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Neuron-Specific Expression of a Hamster Prion Protein Minigene in Transgenic Mice Induces Susceptibility to Hamster Scrapie Agent

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Summary

To study the effect of cell type–restricted hamster PrP expression on susceptibility to the hamster scrapie agent, we generated transgenic mice using a 1 kb hamster cDNA clone containing the 0.76 kb HPrP open reading frame under control of the neuron-specific enolase promoter. In these mice, expression of HPrP was detected only in brain tissue, with highest levels found in neurons of the cerebellum, hippocampus, thalamus, and cerebral cortex. These transgenic mice were susceptible to infection by the 263K strain of hamster scrapie with an average incubation period of 93 days, compared to 72 days in normal hamsters. In contrast, nontransgenic mice were not susceptible to this agent. These results indicate that neuron-specific expression of the 1 kb HPrP minigene including the HPrP open-reading frame is sufficient to mediate susceptibility to hamster scrapie, and that HPrP expression in nonneuronal brain cells is not necessary to overcome the TSE species barrier.

Introduction

Transmissible spongiform encephalopathies (TSE) are degenerative brain diseases that occur naturally in primates and ruminants, and include scrapie of sheep, bovine spongiform encephalopathy, and several human diseases such as Creutzfeldt-Jakob disease, Kuru, and Gerstmann-Sträussler-Scheinker syndrome. An important feature of TSE diseases is the accumulation in brain of a proteinase K–resistant protein, known as PrP-res or PrP^{Sc}, which appears to be strongly associated with the pathogenic process (Bolton et al., 1982; Prusiner, 1982; Diringer et al., 1983). PrP-res is posttranslationally derived from a normal host proteinase K–sensitive PrP molecule (PrP-sen; Borchelt et al., 1990; Caughey and Raymond, 1991; Stahl et al., 1993) by an as yet undefined mechanism. Brain preparations highly enriched for scrapie infectivity contain large amounts of PrP-res, but no scrapie-specific nucleic acid has been detected (Alper et al., 1978; Hunter, 1979; Latarjet, 1979; Dees et al., 1985; Bellinger-Kawahara et al., 1987). As a result, it has been suggested that PrP-res is the etiologic agent of TSE (Prusiner, 1982). Although the precise nature of the causative agent remains unresolved, several studies have established an important role for PrP in TSE pathogenesis. For example, there is a close linkage between the PrP gene and a gene controlling the length of the scrapie incubation period in mice and sheep (Carlson et al., 1988; Hunter et al., 1989; Race et al., 1990). Also, mutations in the human PrP gene are associated with occurrence of familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and Fatal Familial Insomnia (Doh-ura et al., 1989; Hsiao et al., 1989, 1991a, 1991b; Goldgaber et al., 1989; Goldfarb et al., 1990). In addition, PrP null mice devoid of PrP-sen are resistant to experimental scrapie, indicating that expression of PrP-sen is an absolute requirement for scrapie agent replication and disease induction (Bueler et al., 1993).

PrP may also play a critical role in interspecies transmission of TSE diseases. Many species show resistance to disease induction by TSE agents derived from other species. This resistance or "species barrier" is manifested either by total lack of disease induction or by a prolonged incubation period prior to onset of clinical disease. For instance, Chandler mouse scrapie can be transmitted to Syrian hamsters with an average incubation period of 378 days, but in mice the incubation period is 120 days (Kimberlin and Walker, 1978; Kimberlin et al., 1987). Conversely, the 263K hamster scrapie strain has a 72 day incubation period in Syrian hamsters, but does not cause clinical disease in mice. Of particular concern is whether or not various strains of TSE in animals can be transmitted to humans. Epidemiological studies indicate that transmission from scrapie-infected sheep to humans does not occur. However, the recent epidemic of bovine spongiform encephalopathy in Great Britain has raised worries that this apparently new strain of TSE might be infectious to humans.

These concerns have highlighted the importance of understanding the basis for species barriers in the transmission of TSE. Genetic studies have indicated that the PrP genotype strongly influences the host susceptibility to TSE agents (Carlson et al., 1986; Hunter et al., 1987; Race et al., 1990). Transgenic mice with a 40 kb transgene expressing high levels of hamster PrP (HPrP)–sen are susceptible to disease when inoculated with the hamster scrapie agent, while normal mice are resistant (Scott et al., 1989; Prusiner et al., 1990). This suggests that transmission of scrapie may be dependent on interactions between the host PrP-sen and the PrP-res associated with the inoculated agent. The importance of direct PrP-sen–PrP-res interactions in PrP-res formation has now been documented in cell-free reactions (Kocisko et al., 1994, 1995). Furthermore, interactions between PrP molecules from different species have been found to inhibit generation of PrP-res in both scrapie-infected cells (Priola et al.,

1994) and in cell-free systems (Kocisko et al., 1994, 1995), and such inhibitory interactions may provide a biochemical explanation for the species barrier.

The normal PrP-sen precursor of PrP-res appears to be expressed in a wide variety of cells and tissues (Oesch et al., 1985; Caughey et al., 1988; Brown et al., 1990), and it is unclear which of these sites might be involved in the possible effect of PrP on TSE agent replication and PrP-res formation following interspecies transmission. TSE agents are known to replicate in both lymphoreticular organs and brain, but the precise cell types involved are not known. PrP-sen expression has recently been demonstrated in astrocytes and oligodendrocytes (Moser et al., 1995), and astrocytes have been found to be the earliest site of PrP-res accumulation in the brain (Diedrich et al., 1991). Together, these results argue for the direct involvement of glia in scrapie agent propagation. However, in mouse and hamster brain, high levels of PrP mRNA and PrP-sen protein have also been detected in neurons (Kretzschmar et al., 1986; Brown et al., 1990; Manson et al., 1992, 1994). Therefore, it is unclear whether PrP expression in both neurons and astrocytes is critical to PrP-res formation. In the present work, we show that transgenic mice expressing the hamster PrP (HPrP) gene under control of the neuron-specific enolase promoter (Forss-Petter et al., 1990) are highly susceptible to the hamster scrapie agent. In these mice, HPrP expression was found exclusively in neurons and not in glial cells or cells within the spleen or lymph nodes. Thus, neuron-specific hamster PrP expression was sufficient to abrogate the TSE species barrier, and hamster PrP expression in lymphoreticular tissues or nonneuronal brain cells including astrocytes was not required to overcome resistance of mice to the hamster scrapie agent. Furthermore, because the transgene used in these experiments contained only 1 kb of hamster DNA including the open reading frame of hamster PrP, the present results demonstrate that susceptibility of these transgenic mice to hamster scrapie is mediated by the hamster PrP gene itself rather than the additional 39 kb of transgene DNA used in previous studies (Scott et al., 1989; Prusiner et al., 1990; Westaway et al., 1994).

Results

Generation of HPrP Transgenic Mice

To investigate the role of HPrP in the interspecies transmission of hamster scrapie, two lines of HPrP transgenic mice were produced. The Tg52NSE line was derived by inoculation of a construct containing the neuron-specific enolase (NSE) promotor plus a 1 kb cDNA containing the HPrP open reading frame (Figure 1A). A second HPrP transgenic line, Tg10, was generated with a cosmid vector containing the HPrP gene plus 40 kb of flanking DNA (Scott et al., 1989). Previously, this cosmid was used to generate another line of transgenic mice susceptible to hamster scrapie (Scott et al., 1989). Following production of transgenic mice by standard techniques, positive founder mice were bred to nontransgenic C57BL/10 mice, and the transgene was maintained in heterozygous form by selection at each breeding cross. By comparative slot blot analysis, the transgene copy number was approximately 8 for Tg52NSE and 12 for Tg10 mice (data not shown).

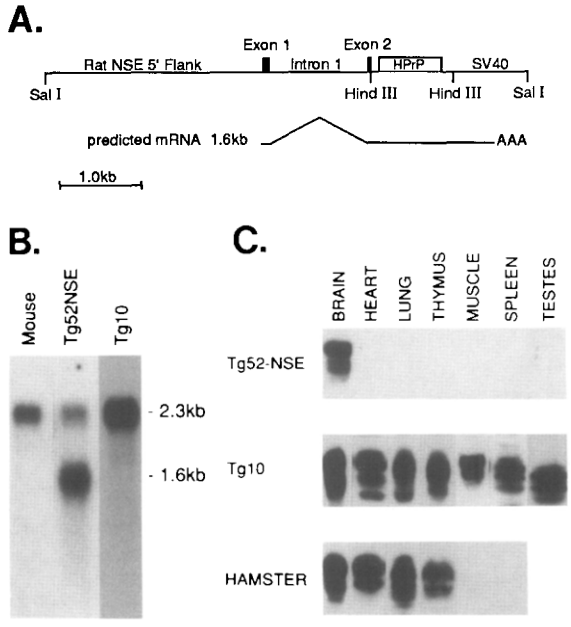

Figure 1A. Structure of NSE-HPrP Transgene and Predicted mRNA

(A) 1.0 kb fragment of the hamster PrP (HPrP) cDNA (Robakis et al., 1986a, 1986b) containing the 762 bp open reading frame (boxed) was cloned into the HindIII site of pNSE-Ex 4, which was generated and provided by Dr. Sonja Forss-Petter, Scripps Research Institute, from a previously described genomic clone of the rat NSE gene (Forss-Petter et al., 1990; Mucke et al., 1994). pNSE-Ex 4 contains 2.8 kb of rat NSE flanking sequences upstream of the transcriptional start of exon 1, 54 bp of exon I, a 1.2 kb intron, and 6 bp of exon 2 upstream of the unique HindIII cloning site. Downstream of the HindIII site is a 1 kb fragment of SV40 sequence used as a molecular tag for transgenic RNA in in situ hybridization studies. The poly A signal is located in the middle of the SV40 DNA. The transgene DNA was excised from the bacterial plasmid vector, pUC 19, by digestion with SalI, and the large SalI fragment shown was purified and used for inoculation of mouse cells for generation of transgenic mice.

(B) Expression of HPrP mRNA in transgenic mouse brain. Polyadenylated RNA (2 μg) extracted from brain of a normal nontransgenic mouse, a Tg52NSE, or a Tg10 mouse was separated on an agarose/formaldehyde gel, blotted, and hybridized to a ^{32}P-labeled DNA probe derived from HPrP. Under the conditions used, this probe hybridizes to RNA derived from either mouse PrP (MPrP) or HPrP. The 2.3 kb mRNA common to all three lanes represents MPrP mRNA derived from the endogenous MPrP gene. The HPrP mRNA derived from Tg10 brain is also present at 2.3 kb. The unique 1.6 kb mRNA band in the Tg52NSE lane is derived from the NSE-HPrP construct and is the size expected for the fully spliced transcript.

(C) Western blot detection of HPrP-sen in tissues from a Tg52NSE mouse, a Tg10 mouse, and a normal hamster. Suspensions of various tissues were separated on 15% SDS-polyacrylamide electrophoresis gels. Proteins were transferred to Immobilon nylon membranes and immunoblots performed using antibody 3F4, which recognizes hamster PrP but not mouse PrP. Blots were developed using the Enhanced Chemiluminescence reagent system (Amersham). Immunoreactive bands shown represent HPrP-sen. HPrP-sen was detected in all of the indicated tissues from Tg10 mice. In Tg52NSE mice, HPrP-sen was detected only in brain. Normal hamsters had HPrP-sen in brain, heart, lung, and thymus but not in muscle or spleen. Hamster testes were not assayed.

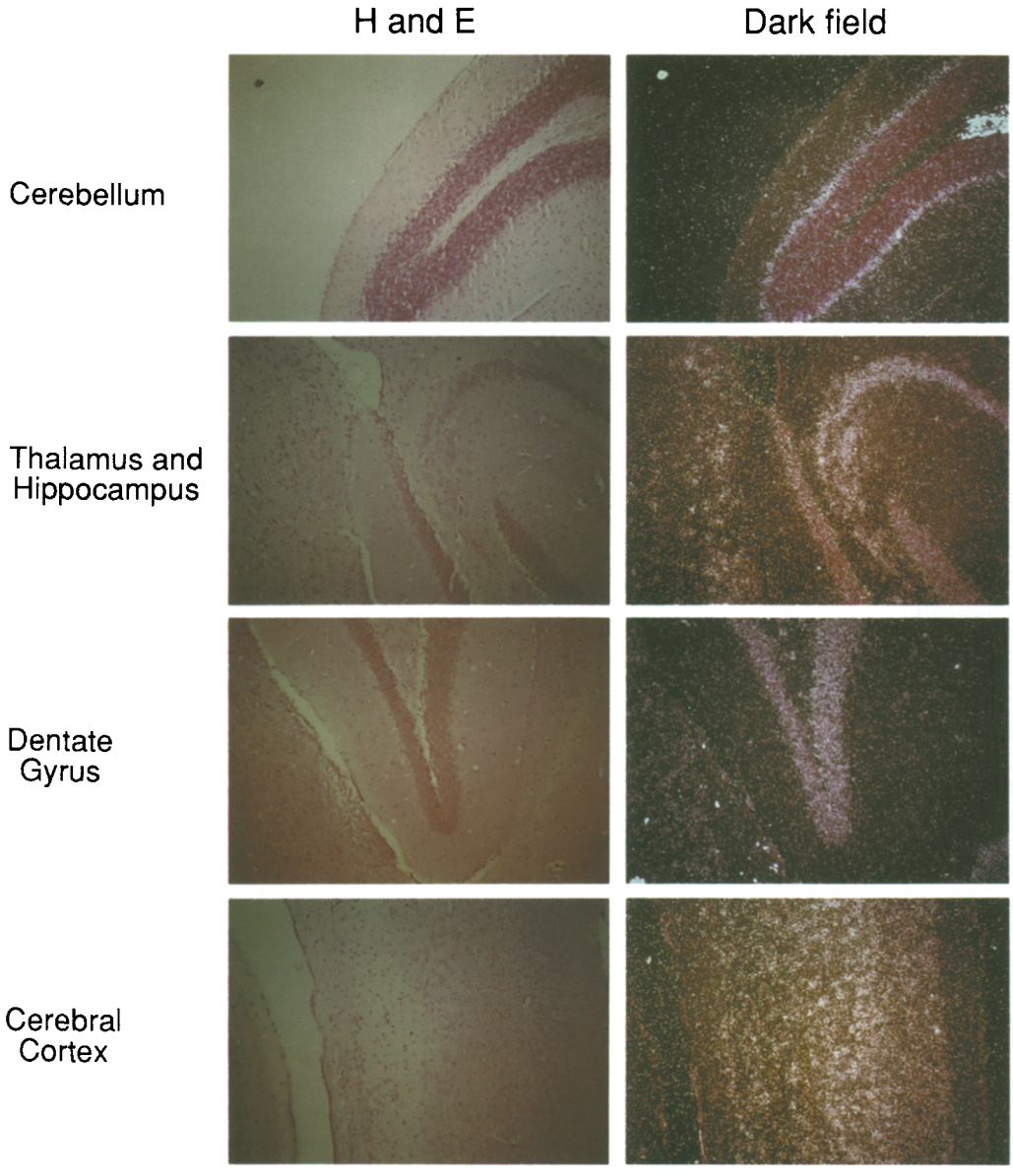

Figure 2. Expression of HPrP mRNA in Various Brain Regions of Tg52NSE Mice

Brain sections were processed for in situ hybridization and light microscopy as described in the Experimental Procedures. The probe used was from a portion of SV40 sequence unique to the transgene (see Figure 1A). Identical fields were photographed with conventional illumination to show histology with hematoxylin and eosin staining ("H and E"; first column) and by dark field illumination (second column) to show the location of silver grains (white dots) corresponding to HPrP mRNA. Strong signals were detected in the band of Purkinje cells adjacent to the granular layer of the cerebellum, and at higher magnification silver grains were localized over individual Purkinje cells (not shown). Granule cell neurons of the cerebellum were also positive, but this was not obvious at the magnification shown here (first row). Regions containing the pyramidal neurons of the hippocampus and the neurons of the dentate gyrus were strongly positive (second and third rows), and individual cells in the adjacent thalamus were also positive (second row, left panel). Several layers of positive cells were also detected in the cerebral cortex (fourth row).

HPrP mRNA Expression in Brains of Transgenic Mice

To analyze HPrP mRNA expression in the brain of transgenic mice, Northern blotting was performed with a probe reactive with both mouse and hamster PrP genes. Tg52NSE mice expressed a 1.6 kb PrP mRNA band corresponding to the predicted HPrP transgene in addition to the 2.3 kb PrP mRNA band expressed in nontransgenic mice (Figure 1B). The intensity of the 1.6 kb HPrP band was 4–5 times higher than that of the 2.3 kb endogenous mouse PrP band. In contrast, Tg10 mice containing the cosmid HPrP transgene showed no new PrP bands distinguishable from mouse PrP by Northern blotting (Figure 1B). However, the increased intensity of the band at 2.3 kb was consistent with expression of a 2.3 kb HPrP mRNA from the cosmid transgene.

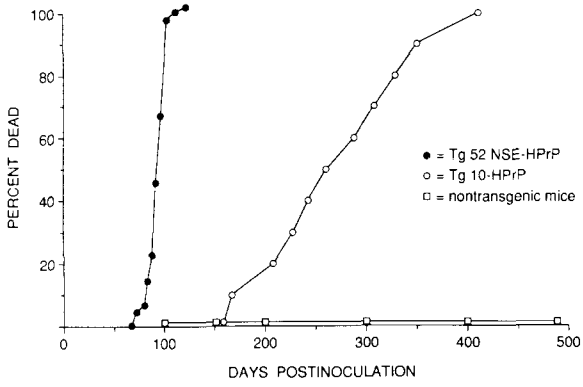

Figure 3. Kinetics of Death of Tg52NSE Mice, Tg10 Mice, and Nontransgenic Mice after Intracerebral Inoculation with Hamster Scrapie Strain 263K

Data are pooled from experiments done at Rocky Mountain Laboratories and Scripps Research Institute (Tg52NSE mice, n = 60; Tg10 mice, n = 65; and nontransgenic mice, n = 25).

HPrP Protein Expression in Transgenic Mouse Tissues

To study tissue specificity of HPrP protein expression, a variety of tissues from both HPrP transgenic mouse lines and normal hamsters was analyzed by immunoblotting using monoclonal antibody 3F4, which has a strong reactivity for HPrP and no reactivity for mouse PrP (Kascsak et al., 1987). In Tg52NSE mice, HPrP protein was detected in brain, but not in six other tissues studied (Figure 1C). In contrast, Tg10 mice and normal hamsters expressed HPrP protein in a variety of other tissues in addition to brain (Figure 1C). Thus, the transgene in Tg52NSE mice showed a restricted pattern of expression as expected from the use of the neuron-specific enolase promoter (Forss-Petter et al., 1990).

Neuronal Localization of HPrP Expression in Tg52NSE Mice

In situ hybridization was used to study localization of HPrP mRNA in Tg52NSE mouse brain. For detection, we used a probe from SV40-derived intervening sequences present only in the HPrP mRNA of the Tg52NSE mice (Figure 1A). HPrP mRNA was detected in a variety of neuronal populations of the brain. Highest expression was seen in Purkinje cells of the cerebellum, neurons of the dentate gyrus, and pyramidal neurons of the hippocampus (Figure 2). Granular layer neurons of the cerebellum, cells in various layers of the cerebral cortex, and cells with large nuclei in the dorsal portion of the thalamus also expressed moderate amounts of HPrP mRNA (Figure 2). These results were consistent with neuron-specific expression of the HPrP transgene in Tg52NSE mice.

Further confirmation of neuronal expression of the HPrP transgene was achieved using primary hippocampal neuron cultures and astrocyte cultures. Using monoclonal antibody 3F4, greater than 90% of neurons from Tg52NSE mice had detectable HPrP protein after in vitro culture for 48 hr. This was observed in 3 out of 5 experiments. In contrast, astrocytes cultured from these same mice never expressed detectable HPrP (data not shown).

Table 1. Comparison of Clinical Disease Induced by the 263K Strain of Hamster Scrapie.

Clinical Data	Transgenic Mice		Syrian Hamsters
	Tg52NSE	Tg10	
Number of animals	60	65	43
Average interval to death[a]	93 ± 8 d	248 ± 64 d	72 ± 3 d
Duration of symptoms	1–3 d	30–90 d	10–15 d
Ataxia	+	+	+
Tremors	–	+	+
Paralysis	–	+	–
Stilted gait[b]	+	–	–
Somnolence	–	–	+

[a] Mean ± standard deviation; d, days. Animals were sacrificed when clinical condition was near terminal. Nontransgenic mice inoculated with hamster 263K scrapie agent showed no signs of disease within a normal life span (2 years).

[b] Stilted gait was characterized by walking on tiptoes.

Susceptibility of Tg52NSE and Tg10 Mice to Hamster Scrapie Agent

To determine the influence of HPrP expression on susceptibility to hamster scrapie agent, Tg52NSE, Tg10, non-Tg mice, and hamsters were inoculated with hamster scrapie strain 263K. Tg52NSE mice all died between 70 and 118 days postinoculation, while non-Tg littermates were clinically normal 400 days postinoculation (Figure 3). Affected mice exhibited a 1–3 day clinical course characterized by a "stilted" gait, mild ataxia, and inactivity (Table 1). Tg10 mice, on the other hand, had a more variable and protracted clinical course lasting several weeks or even months and died between 80 and 405 days postinoculation (Figure 3). The clinical symptoms in Tg10 mice included whole-body tremors, ataxia, and progression to paralysis and death (Table 1). In hamsters, clinical symptoms included ataxia, tremor, and somnolence (Table 1). Clinically ill Tg52NSE mice and Tg10 mice had easily detectable proteinase K–resistant HPrP and histopathological findings typical of scrapie with astrocytosis and spongiosis (data not shown). Furthermore, brain homogenates from hamster scrapie-infected Tg52NSE and Tg10 mice caused scrapie on reinoculation in hamsters, but not in mice (data not shown). In summary, Tg52NSE and Tg10 mice were highly susceptible to hamster 263K scrapie agent, but both the tempo and symptoms of clinical disease were different in the two transgenic strains.

To determine whether the expression of HPrP would modify the pathogenesis of mouse scrapie, Tg52NSE and Tg10 mice were also inoculated with the Chandler strain of mouse-adapted scrapie agent. Tg52NSE mice died 180 ± 3 days postinoculation (n = 8), while normal littermates died 160 ± 3 days postinoculation (n = 11). Tg10 mice died 201 ± 4 days postinoculation (n = 17), while their normal littermates died 164 ± 4 days after inoculation (n = 14). Thus, expression of HPrP in both Tg52NSE and Tg10 mice delayed the onset of clinical disease induced by the mouse scrapie agent, suggesting that expression of HPrP could partially interfere with development of mouse scrapie.

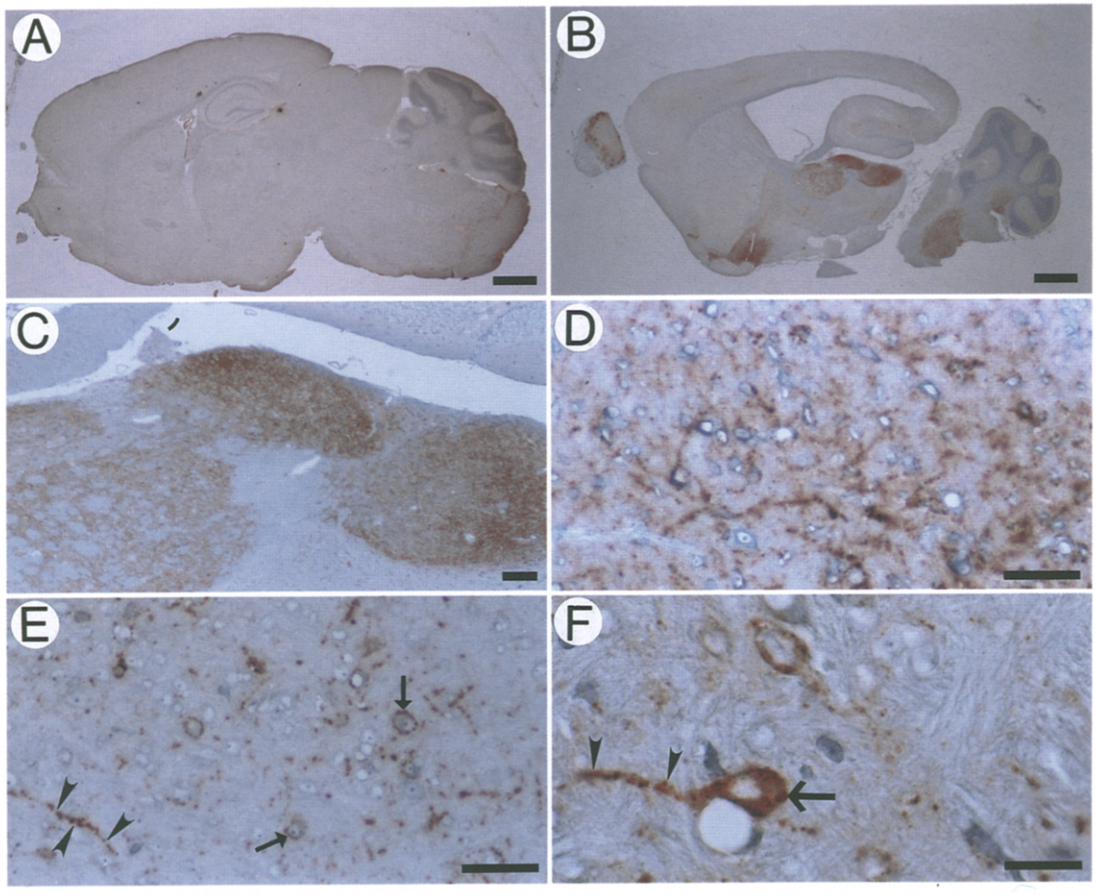

Figure 4. Distribution of HPrP-res in Tg52NSE Brain

Brains from uninfected (A) and hamster scrapie-infected (B–F) Tg52NSE mice were analyzed for HPrP-res by hydrolytic autoclaving and immunostaining using antibody 3F4. HPrP staining was most intense in thalamic nuclei (B and C) and the diagonal band of broca (D) where HPrP was primarily located in the neuropil. Perineuronal (arrows) and "beads-on-a-string" (arrowheads) HPrP staining pattern were found in the anterior hypothalamus (E). In midbrain nuclei (F), intraneuronal HPrP immunostaining was observed in the cell body (arrow) and proximal axons (arrowhead). Enlarged ventricular space in infected mice appeared to be secondary to cortical atrophy, as there was no obvious increased intracranial presence or hydrocephalus on gross examination. Bars in (A) and (B), 1 mm; in (C), 100 μm; in (D) and (E), 50 μm; and in (F), 25 μm.

Pattern of HPrP-res Deposition in Transgenic Mice

The regional and cellular distribution of HPrP-res was determined by immunocytochemistry using antihamster PrP monoclonal antibody 3F4 on brain sections pretreated with hydrolytic autoclaving (Kitamoto et al., 1992). This procedure enhances immunoreactivity of PrP-res and eliminates detection of PrP-sen. The distribution of HPrP-res in brains of Tg52NSE mice infected with hamster scrapie was restricted to specific anatomical regions (Figure 4B) and was not as widespread as reported for hamsters infected with this same scrapie strain (DeArmond et al., 1992). HPrP-res was primarily found in gray matter and was rarely detected in white matter tracts. Intense immunostaining was observed in several thalamic nuclei (Figures 4B and 4C), a diagonal band of Broca (Figures 4B and 4D), the olfactory bulb, and in deep cerebellar nuclei and regions of the midbrain (Figure 4B). A narrow band of HPrP-res immunostaining was also observed in the middle cortical layers of the cerebral cortex (Figure 4B), which was atrophied in 50% of Tg52NSE mice infected with hamster scrapie. There was no detectable HPrP-res staining in the basal ganglia, hippocampus, or cerebellar cortex. Uninfected Tg52NSE mice showed no HPrP immunoreactivity in brain parenchyma and only weak, nonspecific immunoreactivity in the choroid plexus and glial limitans (Figure 4A).

The HPrP-res immunoreactivity in the brains of hamster scrapie–infected Tg52NSE mice had either a diffuse or focal, plaque-like staining pattern. In several brain regions, HPrP-res was located in, or adjacent to, neuronal perikarya. For example, perineuronal HPrP-res staining in the anterior hypothalamic area completely encircled individual neurons (Figure 4E, arrows). A "beads-on-a-string" HPrP-res staining pattern (Figure 4E, arrowheads) also was observed, and these linear structures had a narrow width but could be as long as 120 μm. This pattern was reminiscent of axonal staining, although we could not definitively associate this distribution with nerve cell bodies. However, in the midbrain, HPrP-res staining appeared to be located in the perikaryon and proximal axon of individual neurons (Figure 4F).

Discussion

Previous transgenic mice expressing HPrP were made using a 40 kb cosmid clone containing the HPrP gene (Scott et al., 1989; Prusiner et al., 1990). Because of the large amount of DNA in these transgenes, it was not possible to prove that HPrP expression was the only genetic factor involved in the induction of susceptibility to hamster scrapie in these mice. Furthermore, earlier attempts to produce transgenic mice using only the HPrP open reading frame were not successful (Scott et al., 1989). In contrast, the present experiments succeeded in getting high levels of HPrP expression by using a transgene containing only 1 kb of hamster DNA including the 762 base pair open reading frame of HPrP together with the neuron-specific enolase promoter. Thus, the high susceptibility of Tg52NSE mice to hamster scrapie demonstrates that this HPrP minigene including the open reading frame itself is the critical element in inducing susceptibility to the hamster scrapie agent in vivo.

In the present work, we have studied only one NSE-HPrP transgenic mouse line, Tg52NSE. Therefore, it is conceivable that the susceptibility of this line to hamster scrapie might be a result of site-specific disruption of the function of a normal mouse gene, rather than expression of the HPrP trangene. However, this is highly unlikely since several transgenic mouse lines expressing HPrP with the PrP promoter have been studied (see Figure 3) (Scott et al., 1989; Prusiner et al., 1990), and in all cases HPrP expression level correlates with susceptibility to hamster scrapie. Thus, the HPrP expression level, rather than disruption of an unknown mouse gene, appears to be the critical factor in abrogating the species barrier in this system.

Neuron-specific expression mediated by the NSE promotor has previously been found in several other transgenic mouse models (Forss-Petter et al., 1990; Mucke et al., 1994; Rall et al., 1995). In the present report, neuron-specific HPrP expression was demonstrated by both in vivo and in vitro experiments and was sufficient to render mice susceptible to CNS disease induced by the 263K hamster scrapie strain. Several previous reports indicate that astrocytes and splenic follicular dendritic cells (FDC) may be the earliest sites of PrP-res accumulation following scrapie infection (Diedrich et al., 1991; Muramoto et al., 1993; Moser et al., 1995), and these sites might also be important in restriction of agent replication following interspecies transmission of TSE agents (Muramoto et al., 1993). However, based on the present findings, HPrP expression in astrocytes or FDC was not required to mediate susceptibility of mice to intracerebral inoculation with hamster scrapie. Nevertheless, in addition to neurons, HPrP expression in cells such as astrocytes, FDC, or even other cell types might also be sufficient to overcome the scrapie species barrier, and FDC in spleen and lymph nodes might be particularly involved in interspecies transmission following intraperitoneal inoculation of agent.

Based on recent results involving cell-free interactions between PrP-sen and PrP-res from mouse and hamster (Kocisko et al., 1994, 1995), it seems likely that the species specificity of TSE agents involves direct interactions between PrP molecules, which either facilitate or inhibit the process of PrP-res generation in vivo. For example, incubation of mouse PrP-res with hamster PrP-sen or mouse PrP-sen resulted in generation of protease-resistant products with differing subunit molecular weights (Kocisko et al., 1995). This suggested that the biochemical structures of the intact protease-resistant PrP forms were not identical when PrP-sen from different species were used. Such structural differences might be involved in the species-specific adaptation that often occurs following interspecies transmission of TSE agents.

In contrast to the present and previous (Scott et al., 1989; Prusiner et al., 1990) data with transgenic mice, we have not been successful in infecting mouse neuroblastoma cells in vitro with hamster scrapie, even when clones expressing high levels of HPrP were utilized (Priola et al., 1994). In mouse neuroblastoma cells infected with mouse scrapie, the expression of hamster PrP interfered with generation of mouse PrP-res (Priola et al., 1994), and by analogy similar interactions may also be capable of blocking exogenous infection by the hamster scrapie agent in these cell lines. Although in the present experiments inhibitory effects of HPrP expression on infection of Tg52NSE mice with mouse scrapie resulted in a significantly increased incubation period, all mice eventually developed clinical disease. In the converse experiment of infection of Tg52NSE by hamster scrapie agent, the incubation period was longer than in normal hamster (Table 1), but again all mice were susceptible. At present, we have no adequate explanation for the differences in interspecies infection experiments between in vitro mouse neuroblastoma cell lines and transgenic mice in vivo; however, one important factor might be that the mice are 100,000-fold more sensitive to scrapie infection than are mouse neuroblastoma cells (Race et al., 1988; Race and Ernst, 1992).

The present results indicated that some sites of high HPrP-sen expression in Tg52NSE mice, such as cerebellar cortex and hippocampus, had no detectable HPrP-res deposition. Thus, factors other than level of HPrP-sen expression can also influence HPrP-res accumulation. Such factors might include foreign PrP molecules and cellular glycosaminoglycans, both of which have been noted to influence the biochemistry of PrP-res generation in vitro (Caughey and Raymond, 1993; Caughey et al., 1994; Priola et al., 1994). These same factors might also account for the difference in clinical course and in sites of PrP-res deposition between Tg52NSE mice and normal hamsters after infection with hamster scrapie strain 263K. In addition, non-PrP genetic differences involving major histocompatibility complex genes (Kingsbury et al., 1983; Carp and Callahan, 1986) or other genes (Carlson et al., 1988; Race et al., 1990; Westaway et al., 1991) might also influence patterns of both PrP-res deposition and clinical disease following interspecies TSE agent transmission.

Although experiments with PrP transgenic mice have provided helpful insights into the importance of PrP in scrapie pathogenesis and interspecies transmission, it is

possible that some phenomena observed in transgenic mice are the result of abnormal overexpression of PrP. For example, some transgenic mice expressing mouse or nonmouse PrP have developed a spontaneous degenerative brain disease in the absence of scrapie infection (Hsiao et al., 1990; Westaway et al., 1994). In other instances, transgenic mice expressing an MPrP gene associated with resistance to most mouse scrapie strains had increased sensitivity to mouse scrapie (Westaway et al., 1991). Both of these unexpected examples of neurodegenerative disease in PrP transgenic mice are believed to involve overexpression of PrP, but the detailed pathogenic mechanisms involved in each are not known. Thus, it will be important in the future to confirm conclusions derived from transgenic mice by using PrP null mice, where the mouse PrP gene can be replaced in its normal context in the mouse genome by a single copy of a mutant or foreign PrP gene.

Experimental Procedures

Generation of Transgenic Mice

A 1.0 kb fragment of hamster PrP cDNA containing the 762 bp open reading frame plus 54 bp of upstream sequence and 175 bp of downstream sequence was obtained by digestion with EcoRI and HindIII from pEA974 provided by Dr. N. Robakis (Robakis et al., 1986a, 1986b). After blunting DNA ends with Klenow enzyme, XhoI linkers were added and the fragment was subcloned in pBluescript-KS+ to generate p2-17. This clone was digested with KpnI, ends were blunted with Klenow enzyme, and a HindIII linker was inserted. Subsequently, the SalI site in the polylinkers was eliminated by SalI digestion, blunting with Klenow, and religation. A 1.0 kb HPrP fragment was then excised from this clone with HindIII and inserted into the unique HindIII site of pNSE-Ex 4, which was provided by Dr. Sonja Forss-Petter, Scripps Research Institute (Forss-Petter et al., 1990; Mucke et al., 1994). This plasmid was then digested with SalI to remove the pUC 19 vector sequences to produce the purified DNA fragment shown in Figure 1A, which was used to generate Tg52NSE transgenic mice.

The 40 kb cosmid described by Scott et al. (1989) was kindly provided by Dr. Stanley Prusiner and was used to generate Tg10 mice.

Analysis of Transgenic Mouse DNA

Tail DNA isolated as described (Race et al., 1990) was analyzed by slot blot hybridization using standard techniques with an HPrP cDNA probe (Oesch et al., 1985) or by polymerase chain reaction (PCR). Transgenic mice were identified as those having signal intensity greater than that of non-Tg littermates. To estimate HPrP copy number, successive 10-fold DNA dilutions were blotted, hybridized, and compared to nontransgenic mouse or hamster DNA similarly diluted. Alternatively, a hamster-specific PrP polymerase chain reaction (PCR) assay was utilized. The upper strand primer (5′AACCGTTACCCACCTCAGGGT 3′) did not distinguish mouse PrP from hamster PrP; however, the lower strand primer (3′ATGGTGGGTCATAGTCTTCCTC 5′) contained four base differences (underlined) between mouse and hamster PrP. PCR was done using 50–500 ng of DNA in 25 μl volume with standard buffers. The first cycle was 95°C for 5 min, 50°C for 30 sec and 72°C for 1 min. This was followed by 29 cycles of 95°C for 1 min, 50°C for 30 sec, and 72°C for 1 min. Using normal hamster or HPrP Tg mouse DNA as the template, this assay gave a 530 kb DNA band detectable by ethidium bromide staining. No band was seen when normal mouse DNA was used.

Northern Blots

Polyadenylated mRNA was isolated using the Invitrogen Micro-Fast Track mRNA isolation kit according to the manufacturers instructions. Polyadenylated mRNA was electrophoresed in 6% formaldehyde, 1.4% agarose gels, blotted onto Nylon-1 membrane (GIBCO BRL), and baked 2 hr at 80°C. The membranes were hybridized as described (Caughey et al., 1988). The probe was the same as for slot blot analysis.

In Situ Hybridization

In situ hybridization was performed as described previously (Borrow et al., 1995). Briefly, a ^{35}S-labeled single-stranded RNA probe specific for the SV40 sequence was used to detect expression in sections of brain. Two hundred seventeen bases of SV40 sequence were cloned into the pSP70 plasmid (Promega). The plasmid was linearized by BglII digestion, and a product complementary to the SV40 mRNA was transcribed by the Sp6 polymerase. The quantity of probe generated was calculated by measuring the percentage incorporation of the radioisotope. Paraffin-embedded sections from saline-perfused tissue fixed in Bouins or 10% formalin were deparaffinized by washing twice for 5 min in xylene and twice for 5 min in 100% ethanol. The sections were treated, hybridized, and developed as described (Borrow et al., 1995), with the exception that the hybridization temperature was 55°C.

Western Blots

Immunoblotting was used to detect HPrP-sen and HPrP-res in tissues of hamsters and transgenic and normal mice. For PrP-sen, 20% brain homogenates were made in disposable tube and pestle (Kontes, Vineland, New Jersey) in ice-cold 0.32 M sucrose containing 0.1 μM leupeptin, 0.15 μM apoprotinin, 0.1 μM pepstatin, and 0.001 M phenylmethylsulfonylfluoride (PMSF). The homogenate was sonicated for 2 min then microfuged at 10,000 g for 10 min. Supernatant fluid was carefully removed and added to an equal volume of 2× sample buffer. This solution was vortexed, boiled 5 min and frozen until immunoblots could be run.

PrP-res was isolated from brain as previously described, except brain homogenates were made in disposable tube and pestle (Race and Ernst, 1992). Final pellets were suspended by sonication in sample buffer at a concentration of 3 mg/μl. HPrP-sen or HPrP-res proteins were separated on 15% polyacrylamide gels as described (Race and Ernst, 1992), and proteins were detected using a 1:20,000 dilution of ascites containing hamster-specific monoclonal antibody 3F4 (Kascsak et al., 1987), with the Enhanced Chemiluminescence procedure according to the manufacturers instructions (Amersham, Buckinghamshire, England).

HPrP Expression in Cultured Cells

Astrocytes were prepared from 2 day-old mice as described (Rall et al., 1994). Briefly, brains were removed, and cells were mechanically dissociated and cultured on poly-L-lysine-coated culture flasks. Two days after culturing, the cells were extensively washed to remove debris and shaken overnight at 100 rpm to remove nonastroglial cells (microglia). The cultures were then trypsinized and plated onto glass cover slips for immunostaining. Primary hippocampal neurons were plated as described (Pasick et al., 1994). Briefly, hippocampi were dissected from the brains of embryos 15–17 days old, trypsinized and triturated, and cultured on poly-L-lysine-coated glass cover slips at a density of 500 cells/mm^2. Of these cells, 92%–95% are routinely positive for MAP2, a neuronal marker. For immunostaining, cultures were exposed to a 1:200 dilution of MAb 3F4 ascites fluid for 2 hr at 37°C. After gentle washing, cells were fixed in 50% acetone/50% methanol for 10 min, then washed extensively with buffer and reacted with biotinylated antimouse secondary antibody (Mouse Elite, Vector Laboratories, Burlingame, VT). Following a 1 hr incubation with secondary antibody, the cells were washed, reacted with the avidin-biotin reagent, and incubated with hydrogen peroxide plus diaminobenzidene chromogen, resulting in a brown precipitate over positive cells.

Immunohistochemistry

Brains from clinically ill, age-matched mice were perfused with Bouin's fixative or 10% formaldehyde followed by immersion fixation. Paraffin-embedded sections of brain were submersed in 1.0 mM HCl and autoclaved for 10 min (Kitamoto et al., 1992). Immunostaining for brain HPrP-res was performed using mouse monoclonal antibody 3F4 (Kascsak et al., 1987). Briefly, after blocking, tissue was incubated with 3F4 antibody diluted 1:2,000 in 3% bovine serum albumen in Tris-buffered saline (pH 7.4) for 2 hr at 37°C. Tissue sections were washed and incubated with goat antimouse IgG (1:250, Bio-Rad Laboratories, Hercules, CA.) conjugated with horseradish peroxidase for 1 hr at room temperature. Color development was performed with 0.8 mg/ml of 3-amino-9-ethylcarbazole in 50 mM sodium acetate (pH 5.0) and 0.03% water. Tissue sections were counterstained with Mayer's hematoxylin. MAb 3F4 detects hamster PrP but not mouse PrP (Kascsak

et al., 1987). The hydrolytic autoclaving procedure is specific for PrP-res and does not detect PrP-sen.

Scrapie Infection of Animals

Tg52NSE, Tg10 and non-Tg mice and hamsters were inoculated with either 263K strain of hamster scrapie agent (Marsh and Kimberlin, 1975) or the Chandler strain of mouse scrapie agent (Eklund et al., 1967). Mice received 10^8 LD50 of hamster agent or 10^6 LD50 mouse scrapie agent intracerebrally in a volume of 50 μl.

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