Iranian Biomedical Journal 14 (4): 127-135 (October 2010)

Expression of *hsp90α* and *hsp90β* during *Xenopus laevis* Embryonic Development

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Received 27 June 2010; revised 7 September 2010; accepted 6 October 2010

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[DOR: 20.1001.1.1028852.2010.14.4.1.4]

[DOI: -]

ABSTRACT

Background: Members of the eukaryotic Hsp90 family function as important molecular chaperones in the assembly, folding and activation of cellular signaling in development. Two *hsp90* genes, *hsp90α* and *hsp90β,* have been identified in fish and homeothermic vertebrates but not in poikilothermic vertebrates. In the present study, the expression of *hsp90α* and *hsp90β* genes in *Xenopus laevis*, which is phylogenetically positioned between zebrafish and mammals, has been addressed. **Methods:** Partial *Xenopus hsp90α* and *hsp90β* cDNA were identified and isolated using RT-PCR, and a full-length *Xenopus hsp90β* cDNA was isolated from an embryonic cDNA library. Northern-blot analysis was used to study the expression of *hsp90α* and *hsp90β* genes in total RNA of the embryos and *in situ* hybridization was used to compare the expression of these genes with that of *hsp70* and *MyoD* genes in *Xenopus* embryogenesis. **Results:** Northern-blot analysis revealed that the *hsp90β* gene was strongly expressed constitutively at all stages of embryogenesis, but weakly induced following the heat shock. In contrast, the *hsp90α* gene was weakly expressed in embryos at control temperature, but strongly up-regulated following heat shock. *In situ* hybridization results showed that *hsp90α* gene was observed predominantly in cells of the developing somite. Microscopic sections showed that *hsp90α* and *MyoD* mRNA are expressed in similar regions in somite and this pattern was distinct from that of *hsp70* and *hsp90β.* **Conclusion:** These data support the hypothesis that the presence of *hsp90α* and *hsp90β* genes is conserved among vertebrates, and these genes are differentially regulated in a tissue, stress, and development stage-specific manner. *Iran. Biomed. J. 14 (4): 127-135, 2010*

Keywords: *Xenopus, hsp90α, hsp90β*

INTRODUCTION

Cells respond to stresses such as elevated temperature by transiently increasing the synthesis of highly conserved heat shock proteins [1]. Hsp90 is highly abundant in unstressed eukaryotic cells, accounting for 1-2% of cytosolic protein [1]. It is expressed as two isoforms in vertebrates. Two *hsp90* isoform genes (α and β) have been described in zebrafish, chicken, mouse and human [2]. *hsp90α* and *hsp90β* genes were most likely generated by a duplication event that is occurred shortly before the emergence of the teleosts from the rest of the vertebrate lineage [3]. The Hsp90α and Hsp90β homologs in vertebrate species show about 87% identity to each other [3]. Each isoform from different species show more similarity to each other than different isoforms from a single species [3]. Although both the isoforms are expressed at basal levels even under unstressed conditions, various stresses increase the expression of the two isoforms to different degrees [2]. By interacting with different proteins, Hsp90 is involved in several cell functions [1]. Hsp90 has several identified specific interactions with different proteins such as steroid hormone receptors, protein kinases (mitogen-activated protein kinase system) [1] and the basic helix-loop-helix transcription factor (MyoD) [4, 5]. These studies suggest that Hsp90 plays fundamentally important roles during early development [2]. *Xenopus laevis* is a well known vertebrate model system to study developmental

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biology, gene expression, reproductive toxicity, vertebrate heart development and cell migration [6].

In the present study, we cloned *hsp90α* and *hsp90β* cDNA in *Xenopus laevis* and studied the expression pattern of both isomers and compared them with *hsp70* and *MyoD* in different embryonic stages. These results might provide more evidence for the conservity of Hsp90 in vertebrate, different regulation and function of two isoforms of *hsp90*, role of Hsp90 in myogenesis (with the aid of MyoD) and development of different embryonic stages.

MATERIALS AND METHODS

Cloning partial cDNA of Xenopus laevis hsp90α and hsp90β. In order to obtain a *Xenopus hsp90α* and *hsp90β* cDNA probe, a PCR-based strategy was followed as described by Krone *et al.* [7]. Total RNA was extracted from *Xenopus* tailbud embryos with TRIzol Reagent (Gibco BRL, Montreal, Canada). The isolated RNA were hybridized with oligo-dt and used as templates for for the synthesis of cDNA with reverse transcriptase enzyme (Gibco BRL, Montreal, Canada). Polymerase chain reaction was performed with two degenerate oligonucleotide primers for conserved amino acid sequences (YSNKEI & QFGVGFY) present in both Hsp90α and Hsp90β proteins. Primer neuclotides were linked to additional nucleotide sequence for *Not* I restriction enzyme. A PCR product of about 330 bp was purified from agarose gels by the active filter-paper method [8]. The PCR product was digested with *Not* I, and then was cloned into the *Not* I-digested pBluescript II plasmid. The cloned PCR products were sequenced by the dideoxy chain-termination procedure [9] using sequenase version 2.0 T7 DNA sequensing kit (Amersham, Montreal, Canada).

Screening Xenopus laevis cDNA library. The *Xenopus* stage 42 cDNA library was a generous gift from Michael W. King (IUSM-Terre Haute at Indiana State University, USA). The protocol used for cDNA screening was adapted from Current Protocols in Molecular Biology. After titrating the cDNA library, four 20 X 20 cm plates (NZCYM + top agar + tetracycline) with a total pfu of 40,000 were plated and then the plaques were transferred on nitrocellulose membranes. The *hsp90α/β* partial fragments were used as template to synthesize a [α-32p]-dCTP labeled probes to hybridize the membrane from the plates [10].

Xenopus laevis embryo collection and care. *Xenopus laevis* frogs were purchased from Xenopus I (Ann Arbour, MI, USA). *Xenopus* eggs were fertilized and cultured in Steinberg's solution as described previously [11]. Embryo stages were determined according to Nieuwkoop and Faber [12], and only normally developing embryos were used in all experiments. In each stage, one group (N) of embryos was incubated at 18°C and the other groups (H) were heat shocked at 33°C for 1 h.

Northern-blot analysis. The cloned *Xenopus hsp90α* or *hsp90β* fragments were used as the template to prepare probes. The cDNA probes were labeled with dCTP using the random primed DNA labeling kit (NorthernMax, Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA [15 µg), isolated from embryos by TRIZOL Reagent, was subjected to gel electrophoresis on a horizontal 1.2% (w/v) formaldehyde-agarose gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was baked at 80°C for 2 h. Prehybridization of RNA blots was performed at 65°C for 3 h in 50% sodium phosphate buffer 0.1 M, 35% SDS 20%, 2 µl/ml EDTA 0.5 M. The prehybridization buffer was removed and the hybridization was carried out in prehybridization buffer containing the [α-32p]-dCTP labeled probe at 65°C for 48 h. The blots were washed, then covered with plastic wrap and finally exposed to Kodak X-OMAT film at -70°C. The two *hsp90α* and *hsp90β* probes were tested, but they didn not show any cross-reactivity (data not shown). After stripping, the membranes were re-probed with random-primed chicken 18 rRNA probe to verify RNA loading equivalency between lanes [13].

In situ hybridization. The *in situ* hybridization protocol used was a modification of the methods of Harland [14] with minor modifications. Digoxigenin-11-UTP labeled sense and anti-sense RNA probes of *hsp90α*, *hsp90β*, *hsp70* or *MyoD* genes were synthesized by *in vitro* transcription reactions using linearized plasmid of cDNA fragments as template. Hybridization was carried out with fresh hybridization buffer containing the probe (1 µg/ml) in water bath with shaking at 60°C for 40 h. After washing with PBS, the embryos were preincubated with blocking solution for 2-4 h. This process was followed by incubation with 1:2000 dilution of alkaline phosphatase-coupled anti-digoxygenin antibodies (Boehringer-Mannheim,

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Germany) in alkaline phosphatase buffer (APB) with shaking at 4°C for 24 h. Afterward, the embryos were incubated with staining buffer containing 250 μg/ml 5-bromo-4-chloro-3-indolyl phosphate and 225 μg/ml nitro blue tetrazolium. Reaction was stopped at a suitable signal to noise ration by re-fixing in 4% paraformaldehyd in PBS and 0.05% Tween-20 for 1 h, then washed and kept in PBS and 0.05% Tween 20. For photography, the embryos were dehydrated in 100% methanol, and then cleared in a solution of benzyle alcohol: benzyl benzoate (1:2). The pictures were taken with a dissecting microscope (Zeiss Wild Heerbrugg, Germany) and a digital camera (Nikon COOLPIX 990, Japan). Some of the embryos were embedded in paraffin and 10-μm sections were prepared [7].

RESULTS

***Cloning hsp90α* and *hsp90β* genes of *Xenopus laevis*.** Using RT-PCR technique, an amplification product with the expected size of 330 bp was cloned into pBluescript II and the resulting clones were analyzed by Sanger (dideoxy) sequence analysis. Clones were divided into two groups based on the DNA sequence analysis (DNA sequence of all members within each group being identical to each other). Based on Genbank using the program BlastX, it was concluded that the clones represent *Xenopus* cDNA encoding *hsp90α* and *hsp90β* (data not shown). The *hsp90α* sequence was submitted to Genbank under accession number AY785159, whereas the *hsp90β* had been cloned and previously reported [15]. By screening the cDNA library, full-length *hsp90β* was cloned and submitted to Genbank under accession number AY785160.

Northern-blot analysis. Total RNA isolated from control and heat shocked embryos was subjected to Northern-blot analysis. Developmental stages examined were 16, 19, 30, 32, 39 and 44. All stages of embryos showed high levels of *hsp90β* expression at control temperature and after heat shock, the expression increased moderately (Fig. 1). Levels of *hsp90α* mRNA were low, often apparently undetectable at control temperature in all stages of embryogenesis examined. However, levels increased substantially during a 1-h heat shock at 33°C. Therefore, the expression of *hsp90α* mRNA transcript was different from that of *hsp90β*.

Whole mount in situ hybridization. This technique was employed to visualize the spatial patterns of *hsp90α* and *hsp90β* mRNA accumulation in whole *Xenopus* embryos at specific developmental stages. In stage 20 of control embryos, *hsp90β* mRNA was widely expressed, with particularly strong expression evident in the developing central nerve system, eye, and more caudal portions of the embryos (Fig. 2). Weaker expression was evident within the region of the somites, although the typical segmented pattern of somites was not apparent. A somewhat stronger signal was observed in these tissues following the heat shock, although the relative increase in signal was variable among different embryos. At the stage 28, a strong signal was observed in both the somites and in the head at control temperatures, and the signal did not change or increased only slightly within these tissues in heat-shocked embryos.

The tissue-specific pattern of *hsp90α* mRNA expression differed appreciably from that observed for *hsp90β*. At control temperature, stage 20 embryos showed no *hsp90α* mRNA signal other than a very weak expression in the head, whereas expression was elevated throughout the embryo following the heat shock (Fig. 2). At stage 30, a distinct segmental pattern of expression was evident within the somite. A significant stronger signal observed in these tissues following heat shock.

A *Xenopus laevis hsp70* anti-sense riboprobe was used as a positive heat shock control in these experiments. Previous studies indicated that hsp70 expression is heat inducible [16]. In agreement with these studies, our results showed that at control temperature no expression was seen in stage 30 except a signal in eye but after heat shock a very strong expression was observed within the head and somites.

Expression of *MyoD* is a well characterized marker of formation of early striated muscle in *Xenopus* and other vertebrates [17]. Expression of *Xenopus MyoD* (*XmyoD*) was studies in whole mount *in situ* hybridization as well (Fig 2). As shown in Figure 2, the expression pattern of *MyoD* is similar to *hsp90α* in the head and especially in somites of control embryos. In contrast to *hsp90α*, the *MyoD* signal does not increase following heat shock, instead appears to decline relative to that observed at control temperatures. This is not unexpected since *MyoD* is not a Hsp, and has not been reported to exhibit heat-inducible expression in other studies.

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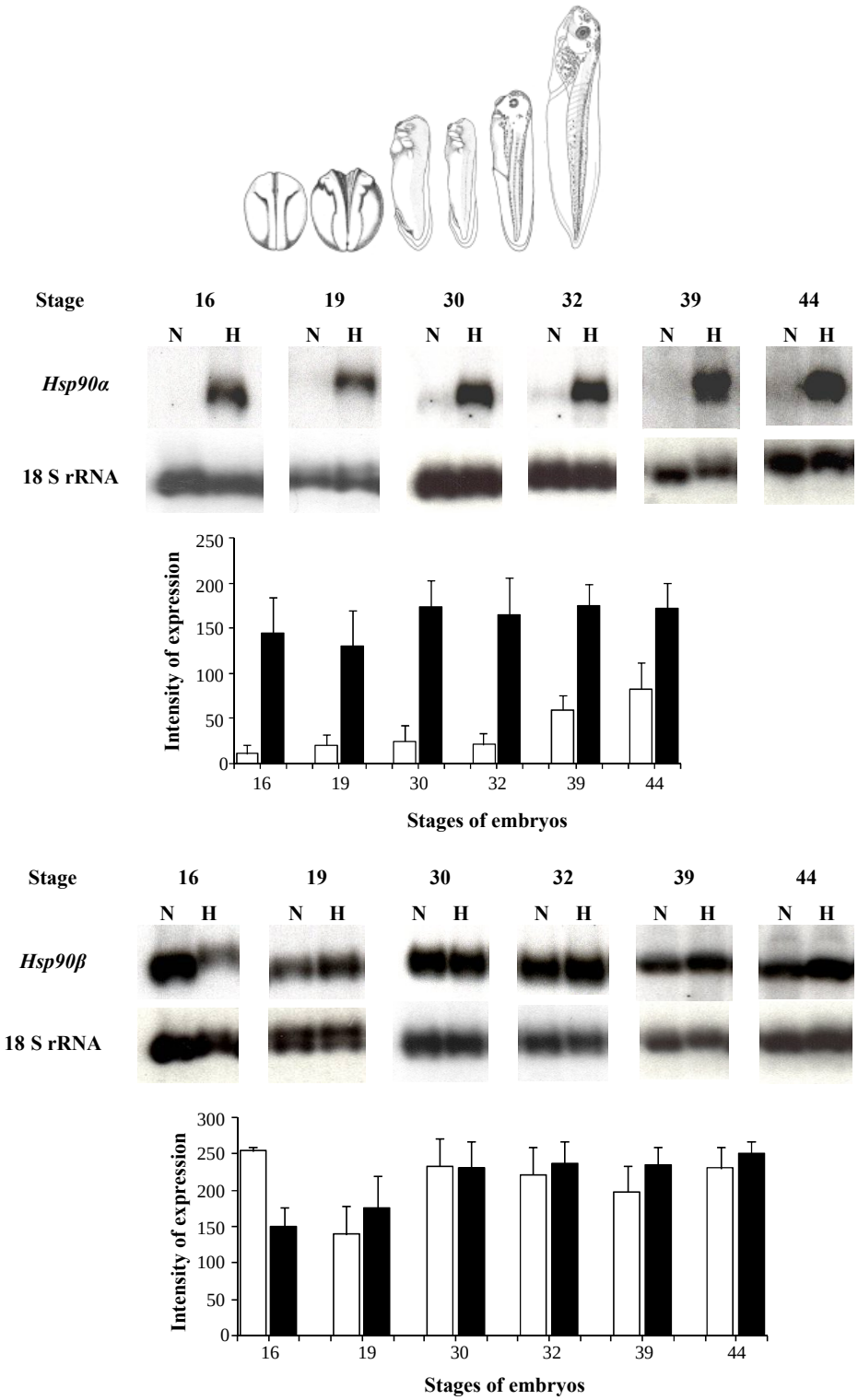

Fig. 1. Different expression of *hsp90α* and *hsp90β* genes in *Xenopus laevis* embryos. Embryos at different stages of development were heat shocked at 33°C for 1 h (H) or incubated at control temperature 18°C (N). Total RNA (15 mg) was analyzed by Northern-blotting using ^{32}P-labeled *Xenopus hsp90α* or *hsp90β* probes. The same RNA samples were used for both probes and then the same membranes were also probed with labeled chicken 18 S rRNA in order to assess loading of RNA in all lanes. Using Photoshop program, density of the expression bands were measured and a graph was made using Excel program. In graphs, the expression of genes at control and heat shock temperature are shown in white and black bars, repectively.

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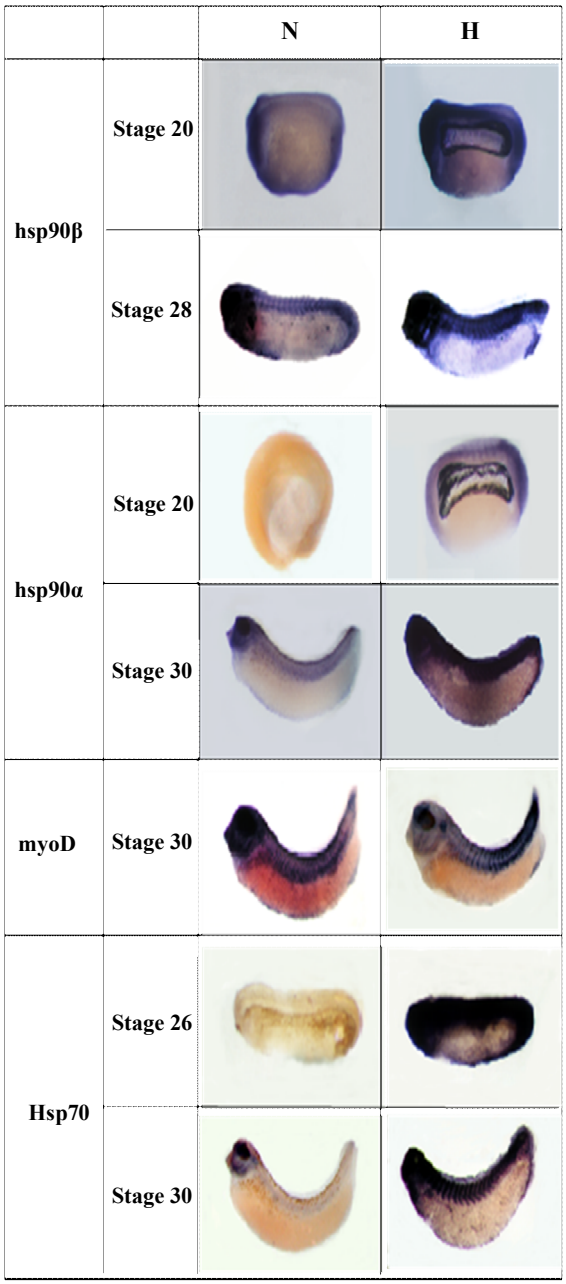

Fig. 2. Strong heat inducibility of *hsp90α* expression. Albino embryos, incubated at 18°C (N) or heat shocked for 1 hour at 33°C (H), were examined by whole mount hybridization using digoxygenin-labeled *hsp90α*, *hsp90β*, *X-MyoD*, or *X-hsp70* anti-sense probes. *hsp90β* is constitutively expressed in non-heat shocked embryos (N) and after heat shock its expression does not change significantly. *hsp90α* expression increased severalfold after heat shock. *Hsp70* is not expressed in non-heat shocked embryos (except in the eyes of stage 30 embryo), but after heat shock it expressed strongly throughout the embryo. *MyoD* expression in head and somites of non-heat shocked embryo decreased after heat shock.

DISCUSSION

The partial *Xenopus laevis hsp90α* and *hsp90β* cDNA were cloned using the same degenerate primers which were used to clone zebrafish and chicken *hsp90α* and *hsp90β* [7]. This shows that Hsp90 sequence is highly conserved between different species at least in this region. In deduced amino acid sequence, these two fragments are 91% identical, but in nucleotide sequence they have more discrepancy and are 79% identical. This difference in nucleotide sequence was sufficient to allow the use of these fragments as probes in cDNA library screening. In our study, by screening a cDNA library, a full-length *Xenopus laevis hsp90β* cDNA was cloned. An alignment with Hsp90α and Hsp90β of human, mouse, chicken, zebrafish and the *Xenopus laevis* was done with DNA Star program. Hsp90β showed that *Xenopus* Hsp90β is highly similar to Hsp90β from other species including zebrafish (data not shown).

Developmental stages examined in Northern-blot analysis provided a broad range of embryos in which a variety of different developmental processes and cell differentiation events are occurring. This study showed that these two genes have completely different constitutive expression patterns in all examined embryonic stages (Fig. 2). The constitutive expression of *hsp90β* was high, but the expression of *hsp90α* was very low or barely detectable. Following the heat shock, the expression of *hsp90β* did not increase significantly, while *hsp90α* expression increased severalfold. Similar differential expression of *hsp90α* and *hsp90β* at control and heat shock temperatures occurs in zebrafish [2], whereas in chicken, the *hsp90β* gene has been reported to be expressed strongly constitutively with no increase following the heat shock [18]. In addition, *hsp90α* higher inducibility has been shown in sole fish [19], salmon [20] and common carp [21] as well. Despite these differences, a common theme that emerges from this and other studies is that *hsp90β* is usually expressed widely at control temperatures, and the *hsp90α* gene exhibits much stronger heat-inducible expression in vertebrates.

Whole mount *in situ* hybridization results in our experiment is in agreement with Northern-blot analysis that *hsp90α* is more strongly induced following heat shock (Fig. 2). The expression of *hsp90α* and *hsp90β* in zebrafish somites shows the same pattern i.e. *hsp90α* expression is segmental, while *hsp90β* shows a uniform expression [2]. The

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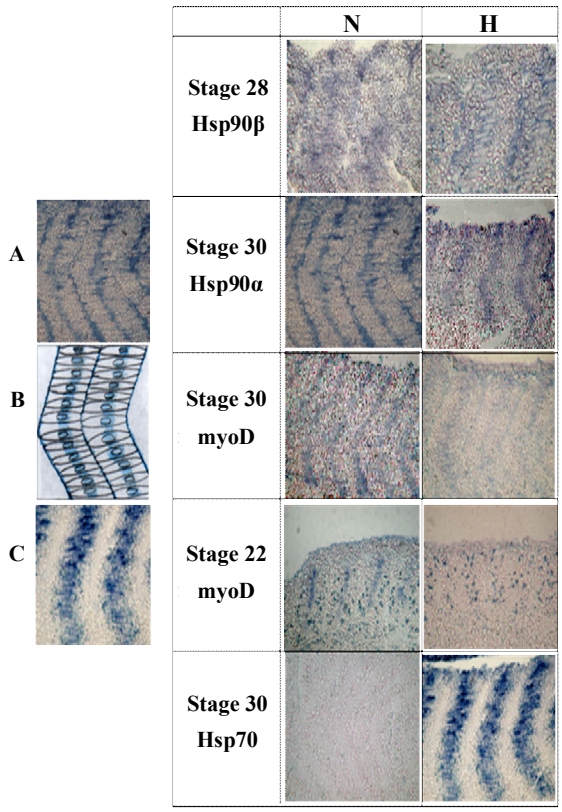

Fig. 3. Similar expression patterns of *hsp90α* and *myo-D* in somites of *Xenopus laevis* embryos. Non-heat shocked (N) and heat shocked (H) Albino embryos were examined by whole mount hybridization using different digoxygenin-labeled anti-sense probes. The embryos were blocked in paraffin and microscopic sections were prepared by microtome (10 μm thick). The left panel shows a schematic diagram of cellular arrangement in two adjacent somites (B), aligned with actual microscopic sections (A and C). The right panel, shows that the expression pattern of *hsp90α* and *myoD* before heat shock is similar i.e. there is a thick band of expression in center of the cells where the nuclei are located and a thin band of expression in the border of somites. *hsp90β* shows more uniform expression and *hsp70* expresses only in a thick band in the middle of somites. The expression of *MyoD* in the beginning of somite formation in stage 22 is shown as well.

expression of Hsp90α and Hsp90β was also studied by immunohistochemistry in 10 days old rat embryos [22]. Hsp90α was induced in heat-shocked embryos beginning at 1 h, predominantly in a neural tube and an optic vesicle. A moderate but increased expression was observed in the somites of heat-exposed embryos at 3 and 5 h. Hsp90β was not heat-inducible and expressed extensively in control and heat-exposed embryos [22]. In mouse embryos, the *hsp90α* and *hsp90β* genes also exhibit differential spatial regulation within the somatic and germ cell compartments of the developing testis [23]. Overall, the different patterns of *hsp90α* and *hsp90β* expression in different species clearly show that these two genes are subjected to different mechanisms of regulation within developing embryos, and they may have unique functional roles.

So far, our results showed that *hsp90α* and *hsp90β* genes are subjected to different mechanisms of regulation within developing embryos. Furthermore, the comparison of *hsp90α* and *hsp90β* expression in whole mount *in situ* hybridization showed that *hsp90α* expression is more cell specific with higher expression in somite area; in contrast, the expression of *hsp90β* was more concentrated in the head and does not show a segmental pattern in the somites. The segmental pattern of *hsp90α* expression in somites was evident in stage 30 as well. The comparison of *hsp90α* and *MyoD* expression in whole mount *in situ* hybridization suggests that *hsp90α* mRNA appeared to be localized predominantly to the developing somites along with *MyoD* during normal development at control temperatures. In order to examine this relationship more closely, embryos at stage 30 were subjected to whole mount *in situ* hybridization, followed by the preparation of 10-μm tissue sections. The sections were observed on a compound microscope in order to localize more specifically transcripts within the somites.

The somites are composed of blocks of dorsal mesodermal cells adjacent to the notochord. These transient structures define the segmental pattern of the embryo, and subsequently give rise to vertebrae and ribs, dermis of the back, skeletal muscles of the back, body wall and limbs. In *Xenopus*, each somite is composed of a group of long myocyte cells that stretch the width of the somite from the anterior to the posterior somitic border [24] (Fig. 3, left panel B). The nuclei of these somitic myocytes lie in the center of the cells, and thus appear to line up along the central portion of the somite [24] (Fig. 3, left panel B).

The expression of *MyoD* in developing somites in stage 22 showed a random expression in the presumptive somitic cells as well as a band of expression in the area of the nuclei (Fig. 3). No *MyoD* expression can be seen in the border of the developing somites at this stage (Fig. 3). In stage 30 embryos, approximately 24-25 somites have been segregated [12]. The expression of *MyoD* in somites was intense and revealed the expected segmental pattern (Fig. 3). At this stage, the expression of *MyoD* is localized in a distinct band in the area of the myocyte nuclei, and a thinner band of expression in the border of the somite.

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Interestingly, *hsp90α* expression in stage 30 at control temperatures showed the same expression pattern as that of *MyoD* (a thick band in the nuclear region and a thin expression band in the border of the somites, Fig. 3). In contrast, *hsp90β* showed more uniform expression in somitic cells and *hsp70* showed only a very thick band of expression in the center of the somite. Furthermore, no *hsp70* expression was seen in the border of the somites.

The similar tissue-specific localization of *hsp90α* and *MyoD* mRNA in somitic cells suggests that the *hsp90α* gene is expressed as a part of the myogenic program in *Xenopus* embryos. This is supported by the similar localization of these mRNA to the perinuclear region and the cell periphery, which is commonly observed in muscle-specific mRNA but not housekeeping mRNA in muscle fibers. Nuclear localization of *XMyoD* in presumptive mesoderm has been shown before [25]. After nuclear localization, MyoD can activate its own transcription and commit a cell to the myogenic lineage [17]. Whole mount *in situ* hybridization of stage 18-19 and 21-22 with anti-sense probes against XMRF4, E3 and XMyoD showed a perinuclear expression like our result [26]. Moreover, the whole mount immunostaining showed a uniform expression of MyoD protein in developing somites, but in developed somites its expression was only perinuclear [27]. Myosin heavy-chain mRNA also exhibits perinuclear localization in both myoblasts and myotubes, and specific sequences within the myosin heavy-chain mRNA 3'-UTR could similarly localize B-globin mRNA at the perinuclear region [28].

In the microscopic sections of whole mount *in situ* hybridizations, a thin band of *hsp90α* and *MyoD* mRNA expression was detected in the intersomitic boundaries (Fig. 3, left panel). Several proteins are expressed in the intersomitic boundaries. In mice and zebrafish, presumptive somites become surrounded by a Fibronectin-rich matrix during somitogenesis [29]. FAK is also accumulates in somite boundaries and is required for somite formation, FN matrix deposition and localization of Xena to the cell cortex [30]. Xena is localized to cell borders in the presomitic mesodrm and later localized in the intersomitic and intermyotomal junctions [30]. In addition, Xena colocalizes with β1-integrin, vinculin and Fak [30]. Other proteins that accumulate in the boundaries of somites are X-Delta-2 [31], deltaD [32], Desmin [33] and β-catenin [34]. Beside these proteins, there are a few reports of expression and secretion of *hsp90α* to the extracellular matrix. *hsp90α* isoform is expressed and secreted to the extracellular matrix of fibrosarcome cells where it interacts and induces the matrix metalloproteinase 2 increasing tumor cell invasiveness [35]. In wounded tissue, hypoxia induces the expression and secretion of *hsp90α* to extracellular matrix, which induces migration of human dermal fibroblast and result in acceleration of wound healing [36]. These data shows that several proteins are expressed and finally located in the intersomitic boundaries or perinuclear area. Although there is a lack of research on direct interaction of these proteins with Hsp90, some of them are sensitive to Hsp90 inhibitory factors like geldanamycin. Geldanamycin stimulats the proteolytic degradation of Fak in all cell lines examined and markedly reduces the half life of the newly synthesized FAK proteins without significantly altering the level of FAK mRNA [37]. Biochemical studies have demonstrated that mouse Hsp90 is able to rescue the DNA-binding ability of heat-inactivated MyoD homodimers [4] and MyoD/E12 heterodimers [5]. Finally, pharmacologic inhibition of Hsp90 activity using geldanamycin inhibits myotube formation in C2C12 myoblasts cell line [38], and prevents formation of muscle pioneers in the somites of zebrafish embryos [39]. Therefore, the expression of *hsp90α* in the border of *Xenopus* somites or in perinuclear area in our study might be related to a chaperoning activity of Hsp90α for MyoD, β-catenin, fibronectin, Xena, B1 integrin, or Fak. Either these proteins need Hsp90 directly for proper function or they need a pathway, which is regulated by Hsp90.

Therefore, this study showed, for the first time, the different expression patterns of *hsp90α* and *hsp90β* in *Xenopus* embryo. This and other studies emphasize the different regulation and function of these two genes in embryogenesis. The similar subcellular localization of *hsp90α* and *XMyoD* mRNA and other supporting studies suggest an important role for *hsp90α* in myogenesis.

ACKNOWLEDGMENTS

We thank Dr. Michael W. King (IUSM-Terre Haute at Indiana State University, USA) for the gift of *Xenopus laevis* cDNA library. Also, we wish to thank Dr. Cristofre Martin (School of Medicine, St. George's University,Grenada) for his valuable advice while working in this lab. This work was supported by

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Natural Science and Engineering Reasearch Council of Canada (NSERC) through a grant to Patick H. Krone and Kashan University of Medical Science, Kashan, Iran.

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