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Mechanisms of Development 51 (1995) 317–327

# Expression and targeting of Syrian hamster prion protein induced by heat shock in transgenic *Drosophila melanogaster*

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Received 14 February 1995; accepted 15 March 1995

**Abstract**

To evaluate the fruit fly as a model for studying neurodegenerative diseases caused by prions, transgenic flies were generated by introducing the Syrian hamster prion protein (SHaPrP) gene into the *Drosophila melanogaster* germ line by P element-mediated transformation. Nine transgenic lines were isolated; induction of transgenes that had been placed under the control of the *Drosophila* heat shock promoter, hsp 70, resulted in the synthesis of full-length SHaPrP. The relative molecular weight of the recombinant protein was lower than that of authentic SHaPrP due to incomplete processing of Asn-linked CHOs. To determine the cellular localization of SHaPrP, *Drosophila* Schneider line 2 cells were transfected with the same constructs used for fly transformation. Heat shock induced SHaPrP was anchored to the surface of S2 cells by a glycolipid, demonstrating that the carboxy-terminal glycolipidation signal of SHaPrP is recognized by this evolutionarily distant host. When SHaPrP was synthesized in transgenic flies constitutively by subjecting them to heat pulses continuously, no difference in their lifespans compared with controls was detected. Furthermore, expression of SHaPrP for 20 days did not produce protease resistant SHaPrP, which is the major and possibly only component of the infectious prion. In contrast to transgenic mice overexpressing SHaPrP, which develop a profound neuromyopathy, no disease phenotype was associated with expression of SHaPrP over the entire lifespan of transgenic flies.

*Keywords:* Transgenic flies; Prion protein; Heat shock proteins; *Drosophila*

## 1. Introduction

While transgenetic studies have yielded a wealth of new knowledge about infectious, genetic and sporadic prion diseases (Prusiner and Hsiao, 1994), the laborious production of Tg mice limits the number of studies that can be performed. The relatively long gestation period of mice coupled with the need to do microinjections of fertilized embryos prevents the creation of the very large numbers of different Tg mice that would yield the greatest amount of new information. Assays that permit screening of a multitude of possible phenotypes in genetic experiments are generally the most informative. While the limited number of mice expressing different transgenes is definitely a liability, experiments with Tg mice expressing foreign and mutant prion protein (PrP) molecules have been extraordinarily useful in advancing our understanding of prion biology (Prusiner 1994). It is important to stress that transgenetic studies can readily yield an incomplete, and sometimes erroneous, interpretation of the data if the number of lines of mice examined expressing a particular construct is inadequate. Defining an adequate number of lines is difficult, but certainly comparisons of lines expressing high and low levels of a given PrP transgene have proved to be quite helpful (Hsiao et al., 1994; Prusiner et al., 1990).

Experiments with the production of transgenic mice harboring a SHaPrP gene have convincingly demonstrated that the PrP transgene is able to modify neuropathology, incubation time and production of species-specific prion infectivity (Prusiner et al., 1990;

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*SSDI* 0925-4773(95)00379-F

Scott et al., 1993). Furthermore, gene targeted mice devoid of PrP are resistant to scrapie and do not support prion propagation (Büeler et al., 1993; Prusiner et al., 1993).

Thus, despite the elegance of the transgenic mice studies, the limitations and drawbacks noted above, in addition to prolonged periods of observation requiring up to two years in some experimental settings, call for the development of better experimental systems. The fruit fly *Drosophila melanogaster* is especially well suited because it is the multicellular organism that has been best characterized genetically and that can be transformed with foreign genes most efficiently. Several recent reports described the successful expression of foreign coding regions introduced into the germline of *Drosophila* (Drain et al., 1991; Phillips et al., 1990; Rancourt et al., 1987; Reveillaud et al., 1991). A search for PrP-related sequences led to the detection of a 5.6 kb restriction fragment of *Drosophila* DNA related to the hamster PrP cDNA (Westaway and Prusiner, 1986). However, numerous attempts to clone such a putative fly PrP homologue have failed and have given rise to the question of the specificity of the observed hybridization signal in genomic *Drosophila* DNA. Interestingly, a recent attempt to clone a *C. elegans* PrP gene homologue led to the isolation of a *C. elegans* heterogeneous nuclear ribonucleoprotein (hnRNP) cDNA (Iwasaki et al., 1992), which supports the view that the cross-hybridization is due to the very GC-rich region of the SHaPrP cDNA.

We decided to explore the possible consequences of expressing mammalian prion proteins in *Drosophila*. If a characteristic nervous system dysfunction could be identified in transgenic flies, it would provide a system with which to assay efficiently many PrP gene constructions.

## 2. Results

### 2.1. Incorporation of SHaPrP into the genome of Drosophila melanogaster

An expression vector containing the SHaPrP open reading frame (ORF) inserted downstream of the native *Drosophila* hsp70 gene promoter was constructed. Embryos of the genotype Df(1)w$^{67c23}$ were microinjected with the construct together with the helper plasmid pπ25.7wc. Transformed flies were identified by their orange or red eyes, and Southern blot analysis was used to confirm their genotype (Fig. 1). Seven lines designated P[SHaPrP-hs]10, 11, 30, 31, 32, 40, and 50 were found with single inserts at different positions in the *Drosophila* genome.

### 2.2. Heat-inducible synthesis of full-length SHaPrP in Drosophila melanogaster

Flies from each transgenic line were subjected to heat shock at 37°C for 2 h followed by a 1.5 h recovery period at 25°C and fly extracts were analyzed by Western blot with SHaPrP-specific monoclonal antibodies (Fig. 2). The immunoblots demonstrate the synthesis of PrP in six transformed lines. Line P[SHaPrP-hs]20, which tested negative for PrP on Southern blot despite having a slight orange eye color, did not show PrP expression on Western blot.

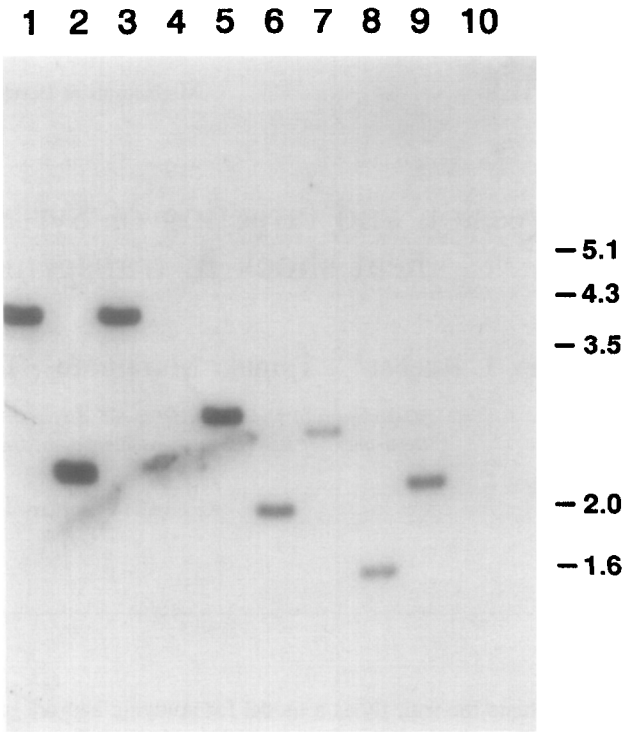


Fig. 1. Southern blot analysis of transgenic flies. DNA was extracted from flies of the indicated transformed lines digested with Eco RI, and blotted onto a nylon membrane after electrophoresis on a 0.8% agarose gel. The membrane was hybridized with a Bam HI-Bgl II restriction fragment containing the whole SHaPrP open reading frame. Sizes of molecular weight markers are in kb. Lanes 1–9, P[SHaPrP-hs] lines 10, 11, 12, 20, 30, 31, 32, 40, 50; lane 10, P[CaSpeR-hs] control transgenic flies obtained from an embryo microinjected with the unrecombined plasmid pCaSpeR-hs and pπ25.7wc helper plasmid.

The pattern of immunoreactive PrP showed three major bands of 26, 28, and 30 kDa apparent molecular mass. This is in agreement with earlier studies of PrP expression in baculovirus infected *Spodoptera* cells (Scott et al., 1988). The observation that all the recombinant PrP species synthesized in transgenic flies migrate faster than the 33–35 kDa protein produced in Syrian hamster brain led us to investigate the possibility of *N*- or *C*-terminal degradation of the recombinant protein. We carried out further immunoblots using monospecific antisera raised against synthetic peptides corresponding to both the *N*- and *C*-terminal amino acid sequence of SHaPrP. These monospecific polyclonal antibodies recognized the same pattern of PrP (Figs. 3B, 3C, 3E) as the monoclonal hamster-specific antibody 13A5 (Fig.

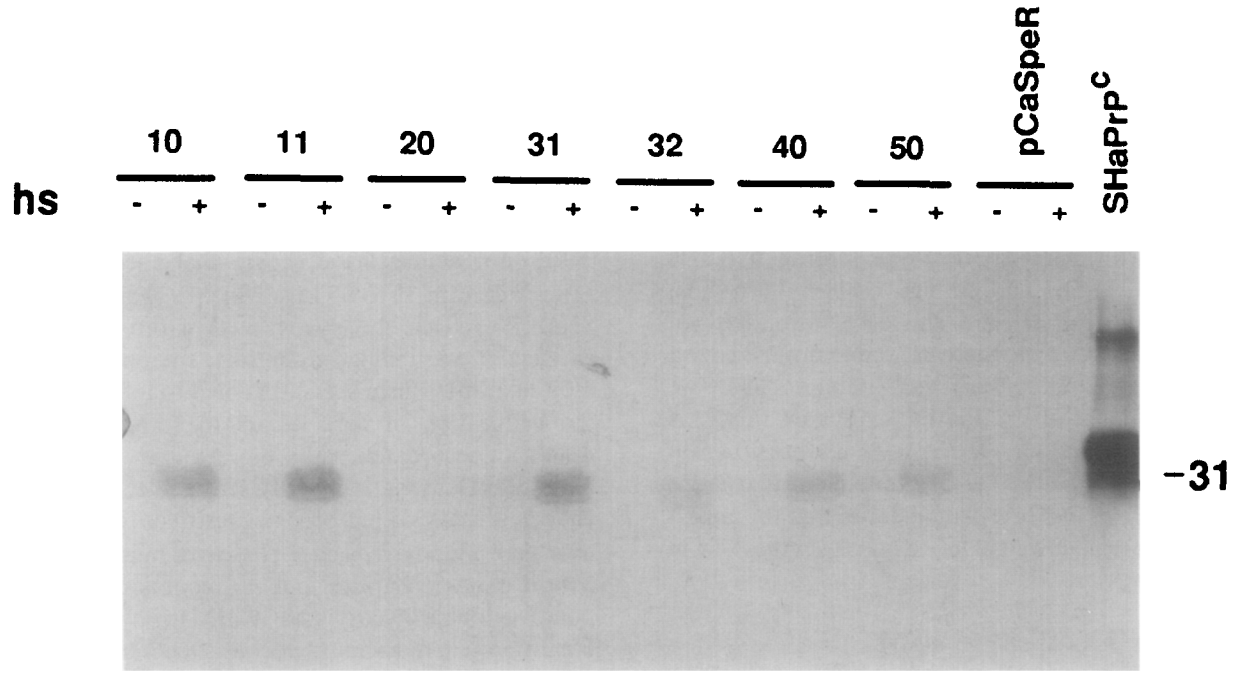


Fig. 2. Western blot analysis of total transgenic fly extracts. Total fly extracts (30 flies per lane) were electrophoresed in a 12% SDS polyacrylamide gel and blotted onto nitrocellulose. SHaPrP was detected using hamster specific monoclonal antibodies 13A5 and 3F4 hybridoma supernatants at a 5-fold dilution followed by immunostaining with the Protoblot alkaline phosphatase system according to the procedure suggested by the manufacturer (Promega). Flies were heat shocked (hs), as indicated, by incubating the vials for 2 h at 37°C followed by a 1.5 h recovery period at 25°C. P[SHaPrP-hs] lines 10, 11, 20, 31, 32, 40, 50, P[CaSpeR-hs] and purified SHaPrP (Pan et al., 1992).

3A). Furthermore, the binding of anti-P1 to these PrP species was effectively blocked using excess P1 peptide, providing further evidence of the authenticity of the bands (Fig. 3D). This suggests that the recombinant fly PrP is full length.

### 2.3. *Glycosylation of SHaPrP in Drosophila melanogaster*

Mammalian SHaPrP is posttranslationally modified by asparagine-linked complex-type oligosaccharides (Endo et al., 1989; Haraguchi et al., 1989). PrP from

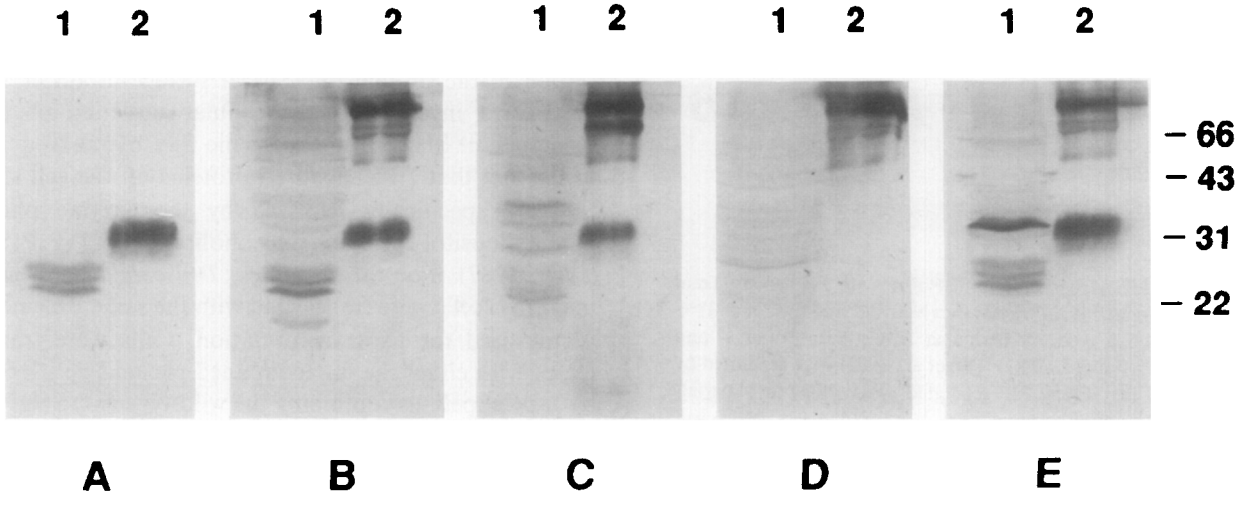


Fig. 3. Western blot showing the binding of anti-PrP peptide antisera to SHaPrP synthesized in transgenic flies. Total extracts of transgenic P[SHaPrP-hs] flies heat shocked for 2 h at 37°C followed by a 1.5 h recovery period at 25°C (lane 1) and purified SHaPrP (Pan et al., 1992) (lane 2) were separated on a 12% SDS polyacrylamide gel and analyzed by Western blotting with the monoclonal hamster PrP specific antibody 13A5 (A), anti-P2 antiserum (specific for *N*-terminus of SHaPrP) (B), anti-P1 antiserum (specific for *N*-terminus of SHaPrP 27–30) (C), anti-P1 preabsorbed with P1 (D), and anti-P3 antiserum (specific for C-terminus of SHaPrP) (E). The high-molecular weight bands in lane 2 of (B), (C), (D), and (E) reflect nonspecific staining by these monospecific antisera since they are not detected by the highly specific monoclonal antibody 13A5. Furthermore these bands are also stained with anti-P1 preabsorbed with P1. Molecular weight markers are depicted in kilodaltons.

hamster brain or cultured hamster brain cells displays extensive size heterogeneity on immunoblots, which has been shown to be due to a variable degree of glycosylation at the two asparagine residues (Rogers et al., 1990; Taraboulos et al., 1990). The different forms represent the double glycosylated form as the most prominent species running at an apparent molecular mass of 33–35 kDa, and two minor species representing a single glycosylated (29 kDa) and an unglycosylated (26 kDa) PrP form. The significance of this observation has not been investigated to date. Since recombinant PrP gave a distinct three band pattern on immunoblots, we suspected a similar aberration in Asn-linked sugars as for the native protein. To test this we performed enzymatic deglycosylation of both recombinant and native PrP with PNGase F. Fig. 4 demonstrates that the apparent lower molecular weight of SHaPrP synthesized in transgenic flies is indeed due to a difference in the carbohydrates since the molecular masses of both proteins were reduced to 26 kDa when completely deglycosylated. Furthermore, these results strongly indicate that the three PrP immunoreactive bands seen on immunoblots of heat-shocked transgenic fly extracts represent double, single, and unglycosylated PrP molecules.

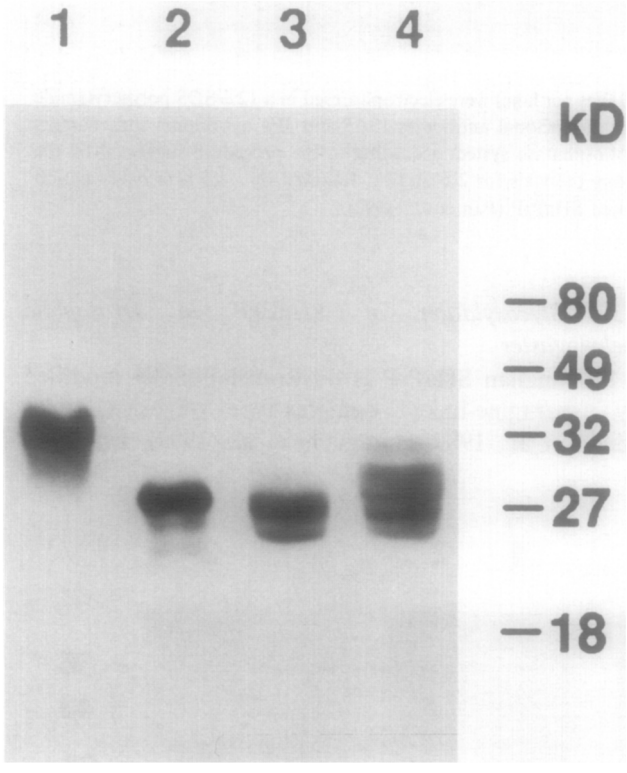


Fig. 4. Enzymatic deglycosylation of SHaPrP with PNGase F. Total extracts of transgenic P[SHaPrP-hs] flies heat shocked for 2 h at 37°C followed by a 1.5 h recovery period at 25°C (lanes 3 and 4) and purified $SHaPrP^C$ (lanes 1 and 2) (Pan et al., 1992) were separated by preparative SDS-PAGE and PrP gel-eluted as described in Materials and methods. The samples were divided into two aliquots, PNGase F (33 units/l) was added to one aliquot (lanes 2 and 3) and the samples incubated for 18 h at 37°C. The proteins were precipitated at −20°C with 15 volumes of ethanol and prepared for SDS-PAGE. Proteins were electroblotted to nitrocellulose, and immunostained with a 5-fold dilution of 13A5 and 3F4 hybridoma supernatants and the blots developed with the chemiluminescence-based ECL detection system according to the procedure provided by the manufacturer (Amersham). Molecular weight markers are indicated on the right in kilodaltons.

Previous studies have shown that the broad band extending from 33 to 35 kDa which is characteristic for $SHaPrP^C$ reflects the glycosylated mature form of PrP (Rogers et al., 1990). In contrast, the bands of lower molecular mass have been attributed to intermediates in the maturation of core sugars to complex carbohydrates. In accordance with this view is the fact that the 33 to 35 kDa band is initially sensitive to digestion by endoglycosidase H but becomes resistant to this enzyme over time. Endoglycosidase H cleaves high-mannose or hybrid oligosaccharides, but not complex sugars. The putative double glycosylated 30 kDa form of the recombinant protein does not appear as such a broad band as the corresponding form of the native protein, suggesting that the carbohydrates are not of the complex type. We therefore carried out endoglycosidase H digestions on both the native and the recombinant protein. We found that both proteins showed complete resistance to digestions by endoglycosidase H, which indicates that the sugars on the recombinant protein are not of a high-mannose type (results not shown).

### 2.4. *Cellular targeting of mammalian PrP in Drosophila* Schneider 2 cells

All mammalian and avian PrP sequences investigated so far contain a *C*-terminal hydrophobic signal sequence which is removed on attachment of a phosphatidylinositol glycolipid (GPI) anchor (Stahl et al., 1987). This modification, which presumably takes place in the endoplasmic reticulum, is essential for the proper localization of the protein on the cell surface (Stahl et al., 1990). We investigated the cellular targeting of the recombinant SHaPrP in transgenic flies by making use of the fact that GPI anchored proteins on the cell surface are specifically removed by the enzyme phosphatidylinositol-specific phospholipase C (PIPLC) (Low, 1987). For these studies, *Drosophila* Schneider line 2 (S2) cells were transfected with the same P element vector used for fly transformation. Cells were transfected with either the unrecombined plasmid pCaSpeR-hs or pCaSpeR-hs containing the wild-type (wt) SHaPrP ORF. In order to achieve stable transfections, the cells were cotransfected with the PC4 plasmid, which encodes a *Drosophila* α-amanitin-resistant RNA polymerase II gene (Jokerst et al., 1989). Poly (A)+ RNA extracted from pools of heat shocked α-amanitin-resistant cells was probed with the entire SHaPrP ORF. Fig. 5A demonstrates the inducibility of SHaPrP-specific transcripts in cells transfected with pSHaPrP-hs versus

cells transfected with pCaSpeR-hs alone. The hybridization signal close to the wells of the gel is probably due to contamination of the RNA with genomic DNA as a result of the one-step oligo(dT)-affinity purification procedure. On prolonged exposure, a faint signal for the PrP transcript was visible in non-heat shocked PrP transfected cells, probably representing constitutive expression from the heat shock promoter. Furthermore, we observed a pattern of bands with PrP reactive monoclonal antibodies on immunoblots of heat induced cell extracts identical to those found in extracts of transformed heat shocked flies (Fig. 5C). A small amount of PrP was detected in non-heat shocked cells, which is consistent with the basal level of transcription from the hsp70 promoter.

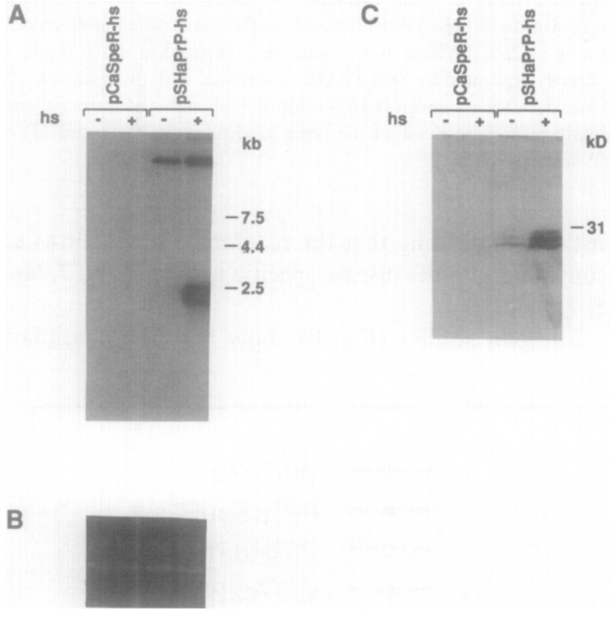


Fig. 5. Inducible expression of SHaPrP in stably transfected S2-SHaPrPhs cells. Schneider line 2 cells were transfected with pSHaPrP-hs or with the unrecombined plasmid pCaSpeR-hs as described in Materials and methods. They were subjected to heat shock by incubating the cells at 37°C for 1 h followed by a recovery period of 1 h at 25°C (+hs) or left at 25°C for the whole period (−hs). (A) Poly(A+) RNA was isolated from the cells, fractionated on a 1% formaldehyde–agarose gel (1.5 μg/ lane), and blotted onto nitrocellulose membranes. The filter was hybridized with a cDNA restriction fragment containing the whole SHaPrP open reading frame. The final wash of the blot was done in 0.1 × SSC, 0.1% SDS at 68°C for 15 min. Sizes of molecular weight markers are in kb. Prolonged exposure of the blot resulted in the appearance of a faint band corresponding to the PrP transcript in the non-heat shocked cells transfected with pSHaPrP-hs. This represents constitutive expression from the heat shock promoter. (B) Photograph of the ethidium bromide stained gel prior to transfer, to demonstrate normalized loading of the samples. (C) $5 \times 10^6$ cells were concentrated, resuspended in TE (250 mM Tris–HCl (pH 7.4), 5 mM EDTA) and lysed by 3 cycles of freeze–thawing. The lysate was cleared by centrifugation, the supernatant fractionated on SDS-PAGE and proteins detected by Western blotting using 5-fold dilutions of 13A5 and 3F4 hybridoma supernatants and the chemiluminescence-based ECL detection system according to the procedure provided by the manufacturer (Amersham).

When pools of PrP transfected cells were metabolically radiolabeled, heat shocked and then exposed to PIPLC, partial release of labeled PrP into the medium was observed (Fig. 6). The inefficiency with which bacterial PIPLC cleaves GPI-anchored proteins has been well documented for a number of vertebrate proteins (Low et al., 1988) and for the *Drosophila* neural cell adhesion molecule fasciclin I (Hortsch and Goodman, 1990). Small amounts of labeled PrP were also detected in the medium of cells without PIPLC treatment. It seems possible that this is either a constitutively secreted form of the protein or the GPI-anchored form cleaved by endogenous phospholipases (Hortsch and Goodman, 1990). To summarize, these findings confirm that recombinant PrP synthesized in *Drosophila* cells is correctly targeted to the cell surface where it is anchored by a phosphatidylinositol-containing glycolipid.

### 2.5. Phenotypic analysis of transgenic flies

In order to detect a phenotype associated with the expression of SHaPrP in transgenic *Drosophila*, we carried out lifespan studies by subjecting flies to heat pulses given at regular intervals during their whole lifespan. This heat treatment resulted in almost constitutive ex-

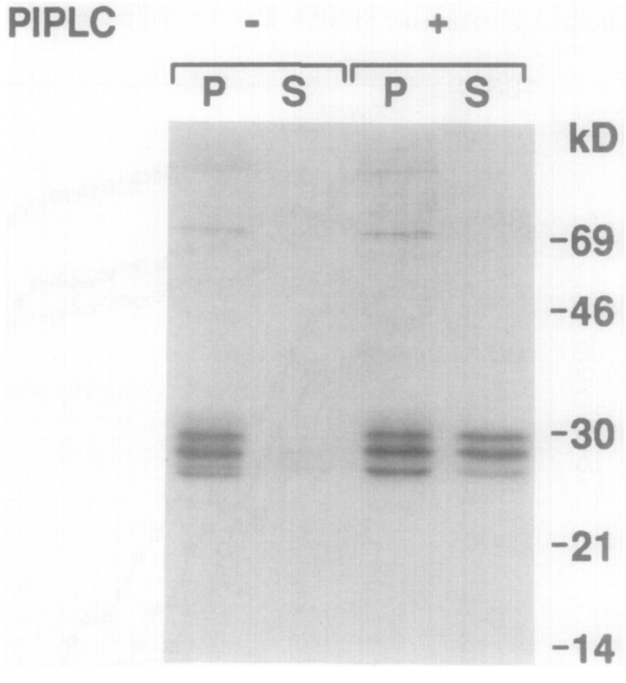


Fig. 6. Phosphatidylinositol-specific phospholipase C releases SHaPrP from the surface of heat shocked S2-SHaPrP-hs cells. S2 cells transfected with pSHaPrP-hs were heat shocked at 37°C for 1 h and then metabolically radiolabeled with [$^{35}$S]methionine as described in Materials and methods. The cell suspension was then incubated with (+) or without (−) PIPLC (0.77 units/ml) for 90 min at 25°C. The cells were pelleted in a microfuge and aliquots of the supernatant (S) and the pellet (P) fractions were used for immunoprecipitation of labeled PrP with SHaPrP-specific monoclonal antibody 3F4 and protein A. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

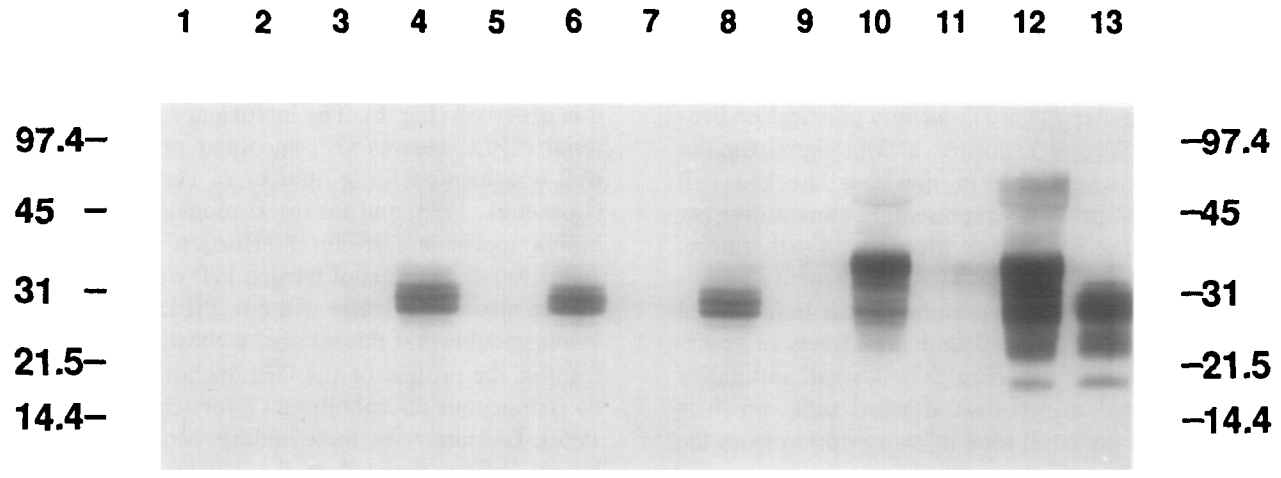


Fig. 7. Western blot showing the constitutive expression and protease sensitivity of SHaPrP in whole extracts from flies treated with continuous heat pulses for up to 20 days. Control transgenic P[CaSpeR-hs] (lanes 1–3) and transgenic P[SHaPrP-hs] (lanes 4–9) flies were raised under conditions of continuous heat pulses of 15 min at 37°C followed by a period of 40 min at 25°C. Whole fly extracts were prepared at day 0 (lanes 1, 4, 5), day 10 (lanes 2, 6, 7) and day 20 (lanes 3, 8, 9) and aliquots were either directly separated by SDS-PAGE or digested with proteinase K (50 μg/ml; 1 h; 37°C) prior to electrophoresis in 12% SDS–polyacrylamide gels (lanes 5, 7, 9). As controls 10% brain homogenates of normal (lanes 10 and 11) or scrapie infected Syrian hamster (lanes 12 and 13) were digested with proteinase K under the same conditions (lanes 11 and 13) or left untreated (lanes 10 and 12). The blot was probed with the polyclonal PrP antisera R073.

pression of PrP as demonstrated by Western blot of whole fly extracts prepared at day 0 (Fig. 7, lane 4), day 10 (lane 6) and day 20 (lane 8). None of the protein synthesized in transgenic SHaPrP flies showed protease resistance, indicating that the recombinant protein is not converted into the disease specific isoform (Fig. 7, lanes 5, 7, 9).

Lifespan studies (Fig. 8) show that the continuous

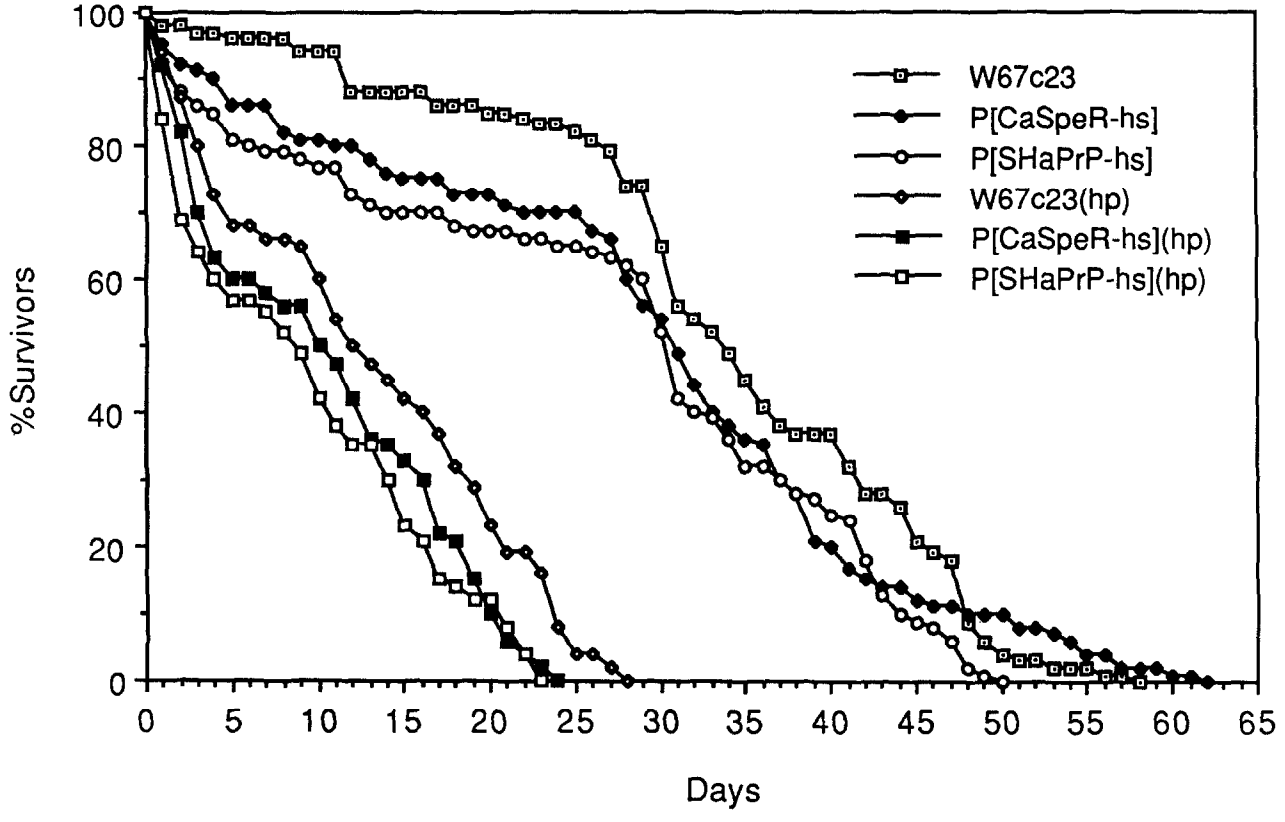


Fig. 8. Survival curves for $w^{67c23}$, control transgenic P[CaSpeR-hs] and transgenic [SHaPrP-hs]31 flies. 100 freshly hatched flies for each strain were either kept at 25°C or exposed to continuous heat pulses of 25 min at 37°C followed by a recovery period of 50 min at 25°C. Surviving flies were counted daily and are plotted as a percentage of the total. The survival curve for each strain of flies kept at 25°C is shown as W67c23, P[CaSpeR-hs] or P[SHaPrP-hs] in the figure, whereas those under heat pulse are shown as W67c23(hp), P[CaSpeR-hs](hp) or P[SHaPrP-hs](hp).

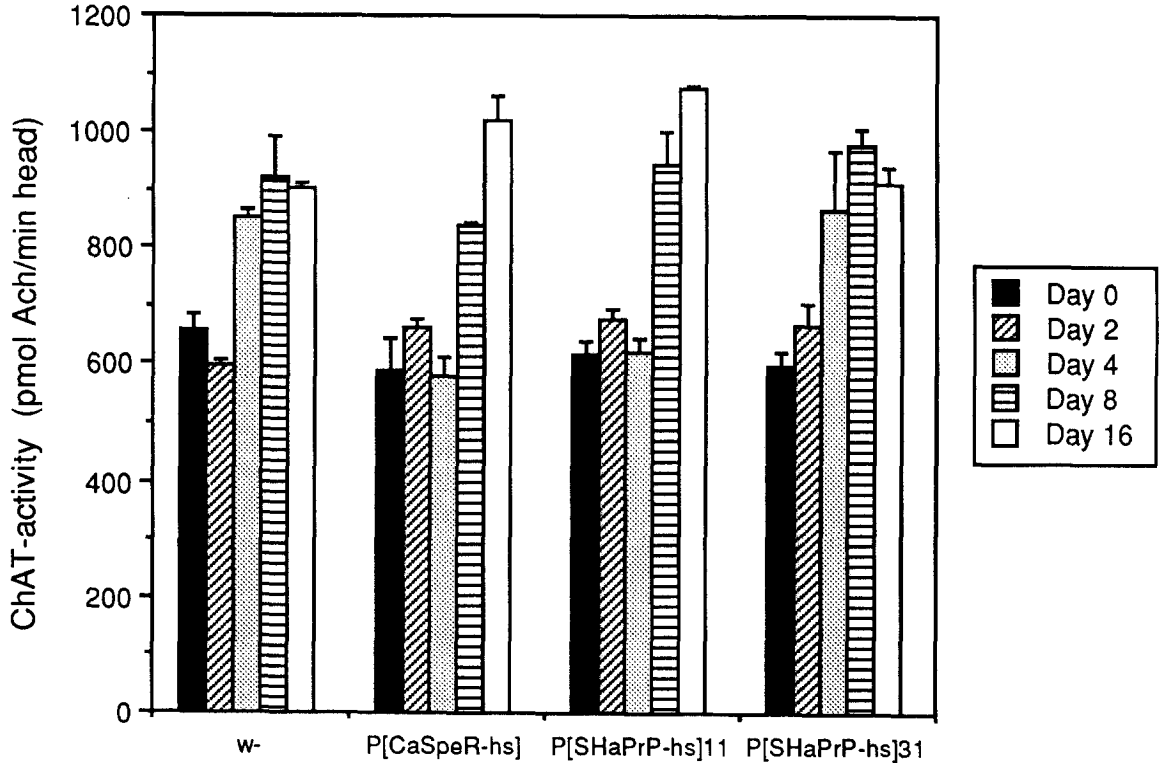


Fig. 9. ChAT activity in $w^{67c23}$, control transgenic P[CaSpeR-hs] and two lines (11 and 31) of transgenic P[SHaPrP-hs] flies. *Drosophila* head extracts were prepared at various days of growth under conditions of continuous heat pulses as described in Material and methods. ChAT activity was measured on an aliquot of the extract as described and is expressed as pmol of acetylcholine formed per min per head at 37°C. Each bar represents the mean value (± S.D.) of triplicate assays.

heat pulses had a dramatic effect on the viability of all fly lines tested per se when compared with the flies kept at 25°C during the whole experiment. However, no significant differences were found in the survival of SHaPrP expressing flies compared with control transgenic flies. These results suggest that the constitutive expression of SHaPrP in transgenic flies does not affect their viability or produce any other obvious phenotype. However, the possibility of a subtle phenotype cannot be ruled out. Considering the brain specific disease phenotype caused by abnormal prion proteins, we investigated the activity of choline acetyltransferase (ChAT), a marker for differentiated cholinergic neurons. Earlier reports showed an increase in both ChAT activity and PrP mRNA in developing septal cholinergic neurons, thereby raising the possibility that these proteins might be coordinately controlled in the early postnatal development of the hamster brain (Mobley et al., 1988). Interestingly, a continuous decrease in ChAT activity was found on infection of PC-12 cells with the 139A scrapie isolate when compared with mock infected cells (Rubenstein et al., 1991). It is well established that acetylcholine is an important neurotransmitter in arthropods (Klemm, 1976). High levels of the biosynthetic enzyme ChAT are present in the insect nervous system (Dewhurst et al., 1970).

We measured the ChAT activity in *Drosophila* head extracts prepared from flies exposed to the continuous heat pulse treatment as described for the lifespan studies. We found an increase in ChAT activity over the 16-day period analyzed in all lines tested, irrespective of their genotype (Fig. 9). This increase was most significant between day 2 and day 8. We conclude that the observed increase in ChAT activity is due to the heat treatment since it is found to the same extent in $w^{67c23}$ flies and in control transgenic flies. A similar effect was reported for wt Canton S flies raised at 32°C instead of 18°C (Salvaterra and McCaman, 1985).

## 3. Discussion

In this report, we describe the evaluation of the fruit fly *Drosophila melanogaster* as a possible new model system for studying the mechanisms underlying neurodegenerative diseases caused by prions. We introduced the wt SHaPrP ORF into embryos of *Drosophila melanogaster* by P element-mediated germline transformation. Modular PrP ORF cassettes were fused to the *Drosophila* hsp 70 gene promoter within the 5′ untranslated region and with the hsp 70 polyadenylation site distal ORF. This inducible promoter system was chosen to allow us to determine whether any lethal effect

is associated with the expression of the heterologous gene.

Six transgenic lines homozygous for the transgene were established and tested positive for inducible expression of SHaPrP. Although the protein showed a reduced molecular weight compared with native SHaPrP, it was shown that this is not due to protein degradation but a direct result of the different degree of glycosylation. An aberrant glycosylation has been described for most vertebrate glycoproteins expressed in insect cells, especially for the very widely used baculovirus expression system. However, there is still some controversy as to whether insect cells are capable of assembling Asn-linked complex-type oligosaccharides or are restricted to the synthesis of high-mannose-type glycans. A recent report described the interesting finding that lepidopteran insect cells synthesize high mannose-type oligosaccharides under normal conditions, but are able to switch to process the sugars on viral infection of the cells (Davidson et al., 1990). Nevertheless, different insect cells may not behave similarly in this regard.

The carbohydrates on PrP synthesized in transgenic flies displayed an endoglycosidase H-resistant structure (data not shown). Fasciclin I, a *Drosophila* glycoprotein, was found to have one high-mannose type *N*-linked sugar chain, whereas the second carbohydrate chain had a endoglycosidase H-resistant structure (Hortsch and Goodman, 1990). To summarize, our results demonstrate that PrP synthesized in transgenic flies displays a pattern of polypeptides indistinguishable from that seen in the baculovirus expression system (Scott et al., 1988).

PrP is anchored in the plasma membrane of mammalian cells by a phosphatidylinositol glycolipid moiety (Stahl et al., 1987). Although this mode of membrane attachment has been described for a steadily increasing number of vertebrate proteins, it has been reported for only a few *Drosophila* membrane proteins (Gnagey et al., 1987; Hortsch and Goodman, 1990; Krantz and Zipursky, 1990). Our findings that SHaPrP synthesized in *Drosophila* cells is targeted to the cell surface where it is attached by a GPI anchor confirm that the signal for the addition of the glycolipid tail is conserved among many eukaryotes.

In view of the finding that overexpression of wt PrP causes a novel neurologic syndrome in transgenic mice (Westaway et al., 1994), we studied the effect of high constitutive expression of SHaPrP from the heat shock promoter on the survival and ChAT-activity of adult transgenic flies. We were unable to detect any abnormalities associated with basal PrP expression from the uninduced hsp 70 promoter or with constitutive PrP expression from heat shocks given at regular intervals during all developmental stages. This might be a consequence of ectopic expression of PrP, or alternatively reflects the inability of the host to recognize the foreign protein. However, this does not rule out the possibility of a phenotype too subtle to be observed with our assay systems.

The results from recent studies with mice expressing a chimeric human/mouse PrP transgene demonstrate that a mouse specific factor other than PrP is necessary for $PrP^{Sc}$ formation and attendant CNS degeneration after inoculation with brain extracts from patients who died of CJD (Telling et al., 1994). Whether or not similar chimeric mammalian *Drosophila* PrP transgenes will be required for development of disease phenotype remains to be established. The problem with testing this hypothesis is that despite considerable effort, no *Drosophila* homologue of PrP has been identified to date. On the other hand, if mammalian PrP expressing fruit flies were susceptible to mammalian prions and would develop a disease-specific phenotype, they would offer a model system, rewarding in many ways, that provided a readily manipulatable system for the study of neurodegenerative diseases caused by prions.

## 4. Materials and methods

### *4.1. Expression vector constructions*

The P element vector pCaSpeR-hs (Pirotta, 1988) was used to express SHaPrP under the control of the heat shock inducible promoter from the hsp 70 gene. A SHaPrP ORF cassette was excised from the plasmid p805aSHaPrP (Scott et al., 1988, 1992) as a 0.8-kb BamHI–XhoI fragment, the XhoI site was made blunt and the fragment cloned into pCaSpeR-hs cut with BglII and StuI to produce the plasmid pSHaPrP-hs. All subcloning steps were performed using standard procedures (Sambrook et al., 1989).

### *4.2. P element mediated transformation*

P element mediated germline transformation of *Drosophila melanogaster* was done essentially as described by Rubin and Spradling (1982). Embryos of the white eyed fly strain $w^{67c23}$ were isolated and microinjected with the P element expression constructs at 0.5 mg/ml together with the helper plasmid pπ25.7wc at 0.1 mg/ml (Karess and Rubin, 1984). $G_0$ injected adults were individually backcrossed to the recipient strain and the $G_1$ progeny screened for the w+ transformation-positive marker (Klemenz et al., 1987). Transformed lines homozygous for the transgene were established from orange eyed $G_1$ flies as described by Klemenz et al. (1987).

### *4.3. Cell culture transfections*

*Drosophila* embryonic Schneider line 2 (S2) cells (Schneider, 1972) were grown at 25°C in Schneider medium (Cell Culture Facility, University of California, San Francisco) supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, and 100

μg of streptomycin per ml. The transfection procedure employed was as described previously (Snow et al., 1989), using the method of Wigler et al. (1979) for the preparation of the calcium phosphate/DNA precipitates. The cells were transfected with 20 μg each of the plasmids pPC4 (Jokerst et al., 1989) and pSHaPrP-hs or pCaSpeR-hs. Stably transfected cells were selected with 5 μg of α-amanitin per ml medium and passaged every 7 to 10 days after a 1:10 dilution in fresh medium. The α-amanitin treatment was discontinued after 5 passages.

*4.4. Southern blot analysis*

Southern blot analysis of each transformed line was carried out according to standard protocols (Sambrook et al., 1989). Briefly, genomic DNA was extracted from G2 adult flies according to the protocol of Steller and Pirotta (Sambrook et al., 1989), digested with *Eco*RI, and fractionated by electrophoresis on 0.8% agarose gels. The gels were depurinated, transferred by standard procedures to nylon membranes (Schleicher & Schuell, Keene, NH) and crosslinked to the membrane by UV irradiation (Stratagene UV Stratalinker 1800). Hybridization was carried out at 42°C for 40 h using a $^{32}$P-labeled 0.85 kb BglII-*Eco*RI fragment isolated from the plasmid pSPPrPSV12 (Hay et al., 1987) and labeled with the random primer method (Feinberg and Vogelstein, 1984). The composition of the hybridization buffer was 50% formamide, 6 × SSC, 10 × Denhardt's solution, 0.5% SDS, and 0.1 mg salmon sperm DNA per ml. The final wash was performed in 0.1% SDS, 0.1 × SSC for 20 min at 55°C.

*4.5. Northern (RNA) blot analysis*

Poly(A)+ RNA was isolated from heat-shocked cells by a one-step procedure employing oligo(dT)-affinity purification (Aviv and Leder, 1972), according to the protocol provided by the manufacturer (Fast-Track, Invitrogen). The RNA was separated on a 1% formaldehyde–agarose gel and blotted onto nitrocellulose (BA 85, Schleicher & Schuell). Filters were hybridized to the same random primed $^{32}$P-labeled probe used for Southern blotting in 50% formamide, 3 × SSC, 5 × Denhardt's solution, 50 mM HEPES (pH 7.6), 0.5% SDS, 0.1 mg heparin per ml, and 0.1 mg salmon sperm DNA per ml. The final wash was carried out in 0.1% SDS, 0.1 × SSC for 15 min at 68°C.

*4.6. Western immunoblot analysis*

Whole fly extracts were prepared by homogenizing the flies in 4 volumes (w/v) of 50 mM Tris–HCl (pH 7.5), 10 mM NaCl, 0.2% Triton X-100 and 1 mM PMSF, followed by centrifugation of the homogenate for 10 min at 12 000 × *g*. Aliquots of the supernatant were electrophoresed on SDS–polyacrylamide gels (SDS-PAGE) (Laemmli, 1970) and the proteins were electrotransferred to nitrocellulose membranes for 2 h. Visualization of PrP using monoclonal or polyclonal antibodies has been detailed previously (Raeber et al., 1992).

*4.7. PNGase F digestions*

Whole fly extracts prepared as described above and partially purified Syrian hamster brain $PrP^C$ (Pan et al., 1992) were separated by preparative SDS-PAGE. Strips of the gels containing PrP, as estimated from the size of prestained molecular weight markers (Bio-Rad, Hercules, CA) run in parallel lanes, were cut out and the proteins eluted in 10 mM $NH_4HCO_3$, 0.1% SDS, 1 mM $NaN_3$ for 16–20 h at 4°C. Zwittergent was added to a final concentration of 0.25%, the samples were divided into two aliquots and recombinant PNGase F (New England Biolabs, Beverly, MA) was added to one aliquot. After incubation for 18 h at 37°C, the proteins were precipitated at −20°C with 15 volumes of ethanol, the pellets resuspended in SDS sample buffer and boiled for 3 min. Aliquots were analyzed by Western blotting as described above.

*4.8. Metabolic labeling of S2 cells*

Typically, cells were grown in 150 cm$^2$ T flask to a density of 2 to 5 × 10$^6$ cells per ml. 2 × 10$^8$ cells were concentrated (at three quarters speed in a clinical centrifuge) and washed in 2 ml of PBS. The cells were concentrated once more and resuspended in 2 ml of Schneider medium without methionine and yeast hydrolysate and incubated for 30 min at 25°C. After a further concentration of the cells, they were resuspended in 1 ml of the above medium and transferred to a 35 mm-diameter cell culture dish where the incubation was continued for 30 min at 37°C. [$^{35}$S]methionine (0.5 mCi) (Amersham, Arlington Heights, IL; >1000 Ci/mmol) was added to the cell suspension and incubation continued for 1 h. Non-heat shocked control cells were continuously kept at 25°C. Labeled cells were concentrated, washed with 2 ml of PBS and further processed for PIPLC treatment.

*4.9. PIPLC treatment of S2 cells*

PIPLC digestions were performed as described by Krantz and Zipursky (1990) with the following modifications. Briefly, labeled cells were washed once in incubation buffer (15 mM HEPES (pH 7.5), 150 mM NaCl, 0.1 mg bovine serum albumin per ml) sedimented in a microfuge at 5000 rev./min and resuspended at a concentration of 5 × 10$^7$ cells per ml of incubation buffer. This cell suspension (0.5 ml) was incubated with or without the addition of 0.77 units of PIPLC (Boehringer Mannheim, Indianapolis, IN) per ml for 90 min at 25°C. Cell suspensions were centrifuged in a microfuge at 15 000 × *g* for 5 min and aliquots of pellet and super-

natant fraction prepared for immunoprecipitation of labeled PrP.

*4.10. Immunoprecipitation of radiolabeled PrP*

Immunoprecipitation of cell media was performed in incubation buffer (see above) supplemented with 1 mM EDTA, 0.1 mM PMSF, and 0.5% Nonidet P40. Cells were lysed in 50 mM Tris–HCl (pH 8), 200 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.5% Nonidet P40 and 5 units of aprotinin per ml for 15 min on ice, nuclei and cell debris removed by centrifugation (1000 × *g*, 5 min, 4°C) and cell extracts used for immunoprecipitation. Cell media or extracts were incubated with a 1:500 dilution of the SHaPrP-specific monoclonal antibody 3F4 (Kascsak et al., 1987) overnight at 4°C. Fifty microliters of a 10% aqueous slurry of protein A-agarose (Pierce, Rockford, IL) were added and incubation continued for 1 h at 4°C. Immune complexes formed with protein A–agarose were washed three times with 50 mM Tris–HCl (pH 8), 500 mM NaCl, 5 mM EDTA, 0.5% Nonidet P40, and once with 50 mM Tris–HCl (pH 8), 150 mMNaCl, 5 mM EDTA and boiled in SDS sample buffer for 3 min. Following SDS-PAGE, the gels were fluorographed, dried and exposed to Kodak X-Omat AR film at −80°C.

*4.11. Lifespan measurements*

Flies from the strain $w^{67c23}$, control transgenic P[CaSpeR-hs] and transgenic P[SHaPrP-hs]31 flies were used for lifespan measurements. One hundred flies were collected within 24 h of eclosion and placed in vials (10 per vial, separated by sex). The flies were either kept at 25°C or subjected to continuous heat pulses of 25 min at 37°C followed by a recovery period of 50 min at 25°C. These heat pulses were delivered by a programmable thermocycling incubator (Biotherm Corporation, Fairfax, VA). The adults were transferred to new vials with fresh medium every 3 days and scored for survivors every day.

*4.12. Choline acetyltransferase (ChAT) assay*

Newly eclosed flies from the wt strain $w^{67c23}$, control transgenic P[CaSpeR-hs] line and transgenic P[SHaPrP-hs]11 and 31 lines were subjected to multiple heat pulses as described for lifespan measurements. At selected timepoints, three flies were anesthetized with ether, decapitated, the heads pooled and homogenized on ice in a glass homogenizer in 0.1 ml of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl and 0.5% Triton X-100. ChAT activity was measured in triplicates on a 2 μl-aliquot of the homogenate by the procedure of Fonnum (1975) with modifications as described previously (Raeber et al., 1989).

## Acknowledgments

The authors thank Dr. C. Goodman for providing the plasmid pPC4. This work was supported by grants from the National Institutes of Health (NS14069, AG02132, NS22786, AG10770 and AG08967) and the American Health Assistance Foundation, as well as by a gift from the Sherman Fairchild Foundation. A.J.R. was supported by a postdoctoral fellowship from the Schweizerische Gesellschaft für Medizinisch-biologische Stipendien.

## References

Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
Büeler, H., Aguzzi, A., Sailer, A., Greiner, R.-A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Cell 73, 1339–1347.
Davidson, D.J., Fraser, M.J. and Castellino, F.J. (1990) Biochemistry 29, 5584–5590.
Dewhurst, S.A., McCaman, R.E. and Kaplan, W.D. (1970) Biochem. Genet. 4, 499–508.
Drain, P., Folkers, E. and Quinn, W.G. (1991) Neuron 6, 71–82.
Endo, T., Groth, D., Prusiner, S.B. and Kobata, A. (1989) Biochemistry 28, 8380–8388.
Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
Fonnum, F. (1975) J. Neurochem. 24, 407–409.
Gnagey, A.L., Forte, M. and Rosenberry, T.L. (1987) J. Biol. Chem. 262, 13 290–13 298.
Haraguchi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarantino, A., Borchelt, D.R., Teplow, D., Hood, L., Burlingame, A., Lycke, E., Kobata, A. and Prusiner, S.B. (1989) Arch. Biochem. Biophys. 274, 1–13.
Hay, B., Barry, R.A., Lieberburg, I., Prusiner, S.B. and Lingappa, V.R. (1987) Mol. Cell. Biol. 7, 914–920.
Hortsch, M. and Goodman, C.S. (1990) J. Biol. Chem. 265, 15 104–15 109.
Hsiao, K.K., Groth, D., Scott, M., Yang, S.L., Serban, H., Rapp, D., Foster, D., Torchia, M., DeArmond, S.J. and Prusiner, S.B. (1994) Proc. Natl. Acad. Sci. USA 91, 9126–9130.
Iwasaki, M., Okumura, K., Kondo, Y., Tanaka, T. and Igarashi, H. (1992) Nucl. Acids Res. 20, 4001–4007.
Jokerst, R.S., Weeks, J.R., Zehring, W.A. and Greenleaf, A.L. (1989) Mol. Gen. Genet. 215, 266–275.
Karess, R.E. and Rubin, G.M. (1984) Cell 38, 135–146.
Kascsak, R.J., Rubenstein, R., Merz, P.A., Tonna-DeMasi, M., Fersko, R., Carp, R.I., Wisniewski, H.M. and Diringer, H. (1987) J. Virol. 61, 3688–3693.
Klemenz, R., Weber, U. and Gehring, W.J. (1987) Nucl. Acids Res. 10, 3947–3959.
Klemm, N. (1976) Prog. Neurobiol. 7, 99–169.
Krantz, D.E. and Zipursky, S.L. (1990) EMBO J. 9, 1969–1977.
Laemmli, UK (1970) Nature 227, 680–685.
Low, M.G. (1987) Biochem. J. 244, 1–13.
Low, M.G., Stiernberg, J., Waneck, G.L., Flavell, R.A. and Kincade, P.W. (1988) J. Immunol. 113, 101–111.
Mobley, W.C., Neve, R.L., Prusiner, S.B. and McKinley, M.P. (1988) Proc. Natl. Acad. Sci. USA 85, 9811–9815.
Pan, K.M., Stahl, N. and Prusiner, S.B. (1992) Protein Sci. 1, 1343–1352.
Phillips, J.P., Xin, J.H., Kirby, K., Milne, C.P., Jr., Krell, P. and Wild, J.R. (1990) Proc. Natl. Acad. Sci. USA 87, 8155–8159.

Pirotta, V. (1988) In Rodriguez, R.L. and Denhardt, D.T. (eds.), Vectors, a Survey of Molecular Cloning Vectors and their Uses. Butterworths, Boston, MA. pp. 437–456.
Prusiner, S.B. (1994) Annu. Rev. Microbiol. 48, 655–685.
Prusiner, S.B., Groth, D., Serban, A., Koehler, R., Foster, D., Torchia, M., Burton, D., Yang, S.L. and DeArmond, S.J. (1993) Proc. Natl. Acad. Sci. USA 90, 10 608–10 612.
Prusiner, S.B. and Hsiao, K.K. (1994) Ann. Neurol. 35, 385–395.
Prusiner, S.B., Scott, M., Foster, D., Pan, K.M., Groth, D., Mirenda, C., Torchia, M., Yang, S.L., Serban, D., Carlson, G.A., Hoppe, P.C., Westaway, D. and DeArmond, S.J. (1990) Cell 63, 673–686.
Raeber, A.J., Borchelt, D.R., Scott, M. and Prusiner, S.B. (1992) J. Virol. 66, 6155–6163.
Raeber, A.J., Riggio, G. and Waser, P.G. (1989) Eur. J. Biochem. 186, 487–492.
Rancourt, D.E., Walker, V.K. and Davies, P.L. (1987) Mol. Cell. Biol. 7, 2188–2195.
Reveillaud, I., Niedzwiecki, A., Bensch, K.G. and Fleming, J.E. (1991) Mol. Cell. Biol. 11, 632–640.
Rogers, M., Taraboulos, A., Scott, M., Groth, D. and Prusiner, S.B. (1990) Glycobiology 1, 101–109.
Rubenstein, R., Deng, H., Scalici, C.L. and Papini, M.C. (1991) J. Gen. Virol. 72, 1279–1285.
Rubin, G.M. and Spradling, A.C. (1982) Science 218, 348–353.
Salvaterra, P.M. and McCaman, R.E. (1985) J. Neurosci. 5, 903–910.
Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 2nd Edn.
Schneider, I. (1972) J. Embryol. Exp. Morphol. 27, 353–365.
Scott, M., Butler, D., Bredesen, D., Wälchli, M., Hsiao, K. and Prusiner, S.B. (1988) Protein Eng. 2, 69–76.
Scott, M., Groth, D., Foster, D., Torchia, M., Yang, S.L., DeArmond, S.J. and Prusiner, S.B. (1993) Cell 73, 979–988.
Scott, M.R., Köhler, R., Foster, D. and Prusiner, S.B. (1992) Protein Sci. 1, 986–997.
Snow, P.M., Bieber, A.J. and Goodman, C.S. (1989) Cell 59, 313–323.
Stahl, N., Borchelt, D.R., Hsiao, K. and Prusiner, S.B. (1987) Cell 51, 229–240.
Stahl, N., Borchelt, D.R. and Prusiner, S.B. (1990) Biochemistry 29, 5405–5412.
Taraboulos, A., Rogers, M., Borchelt, D.R., McKinley, M.P., Scott, M., Serban, D. and Prusiner, S.B. (1990) Proc. Natl. Acad. Sci. USA 87, 8262–8266.
Telling, G.C., Scott, M., Foster, D., Yang, S.L., Torchia, M., Sidle, K.C.L., Collinge, J., DeArmond, S.J. and Prusiner, S.B. (1994) Proc. Natl. Acad. Sci. USA 91, 9936–9940.
Westaway, D., DeArmond, S.J., Cayetano-Canlas, J., Groth, D., Foster, D., Yang, S.L., Torchia, M., Carlson, G.A. and Prusiner, S.B. (1994) Cell 76, 117–129.
Westaway, D. and Prusiner, S.B. (1986) Nucl. Acids Res. 14, 2035–2044.
Wigler, M.A., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) Proc. Natl. Acad. Sci. USA 76, 1373–1376.