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The prion protein gene: a role in mouse embryogenesis?

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Summary

The neural membrane glycoprotein PrP (prion protein) has a key role in the development of scrapie and related neurodegenerative diseases. During pathogenesis, PrP accumulates in and around cells of the brain from which it can be isolated in a disease-specific, protease-resistant form. Although the involvement of PrP in the pathology of these diseases has long been known, the normal function of PrP remains unknown. Previous studies have shown that the PrP gene is expressed tissue specifically in adult animals, the highest levels in the brain, with intermediate levels in heart and lung and low levels in spleen. Prenatally, PrP mRNA has been detected in the brain of rat and hamster just prior to birth. In this study we have examined the expression of the PrP gene during mouse embryonic development by *in situ* hybridisation

and observed dramatic regional and temporal gene expression in the embryo. Transcripts were detected in developing brain and spinal cord by 13.5 days. In addition, PrP gene expression was detected in the peripheral nervous system, in ganglia and nerve trunks of the sympathetic nervous system and neural cell populations of sensory organs. Expression of the PrP gene was not limited to neuronal cells, but was also detected in specific non-neuronal cell populations of the 13.5 and 16.5 day embryos and in extra-embryonic tissues from 6.5 days. This cell-specific expression suggests a pleiotropic role for PrP during development.

Key words. PrP gene, *in situ* hybridisation, mouse embryo.

Introduction

Scrapie, Creutzfeldt-Jakob disease and bovine spongiform encephalopathy are transmissible neurodegenerative disorders associated with alterations in the neural membrane protein PrP (Oesch et al., 1985; review: Hope and Manson, 1991). In unaffected animals, PrP is a protease-sensitive cell surface glycoprotein anchored in the membrane by a glycoinositol phospholipid (Stahl et al., 1987). During the course of these diseases PrP aggregates and accumulates in and around cells of the brain as protease-resistant deposits.

Allelic forms of PrP protein are associated with the incidence of Gerstmann-Straussler (GSS) and Creutzfeldt-Jakob disease (CJD) in humans (Doh-ura et al., 1989; Collinge et al., 1989; Owen et al., 1990) and amino acid differences in PrP have been linked to different incubation periods of scrapie in mice (Westaway et al., 1987), hamsters (Lowenstein et al., 1990) and sheep (Goldmann et al., 1991). Recent experiments have shown that transgenic mice expressing multiple copies of a mutant PrP associated with GSS develop a spontaneous neurodegenerative disease with similar pathology to GSS (Scott et al., 1989; Hsiao et al., 1990).

Although the involvement of PrP in the pathology of

these diseases is now well documented and a role for PrP in their transmissibility is suspected, the normal function of PrP is not known. The conservation of PrP structure in mammalian species suggests an important role for PrP in cell metabolism. Its location on the neural cell membrane has led to suggestions that, like the N-CAMs and cadherins, it may function as a neural cell receptor or as a cell adhesion molecule directing and/or maintaining the architecture of the nervous system (Hope and Manson, 1991). Others have suggested PrP may be an astroglial cell proliferation factor, based on the production of a GFAP-stimulating factor in cultured cell lines from CJD-infected brains (Oleszak et al., 1988) and the gliosis which occurs during the development of neurodegenerative transmissible diseases (DeArmond et al., 1987).

PrP is expressed in specific tissues of adult animals. High levels of PrP mRNA have been detected by Northern analysis in the brain, intermediate levels are found in heart and lung of hamsters and mice, whereas liver and spleen have low or barely detectable levels (Oesch et al., 1985; Caughey et al., 1988). *In situ* hybridisation studies have indicated that expression of PrP within the brain is limited to the neuronal cells (Kretzschmar et al., 1986; Manson et al., 1992). The DNA sequences and trans-acting factors involved in

tissue-specific PrP gene expression have not yet been identified, but there is evidence that nerve growth factor may increase the level of PrP mRNA in both in vivo (Mobley et al., 1988) and in vitro studies (Wion et al., 1988). This may indicate a role for PrP in neuronal differentiation or survival.

Postnatally, PrP gene expression has been shown to be developmentally regulated in the brain. Levels of PrP mRNA were found to increase in the brain during the immediate postnatal period in the hamster (McKinley et al., 1987; Mobley et al., 1988) and in the rat (Liederburg, 1987). In mouse brain, PrP mRNA was detectable at birth and increased 4-fold from birth to day 20 to a level which was maintained throughout adult life (Lazarini et al., 1991). Differential timing of PrP gene expression in different regions of hamster brain has also been shown (Mobley et al., 1988). Early postnatal expression was found in the brainstem and neocortex, intermediate expression in the hippocampus and thalamus and delayed expression in the septum (basal forebrain). Previous studies have either failed to detect PrP mRNA prenatally (McKinley et al., 1987) or detected it only in the brain immediately before birth (Mobley et al., 1988; Liederburg, 1987).

In order to establish the developmental timing of PrP expression and to address whether PrP has a function in embryonic development, we have examined the expression of PrP by in situ hybridisation in mouse embryos. This study has shown that the PrP gene is transcribed both tissue specifically and temporally during embryonic development. Transcripts were not only detected by 13.5 days in the developing brain and spinal cord but were also found in the peripheral nervous system. Detection of PrP transcripts in specific non-neuronal cells and in extra-embryonic tissues suggests a pleiotropic role for PrP during embryogenesis.

Materials and methods

Embryos

All embryos were derived from matings between (C57BL/Ws × CBA/Ca) F1 mice. Embryonic age was determined according to vaginal plug (day 0.5). Embryos were fixed overnight in periodate-lysine paraformaldehyde (2%), dehydrated with ethanols and impregnated in wax during a 16 hour processing cycle. Sagittal and transverse sections of 6 µm thickness from 6.5, 9.5, 13.5 and 16.5 day embryos were mounted on slides coated with Vectabond (Vector laboratories). At least 3 embryos were examined at each stage of development.

Probes for in situ hybridisation

Probe A was prepared by subcloning a 900 bp *KpnI-EcoRI* fragment from exon 2 of the mouse PrP gene (from D. Westaway) into T3T718U phagemid (Pharmacia Ltd). T7 and T3 polymerase were used to synthesise both strands of RNA from the construct after linearisation with the appropriate restriction enzyme. ³⁵S-UTP (Amersham International, 410 Ci/mmol) was incorporated into the single stranded RNA molecules synthesised. The RNA strands were reduced to fragments of approximately 150 bp by incubation in bicarbon-

ate buffer at 60°C. All results were confirmed using RNA synthesised from a different subclone from exon 2 of PrP, a 136 bp *PvuII* fragment cloned into T3T718U phagemid (probe C) (Manson et al., 1992). The specificity of the probes was confirmed by genomic Southern and Northern blot analysis (results not shown).

In situ hybridisation

Slides were deparaffinised, fixed in 4% paraformaldehyde, digested with proteinase K and acetylated with triethanolamine. The slides were then dehydrated and hybridised with 1 × 10⁶ cts/min of ³⁵S-labelled riboprobe at 55°C overnight. Sense and antisense RNA probes were used on adjacent sections. The slides were washed in hybridisation buffer at 55°C for one hour, treated with ribonuclease A at 37°C for 30 minutes and rinsed in 2 × SSC/0.1 × SSC and dehydrated (Davidson et al., 1988). The slides were dipped in Ilford K5 emulsion dried and stored for 7-21 days at 4°C. The slides were developed, air dried and stained with Giemsa.

Results

In situ localisation of PrP in mouse embryos

PrP RNA was detected in sections of mouse embryos using in situ hybridisation with ³⁵S-labelled single stranded RNA probes derived from a mouse genomic clone of PrP (Westaway et al., 1987). Sense is used to describe transcripts which are identical to the primary transcripts of the PrP gene and antisense indicates the complementary orientation which therefore hybridises with the primary transcript.

Initially the hybridisation pattern was characterised using probe A derived from exon 2 of the mouse PrP gene. All results were confirmed using single stranded RNA derived from probe C which contains a different PrP sequence to probe A (Manson et al., 1992). Sagittal and transverse serial sections were hybridised to sense or antisense RNA and hybrids were detected with nuclear track emulsion and autoradiography.

PrP gene expression in the CNS

No PrP transcripts could be detected by in situ hybridisation in 6.5 or 9.5 day old mouse embryos but by 13.5 days expression of the PrP gene was widespread. Transcripts were found both within the CNS and in specific areas outside the CNS of the developing mouse (Fig. 1A). PrP gene expression was detected throughout the developing brain in both the 13.5 and 16.5 day embryos, in the olfactory lobe, the telencephalic cortex of the forebrain, the mantle and ventricular zones of the mesencephalon and the rhombencephalon and in the mantle layer of the spinal cord. Proliferation zones of undifferentiated cell populations and regions containing differentiated neuronal and glial cells throughout the brain were therefore shown to express the PrP gene.

The peripheral nervous system

PrP transcripts were detected in ganglia and nerves of both the central and peripheral nervous systems. Transcripts were detected in cranial and sympathetic ganglia including the trigeminal ganglion (fifth cranial

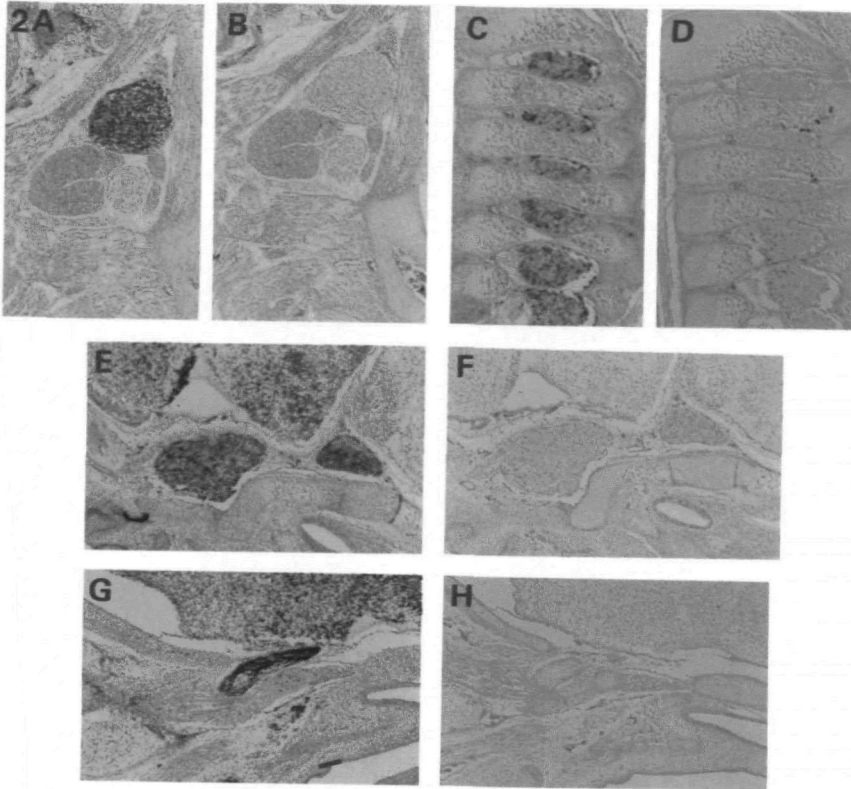


Fig. 2. PrP gene expression was detected in the CNS and peripheral nervous system by in situ hybridisation and bright field microscopy. (A) PrP transcripts were detected in the superior cervical sympathetic ganglion of a 16.5 day embryo hybridised with the antisense RNA probe. PrP transcripts were also detected in (C) dorsal root ganglia where large numbers of silver grains were associated with the large neuronal cells and (E) in the trigeminal ganglion (fifth cranial nerve). In this section hybridisation was also detected in the pituitary situated to the right of the trigeminal ganglion and in the brain. Hybridisation with the antisense RNA probe also detected PrP transcripts throughout nerve axons of both the CNS and peripheral nervous system, clearly seen here (G) in the optic nerve (second cranial nerve). (B,D,F,H) Adjacent sections hybridised with the sense RNA probe showed only background hybridisation. Magnification, approx. $\times 25$.

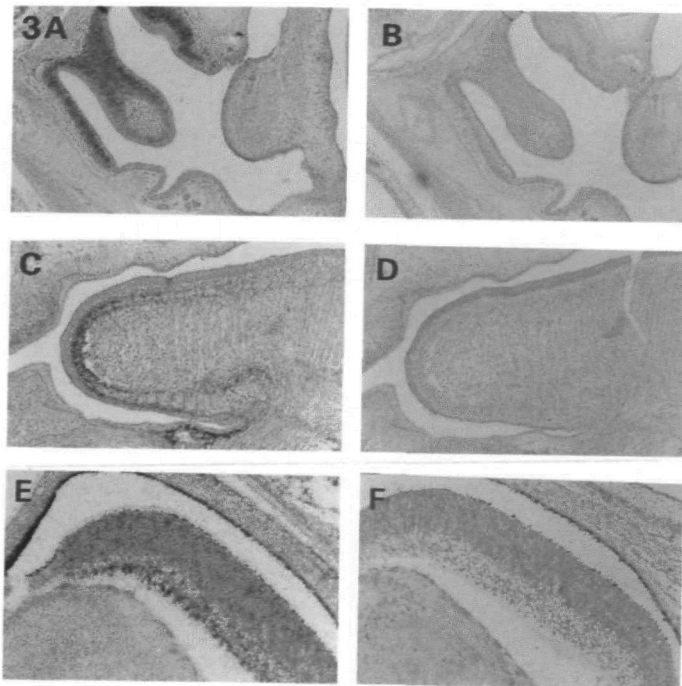


Fig. 3. PrP gene expression was detected associated with neural cell populations of sense organs and the intestine. In a 16.5 day embryo, hybridisation was detected using the antisense RNA probe (A) in the epithelium of the olfactory system where it was localised to the region containing large numbers of neurones. Hybridisation was also detected using the antisense RNA probe (C) in the extensive submucous nerve plexus on the dorsum of the tongue and (E) in the inner nuclear layer of developing neurones in the retina. The pigment layer of the retina is unlabelled but contains large numbers of pigment granules which give the appearance of hybridisation. Only background hybridisation was detected using the sense RNA probes on adjacent sections of (B) the olfactory mucosa of the 16.5 day, (D) the tongue of the 16.5 day embryo and (F) the retina. The sections were stained with Giemsa and examined by bright field microscopy. Magnification, approx. $\times 25$.

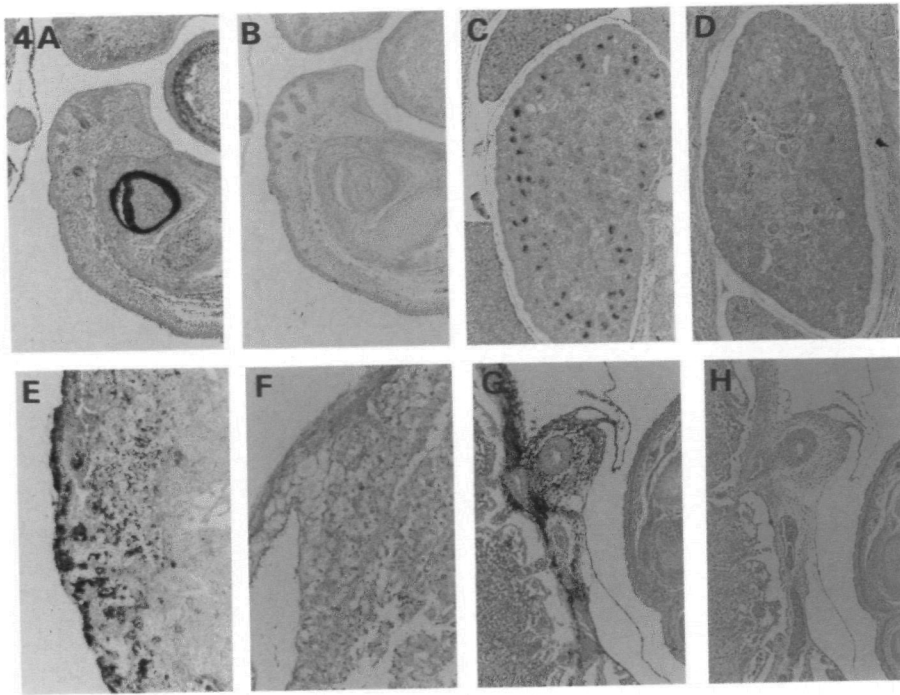


Fig. 4. PrP gene expression was detected in non-neuronal cell populations. Hybridisation was detected in a 16.5 day embryo, using the antisense RNA probe and bright field microscopy (A) in inner and outer epithelium layers of the tooth primordium and (C) the developing metanephric cap derivatives of the kidney. The PrP gene was also expressed in extra-embryonic tissue. PrP transcripts were detected (E) in the maternal decidua and (G) the amnion, mesodermal layer of the yolk sac and the umbilical cord of a 16.5 day conceptus. (B,D,F,H). Only background hybridisation was detected using the sense RNA probe on adjacent sections. Magnification approx. $\times 25$.

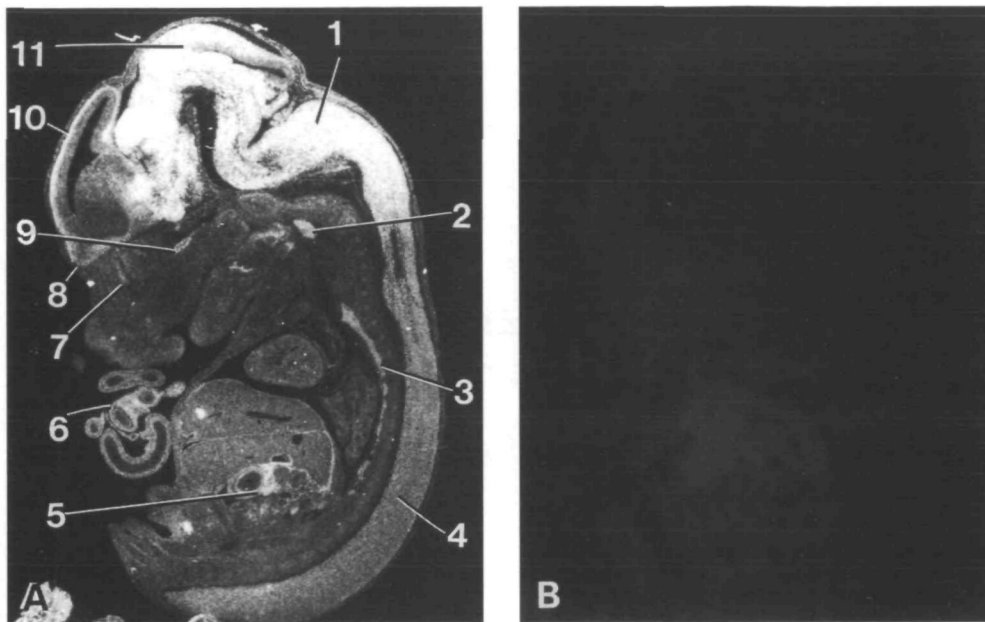


Fig. 1. Expression of the PrP gene in 13.5 day mouse embryo detected by in situ hybridisation (A) Dark field microscopy was used to visualise sagittal sections hybridised with ^{35}S -labelled antisense RNA probe. Expression of the PrP gene was detected throughout the developing brain, in the telencephalic cortex of the forebrain and the mesencephalon, rhombencephalon and mantle layer of the spinal cord. Hybridisation was also clearly detectable in the optic nerve, the superior cervical sympathetic ganglia, sympathetic trunks and the submucosal layers of the intestine 1, rhombencephalon; 2, superior cervical sympathetic ganglion; 3, sympathetic trunk and ganglia; 4, spinal cord; 5, gastro-duodenal junction; 6, midgut within physiological umbilical hernia; 7, olfactory epithelium; 8, olfactory lobe; 9, optic nerve; 10, telencephalic cortex; 11, mesencephalon. (B) Only background hybridisation was detected using the ^{35}S -labelled sense RNA probe.

nerve) (Fig. 2E), the superior cervical sympathetic ganglion (Fig. 2A) and sympathetic trunk and ganglia (Fig. 1). Hybridisation was also detected throughout dorsal root ganglia but the highest numbers of silver grains were located over large neuronal cells (Fig. 2C). Axons of cranial nerves, most strikingly the optic nerve (Fig. 2G) and spinal nerves (Fig. 1) as well as in the sympathetic nerve trunks, were also found to contain PrP transcripts. The PrP gene may be expressed in the glial cells which surround these axons or alternatively, PrP mRNA could be transported from the neural cell body through the axons. Recent studies have contradicted the widely held view that axons do not contain mRNA. There is evidence for transport of vasopressin and oxytocin mRNA along axons from the hypothalamus to the nerve terminals of the posterior pituitary (Mohr et al., 1991). Whether ribosomes are also found within these axons to allow translation of the mRNA remains to be established.

PrP gene expression in sense organs

Neuronal cell populations are candidates for expression of the PrP gene within several organs. In the olfactory epithelium (Fig. 3A) hybridisation was detected at both 13.5 and 16.5 days and was associated with the nervous layer containing large numbers of sensory neurones. Hybridisation was also detected at both 13.5 and 16.5 days in the tongue. The transcripts appear to be associated in the extensive submucosal nerve plexus on the dorsum of the tongue (Fig. 3C). Transcripts were

detected in the retina by 16.5 days and were associated with the inner nuclear layer of differentiating neural cells (Fig. 3E), whereas at 13.5 days only a diffuse low level of hybridisation could be detected in the retina.

The intestine

PrP gene expression was also detected within the submucosal and muscularis mucosa layers of the intestine. Hybridisation in the intestine could be detected at both 13.5 (Fig. 1) and 16.5 days. Transcripts in these regions of the intestine may be associated with the sympathetic plexus (and possibly also parasympathetic ganglia) in this location.

Non-neuronal cells expressing PrP gene

The PrP gene is expressed in non-neuronal cells. At 13.5 days PrP gene expression was detected in early stages of differentiation of dental lamina. By 16.5 days, hybridisation in tooth buds was higher than in any other area of the embryo and was present in both inner and outer epithelial layers (Fig. 4A), which give rise to the enamel-forming cells. PrP gene expression was also detected in specific cells of the kidney in the 16.5 day embryo (Fig. 4C). The cells expressing PrP in the kidney are in the early stages of differentiation into the embryonic nephron. These cells have also been shown to express nerve growth factor receptor during rat embryogenesis (Sariola et al., 1991), which is consistent with a regulatory role for NGF in PrP expression in the

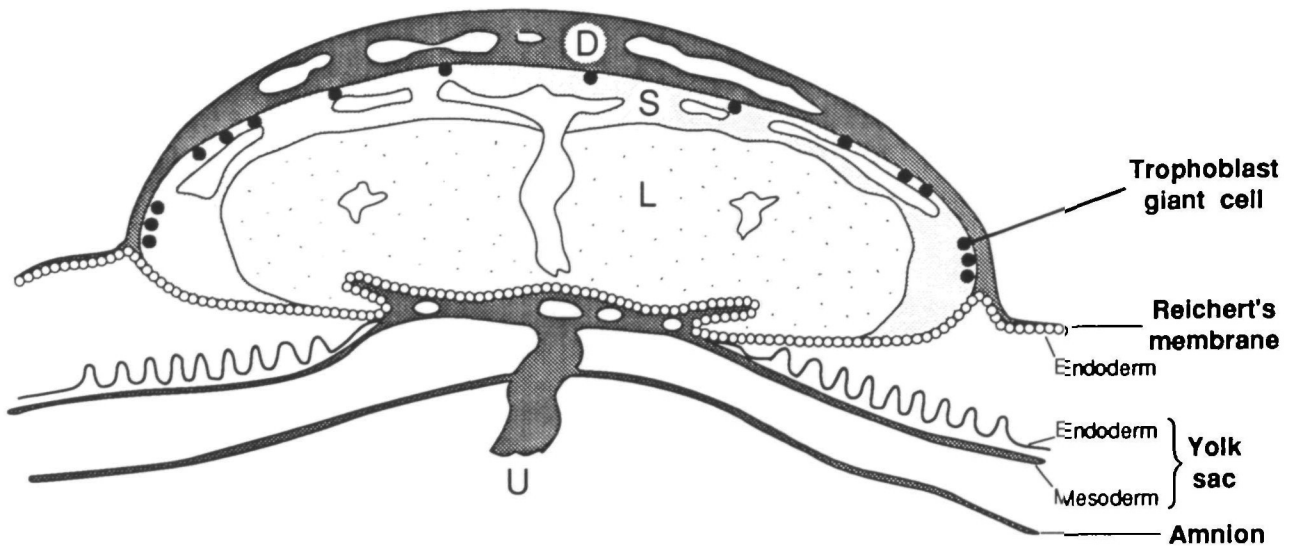


Fig. 5. Diagram of 16.5 day mouse placenta showing outer layer of maternal decidua (D) plus the spongiotrophoblast (S) and labyrinthine trophoblast (L) layers, both of which are derived from the zygote. Secondary trophoblast giant cells (shown as filled circles) are found at the boundary between the maternal and 'fetal' parts of the placenta. The umbilical cord (U) and three extra-embryonic membranes (Reichert's membrane, visceral yolk sac and amnion) are attached to the inner part of the placenta as shown. PrP expression was found in the regions that are shaded dark grey (maternal decidua, umbilical cord, amnion and yolk sac mesoderm), which are all derivatives of the primitive ectoderm lineage.

embryo as well as in the adult (Mobley et al., 1988; Wion et al., 1988).

PrP gene expression in extra-embryonic tissue

The PrP transcripts were detected in maternal decidual cells surrounding the embryos and their membranes at the earliest stage examined, 6.5 days and 8.5 days. By 13.5 days PrP transcripts could be detected in the maternal cells of the placenta and expression in these cells could also be detected in the 16.5 day embryo (Figs 4E and 5). Expression of the PrP gene was also detected in other extra-embryonic tissues of the 13.5 and 16.5 day conceptus. The amnion, umbilical cord and the mesodermal layer of the yolk sac all hybridised to the PrP probe (Fig. 4G). Thus expression in the extra-embryonic membranes occurs in derivatives of the primitive ectoderm lineage, which also produces the entire fetus, but is absent from the derivatives of the primitive endoderm (Reichert's membrane and yolk sac endoderm) and trophoderm (placental trophoblast). A diagrammatic representation of the extra-embryonic hybridisation detected at 16.5 days is shown in Fig. 5.

Anti-PrP

In all experiments adjacent sections were hybridised to sense PrP probes (Figs 2-4). No evidence for any expression from an anti-PrP gene (Goldgaber, 1991) was obtained in these sections. Due to the limits of sensitivity of in situ hybridisation, this result clearly does not rule out the presence of such transcripts but suggests if they are present it is at a very low level (Manson and Hope, 1991; Hewinson et al., 1991; Manson et al., 1992).

Discussion

PrP gene expression has been detected throughout the developing neural tube from 13.5 days of development in mouse embryos. The expression of PrP did not appear to be restricted to areas of differentiated neural cells but its widespread expression throughout the brain suggests that PrP transcripts were also present in undifferentiated cell populations. The PrP gene continues to be expressed in most if not all of the neurones of the adult brain. High levels are found in the pyramidal and dentate granular cells of the hippocampus, Purkinje cells of the cerebellum and in large neurones of the cortex, medulla and septum (Kretzschmar et al., 1986; Manson et al., 1992). The expression of the PrP gene in the developing neural tube and its continued expression in the adult brain suggests that PrP may be critical to neural function in the mammalian central nervous system. PrP protein is located on the outside of the neural cell membrane, attached by a glycoinositol phospholipid (GIP) anchor (Stahl et al., 1987). As a neuronal surface glycoprotein, it may be involved in interactions between cells or with extracellular matrix proteins. This would be similar to the role proposed for the A4 amyloid precursor protein, another membrane glycoprotein, implicated in the onset of amyloidosis and dementia (Shivers et al., 1988). Such interactions may be required to promote neuronal cell differentiation during development of the CNS and to maintain neuronal cell function in the differentiated neurones.

PrP gene expression was not limited to the CNS. Transcripts were also detected in ganglia and nerves of the sympathetic nervous system and in neuronal cell populations of the sensory organs. The role of PrP in

neuronal differentiation or function does not appear to be limited to the CNS but may have a similar function throughout the peripheral nervous system. In early postnatal development, PrP gene expression can be regulated by nerve growth factor (Mobley et al., 1988) which has been ascribed a role in CNS, sensory and sympathetic neuronal survival (Williams et al., 1986; Kromer, 1987). Conversely, the accumulation of PrP in scrapie and related diseases is associated with neurone loss in the CNS (DeArmond et al., 1987; Bruce et al., 1989). PrP may therefore be involved in complex pathways which determine neurone fate. Its expression or aberrant metabolism may result in either neuronal death or survival, during the development of the central and peripheral nervous systems.

A further link between PrP gene expression and NGF is indicated by the expression of PrP in the embryonic nephrons of the kidney at the same time that NGF receptor expression has been shown in rat embryonic nephrons (Sariola et al., 1991). The function of PrP in these cells and elsewhere may be to provide cell-cell adhesion at sites of morphogenesis during development. Alternatively PrP may be part of a cell signalling system required for the differentiation of specific cells.

It may be that different mature forms of the PrP protein are found in different cell types of the developing embryo, resulting in altered cellular locations and functions of the protein. In adult lung and heart a second mRNA, smaller than the more abundant 2.4 kb mRNA, has been detected (Robakis et al., 1986) which allows for the possibility of different protein forms of PrP in these tissues.

The widespread expression of PrP during mouse embryogenesis revealed in this study suggests that understanding the function of PrP will provide not only insight into events which lead to neurodegeneration and transmission of scrapie-like diseases but will also allow investigation of fundamental developmental processes during mouse embryogenesis.

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Note added in proof

Targetted deletion of the PrP gene from embryonic stem cells by homologous recombination has allowed the production of mice (Prn-p^{0/0}) in which the PrP gene is absent (Beuler et al., *Nature* in press). These mice develop and behave normally with no apparent defects. This shows other proteins may be able to take over the pleiotropic functions of PrP in the Prn-p^{0/0} mouse.