Isolation of a novel inducible rat heat-shock protein (HSP70) gene and its expression during ischaemia/hypoxia and heat shock

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Most of the members of the mammalian heat-shock protein (HSP) gene family have been studied and isolated from human and mouse cells. Few studies have concentrated on the HSPs of rat, a commonly used experimental animal. We have isolated and characterized a novel inducible rat HSP70 gene using an HSP70 cDNA sequence obtained from an ischaemic rat heart cDNA library. The isolated rat HSP70 gene was found to be a functional gene, as indicated by RNAase-protection and Northern-blot analysis. The deduced amino acid sequence of the inducible rat HSP70 exhibits a high degree of similarity to previously isolated mammalian inducible HSP70 gene products. Expression of the inducible HSP70 gene in rat myogenic cells (H9c2) is markedly

increased after relatively short periods of hypoxia as well as by heat shock. Two heat-shock elements (HSE) are present in the rat HSP70 promoter. Transient transfection of rat HSP70 promoter/chloramphenicol acetyltransferase constructs into H9c2 cells shows that the presence of either of the two HSEs is sufficient for heat-shock inducibility. In contrast, induction of the rat HSP70/chloramphenicol acetyltransferase constructs by hypoxia is only detectable when both HSEs are present. This leads us to conclude that the induction of HSP70 by hypoxia and heat shock occurs through the same regulatory HSEs but the activation of the inducible HSP70 gene by heat shock is several-fold higher than by hypoxia.

INTRODUCTION

The heat-shock proteins (HSP), a group of proteins found in all organisms examined to date, are sharply induced after an increase in temperature (42 °C in mammals) or heat shock and other noxious stresses resulting in protein misfolding (for review, see ref. [1]). In addition, HSPs are involved in the phenomenon known as acquired thermotolerance. Thermotolerance is defined as a transient resistance to cytotoxic effects from a subsequent lethal heat shock induced by a short exposure to a non-lethal heat treatment [2]. The most predominant of the HSPs expressed after an increase in temperature is the inducible HSP70 (HSP70i). Several studies have demonstrated that HSPs, especially HSP70i, are readily synthesized in the heart during ischaemia/reperfusion [3–6]. This has prompted many investigators to pursue the study of HSP induction as a possible adaptive response against myocardial ischaemia (for review, see refs. [7–9]). A large proportion of these studies have been carried out on isolated perfused rat hearts or cultured neonatal rat cardiomyocytes [10,11]. The rat HSP70i gene has not been cloned, at present, which has limited some studies attempted in rat cardiac cells. Heat-shocked and ischaemic rat hearts exhibit an increase in the expression of two different mRNAs (approximately 2.7 and 2.5 kb in size) for the HSP70i gene on Northern blots. It has been previously established that there are at least three different genes that express HSP70i in rodents [12]. One of the mouse HSP70i genes has been cloned [13].

In the present study, we isolated a partial cDNA clone for a novel rat HSP70i gene from a cDNA library constructed from rat ischaemic heart mRNA by using the inducible human HSP70 gene [14] as a probe. The rat HSP70i cDNA was then used to isolate the corresponding genomic clone from a rat genomic library. The rat HSP70i gene has been characterized and found to be a functional gene corresponding to the 2.5 kb mRNA

detected in Northern blots from RNA of stressed rat cells. In addition, we have characterized the expression of the rat HSP70i by generating promoter/chloramphenicol acetyltransferase (CAT) constructs which were used to transiently transfect the rat myogenic cell line H9c2. HSP70i promoter/CAT constructs including two functional heat-shock elements (HSE) are fully inducible by heat shock as well as by hypoxia. The rat HSP70i promoter possesses both HSEs located within 200 bp of the start of transcription. Interestingly, deletion of the distal HSE from the rat HSP70i promoter reduces heat-shock inducibility and practically abolishes inducibility by hypoxia. Further delineation of the response of the rat HSP70i to heat shock and hypoxia demonstrated that, whereas one HSE was sufficient to confer heat-shock inducibility to a heterologous promoter, it was not sufficient for induction by hypoxia, although both HSEs were found to form a heat-shock factor (HSF)–HSE complex by themselves with extracts from hypoxic cells. Interestingly, we found that the same form of the HSF (HSF1) that binds to the HSE during heat shock does so also during hypoxia. It would then seem that, in the case of hypoxia, binding of HSF to the HSE is not necessarily accompanied by transcriptional activation. However, dimerization of the HSE providing a continuous array of eight NGAAN motifs upstream of a heterologous promoter conferred inducibility by both heat shock and hypoxia. In summary, heat-shock and hypoxia inducibility of the rat HSP70i is mediated through the same response elements (HSE) and HSF (HSF1), but the actual mechanism of activation of transcription is not necessarily the same.

MATERIALS AND METHODS

Animal preparation

Ischaemia was induced in the rat heart as previously described [5]. Briefly, Sprague–Dawley rats were anaesthetized with an

Abbreviations used: HSP, heat-shock proteins; HSP70i, inducible heat-shock protein 70; rHSP70, rat heat-shock protein 70; HSE, heat-shock element; HSF, heat-shock factor; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; TTR, triple tandem repeat; 1 × SSC, 0.15 M NaCl + 0.015 M sodium citrate; DMEM, Dulbecco's modified Eagle's medium; poly(A)⁺, polyadenylated.

The nucleotide sequence data presented in this paper have been submitted to the GenBank/EMBL DataBank with accession number X75357.

intramuscular injection of 50 mg of ketamine mixed with 5 mg of xylazine/kg body weight. Animals were ventilated with a respirator and kept at 37 °C. A left lateral thoracotomy was performed, the pericardium was opened and the left anterior descending coronary artery was ligated. A cyanotic zone became apparent within a few minutes of ligation. Control and ischaemic myocardial tissue of the same heart were excised after ligation of the left anterior descending coronary artery for 30 min. Alternatively, animals were anaesthetized and kept immersed in a water bath at 43 °C for 45 min (heat shock) or control animals were sham-operated without ligation of the left anterior descending coronary artery. Myocardial tissue was frozen quickly in liquid N₂ after removal from the animals and stored at -70 °C. Animal procedures were conducted following NIH guidelines and were approved by the University of California at San Diego Animal Use Committee.

DNA constructs, cDNA and genomic library screening

Poly(A)⁺ RNA was prepared from total RNA of a rat ischaemic heart and subsequently used to synthesize cDNA using standard procedures [15]. The cDNA library generated was cloned into λZAP (Stratagene). The cDNA library was amplified once, and approximately 2 × 10⁵ recombinants were screened with the coding region of the human HSP70i gene [14]. The DNA probe was labelled by random hexamer priming in the presence of [³²P]dCTP [15] to a specific radioactivity of 10⁸ c.p.m./μg. Positive plaques were purified by standard methods [16]. A recombinant containing a partial cDNA sequence for the rat HSP70i gene was isolated in this manner and used to screen a λDASH1-1 genomic DNA library from Wistar rat (Stratagene). Approximately 10⁶ recombinants were screened. Six λDASH recombinants that were positive on hybridization with the cDNA clone were isolated and characterized. Two of the λDASH recombinants containing 15 kb of genomic rat DNA were found to be identical and to contain the genomic rat HSP70i corresponding to the partial cDNA clone. The genomic rat HSP70i was found to be contained in a 5.5 kb fragment (*Eco*RI-*Eco*RI) which was subcloned into pBluescript II SK⁺ (Stratagene). The generation of a series of overlapping subclones of genomic rat hSP70i by using different restriction enzymes in plasmid pT₇/T₃-19 allowed for the sequencing on double-stranded denatured plasmid DNA using a Sequenase kit (US Biochemical Corp.). Approximately 3 kb of the 5.5 kb *Eco*RI-*Eco*RI fragment was sequenced and found to contain the complete rat HSP70i gene. All nucleotide sequences presented were obtained by sequencing both strands of the DNA.

The construction of expression plasmids containing the upstream promoter elements of the rat HSP70i gene fused to the bacterial gene encoding CAT was performed by standard methods [15] and used the promoterless vector pBLCAT3 as the recipient of the promoter regions [17]. Construction of rHSP70i/-880CAT was performed by introducing a *Pst*I (-880) to *Ava*I blunt-ended (+80) fragment from the rat HSP70i into the *Pst*I-*Bgl*II blunt-ended sites of pBLCAT3. Construction of rHSP70i/-580CAT and rHSP70i/-197CAT was achieved by digesting rHSP70i/-880CAT with *Hind*III and *Bgl*II (-580) for rHSP70i/-580CAT and *Hind*III and *Xba*I (-197) for rHSP70i/-197CAT, followed by blunt-ending using DNA polymerase I (large fragment, Klenow) and ligating with T4 ligase to recircularize the plasmid and obtain the respective 5' end deletions of the rat HSP70i promoter. Constructs -282/-68CAT, -282/-172CAT and -172/-68CAT were made by isolating the *Nco*I fragment from the promoter of the rat HSP70i (-282 to -68). The *Nco*I fragment was blunt-ended with DNA

polymerase I (Klenow). Half of the material was then digested with *Kpn*I (-172) generating two fragments, *Nco*I-*Kpn*I (-282/-172) and *Kpn*I-*Nco*I (-172/-68). These two smaller fragments were then blunt-ended using T4 polymerase. All three fragments were independently introduced into the blunt-ended *Sal*I and *Bam*HI sites of Dm HSP70/-50CAT [18], an expression vector containing the *Drosophila* HSP70 TATA box, the start of transcription and 5'-end-transcribed non-translated region (-50 to +80) ligated to the bacterial CAT gene. The HSE/TKCAT constructs were made by introducing the oligonucleotides used in the mobility-shift assay which possess *Xba*I-compatible ends into the *Xba*I site of pBLCAT2 [17]. All plasmid DNA constructs used were checked by DNA sequencing before use in transfection studies.

RNA analysis

Total RNA was prepared from ischaemic, heat-shocked and control rat heart tissue as described previously [19]. Total RNA from H9c2 cells that had been treated with heat, hypoxia or left untreated was prepared by the guanidinium chloride method [20]. Northern-blot analysis was carried out on 10 μg of total RNA from each sample which were fractionated on a 1% formaldehyde/agarose gel, blotted on to a Nylon membrane (Nytran), and subsequently hybridized with specific DNA probes [21]. The DNA probes used were those for the human HSP70i gene, a *Bam*HI-*Hind*III fragment of plasmid pH 2.3 [14] containing the complete protein-coding region of the gene. In addition, the coding region as well as the 5' and 3' transcribed untranslated regions of the rat HSP70i gene were used as probes for Northern-blot analysis. The DNA fragments were labelled using [³²P]dCTP and the multiprime DNA-labelling system (Amersham). Northern blots were hybridized at 42 °C overnight and subsequently washed with 2.0 × SSC/0.1% SDS at 55 °C for the human HSP70i probe and 0.1 × SSC/0.1% SDS at 65 °C for the rat HSP70i probes and exposed to X-ray film for 14-16 h. The results shown are representative of three separate Northern-blot analyses which yielded similar results.

RNAase-protection assays were performed as described by Melton et al. [22]. Total RNA (10 μg) was co-precipitated with 500 000 c.p.m. of the radioactively labelled riboprobe. The riboprobes used were synthesized using pT₇/T₃-rHSP70XP and pT₇/T₃-rHSP70i/-197CAT. The pT₇/T₃-rHSP70XP consisted of the 5'-end region of the rat HSP70i from *Xba*I (-197) to *Pvu*I (+233), cloned into the *Xba*I-*Sma*I sites of pT₇/T₃-19 (Bethesda Research Laboratories). The pT₇/T₃-rHSP70i/-197CAT consisted of the 5'-end region of the rat rHSP70i promoter from *Xba*I (-197) to *Ava*I (+80) ligated to the CAT-coding region from *Bgl*II to *Eco*RI of pBLCAT3 [17] and cloned into the *Xba*I-*Eco*RI sites of pT₇/T₃-19. Radioactively labelled riboprobes were synthesized by digesting pT₇/T₃-rHSP70iXP and pT₇/T₃-rHSP70i/-197CAT with *Hind*III and using T3-polymerase as previously described [22].

Cell culture and transfection

The embryonic rat heart-derived cell line H9c2 was obtained from the American Type Culture Collection (CRL 1446). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (penicillin/streptomycin/fungizone; Gibco) and 10% fetal calf serum. Heat-shock treatment of the H9c2 cells was carried out by floating a sealed culture dish (10 cm) in a 42 °C water bath for 1 h. Hypoxia was achieved by using an anaerobic jar, GasPak System (BBL Microbiology Systems, Becton Dickinson and Co.) equipped with a GasPak H₂- and CO₂-generating envelope and

a Methylene Blue indicator to monitor O₂ depletion. The GasPak System jar is capable of depleting the concentration of O₂ down to 0.2–0.6% in 60 min and providing a 5–7% CO₂ atmosphere [23]. H9c2 cells were placed in 5 ml of glucose- and serum-free DMEM and submitted to hypoxic conditions in the anaerobic jar at 37 °C for the time periods indicated, whereas controls in glucose- and serum-free DMEM were left at normoxic conditions at 37 °C for the total length of the experiment. On completion of the various stress treatments, cells were used directly to prepare total RNA.

Plasmid DNA transfection of H9c2 cells was performed by calcium phosphate–DNA co-precipitation as described by Chen and Okayama [24]. Cells were transfected with 20 µg of plasmid DNA per plate (10 cm) and the precipitate was allowed to stand overnight. The following day, the medium was changed on the transfected cells, and plates were heat-shocked, placed under hypoxic conditions or left untreated. Subsequently, cells were placed in complete media (DMEM/antibiotics/10% fetal calf serum) and harvested for CAT assay after 24 h of recovery.

Cytoplasmic extract preparation and CAT assay

Cell cultures transfected with DNA and subjected to heat shock or hypoxia or left untreated were harvested for cytoplasmic extracts as follows. Cell monolayers were washed twice with ice-cold PBS [15]. Dishes were thoroughly aspirated, and 200 µl of ice-cold 0.25 M Tris/HCl, pH 8.0, was then added to the cell monolayer and cells were collected by scraping with a rubber policeman. Such suspensions were subjected to three cycles of freeze–thaw in a solid CO₂/methanol bath and a 37 °C water bath respectively before a 10 min spin in an Eppendorf Microfuge [9880 g (11 000 rev./min)]. Supernatants were collected, and protein content was determined by the method of Bradford [25]. CAT assays were performed with 200 µg of protein/assay as described by Gorman et al. [26] in a 2 h incubation. After autoradiography, [¹⁴C]chloramphenicol and its acetylated derivatives were specifically excised from the thin-layer plates; radioactivity was determined by liquid-scintillation counting. CAT activity (pmol/min per mg of protein) was determined for each sample and fold induction during heat shock and hypoxia was determined over samples from untreated cells (control).

Mobility-shift DNA-binding assay

H9c2 cells in 10 cm dishes were heat-shocked (42 °C) for 60 min or controls left untreated. H9c2 cells were also submitted to a time-course experiment under hypoxic conditions (0.5, 1, 2, 3 and 4 h) in 5 ml of glucose- and serum-free DMEM; controls were maintained in the same media but under normoxic conditions for 4 h. Immediately after the respective treatments, whole cell extracts were prepared by washing the cells twice in ice-cold PBS. Cells were then scraped into EBC buffer (50 mM Tris/HCl, pH 8, 120 mM NaCl, 0.5% Nonidet P40, 1 mM phenylmethanesulphonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml chymostatin and 1 µg/ml pepstatin), incubated on ice for 5 min, vortexed for 1 min and centrifuged for 10 min at 4 °C. The resulting supernatants were separated into portions, quick-frozen in liquid nitrogen and stored at –70 °C. Protein concentration of the whole cell extract was determined by the Bradford assay (Bio-Rad).

The DNA probes used for the mobility-shift assays were double-stranded synthetic oligonucleotides containing HSE I and II of the rat HSP70i gene. The rat HSP70i promoter contains the canonical HSE repeats NGAAN and its complement:

HSE I synthetic oligonucleotide (24 bp):

5'-CTAGCGAAACTGCTGGAAGATTCT-3'

HSE II synthetic oligonucleotide (25 bp):

5'-CTAGCAGATCCGTCTAGAGAGTTCT-3'

A non-specific double-stranded synthetic oligonucleotide was used to determine the specificity in binding of the HSF to the HSE I and HSE II oligonucleotides. This non-specific DNA probe corresponds to a region known as the triple tandem repeat (TTR) which is found in the transcribed non-translated 5' region of both the mouse [13] and the rat HSP70i. The TTR oligonucleotide contains no HSE repeats:

TTR oligonucleotide (36 bp): 5'-TCGACCAGAGCA-TCCCCGCCGCAAGCGCACCTTCC-3'

The synthetic oligonucleotides were radioactively labelled by filling in the protruding 5'-ends using a DNA polymerase I large fragment (Klenow) in the presence of [α -³²P]dCTP, dATP, dGTP and dTTP. The radioactively labelled oligonucleotide was purified from free nucleotides by two 2.5 M ammonium acetate/ethanol precipitations. Binding reactions (15 µl) were carried out by incubating 20 ng of the radiolabelled DNA probe (10⁸ c.p.m.) with 3 µg of poly(dI-dC) and 1 µg of heat-denatured herring sperm DNA in a buffer containing 4% Ficoll, 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.25 µg/ml BSA (Sigma) and 15 µg of the whole cell extract which was added last. In the competition experiments shown, 1 µg of non-radiolabelled competitor oligonucleotide (HSE I, HSE II or TTR) was incubated for 15 min at 0 °C with the whole cell extract before addition to the binding reaction mixture. The mixture was incubated at room temperature for 30 min after which loading buffer (4% glycerol, 0.045 M Tris/borate/0.01M EDTA, 0.2 µg/ml xylene cyanol, 0.2 µg/ml Bromophenol Blue) was added and the mixture loaded on to a 5% acrylamide gel which was run at 100 V for approx. 3 h. Gels were then soaked in 5% glycerol for 20 min at room temperature, dried on to a 3 MM filter paper and exposed to Kodak X-ray film for 14–16 h.

Mobility-shift DNA-binding assays using antisera against HSF1 and HSF2, kindly provided by Dr. K. D. Sarge and Dr. R. I. Morimoto, were performed as previously described [27].

RESULTS

Expression of HSP70i in the rat heart during ischaemia

Total RNA was prepared from the right and left ventricles of hearts from rats that were subjected to ischaemia by ligation of the left anterior descending coronary artery for 30 min and sham-operated rat hearts (controls). The RNA was used for Northern-blot analysis using a human HSP70i DNA probe [14]. Figure 1 shows the result of such an analysis. Whereas all heart tissue showed expression of the constitutive HSP70 gene (HSP70c, 2.3 kb), only rat hearts made ischaemic expressed the two HSP70i mRNAs (HSP70ia and HSP70ib, 2.7 and 2.5 kb; see below) in the left ventricles. Hybridization with the human HSP70i probe gives a signal on Northern blots with all three HSP70 mRNAs and on many occasions also cross-hybridizes with the 18 S rRNA as seen in Figure 1.

Isolation and characterization of the rat HSP70i gene

In order to obtain a DNA probe specific for the inducible forms of the HSP70 genes of the rat heart during stress (ischaemia/hypoxia), we constructed a cDNA library from poly(A)⁺ mRNA from the left ventricle of an ischaemic rat heart like the one shown in Figure 1. A partial cDNA clone for rat HSP70i was isolated from this cDNA library using human HSP70i as a

Figure 1 Northern-blot analysis of total RNA from ischaemic and control rat hearts

Odd-numbered lanes contain RNA from the right ventricle and even-numbered lanes contain RNA from the left ventricle of the rat hearts. Lanes 1–4 contain RNA from control (sham-operated) rat hearts and lanes 5–8 contain RNA from rat hearts made ischaemic for 30 min by ligation of the left anterior descending coronary artery. The Northern blot was hybridized with the human HSP70i gene [14] and subsequently with an oligonucleotide specific for the rat 28 S rRNA to assess loading differences.

Figure 2 Restriction map and sequence scheme of the rat HSP70ib gene

(a) Restriction map of the λ DASH subcloned 5.5 kb genomic fragment containing the rat HSP70ib gene. B, *Bgl*I; B', *Bgl*II; D, *Dra*I; E, *Eco*RI; H, *Bam*HI; P, *Pvu*I; P', *Pvu*II; R, *Rsa*I; S, *Sac*I; S', *Sma*I; T, *Pst*I; X, *Xba*I; X', *Xho*I. (b) Sequencing scheme used to determine the nucleotide sequence of the rat HSP70ib gene. (c) Restriction map of the partial cDNA clone for the rat HSP70ib gene.

probe. The partial cDNA clone for the rat HSP70i gene was then used to isolate the corresponding genomic clone from a rat genomic library as described in the Materials and methods section. Figure 2 shows the restriction map of both the partial cDNA (Figure 2c) and genomic (Figure 2a) clones for rat HSP70i. These restriction sites permitted the generation of a series of subclones which were used to sequence HSP70i. The scheme used to sequence both strands of the genomic and cDNA clones is shown in Figure 2(b). The complete nucleotide sequence of the rat HSP70i gene is presented in Figure 3. Regions in the 5' end contain several canonic basal promoter elements (Sp1, CCAAT, TATA box). In addition, a proximal and distal region in the promoter contain several perfect and imperfect matches to the heat-shock modular unit NGAAN and are referred to as HSE I and HSE II. The start of transcription determined by RNAase-protection and primer-extension assays is indicated by +1. It is important to point out that the promoter region of the rat HSP70i gene exhibits a high degree of similarity to those of the human and mouse HSP70i. A comparison of the deduced

amino acid sequence of rat HSP70i with the human and mouse HSP70i amino acid sequences is shown in Figure 4.

One concern during the isolation of the rat HSP70i gene was to determine which of the inducible HSP70 genes we had cloned. Figure 5 shows the different hybridization patterns obtained using the human HSP70i probe (lanes A), the 5' end (lanes B) and the 3' end (lanes C) of the transcribed untranslated regions of rat HSP70i on a Northern blot containing RNA from the heart of a heat-shocked and a control rat. The results in Figure 5 indicate that the 5' ends of both rat HSP70i genes cross-hybridize whereas the 3' ends of the HSP70i genes are divergent enough not to cross-hybridize. This demonstrates that the rat HSP70i clone corresponds to the 2.5 kb mRNA for the inducible form of the HSP70 gene as detected on Northern blots. This result agrees with the predicted size of the mRNA from the sequenced rat HSP70i which we have designated HSP70ib so as to distinguish it from the other inducible gene that generates the 2.7 kb mRNA or HSP70ia.

RNAase-protection assays and primer-extension assays were used to determine the start of transcription of the gene (results not shown). A strongly protected RNA fragment was detected in RNA from rat heat-shocked and ischaemic hearts but not in control hearts using the 5'-end region of the hSP70ib gene. This confirms that the isolated rat HSP70i gene is truly a functional HSP70i gene. Interestingly, a minor start of transcription site, 30 nucleotides upstream of the major start site, was observed in all ischaemic rat heart RNA samples tested.

Expression of the rat HSP70ib in the rat H9c2(2-1) cell line

One of the major causes of ischaemia is depletion of O₂ (hypoxia) to the cardiac cell. For this reason, we decided to study the induction of rat HSP70ib during hypoxia in the H9c2 cell line. Figure 6 shows a Northern blot of RNA from H9c2 cells subjected to hypoxic conditions. The transient induction of the two rat HSP70i mRNAs can be observed to occur predominantly between 2 and 4 h of hypoxia. In addition, a plasmid DNA containing the promoter region (–880 to +80) of the rat HSP70i gene ligated to a reporter gene (bacterial CAT) transiently transfected into H9c2 cells was found to be expressed in a similar fashion to the endogenous HSP70i gene by using an RNAase-protection assay (results not shown).

Transiently transfected H9c2 cells were also assayed for expression of the rat HSP70i/CAT construct by the enzymic CAT assay. Figure 7(a) shows the results obtained from H9c2 cells transfected with the rHSP70i/–880 CAT construct, as well as with two additional rHSP70i/CAT constructs that had the rat HSP70i promoter region deleted down to –580 and –197, and submitted to heat shock or hypoxia or left untreated. The data shown are a summary of the CAT assay results from three independent experiments. Interestingly, whereas constructs rHSP70i/–880 CAT and rHSP70i/–580 CAT exhibited inducibility by both heat shock and hypoxia, construct rHSP70i/–197CAT was inducible by heat shock but showed little or no inducibility by hypoxia. Close examination of the region affected by deletion of the rat HSP70i promoter down to –197 shows that the distal region (HSE II) containing the HSE repeats has been partially deleted. This indicates that disturbing the distal HSE II region affects the ability of rat HSP70i to respond to heat shock and hypoxia. To delineate the relative involvement of the distal and proximal HSE regions in induction of HSP70i during heat shock and hypoxia, we subcloned the promoter region containing both distal and proximal HSEs, as well as a region containing each one by itself, upstream of a basal promoter with exclusively a TATA box element (Dm HSP70/–50CAT) [18].

-285 ccattggcaacactgtcacaacagcgaagcaactctctaccacccccccgctcaggatc
-225 caattctgtccagcgaagccacagctccgtctctagagaagttctgacagggcggtaccctcaaca
HSE II Sp1
-162 tggattactcatgggagggcgaggaagctctcaacagacccggaactcgtctgaggaattcttgcc
HSE I
-99 ccaaggctctctccgctcgtctgattggccatcggggggtggcgaggcgagcctcgt
CAT Sp1
-36 taaagggcgaggcgccgcgcagcaccagatctctctctaatctgacagaaacagtttc
TATA +1
27 tgggtccactcgcagagaagcagagaagcagagcaagcgccgcttcgaacctcgggcaaga
90 ccagcctctccagagacatcccaacgcgaagcgacccctctccagagcataccacgcggag
153 cgcacccctccccagagcatccccgcgcgcaagcgcaacctccagaagcagacgcagcagc
201 ATG GCC AAG AAA ACA GCG ATC GGC ATC GAC CTG GGC ACC ACC TAC TCG
M A K K K T A G I D G T T Y S
249 TGC GTG GGC GTG TTC CAG CAC GGC AAG GTG GAG ATC ATC GCC AAC GAC
C V G V F Q H G V E I T A H D
297 CAG GGC AAC CGC AGC ACC CCC AGC TAC GTG GGC TTC ACC GAC ACC GAG
Q G M R T F V A F T D T E
345 CCG CTC ATC GGG GAC GGC AAC AAG CAG GTG GCG TTC AAC CCG CAG
R L I G D A A K N Q V A L N P Q
393 AAC ACC GTG TTC GAC GGC AAC GGC CTG ATC GGC CGC AAG TTC GGC AAC
N T V F D A N A G L I G R K F G D
441 CCG GTG GTG CAG TCG GAC ATG AAG CAG TGG CCG TTC CAG GTG GTG AAC
P V V Q S D M K H W P F Q V V N
489 GAC GGC GAC AAG CCC AAG GTG CAG GTG AAC TAC AAG GGA AAG AAC CGG
D G D K P K V N Y K G K H R
537 TCG TTC TAC CCG GAG GAG ATC TCG TCC ATG GTG CTG ACC AAG ATG AAG
S F V P E E I S S H V L T K M K
585 GAG ATC GGC GAG GCG TAC CTG GGC CAG CCG GTG ACC AAC GCG GTG ATC
E I A E A G Y L G H P V T N A V I
633 ACC GTG CCC GCC TAC TTC AAC GAC TCG CAG CGG CAG GCC ACC AAG GAC
T V P A Y R N D S C Q R Q A T K D
681 GCG GGC GTG ATC CCG GGT CTG AAC GTG CTG CCG ATC ATC AAC GAG CCC
A G V I A G L R I I N E P
729 ACG GCG GCC GGC ATC GGC TAT GGC CTG CAG CGG ACC GGC AAG GGC GAG
T A A A D R T G G GGC GGC ACC GGC TAC GAG GTG TCC
777 CCG AAC GTG CTC ATC TTC GAC CTG GGC GGC AGG TTC GAG GTG TCC
R N V L I F D L G G G R F D V S
825 ATC CTG ACG ATC GAC GAC GGC ATC TTC GAG GTG AAG GGC ACC GCG GGC
I L T I D D G I F E V K A T A G
873 GAC ACG CAC CTG GGC GGC GAG CAG TTC GAC AAC CCG CTG GTG AGC CAC
D T H L G G E D F D N R L V S H
921 TTC GTG GAG GAG TTC AAG AGG AAG CAG AAC GAG ATC AGC CAG AAC
F V E E F K R K H K K K D I S Q N
969 AAG GCG GCG GTG CCG CCG CTG GGC GAG GGC GTG GAG AGG GGC AAC AGG
K R A V R P L A D S V E R A K R
1017 ACG CTG TCG TCC AGC ACC CAG GCC AGC CTG GAG ATC GAC TCT CTG TTC
T L S S S T A Q A S L E I D S L F
1065 GAG GGC ATC GAC TTC TAC TCC ATC AC GCG GCG CCG TTC GAG GAG
E G I D F Y T C S I T R A R F E E
1113 CTG TGC TCG GAC CTG TTC GGC AGC CTG GAG CCC GTG GAG AAC GGC
L E F V E E E E E E E E
1161 CTG CCG GAC GGC AAG GTG GAC AAG GCG CAG ATC CAC GAC CTG GTG CTG
L R D A K L D K A Q I H D L V L Q
1209 GTG GGC GCG TCG ACG GCT CCC AAG GTG CAG AAG CTG CTG CAG GAC
V G S T R I P K V Q K L L Q D
1257 TTC TTC AAC GGC CCG GAC CTG AAC AAG AGC ATC AAT CCG GAC GAG CCG
F N G R D L N K S I N P D E A
1305 GTG GGC TAC GGC GCG GCG CTG CAG GCG GCC ATC CTG ATG GGC GAG AAG
V A Y G A V Q A A I L M G D K
1353 TCG GAG AAC GTG CAG GAC CTG CTG CTG GAC GTG GCG CCG CTG CCG
S F L D V L D V L D V L D V L D
1401 CTG GGT CTG GAG ACG GGC GGC GGC GTG ATG ACG GCG CTC ATC AAC GCG
L G L E T A G G V M T A L I K R
1449 AAC TCC ACC ATC ACC ACC AAG CAG ACG CAG ACC TTC ACC ACC TAC TCG
N S T I P T K Q T Q T F T Y S
1497 GAC AAC CAG CCC GGC GTG CTG ATC CAG GTG TAC GAG GGC GAG AAG GGC
D N Q P G V L I Q V Y E G E R A
1545 ATG ACG CCG GAC AAC AAG CTG CTG GGC GCG TTC GAG TTG AGC GGC ATC
H T R C D N N L L G R E L S G I
1593 CCG CCG GCT CCG AGG GGC GTG CCC CAG ATC GAG CTG ACC TTC GAC ATC
P P P R G V P Q I E V T F D I
1641 GAC GGC AAC GGC ATC CTG AAC GTG ACG GCG CAG AAG AGC ACC GGC
D A N G I L N V T A T D K S T G
1689 AAG GGC AAC AAG ATC ACC ATC AAC CAG AAG GGC CCG CTG AGC AAG
K A N K I T I T N D K G R L N S
1737 GAG GAG ATC GAG CCG ATC GTG CAG GAG CCG GAG CCG TAC AAG CCG GAG
E E I E R M V Q E A E R Y K A E
1785 GAG GAG GTG CAG CCG GAG AGG GTG GCT GCC AAG AAT CCG CTC GAG TCC
D E V A K N R V A L S A L S
1833 TAT GCT TAC AAC ATG AAG AGC CCG GTG GAG GAG GAG GCT ATC AAG GCG
Y A F N H K S A V E D E G L K K G
1881 AAG ATC ACG GAG GCT GAC AAG AAG AAG GTG CTG GAC AAG GCG CAG GAG
K I S E A D K K K V L D K C Q E
1929 GTC ATC TCC TGG CTG GAC TCT AAC ACG CTG GCT GAG AAG GAG GAG TTC
V I S W L D S N T L A E K E A E
1977 GTG CAC AAG CCG GAG GAG CTG GAG CCG GTG TGC AAC CCG ATC ATC AGC
V H K R E L R V C N P I I S
2025 GGG CTG TAT CAG GGT GCG GGT GCT CCC GGC GCT GGC GGC TTC GGC GGC
G L V O G A G A P G A G G F G A
2073 CAG GCG CCC AAG GGA GGC TCT GGC TCG GGC CCC ACC ATC GAG GAG GTG
Q A P K G S G S G P T I E E V
2134 GAT TAG aggcctttctggtctcaggggtgttggtcagagacagactctgagtggtgctg
D *
2197 tgcacgattcttatacagttactctctctccggagttcagtttaaagttacacgcttttat
2260 acggttaattgattgagttgttgcattttgtatctcgtggtgttttttatataattcaaat
2323 aggttgcatcttcttgcgttttaacttaagtagctgtgtaaaatggtgttctctctgcga
2386 acacactcagcactgcacccctgttgcagttttttctctctcctacacaaactgagaaaaa
2449 agttatctcttctgaacttaaacattcaagataaaatgctacaagt
Poly(A)⁺

Figure 3 Nucleotide sequence and deduced amino acid sequence of the rat HSP70ib gene

Nucleotide sequence and deduced amino acid sequence of the rat HSP70ib gene. The start of transcription determined by primer extension and RNAase-protection assays is indicated (+1) which results in an mRNA of 2494 bp in agreement with the 2.5 kb mRNA seen in Northern blots for the HSP70ib gene. The most significant features of the rat HSP70ib promoter are underlined, such as a TATA box 32 bp upstream from the start of transcription, CAT box, two Sp1-binding sites and two HSE regions, one proximal (HSE I) and another distal (HSE II). The poly(A)⁺ signal at the 3' end of the gene is also underlined. Numbers to the left correspond to the nucleotide sequence and numbers to the right the deduced amino acid sequence.

Figure 7(b) shows a diagram and summary of three independent experiments for these constructs. Construct -282/-68CAT, which contains both distal and proximal HSEs, responded well to heat shock and hypoxia. In contrast, although the response of constructs containing only the distal HSE (-282/-172 CAT) or the proximal HSE (-172/-68 CAT) to heat shock was affected, they were still responsive, whereas their response to hypoxia was almost non-existent.

Further analysis of the involvement of HSEs in the heat-shock and hypoxic response of rat HSP70i was carried out by generating synthetic oligonucleotides to the two HSE-containing regions. These oligonucleotides were designated HSE II and HSE I corresponding to the distal and proximal HSE regions respectively. The synthetic oligonucleotides were inserted upstream of the Herpes simplex virus thymidine kinase (TK) promoter in the expression vector pBLCAT2 [17]. In both cases, we obtained constructs containing one copy of the oligonucleotides (HSE I/TK CAT, HSE II/TK CAT) or two copies of the oligonucleotides in tandem and in the appropriate orientation [HSE I(2)/TK CAT, HSE II(2)/TK CAT]. Figure 7(c) presents a representation and summary of the results obtained in three independent experiments with these constructs. Similarly to that observed before, one HSE region is sufficient to confer heat inducibility but only when the HSE region is duplicated is any hypoxia inducibility observed.

Binding of HSF to HSEs of the rat HSP70ib promoter during heat shock and hypoxia

Mobility-shift assays were carried out to determine if the lack of inducibility during hypoxia of the rHSP70i/CAT constructs containing only one HSE region was due to lack of HSF interaction with HSE. The synthetic oligonucleotides HSE I and HSE II were first tested for their specificity to bind HSF using whole cell extracts from H9c2 cells that had been previously heat shocked (42 °C, 1 h). Figure 8(a) shows a mobility-shift assay in which both HSE I and HSE II form an HSF-HSE complex with heat-shocked H9c2 cell extracts in the absence of a competitor DNA fragment or in the presence of a 50-fold molar excess of a non-specific (no HSE repeats) competitor oligonucleotide (TTR), a region of the transcribed non-translated rat HSP70 gene. However, competition of the HSE oligonucleotide with a 50-fold molar excess of itself or with the other HSE oligonucleotide abolished the formation of the HSF-HSE complex. Figure 8(b) shows the result of incubating both HSE I and HSE II with H9c2 cell extracts exposed to a hypoxia time course. Interestingly, one can observe the formation of an HSF-HSE complex for both HSE I and HSE II with extracts from cells subjected to hypoxia for 0.5–3 h, although we were unable to detect any major increased CAT activity from rat HSP70i/CAT constructs containing only one of these HSE regions during hypoxia.

A recent report has shown the existence of several different forms of the HSF mammalian cells [27]. It has also been shown that the HSF1 form is involved in the HSF-HSE complex during exposure of the cell to heat shock. We have explored the possibility that the difference observed in the regulation of rat HSP70i during heat shock and hypoxia may be due to the binding of distinct HSF forms to the HSEs during these two types of stress. Figure 8(c) presents mobility-shift assays using the HSE I oligonucleotide that had been allowed to react with heat-shocked and hypoxic H9c2 whole cell extracts that had previously reacted with preimmune, anti-HSF1 or anti-HSF2 serum. A supershift or increase in mobility of the HSF-HSE complex was observed only when antiserum against HSF1 was used. This indicates that the HSF form involved in the induction

Rat HSP70i	MAKKTATGIDLGTTSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVALNPQNTVFDANGLIGRKFGDPVVQSDMKHWPQ
Human HSP70i	---AA-V-----KR-----A-----
Mouse HSP70i	---N-----KR-----A-----
Rat HSP70i	VVNDGDKPKVQVNYKGNRSFYPEEISSMVLTKMKEIAEAYLOHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNLRIINEPTAAAIAYGLDR
Human HSP70i	-I-----S---ETKA-----Y-----
Mouse HSP70i	-----ES---F-----
Rat HSP70i	TGKGERNVLI FDLGGGRFDVSILTIDGIFEVKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRAVRPLADGVERAKRTLSSSTQAS
Human HSP70i	-----T-----N-----R-RTAC-----G---
Mouse HSP70i	-----T-----R-RTAC-----
Rat HSP70i	LEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPKVQKLLQDFNCRDLNKSINPDEAVAYGAAV
Human HSP70i	-----S-----G-----
Mouse HSP70i	-----M-----
Rat HSP70i	QAAILMGDKSENVQDLLLDVAPLSLGLTAGGVMTALIKRNTIPTKQTQFTTYSNQPQVLIQVYEGERAMTRDNNLLGRFELSGIPAPR
Human HSP70i	-----K-----C-----
Mouse HSP70i	-----
Rat HSP70i	GVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEAERYKAEDVQRERVAAKNALESYAFNMKSAVEDEGLKGIKIS
Human HSP70i	-----KD-----K-----S-----
Mouse HSP70i	-----D-----L-----
Rat HSP70i	EADKKKVLDKCQEVISWLDNSNTLAEKEEFVHKREELERVNPIISGLYQAGAPCAGGFGCAQAPKGG- SGSGPTIEEVD
Human HSP70i	-----A---D---E---K---Q-----G---P---G-----
Mouse HSP70i	-----D-----PK-A-----

Figure 4 Comparison of the rat HSP70ib amino acid sequence with those of the human and mouse HSP70i

Comparison of the deduced amino acid sequences of the rat HSP70ib with the human and mouse HSP70i. Amino acids identical with the rat HSP70ib sequence are shown as (—); absence of a particular amino acid is shown as (-); differing amino acids are shown. The isolated rat HSP70ib amino acid sequence is 95% and 97% identical with the amino acid sequences of the human and mouse HSP70i respectively.

Figure 5 RNA analysis of the isolated rat HSP70i gene

Northern blot of total RNA from heat-shocked (H) and control (C) rat hearts. The Northern blot was divided into three (A, B and C). A, RNA hybridized to a DNA probe to the human HSP70i gene; B, RNA hybridized to the 5' end region (*Xba*I-*Pvu*II); C, RNA hybridized to the 2.5 kb mRNA, which corresponds to the HSP70ib gene. Only the human HSP70 and 5' end rat HSP70ib probes hybridized to the 2.7 kb mRNA, which corresponds to the rat HSP70ia gene. The positions of 28 S and 18 S rRNA are indicated on the right.

of rat HSP70i during hypoxia is the same as that involved in heat-shock induction (HSF1). A similar experiment was performed using the HSE II oligonucleotide which yielded an identical result (results not shown).

DISCUSSION

In the present study, we have isolated a functional rat HSP70i gene and shown that it corresponds to the 2.5 kb mRNA detected in Northern blots of RNA from heat-shocked and ischaemic rat hearts. The deduced rat HSP70i amino acid sequence has a high degree of similarity to the human and mouse HSP70i sequences [13,14]. In addition to the normal elements found in many eukaryotic promoters (TATA box, CAT box, Sp1 sites), we found two regions of the promoter containing several repeats of the canonic HSE sequence NGAAN and its complement NTTTCN

Figure 6 Northern-blot analysis of total RNA from H9c2(2-1) cells subjected to hypoxia

RNA from H9c2 cells subjected to hypoxia for 0, 1, 2, 3, 4 or 5 h (lanes 1-6) was prepared and analysed by Northern blots. A long exposure (4 days) of a representative blot which was hybridized to the rat HSP70ib gene is shown. The positions of the different HSP70 mRNAs are indicated, as well as the positions of the 28 S and 18 S rRNA.

which we have designated HSE II for the distal and HSE I for the proximal region within the HSP70 promoter. It is important to point out that, whereas the distal HSE II only contains one perfect match to the sequence NGAAN, the proximal HSE I contains three perfect NGAAN sequences. These HSE regions have previously been shown to be the binding site of HSF which is directly responsible for the activation of the HSP gene during heat shock.

O₂ depletion is among the major intracellular functional changes associated with ischaemia and, in conjunction with reoxygenation (reperfusion), one of the most severe stresses affecting the cardiac cell. Expression of the HSP70i genes in the ischaemic rat heart can be reproduced in the cell line derived from embryonic rat heart, H9c2, subjected to hypoxia (Figure 6). We have utilized this model to study the regulation of rat HSP70i during hypoxia and heat shock as a means of understanding how

Figure 7 CAT assays of H9c2(2-1) cells transiently transfected with rat HSP70i promoter/CAT constructs

(a) CAT assays of transiently transfected H9c2 cells with rat HSP70i/CAT constructs containing up to -880, -580, -197 nucleotides from the start of transcription (+1). Transfected H9c2 cells were heat-shocked for 60 min at 42 °C, made hypoxic for 4 h in glucose- and serum-free medium in an anaerobic jar or left untreated. A schematic representation of the constructs used and a summary of the results obtained from three independent experiments are shown. **(b)** CAT assays of transiently transfected H9c2 cells with rat HSP70i fragments -282/-68 (containing both HSEs of the promoter), -282/-172 and -172/-68 (containing only one of the HSEs) upstream of the truncated *Drosophila* HSP70 promoter (-50) which contains only a TATA box fused to CAT. Transfected cells were heat shocked, made hypoxic or left untreated. A schematic representation of the constructs used and a summary of the results obtained from three independent experiments are shown. **(c)** CAT assays of transiently transfected H9c2(2-1) cells with constructs containing one or two copies of either HSE II or HSE I upstream of the thymidine kinase (TK)/CAT basal promoter construct. Cells were heat shocked, made hypoxic or left untreated. A schematic representation of the constructs used and a summary of results obtained from three independent experiments are shown. Synthetic oligonucleotides containing HSE II and HSE I sequences were introduced in the appropriate orientation as one or two copies in tandem upstream of the herpes simplex virus TK promoter CAT expression vector (pBLCAT2). Fold induction under heat shock and hypoxia is CAT activity (pmol of acetylated chloramphenicol/min per μ g of protein) for the experimental condition compared with CAT activity for control cells $\pm$ S.E.M.

rat HSP70i genes are regulated during ischaemia and heat shock. Rat HSP70i/CAT constructs transiently transfected into H9c2 cells respond strongly to heat shock and to a lesser extent to

hypoxia as long as more than 200 bp of the promoter is present (Figure 7a). Further delineation of the regulation of rat HSP70i by heat shock and hypoxia showed that most of the regulatory

Figure 8 Mobility-shift gel assays of the HSE I and II oligonucleotides with whole cell extracts from heat-shocked and hypoxic H9c2(2-1) cells

(a) Specificity of the HSF-HSE complex shift is shown using whole cell extracts from heat-shocked H9c2(2-1) cells. A specific HSF-HSE complex was obtained when either HSE I or II was incubated with heat-shocked H9c2(2-1) cell extracts in the absence of any competitor DNA or in the presence of a 50-fold molar excess of a non-specific DNA competitor (TTR) described in the Materials and methods section. No HSF-HSE complex was combined when HSE I or II oligonucleotide was incubated with whole cell extracts in the presence of a 50-fold molar excess of the same unlabelled HSE or the other HSE oligonucleotide. (b) Both HSE I and II oligonucleotides were incubated with whole cell extracts from H9c2(2-1) cells subjected to hypoxia for various periods of time. The appearance of the HSF-HSE complex can be observed in H9c2(2-1) cell extracts exposed to hypoxia for between 0.5 and 3 h and incubated with HSE I or II in a mobility-shift assay. The presence of a non-specific bandshift is observed in all cell extracts used and has previously been seen by others. (c) Mobility-shift assay with HSE I allowed to react with heat-shocked (42 °C, 60 min) and hypoxic (for 2 h) H9c2(2-1) whole cell extracts previously incubated with preimmune serum, antiserum to HSF1 or HSF2.

elements needed are contained in a promoter fragment that includes both HSE regions (Figure 7b). Subdivision of this promoter fragment leaving in place only one HSE, either HSE I or HSE II, reduces the response to heat shock and practically eliminates induction by hypoxia. On the other hand, two copies of either HSE I or HSE II in tandem readily confer inducibility on a TK promoter not only by heat shock but also hypoxia (Figure 7c). Duplication of the HSEs generates a region with eight perfect and imperfect NGAAN sequences which should increase the binding affinity for HSF, according to a recent study [28]. This may explain the ability of the duplicated HSEs to activate transcription more readily during hypoxia. Surprisingly, the induction by heat shock of constructs containing duplicated HSEs remained practically unchanged, compared with constructs containing one HSE. One possible explanation for this result is that the spacing between the HSEs plays an important role, especially during heat-shock induction, as previously shown by others [29]. This can be readily seen if one compares the fold induction in CAT activity during heat shock of construct

–282/–68CAT (Figure 7b) with constructs HSE I(2)/TK CAT and HSE II(2)/TK CAT (Figure 7c). These results point to possible differences in the mechanism of activation during heat shock and hypoxia.

The apparent lack of induction of rat HSP70i/CAT constructs containing only one HSE region during hypoxia led us to question if one HSE region by itself is sufficient to form an HSF/HSE complex under hypoxic conditions. Mobility-shift assays (Figure 8) using whole cell extracts from both heat-shocked and hypoxic H9c2 cells demonstrated the formation of a strong HSF-HSE complex in both cases. Recently it has been shown that several distinct forms of HSF exist in mammalian cells [27]. The HSF1 form was found to be the one responsible for binding to HSE during heat shock. It is then possible that during hypoxia a different HSF form, which might be less active, binds to HSE. We have investigated this possibility and found that HSF1 is also the form that binds to HSE during hypoxia (Figure 8c).

Alternatively, there are several examples in the literature in

which HSF binding and transcriptional activity do not directly correlate [30,31]. This may be due to the requirement for multiple steps in the activation process for transcription. For example, certain stresses induce phosphorylation of HSF which may modulate its transcriptional activity [27]. The exact mechanism that activates HSF during hypoxia is unclear at present, but there is evidence that a decline in intracellular ATP levels during metabolic stress and heat shock may well be the trigger that causes HSF to bind to the HSEs in the promoter of the HSP genes [32]. Our recent results have shown that this is not the case during hypoxia [11]. One explanation could be that hypoxic induction of the HSP70 gene, although mediated through the same regulatory elements (HSE) and factor (HSF1) as heat shock induction does not necessarily share the same mechanism of activation. Another explanation could be that hypoxia may represent a weaker form of stress than heat shock, and therefore, when only one HSE is present, the hypoxic response is too low to be detectable in our system.

A recent report has shown that a stably transfected human HSP70 promoter/CAT construct containing only one HSE region of the human HSP70i gene is inducible during hypoxia in a mouse myogenic cell line [33]. The difference between these results and our results may stem from the different transfection methods used (transient transfection compared with stable transfection). In transient transfections, only a small number of cells will contain the HSP70/CAT construct, and it is therefore possible that a weak response, such as induced by hypoxia, may be undetectable. Alternatively, the difference may be due to the nature of the human HSP70 HSE compared with that of the rat. Further work will be necessary to elucidate the exact mechanism of activation of HSP70i during heat shock and hypoxia. However, the present availability of a cloned rat HSP70i gene will now permit studies on the regulation and postulated protective effect of rat HSP70i against ischaemia in rat cardiac cells.

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