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Molecular Modulation of Expression of Prion Protein by Heat Shock

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Abstract

Prion diseases (also known as transmissible spongiform encephalopathies) are associated with the conversion of the normal cellular form of the prion protein (PrP^{C}) to an abnormal scrapie-isoform (PrP^{Sc}. The conversion of PrP^{C} to PrP^{Sc} is post-translational and is owing to protein conformational change. This has led to the hypothesis that molecular chaperones may be involved in the folding of prion proteins, and hence the disease process. By treating human NT-2 cells with heat-shock stress, we found that both the mRNA levels for prion protein (PrP) and heat shock protein 70 (HSP7O) increased simultaneously after heat treatment. Western-blot analysis of PrP also showed a two-fold increase in PrP protein level 3 after heat treatment. Furthermore, two heat-shock elements (HSEs) were located at the positions of –680 bp (HSE1; G**GAA**CTA**TTC**TT**GAC**AT**TGC**T), and –1653 bp (HSE2; T**GAG**AA**CTC**AG**GAA**G) of the rat PrP (RaPrP) gene promoter. Luciferase reporter constructs of the RaPrP promoter with HSE expressed higher luciferase activity (10- to 15-fold) than those constructs without HSE. Electrophoretic gel mobility shift assay (EMSA) and super-shift assay confirmed the interaction of HSE1 and HSE2 with the heat-shock transcription factor-1 (HSTF-1). These results suggest that cellular stress up-regulates both the transcription and translation of PrP through interaction with the HSEs on the PrP gene promoter, resulting in an increase in protein synthesis.

Index Entries: Prion Protein; heat shock; stress-response protein; heat-shock element; transcriptional regulation.

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Introduction

The cellular prion protein (PrP^c is a 30–35 kDa glycoprotein of unknown function found at the cell surface mainly of neurons (Kretzschmar et al., 1986) and glial cells (Moser et al., 1995). The prion diseases are a group of transmissible and inherited neurodegenerative diseases caused by the conversion of the normal cellular form of prion protein (PrP^c) to an abnormal isoform (PrP^{Sc}). The prion diseases are characterized by spongiform cortical changes, neuronal loss, astrocytosis, and the lack of an inflammatory response (Lantos et al., 1992). However, the pathogenic mechanisms leading to clinical manifestations and death in prion disease are still unknown. It is characteristic of all prion diseases that PrP^{Sc} accumulates in neuronal cells in infected individuals (Bruce et al., 1987). Unlike PrP^c, PrP^{Sc}, which is devoid of nucleic acid extracellularly, is protease-resistant (Bolton et al., 1982). Therefore, PrP^{Sc} may be the agent of transmission or it may be associated with the transmissible agent (Prusiner et al., 1991).

The normal function of PrP in the brain remains unknown. Brown et al. (1999) have found that prion protein possesses similar activity of superoxide dismutase (SOD). Therefore, priori protein expresion may play a role in cellular resistance to oxidative stress (Brown and Besinger, 1998; Brown et al., 2001). In addition, in vivo studies have indicated that mice deficient in PrP ($Prnp^{o/o}$) (Manson et al., 1994) show various abnormalities including reduced LTP (Collinge et al., 1994), altered circadian rhythms (Tobler et al., 1996), and, in one strain, motor disturbances with increasing age of the mice (Sakaguchi et al., 1996).

The well-known example of a cellular adaptive protective response is a cell's response to elevated temperature, or the heat-shock response (Schlesinger et al., 1990), which upregulates a family of proteins, known as heat-shock proteins (HSPs). HSPs, such as heat-shock protein 70 (HSP70), are expressed constitutively and play an important role in protein synthesis and proteolysis. They act as molecular chaperones, preventing inappropriate protein-protein interactions, ensuring proper protein assembly, folding, and transport, and facilitating proteolytic digestion of abnormal or denatured polypeptides (Gething et al., 1992; Baler et al., 1992). Increased expression of HSP has been observed in the later stages of Scrapie-infected mouse brain (Kenward et al., 1994). Moreover, Kato et al. demonstrated that ballooned neurons in the brain of Creutzfeldt-Jakob disease patients reacted positively with antibodies against stress-response protein 27 (Kato et al., 1992).

Over-expression of the β-amyloid precursor protein (β-APP) gene could be a contributing factor to the aberrant processing of this precursor protein, suggesting the importance of investigating factors that induce this expression. Many reports have demonstrated an increase in β-APP mRNA in cultured cells following heat shock (Abe et al., 1991; Ciallella et al., 1994; Dewji et al., 1995, 1996). The regulatory mechanism for this response has been shown to be the activation of the β-APP gene promoter, which contains an HSE (Dewji et al., 1995, 1996). However, the upregulation of β-APP is dependent on the nuclear translocation of heat-shock transcriptional factor (HSTF), which is bound to the HSE in the promoter region of the β-APP gene and then turns on the expression of that gene.

Because of the similar pathogenic mechanisms in prion disease and Alzheimer's disease (DeArmond et al., 1993; Silei et al., 1999), it can reasonably be hypothesized that PrP gene may possess a similar regulatory pattern following heat shock as shown for the β-APP gene. Therefore, we investigated the induction of PrP using human NT-2 cells treated with heat shock followed by Northern- and Western-blot analysis. Subsequently, we cloned two fragments of the RaPrP gene promoter including the possible HSE into a luciferase expression vector. These recombinant constructs were transiently transfected into NT-2 cells. EMSA and supershift assays were used to confirm the interaction of the HSE with the HSTF activated as a result of heat shock.

Materials and Methods

Cells and Cell Culture

Human NT-2 cells (NTERA-2, pleuripotent, embryonal carcinoma cells, ATCC number 1973-CRL) were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose-containing 10% fetal calf serum (FCS), ampicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. The third to eighth passages of the NT-2 subcultures were used in this study. Stocks of NT-2 cells frozen in the third passage were used for other experiments.

Heat-Shock Procedure

Once the cells became confluent in a large flask (about 10^6 cells/mL), they were equally distributed into six smaller flasks (75 cm^2 in size). The six flasks were kept at 37°C in the incubator overnight prior to heat-shock treatment. Five of the six flasks were removed from the incubator for heat-shock treatment, with the remaining flask left in the incubator as a control. The lids of the flasks were quickly closed tightly in order to avoid losing any CO_2 during the heat-shock treatment. The flasks were placed in a 42°C water bath for about 30 min, after which thery were returned to the incubator. After heat shock, the cells were recovered in the incubator for recovery at 37°C for periods of 1, 3, 8, 24, and 48 h. At the completion of the specific recovery period, cells were collected separately from individual flasks in an Eppendorf tube by centrifugation at 1,200*g* for 2 min at 4°C. The supernatant was discarded, and the tubes were quickly frozen at –70°C until extraction of total RNA and protein. Cells from the control flask were collected and frozen in the same manner.

Total RNA Extraction and Northern Blot Analysis

Total RNA was extracted by the guanidine thiocyanate method as previously described (Chomczynski et al., 1987) (RNA isolation kit provided by RNAzo1™B, USA) for Northern-blot analysis, 17 µg of total RNA was electrophoresed onto a 1.2% TBE agarose gel (7 *M* urea, 5X TBE, 10% glycerol, 0.1% bromophenol blue and xylene cyanol), and transferred to nylon membranes (Hybond-N%, Amersham) by capillary action. The membranes were probed with a 228-bp fragment (fragment D) of PrP^CcDNA (Shyu et al., 1996) and a full-length HSP 70 cDNA (Hunt et al., 1985). Since mRNA recovery may vary from sample to sample, a housekeeping gene probe of GAPDH was used to normalize for RNA recovery and loading. The probes were radio-labeled with α [^{32}P]-dCTP by a random-priming method and hybridized on the membranes at 42°C for 20 h. After hybridization, the membranes were washed under stringent conditions and exposed to Hyperfilm™ MP (Amersham) with an intensifying screen at –70°C. Peak optical densities of the bands were determined by densitometry (Molecular Dynamics). Comparisons of experimental and control values of band intensities of autoradiograms were conducted using a paired *t*-test.

Construction of Reporter Plasmids and Transfection of Cells

The RaPrP promoter-luciferase reporter plasmid was constructed as described previously (Saeki et al., 1996). Four of these constructs: praPrP(-2831)-luc, praPrP(-1026)-luc, praPrP(-514)-luc, and pGL2-control (Promega) were used to transfect NT-2 cells. Cells were plated at a density of 1×10^6 in 60-mm plates and grown overnight. Then they were rinsed with serum-free medium and transiently transfected with 6 µg of supercoiled DNA (including 4 µg promoter-luciferase plasmid and 2 µg pCMV β-gal) using the lipofectamine (DC-chol) method. Briefly, the lipofectamine-DNA solution was left at room temperature for 30 min, mixed with enough serum-free medium and added to cells. The cells were incubated for 5 h at 37°C and 5% CO^2, after which the medium was aspirated and replaced with serum-containing medium.

Following transfection, cells were incubated at 42°C for 1 h and allowed to recover for up to 24 h. They were then harvested and lysed, and luciferase activity was determined as previously described (Trejo et al., 1992) using a luminometer (Lumat LB 9501). Luciferase activity was measured in triplicate following normalization by β-gal assay.

Site-Directed Mutagenesis of HSE by Polymerase Chain Reaction (PCR) and Luciferase Assay

In order to obtain a mutant of the heatshock element 1 (HSE1) within the RaPrP gene promoter, the following internal primers with a 5′-end phosphate designed for site-directed mutagenesis were synthesized: 5′-ACACCAGGAAGAAACTATTTTTAACATTG CTGTTTTAATTA G-3′. Two end primers were also synthesized to include 5′-*XcaI* and *SacII* linkers: 5′-GCCGTATACCATAATTTAATTCAT-ACAC-3′ and 5′-ATACCGCGGCCTCAAGTC-CCG-3′. The PCR method included 10XPCR reaction buffer, 20 m*M* of dNTP stock solution, 5 m*M* end primers and 7 m*M* internal primers, and 200 ng of plasmid DNA template of praPrP(-2831)-luc.

The initial cycle of PCR was carried out by heating to 94°C for 5 min to denature template DNA, followed by the addition of 1 µL of Taq polymerase (Gibco-BRL) (1 U/µL) and ampligase. Subsequent amplification (35 cycles) was performed under the following conditions: 94°C for 90 s, 60°C for 90s, and 72°C for 2 min. The PCR product was subcloned into plasmid of praPrP(-2831)-luc. This mutated clone was named praPrP(-2831M)-luc and verified by sequencing. Finally, a luciferase assay was performed as described earlier.

Electrophoretic Gel Mobility Shift Assay (EMSA) and Supershift Assay

Oligonucleotides corresponding to the consensus heat shock element within the RaPrP promoter (Saeki et al., 1996), 5′-ATCT**GA-G**AA**CTC**AG**GAA**GCTG-3′ (from –1667 to –1653) (HSE2) and 5′-GAAG**GAA**C-TA**TTC**TT**GAC**AT**TGC**TGTT-3′ (from –700 to –680) (HSE1) with its complementary strand, were synthesized. The two strands of DNA oligonucleotides were annealed together by heating to 65°C and cooling slowly to room temperature.

Preparation of nuclear extracts was performed as described by Gorski et al. (1986) from NT-2 cells that were heat-shocked at 42°C for 1 h and used immediately. The control nuclear extracts were obtained from NT-2 cells without heat treatment. Binding reactions were performed with 2 µg of protein, ^{32}P-labeled (5,000 cpm) double-stranded oligonucleotide probe, 50 m*M* KC1 and 1 µg poly(dl-dC) (Boehringer Mannheim) in a total volume of 12 µL. In competition experiments, a 100-fold molar excess of unlabeled competitor oligonucleotides was added together with ^{32}P-labeled oligonucleotides. Reactions were carried out for 30 min at-room temperature, and protein-DNA complexes were electrophoretically separated onto nondenaturing 5% polyacrylamide gel. In super-shift assays, polyclonal antibody (PAb) against HSTF-1 (Santa Cruz) was added to the gel mobility shift reaction. Gel was dried and subjected to autoradiography at –70°C.

Results

Heat Shock-Induced Transcription of PrP Gene

To study the effect of heat-shock treatment on the gene transcription of PrP, we carried out Northern-blot analysis. Human NT-2 cells were heat-shocked by placing the flasks in a 42°C water bath for 30 min. Figure 1 A shows the autoradiograms for PrP^{C}, HSP70 and GAPDH after heat-shock treatment. Quantitative analysis of the band intensities for PrP/GAPDH ratios revealed a 1.5° to 2.5-fold increase in PrP mRNA level at both 1 and 3 h after heat shock (Fig. IB). In addition, our previous results showed that the actinomycin

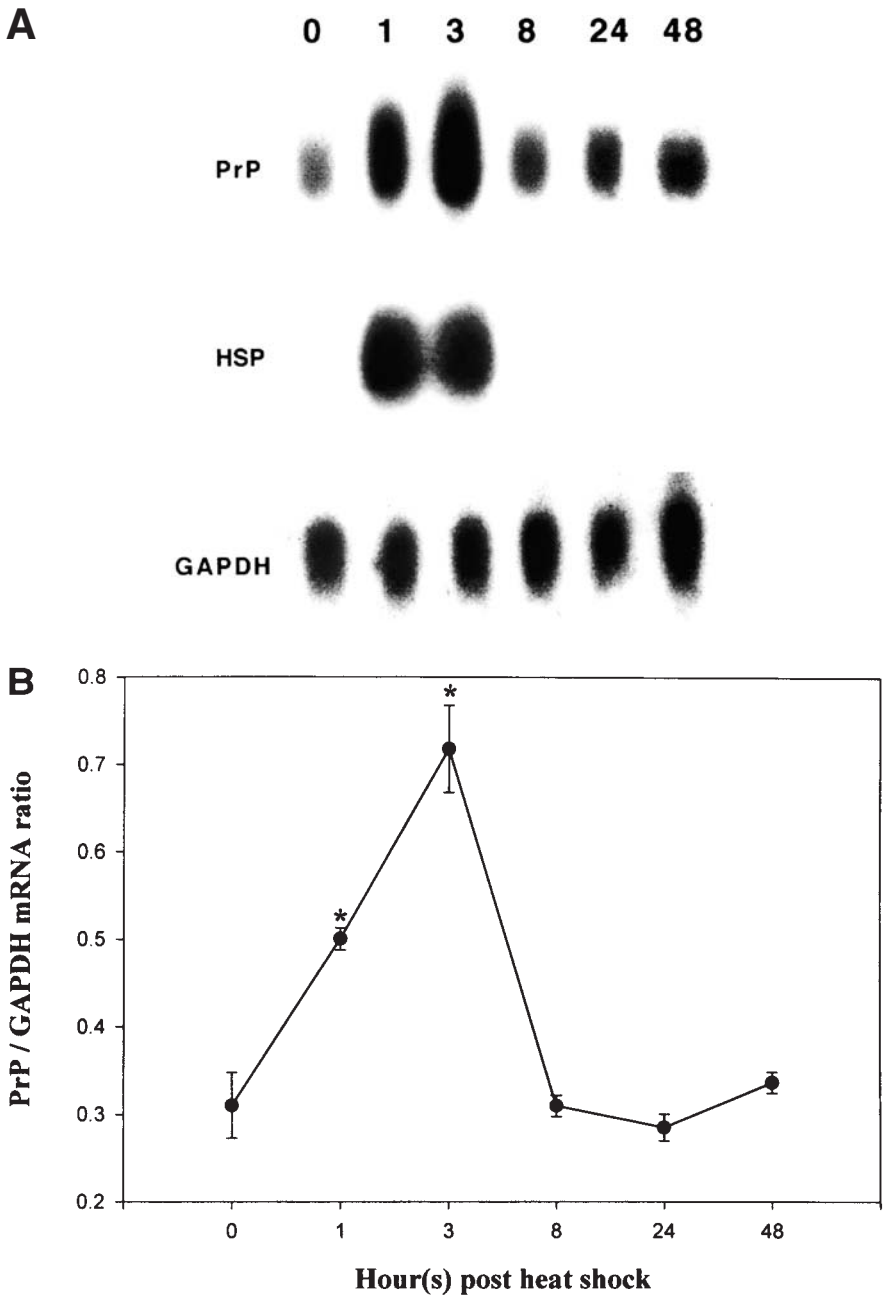

Fig. 1. Heat shock effects on PrP mRNA. **(A)** Northern-blot analysis of PrP (upper), HSP 70 (middle) and GAPDH (lower) after heat shock and recovery for 0, 1, 3, 8, 24, and 48 h, respectively. **(B)** A statistical densitometry plot for the PrP/GAPDH ratios against the time course as shown earlier after heat shock. A 1.5- and 2.5-fold increase of PrP mRNA were observed at 1 and 3 h post-heat shock, respectively ($*p < 0.01$).

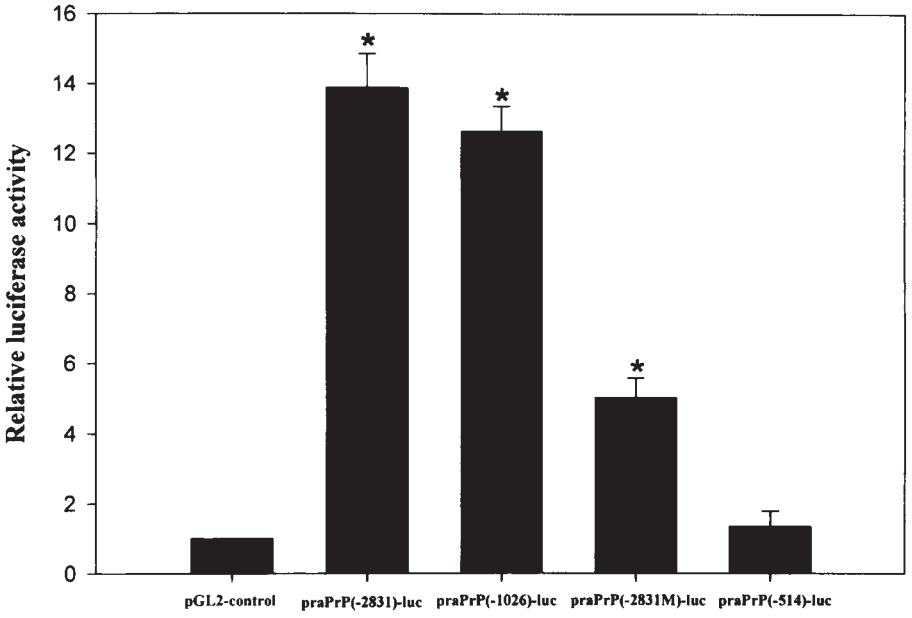

Fig. 2. Lipofectamine-mediated cell transfection and luciferase reporter assay. Following transfection, cells were grown overnight, and were either left untreated or heat-shocked for 1 h at 42°C and progressed to recover for up to 24 h. Luciferase activity was measured in triplicate and expressed as fold induction following normalization by β-gal assay. A ten- to fifteen-fold increase in luciferase activity of praPrP(-2831)-luc and praPrP(-1026)-luc was observed in comparison with praPrP(-514)-luc and pGL2-control. A four- to fivefold increase in praPrP(-2831M)-luc luciferase activity was observed in comparison with praPrP(-5 14)-luc and pGL2-control.

D-mediated inhibition of transcription blocked the heat-shock induction of PrP mRNA. This suggests that the increase in PrP expression was owing to an increase in the RNA synthesis rather than a decrease in RNA degradation.

Heat Shock-Induced Activation of PrP-Luciferase Reporter Gene

Cells were transiently transfected with 6 μg of reporter plasmid and pCMVβ-gal using lipofectamine method. Following transfection, cells were grown overnight, and were either left untreated or heat-shocked for 1 h at 42°C and progressed to recover for up to 24 h. Luciferase activity was measured in triplicate and expressed as fold induction following normalization by β-gal assay. Maximal induction of luciferase activity occurred between 5 and 10 h after heat shock. There was a significant difference between heat-treated and untreated cells. A ten- to fifteen-fold increase in luciferase activity of praPrP(-2831)-luc and praPrP(-1026)-luc was observed in comparison with praPrP(-514)-luc and pGL2-control under conditions of maximal induction (Fig. 2). This suggests that luciferase constructs containing HSE show more luciferase activity than the constructs without HSE.

Site-Directed Mutagenesis of HSE1 and Luciferase Assay

Site-directed mutagenesis of HSE1 by PCR was performed. PCR fragment was subcloned into plasmid of praPrP(-2831)-luc. This mutated plasmid was named praPrP(-2831M)-luc. The luciferase activity of praPrP(-2831M)-luc (Fig. 3) showed a four- to fivefold increase in comparison with praPrP(-514)-luc and pGL2-control (Fig. 2).

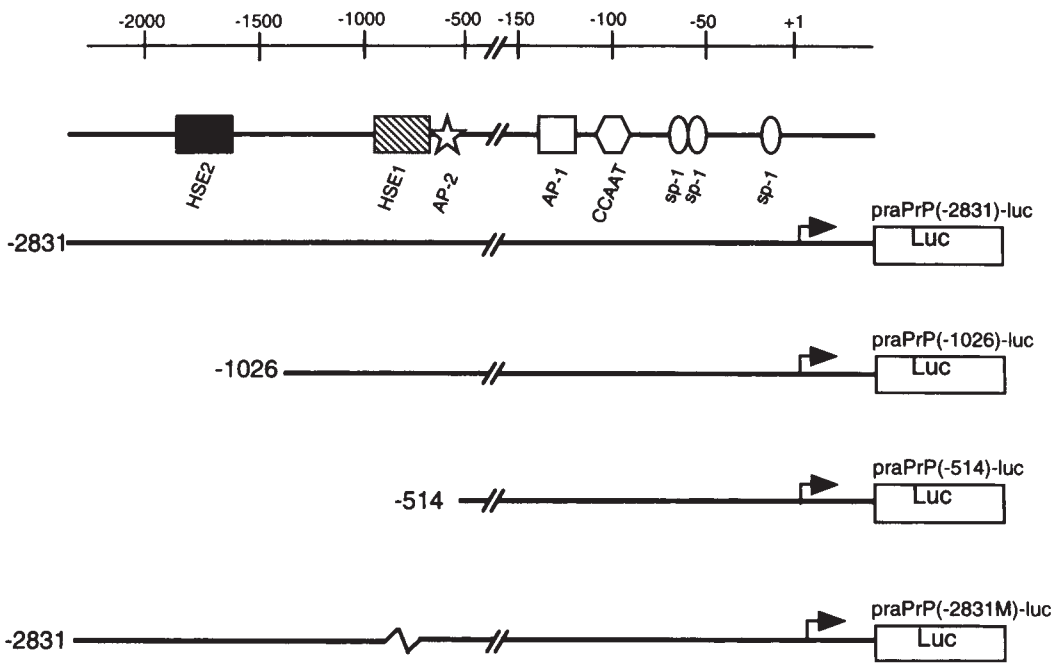

Fig. 3. Schematic representation of the RaPrP gene promoter and the construction of luciferase reporter plasmids. HSE1 and HSE2 are indicated by different shaded squares.

Heat Shock Effects on the Binding Activity of Nuclear Factor to HSE

In order to determine whether heat shock induces nuclear proteins that bind to the HSE consensus sequence present in the RaPrP promoter, EMSA and super-shift assay were performed. Protein-DNA binding activity was examined using HSE oligonucleotide probe. Nuclear extracts were prepared from NT-2 cells treated or not treated with heat shock at 42°C for 1 h. No or low protein binding activity to the HSE oligonucleotide probe was detected in cells without heat treatment (Fig. 4 and Fig. 5, lane b), while strong protein-DNA binding activity to the HSE. oligonucleotide was found in heat-shocked NT-2 cells. (Fig. 5 and Fig. 6, lane c). The heat-induced protein-DNA binding activity was completely inhibited in the presence of 50-fold and/or 100-fold molar excess of an unlabeled HSE oligonucleotide probe (Figs. 5 and 6, lane d). In the presence of the anti-HSTF-1 antibody, the protein-DNA complex cross-reacted with the antibody and super-shifted as shown by the arrow in Figs. 5 and 6 (lane e or f). These results further demonstrate that the specific nuclear protein that binding with the corresponding HSE seemed to be HSTF-1.

Discussion

Alterations in the degradation of the Scrapie-isoform prion protein (PrP^{Sc}) may play a central role in the pathogenesis of prion disease (Bolton et al., 1982; Prusiner et al., 1991). PrP^{Sc} is derived from post-translational modification of a cellular form of prion protein (PrP^{C}). Therefore, a change in the processing of PrP^{C}, might promote the formation of the insoluble PrR^{Sc}, which is deposited in neurons of patients with prion disease. The alteration in expression of PrP mRNA might contribute to these abnormalities, making it important to study the mechanism that induces this expression (Fig. 6).

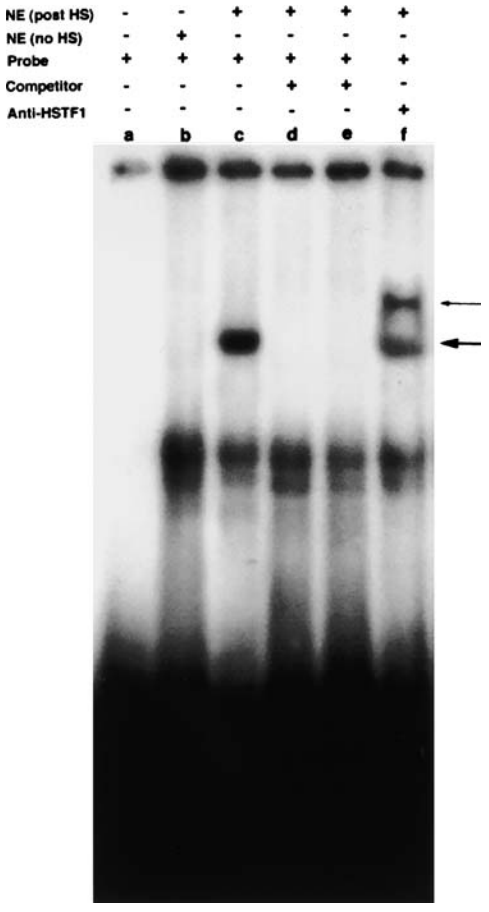

Fig. 4. EMSA and supershift assay for HSE1. Lane **(a)** Labeled HSE oligonucleotide probe only. Lane **(b)** No or low protein binding activity to the HSE oligonucleotide probe was detected in untreated cells. Lane **(c)** Strong protein-DNA binding activity to the HSE oligonucleotide probe was shown in heat-shocked cells. Lane **(d)** and **(e)** Competition experiments were performed using unlabeled HSE oligonucleotide probe. The protein-DNA binding activity was completely inhibited in the presence of 50- and 100-fold molar excess of an unlabeled HSE oligonucleotide probe. Lane **(f)** In the presence of antibody against HSTF-1, the band could be super-shifted, suggesting cross-reaction with the antibody.

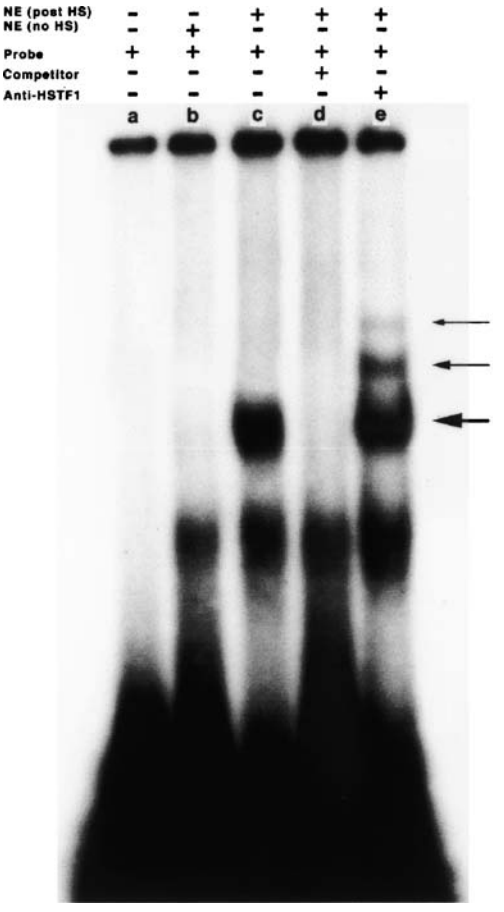

Fig. 5. EMSA and supershift assay for HSE2. Lane **(a)** Labeled HSE oligonucleotide probe only. Lane **(b)** No or low protein binding activity to the HSE oligonucleotide probe was detected in untreated cells. Lane **(c)** Strong protein-DNA binding activity to the HSE oligonucleotide probe was shown in heat-shocked cells. Lane **(d)** Competition experiments were performed using unlabeled HSE oligonucleotide probe. The protein-DNA binding activity was completely abolished in the presence of 100-fold molar excess of an unlabeled HSE oligonucleotide probe. Lane **(e)** In the presence of antibody against HSTF-1, the band could be super-shifted, suggesting cross-reaction with the antibody.

The present study demonstrates that heat shock increases both PrP gene transcription and translation in human NT-2 cells. A 1.5- to 2.5-fold increase in PrP message level was observed after heat shock. This was owing to transcriptional activation because an increase in PrP mRNA level after heat shock was blocked in the presence of actinomycin-D, a transcriptional inhibitor. The PrP protein level also increased following heat shock, indicating

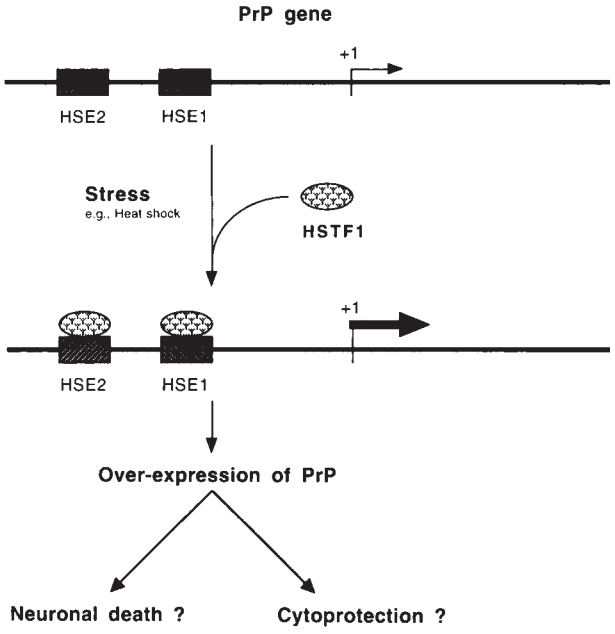

Fig. 6. A hypothetical model of molecular modulation of prion protein by heat shock.

that the stress-induced PrP transcripts were translated into protein products. However, heat-shock proteins and other stress proteins were certainly involved in protecting cells from the deleterious effects of heat and other stresses. Thus, the induction of PrP transcription and translation in heat-shocked human NT-2 cells suggests that it might play a role in the cellular stress response, possibly a protective role that could be categorized as a HSP.

In this study, we demonstrated that there were two heat-shock consensus sequences (HSE) locating at positions -680 bp (HSE1) and -1653 bp (HSE2) within the RaPrP gene promoter. In contrast to the β-APP gene promoter, there was also a HSE located at position –317 bp (Dewji et al., 1995). HSEs are best described as contiguous arrays of variable numbers of the 5-bp sequence nGAAn (n denotes less strongly conserved nucleotides). At least two nGAAn units were needed for high-affinity binding of heat shock factor in vitro, and these might be arranged either head-to-head or tail-to-tail (Perisic et al., 1989). In fact, the HSE in the RaPrP gene promoter contained two sets of three or four nGAAn units, which should predict with high-affmity binding to heat-shock transcription factor (HSTF) as shown in this study.

Another interesting finding was the different biological activities of the two HSEs of the RaPrP gene promoter. HSE1 seemed to play a more dominant role in PrP gene regulation than HSE2 (Fig. 3). Therefore, Konishi et al. assumed that, in addition to HSTF-1, other regulatory factors might be involved in this process (Konishi et al., 1995). Furthermore, Bienz et al. demonstrated that HSE containing extended nGAAn arrays located far from the TATA box in mammalian cells (Bienz et al., 1986). These results imply that transcriptionally active promoter complexes contained six or more heat-shock factor monomers.

Induction of PrP expression should be due to activated HSTF binding to the HSE after heat treatment. In eukaryotes, instead of increasing the concentration of a transcription activator, the heat-shock response is mediated by activation of a pre-existing pool of HSTF that binds to the HSE (Wu et al., 1994). HSTFs have a highly conserved N-terminal DNA binding dormain containing an array of three hydrophobic heptad repeats, known as leucine zippers. For higher eukaryotes, there is a fourth zipper domain near the C-terminal end that appears to interact directly with the more N-terminal leucine zipper array to prevent trimerization under non-stress conditions. Multiple factors can influence the activation of HSTFs. In fact, there are at least four HSTFs found in vertebrates (Wu et al., 1994), potentially allowing for differential activation in response to distinct stresses. For instance, activation of HSTF-1 has been shown to be mediated by numerous stresses including hypoxia, decreased pH, elevated Ca^{2+}, decreased ATP and exposure to reactive oxygen species (Ros). These stresses caused two major changes in HSTF-1. First, the factor multimerizes to form a trimer and acquires the ability to bind HSEs. Second, it undergoes a post-translational modification, e.g., phosphorylation, at multiple positions. Activated HSTF-1 induces transcrip-

tion of heat-shock genes leading to a high level of protein synthesis in the cells (Lis et al., 1993).

Increased expression of PrP may cause various detrimental effects. For instance, mice overexpressing PrP develop peripheral and central nervous system (PNS/CNS) changes (Westaway et al., 1994). Peripheral degeneration, including myopathy, demyelination of axon, and Wallerian degeneration, have been observed. Spongiform degeneration similar to mild Scrapie has also been shown. In addition, a peptide fragment of the prion protein, PrP 106–126, is toxic to neuronal cells. However, PrP 106–126 was more toxic to neuronal cells derived from PrP-overexpressing mice in the presence of microglia (Brown et al., 1998). Therefore, increased PrP expression may potentiate the neurotoxicity of PrP 106–126. Our results demonstrate that heat-shock stress upregulated the PrP expression. This suggests that environmental stress might prompt and enforce the progression of prion disease.

There is much evidence that has demonstrated a close relationship between prion protein and SOD such as evolutionary conserved phenotype suggested by sequence similarity (Brown et al., 1999) and reduced Cu/Zn SOD activity in PrP gene-ablated mice (Brown et al., 1997). It has been suggested that prion protein expression may aid cellular resistance to oxidative stress by influencing the activity of Cu/Zn SOD. The activity of Cu/Zn SOD was investigated in mice with different levels of prion protein expression. Increasing levels of PrP expression were linked to increased levels of SOD activity (Brown et al., 1999). As Peng et al. reported that antioxidant status modulates the synthesis of stress proteins (Peng et al., 2000). They concluded that increase in total antioxidant status (SOD and glutathione peroxidase) would enhance synthesis of heat-shock protein. Therefore, in combination with our result, we could deduce that heat-shock protein, prion protein, and SOD might modulate themselves reciprocally. The definite regulatory mechanism needs to be further investigated.

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