Developmental Toxicology of Cadmium in Living Embryos of a Stable Transgenic Zebrafish Line

Scott R. Blechinger,[1,2] James T. Warren, Jr.,[3] John Y. Kuwada,[4] and Patrick H. Krone[1,2]

[1]Department of Anatomy and Cell Biology; and [2]Toxicology Group, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; [3]Department of Biology, Penn State University, Erie, Pennsylvania, USA; [4]Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA

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. *Environ Health Perspect* **110:1041–1046 (2002). [Online 4 September 2002]**
http://ehpnet1.niehs.nih.gov/docs/2002/110p1041-1046blechinger/abstract.html

Cadmium, which is on the U.S. Environmental Protection Agency's list of priority pollutants (*1*), has been well characterized in terms of its absorption, tissue distribution, mechanism of action, and elimination in many adult vertebrate species, whereas the potential embryonic effects of this heavy metal are poorly understood. Although Cd exposure can give rise to a variety of developmental defects, it has proven difficult to identify toxin-sensitive cells among the wide range of cell populations in a developing embryo. This is due in part to the fact that these cells are continually changing their molecular and physiologic characteristics as they differentiate, and that early-life-stage toxicity assays have been aimed primarily at assessing mortality, hatching success, and other later morphologic end points. This also holds true for a number of other toxins that are known to be embryotoxic or teratogenic. However, it has recently been recognized that the rapidly expanding body of knowledge on molecular and cellular events occurring during embryonic development should be integrated into toxicologic assessment regimes (*2,3*).

Heat shock proteins (Hsps) are a family of highly conserved molecular chaperones that aid in the proper folding, transport, and degradation of cellular proteins. The 70 kDa Hsp subfamily, the best characterized of the Hsps, contains a highly stress-inducible isoform encoded by the *hsp70* gene. A variety of cytotoxic agents including Cd have been shown to up-regulate *hsp70*, and *hsp70* expression has been examined as a potential marker in toxicologic screening for a number of vertebrate species. The most commonly used assays include Northern and Western blots, as well as *hsp70* gene promoters linked to reporter genes in transfected cells (*4–7*). Although such assays can serve as a rapid and accurate means to assess toxin impact in cultured cells, they are of limited value in assessing toxicity or elucidating mechanisms in intact individuals during development.

A number of conditions should first be met to make full use of *hsp70* gene expression as an accurate toxicity biomarker in whole-animal testing (*8,9*). For an ideal *hsp70* model system, a basic knowledge of normal *hsp70* expression under nonstressed conditions is needed to properly assess the significance of results seen after toxin exposure. This is particularly true of early life stages, for which only limited information exists on the basic biology and cell-specific expression of *hsp70* in many of the organisms commonly used for toxicologic research. Toxicant-induced *hsp70* expression must also be dose dependent and should be sensitive enough to assay sublethal doses, which can then be related back to classical toxicity end points (i.e., mortality and dysmorphologies). In addition, the ideal expression assay should be rapid and *in vivo* to allow direct observation of tissue-specific expression in a whole organism.

The zebrafish (*Danio rerio*) offers an attractive system with which to link early-life-stage toxicity assessment in a vertebrate at the molecular, cellular, and whole-organism levels (*10–13*). There is a large body of literature devoted to mechanisms regulating zebrafish embryo development, and a well-characterized morphologic staging series has been published (*14*). Our laboratory has cloned and extensively characterized a number of heat shock genes in zebrafish [reviewed in (*15*)], including analysis of *hsp70* gene expression during normal development and after exposure to environmental stress during embryonic/larval periods (*16,17*). Here we report the establishment of an *in vivo* system that uses *hsp70* gene activation as a measure of Cd toxicity in living early larvae of a transgenic zebrafish line carrying a stably integrated *hsp70*-eGFP (enhanced green fluorescent protein) reporter gene. In this study, we demonstrated that eGFP expression acts as an accurate and reproducible indicator of cell-specific induction of *hsp70* gene expression and that it responds in a dose-dependent manner at concentrations similar to those observed for morphologic indicators of early-life-stage toxicity.

Materials and Methods

Reagents and water. We obtained cadmium chloride (2.5 hydrate) from J.T. Baker Inc. (Phillipsburg, NJ, USA) and prepared a 1 mM Cd stock solution in triple distilled water. Treatment doses were made from dilutions of this stock in carbon filtered system water (total hardness, 177–178 mg/L; alkalinity, 96–97 mg/L as $CaCO_3$; pH 8.1–8.2).

Animals. Adult zebrafish were maintained at 28°C in system water on a 14-hr photoperiod, and embryos were collected and staged

Address correspondence to P.H. Krone, Department of Anatomy and Cell Biology, University of Saskatchewan, Health Sciences Building, 107 Wiggins Road, Saskatoon, Saskatchewan, Canada S7N 5E5. Telephone: (306) 966-4089. Fax: (306) 966-4298. E-mail: krone@duke.usask.ca

We thank Y. Gothilf for advice on modifications to the *in situ* hybridization protocol, L. Doig for water chemistry analysis, and K. Yuen and T. Evans for assistance with sectioning.

We gratefully acknowledge the support of grants from the Canadian Network of Toxicology Centres and the Natural Sciences and Engineering Research Council of Canada (P.H.K.), the National Institute of Health (grant 1R15HD37579-01 to J.T.W. and grant NS3687 to J.Y.K.). S.R.B. was a recipient of scholarships from the Saskatchewan Wheat Pool and the Toxicology Graduate Program, University of Saskatchewan.

Received 14 December 2001; accepted 9 April 2002.

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 (LC_{50}) and median combined adverse effect concentration (EC_{50}) 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. LC_{50} and EC_{50} were calculated from a linear regression of log-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 EC_{50}), and high doses (≥ LC_{50}). 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.

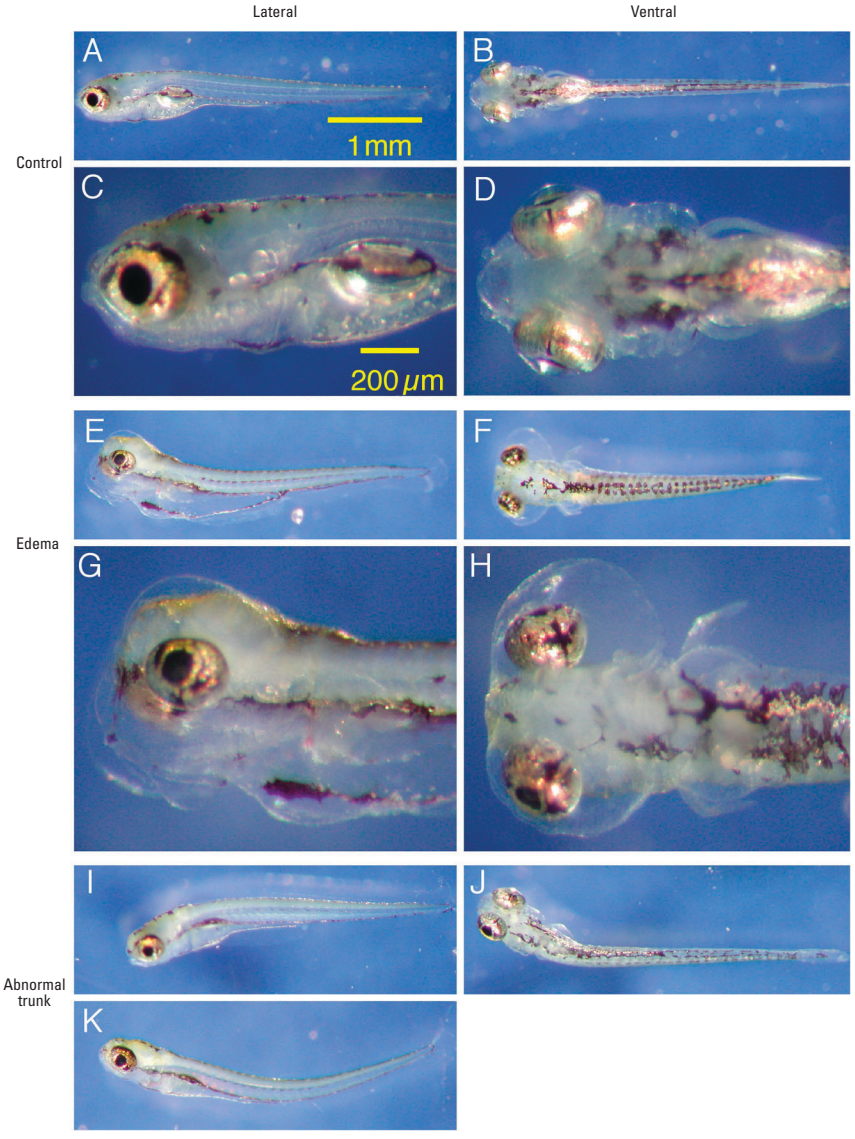

Figure 1. Acute exposure to Cd for 96 hr between 3 and 7 days of age caused increased incidence of edema (*E–H*) and trunk abnormalities such as spinal kyphosis, scoliosis, and lordosis (*I–K*) relative to the control (*A–D*). The scale bar in (*A*) applies also to (*B,E,F*), and (*I–K*); the scale bar in (*C*) applies also to (*D,G,H*).

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-digoxigenen 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 LC_{50} and EC_{50} and their 95% confidence intervals

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 EC_{50}, 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. This 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

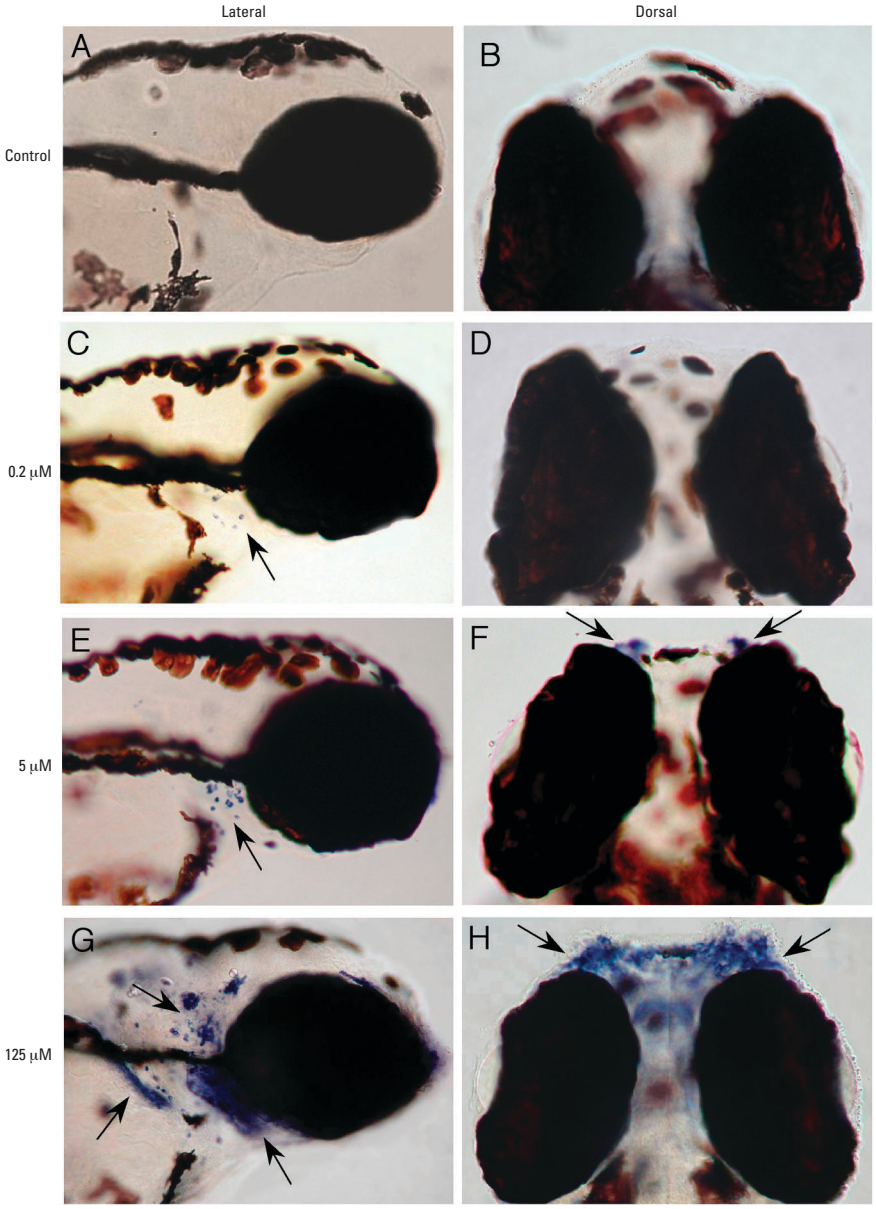

Figure 2. Dose-dependent expression of the endogenous *hsp70* gene after a 3-hr exposure of 80-hr-old larvae to Cd. Expression is detected as a blue-purple hybridization product in Cd-treated larvae (*C–H*) (arrows), while no expression was detected in controls (*A,B*). Magnification: 60× (*A,C,E,G*) and 90× (*B,D,F,H*).

Table 1. Tissues expressing *hsp70* and *hsp70*-eGFP after Cd exposure.

Gene type/treatment (µM Cd)	Percent embryos expressing in tissue					
	Skin	Gill	Olfactory system	Digestive tract	Liver	Pronephros
hsp70						
0 ($n = 45$)	0	0	0	0	0	0
0.2 ($n = 41$)	100	87.8	0	0	0	0
5 ($n = 35$)	97.1	80	91.4	8.6*	0	0
125 ($n = 45$)	100	100	100	100	97.8	77.8
hsp70-eGFP reporter						
0 ($n = 10$)	0	0	0	N/A	0	0
0.2 ($n = 12$)	100	100	0	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*-GFP ($p < 0.0025$), 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).

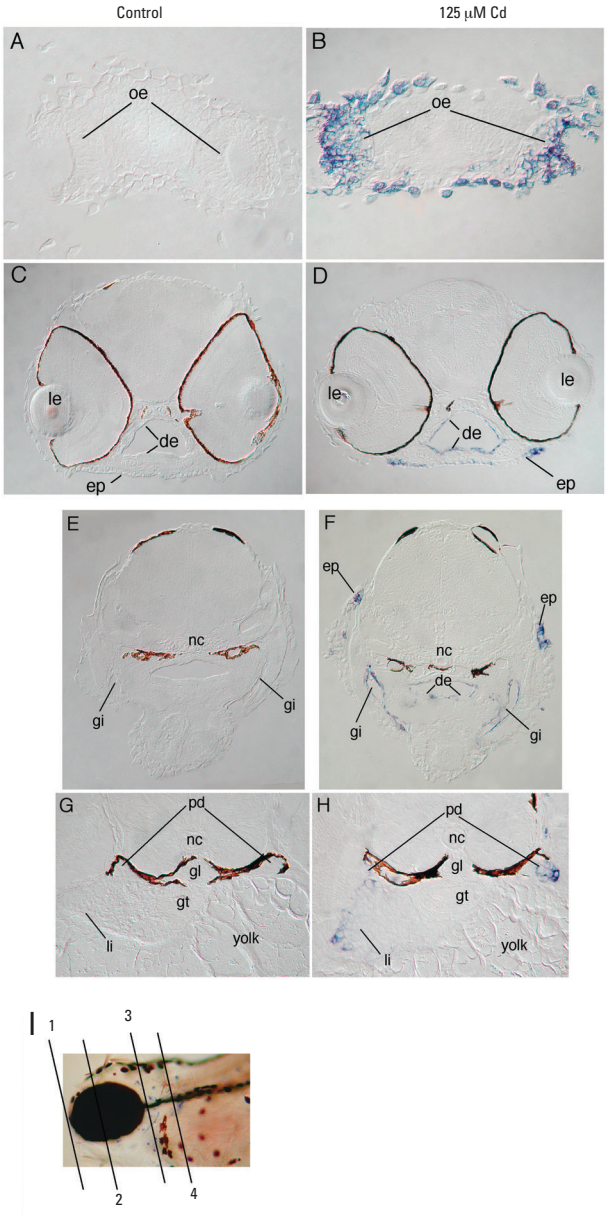

Figure 3. Serial transverse sections of *in situ* hybridized wild-type larvae demonstrating *hsp70* expression after 3 hr-exposure to 125 µM Cd (*B,D,F,H*) relative to untreated controls (*A,C,E,G*). Location of transverse sections 1 (*A,B*), 2 (*C,D*), 3 (*E,F*), and 4 (*G,H*) are shown (*I*). Abbreviations: 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*).

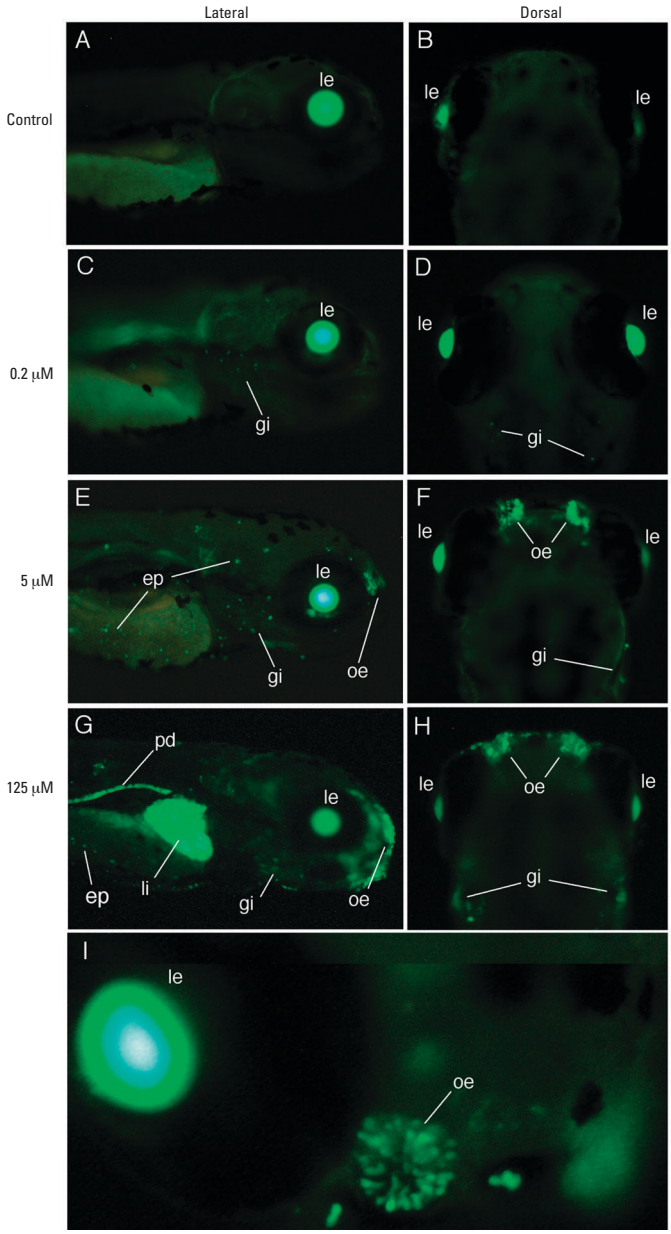

Figure 4. Exposure of *hsp70*-eGFP transgenic larvae to Cd results in a dose-dependent increase in reporter gene expression (eGFP fluorescence) (*C–I*) relative to the control treatment (*A,B*). Abbreviations: ep, skin epithelium; gi, gill; le, lens; li, liver; oe, olfactory epithelium; pd, pronephric 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 neuromasts 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 renewable sentinels for aquatic pollution but had been unable to maintain transgenes beyond the F_2 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 metazoans, 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 $CdCl_2$ (~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 EC_{50} for combined end points in our acute toxicity experiments. Thus, *in vivo* detection of

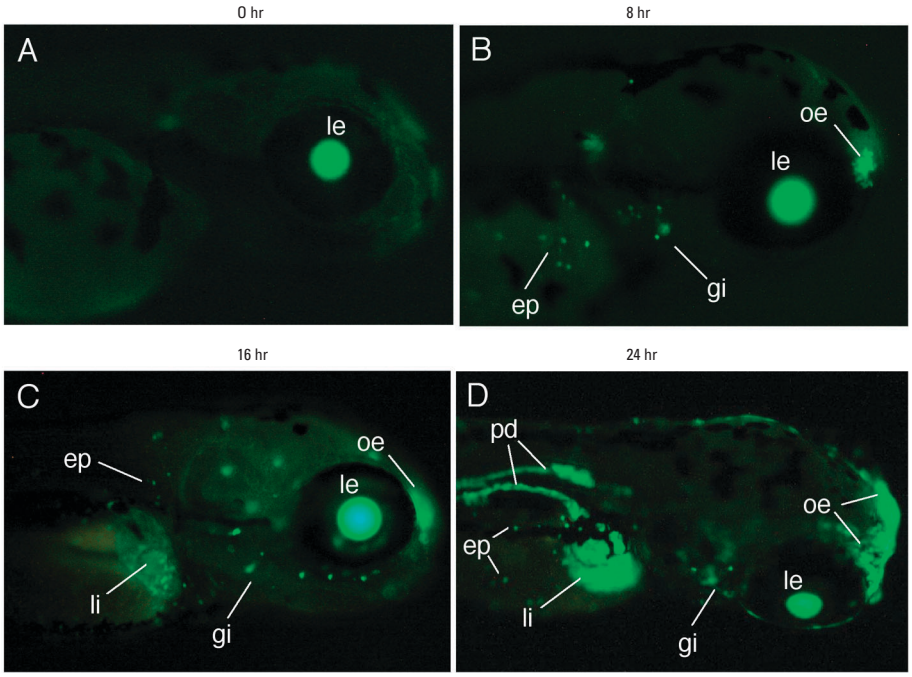

Figure 5. Temporal expression pattern of *hsp70*-eGFP during chase period in clean water immediately after a 3-hr exposure to 125-µM Cd (*A*) and after 8-hr (*B*), 16-hr (*C*), and 24-hr recovery (*D*). Abbreviations: ep, skin epithelium; gi, gill; le, lens; li, liver; oe, olfactory epithelium; pd, pronephric ducts. Residual eGFP fluorescence visible in the lens of all larvae is the result of constitutive early embryonic expression.

eGFP in live transgenic zebrafish is of sensitivity comparable with that of *in vitro hsp70* 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 *hsp16* promoter–β-galactosidase fusion gene, suggesting that HSE-containing promoters could be widely useful in whole-animal transgenic systems (*34*).

The tissues expressing *hsp70* and *hsp70*-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 *hsp70* 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 overwhelms MT synthesis. Whether similar kinetics of Cd uptake and excretion occur in developing fish larvae is unclear. However, this kind of protective effect of MT in both the liver and pronephros could partially explain their relatively diminished sensitivity in terms of Cd-induced *hsp70* expression in our experiments.

Finally, transgenic larvae revealed an interesting temporal pattern of tissue-specific eGFP fluorescence during the chase period after a 3-hr exposure to 125-μM Cd (Figure 5). Exposed tissues such as the developing gills, skin, and olfactory system were the first to show eGFP fluorescence, which expanded to the liver by 16 hr and had finally reached the twin pronephric ducts by 24 hr. This pattern of *hsp70*-eGFP expression may be a reflection of the order in which tissues are exposed to cadmium, with sites of direct exposure and absorbtion (olfactory organ, skin, and gill) expressing first, followed by later expression in tissues of metabolism (liver) and excretion (kidney). At present, we do not know if the lag period between activation of the endogenous *hsp70* gene (Figure 3) and the appearance of fluorescence in transgenic larvae is the result of differential translation rates of eGFP, or differential levels of transgene expression/mRNA stability. Regardless, the ability to monitor *hsp70* gene activation in a living individual animal in real time represents an important feature of this model system. This will allow for the development of rapid toxicologic profiles at the level of the single cell *in vivo* and, importantly, the correlation of sensitive cell populations in early life stages with long-term effects in adult stages for the same individual.

References and Notes

1. ATSDR. Toxicological Profile for Cadmium (Update). Atlanta, GA:Agency for Toxic Substances and Disease Registry, 1999.
2. Bigsby R, Chapin RE, Daston GP, Davis BJ, Gorski J, Gray LE, Howdeshell KL, Zoeller RT, vom Saal FS. Evaluating the effects of endocrine disruptors on endocrine function during development. Environ Health Perspect 107(suppl 4):613–618 (1999).
3. Mantovani A, Stazi AV, Macri C, Maranghi F, Ricciardi C. Problems in testing and risk assessment of endocrine disrupting chemicals with regard to developmental toxicology. Chemosphere 39:1293–1300 (1999).
4. Salminen WF Jr, Voellmy R, Roberts SM. Induction of hsp70 in Hep2G cells in response to hepatotoxicants. Toxicol Appl Pharmacol 141:117–123 (1996).
5. Braeckman B, Smagghe G, Brutsaert N, Cornelis R, Raes H. Cadmium uptake and defense mechanism in insect cells. Environ Res A 80:231–243 (1999).
6. Ait-Aissa S, Porcher J-M, Arrigo A-P, Lambre C. Activation of the *hsp70* promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology 145:147–157 (2000).
7. Tully DB, Collins BJ, Overstreet JD, Smith CS, Dinse GE, Mumtaz MM, Chapin RE. Effects of arsenic, cadmium, chromium, and lead on gene expression regulated by a battery of 13 different promoters in recombinant HepG2 cells. Toxicol Appl Pharmacol 168:79–90 (2000).
8. Bierkens JGEA. Applications and pitfalls of stress-proteins in biomonitoring. Toxicology 153:61–72 (2000).
9. Ryan JA, Hightower LE. Heat shock proteins: molecular biomarkers of effect. In: Molecular Biology of the Toxic Response (Paga A, Wallace KB, eds). Philadelphia:Taylor and Francis, 1999;449–466.
10. Cheng SH, Wing A, Wai K, So CH, Wu RSS. Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. Environ Toxicol Chem 19:3024–3031 (2000).
11. Tanguay RL, Andreasen E, Heideman W, Peterson RE. Identification and expression of alternatively spliced aryl hydrocarbon nuclear translocator 2 (ARNT2) cDNAs from zebrafish with distinct functions. Biochim Biophys Acta 1494:117–128 (2000).
12. Mattingly CJ, McLachlan JA, Toscano WA Jr. Green fluorescent protein (GFP) as a marker of aryl hydrocarbon receptor (AhR) function in developing zebrafish (*Danio rerio*). Environ Health Perspect 109:845–849 (2001).
13. Willey JB, Krone PH. Effects of endosulfan and nonylphenol on the primordial germ cell population in pre-larval zebrafish embryos. Aquat Toxicol 54:113–123 (2001).
14. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310 (1995).
15. Krone PH, Lele Z, Sass JB. Heat shock genes and the heat shock response in zebrafish embryos. Biochem Cell Biol 75:487–497 (1997).
16. Lele Z, Engel S, Krone PH. *Hsp47* and *hsp70* gene expression is differentially regulated in a stress- and tissue-specific manner in zebrafish embryos. Dev Genet 21:123–133 (1996).
17. Blechinger SR, Evans TG, Tang P-T, Warren JT, Kuwada JY, Krone PH. 2002. The heat-inducible hsp70 gene is expressed during normal lens development under non-stress conditions. Mech Dev 112:213–215 (2002).
18. Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish. Eugene, OR:University of Oregon Press, 1995.
19. Gad SC. Statistics and Experimental Design for Toxicologists. 3rd ed. Baton Rouge, LA:CRC Press, 1999.
20. Jowett T, Yan YL. Double fluorescent in situ hybridization to zebrafish embryos. Trends Genet 12:387–389 (1996).
21. Zar JH. Biostatistical Analysis. 4th ed. Upper Saddle River, NJ:Prentice-Hall, 1999.
22. Dave G. The influence of pH on the toxicity of aluminum, cadmium, and iron to eggs and larvae of the zebrafish, *Brachydanio rerio*. Ecotoxicol Environ Saf 10:253–267 (1985).
23. Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, Shoji W. Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127:1953–1960 (2000).
24. Motoike T, Loughna S, Perens E, Roman BL, Liao W, Chau TC, Richardson CD, Kawate T, Kuno J, Weinstein BM, et al. Universal GFP reporter for the study of vascular development. Genesis 28:75–81 (2000).
25. Perz-Edwards A, Hardison NL, Linney E. 2001. Retinoic acid-mediated gene expression in transgenic reporter zebrafish. Dev Biol 229:89–101 (2001).
26. Carvan MJ III, Dalton TP, Stuart GW, Nebert DW. Transgenic zebrafish as sentinels for aquatic pollution. Ann NY Acad Sci 919:133–147 (2000).
27. Westerfield M, Wegner J, Jegalian BG, DeRobertis EM, Puschel AW. Specific activation of mammalian hox promoters in mosaic transgenic zebrafish. Genes Dev 6:591–598 (1992).
28. Adam A, Bartfai R, Lele Z, Krone PH, Orban L. Heat-inducible expression of a reporter gene detected by transient assay in zebrafish. Exp Cell Res 256:282–290 (2000).
29. Mosser DD, Theodorakis NG, Morimoto RI. Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol Cell Biol 8:4736–4744 (1988).
30. Rani AU. Cadmium-induced bioaccumulation in the selected tissues of a freshwater teleost, *Oreochromis mossambicus* (tilapia). Ann NY Acad Sci 919:318–320 (2000).
31. De Smet H, Blust R. Stress response and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure. Ecotoxicol Environ Saf 48:255–262 (2001).
32. Tjalve H, Gottofrey J, Bjorklund I. Tissue disposition of ^{109}Cd in the brown trout (*Salmo trutta*) studied by autoradiography and impulse counting. Toxicol Environ Chem 12:31–45 (1986).
33. Stromberg PC, Ferrante JG, Carter S. Pathology of lethal and sublethal exposure of fathead minnows, *Pimephales promelas*, to cadmium: a model for aquatic toxicity assessment. J Toxicol Environ Health 11:247–259 (1983).
34. Candido EPM, Jones D. Transgenic *Caenorhabditis elegans* strains as biosensors. Trends Biotech 14:125–129 (1996).
35. Glynn AW. Cadmium and zinc kinetics in fish: studies on water-borne ^{109}Cd and ^{65}Zn turnover and intracellular distribution in minnows, *Phoxinus phoxinus*. Pharmacol Toxicol 68:485–491 (1991).