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Zebrafish *Hsp70* is required for embryonic lens formation

Tyler G. Evans,[1] Yoshiyuki Yamamoto,[2] William R. Jeffery,[2] and Patrick H. Krone[1]

[1]Department of Anatomy and Cell Biology, University of Saskatchewan, 107 Wiggins Road, Saskatoon, Saskatchewan, Canada S7N 5E5
[2]Department of Biology, University of Maryland, College Park, MD 20742, USA

Abstract Heat shock proteins (Hsps) were originally identified as proteins expressed after exposure of cells to environmental stress. Several Hsps were subsequently shown to play roles as molecular chaperones in normal intracellular protein folding and targeting events and to be expressed during discrete periods in the development of several embryonic tissues. However, only recently have studies begun to address the specific developmental consequences of inhibiting Hsp expression to determine whether these molecular chaperones are required for specific developmental events. We have previously shown that the heat-inducible zebrafish *hsp70* gene is expressed during a distinct temporal window of embryonic lens formation at normal growth temperatures. In addition, a 1.5-kb fragment of the zebrafish *hsp70* gene promoter is sufficient to direct expression of a gfp reporter gene to the lens, suggesting that the *hsp70* gene is expressed as part of the normal lens development program. Here, we used microinjection of morpholino-modified antisense oligonucleotides (MOs) to reduce Hsp70 levels during zebrafish development and to show that Hsp70 is required for normal lens formation. *Hsp70*-MO–injected embryos exhibited a small-eye phenotype relative to wild-type and control-injected animals, with the phenotype discernable during the second day of development. Histological and immunological analysis revealed a small, underdeveloped lens. Numerous terminal deoxynucleotidyl transferase–mediated dUTP-fluoroscein nick-end labeling (TUNEL)–positive nuclei appeared in the lens of small-eye embryos after 48 hours postfertilization (hpf), whereas they were no longer apparent in untreated embryos by this age. Lenses transplanted from *hsp70*-MO–injected embryos into wild-type hosts failed to recover and retained the immature morphology characteristic of the small-eye phenotype, indicating that the lens phenotype is lens autonomous. Our data suggest that the lens defect in *hsp70*-MO–injected embryos is predominantly at the level of postmitotic lens fiber differentiation, a result supported by the appearance of mature lens organization in these embryos by 5 days postfertilization, once morpholino degradation or dilution has occurred.

INTRODUCTION

Heat shock proteins (Hsps) consist of several families of highly conserved proteins whose expression is upregulated in response to a broad range of environmental stressors, including heat shock, alcohol, heavy metals, and oxidative stress (Feder and Hofmann 1999). Some Hsps are expressed under nonstress conditions and have multiple housekeeping chaperone functions related to the folding and intracellular translocation of newly synthesized proteins and multiprotein complexes. Furthermore, the chaperoning function of several Hsps is required for correct assembly, localization, and activation of a select group of cell surface and intracellular receptors, transcription factors, and other molecules. Many of these molecules are important to embryonic development, and the chaperoning activity of Hsps is required in several different embryonic pathways. For example, Hsp47 is a collagen chaperone essential for early mammalian embryogenesis (Nagai et al 2000), members of the Hsp70 family are required for germ cell meiosis and male fertility in mice (Dix et al 1996), and Hsp90β mutant mice fail to form a fetal placental labyrinth and die at embryonic day 10 (Voss et al 2000). Hsp70 also suppresses apoptosis by directly associating with *Apaf-1* (Beere et al 2000), by antagonism of the apoptosis-inducing factor (Ravagnan et al 2001), or through direct suppression of downstream caspases (Komarova et al 2004).

Vertebrate lens development is initiated through inductive interactions between the optic vesicles and overlying

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Correspondence to: Patrick H. Krone, Tel: 1-306-966-4089; Fax: 1-306-966-4298; E-mail: krone@duke.usask.ca.

surface ectoderm, a series of events that has been most thoroughly investigated in the chicken and mouse (reviewed in Chow and Lang 2001). These interactions cause the cells of the surface ectoderm to elongate and form a lens placode, which invaginates into the optic cup and is constricted to form the lens vesicle. Cell division and differentiation lead to the formation of a mature lens composed of epithelial cells and fiber cells. In fish, unlike other vertebrates, the lens vesicle is composed of a solid sphere of cells that form the primary lens fibers, whereas the secondary lens fibers will subsequently be derived from the lens epithelium. Consequently, the lens consists predominantly of concentric layers of fibers that are formed by the differentiation of cells within the optic vesicle and lens epithelium (Bassnett and Mataic 1997). Lens fiber maturation is further characterized by cell elongation, synthesis of lens-specific proteins, and the degradation of all membrane-bound organelles (Piatigorsky 1981; Bassnett and Mataic 1997). The removal of organelles from lens fiber cells is critical to the proper function of the mature eye and leads to the formation of a transparent region at the center of the lens called the organelle-free zone. This process is thought to occur through programmed cell death pathways because organelle removal displays several features characteristic of apoptosis. Failure of lens fiber cell nuclei to properly degrade is characteristic of several pathological conditions, including human congenital cataracts (Zimmerman and Font 1966; Wride 2000).

Our laboratory is examining the role of Hsps during normal embryonic development of the zebrafish (Krone et al 1997, 2003). For example, we have shown that members of the zebrafish *hsp90* and *hsp47* gene families are constitutively expressed during short windows of somite and notochord development, respectively, and that Hsp90 function is required for normal differentiation of somitic muscle pioneer cells (Lele et al 1999). More recently, we reported that the stress-inducible zebrafish *hsp70* gene is strongly and specifically expressed during a short period of normal embryonic lens formation under nonstress conditions that coincides with the period of lens fiber differentiation (Blechinger et al 2002a, 2002b). Interestingly, constitutive *hsp70* expression has also been detected in the embryonic chicken and human lens (Dash et al 1994; Bagchi et al 2001, 2002), suggesting that it plays a unique role during formation of the vertebrate lens. Here, we have used microinjection of morpholino-modified antisense oligonucleotides (MOs) targeted against *hsp70* messenger ribonucleic acid (mRNA) to examine this question in zebrafish embryos. MOs inhibit translation initiation, and block translation of mRNA in vitro, in tissue culture cells, and in vivo (Summerton and Weller 1997; Summerton 1999; Nasevicius and Ekker 2000; Qin et al 2000). MO treatment has been successfully used in a variety of systems for gene-knockdown studies and represents a viable sequence-specific gene inactivation method in zebrafish (Nasevicius and Ekker 2000; Ekker and Larson 2001). Our data reveal that Hsp70 is required for formation of the zebrafish lens.

MATERIALS AND METHODS

Embryo treatment and manipulation

Breeding, maintenance, and manipulation of zebrafish adults and embryos were performed as described (Westerfield 1995). Heat shock was conducted for 90 minutes in a water bath maintained at 37°C. Embryos and larvae to be sectioned were oriented and embedded in 1.5% agarose and processed in JB-4 methacrylate (Polysciences Inc, Warrington, PA, USA) or paraffin. The resulting sections were stained with methylene blue–azure II–basic fuchsin stain (Humphrey and Pittman 1974), 4′,6-diamidino-2-phenylindole (DAPI), or processed for immunostaining, as described below.

Microinjection of zebrafish embryos

The following MOs were synthesized by Gene Tools, LLC (Corvalis, OR, USA). The start codon (or portion thereof) is underlined, and mismatches of *hsp70*MM-MO are in lower case: *hsp70* #1 (*hsp70*-MO: 5′-GCGATTCCTTTTGG AGAAGACATGA-3′); *hsp70* #2 (*hsp70*-MO2: 5′-ATGATT-GATTTCAAGAAACTGCAGG-3′); standard negative control (5′-CCTCTTACCTCAGTTACAATTTATA-3′); *hsp70* 5-bp mismatch (*hsp70*MM-MO: 5′-GCcATaCCTTTTaGAG AAcACAaGA-3′); *chordin* control (*chordin*-MO: 5′-ATCC ACAGCAGCCCCTCCAT-3′).

Morpholinos were designed based on complementary deoxyribonucleic acid and genomic sequences of the zebrafish *hsp70-4* gene originally identified in our laboratory (Lele et al 1997; Halloran et al 2000; GenBank accession numbers AF006006, AF158020). The original MO was dissolved to a concentration of 22.5 μg/μL with triple distilled water, and the solution was dispensed into 5-μL aliquots for storage at −20°C. Immediately before microinjection, the stock MO solution was diluted with Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and pH 7.6; Nasevicius and Ekker 2000) to the concentration indicated. Initially, embryos were microinjected with a range of MO concentrations to maximize the number of embryos displaying the phenotype while minimizing secondary toxic effects. The *hsp70*-MO, *hsp70*-MO2, *hsp70*MM-MO, and standard control MO solutions were injected into embryos at 3.14 nL/injection using a Narashige IM-300 microinjector. Some MOs carried a 3′ fluoroscein label to allow obser-

vation of MO distribution in injected embryos using fluorescent microscopy. The 2 *hsp70* MOs served as the experimental set, whereas the standard negative control MO and *hsp70*MM-MO served as negative controls. Injection with 3.14 nL Danieau solution served as an additional negative control. The *chordin*-MO has been shown to reproduce the well-characterized *chordino* mutant (Hammerschmidt et al 1996) and was used as a positive control when setting up injection assays.

Western blot analysis

Yolk proteins comigrate with Hsp70 in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Thus, the yolks of embryos 38 hours postfertilization (hpf) were dissected out using fine forceps in phosphate-buffered saline (PBS) containing protease inhibitors before protein extract preparation. Heat shocked embryos were incubated at 37°C for 90 minutes before dissection. Yolk-dissected embryos were placed in 1.5-mL microfuge tubes in PBS plus 0.1% Tween-20 (PBST) containing protease inhibitors and homogenized with a pestle at 4°C. The resulting slurries were further homogenized by sonication. Protein samples were loaded and electrophoresed at 90 V through 8% polyacrylamide-SDS denaturing gels, and separated proteins were electrically transferred to nitrocellulose membranes. The membranes were washed 4 × 5 minutes in 3% milk in PBST, followed by blocking for 2 hours in 3% milk. A rabbit polyclonal antibody raised against recombinant human *hsp70* (Stressgen Biotechnologies Corporation, Victoria, BC, Canada # SPA-812) was diluted to 1:37 500 in 3% milk and incubated with membranes overnight at 4°C. In zebrafish, this antibody detects both the heat-inducible Hsp70 and the cognate member of the Hsp70 family, Hsc70 (SantaCruz et al 1997). After 4 × 5 minutes washes and a 1 hour blocking period in 3% milk, membranes were incubated with goat anti-rabbit horseradish peroxidase–conjugated secondary antibody at 1:500 in 3% milk for 2 hours. After 4 × 5 minute washes in 3% milk, blots were exposed to film, and the film was developed.

Immunohistochemistry

Embryos were dechorionated and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The embryos were rinsed in PBS, embedded in paraffin, and sectioned to 7 μm thickness. The resulting sections were mounted on coverslips, dewaxed, and processed for immunostaining. Briefly, sections were rinsed in PBS, blocked using 3% skim milk powder/0.1% Triton-X (Sigma-Aldrich, Oakville, Ontario, Canada) for 30 minutes, and placed in the primary antibody overnight at 4°C. Primary antibodies used in this study were zl-1 (1:500), zn-5 (1:200), zns-2 (1:50), and znp-6 (1:50) obtained from the University of Oregon Monoclonal Antibody Facility (Eugene, OR, USA). Proliferating cell nuclear antigen (PCNA) primary antibody obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) was used at a 1:2000 dilution. After incubation in the primary antibody, sections were rinsed with PBS, blocked with 3% skim milk powder/0.1% Triton-X, and incubated in biotin-conjugated goat anti-mouse secondary antibody (1:200; Vector Laboratories, Burlington, Ontario, Canada) for 30 minutes at room temperature. Sections were then rinsed with PBS, treated with 0.3% H_2O_2/methanol for 15 minutes, and stained using an ABC detection kit (Vector Laboratories) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase–mediated dUTP-fluoroscein nick-end labeling analysis

The terminal deoxynucleotidyl transferase–mediated dUTP-fluoroscein nick-end labeling (TUNEL) assay protocol described here is from Graham (1998) with minor modifications (Roche Diagnostics, Laval, Quebec, Canada). Embryos were fixed overnight in 4% PFA in PBS at 4°C. Embryos were washed 3 × 30 minutes in PBS with PBST, permeabilized in proteinase K (10 μg/mL in PBST) for 20 minutes, and then washed 2 × 5 minutes in PBST at room temperature. A postfix in 4% PFA in PBST for 30 minutes stopped the permeabilization reaction. Embryos were again washed 2 × 5 minutes in PBST. Embryos were then washed 1 × 5 minutes, followed by 1 × 60 minutes in fresh TUNEL buffer (100 mM cacodylic acid, 2.5 mM cobalt chloride, 0.1 mM dithiothreitol, 100 μg/mL bovine serum albumin, 1% Triton-X, in PBST), and then incubated in the TUNEL reaction mix containing a 9:1 ratio of TUNEL label–TUNEL enzyme (Roche Diagnostics) for 3 hours at 37°C in darkness. As a positive control, embryos were first incubated in 4 μg/mL pancreatic deoxyribonuclease in a polymerase chain reaction buffer (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$) for 1 hour at 37°C before incubation in the TUNEL reaction mix. As negative controls, embryos were incubated in the TUNEL label only. After the reaction, embryos were washed 3 × 30 minutes in PBST at room temperature and stored in PBST at 4°C in a covered canister. Fluorescence was detected using a Nikon Y-FL epifluorescence attachment on a Nikon E-600 microscope.

Lens transplantation

The lens transplantation method described in detail in Yamamoto and Jeffery (2002) was followed in this study.

Table 1 Summary of morpholino injection experiments[a]

Solution injected	Solution concentration[b] (μg/μL)	Number of embryos	Survivors displaying small-eye phenotype[c] (%)	Survival rate at 52 h (%)
chordin-MO	2.25	54	0 (75)[d]	NA
Standard sequence MO	3.46	86	0	61
Danieau solution	NA	175	0	57
hsp70-MO	2.25	123	13	58
hsp70-MO	4.50	107	16[e]	23
hsp70-MO	3.46	486	63	58
hsp70-MO2	3.46	107	53	54
*hsp70*MM-MO	3.46	109	0	63

[a] MO, morpholino-modified antisense oligonucleotides; NA, not available.
[b] 3.14 nL injected per embryo in all experiments.
[c] In all embryos, both eyes are affected equally.
[d] Bracketed numbers refer to percent embryos displaying the typical *chordin* mutant phenotype.
[e] The majority of embryos injected with 4.50 μg/μL *hsp70*-MO concentration displayed nonspecific defects and death as indicated in Results and do not appear in these counts.

RESULTS

Antisense *hsp70* morpholino injection yields a small-eye phenotype in 2-day-old embryos

Two *hsp70*-targeting MOs were designed, one against the 5′–open reading frame (*hsp70*-MO) and the other against the 5′–untranslated region, (*hsp70*-MO2). We initially determined the optimal injection concentrations of MO (Table 1). The majority of studies using MOs in zebrafish embryos have focused on genes expressed during the first day of development and have normally required microinjection of anywhere from 0.5 to 5 ng per embryo to produce a consistent phenotype. We expected that injection of higher amounts of *hsp70*-MO would be required because further MO degradation or dilution occurring between 1 and 2 days of development would reduce the effective concentration during the time period of *hsp70* expression. All embryos in these experiments were raised at the non–heat shock, normal growth temperature of 28°C. Embryos microinjected with a concentration of 3.46 μg/μL *hsp70*-MO (10.9 ng/injection) were indistinguishable from control embryos at 24 hpf, whereas 63% of the 486 assayed embryos displayed a characteristic small-eye phenotype at 52 hpf (Fig 1). Similarly, injection of *hsp70*-MO2 resulted in 53% of embryos displaying an identical, small-eye phenotype at 52 hpf (Table 1). The slightly lower potency of *hsp70*-MO2 is most likely related to the lower GC content of this MO (36%) compared with *hsp70*-MO (44%). The majority of embryos displayed the extreme small-eye phenotype shown in Figure 1A, although we did observe some variation in severity with a minority of the embryos showing a more moderate small-eye phenotype (Fig 1B). This is most likely due to variation in the distribution of the MO in different embryos by this stage of development. Importantly, the phenotype was consistent, easily distinguishable from uninjected and control-injected embryos, and reproducible in 13 separate injection trials with 2 different MOs. Furthermore, the 2 lot numbers of *hsp70*-MO and 1 of *hsp70*-MO2 were all synthesized at different times, eliminating the possibility of any batch-specific contaminants causing the observed phenotype. In addition, the phenotype was consistently produced using different microinjection systems and different operators at both the University of Saskatchewan and the University of Maryland. Small-eye phenotype embryos did not display any other significant abnormalities, although we did observe slight reductions in pigmentation in a proportion of these individuals. Overall growth and development was not affected, as assessed by examination of the overall length, pigment cell distribution, and otic vesicle length (Westerfield 1995). Importantly, injected embryos not displaying the characteristic small-eye phenotype in these experiments deviated from the wild-type condition in only 5.7% of the cases, and in these cases the embryos developed a variety of severe and inconsistent defects (data not shown). Embryos injected with a concentration of 2.25 μg/μL *hsp70*-MO showed a much lower penetrance of the small-eye phenotype, whereas those injected with 4.50 μg/μL exhibited numerous inconsistent toxic effects, including developmental abnormalities and increased death rate (Table 1). Given the data described above, the remaining experiments in this study used microinjection of 3.14 nL of *hsp70*-MO at a concentration of 3.46 μg/μL (10.9 ng/injection).

Injection of *hsp70*-MO resulted in the disappearance of Hsp70 under normal, non–heat shock growth temperatures (28°C) at which these experiments were carried out (Fig 2). Importantly, MO treatment did not affect levels of the closely related heat shock cognate 70 (Hsc70; SantaCruz et al 1997). The identity of the lower band as Hsp70 in Figure 2, and targeting by the MO, were confirmed in heat shocked embryos. Under these conditions, the MO cannot completely eliminate the Hsp70 band, a result that is not surprising considering both the large amount of the protein synthesized after heat shock throughout the embryo and the fact that some MO deg-

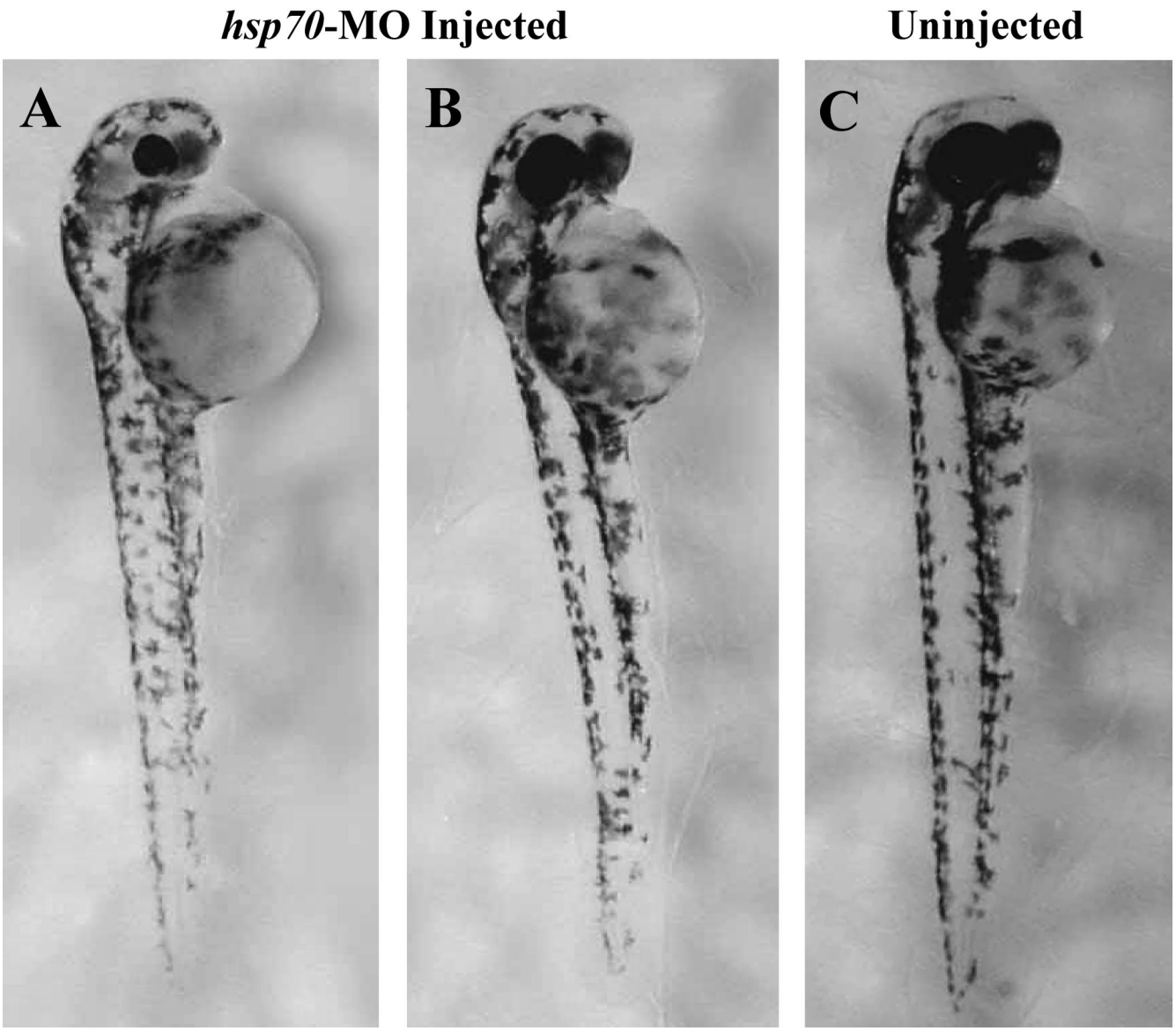

Fig 1. Small-eye phenotype produced by microinjection of *hsp70*–morpholino-modified antisense oligonucleotide (MO) into fertilized eggs. Severe small-eye phenotype (A) and moderate small-eye phenotype (B) are shown relative to an uninjected control embryo (C). All embryos screened as small-eye phenotype lie within this phenotypic range, and in all cases both eyes of these embryos were equally affected.

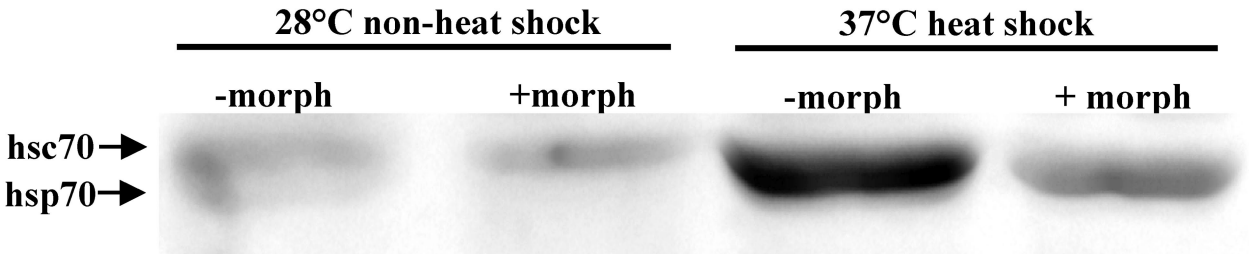

Fig 2. Western blot analysis of Hsp70 knockdown in *hsp70*–morpholino-modified antisense oligonucleotide (MO)–injected embryos. Embryos were allowed to develop until 36.5 hours postfertilization (hpf), and then heat shocked at 37°C for 90 minutes, or maintained at 28°C for an additional 90 minutes. Proteins were isolated from embryos and Western blot analysis carried out as described in Materials and Methods. Hsp70 is a slightly smaller protein than Hsc70 (SantaCruz et al 1997) and can be discerned as a unique band with a slightly greater relative mobility than that of Hsc70. The *hsp70*-MO also causes a significant reduction in the amount of Hsp70 synthesized throughout the embryo after heat shock, an extremely strong and rapid inducer of *hsp70* expression.

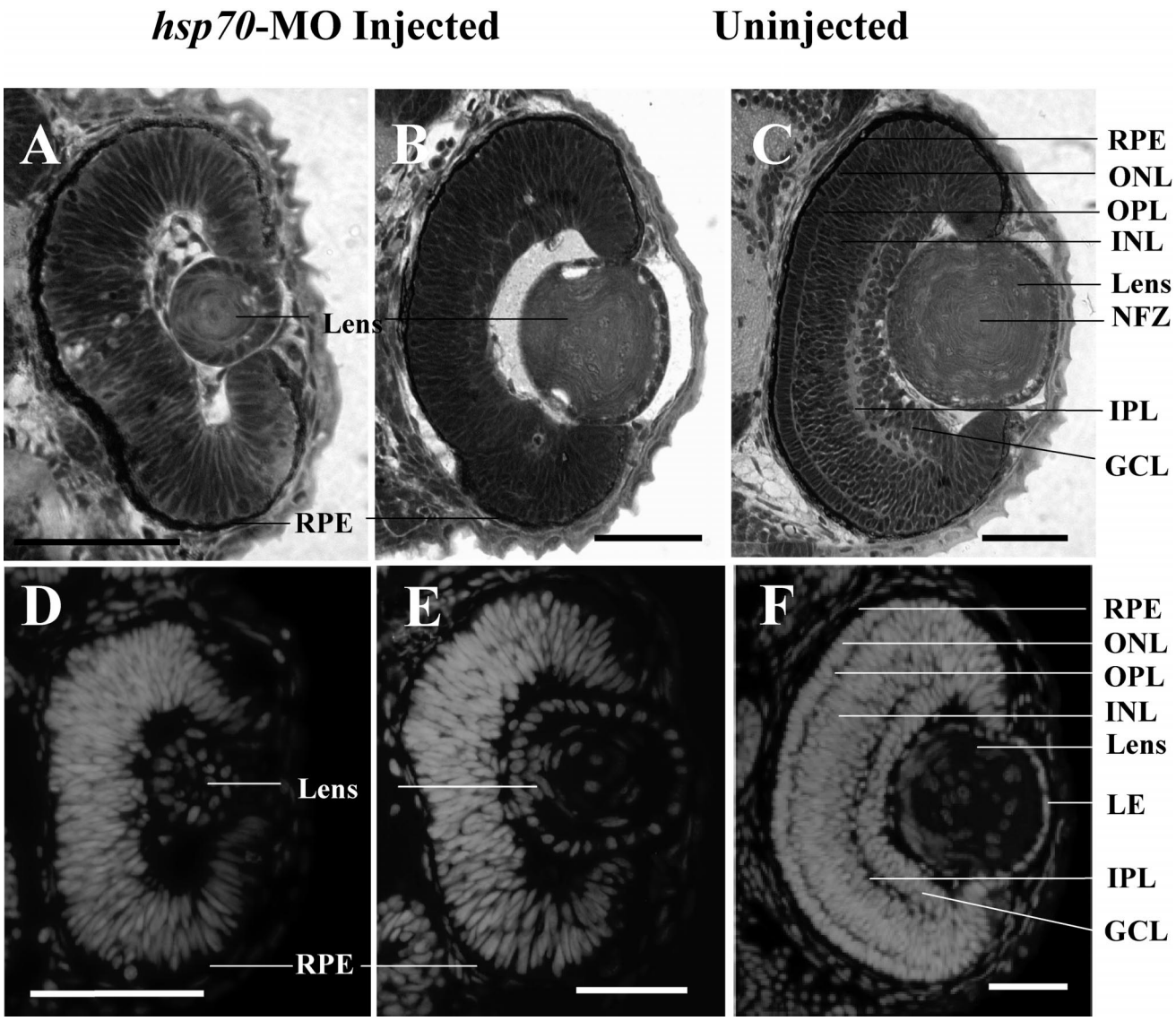

Fig 3. Histological analysis of small-eye phenotype in *hsp70*-morpholino-modified antisense oligonucleotide (MO)–injected embryos and uninjected control embryos at 52 hours postfertilization (hpf). (A,D) Severe small-eye phenotype. (B,E) Moderate small-eye phenotype. (C,F) Uninjected embryos. (A–C) Methylene blue–azure II–basic fuchsin–stained 5.5-μm-thick methacrylate cross sections. (D–F) 4′,6-Diamidino-2-phenylindole (DAPI)–stained 5.5-μm-thick methacrylate cross sections viewed through a fluorescence microscope to observe nuclei. The immature structure of the lens and retina are readily apparent in the small-eye embryos (A,B) relative to uninjected control embryos (C). Note that the nuclear-free zone has begun to appear in the uninjected embryos but is not present in the small-eye lens. RPE, retinal pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFZ, developing nuclear-free zone; LE, lens epithelium. Scale bar = 50 μm.

radation and dilution have occurred by this point in development.

Small-eye phenotype is characterized by an immature and underdeveloped lens

To permit a detailed assessment of the *hsp70*-MO small-eye phenotype, histological analysis was performed on embryos at 52 hpf (Fig 3A–F). Histology revealed that the small-eye phenotype is characterized by an underdeveloped lens. Specifically, the lens is significantly reduced in size, and its cellular structure most closely resembles that of an earlier stage embryo (Fig 3A,B; Easter and Nichola 1996). This morphology is most noticeable in those embryos displaying the severe small-eye phenotype. Severe small-eye embryos had an extremely small lens, did not display the concentric fiber organization characteristic of lenses at this age, and clear separation of lens core and lens epithelium was just becoming apparent (Fig 3A,D). However, these lenses do express the early lens marker zl-1, suggesting that some of the earlier events in lens formation appear to be unaffected in these embryos (Fig

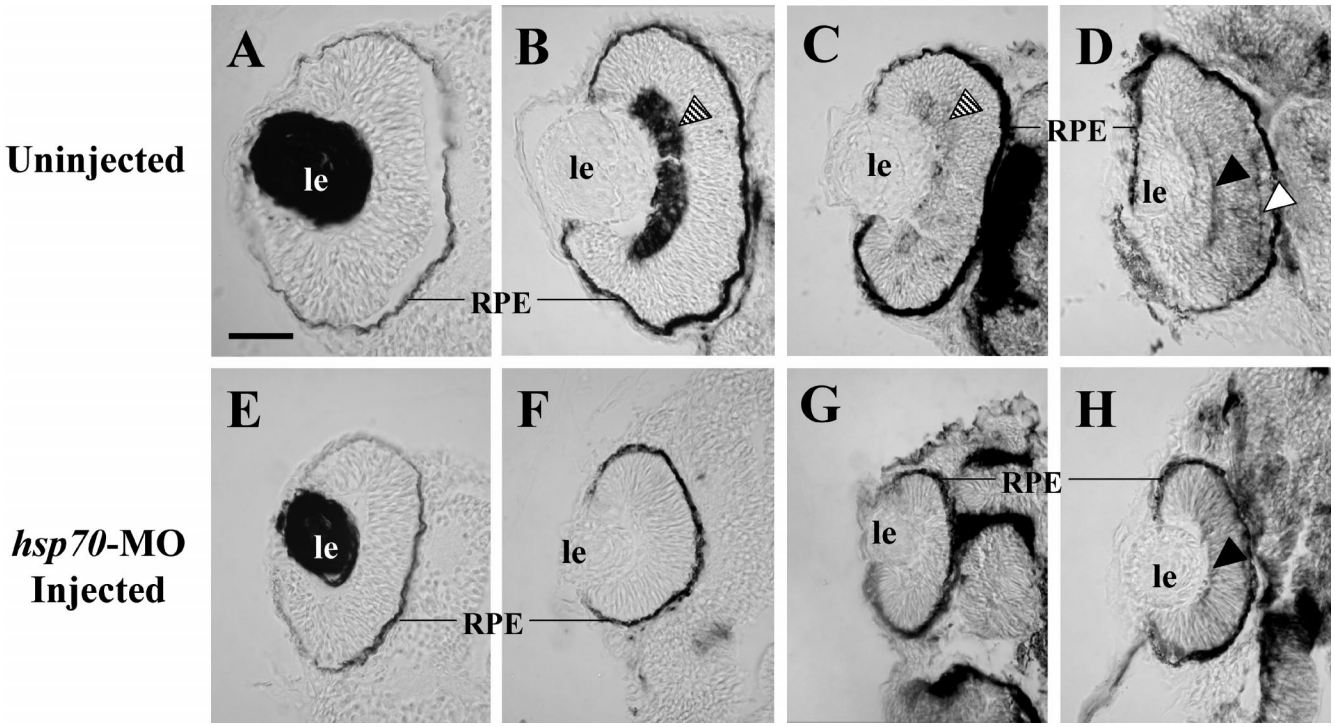

Fig 4. Immunohistochemical analysis of lens and retinal protein expression in wild-type (A–D) and severe small-eye (E–H) paraffin sections. Lens protein zl-1 is expressed in both wild-type (A) and small-eye (E) lenses. Retinal ganglion cells (striped arrowheads), observed by immunostaining with either zn-5 (B,F) or znp-6 (C,G) are absent from the small-eye phenotype retina (F,G), but are readily apparent in uninjected embryos. Similarly, both inner plexiform (black arrowheads) and outer plexiform (white arrowhead) layers are stained with zns-2 in wild-type sections (D), but a similar pattern is not apparent in the eyes of microinjected embryos with the small-eye phenotype (H). Dorsal is to top. RPE, retinal pigmented epithelium. Scale bar = 50 μm.

4A,E). A fraction of embryos displayed a more moderate small-eye phenotype (Fig 3B,E). Although these lenses exhibited some features of more mature lenses, they were still significantly smaller in size, there was no central zone of denucleation yet apparent, there were fewer cells within the lens epithelium and core, and the lens epithelial cells were larger and more cuboidal than is normally expected for a 52-hour embryo (compare panels B, E with C, F in Fig 3).

The retinal organization of *hsp70*-MO–injected embryos was disrupted, and the retinal structure appeared underdeveloped at 52 hpf (Fig 3A,B,D,E). Specifically, the small-eye phenotype retinas lacked the characteristic laminated structure of wild-type embryos at 52 hpf. Immunostaining further confirmed disruption of retina formation. Retinal ganglion cells appear to be completely absent in the *hsp70*-MO–injected embryos because no staining for the retinal ganglion markers zn-5 or znp-6 was observed (compare panels B,C with F,G in Fig 4). Staining with zns-2, a marker of the inner and outer plexiform layers, was abnormal in that the 2 normally recognizable stripes of expression were not present in the *hsp70*-MO–injected embryos, although some weak staining was apparent immediately adjacent to the lens in some embryos (Fig 4D,H). This suggests that formation of the inner plexiform layer, although delayed, was beginning to occur in the microinjected embryos at this age.

The immature lens phenotype in *hsp70*-MO–injected embryos is lens autonomous

Because we have only been able to detect *hsp70* expression in the lens (Blechinger et al 2002a), we predicted that the small-lens phenotype described in this study would be lens autonomous. We tested this using lens transplantation. This technique has been used successfully to reveal that signals from the lens play a critical role in cavefish eye degeneration (Yamamoto and Jeffery 2000) and to demonstrate the autonomy of lens defects in zebrafish mutants (Vihtelic et al 2001). Lenses isolated from one eye of *hsp70*-MO–injected embryos (donors) were transplanted into the optic cup of uninjected, wild-type embryos (hosts) at 30–34 hpf, and transplant embryos were allowed to develop to 52 hpf. Because the *hsp70*-MO small-eye phenotype is not visibly discernable at 34 hpf, all donor embryos were allowed to develop to 52 hpf, and development of their remaining lens in the unmanipulated contralateral eye was assessed. Only those host embryos receiving lenses from donor-microinjected embryos that displayed a typical small-eye phenotype in their remain-

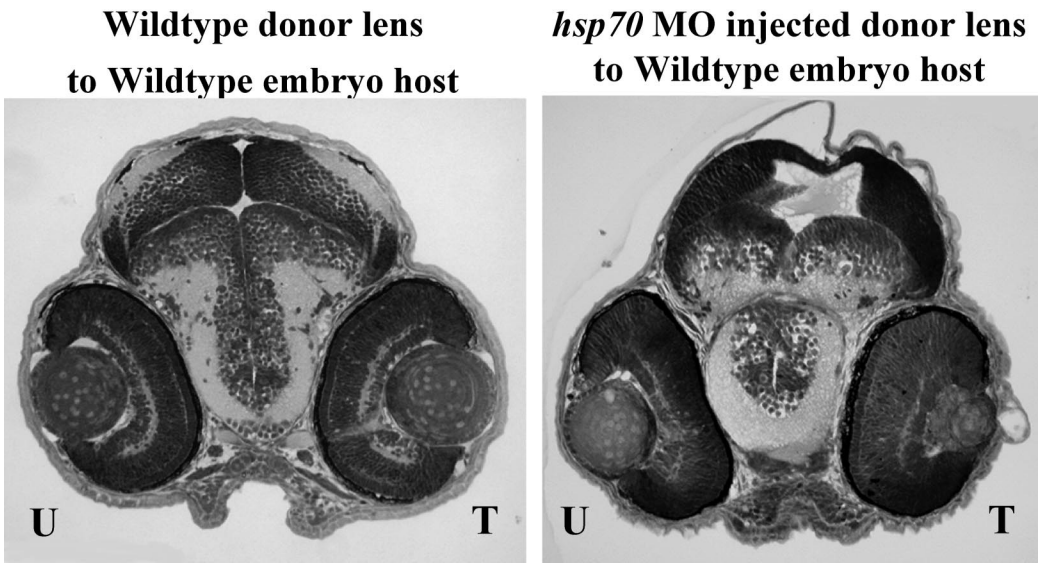

Fig 5. Histological analysis of lens transplant experiments at 52 hours postfertilization (hpf). Lenses were isolated from donor embryos and inserted into the optic cup of host embryos between 30 and 34 hours of age. The right eye received the transplanted lens whereas the left side served as an unmanipulated control in all cases. Note that transplanted lenses from an *hsp70*-MO–injected donor embryos still exhibit the typical characteristics of the small-lens phenotype after transplant into a wild-type retina. T, transplant side; U, unmanipulated control side.

ing eye were assessed further. Initial whole-embryo observation revealed that transplanted lenses in host embryos were small and hardly visible within the surrounding optic cup. In contrast, the contralateral control eye on the host embryos exhibited normal eye morphology at 52 hpf. Sections through transplanted embryos at 52 hpf revealed that the lens retained the cellular characteristics of the small-eye phenotype (Fig 5). Transplanted lenses were extremely small, they did not show the same degree of lens fiber differentiation and lamination that was evident in lenses on the nontransplanted control side, and there was no clear definition between the lens epithelium and core. Thus, the presence of a wild-type retina could not rescue the *hsp70*-MO–dependent lens phenotype, suggesting that the lens phenotype is lens autonomous.

Apoptotic nuclei persist in lenses of *hsp70*-MO–injected embryos beyond 48 hpf

Both apoptosis and breakdown of lens fiber nuclei involve the degradation of chromatin leading to the presence of ''deoxyribonucleic acid (DNA) ladders'' (Appleby and Modak 1977; Bassnett and Mataic 1997; Ishizaki et al 1998), and this fragmented DNA is amenable to labeling through TUNEL analysis (Ishizaki et al 1998). Cole and Ross (2001) previously showed that the developing zebrafish lens also exhibits TUNEL-positive nuclei, with the peak number occurring during the fiber differentiation phase of 24–40 hpf. After this time, apoptotic nuclei become significantly reduced, and by 48 hpf, they are no longer detectable in the lens. Importantly, this corresponds with the time period during which (1) lens fiber differentiation occurs, (2) *hsp70* expression occurs, and (3) the small-eye phenotype of *hsp70*-MO–injected embryos becomes apparent. We carried out a similar analysis on uninjected and *hsp70*-MO–injected embryos at 36 and 52 hpf. In agreement with the data of Cole and Ross (2001), we detected numerous apoptotic nuclei using TUNEL analysis within the lens of uninjected embryos at 36 hpf (Fig 6A) but were unable to detect any by 52 hpf (Fig 6C). However, *hsp70*-MO–injected embryos exhibited significant numbers of TUNEL-labeled nuclei within the lens at 52 hpf (Fig 6D), in addition to the normal peak at 36 hpf (Fig 6B). In all cases, the TUNEL-labeled cells were located specifically within the lens fibers, and labeling was absent from the surrounding lens epithelium. The retinas of small-eye and uninjected embryos contained a comparable number of TUNEL-labeled nuclei (Fig 6A–D), and these levels were in accordance with those reported by Cole and Ross (2001) during normal development. The small lens of *hsp70*-MO–injected embryos does not appear to be correlated with a loss of proliferative capability of cells within the lens epithelial cells because staining for PCNA was comparable within the lens of both microinjected and uninjected embryos (Fig 6E,F).

Recovery of overall lens organization by 5 days postfertilization

We next determined whether the *hsp70*-MO–induced phenotype is permanent or if the lenses of these embryos can recover after degradation or dilution of the MO later in

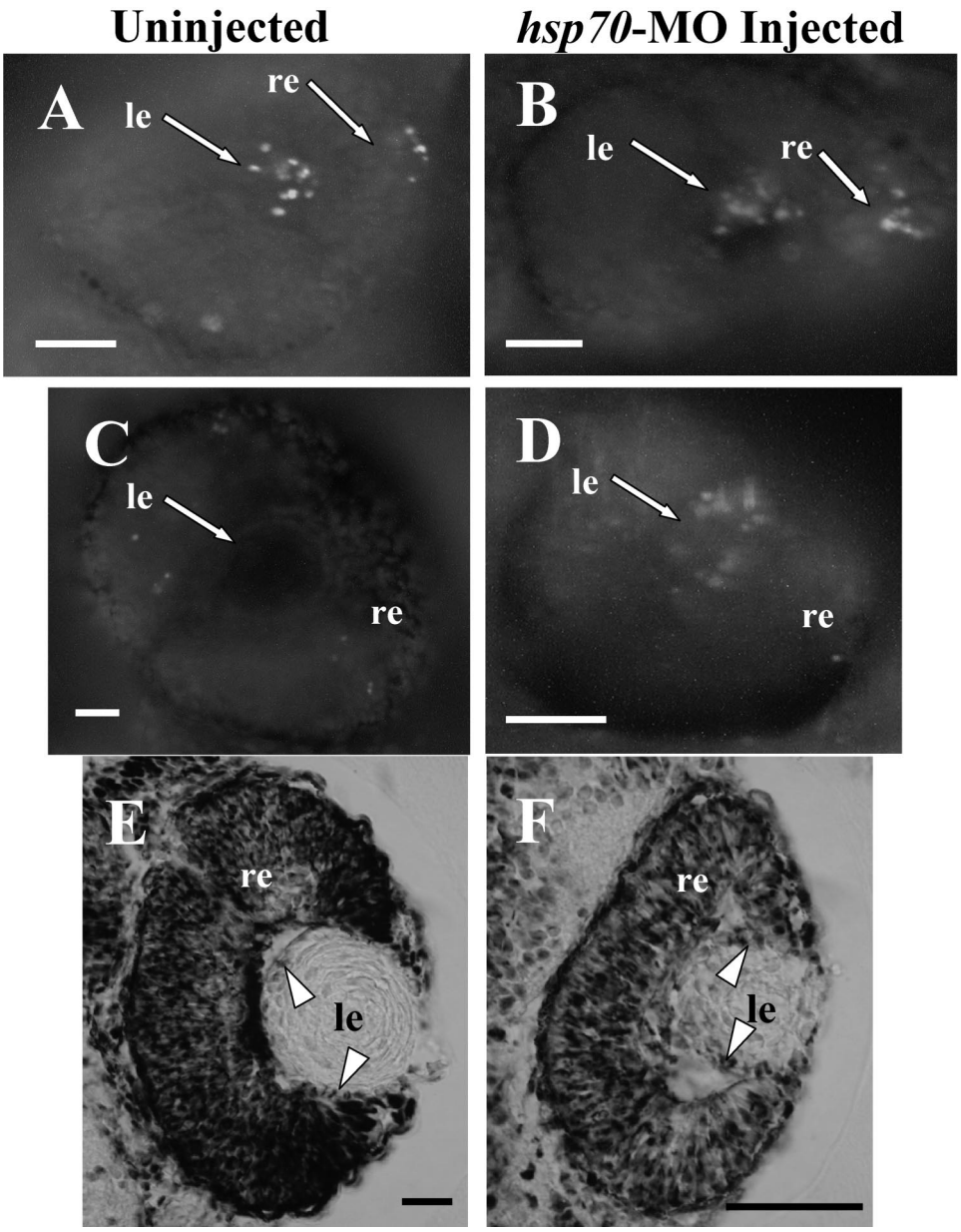

Fig 6. Terminal deoxynucleotidyl transferase–mediated dUTP-fluoroscein nick-end labeling (TUNEL) analysis to detect apoptotic nuclei in uninjected control embryos (A,C) and small-eye phenotype embryos (B,D) at 36 hours postfertilization (hpf) (A,B) and 52 hpf (C,D). The basal level of apoptosis seen in both injected and control embryos (A,B) agrees with the amount of cell death described by Cole and Ross (2001) in the normal development of the eye at 36 hpf. However, by 52 hpf, apoptotic nuclei are no longer apparent in the lens of uninjected control embryos (C) as expected (Cole and Ross 2001) but continue to appear in the small-eye phenotype lens (D). Again, normal developmental apoptosis is observed in the retina of small-eye and control embryos at 52 hpf (C,D). Immunostaining of proliferating cell nuclear antigen (PCNA) in wild-type (E) and small-eye (F) 7-μm paraffin sections at 52 hpf. Although the small-eye lens contains significantly less cells than wild-type controls, this does not appear to be caused by a loss of proliferative capability of lens fiber precursors originating from the lens epithelium (arrowheads). PCNA levels immediately drop as lens epithelial cells are recruited to the region of postmitotic fiber differentiation within the lens. le, lens; re, retina. Scale bar = 50 μm.

development. In embryos injected with fluorescein-tagged *hsp70*-MO, fluorescence was detected in 90% of animals at 48 hpf (data not shown). Only 26% of embryos showed fluorescence at 3 days postfertilization (dpf) and by 4 dpf fluorescence was no longer detectable in any of the injected embryos. We then allowed *hsp70*-MO–injected embryos that displayed the typical small-eye phenotype at 52 hpf to develop until 5 dpf, after which histological analysis was performed. Sections from injected embryos at this age revealed that overall lens and retinal organization of *hsp70*-MO–injected embryos is comparable with uninjected embryos (Fig 7). Although the lens is

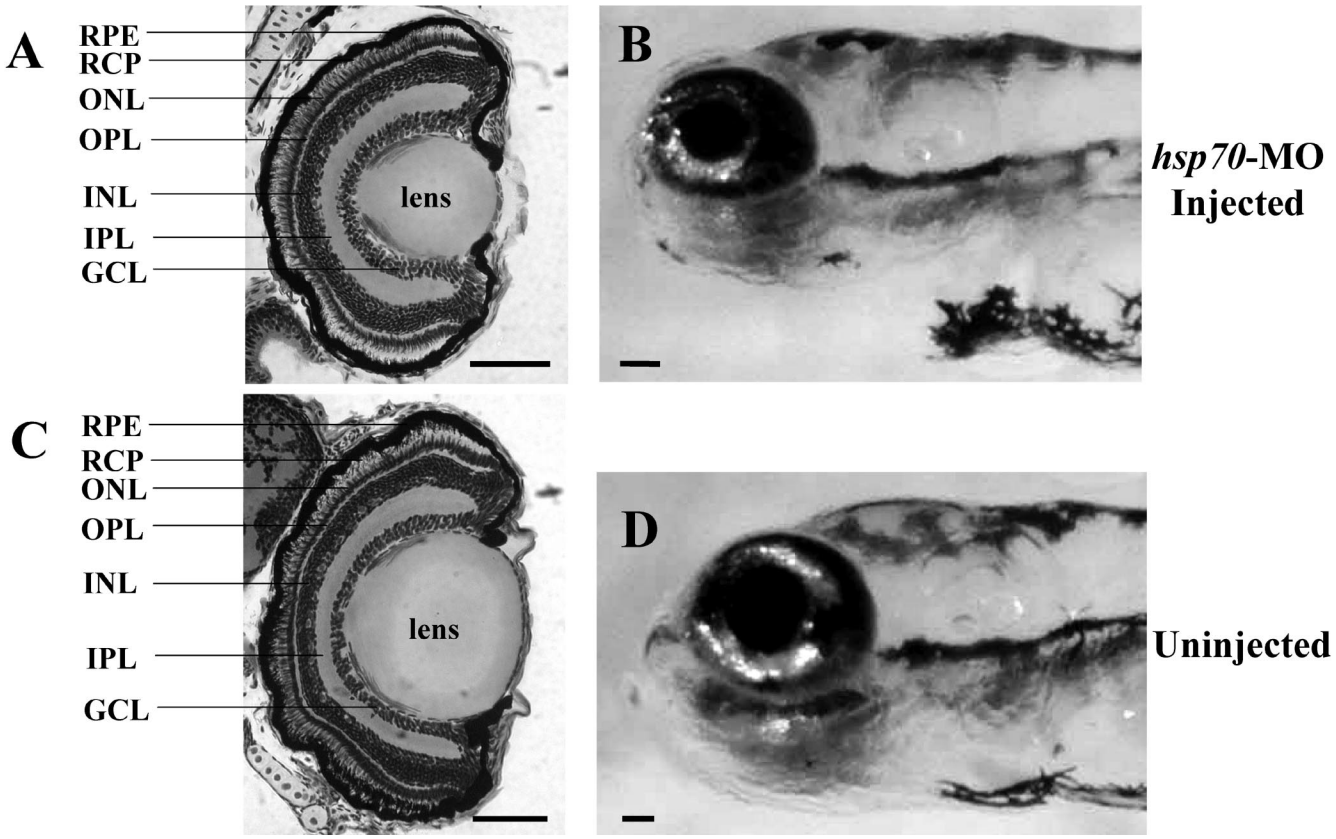

Fig 7. Histological and morphological analysis of small-eye phenotype (A,B) and uninjected control embryo (C,D) at 5 days postfertilization (dpf). The 5.5-μm-thick methacrylate cross sections through *hsp70*-MO injected embryos reveal that, although the eyes remain smaller than uninjected controls, lens and retinal organization are similar to uninjected embryos at this age (A,C). RPE, retinal pigmented epithelium; RCP, rods and cones of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 50 μm.

smaller than in uninjected control embryos at 5 dpf, its organizational structure and the degree of fiber differentiation is indistinguishable from that of a mature lens (Fig 7A,C). Thus, the recovery observed in *hsp70*-MO–injected embryos at 5 dpf correlates closely with dilution or degradation of the MO below detectable levels.

DISCUSSION

The data presented here show that morpholino-modified antisense *hsp70* oligonucleotide microinjection results in a significant reduction in Hsp70 in zebrafish embryos. Furthermore, this leads to a characteristic small-eye phenotype in the majority of embryos but not in embryos injected with any of the control MOs used in this study. The phenotype is characterized by disruption of normal lens development in 2-day-old embryos, with the lenses exhibiting characteristics that indicate they are underdeveloped and immature. These include small size, lack of lens fiber lamination, delayed development of a nuclear-free zone within the lens core, and larger, more cuboidal lens epithelial cells. The appearance of the defect between 24 and 48 hours of development coincides with the time period during which *hsp70* expression occurs specifically within the lens (Blechinger et al 2002a). Furthermore, we demonstrated that the lens defect is lens autonomous and that it results in an extension of the time period over which TUNEL-positive nuclei appear during the process of fiber differentiation. Overall, our data demonstrate that Hsp70 is required during the normal process of lens development under nonstress conditions and that this requirement most likely occurs during the differentiation of postmitotic lens fibers.

The *hsp70*-MO–induced small-eye phenotype is also characterized by an immature retinal organization with a concomitant reduction in cell number. If this effect were directly due to MO, it would have to be a specific effect of the *hsp70*-MO because it was not observed in any of the control experiments. The only location in which we have observed *hsp70* expression under nonstress conditions is the lens, although we cannot discount the possibility of low levels of retinal *hsp70* expression below assay detection limits. Although we cannot conclude that the retinal defects are not due to a direct MO effect in the

retina, we believe a more plausible explanation is that they are a secondary effect caused by the impact of *hsp70*-MO on the lens. A number of studies have demonstrated that the lens plays a central role in the determination and development of eye structures, including the retina. For example, suppression of lens growth by α*A-crystallin* promoter-driven expression of diphtheria toxin specifically to the lens has a significant effect on retinal lamination in zebrafish (Kurita et al 2003). Similarly, lens transplant experiments have revealed that signals originating from the lens are required for the proper degeneration of the cavefish eye and that lenses transplanted from surface fish into cavefish are sufficient to promote retinal and eye development in these normally eyeless organisms (Yamamoto and Jeffery 2000). Because lamination in wild-type retinas receiving *hsp70*-MO–containing lenses was normal at 52 hpf, retinal dependence on the lens in our experiments would have to occur subsequent to 28 hpf, when *hsp70* expression first begins in the lens (Blechinger et al 2002a), but before 34 hpf, when the lens transplants were completed. This is consistent with our inability to detect the retinal ganglion cell layer in 52-hpf *hsp70*-MO–injected embryos, which normally appears at 29 hpf and is formed by 36 hpf (Kay et al 2001; Easter and Malicki 2002).

We could not detect Hsp70 in 38-hpf embryos microinjected with *hsp70*-MO and raised at the normal growth temperature of 28°C. In contrast, levels of the closely related Hsc70 were unaffected by *hsp70*-MO injection (Fig 2). An important consideration in MO use is the extent to which consistent phenotypic penetrance is observed. For example, a *bmp7* MO caused extreme dorsalization in 93% of embryos injected, whereas *chordin* and *no tail* mutations were reproduced at 75% and 98%, respectively (Nasevicius and Ekker 2000). In other cases, a lower degree of penetrance has been observed. For example, MO targeted to *somitabun* and *one-eyed pinhead* displayed only 24% and 49% penetrance, respectively (Lele et al 2001). Thus, the 63% penetrance of the small-eye phenotype observed in 13 independent trials for *hsp70*-MO, and 53% observed in 2 independent trials for *hsp70*-MO2, is well within the range reported in previous studies. In addition, the observed phenotype might lie anywhere on the entire spectrum of phenotypes between complete depletion and only minimal depletion of the target protein. This factor will be influenced not only by MO concentration but also by the amount and stability of protein, amount of MO degradation and diffusion, and localization and time of expression of the targeted mRNA (Heasman 2002). The lens represents a very small fraction of the total cells in the embryo between 24 and 48 hours of development, and it is reasonable to expect some variability in the amount of the MO received by the lens fibers among microinjected embryos.

Zebrafish lens formation begins at 16–20 hpf, with the optic vesicle contacting the overlying head ectoderm. By 24–26 hpf, the lens has detached from the surface ectoderm and invaginates into the optic cup, forming the presumptive lens (Schmitt and Dowling 1994; Easter and Nichola 1996; Li et al 2000). As the lens develops, the central core appears significantly different from the cuboidal epithelium lining the lens periphery. The concentric organization of lens fiber cells becomes apparent by 36 hpf, and by 48 hpf differentiated primary fibers that have lost their nuclei and organelles have begun to appear at the lens core. Subsequent cell differentiation of secondary fibers derived from the lateral lens epithelium eventually leads to the formation of a mature lens that allows for functional vision by 3 dpf (Easter and Nichola 1996; Vihtelic and Hyde 2002). Precisely what role Hsp70 plays during this process is currently unknown. For example, it is possible that Hsp70 functions within the mechanisms by which fiber cells receive and process differentiation cues, within the differentiation process itself, or it may act as a survival factor for differentiating lens fibers. Hsp70 protects cells from a number of apoptotic stimuli, including heat shock, tumor necrosis factor, growth factor withdrawal, oxidative stress, chemotherapeutic agents, ceramide, and radiation (Jaatela et al 1992; Mosser and Martin 1992; Mailhos et al 1993; Simon et al 1995; Samali and Cotter 1996) and is believed to protect cells from stress-induced apoptosis by interacting directly with components of the apoptotic pathway (Beere et al 2000; Garrido et al 2001; Ravagnan et al 2001; Komarova et al 2004). Interestingly, recent evidence suggests that organelle degradation occurring during normal lens fiber differentiation shares several features with classical apoptotic events. For example, both processes involve the degradation of chromatin and subsequent fragmentation of DNA detected by TUNEL analysis (Appleby and Modak 1977; Bassnett and Mataic 1997; Ishizaki et al 1998). Ishizaki et al (1998) also provided several pieces of evidence that lens fiber differentiation involves components of classical apoptosis, including the involvement of cysteine-containing aspartate-specific proteases or caspases. Moreover, overexpression of bcl-*2*, a member of a key family of proteins that regulates apoptosis, disrupts lens fiber cell denucleation, resulting in fibers that are disorganized and contain intact or fragmented nuclei (Fromm and Overbeek 1997). Finally, Wride et al (1999) confirmed these results, demonstrating that several members of the bcl-*2* and caspase families are active during lens fiber denucleation. Thus, 1 possibility is that Hsp70 plays a role in attenuating the apoptotic-like process within differentiating lens fibers. This is consistent with several pieces of evidence from our study. First, numerous TUNEL-positive nuclei remain within the lens of *hsp70*-MO–injected embryos, despite having disappeared from uninjected

embryos. In contrast, TUNEL labeling is absent within the lens epithelium, and these cells express the proliferation marker PCNA. Second, we did not observe the appearance of a nuclear-free zone in the core of the lens of *hsp70*-MO–injected embryos within the expected time frame. If the TUNEL-positive nuclei we observed were present within normally differentiating fibers, we would expect the appearance of a nuclear-free zone comparable with that which appears in the lens of uninjected embryos. This suggests that the cells exhibiting TUNEL-positive nuclei are undergoing complete cell death rather than arresting after organelle degradation and are not contributing differentiated fibers to the lens core. Third, the lens structure of microinjected embryos recovers after MO dilution or degradation. This would be expected because the attenuation of the apoptotic-like process could then resume and occur normally in the newly differentiating fibers that are produced from the lens epithelium.

Several studies in recent years demonstrate that the chaperoning activity of some constitutively expressed Hsps is required at specific periods during normal embryonic development. For example, Hsp47 is a collagen chaperone essential for early mammalian embryogenesis (Nagai et al 2000), and members of the Hsp90 family are required for placental labyrinth development in mouse (Voss et al 2000) and somitic muscle formation in zebrafish (Lele et al 1999). On a broader scale, Hsp90 serves as a capacitor that allows for rapid morphological change during evolution (Rutherford and Lindquist 1998; Ruden et al 2003). However, in all studies that demonstrate an embryonic role for chaperones, the Hsp being examined is expressed constitutively in a number of other embryonic and adult cell types and has numerous chaperoning roles during normal cell growth and development. In contrast, zebrafish *hsp70* gene expression occurs in a wide range of tissues after heat shock or other stresses (Blechinger et al 2002a, 2002b), but expression has been detected in only a single cell type (lens fibers) during a distinct window of time in embryos under nonstress conditions. Further, we have shown here that a specific requirement exists for this normally stress-inducible chaperone in the proper formation of lens fibers. Our data also raise interesting questions regarding how a Hsp that normally provides protection to all cells from the effects of environmental stress is recruited during the evolution of specific developmental pathways.

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