

# PrP Polymorphisms Tightly Control Sheep Prion Replication in Cultured Cells

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**Prion diseases are fatal neurodegenerative disorders of animals and humans that are characterized by the conversion of the host-encoded prion protein (PrP) to an abnormal isoform. In several species, including humans, polymorphisms in the gene encoding the PrP protein tightly control susceptibility of individuals toward this disease. In the present study we show that Rov cells expressing an ovine PrP allele (<sup>VRQ</sup>PrP) associated with high susceptibility of sheep to scrapie were very sensitive to sheep prion transmission and replicated the agent to high titers. In contrast, we did not find any evidence of infection when Rov cells expressed similar levels of a PrP variant (<sup>ARR</sup>PrP) linked to resistance. Our data provide the first direct evidence that natural PrP polymorphisms may affect prion susceptibility by controlling prion replication at the cell level. The study of how PrP polymorphisms influence the genetic control of prion propagation in cultured Rov cells may help elucidate basic mechanisms of prion replication.**

Transmissible spongiform encephalopathies (TSEs) are fatal degenerative disorders of the central nervous system, which naturally affect animals and humans (for a review, see reference 8). TSEs are associated with the posttranslational conversion of the host-encoded prion protein (PrP) to a conformationally altered form (PrP<sup>Sc</sup>). The causative infectious agents, or prions, are thought to be PrP<sup>Sc</sup> itself or a precursor of it (for reviews, see references 22 and 30).

In several species, including mice, sheep, and humans, susceptibility to prion diseases is tightly controlled by the host. The major genetic determinants controlling the length of the incubation period are polymorphisms of *Prnp*, the PrP-encoding gene. In humans, homozygosity for methionine at the polymorphic residue 129 is associated with high susceptibility to the new variant form of Creutzfeldt-Jakob disease (14), whereas homozygosity for valine is overrepresented among early cases of iatrogenic Creutzfeldt-Jakob disease in the United Kingdom (7). In sheep, variations at three amino acid positions of PrP are predominantly linked to scrapie susceptibility (for a review, see reference 17). The <sup>136</sup>V<sup>154</sup>R<sup>171</sup>Q allele (V, R, and Q stand for valine, arginine, and glutamine, respectively) is associated with extremely high susceptibility to scrapie: in naturally infected flocks, sheep homozygous for <sup>VRQ</sup>PrP are almost always affected with scrapie. In contrast, the <sup>136</sup>A<sup>154</sup>R<sup>171</sup>R variant (A for alanine) is associated with resistance to the clinical disease. No clinical scrapie case has been reported in hundreds of sheep homozygous for <sup>ARR</sup>PrP from naturally infected flocks in Europe and the United States, and a single case was reported in Japan (12). Experimental challenge of VRQ- and ARR-encoding sheep with ovine and bovine prions further substantiated the dramatic opposite effects of these two alleles

on disease susceptibility, since sheep homozygous for <sup>ARR</sup>PrP could not be infected by either agent (11). Although the link between specific alleles of PrP and susceptibility to the disease has been well documented during the last decade, especially in sheep, how a few amino acid substitutions can so profoundly affect prion pathogenesis is largely unknown. The development of new experimental models may help elucidate the mechanisms underlying the effects of these polymorphisms.

We recently reported that the stable transfection of a tetracycline-regulatable ovine PrP gene in a rabbit epithelial cell line resulted in the efficient propagation of a natural sheep scrapie agent (28). This scrapie cell culture model, named Rov, represented a unique opportunity to investigate the effect of PrP polymorphisms on the susceptibility to prion infection. In the present study, we examined 2 PrP alleles (<sup>VRQ</sup>PrP and <sup>ARR</sup>PrP) with opposite disease linkage and show that these alleles also have strikingly opposite effects on sheep prion replication in cultured Rov cells.

## MATERIALS AND METHODS

**Isolation of Rov cells expressing ARR and VRQ PrP variants.** The complete coding sequence of the ARR variant of ovine PrP was PCR cloned from sheep genomic DNA in the pTRE plasmid (Clontech) and verified by DNA sequencing. Transfection of RK13 cells and selection of clones for inducible expression of the specified ovine PrP variant were as described previously (28).

**Scrapie strain.** The PG127 isolate originally used to infect Rov cells is from a naturally scrapie-affected sheep (28). This isolate was transmitted to *Prnp*<sup>0/0</sup> transgenic mice (TgOv) expressing the VRQ allele of ovine PrP (29), and the resulting infectious material was used in the present study. Brain homogenate and extracts from infected cells were prepared as described previously (28). When indicated, infectious brain homogenate and cell extracts were diluted in homogenate from healthy brain and in 5% glucose solution containing 5% bovine serum albumin (BSA), respectively.

**Transmission of sheep prion to Rov cells and PrP<sup>Sc</sup> detection.** Inoculation of Rov cells and isolation of sedimentable, proteinase K (PK)-resistant, Rov-derived PrP<sup>Sc</sup> were as described previously (28). Western blots were stained either with 2D6 (25) or 4F2 (18) monoclonal antibodies (MAbs), as indicated.

**Mouse bioassay.** The bioassays were performed on the tg301 line of TgOv mice expressing the VRQ allele of ovine PrP and nullizygous for the mouse *Prnp*

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gene, as described previously (29). Briefly, animals were inoculated intracerebrally with 20  $\mu$ l of inoculum and examined for neurologic disease every 2 days and then daily when clinical signs of scrapie were detected.

**Immunostaining of PrP on living Rov cells.** Immunofluorescence analysis on living Rov cells expressing the VRQ or the ARR allele of PrP was performed at 4°C, with the MH44 anti-PrP polyclonal antibody (20), as described previously (28).

## RESULTS

**Rov cells as a candidate system to study the effect of PrP polymorphisms on prion infection.** Rov cells were obtained by transfecting the VRQ allele of ovine PrP in the RK13 epithelial cell line. Previously, we have shown that doxycycline-mediated expression of the <sup>VRQ</sup>PrP in the Rov9 clone resulted in the efficient replication of the sheep scrapie agent in the exposed cultures (28). Additional transfected cell clones expressing <sup>VRQ</sup>PrP have now been obtained, and nine of them (including the already-described Rov9) were exposed to a strain of sheep prions propagated in a <sup>VRQ</sup>PrP animal (see Materials and Methods). The success of these transmissions was assessed by immunodetection of Rov-derived PrP<sup>sc</sup>, one passage post-inoculation (p.i.). Figure 1A (lanes 1 to 5) shows the relative levels of normal PrP in five inoculated VRQ-Rov clones, and Fig. 1B (lanes 1 to 5) shows the amounts of abnormal PrP in the corresponding PK-digested cell lysates. Abnormal PrP was readily detected in all inoculated VRQ-Rov clones. Since PrP<sup>sc</sup> from residual inoculum is not detected under these experimental conditions (28; see also Fig. 1B, lanes 6 to 9), the presence of cell-derived PrP<sup>sc</sup> indicated that all VRQ-Rov clones have been successfully infected. However, a marked difference in the amount of PrP<sup>sc</sup> accumulated in the different VRQ-Rov clones was observed (Fig. 1B, lanes 1 to 5) that was found not to correlate with the level of PrP expression (Fig. 1A, lanes 1 to 5). The different VRQ-Rov clones might synthesize slightly different subsets of PrP glycoforms, which in turn could influence prion replication. The availability of MAbs recognizing distinct subsets of PrP glycoforms (V. Beringue and S. Hawke, unpublished data) should allow investigating this possibility. These infected cultures were expanded for 2 months. Accumulation of PrP<sup>sc</sup> in each of the individual cultures was evidenced at each passage (data not shown) until the sixth passage (Fig. 1C, lanes 1 to 5), indicating that all of these Rov clones were stably infected. Similar results were obtained with the four other VRQ-Rov clones (data not shown).

Prion titer associated with infected VRQ-Rov extract was determined by endpoint titration in transgenic mice expressing the ovine <sup>VRQ</sup>PrP (TgOv mice), previously shown to be highly sensitive to sheep scrapie (29). Infected VRQ-Rov cultures, grown for 3.5 months (14 passages p.i.), accumulated ca. 2 LD<sub>50</sub> (50% lethal doses) per cell (Table 1). In a second set of experiments, we determined the sensitivity of Rov cells to transmission of sheep prion by exposing VRQ-Rov cells to serial dilutions of the ovine strain of prion and looking for PrP<sup>sc</sup> accumulation at five passages p.i. Under these conditions, transmission was still detectable after inoculation with a 10<sup>-3</sup> dilution of 10% brain homogenate, corresponding to 12.5  $\mu$ g of infectious brain (Fig. 2). For comparison, from 2 to 20  $\mu$ g of Chandler- or RML-infected brain material is required to detect infection of permissive murine N2a and GT1 cells (3,

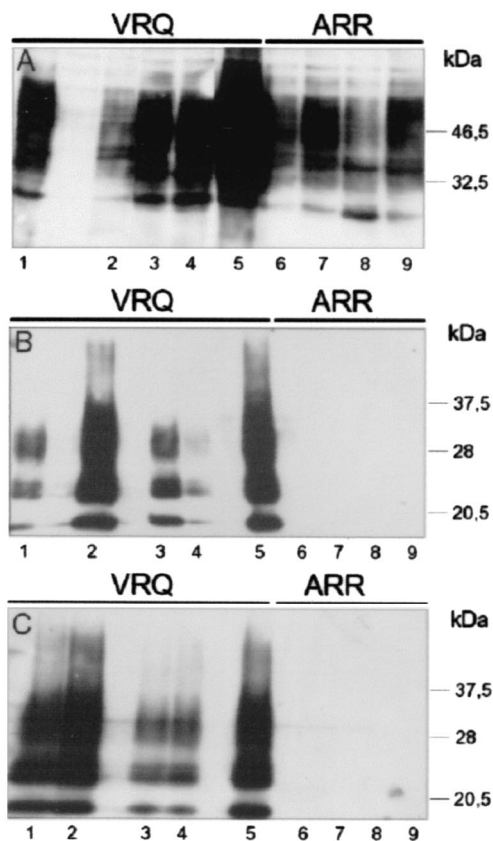


FIG. 1. Analysis of normal and abnormal PrPs in Rov clones expressing the VRQ or the ARR allele of ovine PrP after exposition to sheep prion. Transmission of sheep prion obtained from a <sup>VRQ</sup>PrP animal to nine Rov clones was tested by incubating confluent monolayers expressing the VRQ (lanes 1 to 5) or the ARR (lanes 6 to 9) PrP allele with infectious brain homogenate for 2.5 days. The inoculum was removed, and the cells were washed and passaged 2.5 days later. Cultures were then subcultured once a week at a one-fourth dilution. Parts of the cultures were lysed and analyzed for PrP at either one (A and B) or six (C) passages p.i. Lane 1, Rov9; lane 2, RovC79; lane 3, RovA7; lane 4, RovA1; lane 5, Rov5; lane 6, RovG2; lane 7, RovE2; lane 8, RovG4; lane 9, RovE5. Some lanes were not loaded. (A) Normal PrP in the different clones at one passage p.i. Proteins (the equivalent of 25  $\mu$ g) were methanol precipitated, and PrP was analyzed by immunoblotting with 4F2 MAb directed against the NH<sub>2</sub> terminus region of PrP. Since most of the Rov-derived PrP<sup>sc</sup> undergoes a proteolytic cleavage that removes a portion of the NH<sub>2</sub> terminus region (unpublished observation), accumulated PrP<sup>sc</sup> in Rov cells cannot be detected by 4F2 MAb. (B and C) Abnormal PrP was isolated from PK-digested cell lysates (the equivalent of 250  $\mu$ g of proteins) at one (B) or six (C) passages p.i. The level of PK resistance of PrP<sup>sc</sup> generated in infected VRQ-Rov cells is high enough to sustain digestion with high amounts (50  $\mu$ g/ml) of PK (28). However, in these experiments, low concentrations of PK (ca. 5  $\mu$ g/ml) were used to detect any abnormal PrP with possible low levels of PK resistance. Immunoblots were stained with 2D6 MAb raised against the COOH terminus region of PrP.

19). The relative sensitivity of Rov cells is therefore roughly similar to that of these two widely used neuronal cell models. Finally, transmission of sheep prion to VRQ-Rov cells was found to be very reliable. All of the different VRQ-Rov clones tested so far ( $n = 9$ ) were readily infected, and all transmission attempts to the Rov9 clone (>50) have been successful, based

TABLE 1. Bioassay in TgOv mice of VRQ- and ARR-expressing Rov cells challenged with sheep prion

Ovine PrP allele	Infected culture <sup>a</sup>			Mean survival time ( <i>n/n</i> <sub>0</sub> ) <sup>b</sup> ± SD
	Clone	Passage p.i.	Dilution	
VRQ	Rov9	14	1/10 <sup>6</sup>	89 ± 2.4 (5/5)
VRQ	Rov9	14	1/10 <sup>7</sup>	104.5 ± 2.1 (2/5)
VRQ	Rov9	14	1/10 <sup>8</sup>	>300 (0/5)
VRQ	RovA7	8	1/1	57.3 ± 0.73 (4/4)
ARR	RovE2	8	1/1	>420 (0/5)
ARR	RovG4	8	1/1	>450 (0/7)
None	NA <sup>c</sup>	8	1/1	>420 (0/6)

<sup>a</sup> Mice were inoculated intracerebrally with 20 µl of cell extracts from infected Rov or parental RK13 cultures at the indicated passage postinoculation, either undiluted ( $3 \times 10^6$  cells per mouse) or diluted as indicated. Two distinct infected VRQ- and ARR-Rov clones were bioassayed. The titer of infected Rov9 culture, determined according to Kärber's method, was 2.65 LD<sub>50</sub> per cell (i.e., 10<sup>6.9</sup> LD<sub>50</sub> in  $3 \times 10^6$  cells).

<sup>b</sup> Mean days to the death ± SEM; *n/n*<sub>0</sub>, number of terminally ill/number of inoculated animals.

<sup>c</sup> NA, not applicable.

on the biochemical detection of abnormal PrP accumulation at the very first passage after inoculation (i.e., within 2 weeks p.i.).

These data showed that expression of the VRQ allele of PrP conferred to Rov cells sensitivity to low doses of sheep prion and consistently allowed stable and efficient replication of the agent.

**Rov cells expressing the ARR allele are resistant to infection with sheep prion.** Rov clones expressing the <sup>ARR</sup>PrP allele associated with sheep resistance to the clinical disease were generated. Four clones expressing the ARR allele were selected. They expressed this PrP variant at levels similar to VRQ-PrP in VRQ-Rov cells (data not shown; see also Fig. 1A) and allele-specific glycosylation pattern differences were not detected through Western blot analysis in the present study (Fig. 1A and data not shown). All of the four ARR-Rov clones were exposed to the strain of sheep prion used for VRQ-Rov infection. As described above, cultures were tested for the presence of normal and abnormal PrPs, at one passage p.i. (Fig. 1A, lanes 6 to 9; Fig. 1B, lanes 6 to 9, respectively). In contrast to the situation observed with VRQ-Rov cells, no PrP<sup>sc</sup> could be detected in inoculated ARR-expressing clones (Fig. 1B, lanes 6 to 9). Each week, these cultures were passaged and analyzed for PrP<sup>sc</sup>. No abnormal PrP could be observed at six passages p.i. (Fig. 1C, lane 6 to 9) or before (data not shown), indicating that production of PrP<sup>sc</sup> in inoc-

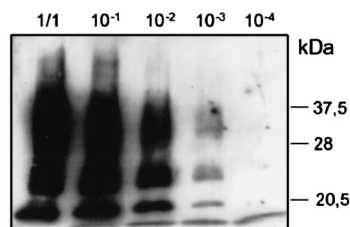


FIG. 2. Sensitivity of VRQ-expressing Rov cells to sheep prion. Rov cells (the Rov9 clone) were exposed to sheep prion (2.5% [wt/vol] brain homogenate) at the indicated dilutions for 7 days. Accumulated PrP<sup>sc</sup> was detected at five passages p.i. with 2D6 MAb.

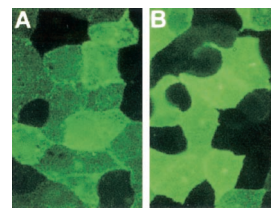


FIG. 3. Immunostaining of PrP in Rov clones expressing the VRQ or the ARR variant of ovine PrP. Confluent monolayers of living cells expressing the VRQ (clone C79) (A) or the ARR (clone E2) (B) PrP allele were labeled with an anti-ovine PrP polyclonal antibody (MH44) and then with fluorescein-conjugated immunoglobulin G second antibodies. No staining was observed when primary antibodies were omitted. Magnification,  $\times 500$ .

ulated ARR-Rov cells was not simply delayed. Extracts from two of the challenged ARR-Rov clones were bioassayed in TgOv mice along with a VRQ-Rov clone infected and passaged in parallel. No infectivity could be demonstrated in the ARR-Rov cultures (Table 1). The fact that each of the nine VRQ-Rov clones but none of the four ARR-Rov could be infected strongly argues against the possibility that the inhibitory effect might be mediated by cell-clone differences unrelated to the sequence of PrP.

There is evidence that normal PrP first transits through the plasma membrane before being converted to abnormal PrP (2, 6). We verified that the <sup>ARR</sup>PrP was expressed at the cell surface of Rov cells as shown previously for the <sup>VRQ</sup>PrP (28). Immunofluorescence analysis of living cells showed that both <sup>ARR</sup>PrP and <sup>VRQ</sup>PrP were present at the outer membrane (Fig. 3). Therefore, the nonpermissiveness of ARR-Rov cells cannot be attributed to a defect of PrP expression at the cell surface.

## DISCUSSION

In this study, with sheep scrapie as an experimental model, we demonstrated that natural polymorphisms of the PrP protein can control prion propagation in permissive cultured cells. Rov cells were highly susceptible to sheep prion when expressing the VRQ allele of PrP. Infected VRQ-Rov cultures produced high titers of sheep prion (ca. 2 LD<sub>50</sub> per cell), a value that compared favorably with titers obtained in two widely used cellular models of mouse prions infection, i.e., neuroblastoma N<sub>2</sub>a (0.004 LD<sub>50</sub> per cell [5]) and neuronal GT1 (0.06 LD<sub>50</sub> per cell [26]) cell lines. The efficient prion propagation in Rov cells could explain why subcloning of the inoculated cultures was unnecessary to assess positive transmission by Western blotting at the very first passage p.i. and to achieve a high percentage (at least 30% [28]) of infected cells. This may explain at least in part why the transmission of sheep prion to VRQ-Rov cells was found to be extremely reliable. Permissive N<sub>2</sub>a and GT1 cell lines are composed of cells with different susceptibilities to infection with the RML strain of murine prions (3, 9), and these marked differences were independent of the level of normal PrP expression (9). In contrast, we found no evidence for the presence of VRQ-expressing cell clones resistant to infection. Prion replication might need some essential cellular factor(s) absent in a significant number of N<sub>2</sub>a and GT1 cells but present in most of the Rov cells or the postulated factor may be missing in Rov cells too, where it

would not be required for the replication of the ovine strain studied.

The reliable transmission of sheep prion, along with the undetectable expression of the endogenous rabbit PrP in these cells (28), makes it feasible to study in-depth relationships between PrP sequence and permissiveness to infection by a reverse genetic approach. As a main result of the present study, we found no evidence of infection in Rov cells expressing the <sup>ARR</sup>PrP-resistant variant. First, no accumulated PrPsc could be detected in ARR-Rov cells exposed to sheep prion, whereas infection of VRQ-Rov cultures with a 1,000-fold-diluted inoculum led to detectable PrPsc. This indicated that <sup>ARR</sup>PrP is at least a 1,000-fold less efficient to support prion replication than is <sup>VRQ</sup>PrP. Second, none of the TgOv mice inoculated with challenged ARR-Rov cultures developed the clinical disease. However, the possibility that low levels of infectivity had been transmitted to these mice, leading to a subclinical level of prion replication (16, 23), is presently being investigated by secondary passages. These findings provide the first ex vivo evidence for a direct link between prion replication and natural PrP allelic variation. Since sheep homozygous for <sup>ARR</sup>PrP are resistant to the various natural strains of sheep scrapie from the field tested so far, it is very unlikely that inhibition of prion replication observed in Rov cells would be restricted to the ovine strain used in the present study. An efficient way to modulate susceptibility to prion disease is to modulate the incubation period by increasing or decreasing the level of PrP expression (10, 21). Since the influence of the PrP genotype on PrP levels in sheep tissues or in particular cell types remains to be established (20), the possibility that PrP polymorphisms could influence the amount of the protein, directly or indirectly, remains open. Our study demonstrating a dramatic effect of PrP polymorphism on prion replication in cells expressing similar levels of different PrP variants implies that PrP polymorphisms may control susceptibility to the disease by conferring distinct functional and/or biochemical properties to the different variants rather than affecting their amounts.

What hypothesis can be made about the cellular mechanisms controlling susceptibility to prion disease through PrP polymorphisms? One possibility could be that some PrP variants might be more prone to be converted to abnormal PrP than others. This notion has been supported by cell-free conversion of sheep PrP alleles. In these assays, guanidium-denatured ovine PrP, purified after solubilization of uninfected cell cultures, was converted to PK-resistant forms upon addition of PrPsc obtained from scrapie-infected sheep brain tissue, and indeed <sup>VRQ</sup>PrP forms were more efficiently converted than <sup>ARR</sup>PrP (4). However, as no generation of infectivity has been demonstrated in cell-free conversion assays (15), it is unclear if conversion reaction truly represents TSE-agent propagation, i.e., if newly converted abnormal PrP can in turn promote conversion of new PrP molecules, as could be expected for a PrPsc-mediated infectious process. Several lines of evidence indicate that the cellular context of PrP is of crucial importance: association of PrP with detergent-resistant microdomains, or rafts, is important in the formation of PrPsc (for a review, see reference 13), and a recent study raised the possibility that insertion of PrP and PrPsc in the same contiguous membrane might be critical for conversion to occur (1). PrP,

however, is not the only player in prion replication. Host factors other than PrP, yet to be identified, are necessary for efficient prion infection (9, 24, 27), and some of them could interact differently with distinct PrP alleles. The possibility that <sup>VRQ</sup>PrP and <sup>ARR</sup>PrP could be recognized and processed differently by Rov cells deserves attention. It has been reported that recombinant VRQ and ARR PrP variants, produced in bacteria, have distinct biochemical and structural properties (25), the biological significance of which needs to be appreciated in mammalian cells. Therefore, we consider TSE cellular models, such as Rov, to be relevant tools for studying the role of PrP polymorphisms on prion infection and to elucidate why the ARR variant, in interaction with the cellular machinery, does not lead to prion multiplication.

In conclusion, we have shown here that the genetic control of susceptibility to prion disease via PrP polymorphisms can be modeled in a cell culture model of scrapie replication. Our data provide evidence that PrP polymorphisms may act on susceptibility by primarily affecting the level of scrapie replication in the infected animal and that Rov cells represent attractive tools to investigate the underlying mechanisms. More generally, both infected and uninfected Rov cells could be used to study PrP structure-function relationships through a reverse genetic approach.

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