Research report

Basal expression of stress-inducible hsp70 mRNA detected in hippocampal and cortical neurons of normal rabbit brain

Jane Foster, Ian Brown *

Department of Zoology, Scarborough Campus, University of Toronto, 1265 Military Trail, West Hill, Ont., Canada, M1C 1A4

Accepted 20 February 1996

Abstract

In response to stresses, such as elevated temperature, cells increase synthesis of a group of highly conserved proteins known as heat shock proteins (hsps). Here, we report detection of basal expression of the stress-inducible hsp70 mRNA species in neurons of the normal rabbit brain. By regional Northern blot analysis, basal levels of hsp70 mRNA were observed in control hippocampus, cortical layers, thalamus, and kidney. Using radioactive in situ hybridization, similar patterns of expression were noted for constitutive hsc70 mRNA and hsp70 mRNA in the unstressed rabbit forebrain. Non-radioactive (DIG) in situ hybridization allowed localization of both heat shock mRNA species to hippocampal neurons. In addition, a dual in situ hybridization protocol, which allowed colocalization of two mRNAs to a single cell, demonstrated that hsp70 and hsc70 mRNAs are expressed in the same hippocampal and cortical neurons.

Keywords: Heat shock protein; Hyperthermia; hsc70; In situ hybridization

1. Introduction

Heat shock proteins (hsps) are a group of highly conserved proteins which are classified into families based on their molecular weight [17]. hsc70 [10] and hsp70 [44] proteins are members of the mammalian hsp70 multigene family [17,42]. In general, hsc70 has been reported to be constitutively expressed [34], while hsp70 is considered to be stress-inducible [26]. hsc70 and hsp70 proteins are closely related. They share 80% amino acid identity [5], and two of three functional domains are highly conserved, i.e. the ATP binding domain and the substrate binding domain [5,37,40]. hsc70 and hsp70 proteins perform cellular functions as molecular chaperones in unstressed cells and in response to stress [3,14–16,43]. In the nervous system, hsc70 mRNA is constitutively expressed at high levels [8,35]. Following trauma, such as hyperthermia, hsp70 mRNA is rapidly induced (for review see [6,7]).

Our laboratory has investigated constitutive and hyperthermia-inducible expression of hsc70 and hsp70 mRNA within the rabbit cerebellum, brain stem, and spinal cord. We have demonstrated constitutive neuronal expression of hsc70 mRNA within these brain regions and have also

shown that 1 h of hyperthermia results in a strong glial induction of hsp70 mRNA [13,20–23]. As reported herein, Northern blot analysis of total RNA isolated from brain regions revealed low levels of hsp70 mRNA in unstressed rabbit tissue. In situ hybridization with ³⁵S-labelled hsc70 and hsp70 riboprobes confirmed this basal expression in control forebrain regions. The present report identifies cell types which demonstrated basal expression of hsp70 mRNA in the normal brain. The results were obtained using a dual non-radioactive and radioactive in situ hybridization protocol [30] which allowed colocalization of two message populations within the same cells. In addition, this protocol uses post-fixation of cryostat tissue sections which enhanced detection of basal expression of hsp70 mRNA. We examine hsp70 and hsc70 mRNA expression in cortical layers and the hippocampus. To aid in the identification of neurons in these regions, neuron-specific enolase (nse) was used as a neuronal marker.

2. Methods*2.1. Treatment of animals*

The body temperature of adult male New Zealand white rabbits (mean weight = 1.9 kg) was elevated 2.5–3°C

* Corresponding author. Fax: (1) (416) 287-7642.

above normal (39.6°C) by the intravenous injection of LSD at 100 µg/kg as previously described [11]. We have shown that the induction of hsp70 mRNA in the rabbit brain is due to hyperthermic effects of this drug [20]. The colonic temperature of the animals was monitored, prior to injection and at 15-min intervals, with a rectal thermistor probe. The body temperature reached maximum temperature at 1 h post-LSD injection, at which time animals were killed.

2.2. Synthesis of riboprobes

The subclone pH 2.3, a 2.3 kb *HindIII*–*BamHI* fragment of the human *hsp70* inducible gene in pGEM1, was obtained from R. Morimoto [44]. An 843 bp insert was digested out with *Clal* and *HindIII* and subcloned into the vector pBluescript KS for preparation of hsp70 riboprobes. Linearization with *Clal* or *HindIII* and subsequent in vitro transcription incorporating ³²P-, ³⁵S- or digoxigenin-labelled UTP using T7 or T3 polymerases produced antisense and sense hsp70 riboprobes, respectively.

The subclone pH 7.6, a 600 bp *EcoRI* fragment from the human constitutive hsc70 related protein p70 in pGEM1 was obtained from R. Morimoto [41]. The 600 bp *EcoRI* fragment was digested out and subcloned into the vector pBluescript KS for preparation of hsc70 riboprobes. Production of antisense and sense hsc70 riboprobes was achieved by linearization with *HindIII* or *BamHI*, followed by in vitro transcription incorporating ³²P-, ³⁵S- or digoxigenin-labelled UTP by T7 or T3 polymerases, respectively.

The subclone pCD69, a 270 bp cDNA encoding neuron-specific enolase (nse) was obtained from J. Gregor Sutcliffe [12]. A 563 bp insert was digested out with *PstI* and *HindIII* and subcloned into the vector pSP72 for preparation of [³⁵S]UTP labelled nse riboprobes. The plasmid was linearized with *PstI* or *HindIII* and transcribed with SP6 or T7 polymerases, respectively, to generate antisense and sense nse riboprobes.

2.3. Northern blot analysis

Total RNA was isolated from control and hyperthermic thalamus, cortical layers (included corpus callosum and layers I–VI), hippocampus, kidney and liver. RNA samples were run on a formaldehyde/agarose gel (5 µg RNA/lane), blotted overnight to a nylon membrane, and fixed under ultraviolet light. Parallel blots were hybridized with ³²P-labelled hsc70 or hsp70 riboprobes. Equal loading was determined by methylene blue staining.

2.4. Tissue preparation

Animals were anaesthetized with pentobarbitol (80 mg/kg) injected via the marginal ear vein. Brains were

removed, frozen in OCT embedding compound and stored at –70°C until use. Data shown are representative of three control and three hyperthermic rabbits. A series of 12 µm frozen sections were thaw mounted on gelatin-coated glass slides (1% gelatin, 0.5% chromium potassium sulphate) and air dried.

2.5. Pretreatment of sections

Tissue sections were fixed in 4% formalin (4% formaldehyde in phosphate buffered saline) for 5 min, rinsed for 2 min in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), incubated for 10 min in TEA/NaCl/AA (0.1 M triethanolamine, 0.9% sodium chloride, 0.5% acetic anhydride) and rinsed in 2 × SSC. Slides were dehydrated in increasing ethanol concentrations, incubated in chloroform for 5 min, to delipidate tissue, followed by 1 min in absolute alcohol, 1 min 95% alcohol and air dried [30].

2.6. Radioactive in situ hybridization

Tissue sections were hybridized for 5 h, at 55°C, with 100 µl of hybridization buffer (50% formamide, 10% dextran sulphate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50 mM dithiothreitol, 0.5 mg/ml yeast tRNA, 0.64 mg/ml herring testis DNA, 100 mM sodium acetate, 750 mM NaCl, 75 mM sodium citrate, pH 7.0), containing 2 × 10⁶ c.p.m. of one of the following [³⁵S]UTP labelled riboprobes; hsc70 antisense or sense, hsp70 antisense or sense. Slides were rinsed in Tris/NaCl buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0), incubated for 30 min in RNase buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5) containing 20 µg/ml RNase A, and washed for 1 h at 37°C in RNase buffer containing 0.3 M β-mercaptoethanol followed by 1 h wash at 70°C in 0.1 × SSC, 0.3 M β-mercaptoethanol. The slides were dehydrated through an ethanol series containing 0.33 M ammonium acetate, air dried and set up with autoradiographic X-ray film.

2.7. Non-radioactive in situ hybridization

Tissue sections were hybridized for 5 h, at 55°C, with 100 µl of hybridization buffer containing 1 µl of one of the following DIG-UTP labelled riboprobes; hsc70 antisense or sense, hsp70 antisense or sense. Slides were rinsed in Tris/NaCl buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0), incubated for 30 min in RNase buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5) containing 20 µg/ml RNase A, and washed for 1 h at 37°C in RNase buffer followed by 1 h wash at 70°C in 0.1 × SSC. After post-hybridization washes, slides were rinsed in 2 × SSC and then incubated in blocking solution (2 × SSC, 6% fetal calf serum, 0.05% Triton X-100) for 1.5 h at room temperature. After blocking, slides were

Fig. 1. Northern blot analysis with hsc70 and hsp70 riboprobes. Total RNA was isolated from control (C) and 1 h hyperthermic (HS) thalamus, (THA) cortical layers (CL), hippocampus (HP), kidney (KID), liver (LIV) and subjected to Northern blot analysis. Blots ($5 \mu\text{g}$ RNA/lane) were hybridized with ^{32}P -labelled hsc70 or hsp70 riboprobes. The hsc70 riboprobe (Panel A) detected a 2.5 kb mRNA species in control and heat shock tissues. The hsp70 riboprobe (Panel B) detected a 2.7 kb mRNA species with no cross hybridization to the constitutively expressed 2.5 kb mRNA species. hsp70 mRNA was detected in control thalamus, hippocampus, kidney, and at low levels in cortical layers. An induction of hsp70 mRNA was observed in 1 h hyperthermic animal. Equal loading of mRNA was verified by methylene blue staining.

Fig. 2. Expression patterns of hsc70 and hsp70 mRNAs in the control and hyperthermic rabbit brain. Control and heat shock brains were processed for in situ hybridization with ^{35}S -labelled hsc70 or hsp70 riboprobes. As shown in panel A, strong hsc70 mRNA expression was observed in control cortical layers (CL), hippocampus (HP) and thalamic (THA) regions. A similar hsc70 mRNA pattern of expression was observed in 1 h hyperthermic tissue (Panel C). In the control forebrain expression of hsp70 mRNA (Panel B) was detected in the hippocampal region (HP), cortical layers (CL) and thalamic regions (THA). As shown in panel D, following 1 h of hyperthermia, hsp70 mRNA was induced in cortical layers (CL) and in fibre tracts such as the corpus callosum (CC) and fimbria (F). Bar = 2.5 mm.

Fig. 3. Expression of hsc70 and hsp70 mRNAs in control hippocampal neurons. Non-radioactive in situ hybridization using DIG-UTP labelled hsc70 (A) or hsp70 (B) riboprobes revealed constitutive expression of hsc70 and hsp70 mRNA species in hippocampal neurons (arrows) of control animals. The black precipitate indicates mRNA localization. Bar = 16.3 μ m.

incubated with anti-dioxigenin-alkaline phosphatase (1:500) in blocking solution for 24–36 h at 4°C. Sections were washed three times for 30 min in Buffer A (100 mM

Tris, pH 8.0, 100 mM NaCl), 5 min in AP Buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20), and 5 min in AP Buffer with 5 mM lev-

Fig. 4. hsc70 mRNA and hsp70 mRNA were expressed in the same cells of the hippocampus. Dual in situ hybridization of tissue sections with ^{35}S -labelled hsc70 and DIG-labelled hsp70 riboprobes revealed identical patterns of expression for these two message populations in the hippocampus. hsc70 mRNA expression is visualized by darkfield microscopy in panel A. hsp70 mRNA expression is revealed by brightfield microscopy in panel C. Higher magnification is shown in panels B and D. Panel D focuses on the black precipitate (hsp70 mRNA) and panel B focuses on the overlying silver grains (hsc70 mRNA). Arrows indicate cells which showed colocalization of hsc70 mRNA and hsp70 mRNA. Bar (A,C) = 800 μ m; (B,D) = 10.6 μ m.

amisole. Slides were incubated in NBT/BCIP colour reagent in AP Buffer with 5 mM levamisole for 4–6 h. Slides were rinsed in Buffer A to halt the colour reaction, rinsed in water, dehydrated in 70% ethanol, air dried, and mounted in 50% permount/50% xylene.

2.8. Dual in situ hybridization

Each tissue section was hybridized for 5 h, at 55°C, with 100 μ l of hybridization buffer containing 1 μ l of DIG-labelled riboprobe, 1×10^6 c.p.m. of 35 S-labelled riboprobe. Combinations of riboprobes included: [35 S]NSE and DIG-hsc70; [35 S]NSE and DIG-hsp70; and [35 S]hsc70 and DIG-hsp70. Following hybridization, slides were washed in four changes of $4 \times$ SSC, for 15 min at room temperature, incubated for 30 min in RNase buffer containing 20 μ g/ml RNase A, and washed sequentially for 15 min at room temperature in: $2 \times$ SSC, 10 mM dithiothreitol; $1 \times$ SSC, 10 mM dithiothreitol; $0.5 \times$ SSC, 10 mM dithiothreitol. Slides were then washed for 15 min at 55°C in $0.1 \times$ SSC, 10 mM dithiothreitol.

Following washes, non-radioactive signal was detected by antibody detection procedures described previously for the non-radioactive protocol. After incubation in NBT/BCIP colour mixture, slides were transferred to Buffer A to halt the colour reaction, rinsed in water, dehydrated in 70% ethanol, and air dried. Slides were dipped in 1% parlodion dissolved in acetone and processed with NTB2 Kodak emulsion to detect the radioactive signal [30].

3. Results

3.1. Northern blot analysis

The expression of constitutive hsc70 mRNA and stress-inducible hsp70 mRNA was examined by Northern blotting in three regions of the rabbit forebrain, namely the thalamus (THA), cerebral cortical layers (CL), and hippocampus (HP) using riboprobes that we have previously shown to be specific in discriminating hsc70 and hsp70

Fig. 5. Colocalization of neuron-specific enolase mRNA confirmed constitutive expression of hsc70 mRNA in hippocampal neurons. Tissue sections were hybridized with 35 S-labelled nse and DIG-labelled hsc70 riboprobes. Darkfield microscopy revealed neuron-specific enolase (nse) mRNA expression in neuronal-enriched regions of the hippocampus (A). Brightfield microscopy of the same tissue section revealed a similar pattern of expression for hsc70 mRNA (C). Higher magnification in panel B focuses on overlying silver grains (nse) while panel D focuses on black precipitate (hsc70 mRNA). Arrows indicate colocalization of nse mRNA and hsc70 mRNA in the same hippocampal cell. Bar (A,C) = 800 μ m; (B,D) = 10.6 μ m.

mRNA [8,22]. As shown in Fig. 1A, the constitutive hsc70 riboprobe detected a 2.5 kb mRNA species in the three brain regions, kidney (KID), and liver (LIV) of both control (C) and 1 h hyperthermic rabbits (HS). In contrast, the inducible hsp70 riboprobe detected a robust induction of a 2.7 kb mRNA species in brain regions, kidney, and liver following 1 h hyperthermic treatment (Fig. 1B). hsp70 mRNA was not detected in control liver, however, low basal levels of this mRNA species were noted in the hippocampus, cortical layers, thalamus, and kidney of control animals. These findings have been confirmed at the protein level since Western blotting studies detect the presence of hsp70 isoforms in the control rabbit brain [24].

3.2. *In situ* hybridization with ^{35}S -labelled riboprobes

Following the Northern blot detection of basal expression of stress-inducible hsp70 mRNA in forebrain regions of the control rabbit (Fig. 1B), *in situ* hybridization studies were undertaken to determine the cellular pattern of this

expression. As shown in Fig. 2A, hybridization with an ^{35}S -labelled hsc70 riboprobe revealed constitutive hsc70 expression in neuronal-enriched areas such as the hippocampus (HP), thalamus (THA), and cortical layers (CL) in control animals. This pattern of hsc70 mRNA expression did not differ following 1 h of hyperthermia (Fig. 2C).

The ^{35}S -labelled hsp70 riboprobe detected basal expression of the hsp70 mRNA species in the hippocampus, cortical layers, and thalamus of the control animal (Fig. 2B), a pattern comparable to that observed for hsc70 mRNA in Fig. 2A. Following 1 h of hyperthermia, a robust induction of hsp70 mRNA was apparent in fibre tract regions enriched in glial cell bodies such as the corpus callosum (CC) and fimbria (F) (Fig. 2D), as we have previously reported [35]. We performed *in situ* hybridization using tissue which was postfixed following preparation of cryostat sections, since this procedure enhanced the detection of the low abundance hsp70 mRNA species in control brain tissue.

Fig. 6. Colocalization of neuron-specific enolase mRNA confirmed constitutive expression of hsp70 mRNA in hippocampal neurons. Tissue sections were hybridized with ^{35}S -labelled nse and DIG-labelled hsp70 riboprobes. Darkfield microscopy revealed nse mRNA expression in neuronal-enriched regions of the hippocampus (A). Brightfield microscopy of the same tissue section revealed a similar pattern of expression for hsp70 mRNA (C). Panel B focuses on overlying silver grains (nse) and panel D focuses on black precipitate (hsp70 mRNA). Arrows indicate colocalization of nse mRNA and hsp70 mRNA in hippocampal neurons. Bar (A,C) = 800 μm ; (B,D) = 10.6 μm .

3.3. Cellular localization of *hsc70* and *hsp70* mRNA species in the hippocampus

To investigate the cell types in the hippocampus of the control rabbit which showed basal expression of *hsp70* mRNA, a non-radioactive in situ hybridization procedure using digoxigenin (DIG)-labelled riboprobes was employed. This method allowed better localization of signal to individual cells compared to in situ hybridization with ^{35}S -labelled riboprobes. Fig. 3 shows adjacent tissue sec-

tions hybridized with either *hsc70* or *hsp70* DIG-labelled riboprobes (left and right panels, respectively). The presence of mRNA is visualized as a black precipitate. These results suggested that hippocampal neurons (arrows) in the control rabbit brain expressed *hsc70* mRNA (Fig. 3A) and *hsp70* mRNA (Fig. 3B).

To establish whether *hsc70* and *hsp70* mRNA were expressed in the same cell type, a dual in situ hybridization protocol was employed. This protocol allowed hybridization of ^{35}S -labelled *hsc70* and DIG-labelled *hsp70* ribo-

Fig. 7. *hsc70* and *hsp70* mRNAs were constitutively expressed in cortical neurons. Tissue sections were hybridized with both ^{35}S -labelled *nse* and DIG-labelled *hsc70* riboprobes (A,B) or both ^{35}S -labelled *nse* and DIG-labelled *hsp70* riboprobes (C,D). Darkfield microscopy revealed neuron-specific enolase (*nse*) mRNA expression in cortical neurons (A,C). Brightfield microscopy of the same tissue sections revealed a similar pattern of expression for *hsc70* mRNA (B) and *hsp70* mRNA (D) in cortical layers. Tissue sections are unstained and the black precipitate in B and D revealed mRNA localization. Bar = 1.6 mm.

probes to the same tissue section. As shown in Fig. 4, the distribution of the two mRNA species was similar in the control hippocampus as viewed in panel A for hsc70 mRNA by darkfield microscopy and in panel C for hsp70 mRNA by brightfield microscopy. Higher magnification (right panels in Fig. 4), revealed that cells (indicated by arrows) which expressed hsc70 mRNA (panel B focuses on silver grains) also showed the presence of hsp70 mRNA (panel D focuses on the black precipitate).

To confirm that the hippocampal cells expressing hsc70 mRNA and hsp70 mRNA were neurons, dual in situ hybridization was carried out on tissue sections using a ^{35}S -labelled neuron-specific enolase (nse) riboprobe as a neuronal marker and either a DIG-labelled hsc70 riboprobe (Fig. 5) or a DIG-labelled hsp70 riboprobe (Fig. 6). The overall distribution of nse mRNA in the hippocampus paralleled that observed for hsc70 mRNA and hsp70 mRNA (compare panels A and C in Figs. 5 and 6). At the cellular level (right panels in Figs. 5 and 6), colocalization of neuron-specific enolase with cells expressing hsc70 mRNA and hsp70 mRNA (Fig. 5B and D and Fig. 6B and D,

respectively) confirmed that both species of heat shock mRNAs were present in the hippocampal neurons of the control rabbit brain. These results demonstrate basal expression of hsp70 mRNA in hippocampal neurons of the unstressed rabbit which also express hsc70 mRNA.

3.4. Expression of hsc70 and hsp70 mRNA in cortical layers

In the Northern blot analysis (Fig. 1), low levels of hsp70 mRNA were also detected in cortical layers of the forebrain of the control rabbit. Dual in situ hybridization with ^{35}S -labelled neuron-specific enolase (nse) riboprobe and DIG-labelled hsc70 or hsp70 riboprobes demonstrated that the distribution of nse mRNA (Fig. 7A and C) in cortical layers of the control rabbit brain was similar to that observed for hsc70 mRNA (Fig. 7B) and hsp70 mRNA (Fig. 7D). At the cellular level, colocalization of nse mRNA with hsc70 mRNA (Fig. 8A and B) and hsp70 mRNA (Fig. 8C and D) demonstrated that these heat shock mRNAs are expressed in cortical neurons. In addition, we

Fig. 8. Constitutive expression of neuron-specific enolase, hsc70, and hsp70 mRNAs in cortical layers. Tissue sections were hybridized with either ^{35}S -labelled nse and DIG-labelled hsc70 riboprobes (A,B) or ^{35}S -labelled nse and DIG-labelled hsp70 riboprobes (C,D). Colocalization of nse and heat shock mRNAs confirmed expression of hsc70 and hsp70 mRNA in cortical neurons. Left panels focus on overlying silver grains (nse mRNA), right panels focus on black precipitate (B, hsc70 mRNA; D, hsp70 mRNA). Bar = 14.6 μm .

Fig. 9. Dual in situ hybridization colocalized hsc70 and hsp70 mRNA expression in control cortical layers. Tissue sections were hybridized with ^{35}S -labelled hsc70 and DIG-labelled hsp70 riboprobes. Darkfield microscopy revealed constitutive hsc70 mRNA expression in cortical layers (A). Brightfield microscopy of the same tissue section showed a similar hsp70 mRNA expression pattern (black precipitate) (B). Higher magnification showed these mRNAs were colocalized in the same cell. Panel C focuses on the overlying silver grains (hsc70 mRNA) and panel D focuses on the black precipitate (hsp70 mRNA). Bar (A,B) = 1.6 mm; (C,D) = 10.6 μm .

hybridized ^{35}S -labelled hsc70 and DIG-labelled hsp70 riboprobes to the same tissue sections. This dual in situ hybridization showed colocalization of hsc70 mRNA and hsp70 mRNA in the same cortical cells (Fig. 9). These results indicate that cortical neurons in the unstressed rabbit brain demonstrate basal expression of hsp70 mRNA in addition to expressing hsc70 mRNA.

4. Discussion

In this study, expression of hsc70 and hsp70 mRNA was characterized in the unstressed rabbit brain. Both Northern blot analysis and radioactive in situ hybridization experiments revealed basal expression of hsp70 mRNA in

control forebrain regions including hippocampus, cortical layers, and thalamus. Our ability to detect the presence of the stress-inducible hsp70 mRNA species in the normal rabbit brain was a result of modified tissue preparation with respect to fixation and freezing. Earlier investigations in our laboratory used tissue that was perfused with 4% phosphate-buffered paraformaldehyde prior to freezing [13,20–23], while in the present investigation, fresh tissue was immediately frozen in OCT embedding compound, and sectioned on a cryostat. Sections were postfixed with 4% formalin following sectioning. Control tissue sections hybridized with ^{35}S -labelled heat shock riboprobes showed similar expression patterns for hsc70 and hsp70 mRNAs. Non-radioactive in situ and dual DIG/ ^{35}S in situ hybridization protocols were employed to characterize the

cell types expressing these heat shock mRNAs in control cortical layers and hippocampus.

The presence of hsc70 and hsp70 mRNAs was observed in hippocampal neurons by non-radioactive in situ hybridization. To confirm that these two message populations were colocalized, we utilized a dual in situ hybridization protocol [30]. Hybridization of tissue sections with ³⁵S-labelled hsc70 and DIG-labelled hsp70 riboprobes revealed expression of both message populations in the same hippocampal cells. Using the dual in situ hybridization protocol, we also observed expression of hsc70 and hsp70 mRNAs in cells in the cortical layers. At high magnification, colocalization of hsc70 and hsp70 mRNAs to individual cells was observed. Dual in situ hybridization experiments were extended to include hybridization with a neuron-specific enolase (nse) riboprobe. Hybridization with nse-hsc70 and nse-hsp70 riboprobe combinations localized hsc70 mRNA and hsp70 mRNA to hippocampal and cortical neurons.

Detection of basal levels of hsp70 mRNA in control brain regions is consistent with observations using one- and two-dimensional Western blot analysis in our laboratory which demonstrated the presence of hsp70 protein in control rabbit brain [24]. Other researchers have also reported basal expression of hsp70 in unstressed cells. Results from tissue culture show high levels of hsc70 and low levels of hsp70 in control HeLa cells [41]. Western blot analysis of neural and non-neural tissues revealed hsp70 protein in control rat and mouse tissues [18,36,39] and Northern blot analysis has revealed hsp70 mRNA in control rat brain [19]. These reports examined overall levels of hsp70 mRNA and protein by Northern blot and Western blot analyses. In the present study, basal hsp70 mRNA expression is examined at the cellular level and localized to neurons within the control rabbit brain which also express hsc70 mRNA.

In our current investigation we observed constitutive expression of hsc70 mRNA in neurons of the normal rabbit forebrain. These observations are consistent with previous observations in our laboratory which reported constitutive neuronal expression patterns for hsc70 mRNA in the control rabbit cerebellum, brain stem and spinal cord [13,21,22]. It is well-established that hsc70 protein is abundant in brain and it comprises 1% of soluble protein content [33]. Brain tissue is often the source used for purification of hsc70 protein [9,10,25,31,38]. The presence of hsp70 mRNA in normal rabbit brain and its localization to neurons is a novel observation. Although regulation of basal expression of hsp70 is not understood, considerable attention has been given to investigating the transcriptional regulation of hsp70 following stress. In mammalian cells, transcription of hsp70 mRNA following stress results from the activation of the heat shock transcription factor (HSF1) [32]. In the unstressed cell, HSF1 is found in an inactive, non DNA-binding, monomer form (reviewed in [27,28]). Levels of hsp70 mRNA in control neurons and other

control cell types may be regulated by basal transcription elements in the hsp70 promoter and not through HSF1 activation.

The function of hsp70 protein in the unstressed neuron is unknown. hsp70 and hsc70 protein have been shown to be functionally similar [9] and it has been suggested that constitutively expressed hsp70/hsc70 protein may play a role in regulating HSF1 activation by binding to the monomer form to maintain it in an inactive conformation [1,2]. In addition, hsc70/hsp70 proteins associate with newly synthesized proteins [3,29] and are involved in protein folding and translocation to different cellular compartments [3,4,14–16,43]. We observe constitutive expression of both hsc70 and hsp70 mRNAs in forebrain neurons of the unstressed rabbit. Constitutive hsc70 mRNA is found in many normal cell types while the detection of basal expression of hsp70 mRNA is limited to certain cell types. This basal expression of the stress-inducible hsp70 mRNA may result from alternative heat shock gene regulation strategies adopted by certain cell types, including neurons.

Acknowledgements

This research was supported by grants to I.R.B. from the Medical Research Council of Canada.

References

- [1] Abravaya, K., Myers, M.P., Murphy, S.P. and Morimoto, R.I., The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression, *Genes Dev.*, 6 (1992) 1153–1164.
- [2] Baler, R., Welch, W.J. and Voellmy, R., Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor, *J. Cell Biol.*, 117 (1992) 1151–1159.
- [3] Becker, J. and Craig, E.A., Heat-shock proteins as molecular chaperones, *Eur. J. Biochem.*, 219 (1994) 11–23.
- [4] Beckman, R.P., Mizzen, L.A. and Welch, W.J., Interaction of hsp 70 with newly synthesized proteins: implications for protein folding and assembly, *Science*, 248 (1990) 850–854.
- [5] Bhattacharyya, T., Karnezis, A.N., Murphy, S.P., Hoang, T., Freeman, B.C., Phillips, B. and Morimoto, R., Cloning and subcellular localization of human mitochondria hsp70, *J. Biol. Chem.*, 270 (1995) 1705–1710.
- [6] Brown, I.R., Induction of heat shock (stress) genes in the mammalian brain by hyperthermia and other traumatic events: a current perspective, *J. Neurosci. Res.*, 27 (1990) 247–255.
- [7] Brown, I.R., Induction of heat shock genes in the mammalian brain by hyperthermia and tissue injury. In R.J. Mayer and I.R. Brown (Eds.), *Heat Shock and the Nervous System*, Academic Press, London, 1994, pp. 31–53.
- [8] Brown, I.R. and Rush, S.J., Expression of heat shock genes (hsp70) in the mammalian brain: distinguishing constitutively and hyperthermia-inducible mRNA species, *J. Neurosci. Res.*, 25 (1990) 14–19.
- [9] Brown, R.C., Martin, R.L., Hansen, W.J., Beckman, R.P. and Welch, W.J., The constitutive and stress inducible forms of hsp70 exhibit functional similarities and interact with one another in an ATP-dependent fashion, *J. Cell Biol.*, 120 (1993) 1101–1112.

- [10] Chappell, T.G., Welch, W.J., Schlossman, D.M., Palter, K.B., Schlesinger, M.J. and Rothman, J.E., Uncoating ATPase is a member of the 70 kdalton family of stress proteins, *Cell*, 45 (1986) 3–13.
- [11] Cosgrove, J.W. and Brown, I.R., Heat shock protein in the mammalian brain and other organs following a physiologically relevant increase in body temperature induced by LSD. *Proc. Natl. Acad. Sci. USA*, 80 (1983) 569–573.
- [12] Forss-Petter, S., Danielson, P. and Sutcliffe, J.G., Neuron-specific enolase: complete structure of rat mRNA, multiple transcription start sites, and evidence suggesting post-transcriptional control, *J. Neurosci. Res.*, 16 (1986) 141–156.
- [13] Foster, J.A., Rush, S.J. and Brown, I.R., Localization of constitutive and hyperthermia-inducible heat shock mRNAs (hsc70 and hsp70) in the rabbit cerebellum and brain stem by non-radioactive in situ hybridization, *J. Neurosci. Res.*, 41 (1995) 603–612.
- [14] Georgopoulos, C. and Welch, W.J., Role of the major heat shock proteins as molecular chaperones, *Annu. Rev. Cell Biol.*, 9 (1993) 601–634.
- [15] Gething, M.-J. and Sambrook, J., Protein folding in the cell, *Nature*, 355 (1992) 33–45.
- [16] Hartl, F.-U., Hlodan, R. and Langer, T., Molecular chaperones in protein folding: the art of avoiding sticky situations, *Trends Biochem. Sci.*, 19 (1994) 20–25.
- [17] Lindquist, S. and Craig, E.A., The heat shock proteins, *Annu. Rev. Genet.*, 22 (1988) 631–677.
- [18] Locke, M., Noble, E.G. and Atkinson, B.G., Inducible isoform of HSP70 is constitutively expressed in a muscle fiber type specific pattern, *Am. J. Physiol.*, 261 (1991) C774–C779.
- [19] Longo, F.M., Wang, S., Narasimhan, P., Zhang, J.S., Chen, J., Massa, S.M. and Sharp, F.R., cDNA cloning and expression of stress-inducible rat hsp70 in normal and injured rat brain, *J. Neurosci. Res.*, 36 (1993) 325–335.
- [20] Manzerra, P. and Brown, I.R., Time course induction of a heat shock gene (hsp70) in the rabbit cerebellum after LSD in vivo: involvement of drug-induced hyperthermia, *Neurochem. Res.*, 15 (1990) 53–59.
- [21] Manzerra, P. and Brown, I.R., Distribution of constitutive- and hyperthermia-inducible heat shock mRNA species (hsp70) in the Purkinje layer of the rabbit cerebellum, *Neurochem. Res.*, 17 (1992) 559–564.
- [22] Manzerra, P. and Brown, I.R., Expression of heat shock genes (hsp70) in the rabbit spinal cord: localization of constitutive and hyperthermia-inducible mRNA species, *J. Neurosci. Res.*, 31 (1992) 606–615.
- [23] Manzerra, P., Rush, S.J. and Brown, I.R., Temporal and spatial distribution of heat shock mRNA and protein (hsp70) in the rabbit cerebellum in response to hyperthermia, *J. Neurosci. Res.*, 36 (1993) 480–490.
- [24] Manzerra, P., Rush, S.J. and Brown, I.R., Constitutive expression of heat shock protein 70 (hsp70) in the rabbit nervous system, *Neurosci. Abs.*, 20 (1994) 1506.
- [25] Mifflin, L.C. and Cohen, R.E., hsc70 moderates the heat shock (stress) response in *Xenopus laevis* oocytes and binds to denatured protein inducers, *J. Biol. Chem.*, 269 (1994) 15718–15723.
- [26] Milarski, K.L. and Morimoto, R.I., Mutational analysis of the human HSP70 protein: distinct domains for nucleolar localization and adenosine triphosphate binding, *J. Cell. Biol.*, 109 (1989) 1947–1962.
- [27] Morimoto, R.I., Cells in stress: transcriptional activation of heat shock genes, *Science*, 259 (1993) 1409–1410.
- [28] Morimoto, R.I., Jurivich, D.A., Kroeger, P.E., Mathur, S.K., Murphy, S.P., Nakai, A., Sarge, K., Abravaya, K. and Sistonen, L.T., Regulation of heat shock gene transcription of a family of heat shock factors. In R.I. Morimoto, A. Tissieres and C. Georgopoulos (Eds.), *The Biology of Heat Shock Proteins and Molecular Chaperones*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1994, pp. 417–455.
- [29] Palleros, D.R., Welch, W.J. and Fink, A.L., Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 5719–5723.
- [30] Peterson, S.L. and McCrone, S., Characterization of the receptor complement of individual neurons using dual-label in situ hybridization histochemistry. In J.H. Eberwine, K.L. Valentino and J.D. Barchas (Eds.), *In Situ Hybridization in Neurobiology: Advances in Methodology*, Oxford University Press, New York, 1994, pp. 78–95.
- [31] Sadis, S., Raghavendra, K. and Hightower, L.E., Secondary structure of the mammalian 70-kilodalton heat shock cognate protein analyzed by circular dichroism spectroscopy and secondary structure prediction, *Biochem.*, 29 (1990) 8199–8206.
- [32] Sarge, K.D., Murphy, S.P. and Morimoto, R.I., Activation of heat shock gene transcription by HSF1 involves oligomerization, acquisition of DNA binding activity, and nuclear localization and can occur in the absence of stress, *Mol. Cell. Biol.*, 13 (1993) 1392–1407.
- [33] Schlossman, D., Schmid, S., Braell, N. and Rothman, J., An enzyme that removes clathrin: purification of an uncoating ATPase, *J. Cell Biol.*, 99 (1984) 723–733.
- [34] Sorger, P.K. and Pelham, H.R.B., Cloning and expression of a gene encoding hsc73, the major hsp70-like protein in unstressed cells, *EMBO J.*, 6 (1987) 993–998.
- [35] Sprang, G.K. and Brown, I.R., Selective induction of a heat shock gene in the fibre tracts and cerebellar neurons of the rabbit brain, *Mol. Brain Res.*, 3 (1987) 89–93.
- [36] Tanguay, R.M., Wu, Y. and Khandjian, E.W., Tissue-specific expression of heat shock proteins of the mouse in the absence of stress, *Dev. Genet.*, 14 (1993) 112–118.
- [37] Tsai, M.-Y. and Wang, C., Uncoupling of peptide-stimulated ATPase and clathrin-uncoating activity in deletion mutant of hsc70, *J. Biol. Chem.*, 269 (1994) 5958–5962.
- [38] Ungewickell, E., The 70-kDa mammalian heat shock proteins are structurally and functionally related to the uncoating protein that release clathrin triskelia from coated vesicles, *EMBO J.*, 4 (1985) 3385–3391.
- [39] Vamvakopoulos, N.O., Tissue-specific expression of heat shock proteins 70 and 90: potential implication for differential sensitivity of tissues to glucocorticoids, *Mol. Cell. Endocrinol.*, 98 (1993) 49–54.
- [40] Wang, T.-F., Chang, J.-H. and Wang, C., Identification of the peptide binding domain of hsc70, *J. Biol. Chem.*, 268 (1994) 26049–26051.
- [41] Watowich, S.S. and Morimoto, R.I., Complex regulation of heat shock- and glucose-responsive genes in human cells, *Mol. Cell. Biol.*, 8 (1988) 393–405.
- [42] Welch, W.J., Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease, *Physiol. Rev.*, 72 (1992) 1063–1081.
- [43] Welch, W.J., Heat shock protein functioning as molecular chaperones: their roles in normal and stressed cells, *Phil. Trans. R. Soc. London, B Ser.*, 339 (1993) 327–333.
- [44] Wu, B., Hunt, C. and Morimoto, R.I., Structure and expression of the human gene encoding major heat shock protein hsp70, *Mol. Cell. Biol.*, 5 (1985) 330–341.