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by Pharmacologic Inhibition of Hsp90

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Members of the Hsp90 family of molecular chaperones play important roles in allowing some intracellular signaling molecules and transcription factors to reach and maintain functionally active conformations. In the present study, we have utilized the specific Hsp90-binding agent, geldanamycin, to examine the requirement for Hsp90 during zebrafish development. We show that geldanamycin interacts with both the α and the β-isoforms of zebrafish Hsp90 and that geldanamycin-treated embryos consistently exhibit a number of defects in tissues which express either one of these genes. Within the somites, geldanamycin treatment results in the absence of *eng-2*-expressing muscle pioneer cells. However, early development of adaxial cells, which give rise to muscle pioneers and which strongly express the *hsp90α* gene shortly before muscle pioneer formation, appeared unaffected. Furthermore, development of the notochord, which provides many of the signals required for proper somite patterning and which does not express detectable levels of either *hsp90α* or *hsp90β* mRNA, was similarly unaffected in geldanamycin-treated embryos. The data are consistent with there being a temporal and spatial requirement for Hsp90 function within somitic cells which is necessary for the formation of *eng-2*-expressing muscle pioneers and possibly other striated muscle fiber types.

Key Words: **muscle development; zebrafish; Hsp90; geldanamycin; MyoD.**

INTRODUCTION

Numerous biochemical and cell culture studies have shown that members of the highly conserved Hsp90[3] family of molecular chaperones interact with and modulate the activity of several important cellular signaling molecules and transcription factors such as steroid receptors (Catelli *et al.*, 1985; Sanchez *et al.*, 1985; Pratt, 1992, 1993; Smith and Toft, 1993), $pp60^{v\text{-}src}$ kinase (Schuh *et al.*, 1985; Brugge, 1986), and the myogenic bHLH protein MyoD (Shaknovich *et al.*, 1992; Shue and Kotz, 1994). Genetic 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 (Picard *et al.*, 1990; Bohen and Yamamoto, 1993; Xu and Lindquist, 1993; Nathan and Lindquist, 1995; Nathan *et al.*, 1997). Recent data suggest that the apparent specificity of Hsp90 for only a small subset of cellular proteins may be due to its role in stabilization of otherwise inherently unstable conformations which some signaling molecules exhibit (reviewed in Csermely *et al.*, 1998).

Complex spatial and temporal patterns of *hsp90* gene expression have been reported during embryogenesis in a variety of vertebrate and nonvertebrate animal systems (Morange, 1997a,b). For example, maternally synthesized *hsp83* mRNA in *Drosophila* embryos is localized to the posterior pole by a novel mechanism which involves general degradation throughout the embryo and localized pro-

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[3] Abbreviations used: hsp, heat shock protein; GA, geldanamycin; PKA, protein kinase A.

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tection at the posterior (Ding *et al.,* 1993; *hsp83* is the single member of the Hsp90 gene 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 (Ding *et al.,* 1993). Studies such as these have led to the suggestion that Hsp90 plays fundamentally important roles during early embryonic development. In this light, the capacity of Hsp90 to act in a regulatory fashion in intracellular signaling pathways is particularly intriguing and work in *Drosophila* suggests that it may play a similar role within the developing embryo. For example, heterozygous mutations in the *Drosophila hsp83* gene impair signaling by the sevenless receptor protein tyrosine kinase, which is required for determination of the R7 photoreceptor neuron during development of the compound eye (Cutforth and Rubin, 1994), and inhibit embryonic Raf-mediated signaling (van der Straten *et al.,* 1997). More recently, Rutherford and Lindquist (1998) have put forth the intriguing hypothesis that Hsp90's role in embryonic signal transduction cascades may allow it to function as a molecular mechanism which links the process of evolutionary change in *Drosophila* embryos to environmental stress.

Our laboratory has been examining the expression and function of heat shock proteins during early embryonic development of the zebrafish (Krone and Sass, 1994; Pearson *et al.,* 1996; Sass *et al.,* 1996; Lele and Krone, 1997; Lele *et al.,* 1997; Krone *et al.,* 1997). We have previously shown that the zebrafish members of two heat shock gene families, namely *hsp47* and *hsp90,* exhibit complex spatial and temporal patterns of expression and, importantly, that they are coexpressed with genes encoding putative targets of their chaperoning activity (Lele and Krone, 1997; Sass *et al.,* 1996). The latter study provided the first evidence which established a strong link between *hsp90* gene expression and the process of myogenesis *in vivo* and demonstrated strong constitutive *hsp90α* expression in developing muscle cells within the somites of early embryos (vertebrates synthesize two closely related members of the Hsp90 family known as Hsp90*α* and Hsp90*β*; reviewed in Gupta, 1995). Both the *hsp90α* and the *myoD* genes are concomitantly down-regulated following differentiation of these cells into striated fibers. In addition, we have recently reported that expression of *hsp90α* mRNA is enriched in developing chicken somites (Sass and Krone, 1997). These studies strongly suggested that the *hsp90α* gene may play a role in the normal process of myogenesis and were particularly interesting in light of studies which had reported the interaction of mammalian Hsp90 with several signaling molecules and transcription factors important to vertebrate skeletal muscle development (Miyata and Yahara, 1992; Shaknovich *et al.,* 1992; Shue and Kohtz, 1994; Johnson *et al.,* 1996).

Despite extensive analysis of Hsp90 expression during vertebrate embryogenesis, a temporal requirement for Hsp90 function during development of any one cell type within the context of the whole vertebrate embryo has not been demonstrated. Studies of Hsp90 function during vertebrate embryogenesis are complicated by several factors. These include the multigene nature of the vertebrate *hsp90* family, including the presence of several pseudogenes (Gupta, 1995), and the fact that Hsp90 probably plays multiple roles at different time points during development. Thus, traditional loss-of-function methodologies for the assessment of embryonic gene function such as microinjection and gene knockouts, which usually target only the first major developmental event in which a gene is involved, will typically result in early lethality for *hsp90*. Recently, the fungal benzoquinoid ansamycin geldanamycin (GA), which was originally identified as a naturally occurring antitumor agent (DeBoer *et al.,* 1970), has been shown to interact in a specific manner with Hsp90 in a number of organisms (Whitesell *et al.,* 1994; Smith *et al.,* 1995; Whitesell and Cook, 1995; Stebbins *et al.,* 1997). GA appears to interact with the ATP-binding domain of Hsp90 (Grenert *et al.,* 1997; Prodromou *et al.,* 1997; Sullivan *et al.,* 1997) and, importantly, causes a specific loss of function to a number of Hsp90-dependent signal transduction proteins both *in vitro* and *in vivo* (Schulte *et al.,* 1995, 1996; Hartson *et al.,* 1996; Thulasiraman and Matts, 1996; Uma *et al.,* 1997; Czar *et al.,* 1997; Dittmar and Pratt, 1997; Segnitz and Gehring, 1997; Stancato *et al.,* 1997). As well, the crystal structure of the Hsp90/GA complex has now been published (Stebbins *et al.,* 1997). Importantly, conserved amino acid residues of Hsp90 in a diverse variety of species are clustered around the GA binding pocket and previously characterized functional mutations in yeast and *Drosophila* Hsp90 map to either this pocket or the immediate vicinity of it (Stebbins *et al.,* 1997). The establishment of GA as a specific inhibitor of Hsp90 has allowed questions regarding the function of this protein *in vivo* to be addressed in eukaryotic cells which were previously only possible in yeast. In the present study, we have utilized GA to examine the requirement for Hsp90 during the early development of zebrafish embryos.

MATERIALS AND METHODS

Embryo Manipulation and Treatment

Adult zebrafish were maintained according to standard methods (Westerfield 1995) and staged as described by Kimmel *et al.* (1995). Geldanamycin, geldampicin, or forskolin was dissolved in 100% DMSO and stored in the dark at −20°C. Manually dechorionated embryos were incubated in system water with 20 μg/ml GA or geldampicin or 250 μM forskolin and photographed using a Zeiss Photomicroscope with Normarski optics. Controls in all experiments consisted of embryos incubated in system water only and in system water containing DMSO at the same concentration as treatment groups. Higher concentrations of GA were lethal, whereas treatment at 5 or 10 μg/ml resulted in a reduction in severity of the phenotype (see Results). Similarly, concentrations of forskolin below 250 μM reduced the severity of the observed phenotype, whereas those above 500 μM resulted in increased lethality.

GA-Affinity Chromatography

Lysates obtained from adult trunks were adjusted to approximately 2 mg/ml protein and preincubated with either soluble GA (30 μg/ml) or DMSO (vehicle control) for 3 h at 4°C. Lysates were clarified by brief centrifugation and incubated in the presence of nonderivatized or GA-derivatized resin for 3 h and washed five times with lysis buffer. GA resins were prepared as previously described (Whitesell *et al.,* 1994) and blocked for 1 h in 10 mM Pipes · NaOH, 150 mM NaCl, 0.05% Tween 20, 10 mg/ml BSA, pH 7.0, prior to use. Bound materials were eluted by boiling in SDS–PAGE sample buffer and were separated by SDS–PAGE. Eluted proteins were visualized by silver staining or Western blotting using anti-Hsp90 antiserum (Ullrich *et al.,* 1986).

Rabbit reticulocyte lysates were used for coupled transcription/translation protein synthesis (Craig *et al.,* 1992) in reactions programmed with the plasmid pCS2$^+$ (gift from R. Rupp) containing either the zebrafish *hsp90α* or *hsp90β* cDNA. These cDNAs, which are described fully elsewhere (Lele *et al.,* unpublished data; GenBank Accession Nos. AF068772 and AF068773), were isolated from a postsomitogenesis embryonic library (gift from D. Grunwald, R. Riggleman, and K. Helde) using the previously described PCR-generated zebrafish *hsp90α* and *hsp90β* cDNA fragments as probes (Krone and Sass, 1994). Reactions containing ^{35}S-labeled Hsp90 were preincubated with either soluble GA (50 μg/ml) or DMSO (vehicle control) prior to incubation with the GA resin for 1 h. After binding, GA resins were washed three times with 10 mM Pipes · NaOH, 150 mM NaCl, 0.05% Tween 20, pH 7.0. Materials remaining bound to the GA resin were subsequently eluted by boiling in SDS–PAGE sample buffer, analyzed by reducing SDS–PAGE on 8% gels, and detected using autoradiography.

Whole-Mount in Situ Hybridization

Digoxigenin-11–UTP (Boehringer Mannheim) labeled sense and antisense RNA probes were synthesized by *in vitro* transcription. The *in situ* hybridization protocol of Puschel *et al.* (1992) was used with minor modifications (Akimenko *et al.,* 1994; Sass *et al.,* 1996; Lele and Krone, 1997). As templates, we used the previously described cDNAs encoding zebrafish *myoD* (Weinberg *et al.,* 1996), *col2a1* (Yan *et al.,* 1995), *α-tropomyosin* (gift from R. Riggleman), *sonic hedgehog* (*shh;* Krauss *et al.,* 1993), *echidna hedgehog* (*ehh;* Currie and Ingham, 1996), *engrailed-2* (*eng-2;* Ekker *et al.,* 1992), *axial* (Strahle *et al.,* 1993), and *no tail* (*ntl;* Schulte-Merker *et al.,* 1994a,b).

RESULTS

Zebrafish Hsp90s Bind in a Specific and Competable Manner to Geldanamycin

To determine whether zebrafish Hsp90s interact with GA and whether other zebrafish proteins also bind GA, GA-affinity assays were performed with extracts prepared from adult zebrafish tissues. Numerous studies have shown that a wide range of vertebrate cell types both *in vivo* and in culture express both the α and the β isoforms of Hsp90 to varying degrees under nonstress conditions (see Morimoto *et al.,* 1994, for reviews). Furthermore, we have found that, while muscles of the trunk significantly down-regulate *hsp90α* gene expression following their formation (Sass *et al.,* 1996), expression of both *hsp90α* and *hsp90β* is detectable using Northern blot analysis during late stages of embryogenesis and in adult tissues and cultured zebrafish cells (data not shown). As shown in Fig. 1A, only a single zebrafish protein is observed to bind to GA-derivatized resin in a competable fashion. This GA-binding protein had an apparent MW of 90 kDa and was thus speculated to represent one or more zebrafish Hsp90 isoforms.

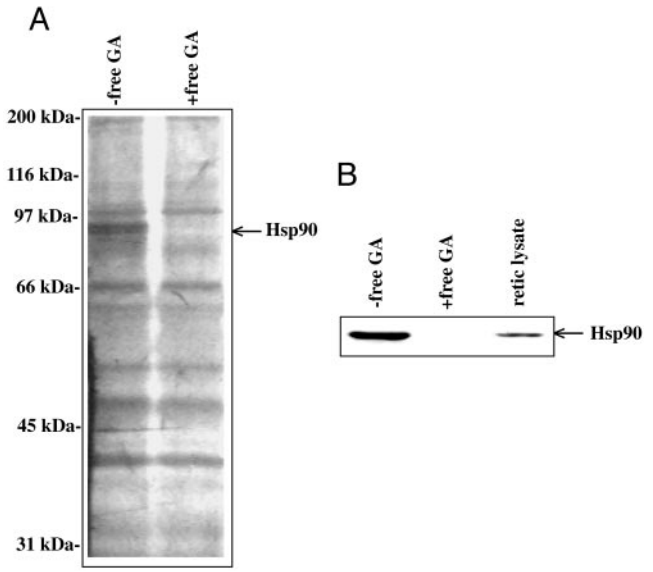


FIG. 1. Specific interaction of zebrafish Hsp90 with solid-phase geldanamycin (GA). Using Western blot analysis with anti-Hsp90 antibody, Hsp90 was detected to bind to GA-derivatized resin (B, lane 1) and this interaction was completely inhibited by preincubation with free GA prior to application to the GA resin (lane 2). Zebrafish Hsp90 comigrated with rabbit reticulocyte lysate Hsp90 detected using the same antibody (lane 3). No other proteins interacted with the resin in a specific manner as determined by silver staining of SDS–polyacrylamide gels (compare lanes 1 and 2 of A).

To confirm this identification, proteins recovered from GA-binding reactions were analyzed by Western blotting with polyclonal antiserum raised against mouse Hsp90 (Ullrich *et al.,* 1986). This anti-Hsp90 antiserum recognizes a 90-kDa protein occurring in unfractionated zebrafish lysates and which is retained on GA resins in a specific (competable) fashion (Fig. 1B). No other proteins were observed to be specifically bound to the GA resin (Fig. 1A). Because protein extracts derived from early embryos consisted predominantly of a 90- to 110-kDa protein (tentatively identified as the yolk cleavage product of vitellogenin (Selman *et al.,* 1993; data not shown)) which severely compromised electorphoretic analyses, such assays using lysates derived from zebrafish embryos were not feasible. Nonetheless, assays with lysates from adult tissues detected a 90-kDa protein with antigenic similarity to mam-

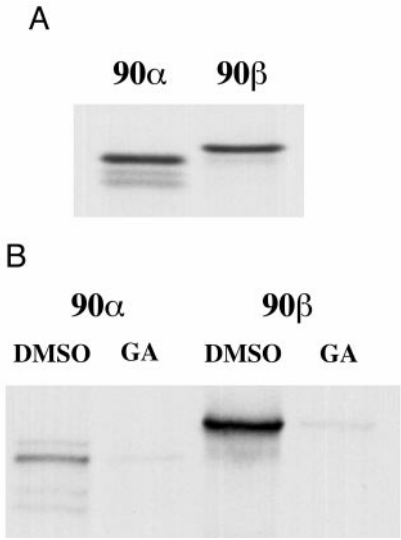


FIG. 2. *In vitro*-translated Hsp90α and Hsp90β interact with GA-derivatized resin in a specific manner. Coupled *in vitro* transcription/translations were carried out and the ^{35}S-labeled products subjected to GA-affinity column analysis as described under Materials and Methods. Both translation products, which have an M_r consistent with their identity as Hsp90 (A), bind to GA-derivatized resin (B) in a manner which is competed with free GA but not DMSO (compare lane 2 to lane 1 and lane 4 to lane 3).

malian Hsp90 that bound to GA resins in a specific (competable) fashion, suggesting that one or more zebrafish Hsp90s bind specifically to GA.

To confirm that both zebrafish Hsp90 isoforms bind to GA, zebrafish Hsp90α and Hsp90β were translated in rabbit reticulocyte lysate, concomitantly radiolabeled with [^{35}S]methionine, and subsequently analyzed on high-resolution SDS–PAGE gels. Translation reactions programmed with template for Hsp90β synthesized a single ^{35}S-labeled gene product whose M_r was consistent with its identity as zebrafish Hsp90β (Fig. 2A). Reactions programmed with message for Hsp90α similarly synthesized ^{35}S-labeled zebrafish Hsp90α, although some minor translation products were also observed (Fig. 2A). Importantly, when zebrafish ^{35}S-labeled Hsp90 gene products were assayed for binding to immobilized GA, both Hsp90α and Hsp90β were observed to bind to immobilized GA in a specific, competable fashion (Fig. 2B). This specific binding was consistent with numerous demonstrations that Hsp90s from various species bind specifically to GA (see Introduction). Furthermore, comparison of the deduced zebrafish Hsp90α and Hsp90β sequences to the groups of amino acid residues which make up the human Hsp90 GA-binding pocket (Stebbins *et al.*, 1997) revealed 98 and 92% identity, respectively (data not shown). Importantly, the two zebrafish proteins exhibit complete conservation of those amino acid residues in and around the GA-binding pocket which are also conserved in the amino-terminal domains of Hsp90 from diverse species such as yeast, *Drosophila*, and human (Stebbins *et al.*, 1997). Given this amino acid identity and the results presented in Fig. 2, we conclude that both Hsp90α and Hsp90β from zebrafish interact with GA in a specific manner. While these data do not allow us to unequivocally conclude that the embryonic forms of Hsp90 also interact with GA, the data presented below strongly suggest that such an interaction occurs in GA-treated embryos.

Geldanamycin Treatment of Zebrafish Embryos Gives Rise to a Characteristic Phenotype

To examine the effects of GA treatment on zebrafish development, embryos were incubated in a solution of GA in system water. GA treatment initiated at the midblastula stage was rapidly lethal in over 95% of the embryos with the few that survived exhibiting developmental arrest during gastrulation (data not shown). This is consistent with the expression pattern of the *hsp90β* gene, which is detectable in all cells of late blastula- and early gastrula-stage embryos following the midblastula transition (Figs. 4A and 4B). When the same treatment was initiated later in development following the onset of gastrulation, the death rate of embryos up to 20 h of age was comparable to that observed in either DMSO-treated or untreated controls (Table 1). However, 78% of the embryos exhibited a predominant phenotype characterized by a shortened trunk

TABLE 1
Summary of Data from Geldanamycin-, Geldampicin-, and Forskolin-Treatment Experiments

Treatment	Number of embryos treated	Survivors at 20 h (% of treated)	Embryos exhibiting abnormal phenotype[a] (% of survivors)
Control	492	427 (86%)	0
Geldanamycin at 50% epiboly	1223	988 (81%)	767 (78%)
Geldampicin at 50% epiboly	91	83 (91%)	0
Forskolin at 50% epiboly	264	200 (76%)	163 (82%)

[a] Embryos exhibiting typical trunk/tail morphology for geldanamycin and forskolin treatments as described under Results.

and tail, aberrations in somite shape and size, and a lack of yolk tube extension below the developing tail (Table 1; Fig. 5, compare 5A, 5C, and 5E with 5B, 5D, and 5F). The aberrations in somite shape and size were most prominent in the caudal portion of the trunk and in the tail (compare somites at arrow in Figs. 5C and 5D and asterisks in 5E and 5F). The total number of somites which formed in GA-treated embryos was 75–85% of controls. Most of the embryos died by about 24 h of age. When treatment was initiated later in development following the onset of anterior somite formation, we observed a reduction in both the incidence and the severity of the phenotype, with effects being confined primarily to the caudal portion of the tail.

To ensure that the defects were due specifically to geldanamycin and not a more general effect of benzoquinone ansamycins, we carried out parallel experiments using geldampicin. Geldampicin is identical to geldanamycin except for a single side-chain substitution (Whitesell *et al.*, 1994). However, the competition efficiency of free geldampicin in Hsp90-binding assays using GA-derivatized resins is approximately two orders of magnitude below that of free GA, a result which is consistent with the difference in the abilities of these two compounds to inhibit the activity of specific Hsp90 substrates (Whitesell *et al.*, 1994; Grenert *et al.*, 1997). In agreement with these studies, we could detect no phenotypic differences between control embryos and those treated with geldampicin beginning at 50% epiboly (Table 1). This confirmed that the GA-induced phenotype was not due to a general effect of benzoquinone ansamycins and, furthermore, suggested that it arose from a specific GA/Hsp90 interaction in treated embryos.

Embryos exposed to GA also exhibited several other abnormalities. The embryos did not extend around the yolk but rather remained on top of it, even though the cell movements of epiboly which engulf the yolk were completed. In the small proportion of less severely affected embryos, which grew until 2 days of age, pigmentation was dramatically reduced, the eyes were smaller, and the forebrain and midbrain did not appear to develop fully. These embryos also displayed defects in the development of the circulatory system. Thus, despite the presence of a beating heart, proper circulation was never established. Perhaps due to the lack of circulation, cells in the tails of these mildly affected embryos eventually became necrotic and the embryos died. The GA-induced defects are consistent with the constitutive expression patterns of the *hsp90α* and *hsp90β* genes. The *hsp90β* gene is expressed in the developing brain, tail bud, cells surrounding the posterior margin of the yolk tube, and several other tissues (Fig. 4; Sass *et al.*, 1996). In contrast, the *hsp90α* gene is strongly expressed in early embryos within the somitic slow muscle progenitors known as adaxial cells, which lie immediately adjacent to the notochord (solid arrow in Fig. 3; Sass *et al.*, 1996). In other work, we have found that the *hsp90α* gene is also strongly expressed during the early differentiation of fast fibers, which form later during development at a time when the gene has already been down-regulated in the formed slow muscle fibers (C. C. Martin and P. H. Krone, unpublished). The somitic phenotype induced by GA was particularly interesting in relation to the expression of *hsp90α* in developing muscle cells and is explored in subsequent sections of this study.

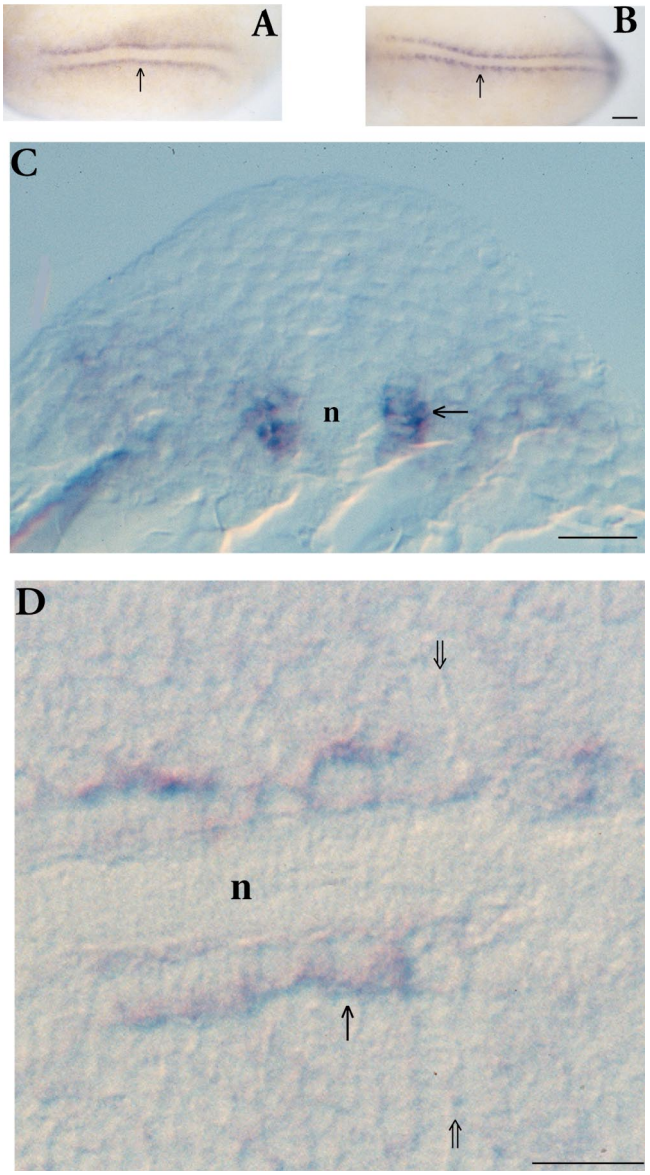


FIG. 3. Expression of *hsp90α* during early somite development as detected by *in situ* hybridization analysis. (A) 3- to 4-somite stage, dorsal view. (B) 16 somite stage, dorsal view. (C) Cross section through unsegmented paraxial mesoderm prior to somite formation. (D) Longitudinal section through the dorsal–ventral midline at the location of the most recently formed somite. Strong constitutive expression of *hsp90α* is confined to the adaxial cells (closed arrows). Very low levels of *hsp90α* mRNA are sometimes detectable in the lateral presomitic cells which lie distal to the adaxial cells (C). Open arrows in D indicate the most recently formed somite furrow. n, notochord. Scale bar in A and B, 100 μm. Scale bar in C and D, 50 μm.

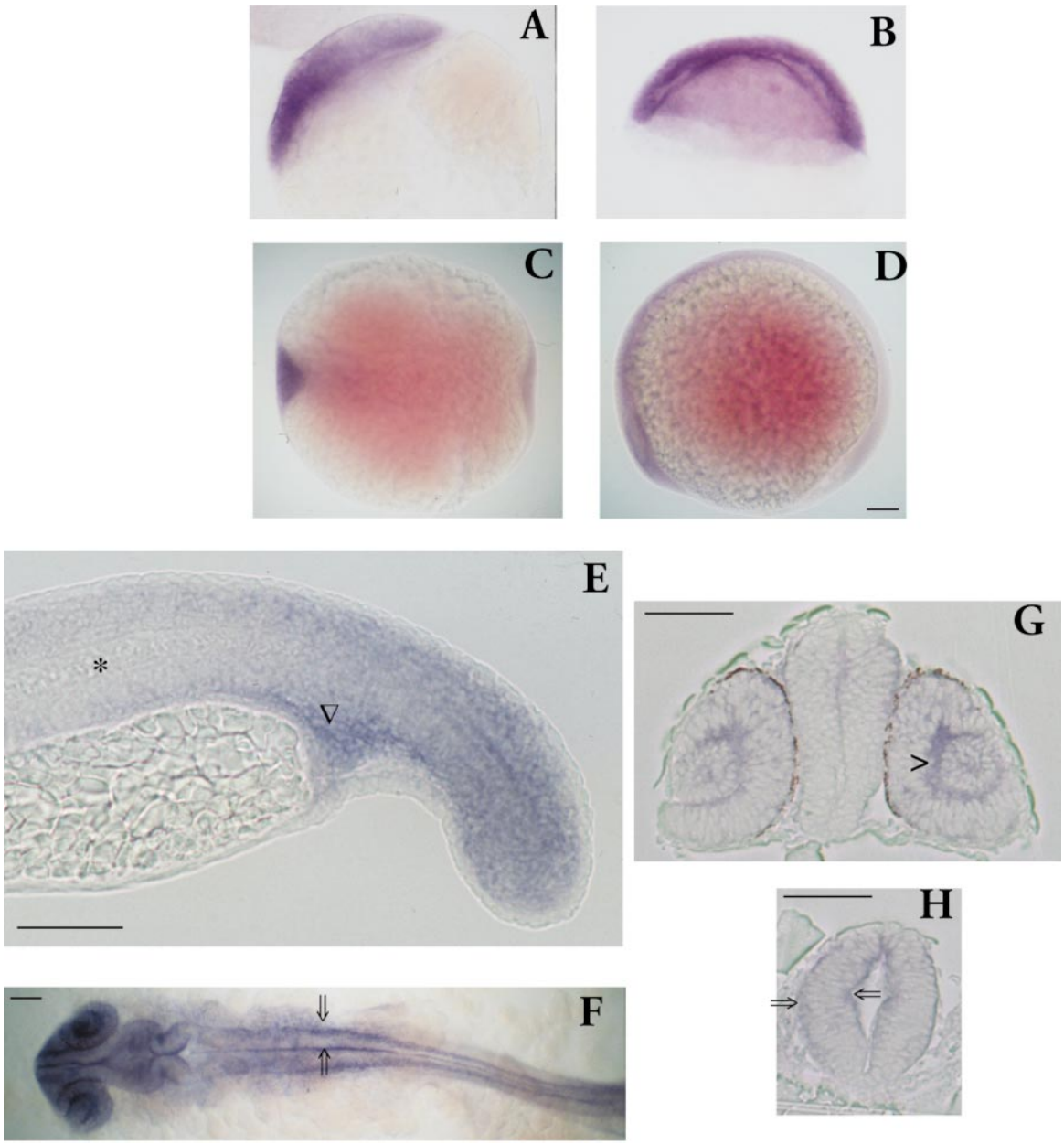
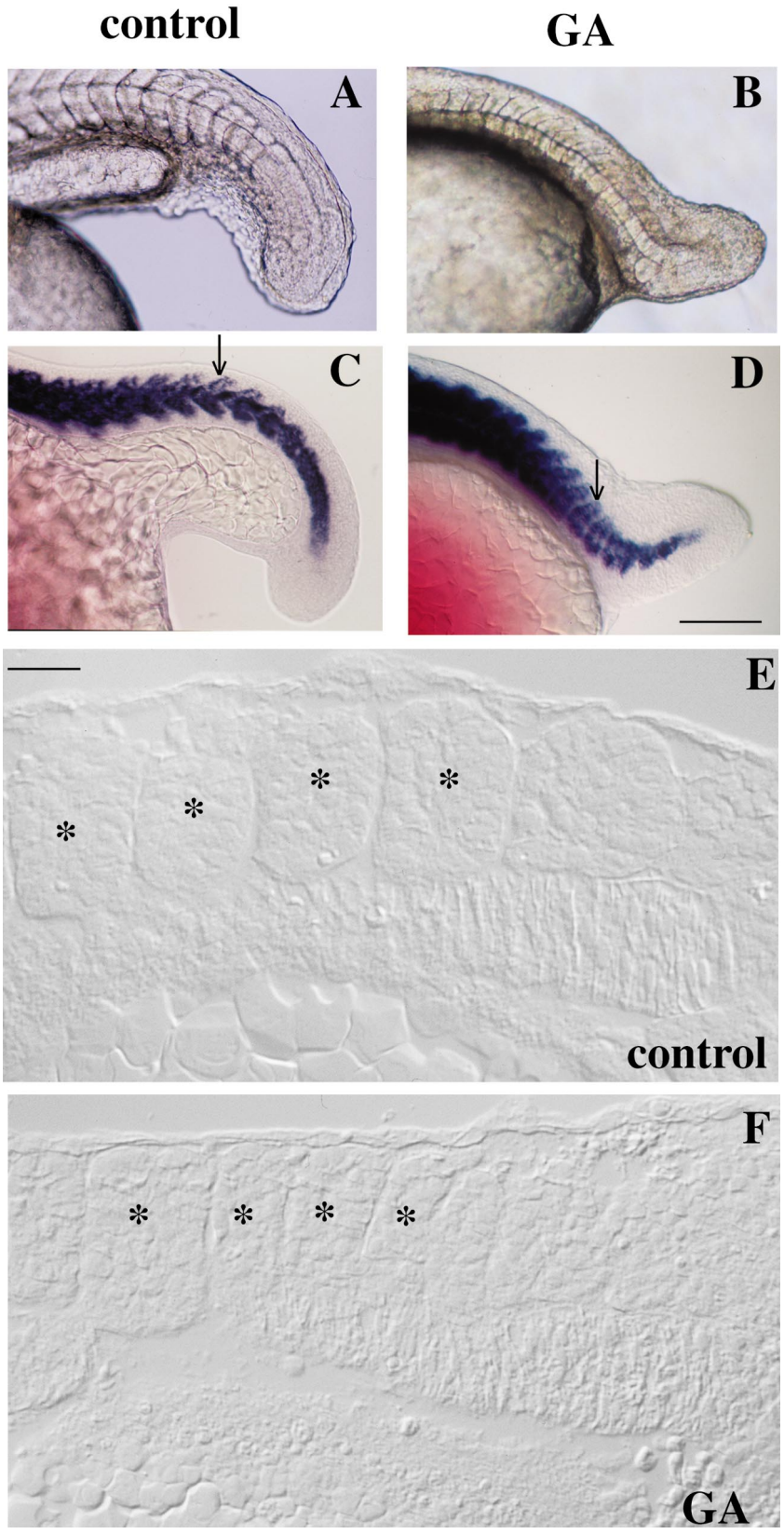


FIG. 4. Expression of *hsp90β* in zebrafish embryos as detected by *in situ* hybridization analysis. (A) Sphere stage. (B) 30% epiboly. (C and D) Bud stage. (E–H) 20 h. (A, B, D, and E) Lateral view. (C) Ventral view. (F) Dorsal view. (G and H) Cross section through the forebrain and midbrain regions, respectively. *hsp90β* expression is widespread in blastula-stage embryos (A and B). During late gastrulation and early somitogenesis, *hsp90β* mRNA is detectable predominantly in the anterior structures of the embryo (C and D). At 20 h of development, the strongest expression of *hsp90β* is within the CNS, particularly in cells along the central canal and the exterior margins (open arrows in F and H) as well as the developing retina (G). In addition, moderate expression is observed in the tail bud and in cells immediately caudal to the yolk ball extension (triangle in E). No *hsp90β* mRNA is detectable in the notochord (asterisk in E). Scale bar, 100 μm.

Development of the Notochord in the Trunk and Tail of GA-Treated Embryos

The somitic phenotype induced by GA is similar to that previously described for embryos which fail to form a notochord when carrying homozygous mutations in the *ntl* (Halpern *et al.*, 1993) and *floating head* (*flh;* Halpern *et al.*, 1995; Talbot *et al.*, 1995) genes. To monitor notochord formation, we initially utilized a zebrafish *col2a1* antisense



probe which detects type II collagen mRNA (Yan *et al.*, 1995). This mRNA is expressed in a spatiotemporal wave during formation of the notochord, with cells in the most posterior portion of the notochord being the last to express it. As shown in Figs. 6A–6D, expression of the *col2a1* gene was unchanged in the notochord of embryos treated with GA beginning at 50% epiboly and the cells exhibited the characteristic vacuolated, "stack of pennies" appearance (Kimmel *et al.*, 1995). As well, down-regulation of *col2a1* expression in the anteriormost mature cells of the notochord occurred in the same spatiotemporal pattern in both control and treated embryos, suggesting that the relative developmental age of these cells was unaffected. Furthermore, we were unable to detect any aberrant patterns in the expression of members of the hedgehog gene family, *shh* and *ehh*, which are expressed by the notochord early during embryogenesis and play critical roles in the patterning of the somites in zebrafish and other vertebrates (Krauss *et al.*, 1993; Chiang *et al.*, 1996; Currie and Ingham, 1996; Hammerschmidt *et al.*, 1996; Blagden *et al.*, 1997; Du *et al.*, 1997; Figs. 6E–6H), nor in the expression of other notochord-specific genes such as *axial* or *no tail* (data not shown). We did, however, observe that the notochord in GA-treated embryos sometimes developed folds, particularly at the location where the tail protrudes off the yolk, and the hypochord was present as only patches of cells along the A–P axis (see open arrows in Fig. 6D). Expression of *col2a1* (solid arrow in 6C and 6D) and *shh* (solid arrow in 6E and 6F) in the floor plate also appeared normal in GA-treated embryos, suggesting that early development of this tissue was also unaffected.

Muscle Pioneer Development Is Impaired in GA-Treated Embryos

The data presented thus far suggest that a specific GA–Hsp90 interaction disrupted development of one or more cell types of the paraxial mesoderm to give rise to the observed somite phenotype. In *flh* and *ntl* embryos, the somitic defects are thought to involve at least in part a failure of development of a group of slow muscle fibers known as the muscle pioneers. Muscle pioneers are the earliest muscle cells to differentiate in zebrafish, becoming morphologically distinct at around 13 h, simultaneous with somite differentiation (Felsenfeld *et al.*, 1991). About two to six muscle pioneers develop per somite at the future location of the horizontal myoseptum; they can be distinguished as they are the only muscle cells which express high levels of the *engrailed-2* gene (Hatta *et al.*, 1991; Ekker *et al.*, 1992). In all embryos examined by *in situ* hybridization, the *eng-2*-expressing muscle pioneers were either completely absent or severely reduced following GA treatment beginning at 50% epiboly (Fig. 7, compare 7A and 7D with 7B and 7E). However, *eng-2* expression at the midbrain/hindbrain boundary was unaffected, indicating that GA treatment did not result in general suppression of *eng-2* activation throughout the embryo (asterisks in Figs. 7A, 7B, and 7C). These data indicate that GA prevented proper expression of the muscle pioneer phenotype in treated embryos.

The Hsp90-Dependent Step in Muscle Pioneer Formation Occurs Subsequent to Adaxial Cell Formation

Adaxial cells in zebrafish are the progenitors of both muscle pioneers and other slow muscle cells known as non-muscle-pioneer slow muscle and derive from paraxial mesoderm which lies directly adjacent to the notochord (Devoto *et al.*, 1996; Du *et al.*, 1997; Blagden *et al.*, 1997). These cells can be distinguished as they are the earliest cells which express *myoD* (Weinberg *et al.*, 1996; Devoto *et al.*, 1996); their development is thought to involve, at least in part, suppression of protein kinase A (PKA) activity in somite cells by hedgehog signaling from the notochord (Devoto *et al.*, 1996; Hammerschmidt *et al.*, 1996; Ungar and Moon, 1996; Weinberg *et al.*, 1996; Blagden *et al.*, 1997; Du *et al.*, 1997; Schauerte *et al.*, 1998). Forskolin, which raises intracellular levels of cAMP through its activation of the catalytic subunit of adenylate cyclase, has been used successfully to antagonize hedgehog activity in mouse in a manner similar to overexpression of constitutively active PKA (Fan *et al.*, 1995). As shown in Table 1 and Figs. 7C, 7F, and 8C, forskolin treatment of zebrafish embryos beginning at 50% epiboly resulted in a phenotype with a shortened body axis and complete inhibition of both *eng-2*-expressing muscle pioneer and *myoD*-expressing adaxial cell formation. In addition, forskolin treatment resulted in forebrain

FIG. 5. Trunk/tail phenotype of 18-h-old embryos treated with geldanamycin beginning at 50% epiboly (5–5.5 h). (A, C, and E) Control. (B, D, and F) GA. (A–D) Lateral view of whole embryos. (E and F) Longitudinally oblique sections through the four most recently formed somites and the unsegmented paraxial mesoderm. (A, B, E, and F) Unstained. (C and D) *In situ* hybridization detection of *α-tropomyosin* mRNA. Anterior is to the left in all. Seventy-eight percent of the GA-treated embryos exhibited a shortened trunk/tail phenotype and the somites did not form their characteristic full chevron shape as described under Results (compare arrows in C and D, which indicate somites at comparable positions along the anterior–posterior axis). In addition, somites of treated embryos typically assumed a block-like shape and varied significantly in size within any one embryo (compare somites indicated by asterisks in E and F, which are the four most recently fully formed in both cases). The closely related benzoquinone ansamycin geldampicin, which is ineffective as an Hsp90-binding agent, had no detectable effect on embryonic development. In contrast to embryos treated with GA beginning at gastrulation, treatment initiated at the blastula stage was rapidly lethal. Scale bar in A–D, 100 μm. Scale bar in E and F, 25 μm.

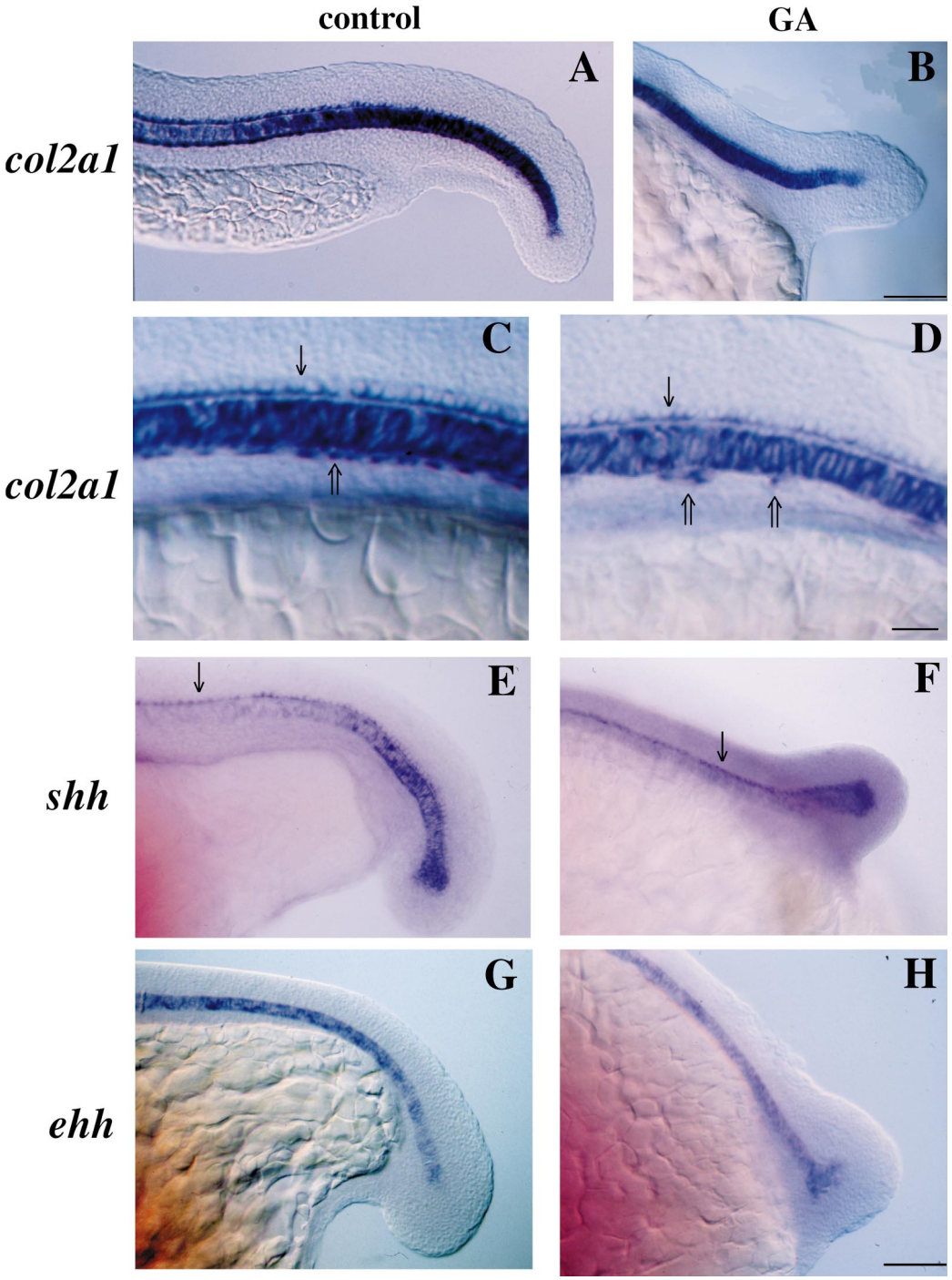


FIG. 6. Expression of notochord and floor plate markers is normal in GA-treated embryos as detected using whole-mount *in situ* hybridization. (A, C, E, and G) Control. (B, D, F, and H) GA treatment beginning at 50% epiboly. (A–D) *col2a1* mRNA. (E and F) *shh*. (G and H) *ehh*. Anterior is to the left and dorsal to the top in all. All three markers were expressed normally in the notochord of GA-treated embryos as were other markers of notochord formation such as *axial* and *ntl* (data not shown). As well, a single, uninterrupted row of floor-plate cells which express both *col2a1* and *shh* developed immediately above the notochord in both control and GA-treated embryos (arrows in C–F). However, treated embryos exhibited large gaps in the row of *col2a1*-expressing hypochord cells (open arrows in D) which normally lie as a continuous row immediately below the notochord (open arrow in C). Control embryos in A and C are at the 23- to 24-somite stage and 15-somite stage in E and G. GA-treated embryos are the same age as control embryos. Scale bar in A, B, and D–G, 100 μm. Scale bar in B and C, 50 μm.

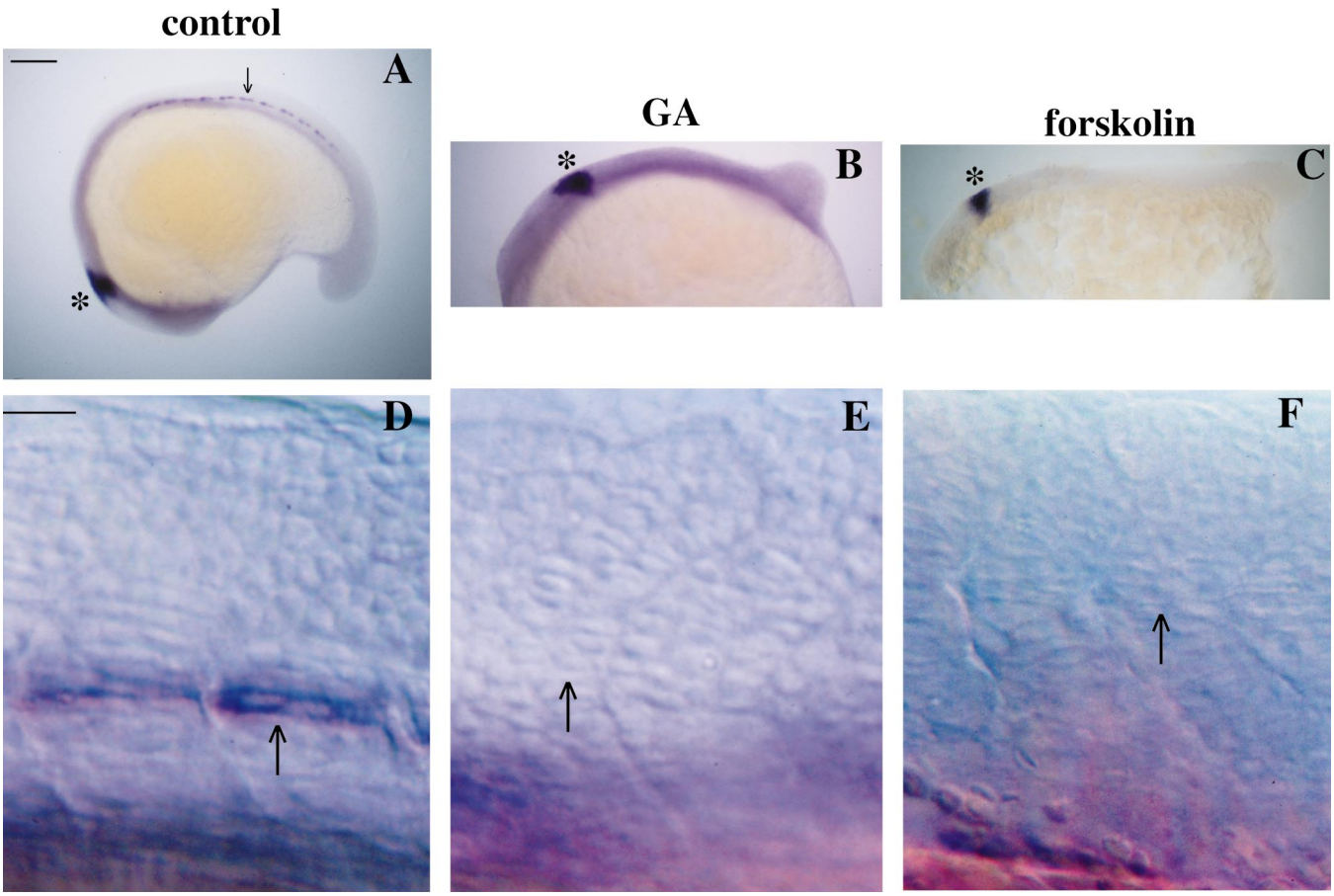


FIG. 7. GA and forskolin inhibit formation of *eng-2*-expressing muscle pioneer cells. (A and D) Control. (B and E) GA treatment beginning at 50% epiboly. (C and F) Forskolin treatment beginning at 50% epiboly. (A–C) Whole 18-h-old embryos. (D–F) Two anterior somites at comparable positions within the trunk of these embryos. *In situ* hybridization for the detection of *eng-2* mRNA was carried out as described under Materials and Methods. All embryos are lateral views with anterior to the left and dorsal to the top. Muscle pioneers, which can be distinguished from other striated muscle cells by high levels of *eng-2* expression, normally develop at the D–V midline of the myotome at the future location of the horizontal myoseptum (arrows in A and D) but are absent from this location in GA-treated and forskolin-treated embryos (arrows in E and F). However, activation of *eng-2* expression in cells of the midbrain/hindbrain boundary is unaffected by GA or forskolin treatment (asterisks in A–C). Scale bar in A–C, 100 μm. Scale bar in D–F, 20 μm.

defects, such as fused eyes, which are also seen in embryos expressing constitutively active PKA (Hammerschmidt *et al.*, 1996; Ungar and Moon, 1996). In contrast, *myoD* expression in adaxial cells was unaffected by GA treatment (Fig. 8, compare 8B and 8C). In the lateral presomitic cells, which later give rise to fast muscle fibers (Devoto *et al.*, 1996), *myoD* continued to be expressed in an apparently normal fashion following both GA and forskolin treatment. In other experiments, we have found that GA treatment had no effect on the expression of several other early markers of adaxial cell formation including *α-tropomyosin* and *snail-1* (data not shown). Thus, the GA-sensitive step during the formation of muscle pioneers exists downstream of the down-regulation of PKA activity and subsequent activation of *myoD*, *α-tropomyosin*, and *snail-1* expression within developing adaxial cells of the somite.

DISCUSSION

We have investigated the requirement for members of the Hsp90 family of molecular chaperones during early development of the zebrafish using the Hsp90-binding agent, geldanamycin. Our data show that GA interacts in a highly specific and competable fashion with both the α and the β isoforms of zebrafish Hsp90 *in vitro*. Furthermore, we were unable to detect an interaction of GA with other zebrafish proteins, a result which is consistent with demonstrations of GA specificity in other organisms (see Introduction). While the interaction of GA with both Hsp90α and Hsp90β was not unexpected based on deduced amino acid sequence comparison of the mammalian GA-binding domain with the zebrafish proteins, to our knowledge experimental demonstration of the interaction of GA with both isoforms from

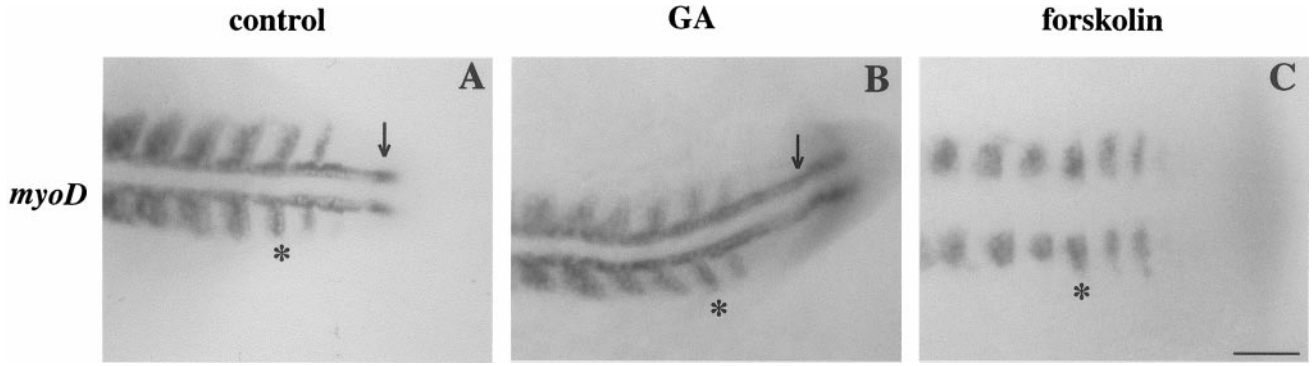


FIG. 8. Adaxial cells form in GA-treated but not in 14-h-old forskolin-treated embryos. (A) Control. (B) GA. (C) Forskolin. Adaxial cells are demonstrated based on their early, strong expression of *myoD*. Adaxial cells (arrow) could be detected directly adjacent to the notochord prior to somitogenesis in both control and GA-treated embryos but did not form in embryos treated with forskolin. In contrast, the *myoD*-expressing lateral presomitic cells which will go on to form fast muscle fibers were normal in all three groups of embryos (asterisks in A–C). Treatment with GA and forskolin was initiated at 50% epiboly. Scale bar, 50 μm.

a single vertebrate has not been reported. The majority of biochemical studies of Hsp90 function have not differentiated between the two isoforms and, for the most part, they appear to be interchangeable with respect to their biochemical activities *in vitro*. However, they do exhibit some unique biochemical characteristics such as stability of dimer formation (Minami *et al.*, 1991, 1994; Nemoto *et al.*, 1995), possible phosphorylation sites (Lees-Miller and Anderson, 1989), and intracellular localization (Perdew *et al.*, 1993). Furthermore, they exhibit remarkably different patterns of expression, with *hsp90α* being strongly inducible following heat shock and other stresses and *hsp90β* being expressed more widely at normal growth temperatures (reviewed in Morange *et al.*, 1997a,b). In zebrafish, we have shown that the *hsp90α* gene is expressed during the normal development of somitic muscle cells but is not expressed as part of the mature muscle phenotype (Fig. 3; Sass *et al.*, 1996; C. C. Martin and P. H. Krone, unpublished data). However, the gene is strongly inducible in all cells of the embryo following heat shock (Sass *et al.*, 1996). In contrast, the *hsp90β* gene is very weakly heat inducible (Krone and Sass, 1994; Sass *et al.*, 1996) yet is expressed in a relatively wide range of tissues during normal development (Fig. 4). Thus, it is possible that the two genes are responsible for different yet related cellular chaperone functions *in vivo*. Alternatively or in addition to this, the ability of embryos to differentially regulate expression of the two isoforms may allow for more precise control of Hsp90 activity in different cell types.

Embryos treated with GA exhibited several characteristic defects which support the occurrence of a specific interaction of GA with the α and β isoforms of Hsp90 *in vivo*. A number of results from this study and others argue in support of this. First, the degenerate PCR-based cloning strategy which we originally used to identify and clone Hsp90 cDNAs expressed in developing zebrafish embryos utilized primers designed around the highly conserved GA-binding amino terminus of Hsp90 from a wide range of species (Krone and Sass, 1994). Indeed, the primers themselves corresponded to the two stretches of highest amino acid identity within this region, which are also constituents of the GA-binding pocket (Grenert *et al.*, 1997; Stebbins *et al.*, 1997). Thus, the screen would have been expected to isolate other GA-binding members of the Hsp90 family which are expressed during embryogenesis, but only *hsp90α* and *hsp90β* were identified. Second, the defects correspond to tissues which express either the *hsp90α* or the *hsp90β* gene; tissues which do not express either of these genes at detectable levels, such as the notochord, are relatively unaffected (see also below). Third, treatment of embryos with geldampicin, a closely related analog of GA which is ineffective as an Hsp90 binding agent, had no effect. Thus, the phenotype is not caused by a toxic effect of benzoquinone ansamycins in general on development. Fourth, no other zebrafish proteins interacted with GA in a specific and competable fashion in our assay. Taken together with further evidence pertaining to defects in muscle pioneer development, these data provide strong support that the GA-induced embryonic defects are due to a specific interaction of GA with Hsp90α and Hsp90β *in vivo*.

While our experiments demonstrate that members of the zebrafish Hsp90 family are likely to play multiple roles during the development of different cell types and that Hsp90 is an essential protein for embryonic development, it is interesting that several tissues were relatively unaffected by GA treatment. Previous studies using yeast and cultured cells have shown that Hsp90 is an abundant protein in a variety of cell types, with some estimates indicating that it comprises up to 1–2% of the total cytosolic protein (reviewed in Csermely *et al.*, 1998). In yeast, a functional copy of the *hsp90* gene is essential for survival (Borkovich *et al.*, 1989). These and other observations have led to the sugges-

tion that high levels of Hsp90 are essential for eukaryotic cell viability. However, the data obtained using GA treatment and that demonstrating spatiotemporal specificity of Hsp90 gene expression in this and other studies from our laboratory (Sass *et al.,* 1996; J. B. Sass and P. H. Krone, unpublished) suggest that not all cells of the zebrafish embryo may require Hsp90 at all times for their proliferation and/or differentiation or that different cell types have dramatically different threshold requirements for Hsp90. For example, the notochord in GA-treated embryos developed normally, a result which is consistent with our inability to detect expression of either the *hsp90α* or the *hsp90β* gene in this tissue. Similarly, the apparently normal early development of adaxial cells is consistent with the fact that strong activation of *hsp90α* gene expression is not detectable within these cells until after the activation of *myoD* expression (Sass *et al.,* 1996; J. B. Sass and P. H. Krone, unpublished). An alternative explanation is that other chaperone molecules which exhibit Hsp90-like activities may substitute for Hsp90 in some cell types. For example, Cdc37 has recently been shown to have chaperone properties similar to those of Hsp90 yet has specific functions in signal transduction pathways which diverge *in vivo* (Kimura *et al.,* 1997). Distantly related members of the Hsp90 family have also been reported recently (hsp75 and TRAP-1; Song *et al.,* 1995; Chen *et al.,* 1996); based on comparative sequence analysis, GA would not be predicted to inhibit the activity of these molecules.

The GA-induced trunk/tail phenotype was typified by the presence of aberrations in shape and size of somites, a shortened trunk and tail, and disruption in proper muscle pioneer formation. Furthermore, the Hsp90-dependent process(es) defined in this study occurs downstream of both notochord development and subsequent formation of *myoD*-expressing adaxial cells, a subset of which gives rise to the muscle pioneers. Our data suggest that Hsp90, and most likely Hsp90*α* (see below), is required during the specification and/or differentiation of muscle pioneers from adaxial cells and that many of the signaling and differentiation events occurring in the pathway prior to this point appear to be unaffected. However, the data do not discount the possibility that Hsp90 also plays a role in the formation of other somitic muscle fiber cell types which differentiate later during formation of the zebrafish myotome (Devoto *et al.,* 1996; Blagden *et al.,* 1997). For example, a subset of adaxial cells undergoes a wave of migration to the surface of the myotome after the 20-somite stage of development and forms a layer of superficial slow muscle fibers known as the non-muscle-pioneer slow muscle (Devoto *et al.,* 1996; Blagden *et al.,* 1997). In contrast, fast muscle fibers are derived from the somitic cells which initially lie lateral to the adaxial cells and begin to differentiate after the wave of migration of the non-muscle-pioneer slow muscle has been completed. The fact that all adaxial cells as well as developing fast muscle fibers later in development strongly express the *hsp90α* gene (this study; Sass *et al.,* 1996; C. C. Martin and P. H. Krone, unpublished) supports a role for Hsp90 in their formation.

Several other pieces of evidence from this and other studies support the hypothesis that Hsp90 function is required during the formation of muscle pioneers from adaxial cells. First, the *hsp90α* gene is strongly expressed in adaxial cells under nonstress conditions (Sass *et al.,* 1996; Fig. 3). Importantly, *hsp90α* expression either coincides with or precedes the expression of other muscle regulatory genes such as myogenin and members of the *MEF-2* family in these cells (Weinberg *et al.,* 1996; Sass *et al.,* 1996; Ticho *et al.,* 1996; J. B. Sass and P. H. Krone, unpublished). Second, *hsp90α* expression is coordinately down-regulated with *myoD* following somitic striated muscle fiber differentiation as would be expected for a gene involved in fiber development but not the maintenance of the mature fiber phenotype (Sass *et al.,* 1996). Third, *hsp90α* expression is activated shortly following *myoD* in midline cells of *flh* mutants which would normally form notochord but are respecified to a paraxial mesoderm fate (J. B. Sass and P. H. Krone, unpublished). These cells have been shown to express characteristics typical of adaxial cells (Halpern *et al.,* 1995; Talbot *et al.,* 1995; Melby *et al.,* 1996; Blagden *et al.,* 1997). Fourth, Hsp90 interacts with a number of molecules involved in signal transduction pathways and the regulation of gene expression in other vertebrates, including several which play important roles during muscle development. In the latter category, biochemical studies have shown that murine Hsp90 can stimulate the DNA binding activity of MyoD *in vitro* (Shaknovich *et al.,* 1992; Shue and Kohtz, 1994). While the precise role of MyoD in zebrafish muscle development has yet to be elucidated, it is likely to play a fundamental role in this process given its importance in other vertebrates (recently reviewed in Firulli and Olson, 1997; Rawls and Olson, 1997) as well as its pattern of expression in zebrafish (Weinberg *et al.,* 1996). Hsp90 also interacts with casein kinase II, which has recently been implicated as a potential regulator of MyoD activity during mammalian myogenesis (Johnson *et al.,* 1996). Interestingly, we have recently reported that *hsp90α* mRNA is also enriched in the developing somites of chicken embryos (Sass and Krone, 1997), a result which suggests that elevated levels of Hsp90 are required for aspects of muscle development in other vertebrates as well.

Rutherford and Lindquist (1998) recently showed that impairment of Hsp90 function in *Drosophila,* by both mutagenesis and feeding of GA, resulted in the appearance of a wide array of morphological variations in a small but significant number of different lines of adult flies. Interestingly, upon selection the traits uncovered in each line became independent of Hsp90 function. The authors suggest that Hsp90 may function as a molecular capacitor for evolution whereby transient decreases in Hsp90 levels cause the appearance of stress-damaged proteins requiring Hsp90 chaperone activity. The titration of Hsp90 by these proteins can then uncover variants in signaling molecules for selection to act upon. Whether such a mechanism exists

in vertebrates, which express two different Hsp90 genes, is not known. However, the consistency and penetrance (almost 80%) of the GA-induced phenotype in zebrafish embryos indicates that we are not observing a similar mechanism at work in the present study. Further, we have observed the same phenotype and high penetrance in embryos obtained from different wild-type sources, a result not seen in the *Drosophila* study. Indeed, the data presented here support the suggestion that Hsp90 isoforms in zebrafish embryos may not simply provide housekeeping chaperone function to all cells and tissues, but rather may fulfill more specific roles in allowing some cell types to respond to or interpret signal transduction events.

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