Expression, Regulation And Possible Function Of Heat Shock Genes (*Hsp47, Hsp70, Hsp90α And Hsp90β*) During Normal Development And Under Stress-Conditions In Zebrafish

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Anatomy and Cell Biology University of Saskatchewan Saskatoon

By

Zsolt Lele

Fall 1998

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SUMMARY OF DISSERTATION
Submitted in partial fulfillment of the requirements for the
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EXPRESSION. REGULATION AND POSSIBLE FUNCTION OF HEAT SHOCK GENES (HSP47, HSP70, HSP90α AND HSP90β) DURING NORMAL DEVELOPMENT AND UNDER STRESS-CONDITIONS IN ZEBRAFISH

The objectives of my thesis work to examine the similarities and differences that exist among the control and stress induced expression patter of the above mentioned heat shock genes during zebrafish embryogenesis. Northern blot analysis revealed stress- and gene-specific differences in the heat and ethanol-induced activation of these genes. Furthermore, spatial differences in the mRNA distribution of hsp genes following heat shock and ethanol-treatment were identified using whole-mount in situ hybridization.

The second aspect of my thesis deals with constitutive expression of *hsp47* during the embryonic development of zebrafish. Based on biochemical evidence, Hsp47 is regarded as a collagen-specific chaperone. I show that strong expression of *hsp47* mRNA is co-incident predominantly with expression of the type II collagen gene (*col2a1*) in a number of chondrogenic and non-chondrogenic tissues including the notochord, otic vesicle and developing fins. Notochordal expression of both genes is disrupted in *floating head (flh)* and *no tail (ntl)* embryos, which lack properly differentiated notochords. Surprisingly, no *hsp47* mRNA is detectable in the strongly *col2a1*-expressing cells of the floor plate and hypochord, indicating that the two genes are not strictly co-regulated.

In the third part of my thesis I examined the possible roles Hsp90 might play during zebrafish myogenesis as it was suggested by our previous studies that revealed coordinate expression of *hsp90α* and *myoD*, one of the major myogenic regulatory factors (J. Sass, PhD thesis). Utilizing the Hsp90-binding agent, geldanamycin (GA), I show that GA-treatment during zebrafish gastrulation and early somitogenesis specifically disrupts the development of a group of muscle cells known as the muscle pioneers. However, development of the notochord, which provides many of the signals required for proper somite patterning, is normal in GA-treated embryos. Furthermore, the PKA-dependent mechanism by which responding cells of the paraxial mesoderm are thought to be specified appears to be intact in treated embryos and the *myoD*-expressing adaxial cell progenitors of muscle pioneers develop. The data indicate that Hsp90 is required during the conversion of adaxial cells into muscle pioneers and that many of the signaling and differentiation events occurring in the pathway prior to this point are unaffected.

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ABSTRACT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

Ah - aryl hydrocarbon receptor

ATP - adenosine triphosphate

bHLH - basic helix-loop-helix

BiP - immunoglobulin heavy chain binding protein

BMP-4 - bone morphogenetic protein 4

bp - base pair

°C - degree Celsius

cAMP - cyclic adenosine monophosphate

cDNA - complementary DNA

CHBF - constitutive heat shock element binding factor

CNS - central nervous system

C-terminal - carboxy-terminal

CTP - citidine triphosphate

Cyp-40 - cyclophilin 40

Da - daltons

DMSO - dimethyl-sulfoxide

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphate

DTT - dithiothreitol

ECM - extracellular matrix

E. coli - *Escherichia coli*

EDTA - ethylenediamine tetraacetic acid disodium salt

EGF - epidermal growth factor

ehh - echidna hedgehog gene

ER - endoplasmic reticulum

Erk - extracellular signal regulating kinase

Erp99 - endoplasmic reticular protein 99

FK504 - immunosupressant drug

FKBP - FK504-binding protein

flh - *floating head* mutant zebrafish strain

g - unit of gravity

GA - geldanamycin

Gly - glycin

GP - geldampicin

GR - glucocorticoid receptor

GroEL - bacterial Hsp60

GroES - bacterial Hsp10

Grp - glucose-regulated protein

Hsc - heat shock cognate protein

HSE - heat shock element

HSF - heat shock factor

Hsp - heat shock protein

IPTG - Isopropyl-b-D-thio-galactopyranoside

K - potassium

Kar2 - karyogamy gene 2

kb - kilobase

M - molar

MAP - mitogen activated protein

MAPK - mitogen activated protein kinase

MBT - midblastula transition

Mg - magnesium

MP - muscle pioneer cell

MRF - myogenic regulatory factor

mRNA - messenger RNA

NGF - neural growth factor

NLS - nuclear localization signal

N-terminal - amino-terminal

ntl - *notail* mutant zebrafish strain

NTP - nucleoside triphosphate

OD - optical density

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

PDGF - platelet-derived growth factor

PDI - protein disulfide isomerase

PFA - paraformaldehyde

PKA - protein kinase A

PPI - peptidyl-prolyl isomerase

RACE - rapid amplification of cDNA ends

Rb - retinoblastoma protein

RNA - ribonucleic acid

RNAse - ribonuclease

RT - room temperature

RT-PCR - reverse transcription polymerase chain reaction

SDS - sodium-dodecylsulfate

shh - sonic hedgehog gene

SSA, SSB, SSC - stress seventy subfamily A, B, C

SSC - standard saline citrate

Sec - *Saccharomyces* endoplasmic complex

Ser - serine

TBP - TATA-binding protein

TCP1 - tailles complex protein 1

Thr - threonine

TRiC - TCP1 ring complex

Tyr - tyrosine

UTR - untranslated region

UV - ultraviolet

X-Gal - 5-bromo-4-chloro-3-indolyl-b-D galactopyranoside

Zn - zinc

1.0. LITERATURE REVIEW AND INTRODUCTION

In 1962, Ritossa found that puffs formed at various locations on the giant, polytene chromosomes in the salivary glands of *Drosophila* following heat shock treatment. This phenomenon, which appeared to be consistently repeatable, suggested changes in the chromosomal structure and therefore possibly in the transcription of genes located at these loci. Since this pioneering experiment heat shock gene activation has been thoroughly analyzed and described in great detail. The proteins produced as a result of this gene activation have been named heat shock proteins. Many of these proteins are involved in the folding and transport of newly synthesized proteins. As well, they help to protect and refold proteins partially denatured by heat or other chemical agents.

In the present study I set out to address several different questions which involved various aspects of expression, regulation and possible functions of heat shock genes in the zebrafish (*Danio rerio*). Genes examined in my research included *hsp47, hsp70, hsp90α* and *hsp90β*. The main lines of this study were:

I. Examination of heat shock protein gene expression in control, heat-shocked and ethanol-treated zebrafish embryos.

II. Characterization of the mRNA distribution of *hsp47* and *col2a1* genes during zebrafish development.

III. Analysis of Hsp90 function during zebrafish development

1.1. Regulation of the heat shock gene expression in eukaryotes

Although there is some evidence for the existence of posttranscriptional regulation mechanisms (Panniers, 1994; Jacquier-Sarlin et al., 1995; Wheeler et al., 1995; Hess and Duncan, 1996), regulation of heat shock gene expression is thought to occur primarily at the transcriptional level. This process is mediated by a transcription factor, called heat shock factor (HSF) which binds to a conserved element found in the promoter of heat-inducible genes, termed the heat shock element (HSE).

1.1.1. The heat shock element

The proximal upstream (5') regulatory elements of heat shock protein (hsp) genes are similar to those of other genes including the presence and location of TATA-box and transcription start regions. However, every hsp gene has at least one but in most cases more heat shock elements which provide a binding sites for the HSFs. All heat shock elements possess a simple, repeating 5-bp sequence 5'-nGAAn-3', where the repeats are contiguous but arranged in alternating orientations, (i.e. 5'-nGAAnnTTCnnGAAn-3') (Pelham, 1982; Pelham and Bienz, 1982; Amin et al., 1988; Xiao and Lis, 1988; Lis et al., 1990). The number of 5-bp units in a functional heat shock element can vary. Three repeats are considered to be a minimum for full activity (such as found in *hsp70* genes of *Drosophila)* but in the *hsp83* gene of the same organisms 8 contiguous repeats exists in a single heat shock element (Lis et al., 1990). The start of the HSE can vary beginning with either 5'-nGAAn-3' or 5'-nTTCn-3'. Also a heat shock element can tolerate a 5-bp insertion between repeating units, provided the phase of the repeat is maintained (Amin et al., 1988). The G in position 2 (numbering shows 5' to 3' direction) as well as the A`s found in positions 3 and 4 are conserved throughout eukaryotic evolution. Single base substitutions either eliminate (guanosines) or seriously reduce (adenosines) the heat inducibility of the promoter. Position 1 is not indifferent either as when the promoter contains an A in this position

it results in stronger activation than found with other nucleotides (Fernandes et al., 1994). In contrast base changes at position 5 have no effect on HSF-mediated transcription driven by the altered heat shock gene promoters (Fernandes et al., 1994).

1.1.2. The eukaryotic heat shock factors: general features and organization

HSFs belong to the leucine zipper family of transcription factors which share a common winged helix-turn-helix DNA-binding motif (for review see Wu, 1995). From yeast and *Drosophila* only one HSF has been cloned; in vertebrates and plants several different homologues have been identified. (4 in mammals: Sarge et al., 1991; Rabindran et al., 1991; Schuetz et al., 1991; Nakai et al., 1997; 3 in chicken: Nakai and Morimoto, 1993; 3 in tomato: Scharf et al., 1990).

The amino acid sequences of different HSFs strikingly differ except for two conserved domains, the DNA-binding and the trimerization domains. Within a species (e.g. comparing mouse HSF1 and HSF2) overall homology is 40% at the amino acid level (Sarge et al., 1991); approximately the same that was found in interspecies comparisons (eg. between mouse HSF1 and *Drosophila* HSF1). Also, HSFs of different species vary significantly in size (from 301 amino acids in length in one of the tomato HSFs to 529 AA in human HSF1 and 833AA in yeast; Morimoto et al.,1994).

Human HSF1 and HSF2 are differentially activated and can synergistically induce hsp gene transcription (Sistonen et al., 1994). HSF1 is induced by heat and other forms of stress while HSF2 is activated during hemin-induced differentiation of human erythroleukemia cells. Also HSF2 shows tissue-dependent activation in mouse testes under normal (non-stress) conditions and it was suggested to play a role in activating the testis-specific *hsp70-2* gene (Goodson et al., 1995). HSF3 which is relatively uncharacterized, is also activated in the absence of cellular stress by binding directly to the product of the *c-myb* protooncogene complex through their DNA-binding domains. This stimulates nuclear entry and transcriptional activation during cellular proliferation (Kanei-Ishi et al., 1997). HSF4

which does not possess the C-terminal heptad repeat (see below), also lacks the properties of a transcriptional activator. It shows tissue-specific expression in human heart, brain, skeletal muscle and pancreas and its constitutive overexpression results in reduced expression of endogenous hsp genes (Nakai et al., 1997), suggesting a tissue-specific inhibitory role in the heat shock response.

Recently, higher chromatin structure has received attention as an important factor during transcriptional regulation (for review see Vermaak and Wolffe, 1998). During HSF-mediated activation of *hsp70* genes the role of nucleosomal arrays has been examined in detail (Landsberger and Wolffe., 1995/a,b) and, as a result, a repressor function for nucleosomes in the regulation of *Xenopus hsp70* promoter activity has been proposed (Landsberger et al., 1995). Coinciding with this theory another study showed that HSF establishes a nucleosome-free region over the TATA-initiation site of the yeast *hsp82* gene (Gross et al., 1993). Recently a report concluded that the GAGA-factor, TFIID and RNA polymerase II combine to a functional transcriptional complex which is assembled on the initiation site of hsp genes but transcription is paused after a few nucleotides. After heat shock, HSF binding releases RNA polymerase II from this repressed state thereby allowing the extremely fast activation of hsp gene expression (Shopland et al., 1995). In this model, the GAGA factor is responsible for the displacement of nucleosomes from the transcription initiation and HSF-binding sites.

1.1.3. Structure and autoregulation of HSF activity

The general structural features of HSFs include an N-terminal DNA-binding domain, and an adjacent region of similarly conserved hydrophobic heptad repeats which are responsible for the trimerization of HSF monomers (leucine zippers). A feature found only in vertebrates to date (except in HSF4) is another leucine zipper at the C-terminal of the protein which has been demonstrated to have a role in the negative autoregulation of HSF DNA-binding activity through intramolecular interaction with its N-terminal counterparts (see below; Rabindran et al., 1993).

As mentioned previously HSF1 is responsible for stress-induced activation of heat shock genes. In every multicellular organism studied so far, HSF1 protein is synthesized constitutively and stored in a latent form under normal conditions. However, there are differences in the regulation of HSF1 between yeast and metazoa. In yeast, HSF exists in a constitutively trimeric, DNA-binding, yet transcriptionally inactive form (Sorger and Nelson, 1989). The induction of hsp gene transcription after heat shock is thought to be regulated by the phosphorylation and activation of yHSF (Sorger, 1990). However, results by Hoj and Jakobsen (1994) showed that phosphorylation of a short element in yeast HSF resulted in its enhanced deactivation, suggesting that phosphorylation of HSF in yeast serves as a negative feedback loop regulating the half-life and thereby the activity of HSF1 rather than being involved in its activation.

Stress-dependent phosphorylation of HSF1 is also present in higher eukaryotes although its function is still unclear since it is not required for the transcription of hsp genes after heat shock (Sarge et al., 1993). Since treatment of cells with the phosphatase inhibitor okadaic acid and constitutive phosphorylation of Ser 303 and 307 residues resulted in the prolongation of the DNA-binding state of HSF1 (Mivechi et al., 1994) it was proposed that phosphorylation might be required in regulating the duration of the activation period. Mivechi and Giaccia (1995) have demonstrated that exposure of NIH3T3 cells to elevated temperature induced the activity of the MAPK-mediated pathway. Induction of Erk-1, the terminal kinase of this pathway resulted in phosphorylation of HSF1, which in turn delayed heat-induced HSF1 activation and *hsp70* expression. Using deletion analysis the same group recently identified the region between amino acids 280 and 308 in the previously described regulatory domain of HSF1, including three serine residues which are potential sites of Erk-1 phosphorylation (Kim et al., 1997). These results confirm that HSF1 can be phosphorylated in a MAPK-dependent manner which negatively regulates its activity and the heat shock response.

In higher eukaryotes, activation of HSF1 is regulated by its transition from the monomeric to trimeric form. The HSF1 monomers are thought to exist in an intramolecular loop state where the N-

terminal and C-terminal leucine-zippers bind to each other preventing trimerization and activation of transcription. After heat shock, these intramolecular links are resorganized and trimerization occurs which, in turn leads to nuclear translocation and activation of hsp mRNA synthesis (Zuo et al.,1994). However, the question of whether HSF1 is a nuclear or cytosolic protein prior to heat shock is a matter of debate and conflicting reports have appeared supporting both hypotheses (Westwood et al., 1991; Westwood and Wu, 1993; Wu, 1995; Orosz et al., 1996; Martinez-Balbas et al., 1995; Baler et al., 1993; Sarge et al., 1993). One of the excellent model systems to study HSF1 activation is the activated oocyte of *Xenopus laevis*. Recently direct evidence has been presented that *Xenopus* HSF1 is a nuclear protein prior to heat-induced activation (Mercier et al., 1997). Western blot analysis showed the presence of HSF1 in isolated germinal vesicles but not in the remaining enucleated oocyte cytoplasm. In correlation with this data HSF binding was detectable in extracts made from the germinal vesicles but not from the enucleated oocytes after heat shock. Contradictory results of various research groups it is possible that localization of non-activated HSF1 monomers is a species specific feature and therefore should not be included in the general description of the heat shock response.

A model for HSF1 regulation has been proposed in which the main role is played by Hsp70, one of the most abundant heat shock proteins in the cell. Hsp70 binds to HSF monomers under control conditions (Mosser et al., 1990; Abravaya et al.,1992; Mosser et al., 1993). Recently it was demonstrated that Hsp70 and HSF1 monomers form a complex in non-activated cells (Baler et al., 1996) and that Hsp70 and its cochaperone, the Hdj protein interact directly with the transactivation domain of HSF1 and repress heat shock gene expression (Shi et al., 1998). Most stress conditions which activate heat shock gene expression compromise the structural integrity of proteins, exposing otherwise hidden hydrophobic domains. Binding of Hsp70 to these damaged proteins is thought to protect these interactive domains and may prevent proteins from aggregating. Stabilizing damaged proteins in a partially folded state could provide the opportunity for their refolding or degradation. This interaction however depletes the pool of free

Hsp70s in the cell which, in turn, results in the removal of Hsp70 molecules from the HSF1 monomers. This allows HSF1 trimerization and the subsequent activation of heat shock gene transcription (among them *hsp70*s). Once pools of free Hsp70 have been replenished unbound Hsp70 would repress transcription, by restoring the monomer HSF-Hsp70 interactions (Baler et al., 1992). This hypothesis is strengthened by experiments in which inhibition of ubiquitin-mediated proteasome activity led to elevated levels of aberrant proteins in the cell and also induced the heat shock response (Bush et al., 1997). Also Hsp70 overexpression in cells inhibits the heat-induced activation of HSF1 (Baler et al., 1996). Further support comes from mutant analysis in yeast which it was found that a heat shock factor mutant with reduced activity can suppress a non-functional yeast *hsp70* mutant which constitutively activated the heat shock response (Halladay and Craig, 1995).

Recently Hsp90 has also been shown to have a role in the process of HSF1 regulation and consequently in the heat shock response (Ali et al., 1998). These authors used anti-Hsp90 antibodies as well as the specific Hsp90-binding agent geldanamycin (see chapter 1.2.6.3.) in microinjection experiments of *Xenopus* oocytes. Coimmunoprecipitation demonstrated the existence of HSF1-Hsp90 heterocomplexes from heat shocked as well as from non-stressed nuclear extracts. Both geldanamycin and microinjection of antiHsp90 antibodies delayed the disassembly of HSF1 trimers during recovery from heat shock as shown by gel retardation assays. However, both agents specifically inhibited transcription from a construct containing a CAT reporter gene driven by *hsp70* promoter. Hsp90 antibody injections also activated HSF1-HSE binding which could be prevented by the addition of excess Hsp90. The authors proposed a regulatory model in which Hsp90 participates in modulating HSF1 activity at different points of its activation-deactivation process. Since Hsp90 is involved in the regulation of several important signal transduction molecules, this model also provides a link between normal cellular signaling and the stress-response. It is likely that both of the above mentioned models are involved in HSF1 regulation and that Hsp90, Hsp70 and other chaperones form a complex similar to the one found

for other molecules (eg. steroid receptors, kinases, discussed in detail later).

HSF2 and HSF3, which are not involved in the stress-responsive activation of hsp genes, are also regulated differently. They exist as dimers under normal conditions; upon induction however (ie. hemin-induction of HSF2; c-Myb binding of HSF3) they form trimers similar to that of HSF1 and bind to the same heat shock elements, thereby activating hsp gene transcription (Morimoto et al., 1994 and Nakai et al., 1995). As mentioned earlier, HSF4 appears to represent an inhibitory form of HSF which is a constitutively active, DNA-binding but not transcription-activating trimer (see above and Nakai et al., 1997).

Liu and coworkers (1993, 1995) have reported the presence of another constitutive HSE-binding factor (termed CHBF) in rodent cells. Upon heat shock, the level of CHBF-HSE interaction decreases in parallel with the increase in HSF1-HSE binding. Since then, CHBF has been identified by monoclonal antibodies as the rodent homologue of the human Ku autoantigen heterodimer (Ku70/Ku80) and shown to compete with HSF1 for HSE binding in certain cell types (Kim et al., 1995; Yang et al., 1996). Stable overexpression of both members of this heterodimer or of the Ku70 subunit alone specifically inhibits *hsp70* expression but not that of any other hsps after heat shock in rodent cell lines. As Ku80 overexpression alone failed to produce the same result it was proposed that only Ku70 has a role in negatively regulating *hsp70* expression after heat shock (Yang et al., 1996). Recently constitutive heat shock element binding activity has been reported by Gordon et al. (1997) in *Xenopus* stage I and II oocytes but was not found at later stages. This constitutive complex was unaffected by heat and it is not presently clear whether it is a homologue of the mammalian Ku autoantigen. Alternatively it may represent an entirely new HSF which is active only during early oogenesis or a known HSF being regulated in a different way.

1.2. Heat shock proteins and molecular chaperones

Molecular chaperones are a class of proteins that interact with the non-native conformations of other proteins. They are key mediators of protein folding in the cell under normal growth conditions as well as under stress and are also involved in protein translocation and degradation. Their common feature is the ability to recognize exposed hydrophobic sequences or surfaces of proteins which in their natural state are buried inside the molecule. It has to be stressed that they do not typically function as protein-folding catalysts as they increase the final yield of the folding reaction rather than its speed.

Molecular chaperones comprise several protein families that are structurally unrelated. Many of these proteins are also classified as heat shock proteins (hsps) as their expression can be induced by a variety of stresses such as heat, oxidative stress, transition metal ions and amino acid analogues. Despite having common members, these two groups of proteins should be clearly distinguished as they both include proteins which are not members of the other class. Such chaperones are for example the peptidyl-disulfide isomerases and peptidyl-prolyl isomerases and such heat shock proteins are the members of the high molecular weight Hsp family which show closest homology to certain bacterial proteases.

Heat shock proteins were traditionally separated into families by their relative mobility on SDS polyacrylamide gel electrophoresis. Later it was found that members of the same family showed high sequence identity. Hsp families include:

Hsp15-30 (low molecular weight)
Hsp60
Hsp70
Hsp90
Hsp100 (high molecular weight)

Other heat shock proteins include Hsp47, a collagen-specific chaperone which is the only member of its family and Hsp40, the eukaryotic homologue of the bacterial DnaJ protein which acts as a cochaperone of Hsp70 in folding processes.

1.2.1. Small (15-30 kDa) heat shock proteins

The small heat shock protein (sHSP) family encompasses proteins that share structural features common with the lens protein α-crystallin and are present in every eukaryotic organism examined to date (for review see Arrigo and Landry, 1994). sHSPs are less highly conserved than members of any of the higher molecular weight hsp families. Their number is variable from 1 in yeast to 3 in mammals, 4 in *Drosophila* and more than 20 in plants. The major characteristic of all sHSPs is that they have a molecular weight between 15-30kDa and share a common domain termed the α-crystallin domain consisting of approximately 80 amino acid residues in the C-terminal half of the protein. This domain, unlike the rest of the protein, is highly conserved between individual sHSPs of a species as well as between distant species. For example the α-crystallin domain of the human HSP27 shares 70 percent homology with the corresponding sequences from *Drosophila* (Arrigo and Landry, 1994). sHSPs also generate interest as one of the heat shock proteins which are expressed differentially during development and the cell-cycle and their expression is correlated with differentiation and oncogenic status (Arrigo and Tanguay, 1991; Walsh et al., 1993; Arrigo and Landry, 1994; Michaud et al., 1997/a). In mammalian cells, their basal level of expression is highly variable but they are one of the most strongly heat-inducible hsp genes (Landry et al., 1991; Klementz et al., 1993).

sHSPs are also unique among heat shock proteins in that their induction is not only a result of enhanced transcription mediated by HSF1-HSE interaction described earlier but also they are regulated at the posttranscriptional level (Ohan and Heikkila, 1995). Their mRNAs contain sequences which promote preferential translation over normal mRNA (Hultmark et al., 1986). Also heat shock induces a block in splicing of other mRNAs, a process which does not affect sHSP transcripts (Yost and Lindquist, 1986). Another important feature of sHSPs is the fact that they are heavily phosphorylated in response to addition of growth factors in mammalian cells (Chambard et al., 1983), indicating a possible role for them in response to changes in the

proliferation state of cells. While heat-inducible activation of the protein kinase C-dependent MAP kinase pathway has been shown to be responsible for the phosphorylation of small heat shock proteins in mammalian cells (Engel et al., 1995), but the functional significance of this heavy phosphorylation is still unclear.

Tissue and stage-specific expression of small hsps during normal development has been characterized in detail in *Drosophila* (Arrigo and Mehlen, 1994; Marin et al., 1993; Tanguay et al., 1993; Michaud et al., 1997/a,b), in *Xenopus* (Krone and Heikkila 1988,1989; Ali et al.,1993) and in mouse (Gernold et al., 1993; Loones et al., 1997; Benjamin et al., 1997). However, their role (other than the obvious role played by α-crystallin in the development of the lens) during embryogenesis is still unclear.

1.2.2. Hsp 60 family

Members of the Hsp60 family mediate the folding of many different proteins in vivo and in vitro and they are often referred to as chaperonins (Hemmingsen et al., 1988; Ellis and van der Vies, 1991). Chaperonins include the well-studied GroEL/GroES complexes in bacteria, as well as the closely related Hsp60/Hsp10 complexes of mitochondria and chloroplasts, and the more distantly related TCP1/TriC complex found in the eukaryotic cytosol (for review see Frydman and Hartl., 1994). These are all large, oligomeric, double toroidal complexes. GroEL/GroES, the best characterized chaperonin so far, is composed of two heptameric rings of 57kDa subunits (GroEL) stacked back to back (Hendrix, 1979). This structure is capped by a single heptameric cap, formed by a 10 kDa GroES subunit which associates or dissociates depending on the state of the complex during the protein folding cycle. Electron microscopic studies have shown that the unfolded proteins can be found in the lumen of the complex (Langer et al., 1992; Ishii et al., 1994). Chaperonins interact preferentially with partially folded proteins which are in the so called "molten globule" state in which they have all their secondary structures formed (α-helix, β-sheets, β-barrel) but these domains are organized only in a loosely folded tertiary state allowing their quick rearrangements (Mendoza et

al., 1992; Landry et al., 1992). Following biochemical analysis of GroEL/GroES function, a cyclic model was proposed by Weissmann et al. (1994). In this model, binding of an unfolded protein to GroEL/GroES results in dissociation of ADP and GroES. This causes binding of ATP which weakens the interaction of GroEL with the substrate and induces GroES to rebind. ATP hydrolysis results in the release of the protein into the cavity of the complex to fold. The restoration of the ADP-bound form starts the cycle from the beginning. If the folding is completed, the released protein will exit the lumen into the cytosol allowing the next substrate to enter. The Hsp60/Hsp10 and TCP1/TriC complexes, the latter of which has an eightfold rather than a sevenfold symmetry, are believed to work in a similar cyclic manner (Willison and Kubota, 1994).

It has been proposed, based on what was described in bacteria, that the sequential activity of Hsp70 (see below) and the chaperonins are responsible for the folding of every cellular protein. This is likely to be true for mitochondrial and chloroplastic proteins as both chaperones are abundant in these compartments of the cell (Bertsch et al., 1992; Lubben et al., 1990). However, the eukaryotic cytosolic TCP1/TriC complex is of too low abundance in many cell types for handling the bulk of cytosolic protein folding. Also, evidence indicates that this complex is specifically responsible for the folding of cytoskeletal proteins such as actin and tubulin (Hartl, 1996). These facts combined with the complete lack of an Hsp60 homologue in the endoplasmic reticulum required a reconsideration of the main role that was proposed for the chaperonins in protein folding processes occurring in these compartments. Recently, evidence has suggested that Hsp90 homologues might act as the main cytosolic and ER-resident chaperones interacting with Hsp70 in the folding (and sometimes in the regulation) of proteins (discussed later).

1.2.3. Large molecular weight (>100kDa) heat shock proteins

The Hsp100 proteins comprise a highly conserved family. Their discovery has been relatively recent but homologues have been isolated from bacteria, yeast, mammals and plants (Gottesmann et al.,

1990; Parsell et al., 1991; Squires et al., 1991). The family is divided into subfamilies (ClpA,ClpB,Clpc) based on the organization of its two main conserved ATP-binding domains (for review see Parsell and Lindquist, 1994). Homology of these domains at the amino acid level is high and it can reach 80% between different species. The most well characterized member of this family is the yeast Hsp104 protein. It forms a hexameric ring-like strucure, not unlike the Hsp60 double toroid except that Hsp104 subunits assemble into a single ring (Parsell et al., 1994). The two ATP-binding domains serve different purposes, with the C-terminal domain being involved in the assembly of the homo-oligomer complex, while the N-terminal domain is required for the ATP-ase activity of the protein. The E.coli homologue of Hsp104 has only one nucleotide binding domain which may possess both functions (Gottesman et al., 1990).

Members of the ClpB subfamily (such as yeast Hsp104) are all heat-inducible and they have been implicated to play a role in the acquisition of thermotolerance (Squires and Squires, 1992). Acquired thermotolerance is a phenomenon whereby exposure to mild hyperthermia results in a much stronger viability against a subsequent strong, normally lethal heat shock. For example yeast cells grown at 25°C survive exposure to 50°C 1,000-10,000-fold better when pretreated at 37°C for a brief period (Sanchez and Lindquist, 1990). This tolerance is severely compromised by mutations in the Hsp104 protein. These data, together with the fact that the mutation has only a modest effect on survival rate at 44°C, suggest a role for Hsp104 in the management of extreme heat-stress conditions (Sanchez et al., 1992). Mutation in Hsp104 also severely depletes the ability of yeast cells to cope with stress caused by 20% ethanol, but surprisingly similar experiments with heavy metals (cadmium, arsenite) did not give the same result, indicating that the damage caused by heat and ethanol is somehow different from that caused by these other hsp-inducing agents (Sanchez et al., 1992). A role for the heat-inducible Hsp100 proteins in the proteolysis of stress-damaged proteins was also suggested based on the activity of a constitutively expressed, bacterial ClpA member which serves as a regulator of the bacterial ClpP protease. Attempts to demonstrate a similar proteolytic role for the heat-inducible Hsp100

family members in eukaryotes have so far failed (Woo et al., 1992; Parsell et al., 1993).

1.2.4. Hsp70 family

1.2.4.1. General structure of Hsp70 proteins

Of all the hsp gene families, Hsp70 is the most thoroughly studied and best characterized. In eukaryotes, *hsp70* genes exist as part of a multigene family whose members are expressed under a variety of physiological conditions. In humans the *hsp70* family encompasses at least 11 genes which encode for both cognate (constitutively active, hsc70s) and highly inducible members (for review see Tavaria et al., 1996). They are found in most if not all cellular compartments including nuclei, mitochondria, chloroplasts, endoplasmic reticulum (ER) and cytosol (Craig et al., 1994). All *hsp70* genes isolated to date show a high degree of identity having at least 50% overall identity at the amino acid level (Boorstein et al., 1994). Homology is much higher among Hsp70 proteins located in the same cellular compartment of different species (human and chicken ER-located Hsp70s) than among Hsp70s of the same species found in different cellular compartment (eg. chicken cytoplasmic and chicken ER Hsp70s).

All Hsp70s have two main functional domains: the N-terminal ATP-ase domain and the C-terminal peptide-binding domain (Milarsky and Morimoto, 1989; Morshauser et al., 1995). The ATP-ase domain forms two lobes with a cleft between them and ATP binds to a pocket formed at the base of the cleft (Flaherty et al., 1990). The C-terminal fragment of Hsp70 has thus far resisted attempts to reveal its structure by X-ray crystallography but computer-assisted structural predictions have revealed a peptide-binding groove-like domain formed by α-helices which showed significant similarity to that found in the human class I and class II major histocompatibility antigens (reviewed in Hightower et al., 1994). Hsp70s fulfill a variety of functions in the cell: they stabilize newly synthesized, unfolded precursor proteins prior to their folding and assembly into multimolecular complexes; facilitate protein import into the mitochondria, chloroplasts and ER; they

maintain newly translocated proteins in an unfolded state before folding and assembly in the organelles; they uncoat clathrin-coated vesicles and they are also involved in the resolution of protein aggregates by targeting them towards degradation. In the following segments I will give a brief description of the characteristics of the Hsp70s found in each intracellular compartment.

1.2.4.2. The cytosolic chaperone machine

In the past several years extensive evidence has accumulated that cytosolic Hsp70s are closely associated with the synthesis, folding and transportation of proteins. All organisms studied so far have at least one Hsp70 in their cytosol. Mammalian cells also have several inducible forms (including Hsp68, Hsp72, etc.). The abundant constitutive form called Hsp73 is sometimes also referred to as heat shock cognate protein 73 (Hsc73). They are closely related (>90% identical) and able to functionally substitute for each other. Interestingly there are also two groups of Hsp70 homologue proteins in yeast (termed SSA and SSB proteins). Proteins belonging to different groups are only about 65% identical and are not functionally redundant (Craig and Jacobsen 1984).

Hsp70s work in cooperation with other proteins to carry out their chaperoning function (for review see Frydman and Hohfeld, 1997). They interact with polypeptides with an extended, unfolded conformation by recognizing 7-9-mer peptide segments with a net hydrophobic character (Flynn et al., 1991; Blond-Elguindi et al., 1993) and execute a regulated cycle of peptide binding and release, which facilitates the acquisition of the active comformation of polypeptides. Among the proteins which assist in this cycle is Hsp40, the eukaryotic homologue of the bacterial DnaJ protein. Hsp40 homologues among eukaryotes are only partially conserved, having a maximum of 30% identity (Raabe and Manley, 1991) between species. These proteins are thought to initially bind non-native proteins and target them to Hsp70 and also stimulate Hsp70`s ATPase activity. In mammals, Hip (p48), another chaperone protein, prevents the dissociation of ADP, thereby stabilizing the complex. This requires the presence of Hsp40 and Hsp70

in the complex containing the substrate to bind to Hip (Hohfeld et al., 1995). Deletion analysis revealed that binding of Hip to the ATPase domain of Hsp70 occurs via its (Hip's) tetratricopeptide domain (Prapapanich et al., 1996).

Another recently discovered co-chaperone molecule is named Hop (p60). It has no bacterial homologue and is responsible for the initiation of the substrate-chaperone complex dissociation and stimulation of ADP-ATP exchange resulting in the completion of the cycle. At this point the protein can be passed on to the next chaperone molecule if the conditions are right or can be rebound by Hsp70 for further protection from misfolding. In mitochondria and chloroplasts, the partially folded protein is transferred to the Hsp60/Hsp10 complex for the completion of its folding (see chapter 1.2.2. for details). For reasons mentioned in Chapter 1.2.2., it appears that in the ER proteins are passed from the Hsp70 homologue (named BiP for binding protein) to the constitutively expressed Grp94 which shows homology with members of the Hsp90 family for further folding (Melnick et al., 1994; Jakob and Buchner, 1994). With the recent description of the Hip and Hop proteins a connection has been made between the Hsp70 and Hsp90 chaperoning systems. In several cases (eg. steroid receptors and tyrosine-kinases) these two chaperone machines cooperate in a sequential fashion (Jacob and Buchner, 1994; Bohen and Yamamoto, 1994) not only to achieve complete folding but also to regulate the substrate's activity. For example the progesterone receptor is held by the Hsp70-Hsp40 complex when binding of Hip results in the dissociation of Hsp40 and in the stabilization of the complex. This is followed by the binding of an Hsp90 dimer which appears to be linked together with Hsp70 by the simultaneously binding Hop protein (Chen et al., 1996/a). Since Hop stimulates the ADP-ATP exchange on Hsp70 it causes Hsp70 to dissociate from the complex. The final maturation of the receptor is carried out by additional peptidyl-prolyl isomerases (FKBP52, Cyp-40) and p23 which join the complex at this point (Bose et al., 1996; Freeman et al., 1996; see also in chapter 1.2.6.2.). This final complex then keeps the progesterone receptor in a ligand-binding active state until the binding of the hormone after which the hormone-receptor complex dissociates.

Cytosolic Hsp70s also carry out important roles in transportation of newly synthesized proteins. They stimulate the translocation of newly synthesized M13 phage procoat protein into microsomes (Zimmerman et al., 1988) and the import of preornithine carbamoyl carboxylase into mitochondria (Peralta et al., 1993). Also antibodies against Hsp70 proteins were shown to inhibit peroxysomal protein import (Walton et al., 1994).

Molecular chaperones also stimulate the breakdown of proteins and it has been hypothesized that when they fail in their function of protein folding, they facilitate the degradation of the mishandled protein (Craig et al., 1994; Sherman and Goldberg, 1992). Although this "refold or degrade" hypothesis remains to be critically tested it has been shown that molecular chaperones indeed facilitate the degradation of proteins. In prokaryotes the main heat shock protein to carry out such a function is the Clp protease, the bacterial homologue of the eukaryotic hsp100 family (see chapter 1.2.3. for details). In eukaryotes, components of the ubiqitin-dependent proteolytic pathway are induced by heat shock. This includes ubiquitin, two ubiquitin carrier proteins and certain subunits of the 26S proteasome, where ubiquitin-marked proteins are transported for degradation (Parsell and Lindquist, 1993). It was shown that inhibition of this proteolytic pathway led to induction of the heat shock response suggesting that the Hsp70 regulated HSF1 induction theory is indeed true (see chapter 1.1.3.; Bush et al., 1997). Also a selective pathway responsible for the proteolysis of cytosolic proteins (Dice et al., 1994) requires Hsc73 binding to the KFERQ lysosomal targeting sequence, assisting in the transport of cellular proteins marked for degradation (Chiang et al., 1989).

1.2.4.3. Hsp70 of the endoplasmic reticulum

The ER contains a single Hsp70 protein named BiP (grp78) in mammalian cells (Haas and Wabl, 1983; Lee et al., 1981) and Kar2 in yeast (Normington et al., 1989). Early studies have demonstrated that it binds strongly to denatured or misfolded proteins (Bole et .al., 1986, Gething et al., 1986) in an ATP dependent manner (Munro and Pelham, 1986). Beside the above described (chapter 1.2.4.1.) N-terminal

ATP-ase domain and C-terminal peptide-binding domains they also have a typical carboxy-teminal ER retention sequence (KDEL; Munro and Pelham, 1987). This feature prevents them from being transported to the trans-Golgi apparatus and instead causes their recycling back to the ER by a receptor-mediated mechanism. In unstressed cells the majority of BiP molecules are present as homo-oligomeric complexes posttranslationally modified by phosphorylation and ADP-ribosylation (Welch et al., 1983, Hendershot et al., 1988; Leustek et al., 1991). Conditions that result in increased concentrations of unfolded polypeptides in the ER promote the reversal of these modifications (Hendershot et al., 1988; Leustek et al., 1991) and the dissociation of the BiP oligomers (Freiden et al., 1992). BiP is found in a complex with other proteins (Sec63,Sec66,Sec67 in yeast; Sec=*Saccharomyces* endoplasmic complex) which are involved in the import of secretory precursor molecules synthesized on the ribosomes of the rough ER. Recently it was shown that Sec62p, Sec71p and Sec72p form a translocon subcomplex that engages secretory precursors at the membrane site of ER translocation machinery. Binding of precursors depends on the presence of an intact signal sequence and occurs only in the absence of ATP. In the presence of ATP the precursor is released from the complex in a reaction mediated by BiP. This release process also requires an interaction between BiP and Sec63p, the ER homologue of the bacterial DnaJ cochaperone (Lyman and Schekman, 1997).

1.2.4.4. Mitochondrial Hsp70

Most mitochondrial proteins are synthesized as precursors with positively charged N-terminal targeting sequences. Their import and correct folding requires the coordinated activity of cytosolic and mitochondrial chaperone molecules (for review see Brodsky, 1996). Following their synthesis in the cytosol, mitochondrial proteins are maintained in an unfolded conformation by cytosolic Hsp70 and Hsp40. The unfolded precursors then bind to receptor proteins on the outer membrane of the mitochondria at sites where the inner and outer membranes are in close proximity (termed contact sites). This

receptor complex contains at least six different proteins which mediate binding and insertion of precursors into one of the translocation pores found in the outer membrane (Kiebler et al., 1993). The targeting sequences are then brought into contact with components of the inner membrane. This step of protein transport requires the presence of a $\Delta\psi$ membrane potential in the inner membrane which may exert an electrophoretic effect on the positively charged signal sequence of the protein (Martin et al., 1991). Further translocation into the matrix does not require this membrane potential, only ATP. Recently two models have been proposed to explain transport across the inner membrane. In the first model, matrix Hsp70 molecules drive the translocation by binding to increasingly longer segments of the protein or by interacting with the recently imported regions of the peptide (brownian ratchet model, Simon et al., 1992). The second model (Hsp70 motor model) suggests that lumenal Hsp70s are force generating motors pulling and releasing the translocating peptide during each cycle of ATP hydrolisis (Glick, 1995). Further studies are necessary to determine the mechanism by which mitochondrial proteins are imported in vivo.

1.2.4.5. *hsp70* expression during development

Although members of the Hsp70 family are the most well characterized among all heat shock proteins, their expression during embryogenesis still requires further examination. Data about heat shock gene expression during development have been collected using various methods and model species therefore providing only partially comparable results. The three basic methods used in analyzing *hsp70* gene expression are : 1. determination of mRNA distribution among tissues by Northern-blot analysis or in situ hybridization experiments; 2. localization of heat shock proteins by Western-blot analysis or immunocytochemistry; 3. study of transgenic animals with reporter genes under the control of hsp gene promoters. Among the most thoroughly studied model systems are the African clawed frog (*Xenopus laevis*) and the mouse, but some partial information is also available concerning *hsp70* gene expression in the fruitfly (*Drosophila*

melanogaster), in the amphibian *Pleurodeles waltl* and in zebrafish (*Danio rerio*).

hsp70 expression in oogenesis is one of the better characterized phenomena which has been studied intensively in amphibians. A number of reports have established the fact that *hsp70* genes showed constitutive expression during oogenesis in *Xenopus laevis* (Bienz, 1984; Bienz and Gurdon, 1982; Browder et al., 1987; Davis and King, 1989; Horrell et al., 1987; Heikkila et al.,1987; Uzawa et al., 1995). *hsp70* mRNA accumulates in stage III oocytes and is maintained at a constant level throughout oocyte maturation, fertilization and early cleavage stages (Horrell et al., 1987). Supporting these data, a microinjected *hsp70* gene is constitutively expressed in oocytes (Bienz, 1984. 1986, Krone, 1990). Surprisingly, along with the continuous expression, the introduced *hsp70* (and *hsp30*) genes were not stress-inducible. A similar result is seen in the newt, *Pleurodeles waltl* in that *hsp70* which is strictly inducible in somatic cells but showed high constitutive levels of *hsp70* mRNA as well as protein in stage II-VI oocytes (Billoud et al., 1993). Gradual translocation of Hsp70-related proteins from the cytoplasm to the nucleus during this time was also observed and it was proposed that Hsp70 might play a role in processes by which stage VI oocytes acquire competence to undergo hormonally stimulated maturation. Hsc70, the cognate member of the Hsp70 family, was also found in developing oocytes of the mouse in an earlier study but detailed characterization of its expression during oogenesis was not provided as the study focused mainly on the differences in heat-inducibility at various stages (Curci et al., 1991).

Despite the intensive work describing the expression of *hsp70* genes in various organisms, its function during development had not been established. This has changed recently, when detailed studies carried out in the mouse established the role of Hsp70-2 during spermiogenesis (for a detailed review see Sarge and Cullen, 1997). Hsp70-2 had been shown to be a testis-specific heat shock protein which was expressed at high levels in spermatocytes at the pachytene stage of meiosis (Zakeri et al., 1988; Allen et al., 1988). Recently it was identified as a component of the synaptonemal complex in prophase nuclei of spermatogenic cells (Allen et al., 1996). Moreover mice carrying the

hsp70-2 -/- knockout mutation did not synthesize Hsp70-2, lacked postmeiotic spermatids and mature sperm and therefore were sterile (Dix et al., 1996). Similar problems during meiosis of oocytes in females were not observed indicating that Hsp70-2 affects a spermiogenesis-specific step during meiosis. It has been reported that CDC2 kinase activity is present mostly during the pachytene stage of meiosis in spermatocytes and it was proposed that Hsp70-2 might affect CDC2 kinase activity in these cells. Indeed in *hsp70-2* -/- knockout mice primary spermatocytes failed to complete meiosis I and underwent apoptosis during postnatal testis development (Mori et al., 1997). Coimmunoprecipitation analysis and in vitro reconstitution experiments showed that Hsp70-2 interacts with CDC2 in the testis. Furthermore, the targeted mutagenesis also eliminated the kinase activity of CDC2 in mutant mice. This activity was restored after addition of recombinant Hsp70-2 to freshly prepared testis extracts (Zhu et al., 1997).

hsp70 expression during embryogenesis has been studied most thoroughly in mice. At the two-cell stage, which in mouse coincides with the activation of the embryonic genome, a spontaneous burst of expression of both *hsp70* and *hsc70* genes was observed by ^{3}H-thymidine incorporation and two-dimensional protein electrophoresis (Bensuade et al., 1983; Howlett and Bolton, 1985). RT-PCR analysis, 2D protein electrophoresis and microinjection experiments using *hsp70* and *hsc70* promoter driven reporter genes confirmed this immediate early expression (Manejwala et al., 1991; Bevilacqua and Mangia, 1993; Thompson et al., 1994, 1995; Bevilacqua et al., 1995; Christians et al., 1995). Interestingly, similar patterns of expression were found in both gynogenetic and androgenetic embryos indicating that *hsp70* and *hsc70* expression is the result of gene activity in both the maternal and the paternal genome (Barra and Renard, 1988). Recently Christians and coworkers (1997) have found that transgenic mouse embryos with mutagenized HSE elements in their *hsp70* genes did not show this early *hsp70* expression and concluded that HSF1, which is already present at this stage of development plays a role in this immediate *hsp70* gene induction.

After this spontaneous burst of gene activity there is a clear difference between the expression of *hsp70* and *hsc70* genes. Cognate 70 kDa heat shock proteins are continuously synthesized during the remainder of embryonic development (Morange et al., 1984; Giebel et al., 1988) and are detectable in both embryonic and extraembryonic tissues (Mezger et al., 1991). In contrast *hsp70* expression is repressed and becomes hardly detectable by the 4-cell stage (Thompson et al., 1994; Christians et al., 1995) as shown by RT-PCR and microinjection experiments. As several other non-related genes showed a similar expression pattern during this stage of mouse development (Latham et al., 1991; Schultz et al., 1995) it was proposed that this 4-cell stage repression is not restricted to the heat shock regulatory pathway.

Later during mouse development, non-induced *hsp70* expression is detectable from day 9 in the placenta and from day 15.5 in the embryo itself (Kothary et al., 1987). This embryonic expression is restricted mainly to the neural tube during its closure and neuroectoderm differentiation (Walsh et al., 1997). *hsc70* also shows stronger expression in neural vs. non-neural tissues during mouse development, a pattern which is also present in adult tissues of rabbits (Manzerra et al., 1997)

Finally, there have been several studies involving the characterization of the aquisition of heat-inducible *hsp70* expression during early embryogenesis in *Xenopus*. The heat-inducibility of *hsp70* gene expression starts at around the late blastula period after MBT (midblastula transition) in *Xenopus* and after zygotic genome activation in mice (Bienz, 1984; Browder et al., 1987; Krone and Heikkila, 1988, 1989; Heikkila et al., 1987), despite the fact that HSF1-binding is detectable throughout early *Xenopus* development including pre-MBT stages (Ovsenek and Heikkila, 1990; Karn et al., 1992). It was proposed that the fast cell-cycle (characterized by the lack of G1 and G2 phases) and the quick changes in chromatin structure occurring in the pre-MBT embryos prevent transcription (Newport and Kirschner, 1982/a,b). The lengthening of the cell-cycle and the inclusion of G1 and G2 stages allow the restoration of heat inducibility after midblastula transition (Kimelman et al., 1987).

hsp70 expression in control or stressed zebrafish embryos had not been studied prior to my research. Recently a paper by Santacruz et al., (1997) described ubiquitous *hsc70* gene expression in normally developing embryos with some enhanced mRNA levels in the CNS and in some of the somites, but this publication did not include data for spatial distribution of *hsp70* mRNA.

1.2.5. Hsp47

Hsp47 is encoded by a single gene in every species examined to date. It is a unique chaperone protein in the sense that it is a "dedicated" chaperone. All studies examining its function found that it has a specific substrate, carrying out its chaperoning activity on newly synthesized procollagen chains. To better understand the function Hsp47 plays during collagen synthesis and triplex formation, I will give a brief introduction to proteins belonging to the family of collagens in the following chapters.

1.2.5.1. Collagen structure, function and synthesis

The word collagen (derived from the Greek kollagene, meaning glue forming) describes a family of structurally related proteins that are located in the extracellular matrix of connective tissues. They are arguably the most abundant structural protein in the animal kingdom. Collagens have likely played a critical role in the evolution of large complex organisms where they provide a flexible, yet solid scaffold for the provision of shape and form, for the attachment of macromolecules, glycoproteins, hydrated polymers and inorganic ions, and for other cells. These proteins comprise three polypeptide chains (α-chains), distinguished by the α1, α2, α3, etc. names, which form a unique triple-helical structure. The triple helix structure lends stability against cleavage by proteases (except collagenase). In order for the three α-chains to wind into a triple helix they must have the smallest amino acid, glycine at every third residue along each chain. Thus, each of the three chains has the repeating structure Gly-X-Y where X and Y can be any amino acid but are frequently the imino acids proline and hydroxy-

proline (Kielty et al., 1993). More than twenty genetically distinct collagens exist in animals. These proteins occur as supramolecular assemblies that range in morphology from rope-like fibrils that provide scaffold and maintain the integrity of tendons, bones and ligaments, to net-like sheets in the basement membranes underlying epithelial and endothelial cells. To distinguish one collagen type from the other, vertebrate collagens are assigned Roman numerals in order of their discovery. Collagen types I, II, III, V and XI self-assemble into cross -striated fibrils (referred to as fibril-forming collagens) and collectively are the most abundant collagens in vertebrates (Prockop and Kivirikko, 1995). The fibril-forming collagen molecules consist of an uninterrupred helix approximately 300nm in length and 1.5nm in diameter flanked by short (20-30nm long) extrahelical telopeptides on both sides (Kadler et al., 1996). The telopeptides do not have the Gly-X-Y repeats and therefore do not adopt the helical structure but are critical in the timing of fibril formation.

The biosynthesis of collagen molecules is a multistep process which has been traditionally divided into two major stages. The first includes the intracellular steps which produce triple-helical procollagen molecules. In the second, extracellular stage, proteolytic enzymes digest the flanking non-helical regions of the procollagen molecule resulting in fibril formation. Translation of the pre-procollagen molecules starts on free ribosomes which are transported to the ER by the classical signal recognition particle-mediated mechanism. Here the N-terminal signal peptide is removed by signal peptidase and the nascent procollagen α-chains undergo specific modification such as hydroxylation and glycosylation on lysine, hydroxylisine and proline residues (Bornstein, 1967). These modifications appear to occur co-translationally during pauses in collagen chain elongation (Gura et al., 1996) rather than postranslationally (Uitto and Prockop, 1974). The hydroxyl groups of hydroxylysine serve important functions in providing attachment sites for glycosyl residues and in the formation of interchain covalent crosslinks. Vertebrate collagens contain O-linked monosaccharide galactose and disaccharide glycosyl-galactose that are covalently linked to the hydroxylysil residues in the triple helical domain of proα-chains

(Butler, 1978). Conversely, the N- and C-terminal propeptides of collagen molecules contain mostly N-linked oligosaccharides such as glucosamine, mannose and galactosamine (Clark et al., 1978; Clark, 1979). Assembly of proα-chains into procollagen occurs after the folding of the C-terminal propeptides into individual chains, followed by intrachain disulphide bonding, association of the C-propeptides and interchain disulphide bonding. After this is completed, triple helix formation advances in a C-terminal to N-terminal direction in a zipper-like fashion (Engel and Prockop, 1991). The extracellular steps in collagen biosynthesis involve the removal of the N-terminal and C-terminal propeptides by procollagen peptidases; metalloproteinases which require Zn^{2+} and Ca^{2+} for their proper function (Hojima et al., 1985; 1989). The removal of the terminal propeptides results in fibril formation, a process driven by entropy (Kadler et al., 1987) which can occur in C to N or N to C fashion resulting in unipolar or bipolar fibrils (Kadler et al., 1996).

Type I collagen is found throughout the body except in cartilagenous tissues. It occurs in bones, tendons, skin and developing heart (for review see Kuhn, 1978; Swiderski et al., 1994) and it is also synthesized in response to injury. Type II collagen is found mainly in cartilage, but also occurs in the developing cornea and vitreous humor. In regions which undergo endochondral ossification there is a switch from type II to type I collagen expression at the onset of calcification. Type III collagen occurs in in the walls of arteries and other hollow organs and it is usually coexpressed with type I collagen. Type V and type IX collagen are relatively minor components of various tissues and occur as heterotypic fibrils with type I and type II collagens respectively.

Among the non fibril-forming collagens, type IV is the most important in establishing the meshlike network of basement membranes found under epithelial and endothelial cells. Type VII collagen forms the fine anchoring filaments which attach basement membranes to specialized anchoring plaques found deep in the underlying connective tissue.

1.2.5.2. Collagen expression during development

During evolution of multicellular organisms, collagen probably played a critical role in providing a large scaffold which helped to define shape. As a major part of the extracellular membrane (ECM), collagens also provided attachment sites for other cells and various macromolecules such as glycoproteins, proteglycans and molecules involved in signaling. Last but not least, inorganic ions are also attached to the matrix which cause water retention and subsequent formation of a rigid hyperosmotic ECM.

Despite the thorough characterization of collagen distribution in adult organisms, their expression has not been studied in similar detail during vertebrate development. As important molecules of the ECM, they probably play a role in cell movements, cell-cell adhesion, cell proliferation and inductive interactions. Among the fibril-forming collagens, expression of type II is the most thoroughly characterized during development. As it constitutes up to 80% of total collagen synthesized by hyaline cartilage, it was hypothesized that its expression would be restricted to chondrogenic tissues during development. Studies that examined its expression in basically every important vertebrate developmental model organism proved otherwise as several non-chondrogenic tissues express type II collagen including skin, muscle, lung, gut, liver, brain and heart (Kosher and Solursh, 1989; Fitch et al., 1989; Su et al., 1991; Cheah et al., 1991; Ng et al., 1993; Lui et al., 1995; Yan et al., 1995; Krengel et al., 1996). Also, In *Xenopus* embryos, it was also shown to be expressed shortly after gastrulation in the notochord, the somites and in the floor plate (Su et al., 1991). By stage 31 (late somitogenesis) *col2a1* mRNA was also detected in the ventral neural tube and in the developing vertebrae. In tadpoles, transcripts were largely restricted to chondrogenic regions of the head and trunk.

In the zebrafish (Yan et al., 1995), *col2a1* expression appears first in the notochord progenitors, at around 10 hrs of age. mRNA levels remain high in the notochord up to 18 hrs when it starts to decay in an anterior-posterior fashion and completely disappears by 28 hrs. A similar expression pattern is detectable in the floor plate (the

ventralmost cells of the neural tube) and in the hypochord (single row of cells just below the notochord, with unknown function). However, *col2a1* expression is delayed in these tissues compared to that found in the notochord by 2.5 hours. In addition to these areas, *col2a1* mRNA is also detectable in the pharyngeal arches, the epithelium of the otic capsule and in the mesenchyme of the neurocranium.

1.2.5.3. Hsp47 as a collagen-specific chaperone

Hsp47 (also known as colligin or GP46) was first described as a 47 kDa protein from murine parietal endodermal cells (Kurkinen et al., 1984), where it was coexpressed specifically with collagen types I and V. Since then, it has been cloned from a variety of vertebrate organisms including chicken, mouse, rat, human and most recently zebrafish (Nagata and Yamada, 1986, Nandan et al., 1990; Clarke and Sanwal, 1992; Takechi et al., 1992; Hosokawa et al., 1993; Pearson et al., 1996). Based on sequence analysis, Hsp47 is considered to be a member of the serine protease inhibitor (serpin) superfamily (Clarke et al., 1991; Hirayoshi et al., 1991). In humans Hsp47 and other serpins show 30% homology with each other (Clarke and Sanwal, 1992). However, Hsp47 lacks the typical reactive site of serpins (Hirayoshi et al., 1991) suggesting that it is not an active serin protease inhibitor. This was confirmed when it was demonstrated that indeed recombinant mouse Hsp47 could not function as a substrate for a variety of serine proteases. However, results from Jain et al., (1994) showed that Hsp47 protected collagen from proteolytic cleavage by the contents of liver microsomes, leaving this question open for debate.

Hsp47 is localized to the ER of various tissues as confirmed by immunfluorescence and immunoelectron microscopy (Saga et al., 1987; Nakai et al., 1990). Direct evidence for the interaction between Hsp47 and collagens came from experiments in which a chemical cross-linker was used to create covalent bonds between attached proteins (Nakai et al., 1992). Hsp47 was found to be coprecipitated with newly synthesized procollagen chains in these studies. The proteins were also coprecipitated when cells were treated with α–α-dipyridyl, an iron-chelating agent that blocks lysine and proline hydroxylation thereby

preventing triple helix formation of collagens. Hsp47 is found associated with polysome-bound pro1α procollagen chains along with other chaperones such as Grp78, Grp94 and PDI (protein disulfide-isomerase) and these chaperones are proposed to function in a series of coupled or successive reactions during procollagen production (Melnick et al., 1994; Hammond and Helenius, 1994; Ferreira et al., 1994) and in triple helix formation (Natsume et al., 1994; Sauk et al., 1994; Hu et al., 1995). Biochemical analysis has established a relatively low (10^{-2}/sec) dissociation constant and a relatively high association rate constant ($2x10^4$/Mol sec) between Hsp47 and various collagens (Natsume et al., 1994) indicative of a transient interaction. Hsp47 and procollagen traverse to the pre-Golgi intermediate vesicles from the ER together (Smith et al., 1995/a), indicating that Hsp47 might also have a role in the transportation of procollagen triple helices. Consistent with this hypothesis is the fact that all Hsp47s isolated up to date contain an KDEL ER-retention sequence (Saga et al., 1987; Nakai et al., 1990; Hirayoshi et al., 1991; Takechi et al., 1992; Pearson et al., 1996) which is responsible for the recycling of Hsp47 molecules to the ER, possibly mediated by KDEL-receptors (Satoh et al., 1996).

The role of Hsp47 as a collagen-specific chaperone is further supported by expression studies which have revealed coordinate expression of these genes in a number of collagen secreting cell-lines (Nakai et al., 1990; Takechi et al., 1992; Clarke et al., 1993; Satoh et al., 1996). For example, the synthesis of Hsp47 and type I collagen coordinately decreases after malignant transformation of fibroblasts (Nagata et al., 1988; Nakai et al., 1990). Conversely, the synthesis of both Hsp47 and type IV collagen markedly increases during the differentiation of the mouse teratocarcinoma cell line F9 (Kurkinen et al., 1984; Takechi et al., 1992). Hsp47 and Type I and III collagens are clearly induced during the progression of rat liver fibrosis caused by carbon terachloride administration (Masuda et al., 1994).

To date the majority of the studies examining Hsp47 have focused on its interaction with collagens as well as its expression using biochemical assays and cultured cells. There are few studies which have compared *hsp47* expression to that of different collagen genes during development in vivo. Miyaishi et al. (1992) examined Hsp47

distribution among various chicken tissues while others have compared *hsp47* expression to that of type I collagen in developing femurs and during tooth eruption in mice (Schroff et al., 1993; 1994). Also Hsp47 has been shown to be present in both neuroectoderm and mesoderm cells as well as in neural crest cells during neural tube closure and neural crest cell migration in mouse (Li et al., 1993) but collagen synthesis in these animals was never examined. Despite these efforts, a thorough analysis of *hsp47* and collagen gene expression in early stages of development has been lacking prior to this study.

1.2.6. Hsp90 family

Members of the *hsp90* gene family have been isolated from a wide range of organisms including the bacteria (HptG; Bardwell and Craig, 1987), *Drosophila* (Blackman and Meselson, 1986), yeast (Finkelstein and Strausberg, 1983; Borkovich et al., 1989) and numerous vertebrate and plant species (for review see Gupta , 1995). In eukaryotic cells, specific Hsp90 homologues exist in the cytosol and in the endoplasmic reticulum. The latter protein is often referred to as Grp94 (glucose-regulated protein; Sorger and Pelham, 1987) or Erp99 (endoplasmic reticular protein; Mazzarella and Green, 1987) and works in cooperation with BiP and Hsp47 in collagen maturation and possibly carries out other functions in the ER (see chapter 1.2.5.3). Two Hsp90 homologues exist in the cytosol of vertebrate and yeast cells. *hsp90α* (*hsp86* in mouse) is expressed only at a very low level constitutively but it is highly inducible by heat shock, whereas *hsp90β* or heat shock cognate 90 (*hsc90*; *hsp84* in mouse) is strongly expressed under normal conditions and only slightly inducible by various stresses. To date no mitochondrial homologue of Hsp90 has been found. However, cloning of a plastid-specific Hsp90 homologue has recently been reported (Schmitz et al., 1996). Also, a novel cytoplasmic Hsp90 homologue has been identified with a molecular weight of 75 kDa (Hsp75; Chen et al., 1996/b). Its homology with Hsp90α and Hsp90β, the other two cytosolic Hsp90 proteins is significantly lower and its origin during evolution is still unclear. It is expressed ubiquitously in the cytoplasm and relocated to the nucleus after heat shock. Also, p75 binds to the cell-cycle

regulatory retinblastoma (Rb) protein in vitro, and has the ability to refold denatured Rb protein into its native conformation. Considering recent evidence indicating the involvement of Hsp90 in the HSF1 regulatory cycle during heat shock, and the fact that the other Hsp90s did not show any stress-dependent translocation, this protein might be a good candidate that travels with the complex to the nucleus after trimerization.

The various Hsp90 proteins (except for Hsp75) show a remarkable homology over their entire length (eg. minimum of 40% identity between *E. coli* and vertebrates plus an additional 16-20% conservative amino acid replacement). The α and β group of cytosolic homologues in vertebrate species show about 87% identity to each other. This is much higher than the identity they show to the ER homologue from the same species (maximum is about 50% identity). Also, Hsp90α has higher sequence identity to Hsp90α of other species than to Hsp90β of the same species. Detailed phylogenetic analysis based on Hsp90 sequences indicates that the cytosolic and endoplasmic reticular forms constitute paralogous gene families which arose by a gene duplication event that took place very early in the evolution of eukaryotic cells (Gupta, 1995). An additional gene duplication event occured shortly after the emergence of the vertebrate line which is responsible for the appearance of the cytosolic Hsp90α and Hsp90β homologues in vertebrates (Krone and Sass, 1994). Sequence analysis has also revealed that the presence of the two yeast cytosolic homologues which are not the result of this event but rather a duplication that affected only members of the Fungi kingdom (Gupta, 1995).

1.2.6.1. Hsp90 structure

Cytosolic Hsp90 in eukaryotes can represent up to 1-2% of the total cellular proteins under normal growth conditions (Buchner, 1996). Hsp90α and Hsp90β are present mainly as α–α and β–β homodimers (Minami et al., 1991). In addition, a minor population of Hsp90β but not Hsp90α exists as monomers. An electron microscopic study has revealed that the Hsp90 dimers have two heads linked by a

flexible portion (Koyashu et al., 1986). Deletion analysis experiments have revealed that dimer formation is mediated by the carboxy-terminal 191 amino acids in which the C-terminal of one subunit associates with the adjacent region of the other subunit (Nemoto et al., 1995). In other experiments, removal of the C-terminal 49 amino acid residues were sufficient to inhibit dimer formation. In contrast, loss of 118 amino acid residues on the N-terminal of Hsp90 did not affect dimerization (Minami et al., 1994). Hsp90 also has an N-terminal ATP-binding site (Prodromou et al., 1997), possesses a relatively weak ATPase and has a serine-threonine autophosphorylation activity (Csermely and Kahn., 1991; Nadeau et al., 1993). The significance of these features is still unclear since the ATPase activity does not appear to be necessary for dimerization (Nadeau et al., 1993). Recently it has been shown that Hsp90 possesses two chaperone (peptide-binding) sites located in the N- and C-terminal fragments of the protein. The C-terminal fragment binds to partially folded proteins in an ATP-independent manner, while binding of denatured proteins to the N-terminal fragment is enhanced by ATP-binding (Scheibel et al., 1998).

1.2.6.2. Hsp90 as a chaperone

As discussed earlier, 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 fact that the ER lacks an Hsp60 homologue and the low amount of cytosolic TriC protein (Hsp60 homologue) is unlikely to be able to cope with the enormous bulk of proteins synthesized on free ribosomes, suggested Hsp90 as a potential chaperone to carry out the folding process in these compartments (for review see Frydman and Hohfeld, 1997; Johnson and Craig, 1997). Indeed Hsp90 has several features which make it a plausible candidate for this function. It has an ATP-binding site as well as ATP-ase activity (see above), basic requirements for all other chaperones. Also Hsp90 appears to bind substrates which are in the "molten globule state", possessing secondary and some tertiary structure (Jakob et al., 1995). In vitro and in vivo chaperoning activity of Hsp90 for artificially

denatured substrates has been reported under both control and stress conditions (Wiech et al., 1992; Jakob et al., 1995; Yonehara et al., 1996; Thulasiraman and Matts, 1996; Hartson et al., 1996; Dittmar and Pratt, 1997). Moreover, inhibition of Hsp90 function facilitates protein degradation in certain cells, suggesting that Hsp90 may be part of a protein quality control system that causes either enhanced refolding or degradation of cellular proteins during recovery from stress (Schneider et al., 1996). Further, degradation of mutant forms of the cell-cycle regulatory protein p53 by the 20S proteasome required the presence of Hsp90 (Whitesell et al., 1997). These results, along with recent data for its role in steroid receptor maturation and in Src and Raf-mediated signaling (see below), suggest that a folding pathway involving transfer of substrates from Hsp70 to an Hsp90-containing complex (see chapter 1.2.6.4.) indeed exists.

1.2.6.3. Geldanamycin, a specific Hsp90-binding agent

Research to identify the in vivo function of Hsp90 in cells recently received unexpected help from pharmacologists and biochemists who set out to find kinase inhibitors which might be useful as anticancer drugs. These researchers have identified several actinomycete fermentation products of the benzoquinone ansamycin class (Figure 1.), namely geldanamycin (GA), herbimycin A and macbecin (17-hexamethylene-17-demethoxy-geldanamycin) as potential candidates (Sasaki et al., 1970; Omura et al., 1979, Muroi et al., 1980; Uehara et al., 1985). Members of this class of agents feature a benzoquinone group with various side chains embedded into the ring of a classical ansamycin molecule which can itself bear various side groups. These agents all inhibit the growth of fibroblasts transformed by a variety of oncogenes such as *src, yes, fps* or *erbB1* and also revert the morphology of these cells from transformed to normal (Uehara et al., 1985; 1988; Murakami et al., 1988). Although originally described as tyrosine-kinase inhibitors, these molecules were found inactive when added directly to purified tyrosine kinases at concentrations up to 1500 times greater than their effective in vivo dose (June et al., 1990; Whitesell et al., 1992). Additionally, several attempts to demonstrate

Figure 1. Chemical composition of geldanamycin and related benzoquinone ansamycins. (Adopted from Whitesell et al., 1994). GA: geldanamycin; HA: herbimycin A; HD: 17-hexamethylene-17-demethoxy-geldanamycin (macbecin); GM: geldampicin

direct association of ansamycins with tyrosine-kinases in vitro and in vivo have been unsuccessful (Whitesell et al., 1994; Miller et al., 1994). Thus, they were hypothesized to exert their inhibitory activity indirectly by somehow destabilizing these enzymes (Uehara et al.,1989). Consistent with this hypothesis is the fact that binding of geldanamycin to Hsp90 and destabilization of Hsp90-pp60$^{v\text{-}src}$ complex occurs at nanomolar concentrations (Whitesell et al.,1994). These levels are similar to the concentrations of ansamycins which are effective inhibiting the above mentioned kinases in vivo in cell cultures. Recently the crystal structure of the geldanamycin-Hsp90 complex has been described in detail (Stebbins et al., 1997). There is currently a debate about whether the GA-binding region of Hsp90 is involved in substrate-binding (Stebbins et al., 1997) or whether is it the ADP-ATP switch domain that regulates conformation changes (Grenert et al., 1997, Prodromou et al., 1997).

Using benzoquinone ansamycins researchers have been able to pinpoint several molecules and groups of molecules that require Hsp90 for their proper function. Among these were the subset of the steroid receptor family (Smith et al., 1995/b; Chen et al., 1996/a; Whitesell and Cook, 1996; Segnitz and Gehring, 1995), tyrosine- (Whitesell et al., 1994; Miller et al., 1994) and serine-threonine kinases (Schulte et al., 1995; 1996), and reverse transcriptase (Hu and Seeger, 1996). In the following sections, I will discuss the role Hsp90 plays in the regulation of these and other (bHLH transcription factors) important signal transduction molecules.

1.2.6.4. Role of Hsp90 in the maturation of steroid receptors

During the last decade, there have been three lines of independent investigation which led to the conclusion that Hsp90 is involved in steroid receptor function. First, biochemical analyses have shown that a subclass of the superfamily of steroid receptors (including glucocorticoid, mineralcorticoid and progesterone receptors) is associated with Hsp90 (Ziemiecki et al., 1984; Howard and Distelhorst, 1988). Similar experiments have also revealed that other steroid receptors including the thyroid and retinoic acid receptors do not show

similar stable association (Dalman et al., 1990; 1991). Second, functional evidence from mutagenesis screens in *Saccharomyces cerevisiae* carried out to isolate mutants with decreased steroid receptor function yielded several mutants in which the *hsp90* gene was altered (Bohen and Yamamoto, 1993). In correlation with this, *hsp90* mutagenesis experiments demonstrated significantly reduced glucocorticoid receptor and src tyrosine kinase function in yeast cells (Nathan and Lindquist, 1995). Finally, evidence regarding Hsp90 function was provided by the application of geldanamycin in experiments examining steroid receptor function in vitro and in cell cultures (Smith et al., 1995/b; Johnson and Toft, 1995; Whitesell and Cook, 1996; Czar et al., 1997, Segnitz and Gehring, 1997; Bamberger et al., 1997). The results of these studies as well as reconstitution analyses (Segnitz and Gehring, 1995; Dittmar and Pratt, 1997) have provided a thorough understanding of the role Hsp90 plays in steroid receptor maturation. As I will describe below, the components of the complex and the mechanism of the process are similar to that of the cytosolic Hsp70-Hsp90 folding machinery. The major difference is that the association between the steroid receptor and the Hsp90-containing aporeceptor complex is fairly stable compared to the one found in the general folding process. The role of this stability is thought to be to keep the receptor in a ligand-binding, active state for an elongated period of time.

Steroid receptors are recovered from hormone-free cells as large, 9S complexes (Toft and Gorski, 1966). This 9S complex does not bind DNA but it can be transformed to a 4S DNA-binding form by hormone addition. Over the last 5 years the components of this heterocomplex have been described in considerable detail (Pratt and Toft, 1997) and include Hsp90 (in a dimeric form), Hsp70, p60 (Hop), p23 and a variable PPI (peptidyl-prolyl isomerase) component (FKBP51-FKBP54, Cyp-40). Its assembly requires ATP, Mg^{2+} and K^{+}. Receptor truncation (Pratt et al., 1988) and protein cleavage (Denis et al., 1988) studies have established that Hsp90 interacts with the hormone-binding domain of the steroid receptor and that the receptor immediately loses its ability to bind its ligand if Hsp90 dissociates (Bresnick et al., 1989). Once the hormone is bound to the receptor, it remains bound even after dissociation of Hsp90, but if the hormone-binding site is unoccupied at

the time of Hsp90 dissociation, the resulting receptor cannot bind hormone (Scherrer et al., 1990; Hutchinson et al., 1992, 1994; Dittmar et al., 1996).

The interaction between the steroid receptor, Hsp90 and other members of the complex has recently been the focus of several studies. The most thoroughly examined interaction is the one between Hp90 and Hsp70 and this binding provides a source of debate between two groups. While Chen et al., (1996/a) support a model in which the two proteins are bound together by p60 (Hop), Czar et al., (1994) present an opinion where the two heat shock proteins bind directly to the receptor, allowing that p60 is a necessary component for optimal interaction. Another member of the complex is p23, a unique and ubiquitous acidic protein (Johnson et al., 1994) which binds to the N-terminal of Hsp90 directly in an ATP-dependent manner (Johnson and Toft, 1995) and stabilizes the complex. In addititon, there are several immunophilins involved in the final step of steroid receptor activation, among them FKBP52 (and in some cases FKBP51 and FKBP54) and Cyclosporin A-binding protein 40 (Cyp-40). These molecules are ubiquitous and conserved proteins that bind immunosupressant drugs such as FK506 and cyclosporin. All of them have peptidyl-prolyl isomerase activity, suggesting that they may have a direct role in protein folding (Peattie et al., 1992; Ratajczak et al., 1993; Kieffer et al., 1993). Recently Freeman and coworkers (1996) have shown that Cyp-40 has chaperone features independent of Hsp90, although in refolding assays it could not completely refold a denatured protein by itself. These molecules bind to Hsp90 through their tetratricopeptide domain found at their C-terminal (Hoffmann and Handschumacher, 1995). There is some evidence which shows that FKBP52 is associated with the microtubule network in interphase cells and with the mitotic apparatus (centrosome, spindles) during cell division (Perrot-Applanat et al., 1995) although the functional significance of this is still unclear.

Immunoadsorption experiments have led to two different models of complex formation. Pratt and coworkers proposed that all of the components are preassociated in an aporeceptor complex which they call the foldosome (Pratt, 1997). In contrast, an earlier model by

Smith (1993/a) suggests that there is an ordered step by step mechanism of component assembly in the complex during its interaction with the steroid receptor. These models will require further examination to establish the real version of events.

1.2.6.5. Functions of Hsp90 in regulating bHLH transcription factors

Hsp90 is implicated in the regulation of two transcription factors belonging to the basic helix-loop-helix family: the dioxin receptor and the muscle regulatory factor, MyoD.

The dioxin receptor mediates activation of cytochrome-P-450, an aryl hydrocarbon hydroxylase enzyme, which is involved in xenobiotic metabolism (Poellinger et al., 1992). Since the receptor binds a variety of halogenated hydrocarbons it is sometimes referred to as the aryl-hydrocarbon (Ah) receptor. It has a basic helix-loop-helix DNA-binding domain and in its inactive state is localized in the cytoplasm (Polenz et al., 1994). It separates as a 9S receptor-Hsp90-containing heterocomplex in cell fractionation experiments (Perdew, 1988; Wilhelmsson et al., 1990), not unlike the complex involved in steroid receptor maturation. Hsp90 also binds to the ligand-binding domain and in so doing maintains the receptors in an active, ligand-binding state. Activation by the appropriate ligand induces Hsp90 dissociation and receptor translocation to the nucleus where they bind to their respective transcription elements. However, there are also several differences in the mechanisms by which these receptors work in contrast to the steroid receptor. First, after activation and nuclear translocation, the dioxin receptor heterodimerizes with another bHLH transcription factor called Arnt to form the active transcription factor (Hankinson, 1994). It was also shown that Hsp90 not only binds to the hormone-binding domain but also to the bHLH domain of the dioxin receptor to prevent its premature dimerization (Whitelaw et al., 1993; Antonsson et al., 1995; Whitelaw et al., 1995). Ligand-induced receptor activation induces the release of Hsp90 and concomitant unmasking of the dimerization and DNA-binding activities of the receptor (Pongratz et al., 1992; Whitelaw et al., 1993; Wilhelmsson et al., 1990). Recently a similar activation mechanism involving an Hsp90-containing

heterocomplex has been described for another bHLH/PAS transcription factor called Sim (McGuire et al., 1996).

The idea that Hsp90 might be involved in regulating the myogenic regulatory factor, MyoD (see Section 1.5. for details) rose when Shaknovich et al., (1992) isolated a murine *hsp84* cDNA clone after screening a cardiac expression library with an antibody made against an amphipathic helix peptide. In vitro expression and gel shift analysis proved that ectopically expressed recombinant or native Hsp90 converted inactive MyoD into an active, DNA-binding conformation. The conversion occurs through a proposed transient complex which the authors could not detect due to its instability. The activation process occurs in a dose-dependent fashion and does not require ATP. MyoD is believed to be biologically active when it heterodimerizes with other members of the E-box family like, E12, E47 or E2A (Lassar et al., 1991; Chakraborty et al., 1992). Examining this complex, Shue and Kohtz (1994) reported that Hsp90 folded E12/MyoD heterodimers as well as E12/E12 homodimers into their active conformation. The authors also demonstrated using deletion mutant analysis that the presence of a carboxy-terminal 48 amino acid region is necessary for Hsp90 to be active. These results when combined with expression data (discussed in Section 1.2.6.7.) strongly suggest that Hsp90 plays a role in myogenesis.

1.2.6.6. Hsp90 and protein kinases

Hsp90 has been shown to affect the function of a wide variety of tyrosine and serine/threonine kinases. Recent developments in signal transduction research have demonstrated a close link between most of the kinases that require Hsp90 for their activity. A variety of receptors for polypeptide ligands (including those for insulin, EGF, PDGF, NGF) transduce signals by activating the MAP (mitogen-activated protein) kinase family of serine/threonine kinases (also called Erks for extracellular signal-regulated kinases) (for review see Crews and Erikson, 1993). The receptors themselves are tyrosine kinases that undergo ligand-induced autophosphorylation leading to activation of membrane-bound Ras protein. Subsequent binding of Ras to the Raf-1

serine/threonine kinase leads to phosphorylation of another kinase called Mek (MAP kinase kinase) by Raf-1. Mek in turn phosphorylates and activates Erk (MAPK) which is the terminal effector in this pathway and phosphorylates transcription factors (such as Jun, Elk, c-Myc) that regulate gene expression and thereby, the proliferation and differentiation state of cells. This receptor-mediated signaling system can be short-circuited by the viral oncoprotein v-Src, which can activate Raf-1 directly (Williams et al., 1992; Pumiglia et al., 1995). v-Src and related kinases (including yes, lck, fyn) form a subfamily of nonreceptor tyrosine kinases (Parsons and Weber, 1989) most of which have been shown to require Hsp90 for their function. As v-Src is the most thoroughly studied of them, I will initially describe its relationship with Hsp90. Immediately after translation, $pp60^{v\text{-}src}$ can be coimmunoprecipitated with Hsp90 (Brugge, 1986). In this complex $pp60^{v\text{-}src}$ is unphosphorylated, incapable of autophosphorylation and inefficient in phosphorylating exogenous substrates (Brugge, 1986). Hsp90 binds to the catalytic domain of v-Src, masking its kinase activity until it reaches its destination in the cell membrane (Jove et al., 1986). Earlier reports have shown that v-Src overexpression in yeast induces growth arrest. Genetic experiments have demonstrated that a mutation which lowers the level of *hsp90* expression relieved this cell-cycle arrest and rescued growth of the cells (Xu and Lindquist, 1993). Furthermore, the eight point mutations created by Nathan and Lindquist (1995) in yeast not only decreased the activation of ectopically expressed mammalian glucocorticoid receptor (see previous chapter) but also resulted in much lower v-Src activity. The v-Src complex is somewhat different from the one found in association with steroid receptors. It does not contain the FKBP52 immunophilin; instead it has a protein termed p50, the function of which in the complex is controversial (Whitelaw et al., 1991). As the complex is responsible for targeting v-Src to the plasma membrane it was suggested that p50 might have a role in specifying this activity of the complex (Owens-Grillo et al., 1996; Stancato et al., 1993). However, p50 has recently been identified as the mammalian homologue of the yeast Cdc37 cell-cycle regulatory protein. It is found in a complex that also contains CDK4 (Dai et al., 1996), one of the major cyclin-dependent kinases regulating

G1-S transition, thereby providing a link between these signal transduction pathways, Hsp90 and the regulation of cell proliferation and differentiation (Stepanova et al., 1996). Furthermore, Cdc37 not only acts as an accessory factor in the complex but also has its own chaperoning capabilities, although Cdc37 and Hsp90 are not fully interchangeable (Kimura et al., 1997).

Two other proteins of the MAP signaling pathway, Raf-1 (Stancato et al., 1993; Wartmann and Davis, 1994) and Mek (MAPKK, Stancato et al., 1997) are also recovered from the cytosolic fraction of cells in native complexes with Hsp90. As described earlier, activated Ras binds to the N-terminal regulatory domain of Raf-1 (Zhang et al., 1993) in a transient manner . This serves to recruit Raf-1 to the cell membrane (Traverse et al., 1993) a step that is necessary for Raf-1 activation. Hsp90 binds to the C-terminal catalytic domain of Raf-1 (Stancato et al., 1993) and has been proposed to have a role in targeting the complex to the cell membrane (Pratt, 1993). The complex is similar to the one regulating v-Src activity and contains p50 instead of the FKBP52 found in steroid receptor complexes (Stancato et al., 1993). It is likely that FKBP52 and p50 exist in separate heterocomplexes and somehow the substrates (steroid receptors or protein kinases respectively) are selective for between these alternatives.

Disruption of the Raf-1-Hsp90 complex results in destabilization and loss of Raf-1 from the cell despite increased Raf-1 synthesis. It also causes the disruption of Raf-1-Ras association and prevents transport of Raf-1 to the plasma membrane (Schulte et al., 1995). Further examination of the downstream events of the pathway have revealed that this destabilization results in the disruption of the Raf-1-MEK(MAPKK)-MAPK-activated protein signaling as demonstrated in NIH3T3 cells by Schulte and coworkers (1996).

Finally, a developmentally important protein tyrosine kinase called Sevenless has also been shown to require Hsp90. Sevenless is necessary for the differentiation of the R7 photoreceptor neuron development of the compound eye of *Drosophila* (Tomlinson and Ready, 1986; Banerjee et al., 1987; Hafen et al., 1987). Like v-Src, Sevenless uses the Ras-Raf-1-MAPK signaling pathway to transduce its signal to the photoreceptor precursor cells resulting in the activation of

R7 cell differentiation fate. In the absence of Sevenless the R7 precursor cell adopts an alternative, nonneuronal fate, becoming a lens-secreting cone cell (Zipursky and Rubin, 1994). In a mutagenesis screen which was set up to isolate enhancer mutations of *sevenless,* Cutforth and Rubin (1994) isolated *hsp83* as an enhancer of *sevenless.* Interestingly the cell-cycle regulatory gene *cdc37* also appeared as a *sevenless* enhancer in the screens, a result consistent with the recent discovery that Cdc37 is the yeast homologue of the mammalian p50 member of the Hsp90 heterocomplex (Stepanova et al., 1996). These results also confirm and partially explain earlier reports which showed cell-cycle-dependent expression and activity of Hsp90 in a variety of cells and tissues (Jerome et al., 1993; Lovric et al., 1994; Galea-Lauri et al., 1996; Walsh et al., 1993). A similar genetic screen in *Drosophila* was set up to identify supressor mutants of *wee1,* which codes for a tyrosine kinase involved in the phosphorylation of Cdc25, the main regulatory protein of the G2-M cell-cycle checkpoint (Aligue et al., 1994). Surprisingly an *hsp90* mutant was isolated which could suppress the cell-cycle arrest caused by Wee1 overproduction. Moreover Wee1 and the mutant Hsp90 coimmunoprecipitated showing that the two proteins interact directly. The mutant cells in the absence of Wee1 overproduction underwent premature mitosis. These data strongly indicate that formation of active Wee1 requires functional Hsp90 in *Drosophila* embryos.

1.2.6.7. Expression of *hsp90* genes during embryogenesis

In the past sections I have presented an overview of the evidence gathered which suggest Hsp90 as a potentially important molecule in cell-cell signaling processes. As these pathways are likely to be involved in embryonic development, Hsp90 is likely to play a role in these inductive interactions occuring during embryogenesis. Below, I will summarize what is known about the spatio-temporal pattern of *hsp90* expression in various model animal systems.

Until 1994, expression of *hsp90* genes during normal development had only been characterized in detail in invertebrate organisms (for review see Heikkila, 1993/a,b). The only cytosolic *hsp90*

homologue (named *hsp83*) in *Drosophila* is expressed in the ovary and in the nurse cells of the oocytes and its mRNA is subsequently deposited into the oocyte (Zimmerman et al., 1983). The maternally synthesized transcripts are localized to the posterior pole of the embryo by a specific mechanism which involves general degradation and local (in the posterior region) protection (Ding et al., 1993). These authors suggested that the gene *oskar* was involved in this posterior protection as mislocalization of *oskar* mRNA to the anterior pole of the embryo led to the anterior protection of *hsp83* mRNA. Later in development, high levels of maternal *hsp83* mRNA are also present in pole cells during their migration in the gonads of the embryos, larvae and adults (Zimmerman et al., 1983; Xiao and Lis, 1989). Zygotic *hsp83* expression commences at the syncytial blastoderm stage and is regulated by the anterior morphogen, bicoid. During metamorphosis *hsp83* mRNA levels correlate with peaks in ecdysone titer suggesting hormone-regulated transcription of this gene (Thomas and Lengyel, 1986).

Dalley and Golomb (1992) have examined *hsp90* expression in another popular model organism, *Caenorhabditis elegans*. Elevated levels of *hsp90* expression were reported in the dauer larva stage, which is a developmentally arrested form during embryogenesis and occurs due to unfavourable growth conditions. *hsp90* mRNA levels decline sharply after reinstatement of a food source, which also results in the larvae reentering the normal developmental pathway.

In vertebrates, reports on *hsp90* expression were restricted primarily to studies examining stress-induced expression. However, recent developments in analyzing Hsp90 functions in biochemical experiments (see previous chapters) indicated that Hsp90 interacts with several important signaling molecules. These results renewed the interest in studying *hsp90* expression under normal conditions in vertebrates. One of the most thoroughly studied organs is the mammalian brain. Widespread constitutive expression of *hsp90* has been found in the brain of adult rats and rabbits (Izumoto and Herbert, 1993; Itoh et al., 1993; Gass et al., 1994; Quarishi and Brown, 1995). It has also been demonstrated that *hsp90* mRNA distribution was restricted to the neurons and not to the glial cells in the adult rabbit brain (Quarishi and Brown, 1995). Moreover this pattern of *hsp90* expression

does not change after heat shock indicating that it is likely to be the result of *hsp90β* (*hsc90*) gene activity. As the study was carried out using polyclonal antibodies further examination is required to confirm this hypothesis.

Another adult organ which was scrutinized for the presence of *hsp90* mRNA is the mammary glands of pregnant and lactating mice (Catelli et al., 1989), where elevated levels of *hsp90* mRNA and protein were also observed. This phenomenon is likely related to the recent discoveries proving Hsp90`s role in steroid receptor function (see chapter 1.2.6.4.).

The only previous studies describing *hsp90* expression under normal conditions found that the two members of the mouse *hsp90* gene family, are relatively abundant in the testis and ovary (Lee, 1990; Gruppi et al., 1991). *hsp86* (the inducible form of *hsp90* in mouse) mRNA was found at high levels in germ cells while *hsp84* (the constitutive *hsp90* gene) mRNA occured primarily in the somatic compartment. Also immunoprecipitation analysis revealed that Hsp70 was coprecipitated with both forms of Hsp90 in cell homogenates (Gruppi and Wolgemuth, 1993).

Also, in the last few years the spatio-temporal pattern of *hsp90* expression has been characterized during embryogenesis in several vertebrate model organisms, although as I demonstrate below the data collected so far is restricted primarily to a few tissues and developmental stages. The first step in this line of research was the investigation of *hsp90* expression in developing oocytes. Strong and continous expression of both *hsp90* genes has been described in developing oocytes of the amphibia *Pleurodeles waltl* (Coumailleau et al., 1995). The possible role for Hsp90 in oogenesis came from a study which found that a complex containing Hsp90, Hsp70 and other proteins bound to centrin in arrested oocytes (Uzawa et al., 1995). Centrin is a Ca^{2+}-binding protein associated with centrosomes and mitotic spindles (Baron et al., 1994; Errabolu et al., 1994). It was also shown that the Hsp90-containing complex dissociates from centrin after activation of the oocytes suggesting that it might be responsible for keeping the centrin in an inactive state (in which it is inaccessible to centrosomes), thereby preventing premature activation of the oocytes.

These reports provided only a limited picture of *hsp90* expression and offered no insight into *hsp90* expression during vertebrate embryonic development. Recent studies examining this question in *Pleurodeles* and zebrafish have presented a more detailed analysis of *hsp90α* and *hsp90β* mRNA distribution during embryogenesis. In *Pleurodeles* two *hsp90β* transcripts with different sizes (2.5 and 3kb) were characterized during development which was surprising as no reports on the existence of two *hsp90β* genes have been published previously in other vertebrate (Coumailleau et al., 1997). It is likely however that in fact the two mRNAs represent *hsp90α* and *hsp90β* respectively as the authors used a heterologous, chicken *hsp90* cDNA probe. This is supported by the fact that *hsp90α* and *hsp90β* homologues in the zebrafish show a similar size difference (Krone and Sass, 1994). Coumailleau and coworkers also examined the spatial distribution of *hsp90β* mRNA in neurula and tailbud stage embryos of *Pleurodeles*, where they found strong *hsp90β* (*hsc90*) expression in the neural tube, eye, branchial arches and in the differentiating somites. Furthermore, Hsp90β underwent nuclear translocation in the cells of the invaginating dorsal blastopore lip during gastrulation. Unfortunately, this report contains several questionable results due to technical problems (such as the use of heterologous probes) and the data has to be considered carefully when drawing conclusions.

Our laboratory has previously reported the PCR assisted cloning of zebrafish *hsp90α* and *hsp90β* cDNA fragments. Whole-mount in situ hybridization analysis revealed strong somite-specific expression of *hsp90α* during normal zebrafish embryogenesis (Sass et al., 1996). *hsp90α* mRNA appearance and distribution followed that of the myogenic regulatory factor gene, *myoD* with a 1.5 hr delay. Furthemore *hsp90α* expression was down-regulated along with *myoD* in differentiated muscles of the trunk at a time when levels of mRNA encoding the muscle structural protein *α-tropomyosin* remained high. Similar coordinate expression was also found in the pectoral fin bud of two-day-old embryos. Moreover, this pattern of *hsp90α* and *myoD* coexpression was also detected during chicken somitogenesis (Sass and Krone, 1997) and in *floating head*, *notail* and *spadetail* mutant

zebrafish embryos (Sass and Krone, unpublished, detailed later). In combination, these data strengthen the argument that *hsp90α* may have a role in muscle differentiation of vertebrate embryos.

1.3. Zebrafish as a model vertebrate system

The zebrafish (*Danio rerio*) is a small tropical freshwater fish, well known to home aquarium enthusiasts. It has long been used in fisheries biology and toxicology studies (Viktor et al., 1982; Ruoppa and Nakari, 1988), but recent developments have also made it a powerful developmental model system. The main advantages and disadvantages of zebrafish as a model system are summarized in Table 1. (modified from Lele and Krone,1996).

One of its most significant features is the large number of embryos available at relatively low cost. Also, space requirements are much smaller than any other widely used vertebrate model system and embryos can be produced on a daily, inducible basis (spawning is regulated by appropriate photoperiod). These features combined with the rapid development and short generation cycle (3 months) provide a powerful tool to study developmental processes and their regulation. Additionally, as a result of Kimmel, Westerfield and coworkers (Oregon, USA) an excellent, comprehensive and frequently updated laboratory resource manual (Westerfield, 1995) as well a thoroughly characterized developmental staging series have been published (Kimmel et al., 1995). The developing embryos are completely translucent which makes the use of the expensive albino embryos in whole mount in situ hybridization experiments (as required with *Xenopus*) unnecessary. On the negative side, techniques available in mouse such as the powerful gene knockout technology are not developed in the zebrafish. Also, there is a paucity of veterinary knowledge as compared to mammalian systems, which can only be partly compensated by the cheapness of the replacement animals. Another ambigous question is the evolutionary position of fishes which can be an advantage or a disadvantage depending on the type of question targeted. As the closest vertebrate link to invertebrates, teleosts (bony fishes) represent a good candidate for homology-

Advantages	Disadvantages
low cost maintenance	lack of extensive veterinary knowledge
low space requirement on a per animal basis	lack of certain molecular techniques (e.g. gene knockout technology has not yet been developed)
rapid generation cycle (egg to mature adult in 2-3 months)	evolutionary position (primarily a problem for some forms of medically-related research)
large number of offspring	
well characterized developmental staging series (Kimmel et al., 1995)	
rapid development (egg to hatching in 2-3 days)	
embryos well suited for experimental manipulation and microinjection	
translucent embryos	

Table 1. Advantages and disadvantages of zebrafish as a model system.

based cloning of genes which had been isolated previously from invertebrates. But the evolutionary distance and the consequent

anatomical differences between fishes and mammals sometimes make it difficult to interpret data obtained in fishes for human application.

There is one powerful technique however for targeting questions concerning the regulation of development which can only be carried out in zebrafish of all the current vertebrate model systems (Concordet and Ingham, 1994). This technique is saturation mutagenesis, a method which uses chemicals or gamma rays to randomly introduce mutations into every locus of the genome. The number of induced mutations/genome can be changed by increasing or decreasing the dose of the chemicals or radiation. In this way, every gene involved in regulating embryonic development can be individually mutated and characterized, provided that the offspring of large numbers of treated individuals are examined. This experiment has carried out been earlier in *Drosophila,* making it the single most important invertebrate developmental model system. The results of these experiments led to the isolation and functional characterization of several of the most important genes involved in regulating development. However, the complexity of vertebrate embryogenesis and the size of their genome when compared to invertebrates suggest that there are a number of novel genes involved in the vertebrate developmental regulation process. These novel genes can not be identified by conventional methods such as homology-based cloning. Describing induced mutations, combined with high resolution mapping and positional cloning techniques can provide a solution to this problem. As a result of the cheapness, high reproduction rate and rapid development of zebrafish, two saturation mutagenesis experiments have been carried out and their preliminary results were recently reported in a special issue of the journal Development (Haffter et al., 1996; Driever et al., 1996). These reports confirmed earlier expectations producing hundreds of mutations affecting development most of which occured in so far unidentified genes. Now, it is only a matter of time, manpower and cost to sort through all these mutations and isolate the genes affected which will no doubt take us closer to the complete understanding of embryonic development at the molecular level.

1.4. Notochord development, somitogenesis, and early muscle differentiation in zebrafish embryos

Gastrulation in the zebrafish begins at around 5.5 hrs of age. At this point, the animal cap covers half of the large yolk cell (50% epiboly) and major cell movements begin which result in the formation of the three germ layers. Cells start to involute at the margin of the animal cap (marginal cells) forming a visible thickening known as the germ ring. At the same time, there is a significant cell migration towards the future dorsal side of the embryo (cell conversion). This latter process results in the local thickening of the involuting marginal hypoblast called the shield which is the equivalent of the blastopore lip in *Xenopus* and of the Hensen`s node in amniotes. Simultaneously with the convergence and extension movements a microtubule-driven animal cap cell migration begins at the marginal zone of the embryo. This process, called epiboly, eventually leads to the complete engulfment of the yolk cell.

Fate maps of 50% epiboly stage embryos created by single cell labelling and tracing experiments show that future notochord cells (along with the anterior plate mesoderm) are localized at the dorsalmost marginal cells of the embryo. As gastrulation begins these cells are the first to involute and migrate towards the anterior pole of the embryo. By 11 hrs, after the yolk plug disappears at the conclusion of the epiboly, the notochord can be distinguished as a separate structure. As somitogenesis begins, the notochord differentiates in an anterior-posterior fashion. Some of its cells vacuolate and swell to become the structural elements, whereas others form the notochord sheath, an epithelial monolayer that surrounds the organ (Kimmel et al., 1995). As development progresses, the notochord loses its signaling function regresses and becomes part of the vertebrae (nucleus pulposus).

In the zebrafish, two genes that are required for notochord development have been identified, *no tail (ntl)* and *floating head (flh)*. Loss of function mutations in *flh* cause a lack of notochord formation along the entire length of the embryo (Talbot et al., 1995) due to a shift in axial mesoderm fate to paraxial mesoderm (Halpern et al., 1995). The

no tail gene (the zebrafish homologue of the mouse *Brachyury (T)* gene) is also required for notochord formation (Halpern et al., 1993, Schulte-Merker et al., 1994). In contrast to *flh*, mutant *ntl* embryos have undifferentiated notochord precursor (axial mesoderm) separating the somites. The floor plate is also present though only as a discontinous line of cells, whereas muscle pioneer cells (see below) in the somites do not develop. As a result of these features it was proposed that *ntl* represents a gene which is downstream of *flh* in the notochord differentiation pathway.

In all vertebrates, skeletal muscle of the trunk and limbs arises from segmented arrays of paraxial mesoderm termed somites. Somites are partitioned into dorsal and ventral domains that contain progenitors for individual structures of the developing embryo. The ventral portion of the somite undergoes a mesenchymal transition and gives rise to the cells of the sclerotome which will contribute chiefly to the formation of the axial skeleton. The dorsal aspect of the somite remains epithelial and forms the two-layered dermomyotome. The inner, cellular compartment of the dermamyotome, which gives rise to muscle cells, is called the myotome whereas the remaining outer cell layer is responsible for forming the dermis of the skin. The myotome is further patterned to produce specific pools of progenitor myoblasts that contribute to different aspects of the developing musculature.

In amniotes, the developing musculature is divided into two separate populations; epaxial muscles, which attach to the vertebral column, and hypaxial muscles which migrate from the forming myotome to contribute to the muscles of the appendicular skeleton and ventral-lateral body wall (Ordahl and LeDourain, 1992). These cells derive from different regions of the dermomyotome in a strict temporal order. Specifically, epaxial muscles differentiate first from the medialmost cells of the dermomyotome while hypaxial myoblasts are specified later from migratory cells of the lateral dermomyotome, differentiating only at their destination.

Within the teleost myotome distinct myoblast populations can also be discerned that arise in a temporally and spatially separable order (Currie and Ingham, 1998). Teleosts lack a migratory hypaxial-like population of myoblasts, except for a few muscle progenitor cells which

putatively migrate to populate the fin rudiments. Therefore the majority of the teleost myotome forms the large axial muscles of the trunk which generate the force for the wave-like forward motion of fishes. Once the proper cells of the somites are committed to the myogenic lineages, differentiation of zebrafish axial muscles occur in two separable waves. The most medial cells of the zebrafish myotome, termed the adaxial cells due to their topographical position next to the notochord, are distinguished from the rest of the presomitic mesoderm by their early expression of myogenic bHLH regulatory genes (*myoD, myogenin*) and their large cubodial morphology (Devoto et al., 1996; Weinberg et al., 1996). From the adaxial cells arise the earliest striated cells of the zebrafish myotome termed muscle pioneers (MP; Felsenfeld et al.,1991) which are distinguished from other muscle cells by their expression of *engrailed (eng)*1and 2 genes (Ekker et al., 1992; Hatta et al., 1991). The non muscle pioneer adaxial myoblasts express slow twitch muscle characteristics, prior to migrating through the entire extent of the myotome to form a layer of subcutaneous slow twitch muscle (Devoto et al., 1996, Blagden et al., 1997). The remainder of the myotome differentiates as fast twitch muscle behind this wave of migrating slow muscle cells. Muscle pioneer cells, despite differentiating as slow twitch muscle cells, fail to migrate and remain axial up to 48 hrs in development, after which they extend or migrate to the surface and are thought to eventually give rise to the horizontal myoseptum.

1.5. Molecular mechanisms underlying somite and muscle differentiation

The molecular mechanisms that control formation and differentiation of the myotome have been examined in some detail (for a recent review see Rawis and Olson, 1997). The decisive step in the entry of somitic cells towards the muscle lineage is the induction and expression of myogenic regulatory factor (MRF) genes. These genes have been cloned and characterized in a wide variety of vertebrate organisms where they are expressed in a species-specific temporal order and act at multiple points of the cell cycle to control muscle cell

determination and differentiation (Lyons and Buckingham, 1992). Four MRF genes have been identified so far named *MyoD, myogenin, myf-5* and *MRF4*. The protein products of these genes are members of the basic helix-loop-helix (bHLH) superfamily of transcription factors and bind to specific regions of the DNA called the E-box. The E-box has a consensus sequence 5'-CANNTG-3' (N can be any nucleotide) found in the promoter of almost all muscle-specific genes including the MRFs themselves (Buskin and Hauschka, 1989; Edmondson et al., 1992). In the zebrafish, *myoD* and *myogenin* have been cloned and their expression characterized (Weinberg et al., 1996). Similar to the avian embryo, *myoD* is the first of these genes to be expressed in the paraxial mesoderm of zebrafish (Pownall and Emerson., 1992; Weinberg et al., 1996). Gene knockout experiments in mice have revealed a partial redundancy between *myf5* and *myoD* as targeted mutagenesis of one of these genes have had only little effect on myogenesis (Rudnicki et al.,1992; Braun et al., 1992) while double *myoD-myf-5* knockout experiments resulted in almost complete elimination of axial muscles (Rudnicki et al., 1993). On the other hand elimination of *myogenin* results in the lack of differentiated muscles suggesting that it functions downstream of the other two genes and is involved in the differentiation rather than in the determination step of the pathway (Hasty et al., 1993).

MEF2 (muscle enhacing factors) proteins comprise another group of transcription factors which is believed to be involved in muscle differentiation. These proteins share a conserved 56 amino acid region termed the MADS-box motif (for review see Duprey and Lesens, 1994). In the zebrafish, MEF2 proteins appear in the nuclei of the adaxial cells shortly after MyoD suggesting that it may be involved in muscle differentiation (Ticho et al., 1996). Supporting this hypothesis, other experiments revealed that MRFs and MEFs enhance each others` expression (Edmondson et al., 1992; Chambers et al., 1994; Cserjesi and Olson, 1991). Moreover, myogenin and MEF2 proteins have been shown to bind DNA cooperatively (Funk and Wright, 1992).

Recent information on the molecular mechanisms that regulate formation of the three distinct cell populations of the zebrafish myotome has arisen from mutagenesis experiments. Zebrafish

embryos homozygous for mutations which disrupt axial mesoderm formation (eg. *ntl, flh*) also have concomitant defects in the specification of adaxially derived slow muscle cells while lateral fast twitch muscle forms normally (Halpern et al., 1993; Talbot et al., 1995 Odenthal et al., 1996; van Eden et al., 1996) indicating that a notochord-derived signal(s) is necessary for proper adaxial cell differentiation. Members of the Hedgehog family of secreted glycoproteins are expressed within the axial mesoderm and have been shown to mimic its inducing activities, simultaneously acting to specify the formation of adaxial cells, the specification of slow muscle cell fate and the induction of MP cells from within the adaxial precursor cell population (Currie and Ingham 1996, Blagden et al., 1997). Also, a BMP4-like signal is proposed to antagonize Hedgehog signals in the dorsal and ventral regions of the myotome and thereby restrict differentiation of muscle pioneers to the region of the somite lateral to the notochord along the dorsal-ventral axis (Du et al., 1997). Although the Hedgehog signaling pathway has been described in detail in *Drosophila* there is not much known about it in teleosts. The zebrafish homologue of *patched*, the putative Hedgehog receptor has been cloned and its mRNA is present in areas surrounding *sonic hedgehog*-expressing tissues of the embryo (Marigo et al., 1996). Also, protein kinase A (PKA) has been shown to be involved in the pathway since injection of dominant negative mutant PKA mRNA into fertilized eggs resulted in a similar phenotype as Shh overexpression (Ungar et al., 1995; Hammerschmidt et al., 1996). Currently it is not known whether it acts directly or indirectly to influence Hh-signaling. Recently, large scale mutagenesis screens of the zebrafish genome carried out independently in two laboratories have defined a common phenotypic class of mutations, termed the *you*-type mutants. These mutations affect differentiation of axially derived muscle cells in embryos whereas formation of the axial mesoderm is normal, suggesting that they may alter genes that act in a stepwise manner to specify the individual muscle cell types of the zebrafish myotome (van Eden et al., 1996). This notion has been reinforced with the finding that individual members of the *you-type* mutant group carry alterations within the gene that encodes one of the Hh secreted

signals itself and a gene known to be required for Hh signal transduction in *Drosophila* (P. Haffter and A. Schier unpublished data).

1.5. Experimental Objectives

Prior to the beginning of my work, other members of the laboratory had cloned several heat shock genes from zebrafish, including *hsp47* (Pearson et al., 1996), *hsp70* (Shane Engel, published in Lele et al., 1997), *hsp90α* and *hsp90β* (Krone and Sass, 1994). These full-length (*hsp47*) or partial length (*hsp70, hsp90s*) cDNAs then served as probes in the preliminary characterization of heat-inducibility of these genes. The Northern-blot experiments demonstrated similar expression patterns for *hsp47, hsp70* and *hsp90α* with very low (often undetectable) mRNA levels in control samples and high inducibility following heat shock. *hsp90β* on the other hand was constitutively expressed under normal conditions and was only slightly inducible after heat shock.

Based on the results of these preliminary characterizations, my first goal was to provide detailed descriptions of the temporal patterns of expression of these hsp genes during continous heat shock and ethanol treatment using Northern-blot analysis. Ethanol was chosen as another well-studied agent which is known to induce the stress response in other organisms. As an extension of this work I also examined tissue-specific differences in the inducible expression of *hsp47* and *hsp70* genes using whole-mount in situ hybridization in zebrafish embryos.

My second main objective was to study the expression of *hsp47*, a collagen-specific chaperone during the embryogenesis of zebrafish. I used the only cloned zebrafish collagen cDNA, *col2a1* in these experiments to compare the spatio-temporal pattern of expression of these genes and to analyse the similarities and possible differences in their tissue-specificity.

The third line of my studies focused on Hsp90 and its possible role during myogenic determination and differentiation in zebrafish embryos. There are several independent lines of evidence suggesting that Hsp90 might have a role in muscle differentiation processes (see

chapter 1.2.6.7. for details). These earlier results strongly suggest that *hsp90α* plays a specific role in the normal process of myogenesis. In an attempt to narrow down Hsp90's role in this complicated pathway I treated live embryos at various developmental stages with the recently described specific Hsp90-binding agent, geldanamycin. I used several specific genes as markers for characterizing the development of mature muscles (*α-tropomyosin*), muscle precursors (*myoD, myogenin, eng-2*) as well as other important tissues (notochord, floor plate, etc.) that might play an inductive role during myogenesis.

In summary, the main goals of my study were:

I. Examination of heat shock protein gene expression in control, heat-shocked and ethanol-treated zebrafish embryos.

II. Characterization of the mRNA distribution of *hsp47* and *col2a1* genes during zebrafish development.

III. Analysis of Hsp90 function during zebrafish development

The results of my work were reported in (and are being submitted to) several peer-reviewed papers in international journals. These are: Lele and Krone, 1996, 1997; Lele et al., 1997; Krone et al., 1997; Lele et al., 1998 (2 papers submitted).

2.0. MATERIALS AND METHODS

2.1. Zebrafish maintenance, breeding and embryo manipulations

Wild-type zebrafish (*Danio rerio*) stocks used in all experimental procedures were purchased in bulk from a local aquaria supplier (Speers Seed Store, Ltd., Saskatoon, Canada). Animals were maintained at 28.5°C under a 14h photoperiod. Fish were fed once daily with either live brine shrimp, frozen blood worms (*Chironomus* Larvae), or tropical fish dry flake food (Nutrafin, by Tetra).

Care of embryos and adults was as described in Westerfield (1995). In brief, embryos were collected after allowing the fish to spawn in a 15 or 20-gallon tank into "spawning baskets" (plastic containers laden with marbles and fitted with a lid of green plastic mesh and artificial plants). The egg collectors were placed in the tank in the evening and eggs were collected the following morning. Eggs were laid and fertilized within an hour of the morning lights turning on, providing large populations of developing embryos. Following collection, embryos were rinsed several times with charcoal-filtered water and then allowed to develop in a dry incubator at 28.5°C in charcoal-filtered dechlorinated water (herein referred to as "system water"). The water was changed every 5-8 hrs for the first 24 hrs, and dead or dying embryos were removed to prevent contamination of the remaining embryos. After this, system water was changed daily, and dead embryos were removed.

Embryos were maintained at 28.5°C, and staged according to the staging series described in Westerfield (1995) and in Kimmel et al. (1995). In this dissertation all developmental ages refer to hours post-fertilization at 28.5°C, unless otherwise indicated.

no tail (*ntl*), and *floating head* (*flh*) embryos displaying two distinct mutant phenotypes were received as a gift from Dr. E. Weinberg (Univ. Pennsylvania, PA) and used in whole mount in situ

hybridization experiments. The heterozygotes were indistinguishable from wild-type siblings (approximately one-fourths of the embryos), and were considered wild-type embryos in this study with respect to notochord and somite development and gene expression. Prior to shipping, embryos were fixed in 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS), followed by methanol fixation. Embryos were washed and stored in 1X PBS in microcentrifuge tubes. The mutant phenotypes are described in detail in the appropriate Results section of this dissertation.

For heat shock treatment, embryos were collected at designated stages of development, placed in a 35 mm petri dish in "system-water", wrapped in parafilm, and submerged in a water bath set to the proper temperature for the required time. Ethanol-treated embryos were changed into "system water" containing the appropriate concentration of ethanol and were grown at 28.5°C for the indicated time. Control embryos remained in "system water" at 28.5°C. Following treatment, both heat shocked, ethanol-treated and same-stage control embryos were frozen until RNA isolation. Embryos which were to be subjected to in situ hybridization analysis were fixed with 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) for 1 h at 4°C. Following fixation, embryos were washed in two changes of PBS, and then dehydated in two changes of methanol. Embryos were stored in 1.5 ml microcentrifuge tubes in methanol at -20°C for at least one hour, and as long as 3 months.

Geldanamycin (gift of Developmental Therapeutics Program, National Cancer Institute), geldampicin (gift of Dr. Kenneth Rinehart, University of Illinois) or forskolin (BIOMOL Research Laboratories) were dissolved in 100% dimethyl-sulfoxide (geldanamycin and geldampicin: 5mg/ml; forskolin: 10 mM) and stored in the dark at -20°C. Embryos were soaked in 0.1mg/ml Pronase E (Sigma) solution for 20 min. to partially digest the chorion. After this embryos were washed in "system water" 10 times to completely remove the remnants of the enzyme. The weakened chorions were then removed manually using two pairs of forceps. Manually dechorionated embryos were incubated in 20 μM GA or geldampicin or 250 μM forskolin solution diluted in "system water". Controls consisted of embryos incubated in

system water only and system water containing DMSO at the same concentration as treatment groups. Embryos that were to undergo in situ hybridizations were fixed in 4% paraformaldehyde as described above. Higher concentrations of GA were lethal whereas treatment at 5 or 10 μg/ml resulted in a reduction in both the number of abnormal embryos and the severity of the phenotype. Similarly, treatments with concentrations of forskolin below 250 μM reduced the consistency of the observed phenotype whereas those above 500μM resulted in increased lethality.

2.2. Cloning and recombinant techniques

Restriction enzymes were obtained from Boehringer-Mannheim or GIBCO-BRL. Enzyme digests were performed according to the manufacturer`s protocols using high, medium, or low salt 10X buffers provided with each restriction enzyme. Unless otherwise stated, all digestion reactions were carried out in microtubes containing DNA in dH_2O combined with 1/10 volume enzyme, 1/10 volume buffer, and dH_2O to final volume. All reactions were carried out for at least 2x2 hrs in a water bath at the required temperature (usually 37°C) and fresh enzyme was added at the beginning of the second two hour period, if it was necessary according to the manufacturers instructions.

For the transformation of plasmid DNA into bacterial hosts, cultures of XL-1 Blue *E. Coli* were established in 5 ml 1X YT broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with tetracycline (15 μg/ml). Cultures were grown overnight at 37°C in a rotary shaker water bath at 300 rpm. Growth phase XL-1 Blue bacteria for transformation were established by inoculating 5 ml 1X YT broth/tetracycline (15 μg/ml) with 100 μl of overnight XL-1 Blue and allowing this subculture to grow for approximately two hrs at 37°C, 300 rpm. Transformation competent log growth phase XL-1 Blue bacteria were established by pelleting the cells at 2000 g for 10 min in a clinical tabletop centrifuge, and resuspending the bacteria in one-half volume (2.5 ml) ice-cold 50 mM $CaCl_2$. Cells were left on ice for 1 hr, and then repelleted as above and resuspended on ice in 500 μl of 50 mM $CaCl_2$.

Transformation reactions were carried out as described in Sambrook et al. (1989). 100 µl of transformation competent bacterial cells were incubated with at least 100 ng of circular plasmid in a 1.5 µl microcentrifuge tube and incubated on ice for 1 hr. Negative control reactions consisted of 100 µl of competent bacterial cells incubated with 10 µl sterile dH_2O and were treated exactly as experimental reaction tubes. Following 1 hr on ice, reaction tubes were incubated in a water bath at 42°C for exactly 2 min, placed at room temp for 10 min, and then incubated in an air-incubator at 37°C for 1 hr with the addition of 500 µl of pre-warmed YT broth. Aproximately 100-200 µl of each transformation reaction was then spread directly onto YT agar growth plates (1.2% Bacto-agar (Difco) containing 75 µg/ml ampicillin, 15 µg/ml tetracycline, 670 µM IPTG (isopropyl-ß-D-thiogalactopyranoside (Boehringer-Mannheim)), and 60 µg/ml X-Gal (5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (Boehringer-Manneheim) for blue/white color screening) and incubated inverted overnight at 37°C in an air-incubator. Following incubation, plates could be stored wrapped in parafilm at 4°C for up to several weeks. Putative clones carrying recombinant plasmid (white clones, if blue/white screening is applicable) were identified and plasmids were isolated following the plasmid isolation procedure described below, and subjected to restriction enzyme digestion and agarose gel electrophoresis to confirm the presence, size, and orientation of the cDNA insert.

Plasmid DNA was isolated according to the method described by Birnboim and Doly (1979) as modified by Sambrook et al. (1989). Glass tubes containing 5 ml of YT broth containing the appropriate antibiotic (usually ampicillin at a concentration of 75 µg/ml) were inoculated with the strain of *E.coli* carrying the desired plasmid (usually XL-I Blue) and grown overnight at 37°C in a water bath with a rotary shaker at 300 rpm. The pBluescript II plasmids were isolated from XL-1 Blue host cells via the alkaline lysis method of plasmid isolation (also called the 'rapid' plasmid preparation). Cells from 1.5 ml of the overnight culture were pelleted at 10,000 rpm (12,000xg) for 1 min in a 1.5 ml microtube. The YT medium was removed and the cells were resuspended in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)). Bacteria were lysed by the addition

of 200 µl of freshly prepared ice-cold lysis buffer (Solution II; 0.2 N NaOH, 1% SDS). Cytoplasmic components (proteins) and chromosomal DNA were precipitated by the addition of 150 µl of ice-cold solution III (5M potassium-acetate). Cellular debris was pelleted by centrifugation at 12,000xg for 5 min. The plasmid-containing supernatant was transferred to a fresh microtube and re-centrifuged an additional 5 min to insure removal of all debris. The supernatant was again transferred to a fresh microtube and plasmid was precipitated by the addition of >2 volumes of 95% ethanol and then left to incubate at room temperature for several minutes. Plasmids were recovered by centrifugation at 12,500 g for 5 min. The plasmid pellet was washed in 70% ethanol and recentrifuged for an additional 5 min. The ethanol was removed and plasmids were then resuspended in a solution of 0.25 mg/ml RNase A to digest contaminating RNA, and incubated for 30 min at room temperature. Alternatively, the plasmid pellet was resuspended in a dilute RNAse A solution (10 µg/ml) and immediately subjected to a restriction digest, if desired. Following RNase A treatment the plasmid solution was made up to a final volume of 200 µl with sterile dH_2O, and extracted with an equal volume of phenol (equilibrated to pH>7.8), followed by extraction with chloroform. The aqueous supernatant containing the plasmids was removed and the plasmid DNA was precipitated by the addition of 1/10 volume 3M sodium acetate (20 µl) and 2.5 volumes of cold absolute ethanol (500 µl). The plasmid solution was stored overnight at -20°C. The plasmids were then pelleted by centrifugation at 13,000 g for 20 min and resuspended in 20 µl sterile dH_2O.

2.3. RNA isolation

Total precipitable RNA was isolated from embryos using Trizol reagent according to the manufacturer`s protocol (Gibco BRL, cat. # 15596-026, Burlington, ON). To approximately 100 embryos 100 µl of Trizol reagent was added. After addition of Trizol, the embryos were homogenized using microtubes and pestles made by VWR-Canlab (Missasauga, ON, Canada) 400µl more Trizol reagent was added and the suspension was pulled through a 21 gauge needle followed by passage

through a 26 gauge needle (10-15 times) to insure complete cellular disruption. The resultant lysate was centrifuged for 10 min at 12,000xg at 4°C to pellet insoluble debris. Supernatant was transferred to a new 1.5 ml microtube and 200 µl chloroform was added (the pellet was discarded). The solution was mixed by vigorous shaking and then incubated at room temperature for several minutes. The aqueous phase (upper, clear fraction) and organic phase (lower, red fraction) were separated by centrifugation for 10 min at 12,500 g in a clinical centrifuge. The RNA partitioned to the aqueous phase (upper), which was drawn off with a pipette and transfered to a new, sterile 1.5 ml microtube to which equal volume (aprox. 250 µl) of isopropyl alochol was added. Contents were mixed by brief vortexing and then incubated at room temperature for 10 min to allow the RNA to precipitate. The sample was centrifuged for 15 min at 14,000 g at 4°C to pellet the RNA. The pellet was washed with 80% ethanol and centrifuged for an additional 15 min at 7,500 g at 4°C. The RNA pellet was resuspended in 50 ml of deionized formamide and stored at -80°C. In this form the RNA can be used for Northern blot hybridization analysis.

The RNA concentration was determined by measuring optical density (OD) at 260 nm and 280 nm in a 1 cm Canlab blue label semi-micro absorbtion cell (220-2500 nm) with a Beckman DU-7 UV/visible spectrophotometer. Concentration calculations were based on the fact that 40 mg of RNA dissolved in 1 ml of water has an absorbance at 260 nm of 1 OD unit. Alternatively, RNA concentration was estimated based on subjecting a sample of RNA to gel electrophoresis on a horizontal 1.0% (w/v) formaldehyde-agarose gel (see below) and comparing the sample RNA with the concentration of an RNA marker ladder (Bethesda Research Laboratories (BRL), Bethesda, MD) of known concentration. RNA is visualized on a UV light box following staining with ethidium bromide (1 µg/ml) for 45 min and then destaining overnight in deionized water.

2.4. Northern blot analysis

The cDNA probes were labelled with [α-^{32}P] dCTP using the random primed DNA labelling kit according to the manufacturer's

protocol (USBiochemicals, 70200, Cleveland, OH). Between 50-100 ng of DNA probe were adjusted to 10 µl with dH_2O, denatured by heating for 10 min at 90-95°C, and then immediately cooled on ice for several minutes. To this solution was added 3 µl of a 1:1:1 mixture of dATP, dGTP, and dTTP, 2 µl of 10X reaction mixture (Klenow reaction buffer), 5 µl of [α-^{32}P] dCTP (50 µCi), and 1 µl of Klenow enzyme (2 U). The reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 1/10 volume 0.5 M EDTA (pH 8.0).

The radio-labelled probe was subsequently separated from unincorporated radio-labelled nucleotides by passing through a Sephadex G25 resin column (Pharmacia). The column was packed in a 1 cc syringe in TE buffer, with glass wool at the base. Prior to loading the probe into the column, the column was centrifuged several times to pack the Sephadex beads, loaded with 100 µl of water and then centrifuged for 10 min at 2,500 rpm to pack and dry the column. A collecting tube (1.5 ml microcentrifuge tube) was placed under the column and the unit was set into a 15 ml Falcon tube. The radiolabelled probe was made up to 100 µl with sterile dH_2O and loaded into the packed column. The column apparatus was centrifuged 1 min at 2000 rpm and labelled probe was collected in the microcentrifuge collecting tube. A Geiger counter was used to confirm the presence of the radio-labelled probe. The probe was then transferred to a fresh tube and stored at -20°C until needed.

A total of 10 µg of total RNA was subjected to gel electrophoresis on a horizontal 1.2% (w/v) formaldehyde-agarose gel (Sambrook et al., 1989) and transferred to a nitrocellulose filter according to the method of Thomas (1983). Lyophilized RNA samples were dissolved in 2.4 µl sterile distilled dH_2O, 1 µl 10X MOPS buffer (0.2 M 3-(morpholino)propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0), 1.6 ml 37% (v/v) formaldehyde, and 5 µl deionized formamide. RNA was denatured at 65°C for 5 min followed by cooling on ice for 10 min. Two µl of 0.1% (w/v) bromophenol blue/50% (v/v) glycerol loading buffer were added and the samples loaded onto the formaldehyde-agarose gel. Gels were run in 1X MOPS buffer at 5 V/cm with the buffer level to the edge but not covering the top of the gel. The buffer at the two electrodes was mixed periodically to

overcome the pH change which occurs during electrophoresis. RNA transcript sizes were determined by comparison with the migration of an RNA marker ladder (0.24-9.5 kb, Gibco/BRL, Cat# 15620-016). Following electrophoresis, the entire gel was stained in ethidium bromide (1 mg/ml) for 45 min, destained overnight in deionized water, visualized on a UV light box, and photographed with a ruler beside it using a Polaroid camera. The marker lane was cut off, and the portion of the gel to be transferred to nitrocellulose was soaked twice in distilled water for 5 min and once in 10 mM sodium phosphate buffer (pH 7.0) for 10 min. Gels were placed on a sheet of Whatman 3 MM paper which had been soaked in 20X SSC (3 M sodium chloride, 300 mM sodium citrate, pH 7.0) and placed on a glass plate over a Pyrex baking dish containing 20X SSC. The ends of the 3 MM paper were dipped into the 20X SSC to allow the flow of the buffer to the gel. Nitrocellulose which had been cut to the size of the gel and presoaked in 5X SSC was placed on the gel and air bubbles between the gel and the nitrocellulose were removed by rolling with a sterile 10 ml pipette. This was overlaid with 3 sheets of 3 MM paper (Whatman) slightly smaller than the filter and a 10 cm stack of paper towels, also cut slightly smaller than the 3MM papers. A 500 g weight was then placed on top of the paper towels, and the entire set-up was covered in plastic wrap and the transfer of the RNA was allowed to proceed overnight. The nitrocellulose filter was then peeled away from the gel, air-dried for 1 h, baked at 80°C for 2 h and stored at room temperature.

All prehybridizations and hybridizations were carried out in a Tekstar Jr. Hybridization Oven (BioCan Scientific) according to the manufacturer's instructions. Prehybridization of RNA blots was performed for 12 h at 42°C in 50% (v/v) formamide, 5X SSC, 10 mM sodium phosphate buffer (pH 7.0), 2.5X Denhardt's solution (0.05% (w/v) BSA, 0.05% (w/v) polyvinylpyrrolidone (Sigma), 0.05% (w/v) Ficoll (type 400, Sigma; Denhardt, 1966), and 250 µg/ml denatured calf thymus DNA. The prehybridization buffer was removed and hybridization reactions were carried out at 42°C for 48 hrs in prehybridization buffer containing 4X SSC instead of 5X SSC, 7.5% (w/v) dextran sulfate (Pharmacia, Baie D'Utre, Quebec) and the [α-^{32}P]-dCTP labeled probe (boiled for 5 min to denature and then cooled on

ice prior to addition) to a final concentration of 5 x 10^5 cpm/ml. The blots were then washed four times over a 15 min period at room temperature in 1X SSC, 0.1% (w/v) SDS followed by two 15 min washes in 0.5X SSC, 0.1% (w/v) SDS at 42°C, and two 10 min washes in 0.1X SSC, 0.1% (w/v) SDS at 42°C. The blots were then set on a sheet of plexiglass, covered in plastic wrap, and exposed to Kodak XAR-5 or Kodak BioMax film at -70°C using a Cronex intensifying screen. Following exposure, the radioactive signal on blots which were to be rehybridized were allowed to decay for 2 months, during which time they were stored at 4°C in plastic wrap behind a plexiglass shield.

2.5. GA-affinity chromatography and Western-blotting

GA-affinity chromatography and Western-blot analysis were carried out by Steve Hartson and Robert Matts (Oklahoma State University, Stillwater, USA). The GA-affinity chromatography columns were prepared by Luke Whitesell (University of Arizona Health Sciences Center, Tucson, USA) as previously described (Whitesell et al., 1994).

Protein extracts derived from early embryos consisted predominantly of a 90 kDa protein, tentatively identified as vitellogenin, that bound non-specifically to both GA-derivatized and non-derivatized resin. Since the overwhelming abundance of this yolk protein compromised the detection of Hsp90 by Western blot analysis, lysates were subsequently obtained from the trunks of adult zebrafish and subjected to GA-binding assays as previously described (Whitesell et al., 1994). Lysates were adjusted to approximately 2 mg/ml protein and preincubated with either soluble GA (30 µg/ml) or DMSO (vehicle control) for 3 hr at 4°C. Lysates were clarified by brief centrifugation and incubated in the presence of non-dervivatized or GA-derivatized resin for 3 hr and washed five times with lysis buffer. Bound materials were eluted by boiling in SDS-PAGE sample buffer and separated by SDS-PAGE. Eluted proteins were visualized by silver staining or Western blotting according to Sambrook et al. (1989) 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 of R. Rupp) containing full-length zebrafish *hsp90α* or *hsp90β* cDNAs. These cDNAs, which are described fully elsewhere (Lele et al., submitted; Genbank accession numbers AF068772, AF068773) were isolated from a post-somitogenesis embryonic library (gift of J. 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 hour. 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.

2.6. Whole-mount in situ hybridization analysis of zebrafish embryos

2.6.1. Template preparation

Digoxigenin-11-UTP (Boehringer Mannheim Biochemicals, BMB) labeled sense and antisense RNA probes were synthesized by in vitro transcription reactions using linearized cDNA fragments as template (described in Section 2.5.2.). Sense RNA was generated and tested in tha case of genes isolated by our laboratory previously (*hsp47, hsp70, hsp90α* and *hsp90β*). Templates for the following transcription reactions were prepared as outlined below:

i. *α-tropomyosin*:

A cDNA clone containing the zebrafish *α-tropomyosin* coding region was a gift of B. Riggleman (Weinberg et al., 1996). Sense probe was synthesized using *Sal* I digested cDNA template, and transcribed

using T7 RNA polymerase. Antisense probe was synthesized from *Eco* R I digested template, and transcribed with T3 RNA polymerase.

ii. *myoD*

The zebrafish *myoD* riboprobe was synthesized from a cDNA clone containing the complete 1.6 Kb *myoD* coding region ligated into pBluescript SK- (gift of E. Weinberg, see Weinberg et al., 1996). Template cDNA was made by digesting with *Xba* I, and then used to synthesize antisense RNA by transcribing with T7 RNA polymerase.

iii. *myogenin*

The 1.3 kb coding region of the *myogenin* cDNA clone was isolated from zebrafish by B. Riggleman and ligated into pBluescript SK- (Weinberg et al., 1996). Antisense probe was synthesized using template cDNA linearized with *Xba* I and transcribed with T7 RNA polymerase.

iv. *hsp90α and hsp90β*

The *hsp90α* and *hsp90β* PCR-amplification products were cloned into the *Not* I site of pBluescript II (Krone and Sass, 1994). The plasmids were linearized by digestion with *EcoR* I or *BssH* II and then transcribed with T3 and T7 RNA polymerase respectively in order to obtain sense and antisense RNA probes.

v. *hsp47*

The zebrafish *hsp47* is a PCR fragment cloned by D. Pearson in our laboratory (Pearson et al., 1996). It is cloned into the *Not* I site of pBluescript II SK-. We used *Sst* I (*Sac* I) to linearize the plasmid and T7 to synthesize the antisense probe.

vi. *col2a1* (type II collagen)

col2a1 is a partial cDNA provided by J. Postlethwait (Univ. of Oregon, Eugene). It contains a 1.8 kb fragment cloned into Bluescript SK- (Yan et al., 1995). To make antisense probe I used *Hind* III to linearize it and T3 RNA polymerase to synthesize the RNA.

vii. *sonic hedgehog*

sonic hedgehog is a full-length cDNA generously provided by P. Ingham (Univ. of Sheffield, UK). The 1.7 kb clone is in the EcoRI site of pUC18 (Krauss et al., 1993). To make antisense probe we used *Hind* III to linearize and T7 RNA polymerase to synthesize the probe.

viii. *echidna hedgehog*

echidna hedgehog was also a gift of P. Ingham (Univ. of Sheffield, UK). It is a full-length cDNA cloned into *Hind* III-*EcoR*I sites (5`-3` respectively) of pUC18 vector (Currie and Ingham, 1996). We cut it with *Hind* III and transcribe it with T7 RNA polymerase to obtain antisense probe.

ix. *notail*

notail, the zebrafish homologue of the mouse Brachyury (T) gene was provided by C. Nusslein-Volhard (Max-Planck Institute of Developmental Biology, Tubingen, Germany). (Schulte-Merker et al., 1992, 1994) To make antisense probe we used *Xho* I to linearize and T7 RNA polymerase to synthesize the probe.

x. *eng-2*

eng-2 is a full-length 2.6kb cDNA provided by M. Ekker (Loeb Institute, Ottawa, Canada). It is cloned into the *EcoR* I site of Bluescript KS- plasmid (Ekker et al., 1992). I used *Xho* I to linearize it and T7 RNA polymerase to synthesize the antisense probe.

xi. *axial*

axial is the zebrafish homologue of the mouse *HNF3-β* gene. It is a full-length (1.75kb) cDNA cloned into the *Not* I site of pBluescript (Stråhle et al., 1993). I used *Dra* I enzyme to linearize the plasmid and T3 RNA polymerase to transcribe the antisense probe.

2.6.2 In vitro transcription reaction

Each in vitro transcription reaction was performed in a 500 µl Eppendorf tube. To the tube was added 5 µl of 5X T3/T7 transcription

buffer (GIBCO BRL), 2.5 µl 10X nucleotide triphosphate (NTP) labelling mixture (10 mM each of ATP, CTP, and GTP, 6.5 mM UTP, and 3.5 mM of DIG-UTP), 2.5 µl 0.1 M dTT (GIBCO BRL), and 0.5 µl RNasin (RNase inhibitor, Promega). Triple-distilled autoclaved water was added to the mixture to a final volume of 25 µl, making allowances for the cDNA template yet to be added. At room temperature, 1 µg of linearized cDNA template and 1 µl of the appropriate RNA polymerase were added to the reaction tube, and the tube was incubated in a water bath at 37°C for two hours to allow transcription to take place. Following the incubation period, 0.8 µl of 200 mM NaCl and 2.4 µl of RQ1 DNase (Promega) were added to stop the reaction and digest the DNA template. After an additional 15 min. at 37°C the RNA was precipitated by the addition of 1.2 µl 0.2 M EDTA, 1.5 µl 4 M LiCl, and 45 µl 95% cold ethanol to the reaction mixture. The tube was left overnight at -20°C to allow all the RNA to precipitate.

The following day, the tube was centrifuged at 13,000 rpm for 30 min at 4°C, washed in 70 % ethanol, and repelleted for 15 min. The pellet was resuspended in 20 µl of triple-distilled autoclaved water. Of this sample, 17 µl was stored at -70°C and used as riboprobe for in situ hybridization reactions, and 3 µl was run on a formaldehyde gel in order to visually determine the approximate concentration of RNA in the sample.

2.6.3. Whole mount in situ hybridization

The in situ hybridization protocol used was the method of Puschel et al. (1992), with minor modifications (Akimenko et al., 1994), and subsequent changes made by J. Sass in our laboratory. Embryos were mechanically dechorionated using fine dissecting forceps, and rehydrated by washing in 75%, 50%, and 25% methanol at room temperature (RT). Embryos were washed in four changes of PBST (PBS with 0.1% Tween 20). Embryos at stages earlier than 20 h post-fertilization were not pre-digested with proteinase K. At later stages embryos were subjected to proteinase K digestion (25 µg/ml, Boehringer-Mannheim) in PBST at RT for 2 min (20 h embryos), 3 min (24 h embryos), 4 min (32 h embryos), or 5 min (40 and 48 h embryos).

Following proteinase K digestion embryos were washed in two changes of PBST, post-fixed in 4% PFA/PBS for 20 min, and again washed in PBST. Pre-hybridization was carried out at 65°C for at least 1 h in hyb-mix (50% formamide, 5x SSC, 0.1% Tween 20, 50 µg/ml heparin, 100 µg/ml yeast tRNA, 9.2 µM citric acid, pH 6.0, and double-distilled water (DDW) to final volume). Hyb-mix was removed and hybridization with probe (100 ng/200 µl hyb-mix) was carried out overnight at 65°C in 500 µl microcentrifuge tubes. The following 10 min washes were carried out at 65°C: 75% hyb-mix/25% 2x SSC, 50% hyb-mix/50% 2x SSC, 25% hyb-mix/75% 2x SSC, 100% 2x SSC. Two 30 min washes were done at 60°C in 0.2x SSC. The following additional 5 min washes were then carried out at room temperature: 75% 0.2x SSC/25% PBST, 50% 0.2x SSC/50% PBST, 25% 0.2xSSC/75% PBST, 100% PBST. Embryos were then preincubated for 1-3 h in a blocking solution (2% newborn calf serum, 2 mg/ml BSA in PBST) and then for 2-3 hours with a 1:20 dilution of alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer-Mannheim). The antibody was previously preadsorbed at a dilution of 1:100 for 1 hr at RT against a batch of fixed 4 day-old embryos, homogenized in a blocking solution containing 2% calf serum and 2 mg/ml BSA in PBST. Following antibody binding, embryos were washed in several changes of PBST for 1.5 hrs, and then in several changes of alkaline phosphatase buffer (100 mM Tris HCL pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20, 1 mM levamisol in DDW). The embryos were incubated in staining buffer (alkaline phosphatase buffer containing 250 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 225 µg/ml nitro blue tetrazolium (NBT)), and the reaction stopped at a suitable signal to noise ratio. Sense and antisense reactions within the same experiment were stopped at the same time, usually after 30-45 min of staining. Immediately following the staining reaction, embryos were washed in PBST and post-fixed in 4% PFA/PBS for 30 min at RT. Embryos were washed in PBS and could then be stored in PBS at 4°C for several days.

Each experiment which depicts wild-type embryos subjected to whole mount in situ hybridization in this dissertation was repeated at least four times. Further, in each individual experiment at least twenty-five individual embryos were visualized per treatment group.

Results varied slightly among individual embryos with respect to signal intensity.

2.6.4. Photography of embryos

Whole embryos were dehydrated in 100% methanol, and then cleared for photography in benzyl benzoate: benzyl alcohol (2:1). Embryos were mounted on glass slides in a solution of benzyl alcohol:glycerol (1:5). This solution both cleared embryos and provided a viscous medium in which the embryos could be rotated and their position maintained during viewing and photography. Embryos were photographed on a Carl Zeiss Photomicroscope using Planapo objectives of 2.5x, 6.3x, 10x, 16x and 25x. The microscope contained internal zoom lenses which provided additional increases of magnification at 1.25x, 1.6x, and 2x, in addition to a further 3.2x magnification provided by the internal camera. All slides were taken with EPY color tungsten slide film, ASA 64. The slides were scanned using a slide scanner and Adobe photoshop running on a Power Mac computer.

2.6.5. Embedding and sectioning of zebrafish embryos

Zebrafish embryos that were subjected to whole mount in situ hybridization were stored in 1X PBS (phosphate-buffered saline) in preparation for embedding in JB-4 resin (Polysciences Inc., Warrington, PA). Embryos were washed with several changes of 70% ethanol, and then dehydrated by washing 15 min in 80% ethanol, 15 min in 95% ethanol, and two 30 min washes in absolute ethanol. Embryos were subjected to two 30 min washes in JB-4 embedding Solution A (Cat. # 0226A; Acrylic monomer, n-Butoxyethanol) with 9 mg/ml catalyst (Cat. # 02618; 70% wet benzoyl peroxide), and embedded in a mixture of Solution A and JB-4 embedding Solution B (Cat.# 0226B; N,N-Dimethylaniline, Poly (ethylene glycol)), at 25:1 respectively. A plastic tray containing small wells provided moulds which contained approximately 50 embryos, and were filled with JB-4 resin. A chuck was gently placed in the well(s) containing JB-4 resin prior to

polymerization. The plastic tray was then wrapped in aluminum foil to keep light out, and the JB-4 resin was allowed to harden at room temperature overnight in darkness.

Embryos were sectioned on a Sorvall Porter-Blum JB-4 microtome with a glass knife. Glass knives were cut from 1/2" thick glass strips (Marivac, Halifax, Canada) using a Sorvall glass knife maker. All tissue sections were 8-10 μm in thickness. Sections were mounted by "picking up" wet sections with a glass slide as they floated on the surface of a water bath. Sections were not subjected to any subsequent histological staining. All sections were visualized and photographed on a Carl Zeiss Photomicroscope fitted with Nomarski optics as described above.

3.0. RESULTS

3.1. Analysis of the temporal pattern of *hsp47, hsp70, hsp90α and hsp90β* gene expression in control and stressed embryos

3.1.1. Developmental stage and temperature dependency of *hsp70* gene expression.

Members of this laboratory have recently reported the degenerate PCR-based cloning and initial characterization of full-length cDNA probes encoding for *hsp47* and partial length (PCR-fragment) *hsp90α* and *hsp90β* cDNA clones (Krone and Sass, 1994; Pearson et al., 1996). In order to more fully assess the stress-response to hyperthermia and ethanol exposure I used these clones along with the recently published partial length *hsp70* cDNA fragment (cloned by Shane Engel) as probes in Northern-blot analysis experiments.

Initial analysis of control and heat-induced expression of *hsp47, hsp90α* and *hsp90β* genes during early stages of zebrafish development has been previously carried out in our laboratory (Krone and Sass, 1994; Pearson et al., 1996). To characterize the accumulation of *hsp70* mRNA during the same period of development, I carried out Northern-blot analysis using the *hsp70-4* cDNA fragment as a probe (Figure 2). Developmental stages examined were gastrula (lanes 1-3), mid-somitogenesis (lanes 4-6), post somitogenesis (lanes 7-9) and 3-day-old hatched larvae (lanes 10-12). The pattern of accumulation of *hsp70* transcript was similar to that observed for *hsp47 and hsp90α* mRNA in previous studies (Krone and Sass, 1994; Pearson et al., 1996). Levels of *hsp70* mRNA were low often undetectable at control temperatures in all stages of embryogenesis examined. However, levels increased substantially during a 90 minute heat shock at 34°C (lanes 2,5,8) and even more dramatically at 37°C (lanes 3,6,9). Equivalent quantities of RNA were present in all samples as determined by hybridization to an

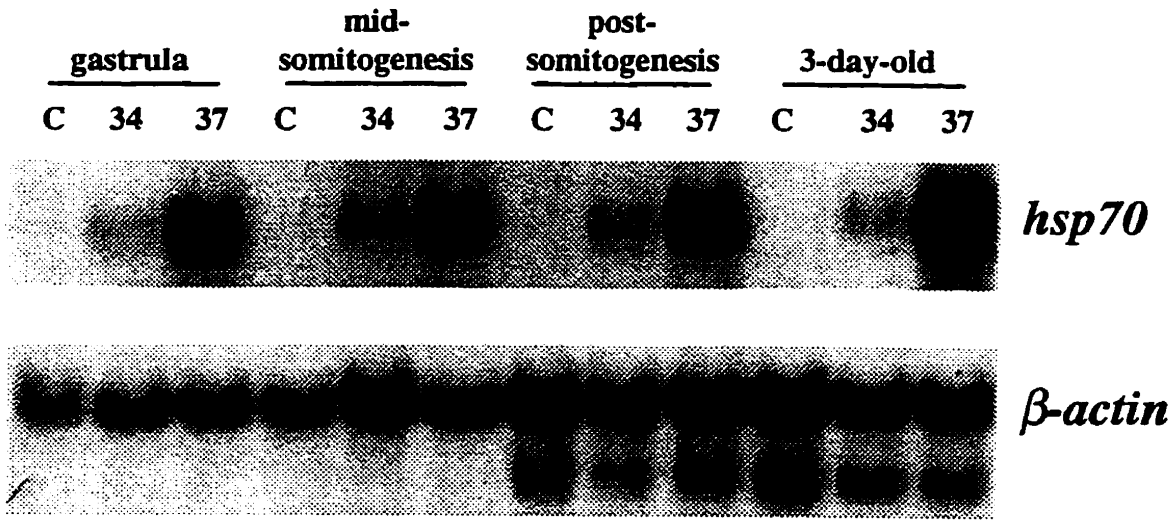
actin cDNA probe and ethidium bromide staining (Krone and Sass, 1994; Pearson et al., 1996).

3.1.2. Expression of *hsp47, hsp70, hsp90α and hsp90β* genes in control and heat-shocked zebrafish embryos

The above experiment and previous studies (Krone and Sass, 1994; Pearson et al., 1996) suggested that the *hsp47, hsp70* and *hsp90α* genes are subject to similar mechanisms of heat-inducible regulation in that they are expressed at low levels constitutively but are all strongly induced following heat shock. *hsp90β* expression showed an entirely different expression pattern, with high levels of its mRNA present in control embryos and heat shock inducing its transcription only slightly (Krone and Sass, 1994). In order to more fully compare the response of these genes to hyperthermic conditions, I examined the temporal pattern of *hsp47, hsp70,* and *hsp90* gene expression in two-day old larvae during continous exposure to 37°C. As shown in Figure 3, high levels of *hsp70* mRNA were apparent within 30 minutes of exposure to 37°C (lane 4) and these high levels persisted until four hours (lane 7). Longer exposure (up to 12 hrs; data not shown) revealed that *hsp70-4* expression decayed to control levels by 8 hrs. In contrast *hsp47* and *hsp90α* mRNA levels did not reach their maximum until between 1 and 2 hours into the heat shock period (lane 6). As well, they began to decline by four hours, suggesting gene specific differences in the transcriptional regulation or in the mRNA stability among zebrafish *hsp* genes. A similar temporal pattern of the response was seen for *hsp90β* mRNA, with a maximum expression reached after 2 hrs followed by a decline back to control levels by 4 hrs. However in agreement with our previous results (Krone and Sass, 1994) the levels of *hsp90β* mRNA unlike the other *hsp* genes examined, showed strong constitutive expression in control embryos and increased only slightly after heat shock.

Interestingly, I consistently observed a significant increase (150-200 nucleotides) in the size of *hsp90α* mRNA following exposure to heat shock (compare lanes 3 and 4) in two independent experiments. The larger transcript persisted until maximum levels of *hsp90α*

Figure 2. Examination of *hsp70-4* mRNA accumulation at different stages of zebrafish embryogenesis using Northern-blot analysis. Equal amounts of total RNA (10 μg) were loaded into each lane as shown by hybridization with *β-actin*. Embryos were maintained at the indicated temperature for 90 minutes. Lane 1. gastrula, 28.5°C. Lane 2, gastrula, 34°C. Lane 3, gastrula, 37°C. Lane 4, 12-14 somite, 28.5°C. Lane 5, 12-14 somite, 34°C. Lane 6, 12-14 somite, 37°C. Lane 7, post-somitogenesis (prim-20), 28.5°C. Lane 8, post-somitogenesis (prim-20), 34°C. Lane 9, post-somitogenesis (prim-20), 37°C. Lane 10, 3-day hatched larvae, 28.5°C. Lane 11, 3-day hatched larvae, 34°C. Lane 12, 3-day hatched larvae, 37°C.


gastrula
mid-
somitogenesis
post-
somitogenesis
3-day-old
C 34 37 C 34 37 C 34 37 C 34 37
hsp70
β-actin

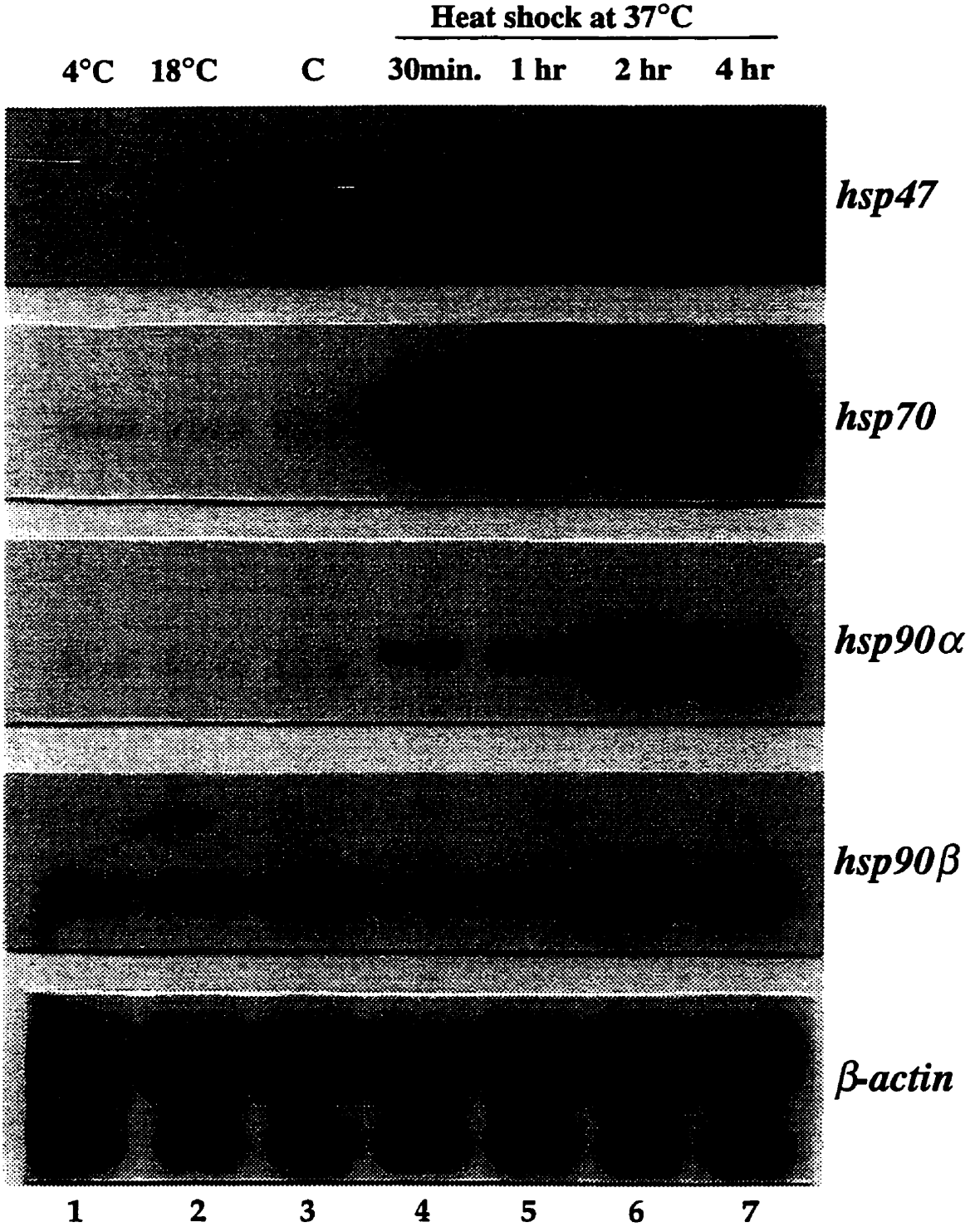
mRNA were reached at 2 hrs. A less substantial size increase for *hsp47* and *hsp90β* was also observed but only at the two hour time-point. This latter result combined with the fact that *hsp70* mRNA and the positive control *actin* mRNA did not show any size increase eliminates the possibility of a gel electrophoresis artifact. Our attempts to investigate whether this size increase was caused by polyadenylation using RNAse H analysis did not lead to a conclusive result due to the insolubility of the RNA pellet after the RNAse H treatment, phenol-chlorofom extraction and ethanol or isopropanol precipitation.

Several studies have shown that *hsp70* and *hsp90* genes in certain mammalian cells (brown adipocytes, keratinocytes) are induced by cold-shock treatment (Holland et al., 1993; Matz et al., 1995; Laios et al., 1997). In order to examine this possibility in zebrafish, 2-day-old embryos were maintained at either 4°C or 18°C for 90 minutes after which total RNA was isolated and subjected to Northern-blot analysis. The cold shock failed to induce an increase in mRNA levels of any of the heat shock genes examined (Figure 3. lane 1 and 2). Even extended treatments up to 4 hrs did not result in heat shock gene induction (data not shown), suggesting that these genes are not subject to cold-inducible upregulation. Despite symptoms indicative of severe hypothermia (lack of spontaneous movements, dramatically slowed heart rate) the embryos survived the experiment, even at the extremely low temperature of 4°C, with no apparent effects on subsequent development (data not shown).

3.1.3. Induction of *hsp47, hsp70, hsp90α and hsp90β* expression by ethanol

The data described above demonstrated that expression of the zebrafish *hsp47, hsp70* and *hsp90α* genes is strongly induced following exposure to elevated temperature, but that their respective mRNAs display different temporal patterns of accumulation during continuous heat shock. Furthermore, a consistent and significant change in *hsp90α* mRNA size occurs immediately following exposure of embryos to heat shock. I was next interested in determining if similar expression patterns of these genes occurred during continous exposure to ethanol,

Figure 3. *Hsp47, hsp70, hsp90α, hsp90β* and *β-actin* gene expression in 2 day old zebrafish embryos during continuous exposure to heat shock at 37°C and to cold shock at 4°C and at 18°C (latter two, exposure for 90 minutes). In order to examine the time course of expression of heat shock genes, embryos were maintained at 37°C and samples removed for RNA isolation and Northern blot analysis after 30 minutes, 1 hour, 2 hours and 4 hours. Lane 1, 4°C, 90 min.; Lane 2, 18°C, 90 min.; Lane 3, control (28.5°C); Lane 4, 37°C, 30 minutes; Lane 5, 37°C, 1 hour, Lane 6, 37°C, 2 hours; Lane 7, 37°C, 4 hours.

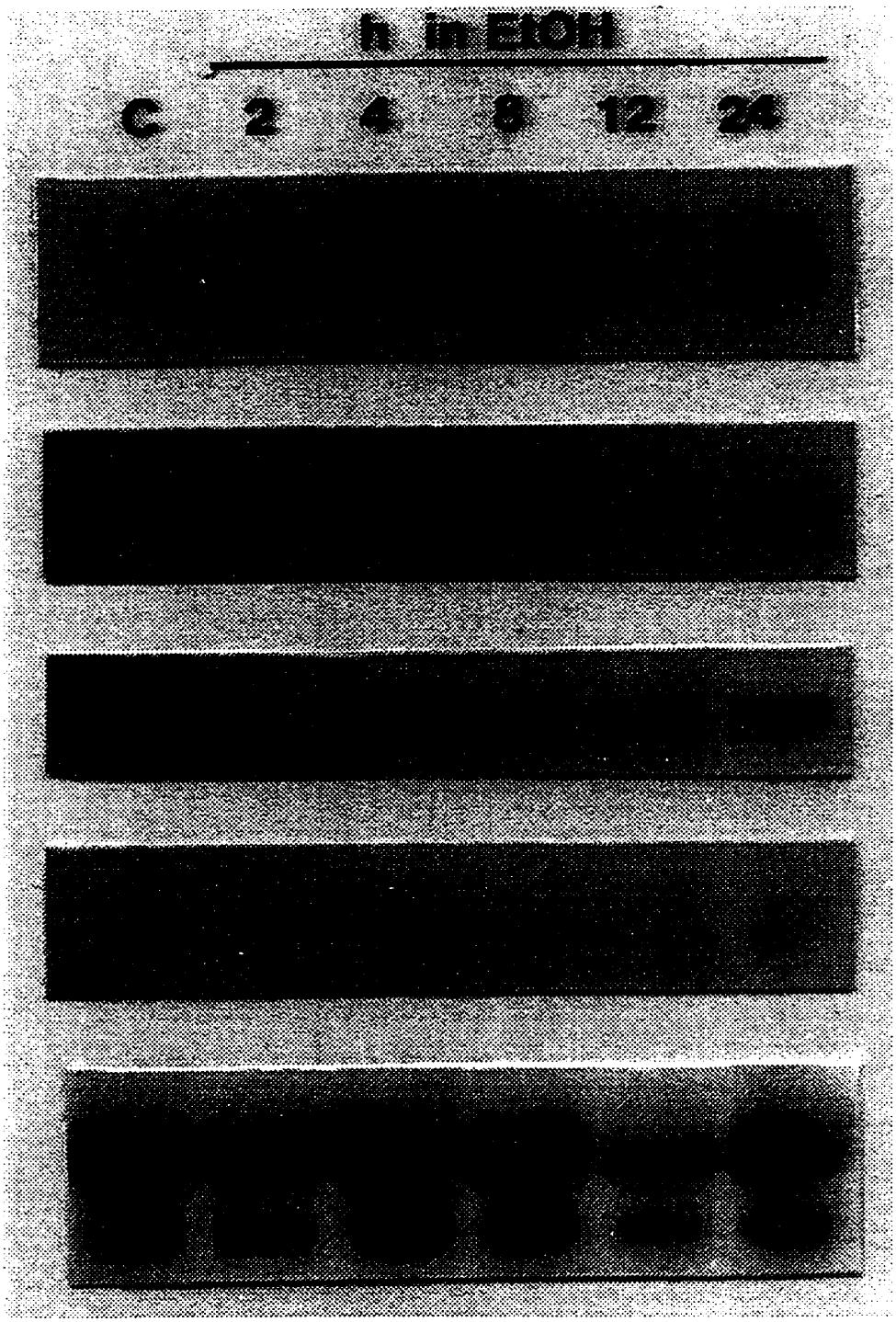

Heat shock at 37°C
4°C
18°C
C
30min.
1 hr
2 hr
4 hr
hsp47
hsp70
hsp90α
hsp90β
β-actin
1
2
3
4
5
6
7

another agent which is known to be a teratogen for zebrafish embryos (Laale, 1971) and to induce the expression of heat shock genes in other poikilothermic vertebrate embryos (Heikkila et al., 1987). In initial experiments examining only *hsp70* expression after exposure to ethanol concentrations ranging from 1% to 8%, embryos exposed to 4% ethanol exhibited induction of *hsp70* expression as well as a good (85%) survival rate (data not shown). Therefore I used 4% as our concentration of choice in subsequent analyses. Figure 4. shows the effect of exposure to 4% ethanol on *hsp47*, *hsp70* and *hsp90* gene expression in two-day-old embryos. Ethanol treatment resulted in the dramatic upregulation of *hsp47* gene expression to levels comparable to those observed following heat shock. Maximum levels of *hsp47* mRNA were reached between four and eight hours of exposure (lanes 3 and 4) after which expression declined to background levels by 24 hrs (lane 6). Compared to *hsp47*, the same treatment induced the expression of *hsp70*, *hsp90α* and *hsp90β* genes to a lesser extent and did not result in an increase of *hsp90α* mRNA size similar to that observed during heat shock. These data indicate that the response of embryos to ethanol differs substantially from that which occurs during hyperthermia. The results also suggest that genes belonging to different zebrafish hsp families may vary in the level of their response to the same stress.

3.2. Spatio-temporal pattern of *hsp47* expression during zebrafish development

The previous experiments have not revealed constitutive expression of *hsp47* in Northern-blot analysis of total RNA, which was surprising given the previous description of this protein as an important collagen-specific chaperone molecule and the fact that diverse types of collagens are likely to be synthesized by this developmental stage (48hrs). I hypothesized that the Northern-blot method was simply not sensitive enough to detect the *hsp47* signal at early stages of development.

Figure 4. *Hsp47, hsp70, hsp90α, hsp90β* and *β-actin* gene expression in 2 day old zebrafish embryos during continuous exposure to ethanol. Embryos were incubated in 4% ethanol at 28.5°C and samples removed for isolation of total RNA and subsequent Northern blot analysis. Lane 1, control; Lane 2, 2 hours; Lane 3, 4 hours; Lane 4, 8 hours; Lane 5, 12 hours; Lane 6, 24 hours.

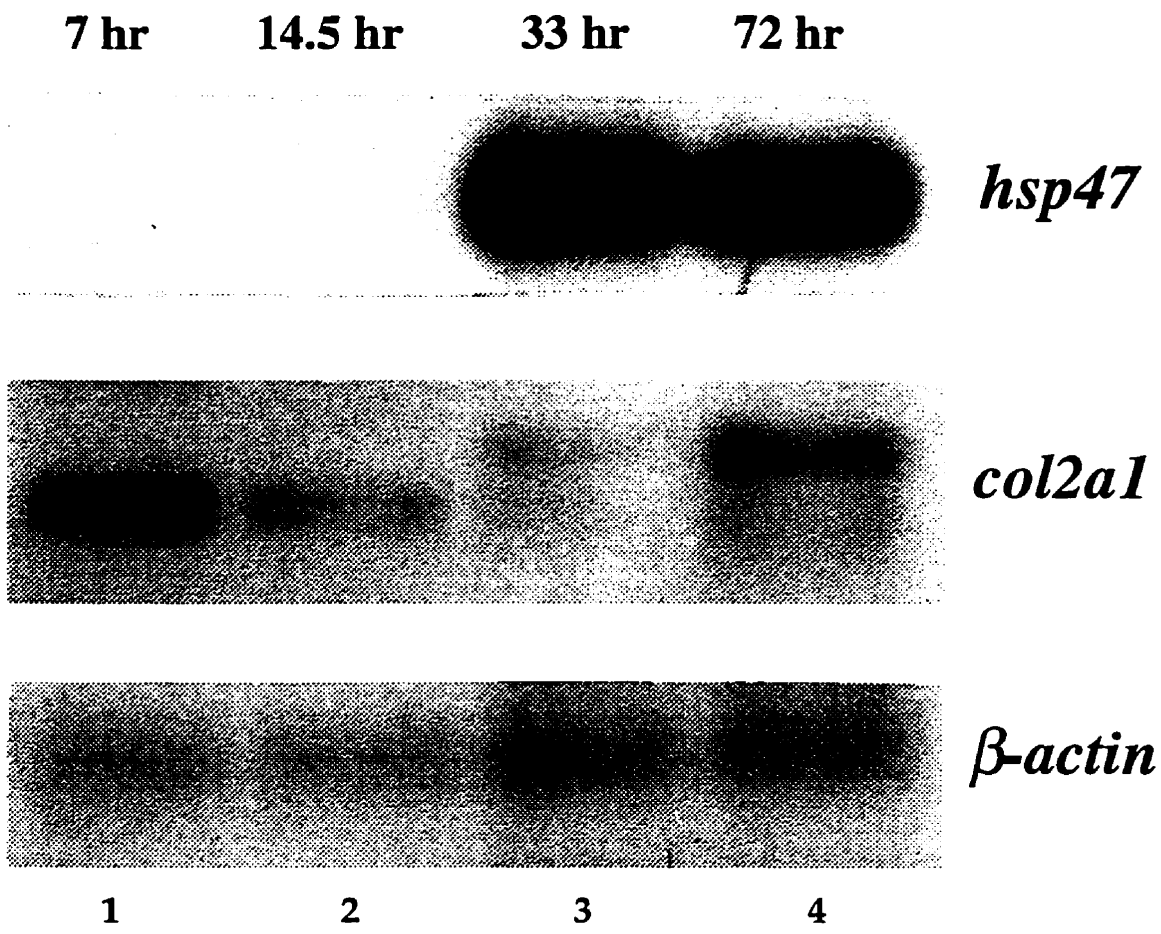

h in EtOH
C
2
4
8
12
24
hsp47
hsp70
hsp90α
hsp90β
β-actin
1
2
3
4
5
6

3.2.1. Temporal pattern of *hsp47* and *col2a1* expression

To examine whether the low sensitivity of the total RNA containing Northern-blot analysis was responsible for the lack of *hsp47* signal in samples made from earlier stage embryos, I carried out Northern-blot experiments using poly(A)$^+$ mRNA in cooperation with D. Pearson and J. Sass. This approach allowed us to load approximately 50 times more mRNA into each lane, thereby increasing the sensitivity of the method dramatically.

Figure 5 shows the results of this Northern blot analysis using a poly(A)+ blot. Equal loading is shown by hybridization to a zebrafish *actin* probe (Fig 5/c). As this experiment was the starting point of my studies in examining possible coexpression between *hsp47* and collagen gene expression during zebrafish development, I also used the only available zebrafish collagen cDNA probe, *col2a1* (coding for the proalpha1 chain of type II collagen; provided by J. Postlethwait, Univ. of Oregon, USA). This experiment demonstrated that *hsp47* mRNA is present as early as 33 hr (Fig5/a, lane 3) but even with the increased sensitivity given by the use of poly(A)$^+$mRNA, I failed to detect its presence in mid-gastrula (7 hrs; lane 1) and in mid-somitogenesis (14hrs; lane 2) stage embryos. Two different *col2a1* transcripts were recognized by the C-terminal/3'-untranslated region-specific probe which was used to hybridize the same blot. A smaller transcript of approximately 3.9 to 4.0 kb was the predominant mRNA species present in gastrula (lane 1) and mid-somitogenesis (lane 2) stage embryos. Following somitogenesis, a second type II collagen transcript of 4.1-4.2 kb appeared and became the predominant species by 3 days of development (lane 3). As well, two minor transcripts of over 6 kb in size, which mirrored the 3.9-4.0 kb species in their temporal appearance were also detected in some blots but are not shown in Fig. 5/b. The data suggest that *col2a1* RNA in zebrafish may be subject to alternative splicing as has been demonstrated for other vertebrate type II collagen genes (Ryan and Sandell, 1990; Metsaranta et al., 1991; Nah and Upholt, 1991; Su et al., 1991). Interestingly, a sharp increase in the level of

Figure 5. Northern blot analysis of poly $(A)^+$ RNA isolated from zebrafish embryos at different stages of embryonic development. Equal quantities of RNA were separated by gel electrophoresis through formaldehyde-agarose gels and hybridized to probe indicated as described in Section 4. Lane 1, 7 h (60-70% epiboly); Lane 2, 14.5 h (mid-somitogenesis); Lane 3, 33 h (prim 20); Lane 4, 72 h (protruding mouth).


7 hr
14.5 hr
33 hr
72 hr
hsp47
col2a1
β-actin
1
2
3
4

hsp47 mRNA was observed in prim-20 embryos (Fig5/a, lane 3), a time which coincided with the appearance of the larger *col2a1* transcript.

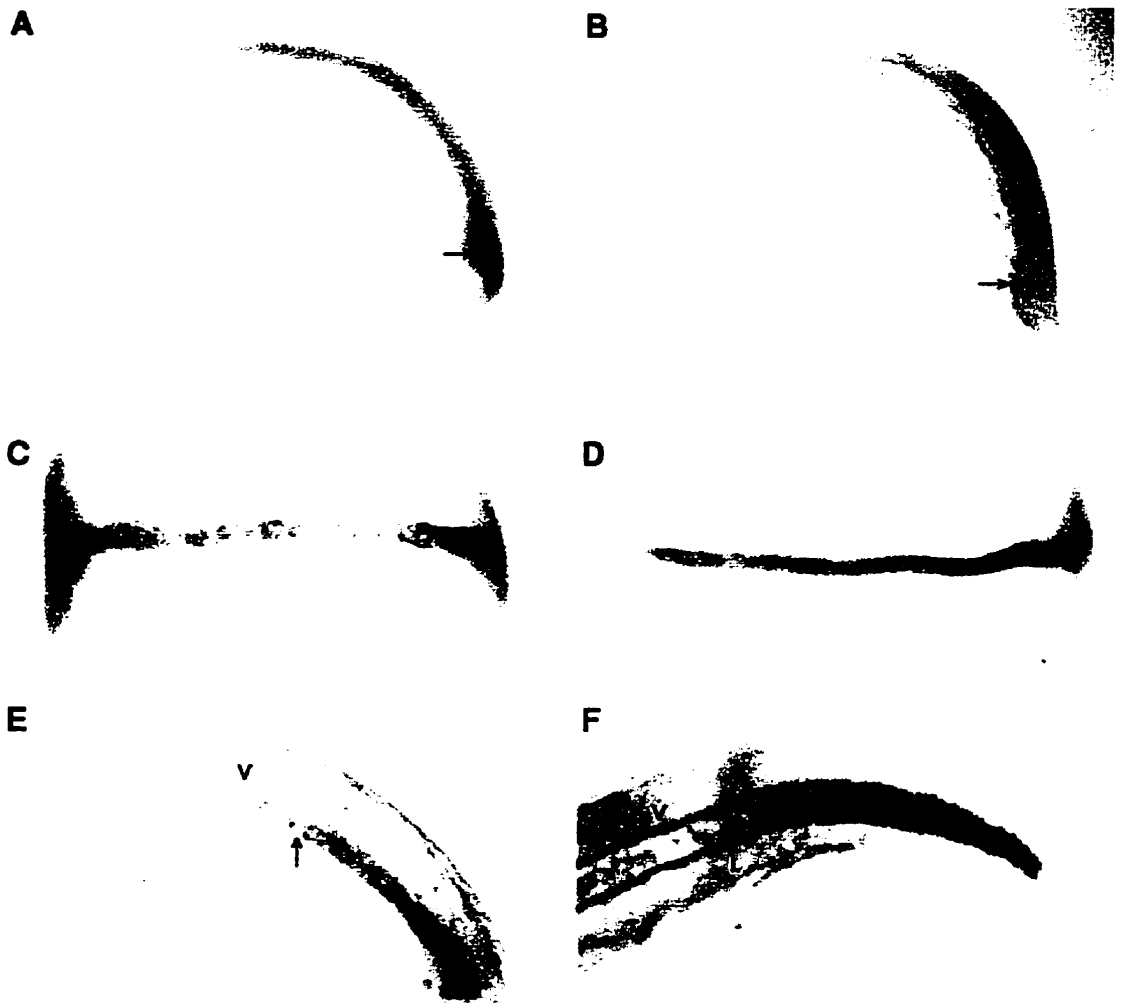
3.2.2. Coexpression and differences in the expression of *hsp47* and*col2a1* during zebrafish development

The low levels of *hsp47* mRNA which could be detected only in poly(A)$^+$ mRNA containing Northern-blot analysis suggested that its expression might occur in a tissue-specific fashion in the developing zebrafish embryos. To address this question I carried out whole-mount in situ hybridization analysis at various stages of zebrafish embryogenesis. I also used the *col2a1* probe in similar experiments to determine if there is a correlation between the expression of these genes which would be expected based on earlier biochemical data identifying Hsp47 as a collagen-specific chaperone (Nagata, 1996).

3.2.2.1. *hsp47* and *col2a1* are co-expressed in non-chondrogenic tissues at gastrula and segmentation stages and are co-ordinately down-regulated in the notochord of 1 day old embryos

Since earlier experiments in our laboratory have proven the superior sensitivity of whole-mount in situ hybridization as compared to the Northern-blot method I carried out such analysis in epiboly and segmentation stage embryos. As shown in Figure 6, panel A, *hsp47* mRNA was detectable as early as six hours of development (gastrulation stage). Expression was first observed within the hypoblast, the inner cell layer of the involuting dorsal marginal zone (called embryonic shield, which is the equivalent of the dorsal blastopore lip in *Xenopus* and Hensen`s node in amniotes). The expressing cells in the shield likely represent those of the notochord rudiment, the chordamesoderm (Kimmel et al., 1995; see results below). Hypoblast cells lacked similarly distinct and strong accumulation of *col2a1* mRNA (panel B). By 11-12 hours of development however, the developing notochord was strongly stained by both the *hsp47* and *col2a1 probes.* (Fig. 6, panels C and D; see also Yan et al., 1995). Finally, *hsp47* mRNA was also detectable in paraxial mesoderm of the embryo

Figure 6. Comparison of *hsp47* (A,C,E) and *col2a1* (B, D, F) expression patterns in early zebrafish embryos using whole mount in situ hybridization analysis as described in Section 4. A, B: 6 h. C, D: 11h. E, F: 24h. A, B, E, F: lateral view. C, D: dorsal view. Strong expression of *hsp47* but not *col2a1* is detectable as early as six hours 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).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V
F
V

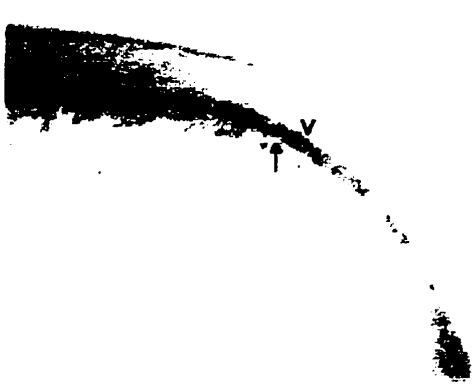
but at much lower levels. This is not unexpected since Hsp47 is proposed to be the specific chaperone of other types of fibril-forming collagens.

These data revealed that *hsp47* and *col2a1* are co-ordinately expressed within the developing notochord of 11-12 hour old embryos. Yan et al. (1995) have previously shown that the concentration of *col2a1* transcripts in midline cells reaches its peak by approximately 21 hours. After this time, levels of the transcript decline in a rostral-caudal direction and by approximately 24 hours they are detectable within the notochord only in the developing tail. As shown in panels E and F of Fig. 6, *hsp47* transcript levels decline in a similar rostral to caudal sequence as *col2a1* and messenger RNA levels of both genes are detectable only in the most caudal part of the notochord by 24 hours of development. This suggests that expression of these genes may be down-regulated by similar mechanisms in cells of the notochord.

3.2.2.2. *col2a1* but not *hsp47* is expressed in the floor plate and hypochord

The results presented thus far indicate that *hsp47* is strongly expressed in cells of the notochord which are also expressing *col2a1*. However, by the 14 somite stage (15 hr), two additional groups of cells just dorsal and ventral to the notochord also express *col2a1*(Yan et al., 1995; Fig. 6. panel F and Fig. 7. panel B). One of these is the floor plate, which is composed of the ventralmost cells of the developing neural tube. It is a single row of wedge-shaped cells which bifurcates and widens to a multicell width layer as it approaches the head region. The other *col2a1* expressing tissue is the hypochord, which is of mesendodermal (hypoblast) origin but its function during development is currently unclear. It is also a single line of flat cells, which lies just ventral to the notochord. Expression of *col2a1* in these tissues is maintained for a longer period of time than notochordal expression and can be detected in embryos well over 24 hours of age (Yan et al., 1995; see Fig. 6, panel F). Interestingly, I was unable to detect *hsp47* mRNA within either the floor plate or hypochord of 15 hour and 24 hour old embryos (see arrow and arrowhead in panel E of Fig.

Figure 7. Expression of both *hsp47* (A, C) and *col2a1* (B, D) in *flh* embryos. Expression of both genes was detected using whole mount in situ hybridization analysis using antisense RNA probes. A, B: wild-type. C, D: *flh*. All embryos were obtained from the same cross and are shown in lateral view. arrowhead: floor plate cells. arrow: hypochord.



A

B

C

D

6). This was not due to a lower level of *hsp47* mRNA being present in these tissues since a signal was still not detectable following heavy overstaining of embryos (data not shown).

The non-coordinate expression of *hsp47* and *col2a1* is particularly evident in the floor plate of *flh* embryos, which is present as a disrupted line of cells distributed along the A-P axis (Figure 7). In these embryos, axial mesoderm cells initially express the usual early markers of axial mesoderm such as *axial* and *twist* but then become respecified to form paraxial mesoderm derivatives and the notochord does not form (Halpern et al., 1995; Talbot et al., 1995). As expected, neither *hsp47* nor *col2a1* are expressed in the axial tissues at the normal location of the notochord in 15 hour old *flh* embryos. However, *hsp47* mRNA is also clearly absent from floor plate cells which strongly express *col2a1* in mutant embryos (arrowhead in panel D; *flh* embryos do not develop a hypochord). A similar result was obtained for *hsp47* with 15 hour old *no tail* (*ntl*) embryos (Halpern et al., 1993), which also exhibit a disruption of proper notochord formation (data not shown). The expression of *col2a1* without the concurrent expression of *hsp47* in these tissues was a very surprising result and clearly indicates that the two genes are not strictly co-regulated in all tissues of developing embryos.

3.2.2.3. *hsp47* and *col2a1* are widely co-expressed in 24 and 48 hour old embryos

Following the first day of development, *col2a1* and *hsp47* begin to be expressed in a wide array of developing tissues. In most cases co-expression is obvious (see below); however there are several tissues which express only one of the two genes. Within the trunk, I have been able to detect *col2a1* mRNA in the floor plate and hypochord of embryos up to 50 hours of age (data not shown). Similar experiments revealed that *hsp47* mRNA remained undetectable in these regions throughout this period of development (data not shown). Conversely *hsp47*, but not *col2a1* mRNA, was detected in the developing lens of the eye (arrowhead in panels A and B of Fig. 8).

Figure 8. Expression of *hsp47* (A, C, E, G) and *col2a1* (B, D, F, H) in the head of 24 and 48 hour old embryos detected using whole mount in situ hybridization analysis. Only *hsp47* is expressed in the developing lens (arrowhead in panels A and B) whereas both genes are expressed in the otic vesicle (arrow in panels A and B), otic capsule (E, F), ventricular neuroepithelium (C, D), and developing chondrocranium (open arrow in panels G and H), among other tissues. A, B, G, H: dorsal view. C, D: optical cross-section. E, F: JB-4 embedded 8μm thick section.

C
D
G
H
B
F
A
E

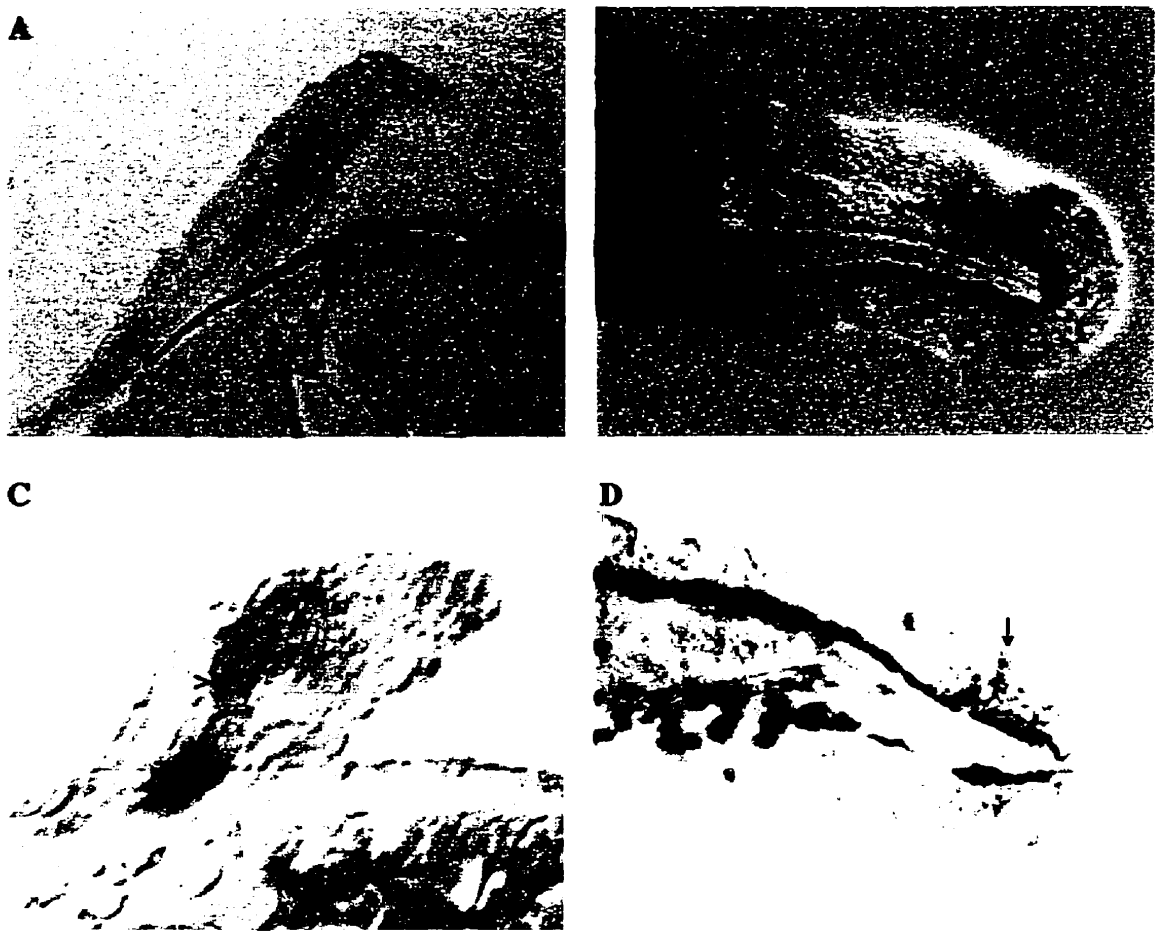
One of the most obvious regions of co-expression of *hsp47* and *col2a1* in older embryos is the developing ear. The otic vesicle, which is well developed by 24 hours, is strongly stained with both probes (arrow in Fig. 8. panels A, B). The co-expression appears at 18 hours and persists at least up to 3 days of development. Optical sectioning using DIC (Nomarski) optics revealed that both transcripts accumulate primarily within the epithelial cells of the vesicle. At 48 hours, strong co-expression is also detectable in the mesenchymal cells which condense to form the cartilaginous otic capsule surrounding the ear (Fig. 8, panels E, F). A number of other tissues in the head also exhibit co-expression of *hsp47* and *col2a1*, including the ventricular neuroepithelium (Fig.8. panels C, D) and the developing chondrocranium (panels G, H). These are all tissues in which *col2a1* expression has been observed in other vertebrates. Within the pectoral fin bud, *hsp47* mRNA was detected in *col2a1*-expressing cells which form the precartilage core of the fin (arrowhead in Fig. 9, panels A, C). As well, expression of both genes was detectable within the mesenchymal cells at the caudalmost region of the median fin fold and within condensations of cells along the fin fold rostral to this region (panels B and D). These condensations most likely represent regions at which the collagenous strengthening rays (actinotrichia) will form (Kimmel et al., 1995).

3.3. Tissue- and stress-specific differences in *hsp47* and *hsp70* expression after heat shock and ethanol treatment in zebrafish embryos.

After finding tissue-specific expression of *hsp47* in developing zebrafish embryos I was next interested in determining whether stress-induced expression of this gene would show any similar tissue-specificity. I also analysed the expression of the *hsp70* gene, since we knew from previous in situ hybridization experiments that under normal conditions it is not expressed in the developing embryos.

The Northern-blot analysis revealed differences in the temporal pattern of expression of these genes after heat shock and ethanol treatment. I wanted to determine if this was due to any tissue-specific differences in their stress-induced expression pattern. To examine

Figure 9. Expression of *hsp47* (A, B) and *col2a1* (C, D) in the developing pectoral fins (A, C) and caudal most region of the median fin fold (B, D) of 48 hour old embryos detected using whole mount in situ hybridization analysis. Both genes are expressed in the precartilage core of the pectoral fins (arrowhead in A, C) and within the mesenchymal cells of the median fin fold (panels B, D). The arrow in panels B and D indicates the cell condensations which express both genes and most likely represents the developing collagenous strengthening rays (actinotrichia).


A
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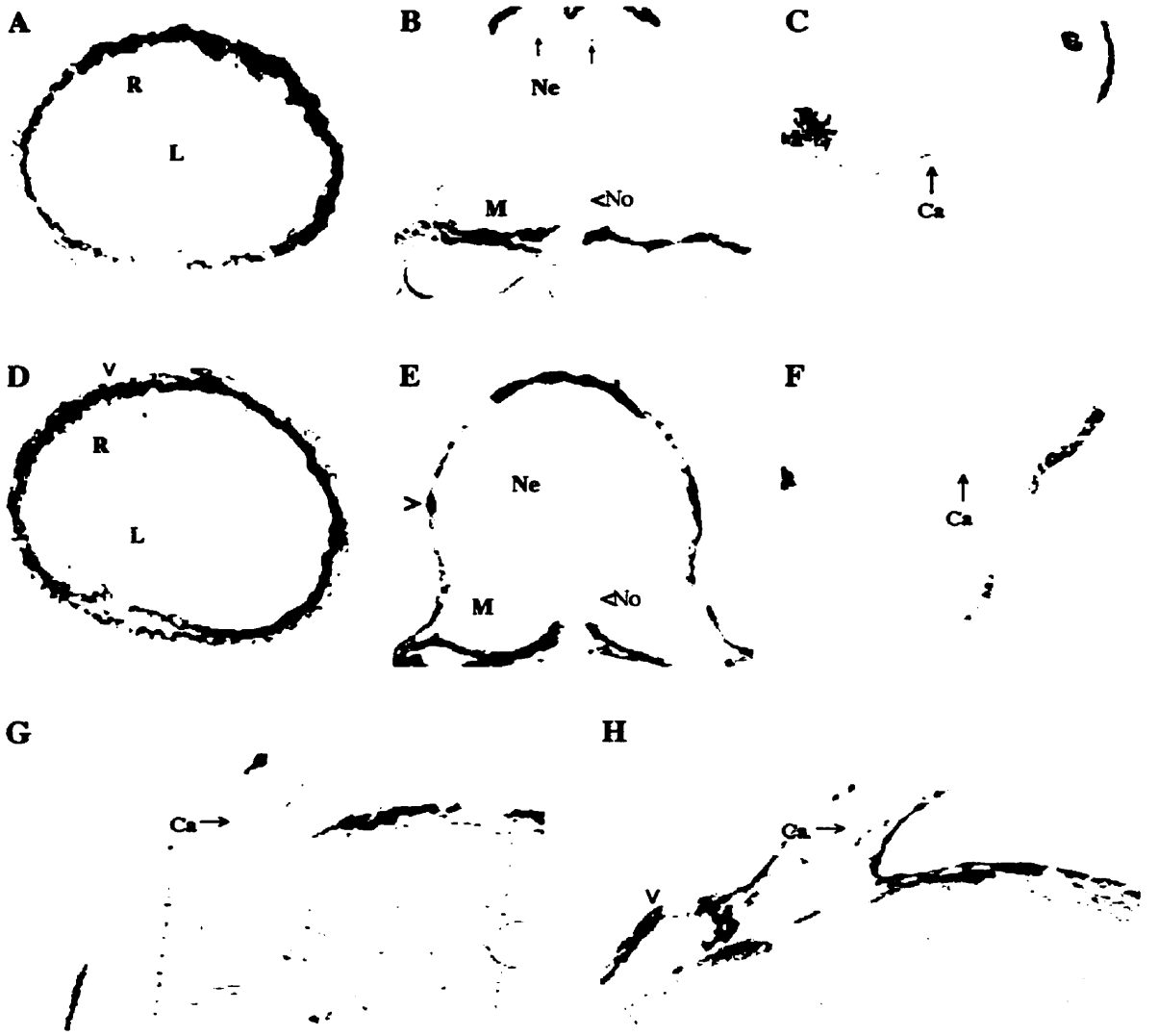
D

expression induced by heat shock and ethanol treatment in more detail, I carried out whole-mount in situ hybridization analysis on 2 day old embryos in order to characterize the three dimensional expression patterns of these genes. Figure 10 and 11 shows the results of this analysis. Following a four hour exposure to 4% ethanol, high levels of *hsp70* mRNA were detectable primarily in the embryonic head, whereas heat shock resulted in dramatic accumulation of *hsp70* mRNA along the entire embryo (data not shown). Both heat shock and ethanol resulted in increased levels of *hsp47* mRNA in the head and the connective tissue septa which separate the myotomes in the trunk and tail (datsa not shown).

To analyse the expression pattern in more detail, I embedded the stained embryos in JB4 polymer (Polysciences Inc.) and sectioned them at 8μ. Comparison of the sectioned embryos following whole mount in situ hybridization revealed several similarities in the distribution of *hsp70* mRNA after the two stress treatments (Figure 10.). Low levels of mRNA were detectable in the outermost cells of the developing lens in both heat-shocked and ethanol-treated embryos (panels A,D). Similar expression was also observed in the mesenchymal tissues of the pectoral fin and head, excluding the precartilagenous cells (panels C, F, G, H). No *hsp70* mRNA was apparent in the striated muscles of the trunk or in the notochord (panels B, E).

In contrast, there are several distinct differences in the inducibility of *hsp70* by heat or ethanol. The most striking example is the epidermal epithelial cells, which exhibit very high levels of *hsp70* mRNA throughout the embryo only after heat shock (large arrowhead in panels D, E, F, and H). This epithelial expression is likely responsible for the considerably higher *hsp70* mRNA levels that were observed in the Northern blot analysis. The *hsp70* mRNA distribution is also different in the eye. Whereas expression in ethanol-treated embryos is limited to the outermost cells of the lens, heat shock resulted in additional strong expression in the cells of the retina (compare panels A and D). In contrast, ethanol but not heat shock resulted in the appearance of *hsp70* mRNA in certain, unidentified cells of the spinal cord (arrows in panel B). The results clearly demonstrate that the

Figure 10. Stress-specific differences in the distribution of *hsp70* mRNA following ethanol treatment (A-C, G) and heat shock (D-F, H) of two day-old embryos. For heat shock, embryos were incubated at 37°C for two hours. Ethanol treatment was carried out in 4% ethanol for 4 hours. Following hybridization, embryos were embedded in JB-4 resin and sectioned as described in Materials and Methods. *Hsp70* expression is not detectable in control embryos. Panels A, D: sagittal section of the eye, Panels B, E: cross section of the trunk caudal to the otic capsule. Panels C, F: mediansagittal section of the head through the mouth, anterior to the right. Panels G, H: Sagittal section of the pectoral fin. R, retina; L, lens; Ne, neural tube; M, striated trunk muscle; No, notochord; ca, precartilagenous cells. Small arrows denote *hsp70*-expressing cells in the neural tube. Large arrowheads mark the *hsp70*-expressing epithelial cells. Dark brown-black pigment cells are visible in all sections underlying the developing epithelial cells which exhibit heat-inducible *hsp70* gene expression.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Ca
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Ca
V

Figure 11. *Hsp47* expression in control (A-C), ethanol-treated (D-F) and heat-shocked (G-I) two day-old zebrafish embryos. All conditions as described in Figure 6. Panels A,D,G: Sagittal section of the pectoral fin. Panels B,E,H: Mediansagittal section of the head through the mouth, anterior to the right. Panels C,F, I: Cross section of the trunk at the level of the otic vesicle. ca: precartilagenous cells; hb: hindbrain; ov: otic vesicle. Large arrowhead in H,I marks the epithelial cells which do not express *hsp47* but exhibit strong, heat-inducible expression of *hsp70* mRNA (see Figure 11). Dark brown-black pigment cells are also visible in immediately underlying the developing epithelial cells which express *hsp70* mRNA following heat shock (open arrows).

A
ca
B
ca
C
D
ca
E
v
ca
F
hb
ov
G
ca
H
v
ca
I
hb
ov

zebrafish *hsp70* gene is expressed in different spatial patterns in a stress-specific manner.

In order to characterize *hsp47* mRNA expression in more detail, I next examined the distribution of the transcript in sections of embryos following whole mount in situ hybridization (Figure 11.). *Hsp47* mRNA in control embryos is detectable in the precartilagenous cells of the fin bud and the chondrocranium (panels A, B), as well as both in the otic vesicle itself and in the precartilagenous cells which will differentiate into the cartilage capsule surrounding this structure (panel C; see also Figure 8.). Following either a 2 hour heat shock at 37°C or a 4 hour exposure to 4% ethanol, *hsp47* mRNA levels significantly increased in the precartilagenous cells of the fin bud and head (panels D, E, G, H). Furthermore, strong accumulation of *hsp47* transcripts became apparent in the mesenchymal tissues surrounding these developing chondrogenic cells. Elevated *hsp47* expression was also evident in the otic capsule and associated populations of mesenchymal cells of the head (panels F, I). In all cases, *hsp47* mRNA levels appeared only slightly higher in heat-shocked embryos than those exposed to ethanol, thus confirming the data obtained from Northern-blot analysis. Importantly, heat shock did not result in any detectable accumulation of *hsp47* mRNA in the cells of the epidermis which exhibit very strong heat-inducible accumulation of *hsp70* transcripts (compare large arrowheads in Figure 11, panels H, I with those in Figure 10, panels E, F). Thus, *hsp47* expression occurs in a spatial and stress-specific pattern which differs significantly from that of *hsp70* indicating the presence of tissue-specific stress-induced regulatory mechanisms for these genes.

This concludes the portion of my thesis that deals with the stress-inducibility of various heat shock genes in zebrafish. I produced results that confirm previous reports on the heat-inducibility (or the lack of it) of these genes. More importantly I have established that different heat shock genes are subject to stress and tissue-dependent regulatory mechanisms in zebrafish embryos. I also found that under normal conditions in the developing embryo the expression pattern of *hsp47* shows extensive similarity to the gene encoding one of its

potential substrates *col2a1*. This latter result will undoubedly open new possibilities in our laboratory`s research in this field.

3.4. Examination of Hsp90 function during zebrafish development

In the recent years several discoveries have been made which suggest that Hsp90 might play an important role during vertebrate development. For example, numerous biochemical and cell culture studies have shown that members of the Hsp90 family 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, 1993; Smith and Toft, 1993), $pp60^{v\text{-}src}$ kinase (Schuh et al., 1985; Brugge, 1986), dioxin (Ah) receptor (Antonsson et al.,1995; Whitelaw et al., 1995) and MyoD (Shaknovich et al., 1992; Shue and Kotz, 1994). There are also several lines of independent, though indirect evidence suggesting that Hsp90 might have a role in muscle differentiation processes. The first indication was provided by biochemical analysis which showed the in vitro interaction between Hsp90 and MyoD, one of the main regulatory factors involved in myogenesis. It is a bHLH transcription factor which can activate the myogenic differentiation pathway in vitro (for review see Emerson, 1990). The sequence-specific binding activity of both in vitro synthesized MyoD and MyoD/E12 heterodimers, the latter of which is thought to be the in vivo active form of the transcription factor, was enhanced in the presence of Hsp90 (Shaknovich et al., 1992; Shue and Kohtz, 1994). In addition, another Hsp90 substrate, casein-kinase II has been implicated as a regulator of MyoD function during mammalian muscle differentiation (Miyata and Yahara, 1992; Johnson et al., 1996).

These in vitro experiments are especially interesting in the light of studies carried out in our laboratory by Sass and coworkers (1996), which showed that constitutive expression of *hsp90α* is restricted to a subset of cells which also express *myoD* in the developing somites of zebrafish embryos. Furthermore, *hsp90α* is downregulated along with *myoD* in the mature muscles of the trunk at a time when expression of the muscle structural protein *α-tropomysin* remained high. Coordinate

misexpression of both *myoD* and *hsp90α* has been reported in several mutant strains of zebrafish during somitogenesis (Sass and Krone, in preparation). In these mutant embryos *hsp90α* expression followed that of *myoD*. Moreover, enhanced expression of *hsp90* was observed in developing infantile myofibers and in regenerating fibers of Duchenne's muscular dystrophy patients which are both likely sites of *myoD* expression (Bornmann et al., 1995, 1996). In combination, these results strongly suggested that *hsp90α* plays a specific role in the normal process of myogenesis.

3.4.1. Expression of *hsp90β* during zebrafish development

As developmental expression of zebrafish *hsp90α* has been earlier reported by our laboratory (Sass et al., 1996), it was a natural step to extend these observations to *hsp90β*, which as Northern-blot analysis had shown, is strongly expressed throughout zebrafish development under normal conditions. Thus, I next examined the spatial pattern of its expression using whole-mount in situ hybridization analysis.

In the 3.5 hour old embryo which just passed the midblastula transition (MBT, activation of the embryonic genome), *hsp90β* mRNA is present ubiquitously (Figure 12./A). At the onset of gastrulation (50% epiboly, 6hr pf.) this homogenous expression remains (Figure 12./B). However, by the time epiboly is almost completed (8.5 hr pf.) *hsp90β* becomes primarily restricted to the anterior half of the embryo (Fig. 12./C,D). This restriction is only transient; by the time somitogenesis is completed (24 hr pf.) *hsp90β* expression reappears in the posterior part of the embryo (Figure 13./A). At this time, elevated *hsp90β* mRNA levels can be detected in the tip of the tail, in the developing urogenital region and in the neural tube. The notochord on the other hand does not exhibit *hsp90β* expression (arrowhead Fig 13./B). As it can be seen in Figure 13./B,C, high levels of *hsp90β* mRNA demarcate the neuroepithelial layer of the central (neural) canal and the ventricles which eventually develop into the lining cells of these regions. The outermost cell layer of the neural tube also strongly expresses *hsp90β* (arrowhead Fig. 13./C) but it will require further analysis in sections of

Figure 12. Expression of *hsp90β* in early stages of zebrafish embryogenesis. Panel A: 512-cell stage (early-midblastula; 2.75 hr). Panel B: 50% epiboly (beginning of gastrulation; 5.5 hr). Panel C and D: 80% epiboly (8.5 hr). The early ubiquitous expression becomes restricted to the anterior part of the developing embryos as epiboly progresses.

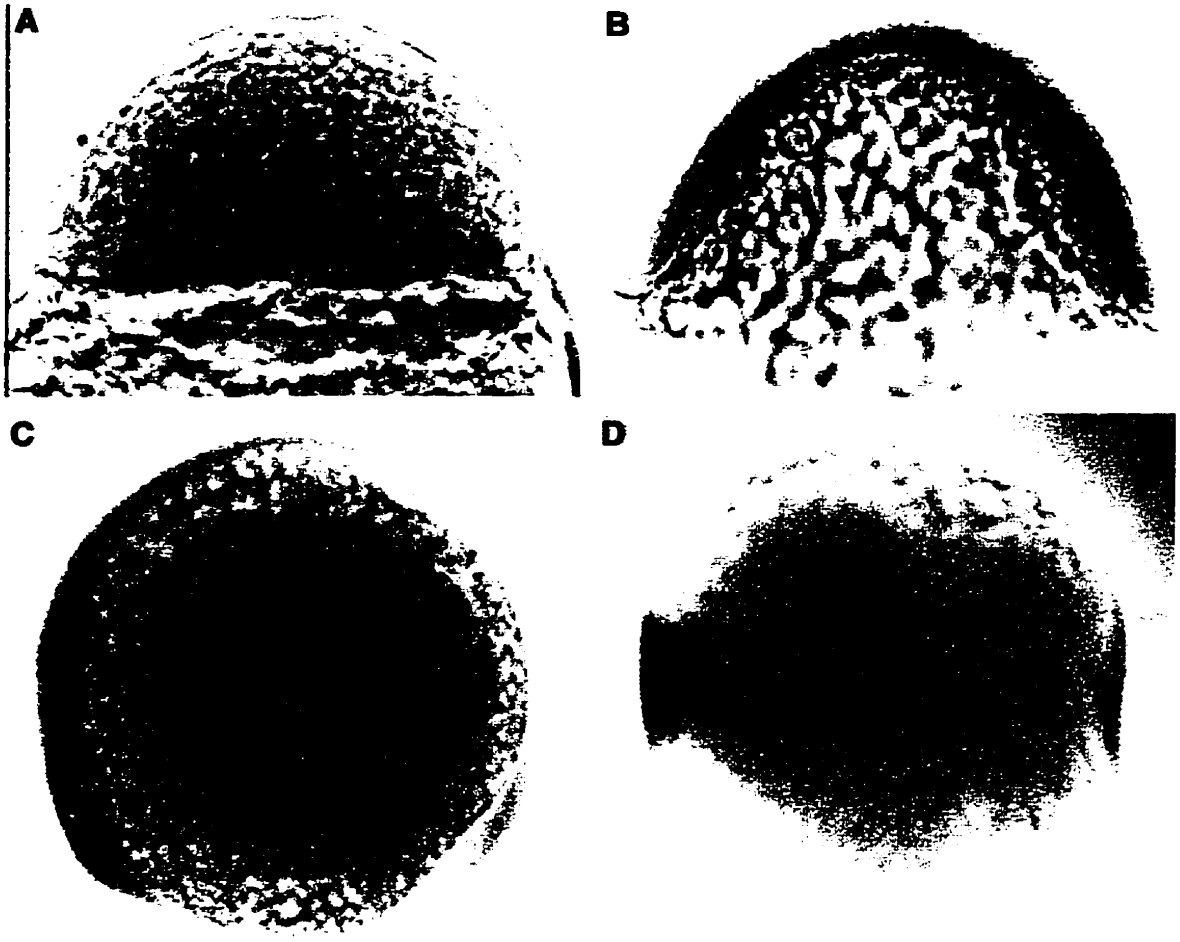
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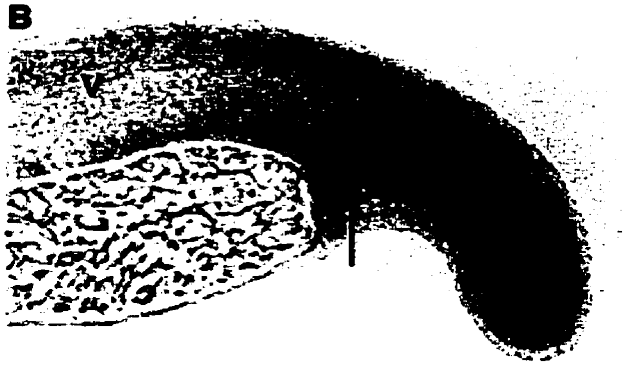
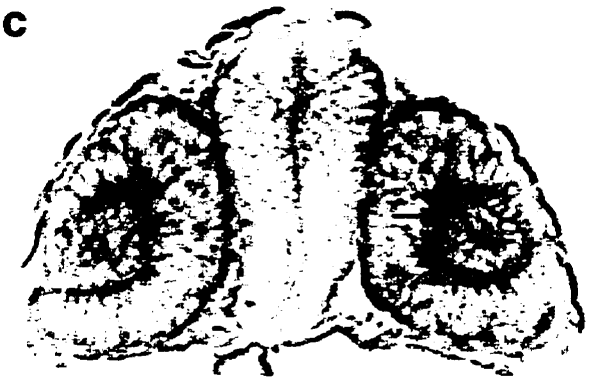
Figure 13. Expression of *hsp90β* in 24 hr old zebrafish embryos. A: Dorsal view of a 24 hr old embryo. B: Lateral view of the tail. C: Cross-section of the head at the level of the eyes. D: Cross-section of the midbrain ventricle. Expression is detectable in the developing urogenital region (arrow in panel B); in the neural retina and lens of the eye (arrow in panel C) and in the outermost and innermost cells of the midbrain (arrowhead and arrow in panel D). No *hsp90β* mRNA is detectable in the notochord (arrowhead in panel B).

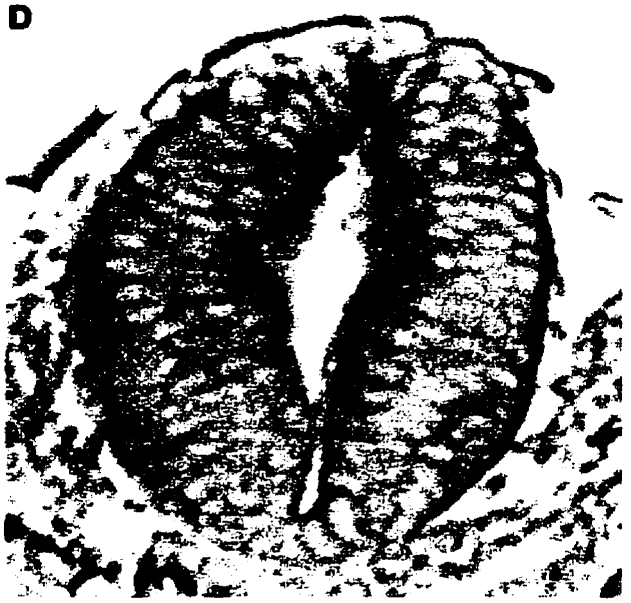
B



C



D

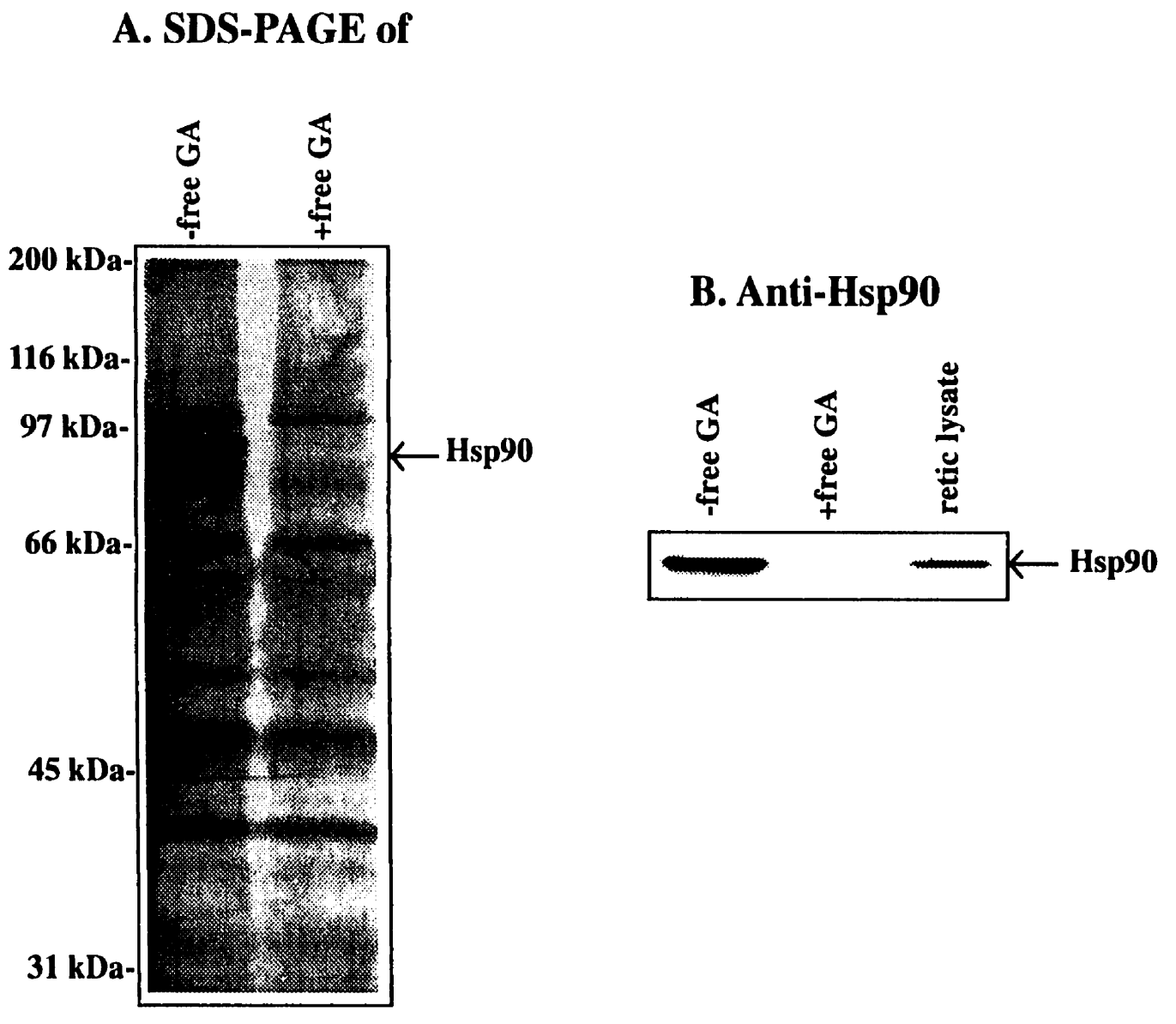


embryos to identify the specificity of the expression and the nature of the expressing cells. The mRNA distribution is especially interesting in the developing eye, where it is present in extremely high levels in the retinal layer of the optic cup as well as in the outermost layer of the forming lens (Figure 13./D). These data suggest that *hsp90β* might be responsible for carrying out the general chaperoning functions attributed to Hsp90 in cells under normal conditions.

3.4.2. Geldanamycin binds zebrafish Hsp90s specifically

To study the effect that elimination of Hsp90 function has on developing zebrafish embryos, I used the benzoquinone ansamycin, geldanamycin (GA). It was shown to interact in a specific manner with mammalian Hsp90 and to inhibit the activity of a number of Hsp90 substrates both in vitro and in cell culture (see chapter 1.2.6.3.), but no similar studies have been carried out regarding its interaction with zebrafish Hsp90. Given the high degree of amino acid identity of Hsp90s between zebrafish and other vertebrates (Krone and Sass, 1994), I predicted that GA would also interact in a specific manner with zebrafish Hsp90. To investigate this question, GA-derivatized resin was prepared and used for zebrafish Hsp90-binding studies (Figure 14, in collaboration with S. Hartson, B. Matts, Oklahoma State Univ., Stillwater, USA; and L. Whitesell, Univ. of Arizona Health Sciences Center, Tucson, USA; Whitesell et al., 1994). Initial work revealed that a polyclonal antibody against mouse Hsp90 (Ullrich et al., 1986) also detected a 90 kDa protein in zebrafish which co-migrates with mammalian Hsp90 (data not shown; see also Figure 14, panel B). This protein, which I suggest to be zebrafish Hsp90, binds strongly to the GA-derivatized resin (panel B), and the interaction is completely inhibited by preincubation of the protein extract with free GA (panel B). Furthermore, Hsp90 is the only detectable zebrafish protein which exhibits competable binding as determined by silver staining of polyacrylamide gels (panel A). Thus, I concluded that zebrafish Hsp90 interacts in a specific manner with free geldanamycin.

Figure 14. 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 (panel B, lane 1) and this interaction was completely inhibited by pre-incubation with free GA prior to application to the GA-resin (lane 2). Zebrafish Hsp90 co-migrated 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 polyacrylamide gels (compare lanes 1 and 2 of panel A).


A. SDS-PAGE of
-free GA
+free GA
200 kDa-
116 kDa-
97 kDa-
66 kDa-
45 kDa-
31 kDa-
Hsp90
B. Anti-Hsp90
-free GA
+free GA
retic lysate
Hsp90

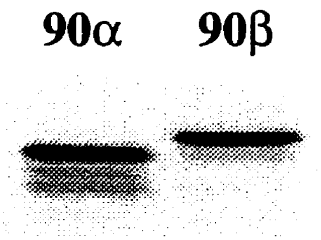
One question which remained was whether geldanamycin interacts with both Hsp90α and Hsp90β isoforms. Since there are no antibodies that can distinguish between the two proteins presently, an alternative approach had to be employed. To overcome this problem both full-length cDNAs were isolated (Lele et al., in preparation) and cloned into the expression vector pCS2 (gift of Ralph Rupp). These clones were expressed separately in rabbit reticulocyte lysate and GA-resin assay were carried out. Figure 15. demonstrates that both Hsp90α and Hsp90β was bound specifically by geldanamycin-resin. This result also underscores the findings which demonstrated that GA induced alterations in tissues where either *hsp90α* or *hsp90β* was expressed (somites, circulatory system, brain) under normal conditions. On the other hand, development of the notochord which did not show expression of either of these genes was not affacted.

3.4.3. Effects of geldanamycin on the development of zebrafish embryos

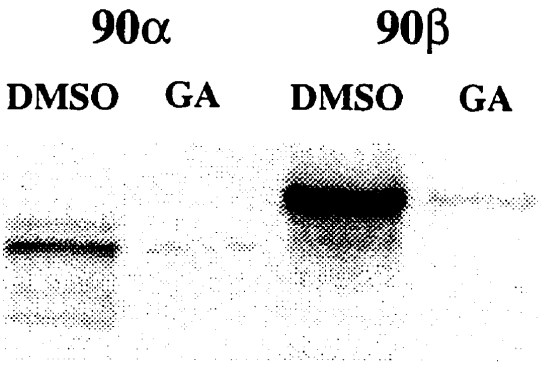
To examine the effects of GA treatment on zebrafish development, embryos were incubated in a solution of GA in system water (see Materials and Methods) for a number of different temporal and concentration (data not shown) treatment regimes. The results of these experiments are summarized in Table 2. GA treatment (20 μM) initiated at the mid-blastula stage was rapidly lethal in over 95% of the embryos with the few that survived exhibiting developmental arrest during gastrulation (data not shown). When the same treatment was initiated slightly later in development, at the onset of gastrulation (50% epiboly; 5-5.5 hr stage), the death rate of embryos up to 20hrs of age was comparable to that observed in either DMSO-treated or untreated controls (Table 2.). However, 78% of the embryos exhibited a distinct phenotype characterized by a severely shortened trunk and tail with block-shaped or U-shaped somites (Figure 16, compare panels A and C with B and D). The somite shape change is especially evident in those embryos stained for muscle-specific *α-tropomyosin* mRNA, the expression of which outlines the shape of the somites in normal and

Figure 15. 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-labelled products subjected to GA affinity column analysis as described in Section 4. Both translation products, which have an M_r consistent with their identity as Hsp90 (panel A), bind to GA-derivatized resin (panel B) in a manner which is competed with free GA but not with DMSO (compare lane 2 to lane 1 and lane 4 to lane 3).

A. Translation products



B. GA resin



treated embryos (compare panels C and D). Most of these embryos die by about 24 h of age.

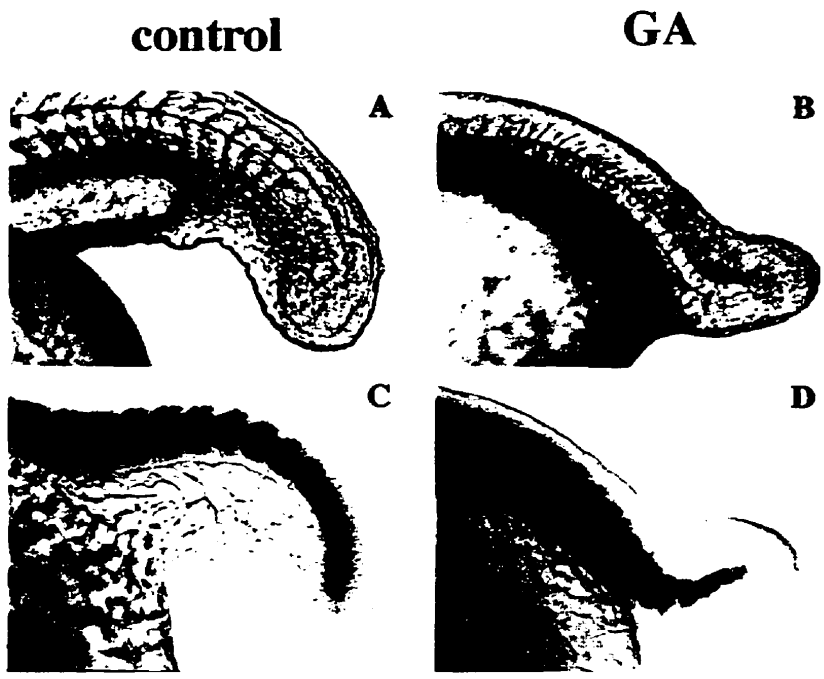
Table 2: Summary of data from geldanamycin, geldampicin and forskolin treatment experiments.

Treatment	Number of embryos treated	Survivors at 20 hours (% of treated)	Embryos exhibiting abnormal phenotype[1] (% 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%)

1. embryos exhibiting typical severe trunk/tail phenotypes for geldanamycin and forskolin treatments.

To ensure that the defects were due specifically to geldanamycin and not to a more general toxic effect of benzoquinone ansamycins, I carried out parallel experiments using geldampicin (gift of K. Rinehardt, University of Illinois). Geldampicin has an almost identical structure to that of geldanamycin's except for a single sidechain substitution (see Fig. 1. and 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

Figure 16. Trunk/tail phenotype of 18 hour old embryos treated with geldanamycin beginning at 50% epiboly (5-5.5 hours).
A and C: control. B and D: GA. While GA-treated embryos exhibited a comparable survival rate as controls prior to the first day of development, seventy-eight percent exhibited a shortened trunk/tail phenotype and the somites did not form their characteristic chevron shape (panel B). Expression of muscle *α-tropomyosin* was normal in treated embryos (compare panels C and panel D) 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.


control
GA
A
B
C
D

in the ability of these two compounds to inhibit the activity of specific Hsp90 substrates (Whitesell et al., 1994; Grenert et al., 1997). In agreement with these data, I could detect no phenotypic differences between control embryos and those treated with geldampicin beginning at 50% epiboly (Table 2). These data indicate that the geldanamycin-induced phenotype observed in our experiments is not due to the toxicity of benzoquinone ansamycins in general but instead is consistent with it arising from a specific GA/Hsp90 interaction which occurs during gastrulation. Furthermore, this interaction appears to disrupt a critical function of Hsp90 which is required for proper somitogenesis and trunk/tail formation.

Embryos exposed continuously to GA also exhibit several other abnormalities. The embryos do not extend around the yolk but rather remain on the top of it, even though the cell movements of epiboly which engulf the yolk are completed. The yolk sac extension known as the yolk tube, which normally extends below the developing tail, does not form in GA-treated embryos and the tail lifts vertically off of the yolk (Figures 16 and 18). In the few less severely affected embryos, which grow and live until two days of age, pigmentation is dramatically reduced and the eyes are much smaller. These embryos also display defects in the development of the circulatory system including lack of the dorsal aorta. Thus, despite the presence of a beating heart, proper circulation is never established. Perhaps due to the lack of circulation, cells in the tail of these mildly affected embryos eventually become necrotic and the embryos die. These circulation defects are currently being investigated in more detail.

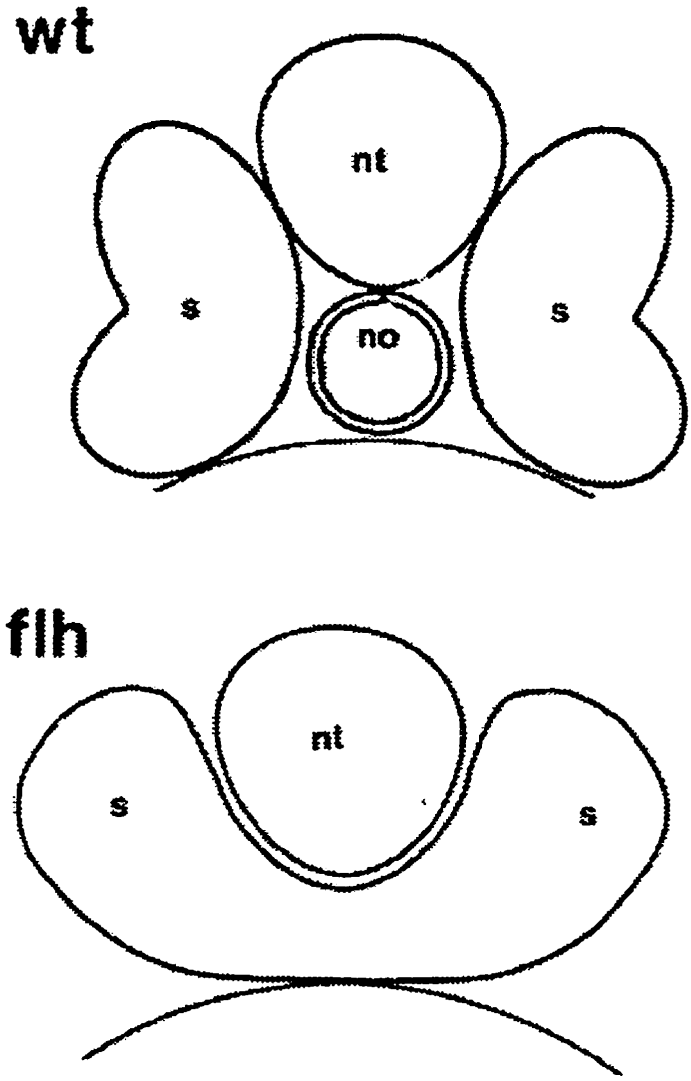
3.4.4. Development of the notochord in the trunk and tail of geldanamycin-treated embryos

The somitic phenotype induced by applying GA at 50% epiboly is very similar to that previously described for embryos carrying mutations in the *no tail* (*ntl*; Halpern et al., 1993) and *floating head* (*flh*; Halpern et al., 1995; Talbot et al., 1995) genes. A notochord does not develop in embryos homozygous for either of these mutations. In *flh* mutants, cells in the region which would normally form the

notochord instead express characteristics of paraxial mesoderm and the somites fuse beneath the neural tube (Figure 17.). In *ntl* embryos, cells in this location appear to be blocked notochord precursors which express some characteristics of early notochord development. Since the notochord plays a crucial role in patterning the somites, the defects in embryos carrying these mutations are thought to arise due to a lack of proper signaling from the notochord. Thus, it was possible that the GA-induced phenotype in our experiments was caused by a lack of proper notochord formation. To monitor development of the notochord, I utilized the zebrafish *col2a1* cDNA probe which detects *type II collagen* mRNA (Yan et al., 1995). This mRNA is expressed in a spatiotemporal wave along the notochord during its formation, with cells in the most posterior portion of the notochord being the last to express it (see Fig. 6. or Lele and Krone, 1997). As shown in Figure 18. panels A-D, expression of the *col2a1* gene was unchanged in the notochord of GA-treated embryos and the cells exhibited the characteristic vacuolated, "stack of pennies" appearance (Kimmel et al., 1995). As well, downregulation of *col2a1* expression in the anteriormost mature cells of the notochord was initiated at the same time in both control and treated embryos, suggesting that the relative developmental age of these cells was unchanged. Furthermore, I was unable to detect any aberrant patterns in the expression of a number of other notochord-specific genes such as *axial* or *no tail* (data not shown). I did, however, observe that the notochord in GA-treated embryos sometimes developed folds or even branched into two, particularly at the location where the tail protrudes off the yolk. Expression of *col2a1* in the floor plate also appeared normal in GA-treated embryos (see arrow in panel C and D), suggesting that the development of the ventral neural tube was unaffected. This was also confirmed through the use of another marker of ventral neural tube formation, *sonic hedgehog* (*shh*; arrow in panels E,F; see also below).

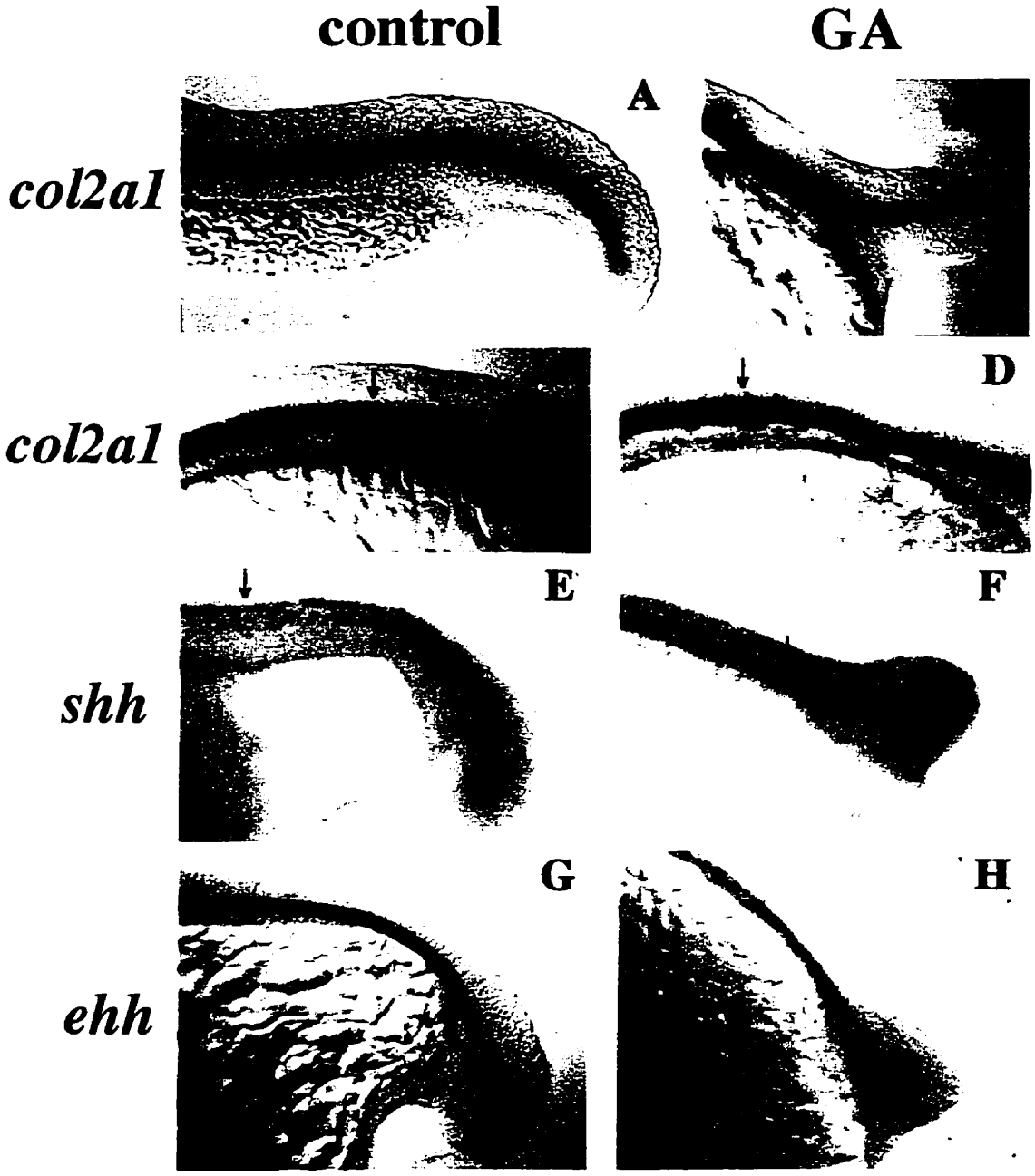
In contrast to the neural tube and notochord, development of the hypochord in GA-treated embryos was impaired. The hypochord is normally a single row of flat, *col2a1*-expressing cells which lie immediately below the notochord the developmental role of which is still unclear (Fig 6; Kimmel et al., 1995; Yan et al.,1995). Although

Figure 17. Simplified model of mesoderm development in *wild-type* and *flh* mutant embryos. (Figure adapted from Halpern et al., 1993.) In *flh* embryos, the cells of the future axial mesoderm have a shift in their fate and become paraxial mesoderm cells. This results in the development of a single fused somite under the neural tube.



(Adapted from Halpern et al., 1993)

Figure 18. Expression of notochord and floor plate markers is normal in 18 hour old GA-treated embryos as detected using whole mount in situ hybridization. A, C, E, and G: Control. B, D, F, and H: GA. A-D: *col2a1* mRNA. E and F: *shh*. G and H: *ehh*. Anterior is to the left and dorsal to the top in all panels. Both *shh* (E, F) and *ehh* (G, H) were also expressed normally in the notochord of GA-treated embryos as were other markers 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 (asterisks in D) which normally lie as a continuous row immediately below the notochord (C).


control
GA
A
col2a1
D
col2a1
E
F
shh
G
H
ehh

poorly understood, the mechanisms responsible for specification of ventral tissues such as the hypochord, dorsal aorta and cardinal vein are thought to involve signaling from the notochord since their development is impaired in *flh* embryos (Halpern et al., 1995; Talbot et al., 1995). Although GA-treated embryos exhibit apparently normal notochord formation, the hypochord was present as only patches of cells along the A-P axis (see asterisks in Fig. 18/D) and the dorsal aorta did not form (see above). Thus, the presence of a properly differentiated notochord is not sufficient for the formation of all ventral axial structures in GA-treated embryos.

3.4.5. Expression of *hedgehog* genes in the notochord of geldanamycin-treated embryos

While the notochord appeared structurally normal and expressed the *col2a1* gene in a normal spatiotemporal pattern, it was possible that expression of putative notochord signaling genes was impaired, resulting in abnormal patterning of surrounding tissues. A number of studies have shown that members of the vertebrate hedgehog gene family such as *shh, indian hedgehog* (*ihh*), *and echidna hedgehog* (*ehh*) are expressed and secreted by the notochord early during vertebrate embryogenesis and play critical roles in the patterning of surrounding tissues such as the somites and neural tube (Krauss et al., 1993; Chiang et al., 1996; Currie and Ingham, 1996; Hammerschmidt et al., 1996; Blagden et al., 1997; Du et al., 1997). As shown in panels E-H of Figure 18, both *shh* and *ehh* were expressed normally in GA-treated embryos, suggesting that signaling by the notochord was not impaired. This is supported by the normal development of the floor plate in these embryos, which numerous studies have suggested is dependent on expression of the *shh* gene by the notochord (see Chiang et al., 1996 and references therein).

3.4.6. Geldanamycin inhibits the formation of *eng2*-expressing muscle pioneer cells

The somitic defects observed in *flh* and *ntl* embryos are thought to arise due to a failure of development of a specific group of slow muscle fibres known as the muscle pioneers (MP). Muscle pioneers are the earliest muscle cells to differentiate in zebrafish, becoming morphologically distinct at around 13 h simultaneously with somite differentiation (Felsenfeld et al., 1991; see chapter 1.4. for more details on myogenesis in zebrafish). About 2 to 6 muscle pioneers develop per somite at the future location of the horizontal myoseptum which can be distinguished as they are the only muscle cells which express high levels of the *engrailed1* and *engrailed2* genes (Hatta et al., 1991; Ekker et al., 1992). Several studies have shown that ectopic expression of genes belonging to the *hedgehog* family of signaling molecules induces the formation of supernumerary muscle pioneer cells, suggesting that hedgehog expression by the notochord plays a fundamental role in their determination. This is supported by the fact that *shh* expression is greatly reduced in *flh* and *ntl* embryos which also lack muscle pioneers. However, I have demonstrated above that *shh* and *ehh* expression occurs in the typical spatiotemporal pattern in the notochord of GA-treated embryos and that floor plate formation (which is dependent on notochord signaling; Smith et al., 1993/b) occurs in an apparently normal fashion. Thus, I next determined whether differentiation of muscle pioneers is impaired following GA treatment. As shown in Figure 19. *eng-2* expressing muscle pioneers were either completely absent or severely reduced in embryos treated with GA beginning at 50% epiboly (compare panels A and D with panels B and E). However, *eng2* expression at the midbrain/hindbrain boundary was unaffected, indicating that GA treatment does not result in a general suppression of *eng2* activation throughout the embryo (asterisk in panels A and B). These data suggested that the somitic defects observed in GA-treated embryos were due at least in part to a lack of muscle pioneer formation. Furthermore, this defect appears to be due to inhibition of an Hsp90-dependent step which occurs downstream of signaling by the notochord.

Figure 19. GA and forskolin give rise to similar overt somitic phenotypes and inhibit formation of *eng2*-expressing muscle pioneer cells.. A, D: Control. B, E: GA. C, F: forskolin. Panels A-C show whole 18 hour old embryos whereas panels D-F show two somites within the trunk of the same embryos. In situ hybridization for the detection of *eng2* mRNA was carried out as described in Section 4. 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 *eng2* expression, normally develop at the D-V midline of the myotome at the future location of the horizontal myoseptum (arrows in A, D) but are absent from this location in GA-treated and forskolin-treated embryos (arrow in E, F). However, activation of *eng2* expression in cells of the midbrain/hindbrain boundary is unaffected by GA or forskolin treatment (asterisks in A-C).

control
GA
forskolin
A
B
F
E

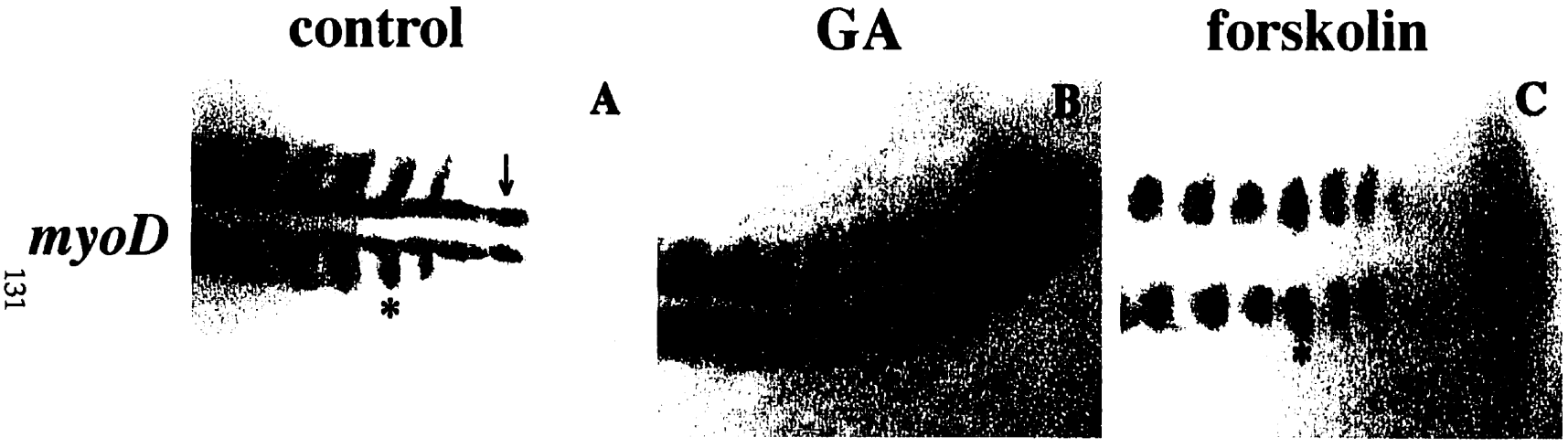
3.4.7. Hsp90 is necessary in the muscle pioneer differentiation pathway downstream of PKA-dependent adaxial cell formation

The data presented above indicate that the GA-sensitive step in somite formation occurs downstream of normal notochord development but prior to differentiation of muscle pioneers. The Hedgehog signaling pathway in *Drosophila* and vertebrates involves protein kinase A (PKA) within the responding cell, which is thought to suppress the activity of Hedgehog target genes in the absence of Hedgehog signaling (Fan et al., 1995; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995; Hammerschmidt et al., 1996; Ungar and Moon, 1996; Du et al., 1997). This is supported by microinjection studies which have shown that a dominant negative isoform of PKA mimics overexpression of hedgehog, thereby stimulating the formation of supernumerary muscle pioneers in zebrafish (Hammerschmidt et al., 1996; Ungar and Moon, 1996). Conversely, microinjection of a constitutively active isoform of PKA gives rise to a somite phenotype very similar to that obtained with GA, including inhibition of muscle pioneer development (Hammerschmidt et al., 1996; Du et al., 1997). However, these embryos are also cyclopic (fused eyes), a feature not apparent in GA-treated embryos suggesting that Hsp90 does not directly affect the PKA-dependent step of the pathway. In order to further examine the relationship between Hedgehog signaling and GA treatment, I utilized the adenylate cyclase activator forskolin. This compound interacts with and activates the catalytic subunit of adenylate cyclase, thereby raising intracellular levels of cAMP which activates PKA, and has been used successfully to antagonize hedgehog activity in mouse (Fan et al., 1995). I reasoned that upregulation of adenylate cyclase activity in cells of the zebrafish embryo should give rise to the same phenotype as the constitutively active subunit of PKA. As shown in Table 2. and Figure 18, panels C and F, forskolin treatment did indeed give rise to a phenotype with a dramatically shortened body axis and complete inhibition of *eng2*-expressing muscle pioneer formation. In addition, forskolin treatment resulted in forebrain

defects such as fused eyes (data not shown) which are also seen in embryos expressing constitutively active PKA (Hammerschmidt et al., 1996; Ungar and Moon, 1996). Taken together, these data indicated that the phenotype induced by forskolin is most likely the result of an upregulation of PKA caused by elevated intracellular cyclic AMP levels. Furthermore, the forskolin-induced trunk/tail phenotype was very similar to that obtained with GA treatment, suggesting that GA could be affecting PKA-dependent hedgehog signal reception during muscle pioneer development.

In order to examine this possibility, I monitored the development of adaxial cells in GA-treated and forskolin-treated embryos (Figure 20.). Adaxial cells are the progenitors of both muscle pioneers and other slow muscle cells and derive from paraxial mesoderm which lies directly adjacent to the notochord (Devoto et al., 1996; Du et al., 1997; Blagden et al., 1997; also see chapter 1.4. of Introduction). These cells can be distinguished as they are the earliest cells which express *myoD* (Weinberg et al., 1996). Inhibition of Hedgehog signaling by expression of constitutively active PKA impairs adaxial cell development resulting in the failure of muscle pioneer formation described above (Hammerschmidt et al., 1996; Du et al., 1997). As expected, I observed that stimulation of adenylate cyclase activity also abolished the formation of *myoD*-expressing adaxial cells whereas the lateral presomitic cells, which will later give rise to fast muscle fibres (Devoto et al., 1996), were unaffected (Figure 20. panel C). In contrast, a normal number of *myoD*-expressing adaxial cells formed at their usual location adjacent to the notochord in GA-treated embryos. Formation of the *myoD*-expressing lateral presomitic cells was similarly unaffected. These experiments define the requirement for Hsp90 as being downstream of PKA-dependent hedgehog signaling mechanisms which specify development of adaxial cells from paraxial mesoderm but prior to the differentiation of a subset of these adaxial cells into muscle pioneers.

Figure 20. Adaxial cells form in GA-treated but not 14-hour-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 fibres were normal in all three groups of embryos (asterisks in panels A-C).


control
GA
forskolin
A
B
C
myoD

4.0. Discussion

In the present study I set out to address several different questions regarding various aspects of expression, regulation and possible functions of heat shock genes in the zebrafish (*Danio rerio*). Genes examined in my research included *hsp47, hsp70, hsp90α* and *hsp90β*. Full-length (*hsp47*) or partial (*hsp70, hsp90α, hsp90β*) cDNA sequences had been cloned in our laboratory (Pearson et al., 1996; Krone and Sass, 1996, Lele et al., 1997) and were used as probes in Northern-blot analysis and as a template for antisense mRNA probe synthesis by in vitro transcription for whole-mount in situ hybridization experiments. The main lines of my study were:

4.1. Examination of heat shock protein gene expression in control, heat-shocked and ethanol-treated zebrafish embryos.

4.2. Characterization of the mRNA distribution of *hsp47* and *col2a1* genes during zebrafish development.

4.3. Analysis of Hsp90 function during zebrafish development

As these topics are relatively independent from one another I will discuss the results in three separate chapters. Finally, in chapter 4.4. I will present possible further directions these studies could lead to, including some projects that are already in progress.

4.1. Examination of heat shock protein gene expression in control, heat-shocked and ethanol-treated zebrafish embryos.

Previous studies in our laboratory have described the expression and heat-inducibility of *hsp90α, hsp90β* and *hsp47* genes during various stages of zebrafish development (Krone and Sass, 1994; Pearson

et al., 1996). These experiments demonstrated that *hsp47* and *hsp90α* are expressed in similar patterns as their respective mRNAs were not (or only barely) detectable in control embryos but underwent strong induction following heat shock at every developmental stage examined. Moreover a temperature-dependent effect was observable as a 37°C heat shock resulted in a higher level of expression than at 34°C. *hsp90β* on the other hand, showed strong constitutive expression in control embryos and its mRNA levels were elevated only sligthly after heat shock. This supports the earlier, sequence analysis-based assumption that it represents a constitutive member of the zebrafish *hsp90* gene family. To make the comparison of various heat shock gene expression patterns after heat-induction complete, I carried out Northern-blot analysis using the *hsp70* cDNA probe isolated by Shane Engel earlier in our laboratory. In this experiment *hsp70* expression resembled that of *hsp47* and *hsp90α*. No constitutive *hsp70* expression was detected at control temperatures but its mRNA levels rose significantly after heat shock.

These preliminary studies indicated that there are both similarities as well as significant differences in the regulation of zebrafish heat shock genes at the mRNA level (eg. *hsp90β* vs. the other hsp genes). However, each gene behaved in a similar manner after heat-induction in every developmental stage examined, unlike what has been reported in mouse and *Xenopus* embryos in earlier reports (see Section 1.2.4.5. for details, Curci et al., 1991; Bienz, 1984; Krone and Heikkila, 1988). This suggests that there are no differences in the regulation of individual hsp genes between gastrulation (6hrs) and hatching (48hrs) of the embryos.

In contrast to previous studies carried out in *Xenopus* which reported coordinate transient accumulation and decay of both *hsp30* and *hsp70* mRNAs during continuous heat shock of tadpoles (Krone and Heikkila 1988), I found gene-specific differences in the zebrafish. First, mRNA levels of *hsp70* increased after only 30 min. of heat shock while the rest of the hsp genes required 1-2 hours to peak. This suggests that Hsp70 may fullfill an immediate chaperoning role after heat shock, perhaps binding to partially denatured proteins and thereby preventing their aggregation whereas the other heat shock proteins are

required at a later step, possibly in the refolding of these partially denatured proteins. Also *hsp70* gene expression remained high until 4 hrs into the treatment and decayed to control levels only after 8 hrs whereas the increase in *hsp47*, *hsp90α* and *hsp90β* mRNA levels were more transient as elevated expression was only present in the 1 and 2 hr heat-shock samples and background expression was restored by 4 hours. Further, the weak induction of *hsp90β* strengthened the previous findings by Krone and Sass (1994) regarding *hsp90β* being a cognate heat shock protein gene as it showed strong constitutive expression in control embryos.

Heat-inducible induction of hsp gene expression in other systems is believed to be regulated primarily at the transcriptional level through the activation and sequence-specific binding of heat shock factor 1 (HSF1) to the heat shock elements (HSE) found in the promoter of hsp genes (Fernandes et al., 1994; Morimoto et al., 1994). It is currently unclear however whether the observed differences in the accumulation pattern of different heat shock mRNAs in zebrafish are also due to transcriptional regulation by HSF1. Heat shock experiments and similar Northern-blot analysis in actinomycin D-treated (inhibitor of RNA polymerase II mediated transcription) embryos could assist in determining whether the regulation is transcriptional or posttranscriptional. It would require deletion analysis of these promoters in transgenic animals to see what transcriptional elements (possibly HSEs) are responsible for these differences. Alternatively the differences in their expression patterns could be the result of disruption of mRNA splicing and subsequent degradation of the unspliced precursors. Splicing mechanisms have previously been shown to be susceptible to inactivation by heat shock (Yost and Lindquist, 1986). Furthermore strongly heat-inducible members of the *hsp70* gene family in other vertebrates do not contain introns (Yost and Lindquist, 1986), whereas the vertebrate *hsp47* and *hsp90α* genes isolated to date have several introns (Vourc`h et al., 1989; Hosokawa et al., 1993).

A significant (150-200bp) and reproducible increase was found in the size of *hsp90α* mRNA following exposure of embryos to continous heat shock (see Section 4.4. for details). There are several possible explanations of this phenomenon including alternative splicing and

polyadenylation. These possibilities will be discussed later in the Future directions section.

Both stress- and gene-specific differences in heat shock gene regulation were demonstrated in these experiments. Following ethanol treatment *hsp47* was strongly induced whereas *hsp70*, *hsp90α* and *hsp90β* showed only a slight increase in their mRNA levels. This suggests that, in general, ethanol is a weaker stress response-inducing agent than heat and that it might affect proteins that are targets of Hsp47. Whether these proteins include only various types of collagens, in correlation with the proposed role of Hsp47 in the assembly of procollagen molecules under normal conditions remains to be seen.

The temporal pattern of mRNA accumulation induced by ethanol was much slower compared to that found after heat shock, requiring 4-8 hours to reach maximum levels of mRNA accumulation. This similar induction time among hsp genes suggests a common mechanism in their activation after ethanol treatment. The results also suggest that the response of embryos to continous ethanol exposure differs substantially from that which occurs during hyperthermia, indicating the presence of different regulatory mechanisms in the activation of these genes after different stresses. Alternatively it is possible that the degree of stress perceived by the cell is higher during heat shock. Considering the fact that activation of these genes after heat shock occurs through trimerization, nuclear translocation and thereby activation of heat shock factor 1 (HSF1), it is possible that induction of these genes by ethanol utilizes a separate transcriptional pathway not involving HSF1. Alternatively the presence of posttranscriptional regulatory mechanisms (differences in splicing, cytoplasmic polyadenylation of hsp mRNAs, resulting in their stabilization) can also explain the variations in these expression patterns. Further experiments using actinomycin D (an inhibitor of RNA polymerase II-mediated transcription) could assist in determining whether the differences in mRNA accumulation are regulated at the transcriptional or posttranscriptional level.

Characterizing the temporal pattern of expression during various stress treatments raised the question of whether these genes show tissue-specific mRNA distribution in heat shocked or in ethanol

treated animals. Previous results, which showed that *hsp90α* was induced ubiquitously throughout the embryo (Sass et al., 1996) argued against the idea of the existence of a tissue-specific stress-response. Surprisingly however, whole-mount in situ hybridization analysis using *hsp47* and *hsp70* antisense mRNA probes demonstrated preferential sites of accumulation of these mRNAs in 2-day-old zebrafish embryos. The overall level of *hsp47* and *hsp70* expression observed using Northern-blot analysis correlated nicely with that detected in the whole-mount in situ hybridization experiments. In control embryos *hsp70* mRNA is not detectable, while *hsp47* mRNA is present in a wide array of tissues (see Results chapter 3.2.2.). The lack of *hsp70-4* mRNA in control embryos suggests that this gene is not likely to have a function in the regulation of zebrafish development under normal conditions. In contrast, the product of the *hsp47* gene, is likely to play a major role in the posttranslational processing of various types of collagens (for review see Nagata, 1996) and therefore it is likely to be important for the development of a number of embryonic tissues. Our in situ hybridization analyses have revealed that *hsp47* showed enhanced expression in tissues (cartilage, mesenchyme) that are all likely to produce large amounts of fibril-forming collagens. Given the proposed role of Hsp47 as a collagen-specific chaperone demonstrated in biochemical experiments (Nagata, 1996) and the fact that *hsp47* is induced in areas which are all potential sites of collagen synthesis, it is possible that *hsp47* upregulation is part of a specific protective mechanism for collagen-secreting cells. While biochemical evidence indicating a role for zebrafish Hsp47 in collagen processing and/or protection is not yet available, the high degree of sequence conservation between the zebrafish and mammalian Hsp47 proteins, particularly in putative functional domains, indicates that it is likely to carry out such a function.

The activation of *hsp47* gene expression in mesenchymal cells surrounding the precartilagenous cells which express the gene at control temperatures is particularly interesting in light of a recent study by Kulyk and Hoffmann (1996). These authors demonstrated that ethanol is a potent stimulator of cartilage differentiation in chick mesenchymal micromass cultures. Ethanol was found to increase the

expression of cartilage specific proteoglycan and type II collagen genes in micromass cultures and to result in the elaboration of cartilage extracellular matrix components. Given the co-expression of genes encoding Hsp47 and type II collagen which I demonstrated in a number of embryonic tissues (see Results chapter 3.2.2. and next chapter of the Discussion), this could provide an alternative explanation for *hsp47* upregulation in the mesenchymal cell populations surrounding precartilagenous cell condensations in ethanol-treated embryos. Unfortunately, there is currently no evidence available which would indicate that ethanol treatment results in enhanced chondrogenesis in mesenchymal cell populations of zebrafish embryos.

4.2. Characterization of the mRNA distribution of *hsp47* and *col2a1* genes during zebrafish development.

The Northern-blot experiments using total mRNA carried out earlier could not detect basal levels of *hsp47* mRNA during the first two days of development. Since other studies have shown the presence of type II collagen at this time in zebrafish embryos (Yan et al., 1995) and at comparable stages in other vertebrate embryos (Su et al., 1991; Ng et al., 1993) I carried out Northern-blot analysis using poly(A)+ mRNA, which showed *col2a1* as well as *hsp47* expression in 33hr-old, but not in younger embryos. The fact that *col2a1* mRNA was also likely to be present at earlier stages combined with previously reported biochemical data showing that *hsp47* is a collagen-specific chaperone suggested that even this technique might not be sensitive enough to detect low levels of *hsp47* mRNA in earlier developmental stages. Therefore, I elected to use the more sensitive in situ hybridization method to examine whether *hsp47* mRNA shows tissue-specific expression and whether it is coordinately synthesized with that of type II collagen, a fact that would support its role as a collagen-specific chaperone in zebrafish embryos. Type II collagen is the major cartilage extracellular matrix (ECM) protein, constituting up to 80% of all collagens secreted by this tissue. In addition, studies examining the expression of type II collagen genes during embryonic development have revealed the presence of *col2a1* mRNA within a number of non-

chondrogenic tissues (Cheah et al., 1991; Kosher and Solursh, 1989; Su et al., 1991; Yan et al., 1995). This has led to the suggestion that type II collagen may play a role in matrix-mediated morphogenetic mechanisms in addition to its structural role. Thus, the proper processing of type II collagen molecules within the ER could be extremely important for normal embryonic development. Earlier studies have revealed coordinate regulation of Hsp47 and different types of collagens, among them type II, in various cell lines and tissues (Miyaishi et al., 1992: Takechi et al., 1992; Clarke et al., 1993; Shroff et al., 1993, 1994). The data obtained in these studies suggested that *hsp47* and collagen genes might share transcriptional regulatory elements which direct their coordinate expression. The temporal and spatial expression data presented in our study support a model in which the *hsp47* gene in zebrafish embryos is co-ordinately regulated with *col2a1*, in a number of developing tissues in vivo. This is true for both chondrogenic and non-chondrogenic tissues and suggests that a common mechanism may be at work in the co-expressing tissues. Alternatively, given the wide range of tissues which exhibit co-expression, it is possible that multiple factors are involved and that the actual mechanism in operation depends on the cell types in which the two genes are being expressed. For example, the expression of the gene encoding the transcription factor Twist within the notochord has previously indicated that it might be a possible regulator of *col2a1* expression (Yan et al., 1995). Thus, it is possible that Twist could play a role in co-regulating *col2a1* and *hsp47* in the notochord whereas cartilage-specific factors could regulate these genes in chondrogenic cells. However, the expression of *hsp47* but not *col2a1* within the hypoblast suggests that other mechanisms would also have to be at work, at least within the notochord. Cloning of the *hsp47* and *col2a1* promoters from zebrafish would allow the identification of potential common regulatory elements in order to begin to address this question.

Although a wide array of tissues coordinately express *col2a1* and *hsp47*, several important exceptions were observed in our study. Of particular interest is the high level of *col2a1* expression within cells of the floor plate and hypochord in both wild-type and *flh* mutant embryos, which did not exhibit detectable levels of *hsp47* mRNA. This

lack of co-ordinate expression was unexpected and clearly indicates that transcription of the *col2a1* and *hsp47* genes is not strictly co-regulated. Furthermore, it supports a model in which the regulation of these genes is subject to multiple and non-overlapping mechanisms which differ in a tissue-specific manner. Alternatively, it is possible that *hsp47* is coregulated with another gene encoding an extracellular matrix molecule which is expressed in some but not all *col2a1*-expressing cells.

The expression of *hsp47* in some cells which did not express *col2a1* was not as surprising. This is most easily explained by the fact that Hsp47 has been proposed to act as a molecular chaperone for a wide variety of fibril-forming collagens many of which are expressed in a broad range of cell types during embryonic development. This is particularly true for type I collagen, which is the most ubiquitously expressed of the collagens and for which the greatest amount of evidence regarding a specific role for Hsp47 in post-translational processing has been obtained (Nagata, 1996). Indeed, it is very surprising that I was unable to detect high levels of *hsp47* expression throughout most cells of the embryo. Perhaps the processing of a type II collagen triple helix requires a greater number of Hsp47 molecules than that of most other collagens. Unfortunately, a direct comparison of the number of Hsp47 molecules required to properly fold different forms of collagens has not been reported. Alternatively, cells expressing type II collagen during development may be required to synthesize very large amounts of triple helical molecule within a short time frame. This latter possibility would be expected if type II triple helixes do indeed play a role in matrix-mediated morphogenesis, a role which would predict that molecules have to appear within a short time frame during development and in relatively large amounts. It is also possible that translation of *hsp47* mRNA does not occur at the same speed in different tissues. Finally, the possibility exists that Hsp47 interacts with another collagenous component of the extracellular matrix distinct from type II collagen which is expressed in all *hsp47*-expressing cells but not in all *col2a1*-expressing cells.

The reasons for the lack of *hsp47* gene expression in cells of the floor plate and hypochord are currently unclear. Previous studies have revealed that *col2a1* mRNA is also expressed within the ventral neural

tube of other vertebrates and that type II collagen immunoreactivity is present within the basal lamina of cells in the floor plate/ventral neural tube (Kosher and Solursh, 1989: Cheah et al., 1991; Su et al., 1991; Lui et al., 1995). It is not known if *hsp47* expression is also excluded from these cells in these organisms. The data obtained in the present study suggest that Hsp47 may be dispensable for processing of some collagen molecules containing the *col2a1* gene product in cells of the floor plate and hypochord. There is no evidence presently that would prove that type II collagen forms triple helices in these tissues. Experiments similar to those carried out in the case of type I collagen (Satoh et al., 1996) are necessary to prove this point. Also, the three α1(II) chains of the type II collagen homotrimer and the α3(XI) chain of the type XI collagen heterotrimer both appear to to the product of the *col2a1* gene in humans (Kadler, 1996). Further experiments aimed specifically at the assembly of homotrimeric and heterotrimeric triple helixes containing products of the *col2a1* gene must be carried out before any conclusions can be drawn regarding conditions, under which triple-helix assembly can occur in the absence of Hsp47.

The data obtained using Northern blot analysis revealed that two major transcripts of the *col2a1* gene are present during embryonic development of zebrafish. Previous studies in other vertebrates have revealed the existence of two alternative forms of *col2a1* mRNA, designated IIA and IIB (Ryan and Sandell, 1990; Metsaranta et al., 1991; Nah and Upholdt, 1991; Su et al., 1991). These two transcripts arise by alternative splicing which results in a final mRNA that either contains exon 2 (type IIA) or has this exon removed (type IIB). Type IIB is the predominant transcript expressed in mature chondrocytes whereas type IIA is present in prechondrogenic and other cells (Nah and Upholdt, 1991; Su et al., 1991; Ng et al., 1993; Sandell et al., 1994). At present, we cannot determine if the two transcripts detected on our Northern blots represent type IIA and IIB of the zebrafish gene since the *col2a1* cDNA clone contains only the carboxy-terminal coding and 3' untranslated regions. The size difference of the two transcripts, however, is in agreement with what was found in type IIA and IIB transcripts in other systems. It is unlikely that these transcripts represent cross-hybridization to the mRNA products of other collagen genes in

zebrafish since the conditions used can clearly distinguish mRNA species with a greater degree of identity (80%) than has previously been observed for different collagens within the same species (Krone and Sass, 1994; Kadler, 1996). Of particular interest to the present study is the substantial increase in *hsp47* mRNA which occurred concurrently with the appearance of the larger transcript in embryos following somitogenesis. This is also the time at which co-expression of *hsp47* and *col2a1* becomes apparent in several tissues other than the notochord, including mesenchymal cells which will later undergo chondrogenesis. It will be interesting to determine if similar changes in *hsp47* mRNA dynamics accompany the appearance and/or disappearance of alternatively spliced forms of type II collagen mRNA in other vertebrates.

Our results, when taken together with the biochemical and cell culture evidence regarding the interaction of Hsp47 with fibril-forming collagens, suggest that Hsp47 may be a necessary component of the post-translational processing machinery of *col2a1*-expressing cells in a number of different embryonic tissues. Whether it is directly involved in the processing of type II collagen triple helices or another component of the extracellular matrix in co-expressing cells remains to be determined.

4.3. Analysis of Hsp90 function during zebrafish development

Recently, biochemical evidence has been presented from numerous laboratories which indicates that Hsp90s play a role in a wide variety of cellular signaling pathways involving steroid receptors, tyrosine and serine-threonine kinases. The question of whether Hsp90α or Hsp90β (or both) is responsible for regulating these signal transduction molecules has not been adressed to date. Our Northern-blot experiments suggested that Hsp90β is the more likely candidate as a general chaperone, as its expression was much higher in control samples than that of *hsp90α* (J. Sass. personal communication). Also, previous studies in our laboratory have demonstrated a strictly somite-specific expression pattern of *hsp90α* in zebrafish embryos (Sass et al., 1996). Coordinate distribution of its mRNA with that of *myoD*, one of

the bHLH myogenic regulatory factors in wild-type as well as in *flh, ntl* and *spd* mutant embryos (Sass and Krone unpublished data) suggested a specific role for Hsp90α in muscle differentiation. However, the signaling molecules mentioned above are likely to play an important role in other tissues as well. To address this contradiction I carried out whole-mount in situ hybridization which demonstrated a ubiquitous expression for *hsp90β* in early stages of zebrafish embryogenesis thereby supporting its role as the Hsp90 homologue involved in the regulation of the above mentioned signal transduction processes. The data on *hsp90β* expression suggest a hypothesis in which Hsp90β would act as a "housekeeping" general chaperone of the earlier described (and possibly other, so far unidentified) signaling molecules, whereas Hsp90α would work in a more selective manner during early myogenic differentiation and would also probably be involved in protection of cellular proteins after stress as indicated by its strong inducibility throughout the embryo after heat shock (Sass et al., 1996).

To identify various steps and pathways during development at which either of these Hsp90s are required, I utilized the specific Hsp90-binding agent geldanamycin (GA). We established that, when administered at the start of gastrulation, GA treatments result in altered development of several tissues including the tail, the somites, and the circulatory system. Since previous results by others (see below in detail) have already pointed to myogenesis as a possible site of Hsp90 action I focused my efforts on the potential role of Hsp90 during early muscle development.

Several pieces of independent, though indirect evidence support the hypothesis that Hsp90 plays a role during the specification and/or differentiation of somitic muscle. First, the *hsp90α* gene is strongly and specifically expressed in the somites under non-stress conditions (Sass et al., 1996). Expression of *hsp90α* is first detectable within 1.5 hours following activation of *myoD* expression in the adaxial cells of the somites. Importantly, *hsp90α* expression either coincides with or precedes the expression of other muscle regulatory genes of the *myoD* and *MEF-2* families in these cells (Weinberg et al., 1996; Sass et al., 1996; Ticho et al., 1996; Sass and Krone, in preparation). Second, *hsp90α* expression is coordinately downregulated with *myoD* in 2 day old

embryos following fibre differentiation as would be expected for a gene involved in fibre development but not the maintenance of the mature fibre phenotype (Sass et al., 1996). In contrast, expression of the structural gene *α-tropomyosin* remains high in muscle fibres at this age. 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 (Sass and Krone, in preparation). 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, enhanced levels of Hsp90 have been reported in developing human infantile myofibers and in regenerating fibers of Duchenne's muscular dystrophy patients which are also both sites of increased *myoD* expression (Bornmann et al., 1995, 1996). Fifth, Hsp90 interacts with a number of molecules involved in signal transduction pathways and the regulation of gene expression in other vertebrates, including some 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 function 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 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). When combined with the results reported in the present study, these observations provide evidence that Hsp90 is likely to play a fundamentally important role during early muscle differentiation.

The results of my investigation however suggested that geldanamycin affected primarily a small subset of cells within the somites called the muscle pioneer cells, which failed to develop properly after GA-treatment. Devoto et al. (1996) have elegantly demonstrated that adaxial cells in zebrafish give rise to two types of slow muscle, the muscle pioneers and non-muscle pioneer slow fibres, whereas fast muscle arises from the lateral presomitic cells which lie distal-lateral to the adaxial cells. Shortly after the development of the

adaxial cells, non-muscle pioneer slow muscle cells migrate radially outward past the lateral presomitic cells and assume a final position as a monolayer on the surface of the myotome. In contrast, the subset of adaxial cells which give rise to muscle pioneers remain in contact with the notochord and eventually become a thin layer of elongated cells each of which is thought to extend out laterally to the surface of the myotome. More recently, a multi-step model for muscle pioneer formation has been put forward by Du et al. (1997). In this model, adaxial cells are initially specified from paraxial mesoderm by Sonic hedgehog emanating from the notochord. The subset of these adaxial cells which will form the muscle pioneers then remain adjacent to the notochord and would require subsequent additional exposure to one or more hedgehog family members (Echidna hedgehog; Currie and Ingham, 1996) in order to differentiate. Also a BMP4-like (member of the TGF-β superfamily of transcription factors) signal is proposed to antagonize hedgehog signals in the dorsal and lateral regions of the myotome and thereby restrict differentiation of muscle pioneers to the region of the somite lateral to the notochord along the dorsal-ventral axis (Du et al., 1997). The data in the present study clearly support such a multi-step model for specification and differentiation of muscle pioneers, with Hsp90 being required downstream of adaxial cell formation.

Taken together, the effects of GA on the development of zebrafish embryos are most consistent with a model in which GA inhibits Hsp90 function specifically required for adaxial cells to interpret and/or respond to intercellular signals required for their differentiation into muscle pioneers. While the precise role of Hsp90 in this process is unclear, there are several potential steps in which it may be involved. As discussed above, one potential target molecule of Hsp90 is MyoD. In this scenario, Hsp90 would be required in adaxial cells in order to stimulate the activity of MyoD which, in turn, would be involved in initiating the cascade of events which leads to muscle pioneer differentiation. Evidence in support of this includes the fact that *hsp90α* expression is activated in adaxial cells shortly after *myoD* and co-incident with or prior to the activation of other muscle regulatory genes. As well, both *myoD* and *hsp90α* are co-ordinately

downregulated following muscle fibre differentiation (Sass et al., 1996). However, it will require further biochemical analysis to determine whether Hsp90 actually regulates MyoD in the adaxial cells.

As discussed in the Results, the trunk and tail phenotype of GA-treated embryos is reminiscent of that previously described for embryos lacking a notochord due to the mutations *flh* and *ntl* , which affect the zebrafish homologues of the frog *Xnot* and mouse *Brachyury* genes, respectively. Like homozygous *flh* embryos, GA-treated embryos also exhibited impaired development of the hypochord. However, in contrast to *flh* and *ntl* embryos, GA-treated embryos displayed a well-differentiated notochord which expressed a number of marker genes such as *col2a1, axial, ntl, shh,* and *ehh*. Consistent with this observation, the notochord cells in GA-treated embryos displayed the typical vacuolated morphology (Kimmel et al., 1995). Also, *col2a1* expression was downregulated in the same anterior-posterior temporal pattern as in control embryos, suggesting that GA caused no alterations in the normal developmental sequence of events within the notochord. Normal differentiation of the notochord in GA-treated embryos is consistent with our inability to detect expression of either the *hsp90α* (Sass et al., 1996) or *hsp90β* genes in this tissue. Similarly, the development of adaxial cells is consistent with the fact that strong expression of the *hsp90α* gene is not detectable within the paraxial mesoderm prior to adaxial cell formation and activation of *myoD* expression (Sass et al., 1996; Sass and Krone, in preparation).

From these results it is clear that the Hsp90-dependent step is not represented by the *flh* and *ntl* genes. However, large scale mutagenesis screens have recently identified mutations in several other genes which give rise to phenotypes similar to GA-treated embryos (van Eden et al., 1996). A series of zygotic mutations isolated in this study clearly support the multi-step model for muscle pioneer formation. Embryos carrying mutations in these genes, named the *you*-type genes because of the U-shaped somites, have no or a reduced myoseptum and a reduction in the number of *engrailed*-expressing muscle pioneers yet form a morphologically normal notochord. Four of these mutants, namely *you, yot, syu,* and *con,* also exhibit circulation defects and formation of the dorsal aorta is delayed or does not occur at all similar

to what we found in GA-treated embryos. However, the mutations *syu, you, con* clearly affect notochord signaling and are involved in directing adaxial cell formation from paraxial mesoderm. These results are consistent with recent mapping data (P. Haffter, M Hammerschmidt, personal communication) which identified *syu* as a mutation affecting the gene coding for Shh and *you* as a mutation affecting the *gli2* gene which codes for a transcription factor also involved in the Hedgehog-signaling pathway. Both of these genes affect notochord signaling and signal transduction, resulting in aberrant somite differentiation.

Another you-type mutant *ubo,* on the other hand affects the subsequent step, the differentiation of muscle pioneers from adaxial cells. Considering its similarity to the phenotype of GA-treated embryos and the fact that it represents a gene that functions downstream of hedgehog-signaling, *ubo* is a good candidate for the Hsp90-dependent member of the pathway within adaxial cells. Alternatively it might represent a mutation in the zebrafish *hsp90α* gene itself. Clearly, further investigations into the relationship between these mutants and GA-treated embryos, including the positional cloning of the *you*-type genes, will have to be carried out before any conclusions can be drawn.

Hsp90 has been shown to be an abundant protein in a wide variety of cell types, with some estimates indicating that it comprises up to 1% of the total cytosolic protein. In yeast, a functional copy of the *hsp82* gene is essential for survival (Borkovich et al., 1989). These and other observations have led to the suggestion that Hsp90 is required for eukaryotic cell viability. However, the data presented in this study suggest that not all cells of the zebrafish embryo may require Hsp90 for their proliferation and/or differentiation or that different cell types have dramatically different threshold requirements for Hsp90. Alternatively, it is possible that other chaperone molecules which exhibit Hsp90-like activities may substitute for Hsp90 in some cells. For example, Cdc37 has recently been shown to have chaperone properties remarkably similar to Hsp90 yet has specific functions in signal transduction pathways which diverge in vivo (Kimura et al., 1997). It is also possible, although yet to be proven, that other heat shock proteins can take over the functions played by Hsp90 in the cell.

To date, very little information regarding the function of Hsp90 in vivo in the context of a complex, multicellular, vertebrate embryo has been available. This has been due in part to the multi-gene nature of the vertebrate *hsp90* family (Gupta, 1995) and to the fact that Hsp90 probably plays multiple roles at different time points during development (Rutherford and Zuker, 1994). For example, traditional loss of function methodologies for the assessment of embryonic gene function usually target only the first major developmental event in which a gene is involved and will typically result in early lethality for *hsp90* such as that observed in embryos treated with GA beginning at the mid-blastula stage (see Results). The approach utilized in the present study overcomes many of these difficulties and allows for the targeting of Hsp90 inhibition to specific time points during embryonic development, thereby avoiding early lethality. The data obtained using this approach are, to our knowledge, the first demonstration of a requirement for Hsp90 at a defined point during the development of a specific cell type in a vertebrate embryo. Further, they support the suggestion that Hsp90 may not simply provide housekeeping chaperone function to all cells and tissues, but rather may fulfill regulated roles in interpreting and responding to signal transduction events. Temporally-targeted, GA-mediated disruption of Hsp90 function in zebrafish will allow us to further address the specific Hsp90-mediated event(s) required for the formation of muscle pioneers and other cell types in the context of the complex intercellular interactions which occur during vertebrate development. Indeed, such information regarding the effects of GA on vertebrate development will become increasingly important since this compound has already been selected for preclinical development as an antitumour agent (Supko et al., 1995).

4.4. Future directions

My work has led to several conclusions which, when combined with further experiments, will enable us to understand the regulation and possible functions of different heat shock proteins. Some of these experiments are already under way and here I will attempt to

summarize preliminary results as well as try to reveal the logical directions this research can lead to in the near future.

The first line of questioning revolves around the stress-induced regulation of heat shock genes. Several questions have emerged from the work regarding *hsp90* which center on the difference in the constitutive expression and heat-inducibility of *hsp90α* and *hsp90β* genes. Cloning and analysis of 5'-regulatory sequences, followed by transient expression assays could assist in identifying the promoter elements responsible for these differences. I already started the cloning process by screening a zebrafish genomic library using *hsp90α* and *hsp90β* PCR-fragments as probes. Primary and secondary screenings have been completed and several fragments of the *hsp90β* gene have been subcloned into Bluescript SK- plasmid. Partial sequencing of one of these fragments has confirmed that it contains sequences close to the 5'-region of the *hsp90β* gene. Based on homology analysis of other vertebrate *hsp90β* genes the fragment is likely to contain 500-1000bp of the 5'-upstream regulatory region of zebrafish *hsp90β*. Similar subcloning will be carried out for fragments of positive clones from the secondary screening for *hsp90α* gene including hopefully the *hsp90α* promoter. The differences in the presence of heat shock elements in promoters of these genes will be analyzed to determine if it correlates with their different heat-inducibility. Also elements that are responsible for the somite-specific expression of *hsp90α* could be identified by deletion mutagenesis experiments. Promoters of different lengths will be cloned in a vector containing a reporter gene (*lacZ*). Microinjection of these constructs combined with the proper assays will allow us to follow the tissue-specificity of expression driven by various parts of the promoter. Considering the complete overlap of *myoD* and *hsp90α*- expressing tissues in the embryos and the fact that *myoD* is expressed prior to *hsp90α* it is possible that E-boxes (binding sites for bHLH muscle regulatory factors) in the promoter of *hsp90α* could direct its somite-specific expression.

Northern-blot analysis has revealed a significant (150-200bp), distinct and reproducible size increase in *hsp90α* mRNA after heat-shock. One possible explanation for this phenomenon is that mRNA splicing is interrupted, leading to accumulation of a larger, unspliced

hsp90α transcript. However, *hsp90α* genes in all other vertebrates examined to date contain several introns the total length of which far exceeds the size increase observed after heat shock (Vourc'h et al., 1989; Walter et al., 1989) and preliminary evidence obtained in our laboratory also indicates the presence of at least one of these introns in the zebrafish *hsp90α* gene (data not shown). Furthermore, a size change in *hsp90α* mRNA following heat shock is not observed in other animal systems, suggesting that inhibition of splicing of *hsp90α* RNA does not occur in these systems (Vourc'h et al., 1989; Ullrich et al., 1986). An alternative explanation is alternative splice site selection following heat shock as has been shown to occur for mouse *hsp47* RNA, a question that could be targeted by RNAase protection assays (Takechi et al., 1994). However, preliminary results from our laboratory using RACE-PCR (Frohman, 1990; Borson et al., 1992) suggest the presence of two alternative forms of *hsp90α* which differ in their 5'-untranslated region rather than in their intron-exon structure (S. Hadfi, unpublished data). Whether this variation in the 5'-UTR is responsible for the size change after heat shock is not currently clear as our PCR analysis detected the presence of both transcripts in control as well as in heat-shocked RNA samples. A third possible mechanism which could generate the observed size increase would be that the zebrafish *hsp90α* mRNA is subject to heat-induced polyadenylation. Such a mechanism has been shown to occur in the *hsp21* gene of *Arabidopsis thaliana* (Osteryoung et al., 1993) and we are currently undertaking experiments to ascertain if it exists in the processing of zebrafish *hsp90α* gene. Unfortunately the results of the RNAse H treatment experiments were inconclusive due to the insolubility of the digested and precipitated RNA, but there is also the possibility to pursue this question by 3'-RACE-PCR, which can be used to analyse the length of the poly(A)-tail of mRNAs (Sheflin et al., 1995).

Promoter cloning and analysis could also answer the questions raised by the in situ hybridization experiments concerning *hsp47* and *col2a1* expression. The significant overlap of expression patterns suggests that they share common regulatory elements, which could be identified in such a study. The transcriptional elements that might be responsible for the minor differences in their mRNA distribution

could also be identified and their characterization could further enhance our understanding of the tissue-specific regulation of these genes. Analysis of the upstream sequences could shed some light on the tissue-specific activation of *hsp47* after stress. It would also be interesting to know whether the promoter of *col2a1* gene has a heat shock element. Since Kulyk and Hoffmann (1996) found evidence of increased chondrogenic differentiation in micromass cell cultures after ethanol treatment, it is possible that such stress-induced differentiation also gives rise to an increase of *col2a1* expression.

Another important question raised during these in situ hybridization studies is why *hsp47* mRNA is present in a relatively limited array of tissue. Based on the expression pattern of type I collagen (which requires Hsp47 for its proper folding) in a wide variety of tissues of other vertebrates, I would expect *hsp47* to be widely expressed throughout the embryo. This question could be addressed initially through the cloning of the zebrafish homologue of type I collagen cDNA, then analyzing its expression pattern by in situ hybridization and comparing it to that of *hsp47*.

Analysis of Hsp47 function to date has been limited to in vitro studies and coexpression data with collagen genes. Carrying out an in vivo deletion mutagenesis and overexpression of the deleted mutants of Hsp47 in zebrafish could provide evidence for Hsp47 being a chaperone of various fibril-forming collagens. Such an analysis using the full-length *hsp47* cDNA (Pearson et al., 1996) is already under way by Steven Sperber in our laboratory. The various mutated *hsp47* cDNA clones including one which lacks the C-terminal RDEL ER-retention sequences will be transcribed in vitro and mRNA microinjections will be carried out to see what, if any effect the mutant Hsp47 molecules will have on development of various organs (notochord, lens of the eye, etc.) that showed strong continuous *hsp47* expression during normal development.

Finally, future experiments employing the specific Hsp90-binding agent geldanamycin should assist in elucidating the possible role(s) Hsp90 plays during embryonic development of the zebrafish. I have analyzed in detail the effect of geldanamycin on somitogenesis and muscle differentiation and found that Hsp90 is a potential

candidate to interact with and regulate proteins involved in modifying and transducing the Hedgehog signals emanating from the notochord. However, further experiments are required to clarify Hsp90's role in the myogenic pathway. As Hsp90 has been shown to regulate the function of steroid receptors, tyrosine and serine-threonine kinases, the next logical step is to look for potential targets of Hsp90 function in the hedgehog signaling pathway. This signal transduction cascade is most thoroughly characterized in *Drosophila* and these studies have revealed that a serine-threonine kinase, named Fused is required for correct Hedgehog signal transduction (Preat et al., 1990; Therond et al., 1993) although the exact role it plays remains to be determined. It acts downstream of PKA activity in the Hh signaling pathway and therefore represents a good candidate for the HSP90 target molecule since in our studies Hsp90 was also found to act downstream of the PKA-dependent step. One possibility is to clone the zebrafish *fused* cDNA by PCR based on homology to its *Drosophila* counterpart and analyse its expression pattern to see whether it possesses the same somite-specificity as *hsp90α*. This approach could eventually lead to biochemical studies in which coimmunoprecipitation, GA-resin binding studies and kinase assay would be utilized to determine if Fused and Hsp90 interact directly and whether Hsp90 is necessary for the kinase activity of Fused.

Another interesting project could be to generate monoclonal antibodies that are able to distinguish between Hsp90α and Hsp90β. The recently developed phage display library method provides a quick way to isolate such antibodies. Microinjection of these antibodies into 1-4 cell stage embryos can help us in distinguishing the Hsp90α and Hsp90β-dependent developmental pathways during embryogenesis.

There are several, so far uninvestigated developmental defects in zebrafish embryos caused by GA-treatment. One of the two most characteristic problems in GA-treated embryos is the missing dorsal aorta and therefore the lack of circulation in the trunk and tail. To narrow down the cause of the circulation defects we plan to use various antisense RNA probes made from genes that have been demonstrated to function during hemopoesis and vessel formation. The other significant feature of GA-treated embryos is the extremely shortened tail which everses from the yolk sac prematurely. This

would be best examined by single-cell labelling experiments to determine the alterations in cell migratory patterns during tail elongation and eversion, but unfortunately our laboratory currently does not have the technical background to perform such experiments.

Utilizing geldanamycin and the expanding knowledge of zebrafish development that has been gathered during the last decade by scientists around the world, we will undoubtedly be able to enhance our knowledge on the role Hsp90α and Hsp90β play during zebrafish development.

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