Cloning and expression analysis of an inducible *HSP70* gene from tilapia fish

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Received 13 March 2000; received in revised form 18 April 2000

Edited by Ned Mantei

Abstract We isolated and characterized the tilapia (*Oreochromis mossambicus*) *HSP70* gene, highly homologous to other *HSP70* genes. A dramatic increase of tilapia *HSP70* mRNA levels was observed after heat shock of whole animals in all organs tested. Reporter constructs were tested for transient expression in carp cells and in microinjected zebrafish embryos. The entire isolated regulatory region (−851/+157) was able to mediate heat shock inducible expression of the reporter gene, with no preference for a particular tissue. Our studies represent the first transcriptional analysis of a *HSP70* promoter from fish, revealing a powerful tool to direct controlled, tissue-independent gene expression in fish.

Key words: *HSP70*; Tilapia; Heat shock; Expression; Zebrafish

1. Introduction

The first description in *Drosophila* of a subset of cellular proteins which are induced upon heat shock (HSP proteins) [1] has triggered a remarkable amount of research on these proteins in various organisms and on their function in stress tolerance. This heat shock response is found universally from bacteria to human and the HSP genes are among the most conserved genes during evolution.

The most important and most studied heat shock proteins form the *HSP70* gene family. They play essential roles in protein metabolism under normal and stress conditions, including de novo protein folding, membrane translocation, degradation of misfolded proteins and other regulatory processes. Their expression is regulated by environmental and physiological stress and non-stressful conditions such as cell growth, development and pathophysiological states [2]. While their binding to an unfolded polypeptide chain results in the stabilization of the unfolded state, their controlled release may allow progression along the folding pathway [3]. Owing to their functions, HSP70s have been included in the large family of chaperones.

The regulation of expression of *HSP70* genes occurs mainly at the transcriptional level. Analysis of the *Drosophila HSP70* gene and comparison of different heat shock regulatory regions led to the identification of a palindromic 'heat shock element' (HSE), CnnGAAnnTTCnnG [4–6]. More recent results suggested the view that HSEs are composed of contiguous arrays of a variable number of the highly conserved sequence nGAAn arranged in alternating orientation [7–9]. These elements bind the *trans*-acting heat shock factors (HSF) which are normally present in the cytoplasm in a monomeric form (with no DNA-binding activity) and which, upon heat stress, form trimers and migrate to the nucleus to bind HSEs with high affinity [10–15].

In fish, several cDNAs encoding HSP70 from rainbow trout [16], medaka [17] or zebrafish [18,19] have been described and a heat-shock-induced increase of mRNA levels was shown. Genomic sequences of a *HSP70* gene family have been reported in the teleost *Fugu rubripes* [20]. However, no information concerning the sequences involved in transcriptional regulation of fish *HSP70* genes was available until now.

Here, we report the isolation and characterization of the tilapia *(Oreochromis mossambicus) HSP70* gene including about 1 kb of regulatory sequences. We show that the tilapia *HSP70* promoter is able to confer heat shock regulation to a reporter gene both in fish cells and in microinjected zebrafish embryos. This promoter thus represents a powerful tool to drive ubiquitous and controlled expression of a gene of interest in fish.

2. Materials and methods

2.1. Oligonucleotides

Synthetic oligonucleotides were obtained from Eurogentec (Seraing, Belgium): library screening PCR probe: forward (H70pf1) 5′-TATGTGGCYTTCACHGAYAC-3′ and reverse (H70pr1) 5′-TGAGDCKYTTGGCRTCMAAV-3′; semi-quantitative RT-PCR: forward (H70pf2) 5′-TCTGCAGCTAAAGGTGTAGC-3′ and reverse (H70pr2) 5′-TTGAAGGGCCAGTGCTTCATG-3′ forward (L18pf1) 5′-TATGTGGCYTTCACHGAYAC-3′ and reverse (L18pr1) 5′-TTGGTCCTGCTCATGAACAG-3′; transcription start site: (fH70pr3) 5′-CTACACCTTTAGCTGCAGAC-3′; reporter gene construction: *Nco*I forward (pH70f1) 5′-CGCCATGGCTG-TCTTCTAGAAAATTCAG-3′ and *Eco*RI reverse (pH70r1) 5′-CG-GAATTCTTTGACTTCGTTTCAAAAAGAGG-3′.

2.2. Probe isolation

2.2.1. Library screening. Degenerate primers (H70pf1 and H70pr1) were designed by comparing several *HSP70* sequences and chosen in the most conserved region. After PCR amplification the 100 bp PCR product was directly cloned into the pCR™II vector (TA-Cloning kit, Invitrogen) and sequenced.

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PII: S0014-5793(00)01538-6

2.3. Library screen

Using the tilapia *HSP70* 100 bp PCR probe described above, 2×10^6 plaques (recombinant λ-GEM11) of a tilapia genomic library [21] were screened. The probe was labeled by random priming (^{T7}QuickPrime™ kit, Pharmacia Biotech) using [α^{32}P]dCTP (3000 Ci/mmol, Amersham). Hybridization and washing were carried out under stringent conditions. After four additional screening cycles, purified positive clones were amplified [22] and the phage DNA was purified (Lambda midi kit, Qiagen, Hilden, Germany). Subcloning was performed into pBluescript II KS+ (Stratagene). Restriction mapping and Southern blotting were performed using standard procedures [23].

2.4. Cap site determination by primer extension

Total RNA from liver was purified using TRIzol reagent (GibcoBRL) and 1 μg was used to perform primer extension experiments using the GeneAmp, Thermostable *rTth* Reverse Transcriptase RNA PCR kit (Perkin Elmer). After 10 cycles of annealing (48°C, 10 min), extension (70°C, 5 min) and denaturation (95°C, 1 min) using the 5′ fluorescein labeled fH70pr3 primer, the reaction was treated as is described in [24] and loaded on an automated sequencer (A.L.F., Pharmacia). The data were analyzed using the ALF manager 3.02 software.

2.5. Semi-quantitative RT-PCR

From a group of adult mature female tilapia *Oreochromis niloticus* (mean weight: 121 ± 26 g, $n=6$) reared in freshwater at 27°C, half were transferred at 37°C for 1 h, whereas a control group was kept at 27°C. All fish were kept at 27°C for an additional 2 h and tissues were sampled. Fish were anaesthetized; organs were dissected and immediately frozen in liquid nitrogen. Poly-A$^+$ RNA was isolated from these tissues using the QuickPrep™ Micro mRNA Purification kit (Amersham/Pharmacia Biotech). RT-PCR experiments were performed using the Titan™ One Tube RT-PCR kit from Boehringer Mannheim. A 283 bp *HSP70* fragment was amplified using the H70pf2 and H70pr2 primers. As a control, a 192 bp fragment of the constitutively expressed L18 ribosomal protein gene was amplified (GenBank number: AF240375) using the L18 primers L18pf1 and L18pr1. Five μl aliquots of the PCR reaction were collected after 18, 20, 22, 24, 26, 28 and 30 cycles to determine the linear range of the reaction, in two independent experiments. Twenty-six cycles were chosen and the *HSP70* and L18 reactions were analyzed in the same slot on a 2% agarose gel.

2.6. Reporter gene construction

The tiHSP70-0.3LacZ expression vector was constructed as follows: a 360 bp PCR fragment, including the appropriate restriction sites at the 5′-end of the primers pH70f1 and pH70r1, was amplified from the pB3-tihsp subclone, and introduced into the expression vector pGCV-Lac-Z (positive control) [25], from which the SV40 promoter had previously been deleted by double digestion with *Sap*I and *Nco*I followed by religation.

The tiHSP70-1.0LacZ expression vector was obtained by inserting the 700 bp *Xba*I fragment from the pB3-tihsp into the *Xba*I site of the tiHSP70-0.3LacZ construct.

The linear fragments containing the promoter, reporter gene and poly-A signal unit for microinjection into zebrafish embryos were excised with *Nco*I and *Bam*HI and purified using the Easy-Pure™ kit (BI0zym bv, the Netherlands). The negative, promoterless control was obtained by excision with the same enzymes directly from the pGCV-Lac-Z vector.

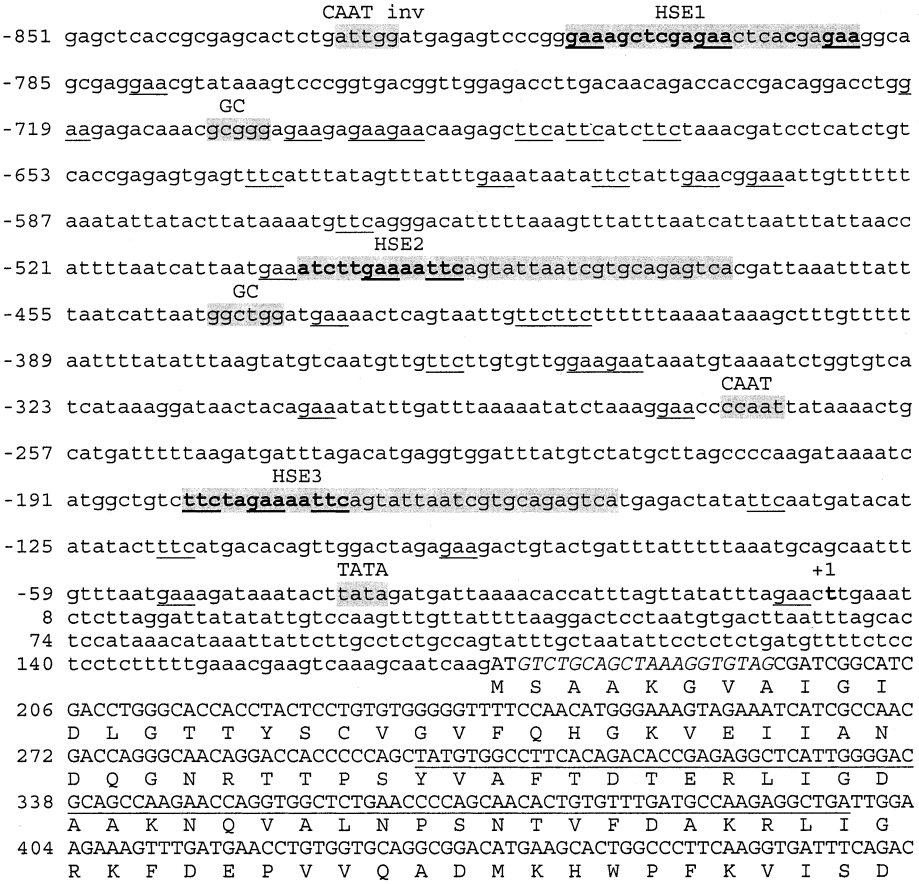

Fig. 1. The nucleotide sequence of the ti*HSP70* gene and the deduced amino acid sequence in the coding region are shown. Upper case letters are used for the coding sequence. The transcription start site is indicated by +1. The putative TATA-box and *cis*-acting elements are boxed and the three putative HSE elements are in bold. Isolated nGAAn or nTTCn sequence units are underlined. The sequence of the PCR probe used to screen the library is underlined (positions +299/+398). The oligonucleotide used to determine the transcription start site is shown in italics. The complete isolated sequence including the coding region and the 3′-end of the gene has been deposited in the EMBL databank (accession number AJ001312).

2.7. Cell culture and transfection experiments

Epithelioma papulosum cyprini (EPC) cells, derived from carp epidermal herpes virus-induced hyperplasia lesions, [26] were grown in BHK-21 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 24°C in 5% $C0_2$. Twenty pmol of purified plasmid DNA was transfected in 800 μl of resuspended cells (1.5×10^6 cells/ml of medium) by electroporation in 4 mm cuvettes using a single pulse (250 V, 1500 μF) delivered by a Easyject apparatus (Eurogentec, Seraing, Belgium). Transfected cells were immediately transferred to four culture dishes (55 mm). After 48 h, the cells were harvested by scraping, washed two times in PBS and resuspended in lysis buffer [27]. Protein concentration and β-galactosidase activity were determined according to [23].

Heat shocks were performed by transferring the plates previously sealed with parafilm in a thermoregulated bath at the appropriate temperature. After the treatment, the cells were returned for 2 h at 24°C and the LacZ activity was determined.

2.8. Microinjection procedure

Fish care and embryo rearing was performed as described by [28]. Eggs were collected and microinjected (300 pl, 5O ng/μl) at the one cell stage, targeting to the cytoplasm/yolk boundary of the zygote. Following microinjection, the embryos were replaced at 28.5°C in small tanks containing 500 ml of Holtfreter's solution to allow correct development. Heat shock treatment was carried out on 1 day old embryos at 40°C for 15 min, followed by a recovery for 2 h at 28.5°C. LacZ expression was determined as previously described [29].

3. Results

3.1. Genomic library screening

A 100 bp probe specific for the tilapia HSP70 coding sequence was obtained by performing a PCR on tilapia DNA using the primers H70pf1 and H70pr1 (Fig. 1). By screening 2.0×10^6 λ-phage plaques from a tilapia genomic library, 104 positive clones were obtained. Eight of them were selected and purified by three additional screenings. Restriction analysis revealed the presence of three different groups of clones. One of them, λ-11tiHSP, containing approximately 15 kb of insert, was chosen for restriction mapping and Southern blot analysis (data not shown). A 3 kb *Sac*I fragment, shown to hybridize with the *HSP70* probe, was subcloned (pB3-tihsp) and subdivided further into three fragments of approximately 1 kb by *Pst*I digestion, which were again subcloned (pB3A-tihsp, pB3Btihsp and pB3C-tihsp) and completely sequenced on both strands. An oligonucleotide hybridizing to the 3′-end of the pB3-tihsp insert was used to sequence the 3′-flanking region using the λ-11tiHSP DNA. The isolated promoter, 5′-untranslated and coding sequence up to +469 is shown in Fig. 1.

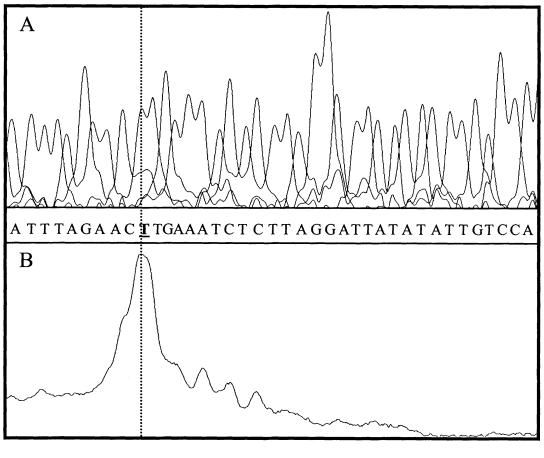

Fig. 2. Transcription start site. Primer extension was performed using adult tilapia liver RNA. Comparison of the primer extension product with the products of a sequencing reaction on the pB3-tihsp is shown.

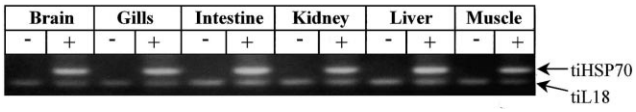

Fig. 3. Expression analysis in different organs. RT-PCR amplification products for a ti*HSP70* fragment (283 bp) and the constitutive control L18 fragment (192 bp) from RNA Poly-A^+ from control (−) and heat shock treated (+) fish are shown. The complete experiment was performed twice with identical results.

3.2. Sequence analysis of the tilapia HSP70 gene

Computer analysis of the isolated sequence reveals a long open reading frame encoding a 640-amino acid protein corresponding to a HSP70 family protein (Fig. 1). Amino acid sequence comparison confirmed the high degree of conserva-

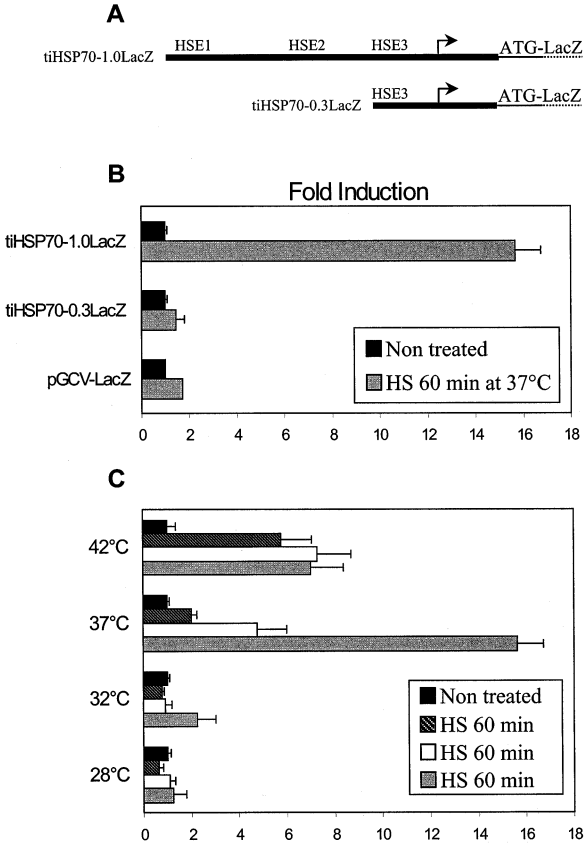

Fig. 4. Transient expression of ti*HSP70* promoter/LacZ in cultured cells. A: Schematic representation of the reporter constructs used. TiHSP70-1.0Lac-Z contains the complete isolated ti*HSP70* control region (−851/+157) fused upstream from the ATG codon of the LacZ gene. TiHSP70-0.3LacZ contains the control region starting just upstream from HSE3 (−182/+157). B: EPC cells transfected with either reporter construct or the pGCV-LacZ (transfection positive control) were subjected or not to a heat shock for 60 min at 37°C. The cells were left 2 h at 24°C for recovery, harvested and lacZ activity was measured. β-Galactosidase activity was normalized to the protein concentration and fold induction relative to the non-treated cells is shown. C: EPC cells transfected with the tiHSP70-1.0LacZ with or without heat shock for 60 min at 28, 32, 37 and 42°C. Triplicate experiments were performed three times, the data are represented as mean ± S.D.

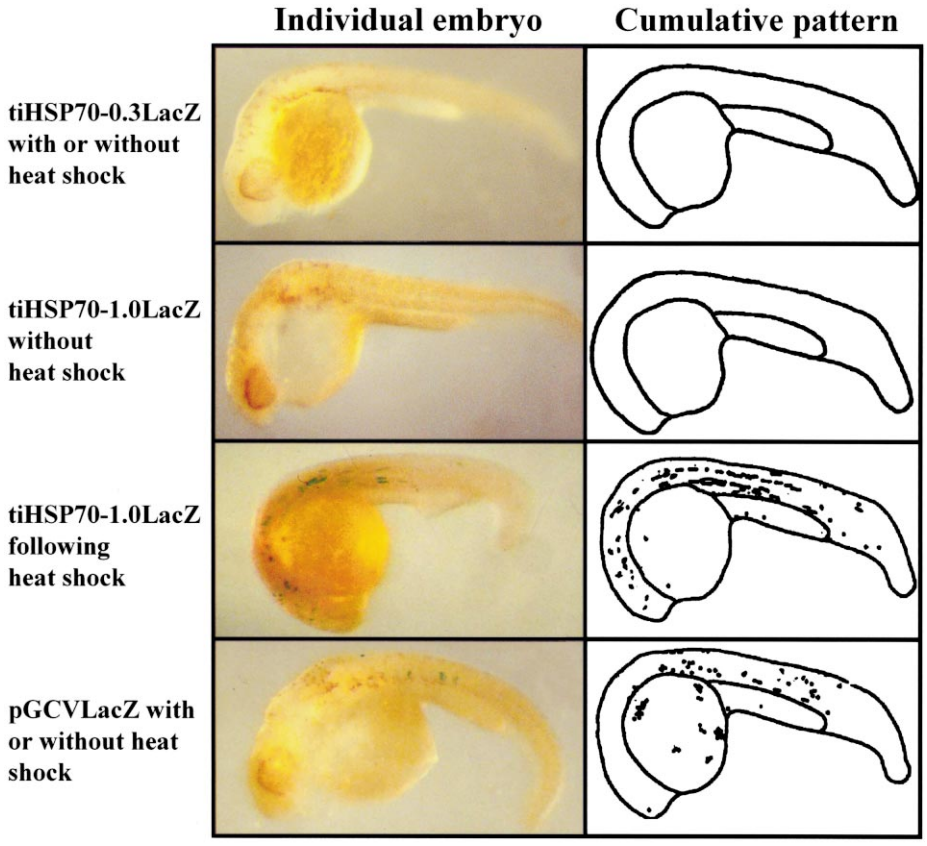

Fig. 5. Transient expression of tilapia *HSP70* promoter-LacZ constructs in 1 day old zebrafish embryos. Zebrafish embryos were microinjected at the one cell stage with the tiHSP70-1.0LacZ; tiHSP70-0.3LacZ or pGCV-LacZ linearized constructs. One day old embryos were subjected to a heat shock at 40°C for 15 min, allowed to recover for 2 h at 28.5°C and stained with X-gal. Untreated controls were stained in parallel. Injection of supercoiled tiHSP70-1.0LacZ led to a stronger expression compared to the linearized fragment, however, survival rates were much lower (data not shown). Cumulative patterns of LacZ expression were obtained by superimposing the expression patterns of all the individual embryos within a same batch.

tion of HSP70 in various species: 83, 84, 87 and 91% identity with human [30], rat [31], *Xenopus laevis* [32] and trout [16] sequences, respectively. The fact that the genomic sequence contains no intron is consistent with what is observed in inducible *HSP70* genes from other species, further supporting the conclusion that the isolated sequence corresponds to an inducible tilapia *HSP70* gene (ti*HSP70*).

The complete coding region of the ti*HSP70* gene and about 1 kb of regulatory sequence, including the 5′UTR region, was isolated. To determine the transcription initiation (CAP) site, a primer extension experiment was performed using a fluorescent oligonucleotide located in the tiHSP70-coding region (see Fig. 1). One clear signal was obtained which, by comparing the migration of the primer extension product with that of the products from a sequencing reaction performed on the pB3-tihsp using the same primer (Fig. 2), allowed to define the start site at 172 bp upstream from the ATG.

Several putative *cis*-acting sequences were found upstream from the CAP site (Fig. 1). In addition to a TATA box at −38, two GC boxes were detected at position −444 and −708, a CAAT box at −272 and an inverted CAAT box at −831 were found. Three elements corresponding to the HSE consensus were found, respectively, at positions −815, −500 and −179. Several additional nGAAn elements were detected in both orientations. Moreover, HSE2 and HSE3 form the 5′-part of a 32 bp direct repeat.

3.3. TiHSP70 gene expression by semi-quantitative RT-PCR

Two adult tilapias were heat shocked at 37°C for 1 h and left to recover for 2 h at normal temperature (27°C). Poly-A$^+$ RNA was extracted from different organs of these fish and of two untreated fish. Ti*HSP70* mRNA levels were assessed in each organ by RT-PCR, using expression of the L18 ribosomal protein as invariant control (Fig. 3). After heat shock, tiHSP70 RNA was readily detected in each organ, while no signal was observed in the control fish, indicating that the ti*HSP70* gene is highly inducible. Induction levels seem to be roughly the same in the different organs tested.

3.4. Transfection experiments

Transient expression studies were performed in carp EPC cells to evaluate the transcriptional regulation of the ti*HSP70* promoter by thermal stress. To that purpose, two constructs containing either the whole available regulatory region (tiHSP70-1.0LacZ) or starting just upstream from HSE3 (tiHSP70-0.3LacZ) were tested (Fig. 4A). Heat shock for 1 h at 37°C resulted in a clear, 16-fold stimulation of β-galactosidase expression only for the tiHSP70-1.0LacZ construct, while the tiHSP70-0.3LacZ or the control pGCV-LacZ constructs were unaffected (Fig. 4B). Different temperature treatments were tested using the inducible tiHSP70-1.0LacZ construct (Fig. 4C). A slight induction was observed after 1 h at 32°C. A heat shock at 37°C resulted in a two-fold stimulation

after 15 min which increased up to 16-fold after 1 h. Higher temperatures (42°C) led to a much faster response (six-fold after 15 min) which did not increase after longer treatment. In this case, detachment of the cells from the plate indicated cellular death.

3.5. Transient expression in zebrafish embryos

The transcriptional activity of the ti*HSP70* promoter was also tested in whole animals by microinjection into zebrafish eggs. When the tiHSP70-0.3LacZ construct was injected, no expression of lacZ was observed even when the embryos were subjected to heat shock (Fig. 5) (a total of 81 individuals were analyzed). In contrast, injection of tiHSP70-1.0LacZ resulted in a strong expression only after heat shock (0/82 positive individuals without heat shock versus 37/83 after heat shock). As expected, no LacZ expression was observed with the promoterless construct (not shown) while the positive control (pGCVLacZ) directed constitutive expression, both with (9/29 positives) or without heat treatment (6/29 positives).

By superimposing the different locations of expression from all the individual embryos within a same batch, a cumulative pattern of expression was obtained. The results (Fig. 5) show that the heat shock-induced expression directed by the ti*HSP70* promoter displays no preference for any particular tissue, the pattern is comparable to that obtained with the SV40 promoter containing pGCVlacZ.

4. Discussion

We report the cloning, sequencing and expression analysis of the ti*HSP70* gene. Furthermore, the first analysis of a fish *HSP70* promoter and its regulation by stress is presented.

Analysis of the deduced amino acid sequence revealed a remarkable homology to *HSP70* genes from other species, as much as 91% similarity with trout *HSP70*. Perfectly conserved regions correspond to the functional domains involved in ATP and peptide binding. In particular, amino acids directly involved in interaction with HSP40 and in ATPase activity [33] are identical to those in other species. Although the C-terminal end appears to be less conserved, the last 8 amino acids, GPTIEEVD, are again identical in all the species. This feature was previously shown to be characteristic of nuclear-cytosolic HSP70s [34].

Another striking feature of the ti*HSP70* gene is the absence of introns. An uninterrupted open reading frame of 1920 bp encoding the entire protein was found. Such a gene structure is characteristic of inducible *HSP* genes, in contrast to the constitutively expressed *HSP* (cognate heat shock *HSC*) genes, and of other stress induced genes such as glutathione *S*-transferase D [35]. Intron splicing is normally required for translocation of most mRNAs from the nucleus to the cytoplasm, however an inhibition of RNA-splicing by stress was shown [36–38]. It was suggested that the absence of introns in stress-induced genes allows to compensate for this inhibition thereby enabling preferential expression of these proteins during cellular stress, the nuclear export signal being probably provided by the mRNA secondary or tertiary structure [39].

Semi-quantitative RT-PCR experiments demonstrate the presence of tiHSP70 mRNA in various tissues only after heat shock. Taken together, these observations strongly suggest that the isolated sequence corresponds to a heat inducible, nuclear-cytosolic ti*HSP70* gene.

Analysis of the 5′ regulatory region showed no evident homologies to other *HSP70* promoters or 5′ leader sequences. However, basal *cis*-acting elements (TATA, CAAT and GCGGG boxes) and putative HSEs are detected, although at different positions and in different arrangements. In the *HSP70* promoters from other species, a CAAT box localized near the TATA box, around −70 [40–42], was shown to be required for full-stress induction [43–45]. The role of the two CAAT boxes in the ti*HSP70* promoter, respectively at −831 and −272, in transcriptional regulation is at present unclear.

The function of the ti*HSP70* promoter was tested in vivo by transient expression in cell culture and microinjected zebrafish embryos. As the 5′ leader sequence was shown to mediate preferential translation of HSP70 mRNAs in several species [46–48], this region was included in our reporter constructs although no obvious sequence conservation was found. Only the longest construct containing the complete isolated regulatory region (tiHSP70-1.0LacZ) was able to mediate high transient expression and only after heat shock both in transfected cells and in developing embryos. These results suggest that the most proximal putative HSE3 is not sufficient to confer heat inducibility, in contrast to what was observed in HSP gene promoters from other species. In addition, as the shorter construct (tiHSP70-0.3LacZ) still contains the 5′ leader sequence, we can rule out that the response observed with tiHSP70-1.0LacZ is due to enhanced translation or stabilization of preexisting mRNAs. Thus, the region between −851 and −182 in the ti*HSP70* promoter mediates transcriptional stimulation upon heat shock; further experiments will show which of the HSE1, HSE2, CAAT or GC-boxes are precisely involved.

Transient expression in EPC cells revealed a time- and 'dose'-dependent expression of the reporter gene, with the highest stimulation at 37°C for 1 h. This response behavior is similar to that observed in other cold-blooded organisms [49,50].

Even if the transient expression of the tiHSP70-1.0Lac-Z transgene after heat shock is mosaic, the cumulative expression patterns of all injected embryos demonstrate no preference for a particular tissue. This observation is in agreement with the expected ubiquitous expression of HSP70 proteins and with our results showing a very homogeneous expression in all the tissues from heat shocked tilapia (Fig. 3). In conclusion, the ti*HSP70* promoter is probably able to confer heat shock response to a reporter gene in the entire animal.

The introduction of novel genes into animals follows two major objectives: (1) gene regulation, expression and developmental studies in vivo and (2) the production of commercially valuable transgenic species benefiting from the acquisition of desirable traits [51]. In this respect, the availability of inducible promoters able to control the expression of a particular gene by a simple stimulus would be very useful. Cellular stress, in particular heat shock, is an easy and non-toxic way to treat an animal. Moreover, the mechanisms mediating stress response are highly conserved. The results reported here demonstrate that the tilapia *HSP70* promoter represents a potentially powerful tool to drive the expression of a transgene of interest in fish under controlled conditions.

Acknowledgements: We are grateful to S.E. Wendelaar Bonga (Nijmegen, The Netherlands) for providing fish material. We would like to thank R. Carpeaux for excellent technical assistance. This work was

supported by the 'Région Wallone' (ULg 1815); the 'Services Fédéraux des Affaires Scientifiques, Techniques et Culturelles' (PAI P4/30 and 'Actions de Recherche Concertées': 95/00-193); the Fonds National de la Recherche Scientifique (FNRS) (−3.4537.93 and −9.4569.95) and the EU (No. BIO4-CT97-0554). M.M. is a 'Chercheur qualifié' at the FNRS. A.M. held fellowships from 'CGRI' and 'ULg patrimoine'.

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