HSEs). This short, highly conserved DNA sequence element is the binding site of heat shock factor (HSF), a transcription factor known to be responsible for activation of heat shock gene transcription in response to elevated temperature or other forms of environmental stress in organisms as diverse as yeast, insects, and man. In zebrafish, HSF normally resides in the cell in an inactive, monomeric form that is rapidly converted to an active, HSE-binding trimeric form after stress exposure. Importantly, HSE-binding activity of HSF can be activated by Cd, arsenite, and other chemical stressors (29). Thus, it is unlikely that activation of the transgene in our study is an indirect, downstream effect of Cd; it is more likely the result of HSF activation immediately after exposure of a cell within the embryo to Cd. This is supported by the fact that we have also observed strong eGFP induction after exposure of transgenic larvae to relevant doses of sodium arsenite, a known inducer of hsp70 expression that also activates HSF activity (data not shown). Given the range of chemical stressors that are known to be capable of inducing hsp70 gene expression in other systems, it is likely that similar relationships will be found between eGFP expression, endogenous hsp70 gene activation, and acute toxicity.

Tissues found to be positive for hsp70 (Figure 3) and hsp70-eGFP (Figure 4) in our experiments are considered target tissues or accumulators of Cd in fish (30–32), and histopathologic lesions in skin, digestive tract, olfactory epithelium, kidney, and gills have been reported after a 96-hr acute exposure to 12 ppm CdCl₂ (~66 µM) (33). This dose is within our exposure range, but expression of hsp70 and hsp70-eGFP was detected in developing tissues after a much shorter period of exposure (3 vs. 96 hr). In addition, we observed eGFP expression at concentrations as low as 0.2 µM, which is lower than those doses at which Cd induces hsp70 expression in cultured cells from a number of different species (0.5–50 µM) (4–7). Furthermore, we observed strong eGFP expression at doses near the EC₅₀ for combined end points in our acute toxicity experiments. Thus, in vivo detection of eGFP expression is a valid approach for examining the effects of Cd toxicity.
eGFP in live transgenic zebrafish is of sensitivity comparable with that of in vitro hp70 expression systems and can clearly be used at relevant concentrations when compared with more classical indicators of embryotoxicity. Heavy-metal–induced reporter gene expression has also been shown to occur at toxicologically relevant concentrations in transgenic adults of the soil nematode Caenorhabditis elegans carrying an hp70 promoter–β-galactosidase fusion gene, suggesting that HSE-containing promoters could be widely useful in whole-animal transgenic systems (34).

The tissues expressing hp70 and hp70-eGFP in our experiments did so in a dose-dependent manner, with the order of sensitivity being gill > skin > olfactory organ > digestive tract > liver > pronephros (Table 1). It is possible that this is due at least in part to the route of exposure because the skin, gill, and olfactory tissue would be directly exposed to waterborne Cd ions. Stromberg et al. (33) found that adult tissues directly exposed to Cd in water (gills, olfactory tissue, and skin) exhibited more severe lesions than did more visceral tissues. Gills are also the osmoregulatory organ in fish and therefore are the principal route of absorption for waterborne Cd (31,35), although it is not known whether the developing larval gill plays a similar role. The liver and pronephros expressed endogenous hp70 and eGFP only at the highest 125-µM dose, indicating a lower degree of sensitivity to Cd relative to the skin, gill, and olfactory tissues. Absorbed Cd is transported via the blood to the liver, where it becomes bound to metallothionein (MT), thereby reducing the toxic effect of Cd (1). Complexes of Cd-MT are then filtered out of the blood in the kidney and reabsorbed in the proximal tubule cells. Reabsorbed Cd-MT is digested by cellular proteases, releasing free Cd, which induces de novo synthesis of MT. It is believed that Cd toxicity occurs in the kidney when Cd overwhels MT synthesis. Whether similar kinet-ics of Cd uptake and excretion occur in the kidney when Cd overwhells MT synthesis is of sensitive cell populations in early life stages with long-term effects in adult stages for the same individual.

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