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Heat shock protein gene expression during embryonic development of the zebrafish

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Abstract. Heat shock genes exhibit complex patterns of spatial and temporal regulation during embryonic development of a wide range of organisms. Our laboratory has been involved in an analysis of heat shock gene expression in the zebrafish, a model system which is now utilized extensively for the examination of early embryonic development of vertebrates. Members of the zebrafish hsp47, hsp70 and hsp90 gene families have been cloned and shown to be closely related to their counterparts in higher vertebrates. Expression of these genes has been examined using Northern blot and whole mount in situ hybridization analyses. Both the hsp47 and hsp90 genes are expressed in a highly tissue-restricted manner during normal development. The data raise a number of interesting questions regarding the function and regulation of these heat shock genes during early zebrafish development.

Key words. Zebrafish; heat shock; myogenesis; developmental biology; hsp90; hsp70; hsp47.

Introduction

Exposure of prokaryotic and eukaryotic cells to elevated temperature or other environmental stresses results in the activation of a small but highly conserved family of genes encoding the heat shock proteins (hsps) [1]. Families of heat shock proteins with relative molecular masses of 80–90, 68–70, 60 and 15–30 kDa are synthesized by most, if not all, eukaryotes. Several heat shock proteins act as molecular chaperones which mediate the correct assembly and localization of intracellular and secreted polypeptides and oligomeric protein structures. The importance of heat shock proteins in the protein folding pathway is reflected in the fact that a number of heat shock genes are expressed at high levels during normal cell growth [1]. Stress conditions which enhance the synthesis of heat shock proteins often give rise to an accumulation of denatured and aberrantly folded proteins within the cell. Thus, the interaction of hsps with abnormal proteins during stress is thought to be an extension of their role under normal, non-stress conditions [2, 3]. In addition to these well-studied proteins, a number of other novel heat shock proteins have been identified in recent years. For example, a 47 kDa collagen-binding heat shock protein (hsp47) has been reported in several eukaryotic organisms [4].

The expression of heat shock genes during embryonic development has long been an area of intensive investigation. Many heat shock genes exhibit complex patterns of constitutive and inducible expression during early embryonic development of both invertebrates and vertebrates [5–7]. However, while significant advances have been made elucidating the role of heat shock proteins as molecular chaperones in protein folding and translocation within the cell, the specific roles which these proteins play during embryogenesis are poorly understood. In addition, the regulatory networks responsible for the complex patterns of heat shock gene expression in developing embryos are only beginning to be elucidated. The zebrafish is a small tropical aquarium fish well known to home aquarium enthusiasts. Many of the features which have made it a popular addition to aquariums are also the reasons it has emerged in recent years as a new model system for the examination of embryonic development of vertebrates; it is readily available, inexpensive, hardy, easy to care for in large numbers, will readily spawn under appropriate photoperiod conditions and the translucent embryos develop rapidly. In this review, we introduce the zebrafish as a model system for the examination of heat shock protein regulation and function during embryonic development and summarize recent advances in the understanding of the heat shock response in this organism.

The zebrafish as a model system for the study of embryonic development of vertebrate organisms

The zebrafish has long been used as a model system in fisheries research and an excellent historical perspective of the use of this teleost in this capacity prior to the 1980's has been prepared by H. Laale [8]. The past decade has seen the zebrafish become solidly established as a model system for the examination of embryonic development [9–11]. It is now utilized extensively for studies examining a wide range of aspects of embryonic development and significant advances have been made on a number of fronts including but not limited to determination of the embryonic axis, cell lineage analysis, formation of the central and peripheral nervous

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systems, muscle development and the differential regulation of gene expression. The reader is referred to a number of references which summarize the current state of research in different areas of zebrafish developmental biology [12–21].

One of the significant features of the zebrafish is the relatively low space requirements of an aquarium facility on a per animal basis. Thus, a colony of zebrafish can be maintained in a fraction of the space which would be required for a comparable colony of mammals or larger fish models such as trout or salmon. Fertilized eggs can be obtained on demand by maintaining the fish on a constant photoperiod schedule. Together, these features allow users to obtain a large number of embryos for experimental manipulation without any seasonal limitations. The embryos themselves develop rapidly (somitogenesis is completed within the first day following fertilization) and a comprehensive staging series has recently been published [10]. Manipulations of environmental conditions are easily carried out since the embryos develop ex utero, a feature which greatly facilitates the study of heat shock proteins. As well, internal morphological changes can be readily monitored in both living and fixed whole embryos due to their translucency. This feature also makes the embryos readily amenable to the technique of whole mount in situ hybridization analysis to rapidly examine the three dimensional patterns of tissue-specific gene expression. Finally, the zebrafish has recently become the focus of a large scale mutagenesis effort aimed at identifying genes which regulate vertebrate embryonic development [13–18]. Similar studies carried out in the fruit fly, *Drosophila*, resulted in the identification of many of the genes which regulate embryonic development of this insect [22]. Remarkably, many of these same genes have counterparts in vertebrates and carry out analogous functions during vertebrate embryonic development. However, it is also clear that the regulation of vertebrate embryonic development will involve genes which do not have insect counterparts and can only be identified through saturation mutagenesis of the vertebrate genome. While a small number of zebrafish strains carrying mutations which affect development are available from small scale mutagenesis experiments [23–26], the large number of rapidly developing embryos required to properly carry out saturation mutagenesis meant that, until recently, this approach was impossible for vertebrates. However, saturation mutagenesis screening of the zebrafish genome is now underway and initial results are extremely promising [13, 15, 18]. Combined with recent studies which have advanced the mapping of the zebrafish genome [27, 28], these studies will provide invaluable information regarding the molecular cascades which regulate development.

Heat shock proteins and the heat shock response in zebrafish

Numerous laboratories have examined the heat shock response in fish and excellent reviews which cover this topic have been published [29–32]. Many of these studies have focused on environmental aspects of stress protein biology using cultured fish cells. For example, the relationship between heat shock protein synthesis and the development of thermotolerance has been well studied [33–35]. The effects of daily and seasonal temperature fluctuations as well as acclimation temperature have also been examined, especially in fish species which are exposed to a wide range of temperatures under normal environmental conditions [36–38]. The latter study is particularly interesting since substantial differences in the complement of heat shock proteins synthesized by very closely related species of the desert fish, *Poeciliopsis*, were observed.

In contrast to the extensive literature base on the fish heat shock response, including the cloning of heat shock genes from several teleosts1 [39–41], very little information is available regarding the expression or function of heat shock proteins during embryonic development of fish. However, a number of studies have examined the effects of elevated temperature and other environmental insults which are known to induce heat shock gene expression in other animal systems on embryonic development of zebrafish. Zebrafish embryos develop normally within a temperature range of approximately 23–33 °C and are usually maintained at 28–29 °C under laboratory conditions. There is a proportional increase in the rate of development with higher temperatures over this range [42, 43]. Exposure to a temperature of 34–35 °C for 2–3 h results in higher death rates as well as increases in a number of different developmental anomalies [43–45]. Embryos are most susceptible to heat shock at early cleavage stages and acquire increased resistance as they progress through the blastula and gastrula stages. As well, brief exposures of 2–20 min to higher temperatures of 37–41 °C give rise to a number of developmental abnormalities [42, 46]. Finally, exposure to ethanol at the blastula stage followed by a return to normal conditions for 24 h gives rise to notochord and spinal cord duplications as well as defects in development of the eye [47].

The advantages of zebrafish as a model developmental system as well as the embryological studies on the effects of environmental stress which have been carried out in the past led us to investigate the regulation and role of heat shock proteins in zebrafish embryos. To this end, we have cloned and characterized cDNA and genomic clones encoding members of the zebrafish hsp47, hsp70 and hsp90 families [17, 48–50]. In order to initially characterize the embryonic heat shock response at the molecular level, Northern blot analysis was utilized

to examine the expression of these heat shock genes at different stages of development under both control and heat shock conditions (table 1). Post-blastula stage embryos maintained at 28.5 °C (control temperature) first exhibit inducible hsp47, hsp70 and hsp90 mRNA accumulation following a one hour heat shock at 34 °C with maximum induction occurring at 37 °C [48–50]. At 40 °C, embryos at all stages of development which we have examined from gastrula through to post-hatching die rapidly and exhibit very weak induction of hsp mRNA. This suggests that heat protective mechanisms cease to be effective in zebrafish embryos at temperatures above 37 °C. Interestingly, these expression patterns appear to correlate with the aforementioned embryological data with respect to stress temperature and the associated level of heat shock gene expression and appearance of developmental anomalies. However, a more thorough investigation of this correlation needs to be carried out before any firm conclusions can be drawn.

Hsp90α and *hsp90β* genes are differentially regulated at both control and heat shock temperatures

Following a preliminary characterization of the zebrafish heat shock response, we initiated a thorough analysis of the expression of the cloned heat shock genes during normal development and following environmental stress. We initially focused on the *hsp90* gene family [48, 50], primarily because extensive biochemical studies have revealed that members of this family play a post-translational regulatory role within cells by interacting with and modulating the activity of several important cellular signaling molecules and transcription factors such as steroid receptors [51–53], $pp60^{v\text{-}src}$ kinase [54], and the basic helix-loop-helix transcription factor myoD [55, 56]. Studies in yeast have extended these observations to show that hsp90 plays a role in signal transduction cascades in vivo. For example, reduction in the activity or levels of hsp90 in *Saccharomyces cerevisiae* specifically compromise the activity of mammalian glucocorticoid receptor and $pp60^{v\text{-}src}$ in strains which express these two signaling molecules [57–60].

Members of the *hsp90* family have been found to exhibit complex spatial and temporal patterns of expression during embryogenesis and gametogenesis in a variety of animal systems [5–7]. For example, maternally synthesized *hsp83* mRNA in *Drosophila* embryos is localized by a novel mechanism which involves general degradation throughout the embryo and localized protection at the posterior pole [61] (*hsp83* is the single identified member of the *hsp90* family in *Drosophila*). In contrast, zygotic expression of the *hsp83* gene is localized specifically to the anterior pole and may be regulated by the anterior morphogen, bicoid [61]. Higher vertebrates express two closely related members of the hsp90 family termed hsp90*α* and hsp90*β* or the corresponding hsp86 and hsp84 in mouse [62]. In mammalian systems, *hsp90α* and *hsp90β* have been shown to be differentially regulated in cells of the testis, uterus and mammary gland [63–65].

While *hsp90α* and *hsp90β* genes had been identified in higher vertebrates, it was not known if these genes arose following the divergence of lower vertebrates such as teleosts. Using a degenerate polymerase chain reaction (PCR)-based approach, we demonstrated that *hsp90α* and *hsp90β* genes are also present in zebrafish and that the two genes were generated by a duplication event which occurred shortly before the emergence of the teleosts from the rest of the vertebrate lineage [48]. The use of gene-specific probes which can discriminate between the *hsp90α* and *hsp90β* messages in Northern blot analysis revealed that the *hsp90α* gene is expressed at low levels constitutively but is strongly heat-inducible in gastrula and later stage embryos following a one hour heat shock at 34 °C or 37 °C [48]. In contrast, the *hsp90β* gene is expressed at much higher levels constitutively and is only weakly inducible following a similar heat shock. These data were the first indication that the two genes are subject to different mechanisms of regulation in zebrafish embryos and may be playing different roles during early embryogenesis.

Table 1. Relative levels and tissue-specificity of zebrafish *hsp47*, *hsp70* and *hsp90* mRNAs.

	Control (28.5 °C)[1]	37 °C, 1 hour[1]	4% ethanol[1]	Known tissue-specificity[2]
hsp90α	+/−	++++	+	putative myogenic cells
hsp90β	++	+++	++	CNS, possibly other tissues
hsp70	−	++++	+	not determined
hsp47	+/−	++++	+++	Type II collagen expressing cells

[1]Assessed by Northern blot analysis in gastrula and later stage embryos.
[2]At control temperatures as assessed by whole mount in situ hybridization analysis.

Constitutive *hsp90α* mRNA is localized to *myo*D-expressing cells during normal development

One of the advantages of zebrafish embryos for the study of gene expression is that they are readily amenable to the technique of whole mount in situ hybridization analysis. This technique allows for rapid determination of three dimensional patterns of gene expression within embryos at all stages of development. Whole mount in situ hybridization analysis with gene-specific probes revealed that constitutive *hsp90α* mRNA is restricted primarily to a small subset of cells within the pre-somitic paraxial mesoderm, somites and pectoral fin buds of developing zebrafish embryos [50].

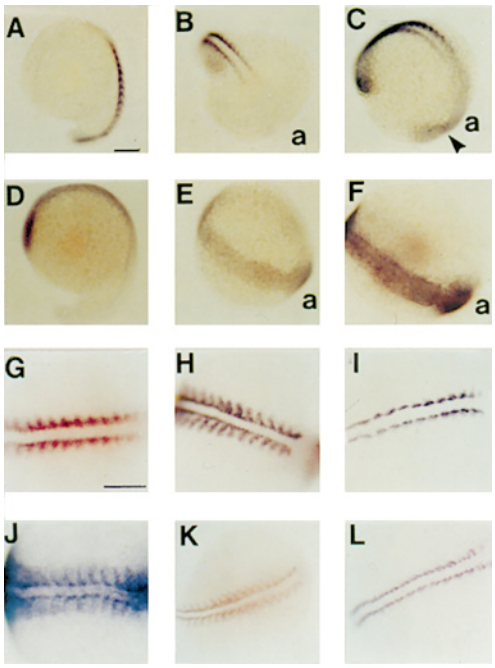

Figure 1. Expression of *hsp90α* (panels A–C, G, J), *myoD* (panels H, K), *α-tropomyosin* (panels I, L) and *hsp90β* (panels D–F) genes in mid-somitogenesis embryos. Whole mount in situ hybridizations were carried out on 16-h embryos using antisense RNA probes. Note that *hsp90α* mRNA is detectable in the head, including both the developing brain and head mesoderm, only following heat shock (arrowhead in panel C). A, B, D, E, G, H, I: control (28.5 °C). C, F, J, K, L: heat shock (37 °C, 1 h). A, D: lateral view. B, C, E, F, G–L: dorsal view. The anterior end of the embryos in panels B, C, E, F is indicated with a lower case letter a. G–L, the anterior end of the embryo is to the left of the panel. (Reprinted from [50] with kind permission from Elsevier Science Ireland Ltd.)

Since these are all potential locations of muscle progenitor cells, the expression pattern of the *hsp90α* gene was compared to that of the *myoD* gene. *MyoD* was originally identified by its ability to convert cultured CH3 10T1/2 fibroblasts to a myoblast phenotype [66]. It belongs to a family of conserved proteins known as myogenic regulatory factors (MRFs), four of which have been identified in mammals: MyoD [66], Myf-5 [67], myogenin [68], and MRF-4 [69]. The *MRF* genes are expressed in skeletal myoblasts and myotubes and are believed to play a pivotal role in the myogenic program [70, 71]. The expression pattern of zebrafish *myoD* suggests that it plays a similar role during early myogenesis [72]. Importantly, the *hsp90α*-expressing cells observed in both the somites and pectoral fin buds of zebrafish embryos also express the *myoD* gene [50] (fig. 1, panels G, H). Furthermore, expression of the *hsp90α* gene is down-regulated along with *myoD* in mature muscles of the trunk at a time when levels of mRNA encoding the muscle structural protein *α*-tropomyosin remain high. No *hsp90α* mRNA is detectable in the developing brain or spinal cord at control temperatures.

In contrast to the highly restricted pattern of expression at control temperatures, the *hsp90α* gene is expressed at high levels throughout the embryo following heat shock [50] (fig. 1, panels C, J). This is true for all stages of development which have been examined. Thus, it is possible that the *hsp90α* gene may be playing a specific role in the normal process of myogenesis in zebrafish embryos in addition to providing protection to all cells of the embryo during periods of environmental stress. In contrast to *hsp90α* mRNA, *hsp90β* mRNA is detectable within the CNS at both control and heat shock temperatures and is not specifically restricted to *myoD*-expressing cells (fig. 1, panels D–F). This further supports the suggestion that these two genes are subject to different mechanisms of regulation within developing embryos and probably play different functional roles.

The restriction of *hsp90α* mRNA to *myoD*-expressing cells in zebrafish embryos is particularly interesting since the sequence-specific DNA-binding activity of murine *myoD* synthesized in vitro is known to be enhanced in the presence of hsp90 [55]. Furthermore, Shue and Kohtz [56] demonstrated that hsp90 can also activate the DNA-binding activity of recombinant myoD/E12 heterodimers, the most probable cellular form of myoD [73], purified in an inactive state from bacterial cells. The authors suggested that hsp90, together with other chaperone-like molecules, could be components of post-translational regulatory circuits which modulate the activity of *myoD* and other bHLH proteins in a non-covalent manner. Thus, it is possible that the constitutive *hsp90α* expression observed in zebrafish embryos is involved in enhancing the DNA-binding activity of *myoD* in those cells which are to undergo myogenesis. Alternatively, hsp90α could play a role in transporting myoD to the nucleus specifically in myoblasts [52, 53, 74]. However, it is not presently known if muscle differentiation in zebrafish embryos is limited only to those cells which co-express *myoD* and *hsp90α*. Such models will have to await more detailed fate mapping studies in which the differentiation of individual cells can be more closely correlated to the spatial pattern of *myoD* and *hsp90α* expression. An alternative explanation is that constitutive *hsp90α* gene expression in muscle progenitors serves as an immediate safeguard for protection of heat-sensitive myogenic regulatory mechanisms. The high level of *hsp90α* mRNA expression seen throughout the embryo following heat shock suggests that the gene plays a role in heat protective mechanisms. Given the rapid development of zebrafish embryos and the temperature-sensitive nature of somito genesis [42], constitutive expression of *hsp90α* may allow development to proceed normally following a sudden temperature increase to non-lethal levels. In this light, it is interesting that murine hsp90 can partially reactivate the DNA-binding ability of *myoD* heat-denatured at physiologically relevant temperatures in vitro [55].

The zebrafish *hsp47* gene

A 47 kDa heat shock protein (hsp47) has been reported in chicken, mouse, rat and human [4, 75–79]. Examination of the putative translation products of hsp47 cDNAs has revealed that hsp47 contains a signature sequence common to the superfamily of serine protease inhibitors (serpins) and, by virtue of its C-terminal RDEL signal sequence, is localized intracellularly to the endoplasmic reticulum (ER). This latter feature, along with studies showing pH-dependent association of hsp47 with procollagen, suggest that it is involved in the processing of nascent procollagen molecules within the ER of collagen-secreting cells [76, 80–82]. In this light, it is interesting that expression of hsp47 appears to be co-regulated with that of several types of collagen. For example, the synthesis of hsp47 and type IV collagen are co-ordinately increased during the differentiation of murine F9 teratocarcinoma cells [79] and co-ordinately decreased following viral transformation of fibroblasts in vitro [83, 84]. Immunocytochemical analysis has shown that hsp47 synthesis is coincident with production of type I collagen during murine tooth development [85, 86] and types I and II collagen in chicken vertebrae [87]. Following heat shock, expression of the mouse *hsp47* gene is induced approximately 4-fold in undifferentiated F9 cells, while in differentiated F9 cells, expression of the gene is induced 1.5 to 2-fold [79]. In chicken embryonic fibroblasts, Hirayoshi et al. [76] have shown that expression of the *hsp47* gene is induced 10-fold relative to the expression of actin two hours after the initiation of heat shock treatment.

Since vertebrate embryos synthesize a diverse range of collagen types in dynamic spatial and temporal patterns [88], it will be important to examine the regulation and function of hsp47 throughout embryonic development. In order to carry out a more thorough examination of *hsp47* gene expression during vertebrate embryogenesis, we cloned and characterized a full length *hsp47* cDNA from zebrafish using a polymerase chain reaction-based approach [49]. In agreement with observations in other vertebrates, Southern blot analysis indicated that only a single *hsp47* gene is present in zebrafish. The deduced translation product shares 72% identity with chicken hsp47, 64% identity with mouse and rat hsp47 and 69% identity with human hsp47. Importantly, zebrafish hsp47 exhibits complete conservation of the ER retention and serpin signature sequences which are characteristic of hsp47 in higher vertebrates, suggesting that it is likely to play a similar role in procollagen processing in zebrafish as it does in chicken and mammal. Using Northern blot analysis, very low levels of *hsp47* mRNA are detectable at the gastrula, mid-somitogenesis, 2-day and 3-day larval stages of development [49]. In contrast, the gene is strongly induced following exposure of embryos to heat shock at the same points during development. The level of induction is much greater than that previously reported for the chicken and mouse *hsp47* genes in cultured cells [76, 79]. Furthermore, ethanol exposure results in a dramatic upregulation of *hsp47* gene expression at a level much greater than that observed for the *hsp70* and *hsp90* genes. We have recently initiated whole mount in situ hybridization analysis using *hsp47* antisense RNA as a probe [90]. The data obtained reveal that *hsp47* mRNA is expressed in a highly tissue-restricted manner in embryos maintained at control temperatures, which is likely why it is barely detectable using Northern blot analysis. The pattern of expression correlates closely in both a temporal and spatial manner with that of the gene encoding type II collagen [89] in a number of tissues such as the notochord and otic capsule [90] (fig. 2 and table 1). However, not all cells expressing the type II collagen gene also express *hsp47*, suggesting that the two are not strictly coregulated during development.

The zebrafish *hsp70* genes

A degenerate PCR-based approach similar to that employed to identify and isolate *hsp47* and *hsp90* cDNA clones resulted in the isolation of genomic clones encoding several different zebrafish *hsp70* genes. Two of

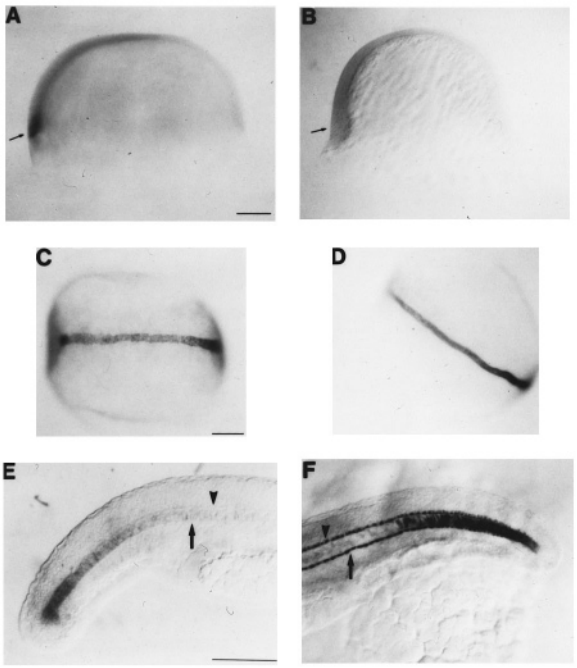

Figure 2. Comparison of *hsp47* (*A*, *C*, *E*) and *col2a1* (type II collagen; *B*, *D*, *F*) expression patterns in early zebrafish embryos using whole mount in situ hybridization analysis. *A*, *B*: 7 h. *C*, *D*: 11 h. *E*, *F*: 24 h. *A*, *B*, *E*, *F*: lateral view. *C*, *D*: dorsal view. Strong expression of *hsp47* but not *col2a1* is detectable as early as 6 h in the hypoblast (small arrow in panel A). Note that cells of both the floor plate (arrowhead) and hypochord *(*arrow*)* express *col2a1* but not *hsp47* in 24-h-old embryos (panels E and F). (Reprinted from [90] with kind permission from Elsevier Science Ireland Ltd.)

these, *hsp70-4* and *hsp70-7*, are strongly heat-inducible at the gastrula, mid-somitogenesis, 2-day or 3-day larval stages as assessed by Northern blot analysis (table 1). Expression of neither gene is detectable at control temperatures. We are currently examining the tissue-specific patterns of expression of these two genes.

Conclusions and future perspectives

Whole mount in situ hybridization analyses have revealed that zebrafish heat shock genes are differentially regulated in a complex tissue-specific manner in developing embryos. Furthermore, the expression patterns of both the *hsp90α* and *hsp47* genes correlate with previously identified biochemical functions of these proteins. The restriction of constitutive *hsp90α* gene expression to myoD-expressing cells is particularly interesting and suggests that hsp90α may play a role during normal muscle formation. Future analyses will examine the expression of the *hsp90α* gene in several mutant strains which exhibit abnormalities in somite formation. Spadetail embryos exhibit inappropriate migration of paraxial mesoderm cells [25], leading to disruption of somite formation and a deficiency of some paraxial mesoderm derivatives. In no tail mutants, the notochord fails to differentiate properly, somites are misshapen and the formation of muscle pioneers which are normally adjacent to the notochord is impaired [23]. Floating head embryos lack a notochord and somites fuse under the neural tube to form single, unpaired myotomes [24, 26]. The latter two strains will also prove valuable in further examination of the notochordal-specific pattern of *hsp47* gene expression.

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