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# Conversion Efficiency of Bank Vole Prion Protein *in Vitro* Is Determined by Residues 155 and 170, but Does Not Correlate with the High Susceptibility of Bank Voles to Sheep Scrapie *in Vivo*[*]

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**The misfolded infectious isoform of the prion protein ($PrP^{Sc}$) is thought to replicate in an autocatalytic manner by converting the cellular form ($PrP^{C}$) into its pathogenic folding variant. The similarity in the amino acid sequence of $PrP^{C}$ and $PrP^{Sc}$ influences the conversion efficiency and is considered as the major determinant for the species barrier. We performed *in vitro* conversion reactions on wild-type and mutated $PrP^{C}$ to determine the role of the primary sequence for the high susceptibility of bank voles to scrapie. Different conversion efficiencies obtained with bank vole and mouse $PrP^{C}$ in reactions with several prion strains were due to differences at amino acid residues 155 and 170. However, the conversion efficiencies obtained with mouse and vole $PrP^{C}$ in reactions with sheep scrapie did not correlate with the susceptibility of the respective species to this prion strain. This discrepancy between *in vitro* and *in vivo* data may indicate that at least in the case of scrapie transmission to bank voles additional host factors can strongly modulate the species barrier. Furthermore, *in vitro* conversion reactions with different prion strains revealed that the degree of alteration of the conversion efficiency induced by amino acid exchanges was varying according to the prion strain. These results support the assumption that the repertoire of conformations adopted by a certain $PrP^{C}$ primary sequence is decisive for its convertibility to the strain-specific $PrP^{Sc}$ conformation.**

Transmissible spongiform encephalopathies (TSEs),[2] or prion diseases, are a group of neurodegenerative diseases, including Creutzfeldt-Jakob disease of humans, scrapie of sheep, and bovine spongiform encephalopathy (BSE) of cattle and are caused by a new class of unusual pathogens termed prions (1). Prion diseases are associated with the accumulation of an abnormal, partially protease-resistant isoform of the cellular prion protein ($PrP^{C}$) in the brain of affected individuals. This disease-related isoform, $PrP^{Sc}$, is identical to $PrP^{C}$ with respect to amino acid sequence and chemical post-translational modifications and, according to the "protein-only" hypothesis, is the major if not the only constituent of the infectious agent (2, 3).

The three-dimensional structure of $PrP^{C}$ is characterized by an unstructured N terminus and a globular C-terminal domain, consisting of three $\alpha$-helices with a short stretch of $\beta$-sheet (4, 5). In contrast to $PrP^{C}$ with its high proportion of $\alpha$-helices, circular dichroism analysis and Fourier transform infrared spectroscopy studies revealed that the predominant structural element of $PrP^{Sc}$ is $\beta$-sheet (6). Reduction of the $\beta$-sheet content in $PrP^{Sc}$ preparations leads to a diminished level of infectivity, suggesting that the conversion from $\alpha$-helices into $\beta$-sheets is the fundamental event in $PrP^{Sc}$ formation as well as for propagating prion infectivity (7, 8). $PrP^{Sc}$ is postulated to replicate in an autocatalytic manner by acting as a conformational template that promotes the conversion of $PrP^{C}$ into its protease-resistant isoform (9). The conversion of $PrP^{C}$ to its protease-resistant state can be modeled in cell-free conversion reactions. Using *in vitro* conversion reactions it has recently been possible to demonstrate the *in vitro* generation of infectivity consolidating the protein-only hypothesis (10, 11).

Transmission of prion diseases between different mammalian species is limited by a species barrier (12). Upon primary transmission from one species to another a prolongation of the mean incubation period, an increased range of incubation periods, and a reduced fraction of inoculated animals succumbing to clinical disease are observed. On second passage to further animals of the same species the incubation period usually is decreased and becomes much more consistent. The degree of alteration of the incubation time between primary and second passage in the new host is used as a measure for the species barrier.

Abrogation of the species barrier has been achieved using transgenic mice expressing PrP genes of other species. Mice expressing hamster $PrP^{C}$ were, unlike wild-type mice, susceptible to hamster prions, demonstrating that the molecular basis of the species barrier mainly resides in differences in the amino acid sequence between $PrP^{Sc}$ of the inoculum and $PrP^{C}$ of the inoculated host (13–15). Reports about the susceptibility of transgenic mice expressing human $PrP^{C}$ to human prions are controversial (16, 17) and have led to the postulation of a "protein X", a putative host-specific cofactor that is supposed to modulate the species barrier by interacting with $PrP^{C}$ (18, 19). So far protein X has not been identified, and its role for the conversion has been questioned (20, 21). Transmission studies with different inbred mouse lines expressing the same $PrP^{C}$ revealed different incubation times, stressing the importance of host-specific factors other than $PrP^{C}$ for the species barrier (22, 23).

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[2] The abbreviations used are: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; CVE, conversion efficiency; GndHCl, guanidine hydrochloride; PK, proteinase K; PrP, prion protein; $PrP^{C}$, cellular form of the prion protein; PrPres, protease-resistant prion protein; $PrP^{Sc}$, scrapie-related isoform of the prion protein; RK13, rabbit kidney epithelial cell line; Scrap UK, British (SsUK3) sheep scrapie case; Scrap Italy, Italian (Ss3) sheep scrapie case.

The existence of different prion strains isolated from the same host that can evoke distinct clinical symptoms and can be distinguished by the pattern of $PrP^{Sc}$ deposition in the brain is a challenge for the protein-only hypothesis (1). Prion strains also vary in the biochemical properties of $PrP^{Sc}$ with respect to the degree of resistance to digestion with proteinase K (PK) and the pattern of glycosylation (24–26). Because different strains can exhibit different incubation times in the same host, it becomes apparent that prion strains and species barriers are related phenomena (27, 28). No nucleic acid has been identified in $PrP^{Sc}$ preparations (29) that could account for the strain specificity. In accordance with the protein-only hypothesis, experimental evidence indicates that different strains are defined by conformational isomers capable of propagating their distinct conformation and the related specific disease phenotypes involved (24, 30, 31).

Early inoculation studies performed by Chandler and Turfrey using wild rodent species (32, 33) revealed that field voles (*Microtus agrestis*) in comparison to mice exhibit very short incubation times after inoculation with scrapie. Recent transmission studies with bank voles (*Clethrionomys glareolus*), another wild rodent species closely related to field voles, also demonstrated a high susceptibility to scrapie (34). The high susceptibility of wild rodent species to TSE agents of a phylogenetic distant animal raises epidemiological concerns, because wild rodent species share the same habitat with domestic animals (35) and therefore might function as an environmental reservoir of infectivity.

Using an *in vitro* conversion assay suitable for the investigation of species barriers (36–38) we analyzed the role of the primary amino acid sequence for the high susceptibility of bank voles to the scrapie agent. We clearly identified specific amino acid residues responsible for the different conversion efficiencies obtained with mouse and bank vole $PrP^{C}$ to several prion strains. Unexpectedly, the high susceptibility of bank voles to sheep scrapie as compared with mice was not reflected by the *in vitro* conversion efficiencies. In addition, we observed strain-specific changes of the conversion efficiency induced by amino acid exchanges, providing experimental evidence for the assumption that it is not the mere similarity of the primary amino acid sequence but rather the structural compatibility between $PrP^{C}$ and $PrP^{Sc}$ that determines conversion efficiency and thereby the extent of the species barrier.

## EXPERIMENTAL PROCEDURES

*TSE Inocula and Animal Experiments*—Bank voles (Istituto Superiore di Sanità breeding colony), C57Bl mice (Charles River, Como, Italy), and golden hamsters (Charles River, Como, Italy) were housed in standard cages and treated according to Legislative Decree 116/92 guidelines, and animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal welfare. All animals were individually identified by passive integrated transponders. For natural sheep scrapie isolates, frozen brain tissue from the medulla oblongata was obtained from two Italian Sarda sheep (Ss3 and Ss5) carrying the ARQ/ARQ PrP genotype (indicating amino acids at codons 136, 154, and 171, respectively, on both alleles) and from one British Suffolk Cross sheep (SsUK3) carrying also the genotype ARQ/ARQ. Brain tissue from the UK scrapie case (PG304/02) was provided by the Veterinary Laboratories Agency in Weybrigde, UK. For the natural goat scrapie isolate, frozen brain tissue from the medulla oblongata was obtained from an Italian Ionica breed goat carrying a genotype homologue to the sheep ARQ/ARQ.

Mouse-passaged TSE strains were supplied by the TSE Resource Centre, Institute for Animal Health, Edinburgh, and hamster-passaged 263K strain was originally donated by Richard H. Kimberlin. The inocula from mouse- and hamster-adapted TSE strains were prepared from individual brains obtained from terminally ill C57Bl mice (ME7) and golden hamsters (263K). New dedicated glassware and instruments were autoclaved at 136 °C for 1 h before use. All samples were homogenized at 10% (w/v) concentration in sterile physiological saline and stored at −80 °C. Groups of 5–15 bank voles, C57Bl mice, or golden hamsters were inoculated by the intracerebral route (20 $\mu$l for mice and voles, 30 $\mu$l for hamsters) into the left cerebral hemisphere under ketamine anesthesia. Beginning 1 month after inoculation, animals were examined twice a week until the appearance of neurological signs and then were examined daily. The animals were sacrificed with carbon dioxide when they reached the terminal stage of the disease. Survival time was calculated as the interval between inoculation and sacrifice.

Vole-passaged strains were newly derived in the Istituto Superiore di Sanità[3] after primary transmission and subsequent passages in bank voles of 139A and 301C (originally passaged in C57Bl mice), of a natural sheep scrapie isolate (Ss3), and of BSE sheep from a Cheviot sheep (AHQ/AHQ) experimentally infected with BSE (brain tissue was obtained from the Neuropathogenesis Unit, Institute for Animal Health, Edinburgh). For the *in vitro* conversion studies, $PrP^{Sc}$ was obtained from brain tissue of the third serial passage of 139A, 301C, Ss3, and BSE in bank voles (vole 139A, vole 301C, vole Ss3, and vole BSE, respectively), which showed survival times ± S.D. values of 76 ± 8, 71 ± 3, 90 ± 4, and 79 ± 5, respectively.

*Generation of Plasmids for $PrP^{C}$ Expression*—To generate plasmids for constitutive expression of the prion protein in mammalian cell culture the entire open reading frame of the *Prnp* gene from different species was amplified using the polymerase chain reaction and cloned into the pCIneo vector (Promega, Mannheim, Germany) after subcloning in either pBluescript II SK(−) (Stratagene, La Jolla, CA) or pGEM-T Easy (Promega). The open reading frame of the *Prnp* gene from bank vole (NCBI Nucleotide Database = NCB Accession AF367624), hamster (NCB Accession M14054), sheep (genotype ARQ, NCB Accession AJ000739), and cattle (NCB Accession AJ298878) was amplified from genomic DNA using the primer pairs 5′-CTC ATT AAG CTT AT(C/T) AGC TGT CAT GGC GAA CCT CAG CTA CTG GCT GC-3′/5′-CAA GCA GGG ATC CCT CCC TCA TCC CAC (G/C/T)AT CAG GAA GAT GAG-3′, 5′-CCC TCT TTA TTC TCG AGA TAA GTC ATC ATG GTG AAA AGC CAC ATA GGC AG-3′/5′-GAA AAC AGT CTA GAT GCC CCT ATC CTA CTA TGA GAA AAA TGA GG-3′, 5′-CTC TTT ATT GAA TTC AGA AGT CAT CAT GGT GAA AAG CCA CAT AGG-3′/5′-GAA AAC AGT CTA GAT GCC CCT ATC CTA CTA TGA GAA AAA TGA GG-3′, and 5′-CTT CAT TAA GCT TAT CAG CCA TCA TGG CGA ACC TTA GCT ACT GGC-3′/5′-CAA GCA GGG ATC CTT CCT TCA TCC CAC CAT CAG GAA GAT GAG-3′, respectively. The open reading frame of the *Prnp* gene from sheep (genotype ARR, NCB Accession AJ000736) was amplified from plasmid pScr23.4 (39) using the primer pair 5′-CTC TTT ATT AAG CTT AGA AGT CAT CAT GGT GAA AAG CCA CAT AGG-3′/5′-GAA AAC AGG AAT TCT GCC CCT ATC CTA CTA TGA GAA AAA TGA GG-3′. To obtain bank vole $PrP^{C}$ with the amino acid substitutions M109I, M109L, N155Y, N170S, and E227D, a site-directed mutagenesis approach was applied using the primer pairs 5′-GCC AAA AAC CAA CAT CAA GCA CGT GGC AGG CGC-3′/5′-GCG CCT GCC ACG TGC TTG ATG TTG GTT TTT GGC-3′, 5′-GCC AAA AAC CAA CCT GAA GCA CGT GGC AGG CGC-3′/5′-GCG CCT GCC ACG TGC TTC AGG TTG GTT TTT GGC-3′, 5′-CCG TGA AAA CAT GTA CCG CTA CCC TAA CCA AGT G-3′/5′-CAC TTG GTT AGG GTA GCG GTA CAT GTT TTC ACG G-3′,

[3] Nonno, R., Di Bari, M. A., Cardone, F., Vaccari, G., Fazzi, P., Dell'Omo, G., Cartoni, C., Ingrosso, L., Boyle, A., Galeno, R., Sbriccoli, M., Lipp, H.-P., Bruce, M., Pocchiari, M., and Agrimi, U. (2006) *PLoS Pathog.* **2,** e12.

5′-CCA GTA CAG CAA CCA GAA CAA CTT CGT ACA CGA TTG C-3′/5′-GCA ATC GTG TAC GAA GTT GTT CTG GTT GCT GTA CTG G-3′, and 5′-GGC CTA CTA CGA CGG GAG AAG TTC CCG GGC CGT GCT GC-3′/5′-GCA GCA CGG CCC GGG AAC TTC TCC CGT CGT AGT AGG CC-3′, respectively. The expression vector for bank vole $PrP^C$ with amino acid exchange at residues 155 and 170 (N155Y/N170S) was generated by a two-step site-directed mutagenesis approach using the primer pairs 5′-CCG TGA AAA CAT GTA CCG CTA CCC TAA CCA AGT G-3′/5′-CAC TTG GTT AGG GTA GCG GTA CAT GTT TTC ACG G-3′, and 5′-CCA GTA CAG CAA CCA GAA CAA CTT CGT ACA CGA TTG C-3′/5′-GCA ATC GTG TAC GAA GTT GTT CTG GTT GCT GTA CTG G-3′. The pCIneo vector for the expression of the prion protein of mouse (NCB Accession U29186) was described previously (40).

*Cell Culture Conditions and Transfection Procedure*—Adherent rabbit kidney epithelial (RK13) cells (41) were chosen for transfection due to the absence of detectable endogenous $PrP^C$ expression (42, 43). RK13 cells were cultivated at 37 °C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (4.5 g/liter glucose, without glutamine) with 10% fetal bovine serum (Pan Biotech, Aidenbach, Germany) supplemented with Glutamax, penicillin, and streptomycin (Invitrogen). Hygromycin B (Roche Applied Science) at a concentration of 0.5 mg/ml was added to the media of cell lines stably transfected with constructs for constitutive expression of the cellular prion protein. For the generation of stable cell lines, RK13 cells were co-transfected with pHA58 (40) and derivatives of pCIneo (Promega) using Lipofectamine 2000 (Invitrogen). Cells resistant to hygromycin were selected with 1 mg/ml hygromycin B, and single cell clones were obtained by limiting dilution. The level of $PrP^C$ expression was monitored by Western blot analysis after SDS-PAGE of cell lysates.

*Radioactive Labeling and Purification of $PrP^C$*—To obtain $PrP^C$ labeled with the sulfur isotope $^{35}S$, cell clones were incubated for 1 h at 37 °C with 5% $CO_2$ in Dulbecco's modified Eagle's medium without cysteine and methionine with 10% dialyzed fetal bovine serum (Pan Biotech) supplemented with Glutamax, penicillin, and streptomycin (Invitrogen). Tunicamycin (27 $\mu$g/ml) was also included in the starvation media to obtain deglycosylated prion protein. After 1-h starvation Redivue-Promix (Amersham Biosciences) containing [$^{35}S$]methionine and [$^{35}S$]cysteine was added in a concentration of 0.23 mCi/ml, and the cells were incubated for 5 h at 37 °C with 5% $CO_2$. Afterward cells were washed once with phosphate-buffered saline and lysed with cold lysis-buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 0.5% Igepal, 0.5% desoxycholate, 5 mM EDTA) containing the protease inhibitor mixture Complete Mini without EDTA (Roche Applied Science). The cell lysate was centrifuged for 5 min at 4 °C at 1000 × *g*, and the supernatant was precipitated with four volumes of cold methanol (4 °C) by spinning at 4000 × *g* for 20 min after incubation at −20 °C overnight. The methanol was carefully removed, and the pellet was resuspended into a detergent lipid protein complex buffer (150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 2% *N*-laurylsarcosine, 0.4% L-$\alpha$-phosphatidylcholine) by sonicating three times for 20 s with 70% output intensity using the ultrasound-generator Sonoplus HD2200-UW2200 with BR30 cup-horn sonicator (Bandelin, Berlin, Germany).

$PrP^C$ was purified from the suspension by immunoprecipitation as described by Caughey *et al.* (44) using either the antibody RA3153 (45) or 3B5 (46). $PrP^C$ was eluted from the protein-A Sepharose beads with 0.1 M acetic acid, transferred into 1.5-ml low-binding tubes (Eppendorf, Hamburg, Germany), and stored at 4 °C. The activity of purified $PrP^C$ samples was measured using the $\beta$-counter TRI-CARB 2900TR (PerkinElmer Life Sciences).

*Purification of $PrP^{Sc}$*—Purification of $PrP^{Sc}$ was performed as described by Hope *et al.* (47). The final pellet resulting from this purification method was resuspended in phosphate-buffered saline with 0.5% zwittergent sulfobetaine 3–14 by subjecting the sample two times for 20 s to ultrasound using the ultrasound generator Sonoplus HD2200-UW2200 with BR30 cuphorn sonicator (Bandelin) at 40% output intensity. The resulting suspension was transferred into 1.5-ml low binding tubes (Eppendorf) and stored at 4 °C. $PrP^{Sc}$ was purified from brains of terminally diseased bank voles after the third serial passage of 139A from mouse (vole 139A), Ss3 from sheep (vole Ss3), 301C from mouse (vole 301C), or BSE passaged in sheep (vole BSE). Brains of ME7-infected CL57/Bl6 mice (*Prnp*-a) were used to prepare $PrP^{Sc}$ from mouse (ME7), and brains of 263K-infected golden hamsters were used to obtain $PrP^{Sc}$ from hamster (263K). In addition, $PrP^{Sc}$ from cattle brain of a British BSE case (BSE) as well as $PrP^{Sc}$ from sheep brain of a British (SsUK3) and an Italian (Ss3) sheep scrapie case (named Scrap UK and Scrap Italy, respectively) were also purified. Brain material of the British cattle BSE (case number 02/00996) and sheep scrapie case (case number PG304/02) was provided by the Veterinary Laboratories Agency in Weybridge, UK. The purity of the preparations and the concentration of $PrP^{Sc}$ were determined by silver staining and Western blot analysis after SDS-PAGE. Brain tissue from scrapie-affected sheep (Ss3 and SsUK3), mice (ME7), and hamsters (263K) used for *in vitro* conversion were also used for *in vivo* transmission studies.

*In Vitro Conversion Reactions*—*In vitro* conversion reactions with purified $PrP^{Sc}$ and $^{35}S$-labeled $PrP^C$ were performed in low binding tubes (Eppendorf) in a reaction volume of 30 $\mu$l as described previously (48). In one reaction 15,000 cpm $^{35}S$-$PrP^C$ were incubated for 3 days at 37 °C with 0.4–1 $\mu$g of $PrP^{Sc}$ in conversion buffer (200 mM KCl, 5 mM $MgCl_2$, 0.625% *N*-laurylsarcosine, 50 mM sodium citrate, pH 6.0). The amount of $PrP^{Sc}$ was optimized in saturation studies to obtain the highest conversion efficiency and was varying according to the prion strain. 90% of the reaction volume was digested with PK for 1 h at 37 °C (20 $\mu$g/ml), and the remaining 10% were left untreated. Further sample preparation was performed as described (48). To obtain detectable amounts of PrPres in conversion reactions with $PrP^{Sc}$ from BSE-affected cattle and scrapie-affected sheep the addition of guanidine hydrochloride (GndHCl) to the reaction buffer was required. Therefore, reactions with BSE and Scrap Italy were performed with 0.4 M and reactions with Scrap UK were performed with 0.7 M GndHCl. For reactions with purified BSE and sheep scrapie $PrP^C$ immunoprecipitated with the antibody 3B5 (46) was used. All other reactions were performed with $PrP^C$ purified with the antibody RA3153 (45).

After electrophoresis of untreated and PK-digested samples gels were incubated in fixing solution (isopropanol, $H_2O$, and acetic acid in a ratio (v/v) of 25:65:10, respectively) for 30 min and subsequently incubated in Amplify (Amersham Biosciences) for additional 30 min. Pretreated gels were dried, exposed to a Fujifilm imaging plate BAS-IP MS 2325 (Raytest, Straubenhardt, Germany), and analyzed using a Fujifilm BAS 1800 II phosphorimaging device (Raytest). Phosphor images were evaluated using the densitometry software AIDA V3.44.035 (Raytest). The band intensity of the samples with ($I_{+PK}$) and without ($I_{-PK}$) PK treatment were measured after background subtraction. With respect to samples treated with proteinase K, only bands within the molecular mass range of 18–24 kDa were used for evaluation. The conversion efficiency (CVE) was calculated using the formula, $CVE\ [\%] = [I_{+PK}/(I_{-PK} \times 10)] \times 100$.

*Graphical Representations of $PrP^C$ and $PrP^{Sc}$*—Graphical representations of $PrP^C$ and $PrP^{Sc}$ were generated using the Visual Molecular Dynamics (VMD) software (49). VMD was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for

**TABLE 1**

**Survival times of bank voles, mice, and hamsters after inoculation of different scrapie isolates**

Groups of 5–15 bank voles, C57Bl mice, or hamsters were injected by the intracerebral route with homogenate prepared from brain tissue of scrapie affected animals. Natural sheep scrapie brain homogenates were generated from two Italian sheep (Ss3 and Ss5) carrying the ARQ/ARQ genotype and from one British sheep (SsUK3) carrying also the genotype ARQ/ARQ. Natural goat scrapie brain homogenate was obtained from an Italian goat (SG1) carrying a genotype homologue to the sheep ARQ/ARQ. The inocula from mouse- and hamster-adapted TSE strains were prepared from terminally ill C57Bl mice (ME7) and golden hamsters (263K). $PrP^{Sc}$ purified from brain homogenate of the Italian Ss3 case (Scrap Italy), the British SsUK3 case (Scrap UK), mouse infected with ME7 and 263K affected hamsters were also used for *in vitro* conversion reactions. Survival times were calculated as the interval between inoculation and sacrifice and mean survival times ± S.D. were calculated. The number of animals showing clinical signs was compared to the number of inoculated individuals (*n*/*N*). In comparison to the survival times the conversion efficiencies (CVE) obtained from *in vitro* conversion reactions (Figs. 5 and 6) are shown.

| Inoculum | | Voles | | | C57Bl Mice | | | | Hamsters | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TSE type | Species | Survival time | Clinical disease | CVE | Survival time[a] | Clinical disease | Infection[b] | CVE | Survival time[a] | Clinical disease | Infection[b] | CVE |
| | | *days ± S.D.* | | | *days ± S.D.* | | | | *days ± S.D.* | | | |
| Scrapie Ss3 | Sheep | 199 ± 28[c] | 9/9 | 1.8 ± 0.3 | 401–857 | 0/9 | 3/9 | 5.4 ± 1.9 | 471–608 | 0/8 | 0/8 | 1.4 ± 0.4 |
| Scrapie Ss5 | Sheep | 200 ± 27[c] | 10/10 | | 611–686 | 0/10 | 1/10 | | | | | |
| Scrapie SsUK3 | Sheep | 236 ± 86 | 14/14 | 2.0 ± 0.5 | | | | 5.6 ± 0.8 | | | | 0.5 ± 0.1 |
| Scrapie SG1 | Goat | 187 ± 20[c] | 6/6 | | 583–793 | 0/8 | 1/8 | | 409–609 | 0/10 | 0/10 | |
| Scrapie ME7 | Mouse | 230 ± 46 | 7/7 | 1.7 ± 0.2 | 169 ± 6 | 22/22 | | 5.4 ± 0.5 | | | | 0.7 ± 0.1 |
| Scrapie 263K | Hamster | 195 ± 26 | 12/12 | 16.3 ± 1.6 | | | | 3.2 ± 0.8 | 79 ± 1 | 7/7 | | 15.1 ± 2.2 |

[a] When animals did not show clinical signs, the range of survival time is reported.
[b] Prion infection in clinically healthy animals dead or sacrificed at more than 250 days post-inoculation was assessed by Western blot analysis or histopathology.
[c] Results of the transmission of scrapie Ss3, Ss5, and SG1 in bank voles were previously reported in Cartoni *et al.* (34).

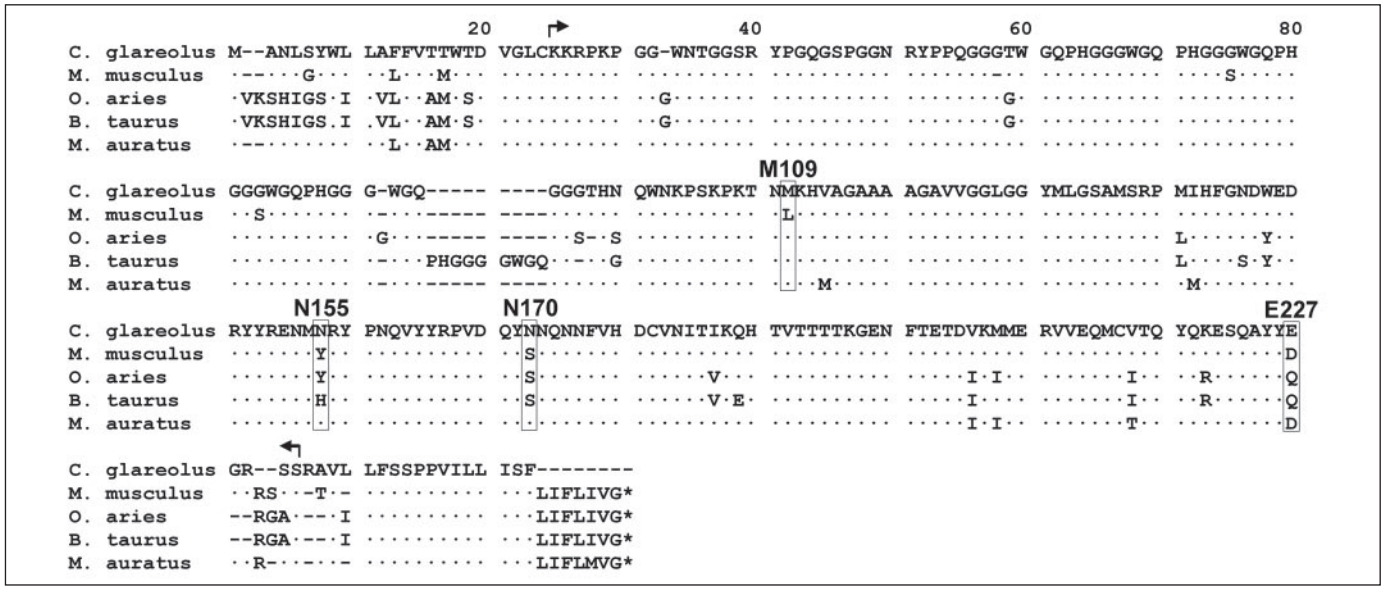


FIGURE 1. **Comparison of the prion protein amino acid sequence from different mammalian species.** Sequence comparison of the prion protein from bank vole (NCBI Nucleotide Database = NCB Accession AF367624) (*C. glareolus*), mouse (NCB Accession U29186) (*M. musculus*), sheep (*Ovis aries*) ARQ-genotype (NCB Accession AJ000739), cattle (NCB Accession AJ298878) (*Bos taurus*), and Syrian hamster (NCB Accession M14054) (*M. auratus*). The amino acid residues that are potentially important for the high susceptibility of the bank vole toward an infection with sheep scrapie are *boxed* (109, 155, 170, and 227). *Arrows* indicate the region of the mature prion protein after cleavage of the endoplasmic reticulum and glycosyl phosphatidylinositol-anchor signal peptide. Amino acid residues identical with the bank vole sequence are marked with *dots*. Lines at the N or C termini of the sequence represent so far undetermined amino acid residues.

Advanced Science and Technology at the University of Illinois at Urbana-Champaign (www.ks.uiuc.edu/Research/vmd).

## RESULTS

Transmission studies revealed that sheep and goat scrapie transmit to bank voles (*C. glareolus*) with very short survival times (34). When challenged with the same scrapie isolates previously transmitted to voles (34), mice (*Mus musculus*, C57Bl inbred strain) were rather resistant to infection, and hamsters (*Mesocricetus auratus*) were fully resistant (Table 1). Bank voles also showed efficient transmission and short survival times after intra-cerebral challenge with a sheep scrapie isolate from the UK (Scrap UK) as well as with the mouse-passaged scrapie ME7 and the hamster-passaged scrapie strain 263K (Table 1).

Because several lines of evidence indicate that the degree of sequence identity of the amino acid sequence of $PrP^{C}$ from the host and the inoculated $PrP^{Sc}$ is the major determinant of the species barrier (13, 14, 36, 37, 50, 51), a comparison of the prion protein sequence from the species involved in our transmission studies is shown in Fig. 1. Amino acid residues 109, 155, 170, and 227 were identified as different between the murine and the bank vole sequence and therefore potentially responsible for the observed differences in susceptibility toward scrapie infection. To investigate the role of these four amino acid residues in detail, the bank vole prion protein sequence was changed at residues 109, 155, 170, and 227 into mouse-specific residues. At residue 109, methionine was changed to leucine (M109L), asparagine at residue 155 to tyrosine (N155Y), asparagine at residue 170 to serine (N155S), and glutamate at residue 227 to aspartate (E227D). With respect to the natural polymorphism of bank voles at residue 109, which influences the incubation time (34), methionine at residue 109 was also changed to isoleucine (M109I). In addition, an expression vector for bank vole $PrP^{C}$ was generated, harboring both changes at residues 155 and 170 (N155Y/N170S). Following expression in RK13 cells all altered $PrP^{C}$ variants

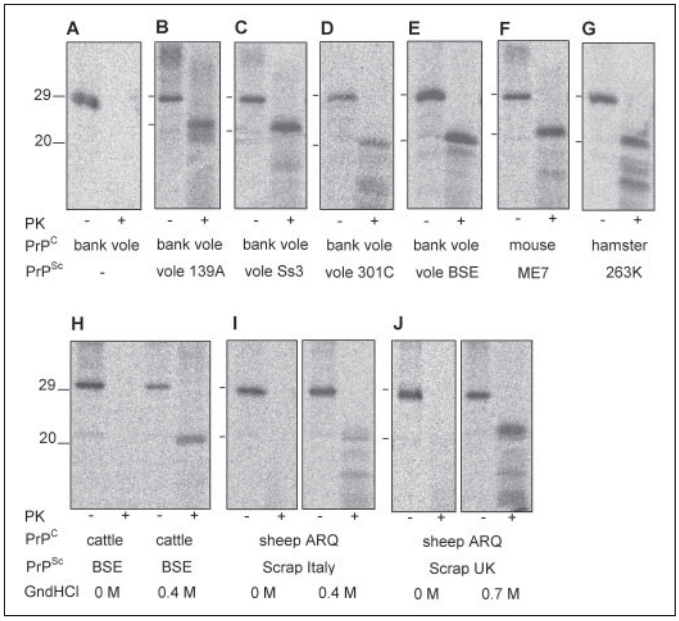


FIGURE 2. **Homologous *in vitro* conversion reactions with different prion strains.** Deglycosylated $PrP^C$ from bank vole (*A–E*), mouse (*F*), hamster (*G*), cattle (*H*), and sheep (*I*, and *J*) was immunoprecipitated from lysates of $^{35}S$-labeled cells and incubated with purified $PrP^{Sc}$. Nine-tenths of the reaction was digested with proteinase K, and one-tenth was left untreated. PK-treated and untreated samples were subjected to SDS-PAGE analysis. Dried gels were exposed to a phosphorimaging plate. *vole 139A*, mouse-passaged scrapie (139A) further passaged in bank voles; *vole Ss3*, Italian sheep scrapie passaged in bank voles; *vole 301C*, BSE-transmitted to mice (301C) and further passaged in bank voles; *vole BSE*, BSE transmitted to sheep and further passaged in bank voles; *ME7*, mouse-passaged scrapie strain; *263K*, hamster-passaged scrapie strain; *BSE*, British cattle BSE case; *Scrap Italy*, Italian sheep scrapie case (Ss3); *Scrap UK*, British sheep scrapie case (SsUK3); *GndHCl*, guanidine hydrochloride. The molecular mass is indicated at the margin (in kilodaltons).

were processed and glycosylated indistinguishably from wild-type $PrP^C$ (data not shown). The altered $PrP^C$ variants as well as wild-type $PrP^C$ from bank vole, mouse, hamster, cattle, and sheep with the genotypes for Ala-136, Arg-154, and Gln-171 (ARQ) and Ala-136, Arg-154, and Arg-171 (ARR) were labeled with [$^{35}S$]methionine and [$^{35}S$]cysteine, purified by immunoprecipitation, and used together with purified $PrP^{Sc}$ from different species for *in vitro* conversion reactions. $PrP^{Sc}$ was obtained from the same scrapie sources used for transmission studies: Scrap Italy (Ss3) and Scrap UK (SsUK3) from sheep, ME7 from mice and 263K from hamsters. The vole-to-vole homologous reaction was investigated with $PrP^{Sc}$ purified from terminally diseased bank voles. Therefore, 139A from mouse, Ss3 from sheep, 301C from mouse, and BSE passaged in sheep were propagated over three passages in bank voles leading to the prion isolates vole 139A, vole Ss3, vole 301C, and vole BSE, respectively. Furthermore, mice are highly susceptible to BSE (52), whereas hamsters (53) and bank voles[4] are resistant. For these reasons we also purified $PrP^{Sc}$ from a British BSE case.

Representative results of homologous *in vitro* conversion reactions, with $PrP^C$ and $PrP^{Sc}$ having the same amino acid sequence, are shown in Fig. 2. Digestion of the samples after the conversion reaction with proteinase K resulted in a truncated protease-resistant prion protein (PrPres) with a molecular mass of ~6–8 kDa lower than $PrP^C$ (Fig. 2, *B–J*). Without the addition of $PrP^{Sc}$ no PrPres was formed (Fig. 2*A*). Fig. 2 (*B–J*) shows that additional protease-resistant fragments with lower molecular weight were detected after the conversion reaction. The pattern of protease-resistant fragments was related to the prion strain and probably due to the formation of folding intermediates with different degrees of protease resistance (37). The occurrence of strain-specific patterns of protease-resistant fragments indicates that strain-specific properties are maintained under the applied reaction conditions. A differential pattern of protease-resistant fragments after *in vitro* conversion has been observed for the distinct hamster-adapted strains Hyper and Drowsy (30, 54).

No PrPres formation was observed when altered bank vole $PrP^C$ variants harboring the amino acid exchanges at residues 109, 155, 170, or 227 were incubated without $PrP^{Sc}$, indicating that the amino acid substitutions did not induce PrPres formation under these experimental conditions (data not shown). In contrast to the prion strains derived from bank voles, mice, and hamsters (Fig. 2, *B–G*) BSE, Scrap Italy, and Scrap UK required the addition of GndHCl to the reaction buffer to obtain PrPres at a detectable level (Fig. 2, *H–J*). The highest conversion efficiency in homologous reactions with BSE and Scrap Italy were obtained at 0.4 M GndHCl and with Scrap UK at 0.7 M GndHCl. In contrast, the addition of GndHCl to reactions with vole 139A yielded a lower conversion efficiency (data not shown). With mouse passaged scrapie strain ME7 PrPres was formed in conversion reactions under non-denaturating conditions (Fig. 2*F*), but addition of GndHCl improved the conversion efficiency slightly (data not shown). To maintain mostly non-denaturating reaction conditions, GndHCl was added only when absolutely required to obtain PrPres at a detectable level (reaction with BSE, Scrap Italy, and Scrap UK).

To investigate the influence of the amino acid exchanges M109I, M109L, N155Y, N170S, N155Y/N170S, and E227D on the conversion of bank vole $PrP^C$ into its protease-resistant isoform, *in vitro* conversion reactions were performed with prion strains passaged in bank voles. From a set of independent conversion reactions the mean conversion efficiencies (CVEs) were calculated and compared with the conversion efficiencies obtained with wild-type $PrP^C$ from bank vole, mouse, and hamster (Fig. 3). To analyze if prion strains display their characteristic properties not only in different patterns of PrPres fragments (Fig. 2) but also in their behavior toward amino acid exchanges, four different bank vole prion strains (vole Ss3, vole 139A, vole 301C, and vole BSE) were analyzed. Fig. 3*A* displays the conversion efficiencies obtained with scrapie-related prion strains (vole Ss3 and vole 139A), and Fig. 3*B* displays the conversion efficiencies of BSE-related strains (vole 301C and vole BSE). The conversion efficiency of the homologous reactions, with $PrP^C$ and $PrP^{Sc}$ having the same primary sequence, obtained with the different prion strains was in the range of 10–35% (Fig. 3, *A* and *B*, *vole*). For all investigated strains the amino acid exchanges at residues 155 (Fig. 3, *A* and *B*, *vole N155Y*) and 170 (Fig. 3, *A* and *B*, *vole N170S*) decreased the conversion efficiency compared with bank vole wild-type $PrP^C$ (vole), whereas the mutation E227D had no significant effect on the formation of PrPres. Mouse-$PrP^C$ and hamster-$PrP^C$ displayed a low conversion efficiency when incubated with bank vole-derived $PrP^{Sc}$. Although $PrP^C$ from hamster has a high degree of sequence similarity with respect to the bank vole sequence, the conversion efficiency obtained with bank vole-derived strains exceeded only slightly the conversion efficiency of mouse $PrP^C$. Alteration of the bank vole sequence in mouse-specific amino acids either at residue 155 (N155Y), 170 (N170S), or at both residues (N155Y/N170S) lowered the conversion efficiency down to the level of the conversion efficiency obtained with mouse $PrP^C$. For all investigated vole prion strains the amino acid exchange M109I (Fig. 3, *A* and *B*, *vole M109I*), which mimics the natural polymorphism of bank voles in its isoleucine variant, when compared with the bank vole wild-type $PrP^C$ (vole) led to a lower conversion efficiency. The exchange toward leucine (Fig. 3, *A* and *B*, *vole M109L*) at this position had a similar effect.

[4] U. Agrimi, manuscript in preparation.

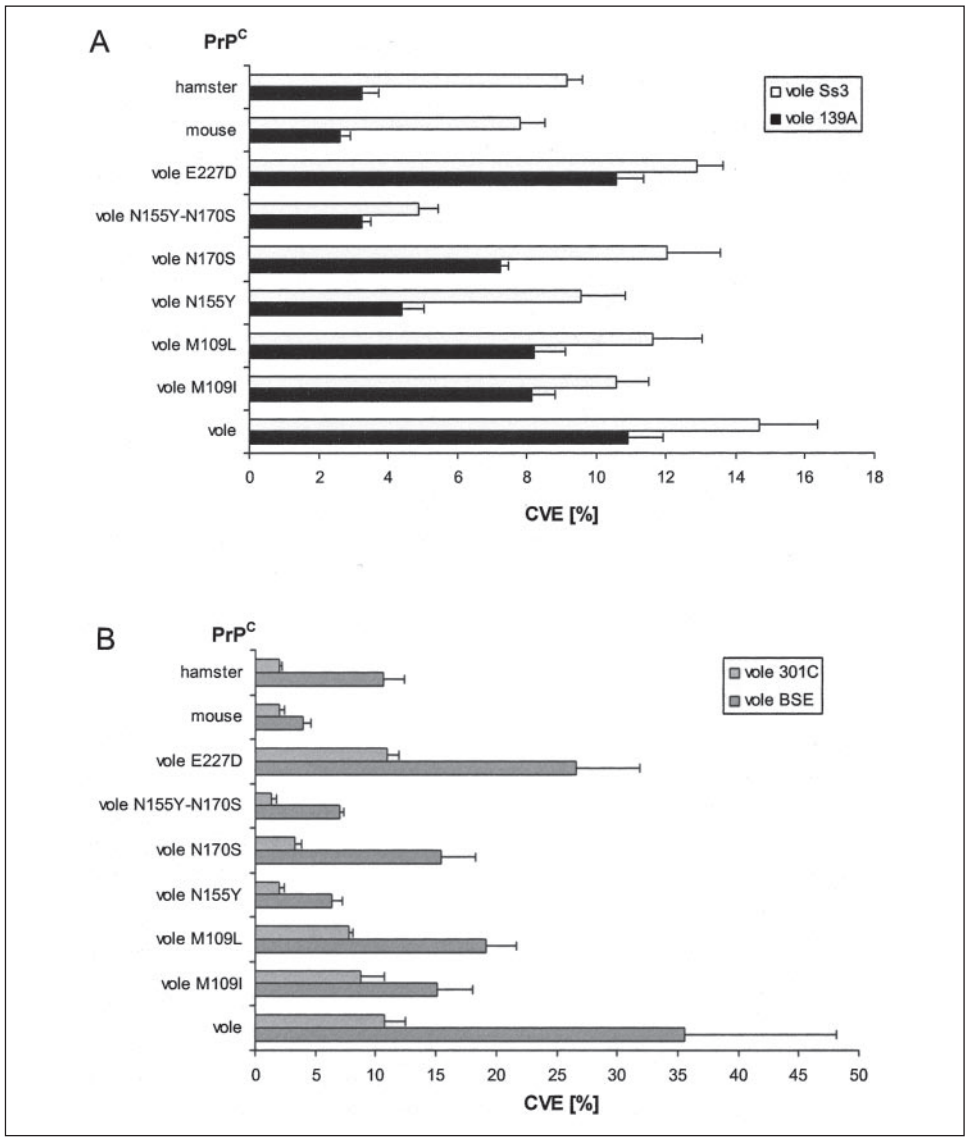


FIGURE 3. **Conversion of altered bank vole $PrP^C$ with prion strains derived from bank voles.** $^{35}S$-$PrP^C$ purified by immunoprecipitation was incubated with purified $PrP^{Sc}$ derived from bank voles infected with different prion strains. Conversion efficiencies (CVE) of reactions with different $PrP^C$/$PrP^{Sc}$ combinations were calculated from band intensities before and after digestion with proteinase K using the formula, CVE [%] = $[I_{+PK}/(I_{-PK} \times 10)] \times 100$. Mean values ± S.E. were determined from a number (*n*) of independent experiments. *A*, conversion by mouse passaged scrapie (139A) and by sheep scrapie from an Italian scrapie case, both further passaged in bank voles (*vole 139A* and *vole Ss3*, respectively); $n \geq 6$. *B*, conversion by BSE passaged in sheep and by the BSE passaged in mice (301C), both further passaged in bank voles (vole BSE and vole 301C, respectively); $n \geq 4$. The type of $PrP^C$ (species and type of mutation) used for conversion is indicated on the *left*.

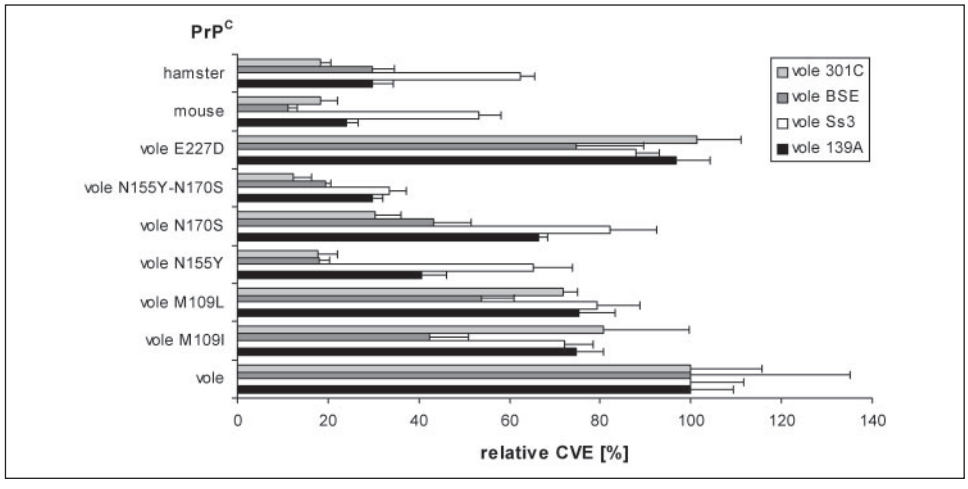


FIGURE 4. **Relative conversion efficiency of conversion reactions with different bank vole-derived prion strains.** Mean values from Fig. 3 and their standard errors were normalized to the homologous reaction, with $PrP^C$ and $PrP^{Sc}$ having the same amino acid sequence, using the formula, relative CVE [%] = $(CVE_{heterologous}/CVE_{homologous}) \times 100$. The type of $PrP^C$ (species and type of mutation) used for conversion is indicated on the *left*.

To compare the degree of alteration of PrPres formation caused by amino acid exchanges between the different bank vole-passaged prion strains, the conversion efficiencies were normalized with respect to the homologous reaction (Fig. 4). Although the primary sequence composition for a specific $PrP^C$/$PrP^{Sc}$ combination was the same, the degree of alteration of the conversion efficiency varied according to the prion strain. For instance, the inhibitory effect on the conversion efficiency of the amino acid exchanges M109I and M109L was more pronounced in

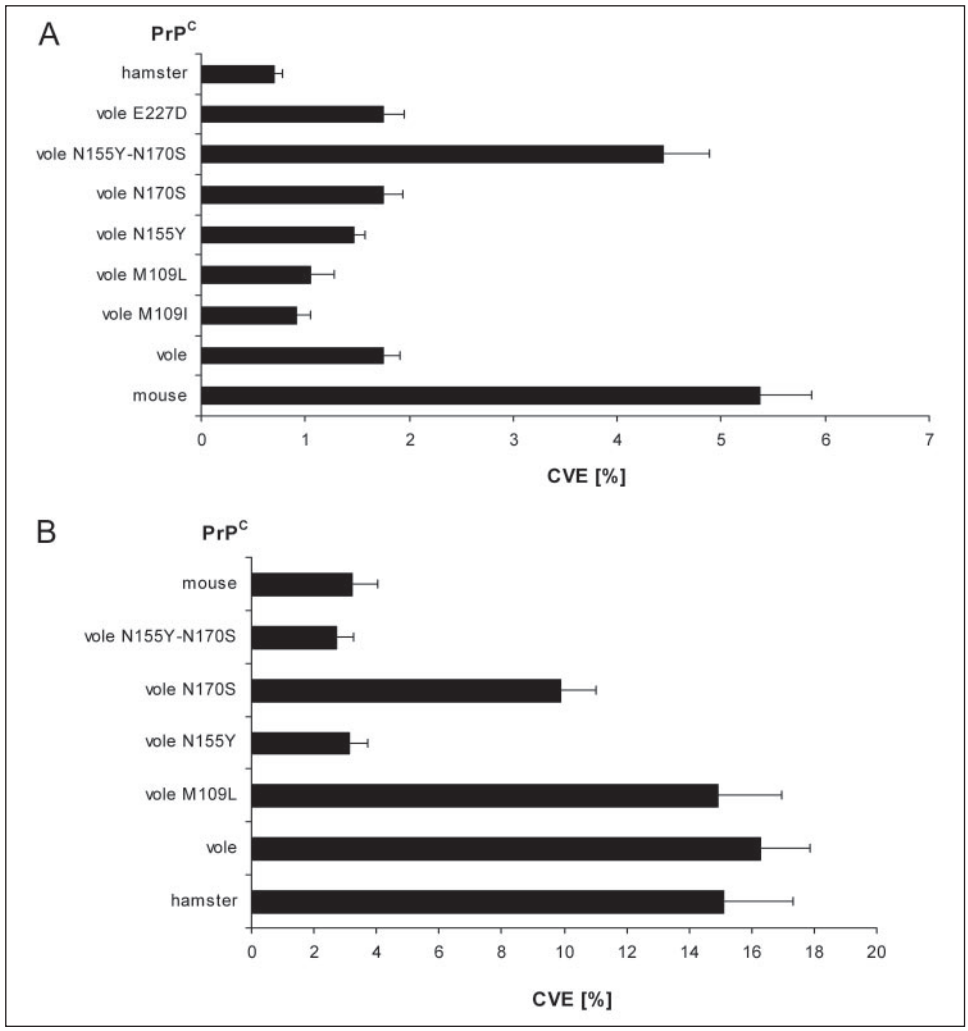


FIGURE 5. **Conversion of altered bank vole $PrP^C$ with mouse and hamster prion strains.** $^{35}S$-$PrP^C$ purified by immunoprecipitation was incubated either with purified mouse-passaged scrapie ME7 (*A*) or hamster-passaged scrapie 263K (*B*). Conversion efficiencies were determined (CVE [%] = $[I_{+PK}/(I_{-PK} \times 10)] \times 100$), and mean values ± S.E. were calculated from a number (*n*) of independent experiments. *A*, $n \geq 6$. *B*, $n = 4$ (except vole M109L: $n = 3$). The type of $PrP^C$ (species and type of mutation) used for conversion is indicated on the *left*.

conversion reactions with vole BSE (CVE was lowered by 50–60%), compared with the other investigated bank vole strains (CVE was lowered by 20–30%). In reactions with vole Ss3 and $PrP^C$ from wild-type mouse or hamster a mean relative CVE of 53 ± 5% and 63 ± 3% was obtained, respectively. In contrast, with vole 139A, vole BSE, or vole 301C the conversion of mouse and hamster $PrP^C$ was remarkably less efficient (11–30%). Significant differences were observed in reactions with the amino acid exchanges at residue 155 (Fig. 4, *vole N155Y*) and 170 (Fig. 4, *vole N170S*). The inhibitory effect on the conversion due to the alteration at either residue 155 or 170 was stronger in reactions with vole BSE and vole 301C compared with reactions with the vole 139A and vole Ss3. In reactions with vole 139A and vole Ss3 the double mutation N155Y/N170S had the strongest inhibitory effect on the conversion efficiency, whereas in reactions with vole BSE and vole 301C the exchange at residue 155 was sufficient to reduce the conversion efficiency to the same level as the alteration at both residues 155 and 170. It is interesting to note that vole 139A and vole Ss3 are scrapie-related strains, whereas vole BSE and vole 301C are related to BSE. Regarding the influence of the amino acid exchanges N155Y, N170S, and N155Y/N170S on the conversion efficiency prion strains could be classified with respect to their origin.

*In vitro* conversion reactions with different strains passaged in bank voles revealed that the degree of inhibition induced by changes in the primary sequence is related to the prion strain (Fig. 4). To further investigate the strain dependence of amino acid exchanges on the conversion efficiency and to compare *in vitro* reactions with *in vivo* transmission properties (Table 1), *in vitro* conversion reactions with the mouse passaged scrapie strain ME7 were performed. As shown in Fig. 5*A*, the importance of residues 155 and 170 for the species barrier between mouse and bank vole could also be demonstrated in conversion reactions with ME7. As expected, homologous reactions with mouse $PrP^C$ (Fig. 5*A*, *mouse*) resulted in a higher conversion efficiency than reactions with bank vole $PrP^C$ (Fig. 5*A*, *vole*) with 5.4 ± 0.5% and 1.7 ± 0.2%, respectively. Compared with bank vole wild-type $PrP^C$ the double mutation N155Y/N170S improved the conversion efficiency up to a level comparable to the level obtained with wild-type mouse $PrP^C$ (Fig. 5*A*, *vole N155Y/N170S*). The single point mutations N155Y and N170S did not significantly alter the PrPres formation (CVE of 1.5 ± 0.1% and 1.7 ± 0.2%, respectively). Similar to reactions with vole-passaged prion strains (Fig. 3, *A* and *B*) compared with wild-type bank vole $PrP^C$ the amino acid exchange at residue 227 did not alter the conversion efficiency significantly (Fig. 5*A*, *vole E227D*). The amino acid exchange at residue 109 (Fig. 5*A*, *vole M109I*) representing the natural polymorphism in bank vole populations resulted in a reduced conversion efficiency of 0.9 ± 0.1%. The amino acid exchange at residue 109 from methionine to leucine (M109L) inhibited PrPres formation upon incubation with mouse derived ME7 (CVE of 1.1 ± 0.2%). This was unexpected, because the change to leucine in the bank vole sequence introduced a mouse-specific amino acid, and therefore one would expect improved conversion efficiency. However, the observation, that an

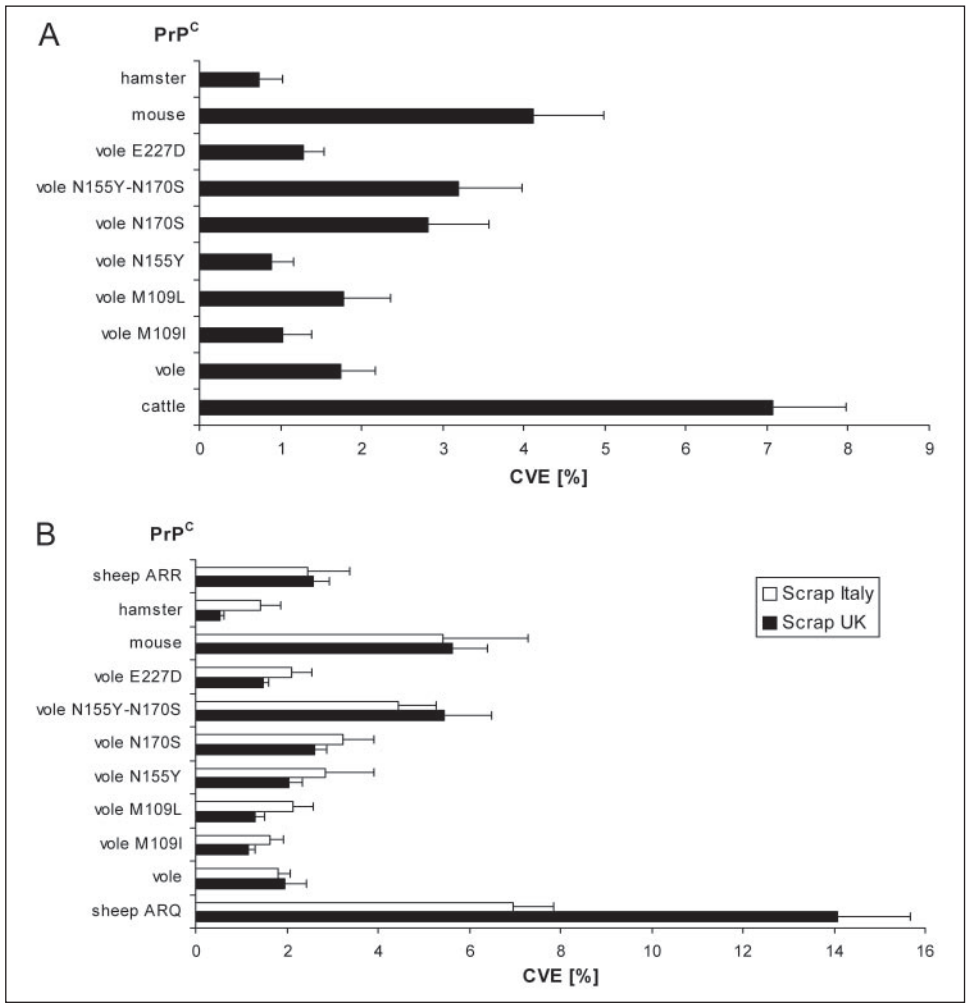


FIGURE 6. **Conversion of altered bank vole $PrP^C$ with cattle BSE and sheep scrapie.** $^{35}S$-$PrP^C$ was purified by immunoprecipitation and incubated either with purified BSE (*A*) or purified sheep scrapie (*B*). For the conversion with BSE 0.4 M GndHCl was included in the reaction buffer. The reactions with purified $PrP^{Sc}$ from a British sheep scrapie case (*Scrap UK*) were performed with 0.7 M GndHCl and for the reactions with purified $PrP^{Sc}$ from an Italian sheep scrapie case (*Scrap Italy*) 0.4 M GndHCl was used. Conversion efficiencies were determined (CVE [%] = $[I_{+PK}/(I_{-PK} \times 10)] \times 100$) and mean values ± S.E. were calculated from a number ($n \geq 6$) of independent experiments. The type of $PrP^C$ (species and type of mutation) used for conversion is indicated on the *left*.

alteration of the bank vole sequence at residues 155 and 170 into mouse-specific residues (N155Y/N170S) led to a lower conversion efficiency upon incubation with bank vole-passaged strains (Fig. 3, *A* and *B*) but improved the PrPres formation in reactions with mouse ME7 (Fig. 5*A*), underscores the notion that PrPres formation strongly depends on the nature of the interactions between $PrP^C$ and $PrP^{Sc}$. Depending on the prion strain, amino acid exchanges can either inhibit or improve the conversion efficiency.

The observed differences in conversion efficiencies between mouse and bank vole $PrP^C$ upon incubation with ME7 are in accordance with the *in vivo* data obtained in transmission experiments with ME7. Bank voles appeared less susceptible to ME7 than mice (Table 1). Also the lower conversion efficiency, compared with bank vole $PrP^C$, obtained with hamster $PrP^C$ is consistent with the *in vivo* transmission data. ME7 can be transmitted to hamsters (27) only with longer incubation periods than those reported here for bank voles (Table 1).

Although the amino acid sequences of hamster and bank vole $PrP^C$ are very similar, the conversion efficiency in reactions with hamster $PrP^C$ and bank vole prion strains was quite low (Fig. 3). In contrast, incubating bank vole $PrP^C$ with hamster-passaged scrapie strain 263K yielded a conversion efficiency (16.3 ± 0.6%) similar to the homologous reaction with hamster $PrP^C$ (Fig. 5*B*). With $PrP^C$ from mouse the conversion was quite inefficient (conversion efficiency of 3.2 ± 0.8%). Mouse-specific alterations introduced into bank vole $PrP^C$ at residue 170 (vole N170S) lowered the conversion efficiency down to 10 ± 1%. Upon amino acid exchange at residues 155 and 170 (vole N155Y/N170S) the conversion efficiency was decreased down to the low level obtained with mouse $PrP^C$, but single amino acid alteration at residue 155 (vole N155Y) had a comparable effect (CVE of around 3%). With respect to the different conversion efficiencies obtained with mouse and bank vole $PrP^C$ in reactions with 263K, it is worth noting that intracerebral inoculation of 263K in mice led only to an asymptomatic infection (55), whereas 263K was successfully transmitted to bank voles (Table 1), underscoring the accordance of *in vitro* and *in vivo* data.

To estimate the role of the differences in the primary sequences of the prion protein from mice and bank voles for the high susceptibility of voles to sheep scrapie *in vitro* conversion reactions were performed with purified sheep scrapie and in addition with cattle BSE. The conversion efficiencies resulting from reactions with BSE are shown in Fig. 6*A*. The homologous reaction with $PrP^C$ from cattle resulted in a conversion efficiency of 7.1 ± 1.0%. In comparison with $PrP^C$ from bank vole and hamster, mouse $PrP^C$ was converted by BSE with a higher conversion efficiency (1.7 ± 0.4%, 0.7 ± 0.3%, and 4.1 ± 0.9%, respectively). This is in accordance with the results of transmission studies that revealed that mice are susceptible to BSE (52), whereas hamsters (53) and bank voles[4] are resistant. By introducing the double mutation N155Y/N170S into the bank vole sequence, the conversion efficiency was enhanced up to a level comparable to the efficiency achieved with mouse $PrP^C$. The single amino acid exchange N170S also led to an enhanced conversion efficiency. Surprisingly, the N155Y exchange led to a reduced conversion efficiency (0.9 ± 0.3%). In comparison with wild-type bank vole $PrP^C$ the

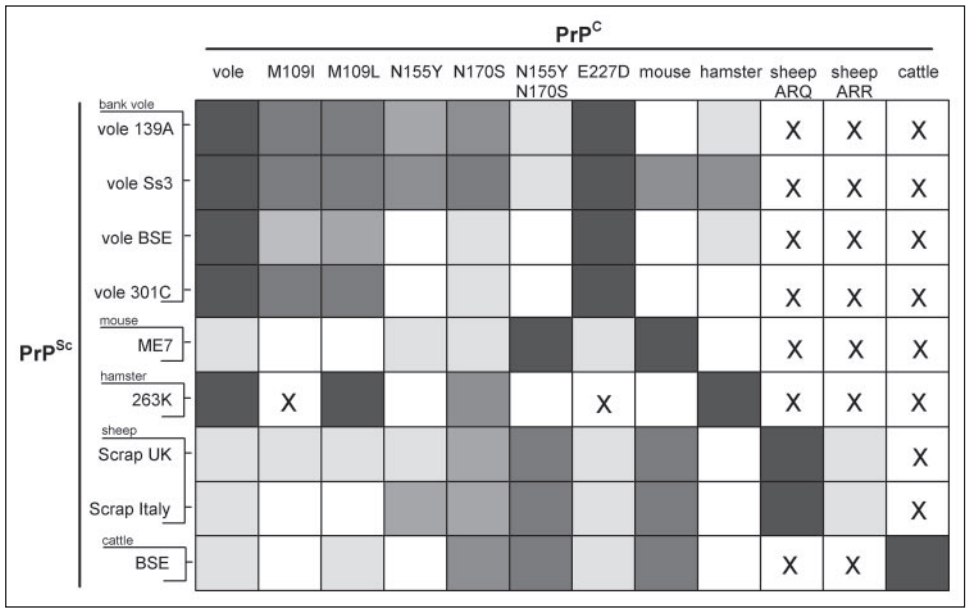


FIGURE 7. **Summary of *in vitro* conversion results.** Graphic representation of mean conversion efficiencies calculated from the results of *in vitro* conversion reactions with different $PrP^C$ variants and $PrP^{Sc}$ from diverse prion strains. In general for each $PrP^C/PrP^{Sc}$ combination 6–12 independent experiments were performed and the conversion efficiencies determined (except for hamster 263K with three to four and vole BSE and vole 301C with four to five independent experiments). In each *row* the height of the conversion efficiency is normalized to the homologous reaction ($PrP^C$ and $PrP^{Sc}$ with the same amino acid sequence), which has the highest conversion efficiency (*black*). *Different gray tones* indicate intermediate conversion efficiencies, and the lowest conversion efficiency is drawn in *white*. M109I, M109L, N155Y, N170S, N155Y/N170S, and E227D: bank vole primary sequence with amino acid exchanges at indicated positions; vole 139A, vole Ss3: prion strains derived from bank voles with relation to sheep scrapie; vole BSE, vole 301C: prion strains derived from bank vole with relation to cattle BSE; ME7: mouse-passaged scrapie; 263K: hamster-passaged scrapie; Scrap UK: British sheep scrapie case (SsUK3); Scrap Italy: Italian sheep scrapie case (Ss3); BSE: British cattle BSE case; *X*: not done.

amino acid exchange at residue 227 (E227D) did not lead to an altered conversion efficiency.

Contrary to the expectations from transmission studies (Table 1), in which bank voles compared with mice displayed a high susceptibility to sheep scrapie, *in vitro* conversion of mouse $PrP^C$ with purified sheep scrapie was more efficient than conversion of $PrP^C$ from bank vole (Fig. 6*B*). This was observed with purified $PrP^{Sc}$ from brain tissue of a sheep scrapie case from Great Britain (Scrap UK) as well as with $PrP^{Sc}$ from a sheep scrapie case from Italy (Scrap Italy). The purifications of Scrap UK and Scrap Italy were performed with the same brain tissue used to obtain the transmission data shown in Table 1 (Scrapie SsUK3 and Ss3, respectively). Amino acid exchanges within the bank vole sequence at residues 155 and 170 improved the conversion efficiency slightly and the double mutation N155Y/N170S enhanced the conversion efficiency up to the level that was obtained in reactions with mouse $PrP^C$. The conversion efficiency of bank vole $PrP^C$ with the mutation E227D was comparable to the conversion efficiency of the bank vole wild-type sequence. As observed with purified BSE, hamster $PrP^C$ was converted by sheep scrapie with low efficiency. In addition to the reaction with sheep $PrP^C$ with the amino acid composition ARQ at residues 136, 154, and 171, conversion reactions were performed with sheep $PrP^C$ with ARR composition. The ARR genotype is associated with enhanced resistance toward scrapie infection (39). In accordance with these findings compared with sheep $PrP^C$ (ARQ) with a CVE of 7.0 ± 0.9% and 14.1 ± 1.6% (Scrap UK and Scrap Italy, respectively) sheep $PrP^C$(ARR) could be converted with sheep scrapie only with a CVE of 2.5 ± 0.9% and 2.6 ± 0.4% (Scrap UK and Scrap Italy, respectively).

## DISCUSSION

Transmission studies with bank voles revealed that compared with mice these rodents are highly susceptible to scrapie (Table 1). To elucidate the role of the primary sequence for this unusual susceptibility we performed *in vitro* conversion reactions following a protocol based on purified $PrP^C$ and $PrP^{Sc}$ (44). The use of purified components minimizes the influence of additional factors other than $PrP^C$ and $PrP^{Sc}$ on the conversion process and newly formed PrPres can easily be distinguished from initial $PrP^{Sc}$ due to the radioactive labeling of $PrP^C$. Sequence comparison between the prion protein of bank voles and mice revealed that differences in amino acid residues 109, 155, 170, and 227 may be responsible for the high susceptibility of bank voles to the scrapie agent (Fig. 1). To elucidate the role of these four amino acid residues bank vole $PrP^C$ was altered at these positions toward mouse-specific residues. The chimeric $PrP^C$ variants were purified and used for *in vitro* conversion reactions with different prion strains.

In the absence of $PrP^{Sc}$ neither wild-type nor any of the chimeric $PrP^C$ variants were converted into the protease-resistant isoform, demonstrating that PrPres formation was strictly dependent on the presence of $PrP^{Sc}$. The graphical overview shown in Fig. 7 that summarizes the conversion efficiencies obtained are in line with the assumption that the conversion is caused by a direct interaction with $PrP^{Sc}$, because the influence of point mutations on the conversion efficiency was dependent on the prion strain. For instance, the amino acid exchange at residues 155 (N155Y) and 170 (N170S) led to a lowered conversion efficiency when incubated with purified $PrP^{Sc}$ derived from bank voles or hamster (Fig. 3, 5*B*, and 7). In contrast, the conversion efficiency was enhanced when the same sequences were incubated with $PrP^{Sc}$ derived from mouse (Figs. 5*A* and 7) and cattle or sheep (Figs. 6 and 7). The observed changes in conversion efficiency therefore cannot be attributed to a general stabilization or destabilization of the $PrP^C$ structure induced by the amino acid exchanges, subsequently leading to a general inhibition or improvement of conversion. In fact, the alterations of the conversion efficiency have to be evaluated with respect to an interaction with $PrP^{Sc}$. These results are in accordance with the postulate of the prion hypothesis that the interaction of $PrP^C$ and $PrP^{Sc}$ plays a fundamental role for the conversion process. The finding that an amino acid exchange at residues 155 and 170 had a severe effect on the conversion efficiency, whereas an exchange at residue 227 did not influence the formation of PrPres demonstrates that sequence similarity at residues 155 and 170 in contrast to a sequence similarity at residue 227 is of specific importance for the interaction and subsequent conversion (Fig. 7).

The observations suggesting that conversion was dependent on the direct interaction of $PrP^C$ with $PrP^{Sc}$ lead to the conclusion that side chains of the amino acids residues that altered the conversion efficiency reside at important interacting surfaces. Although there is no NMR structure for bank vole $PrP^C$ available, because of the high similarity of the globular structure of $PrP^C$ from different mammalian species (56) it is likely that the structure of bank vole $PrP^C$ is similar to the $PrP^C$ structure of the closely related Syrian golden hamster (5). Although being located to different regions of the globular domain of $PrP^C$, residues 155,

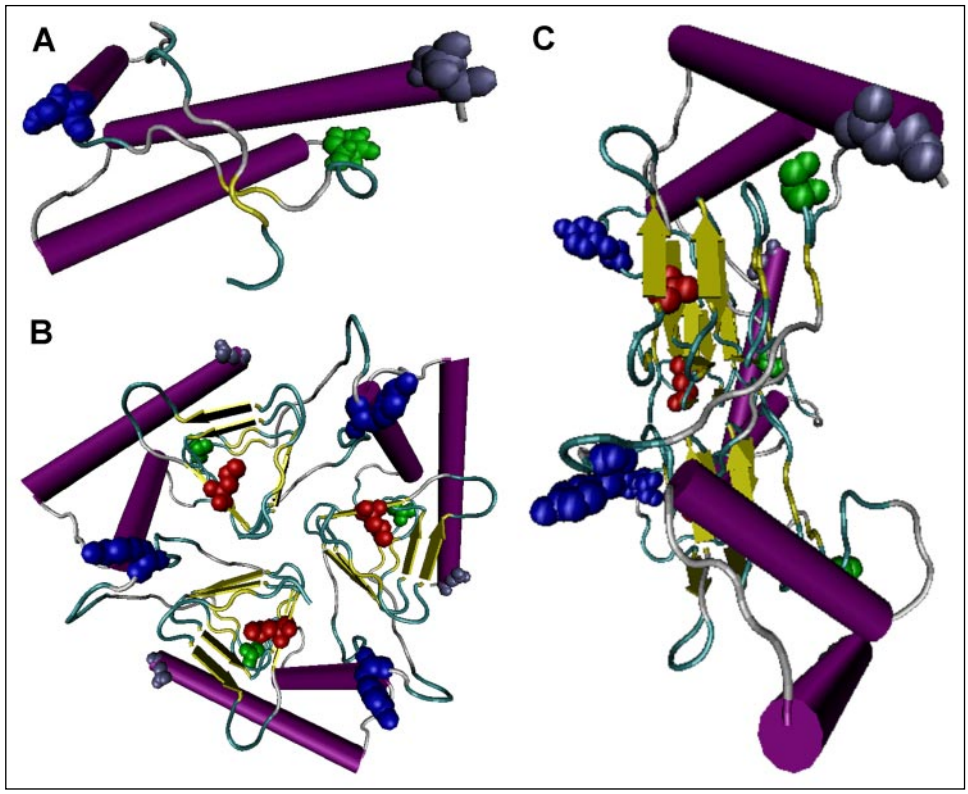


FIGURE 8. **Amino acid positions 109, 155, 170, and 227 within models of $PrP^C$ and $PrP^{Sc}$.** *A*, position of amino acid residues Asn-155 (*blue*), Asn-170 (*green*), and Asp-227 (*ice blue*) in NMR structure of the globular domain of hamster $PrP^C$ (amino acid 125–228) (NCB Accession 1B10). Amino acid residue Met-109 is located in the unstructured N terminus. *B* and *C*, position of amino acid residues Leu-108 (*red*), Tyr-154 (*blue*), Ser-168 (*green*), and Asp-226 (*ice blue*) in trimeric model of mouse-$PrP^{Sc}$ (57). Mouse-specific residues Leu-108, Tyr-154, Ser-169, and Asp-226 correspond to Met-109, Asn-155, Asn-170, and Glu-227 of bank vole prion protein. *B*, view on the top (N terminus). *C*, view from the side. Different secondary structure elements are drawn in different colors (*purple*, $\alpha$-helix; *yellow*, $\beta$-sheet; and *green*, loop).

170, and 227 are exposed on the protein's surface (Fig. 8*A*) and therefore accessible for potential interactions with $PrP^{Sc}$. Amino acid residue 109 is located in the unstructured N terminus and therefore without defined position in the NMR structure.

In a recent three-dimensional model of $PrP^{Sc}$ based on electron micrographs of two-dimensional crystals (57), the corresponding residues are also located on accessible surfaces (Fig. 8, *B* and *C*). Interestingly, residues 109, 155, and 170 are located on surfaces of the $\beta$-helical core structure potentially important for $PrP^{Sc}$-fibril formation. In contrast, amino acid residue 227 is located at the C-terminal end of helix 3, a region neither affected by the $PrP^C/PrP^{Sc}$ conversion nor a region important for the formation of $PrP^{Sc}$ fibrils.

The results of the *in vitro* conversion reactions display the structural relevance of amino acid residues 155 and 170 for the interaction with $PrP^{Sc}$ and for PrPres formation. Although being exposed on the surface of $PrP^C$ amino acid residue 227 is not important for the conversion process. The alteration of the bank vole sequence at position 109 into a mouse-specific residue (M109L) as well as the amino acid exchange methionine to isoleucine (M109I), representing the natural polymorphism of bank voles, lowered the level of PrPres formation with all investigated prion strains (Fig. 7). Even in the reactions with $PrP^{Sc}$ purified from mice infected with ME7 the alteration at position 109 into a mouse-specific residue (M109L) did not improve the conversion efficiency (Figs. 5 and 7). With respect to the location of residue 109 in the unstructured N terminus of $PrP^C$, it is therefore possible that the observed effect on the conversion efficiency of an amino acid exchange at residue 109 is based on a mechanism that is different from the mechanism underlying the effects on conversion efficiency of residues 155 and 170.

As described above, matching amino acid residues between $PrP^C$ and $PrP^{Sc}$ at specific positions are determinants for the conversion efficiency. To investigate the role of the prion strain for conversion efficiency we performed conversion reactions with different strains passaged in bank voles. A comparison of the obtained conversion efficiencies revealed that the conversion is not only dependent on sequence identity at certain residues but also dependent on the prion strain (Figs. 4 and 7). For instance with respect to the amino acid exchanges N155Y, N170S, and the double mutation N155Y/N170S strain-specific effects on the conversion efficiency could be observed. In reactions with the bank vole-derived strains vole 139A and vole Ss3, both related to sheep scrapie, the conversion efficiency was decreased to a much higher extent by the double mutation than by the single amino acid exchanges N155Y or N170S. In contrast, in reactions with the BSE-related prion strains vole BSE and vole 301C the amino acid exchange N155Y was sufficient to reduce the conversion efficiency down to the level obtained with mouse $PrP^C$. Although $PrP^{Sc}$ isolated from bank voles infected with different prion strains (vole 139A, vole Ss3, vole BSE, and vole 301C) has the same primary sequence (the bank vole wild-type sequence) different alterations of the conversion efficiency induced by the amino acid exchanges N155Y and N170S have been observed. Furthermore, the distinct reaction toward amino acid exchanges could be used to classify the different strains with respect to their origin. Although on the one hand prion strains are thought to be conformational isomers (31), on the other hand $PrP^C$ is thought to be able to adopt a certain repertoire of conformations (58). The range of conformations accessible to a particular $PrP^C$ molecule according to this hypothesis will depend on its primary sequence. Some of the conformations of a $PrP^C$ molecule with a specific primary sequence may be compatible with the strain-specific $PrP^{Sc}$ conformation, and therefore, this PrPC molecule will be converted easily to $PrP^{Sc}$, while a $PrP^C$ molecule with a different primary sequence may not adopt any conformation that is structurally compatible with the conformation of a particular $PrP^{Sc}$ strain and will therefore not be converted at all by this prion strain. In this context the observed strain dependence of conversion efficiencies within the framework of identical primary sequences of $PrP^C$ and $PrP^{Sc}$ demonstrates that the conversion efficiency and, therefore, the

species barrier is not simply determined by sequence identity between $PrP^C$ and $PrP^{Sc}$. Rather, our findings support the view that it is determined by the structural compatibility of $PrP^C$ and $PrP^{Sc}$, which in turn is determined by certain important amino acid residues that define the repertoire of possible conformations that can be adopted by a certain $PrP^C$ primary sequence upon interaction with a certain $PrP^{Sc}$ conformation.

This finding is underlined by the results of heterologous conversion reactions with $PrP^C$ and $PrP^{Sc}$ having different PrP sequences. In reciprocal reactions the conversion efficiencies obtained can be quite distinct, although the combination of primary sequences has not changed. Fig. 3 shows that, although bank vole and hamster $PrP^C$ are quite similar in their primary sequence, hamster $PrP^C$ is converted only very inefficiently with $PrP^{Sc}$ derived from bank voles. In contrast, $PrP^C$ of the bank vole was efficiently converted with $PrP^{Sc}$ from hamster. That strikingly different conversion efficiencies can be obtained depending on which sequence is in its misfolded form has also been observed in earlier *in vitro* conversion reactions investigating the mouse/hamster species barrier (36, 37). In addition, alteration of mouse $PrP^C$ at residue 138 into a hamster-specific residue (I138M) prevented the formation of PrPres in scrapie-infected neuroblastoma cells (59), but *in vitro* conversion with mouse $PrP^C$ by hamster $PrP^{Sc}$ proved that identity at position 154/155 and not 138/139 is the major determinant for the conversion efficiency (60). This is reminiscent of what we have observed in conversion reactions with $PrP^C$/$PrP^{Sc}$ from hamster and bank vole. Hamster $PrP^C$ (Met-139 and Asn-155) has a low conversion efficiency with $PrP^{Sc}$ from bank voles (Ile-139 and Asn-155), but bank vole $PrP^C$ is easily converted with hamster $PrP^{Sc}$ (Figs. 3 and 5*B*).

If the observed differences in susceptibility of bank voles and mice to scrapie were determined by the different primary sequences of the prion protein, a higher conversion efficiency should be measured with bank vole $PrP^C$ and sheep scrapie using *in vitro* conversion reactions with purified $PrP^C$ and $PrP^{Sc}$. A correlation between *in vitro* and *in vivo* data has been shown in this study for instance with ME7, 263K, and BSE (Table 1 and Figs. 5 and 6*A*) and previously in other studies (36–38). As shown in Fig. 6*B* the conversion efficiency of bank vole $PrP^C$ upon incubation with purified sheep scrapie is lower than the conversion efficiency obtained with mouse $PrP^C$. A change of the bank vole PrP sequence toward the mouse sequence at residues 155 and 170 (vole N155Y/N170S) improved the conversion efficiency to a level comparable with the level obtained with mouse $PrP^C$. This discrepancy between *in vitro* and *in vivo* data with respect to sheep scrapie has been observed in reactions with the Italian scrapie case (Scrap Italy) as well as with the British scrapie case (Scrap UK). The differences between bank vole and mice with respect to the susceptibility to scrapie thus appear to be unrelated to the different primary sequences. Although a specific inhibitory effect of the chosen experimental reaction conditions on the conversion of bank vole $PrP^C$ cannot be excluded formally, there are no indications that such a trivial explanation of the observed unexpected discrepancies between *in vitro* and *in vivo* data may be valid. Not only have we observed the unexpectedly low conversion efficiency of vole PrP with two independent scrapie cases using two different guanidine hydrochloride concentrations, but also in six (or more) independent reactions per scrapie case. Moreover, the entire dataset, where single amino acid exchanges at positions 155 and 170 show intermediate conversion efficiencies and the double mutation at these positions results in a level of conversion comparable to that obtained with mouse $PrP^C$, suggests the validity of the observations. There are no signs of any particular inhibitory disturbances of these measurements in comparison to all the other measurements presented here, which in their vast majority support a good agreement of *in vitro* and *in vivo* data. Therefore, we propose an explanation of this discrepancy considering additional host factors that modulate the transmission of prions *in vivo*, at least in the case of scrapie infection of bank voles. Such unidentified host factors do not necessarily need to influence the conversion efficiency *in vivo* but could also account for the facilitation of any step in the prion propagation within the animal. Thus these factors could for instance pertain to an increased uptake of prions by certain cell types or to an increased efficiency of cell-to-cell transmission or intracellular transport of $PrP^{Sc}$ as well as to a reduced clearance of prions from cells or from the brain as a whole. Accordingly, our findings and the explanation by additional host factors does not provide any support for the previously postulated cofactor of conversion, protein X (18).

With respect to the different conversion efficiencies in reactions with the same $PrP^C$/$PrP^{Sc}$ combination discussed above we provide experimental evidence that the term "species barrier" is inappropriate. As suggested earlier (58, 61) the barriers to prion transmissibility should be referred to as "transmission barriers." These transmission barriers are determined by the primary sequence, the structural compatibility between the strain-specific $PrP^{Sc}$ conformation, and conformations adoptable by $PrP^C$ according to its primary sequence and, most probably in the case of scrapie transmission to bank voles, also by additional host factors, which in this case would facilitate scrapie propagation in bank voles.

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# Conversion Efficiency of Bank Vole Prion Protein *in Vitro* Is Determined by Residues 155 and 170, but Does Not Correlate with the High Susceptibility of Bank Voles to Sheep Scrapie *in Vivo*

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