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An artificial promoter construct for heat-inducible misexpression during fish embryogenesis

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Abstract

Beside spatial distribution, timing of gene expression is a key parameter controlling gene function during embryonic development. Gain-of-function experiments can therefore have quite opposing results, depending on the time of gene activation. Induction techniques are necessary to control timing in these experiments from outside of the organism. Natural heat shock promoters constitute a simple inducible misexpression system, the main disadvantage is a high background level of expression. We present here a new heat stress-inducible bidirectional promoter consisting of multimerized heat shock elements (HSE). The simplified architecture of this promoter largely improves the properties needed for an efficient induction system: dramatically reduced background activity, improved inducibility, and loss of all tissue specific components. Based on this new artificial promoter, we present a transient induction system for fish embryos. Application of this new induction system for Fgf8 misexpression during embryonic development reveals different windows of competence during eye development. A dramatic early phenotype resulting in loss of the eyes is observed for conventional mRNA injection. Later activation, by using our inducible promoter, uncovers different eye phenotypes like cyclopic eyes. Even after 14 days, an efficient heat stress response could be evoked in the injected embryos. The HSE promoter therefore represents a new artificial heat shock promoter with superior properties, making possible transient experiments with inducible misexpression at various stages of development.

Keywords: Heat shock promoter; Inducible misexpression; Medaka; Injection; Meganuclease; Fgf8; Cyclopic eye; Otic vesicle

Introduction

Complicated gene regulatory networks are active during embryonic development. The resulting timing of gene activity critically determines gene function. This timing determines both the presence of an inducing signal as well as the competence of a tissue to respond to the signal. For example, signal transduction pathways involving Fgf and Wnt family members are known to have numerous functions during embryonic development. Misexpression experiments interfering with these pathways can therefore have quite opposing results depending on the time window of activity. Of major importance for these gain-of-function experiments are therefore effective induction systems that can be controlled from outside of the embryo.

Inducible misexpression systems consist of two components: an inducible transcription factor and a promoter responsive for this transcription factor. In the cases of hormone-inducible systems (Braselmann et al., 1993; No et al., 1996), the tet system (Gossen and Bujard, 1992), lac promoters (Cronin et al., 2001), and the rapalog system (Rivera et al., 1996), one component is affected by an externally added drug and has to be expressed constitutively, whereas the second one containing the inducible promoter together with the gene of interest has to be transcriptionally inactive in the uninduced state. These opposed levels of transcriptional activity for the two components normally prevent a combination within a single DNA construct and require separate integration into the genome. Successful application in vivo therefore normally depends on two transgenic lines that have to be crossed. On the contrary, heat shock protein (HSP) promoters are in-

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duced by endogenous factors, thereby reducing the system to a single ectopic DNA construct. Thus, HSP promoters provide a simple one-component system for inducible misexpression. In mammals, strict control of the body temperature makes the in vivo application of this system difficult, but other systems like insects (D'Avino and Thummel, 1999), frogs (Wheeler et al., 2000), and fish (Halloran et al., 2000) are ideal for the induction of a heat shock response at elevated temperatures.

Heat shock activation is a highly conserved response to cellular stress. Heat shock proteins, which function as chaperonins, help the cell to survive the stress situation (Beckmann et al., 1992). The activation of this response is regulated at the transcriptional level (Morimoto et al., 1992) and heat shock elements (HSE), short sequences present in all HSP promoters have been identified to be essential for stress inducibility (Bienz and Pelham, 1987). HSEs contain multiple copies of the five base pairs sequence NGAAN (Amin et al., 1988), detailed mutational analysis identified AGAAC as the optimal sequence (Cunniff and Morgan, 1993). The number of pentameric units in an HSE can vary, but a minimum of three is required for efficient heat-inducible expression (Fernandes et al., 1994). Positioned upstream of a heterologous promoter, HSEs can confer heat stress inducibility to heterologous promoters (Bienz and Pelham, 1986). Heat shock factor 1 (HSF1) has been identified as the cellular component binding to these sequence elements (Morimoto, 1993). Under normal growth conditions, HSF1 exists as a monomer, in which DNA-binding and transcriptional activities are repressed. In response to heat shock and other chemical, environmental, or physiological stresses, HSF1 undergoes trimerization, binds to the HSE, and exhibits transcriptional activity (Wu, 1995). Several studies have shown that the temperature at which HSF1 is activated is not fixed (Abravaya et al., 1991; Clos et al., 1993). Since HSFs are highly conserved, this implies that additional factors play important roles in regulating the activity of this protein. The binding of HSF1 to HSE is highly cooperative, deviations from the NGAAN consensus sequence are tolerated in vivo because multiple HSEs foster cooperative interactions between multiple HSF trimers (Cunniff and Morgan, 1993; Santoro et al., 1998). Sequence variations of the binding site affect the affinity of HSF1 for the HSE of a particular target gene, thereby fine-tuning the heat shock response. Thus, direct comparison between a natural HSE from the human HSP70.1 promoter and an idealized sequence revealed a 57-fold difference in binding affinity for HSF1 (Cunniff and Morgan, 1993).

Heat shock promoters have extensively been used in different experimental systems. The highly conserved nature of the heat stress response allows the use of heterologous promoters. Thus, *Xenopus* and mouse HSP70 promoters were first tested in the fish system (Adam et al., 2000), later followed by experiments with fish promoters (Halloran et al., 2000; Molina et al., 2000; Scheer et al., 2002). The main problems observed in these experiments were high levels of background activity. On contrary to *Drosophila*, in vertebrates HSP70 promoters are highly expressed during certain stages of development, explaining the high basal level in these experiments (Bevilacqua et al., 1997; Bienz, 1986). Generation of transgenic lines can alleviate this problem, but transient injection experiments are severely hampered by the leakiness of the promoter.

Transient injection experiments constitute a fast gain-of-function method for fish and frog embryos. In fish embryos, mRNA injection at the one-cell stage leads to uniform misexpression in the embryo, whereas injected DNA is subject to distribution phenomena, resulting in mosaic expression. Different modifications have therefore been tested to improve DNA distribution (Fu et al., 1997). The recently introduced meganuclease method results in elevated integration efficiency of the DNA into the genome (Thermes et al., 2002). As a consequence, the integrated DNA is stably transmitted among somatic cells, thereby largely increasing the level of misexpressing cells. Moreover, the number of transgenic offspring is drastically increased.

We present here the generation of a new artificial heat shock promoter consisting of multimerized HSEs. This construct shows improved inducibility and eliminates disturbing background activity. The improved properties of this promoter construct, combined with the meganuclease method, can be used for an efficient inducible misexpression system in transient injection experiments.

Materials and methods

Fish strains and maintenance

Medaka embryos and adults of the Cab inbred strain were used for all experiments (Loosli et al., 2000). Adult fish were kept under a reproduction regime (14 h light–10 h dark) at 26 °C. Embryos were collected daily immediately after spawning. Embryonic stages were determined according to Iwamatsu (1994).

DNA constructs

Multimerized HSEs with the idealized sequence AGAACGTTCTAGAAC (Cunniff and Morgan, 1993), alternatingly separated by 3 and 6 bp, were generated by oligonucleotide ligation. A fragment containing eight HSEs was inserted upstream of a CMV minimal promoter, driving the firefly luciferase gene flanked by 5′ and 3′ globin UTRs and the SV40 polyadenylation (pA) signal. In the opposite orientation, a similar cassette containing gfp instead of the luciferase gene, but the same minimal promoter, UTRs, and the pA signal was inserted, resulting in the gfp:HSE:luc construct. The gfp:HSE:Fgf8 construct was obtained by replacing the luciferase gene with the zebrafish Fgf8 cDNA. This cDNA with the same flanking sequences and pA signal

was used to generate the CMV:Fgf8 construct using the complete CMV promoter/enhancer region of the pCS2 vector. A 1.5-kb fragment of the zebrafish HSP70 promoter (Halloran et al., 2000) was used for the natural heat shock promoter constructs (two versions with luciferase and gfp, respectively).

Microinjection of RNA and DNA into Medaka embryos

Fertilized medaka eggs were microinjected through the chorion into the cytoplasm at the one-cell stage. After injection, the embryos were incubated at 28°C. mRNA was in vitro transcribed using the T7 message machine kit (Ambion) and injected in 1× Yamamoto buffer. DNA was prepared with a Jetstar midiprep kit (Genomed) and also injected in 1× Yamamoto buffer. For the meganuclease system according to Thermes et al. (2002), DNA was co-injected with *I-SceI* meganuclease enzyme (0.5 unit/μl) in 1× *I-SceI* buffer (New England BioLabs). For all experiments, a pressure injector (FemtoJet, Eppendorf) was used with borosilicate glass capillaries (GC100-10; Clark Electromedical Instr.) pulled on a Sutter Instruments P-97. Capillaries were backfilled with the injection solution.

Transgenic lines

To make transgenic lines, the gfp:HSE:luc construct was injected at 10 ng/μl together with *I-SceI* meganuclease (0.5 units/μl) into embryos at the one-cell stage as described by Thermes et al. (2002). For screening, 60 larvae of 14 days were heat treated at 39°C for 1 h and observed under the fluorescent microscope after 1 day. Seventeen larvae were gfp-positive and the eight with the strongest expression were selected. After 8 weeks, the mature fish were crossed with wild-type fish and their F1 progeny was assayed for transgene expression after heat shock. Four of the eight selected fish produced progeny that exhibited gfp fluorescence following heat induction. The average germline transmission rate was different between each founder (10–27%). The founder with the highest germline transmission rate (27%) was selected for analysis of the F1 offspring.

Cell culture

Human HeLa and mouse Cop8 cells were kept under standard cell culture conditions with DMEM medium supplemented with 10% FCS. Cells (1×10^5) were transfected in a 24-well plate with 400 ng DNA (if necessary filled up with pBS plasmid) and 0.5 μl Transfast (Promega) in 200 μl medium without FCS. As an internal reference, 5 ng of a Renilla luciferase expression vector (SV40:Rluc) was co-transfected. After 6 h, the medium was replaced by fresh DMEM + FCS. Heat treatment was applied after 24 h by transferring the plates in a different incubator (without CO_2). The cells were lysed 24 h after the heat shock and luciferase activity measured with the Dual Luciferase Kit (Promega). For normalization, firefly luciferase activity values were subsequently divided by Renilla luciferase values.

Heat-shock treatment and luciferase activity measurement of Medaka embryos

For heat treatment, 10–20 embryos or five larvae were incubated in 0.5 ml of Embryo Rearing Medium (ERM) in a 1.5-ml tube at elevated temperature in a heating block. After this treatment, the embryos were transferred into petri dishes and kept at 28°C. For luciferase activity measurements (usually 24 h after the heat shock), the embryos were transferred individually into 1.5-ml tubes, homogenized with a pestle in 100 μl of lysis buffer, incubated for 15 min on a shaker at RT, and then centrifuged for 5 min at 14,000 rpm (RT). Luciferase activity was determined from the supernatant with the Dual Luciferase Kit (Promega).

Results

Heat stress inducible activation of multimerized heat shock elements (HSE)

The HSE has been identified to provide heat stress inducibility to HSP promoters. Conversely, DNA elements like CCAAT and SP1 boxes are typically found in regulatory regions of house keeping genes, where they confer noninducible ubiquitous expression. The presence of these elements in HSP promoters is therefore thought to be responsible for the basal expression of these genes (Bienz, 1986). We reasoned that an artificial promoter combining HSE sequences with a minimal promoter should retain the inducibility, but at the same time reduce the background activity. An idealized HSE sequence (Cunniff and Morgan, 1993) was multimerized eight times, and since these elements are not orientation dependent, we symmetrically added minimal promoters to both ends. Gfp was selected as an expression marker for living cells on one side and luciferase for sensitive quantification on the other side (Fig. 1E). The resulting construct gfp:HSE:luc should therefore express both marker genes from a bidirectional promoter. For a first test, we injected this construct into medaka embryos. Heat shock at 37°C for 2 h resulted in few gfp-positive cells after 24 h (data not shown), therefore we tested higher temperatures. Indeed, after treatment at 39°C for 2 h, substantial gfp expression could be observed in the embryos (Fig. 1B), whereas a control group did not show ectopic gene activation (Fig. 1A). Luciferase activity measurements for these embryos furthermore demonstrated bidirectional promoter activity of the construct (data not shown). Similarly, an experiment in mouse Cop8 cells

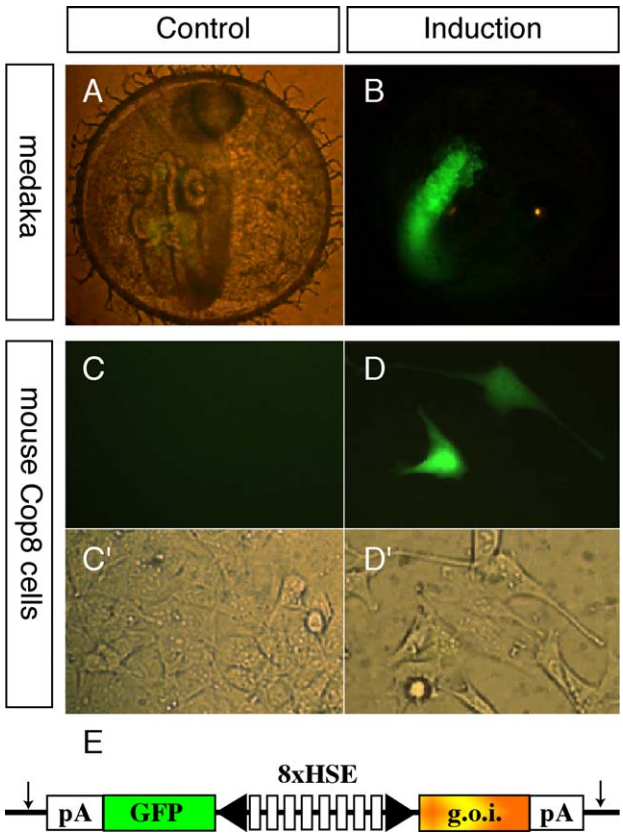

Fig. 1. Activation of the HSE promoter in medaka embryos and in cell culture. The gfp:HSE:luc DNA construct was co-injected with meganuclease into one-cell stage medaka embryos (A and B) or transfected in mouse Cop8 cells (C, C′, D, and D′). The injected embryos were divided into a control group and a test group. Embryos of the untreated control group (A) were gfp-negative. Embryos of the test group were treated at 39°C for 2 h, resulting in strong gfp expression (B). Typical embryos (stage 24) are shown for both groups. After transfection into mouse Cop8 cells, control plates remained gfp-negative (C). Treatment at 44°C for 2 h induced a strong gfp response in the transfected cells (D). C′ and D′ are the corresponding brightfield views for C and D, respectively. A, B, C, and D are fluorescent images, background light was added for image A to visualize the otherwise gfp-negative embryo. A schematic presentation of the HSE promoter is depicted in E. The artificial promoter contains eight multimerized heat shock elements flanked by two minimal promoters in opposed orientation (black arrowhead). Gfp on one side and the gene of interest (luciferase or Fgf8) at the other side are expressed from the bicistronic promoter. The vector is flanked by *I-SceI* meganuclease sites (arrows). Abbreviations: od, oil droplet; pA, SV40 polyadenylation signal; HSE, heat shock element; g.o.i., gene of interest.

confirmed heat stress inducibility of our construct in a different system (Figs. 1C and 1D).

Generation of a HSE transgenic medaka line

To thoroughly analyze the properties of our HSE promoter in vivo, we stably integrated the gfp:HSE:luc construct into the medaka genome. All transgenic embryos of four independent transgenic medaka lines were completely devoid of basal gfp expression at all stages of development (data not shown), but developed strong gfp fluorescence in the whole embryo after heat shock treatment (Figs. 2A–2D). Quantitation revealed similar expression levels and induction rates for all four lines, thereby excluding position effects of transgene integration. All transgenic embryos developed normally. We selected one transgenic line for further experiments. gfp was first detectable under the fluorescent microscope 2.5 h after treatment of the embryos at 39°C (Fig. 2A). The signal intensity increased up to 24 h and due to the stability of the protein persisted for several days (Figs. 2B–2D). Induced expression was seen in all embryonic tissues, including the lens (Fig. 2F), whereas lenses of uninduced embryos lacked any gfp activity (Fig. 2E). Basal gfp expression in the lens is typically observed for HSP70:gfp transgenic zebrafish in the uninduced state and can be explained by a combined effect of high promoter activity and low protein turnover in this tissue (Blechinger et al., 2002; Halloran et al., 2000). Indeed, injection of a zebrafish HSP70:gfp construct (see below) confirmed the preferential activation of the uninduced promoter in the medaka lens (Fig. 2G). Therefore, the HSE promoter can be efficiently induced in all embryonic tissues, without showing any background activity.

Properties of the HSE promoter and comparison with the zebrafish HSP70 promoter

Making use of the high reproducibility of the transgenic line, various conditions for activation of the HSE promoter were tested in a quantitative manner. For this purpose, we used the luciferase gene of the bicistronic promoter construct. Transgenic embryos were collected and incubated at 28°C. Twenty-four hours past fertilization, when the embryos finished gastrulation (stage 19), heat treatment was initiated. Twenty-four hours later, the embryos were lysed and luciferase activity was measured. Even for this highly sensitive marker, activity measurements of uninduced control embryos were close to the detection limit, confirming the low background activity of the HSE promoter. In a first series of experiments, the temperature of heat treatment was varied. Luciferase activity measurements revealed a 9.3-fold increase in promoter activity after treatment at 37°C for 2 h compared to untreated control embryos kept at 28°C (Fig. 3A). The strongest response (up to 680-fold induction) was obtained at 39°C. Decreasing the time between heat shock treatment and lysis of the embryos from 24 to 5 h resulted in a concomitant 5.5-fold reduction of luciferase activity (at 39°C, Fig. 3A). No further increase in induction rates was observed for 41°C, whereas 42°C treatment resulted in extensive death of embryos (data not shown). The same survival rates were obtained for wild-type control embryos, indicating that 2 h at 41°C is the limit for heat treatment of medaka embryos. Using the optimal temperature of 39°C, we then varied the duration of the heat shock treatment. A gradual increase of the induction rate was observed starting from 15 min (13.5-fold) up to 2 h of treatment (680-fold, Fig. 3B).

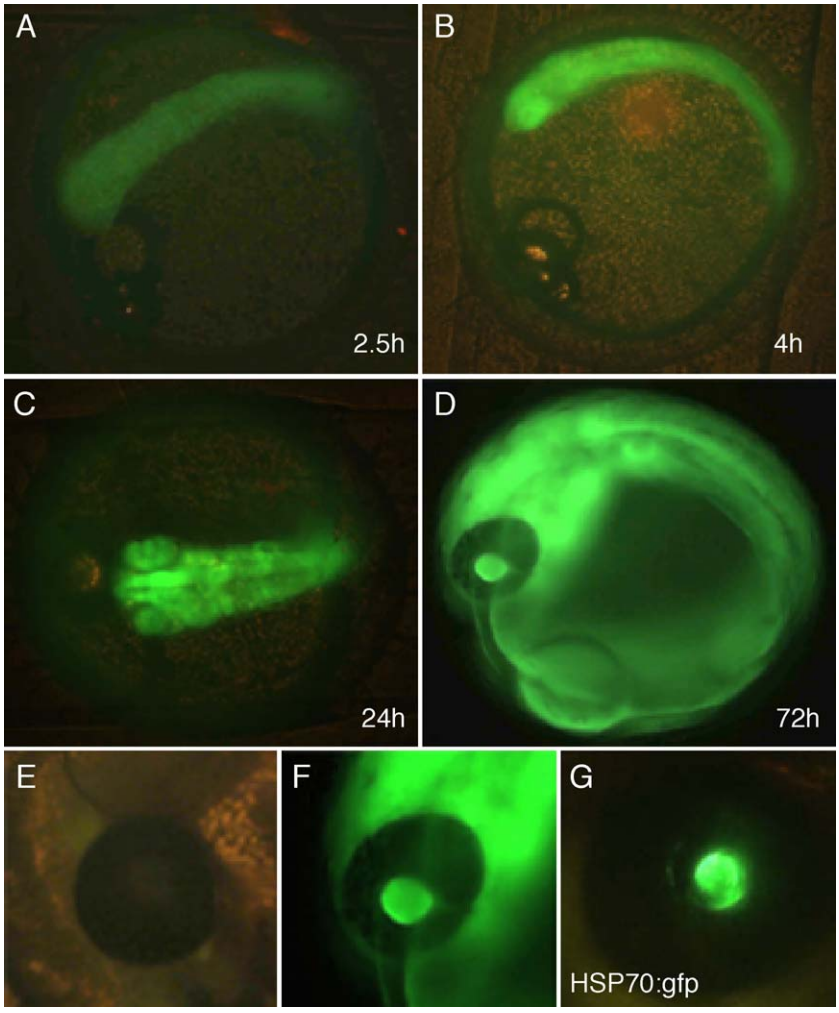

Fig. 2. Stable integration of a HSE construct into the medaka genome. A–D show a time course of gfp expression in a gfp:HSE:luc transgenic line. After heat treatment at 39°C (2 h), gfp expression was first detected after 2.5 h (A). Increasing signal intensity was seen after 4 h (B) and 24 h (C). Gfp activity remained for several days (D, 72 h). Note the reduction of gfp signal in the eye, but not the lens, due to pigmentation. Lenses of uninduced transgenic embryos were background-free also in the lens (E), heat treatment induced a strong response in all tissues, including the lens (F). Wild-type embryos injected with HSP70:gfp developed strong gfp activity in the lens without induction (G).

Therefore, the HSE promoter is highly inducible when stably integrated into the medaka genome, with an optimal activation temperature at 39°C. We next wanted to know whether these results for the transgenic line are likewise valid for DNA injection experiments. Luciferase activity measurements of embryos injected at the one-cell stage with gfp:HSE:luc DNA revealed an average 250-fold induction upon a 2-h 39°C treatment. Taking into account the variabilities of injection experiments, this result is in good agreement with data for the transgenic line (Fig. 3A).

In mammalian cells that show optimized growth rates at 37°C, induction of the heat shock response has been described for 42°C (Abravaya et al., 1991), but elevated activities have been observed for higher temperatures (Török et al., 2003). When we applied this temperature for 2 h to mouse Cop8 cells, we observed a 22-fold activation of luciferase activity for cells transiently transfected with the HSE construct (Fig. 4A). This induction rate is weak compared to the values obtained for medaka embryos. We therefore increased the temperature of the heat shock treatment and indeed observed a 134-fold induction at 43°C. At 44°C, the response was even more pronounced (1020-fold induction, Fig. 4A), but in some experiments, depending on the cell line used, partial cell death was observed at this temperature. To some extent, this toxic effect can be attributed to the high level of transgene expression in these cells, since untransfected control cells survived the treatment better. Similar temperature dependence was observed after stable integration of the construct into human U2OS cells (data not shown). Other types of cellular stress like heavy metal ions likewise lead to strong activation of the construct (100 μM Cd++, data not shown). These data demonstrate a high inducibility of the HSE construct also in cell culture cells. We next wanted to know how these data compare to a natural heat shock promoter. For this purpose, we used a construct containing a 1.5-kb

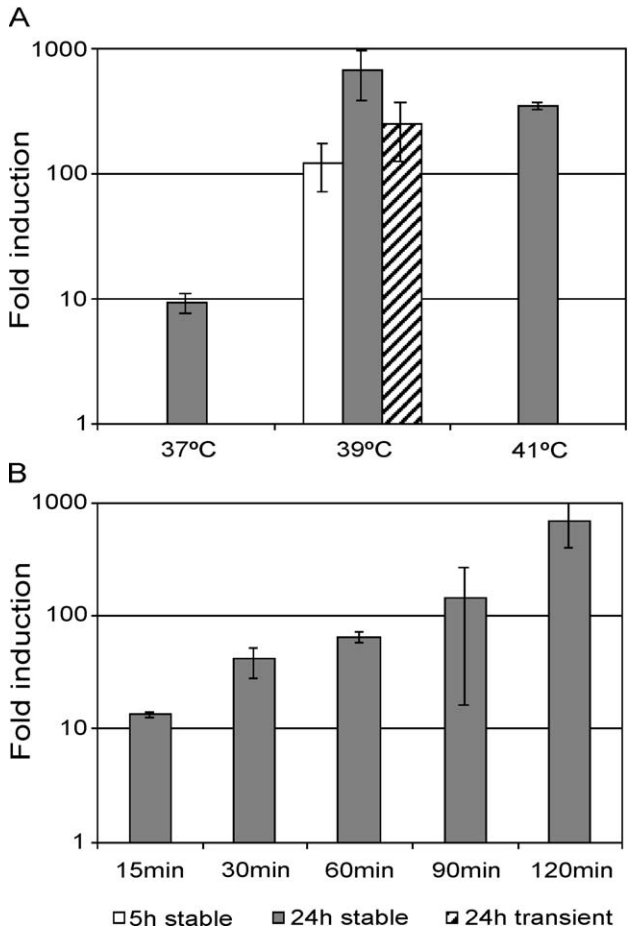

Fig. 3. Quantification of heat shock induction of the HSE promoter in vivo. Luciferase activity measurements were performed with individual transgenic animals and the induction calculated by dividing induced values by basal activity values obtained for uninduced embryos. All embryos were heat treated at stage 19 (two somites). Variation of the heat stress temperature was investigated in A, duration of incubation was 2 h for this experiment. Luciferase activity was measured 5 h (5 h stable) or 24 h after heat treatment (24 h stable). For comparison, a transient injection experiment with the same construct was quantified identically (24 h transient). Note that the induction is displayed in a logarithmic scale. Duration of the heat treatment at 39°C was varied in B, luciferase activity was measured 24 h after induction. For calculation of the values (transgenic embryos), between two and seven independent measurements were taken (five for the uninduced control used as reference) and 17 for the transient medaka experiment (20 for the uninduced control). Error bars shown are ± SD.

fragment of the zebrafish HSP70 promoter (Halloran et al., 2000) driving the luciferase gene. In cell culture experiments, this construct showed high inducibility upon heat treatment. Nevertheless, in all experiments, the absolute numbers of HSP70 promoter induction were clearly below that for the HSE construct under comparable conditions (Fig. 4A). The idealized sequence and the multimerization of the HSE thus increased the inducibility on average 5-fold compared to the natural promoter. Similarly, we observed an improved induction rate for our construct in injection experiments into medaka embryos compared to the HSP70 construct (see below and data not shown).

Beside improving the inducibility, a rationale of our approach was to reduce the background activity of the promoter. To test this, we compared luciferase activity values of the uninduced control cells transfected with both constructs. Due to the complex structure of the HSP70 promoter and known tissue-specific expression characteristics (Basu et al., 2002), we tested different cell lines and in addition quantified medaka injection experiments. In all cases, the HSP70 promoter showed dramatically higher background activity compared to the artificial HSE construct (Fig. 4B). The observed differences were between 13- and 18-fold for cell culture cells (HeLa and Cop8, respectively) and 12-fold for in vivo injection experiments.

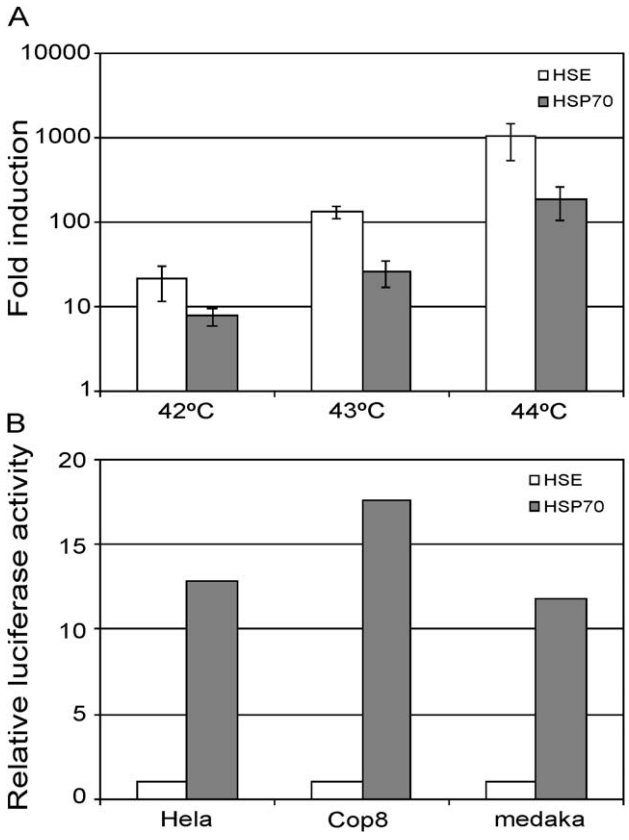

Fig. 4. Quantitative comparison of the HSE promoter with the zebrafish HSP70 promoter. (A) Induction values for the gfp:HSE:luc and the HSP70:luc construct in cell culture after heat treatment at different temperatures. Ten nanograms of the heat shock constructs was transfected together with a Renilla luciferase expression vector as internal reference into mouse Cop8 cells, heat treatment initiated after 24 h, and luciferase activity measured 24 h later. Luciferase activity normalized by Renilla luciferase values was used for the calculation of induction values as described in Fig. 3. Heat treatment was done for 2 h, uninduced control plates were kept at 37°C. For calculation in A, four independent measurements were used (eight for the uninduced control taken as the reference). Error bars shown are ± SD. Basal activity of the two promoters was compared in B, both in cell culture cells (HeLa and Cop8) and in transiently injected medaka embryos. The luciferase activity for the HSE construct was set 1 for each experiment and activity for the HSP70 construct calculated relative to this value. For B, eight independent measurements were made for each value (cell culture) and between 17 (HSE) and 36 (HSP70) for the transient medaka experiment.

Combining the low-background activity with the improved inducibility, the HSE construct shows absolute expression levels comparable with the HSP70 promoter, but with a largely reduced background expression.

A transient misexpression system for medaka embryos based on the HSE promoter

The heat shock promoter has proven to be a valuable tool for inducible misexpression in fish embryos (Halloran et al., 2000). Nevertheless, stable integration into the genome is necessary to overcome the problem of high background activity of this promoter (see below). Compared to simple DNA injection experiments, the generation of transgenic lines is time consuming. Reduced background activity and high inducibility make the HSE promoter an ideal candidate for application in transient experiments. An additional tool that we applied for these experiments was a recently developed method based on the restriction enzyme meganuclease, which leads to more uniform expression after injection of DNA (Thermes et al., 2002).

The DNA construct gfp:HSE:luc was injected into one-cell stage medaka embryos. After 24 h, when they had passed gastrulation (stage 19), the embryos were scored for background expression under the fluorescent microscope. In a typical experiment, 6% of the embryos showed weak gfp activity (Fig. 5). This background activity was restricted to less than 10 cells and depended on the injection conditions. Background values varied between 24% and 0% for the best experiments. Furthermore, the number of gfp-positive cells decreased with time, suggesting that the majority of these cells underwent cell death. The positive embryos were excluded from further analysis and the remaining gfp-negative embryos were divided into two groups. One group served as an uninduced control group, whereas the other group was heat-treated at 39°C for 2 h. None of the embryos of the control group developed any gfp signals during further development. On contrary, 86% of the heat-treated embryos were positive after 24 h (Fig. 5), and more than one-third of these embryos showed strong gfp activity (Figs. 6A–6C). The groups of strong, moderate, and weak gfp activity mainly differed by the number of positive cells, but not the intensity of expression within individual cells. In all cases, misexpression was mainly confined to the embryo (Figs. 6A–6C), whereas strong gfp signals in yolk cells were rarely observed. Taken together, in this typical injection experiment (88 embryos injected), a group of 36 embryos exhibited induced misexpression, out of which 14 showed widespread activation and a control group of 35 uninduced embryos was devoid of any misexpression (Fig. 5).

To directly compare these results, we performed a similar experiment with the zebrafish HSP70 promoter. The same DNA backbone including the gfp gene, flanking UTRs, polyadenylation signal, and meganuclease sites was used for the construct. Upon injection, 64% of the medaka

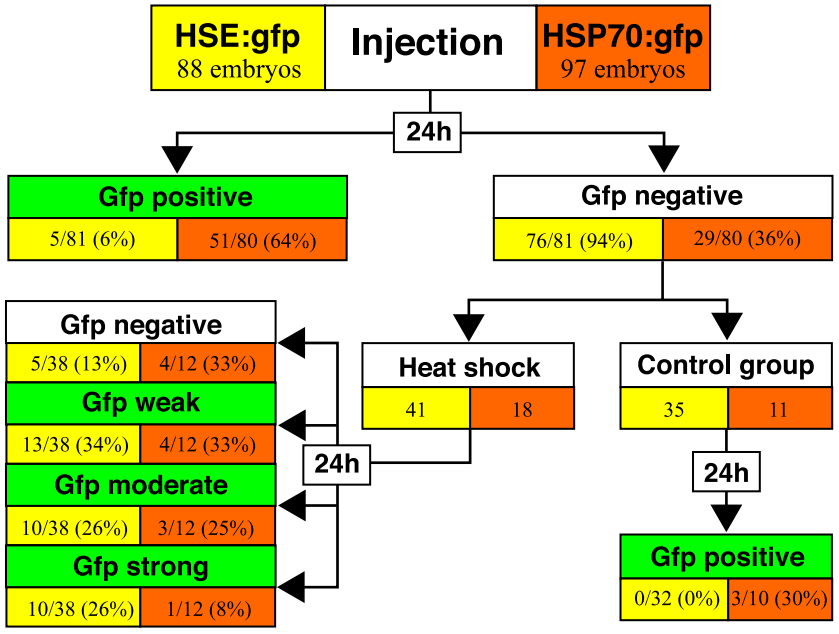

Fig. 5. Comparison between the HSE promoter and the HSP70 promoter in a typical transient experiment. Each vector (HSE:gfp or HSP70:gfp) was co-injected in a concentration of 10 ng/μl with *I-SceI* meganuclease at the one-cell stage (number of injected embryos is indicated). The embryos were examined for gfp expression 24 h after fertilization and gfp-positive embryos separated. Gfp-negative embryos were divided into two groups: a heat shock group and an untreated control group. Embryos in the heat shock group were treated at 39°C for 2 h and then incubated at 28°C, the control group was kept at 28°C. The embryos of both groups were observed for gfp expression after 24 h. The number of embryos for each group is indicated, with the total number of surviving embryos separated by a slash. The percentage was calculated separately 24 h after injection and 24 h after heat shock (dead embryos were not included in the calculation of percentages). The two constructs are differentiated by yellow (HSE) and orange (HSP70) overlay.

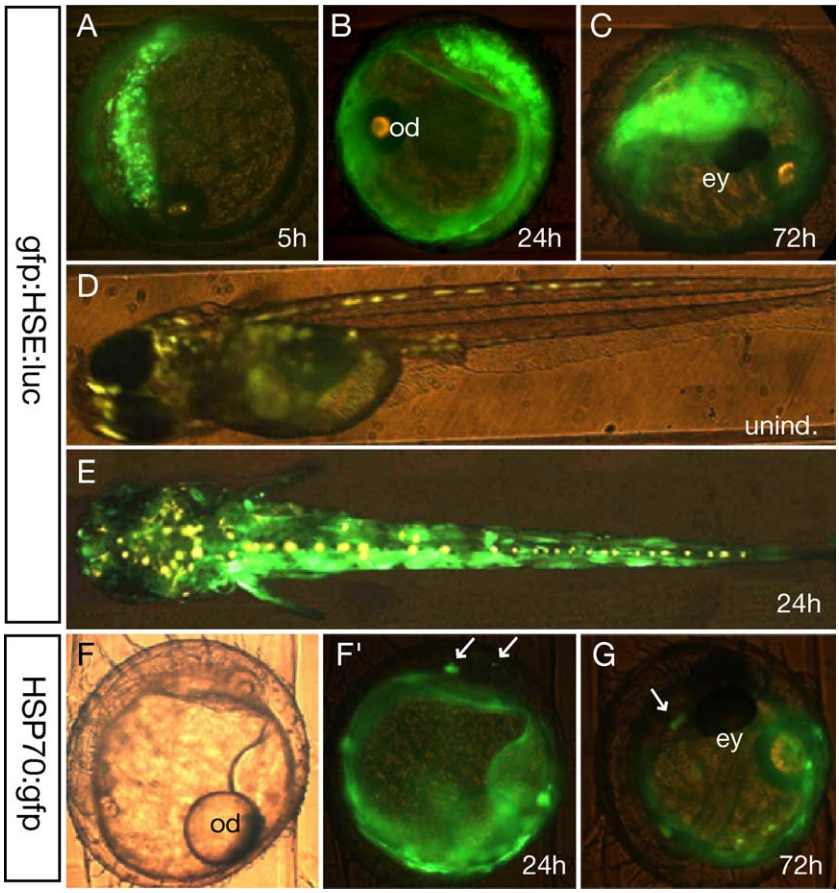

Fig. 6. Transient misexpression with heat stress-inducible constructs. Medaka embryos were co-injected at the one-cell stage with gfp:HSE:luc (10 ng/μl) and meganuclease enzyme. Heat treatment was applied after 24 h at embryonic stage 19 (39°C/2 h). Gfp expression was recorded 5 h (A), 24 h (B), and 72 h (C) after induction. Uninduced embryos were grown until hatching and did not show any gfp expression (D). Yellow staining originates from autofluorescing cells. These larvae were induced (39°C/1 h) and exhibited a strong response after 24 h (E). For comparison, embryos were injected the same way with the HSP70:gfp construct and induced under the same conditions (F, F′, and G). Gfp signals were preferentially observed in yolk cells (F′), gfp-positive cells within the embryo are marked by arrows. (G) The same embryo 48 h later. F is a brightfield view of F′. Abbreviations: od, oil droplet; ey, eye.

embryos showed background gfp activity after 24 h (Fig. 5). In the majority of cases, widespread expression occurred in yolk cells indicating a preference of the HSP70 promoter for this tissue. Excluding the gfp-positive embryos, the remaining embryos were again divided into two groups. Thirty percent of the uninduced control group developed a gfp signal within 24 h, confirming the high background activity of this promoter. In the heat-treated group, 66% of the embryos were positive after 24 h. In all cases, preferential activity was observed for yolk cells, making the detection of positive cells in the embryo difficult (Figs. 6F–6G). In absolute numbers, only 1 out of 97 embryos injected with the HSP70 construct showed strong induced misexpression (Fig. 5). This has to be compared to 14 embryos of this group for the HSE promoter experiment. Therefore, due to the high background activity of this promoter, the evaluation of the uninduced control group is difficult and the number of strongly expressing embryos within one experiment is largely reduced. A transient application of the natural HSP70 promoter is therefore of limited use.

DNA injection typically leads to a mosaic distribution of the expression constructs. The high percentage of embryos with widespread activation of the HSE transgene has to be attributed both to the high inducibility of the promoter and the meganuclease method. Elevated integration rates for the injected DNA constructs into the genome of the early embryo are responsible for the latter effect and greatly diminish the mosaic expression (Thermes et al., 2002). Whereas this results in a gradual shift to more widespread misexpression during early development, a more dramatic difference is observed at later stages. Due to stable integration into the genome of somatic cells, the meganuclease system can lead to continuous misexpression in larvae and adult fish (Thermes et al., 2002). We tested whether the combination of the meganuclease system with the HSE promoter can be used to obtain inducible misexpression at late stages of development. Sixty embryos were injected with the gfp:HSE:luc DNA construct at the one-cell stage and then grown until stage 40 (14 days), where they were all gfp-negative (Fig. 6D). After heat treatment at 39°C for 1 h, 28% of these larvae exhibited moderate or strong gfp

Table 1
Dose dependence of HSE-induced misexpression of Fgf8 in medaka embryos

Concentration gfp:HSE:Fgf8 (ng/μl)	Heat shock 39°C/2 h	Developmental defects[a] (%)	Normal embryos (%)	Dead embryos (%)	Gfp expression, percentage of surviving embryos (%)	Number of embryos
5	+	23	62	14	53	109
	–	0	93	7	0	67
12	+	28	35	36	89	60
	–	2	98	0	2	58
25	+	43	26	30	69	69
	–	20	60	20	5	46

[a] Only strong phenotypes were considered.

expression (Fig. 6E). Therefore, the HSE promoter can be used in combination with the meganuclease system to study late developmental processes by induced misexpression in transient experiments.

Misexpression of Fgf8 with the transient HSE system

For the first application of our inducible misexpression system, we selected the Fgf8 gene. A gfp:HSE:Fgf8 construct, containing the zebrafish Fgf8 cDNA together with the gfp marker gene bidirectionally expressed from the same promoter, was injected into one-cell stage embryos at different concentrations together with meganuclease. At a concentration of 5 ng/μl, 53% of the surviving heat-treated embryos were gfp-positive, whereas no embryo of the uninduced control group showed gfp expression (Table 1). The marker gene expression equaled the developmental defects caused by Fgf8 misexpression. All surviving embryos of the control group appeared normal, whereas 23% of the heat-treated animals developed strong morphological defects (Table 1). Increasing the DNA concentration to 12 and 25 ng/μl directly influenced the frequency of affected (up to 43%) as well as dead embryos (up to 36%), many of which were gfp-positive. Similarly, the elevated amounts of DNA resulted in the appearance of malformed embryos in the control group (up to 20%). Therefore, the extent of developmental effects induced by the HSE system can be influenced by DNA dosage. For highly effective genes like Fgf8, a low concentration is necessary to start induction in embryos where the level of misexpression is below the detection limit, as concluded from the absence of any morphological defects in the control group. On the other hand, higher concentrations can be helpful to detect more dramatic phenotypes due to the high level of misexpression.

The spectrum of observed malformations for Fgf8 misexpression was in good agreement with published roles for Fgf8 in different tissues (Furthauer et al., 1997; Reifers et al., 1998). At a low frequency, we observed the formation of a secondary axis, abnormalities of the pectoral and the tail fin, and problems with the blood circulatory system and the heart. Phenotypes affecting the eyes and the otic vesicles appeared more often and were therefore analyzed in more detail. Inducible misexpression systems offer the advantage to investigate gene function during different time windows, which differ by the responsiveness of individual tissues to various levels of the ectopic gene activity. In the next series of experiments, we systematically varied the time of induction (Table 2). In addition, injection of Fgf8 mRNA and a CMV:Fgf8 construct was included into these experiments. We thus covered ectopic gene activation starting from the one-cell stage (mRNA), mid-blastula stage (CMV:Fgf8), and various time points during and shortly after gastrulation with the HSE induction system. The resulting eye phenotypes are summarized in Table 2.

Table 2
Eye phenotypes observed after Fgf8 misexpression

Construct (ng/μl)	Stage of activation[a]	Developmental defects[b] (%)	Eye defects[c]	Loss of eye	Pigmentation defect in eye	Cyclopic eye	Injected embryos
Fgf8 mRNA (5)	One-cell	15 (37)	4	2	0	0	40
Fgf8 mRNA (25)	One-cell	23 (45)	17	14	0	0	51
CMV:Fgf8 (5)	Mid-blastula (10)	4 (4)	2	1	1	0	91
CMV:Fgf8 (25)	Mid-blastula (10)	29 (39)	20	14	1	0	74
HSE:Fgf8 (25)	Early gastrula (13)	23 (50)	7	0	4	2	46
HSE:Fgf8 (5)	Pre-mid-gastrula (14)	17 (24)	13	6	4	2	70
HSE:Fgf8 (12)	Pre-mid-gastrula (14)	17 (48)	9	0	2	5	35
HSE:Fgf8 (5)	Mid-gastrula (15)	13 (19)	1	n.d.	n.d.	1	67
HSE:Fgf8 (12)	2 somites (19)	3 (12)	1	0	0	0	25

n.d., not determined.
[a] Embryonic stages determined according to Iwamatsu (1994) are written in brackets.
[b] Total number of embryos with visible developmental defects (percent values in brackets).
[c] Total number of embryos with eye defects.

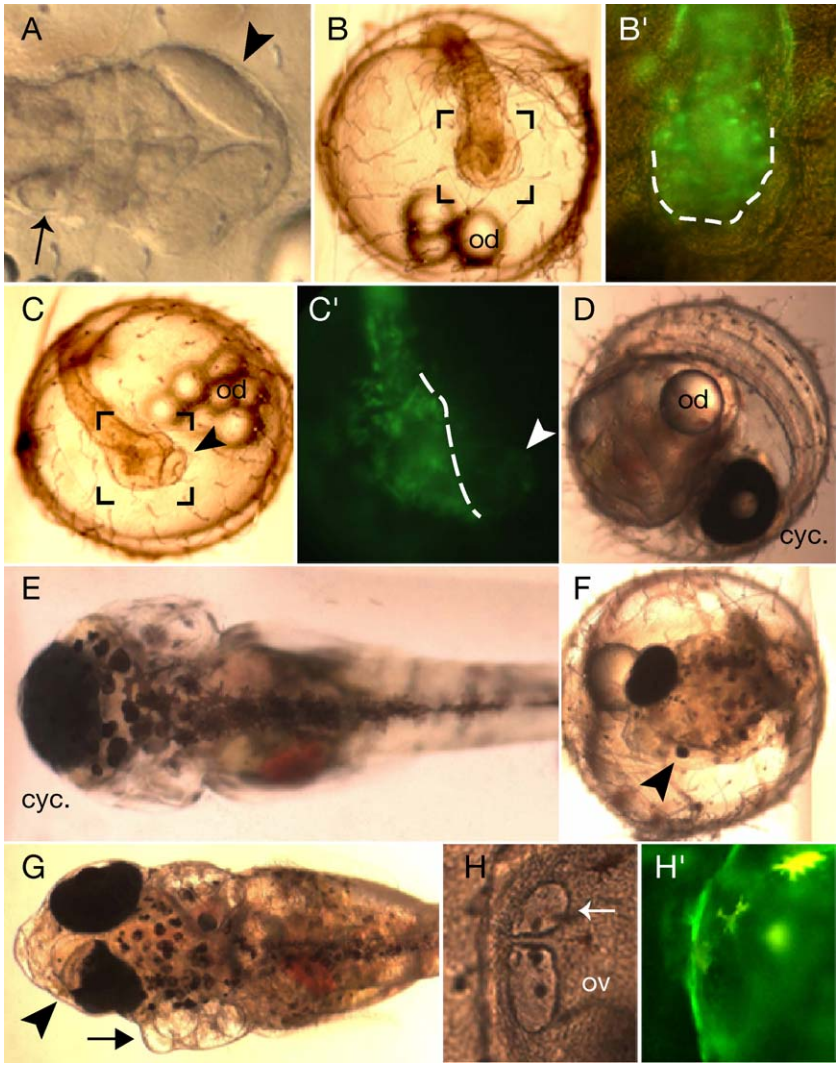

Fig. 7. Phenotypes of medaka embryos misexpressing Fgf8. Injection of Fgf8 mRNA and CMV:Fgf8 results predominantly in a strong eye/forebrain phenotype (A). In most cases, the forebrain is dramatically reduced, but more posterior structures appear normal (otic vesicle marked by an arrow). The midbrain appears dramatically expanded (arrowhead) and structures anterior to the midbrain became lost. B and C show examples of observed eye phenotypes after induction of the gfp:HSE:Fgf8 DNA at stage 14. (B′ and C′) gfp expression in the area encompassing the black square marks in the corresponding images (B and C). Embryos developing cyclopic eyes (B) exhibit misexpression not within the eye, but in the adjacent tissue (B′; dotted line indicates the border between the eye and the forebrain). Loss of one eye (C) correlated with misexpression on the same side of the embryo (C′; the dotted line demarcates gfp-positive and -negative cells), the other eye is marked by arrowheads. D and E show the embryo with the cyclopia phenotype (B) at a later embryonic stage (D) and a larval stage (E). An embryo induced at the two-somite stage (stage 19) exhibited reduced size of one eye marked by an arrowhead (F). A pigmentation phenotype in one eye (arrowhead) and an expanded otic vesicle (arrow) were seen for a larva induced during mid-gastrula (G). Ectopic otic vesicles were repeatedly observed, a magnification of such an embryo is shown in H; the ectopic otic vesicle is marked by an arrow. Note that gfp, marking the Fgf8 misexpressing cells, is not seen within in the vesicle (H′); autofluorescing medaka cells are marked by an arrowhead. Abbreviations: cyc., cyclopic eye; od, oil droplet.

The typical phenotype we observed after injection of Fgf8 mRNA was a complete loss of eyes often accompanied by a dysgenesis of the forebrain (Table 2, Fgf8 mRNA 25 ng). The midbrain was expanded, and in severe cases, structures anterior to the midbrain seemed to be lost completely (Fig. 7A). This dramatic phenotype was seen at a similar frequency upon injection of the Fgf8 DNA expression construct (CMV:Fgf8 25 ng), indicating that this tissue is competent to respond to Fgf8 until the mid-blastula stage, when expression is initiated. On the contrary, activation of the protein at a slightly later stage with the HSE promoter (early gastrulation, Table 2) does not result in this phenotype any more. Whereas various effects on eye size were observed for all stages of activation (Fig. 7F), the complete loss of both eyes was associated mainly with early misexpression. Interestingly different eye phenotypes appeared when Fgf8 expression was induced slightly later. Pigmentation defects in the eye (Fig. 7G) and the formation of cyclopic eyes (Figs. 7B, 7D, and 7E) accumulated for induction times between early and mid-gastrulation (Table 2). Both phenotypes were not observed for mRNA injections. This does not depend on the high dose of protein

obtained after mRNA injection, since a reduction of the amount of injected mRNA leads to the same phenotypes, but at a reduced frequency (Table 2, Fgf8 mRNA 5 ng).

A major advantage of the HSE construct is that misexpressing cells can be traced by their gfp signal. Thus, gfp activity was observed in cells directly adjacent to the cyclopic eyes (Fig. 7B), but interestingly, no gfp signal occurred within these eyes (Fig. 7B′). In other cases, the pattern of gfp appeared more restricted, consequently confining dramatic phenotypes to these parts of the embryo. In Fig. 7C, an example is shown, where misexpression was found in the right half of the embryo resulting in loss of the eye specifically at this side. Our transient approach therefore represents a straightforward approach to follow misexpressing cells or cell clones, and study developmental consequences caused by positional effects.

Based on loss-of-function experiments, Fgf signalling could be associated with multiple steps in ear formation of zebrafish embryos (Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001), which starts during somitogenesis with induction of the otic placode and later the otic vesicle. In the zebrafish, Fgf8 is not expressed in the placode before the 18 somite stage, but experiments based on mRNA injection and Fgf8-bead implantation provided evidence that Fgf8 acts as a placode inducer acting from the hindbrain primordium (Leger and Brand, 2002). However, induction of ectopic otic vesicles through overexpression of Fgf8 alone was not possible with these methods (Leger and Brand, 2002) and also failed in chick embryos (Adamska et al., 2001). Applying the HSE system, we frequently observed both expanded and duplicated otic vesicles after activation of Fgf8 expression in the midgastrula stage (Figs. 7G and 7H). At a much lower frequency, the same phenotype appeared for CMV:Fgf8-injected embryos, but not for mRNA-injected embryos. Consistent with the idea that fgf8 acts as an inducing agent from the distance, gfp-positive cells appeared not within, but adjacent to the otic vesicles (Fig. 7H′). Other phenotypes, like the reduction of the otolith number, were seen preferentially for mRNA-injected embryos.

Fgf8 has multiple roles during various stages of embryonic development. Induced misexpression of Fgf8 with the HSE system is a valuable tool to study these functions. Here, Fgf8 misexpression mainly served to study the basic requirements for a transient inducible system. Many interesting questions concerning Fgf8 gene function might be investigated with this tool in the future.

Discussion

The artificial HSE promoter as an inducible system

Detailed analysis of the properties of HSP promoters revealed the HSEs as the key elements for the heat stress response (Amin et al., 1988). Isolated HSEs have been shown to be able to confer heat stress inducibility to heterologous promoters (Bienz and Pelham, 1986); nevertheless, specific cooperation with other promoter elements in its natural context was assumed to account for full activity in vivo (Bienz, 1986). We extended these experiments by testing whether isolated HSEs are able to provide peak levels of induced expression. The artificial HSE promoter was highly active, both after heat treatment and exposure to heavy metal ions, indicating that the full response to cellular stress can indeed be mediated by HSEs alone.

Three parameters are of major importance for the application of an inducible promoter: (1) low background activity, (2) high inducibility, and (3) lack of tissue-specific expression. In the uninduced state, the activity of the promoter has to be as low as possible to prevent any unspecific effects. Tissue-specific preferences of the promoter can further complicate the situation by increasing the background in certain tissues. Upon induction, the promoter should provide a sufficiently high activity, resulting in ubiquitous expression. Low background activity and high peak levels of expression are quite contradictory properties for a promoter. Quantitation of these two extreme levels and calculation of the inducibility are therefore good measurements for the applicability of the promoter. We tested the HSE promoter in transient experiments and in stable transgenic lines, in cell culture cells, as well as in medaka embryos. Luciferase activity measurements were used to allow sensitive quantitation and gfp expression to follow expression patterns during development. In all these assays, the HSE promoter demonstrated superior properties.

Natural heat shock promoters like the HSP70 promoter have successfully been used for induced misexpression during embryonic development (D'Avino and Thummel, 1999). Leakiness in the uninduced state is the main disadvantage of this promoter and results in high background activity. By reducing the complex structure of heat shock promoters, we were able to dramatically diminish the background expression. A high basal level can be attributed to promoter elements like CCAAT- and SP1 boxes (Bienz, 1986), which are known to provide ubiquitous expression. We therefore expected that the absence of these elements should result in a reduced background level in all cells. Comparison of basal luciferase activity values for both promoters in various cell lines and in vivo, indeed, showed a more than 10-fold average reduction in background activity for the artificial HSE construct.

The direct comparison between the HSE and the HSP70 promoter should provide clear data on the applicability of our construct. Beside a reduced background, the artificial HSE promoter exhibited improved inducibility in all experiments. On average, a 5-fold increase for this important parameter was observed. Up to 1000-fold activation leads to high levels of misexpression even under less favorable conditions including DNA injection, which impose a higher variability to the experiments.

Endogenous heat shock promoters are developmentally regulated (Basu et al., 2002; Bevilacqua et al., 1997). The tissue-specific components of these promoters have not been characterized in detail, but result, for example, in preferential expression of the HSP70 promoter in the yolk (Adam et al., 2000) and the lens (Blechinger et al., 2002). High transcriptional activity in the yolk was in our experiments the predominant problem for a transient application of the HSP70 promoter in medaka embryos. Removal of all noninducible sequences successfully eliminated all tissue-specific components from the HSE promoter, which therefore exhibited equal expression levels throughout the whole embryo. Background expression in the lens could also be eliminated, an important factor for misexpression experiments in the developing eye. These results clearly demonstrate that the elevated background expression in yolk and lens cells does not depend on a high basal level of cellular stress response acting on the HSEs, but depends on other sequence elements in the HSP70 promoter.

Summarizing the improvements we obtained for the artificial HSE promoter compared to the natural version, we could reduce the general background activity, increase the inducibility, and eliminate all tissue specific components.

Optimizing the conditions of heat treatment

As expected, increased temperature and longer exposure to the stress factor result in an elevated response. To obtain high expression levels, excessive cellular stress has to be applied, which can be harmful to the cells and in extreme cases lead to cell death. In particular, mammalian cells seem to tolerate deviations from their optimal growth conditions less well. Activation of the heat stress response was weak (20-fold) at 42°C. At a slightly higher temperature (44°C), this value dramatically increased to 1000-fold activation. This seems to be the limit, since increasing cell death was observed upon extended exposure at this temperature. Fish embryos tolerate different temperatures more easily. Normally kept at 26–28°C, a first heat stress response is seen in medaka embryos at 37°C (10-fold). Again, raising the temperature by only 2°, the induction level jumped to the maximum value of 680-fold. Even upon extended incubation at 41°C, the embryos developed normally and finally started to die at 42°C. Therefore, in vivo application of the heat shock response in medaka embryos is a straightforward approach. Treatment at 39°C leads to optimal induction rates, retaining a reasonable distance to 42°C, where the embryos die.

Comparing these results with the literature, quite similar data have been obtained for zebrafish embryos. For heterologous HSP70 promoters, peak induction values were obtained at 39°C (Adam et al., 2000), and using the endogenous promoter, a temperature of 40°C was found to be optimal (Halloran et al., 2000; Scheer et al., 2002). The HSP70 promoter has also been tried in combination with the Gal4-UAS system (Scheer et al., 2002; C. Grabher and J. Wittbrodt, personal communication). The amplification effect of Gal4-VP16 (Koster and Fraser, 2001) drastically reduces the duration of the heat treatment, allowing short pulses of activation, but does not eliminate the background problem of this promoter, in particular during transient applications (C. Grabher and J. Wittbrodt, personal communication).

A transient misexpression system based on the HSE promoter

Transient misexpression experiments are a fast way to study gene function in vivo. Injection of mRNA, which is translated immediately, often results in dramatic early phenotypes. Application of DNA constructs shifts the initiation of expression to the mid-blastula stage, but to study gene function at later developmental stages, an inducible promoter has to be used, and heat shock promoters represent an attractive single component induction system. Problems with reproducibility of the injection procedure and distribution phenomena affecting the DNA copy number make the transient application of induction systems difficult. Only systems of superior quality can compensate for these problems. When we tested the HSP70 promoter in transient experiments, high background expression was observed. On the contrary, the artificial HSE promoter shows largely reduced background activity together with improved inducibility and can therefore efficiently be used for transient experiments in fish embryos.

In a typical transient experiment, we inject close to 100 embryos. Less than 10 embryos show weak background activation of gfp and are eliminated. The remaining embryos are divided into a control group and a test group. At the required developmental stage, embryos of the test group are heat-treated and obtain high levels of misexpression within a few hours after induction. About 40 misexpressing embryos can be expected. Due to application of the meganuclease method, a high proportion of these embryos shows widespread activation of the transgene. At the same time, the control group can be analyzed. Due to the improved inducibility of the HSE promoter, low amounts of DNA can be injected, which avoids the appearance of any phenotypes before induction. The level of expression can be regulated by the duration of the heat treatment.

A particular advantage of the HSE construct is the coexpression of the gfp marker gene from the same promoter. Most natural promoters have a strong tendency for unidirectional transcription, although some examples for bidirectional expression have been described, among them also HSP promoters (Hansen et al., 2003). In the majority of cases, the transcriptional activity is not evenly distributed, and bicistronic modification of a natural promoter is therefore difficult. Thus, addition of a TATA box to the 5′ end of the actin promoter resulted in uneven expression levels (B.B., N.A. and T.C., unpublished). Bicistronic expression

ideally requires short and symmetric structure of the regulatory sequences, which fits ideally for our artificial construct. Indeed, we observed strong activation of the HSE promoter in both orientations. The level of expression strongly correlated for both orientations, when both gfp signal intensity and luciferase activity were compared (data not shown). Furthermore, double in situ hybridization experiments against gfp and Fgf8 revealed concomitant expression of both genes from the gfp:HSE:Fgf8 construct (data not shown). Co-expression of gfp is an important tool to eliminate embryos with background expression and allows the immediate recognition of promoter activation in the test and the control group. In addition, the exact position of misexpressing cells in the embryo can be determined (see below). Our experimental design therefore provides an efficient tool for gene function analysis in fish embryos.

Misexpression of Fgf8 with the transient HSE misexpression system

Early misexpression of Fgf8 in medaka resulted in a dramatic phenotype. The embryos did not develop eyes and a severe dysgenesis of the forebrain was observed (similar observations were made by M. Carl and J. Wittbrodt, personal communication). Surprisingly, mRNA injection experiments in the zebrafish exhibited a quite different phenotype (Furthauer et al., 1997; Reifers et al., 1998). The embryos showed abnormalities along the dorsoventral axis, and even in the most severe cases, where posterior structures of the embryo became lost, anterior structures like the eyes and the forebrain remained intact. Since we used the zebrafish cDNA for our medaka experiments, Fgf8 protein function cannot account for these differences. Medaka embryos seem to have a divergent competence of the forebrain tissue to react to elevated levels of Fgf8.

Fgf8 is an example of a highly active gene with multiple functions during embryonic development. Early overexpression with mRNA or DNA injection leads to severe phenotypes, which block further analysis of later functions. Application of a transient inducible system solves this problem. Delayed activation of Fgf8 with the HSE system thus prevented the severe early phenotype (complete loss of the eyes) observed after mRNA/DNA injection experiments and allowed the study of late Fgf8 functions, in particular in the eye. We thus observed the formation of cyclopic eyes, which has not yet been described for Fgf8 misexpression experiments. Interestingly, in medaka, a similar phenotype was observed for overexpression of a dominant-negative Fgf receptor, interfering with Fgf signalling (Carl and Wittbrodt, 1999). Therefore, both activating and blocking Fgf8 function lead to the same phenotype. A similar observation was recently made for Fgf8-dependent cell survival in the mouse forebrain by Storm et al. (2003). In these experiments, both reduction of gene dosage and overexpression resulted in the same phenotype (apoptotic cell death).

The otic placode is induced by signals from the neighboring hindbrain during early somitogenesis. Fgf signalling molecules have been implicated in this process, and recent studies suggest that Fgf3 and Fgf8 act in a redundant fashion during ear induction. Combined inactivation of the two genes in zebrafish by using the acerebellar (Fgf8) mutant, morpholino knock-down, or by inhibition of Fgf-Signalling with SU5402 treatment completely blocks ear development (Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001). Gain-of-function experiments further strengthened the role of Fgf family members in this inductive event. Ectopic otic vesicle formation was observed in overexpression experiments for Fgf2 and Fgf3 in *Xenopus* (Lombardo and Slack, 1998), for Fgf3 in chick embryos (Vendrell et al., 2000), and Fgf10 in the mouse (Alvarez et al., 2003). Surprisingly, similar attempts for Fgf8 by mRNA injection and Fgf8-bead implantation failed both in zebrafish (Leger and Brand, 2002) and chick embryos (Adamska et al., 2001). Applying the HSE system, we were now able to induce additional otic vesicles in medaka. Fgf8 misexpression was induced in these embryos during mid-gastrulation, which is in good agreement with previous studies, timing the inductive event to this stage (Heller and Brandli, 1999; Pfeffer et al., 1998). In addition to the exact timing, the expression level and the position of the signal can be of critical importance for successful induction. Indeed, tracing of gfp activity as a marker for misexpressing cells confirmed the action of Fgf8 from a distance in our experiments. On contrary to duplication, we frequently observed malformations of the otic vesicle, which appeared most prominently in mRNA experiments. mRNA injection typically leads to uniform misexpression, suggesting that broad overexpression of Fgf8 including the developing ear might result in this phenotype. Implantation of beads better resembles an inductive event from the distance, but it is difficult to test all possible positions and protein levels. Transient DNA injection experiments, on the other hand, provide a large spectrum of variations both in the expression level and the position of misexpressing cells. Having in addition the option to manipulate the timing of activation, the HSE system is ideal for such experiments. Applying this technique, hundreds of embryos each with slightly different parameters for misexpression can be rapidly scanned within a few experiments. Furthermore, the position and intensity of the gfp signal can be traced in vivo. In the case of Fgf8 inducing otic vesicle formation, it might be interesting to analyze in detail position and expression level of misexpressing cells for inductive events.

Summarizing both the data on quantitation of luciferase and gfp activity, together with the results for inducible misexpression of Fgf8 during embryonic development, the HSE promoter perfectly matches the requirements for a transient inducible system: low background, high inducibility, lack of tissue specificity. Such a system is able to

study gene function during later stages of development, in particular when early overexpression results in dramatic phenotypes. Time windows of competence to react to a signal can rapidly be investigated. Applying this promoter in transient injection experiments in combination with the meganuclease system furthermore extends the spectrum of expression patterns from spot-wise misexpression in single cells, preferentially seen for inductive events from a distance, up to widespread overexpression during all stages of development. Expression level and position of misexpressing cells can readily be followed in vivo.

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