Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 309–317
BBRC
www.elsevier.com/locate/ybbrc

Selection of ovine PrP high-producer subclones from a transfected epithelial cell line ☆

Pierre B. Falanga *, Marie-Christine Blom-Potar, Patrick Bittoun, Michel E. Goldberg, Mireille Hontebeyrie

Unité de repliement et modélisation des protéines, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, France

Received 17 November 2005
Available online 12 December 2005

Abstract

The hallmarks of prion diseases are the conversion of the normal prion into an abnormal protease resistant isoform and its brain accumulation. Purification of the native abnormal prion isoform for biochemical and biophysical studies has been hampered by poor recovery from brain tissue. An epithelial cell transfected with the ovine VRQ allele prion, called Rov9, has been used to select prion high-producer cells by flow cytometry. The representative clone 4 described here produced 6.2 μg of cellular prion protein per mg of total protein extract, representing 8- to 10-fold the amount produced by the Rov9 parental cells. After exposure to the scrapie agent (PG128/98), clone 4 produced 2.6 μg of abnormal isoform per mg of total protein. When infected clone 4 cell cultures were treated with tunicamycin, 80% of the abnormal isoform was deglycosylated. The infectivity of the prions produced in clone 4 cultures was confirmed in a mouse bioassay. Such high-producer clones represent new tools for producing large amounts of glycosylated and/or non-glycosylated PrP^{Sc} and for a powerful screening of clinical samples' infectivity.

Keywords: PrP^{Sc}; Cultured cells; Rov9; Prion diseases; Sheep

Creutzfeldt–Jakob disease in human, scrapie in sheep, and bovine spongiform encephalopathy in cattle defines invariably fatal neurodegenerative disorders [1]. The hallmarks of these transmissible spongiform encephalopathies (TSEs), called "prion diseases," are the conversion of a normal cellular prion protein (PrP^{C}) into a pathological isoform, designated PrP^{Sc}, and its accumulation in the brain.

Difficulties in obtaining appropriate quantities of the purified infectious PrP^{Sc} protein have hampered its biochemical and biophysical characterization. Attempts to extract and purify native PrP^{C} from brain tissue have resulted in poor recoveries [2–5]. An additional difficulty, particularly from a thermodynamic viewpoint, stems from the fact that PrP^{C} and PrP^{Sc} are heavily and very heterogeneously glycosylated and the material obtained from natural sources is heterogeneous and unsuitable for careful biochemical and structural analysis. Furthermore, the presence of large sugar moieties on the PrP molecule is likely to seriously impede the interaction of PrP with mAbs or enzymes used for analytical purposes. Finally, PrP^{Sc} is highly resistant to enzymatic deglycosylation. Thus, unglycosylated PrP^{Sc} cannot be obtained from natural sources, except possibly in the case of recombinant PrP produced by transgenic animals carrying mutations of the *prp* gene at the two glycosylation sites [6–8].

To circumvent the difficulties related to the purification of PrP^{Sc} from the brains of infected animals, and to obtain unglycosylated PrP^{Sc} by use of glycosylation inhibitors, we tried to identify an easy-to-grow cell line producing PrP^{Sc}.

☆ *Abbreviations:* dox, doxycycline; HPRT, hypoxanthine phospho-ribosyl-transferase; PK, proteinase K; PrP, prion protein; PrP^{C}, cellular isoform of the prion protein; PrP^{Res}, proteinase K-resistant prion protein; PrP^{Sc}, scrapie isoform of the prion protein; TSE, transmissible spongiform encephalopathy; ^{VRQ}PrP, ovine PrP allele.

* Corresponding author. Fax: +33 1 45 68 84 32.
E-mail address: pfalanga@pasteur.fr (P.B. Falanga).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.153

Intense research efforts have been made to develop cell culture models that support stable and persistant replication of PrP^{Sc} as well as infectivity. Only a few neuronally derived cell lines, among them N2a mouse neuroblastoma cells, PC12 rat pheochromocytoma cells, spontaneously immortalized hamster brain (HaB) cells, and T-antigen-immortalized hypothalamic neurons (GT1), supported prion infection, for reviews, see [9] and [10]. Recently, a new heterologous model for sheep scrapie was developed by Vilette et al. [11]. These authors have transfected a rabbit epithelial cell line (RK13) with the ovine PrP gene. The transfected cells, named Rov9, were able to support ovine prion propagation in cells from an otherwise refractory species to TSE. However, all these cell lines produce only small amounts of PrP^{C} as well as PrP^{Sc}. We have therefore attempted to derive cloned cells line able to propagate the prion and to produce larger amounts of PrP^{C} and PrP^{Sc}.

This report describes the selection by flow cytometry of PrP^{C} hyperproducer clones derived from Rov9 cells. It describes the characterization of these clones in terms of quantitative amounts of PrP^{C} or PrP^{Sc} produced in uninfected or infected cultures and of their ability to propagate the prion in cell culture and to support infection in vivo. Finally, it also describes culture conditions under which these clones allow the production of unglycosylated PrP^{Sc}.

Materials and methods

Antibodies. Anti-PrP mAb SAF-32 recognizes an epitope in residues 79–92 of the human PrP sequence [12], 12F10 in residues 142–160 [13], and Bar-224 in residues 141–154 [14]. E1-4, an IgG2b mAb against a polysaccharide from *Shigella flexneri*, is used as isotypic control.

Cell culture and infection. The Rov9 cell line is derived from the rabbit kidney epithelial cell line RK13 by transfection with a plasmid carrying the ovine PrP allele (^{VRQ}PrP) under a doxycycline regulated Tet on promoter [11]. Cells were cultured in EMEM (BioWittaker) containing 10% heat-inactivated FBS (HyClone, Perbio Science, France), 2 mM L-glutamine, and penicillin–streptomycin. The cultures were split every week at 1:3 to 1:5 dilutions. Induction of PrP^{C} was obtained by adding 1 μg of doxycycline (dox) per ml of culture medium 24 h before harvesting the cells.

The brain of a Tg338 mouse infected with the prion strain PG128/98 was homogenized at 10% (wt/vol) in a sterile 5% glucose solution; the aliquoted material was frozen at −80 °C until used. After thawing, the homogenate was diluted to 0.125% in complete culture medium, sonicated for 1 min in a cup-horn apparatus, and added (0.25 ml/cm^2) to confluent Rov-9 cells or clones grown for 24 h in the presence of dox. All infected cell cultures were maintained at 37 °C in 5% CO_2 in a biohazard P3 laboratory (Ministry agreement number : 3876).

Flow cytometry and cell sorting. To obtain a single cell suspension, confluent cultures were first incubated for 30 min at 37 °C with 0.02% EDTA in PBS. The cells were then detached by trypsin treatment (1 min, 2.5 mg/ml) and rapidly washed in complete culture medium. Cells were labelled by indirect immunofluorescence using SAF-32 (1 μg/10^6 cells, 1 h at 4 °C) unless otherwise stated. They were washed three times with cold PBS containing 5% of FBS, incubated for 1 h at 4 °C with a goat fluorescein-conjugated affinity purified anti-mouse IgG (heavy and light chains) (Rockland), and washed again three times. Negative controls were obtained either with noninduced Rov9 cells or using the E1-4 mAb (1 μg/10^6 cells, 1 h at 4 °C). Flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACS starPlus, Becton Dickinson, San Jose, California). FITC labelled cells were sorted on the highest fluorescence signal and gated on their forward (FSC-H) and side (SSC-H) scatter signals using a flow cytometry MOFLO (DAKO Cytomation) Cell Sorter. The sorted cells were collected in a tube containing culture medium with 50% FBS. After sorting, the cell suspension was spun down and resuspended in fresh culture medium and either distributed at a ratio of one cell per three wells for limiting dilution cloning in 96-well plates or cultured in bulk flasks.

Assay for infectivity in mice. Infectivity of the PrP^{Sc}-induced infected cells was tested using Tg338 mice [15], provided by the CNRS facility (Centre National de la Recherche Scientifique, Orléans, France), which are knockout for the murine *Prnp* gene and transgenic for the VRQ allele of the ovine Prnp. All animals were housed in level 3 care facilities at the Pasteur Institute (Ministry agreement number A75-15-27). Cultured cells were detached, washed, and resuspended in a 5% glucose solution at 10^8 cells per ml. The cell material was frozen-thawed three times and filtered on a 0.45 μm Millipore filter before injection. Animals were infected intra cerebrally with 20 μl of inoculum, examined every week and then daily after the appearance of neurological disorders. The Kaplan and Meier survival curve of the different groups was analyzed using the log-rank test (GraphPad Prism, version 4, GraphPad). Brains of dead or euthanised animals (at the late stage of the disease) were analyzed for the presence of PK-resistant PrP by immunoblotting.

Quantification of PrP^{C} and PrP^{Sc} in cultured cells. Confluent cultures were lysed for 30 min at 4 °C in TTD lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate), with or without protease inhibitors Pefabloc or ''Complete'' (Roche, France). After a 10 min centrifugation at 430*g* and 4 °C, the supernatant was collected and its total protein concentration was measured by the BCA protein assay (Pierce). Samples were mixed with four times their volume of chilled methanol. The mixture was chilled at −20 °C for 30 min and centrifuged at 18,000*g* at 4 °C for 30 min. The pellet was resuspended in 100 μl of Laemmli buffer, containing 3 M final urea and protein inhibitors, and boiled 5 min at 100 °C. Samples and a range of highly purified recombinant ovine PrP proteins identically processed were analyzed by SDS–12.5% polyacrylamide gel electrophoresis. To obtain a reliable and robust quantification, several different dilutions of the sample were run in the same gel as the standard, thus avoiding artifacts due to saturation of the signal or to a too weak signal. The proteins were transferred onto an Hybond-ECL nitrocellulose membrane (Amersham) in Tris–glycine buffer containing 10% ethanol (vol/vol), 10% SDS (wt/vol) at 20 V for 35 min; the membrane was blocked for 15 min at room temperature with 5% nonfat dried milk in TBST (10 mM Tris–HCl [pH 7.8], 100 mM NaCl, and 0.1% Tween 20). The PrP was detected by immunoblotting with Bar-224 diluted at 1 μg/ml in TBST containing 0.5% nonfat dried milk; the membrane was washed three times with TBST and then incubated with a peroxidase-conjugated affinity-purified goat anti-mouse IgG (heavy and light chains) antibody. After extensive washing, the blots were developed by enhanced chemiluminescence (ECL Plus Western Blotting, Amersham) and scanned on a Typhoon 9400 (Amersham). Images were processed with the ImageQuant (version 5) analysis software from Molecular Dynamics.

To detect the presence of PrP^{Sc} in infected cultures, TTD cell extracts, without any protease inhibitor, processed as above, were digested at 37 °C for 1 h, with 0.16% (wt) of proteinase K (Roche) per weight of toal protein content; digestion was stopped by addition of Pefabloc (1 mM) for 5 min on ice. The samples were then deglycosylated with PNGase F according to the manufacturer's instructions (New England BioLabs, Ozyme). The reaction was stopped by addition of chilled methanol. After cooling and centrifugation, the pellet was resuspended in 100 μl of Laemmli buffer and processed as described above.

Analysis of PrP mRNA by RT-PCR. Total RNA from cultured cells was extracted using RNeasy (Qiagen, France) according to the manufacturer's instructions. Protocols for the reverse transcription of the RNA, for PCR amplification, and for PCR product analysis have been detailed elsewhere [16]. The sequences of the primers used to amplify cDNA from rabbit hypoxanthine phosphoribosyltransferase (HPRT) or rabbit and ovine PrP gene, their location, and the references are indicated in Table 1. The amplification cycle (denaturation step at 94 °C for 1 min, an annealing

Table 1
Primers used for polymerase chain reactions

Molecule	Primer sequence 5′–3′	Location in the sequence	Accession Nos.	Reference
HPRT	Sense: GAC TGA ACG GCT TGC TCG AGA	12–35	AF020294	GenBank
	Antisense: TGA GAG ATC ATC TCC ACC GAT TCA	223–246		
Ovine PrP	Sense: GGA GTG ACG TGG GCC TCT GC	64–83	AJ000738	[29]
	Antisense: TGA GAG ATC ATC TCC ACC GAT TCA	431–456		
Rabbit PrP	Sense: GGA GTG ACG TGG GCC TCT GC	47–66	AFO15603	[30]
	Antisense: CGT TGC CGA AGT GGA TGA GGG	413–433		

step at 55 or 62 °C for 1 min, and an extension step at 72 °C) was repeated 30 times followed by a final extension for 10 min at 72 °C. These conditions were defined as optimal to amplify mRNA from a housekeeping gene, the rabbit HPRT, present at a low level (1–10 copies per cell). A 30 cycle amplification of 10–50 ng cDNA with 100 pmole of primers at 62 °C gave rise to a unique band corresponding to the 325 bp HPRT amplicon. Digestion of 325 bp HPRT amplicon with *Hin*cII resulted, as expected, in two fragments in the range of 42 and 193 bp. Digestion of 393 bp ovine PrP amplicon with either *Bgl*I or *Dra*III resulted in two fragments of 65/328 or 185/208, respectively.

Results and discussion

FACS analysis of Rov9 cells

Preliminary immunofluorescence studies showed that only a small number of Rov9 cells expressed the PrP^C antigen. In addition, double labelling of nuclei with propidium iodide and PrP^C with a specific mAb (SAF-32) revealed that PrP expression was cell cycle independent (data not shown). Fig. 1 shows the fluorescence histogram of the amount of PrP^C present on the cell surface 24 h after dox-induction, as detected with SAF-32 and FITC anti-mouse Ig, including different "negative" controls. Less than 10% of the induced cells expressed high levels of PrP^C (>300 arbitrary units). These results confirmed and extended previous data published by others on the Rov9 cells [11,17]. Permeabilization of the cells to reveal both surface and intracellular PrP^C resulted in an overall increase in fluorescence, but did not change either the bimodal aspect of the histograms or the relative fraction of highly fluorescent cells. This indicated that the heterogeneity reflected differences in overall PrP^C expression rather than transport to the cell surface. Ovine PrP^C expression was detected on the cell surface 4–6 h after induction, reaching a plateau at 12 h and remaining stable for several days (more than 30 days, data not shown). Altogether, these data suggested that the Rov9 cell line contained a mixture of both PrP-producing and non-producing cells and prompted us to try subcloning a more homogeneous PrP-producing cell line using a fluorescence-activated cell sorter (FACS).

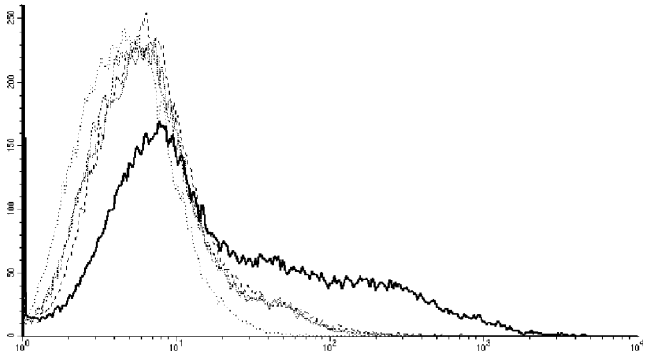

Fig. 1. Analysis of Rov9 cell surface PrP by FACS. Confluent cultures of Rov9 cells were either dox-induced (1 μg/ml) or not for expression of ovine PrP^C and 24 h later cells were analyzed by FACS. Cell suspensions were either incubated with the anti-PrP mAb SAF-32 (bold line) or E1-4, an IgG2b control (dashed line), and then incubated with a FITC-conjugated goat anti-mouse IgG antibody. *X* axis: fluorescence intensity (arbitrary units), *Y* axis: cell number. Other controls: unlabeled cells (dotted line), uninduced cells labeled with the first and second antibodies (point dashed line), induced cells labeled with the second antibody (bold dotted line).

Selection of highly positive cells

To specifically select for a PrP expressing population, induced Rov9 cells were labelled with SAF-32 as the primary antibody and sorted in the FACS using two windows, one in the forward scatter vs side scatter dot plot to eliminate cell debris, and the second in the fluorescence histogram to select for higher FITC labelled cells. The sorted cells (about 10% of the initial population) were cultured without dox and expanded during two passages. The resulting cells, called Trov, were dox-induced and compared to the parent Rov9 population. Fig. 2 shows the histograms obtained with two different anti-PrP mAbs, SAF-32 and 12F10, that recognize two different epitopes, on the N-terminal tail and the core of PrP^C, respectively. In the Trov population, the proportion of positive cells increased to 30%. Nevertheless, the histogram of the sorted cells was not dramatically different from that of the parent cells, using either of two anti-PrP mAbs. The sorting therefore did not lead to more intense fluorescence signals indicative of PrP-hyperproducers. Moreover, the bimodal histogram indicated that although the Trov cells were selected for their positive signal, after two culture passages they still generated cells that were PrP negative after a new dox-induction.

Selection of high-producer clones from the Trov population

In order to improve the sorting procedure and to select for PrP hyper-producing cells, the Trov population was subjected to a second round of sorting, with 12F10, since the fluorescence histogram it provides covers a much wider range than that obtained with SAF-32 (see Fig. 2). The

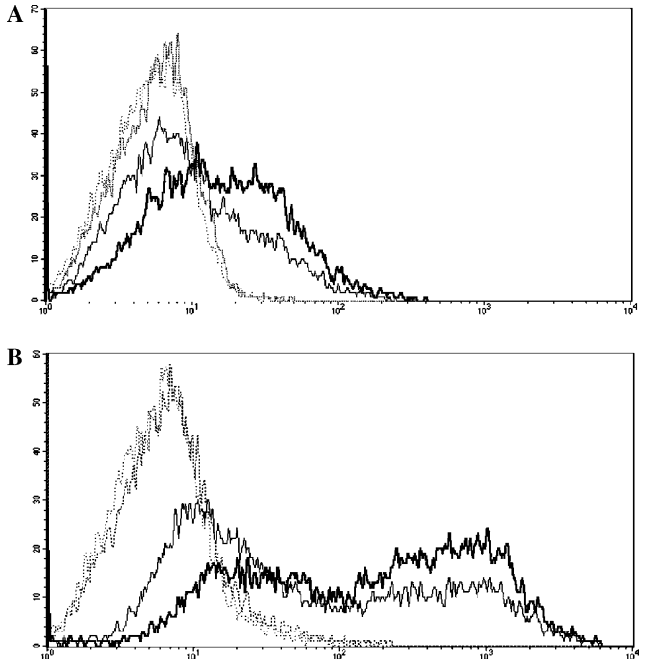

Fig. 2. Analysis of surface PrP on the sorted cells Trov and the parental cell population. Trov cells (bold solid line) after two week expansion in culture and Rov9 cells (solid line) have been induced and labelled with either SAF-32 (A) or 12F10 (B), two mAbs which recognized, respectively, the 79–92 and 142–160 aa regions of the ovine PrP. Other controls were unlabelled clone 4 (dotted line) and clone 4 labelled with E1-4 (dashed line).

sorted population thus obtained was then cloned by limiting dilution.

Two 96-well plates seeded at a ratio of one sorted cell per 3 wells yielded 17 clones from 192 distributed wells, which was 50% lower than expected, possibly due to a heterogeneous initial population. Each clone was expanded and then analysed by FACS after induction. The 17 clones typically fell into three categories: nine clearly expressed PrP^C (see Fig. 3A); two did not express any PrP^C (Fig. 3B); and six showed a bimodal pattern of PrP^C (see Fig. 3C), indicating the presence of both labelled and unlabelled cells. The latter category probably results from the cloning of doublets including both a positive and a negative cell. Such doublets likely form because the trypsin-treated Rov9 cells have a strong propensity to aggregate. Under the fluorescence microscope, cells in PrP^C expressing clones (e.g., clone 4) appear uniformly green, whereas only a minority of the initial Rov9 cells were stained. Cells belonging to the first and second categories remained stable over several generations (i.e., homogeneously positive or negative). In the third category, the proportions of labelled and unlabelled cells evolved with the number of generations, usually leading to an increase in labelled cells.

Representative clones of each category were permeabilized, labelled, and analysed by FACS as described above. Except for an increase in fluorescence observed for all cell lines, the histograms obtained showed the same characteristics as those of the corresponding, non-permeabilized cells, indicating that the differences in amounts of PrP present on the cell surface resulted from the level of PrP expression rather than from the efficiency of the PrP transport to the cell membrane.

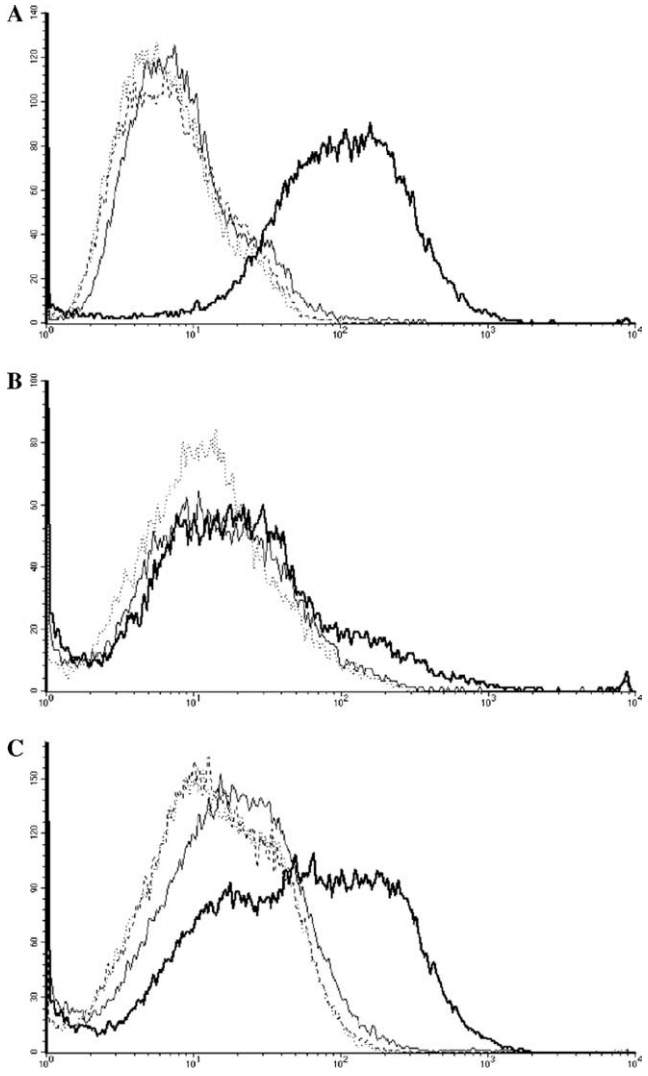

Fig. 3. Pattern of surface PrP molecule on cloned cell population. Trov cells were labelled with 12F10 and a FITC-conjugated goat anti-mouse IgG antibody. Cells expressing the highest FITC fluorescence signal were sorted again and cloned by limiting dilution. Seventeen clones were recovered which showed three classes of PrP expression patterns: positive clone (A), essentially negative clone (B), and intermediate (C). Cells were either induced (solid bold line) or not (solid line) and labelled with SAF-32 or not induced and labelled with E1-4 (dashed line) or unlabelled (dotted line).

Analysis by Western blotting of the PrP^C from a high-producer clone and Rov9 cells

In cell lysates of induced Rov9 and clone 4 (representative of PrP-producer clones, Fig. 3A), ovine PrP^C was detected on immunoblots using SAF-32 and Bar-224, two mAbs recognizing, respectively, the sequences 82–96 and 144–154 in ovine PrP. As shown in Fig. 4, panel A, SAF-32 recognizes three forms of ov-PrP in both Rov9 (lane 1) and clone 4 (lane 4) extracts: unglycosylated (31 kDa),

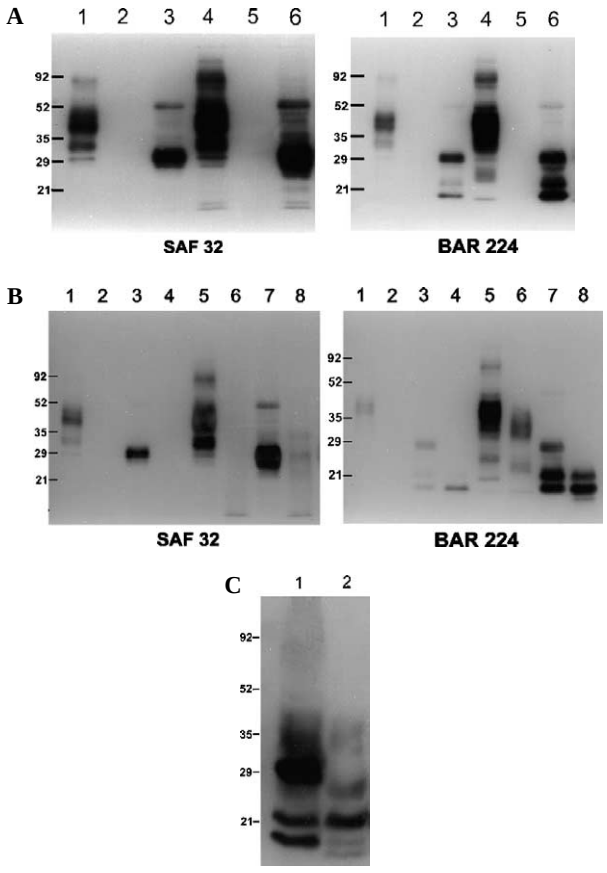

Fig. 4. (A) Electrophoretic pattern of PrP^C in induced Rov9 and clone 4. Cell lysates of dox-induced Rov9 (lanes 1–3) or clone 4 (lanes 4–6) cells were subjected to PAGE, transferred onto a nitrocellulose membrane, and PrP was detected with SAF-32 or Bar-224, two mAbs recognizing, respectively, 82–96 and 145–163 residues of ovine PrP. Total extract (lanes 1 and 4), total extract after proteinase K digestion (lanes 2 and 5) or after PNGase F deglycosylation (lanes 3 and 6). Molecular mass markers are indicated on the left in kDa. (B) Electrophoretic pattern of PrP^{Res} in induced and infected Rov9 and clone 4. Cell lysates of induced and infected Rov9 (lanes 1–4) or clone 4 (lanes 5–8) cells were immunoblotted with SAF-32 or Bar-224. Total extract (lanes 1 and 5), total extract after either proteinase K digestion (lanes 2 and 6) or PNGase F deglycosylation (lanes 3 and 7) or both (lanes 4 and 8). SAF-32 did not detect PrP^{Sc} species, since the epitope recognized (82–96), worn by the unstructurated tail of the PrP^{Sc}, is digested by PK. (C) Production of unglycosylated PrP^{Res} in infected tunicamycin-treated clone 4 culture. A confluent infected culture of dox-induced clone 4 at passage 8 was treated with 8 μg of tunicamycin per ml of culture during 48 h. The cell extract, untreated (lane 1) or treated with PK (lane 2) was analyzed by PAGE and Western blot analysis with Bar 224.

monoglycosylated (34 kDa), and diglycosylated (45 kDa). The three bands resolved into one when cell extracts were deglycosylated with PNGase F (lanes 3 and 6) or disappeared after digestion with PK (lanes 2 and 5). In both Rov9 and clone 4, the PrP^C patterns were similar with diglycosylated > monoglycosylated > unglycosylated forms. Additional polypeptides of 17 and 21 kDa were clearly detected with Bar-224 after deglycosylation (lanes 3 and 6), indicating that PrP^C undergoes limited proteolysis as already described for neuroblastoma cell lines [18]. These additional truncated forms of ovine PrP, detected by Bar-224 but not SAF-32, generated a complex pattern when the deglycosylation step was omitted. All PrP^C entities detected by Bar-224 were found to be sensitive to PK (lanes 2 and 5).

Quantification of the PrP^C produced by Rov9 cells and a high-producer clone

Flasks seeded with either Rov9 or clone 4 were cultured with dox and passaged in parallel at a 1:3 dilution every week. P0 designates the first week of culture under dox and P1, P2, P3..., the following weekly passages. Cell extracts were prepared and treated with PNGase F. To quantify the PrP^C produced by the Rov9 and clone 4 cells, extracts were run on SDS–PAGE and immunoblotted using Bar-224 and a peroxidase-coupled anti-mouse antibody. The blots were then scanned for chemiluminescence and the signals corresponding to the three polypeptides (17, 21, and 29–31 kDa) were summed and compared to signals obtained with a range of known amounts of recombinant ovine PrP^C run in parallel on the same gel. Fig. 5A shows the production of dox-induced PrP^C by Rov9 and clone 4, over 6 passages. These quantitative results confirm the FACS observation, which suggested that clone 4 produced significantly more PrP^C than the Rov9 parent cells. They also indicate that the production of PrP^C increased during the first 2 or 3 weeks and decreased thereafter, an effect more pronounced for clone 4 than for Rov9. The amount of PrP^C, averaged over passages P1, P2, and P3, was estimated to be about 0.065 and 0.625 μg per mg of total protein for Rov9 cells and clone 4, respectively. Thus, cultures of clone 4 clearly lead to hyperproduction of PrP, as compared to Rov9 cells.

The stability of clone 4 was monitored as follows. A non-induced culture was separated into two fractions, which were either stored frozen or cultivated for 12 weeks with a weekly 1:3 dilution. Thawed and passaged cells were both dox-induced for 24 h and analysed by FACS to compare the amounts of expressed PrP. No significant difference between the two histograms was observed. In particular, the two populations appeared homogeneously PrP positive, indicating that the capacity of clone 4 to express high levels of PrP was maintained during 12 passages in the absence of dox. Thus, clone 4 appears to retain its high PrP-producer phenotype when grown without induction.

Similarly, continuous dox-induction of clone 4 cultures during 1, 4, 8, and 12 weeks led to the same patterns of PrP expression as analysed by FACS: the population remained homogeneously PrP positive, and the position of the fluorescence histogram peak did not change significantly. This observation contradicts the apparent decrease in PrP expression after the 3rd passage seen in Fig. 5, panel A using the immunoblot technique. However, the logarithmic scale used in the cytometry experiments may easily mask a factor 2 change in the peak fluorescence, and cytometry can be considered to be a more quantitative

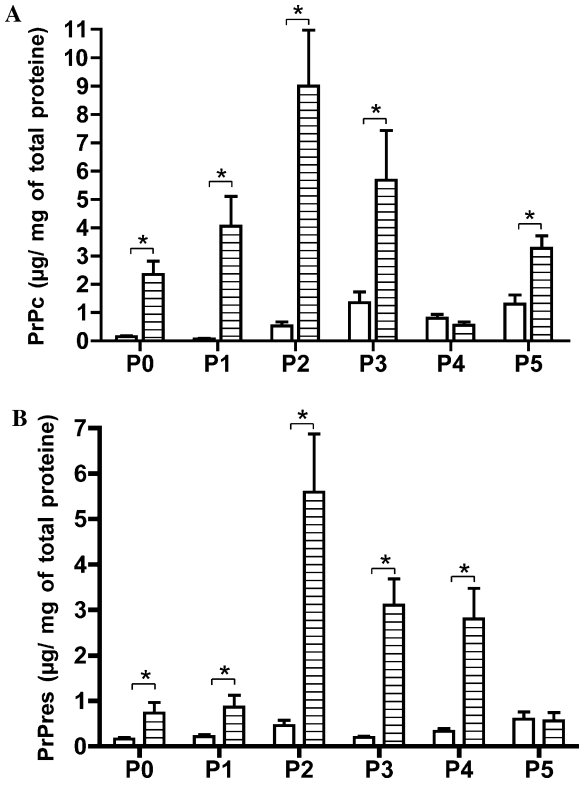

Fig. 5. Quantification of PrP^{C} and PrP^{Res} in clone 4 and Rov9 cells. Rov9 or the clone 4 were either dox-induced (A) or dox-induced and infected (B). Cultures were passaged in parallel at a 1:3 dilution every week. P0 corresponds to the first week after induction and P1, P2, P3..., the first, second, and following passages. For each culture cell lysates were prepared from two independent 25 cm^2 flasks. Uninfected cell extracts (A) were deglycosylated with PNGase F and analyzed by Western blot using Bar-224, and the immunoblots developed by chemiluminescence were scanned. The amount of PrP^{C} in each extract was determined by summing signals obtained for the 17, 21, and 29–31 kDa polypeptides (see Fig. 4A). Infected cell lysates (B) were digested with PK prior to their deglycosylation with PNGase F and analyzed by Western blot. The amount of PrP^{Res} in each extract was determined by summing signals of the 17 and 21 kDa polypeptides (see Fig. 4B). Signals were compared to a range amount of recombinant ovine PrP^{C}.

technique. Nevertheless, it can be concluded that clone 4 is stable for a large number of generations in the absence of induction, and that the level of PrP expression remains very high even after 12 weeks of induction.

Analysis of PrP mRNA expression by RT-PCR

To develop improved cellular models for in vitro studies on prion propagation and PrP^{Sc} production, the cells must express the PrP^{C} of interest. Prion transmission across species requires the conversion of endogenous PrP^{C} to PrP^{Sc}, which depends on the amino acid sequences of the infectious prion and host PrP^{C}. Indeed, rabbits are resistant to infection from prions of all origins tested including TSE [19], probably due to certain incompatible amino acids in the rabbit PrP^{C} sequence. It has been proposed that rabbit PrP^{C} could act as a "dominant negative" inhibitor of prion replication when mixed with ovine PrP, to decrease the conversion of ovine PrP^{C} into PrP^{Sc} [20]. Because the high-producer clones we isolated originated from rabbit cells transfected with a gene encoding ovine PrP, it was important to demonstrate that only ovine PrP, and not endogenous rabbit PrP, was expressed.

Since no specific antibodies were available to distinguish between rabbit and ovine PrP expression in the high-producer clones, mRNA encoding PrP was analysed by RT-PCR. Because of the extensive homology between the ovine and rabbit PrP nucleotide sequences, two pairs of primers were used: a forward oligo, which was identical for the rabbit and ovine genes, and reverse oligos that were different. No PCR product was obtained from the parent RK13 cells with any primer indicating that they did not express rabbit PrP (data not shown). In Rov9 cells and high-producer clones, an RT-PCR product corresponding to the expected size (393 bp) and with the expected *Bgl*1 and *Dra*III restriction sites was obtained with the ovine primers, even when not dox-induced. In contrast, no RT-PCR products of the proper size and expected *Nco*1 restriction were ever obtained with the rabbit specific primers in any of the cell lines or clones (data not shown). These results show that the PrP expressed in the Rov 9 and high-producer cells is exclusively ovine.

Characterization and quantification of $cPrP^{Res}$ in Rov9 and clone 4 infected cultures

Dox-induced clone 4 and Rov9 cells were incubated with infected mouse brain homogenate (0.125% wt/vol in complete culture medium) for 48 h and then cultured with dox and passaged. The presence of PrP^{Res} in cell lysates was monitored by immunoblots with the SAF-32 and Bar-224 mAbs after treatment with PK and/or PNGase. The results shown in Fig. 4, panel B indicate that, as expected, SAF-32 showed no reactivity with PrP^{Res}, while Bar-224 detected its characteristic deglycosylated 17 and 21 kDa PK-resistant peptides (lane 8).

PrP^{Res} was quantified as described above, with Bar-224, by adding the 17 and 21 kDa peptide signals. The amount of PrP^{Res} averaged over passages P0 to P5 was 2.67 µg and 0.32 µg per mg of total protein for clone 4 and Rov9 cells, respectively (Fig. 5B). As observed for PrP^{C} in uninfected cells, PrP^{Res} production diminished after the third passage. Nevertheless, the expression of PrP^{Res} by clone 4 was consistently several fold higher than in Rov9 cells, reaching levels theoretically compatible with the production of milligram quantities. In dox-induced and infected negative clones used as controls (see Fig. 3B), neither PrP^{C} nor PrP^{Res} was detectable on immunoblots, indicating that residual prions from the infected brain inoculum did not contribute to the signals observed.

Most studies aimed at quantifying PrP^{Sc} have used a titering procedure by measuring the LD_{50} per infected cell in a murine model [21]. Using this bioassay the Rov9 cell line was shown to produce 2 LD_{50} per cell [17]. From

our estimate of 0.3 μg of PrP^{Res} per mg of total Rov9 protein, corresponding to 2.6 pg of PrP^{Res} per cell, it can be concluded that 1 mg of PrP^{Res} from this prion strain would be equivalent to 3.8×10^{11} LD_{50}.

Recently, Bate et al. [22] reported production of 2.2 ng of PrP^{Res} per 10^7 scrapie infected neuroblastoma cells (ScN2a). Our calculated production of 20 μg of PrP^{Res} per 10^7 clone 4 cells corresponds to a 10,000-fold increase. Thus, by starting with one T-75 flask of induced infected clone 4, and propagating the culture at a 1:3 dilution per week, it should be possible to obtain 425 mg of total protein containing 1 mg of PrP^{Res} in four weeks.

Production of non-glycosylated PrP^{Res}

In order to obtain material for structural and biochemical analyses, and to screen for new PrP^{Sc} specific mAbs, infected clone 4 was used to produce non-glycosylated PrP^{Res} by culturing Rov9 and clone 4 cells in the presence of tunicamycin, an inhibitor of N-glycosylation. Tunicamycin has been widely used to prevent glycosylation of PrP^{C} and PrP^{Sc} [6,23,24], and did not interfere with transport to the cell surface, in contrast to non-glycosylated PrP obtained after mutation of the asparagines in both N-glycosylation sites [6–8]. Under optimal conditions (48 h with 8 μg/ml of tunicamycin), about 80% of PrP^{Sc} produced by dox-induced clone 4 was estimated to be non-glycosylated (Fig. 4C).

The availability of infectious non-glycosylated PrP^{Sc} opens the door to a variety of investigations. In particular, the protease-resistant core of the scrapie protein has heretofore been poorly immunogenic, possibly because N-glycosylation normally constitutes up to 30% of PrP^{Res} molecular mass, hindering antibody access to parts of PrP^{Sc} subunits not buried in the prion polymer. Thus, 80% unglycosylated PrP^{Res} may be a more suitable immunogen for the induction of antibodies directed against the PrP^{Sc} protein core. Furthermore, milligram quantities of unglycosylated PrP^{Sc} would facilitate physicochemical and structural studies of prions, which have been seriously hampered to date by the heterogeneity of PrP^{Sc} preparations. Optimization of unglycosylated PrP^{Sc} production and purification as described in this report should provide such material.

Infectivity of PrP^{Res} produced by infected cultures

To investigate if PK-resistant PrP produced by dox-induced and infected clone 4 cells was pathogenic, tg338 mice were inoculated intracerebrally with 20 μl of different crude cellular extracts or with a 10% (wt/vol) brain homogenate from a tg338 infected mouse as a positive control (Table 2). Negative controls were inoculated with a crude extract of uninfected dox-induced Rov9 cells, incubated with or without tunicamycin (Rov9 T and Rov9, respectively). All mice inoculated with the scrapie brain or with infected cellular extracts developed typical scrapie symptoms. The length of the incubation period was not statistically significantly different for the various infected cell extracts (data not shown). Animals showing scrapie symptoms were sacrificed at the terminal stage and their brains were analysed for the presence of PrP^{Res} by immunoblot with the Bar-224 mAb. PrP^{Res} was detected in the brains of all animals inoculated with infected extracts. In contrast, none of those inoculated with uninfected extracts developed any neurological symptoms and post mortem analysis of their brains detected no PrP^{Res} (see Table 2). These results clearly indicate that the PrP^{Res} produced by infected Rov9 and clone 4 cultures contained infectious prions, and that infectivity was associated with predominantly non-glycosylated PrP^{Res}. To our knowledge, this is the first report showing that wild-type (no Asp mutations) non-glycosylated PrP is infectious, strongly suggesting that prion glycosylation is not required for infectivity.

In conclusion, we present here the selection of cell clones expressing high levels of PrP derived from a rabbit epithelial cell line transfected with the ovine ^{VRQ}PrP allele (Rov9). The fully characterized clone 4 is stable with time and produces 8- to 10-fold more PrP than the parent Rov9 cell line, corresponding to about 2.6 μg of infectious, protease-resistant PrP per mg of total protein. Thus, for the first time, it becomes feasible to produce milligram amounts of infectious PrP by cell culture, providing homogeneous material for biochemical, biophysical, and structural studies of prions. The availability of cell culture models reproducing high levels of functional PrP^{Sc} prion propagation, responsible for genetic and infectious forms of TSEs, should accelerate studies on the physiology of prion diseases. Indeed, over-

Table 2
Bioassay of cell culture material in ovine PrP tg338.

Inoculum[a]	Infected brain[b]	Rov9 Sc	Clone 3 Sc	Clone 4 Sc	Clone 4 Sc T	Rov9[c]	Rov9 T[c]
Days to death (n/n_0)[d]	91 ± 10 (15/15)	86 ± 8 (19/19)	82 ± 17 (9/9)	71.5 ± 12 (11/11)	73 ± 23 (7/7)	666[e] (0/3)	259 [e] (0/4)

[a] Cells from infected culture flasks were detached, washed, and homogenated at 10^8 cells/ml in 5% glucose solution, tg338 mice were inoculated with 20 μl of homogenate.

[b] tg338 mice were inoculated with 20 μl of homogenate, from infected mouse brain (10% wt/vol in glucose solution). This homogenate was identical to that used as a source of infectious agent to inoculate the different cultures.

[c] Culture of uninfected dox-induced Rov9; uninfected dox-induced Rov9 have been also treated with tunicamycin (Rov9 T).

[d] Mean days ± SE; n, number of terminally ill animals; n_0 number of animals inoculated.

[e] Animals euthanasied without presenting any neurological signs.

expression of PrP^C in cell culture increases the sensitivity of cells to the prion infectious agent [25,26], and host animals harbouring a high copy number of the *Prnp* transgene showed a much shorter incubation time than wild-type animals [15,27,28]. These homogeneous and stable high-producer clones represent a significant improvement over the Rov9 cell line as a tool for diagnostic and infectivity tests, and a major step forward in the production of sufficient purified infectious PrP^{Sc} protein for biochemical and biophysical studies. Current animal bioassays of infectious particles are time consuming (requiring months to years) and are costly in terms of animal supply and maintenance. Finally, from an ethical standpoint, TSE cellular models represent an attractive alternative to the use of animals for screening drugs with potential therapeutic value and for diagnostic applications.

Acknowledgments

Research in the Unité de Repliement et Modélisation des Protéines was supported by the Institut Pasteur, the Centre National de la Recherche Scientifique (U.R.A. 2185) and the French Ministry of Research and Technology (GIS ''Infection à prions'' Project No. A75). We thank Drs. Didier Vilette and Hubert Laude (Unité de Virologie Immunologie Moléculaire, INRA, 78350 Jouy-en-Josas, France) for the kind gift of RK13, Rov9 cells, and the prion strain PG128/98. We also acknowledge Dr. Jacques Grassi (Commissariat à l'Energie Atomique, Service de Pharmacologie et d'Immunologie) for providing SAF-32 12F10, and Bar-224 antibodies and Dr. Armelle Phalipon (Institut Pasteur, Paris) for E1-4 antibody. We also thank Mrs. Anne Marie Balazuc (Plate-forme de cytométrie, Institut Pasteur) for her help in performing the cell sorter experiments. We are grateful to Drs. Shirley Longacre and Françoise Lazarini for helpful comments in preparing the manuscript.

References

[1] J. Collinge, Prion diseases of humans and animals: their causes and molecular basis, Annu. Rev. Neurosci. 24 (2001) 519–550.
[2] E. Turk, D.B. Teplow, L.E. Hood, S.B. Prusiner, Purification and properties of the cellular and scrapie hamster prion proteins, Eur. J. Biochem. 176 (1988) 21–30.
[3] K.M. Pan, N. Stahl, S.B. Prusiner, Purification and properties of the cellular prion protein from Syrian hamster brain, Protein Sci. 1 (1992) 1343–1352.
[4] K.M. Pan, M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R.J. Fletterick, F.E. Cohen, et al., Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins, Proc. Natl. Acad. Sci. USA 90 (1993) 10962–10966.
[5] P. Pergami, H. Jaffe, J. Safar, Semipreparative chromatographic method to purify the normal cellular isoform of the prion protein in nondenatured form, Anal. Biochem. 236 (1996) 63–73.
[6] S. Lehmann, D.A. Harris, Blockade of glycosylation promotes acquisition of scrapie-like properties by the prion protein in cultured cells, J. Biol. Chem. 272 (1997) 21479–21487.
[7] S.J. DeArmond, H. Sanchez, F. Yehiely, Y. Qiu, A. Ninchak-Casey, V. Daggett, A.P. Camerino, J. Cayetano, M. Rogers, D. Groth, M. Torchia, P. Tremblay, M.R. Scott, F.E. Cohen, S.B. Prusiner, Selective neuronal targeting in prion disease, Neuron 19 (1997) 1337–1348.
[8] M. Rogers, A. Taraboulos, M. Scott, D. Groth, S.B. Prusiner, Intracellular accumulation of the cellular prion protein after mutagenesis of its Asn-linked glycosylation sites, Glycobiology 1 (1990) 101–109.
[9] T.C. Blochberger, C. Cooper, D. Peretz, J. Tatzelt, O.H. Griffith, M.A. Baldwin, S.B. Prusiner, Prion protein expression in Chinese hamster ovary cells using a glutamine synthetase selection and amplification system, Protein Eng. 10 (1997) 1465–1473.
[10] J. Solassol, C. Crozet, S. Lehmann, Prion propagation in cultured cells, Br. Med. Bull. 66 (2003) 87–97.
[11] D. Vilette, O. Andreoletti, F. Archer, M.F. Madelaine, J.L. Vilotte, S. Lehmann, H. Laude, Ex vivo propagation of infectious sheep scrapie agent in heterologous epithelial cells expressing ovine prion protein, Proc. Natl. Acad. Sci. USA 98 (2001) 4055–4059.
[12] A.M. Haeberle, C. Ribaut-Barassin, G. Bombarde, J. Mariani, G. Hunsmann, J. Grassi, Y. Bailly, Synaptic prion protein immunoreactivity in the rodent cerebellum, Microsc. Res. Tech. 50 (2000) 66–75.
[13] S. Krasemann, T. Jurgens, W. Bodemer, Generation of monoclonal antibodies against prion proteins with an unconventional nucleic acid-based immunization strategy, J. Biotechnol. 73 (1999) 119–129.
[14] N. Morel, S. Simon, Y. Frobert, H. Volland, C. Mourton-Gilles, A. Negro, M.C. Sorgato, C. Creminon, J. Grassi, Selective and efficient immunoprecipitation of the disease-associated form of the prion protein can be mediated by nonspecific interactions between monoclonal antibodies and scrapie-associated fibrils, J. Biol. Chem. 279 (2004) 30143–30149.
[15] J.L. Vilotte, S. Soulier, R. Essalmani, M.G. Stinnakre, D. Vaiman, L. Lepourry, J.C. Da Silva, N. Besnard, M. Dawson, A. Buschmann, M. Groschup, S. Petit, M.F. Madelaine, S. Rakatobe, A. Le Dur, D. Vilette, H. Laude, Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine prp, J. Virol. 75 (2001) 5977–5984.
[16] J.H. Colle, P.B. Falanga, M. Singer, B. Hevin, G. Milon, Quantitation of messenger RNA by competitive RT-PCR: a simplified read out assay, J. Immunol. Methods 210 (1997) 175–184.
[17] E. Sabuncu, S. Petit, A. Le Dur, T. Lan Lai, J.L. Vilotte, H. Laude, D. Vilette, PrP polymorphisms tightly control sheep prion replication in cultured cells, J. Virol. 77 (2003) 2696–2700.
[18] S.G. Chen, D.B. Teplow, P. Parchi, J.K. Teller, P. Gambetti, L. Autilio-Gambetti, Truncated forms of the human prion protein in normal brain and in prion diseases, J. Biol. Chem. 270 (1995) 19173–19180.
[19] C.J. Gibbs, D.C. Gajdusek, H. Amyx, Strain variation in the viruses of Creutzfeldt–Jakob disease and Kuru, in: S.B. Prusiner, W.J. Hadlow (Eds.), Slow Transmissible Diseases of the Nervous System, Academic Press, New York, 1979, pp. 87–110.
[20] V. Perrier, K. Kaneko, J. Safar, J. Vergara, P. Tremblay, S.J. DeArmond, F.E. Cohen, S.B. Prusiner, A.C. Wallace, Dominant-negative inhibition of prion replication in transgenic mice, Proc. Natl. Acad. Sci. USA 99 (2002) 13079–13084.
[21] S.B. Prusiner, S.P. Cochran, D.F. Groth, D.E. Downey, K.A. Bowman, H.M. Martinez, Measurement of the scrapie agent using an incubation time interval assay, Ann. Neurol. 11 (1982) 353–358.
[22] C. Bate, J. Langeveld, A. Williams, Manipulation of PrPres production in scrapie-infected neuroblastoma cells, J. Neurosci. Methods 138 (2004) 217–223.
[23] A. Taraboulos, M. Rogers, D.R. Borchelt, M.P. McKinley, M. Scott, D. Serban, S.B. Prusiner, Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation, Proc. Natl. Acad. Sci. USA 87 (1990) 8262–8266.
[24] R.B. Petersen, P. Parchi, S.L. Richardson, C.B. Urig, P. Gambetti, Effect of the D178N mutation and the codon 129 polymorphism on

the metabolism of the prion protein, J. Biol. Chem. 271 (1996) 12661–12668.
[25] N. Nishida, D.A. Harris, D. Vilette, H. Laude, Y. Frobert, J. Grassi, D. Casanova, O. Milhavet, S. Lehmann, Successful transmission of three mouse-adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild-type mouse prion protein, J. Virol. 74 (2000) 320–325.
[26] P.J. Bosque, S.B. Prusiner, Cultured cell sublines highly susceptible to prion infection, J. Virol. 74 (2000) 4377–4386.
[27] G.A. Carlson, C. Ebeling, S.L. Yang, G. Telling, M. Torchia, D. Groth, D. Westaway, S.J. DeArmond, S.B. Prusiner, Prion isolate specified allotypic interactions between the cellular and scrapie prion proteins in congenic and transgenic mice, Proc. Natl. Acad. Sci. USA 91 (1994) 5690–5694.
[28] P. Tremblay, Z. Meiner, M. Galou, C. Heinrich, C. Petromilli, T. Lisse, J. Cayetano, M. Torchia, W. Mobley, H. Bujard, S.J. DeArmond, S.B. Prusiner, Doxycycline control of prion protein transgene expression modulates prion disease in mice, Proc. Natl. Acad. Sci. USA 95 (1998) 12580–12585.
[29] W. Goldmann, N. Hunter, G. Benson, J.D. Foster, J. Hope, Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene, J. Gen. Virol. 72 (Pt. 10) (1991) 2411–2417.
[30] R. Rubenstein, R.J. Kascsak, M. Papini, R. Kascsak, R.I. Carp, G. LaFauci, R. Meloen, J. Langeveld, Immune surveillance and antigen conformation determines humoral immune response to the prion protein immunogen, J. Neurovirol. 5 (1999) 401–413.