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IDENTIFICATION AND INDUCTION OF *hsp70* GENE BY HEAT SHOCK AND CADMIUM EXPOSURE IN CARP

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A member of the multi-gene family, encoding 70 kD stress proteins, was identified from the common carp (*Cyprinus carpio*). Homologies, observed at both nucleic acid and amino acid levels, and also the intronless structure of this gene, strongly suggest that it corresponds to a heat-inducible *hsp70* gene in carp. Gene-specific primers were selected and used in RT-PCR reactions to measure the basal *hsp70* mRNA levels and to follow the inducer-specific expression of this gene in different tissues during *in vivo* studies. Carp *hsp70* mRNA is not detectable in the brain and muscle, and its concentration is around the limit of detection in the kidney and liver of unstressed animals. The expression of *hsp70* is induced by elevated temperature and it responds to Cd treatment in a tissue and time-dependent manner.

Keywords: Cadmium – fish – heat shock – *hsp70* expression – liver – muscle

INTRODUCTION

A temperature shift and many other types of environmental stress induce the synthesis of a small set of proteins called heat shock or stress proteins (Hsps). The most deeply studied among them comprise the Hsp70 family. They play essential roles in protein metabolism under both normal and stress conditions; their expression is regulated by environmental and physiological stress and non-stressful conditions such as growth and development. Some family members are constitutively expressed (heat shock cognates; Hsc70s) and are crucial for the chaperoning function of unstressed cells, whereas the inducible forms (Hsp70s) are important for allowing cells to cope with acute stressor insults (for reviews, see [7, 9, 22]).

The family of *hsp70* genes have been characterized in a wide spectrum of organisms, ranging from *Escherichia coli* through *Drosophila* to human (for a review, see [9]). The Hsp70s are among the most conserved proteins during evolution, and demonstrate a 60–78% identity among eukaryotic organisms and a 40–60% identity between eukaryotic Hsp70s and *Escherichia coli* DnaK. One factor that contributes

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to the high degree of homology is that all Hsp70 proteins have a sequence of amino acid residues (consensus VDLGGGDFE) in the primary structure, which is repeated several times throughout the N-terminal half of the protein [6, 21]. Major differences in the published Hsp70 sequences are usually found in the variable C-terminal protein segment.

In fish, several cDNAs encoding Hsp70 from rainbow trout [10], medaka [2] and zebrafish [12] have been described, and the heat-shock-induced increase of mRNA levels has been demonstrated. Furthermore, genomic *hsp70* sequences have been reported in *Fugu rubries* [14] and tilapia [18]. Most of the studies followed the *hsp70* expression in different tissue cultures [1, 17], where the regulation of stress genes might differ substantially from that in fully developed animals, or concentrated on the regulatory elements of the promoter region using lacZ reporter constructs [11, 18].

Herein, we report the identification of the *hsp70* gene in carp, by PCR amplification, using primers designed for conserved regions in both the Hsp and Hsc subfamilies. We also addressed the effects of two types of stressors: heat shock and Cd exposure. Besides heat treatment, Cd was chosen because it is a systemic poison and is often found to induce the synthesis of a set of stress proteins; its action is reported to be cell type-specific [3, 4, 13, 19].

MATERIALS AND METHODS

Animals and treatments

Carp (*Cyprinus carpio*) weighing 800–1000 g, obtained from the Tisza Fish Farm, Szeged, were acclimatized in well-aerated 400-l water tanks at 12–13 °C, under fasting conditions over a 3-week period, in January and February. During the acclimatization, the water was changed twice weekly. In heat shock treatments, fish were exposed to 26 °C for up to 3 h. For treatment with Cd, carp were injected intraperitoneally with 10 mg/kg Cd (cadmium acetate dissolved in water, Fluka), and exposed to it for up to 48 h. In all experiments, 3 or 4 animals were sacrificed at each time point for organ harvesting. The gall-bladders were excised to avoid bile contamination of the liver, and tissues were removed, frozen immediately in liquid nitrogen and stored at –80 °C.

Genomic DNA extraction

Genomic DNA was extracted from carp liver by a modified version of the salting-out procedure [16]. Briefly, approximately 1 mg of frozen tissue was incubated in 0.5 ml of lysis buffer (10 mM Tris HCl pH 8.2, 5 mM EDTA, 0.2% SDS, 200 µg/ml protease K) at 55 °C, overnight. After digestion was complete, 150 µl of saturated NaCl (approximately 6 M) was added and the mixture was shaken vigorously, followed by

centrifugation at 3000 rpm for 15 min. A 0.75 volume of isopropanol was added to the supernatant and inversion was performed several times until precipitation was visible. The precipitated DNA was transferred into 75% ethanol, incubated overnight, collected by centrifugation, dried under vacuum, dissolved in H_2O and stored at –80 °C.

RNA extraction and reverse transcription

Approximately 100 mg of frozen tissue was homogenized in RNAzol B reagent (Tel-Test, Inc.) and the total RNA was prepared according to the procedure suggested by the manufacturer. Total RNA was routinely treated with 100 U of RNase-free DNaseI (Boehringer-Mannheim) to avoid any DNA contamination.

To obtain carp *hsp70*-specific cDNAs, an RT-PCR-based strategy was employed. First-strand cDNA was synthesized by using 5 μg of total RNA as template. The RNA was denatured at 90 °C, mixed with 200 pmol of each dNTP (Boehringer-Mannheim), 200 U of M-MuLV reverse transcriptase (Sigma) and 500 pmol of random hexamer primer in a final volume of 20 μl, and incubated for 10 min at 37 °C, followed by 1 h at 42 °C. The reaction was stopped by heating at 65 °C for 5 min and the product was stored at –80 °C until use.

PCR amplificaton and sequencing

50 ng of genomic DNA or 2 μl of RT reaction product was added to 48 μl of a PCR reaction mixture containing 250 μM of each dNTP, 50 pmol of primers, 1× Sigma PCR buffer/$MgCl_2$ and 5 U of Taq polymerase (Sigma). Amplification was performed in a PTC 150-16 MiniCycler (MJ Research), using 5 cycles of 95 °C for 30 sec, 45 °C for 30 sec, and 72 °C for 90 sec, followed by 25 cycles for β-actin and 30 cycles for hsp70 amplification, in which the annealing temperature was raised to 55 °C. The number of amplification cycles, during which the formation of PCR products were limited by template concentration, were determined in pilot experiments.

When carp-specific primers were used (hsp70-7 and -8, and β-actin-3 and -4), the annealing temperature was 60 °C in all cycles. The amplified products were separated on 1–1.8% agarose gels (Sigma) and isolated from them by using Ultrafree-MC Centrifugal Filter Units (Millipore). The purified, PCR-amplified cDNA was inserted into pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α cells. The recombinant clones were screened for, and the sequence of the inserts was determined. The DNA sequence of *hsp70* gene was determined by sequencing the genomic and cDNA PCR products by the dideoxy termination method on a fluorescent DNA sequencer (sequencing facility of the Biological Research Center, Szeged) with either vector-specific primers (T7, Sp6) or gene-specific primers derived from initial sequences.

Northern blot analysis

Total RNA (20 μg) was size-fractionated by electrophoresis through denaturing 2.2 M formaldehyde/1.2% agarose gel, transferred to a nylon membrane (Amersham) by capillary blotting and cross-linked to the membrane by UV irradiation (GS-Gene linker, Bio-Rad). DNA probe (isolated 280 bp PCR fragment amplified by primer pair 7/8) was labeled with α^{32}P dCTP by random priming using a High Prime DNA Labeling Kit (Boehringer-Mannheim). Hybridization was carried out at 42 °C in 50% formamide/5×Denhardt's reagent/5×SSC for 14–16 h. The post-hybridization washes were performed under high-stringency conditions (68 °C, 0.1×SSC/0.1% SDS). Equal loading of the RNA gels was determined by visualization of the ribosomal RNA bands after staining with ethidium bromide.

Primers

To amplify carp *hsp70*-specific genomic DNA and cDNA, oligonucleotides based on the *hsp70/hsc70* consensus sequence of zebrafish (GI:17061841; GI:1865782), trout (GI:17129570; GI:246719), *Xiphophorus maculatus* (GI:17061837; GI:17061839), xenopus (GI:64796; GI:1326171), chicken (GI:211941; GI:2996407), mouse (GI:193983; GI:309319) and human (GI:386785; GI:13273304) were selected and used in RT-PCR amplifications.

hsp70/hsc70-specific primers:

hsp70 1 forward: 5' GCTGTTGGCATTGACCTGGG 3',
hsp70 2 reverse: 5' TCTGGGTTAATGCTCTTGTT 3',
hsp70 4 reverse: 5' GGTGATGGTGATCTTGTTCT3',
hsp70 6 reverse: 5' GTCAACCTCCTCAATGGTTG 3',

Carp *hsp70*-specific primers (AY120894):

hsp70 3 forward: 5' TTGACAAAGGCAAAGCTTCA3',
hsp70 5 forward: 5' CGCTTTGAAGAGATGTGCTC 3',
hsp70 7 forward: 5' CTAAAGACGCTGGAGTAATC 3'
hsp70 8 reverse: 5' TGCTTCCTCTTGAACTCCTCC 3'.

The sequences of the carp β-actin specific primers were derived from GeneBank entry M24113:

β-actin-3: 5' GCAAGAGAGGTATCCTGACC3',
β-actin-4: 5' CCCTCGTAGATGGGCACAGT3'.

Measurement of hsp70 mRNA levels

At each time point of the experiments, 3–4 fish were used to prepare RNA. RT-PCR reactions for each animal were performed in triplicate to increase the reliability of the measurements. For normalization of the amount of *hsp70* mRNA, the level of carp β-actin mRNA was used as internal standard. Images of the ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot™ Pro Gel Analysis Software (UVP). The relative levels of the *hsp70* mRNAs are expressed as ratios (hsp70/β-actin). The results were submitted to Student's *t*-test analysis, with a probability $P < 0.05$ taken as the limit of significance.

RESULTS AND DISCUSSION

Identification of hsp70 in carp

Primer design

Databases were searched for Hsp70 and Hsc70 protein sequences. All entry pairs corresponding to both the inducible and the constitutive forms from the same fish species were selected, together with several other pairs from evolutionally distant species: Xenopus, chicken, mouse and human. Those entries representing only one of the two forms from a given species were not included in this analysis. The sequences were aligned and regions conserved in both the Hsp70 and Hsc70 primary structures were looked for. These conserved regions were further ordered on the basis of the highest similarity at a nucleic acid level. As a result of this procedure, four regions were selected for primer design, two being close to the ends of the amino- and carboxyl-terminal coding regions, and the other two located internally (Fig. 1B, primers 1, 2, 4 and 6).

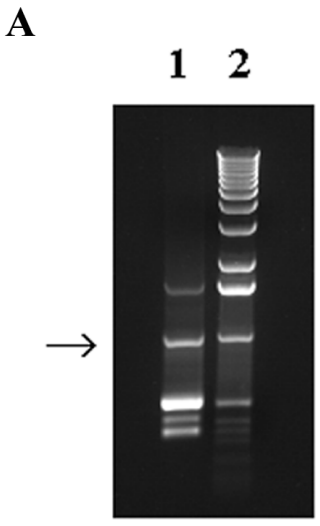
Amplification and sequence determination

Primers complementary to the above selected regions were expected to amplify both *hsp70* and *hsc70*-specific sequences. In order to avoid co-amplification of the two products of nearly identical length, we took advantage of the observation that known *hsc70* genes contain introns at evolutionally conserved positions, while the known *hsp70*s have either no or fewer introns [8, 15, 23]. Therefore, we used genomic DNA as a template in the PCR reactions with primers 1 and 2. The region amplified by this primer pair was expected to have the size of the cDNA for *hsp70*, and a larger size for *hsc70* because of the 3 introns present. The result of the experiment confirmed this expectation: one of the few amplified products had the expected size of about 1 kb., characteristic of the intronless cDNAs (Fig. 1A). This product was isolated

from the gel and inserted into a plasmid vector, followed by sequencing of the inserts of three independent transformants. On the basis of the determined sequence, gene-specific primers 3 and 5 were synthesized (Fig. 1B) and two additional, overlapping segments of the coding region were amplified from genomic DNA template using primer pairs 3/4 and 5/6 (960 bp and 1026 bp, respectively). Isolation, cloning and sequencing of the products of these reactions were performed as previously. For each set of the independent inserts, the three sequences differed at several positions, possibly due to Taq polymerase-generated mutations. In these cases, the two identical nucleotides were accepted as the "real sequence".

Sequence analysis

The sequences of the overlapping regions of the three PCR products were identical, indicating that amplification took place from the same template. From the partial sequences of the three amplicons a single sequence could unambiguously be compiled with a single open reading frame spanning the whole length. Database searches indicated that both the coding region and the encoded protein were most similar to known *hsp70* and *hsc70* cDNAs and proteins, with the highest homology exhibited to the *hsp70* gene (88%) and protein (90%) described from zebrafish (Fig. 1B). The similarity levels were appreciably lower when the carp sequence was compared with zebrafish *hsc70* gene and protein (77% and 83%, respectively). Phylogenetic analyses placed the identified carp sequence in the group of fish Hsp70 proteins, clearly distinguishable from the second group populated by all known Hsc70 proteins described from fish. This second class also contained two additional proteins identified as Hsp70s (Fig. 2).



B

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                    Primer 1 →
Carp hsp70   ———-AIGIDLGTTYSWVGGVQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ   53
ZF   hsp70   MSSPKGI.I.........C..VS.......................NA..........L   60
ZF   hsc70   MS—KGP.V.........C..VF.......................D............   58

Carp hsp70   VAMNPNNTVFDAKRLIGRKFDDPVVQSDMKHWSFQVVSDGGKPKVQVEYKGENKTFYPEE  113
ZF   hsp70   ..................R...H..........K..R.......A..HS..D...N...  120
ZF   hsc70   .....T...L..N..N..RQ...G.........P.N.IN.NSR..........S.S.....  118
                                                  Primer 7 →
Carp hsp70   ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLENINEPTA  173
ZF   hsp70   ................................E.H.........V.....ARI....RP  180
ZF   hsc70   ......T...........KT.S.........S............T.S.....VI......  178
                    Primer 3 →
Carp hsp70   AAIAYGLDKGKASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMV  233
ZF   hsp70   ...........S...........E............................V......T.  240
ZF   hsc70   .........KVGA............S.............S..................  238
                  ← Primer 8
Carp hsp70   NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYTSIT  293
ZF   hsp70   ............................................................  300
ZF   hsc70   ...IT...........D....V..................T.....................  298
                  Primer 5 →
Carp hsp70   RARFEEMCSDLFRGTLEPVEKALRDAKMDKSQINDVVLVGGSTRIPKIQKLLQDFFNRRE  353
ZF   hsp70   ......L.........D.............A..H.......................G..  360
ZF   hsc70   ......LND.......D.............A.......................Y..GK.  358
                  ← Primer 2
Carp hsp70   LNKSINPDEAVAYGAAVQAAILMGDTSGNVQDLLLLDVAPLSLGIETAGGVMTPLIKRNT  413
ZF   hsp70   ................................M.....................A......  420
ZF   hsc70   ......................S..K.E..........T.............V......  418

Carp hsp70   TIPTKQTQTFTTYSANQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTF  473
ZF   hsp70   ..........A...D.........F...G............D..................  480
ZF   hsc70   ..............D.............................................  478
                          ← Primer 4
Carp hsp70   DIDANGILNVSAVDKSTGKENKITITNDKGRLSKEEIERMVQDADKYKAEDDLQREKIAA  533
ZF   hsp70   ............A......Q.....................E..............S.  540
ZF   hsc70   .......M..........................D......E.E.......V..D.VS.  538

Carp hsp70   KNSLESYAFNMKNSVEDENLKGKISEDDKKKVIEKCNEAVSWLENNQLADKEEYEHHLKE  593
ZF   hsp70   ..................D........E...R.V........R..............Q...  600
ZF   hsc70   ..G.........ST....K......DE..Q.ILD....VIG..DK..T.ER..F..QQ..  598
                                                  ← Primer 6
Carp hsp70   LEKVCNPLITKLYQ———GGMPAGGCG—AQAHGRIRAALR-PTIEEVD  635
ZF   hsp70   .......V.S....———.........—...RAASG.SAQG.......  643
ZF   hsc70   .......I.T....SAGGMP....E.MP.GFPG.GAAPGGGSSG.......  649
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Fig. 1A. Representative amplification of carp *hsp70* gene segment from genomic DNA template by primer pair 1/2 (line 1). The arrow indicates the expected size of the product amplified from the *hsp70* template. Line 2: 1 kb molecular weight marker (GIBCO BRL)

Fig. 1B. Deduced amino acid sequence of carp Hsp70 aligned with that of zebrafish Hsp70 and Hsc70 proteins. The carp sequence is taken as reference; zebrafish amino acids are shown only where they differ from carp Hsp70. Dots indicates identities, whereas dashes signify gaps. The positions of the PCR primers used in this work are underlined, and their orientations are indicated by arrows. In Northern hybridization experiments, the segment amplified by primer pair 7/8 was used for probe

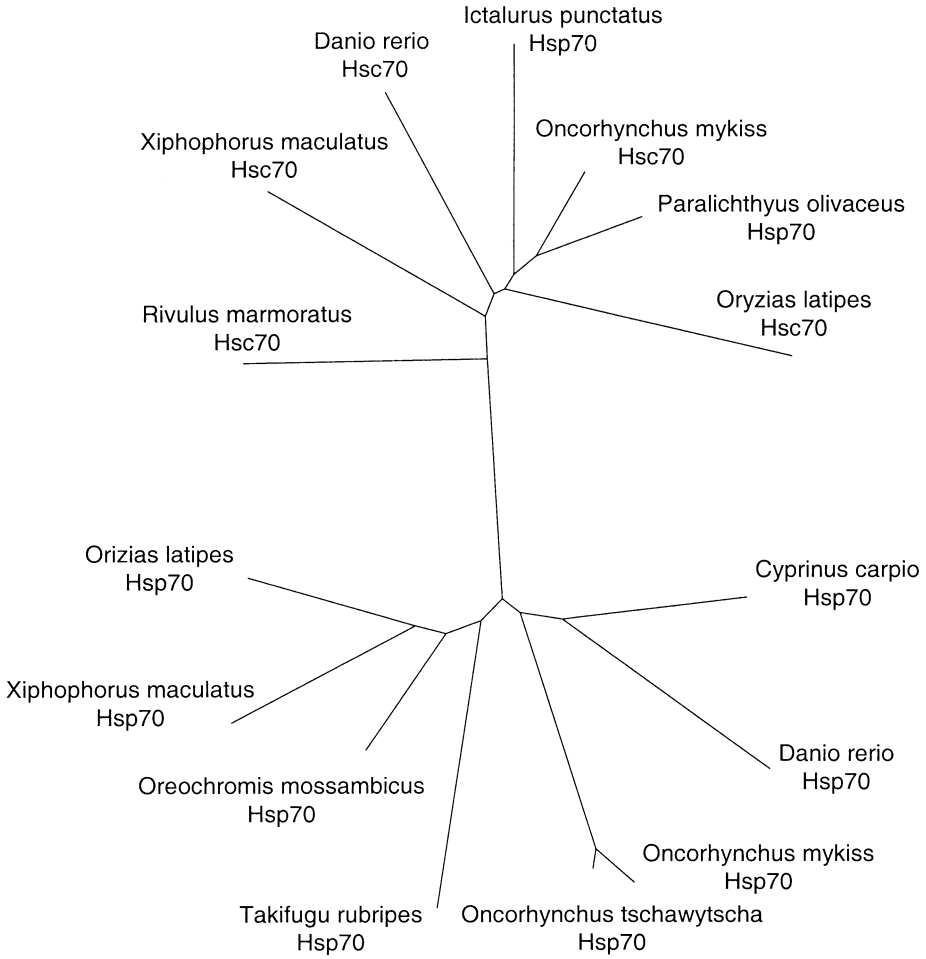

Fig. 2. Phylogenetic analysis of fish Hsp70 and Hsc70 sequences. Full-length sequences were retrieved from the GenBank and aligned with the aid of CLUSTAL W [20]. The unrooted tree was generated by the PHYLIP package [5] and viewed by TREEVIEW [24]. For all analyses the public facility of the Institut Pasteur was used (www.pasteur.fr)

Functional characterization of carp hsp70

The presence of two proteins described as Hsp70 in the group of Hsc70s suggest that unambiguous classification of a gene as inducible or constitutive cannot be based solely on sequence similarities. Therefore, we analyzed the basal expression of the newly identified carp gene and its inducibility by heat shock and by an aspecific stressor, Cd.

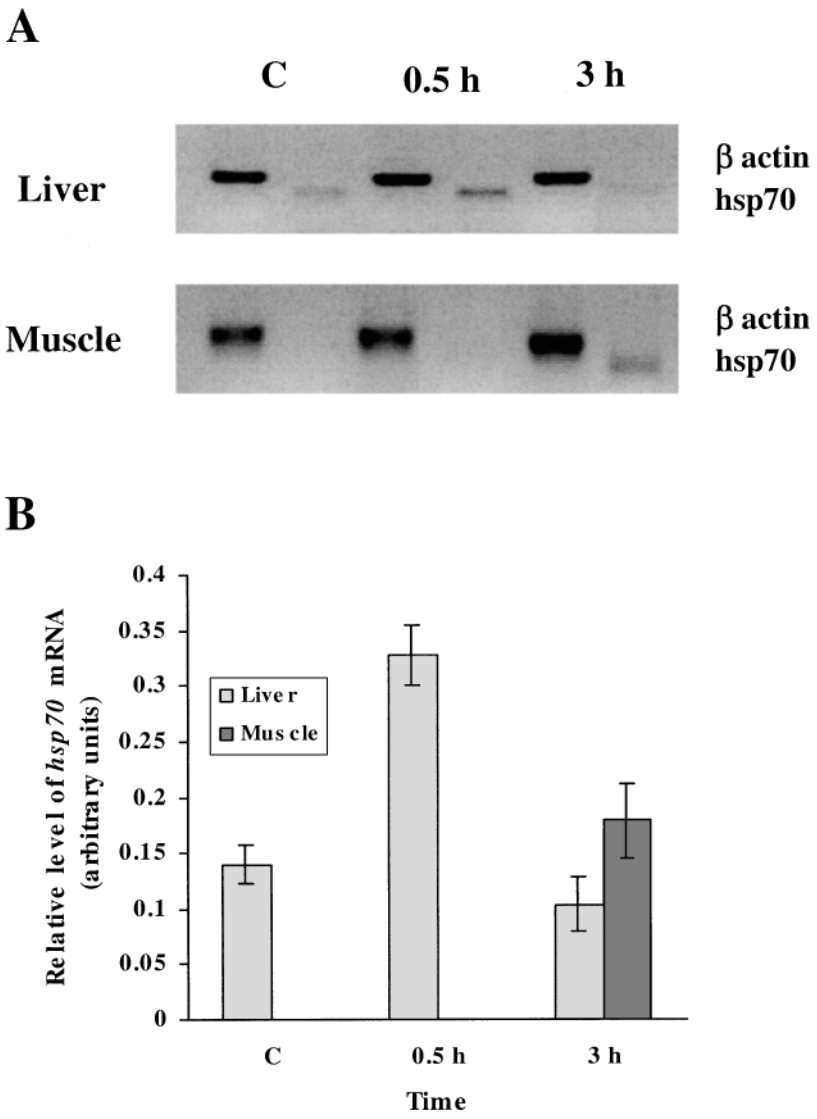

Fig. 3. Induction of *hsp70* by heat shock. A representative result of RT-PCR amplification (A). In parallel with the *hsp70* transcript, β-actin mRNA was amplified and used as an internal control to determine the relative levels of the *hsp70* mRNA. Template RNAs were prepared from the liver and muscle at the time point indicated, and from untreated animals (C). All data are means ±SD from 3–4 measurements on 3–4 fish at each time point (B)

The carp *hsp70*-specific mRNA was not detectable by Northern blot analysis in any of the tissues investigated from unstressed animals (data not shown; for probe, see Fig. 1B). The level of basal expression was also found to be below the level of detectability in the brain and muscle by the more sensitive RT-PCR amplification, and close to the threshold of detection in the liver and the kidney (for the muscle and liver, see Fig. 3, lane 1; data not otherwise shown).

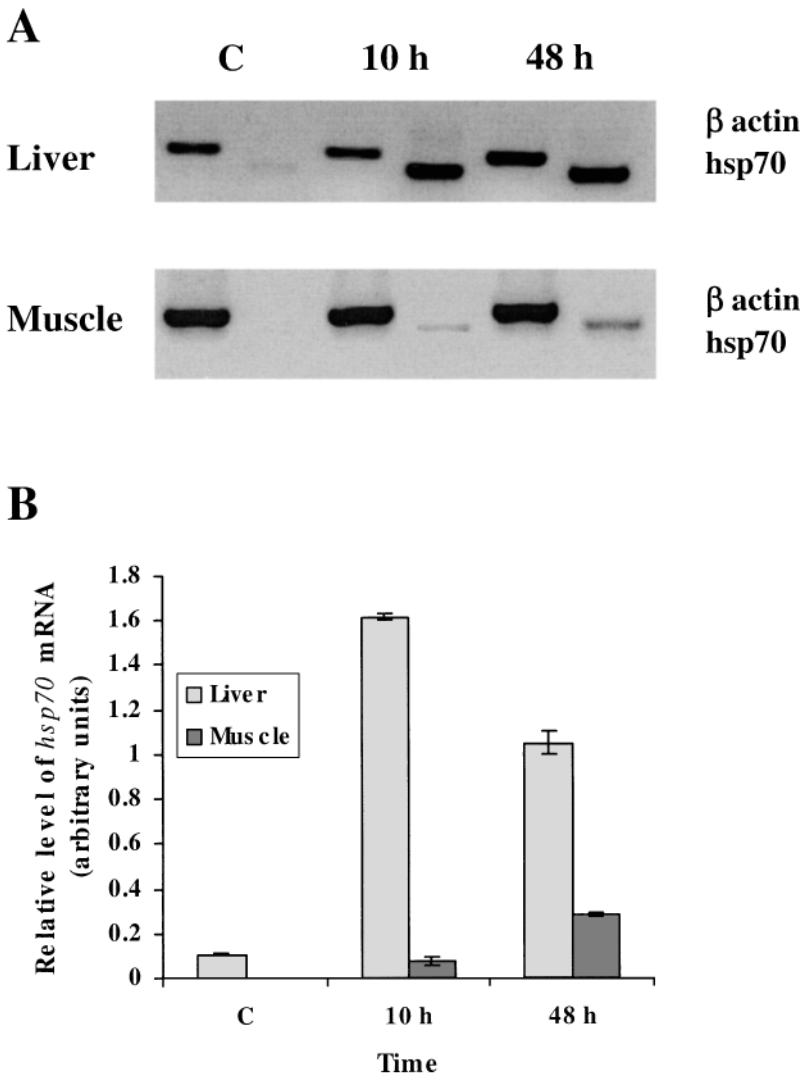

Fig. 4. Time-dependent effect of 10 mg/kg Cd treatment on the accumulation of *hsp70* mRNA in the liver and muscle. A representative amplification is shown in panel A. All data are means ±SD from 3 measurements on 3–4 fish at each time point (B)

Stress-specific change in the expression was investigated in two organs: the liver and muscle. A 14 °C jump in temperature induced the *hsp70* gene in both tissues (Fig. 3). In the liver, transient induction was observed, with the peak of expression (2–2.5-fold) at an exposure of around 30 min. By 3 h, the level of expression was indistinguishable from that for the uninduced stage. Induction was delayed in the muscle: the amount of *hsp70* mRNA was still below the level of detectability at 30 min. However, by 3 h of incubation, induction was detected with a relative level of *hsp70*-specific mRNA about half of that observed in the liver at 30 min.

10 mg/kg Cd injected intraperitoneally strongly induced the *hsp70* expression in the liver (Fig. 4). The induction was again transient, with the highest expression measured at 10 h. At this point an induction of about 15-fold was detected, i.e. 6–7 times higher than that achieved by heat shock. The level of *hsp70* mRNA started to decrease by 48 h, at which point only 10-fold the basal level was measured. The kinetics of the Cd-induced expression in the muscle was similar to that observed during heat shock (Fig. 4). No peak of induction was detected: at 48 h, the level of the *hsp70*-specific message was still rising. Interestingly, Cd was not as potent an inducer in the muscle as in the liver: the relative level measured at 48 h was only slightly more than that measured after 3 h of heat shock (0.29 vs. 0.18).

In summary, we have identified a gene coding for a 70 kD stress protein in common carp. Sequence similarities and the intronless structure suggest that it is a member of the *hsp70* family. The likelihood of this classification is strongly supported by functional analyses: the low basal expression and the inducibility by specific and aspecific stresses are characteristic of the known *hsp70* forms.

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