Displaying the prion protein on virus-like particles: a novel tool towards vaccination

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von
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aus Köln

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Für meinen Ehemann Dennis Nikles

Alles Vergängliche
Ist nur ein Gleichnis;
Das Unzulängliche
Hier wird’s Ereignis;
Das Unbeschreibliche
Hier ist es getan;
Das Ewig-Weibliche
Zieht uns hinan.
(Johann Wolfgang von Goethe, 1831)

…und viel Probieren. Möglichste Genauigkeit und Ausdehnung der Versuche, möglichst wenig Schätzung. Viel arbeiten, wenig publizieren...
(Paul Ehrlich)

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Summary

I.1 German summary (short)

Prionenerkrankungen sind neurodegenerative Erkrankungen, zu denen Scrapie beim Schaf, Bovine Spongiforme Enzephalopathien beim Rind und beispielsweise die Creutzfeld-Jakob-Erkrankung beim Menschen zählen. Bei dem Erreger der Erkrankung handelt es sich um ein infektiöses Protein, das sogenannte Prionen Protein PrP^{Sc}. Die primäre Sequenz des PrP^{Sc} ist identisch zur Sequenz eines weit verbreiteten zellulären Proteins PrP^{c}. Es wird angenommen, dass die Vermehrung durch die Umfaltung des zellulären Gegenspielers PrP^{c} stattfindet. Als möglicher therapeutischer Ansatz gegen diese Erkrankungen wird eine Vakzinierung diskutiert. Eine krankheitsverlangsamende Wirkung durch passive Immunisierung mit spezifischen Antikörpern, die die zelluläre Form des Proteins PrP^{c} erkennen, wurde bereits im Tiermodell gezeigt. Die Erzeugung einer Immunantwort gegen PrP^{c} erfordert die Umgehung der wirtsspezifischen Toleranz gegen das Selbstantigen PrP^{c}. Ziel der vorliegenden Arbeit war die Entwicklung von Impfstoffkandidaten für eine aktive Immunisierung gegen Prionenerkrankungen.

Es wurden Vakzine auf der Basis von Virus-ähnlichen Partikeln (Retropartikel) entwickelt, die vom murinen Leukämievirus (MLV) bzw. vom humanen Immundefizienzvirus (HIV) abgeleitet wurden. Sowohl die Präsentation der zellulären Form PrP^{c} als auch der pathogenen Form PrP^{Sc} wurde adressiert.
Zur Präsentation auf Retropartikeln wurde PrP^{c} entweder an die Transmembrandomäne des Plättchen-abgeleiteten Wachstumsfaktor Rezeptors (PDGFR) oder an den N-terminus des retroviralen Hüllproteins (Env) von MLV fusioniert wurde. In beiden Fällen konnten die entsprechenden PrP^{D}- bzw. PrP^{E}-Retropartikel erfolgreich hergestellt werden, die des Weiteren mittels Immunfluoreszenzfärbung, Western Blot Analyse, Immunogold-Elektronen-mikroskopie sowie ELISA Methoden charakterisiert wurden. Sowohl für PrP^{D}- als auch für PrP^{E}-Retropartikel konnte gezeigt werden, dass PrP^{c} inkorporiert und auf der Oberfläche der viralen Partikel präsentiert wurde, wenn N-terminal trunkierte Formen von PrP^{c} verwendet wurden. Die für PrP^{c} typischen Glykosylierungsmuster konnten durch spezifischen Glykosidase-Verdau nachgewiesen werden. Die präsentierten PrP^{c}-Varianten wurden unter nativen Bedingungen von PrP-spezifischen Antikörpern erkannt. Im Gegensatz zu den N-terminal trunkierten Varianten wurde das komplette PrP^{c} nicht in Retropartikel inkorporiert. Mittels Elektronen-Mikroskopie konnte bei der

Inkorporation von PrP^{c}-Varianten keine Veränderung der charakteristischen retroviralen Morphologie der Partikel festgestellt werden.
MLV-abgeleitete PrP^{D}-Retropartikel wurden anschließend erfolgreich zur Immunisierung von Mäusen eingesetzt. Dabei wurden, im Gegensatz zur Immunisierung mit Vakzinen, die auf bakteriell hergestelltem PrP^{c} basierten, spezifische Antikörpertiter erhalten.
Versuche zur Präsentation der pathogenen Form wurden zum einen durch Konvertieren der Proteinase K-sensitiven in die Proteinase K-resistente Form des PrP auf der Oberfläche der PrP^{D}-Retropartikel durchgeführt. Der Nachweis mittels Proteinase K-Verdau wurde hier speziell für virale Partikel adaptiert, die Umwandlung der PrP^{D}-Retropartikel in Proteinase K-resistent Formen konnte jedoch nicht beobachtet werden. Zum anderen wurde ein PrP^{c}-codierendes replikationskompetentes MLV-abgeleitetes Retrovirus erzeugt. Dieses konnte über sechs Passagen genetisch stabil auf murinen Fibroblasten propagiert werden, zeigte allerdings nach Infektion einer Prionen-infizierten Zelllinie, die PrP^{Sc} stetig propagierte, keine Replikation.
Neben virus-ähnlichen Partikeln standen damit MLV-abgeleitete replikationskompetente Viren zur Verfügung, die PrP^{c} stabil im viralen Genom enthalten und auf ihrem Hüllprotein präsentieren.

Basierend auf Hinweisen aus der Literatur war die Inkorporation von PrP^{c} in Virus-ähnliche Partikel zu erwarten, da es sich um ein Zelloberflächenprotein handelt. Der fehlende Einbau des kompletten PrP in retrovirale Partikel war daher unerwartet. Anders als für die trunkierten PrP^{c}-Varianten war sowohl die Fusion mit PDGFR als auch die N-terminale Env-Fusion für PrP^{c} inhibiert. Das komplette PrP^{c} unterliegt im Vergleich zu trunkierten Formen einem erhöhten Zelloberflächen-Umsatz und weist eine geringere Halbwertszeit auf. Diese beiden Faktoren können einen Beitrag zur verminderten Inkorporation in retrovirale Partikel leisten.
Die in der vorliegenden Arbeit beschriebenen PrP^{D}-Retropartikel stellen das erste erfolgreiche System zur Erzeugung PrP-spezifischer Immunantworten gegen die native Zelloberflächen-Form des PrP^{c} dar. Ein möglicher Mechanismus zur Erklärung basiert auf der Induktion der T-Zellhilfe sowie eines Beitrags des angeborenen Immunsystems. Eine weitere Verbesserung der Immunantwort sollte sich mit den in dieser Arbeit hergestellten MLV- und HIV-abgeleiteten Partikeln, die zusätzlich mit PrP-codierenden Sequenzen ausgestattet wurden sowie mit den replikationskompetenten MLV-Varianten verwirklichen lassen.

I.2 German summary (long)

Prionenerkrankungen sind neurodegenerative Erkrankungen, zu denen Scrapie beim Schaf, Bovine Spongiforme Enzephalopathien beim Rind und beispielsweise die Creutzfeld-Jakob-Erkrankung beim Menschen zählen. Bei dem Erreger der Erkrankung handelt es sich um ein infektiöses Protein, das sogenannte Prionen Protein PrP^{Sc}. Die primäre Sequenz von PrP^{Sc} ist identisch zur Sequenz des zellulären Proteins PrP^{c}. Dieses zelluläre Protein, das in allen Säugetieren stark konserviert ist, zeigt hohe Expressionsraten im zentralen Nervensystem sowie in den Zellen des Immunsystems. Anhand von Experimenten mit PrP^{c}-knock-out Mäusen konnte die Unerlässlichkeit des PrP^{c} für die Entwicklung der Prionenerkrankungen abgeleitet werden. Es wird daher angenommen, dass die Vermehrung durch die Umfaltung des zellulären Gegenspielers PrP^{c} stattfindet.
Als möglicher therapeutischer Ansatz gegen diese Erkrankungen wird eine Vakzinierung diskutiert. Eine krankheitsverlangsamende Wirkung durch passive Immunisierung mit spezifischen Antikörpern, die die zelluläre Form des Proteins PrP^{c} erkennen, wurde bereits im Tiermodell gezeigt. Die Erzeugung einer Immunantwort gegen PrP^{c} erfordert die Umgehung der wirtsspezifischen Toleranz gegen das Selbstantigen PrP^{c}. Das Verständnis der Toleranzmechanismen des Immunsystems ist die Grundvoraussetzung zur erfolgreichen Immunisierung gegen PrP^{c}. Diesbezüglich konnte gezeigt werden, dass die relativ geringe B-Zell-basierte Toleranz gegen PrP^{c} prinzipiell die Entstehung spezifischer Antikörper ermöglicht. Allerdings muss ein zu entwickelndes Vakzin eine hohe T-Zell-Toleranz gegen PrP^{c} überwinden.
Ziel der vorliegenden Arbeit war die Entwicklung von Impfstoffkandidaten für eine aktive Immunisierung gegen Prionenerkrankungen.

Es wurden Vakzine auf der Basis von Virus-ähnlichen Partikeln (Retropartikel) entwickelt, die vom murinen Leukämievirus (MLV) bzw. vom humanen Immundefizienzvirus (HIV) abgeleitet wurden. Sowohl die Präsentation der zellulären Form PrP^{c} als auch der pathogenen Form PrP^{Sc} wurde adressiert.
Zur Präsentation auf Retropartikeln wurde PrP^{c} entweder an die Transmembrandomäne des Plättchen-abgeleiteten Wachstumsfaktor Rezeptors (PDGFR) oder an den N-terminus des retroviralen Hüllproteins (Env) von MLV fusioniert. In beiden Fällen konnten die entsprechenden PrP^{D}- (PrP-PDGFR) bzw. PrP^{E}-Retropartikel (PrP-Env) erfolgreich hergestellt werden, die des Weiteren

mittels Immunfluoreszenzfärbung, Western Blot Analyse, Immunogold-Elektronen-mikroskopie sowie ELISA Methoden charakterisiert wurden.
Sowohl für PrP^{D}- als auch für PrP^{E}-Retropartikel konnte gezeigt werden, dass PrP^{c} inkorporiert und auf der Oberfläche der viralen Partikel präsentiert wurde, wenn N-terminal trunkierte Formen von PrP^{c} verwendet wurden. PrP^{c} wies nach Expression ein typisches Glykosylierungsmuster bestehend aus un-, mono- und diglykosyliertem Protein auf. Dieses typische Muster konnte für beide Präsentations-Systeme durch spezifischen Glykosidase-Verdau nachgewiesen werden. Des Weiteren wurden die präsentierten PrP^{c}-Varianten in Immunfluoreszenz-Färbungen unter nativen Bedingungen von PrP-spezifischen Antikörpern erkannt.
Im Gegensatz zu den N-terminal trunkierten Varianten wurde das komplette PrP^{c} nicht in Retropartikel inkorporiert. Sowohl die Fusion zum PDGFR als auch die N-terminale Präsentation auf dem viralen Env Protein führten zur Inhibition der PrP^{c}-Inkorporation in MLV- und HIV-abgeleitete Partikel. Mittels Western Blot Analyse konnte ein Einfluss bereits auf die Expression der Fusionsproteine in den Zielzellen gezeigt werden, die für komplettes PrP^{c} an sich stark reduziert war und in den aus den Zielzellen entstehenden Partikeln nicht nachgewiesen werden konnten.
Der Vergleich der beiden verschiedenen Präsentationssysteme zeigte unterschiedliche Inkorporationsraten. Dabei konnte für PrP^{D}-Retropartikel, die von MLV abgeleitet waren und N-terminal trunkierte Formen des PrP^{c} präsentierten, die höchste Inkorporationsrate erhalten werden. PrP^{E}-Retropartikel zeigten deutlich reduzierte Einbau-Raten. Generell erfolgte der Einbau in HIV-abgeleitete Partikel weniger effizient als in MLV-Partikel.
Mittels Elektronen-Mikroskopie konnte nach der Inkorporation der PrP^{c}-Varianten keine Veränderung der charakteristischen retroviralen Morphologie der Partikel festgestellt werden. Sowohl PrP^{D}- als auch PrP^{E}-Retropartikel zeigten nach Inkubation mit einem PrP-bindenden monoklonalen Antikörper spezifische Markierungen an der Virushülle. Neben den an ihrer Morphologie erkennbaren Viruspartikel enthielten die Präparationen Vesikel-ähnliche Strukturen. Die in Form und Größe variablen Vesikel enthielten ebenfalls die PrP^{D}- bzw. PrP^{E}-Proteine. Die Vesiklebildung war für PrP^{D}-Retropartikel verstärkt, für PrP^{E}-Retropartikel jedoch nur in geringem Maß zu beobachten.

MLV-abgeleitete PrP^D-Retropartikel, die aufgrund ihrer besonders hohen Inkorporationsrate ausgewählt wurden, wurden anschließend in einer Kooperation mit U.Kalinke und P.Bach (Abt. Immunologie, Paul-Ehrlich-Institut) erfolgreich zur Immunisierung von Mäusen eingesetzt. Dabei wurden, im Gegensatz zur Immunisierung mit Vakzinen, die auf bakteriell hergestelltem PrP^c basierten, spezifische Antikörpertiter erhalten. Die Bindung dieser erhaltenen Antikörper an die sogenannte native Form des PrP, die mittels eines speziellen Bindungsassays auf murinen T-Zellen gezeigt werden konnte, stellte dabei ein wichtiges Kriterium zur Bestimmung der Qualität der erhaltenen Seren dar. Aktuelle Studien zur Immunisierung gegen Prionenerkrankungen zeigten, dass nur derartige Antikörper Protektion zu vermitteln vermögen.

Versuche zur Präsentation der pathogenen Form wurden zum einen durch Konvertieren der Proteinase K-sensitiven in die Proteinase K-resistente Form des PrP auf der Oberfläche der PrP^D-Retropartikel durchgeführt. Der Nachweis mittels Proteinase K-Verdau wurde hier speziell für virale Partikel adaptiert, die Umwandlung der PrP^D-Retropartikel in die Proteinase K-resistente Form konnte jedoch nicht beobachtet werden. Zum anderen wurde ein PrP^c-codierendes replikations-kompetentes ecotropes MLV erzeugt. Es konnte erneut die Beobachtung gemacht werden, dass nur N-terminal trunkierte PrP^c-Varianten in das Virus inkorporiert wurden. Das komplette PrP^c inhibierte die Replikation des Virus. Wurden jedoch trunkierte Varianten eingesetzt, konnte die Virus-Variante über sechs Passagen genetisch stabil auf murinen Fibroblasten propagiert werden. Die genetische Stabilität wurde sowohl anhand von Immunfärbungen der infizierten Zellen als auch anhand der Präparation des Virusgenoms und sich anschließender spezifischer Amplifikation der PrP-codierenden Sequenzen durchgeführt. Des Weiteren wurde der Tropismus der replikations-kompetenten Virus-Variante auf verschiedenen murinen und humanen Zielzellen untersucht. Es wurde ein verstärkter Zelleintritt der PrP^c-tragenden Variante auf humanen T-Zellen beobachtet, die möglicherweise auf das Vorhandensein des PrP zurückzuführen war. Eine erweiterung des Virudtropismus auf humane T-Zellen Die ecotrope Virus-Variante konnte jedoch nicht in den humanen T-Zellen konnte jedoch nicht festgestellt werden.

Nach Infektion einer Prionen-infizierten Zelllinie, die PrP^{Sc} stetig propagierte, konnte, im Gegensatz zur schnellen Ausbreitung in murinen Fibroblasten, keine Replikation beobachtet werden. Diesbezüglich wurde die Integration der Variante

ins Zellgenom und das Vorhandensein viraler RNA nachgewiesen. Allerdings konnte keine Expression des viralen env-Gens gezeigt werden.
Neben virus-ähnlichen Partikeln standen damit MLV-abgeleitete replikationskompetente Viren zur Verfügung, die PrP^{c} stabil im viralen Genom enthalten und auf ihrem Hüllprotein präsentieren. Diese ecotropen Virus-Varianten replizierten auf murinen Fibroblasten, jedoch nicht auf den bisher getesteten scrapie-infizierten Zelllinien.

Der Einbau zellulärer Membranproteine in retrovirale Partikel ist ein bekanntes Phänomen. Die Inkorporation der PrP^{D}-Proteine in Virus-ähnliche Partikel war insofern zu erwarten. Der fehlende Einbau des kompletten PrP^{C} in retrovirale Partikel dagegen war eher unerwartet. Anders als für die trunkierten PrP^{c}-Varianten war der Einbau sowohl der PrP^{c}-Fusion mit PDGFR als auch der Fusion mit Env für das komplette PrP^{c} inhibiert. Das komplette PrP^{c} unterliegt im Vergleich zu trunkierten Formen einem erhöhten Zelloberflächen-Umsatz und weist eine geringere Halbwertszeit auf. Diese beiden Faktoren könnten den beobachteten Effekt erklären.
Ein weiterer Aspekt im Zusammenhang mit der Wechselwirkung von PrP^{c} mit der Enstehung retroviraler Partikel, war die Beeinflussung der viralen Gen-Expression durch PrP^{c}. Aktuelle Publikationen zeigen, dass PrP^{c} die Expression einiger HIV-Gene inhibieren kann. Dies betrifft das virale Hüllprotein, akzessorische Proteine sowie in geringem Maße auch das virale Kapsid-Protein. Ein derartiger Einfluss von PrP^{c} kann ebenfalls zur Erklärung der in dieser Arbeit beobachteten Inhibition der MLV-Replikation und Partikelbildung herangezogen werden. Sobald das komplette PrP^{c} in Zielzellen exprimiert wurde, ob als endogenes, zelluläres PrP oder als Fusionsprotein im PrP^{D}- bzw. PrP^{E}-System, war die Enstehung viraler Partikel und vor allem die Produktion des viralen Hüllproteins stark reduziert. Einzig die Verwendung der N-terminal trunkierten PrP^{c}-Varianten in Zielzellen, die nur geringe Expression des PrP^{c} zeigen, führte zur erfolgreichen Produktion von Viruspartikeln. Diese Beobachtungen deuten auf eine Interferenz des N-terminus von PrP^{c} mit der viralen Genexpression oder der Entstehung und Abschnürung viraler Partikel hin.
Die N-terminale Präsentation Virusfremder Polypeptide kann generell unterschiedliche Auswirkungen auf den viralen Tropismus haben. Nur in Ausnahmefällen wurde eine Tropismuserweiterung (Infektion humaner Zellen

durch ecotropes MLV) beobachtet, üblicherweise kommt es zu einer Tropismus verengung. Letztere kann durch Bindung der Viruspartikel an den zellulären Rezeptor des präsentierten Polypeptids, gefolgt von Endozytose geschehen. Alternativ kann es auch zu einer sterischen Beeinträchtigung der Erkennung des viralen Rezeptors kommen, wenn trimere Polypeptode präsentiert werden. Im Falle des trunkierten PrP^c wurde auf PrP^c-negativen Zellen keine Blockierung des viralen Zelleintritts beobachtet, ein genereller Blockierungsmechanismus durch PrP^c konnte somit ausgeschlossen werden.

Die in der vorliegenden Arbeit beschriebenen PrP^D-Retropartikel stellen das erste erfolgreiche System zur Erzeugung PrP-spezifischer Immunantworten gegen die native Zelloberflächen-Form des PrP^c dar. Ein möglicher Mechanismus zur Erklärung basiert auf der Induktion der T-Zellhilfe. Virus-ähnliche Partikel, die von HIV abgeleitet waren, wurden bereits zur Immunisierung gegen HIV und andere Virus-basierte Erkrankungen eingesetzt. Diese Antigene wiesen stets eine Partikel-abhängige T-Zellantwort der CD4-positiven T-Helferzellen auf. Obwohl dies in der vorliegenden Arbeit nicht getestet wurde, könnte dieser Mechanismus auch bei der mit PrP^D-Retropartikeln ausgeführten Immunisierung zur Erzeugung PrP-spezifischer IgG-Antikörper beigetragen haben. Des Weiteren kommt hierfür auch ein Beitrag des angeborenen Immunsystems in Betracht. Die Stimulierung von Antigen-präsentierenden Zellen kann über sogenannte Toll-like Rezeptoren einen Einfluss auf die T-Zellantwort des Organismus haben. Viral verpackte RNA kann dabei extrazellulär an die Rezeptoren binden und eine verstärkte Immunreaktion bewirken.

Eine weitere Verbesserung der Immunantwort sollte sich aus diesem Grund auch mit den in dieser Arbeit hergestellten MLV- und HIV-abgeleiteten Partikeln, die zusätzlich mit PrP-codierenden Sequenzen ausgestattet wurden, sowie mit den replikationskompetenten PrP-MLV-Varianten verwirklichen lassen.

I.3 English summary

Prion diseases, also called transmissible spongiform encephalopathies, are a group of fatal neurodegenerative conditions that affect humans and a wide variety of animals. To date there is no therapeutic or prophylactic approach against prion diseases available. The causative infectious agent is the prion, also termed PrP^{Sc}, which is a pathological conformer of a cellular protein named prion protein PrP^{c}. Prions are thought to multiply upon conversion of PrP^{c} to PrP^{Sc} in a self-propagating manner. Immunotherapeutic strategies directed against PrP^{c} represent a possible approach in preventing or curing prion diseases. Accordingly, it was already shown in animal models, that passive immunization delays the onset of prion diseases. The present thesis aimed at the development of a candidate vaccine towards the active immunization against prion diseases, an immune response, which has to be accompanied by the circumvention of host tolerance to the self-antigen PrP^{c}.

The vaccine development was approached using virus-like particles (retroparticles) derived from either the murine leukemia (MLV) or the human immunodeficiency virus (HIV). The display of PrP on the surface of such particles was addressed for both the cellular and the pathogenic form of PrP.

The display of PrP^{c} was achieved by either fusion to the transmembrane domain of the platelet derived growth factor receptor (PDGFR) or to the N-terminal part of the viral envelope protein (Env). In both cases, the corresponding PrP^{D}- and PrP^{E}-retroparticles were successfully produced and analyzed via immune fluorescence, Western Blot analysis, immunogold electron microscopy as well as by ELISA methods. Both, PrP^{D}- and PrP^{E}-retroparticles showed effective incorporation of N-terminally truncated forms of PrP^{c} but not for the complete protein. PrP^{c} at this revealed the typical glycosylation pattern, which was specifically removed by a glycosidase enzyme. Upon display of PrP^{c} on retroparticles the protein remained detectable by PrP-specific antibodies under native conditions. Electron microscopy analysis of PrP^{c}-variants revealed no alteration of the characteristic retroviral morphology of the generated particles.

MLV-derived PrP^{D}-retroparticles were successfully used in immunization studies. Contrary to approaches using bacterially expressed PrP^{c}, the immunization of mice resulted in a specific antibody response.

The display of the pathogenic isoform was aimed by two different strategies. The first one was directed at the conversion of the proteinase K (PK) sensitive from of PrP on

the surface of PrP^{D}-retroparticles into the PK resistant form. Albeit specific adaption of the PK digestion assay detecting resistant PrP, no PrP conversion was observed for PrP^{D}-retroparticles. The second approach utilized a replication competent variant of the ecotropic MLV displaying PrP^{c} on the viral Env protein. This MLV variant was stable in cell culture for six passages but did not replicate on scrapie-infected, PrP^{Sc}-propagating neuroblastoma cells.
Thus, besides PrP^{c}-displaying virus-like particles a replication competent MLV variant was obtained, which stably incorporated PrP^{c} at the N-terminus of the viral Env protein.

The incorporation of the cell-surface located PrP^{c} into particles was expected from previously obtained data on protein display in the context of retrovirus-derived particles. Thus, the lack of incorporation observed for the complete PrP^{c} sequence was rather unexpected and was found to be inhibited at both, fusion to PDGFR and the viral Env. In contrast to N-terminally truncated PrP^{c}, the complete PrP^{c} was shown to exhibit increased cell surface internalization rates and half-life times eventually contributing to the observed results.
The PrP-vaccination approach described in this work represents the first successful system inducing PrP-specific antibody responses against the prion protein in wt mice. Explanations at this are based on the induction of specific T cell help or effects of the innate immunity, respectively. MLV-and HIV-derived particles bearing the PrP-coding sequence or being replication competent variants generated during this thesis might help to further improve the PrP-specific immune response.

II

Introduction

II.1 Prion diseases

II.1.1 Prions

Prions are the causative agent for certain neurodegenerative diseases named prion diseases or transmissible spongiform encephalopathies (TSE) (Prusiner, S B 1982), e.g. bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans. The term prion is identical with PrP^{Sc}, which is a pathological conformer of a cellular protein named cellular prion protein PrP^{c} encoded by the Prnp gene (Oesch, B et al. 1985). This protein is widely expressed in mammals, high levels are particularly present in the central nervous system and in cells of the immune system (Ford, M J et al. 2002), and it is essential for the replication of prions and disease development (Bueler, H et al. 1993), (Prusiner, S B et al. 1993).

The precise nature of the transmissible agent, later named prion, has been long debated and was earlier believed to consist of a protein-encapsidated nucleic acid (Weissmann, C 2004). This VIRINO hypothesis asserts that the infectious agent contains an agent-specific nucleic acid enveloped in a host-specified protein explaining the lack of an immune response in disease development. However, although intense efforts have been made, no nucleic acid, long enough to encode even a small protein, could be identified in infectious fractions. This and several more findings finally have provided support for an updated version of the "protein-only" hypothesis (Griffith, J S 1967). A comparison of the different hypothesis is illustrated in **Figure 1** (derived from (Weissmann, C 2004)).

The host protein PrP^{c}, which typically occurs in un-, mono- and diglycosylated forms, is attached to the outer surface plasma membrane by a glycosyl phosphatidyl inositol (GPI) anchor and can be released from the cell surface by cleavage with phosphatidyl inositol specific phospholipase C (PIPLC) (reviewed in (Weissmann, C 2004)). As an example, the murine PrP gene structure is described in Figure 2, including characteristic regions of the protein.

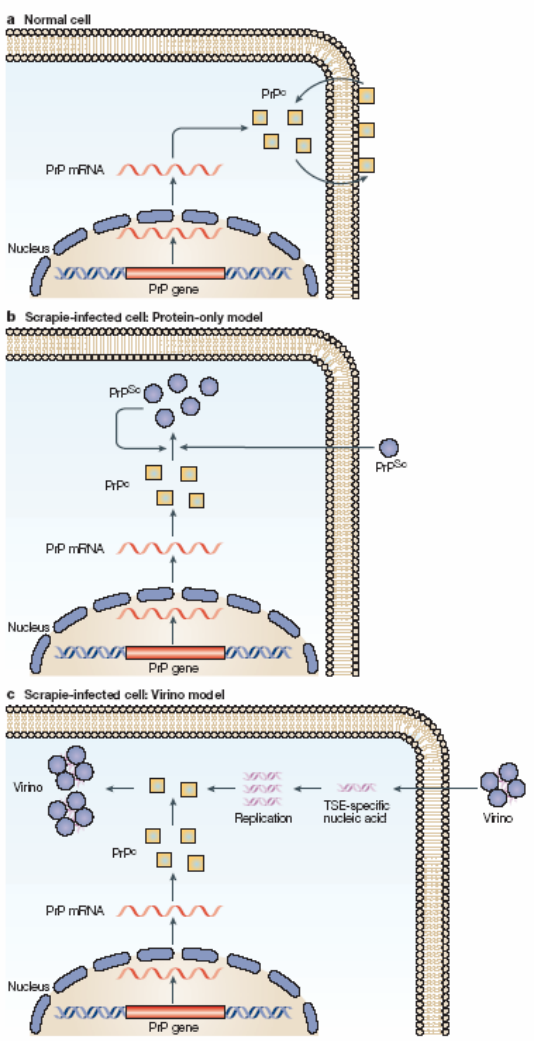


Figure 1 **Models for the propagation of the TSE agent (prion). a** in a normal cell, PrP^{c} (yellow square) is synthesized, transported to the cell surface and eventually internalized. **b** The protein-only model postulates that the infectious entity, the prion, is congruent with an isoform of PrP, here designated as PrP^{Sc} (blue circle). Exogenous PrP^{Sc} causes catalytic conversion of PrP^{c} to PrP^{Sc}, either at the cell surface or after internalization. **c** The virino model postulates that the infectious agent consists of a TSE-specific nucleic acid associated with or packaged in PrP^{Sc}. The hypothetical nucleic acid is replicated in the cell and associates with PrP^{c}, which is thereby converted to PrP^{Sc} (Weissmann, C 2004).

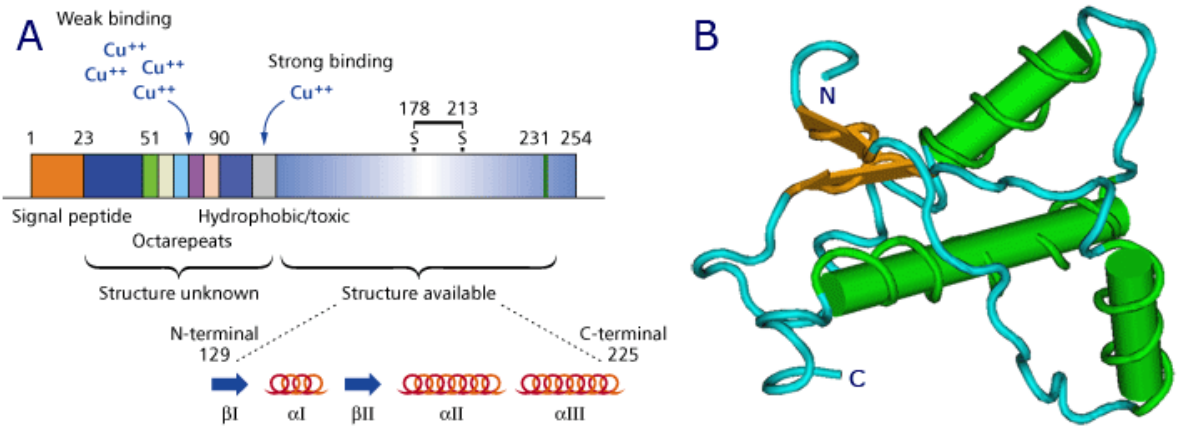


Figure 2 **Anatomy and structure of the prion protein. A)** The cellular prion protein consists of a flexible N-terminal and a globular C-terminal domain. The C-terminal structure containing mainly α-helical arrangements and two small β-sheet secondary elements has been determined. The N-terminus possesses the copper-binding octarepeats; the protein has a signal peptide for membrane targeting and is post-translationally modified by addition of two glycosylations as well as a glycosyl phosphatidyl inositol (GPI) membrane anchor (Jones, I). **B)** The nuclear magnetic resonance (NMR) structure of mouse PrP (121-231) contains a two-stranded antiparallel beta-sheet and three alpha-helices (Riek, R et al. 1996).

The protein is highly susceptible to proteinase K (PK) digestion, whereas the term PrP^{Sc} was originally defined as a form of PrP that is largely resistant to PK digestion, although it is now also used for the designation of the infectious isoform of PrP. The digestion of PrP^{Sc} by PK leads to a characteristic electrophoretic mobility shift, caused by cleavage at residue 87-91 (depending on the prion strain), which results in the so-called PrP27-30. Notably, PK resistance is a relative concept, depending on the concentration of PK, on the PK to protein ratio as well as on the prion strain. Also forms of PrP, which are more resistant to PK but nonetheless remain non-infectious, can exist (Weissmann, C 2004).

The conversion of PrP^{c} to PrP^{Sc} has been proposed to be a self-propagating conversion, for which two models can be assumed as shown in Figure 3 (Weissmann, C 2004).

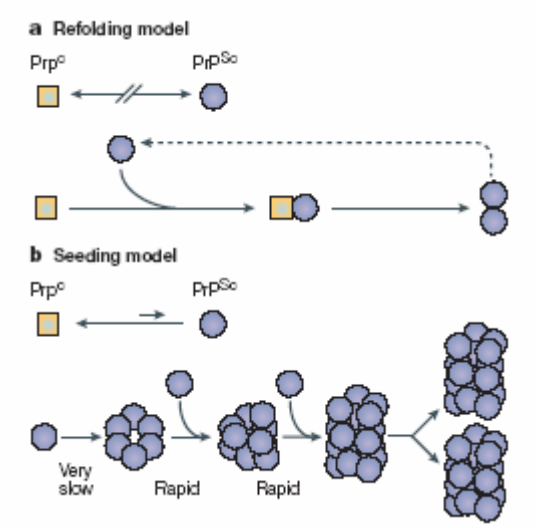


Figure 3 **Models for the conversion of PrPc to PrPSc. a** The refolding model. The conformational change is kinetically controlled, a high activation energy barrier preventing spontaneous conversion at detectable rates. Interaction with exogenously introduced PrP^{Sc} (blue circle) causes PrP^{c} (yellow square) to undergo an induced conformational change to yield PrP^{Sc}. This reaction could be facilitated by an enzyme or chaperone. In the case of certain mutations in PrP^{c}, spontaneous conversion to PrP^{Sc} can occur as a rare event, explaining why familial Creutzfeldt-Jakob disease (CJD) or Gerstmann-Sträussler-Scheinker syndrome (GSS) arise spontaneously, albeit late in life. Sporadic CJD (sCJD) might arise when an extremely rare event (occurring in about one in a million individuals per year) leads to spontaneous conversion of PrP^{c} to PrP^{Sc}. **b** The seeding model. PrP^{c} (yellow square) and PrP^{Sc} (or a PrP^{Sc}-like molecule, shown as blue circle) are in equilibrium, with PrP^{c} strongly favoured. PrP^{Sc} is only stabilized when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. Seed formation is rare; however, once a seed is present, monomer addition ensues rapidly. To explain exponential conversion rates, aggregates must be continuously fragmented, generating increasing surfaces for accretion (derived from (Weissmann, C 2004)).

The seeding or nucleation model has found convincing experimental support in the case of yeast prions, and recently also with mammalian prions (reviewed in (Weissmann, C 2004)).

A few cell lines, including the murine neuroblastoma cell line N2a, can propagate scrapie prions such as the so-called RML murine scrapie prion strain. It is not clear so far, which parameters and properties are rendering cells susceptible to prion infection; differences in the sequence of PrP^{Sc} and PrP^{c} as well as some unknown, eventually epigenic, mechanisms seem to play a role. The capacity of such cells to replicate prions can be described by the recently proposed "dynamic susceptibility" model ((Weissmann, C 2004)), in which the susceptibility depends on the ratio of prion synthesis to degradation and can therefore even lead to elimination of infection (in case the degradation rate is higher than the formation rate).

Under "natural" circumstances, e.g. in sheep scrapie, the prion agent is likely to be transmitted by ingestion of contaminated foodstuff, as it has been shown to be the case in cow-to-human transmissions of the BSE strain in the end of the 21th century in Great Britain (Taylor, D M 2003).

The spread of prions entering an organism via the food chain, has been shown to appear by the infection of immune and lymphoreticular cells and subsequent infection of the peripheral and the central nervous system, a process termed "prion neuroinvasion" as reviewed in (Aguzzi, A et al. 2003). This route of infection is the basis of therapeutic approaches relying on antibody responses to the cellular PrP, as described in the following chapter, which seem to interfere with prion replication already but maybe not exclusively in early states of infection before neuroinvasion takes place.

II.1.2 PrP-vaccination

Humoral immune responses to the prion protein can antagonize prion infection, even when they do not selectively target PrP^{Sc} but PrP^{c} only (Heppner, F L and Aguzzi, A 2004). Chronically scrapie-infected cultures of N2a cells (ScN2a) can rapidly be cured when exposed to a monoclonal PrP antibody or to phospholipase C (PIPLC), pointing to the possibility that depletion of PrP^{c} can interrupt the propagation of PrP^{Sc} (Enari, M et al. 2001). Furthermore, anti-PrP antisera reduce the titer of infectious hamster brain homogenates as it has been shown in an in vitro experiment (Gabizon, R et al. 1988). The formation of PrP^{Sc} is abolished by anti-PrP antibodies in a cell-free system (Horiuchi, M and Caughey, B 1999). The use of antibody antigen-binding fragments (Fabs) in PrP^{Sc}-infected neuroblastoma cells abolished prion replication and reduced the level of pre-existing PrP^{Sc} in a dose-dependent manner (Peretz, D et al. 2001).

Inhibitory effects on prion replication could also be demonstrated in mouse models. In a murine scrapie model, administration of monoclonal antibodies at the point of near maximal accumulation of PrP^{Sc} in the spleen lead to the delay of prion disease onset for more than 300 days when the treatment was continued (White, A R et al. 2003).

A study in which an anti-PrP µ-chain was expressed in Prnp$^{0/0}$ mice followed by introduction of the Prnp^{+} allele did not show suppressed anti-PrP titers, although PrP expression in spleen and brain was present. This finding indicated that PrP, when expressed at physiological levels, can be coexpressed with PrP-specific antibodies without inducing autoimmune disease or haematological disorders

(Heppner, F L et al. 2001). However, protection is restricted to intraperitoneal prion infection and is inefficient when prions are administered intracerebrally (White, A R, Enever, P et al. 2003; Polymenidou, M et al. 2004), most likely due to limited influx of immunglobulins into the CNS.

Various studies including immunization experiments with PrP-derived peptides (Hanan, E et al. 2001; Souan, L et al. 2001; Schwarz, A et al. 2003; Rosset, M B et al. 2004), DNA-based vaccines (Krasemann, S et al. 1996), bacterially expressed recombinant murine PrP (Koller, M F et al. 2002; Sigurdsson, E M et al. 2002; Gilch, S et al. 2003; Polymenidou, M, Heppner, F L et al. 2004) or PrP-PrP polyproteins (Gilch, S, Wopfner, F et al. 2003; Polymenidou, M, Heppner, F L et al. 2004) resulted in low antibody titers, when testet against the corresponding PrP antigens. Different adjuvants including e.g. Freund´s adjuvants and CpG-oligonucleotides were used as summarized in Table 1 (Heppner, F L and Aguzzi, A 2004).

Previous immunization studies could show, that particular peptides seem to be immunogenic in mice, resulting in reduction of the PrP^{Sc} level compared to non-PrP derived peptide immunization, when an ScN2a tumour model was used (Souan, L, Tal, Y et al. 2001). Also, vaccination with recombinant mouse prion protein with complete Freund´s adjuvant could delay the onset of prion disease by a few days (10-15 d) in mice after intraperitoneal exposure to a mouse-adapted prion strain (Sigurdsson, E M, Brown, D R et al. 2002). In the latter, a correlation between the anti-prion protein titers and the incubation period of disease onset was observed but notably, control groups did not receive CFA.

On the contrary, other studies failed to induce an immune response in $Prnp^{+/+}$ mice (Krasemann, S, Groschup, M et al. 1996).

Overall, in all of the above listed studies, anti-PrP titers were rather low, never directed against the native cell surface form of PrP^{c} and the prionostatic *in vivo* effect was only moderate. More importantly, all of these studies were challenged by the observation, that the use of certain adjuvants, e.g. Freund´s adjuvant, is questionable, because an antiprion activity has been shown for the adjuvant itself (Tal, Y et al. 2003).

Table 1 PrP immunization studies in wild-type mice

immunogen	cross-linked	adjuvant	response ag. antigen	anti-native PrP^c response	antiprion *in vivo* effect	Ref.
PrP^{REC}	DnaK	CFA	yes	n.d.	n.d.	(Koller, M F, Grau, T et al. 2002)
PrP^{REC}	____	CFA	yes	n.d.	Yes	(Sigurdsson, E M, Brown, D R et al. 2002)
PrP^{REC}/PrP-PrP	____	CFA	yes	no	No	(Polymenidou, M, Heppner, F L et al. 2004)
PrP^{REC}/PrP-PrP	____	CFA/ TiterMax/ CpG	yes	n.d.	n.d.	(Gilch, S, Wopfner, F et al. 2003)
PrP peptides	KLH	CFA	yes	n.d.	n.d.	(Hanan, E, Priola, S A et al. 2001)
PrP peptides	____	CFA	yes	n.d.	Yes	(Souan, L, Tal, Y et al. 2001)
PrP peptides	KLH	Montanide IMS-1313	yes	n.d.	Yes	(Schwarz, A, Kratke, O et al. 2003)
PrP peptides	____	CpG	yes	n.d.	n.d.	(Rosset, M B, Ballerini, C et al. 2004)
PrP α–helix poly-peptides	____	CFA/ bacterio-phage	yes	n.d.	n.d.	(Arbel, M et al. 2003)
Prnp genes	____	____	no	n.d.	n.d.	(Krasemann, S, Groschup, M et al. 1996)

n.d.: not described

DnaK heat shock protein (Hsp)70 homolog, ***KLH*** keyhole limpet hemocyanine, ***CFA*** complete Freund´s adjuvant

Recently, a correlation between the recognition of native, cell-surface PrP and the effect on prion replication has been proposed (Heppner, F L and Aguzzi, A 2004), underlined by the finding that antibodies, which exclusively bind to

recombinant PrP, are not sufficient to confer prionostatic effects in wild-type mice upon peripheral prion challenge (Polymenidou, M, Heppner, F L et al. 2004). This argument would also explain the modest effects on prion pathogenesis of the above listed studies (Souan, L, Tal, Y et al. 2001; Sigurdsson, E M, Brown, D R et al. 2002; Schwarz, A, Kratke, O et al. 2003), in which binding to native PrP^c has not been examined.

II.2 Virus-like particles

Historically, the field of vaccinology has experienced sustained success in developing prophylactic vaccines against a variety of infectious diseases. Besides attenuated and inactivated microorganisms, also toxoids and protein subunits have been used. Within the last decades, several vaccine components, suitable for the induction of strong cellular immunity have been developed, e.g. attenuated viruses, recombinant viral vectors and DNA based vaccines, as reviewed in (Deml, L et al. 2005). These vaccine components have their advantages in prolonged antigen production within the host cell and immonostimulatory effects of the delivery system. Especially for virus-like particles (VLPs), that include all immunologically relevant structures of viruses but lack unwanted regulatory proteins and genetic information, several groups have shown induction of more effective humoral and cellular immune responses as compared to subunit vaccines, as summarized in (Deml, L, Speth, C et al. 2005).

To date, numerous types of VLPs have been produced utilizing the ability of more than 30 different viruses to self-assemble into highly organized particulate structures (Deml, L, Speth, C et al. 2005). Some of the most prominent among them are hepatitis B virus cores, displaying i.e. influenza, human immunodeficiency, polio- and hantavirus derived immunodominant epitopes (reviewed in (Pumpens, P and Grens, E 2001)), as well as human immunodeficiency virus (HIV) Gag proteins, used as carrier to present foreign polypeptides and immunological epitopes (Deml, L, Speth, C et al. 2005). Detailed knowledge of spatial structures and molecular mechanism of assembly and morphogenesis also led to the use of phage particles, e.g. the RNA phage Qβ (Kozlovska, T M et al. 1996), as display scaffold.

Retrovirus-based VLPs (in this case derived from HIV) have been studied in detail concerning their immunological properties (Deml, L, Speth, C et al. 2005).

Vectors displaying foreign polypeptides were classified into two groups: type I and type II VLPs, differing in the region, in which the antigen is presented. Whereas type I VLPs display via fusion to the Gag polyprotein, type II VLPs present the antigen at the outer particle surface (Deml, L, Speth, C et al. 2005). Incorporation of the foreign polypeptides or proteins can thereby be achieved using either the retrovirally derived envelope (Env) protein or by taking advantage of the fact that retroviruses unspecifically package host membrane proteins into their envelope (Hammarstedt, M et al. 2000; Hammarstedt, M and Garoff, H 2004).

Overall, retrovirus-based VLPs are highly immunogenic subunit vaccines, because they facilitate introduction of antigens in the MHC class I and II processing and presentation pathway and additionally act as "danger signals" to activate the innate immune system. Thus, they initiate strong T_H1-biased humoral and cellular immune responses (Deml, L, Speth, C et al. 2005).

Sendai-virus based nucleocapsid particles have not been used for immunization studies up to date. This virus belongs to the family of paramyxoviridae (enveloped dsRNA viruses) and consists of a nucleocapsid enveloped by a lipid membrane, which contains genomic viral RNA surrounded by the three structural proteins NP, P and L (Choppin, P W and Compans, R W 1970; Finch, J T and Gibbs, A J 1970). The nucleocapsid protein NP is the main component of the nucleocapsid structure, which is composed of a left-handed helix of 1 µm length and 14-17 nm diameter containing 11-13 protein subunits (mainly NP) per winding. The protein subunits exhibit a rotated arrangement, so that they point towards one end of the complex like arrowheads (Finch, J T and Gibbs, A J 1970). The nucleocapsid contains 2400-2800 molecules of NP packaging 6 nucleotides of viral RNA per protein subunit (Hosaka, Y and Hosoi, J 1983) (Figure 4).

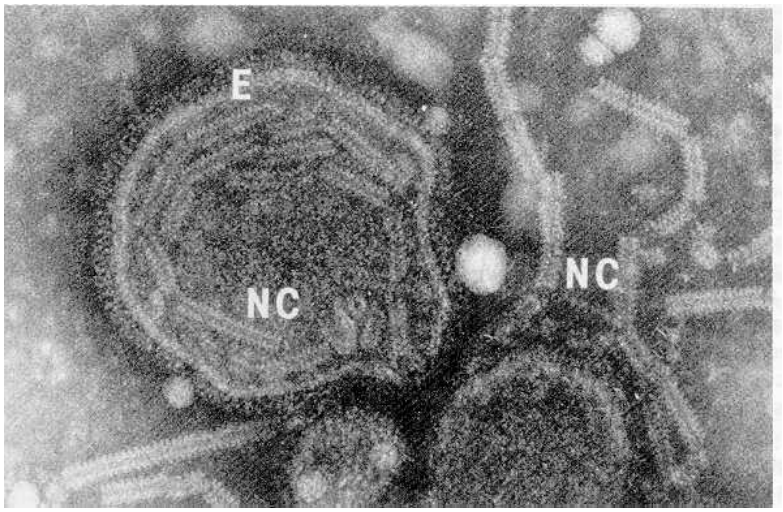


Figure 4 Structure of Sendai nucleocapsid (NC). Fragments of flexible helical nucleocapsids (NC) of Sendai virus, a paramyxovirus, are seen either within the protective envelope (E) or free, after rupture of the envelope. The intact nucleocapsid is about 1,000 nm long and 17 nm in diameter; its pitch (helical period) is about 5 nm. (x200,000) (A. Kalica, N I o H 1996)

The percentage of P and L molecules in nucleocapsids is much lower with about 300 molecules P and 30 molecules L (Lamb, R A et al. 1976). They are not essential for nucleocapsid formation, as NP synthesized in the absence of other viral components assembles into nucleocapsid-like particles. Furthermore it was observed previously, that the highly immunogenic C-terminal part of NP, comprising about 120 amino acids (aa), can be deleted without affecting the NP assembly (Buchholz, C J et al. 1993).

II.3 Retroviruses

II.3.1 Structure and life cycle

Retroviruses are enveloped viruses with a diploid genome, which is packaged as single (+)-stranded RNA of almost 10 kb. The viral genome resembles eukaryotic mRNA (Buchholz, C J, Spehner, D et al. 1993). The viral genes are flanked by so called long terminal repeats (LTRs) which are required for their integration into the host cell genome and for efficient transcription. In addition, a sequence termed psi-site (ψ) ensures efficient packaging of the viral RNA into the particles. The viral genome includes the structural genes gag/pol and env, which are common genes, present in all retroviruses. As an example, the organization (Figure 5) and assembly (Figure 6) of the murine leukemia virus MLV, which belongs to the C-type retroviruses, are briefly described.

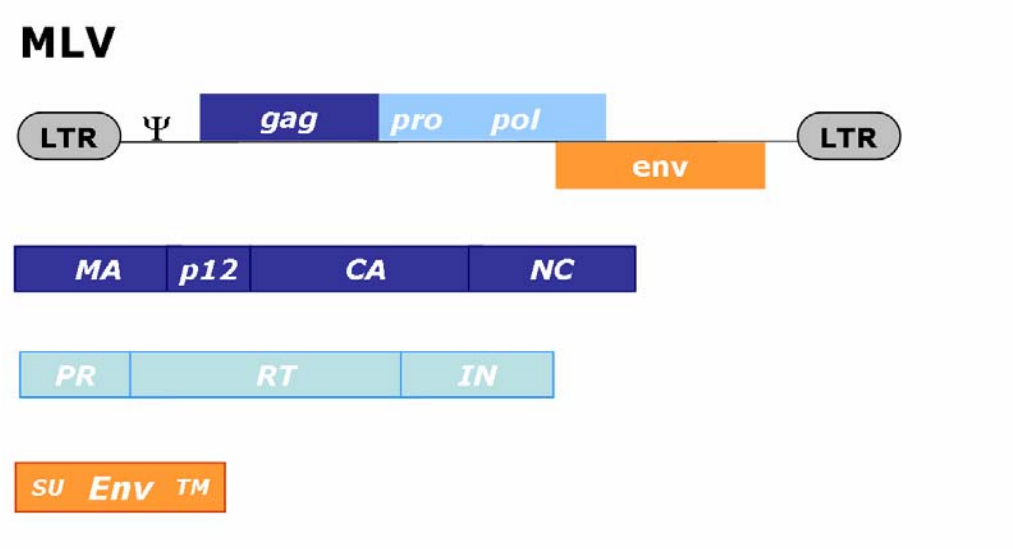


Figure 5 **Genomic organization of the murine leukaemia virus MLV.** The viral genome integrated into the host chromosome consists of two flanking regions named long terminal repeats (LTR). The signal relevant for packaging of the viral RNA is also termed psi-site (Ψ) and is adjacent to the structural genes gag (abbreviation of group-specific antigen) and pro (protease) as well as pol (polymerase). The env (envelope) gene is present in a different reading frame. The gag precursor protein code for the structural proteins matrix (MA), p12, capsid (CA) and nucleocapsid (CA), whereas the pro/pol gene can be divided into the viral protease (PR), the reverse transcriptase (RT) and the intergrase (IN) enzymes. The envelope protein consists of two subunits named soluble unit (SU) and transmembrane unit (TM). (Coffin, J M et al. c1997)

The viral structural proteins including the core forming capsid (CA), the RNA-binding nucleocapsid (NC) and the membrane associated matrix proteins (MA) are encoded within the gag gene. In addition, the pol gene provides all viral enzymes, namely the reverse transcriptase (RT), the protease (PR) and the integrase (IN). The env gene encodes the Env polyprotein, which mediates host cell entry and thereby determines the tropism of the virus. The Env protein consists of two non-covalently linked polypeptides, the surface unit (SU) and the transmembrane domain (TM).

The life cycle of retroviruses starts by infection of the host cell followed by reverse transcription of the viral genome into double stranded DNA. This step is catalyzed by the RT. Afterwards integration of the DNA into the host cell genome is mediated by IN. The resulting so called provirus is transcribed by the host cell machinery in the same way as endogenous genes. Expression is promoted by the viral LTR, which serves as efficient promotor.

The retroviral structural proteins, but not the Env, are translated as polyprotein precursors, which are cleaved by the viral protease into the corresponding proteins. These posttranslational modifications occur during budding or

maturation of already released particles. The polyprotein which is synthesized at the earliest stage of replication is Gag, including CA, MA and NC. The Gag polyprotein has been shown to initiate the budding process even in absence of any other viral protein. Pol is also synthesized as a polyprotein in dependence of Gag expression. The Pol-encoded proteins RT, PR and IN are then directed by Gag to the site of assembly, close to the host cell membrane.

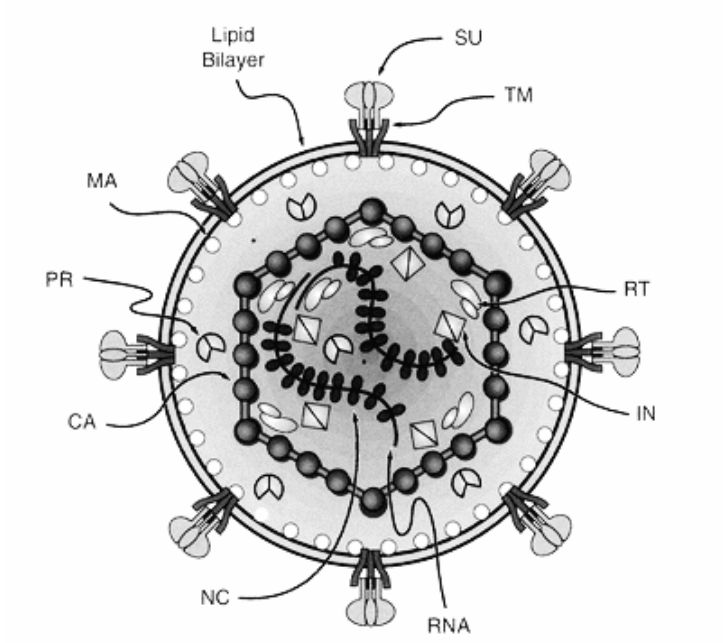


Figure 6 **Schematic cross section through a retroviral particle.** The viral envelope is formed by a cell-derived lipid bilayer into which proteins encoded by the env region of the viral genome are inserted. These consist of the transmembrane (TM) and the surface (SU) components linked together by disulfide bonds. Internal nonglycosylated structural proteins are encoded by the gag region of the viral genome. They are the matrix (MA) protein, capsid (CA) protein, and nucleocapsid (NC) protein. Major products of the pol-coding region are reverse transcriptase (RT) and integrase (IN). The protease (PR) is derived from the pro gene between gag and pol (Coffin, J M, Hughes, S H et al. c1997)

In contrast to these viral proteins, the Env polyprotein is a translation product of spliced viral mRNA. It includes the SU and TM subunits and an additional N-terminal signal peptide, which ensures routing to the secretory pathway. Within the ER, the Env precursor protein is heavily glycosylated at Asn residues and oligomerizes into trimers. Then, upon passage through the Golgi apparatus, cleavage between SU and TM is performed by furin, a cellular protease. Subsequently, the Env trimers are directed to the cell surface by vesicular transport, where they diffuse laterally to the site of budding.

For most retroviruses the budding takes place in so called lipid rafts, as it was observed for HIV (Holm, K et al. 2003). Here, the Env molecules are assembled onto particles containing the gag/pol products and two full length RNA transcripts. These functional particles are subsequently released and the remaining Gag/Pol polyproteins are cleaved during a further maturation step. In conclusion, infectious retroviral particles able to initiate the next replication cycle are generated.

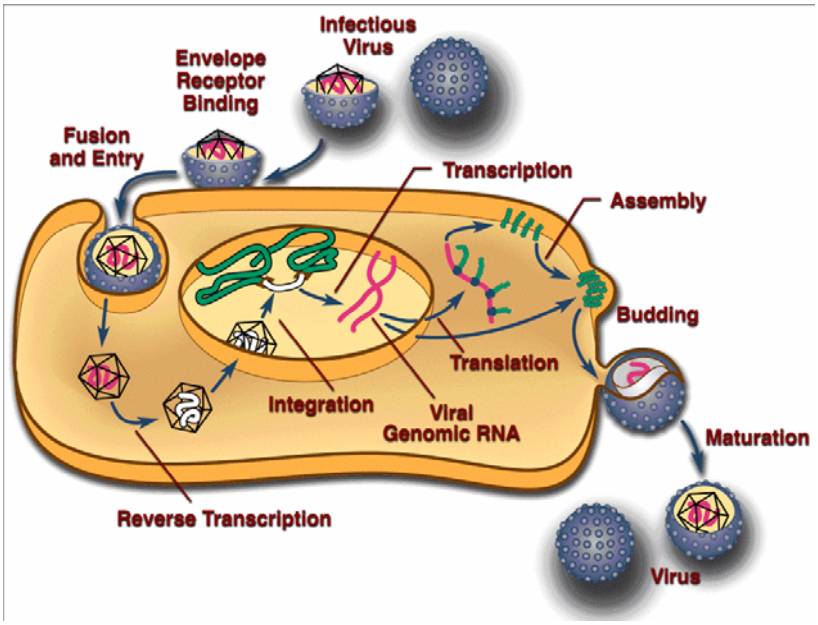


Figure 7 Life cycle of retroviruses. Upon binding to the cell surface receptor, particle and cell membranes fuse. Subsequently, the uncoated particle penetrates the cytosol, where disassembly takes place. Viral RNA is reverse transcribed into DNA which homes to the nucleus. Here, it is stably integrated into the host genome. Thus, transcription and translation of retroviral proteins begins. The Env protein is directed to the cell surface whereas all other retroviral proteins accumulate in the cytosol at the site of budding. Finally, particles are released which undergo a further maturation step before getting infective (Hughes, S H 2002).

II.3.2 Retroviral vectors and display systems

In contrast to retroviruses, retroviral vectors are by definition non-replicative and can thus be used for single round delivery, only. Instead of the wild type (wt) genome, the gene of interest, e.g. therapeutic or marker genes, are packaged into the particles which can then be transferred into target cells, a procedure termed "transduction". For this purpose, the gene of interest has to be flanked by appropriate LTRs and the Ψ-signal, to ensure specific packaging into the applied particle species and subsequent integration into the host genome. Thus,

long term expression of the transgene is achieved, which is a major advantage of retroviral vectors in comparison to other gene delivery systems. A further advantage is the possibility to specifically transduce certain cell types. To alter the tropism of a given vector system, envelope proteins of other viral species can be incorporated into the particle surface, a technique also referred to as pseudotyping. For this purpose, packaging cells are generated that simultaneously express Gag/Pol of one viral species and the Env protein of another. In conclusion, so called pseudotype particles are generated, which incorporate the heterologous Env protein and exhibit the corresponding tropism. This way, highly effective gene delivery systems can be generated.

Since retroviruses tolerate insertions in their envelope protein, foreign polypeptides can be readily displayed at the N-terminus of SU (N-terminal display) represents one possible way to display foreign sequences (Buchholz, C J et al. 1999). Experience has been made with insertions like growth factors, cytokines, antigenic epitopes and antibody molecules. Examples for such N-terminal display approaches are the human epidermal growth factor (hEGF) (Cosset, F L et al. 1995) as well as CD40L (Morling, F J et al. 1997) and others. Also single chain variable fragments (scFvs) have been recently used to establish selection of functional human antibodies from retrovirus-based display libraries (Urban, J H et al. 2005).

II.4 Objective

The primary objective of this thesis was the development of a retrovirus particle based display system for PrP. Such particles will be of high value for the development of vaccination strategies against TSEs and also as tool to address basic questions about the molecular properties of the infectious PrP agent.

Basically, this goal will be addressed by displaying either i) the cellular form of the protein (PrP^{c}) or ii) the pathogenic isoform PrP^{Sc}.

i) To develop a system which allows efficient incorporation of the cellular murine prion protein (PrP^{c}) into either retrovirus-based virus-like particles (shortly referred to as retroparticles) or nucleocapsid-based particles (NPs), three different approaches will be chosen.

High amounts of PrP^{c} in retroparticles could be expected from a commercially available system using the transmembrane domain (TMD) of the platelet derived growth factor receptor (PDGFR). Foreign proteins expressed in this setting are

expressed and targeted to the plasma membrane in particular high amounts (Chesnut, J D et al. 1996; Douglas, J T et al. 1999). The presence of this transmembrane domain on the surface of a retroviral packaging cell line has been demonstrated to facilitate the incorporation of the EGF domain into retroviral vector particles in the host laboratory just before the start of this thesis (Merten and Buchholz, *unpublished data*).

Secondly, PrP^{c} will be displayed on top of the viral Env, as it was previously described for various polypeptides in the host laboratory (see chapter II.3.2).

Addressing the amount of mPrP per particle surface area, the third approach deals with fusion to the Sendai virus nucleocapsid protein (NP). At this, several hundred protein subunits arrange around RNA molecules resulting in substantially dense particles (Buchholz, C J, Spehner, D et al. 1993; Curran, J et al. 1993).

The different particle systems to display the PrP^{c} have to be established, the particles have to be produced in high amounts and subsequently have to be characterized biochemically. Immunization studies using the most promising particle type have to be performed in collaboration with the Division of Immunology at the Paul Ehrlich Institute.

ii) As a second aim of the thesis, the display of the pathologic isoform of the prion protein (PrP^{Sc}) on VLPs and replication competent retroviruses is addressed. Since it is rather difficult to generate or even isolate the pathogenic isoform purely *in vitro*, this approach is by far more ambitious than the display of cellular PrP. Nevertheless, there are in vitro conversion protocols available to generate PrP resistant to proteinase K (PrP^{res}) (Caughey, B 2003), a form which resembles the pathogenic form of PrP.

From this approach strategies for the generation of a PrP^{Sc}-based vaccine are expected as well as novel insights into interactions between PrP and retroviruses.

III

Material and methods

III.1 Molecular biology

III.1.1 Plasmids and vectors

All plasmids used and generated during the thesis are described in detail in the Appendix.

Table 2 Origin of used plasmids and vectors

plasmids	coding region	reference	provided by
cloning plasmids			
phgPrP	murine Prion protein (mPrP)	(Fischer, M et al. 1996)	Fabio Montrasio, Paul-Ehrlich-Institut Langen, Germany
parental plasmids			
pDisplay (pD)	platelet derived growth factor receptor (PDGFR)	©	Invitrogen Catalog no. V660-20
plenti-V5-lacZ	HIV-packagable lacZ protein	©	Invitrogen Catalog no. K4955-10
pGem-NP	nucleocapside protein of the Sendai virus	(Buchholz, C J, Spehner, D et al. 1993)	Christian Buchholz (Paul-Ehrlich-Institut, Langen)
pFB-EASALF	hEGF/amphotropic Env fusion protein	(Peng, K W et al. 1997)	F.-L. Cosset (INSERM U412, Lyon, Frankreich)
pNCA	ecotropic MLV	(Colicelli, J and Goff, S P 1988)	Christian Buchholz (Paul-Ehrlich-Institut, Langen)
retroviral vector plasmids			
pHit60	murine leukemia virus gag/pol genes (MLVgp)	(Soneoka, Y et al. 1995)	A.J. Kingsman (University of Oxford, UK)
pCMVΔR8.2	human immunodeficiency virus gag/pol genes (HIVgp)	(Naldini, L et al. 1996)	U. Blömer (University Hospital, Kiel, Germany)
pMD.G	VSV-G protein	(Naldini, L, Blomer, U et al. 1996)	U. Blömer (University Hospital, Kiel, Germany)
pD-EGF	human epidermal growth factor (hEGF) fused to PDGFR-TMD	(Merten, C A et al. 2003)	Christoph Merten (Paul-Ehrlich-Institut, Langen)
pLP1	human immunodeficiency virus gag/pol genes (HIVgp)	©	Invitrogen Catalog no. K4950-00
pLP2	human immunodeficiency virus ref gene	©	Invitrogen Catalog no. K4950-00

pLPVSVG	VSV-G protein	©	Invitrogen Catalog no. K4950-00
nucleocapside plasmids			
pSCT7	T7 polymerase	(Radecke, F et al. 1995)	Christian Buchholz (Paul-Ehrlich-Institut, Langen)

III.1.2 Polymerase chain reaction

The polymerase chain reaction (PCR) allows to amplify specific DNA sequences of different origins, such as plasmid, genomic or complementary DNA (Saiki, R K et al. 1985; Mullis, K B and Faloona, F A 1987). The amplified fragment can subsequently be used for further molecular biological methods. Current PCR protocols make use of a DNA-dependent DNA polymerase isolated from *Thermophilus aquaticus* (Taq Polymerase), which shows a temperature optimum of 72 °C. Using appropriate buffers, primers, desoxynucleotides and cycling conditions the Taq polymerase can amplify a DNA fragment bordered by the two primer sequences. Under optimal conditions this amplification takes place in an exponential manner.

A typical PCR cycle contains a denaturation step at 94 °C, leading to the dissociation of the double stranded template. The following hybridization step allows primer annealing to the resulting single stranded template. The hybridization temperature T_D is adjusted according to length and composition of the DNA template. It can be calculated roughly corresponding to the Wallace rule (Suggs, S V et al. 1981) as follows: $T_D = 4x(G+C) + 2x(A+T)$. After hybridization, DNA elongation is performed at 70-75 °C. The elongation time is chosen in pursuance with the template length, approximately 1 min/1 kb DNA. By repeating the cycle sequence the template is amplified in an exponential manner.

In this work, the Taq Polymerase (Qiagen, Hilden, Germany) and the Vent proofreading polymerase (NEB, Schwalbach, Germany) with 3´-5´ exonuclease activity have been used. For amplification of PrP fragments a standard PCR mix (100 µl) contained the following ingredients:

1x PCR buffer (20 mM Tris/HCl, pH 7.5; 100 mM KCl; 1.5 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA, 0.5% Tween 20; 0,5% Nonidet P40; 0.01% gelatine)
1-2 µM (+)-primer and (-)-primer, respectively
200 µM of each desoxynucleotid
2 units Taq /Vent polymerase
0.05 µg template (plasmid or genomic DNA)

PCR conditions
94 °C 5 min
94 °C 30 sec
55 °C 45 sec
72 °C 1 min
25 cycles
72 °C 10 min; subsequent cool down to 4°C

All PCR reactions were performed using an PerkinElmer/Eppendorf Mastercycler gradient (PerkinElmer, USA)

III.1.3 Oligonucleotides

Table 3 sequences of used oligonucleotides

oligonucleotide	5´-sequence-3´	published
cloning		
PrP23Sfi+	GTATGGCCCAGCCGGCCAAAAAGCGGCCAAAGC	forward 209
PrP121Sfi+	GACT*GGCCCAGCCGGCC*GTGGGGGGCCTTGGTGGCTACATGC	forward 111
PrP231SacII-	GACTCCGCGGCCTTCCCTCGATGCTGGATCTTCTCCCGTC	reverse PrP
PrP90Sfi+	GTATGGCCCAGCCGGCCCAAGGAGGGGGTAC	forward 142
PrPNotI-	GAGCGCGGCCGCCCTTCCCTCGATGCTGGATCTTCTCCCGTCGTA	
PrP121MscI+	GCGATGGCCACGGCGGTGGTGGATCAATCGAGGGAAGGGTGGGGGGCCTTGGTGGCTACATGC	
PrP121EcoRV+	GCGAGATATCGGCGGTGGTGGATCAATCGAGGGAAGGGTGGGGGGCCTTGGTGGCTACATGC	
PrP231EcoRI-	GGATGAATTCTCATGCTGGATCTTCTCCCGTCGTAATAG	
fullNP-HA+	GTGGTGGCCACTTTGTCACACTACATGGGGCTGAACG	
fullNP-HA-	GCATGAATTCGAGCTCGGTACCTCAATAATCTGGAACATCATATGGATACATATGCTCGAGGATTCCTCCTATCC	

Sequencing and Screening		
CB6	CCCCTAATCCCCTTAATTCTTC	
EASeq	CAAGATGCCTTCCCAAAATTATA	
EMoSeq	GGTAACCAATGGAGATCGGGAGACG	
EMoSeq2	CCAGTCCTCATCAAGTCTA	
CMVforw	CGCAAATGGGCGGTAGGCGTG	
V5reverse	ACCGAGGAGAGGGTTAGGGAT	

III.1.4 Working with plasmid DNA

All DNA restrictions were performed using commercially available restriction endonucleases from New England Biolabs (NEB, Schwalbach, Germany) according to the manufacturer's instructions.

III.1.4.1.1 Standard restriction for preparative purposes

10 µg DNA
10 u restriction enzyme
5 µl 10x buffer (NEB buffer 1-4, corresponding to the used restriction enzyme)
5 µl BSA (NEB; only if required by the applied enzyme)
add 50 µl aqua bidest
The restriction sample was incubated at the temperature optimum of the used restriction enzyme for two hours. In case of double digests the restriction enzymes were added sequentially (2.5 µl at maximum) and incubation was performed at the respective temperature optimum for two hours, each. If two different buffers were required, DNA was purified after the first digest using the Qiagen PCR purification Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) or the GeneClean purification Kit (QBiogene, Irvine, USA), followed by incubation with a new reaction mixture for the second digest.

III.1.4.1.2 Standard restriction for analytic purposes

0.5 µg DNA

5 u restriction enzyme
2 µl Puffer (NEB buffer 1-4, corresponding to the used restriction enzyme)
2 µl BSA (NEB; only if required by the applied enzyme)
add 20 µl aqua bidest
Incubation was performed at the temperature optimum of the applied enzyme for 90 min. Double digests were done as described for preparative purposes.

III.1.4.1.3 Ligation and dephosphorylation of digested DNA

Recombination of DNA is usually performed by ligation of two double stranded nucleic acid molecules, exhibiting complementary overhangs or blunt ends. This reaction can be accomplished using T4 DNA ligase, which catalyzes the formation of phosphodiester-bonds between the fragments, under consumption of ATP. The following reaction mixture has been used as standard sample:
0.05 µg DNA (molar ratio of backbone to insert = 1:2)
2 µl ligase buffer (NEB)
0.5 µl T4-DNA-ligase (200 U)
add 20 µl aqua bidest
To avoid recirculation of linearized plasmids, these were dephosphorylated using alkaline phosphatase (CIP, NEB, Schwalbach, Germany). For this purpose, 10 U CIP were added to the restriction digestion sample after heat inactivation (75 °C for 10 min). Subsequently, the resulting mixture was incubated for 1 h at 37 °C before it was inactivated at 75 °C for 10 min.

III.1.4.1.4 Transformation of competent bacteria

Transformation of *E. coli* (K12-derived safety strains) is the method of choice to amplify plasmid DNA through cellular replication. For this purpose, bacteria have to be pretreated in a special manner to become competent, thus allowing introduction of foreign DNA.
2.5 ml of an over night culture were used to inoculate 100 ml of fresh LB-Media which was subsequently incubated at 37 °C in a bacteria shaker. Cells were allowed to grow up to an OD_{600nm} of about 0.5 – 0.55, hence reaching the logarithmic growth phase. Then the culture was incubated on ice for 5 minutes, divided into two portions and pelleted at 6000 rpm for 10 minutes at 4 °C. The cells were resuspended in 20 ml TFB1-buffer (sterile, filtrated solution of 30 mM

KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerin, pH adjusted to 5.8 with HAc) respectively, incubated on ice for 5 minutes and once again pelleted as above. Subsequently the cells were resuspended each in 2 ml TFB2-buffer (sterile filtrated solution of 10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerine, pH adjusted to 6.5 using KOH-solution) and incubated on ice for 15 minutes. Afterwards the suspension was portioned into Eppendorf tubes of 100 µl and frozen at –80 °C.

To transform *E. coli*, transformation of the chemically competent cells described above has been performed. For this, the cells were thawed on ice and approximately 50 ng DNA were added. After further incubation on ice for 30 min, a heat shock at 42 °C for 45 sec was performed in a thermoblock. Then 250 µl of prewarmed (37 °C) SOC medium (Invitrogen, Carlsbad, USA) was added and the sample was incubated for 60 min at 37 °C. The bacteria suspension was plated onto LB-AMP-plates (1 % (w/v) Bacto-Trypton, 0.5 % (w/v) yeast extract, 1 % NaCl, 50 µg/ml ampicilin 1.5 % (w/v) agar agar).

III.1.4.1.5 Plasmid preparation

Preparation of plasmids from bacteria was performed using the Qiagen plasmid kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The basic principle of this method is binding of DNA to anion exchange columns. Thus, all cellular compounds such as proteins can be washed away whereas the DNA is retained within the columns.

For purification of low amounts of DNA (Miniprep), 5 ml over night cultures were inoculated using LB-AMP-Medium (1 % (w/v) Bacto-Trypton, 0.5 % (w/v) yeast extract, 1 % NaCl, 50 µg/ml ampicillin). The next day, bacteria were harvested at 5000 rpm for 5 min (Minifuge RF, Heraeus, Hanau, Germany). The resulting pellet was lysed using solutions delivered by the manufacturer (P1 & P2). Chromosomal DNA and cellular fragments were excluded by centrifugation (13000 rpm for 5 min in an Eppendorf table centrifuge). Subsequently the supernatant was applied to anion exchange columns (Qiaprep-8-strips) according to the manual.

For extraction of larger amounts of DNA (Maxiprep), 200 µl LB-AMP-Media were inoculated and cultivated over night. Bacterial yield was performed at 5000 rpm for 10 min (JS-13.1-rotor; J2-21-centrifuge; Beckman, Munich, Germany). Afterwards cells were lysed and the remaining cell debris and chromosomal DNA

was removed by centrifugation (10000 rpm for 20 min). The resulting supernatant was subsequently purified via an anion exchange column according to the manufacturer's instructions. Finally, concentration and purity of the DNA was determined photometrically.

III.1.4.1.6 Agarose gel electrophoresis

Agarose gel electrophoresis allows to separate DNA molecules by their size. The principle is based on the properties of polymerized agarose, which acts like a molecular sieve. Since DNA is negatively charged, it migrates through such gels upon application of electric current in a size dependent manner.
For fragments with a size of 1 kb – 14 kb, 1% agarose gels were used, whereas 1.5 – 2 % agarose gels were used for smaller fragments (according to the fragment of interest). The gels were generated by adding the corresponding amount of agarose to 120 ml TAE buffer. The resulting emulsion was then heated in a microwave oven until the solid agarose became solved. 50 µg/ml ethidium bromide were added and the gel was casted into the tray. DNA samples were mixed with 0.2 volumes 5 x sample buffer (30 % glycerin and 1 % brome phenol blue in 5 x TAE buffer) and applied to the gel. As marker, either the 2-log ladder (NEB, Schwalbach, Germany) was used according to the fragment size. Electrophoresis was then performed at 130 V for approximately 45 minutes. Afterwards fragment bands were photographically documented under UV light and, if desired, bands were cut out for purification as described below.

<u>TAE buffer</u>
Tris-Acetat 40 mM
EDTA 1 mM
adjusted to pH 7.5

III.1.4.1.7 Isolation of DNA fragments from agarose gels

Purification of DNA fragments from agarose gels was performed using the Jetsorb gel extraction kit (Genomed, Löhne, Germany) or the GeneClean Purification Kit (QBiogene, Irvine, USA) according to the manufacturer's instructions. These methods are based on the intrinsic adhesion of DNA to glass powder or on binding of DNA to appropriate anionic columns.

After electrophoretic separation, the DNA band of interest was cut out of the gel and transferred into an Eppendorf tube. These samples were purified according to the manual.

III.1.5 DNA and RNA methods

III.1.5.1 Preparation of genomic DNA

Genomic DNA was isolated from cultured adherent cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. This method is based on binding of nucleic acids to silica-gel-membrane columns. In addition, RNA is excluded from purification through RNAse H digestion. Thus, DNA is retained, whereas all other cellular components can be washed away.
Genomic DNA was also prepared on the basis of Phenol/Chloroform extraction using Trizol reagent [Invitrogen Catalogue number 15596018, (Chomczynski, P and Sacchi, N 1987)] according to the manufacturer`s instructions.
Cells were trypsinized within the culture flasks and the resulting cell suspension was centrifuged at 3000 rpm in an Eppendorf table centrifuge. Approximately 1-3.0 x 10^6 cells were resuspended in 200 µl PBS and applied to the purification procedure as described in the manual with the following modification: For elution of the DNA from the columns 50 µl of buffer AE were used instead of 200 µl to increase the DNA concentration for the subsequent PCR.

III.1.5.2 Preparation of RNA from viral particles

Viral RNA was isolated from virus particles using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This method is based on binding of nucleic acids to silica-gel-membrane columns. To be sure that RNA is purified specifically; the procedure was extended to a DNAseI digestion step.

Virus particles from 140 µl filtered (0.45 µm Filter) cell culture supernatants were treated according to the manufacturer's instructions with the following modification:
Column-bound RNA was washed once with 500 µl AW1. Then a mixture of 10 µl DNAse I and 70 µl RDD buffer (available from Qiagen for on-column-digestions)

was pipetted directly onto the membrane. Subsequently the sample was incubated at room temperature for 15 min. Afterwards the column was washed twice with 500 µl AW1 before it was proceeded corresponding to the manual (AW2 washing step).

III.1.5.3 cDNA synthesis of viral RNA

After RNA isolation, cDNA synthesis was performed. During this step the purified viral RNA is transcribed into complementary single stranded DNA by the enzyme reverse transcriptase (RT). The cDNA obtained this way can subsequently be used for amplification within usual PCR protocols, as described above (III.1.2). Reactions for cDNA synthesis were set up as follows:

Mastermix	Sample
4 µl 5x buffer	4 µl RNA solution (within AVE buffer)
1 µl 20 mM dNTPs	1 µl (10 pmol) reverse Primer
0.5 µl RNAsin	2 µl H_2O
0.5 µl RT	
2 µl DTT	
5 µl H_2O	

The sample was incubated at 80 °C for 1 min and subsequently cooled down on ice. Then, the mastermix was added and the resulting mixture was incubated at 37 °C for 1 h. For subsequent PCR reactions, 2 µl of the cDNA sample were used as template.

III.1.5.4 Isolation of cellular RNA

Cellular RNA was isolated on the basis of Phenol/Chloroform extraction using Trizol reagent [Invitrogen Catalogue number 15596018, (Chomczynski, P and Sacchi, N 1987)] according to the manufacturer`s instructions.

III.1.6 Nucleic acid sequencing

Nucleic Acid sequencing was performed at MWG Biotech (Ebersberg, Germany). For this purpose, DNA samples containing approximately 2 µg plasmid DNA were sent to the company via regular mail, where they were sequenced using appropriate primers.

III.2 Cell culture

III.2.1 Cells and media

All cell lines were grown in Dulbecco´s Modified Eagles Medium (DMEM; Gibco BRL, Eggenstein, Germany) supplemented with 10 % fetal calf serum (FCS; Biochrom KG, Berlin, Germany), 2 mM L-glutamine and antibiotics (0.1 mg/ml neomycin, 100 units/ml penicillin, 50 mg/ml streptomycin; Biochrom KG, Berlin, Germany). Cells were cultivated in an incubator (Heraeus, Hanau, Germany) at 37 °C, 5 % CO_2 and saturated water atmosphere. Cells were trypsinized (0.25 % trypsin in PBS, 1 mM EDTA) for passaging. A fraction of the resulting suspension was seeded into new culture flasks and fresh medium was added.

The following cell lines were cultivated:

HT1080	human fibrosarcoma cell line
HEK293	human embryonal kidney cell line
HEK293T	human embryonal kidney cell line expressing the large T antigen
293 STAR	human embryonal kidney cell line expressing HIV gag/pol
NIH3T3	murine fibroblast cell line
N2a	murine neuroblastoma cell line
ScN2a/PK1	scrapie-infected murine neuroblastoma derived from N2a

III.2.2 Freezing and thawing of cultured cells

For storage, cells were kept at –80 °C or, long term storage was performed in liquid nitrogen.

Freezing

Cells were trypsinised and resuspended in DMEM. Subsequently a centrifugation step (1200 rpm for 10 min at 4 °C in a Heraeus centrifuge) was carried out to pellet the cells. These were then resuspended in freezing medium (90 % FCS, 10 % DMSO), divided into cryotube aliquots of approximately 5 x 10^6 cells and frozen.

Thawing

Cryotubes were incubated in a water bath at 37 °C until the ice thawed. Then the cell suspension was immediately transferred into a falcon tube with 15 ml

prewarmed medium. To exclude the cytotoxic DMSO, cells were subsequently centrifuged (1200 rpm for 6 min at room temperature) and resuspended in fresh medium and seeded into appropriate culture flasks.

III.2.3 Cell transfection

To express plasmids within eukaryotic cells, transfection using the Lipofectamin Plus Kit or Lipofectamin 2000 (Invitrogen, Karlsruhe, Germany) was performed. The corresponding method is based on a complex formation between plasmid DNA and liposomes which subsequently fuse with the cellular membrane. Hence, the DNA is taken up by endocytotic mechanisms.

For the generation of VLPs and vector particles as well as virus stocks, 3 x 10^6 cells were seeded into a 175 cm² flask at the day before transfection. After cultivation over night, the transfection was performed as follows: 30 µg of total plasmid (10 µg PrP–fusion protein + 10 µg gag/pol and 10 µg transfer vector; 15 µg PrP–fusion protein + 10 µg gag/pol and 5 µg VSV-G coding plasmid) were either mixed in a triple transfection or (15 µg PrP–fusion protein + 15 µg gag/pol) in a double transfection, respectively. In case of full-length virus production, the virus-coding plasmid was transfected in a single-transfection (30 µg). The resulting plasmid mixture was diluted in 2 ml serum free DMEM, in case of Lipofectamin Plus Kit before 180 µl Plus reagent were added. In parallel, 90 µl Lipofectamin or Lipofectamin 2000 were suspended in another 2 ml serum free DMEM (without any supplements). After incubation for 15 min (Lipofectamin Plus Kit) or 5 Min (Lipofectamin 2000) at room temperature, both samples were mixed. This solution was then incubated for further 15 min (Lipofectamin Plus Kit) or 20 Min (Lipofectamin 2000). Afterwards, medium was exchanged against the transfection mixture (4 ml + 8 ml serum free DMEM) and the cells were incubated for 4 h at 37 °C. Then, the transfection mixture was exchanged against the normal serum-containing DMEM. After 48 and 72 h, VLPs, vectors or the virus-stock were harvested and filtered through a 0.45 µm filter.

III.3 Virological methods

III.3.1 Concentration of retroparticles

VLPs and vectors were further concentrated by low-speed centrifugation [16h-24h, 3600 rpm (Minifuge RF, Heraeus, Hanau, Germany), 4°C] or by ultra-centrifugation [1.5 h, 35000 rpm SW-41 rotor, L8-70M-UZ ultracentrifuge, Beckman, 4°C] through a 30 % sucrose cushion (4 ml cushion, 8 ml supernatant). For standard particle preparations, three T175 flasks were transfected, resulting particles were resuspended after low-speed concentration, in 1 ml PBS Dulbecco + 1 % FCS and divided into 20 aliquots of 50 µl, each.
For immune fluorescence cells were transfected in 6-wells containing coverslips (1.2×10^6 cells per total 6-well, 30 µl Lipofectamin/Lipo2000, 60 µl Plus reagent in 750 µl DMEM each) and analyzed after 48 h as described in chapter III.4.4.

III.3.2 Transduction

Transduction experiments were performed either to determine particle titers or to obtain stably transgene-expressing cell lines.
Infectious titers of vector particles were calculated by the X-Gal assay based on ß-galactosidase transfer via the vector particles. Therefore, 1.25×10^5 HT1080 or N2a target cells were seeded in a 12 well plate and incubated with different dilutions of the filtrate for 4 hours. Transduced cells were fixed two days post transduction in 2 % formaldehyde and 0.5 % glutaraldehyde. After washing with PBS, samples were incubated with the staining solution [4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 80 µg/ml X-Gal (Sigma)] at 37 °C overnight. The next day blue colonies were counted under the light microscope.
Titers were calculated by multiplying the number of surviving or blue colonies in each well with the dilution factor of the particle containing supernatant.
Stably transduced cell lines were obtained via transduction of blasticidin encoding transgenes. From day two post transduction of 1×10^6 N2a, ScN2a and 293 STAR cells in a T75 flask were treated with DMEM containing 4 µg/ml Blasticidin (Invitrogen) for the N2a and 8 µg/ml Blasticidin for the 293 STAR cells for two weeks. (ScN2a cells were not selected on Blasticidin). During this period the medium was changed on every second day to wash away dead cells, and cells

were eventually splitted, if necessary. After two weeks of antibiotic selection cells were analyzed and frozen in aliquots.

III.3.3 Infection

Infection of target cells was performed either to determine the virus titers or to obtain stably infected cell lines.

For this, either or 2.5×10^5 cells in a T25 flask were infected with different multiplicity of infection (moi, virus per cell), ranging from 1×10^{-6} to 20 IU/ml. Titration of virus stocks thereby required serial dilutions from 10^{-3} to 10^{-6} IU/ml. Infection was carried out in supplemented DMEM containing 10 % FCS for 4 h at 37 °C. After 24 h, titration experiments were stopped by fixation of the cells with Methanol (-20°C) for at least 1 h at -20°C and subsequent immune peroxidase assay (IPA, III.4.3) was carried out. Stably infected cells were analyzed at different time points ranging from 1 to 10 days post infection and frozen in aliquots.

III.3.4 Titration

Serial 10fold dilutions (ranging from 10^0 to 10^{-6}) were therefore added in triplica to 5×10^4 NIH-3T3 cells per well in a 24 well and viral capsid was stained 24h after infection by IPA (III.4.3) to ensure single-round replication of the virus. Positive cells were counted in the appropriate dilutions showing infection events from 100-500. Therefore four images were taken at 500fold magnification using Zeiss AxioVision Software and Camera. Positive as well as negative cells were counted in each image area (4×0.25 mm^2). Infectious titers per ml were obtained by extrapolation of the counted area to the size of one well (3 mm^2) including the dilution factor of virus stocks.

III.3.5 Caesium-chloride gradient purification of NPs

HEK-293 FT producer cells were lysed using hypotonic lysis buffer (10 mM Tris-Cl pH 8.0, 1mM EDTA). After 30 min incubation on ice cells were destroyed by 30 knocks in a douncer. Cells were thereby kept at 4 °C and were subsequently loaded on a Caesium-chloride step gradient. An aliquot was kept for Western Blot analysis.

Caesium-chloride (CsCl) step gradients were generated using 40%, 30% and 20% solution of CsCl in TNE buffer (25 mM Tris pH 7.5, 50 mM NaCl 2 mM EDTA). 3 ml of each solution were pipetted consecutively into an ultracentrifuge tube (12.5 ml) starting with 40 % CsCl. On top of the resulting step gradient 0.5 ml 30% Glycerol were added followed by pipetting of 2 ml sample volume in hypotonic lysis buffer. Gradients were centrifuged at 35.000 rpm (Beckmann Ultracentrifuge, Rotor SW41) for 16 h at 4 °C. Gradients were fractionated from the bottom of the tube by insertion of a whole and dropwise collection of 1 ml portions. Analysis was performed by Western Blot analysis of sample aliquots as described in chapter III.4.5 and III.4.6.

III.4 Protein biochemistry

III.4.1 Antibodies

All antibodies, dilutions and manufacturers are summarized as follows:

Table 4 summarization of used antibodies

Primary antibodies	**Origin**	**Dilution WB**	**Dilution IPA**	**Dilution IF**	**Dilution EM**	**Source/ manufacturer**
α-HA (clone 12CA5)	mouse	1:5000		1:500		Roche
α-RLV-SU	goat	1:5000		1:500		Quality, Biotech
α-p24	mouse	1:2000		1:200		Chemicon
α-PrP 6H4	mouse	1:10000	1:1000	1:1000	1:750	Prionics
α-PrP serum	rabbit	1:5000				
α-NP(Sendai) serum	rabbit	1:5000		1:500		
α-p30	goat	1:50000	1:1000	1:1000		Quality, Biotech
α-EGF	mouse	1:5000		1:500	1:500	
Secondary antibodies	**Origin**	**Dilution WB**	**Dilution IPA**	**Dilution IF**	**Dilution EM**	**Source/ manufacturer**
α-mouse-HRP	goat	1:10000	1:1000			Sigma
α-rabbit-HRP	rabbit	1:5000				Dako Cytomation
α-goat-HRP	rabbit	1:10000	1:1000			Dako Cytomation

α-mouse-FITC	rabbit			1:500		Dianova
α-goat-Cy3	rabbit			1:200		Dianova
α-rabbit-FITC	goat			1:200		Dianova
α-mouse-10nmgold	rabbit				1:200	Dianova

III.4.2 Reverse Transcriptase activity assay (RT)

The reverse transcriptase assay (RT) is based on detection of the activity of the viral enzyme reverse transcriptase (RT), which converts retroviral genomic RNA into double stranded DNA during the viral life cycle (see II.3.2).

For detection of viruses and viral particles in cell culture supernatants, special plates, on which a polyA substrate is bound, are used. By use of a dT primer, the enzyme catalyzes incorporation of dUTPs into a newly synthesized strand. Subsequently, the reaction is quantified by antibody binding to UTP, to which the alkaline phosphatase (AP) is covalently linked. The antibody binding is then followed by photometric analysis with an AP-specific substrate at 405 nm. Comparison with a MLV RT standard provides the possibility to determine the amount of RT present in the samples. Since the average amount of RT per particle has been evaluated (Pyra, H et al. 1994), it is possible to calculate particle values present per sample using the following equation

$$n_{CP} = 3 \times 10^9 \text{ [p/units]} \times \text{RT-value [units]}$$

The RT test solutions are commercially available (Cavidi Tech), for detection of MLV particles the C-type RT test, for HIV detection, the lenti RT test has been used.

III.4.3 Immune peroxidase assay (IPA)

The immune peroxides assay is based on the determination of proteins via antibody stain. For this, fixed cells are subjected to first blocking of unspecific interactions by a blocking agent and second, specific antibody stain with an antibody directed against the protein of interest. Subsequently the cells are stained by a second antibody labeled with peroxidase. Detection is carried out by the IPA dye, which contains a dye turning to red colour upon incubation with Peroxidase.

The IPA was used to determine the viral capside p30 protein in the infected cells. Upon counting positive (red stained) cells in the serially diluted virus stock, the calculation of infectious particles per ml is possible. Upon this, the fixation of the

target cells early after the infection is necessary to ensure one positive infection event per infectious particle.

The assay was carried out as follows: methanol-fixed, infected cells were blocked by 2 % BSA solution in PBS (500 µl) for 1 h at RT and subsequently stained by the primary antibody (see Table 4) (300 µl per well) in 1 % BSA/PBS for 1 h at 37°C. Then, cells were washed three times with PBS and stained with the secondary antibody (see Table 4) (300 µl per well) for 1 h at 37°C. Cells were again washed three times with PBS and were then stained with the IPA dye solution:

2 mg 3-amino-9-ethyl-carbazol in 500 µl dimethylformamide

66 µl sodium acetate 3 M

9.5 ml H_2O bidest

filtered through 0.45 µm filter

+ 50 µl H_2O_2 30 %

Thereby, 300 µl per well were added and samples were incubated until red cells could be observed in the microscope (5 to 30 min).

III.4.4 Immune fluorescence (IF)

The principle of immune fluorescence (IF) staining is the detection of proteins via specific antibody stain coupled to a fluorescence antibody. The method is more sensitive than IPA due to the detection limit of a visible dye contra a fluorescent dye. Nevertheless, the staining protocol is similarly based on blocking, staining with a specific first antibody and subsequent stain with a labeled second antibody as described for IPA.

IF was carried out as follows: cells (grown in 6-wells on coverslips) were fixed with 2% formaldehyde in PBS at RT or with Methanol (-20°C) at -20°C, the latter is discussed to render the cell membranes more permeable, after 48 h post transfection. Cells were then blocked with 10 % horse serum in PBS and afterwards stained by the primary antibody (see Table 4) in 5 % horse serum/PBS at 37°C for 1 h or for double-stains with a mixture of the mentioned antibodies in same concentrations (Table 4). After washing (three times with PBS) incubation with an FITC- or Cy3-conjugated secondary antibody (see Table 4) in 5% horse serum/PBS was carried out for 1 h at 37 °C. The cells were again washed three times with PBS, embedded using Mowiol on an object holder and dried for 30 min. Subsequently the cells were analyzed under a light microscope

using a laser light source combined with appropriate fluorescence filters (515 nm FITC, 532 nm Cy3).

III.4.5 SDS-polyacrylamide-gelelectrophoresis

The SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE) (modified after (Laemmli, U K 1970)) allows to separate protein mixtures according to their apparent molecular weight. The basic principle includes binding of multiple molecules of the negatively charged detergent sodium dodecylsulfate (SDS) via hydrophobic interactions to denatured protein molecules. That way, the latter ones acquire an excess of negative charges on their surface and can thus be applied to electrophoresis. For this purpose, the samples are loaded on polyacrylamid gels, which act like molecular sieves, similar to agarose gels.
Samples were mixed with the appropriate amount of 4 x sample buffer (400 mM Tris/Cl (pH 6.8), 800 mM dithiothreitol (DTT), 16 % SDS, 0.8 % bromephenolblue, 25 % glycerin, 40 % ß-mercapto-Ethanol) and loaded either on 10-16 % polyacrylamid gels (see Table 5), within a Biorad Protean II chamber (Biorad).

Table 5 SDS-PAGE ingredients

ingredient	Volume 10 %	Volume 12 %	Volume 16 %	Volume collection gel
acrylamide/ bisacrylamide 30 %	33.3 ml	36.4 ml	48.5 ml	16.5 ml
1 M Tris (pH 6.8)				12.5 ml
1 M Tris (pH 8.8)	39 ml	39 ml	39 ml	
10 % SDS	1.1 ml	1.1 ml	1.1 ml	1 ml
Glycerol 50 %	8.2 ml	8.2 ml	8.2 ml	
aqua bidest.	add 100 ml	add 100 ml	add 100 ml	add 100 ml
10 % APS	50 µl *	50 µl *	50 µl *	50 µl *
TEMED	25 µl *	25 µl *	25 µl *	25 µl *
* volume added to 15 ml gel solution, shortly before use				

Then discontinuous SDS polyacrylamide gelelectrophoresis (SDS-PAGE) was performed according to the manufacturer's instructions using a Biometra P25 power supply (Biometra, Göttingen, Germany) with the following running buffer:

SDS running buffer
36 g glycin
7.75 g Tris
1.25 ml 20 % SDS
add 250 ml aqua
bidest

The RPN 800 Full Range Rainbow marker (Amersham, Little Charlton, UK) was used as protein standard.
Rainbow marker size standards (kD)
250, 160, 105, 75, 50, 35, 30, 25, 15, 10

III.4.6 Western blot analysis

The Western blot technique is a method which enables the transfer of proteins on protein-binding surfaces such as nitrocellulose membranes (Towbin, H et al. 1979). This transfer, also termed blot, enables the specific visualization of proteins of interest by immunostaining. Usually, proteins which have been separated by SDS-PAGE are applied to Western blot analysis.

III.4.6.1 Blot technique

Thereby, the transfer was performed electrophoretically onto nitrocellulose membranes (Immobilon^{TM}-P, Millipore) within a Fastblot-device (Biometra), according to the manufacturer's instructions. For this, membranes were shortly incubated and blotted in transfer buffer (48 mM Tris, 39 mM glycin, 20 % methanol, add 1000 ml Aqua bidest). After blotting at 120 mA for 50 min (1 blot) or 200 mA for 50 min (2 blots), unspecific binding sites were blocked with 10 % horse sera in TBST for at least 45 min. For specific protein staining, antibodies diluted in 5 % horse sera in TBST (10 mM Tris pH 8, 150 mM NaCl, 0.05 % Tween 20) have been used (see Table 4), staining was performed for at least 3 h at RT or overnight at 4 °C. The membranes were washed four times with TBST before they were incubated with the appropriate secondary antibodies (see Table 4). After washing as described above, detection was performed using the enhanced chemoluminiscence kit (ECL, Pierce) according to the manufacturer's instructions.

The two reagents contain a HRP substrate, that emits light during conversion into the product. Hence the signal can be visualized, using chemiluminescence films (Amersham) or in an LumiImager (Roche). Signal were collected for 5 sec – 2 h, depending on the signal intensities.

III.4.6.2 Preparation of cell lysates

For preparation of cell lysates, 1×10^7 cells were incubated with 0.5-1 ml of the the RIPA lysis buffer [50 µl 1 M Tris/pH 8.0, 30 µl 5 M NaCl, 1.5 µl 1 M $MgCl_2$, 100 µl 10 % NP-40, 5 µl 20 % SDS, 40 µl protease-inhibitor-mix (25 x Multicomplete™, Roche), 773.5 µl aqua bidest] directly within the culture flasks or after centrifugation in a Falcon tube at 970 rpm at 4°C. To get rid of DNA contaminations, samples were subsequently filtrated through QIAshredder columns according to the manufacturer's instructions (Qiagen). Protein concentration was measured by use of a Brafdford assay (Biorad) and subsequent photometrical detection at 595 nm. Lysates were mixed with 4 x sample buffer in appropriate amount and incubated at 95 °C for 5-10 min. Usually 50-100 µg of cell lysate were then loaded onto the SDS gel.

III.4.6.3 Preparation of virus and vector lysates

For preparation of viral lysates, supernatant of approximately 3×10^7 packaging cells (confluent T175 flask) was harvested. After filtration through a 0.45 µm filter (Sartorius) the samples were concentrated as described in chapter III.3. The appropriate amount of 4 x sample buffer was added, and the samples were incubated at 95 °C for 5-10 min and subsequently loaded on the SDS gel.

III.4.6.4 Deglycosylation of Western Blot samples

Deglycosylation of particles or lysates was performed by treatment with PNGaseF (NEB Biolabs) according to the manufacturers instructions. This enzyme deglycosylates proteins, as required to obtain distinct bands in Western Blot analysis. After incubation at 37 °C for 1 h, the appropriate amount of 4 x sample buffer was added and the samples were subsequently treated as described above.

III.4.6.5 Factor Xa cleavage

Factor Xa cleavage was carried out on concentrated samples using 1 μl enzyme (10 μg/μl NEB, UK) in 25 μl sample volume supplemented with 2 mM Calciumchloride. Samples were incubated one hour at 25 °C and were subsequently prepared for Western Blot analysis as described in chapter III.4.5.

III.4.7 Prion conversion methods

III.4.7.1 Conversion of retroparticles

The conversion reaction was carried out using the Chandler scrapie strain (Chandler, R L 1961). 25 µl of concentrated particles were therefore incubated with 1µl of 10% brain homogenate of Chandler-infected mice (lysis buffer: 100mM NaCl, 10mM EDTA, 0.5% Nonidet P 40 (Nonidet P-40), 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) overnight at 37 °C in an incubator. Samples were subjected to Proteinase K (PK) digestion and were then analyzed by Western Blot analysis.

III.4.7.2 Proteinase K digestion

Digestion with Proteinase K (PK) was performed using 20 µg/ml PK stocks (Qiagen). Vector and VLP stocks were incubated with appropriate amount of PK (0.5-5 µl in 20 µl sample volume) at 37 °C for 30 min. Reaction was stopped by addition of 4 × SDS sample buffer and incubated for 5-10 min at 95 °C.

III.4.8 Electron microscopy

III.4.8.1 Negative contrast

For detection of PrP displayed on retrovirus particles, virions concentrated from the cell supernatant were used. For immunonegative staining, 20 µl of virus suspension was adsorbed to glow discharged carbon coated formvar grids for 2 minutes. After rinsing in PBS, grids were incubated with 2% BSA for 30 minutes and with the primary antibodies 6H4 or anti-EGF at 1:750 dilution for 1h, respectively. After washing, grids were incubated with a 1:100 dilution of 10 nm gold labeled anti mouse IgG (BioCell, Cardiff, U.K.) for 30 minutes (antibodies: see Table 4). Finally, immunolabeled viruses were negatively stained with 2% uranylacetate or phosphotungstate for 10 seconds.

III.4.8.2 Ultrathin sections

Ultrathin frozen sections of virus producing cells were fixed with a mixture of 2% formaldehyde and 0.1% glutaraldehyde for 1 h. After washing, fixed cells were embedded in warm liquid agarose that after gelling could be cut into small blocks. These blocks were immersed overnight in 2.3 M sucrose containing 10% polyvinylpyrrolidone, frozen in liquid nitrogen and cut into 80-100 nm sections with an ultramicrotome (Ultracut E, Reichert, Vienna, Austria) with cryoequipment. Sections were mounted on carbon coated formvar grids and after thawing washed with PBS. After treatment with 2% BSA, grids were incubated with the 6H4 antibody at a 1:750 dilution. After rinsing in PBS, grids were incubated with anti-mouse IgG (1:100 dilution), coupled to 10 nm gold particles (BioCell). Finally, to embed and stain structures, the grids were floated, sections down, on 1.6% methylcellulose containing 0.2% uranylacetate for 5 minutes. Excess methylcellulose was aspirated before air drying the resulting thin film.
EM preparations were examined in a Zeiss EM 109 or 902 electron microscope and micrographs were taken on Kodak Estar electron microscope film.

IV Results

Basically, this thesis is divided into two major chapters, reflecting the two strategies for TSE vaccination development i) immune response against the cellular form of PrP and ii) immune response against the infective form of PrP. In the first chapter, the display of the cellular isoform of the prion protein (PrP^c) is addressed using three different approaches.

The first one deals with monomeric PrP^c display via fusion to a mammalian transmembrane domain (Display system). The other two approaches attempt trimeric and multimeric display of PrP^c, by selection of trimerizing (Envelope fusion system) or multimerizing (nucleocapsid particles) proteins as scaffolds for display.

The second chapter attempts the display of the scrapie isoform of the prion protein PrP^{Sc}. Either PrP^c displayed on the surface of the particles is converted into PrP^{Sc} (Conversion of retroparticles) using scrapie-infective agent or retroviruses are produced in scrapie-infected cells (Retroviral infection of scrapie-infected cells), aiming at the incorporation of PrP^{Sc} into the viral envelope.

IV.1 Display of the cellular prion protein

IV.1.1 Retroparticles

IV.1.1.1 MLV-derived display retroparticles

To present the cellular isoform of the murine prion protein (mPrP) on the surface of virus-like particles derived from retroviruses, a commercially available expression system was used (pDisplay, Invitrogen). The pDisplay system functions as multi-fusion protein, in which the target sequence is N-terminally flanked by the murine immunglobulin κ chain signal peptide and C-terminally flanked by the transmembrane domain of the platelet derived growth factor receptor (PDGFR) anchoring the protein into the plasma membrane.

The Display system also provides HA and myc tags for immunological detection and a factor Xa cleavage site for preparation of soluble PrP fusion proteins.

Upon insertion of the human epidermal growth factor (EGF) coding sequence into pDisplay, it had been shown, that the resulting fusion protein EGF^D expressed from pD-EGF (Figure 9) is unspecifically incorporated into virus-like particles (VLPs) derived from the murine leukemia virus (MLV) (Nikles, D et al. 2005). EGF^D is

present on the cell surface in high amounts and seems to be incorporated by viral particles in a passive process, while they bud from the cell surface resulting in the production of display retroparticles. In this way, viral particles harbouring the protein of interest in their membranes are produced.

As target cells for the production of PrP-displaying particles, the human kidney derived cell line HEK-293 was selected, especially a new subclone HEK-293FT, which grows faster and expresses the large T antigen (SV40) facilitating optimal viral production (*293FT-cells manual, Invitrogen*). Cotransfection of a plasmid coding for the viral structural proteins Gag/Pol and a plasmid coding for the transmembrane domain PrP-fusion-protein (PrP^D) should allow the production of PrP^D bearing VLPs, which were named PrP^D-retroparticles.

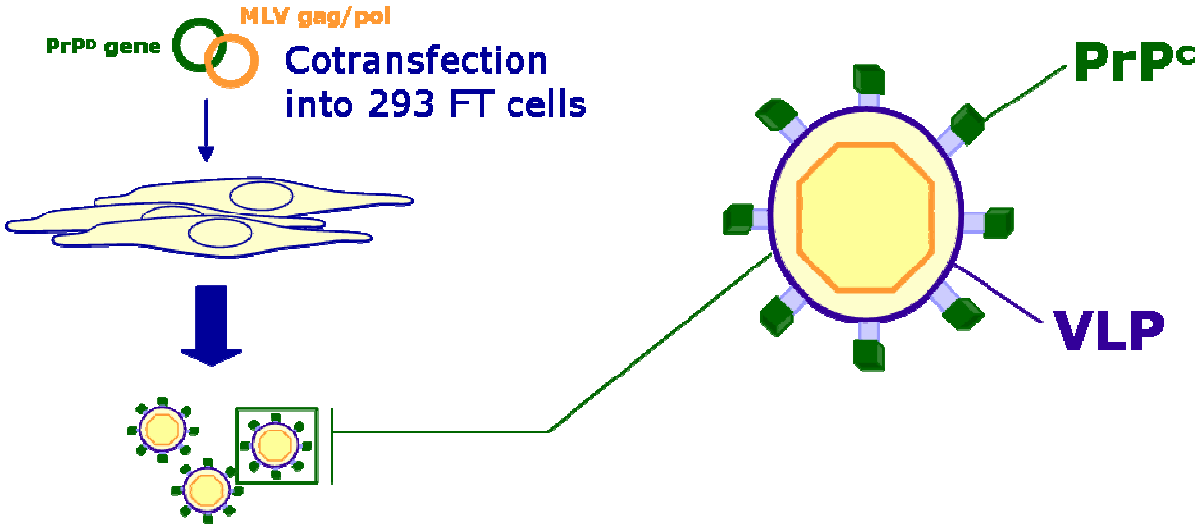


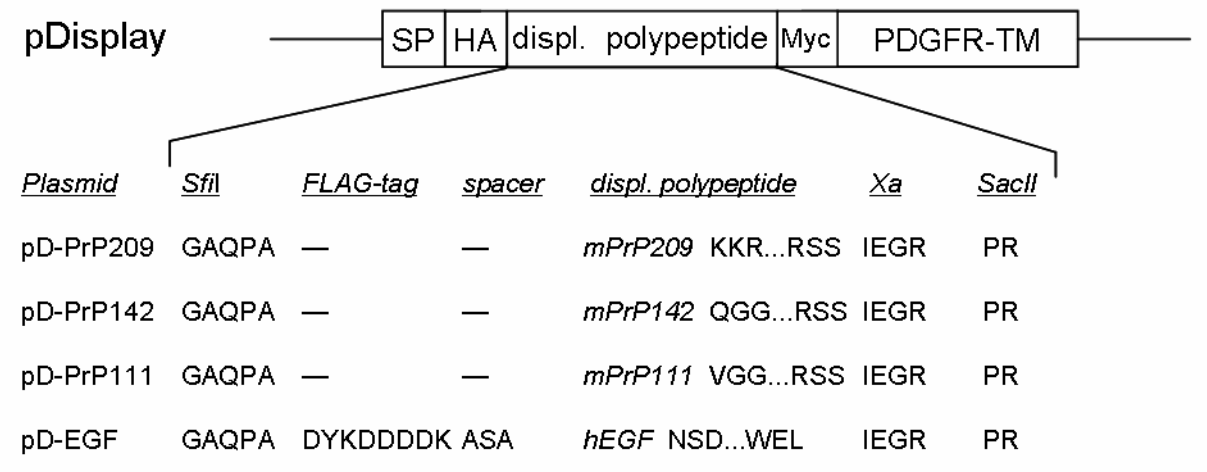
Figure 8 **Production of PrP^D retroparticles.** Plasmids coding for either the PrP^D sequence or the MLV structural proteins Gag and Pol were cotransfected into HEK-293FT producer cells, which will subsequently release VLPs incorporating PrP^D into the viral envelope (PrP^D-retroparticles).

IV.1.1.1.1 Generation of the expression plasmids

The expression plasmids, based on the pDisplay vector system, were constructed by ligation of mPrP DNA fragments coding for aa23-231 (mPrP209), aa90-231 (mPrP142) and aa121-231 (mPrP111) into the *Sfi*I and *Sac*II digested vector, respectively. For each DNA fragment two oligonucleotides were designed containing either the restriction site *Sfi*I (forward primer) or *Sac*II (reverse primer) as well as the factor Xa cleavage signal (reverse primer) and were flanked by homologues nucleotides of the PrP gene (Table 3).

The PrP encoding DNA fragments were synthesised by PCR using the designed oligonucleotides and the phgPrP plasmid (Appendix, VI.1.1) as template. The

resulting PCR fragments were cleaved by the corresponding restriction enzymes *Sfi*I and *Sac*II and inserted into pDisplay. Correct insertion of each of the PCR fragments verified by restriction analysis and DNA sequencing (Appendix, VI.3), resulted in the PrP-fusion-protein expression plasmids pD-PrP209, pD-PrP142 and pD-PrP111 (Figure 9). The corresponding protein PrP^{D142} was especially included as an intermediate candidate between PrP^{D209} and PrP^{D111}, when it was observed that the expression of these two forms showed high variation.



Plasmid	SfiI	FLAG-tag	spacer	displ. polypeptide	Xa	SacII
pD-PrP209	GAQPA	—	—	*mPrP209* KKR...RSS	IEGR	PR
pD-PrP142	GAQPA	—	—	*mPrP142* QGG...RSS	IEGR	PR
pD-PrP111	GAQPA	—	—	*mPrP111* VGG...RSS	IEGR	PR
pD-EGF	GAQPA	DYKDDDDK	ASA	*hEGF* NSD...WEL	IEGR	PR

Figure 9 Schematic representation of pDisplay derived expression plasmids. The pDisplay expression plasmid (Invitrogen) provides the immunoglobuline κ signal peptide (SP), the HA and myc tags, and the transmembrane domain of the PDGF receptor (PDGFR-TM). In addition, a factor Xa cleavage site was inserted N-terminally of the PDGFR-TM.

IV.1.1.1.2 Expression analysis by immune fluorescence

The expression of the different PrP^{D} proteins was examined in HEK-293FT and N2a cells. The latter were used to analyze the gene expression of PrP^{D} in PrP^{c}-expressing cells, because in contrast to HEK-293FT cells, the N2a cell line expresses PrP endogenously. The expression was analyzed after cotransfection of plasmids pD-PrP111 and pD-PrP209 together with the MLV gag/pol coding expression plasmid pHit60 (Appendix, VI.1.7), respectively. pD-EGF cotransfected with pHit60 served as positive control.

Transfected HEK-293FT (Figure 10) and N2a (Figure 11) cells were subjected to an epi-immune fluorescence analysis, performed by immunostaining by the specific anti-PrP antibody (6H4), the anti-EGF antibody or the anti-p30 serum (MLV capsid).

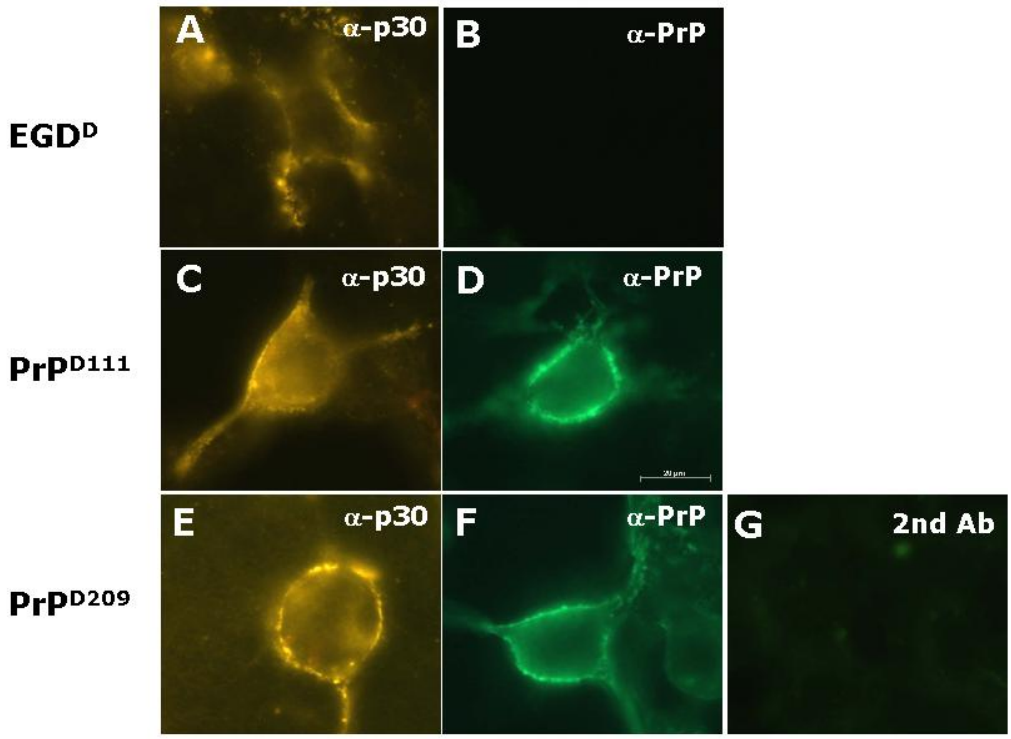


Figure 10 Cell surface expression of Display constructs encoding PrP fusion proteins. HEK-293FT cells were transfected with plasmids pD-EGF and pHit60 (A, B), pD-PrP111 and pHIT60 (C, D, G) or pD-PrP209 and pHIT60 (E, F), respectively. Cells were stained by the anti-PrP antibody 6H4 (B, D, F), with the anti-p30 serum (A, C, E), and bound antibody was detected by FITC-conjugated anti-mouse Ig (B, D, F, G) or Cy3-conjugated anti-goat Ig (A, C, E). Exposure times used for imaging of A-E and G were 20 sec, for F 200 sec. Epi-fluorescence is shown.

Specificity of the staining procedure was demonstrated as follows: HEK-293FT cells transfected with pD-EGF/pHit60 showed no immune fluorescence, upon staining by the 6H4 antibody (Figure 10, B) but showed signals upon staining with anti-EGF antibody (Figure 11, B). Furthermore, cells expressing PrP^{D111} proteins were negative upon staining by the second antibody, only (Figure 10, G and Figure 11 H). Strong and specific immune fluorescence signals were obtained for both, HEK-293FT and N2a cells expressing, the PrP^{D111} protein. This protein was detected to a high extend on the cell surface (Figure 10, D and Figure 11, F). In contrast, the anti-PrP staining in the PrP^{D209} sample was less efficient, and only single cells were antigen positive (Figure 10, F and Figure 11, K).

Both, the PrP samples and the EGF^{D} sample were equally well stained by the anti-p30 antibody detecting the MLV capsid protein (Figure 10 A, C and E as well as Figure 11, A, E and I).

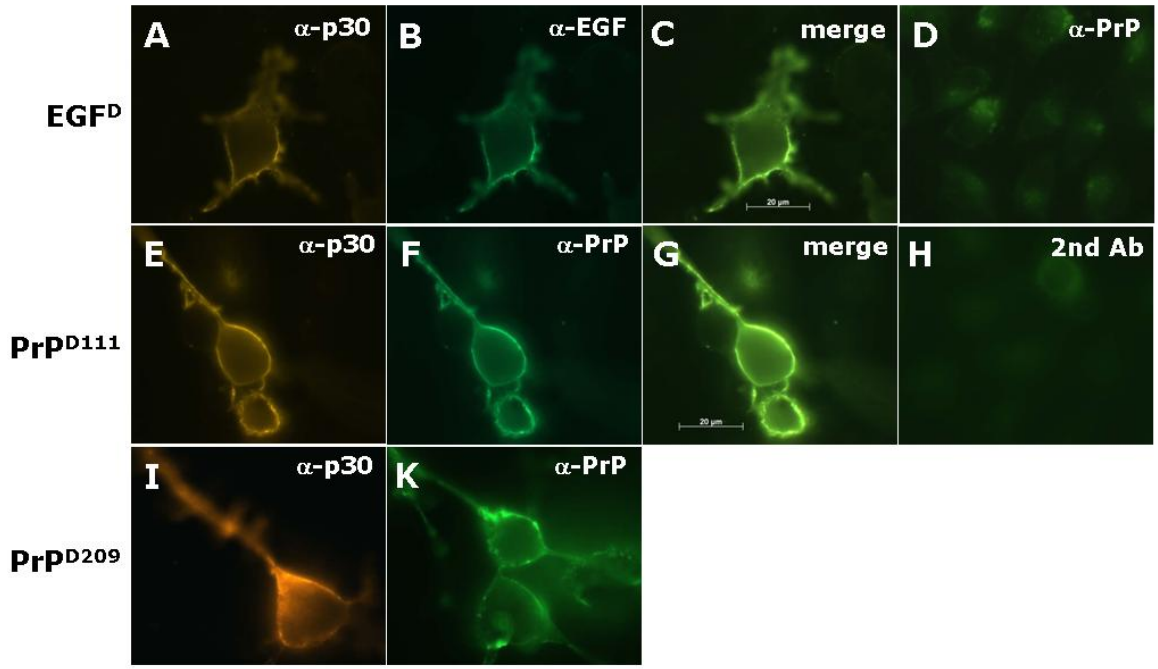


Figure 11 Cell surface expression of Display constructs encoding EGF and PrP fusion proteins on N2A cells. N2A cells were transfected with plasmids pD-EGF and pHIT60 (A-D) or pD-PrP111 and pHIT60 (E-H), respectively. Cells were stained by the anti-PrP antibody 6H4 (D, F), the anti-EGF antibody (B) or the anti-p30 antiserum (A, E), and bound antibody was detected by FITC-conjugated anti-mouse Ig (B, F, D, H) or Cy3-conjugated anti-goat Ig (A, E), respectively. The merge of A and B is shown in C, that of E and F in G. Exposure times are 20 sec except for I and K (200 sec). Epi-fluorence is shown.

Thus, compared to the endogenous PrP levels strong overexpression of the PrP^{D111} protein was achieved in HEK-293FT cells as well as in N2a cells. Upon coexpression of the MLV gag/pol genes, colocalization of the MLV capsid protein with the PrP^{D111} or the EGF^{D} molecules at the cell surface was demonstrated.
These findings suggest differences in the expression of the $PrP^{D}209$ in comparison to the PrP^{D111} proteins. However, the MLV capsid protein expression in these samples is equal, indicating that various expression rates of PrP^{D209} and PrP^{D111} are not due to basic protein expression mechanisms.

IV.1.1.1.3 Expression analysis by Western Blot

To further verify expression of PrP^{D} fusion proteins in the cells, cell lysates were analyzed by Western Blot. This method allows the specific staining of target proteins in dependence of their molecular weight and their separation properties on SDS polyacrylamid gels. The PrP^{D} fusion proteins PrP^{D111} (22.5 kD) and PrP^{D209} (32.3 kD) should be distinguishable from endogenous PrP protein (28.0 kD). In

addition, for PrP a typical band pattern is expected due to mobility shifts of non-, mono- and diglycosylated PrP forms (glycosylation sites: Asn^{180} and Asn^{196}).

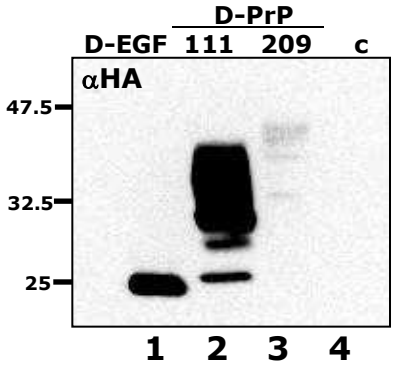


Figure 12 Expression of PrP^D proteins in HEK-293FT derived cell lysates. PrP^D proteins and the EGF^D control were detected by Western blot analysis using 16% polyacrylamide gels. For this, HEK-293FT cells were transfected with the pHIT60 plasmid in combination with either pD-EGF (lane 1), pD-PrP111 (lane 2), pD-PrP209 (lane 3) or an unrelated control plasmid (lane 4). Cell extracts were analyzed with the anti-HA tag antibody. Molecular weights are indicated.

Non-transfected cell lysates of HEK-293FT did not show detectable levels of endogenous PrP (Figure 12, lane 4). Whereas a single specific band is observed for the EGF^D protein, a panel of specific bands are seen for PrP^{D111} as well as for PrP^{D209} as expected for differently glycosylated PrP forms. In line with the results of the immune fluorescence analysis, higher expression levels for the PrP^{D111} protein and significantly lower levels for the PrP^{D209} protein were obtained (Figure 12, lanes 1-3).

IV.1.1.1.4 Expression analysis by electron microscopy

The immune fluorescence data supported the assumption, that PrP^{D111} is present on the cell surface. To further verify this assumption, electron microscopic analysis of cell sections were performed (Figure 13). Visualization of cell-surface located PrP^D was achieved by specific immunostaining with the anti-PrP 6H4 antibody. Secondary antibodies used for detection in electron microscopy were coupled to gold particles of 10 nm diameter. The specificity of the anti-PrP 6H4 staining was approved by using EGF^D expressing cells as negative control, for which no staining was observed (not shown).

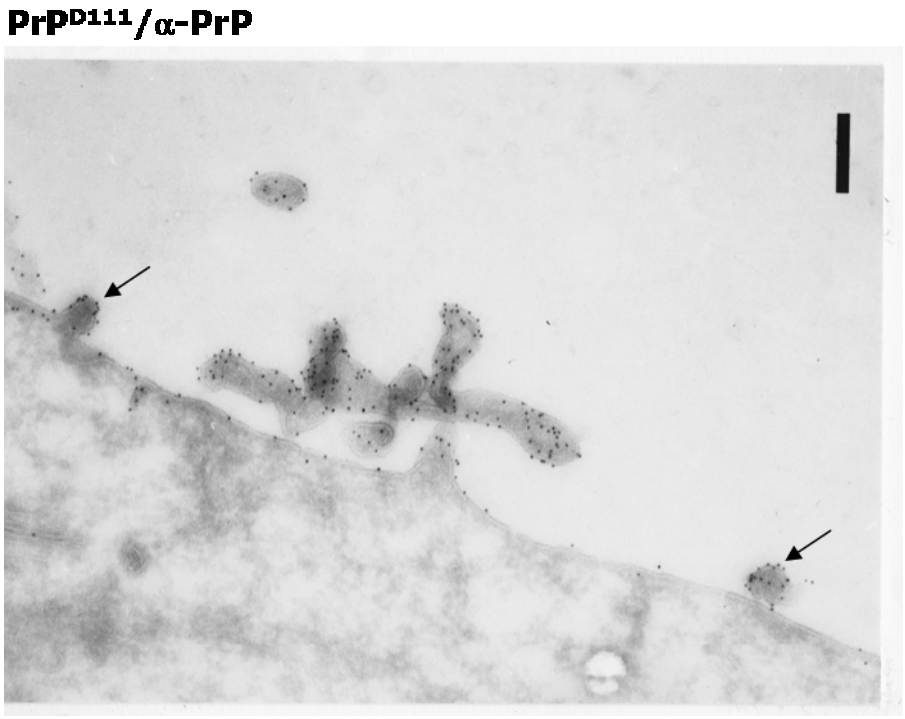


Figure 13 Immunoelectron microscopic analysis of cell sections. HEK-293FT cells were cotransfected with pD-PrP111 and pHit60 coding for MLV Gag and Pol and were then stained by anti-PrP 6H4 and immunogold labelled secondary antibodies. Bar represents 250 nm. Candidates for budding particles are labelled by the arrows.

The analysis for PrP^{D111} expressing cells revealed widely marked cell membranes stained by anti-PrP 6H4 antibody (Figure 13). In addition to membranes, also microvilli and vesicles were stained. The vesicle-like structure on the upper left side (marked by the arrow) of the image may represent a budding retrovirus particle.

Taken together, the expression of EGF^{D}, PrP^{D111}, and to a minor extend of PrP^{D209}, was observed in transfected HEK-293FT and N2a cells.

Determination of protein expression of the fusion proteins and colocalization with viral structural proteins facilitated the assumption that budding particles may incorporate the newly generated PrP^{D} proteins.

This hypothesis was addressed using Western Blot analysis (Figure 14) and electron microscopy analysis (Figure 15) of retroparticle-containing cell culture supernatants.

IV.1.1.1.5 Cosedimentation with particles

Cell culture supernatants from cotransfection of pD-EGF, pD-PrP111, pD-PrP142 or pD-PrP209 plasmids with pHit60, respectively, were harvested and filtered

through a 0.45 µm filter to get rid of cell debris and dead cells. After concentration of retroparticles via either low-speed centrifugation methods or ultracentrifugation methods (III.3), the supernatants were separated on SDS-polyacrylamid gels and were then analyzed via Western Blot.

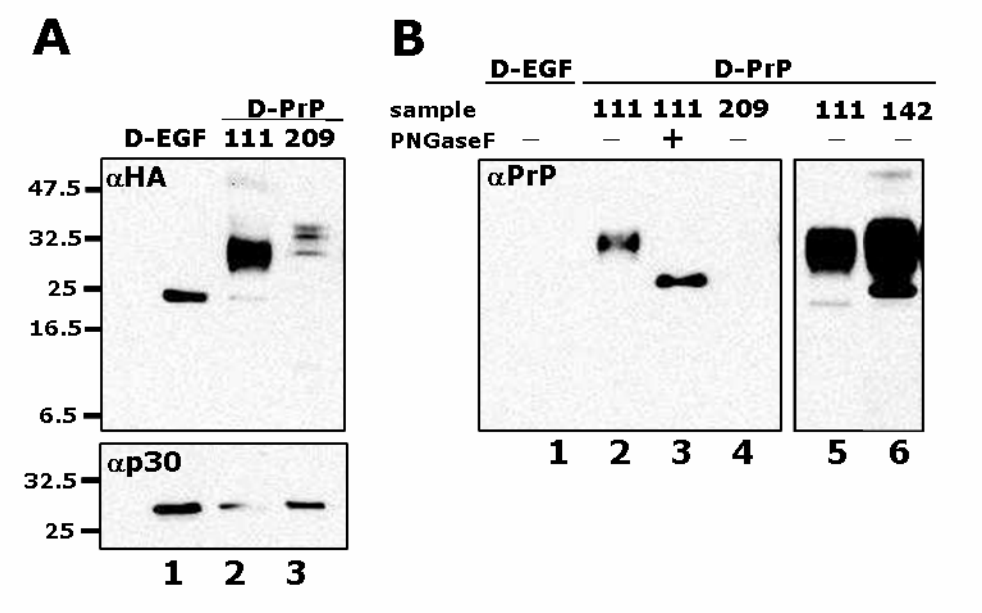


Figure 14 Incorporation of PrP^D fusion-proteins in VLPs. PrP^D proteins and the EGF^D control were detected by Western blot analysis using 16% polyacrylamid gels. A) Supernatants of cells transfected with pHIT60/pD-EGF (lane 1), pHIT60/pD-PrP111 (lane 2) and pHIT60/pD-PrP209 (lane 3) were concentrated by low speed centrifugation. Pellets were resuspended in PBS. Western blot was performed using the anti-HA antibody in the upper blot and the anti-p30 serum to detect the MLV capsid in the lower blot. Volumes loaded corresponded to the following amounts of cell culture supernatant: 0.7 ml (lane 1 upper), 0.35 ml (lanes 2 upper) and 13.5 ml (lane 3 upper), Volumes loaded on the lower blot corresponded to 1 ml of cell culture supernatant, each. B) Supernatants of cells transfected with pHIT60/pD-EGF (lane 1), pHIT60/pD-PrP111 (lanes 2, 3, 5), pHIT60/pD-PrP209 (lane 4) or pHIT60/pD-PrP142 (lane 6) were detected using the anti-PrP antibody 6H4. For PNGaseF digestion, particles were equilibrated in denaturing buffer and incubated in the presence (lane 3) or absence of PNGaseF (lane 2). Volumes loaded on the gels corresponded to 0.7 ml (lane 1), 0.35 ml (lanes 2, 3), 13.5 ml (lane 4), 0.8 ml (lane 5) and 1.2 ml (lane 6) of cell culture supernatants.

The EGF^D expression control was detected by the anti-HA tag antibody as single band at the expected molecular weight (Figure 14 A, lane 1). Particle numbers budding from the transfected producer cells were similar for EGF^D, PrP^{D}209 and PrP^{D}111 as revealed by the anti-p30 staining (Figure 14 A, lower panel).

The proteins PrP^{D111} and PrP^{D209} were specifically detected by the anti-HA tag antibody (Figure 14 A, upper panel, lanes 2 and 3) whereas specific signals upon anti-PrP 6H4 staining were obtained for PrP^{D111} and PrP^{D142} (Figure 14 A, lane 5 and 6), but not for PrP^{D209} (Figure 14 B, lane 4). The signal intensities for the two fusion

proteins PrP^{D111} and PrP^{D142} were similar suggesting that both proteins were equally well incorporated into retroparticles. Thus, the incorporation levels of PrP^{D142} and PrP^{D111} into retroparticles were substantially higher than that of PrP^{D209}.

To assure that the PrP^{D111} band pattern observed in the immunoblot (Figure 14 B, lane 2 and 5) was due to glycosylation of PrP^{D111}, treatment with Peptide:N-GlycosidaseF (PNGaseF) was performed (Figure 14 B, lane 3). A single band was obtained after deglycosylation, verifying that three different glycosylated forms of PrP, representing un-, mono- and deglycosylated PrP, are present in untreated samples (Figure 14 B, lane 2 and 5).

The retroparticles displaying PrP or fragments thereof were named PrP^{D}-retroparticles. Further studies focussed on the analysis of PrP^{D111}-retroparticles only, because they represented the most promising candidate for immunization studies at that time point.

IV.1.1.1.6 Characterization by electron microscopy

The results from Western Blot analysis suggested that the fact that PrP^{D} fusion proteins, especially when PrP is N-terminally shortened, were incorporated into retroparticles derived from MLV, but they did not exclude completely the possibility that signals in the supernatants were due to vesicle-like structures potentially budding from the cell surface. The latter would also penetrate the 0.45 µm filters and would co-sediment with viral particles upon centrifugation of the sample, because of similar weight and density.

Thus, to find out if PrP^{D} proteins were present in retroparticles, the cell culture supernatants were analyzed by electron microscopy (Figure 15). This method had been especially selected to differentiate between VLPs and vesicle-like structures. Vesicles vary in form and size whereas virus-like particles are regular, round-shaped and equal in size.

Low-speed centrifuged cell culture supernatants were immunostained by anti-PrP 6H4 and subsequently stained by a gold-labelled anti-mouse antibody, as described in detail in chapter IV.1.1.1.4.

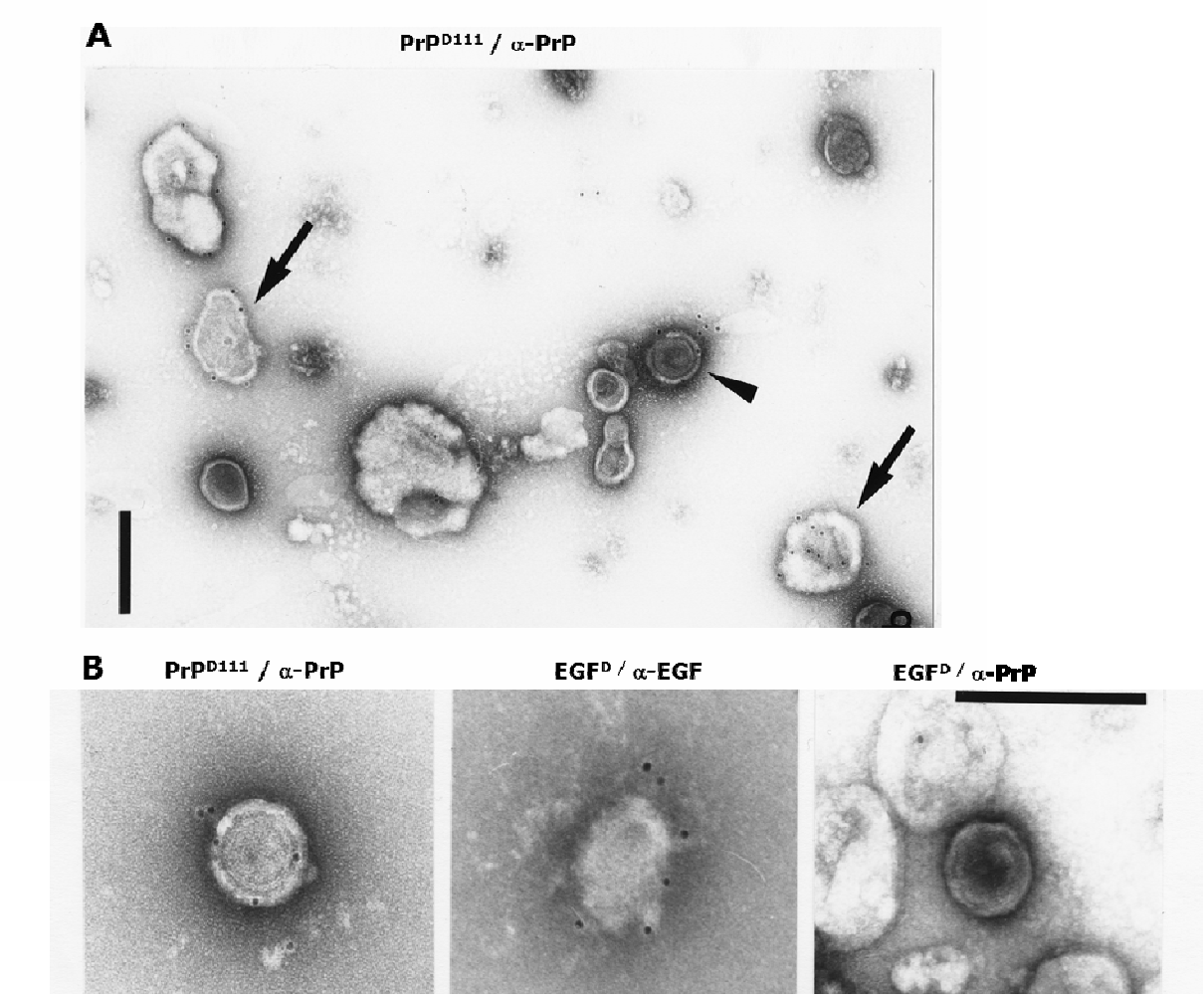


Figure 15 Immunoelectron microscopic analysis of PrP^D-retroparticles. PrP^{D}111-retroparticles harvested from the cell culture supernatant and concentrated by low speed centrifugation were stained with the PrP specific 6H4 antibody (A, B left panel) and a 10 nm gold particle labelled anti-mouse IgG. EGF retroparticles (B middle and right panel) were harvested from pHIT60/pD-EGF transfected 293FT cells and stained with the anti-EGF (middle panel) or the 6H4 antibody (right panel). Bars represent 250 nm. Arrowheads indicate particles with the typical morphology of C-type retroviruses, arrows indicate pleomorphic vesicles. Positive staining events are visible as dots.

The specificity of the anti-PrP 6H4 staining was confirmed by using EGF^D particles as controls. EGF^D retroparticles revealed specific signals upon staining by an anti-EGF antibody (Figure 15 B).

The cell culture supernatants contained morphologically different structures (Figure 15 A). On one hand particles exhibiting the typical morphology of C-type retroviruses were present. On the other hand also pleomorphic vesicle-like structures were detectable. Both types of particles were present in roughly similar amounts and were surrounded by numerous immunogold particles.

These results clearly demonstrated that retroparticles incorporate PrP^{D111} and display the protein on their surface. Moreover, preparations concentrated by low-

speed centrifugation of cell culture supernatant contain vesicles and vesicle-like structures, presenting PrP^{D111} as well.

IV.1.1.1.7 Quantification of particles

Since transient transfection may strongly differ in efficiency, a method to quantify the amount of PrP^{D}-retroparticles released from producer cells was established. For this, the reverse transcriptase (RT) assay has been used, which determines the activity of the RT in stocks of retroviral particles (*C-type RT activity kit, Cavidi Tech*). Various particle preparations concentrated by low-speed centrifugation were diluted and subjected to RT analysis (Figure 16).

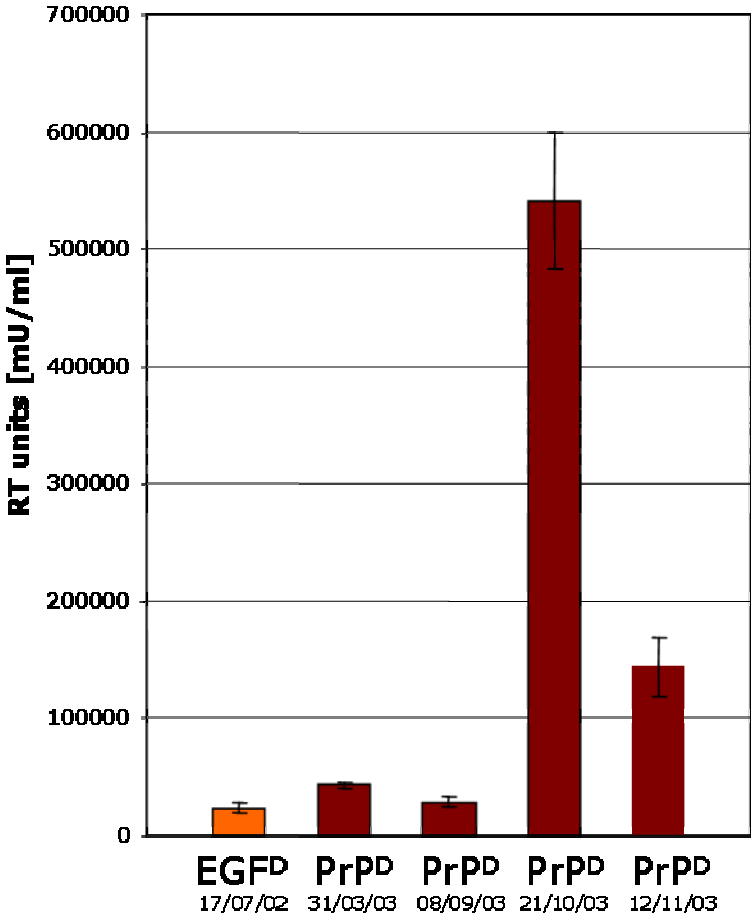


Figure 16 **Quantification of concentrated particle stocks.** Particles were measured in a C-type reverse transcriptase (RT) activity assay. Average values of different PrP^{D}-particle and of EGF^{D}-particle preparations are shown. For particle production, three EGF^{D} (17/07/02), PrP^{D} (31/03/03), six (12/11/03) or twelve (21/10/03) T175 cell culture flasks of HEK-293FT cells were transfected. Average values of at least three measurements are shown.

The amounts of different particle preparations of PrP^{D111}-retroparticles, which were subsequently used in immunization studies, varied between 20-500 RT

units per ml, depending on the amount of cells used for transfection. In addition, the variation may be due to different transfection efficiencies and cell densities as well as the state of the producer HEK-293FT cells. There was basically no difference in the amounts of EGF^{D}-retroparticles compared to PrP^{D111}-retroparticles when they were prepared under similar conditions.

Based on the RT units obtained, particle numbers of the different preparations were calculated as described (Pyra, H, Boni, J et al. 1994) (Figure 17).

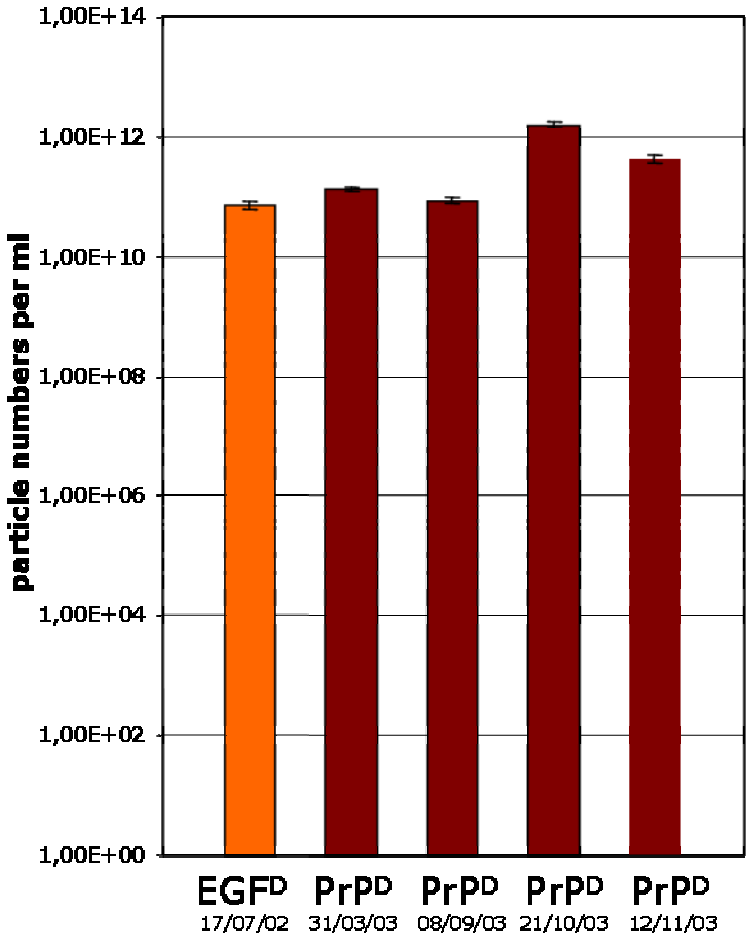


Figure 17 **Calculated particle numbers in concentrated preparations.** Total particle numbers were calculated based on the measured RT units described above. For this, the equation $n_{CP} = 3 \times 10^9$ [p/units] × RT-value [units] was applied (Pyra, H, Boni, J et al. 1994). n_{CP} represents the number of concentrated particles.

Each preparation contained at least 10^{10} PrP^{D111}-retroparticles, verifying that the concentration methods used in this study were successful.

In summary, PrP^{D111}-retroparticles were successfully generated in high quantities. However, particle numbers may not directly reflect the amount of PrP^{D111} protein present in each preparation. Therefore, another method for PrP-quantification had to be established.

IV.1.1.1.8 Quantification of PrP in particle preparations

For mouse immunization experiments a method for quantitative determination of the amounts of PrP, present in different particle preparations, had to be established. As the method of choice ELISA was used to compare PrP-specific antibody binding in a sensitive assay (Figure 18). The particles were analyzed by coating log 3 dilutions of concentrated stocks (100 RT units/ml) on ELISA plates. For this purpose, particles were coated on plastic dishes and subsequently detected using the anti-PrP 6H4 as first and an anti-mouse-HRP conjugated secondary antibody. To quantify the total number of particles anti-p30 sera in combination with a goat-specific secondary HRP-labelled antibody was used.

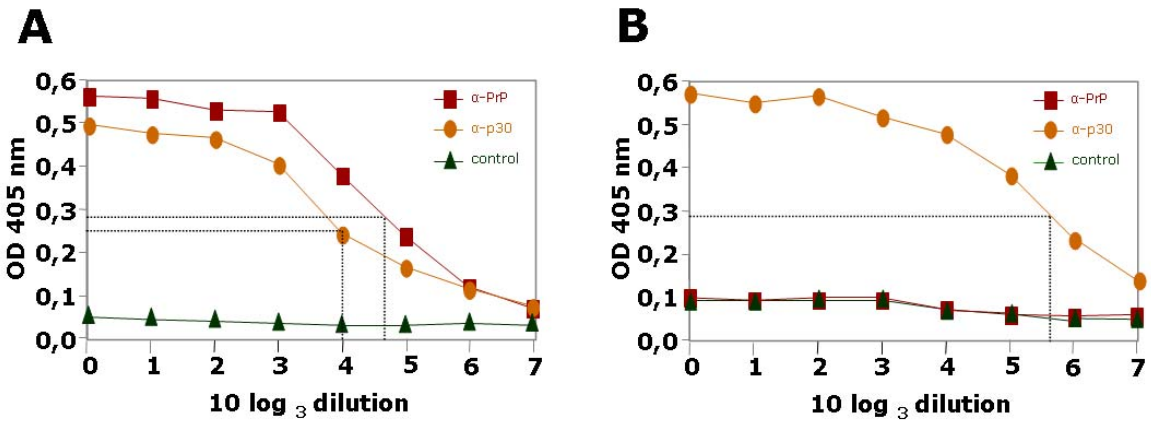


Figure 18 Detection of the amounts of PrP in PrP^{D}-retroparticles. Analysis of PrP^{D111}-retroparticles (A) and of EGF^{D}-retroparticles (B) by ELISA. Concentrated stocks of both particle types were coated to ELISA plates at the indicated dilutions. Virally expressed antigens were detected by use of the anti-PrP monoclonal antibody 6H4 (red rectangles), polyclonal anti-p30 antiserum (orange ovals), or mouse preimmune serum (green triangles). Data represent one out of two experiments with similar results. Half-maximal dilution are indicated by dotted lines. Figure taken from (Nikles, D, Bach, P et al. 2005).

While EGF^{D}-retroparticles showed no 6H4 binding but strong anti-p30 binding (Figure 18, B), PrP^{D111}-retroparticles showed strong binding of both, the PrP-specific 6H4 antibody and of the anti-p30 antibody (Figure 18, A). As dilutions at half-maximum values for PrP^{D111}-retroparticles 800fold dilution detected by anti-p30 serum and 1600fold dilution detected by anti-PrP 6H4 antibody were calculated, whereas EGF^{D}-retroparticles showed 4900fold dilution at half maximum value.
Throughout this study the ELISA was used to standardize particle preparations for immunization purposes.

Based on the studies described above, an optimal protocol for particle production was determined. According to this protocol, which is described in detail in chapter III.3 twelve T175 cell culture flasks were transfected by pDPrP111/pHit60, concentrated and separated into small portions followed by storage until further use in immunization studies.

IV.1.1.1.9 Induction of PrP-specific antibody responses

To verify the antigenicity of the PrP^{D111}-retroparticles, different types of mice were immunized. Firstly, Prnp knock-out mice (*Prnp*$^{o/o}$) were subjected to immunization experiments, in which the immune response was expected to be high, because PrP is no self-antigen in these animals. Since the main goal was to circumvent the tolerance in animals expressing PrP, both *Prnp* heterozygous mice (*Prnp*$^{+/o}$) and wild-type mice (*Prnp*$^{+/+}$) expressing either one or two *Prnp* alleles were included in immunization studies.

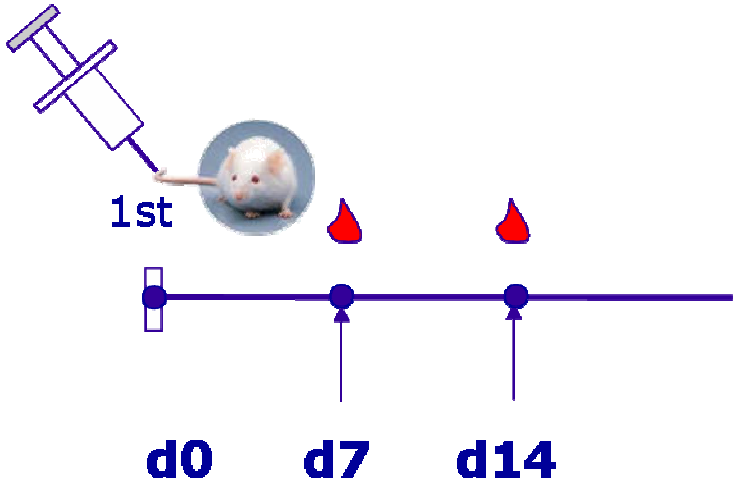


Figure 19 Immunization scheme used for PrP^{D}-retroparticle immunization. $Prnp^{0/0}$, $Prnp^{0/+}$ and $Prnp^{+/+}$ mice were immunized with 10^{11} particles in 200 µl PBS/1% FCS solution using five mice were per group. Bleeding was carried out on day 7 and on day 14 post immunization.

For this purpose, groups of five mice were i.v. immunized with approximately 10^{11} PrP^{D111}-retroparticles and serum was taken weekly (Figure 19).

Already seven days after immunization, *Prnp*$^{o/o}$ mice showed high titers of PrP-specific antibodies as indicated by an ELISA, detecting PrP-specific immunoglobulin of the M, G, and A subclasses (Figure 20).

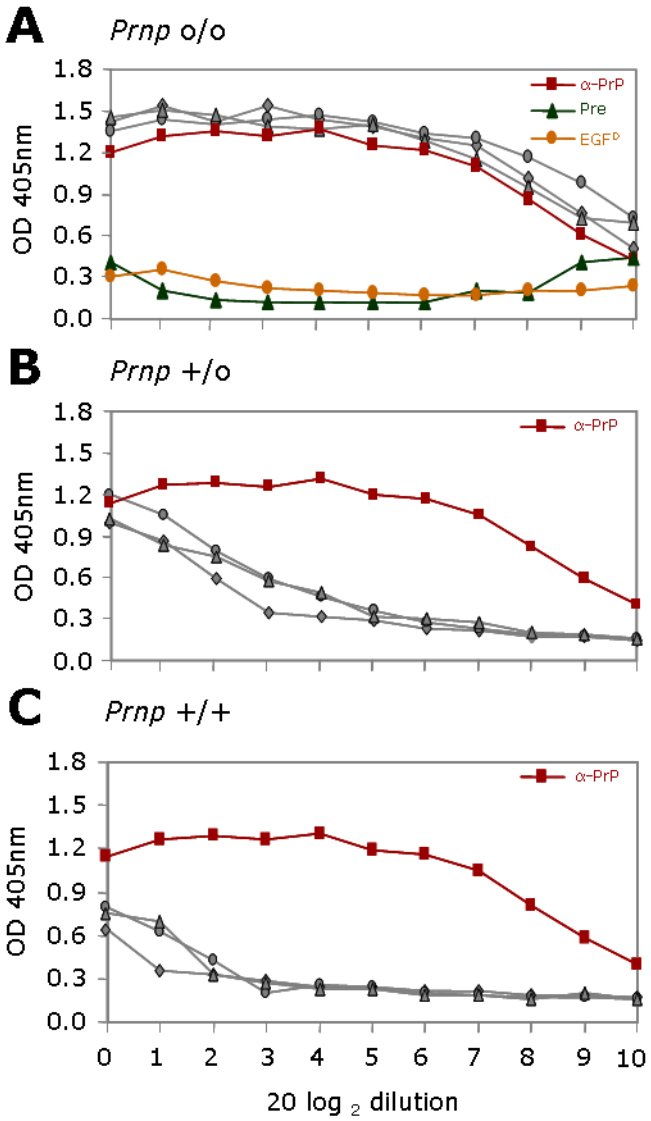


Figure 20 Sera of mice immunized with PrP^{D}-retroparticles specifically bind recombinant PrP in ELISA analysis. PrP^{D111}-retroparticles devoid of adjuvant were i.v. injected into three individual mice of the Prnp$^{o/o}$ (A), Prnp$^{+/o}$ (B), or Prnp$^{+/+}$ (C) genotypes, respectively (open symbols). Seven days after immunization serum samples were taken and tested in log 2 serial dilutions (20-fold predilution) for the presence of PrP-specific total Ig reactive against bacterially expressed PrP$^{REC121-231}$. As controls, the anti-PrP monoclonal antibody 6H4 (filled rectangles), pre-immune serum (filled triangles) or serum from mice injected with EGF^{D}-retroparticles (filled circles) was used. One out of two similar experiments is shown. Figure taken from (Nikles, D, Bach, P et al. 2005)

Interestingly, despite the expression of one or two *Prnp* alleles, also *Prnp*$^{+/o}$ and *Prnp*$^{+/+}$ mice mounted PrP-specific antibody responses, albeit at substantially reduced levels when compared to *Prnp*$^{o/o}$ mice (Figure 20 C). Essentially similar response curves were monitored with sera obtained 14 days after immunization (data not shown). PrP-specific antibody responses in *Prnp*$^{+/+}$ mice were slightly

lower than in *Prnp*$^{+/o}$ mice which might be explained by a higher level of PrP-specific tolerance in *Prnp*$^{+/+}$ mice than in *Prnp*$^{+/o}$ mice.

Next it was studied whether serum antibodies showing PrP-specific binding in an ELISA assay also bind the native form of PrP^{c} as expressed on the cell surface (Figure 21).

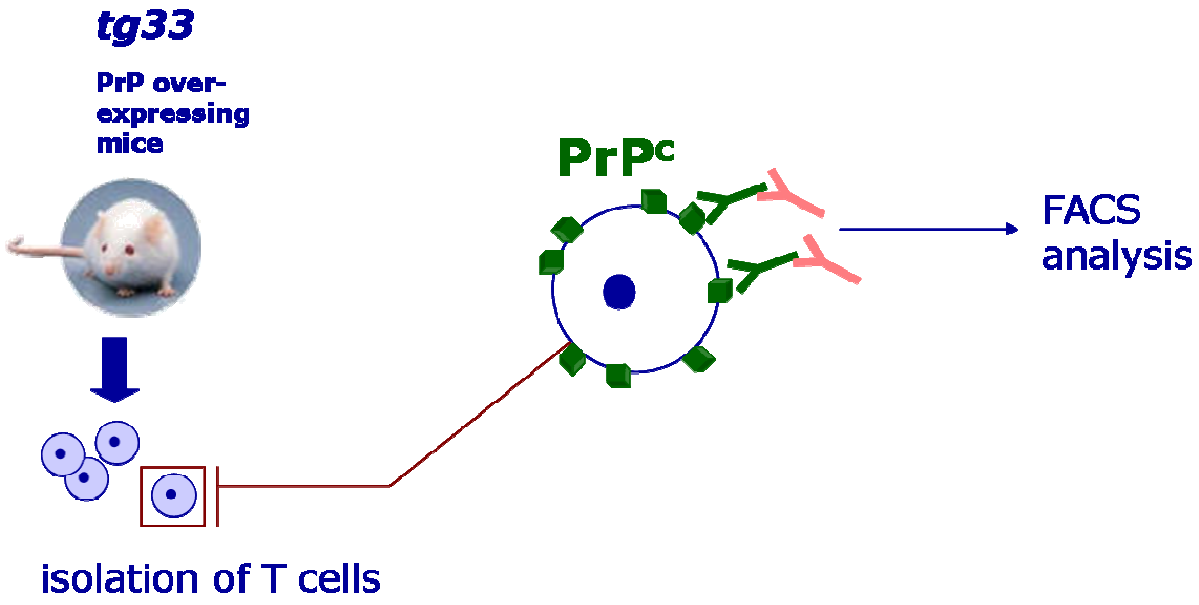


Figure 21 Schematic representation of FACS assay to detect cell surface PrP binding. T cells were isolated out of blood from PrP^{c}-overexpressing mice (tg33). Sera from immunized Prnp$^{0/0}$, Prnp$^{0/+}$ and Prnp$^{+/+}$ mice were analyzed for binding to PrP^{c} on the surface of the isolated T cells. Analysis was performed using FACS method.

To this end, peripheral blood was taken from tg33 mice overexpressing PrP on T lymphocytes (Raeber, A J et al. 1999), and CD3 positive T cells were tested for binding of serum IgM or IgG derived from immunized mice by FACS analysis (Figure 22).

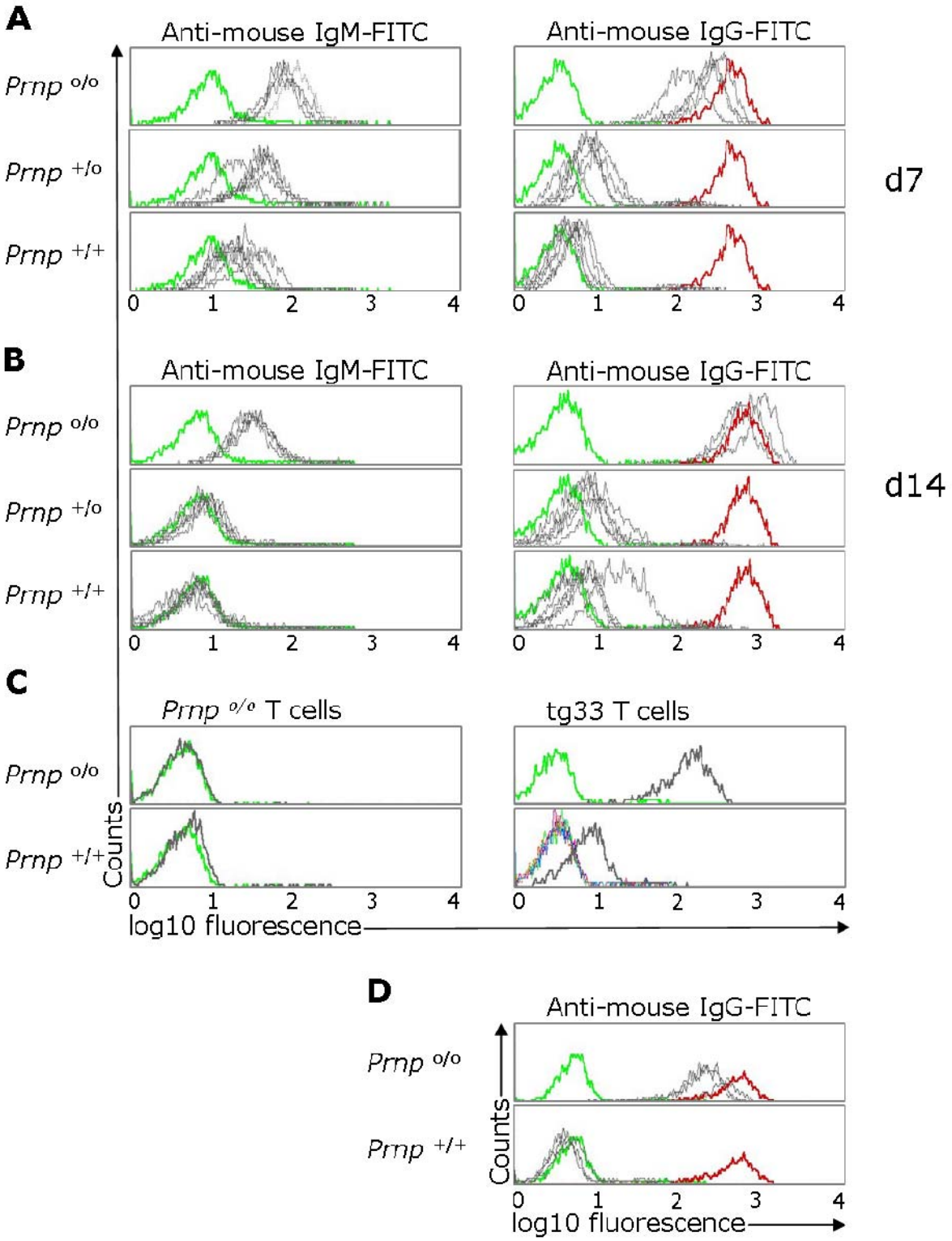


Figure 22 Sera of mice immunized with PrP-retroparticles specifically bind the native form of PrP as expressed on the cell surface of tg33 derived T cells. Serum samples were taken seven (A) or 14 days (B) after i.v. injection of $PrP^{D}111$-retroparticles into $Prnp^{o/o}$, $Prnp^{+/o}$ or wild-type mice ($Prnp^{+/+}$) and analyzed for their reactivity against PrP as expressed on T cells derived from PrP overexpressing tg33 transgenic mice. Five individuals per group were analyzed (grey lines). IgM (left panels) and IgG subtypes (right panels) were determined. Cells incubated with preimmune serum (green) or 6H4 (red) were used as

controls. C) Sera from immunized $Prnp^{o/o}$ or wild-type mice ($Prnp^{+/+}$) were tested for the reactivity of IgG subtypes with T cells from $Prnp^{o/o}$ mice (left panels, grey lines) or with T cells from tg33 mice (right panels, grey lines). Preimmune sera are shown in green in left panels. Preimmune sera from five individual $Prnp^{+/+}$ mice (lower right panel, coloured lines) or pooled preimmune sera from seven $Prnp^{0/0}$ mice (upper right panel, green line) were tested for IgG reactivity against PrP as expressed on tg33 T cells. D) $Prnp^{o/o}$ mice immunized with PrP^{REC} in CFA/IFA mounted significant anti-PrP antibodies, whereas $Prnp^{+/+}$ were unresponsive to immunizations. Bacterially expressed PrP^{REC} emulsified in CFA and IFA was injected into mice of the $Prnp^{o/o}$ and $Prnp^{+/+}$ genotype, respectively (Polymenidou, M, Heppner, F L et al. 2004). 28 days after immunization, serum samples were analyzed for their IgG reactivity against PrP as expressed on tg33 derived T cells. Three individuals were analyzed per group (grey lines). Cells incubated with preimmune serum (green) or 6H4 (red) were used as controls. All histograms (A-D) show PrP-specific binding gated on CD3-positive T cells. Figure taken from (Nikles, D, Bach, P et al. 2005)

Seven days after immunization of *Prnp*$^{o/o}$ mice, serum showed PrP^{c}-specific IgM binding that was slightly decreased by day 14, whereas strong PrP^{c}-specific IgG binding was detected on day 7, that was further increased by day 14, even beyond the binding strength of the positive control 6H4 (Figure 22 A, B). Reminiscent of the above ELISA results, 7 days after immunization of *Prnp*$^{+/o}$ mice, serum showed PrP^{c}-specific IgM binding that was slightly lower than that of *Prnp*$^{o/o}$ mice and slightly higher than that of *Prnp*$^{+/+}$ mice (Figure 22 A), whereas 14 days after immunization PrP^{c}-specific IgM was not detectable, neither in serum of *Prnp*$^{+/o}$ nor *Prnp*$^{+/+}$ mice (Figure 22 B). Compared to *Prnp*$^{o/o}$ mice, PrP^{c}-specific IgG binding was substantially reduced in the serum of *Prnp*$^{+/o}$ and *Prnp*$^{+/+}$ mice, whereas serum of *Prnp*$^{+/o}$ mice showed slightly higher binding than that of *Prnp*$^{+/+}$ mice. Interestingly, some PrP^{c}-specific IgG was still detectable 14 days after immunization (Figure 22 B). To verify the specificity and reliability of the FACS method used for determination of PrP^{c}-reactive antibodies a number of control experiments were performed.

Firstly, N2a neuroblastoma cells and tg33 derived T cells incubated with 6H4 antibody or various different PrP-reactive immune sera showed very similar staining, indicating that the tg33 based FACS method revealed binding of PrP^{c} as expressed on normal cells (data not shown). Secondly, a potential inter-individual heterogeneity with respect to spontaneous PrP-specific antibody titers was assessed. To this end, individual preimmune sera and pools of preimmune sera derived from *Prnp*$^{+/+}$ and *Prnp*$^{o/o}$ mice were assayed for binding to tg33 cells in the tg33 based FACS assay. In no case significant PrP binding of preimmune serum was observed (Figure 22 C, right panel; data not shown). Furthermore, the very narrow scattering of the blots of staining with individual preimmune sera

demonstrated the reliability and reproducibility of the FACS method used (Figure 22 C, bottom right panel). Thirdly, for further verify the PrP-specificity of serum binding, we included *Prnp*$^{o/o}$ derived T cells in the FACS analysis as negative controls. Even if strong binding of tg33 derived T cells was observed, no IgG binding of *Prnp*$^{o/o}$ derived T cells was detected, neither with serum from *Prnp*$^{o/o}$ nor with serum from *Prnp*$^{+/+}$ mice (Figure 22 C, left panels). Finally, sera from *Prnp*$^{o/o}$ and *Prnp*$^{+/+}$ mice immunized with bacterially produced PrP^{REC} were analysed by the tg33 based FACS assay (Gilch, S, Wopfner, F et al. 2003; Polymenidou, M, Heppner, F L et al. 2004). In contrast to the sera obtained from immunizations with PrP^{D111}-retroparticles, we could not detect any antibodies in *Prnp*$^{+/+}$ mice recognising the native cell surface exposed PrP (Figure 22 D). This failure of PrP^{REC} to circumvent tolerance in wild-type animals is in line with recently published data (Polymenidou, M, Heppner, F L et al. 2004).

In conclusion, PrP^{D111}-retroparticles are good B cell antigens as indicated by strong antibody responses induced in *Prnp*$^{o/o}$ mice. Furthermore, PrP^{D111}-retroparticles can induce low but significant native PrP^{c}-specific IgG antibodies in mice carrying one or two *Prnp* wild-type alleles, thus being superior to recombinant, bacterially expressed PrP.

IV.1.1.2 HIV-derived display retroparticles

Virus-like particle production, as described above for the C-type retrovirus candidate MLV, is in principle also possible with lentiviral particles. Since vector systems based on the lentivirus HIV are available and the viral structural proteins coded by gag and pol are sufficient for particle budding, these viral particles can be produced in the same way as MLV derived VLPs. In terms of immunization studies on the basis of PrP^{D}-retroparticles, the advantage of an alternative system, which displays PrP in a different context, lies in the possibility to boost antibody titers against PrP by a prime/boost protocol to enhance immunization efficiencies. For this purpose, HIV-derived VLPs displaying PrP^{D111}, which were subsequently named PrP^{D}-lentiparticles, were generated.

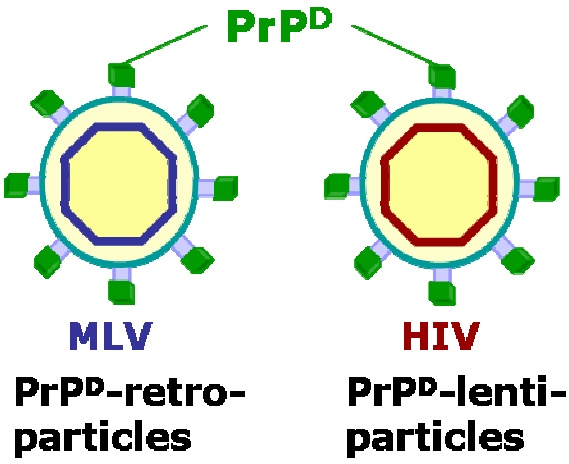


Figure 23 Comparison of PrP^D-retro- and lentiparticles. PrP^D-retroparticles are derived from the murine leukaemia virus MLV (blue capsid). PrP^D-lentiparticles (red capsid) were produced using human immunodeficiency virus derived structural proteins encoded by gag and pol genes.

IV.1.1.2.1 Incorporation analysis by Western Blot

For the generation of HIV-derived PrP^{D}111 lentiparticles, the HIV gag/pol coding plasmid pCMVΔR8.2 (Appendix, VI.1.7) was cotransfected along with pD-PrP111 into HEK-293FT cells to produce HIV-derived VLPs bearing PrP^{D111} on their surface. The expression and incorporation of the fusion protein was addressed by Western Blot analysis.

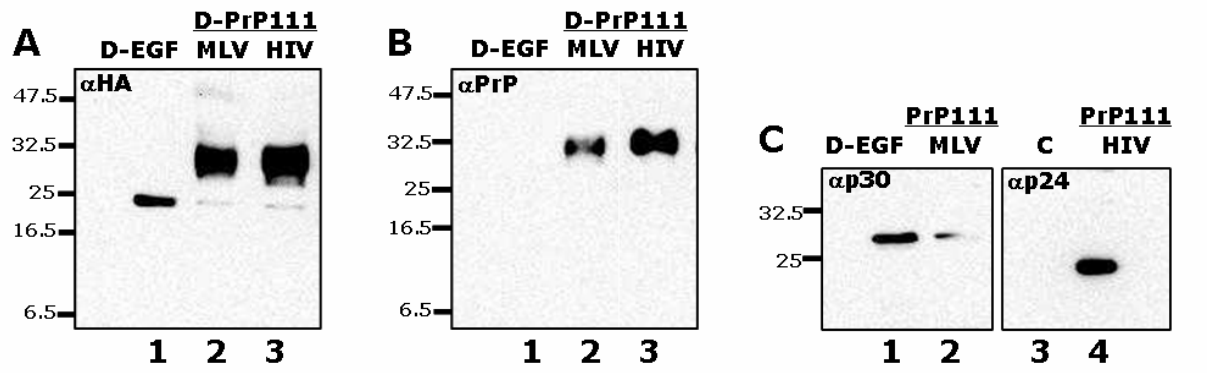


Figure 24 Western Blot analysis of PrP^D-lentiparticles. PrP^{D111} and the EGF^D control were detected by Western blot analysis using 16% polyacrylamid gels. A) and B) Particles from supernatants of cells transfected with pHIT60/pD-EGF (lane 1), pHIT60/pD-PrP111 (lane 2) and pCMVΔR8.2/pD-PrP111 (lane 3) were concentrated by low speed centrifugation. Pellets were resuspended in PBS. Volumes loaded corresponded to the following amounts of cell culture supernatant: 0.7 ml (lane 1), 0.35 ml (lanes 2) and 0.88 ml (lane 3). Western blot was performed using the anti-HA antibody (A) or the anti-PrP 6H4 antibody (B). C) Supernatants of cells transfected with pHIT60/pD-EGF (lane 1, 3), pHIT60/pD-PrP111 (lane 2) and pCMVΔR8.2/pD-PrP111 (lane 4) were loaded. Volumes corresponded to 1 ml of cell culture supernatant, each. Blots were stained by anti-p30 serum (left panel) or an anti-p24 monoclonal antibody (right panel), respectively.

EGF^D was included as molecular weight marker and as specificity control for the anti-PrP 6H4 antibody (Figure 24 A and B, lane 1). Staining against the respective capsid proteins (p30 for MLV and p24 for HIV), was performed to demonstrate presence of lentiparticles (Figure 24 C). The signals present in the supernatant of cotransfected cells were compared to those of MLV-derived PrP^{D111} retroparticles (Figure 24 A, lane 2 and 3). Roughly twice as much supernatant was loaded compared to retroparticles, the signal from PrP^{D111} in lentiparticles, for which the double amount was loaded, was stronger thus pointing to equal well incorporation into lentiparticles. In addition to anti-HA tag antibody stain (Figure 24 A), detection with the anti-PrP 6H4 antibody was also possible (Figure 24 B).

Thus, the Western Blot data indicated the presence of PrP^{D111} fusion protein in lentiparticles.

IV.1.1.2.2 Incorporation analysis by electron microscopy

The incorporation into HIV-deribed lentiparticles was then further investigated by electron microscopy to assure presence of PrP^{D111}. For this, cell culture supernatants of pDPrP111/pCMVΔR8.2 cotransfected HEK-293FT cells were subjected to immunogold labelling analysis (Figure 25).

Control particles derived from HIV bearing EGF^D were not stained by anti-PrP 6H4 (Figure 25 C).

The PrP^{D111} lentiparticles derived from HIV were specifically stained by the anti-PrP 6H4 antibody in combination with the immunogold labelled second antibody (Figure 25 A, B). At this, there were vesicle-like as well as virus-like particles visible in the image as it was observed for MLV-derived VLPs before. Overall, the amount of particles was lower copared to MLV-derived retroparticles and the vesicle to virus-like particle ratio was increased. Nevertheless, both structures were equally well labelled by immunogold in comparison to MLV-derived retroparticles.

Taken together these data verify that PrP^{D111} fusion protein was incorporated into HIV-derived lentiparticles in the same way as it was observed for MLV-derived retroparticles. These particles are available for immunization studies.

Thus, an additional particle type which can be used to boost primary immune responses against PrP^D-retropartciles was generated. Nevertheless, the retroviral capability to package genetic information offers the possibility to combine nucleic

acid and protein vaccination approaches. The hypothesis that particles, which additionally package genetic information of PrP may elicit even higher immune responses led to the design of retroviral vectors.

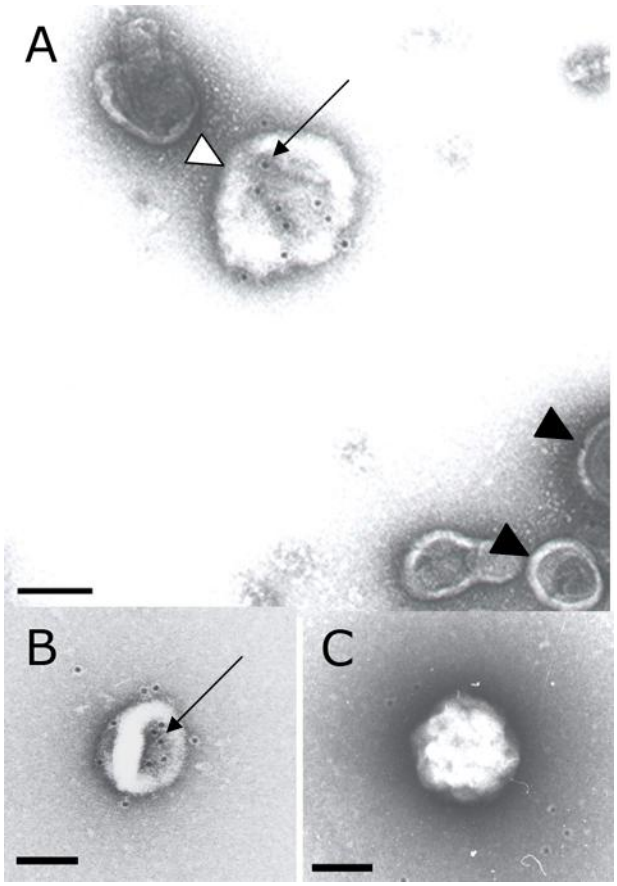


Figure 25 PrP^{D}111 protein is present in HIV-derived VLPs. HIV-derived PrP^{D}111 particles (A, B) and HIV-derived EGF^{D} particles (C) were stained by the anti-PrP 6H4 antibody and a secondary antibody labelled by 10 nm gold-particles. Bars represent 100 nm. Gold-labelling is marked by arrows. Virus-like particles are indicated by filled arrowheads. Vesicle-like structure are indicated by open arrowheads.

IV.1.1.3 Lentiviral vector system

Besides the insertion of PrP into the membrane of particles, the PrP^{D} coding sequence was also packacked into a lentiviral vector. This should allow transfer of the PrP^{D} coding sequence into cells, which can not easily be transfected with plasmids. Moreover, upon transduction with vector particles, stably PrP^{D} expressing cell lines can be established.

The possibility to directly transduce retroviral packaging cell lines, as e.g. Phoenix-gp (Nolan, G), TelCeB15 (Cosset, F L et al. 1995) (both MLV gag/pol producer cells) or 293 STAR (Ikeda, Y et al. 2003) (HIV gag/pol producer cells), will enable the generation of stably PrP^{D}-particle producer cell lines. Transduction

of the HIV gag/pol packaging cell line 293 STAR would lead to a stable cell line generating viral vectors. On the contrary, transduction of Phoenix-gp or TelCeB15 packaging cell lines would lead to stable production of VLPs without packaged mPrP coding sequence. At this, the budding MLV-derived retroparticles do not encapsidate mPrP containng a lentiviral encapsidation signal, because retroviral packaging involves specific encapsidation signals (Berkowitz, R et al. 1996) differing for HIV and MLV (Figure 26).

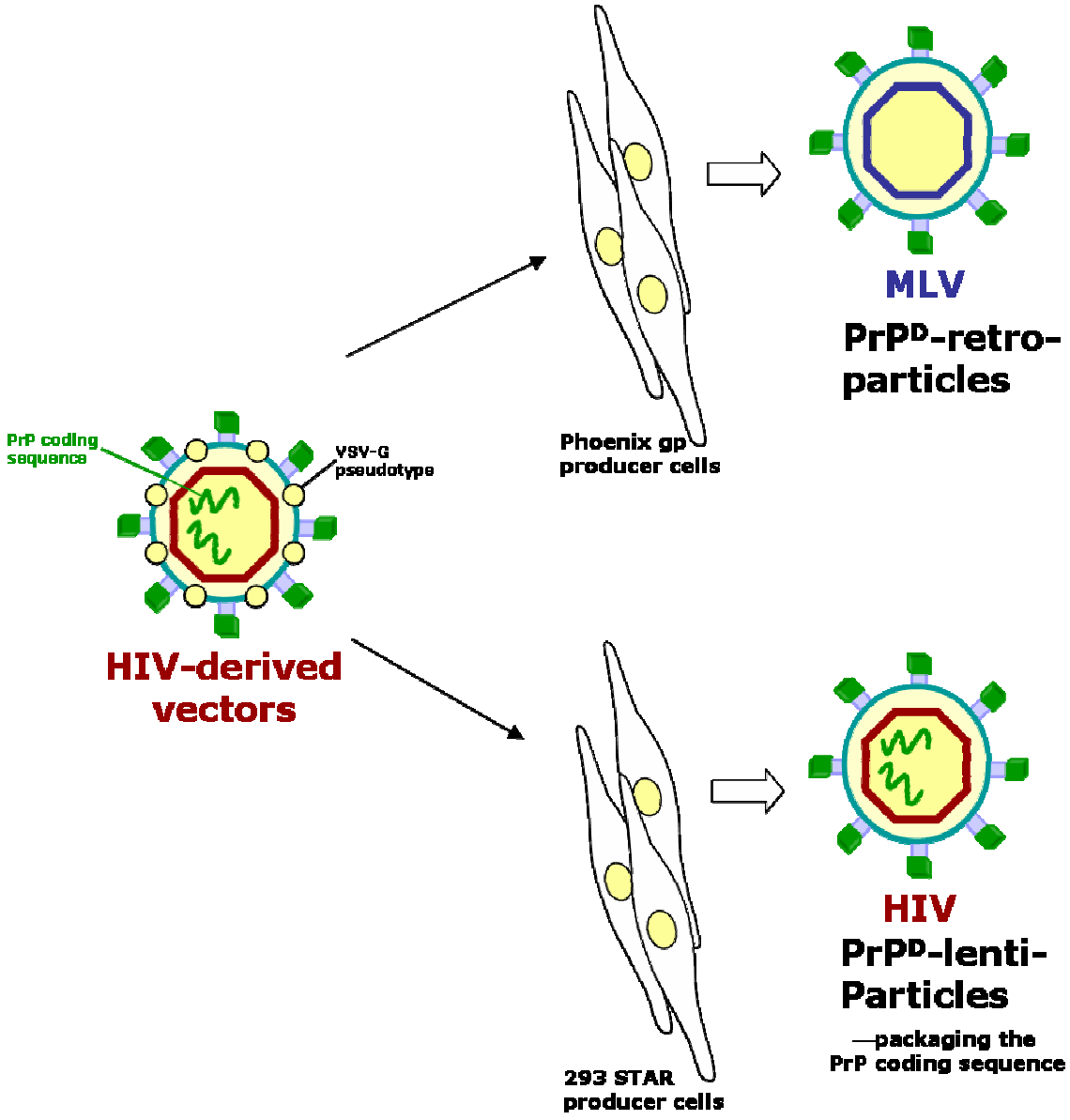


Figure 26 Schematic representation of the PrP^{D}-lentiviral vector system and the resulting producer cell systems. Lentiviral vectors presenting PrP^{D} proteins on their surface are additionally pseudotyped by the vesicular stomatitis virus G protein (VSVG) to ensure transduction of either MLV Gag/Pol producer cells (upper panel) or HIV Gag/Pol producer cells (lower panel). The transduced producer cells will subsequently produce MLV-derived PrP^{D}-retroparticles (upper panel) or HIV-derived PrP^{D}-lentiparticles packaging the PrP coding sequence (lower panel). MLV capsid is shown in blue, HIV capsid is shown in red. PrPD is indicated as green squares.

The newly available lentiviral expression system (Virapower lentiviral system, Invitrogen) allows creation of a replication-incompetent, HIV-1-based lentivirus, which can then be used to deliver and express the gene of interest into mammalian cells. This expression system was used to generate packagable PrP^{D} genes and obtain lentiviral vector particles.

The transfer vector of this system, named plenti6-V5 (Appendix, VI.1.4) bearing the PrP^{D} gene, was cotransfected with an optimized mix of the three packaging plasmids (pLP1, pLP2, and pLP/VSVG (Appendix, VI.1.11). The mix supplies the structural proteins *in trans* that are required to produce the lentiviral vector. As producer cells the HEK-293FT cell line was selected as before.

IV.1.1.3.1 Strategy

The two coding sequences for PrP fusion proteins PrP^{D111} and PrP^{D142} were subcloned into plenti6/V5-lacZ (Appendix, VI.1.4) using the restriction sites *Bst*XI and *Xho*I. The procedure resulted in a construct providing the possibility to package the fusion protein genes in form of viral RNA into HIV derived particles. The genes are flanked by the HIV 5´and 3´LTRs responsible for integration into the host cell genome and the HIV packaging site (Ψ) providing packaging of the viral genome into particles (Figure 27).

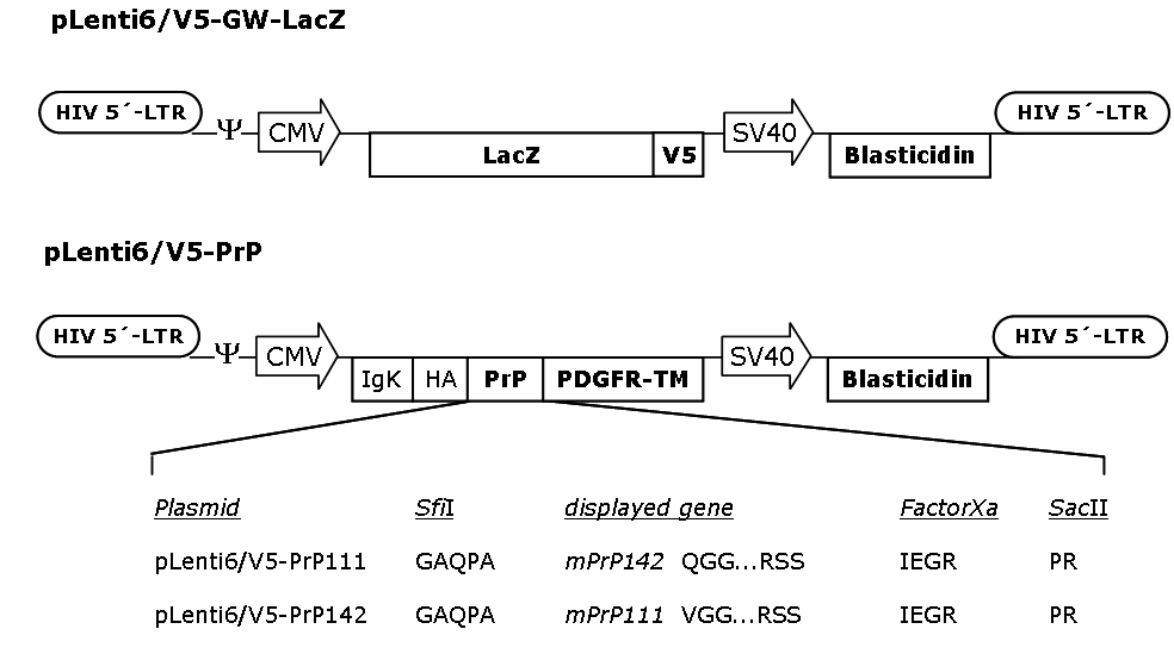


Figure 27 Schematic representation of the plenti6-PrP111 and 142 expression constructs derived from pLenti6/V5-GW-LacZ. The PrP^{D111} and the PrP^{D142} coding sequences were transferred into the plenti6/V5-lacZ vector via the *Bst*XI and the *Xho*I restriction sites. This results in substitution of the lacZ coding sequence against the PrP^{D} coding sequence. The newly generated plasmids bear the capacity of being packaged into lentiviral HIV-derived vectors and to become integrated into target cell genomes.

A stop-codon was added to the 3´end of the parental transferred PrP^D coding sequence, so that the available V5 epitope present in the parental pLenti6/V5-GW-LacZ plasmid was not included in the reading frame. The expression remains under control of the CMV promoter, which is included in the expression plasmid plenti6/V5-GW-LacZ.

IV.1.1.3.2 Packaging

The newly generated plasmids pLenti6-DPrP111 and pLenti6-DPrP142 were cotransfected with the ViraPower packaging mix (Invitrogen), which consists of a mixture of plasmids needed for production of lentiviral vector particles. This mixture includes pLP1 and pLP2 coding for the HIV gag/pol genes and the HIV accessory Rev gene, respectively. The Rev protein interacts with the Rev response element (RRE) on pLP1 to enhance Gag and Pol expression as well as with the pLenti6/V5 expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles. The pLP/VSVG plasmid encodes the envelope G glycoprotein of the Vesicular Stomatitis Virus (VSV) to allow production of pseudotyped particles with a broad host range (Emi, N et al. 1991). The cotransfection of HEK-293FT producer cells was performed according to the ViraPower manual (Invitrogen) followed by the collection of supernatants containing the vector particles.

To assure the packaging of the pLenti-DPrP encoded genes, the supernatants were subjected to RNA isolation. Subsequently the RNA preparations were reverse transcribed using the V5 reverse primer (Table 3). PCR fragments of 889 bp for pLenti-DPrP111 derived RNA and 982 bp for pLenti-DPrP142 derived RNA were the generated using the primer pair CMV forward and V5 reverse.

The cDNA samples were analyzed by gel electrophoresis in comparison to RNA samples, which served as negative controls (Figure 28, lane 2, 5), because the RNA itself cannot act as template in PCR reactions. This control excludes the possibility that signals arose from residual plasmid DNA of the transfection of producer cells. The RNA sample of pLenti-DPrP111 did not give rise to any PCR signal, demonstrating that the RNA sample was free of DNA (Figure 28, lane 2). In contrast to that, the pLenti-DPrP142 sample resulted in a faint RNA signal (Figure 28, lane 5), suggesting that residual DNA was present in this sample.

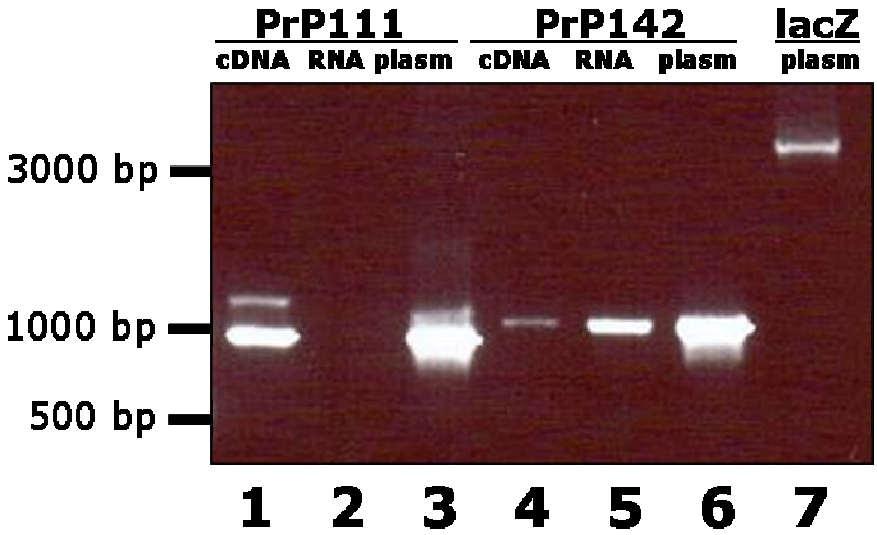


Figure 28 The pLenti-DPrP111 and pLenti-DPrP142-derived coding sequences were packaged into lentiviral vector particles. Vector containing supernatants were subjected to RNA isolation and subsequently reverse transcribed into cDNA. This cDNA was used as a template in analytical PCR analysis with CMVforward and V5reverse oligonucleotides. Besides the two constructs pLenti-DPrP111 (lane 1) and pLenti-DPrP142 (lane 4), also RNA negative controls (lanes 2 and 5, respectively) and plasmid positive controls (lanes 3 and 6) were included. For comparison the parental pLenti6/V5-lacZ plasmid (lane 7) was used as template.

As positive controls the plasmids pLenti-DPrP142 and pLenti-DPrP111 were included as well as the parental plasmid pLenti/V5-lacZ (3393 bp signal), which indicated the expected size of the amplified fragments.

pLenti-DPrP142-derived and pLenti-DPrP111-derived cDNA revealed the expected signals compared to plasmid DNA (Figure 28, lane 1, 3), although an unexpected second higher band was present for pLenti-DPrP142 possibly due to unspecific primer binding in the cDNA sample.

Thus, the coding sequences for the PrP^{D111} and PrP^{D142} fusion proteins were sucessfully packaged into lentiviral vectors, which can be used for transfer into target cells. Furthermore, such particles may be useful to enhance immune responses against virus-derived display particles and to establish immunization protocols.

IV.1.1.3.3 Transduction and stable cell lines

The vector containing supernatants were subsequently used for transduction of the target cell lines N2a and 293 STAR. The neuroblastoma cell line will then stably express PrP^{D} on the surface, thus providing the basis for experiments with the scrapie-infected subclone PK1 available from this cell line. 293 STAR stably produce HIV-derived virus-like particles (Ikeda, Y, Takeuchi, Y et al. 2003) and

should therefore be convertable into a stable PrP^D-vector particle producer cell line upon transduction with PrP^D-(VSV-G)-lentivectors, as it is shown in Figure 26.
The PrP^D-(VSV-G)-lentivectors provide a Blasticidin resistance marker enabling the selection of transduced cells after transduction events. As a prerequisite for successful blasticidin selection, the optimal blasticidin concentration for N2a and 293 STAR cells was determined. For this purpose, cells were exposed to various concentrations of blasticidin ranging from 2 to 10 µg/ml. While for N2a cells 8 µg/ml were sufficient to kill all cells after 10 days of incubation with blasticidin, for the 293 Star cell line 4 µg/ml blasticidin were sufficient.
The cell lines were then transduced with vector stocks of both, pLenti-DPrP111-derived vectors and pLenti-DPrP142-derived vectors, and incubated with the corresponding amounts of blasticidin for 7 days starting from day 2 after transduction. Cells were expanded and used for further analysis. For this, the genomic DNA was isolated from about 3×10^6 cells and subjected to PCR analysis (Figure 29) with the CMVforward/V5reverse primer pair as described for cDNA analysis above.

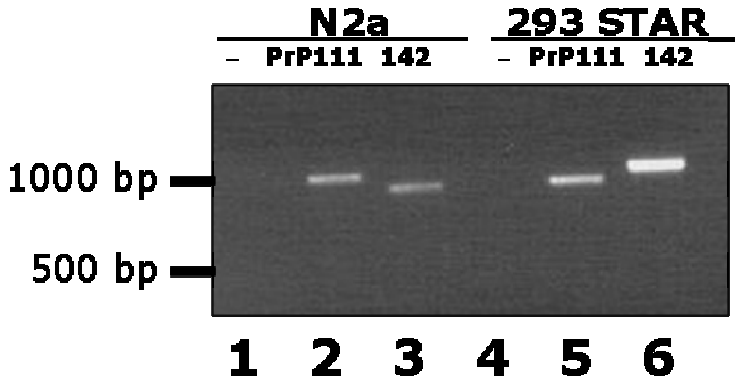


Figure 29 Transduced cell lines integrate Lenti-PrP^D genetic information into their genome. Vector particles were used for the transduction of N2a and HEK 293 Star cells to achieve stable integration of the packaged pLenti-PrP^D coding sequences. Upon isolation of genomic DNA and subsequent analytical PCR using *CMVforward/V5reverse* primers, cells were tested for the integration of the virally packaged genes. pLenti-DPrP111 vectors (lane 3, 5) and pLenti-DPrP142 vectors (lane 2 and 6) were compared to genomic DNA of untransduced N2a (lane 1) and 293 STAR (lane 4) cells.

While the control from untransduced cells remained without signal for both cell lines (Figure 29, lanes 1 and 4), specific bands for pLenti-DPrP111 and pLenti-DPrP142 vector transduced cells appeared at the expected positions (889 bp and 982 bp, respectively) for both cell lines (Figure 29).

Thus, the transduced and blasticidin-selected cell lines N2a and 293 STAR contained integrated PrP^D coding sequences and are available for production of the PrP^D fusion proteins (N2a) or as PrP^D lentivector producer cell line (293 STAR).

So far, display of monomeric PrP^D on the surface of retroparticles and lentiparticles has been achieved.
However, oligo- or multimeric display of PrP may be advantageous for immunization. Attempts in this direction are addressed in the next chapter.

IV.1.1.4 Display of PrP^C on the Env protein

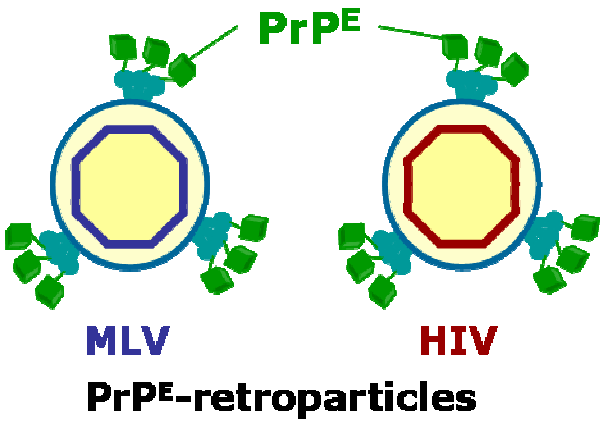


Figure 30 Schematic representation of PrP^E-retroparticles. PrP^C is presented in context of the MLV-derived amphotropic Env protein. The coding sequence was fused to the N-terminal end of the trimeric Env protein (N-terminal display). Cotransfection with either MLV or HIV Gag and Pol genes results in production of corresponding retro- or lentiparticles, respectively.

The display of PrP^C in an oligomeric system offers a distinct way of presenting the protein, because the single PrP molecules are believed to come into close proximity. Here, the amphotropic MLV (aMLV)-derived viral envelope protein (Env), which forms a trimer (Kamps, C A et al. 1991), is utilized as scaffold for the display of N-terminally fused cellular PrP. The N-terminus has previously been shown to tolerate insertions largely without affecting the Env functions (Russell, S J et al. 1993; Cosset, F L, Morling, F J et al. 1995; Finger, C et al. 2005). The aMLV Env protein (includes human cell tropism) was used, because the presentation should be carried out in a trimeric but nonreplicative context. Utilization of the amphotropic instead of the ecotropic Env, which enters murine

cells only, was not relevant in terms of safety aspects (because retroparticle would not mediate infectivity) and was simply due to availability of the corresponding plasmid coding for the amphotropic variant.
Upon cotransfection with MLV structural genes gag/pol, the production of VLPs incorporating the altered viral envelope can be achieved. The resulting particles were named PrP^E-retroparticles (MLV-derived VLPs) or PrP^E-lentiparticles (HIV-derived particles), respectively.

IV.1.1.4.1 Strategy

The fusion proteins of mPrP with the aMLV Env were produced using the parental plasmid pFB-EASALF (Appendix, VI.1.5), coding for aMLV Env, cleaved by the restriction enzymes *Sfi*I and *Not*I. The mPrP gene fragments coding for the aa23-231 (mPrP209) and 121-231 (mPrP111) were inserted by use of *Not*I-containing reverse primers and subsequent PCR with PrP121Sfi+ forward and PrP*Not*-reverse primers (Table 3).
For both constructed plasmids, one positive candidate was selected and insertion of the correct insert was verified by restriction analysis and DNA sequencing (Appendix, VI.5). Resulting expression plasmids (Figure 31) code the PrP-Env fusion protein PrP^E.

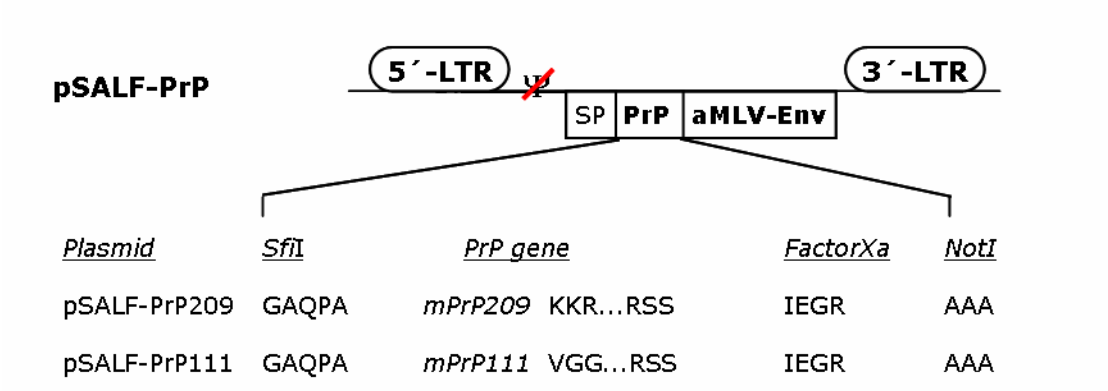


Figure 31 Schematic representation of the pFB-EASALF derived mPrP expression plasmids pSALF-PrP209 and pSALF-PrP111. PrP111 and PrP 209 were inserted at the N-terminal end of the MLV amphotropic envelope gene, which stays under control of the viral promotor (LTR). The signal sequence remains N-terminally to target the protein to the plasma membrane. A factor Xa cleavage site was inserted between the PrP and the Env genes. The MLV packaging signal was deleted previously to reveal an unpackagable construct.

The factor Xa cleavage site was included between the aMLV Env and PrP sequence. At this. it is possible to specifically release PrP from the surface of the produced viral particles.

IV.1.1.4.2 Incorporation into MLV-derived retroparticles

Plasmids pSALF-PrP111 and pSALF-PrP209 were cotransfected with pHit60 (Appendix, VI.1.7) to generate MLV-derived retroparticles bearing the Env-PrP fusion proteins PrP^{E111} and PrP^{E209}, respectively, on their surface. The expression was addressed using Western Blot analysis (Figure 32). After cotransfection of HEK-293FT producer cells with either wild-type Env (wt aMLV Env) (68.1 kD), PrP^{E209} (92.1 kD) or the PrP^{E111} fusion envelope protein (82.3 kD), cell culture supernatants were filtered and concentrated by ultracentrifugation through sucrose cushion or low-speed centrifugation and subsequently analyzed by western Blot. As a control the wt aMLV Env was included in the analysis [pHit456/pHit60] (Figure 32, lanes 1, 2).

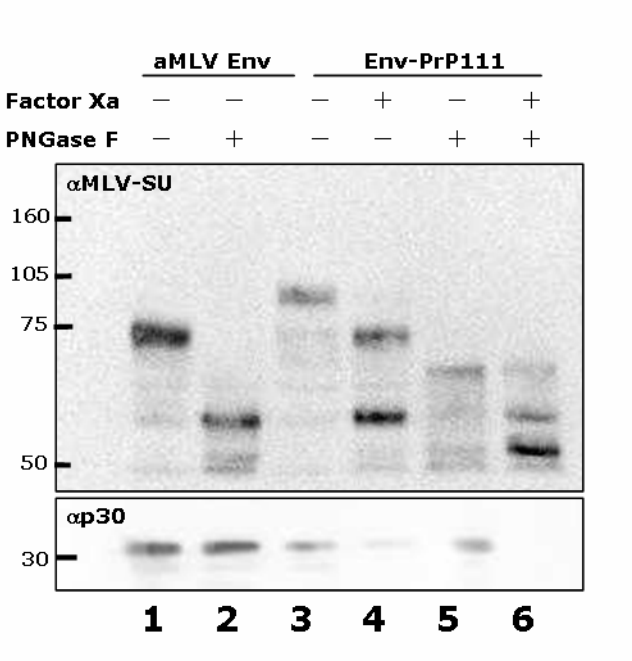


Figure 32 The envelope fusion protein PrP^{E111} is expressed and incorporated into retroparticles. Cell culture supernatants of HEK-293FT cells cotransfected with pSALF-PrP111/pHit60 (lanes 3-6) were separated on 10% SDS polyacrylamide gel and compared to the wild-type Env coded by pHit456 in cotransfection with pHit60 (lanes 1, 2). Samples were incubated with PNGaseF to remove glycosylation (lanes 2, 5-6). The PrP^{E111} samples were treated with factor Xa (lanes 4, 6). Upper panel was stained with an anti-MLV-SU antibody; lower panel was stained with the anti-p30 serum. The amount of supernatants corresponds to either 3 ml for the wild-type Env (lanes 1, 2) or 7.5 ml for the PrP^{E111} protein (lanes 3-6).

PrP^{E111} is incorporated into retroparticles but incorporation rate showed lower efficiencies when p30/Env ratios were compared. The cleavage by factor Xa

protease is functional but a second cleavage site might be present in the PrP^{E111} sequence, as it is indicated by a second band with lower mobility shift compared to single-cleaved protein. The deglycosylation of PrP^{E111} works well, but Env and PrP-glycosylation can not be distinguished.
The PrP^{E209} was analyzed in the same way but revealed no signal upon staining with the MLV-SU reactive antibody.
These results indicated successful incorporation of PrP^{E111} into MLV-derived retroparticles.

IV.1.1.4.3 Incorporation into HIV-derived lentiparticles

Furthermore, the question, whether PrP^{E111} can also be incorporated into HIV-derived particles, was addressed. HIV particles are known to be pseudotyped by the aMLV Env, although the incorporation efficiency is lower than that of wt HIV Env protein (Reiser, J et al. 1996). Therefore the pSALF-PrP111 plasmid was cotransfected with pCMVΔR8.2 (Appendix, VI.1.7) coding for the HIV-derived structural proteins resulting in the production of PrP^{E111}-lentiparticles (Figure 33).
There were no signals obtained in the negative control, i.e. untransfected cells neither when stained with the anti-MLV-SU reactive antibody, the anti-capsid antibodies (p24 and p30) nor with the anti-PrP6H4 antibody (Figure 33, lane 1). PrP^{E111}-retroparticles served as positive controls and resulted in the expected signals (Figure 33, lane 2).
In contrast to the MLV-derived particles, PrP^{E111}-lentiparticles were specifically stained by the p24 antibody but revealed no signal when stained by anti-p30 (Figure 33, lane 3).
For both MLV- and HIV particles, staining against the soluble unit (SU) of the Env resulted in two bands (Figure 33 A , lanes 2, 3). However, only when high amounts were loaded on the gel, the second band appeared (compare to Figure 32, lane 3). As explained before (IV.1.1.4.2), due to cleavage at the factor Xa cleavage site, the lower one most likely represents the SU protein. The PrP^{E111} protein could additionally be stained with the anti-PrP 6H4 antibody, although the signal intensity was weak (Figure 33 D). The band was present only for PrP^{E111}-retroparticles and not for PrP^{E111}-lentiparticles, in which lower incorporation efficiencies might contribute. Incorporation rates for PrP^{E111}-lentiparticles were twofold decreased compared to PrP^{E111}-retroparticles, as it was roughly estimated from Western Blot analysis.

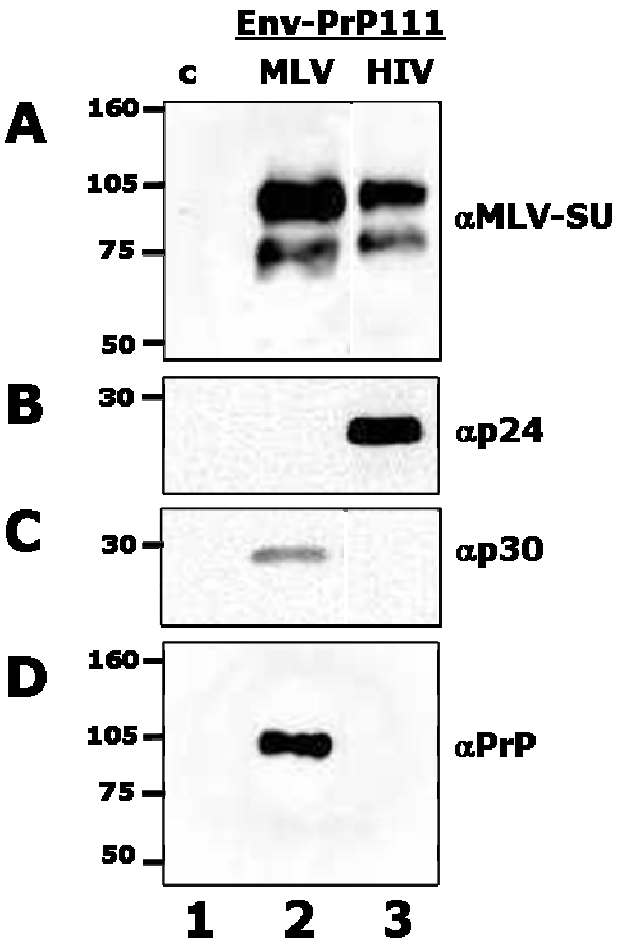


Figure 33 **PrP^{E111} protein is present in HIV-derived lentiparticles.** Cell culture supernatants from both pSALF-PrP111/pHit60 (lane 2) and pSALF-PrP111/pCMVΔR8.2 (lane 3) cotransfection were subjected to Western Blot analyses on 10% SDS gels in comparison to supernatants of untransfected cells (lane 1). Samples were stained with anti-MLV-SU reactive antibody (A), with the monoclonal anti-p24 antibody (B), with the anti-p30 serum (C) or with the anti-PrP 6H4 antibody (D), respectively. Loaded amounts correspond to 10 ml (A-C) or 15 ml (D) cell culture supernatants. PrP^{E111}-lentiparticles signal intensities were compared to PrP^{E111}-retroparticles by estimating peak density using Adobe Photoshop Software.

Taken together, the PrP^{E111} protein was expressed and incorporated upon cotransfection with MLV- and HIV-derived structural proteins to result in the characterized PrP^{E111}-retro- and lentiparticles, respectively, whereas the full length PrP form could not be detected. Western Blot analysis of producer cell supernatants indicated the incorporation of PrP^{E111} into MLV and HIV, a result which was further addressed by electron microscopy as described in the next chapter.

IV.1.1.4.4 Characterization by electron microscopy

Characterization by electron microscopy of immunogold labelled samples was done to verify, that PrP^{E} proteins were displayed on the surface of retro- or

lentiparticles, respectively. Either pSALF-PrP111/pHit60- or pSALF-PrP111/pCMVΔR8.2-cotransfected HEK-293FT cell supernatants were therefore stained by the anti-PrP 6H4 antibody.

The aMLV Env-bearing retroparticles were not stained by the anti-PrP 6H4 antibody. On the contrary, both PrP^{E111}-retro- and lentiparticles show PrP-specific staining (Figure 34 D-F). In line with the Western Blot data, for PrP^{E111}-lentiparticles, much lower numbers of stained particles were found, compared to PrP^{E111}-retroparticles.

This result demonstrates that PrP^{E111} was effectively incorporated into retro-and lentiparticles. These particles are available for immunization studies possibly resulting in enhanced antibody titers.

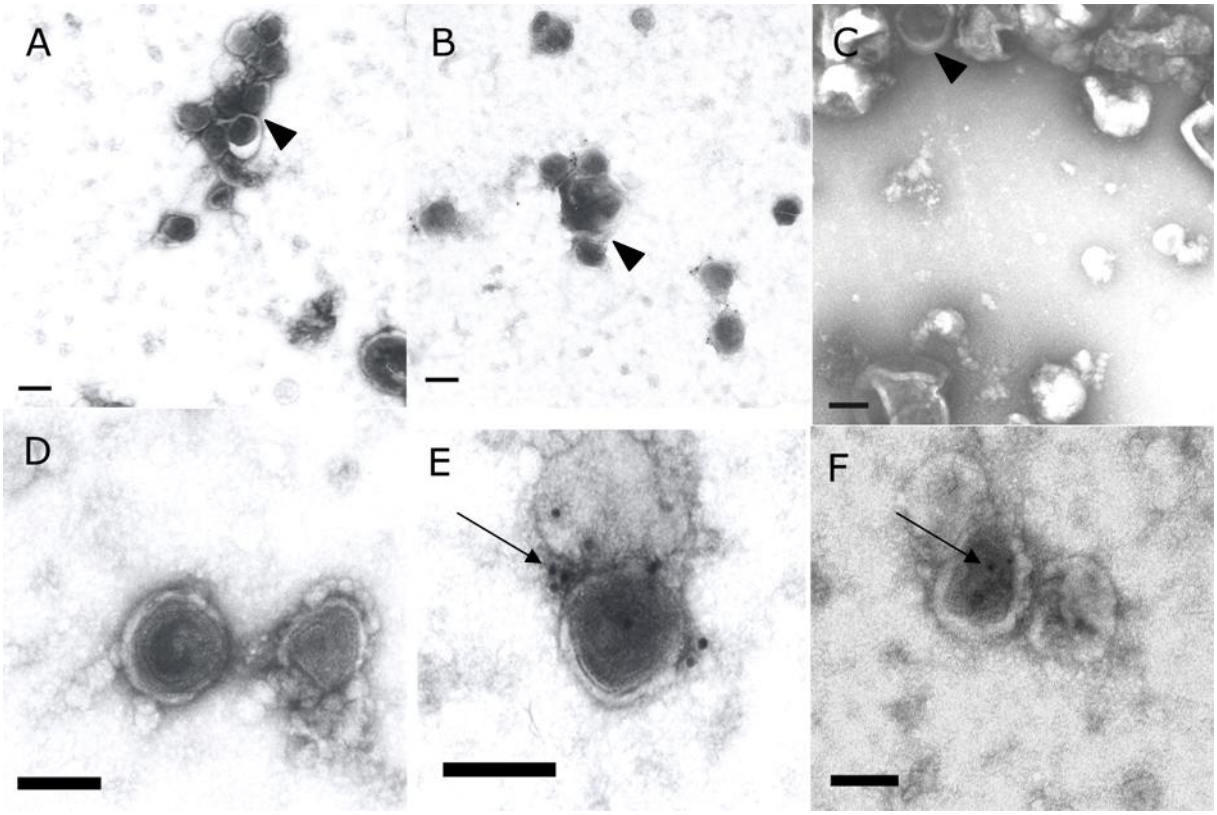


Figure 34 MLV- and HIV- derived particles incorporate the PrP^{E111} fusion protein. Supernatants of cotransfected HEK-293FT producer cells were analyzed by immunogold stains using the anti-PrP 6H4 and an anti-mouse gold-conjugated secondary antibody. Plasmids pHit456/pHit60 (A, D), pSALF-PrP111/pHit60 (B, E) and pSALF-PrP111/pCMVΔR8.2 (C, F) were cotransfected to reveal MLV-derived (A, B, D, E) or HIV-derived (C, F) retro- or lentiparticles, respectively. Upper panels show an overview, whereas magnified particles are shown in the lower panels. Groups of typical retrovirus-derived particles are marked by arrowheads. Gold labelling is marked by arrows.

IV.1.2 Nucleocapsid-like particles

An alternative approach to present oligomeric PrP^c was followed by using the Sendai virus nucleocapsid protein NP as scaffold. Upon expression of NP in eukaryotic cells, the protein self-assembles into dense RNA-protein particles remaining in the cytoplasm of the cell when the other viral proteins are absent. In this case the NP assembles around cellular RNA. The resulting particles can be harvested through cell lysis and subsequent purification of the particles via ultracentrifugation in caesium chloride gradients. The assembly of such NP particles has been examined in detail for the wild-type NP protein (Finch, J T and Gibbs, A J 1970; Lamb, R A, Mahy, B W et al. 1976) and various deletion mutants (Buchholz, C J, Spehner, D et al. 1993; Curran, J, Homann, H et al. 1993). Whereas the N-terminal part of the protein is responsible for RNA binding, the C-terminal part is located at the particle surface, and therefore seems to be optimal for presentation of foreign amino acid sequences.

To investigate the suitability of NP for PrP display, insertion of the Prnp gene at the C-terminal end of NP and subsequent expression analysis had to be carried out. Upon this, it had to be investigated, whether the newly generated fusion-proteins maintained the ability to self-assemble around RNA molecules.

IV.1.2.1.1 Construction

The NP fusion protein expression plasmids were produced starting from plasmid pGem-NP (Appendix, VI.1.6). This plasmid encodes the NP protein under control of the T7 promotor. The PrP^{111} protein had to be fused to either amino acids (aa) 425 or 456 of NP. This was achieved by making use of the restriction sites *Msc*I and *Eco*RI or *Eco*RV and *Eco*RI, respectively. The resulting plasmids were referred to as pGem-NP425-PrP^{111} and pGem-NP456-PrP^{111} (Appendix, VI.7) corresponding to the number of NP aa present in the constructs (Figure 35).

To further examine the influence of the addition of foreign peptides to NP at the C-terminus, an immunological tag, derived from the hemagglutinine A (HA) was fused to the original C-terminus of NP (Figure 35) resulting in the plasmid pGem-NP523-HA (Appendix, VI.7). Thus, a possibility to insert foreign genes via XhoI and NdeI restriction and render the HA-tag intact at the C-terminal end is offered.

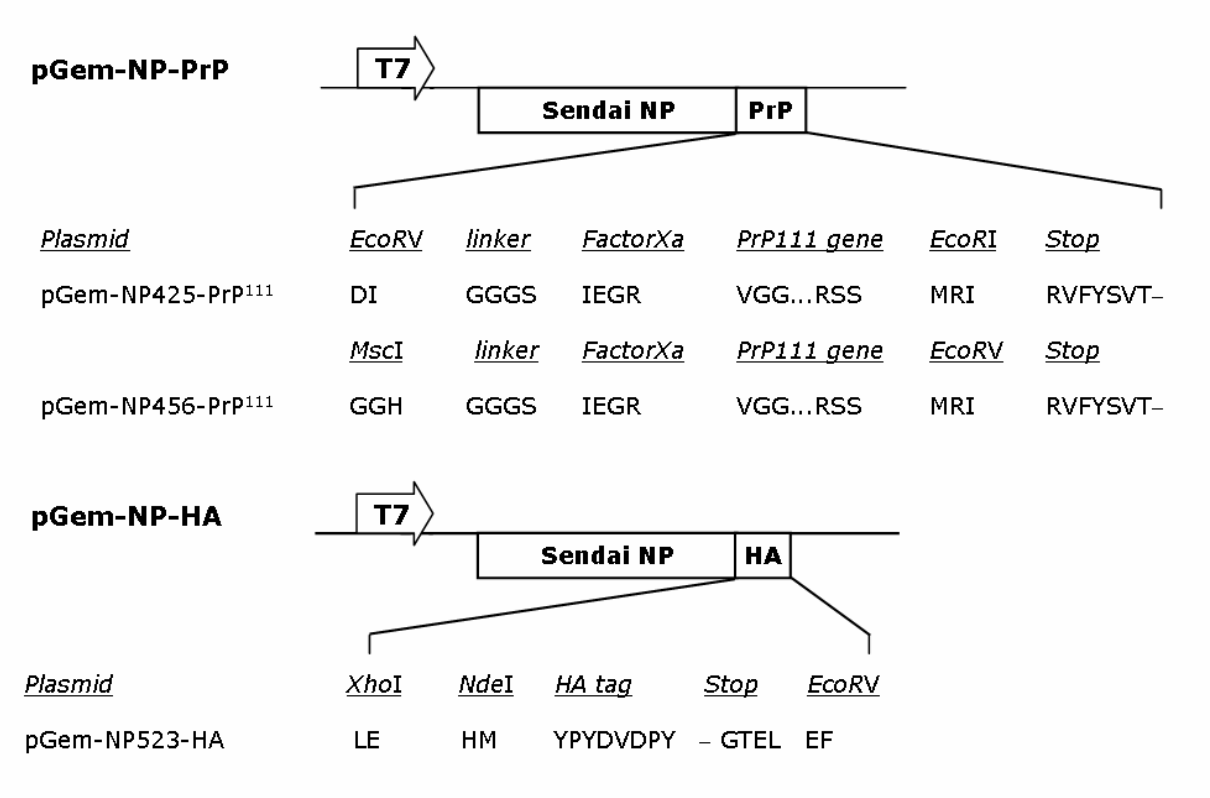


Figure 35 Schematic representation of the NP fusion protein expression plasmids. Either the PrP^{111} coding sequence or the HA-tag gene was fused to the C-terminal part of the Sendai virus NP protein. NP was thereby C-terminally truncated to 425 or 456 aa or left complete (523 aa). The NP gene expression is controlled by the phage-derived T7 promotor.

IV.1.2.1.2 Expression

Expression of the NP fusion proteins was investigated using the vaccinia virus T7 expression system (Fuerst, T R et al. 1986). HEK-293FT producer cells were at first infected by the recombinant vaccinia virus modified vaccinia Ankara (MVA)-T7 providing the T7 RNA polymerase. This phage-derived polymerase is widely used to enable overexpression of recombinant proteins in target cells. The corresponding T7 promotor is highly active and results in huge amounts of proteins, when the polymerase, which is absent in eukaryotic cells, is provided *in trans*. After infection with MVA-T7, the producer cells were transfected with the target plasmids and lysed after 24 hours. For comparison, instead of utilization of the MVA-T7 system, cells were cotransfected by the NP fusion protein plasmids and a T7 polymerase-coding plasmid, namely pScT7 (Appendix

Appendix, VI.1.6). Cell Lysates of both systems were then examined by Western Blot analysis (Figure 36). The molecular weights of NP fusion proteins were calculated as 61.3 kD (NP425-PrP^{111}), 64.7 kD (NP456-PrP^{111}) and 58.5 kD (NP523-HA) compared to 56.8 kD for the wt NP protein. To compare the protein expression levels, wt NP protein is included in the analysis.

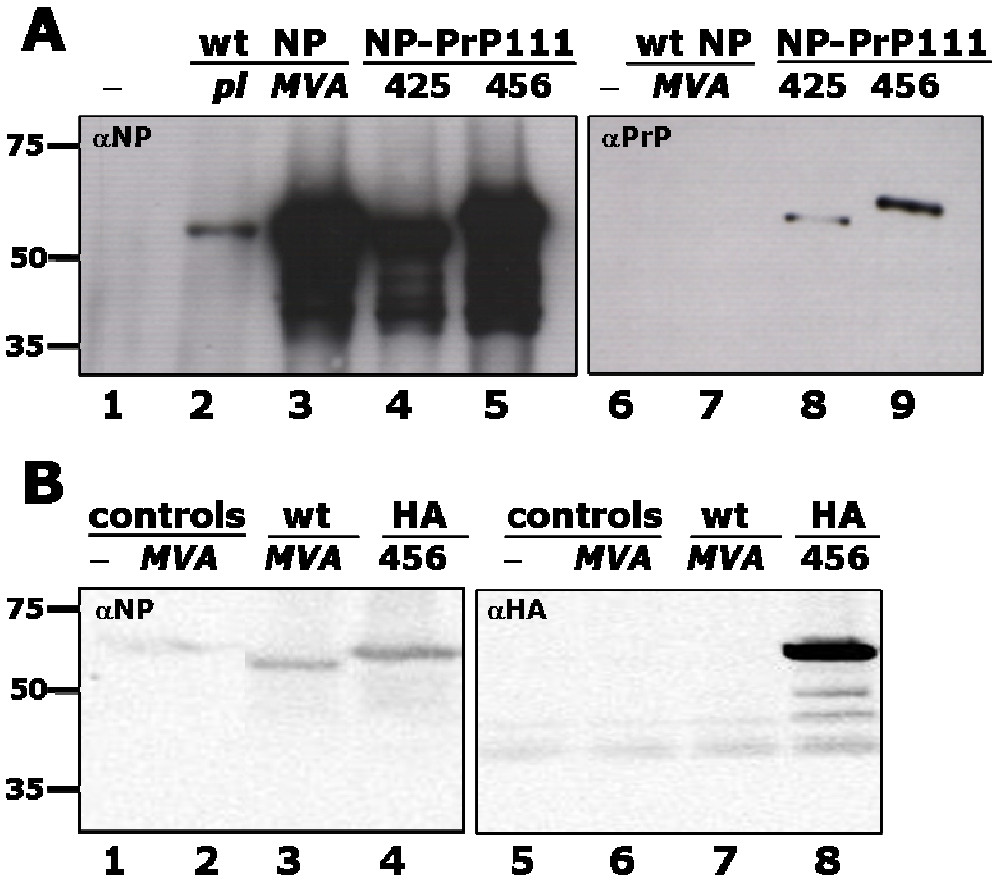


Figure 36 **Expression of the NP fusion proteins using MVA-T7.** MVA-T7-infected HEK-293FT cells were transfected with the pGem-NP-derived plasmids. The resulting proteins NP425-PrP^{111} (A, lanes 4, 8), NP456-PrP^{111} (A, lanes 5, 9) as well as NP523-HA (B, lanes 4, 8) were detected 24 h post transfection in cell lysates by either anti-NP sera (A and B, left panels), the anti-PrP 6H4 antibody (A, right panel) or the anti-HA tag antibody (B, right panel). The wt protein, which was included as a control, was expressed via cotransfection with the T7 polymerase expression plasmid pScT7 (A, lane 2) or via the MVA-T7 system (A, lanes 3, 7; B, lanes 3, 7). As negative controls untreated HEK-293FT cell lysates (A, lanes 1, 6; B, lane 1) and untransfected, MVA-T7 infected cell lysates (B, lane 2) were applied.

No signal was obtained for the wild-type protein when stained by anti-PrP 6H4 or anti-HA tag antibodies (Figure 36).´The PrP fusion proteins NP425-PrP^{111} and NP456-PrP^{111} (Figure 36 A, lanes 4-9) were detectable by both, the anti-NP serum and the anti-PrP 6H4 antibody. The anti-PrP signal is present only in the PrP fusion proteins, demonstrating successful fusion of NP and PrP proteins as well as the retained antigenicity of PrP. Also NP523-HA was detected by both specific

antibodies, namely the anti-NP or the anti-HA antibody (Figure 36 B, lanes 4, 8). As expected from its molecular weight, the NP523-HA showed a reduced electrophoretic mobility when compared to wt NP protein.
In summary, the expression of the different NP fusion proteins was successful in HEK-293FT producer cells, as detected in cell lysates 24 h post transfection. It remained to be detected whether these proteins retained the capability to assemble into nucleocapsid-like particles.

IV.1.2.1.3 Assembly analysis by gradient purification

Next, the assembly of the NP fusion proteins had to be examined. Therefore the cell lysates were purified *via* ultracentrifugation in a caesium chloride gradient. Due to their RNA content, nucleocapsids reveal a high density of about 1.31 g/cm^3. CsCl density gradients were therefore the method of choice to determine the density of the NP-PrP fusion proteins. Whereas non-assembled proteins are unable to migrate into high density fractions of the gradient, assembled NP/RNA particles are present in the 30-40% Caesium chloride layer (Buchholz, C J, Spehner, D et al. 1993). The gradient was established as a step gradient including 40%, 30% and 20% CsCl-solution from bottom to top of the tube. After centrifugation, fractions were collected from the bottom of the tube and individually analyzed for the presence of the NP-PrP proteins *via* Western Blot analysis.
For the wt NP protein included for comparison, the vast majority of protein accumulated in fractions 1-3. Only faint bands were present in the other fractions (Figure 37 D). For the proteins NP456-PrP^{111} and NP425-PrP^{111}, signals were found in fractions number 3 and 4 corresponding to 30-40 % CsCl (Figure 37 B). Additionally, signals were present in the upper fractions arising from unassembled monomeric proteins (Figure 37 A and B, fractions 7-10). The same result was observed for the HA-tag fusion protein NP523-HA, for which assembled proteins are present in fraction 4 (Figure 37 C).

This result indicated that the NP fusion proteins must have been assembled into dense particles. However, the assembly efficiency was much reduced compared to the wt protein. Overall, only about 5-10% of NP4456-PrP^{111}, 10% of NP523-HA and 20-30% of NP425-PrP^{111} appeared in high density fractions.

Thus it remained to be further analyzed, to what extend the NP/RNA nucleocapsid-like particles were build. Therefore the proteins were subsequently examined by electron microscopy.

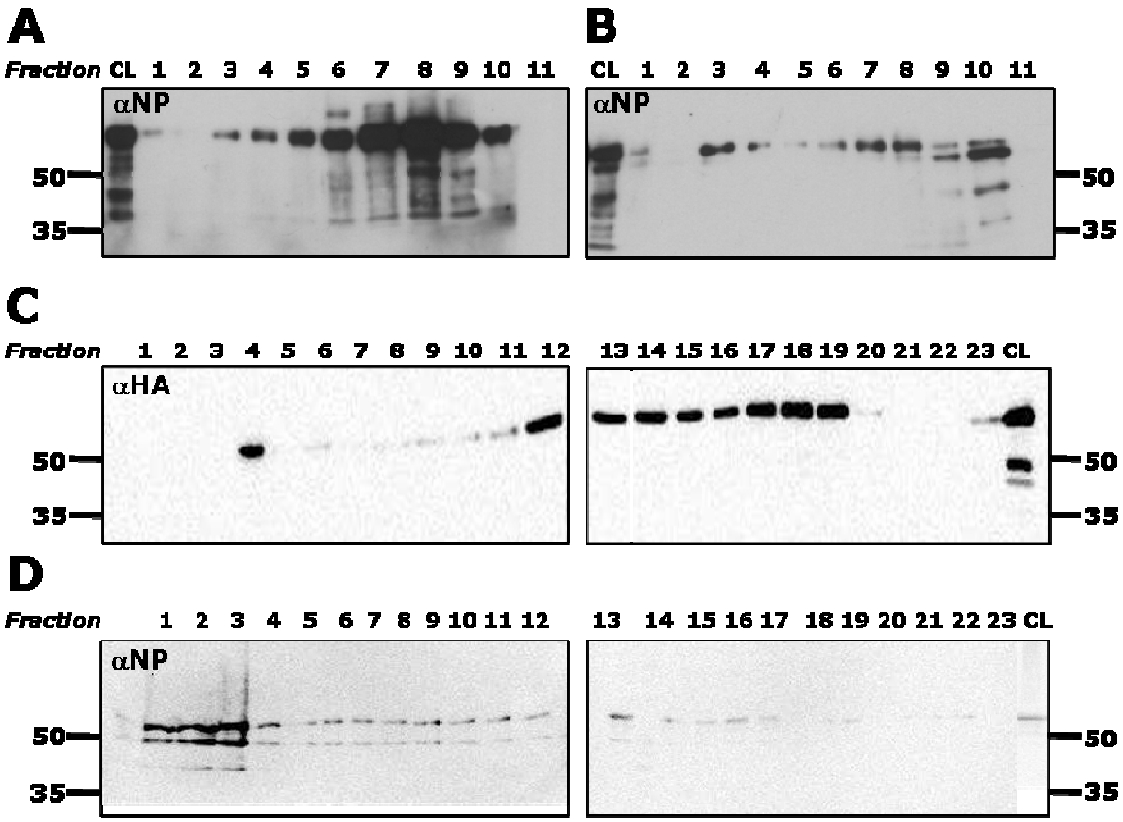


Figure 37 NP fusion proteins were present in high density fractions. The NP fusion proteins were subjected to ultracentrifugation in a CsCl step gradient ranging from 20-40%. Low fraction numbers contain highest density. NP456-PrP^{111} (A) and NP425-PrP^{111} (B) as well as the wt NP (D) were stained by the anti-NP serum. NP523-HA (C) was stained by ant-HA. *CL* cell lysate before ultracentrifugation.

IV.1.2.1.4 Detection of nucleocapsid-like particles by electron microscopy

To further analyze the assembly, the fusion proteins were subjected to electron microscopy immunogold labelling Figure 38. For this purpose, cell lysates were utilized, as CsCl present in the purified gradient samples led to reduced detection efficiency.

Nucleocapsid-like particles could be visualized for the wt NP samples (Figure 38 A), which was stained by anti-NP serum. On the contrary, staining of NP425-PrP^{111} with the anti-PrP 6H4 antibody was negative (Figure 38 B). For both PrP^{111} fusion proteins, only low amounts of nucleocapsid-like particles were detected in comparison to wt samples. Only some multimeric complexes were observed, which could not specifically be stained by the anti-NP (not shown) or the anti-PrP

6H4 antibody. The same was true for NP523-HA, which also revealed low amounts of nucleocapsid-like particles, only. Nevertheless, some huge complexes were found in the latter case, which could be slightly marked by the anti-HA tag antibody (Figure 38 C).

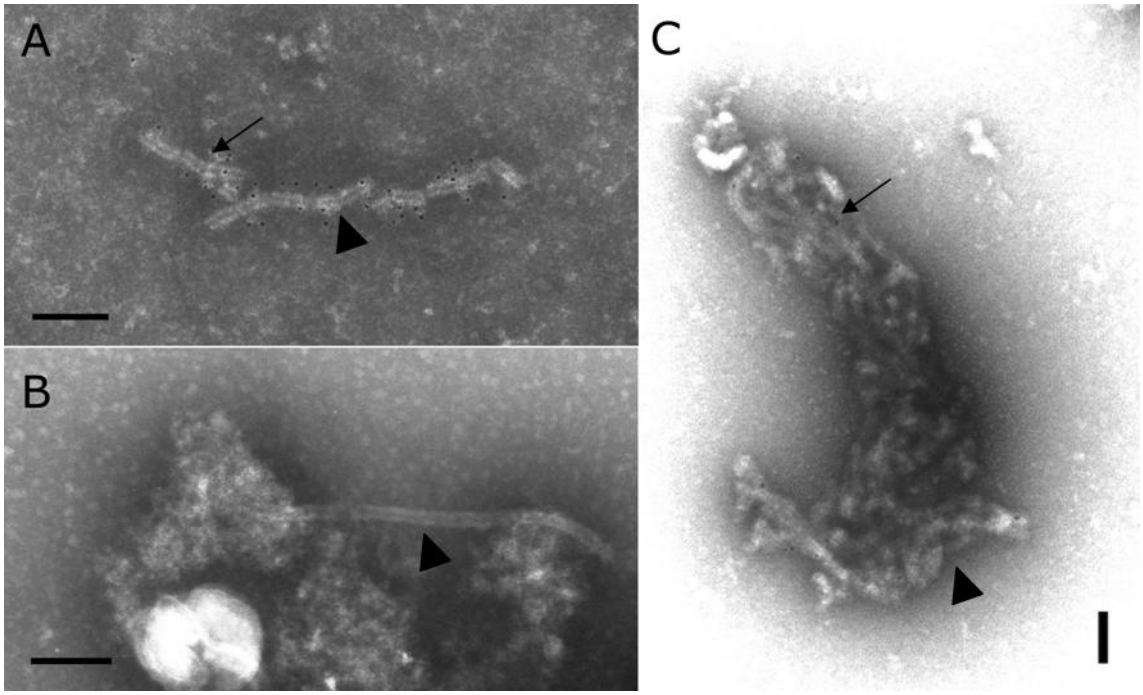


Figure 38 Formation of nucleocapsid-like particles was affected by the insertion of foreign peptides. The assembly of NP fusion proteins was addressed by electron microscopy immunogold labelling using different specific antibodies. The wt NP was stained with the NP-specific serum (A), NP425-PrP^{111} was stained using anti-PrP 6H4 antibody (B) and NP523-HA was stained with the anti-HA-tag antibody (C). Nucleocapsid-like particles are marked by arrowheads; gold-labelling is marked by arrows. Bars represent 100 nm.

This result showed that the insertion of foreign polypeptides and proteins impaired the formation of nucleocapsid-like particles. The suitability of this approach for the generation of PrP particle vaccines therefore remained questionable.

IV.2 Display of prions

The second part of this thesis attempted the display of the pathogenic isoform of PrP (PrP^{Sc} or prion) on the surface of retroparticles. This goal was addressed on one hand *via* conversion of the PrP^{c}, displayed on retroparticles as described in chapter IV.1.1.1. On the other hand the production of replication-competent MLV variants in scrapie-infected cells was pursued.

IV.2.1 Conversion of PrP^{D}-retroparticles

The conversion was examined for the PrP^{D142}-retroparticles (IV.1.1.1). These particles offer the opportunity to work with high amounts of PrP^{c}. Secondly, the PrP protein variant bearing 142 aa has previously been shown to be sufficient to propagate prion infection, while this was not the case for a truncated form of PrP containing only 111 aa (Flechsig, E et al. 2000).

In vitro conversion assays have been carried out extensively as reviewed in (Caughey, B 2003). Upon incubation with PrP^{Sc} or other types of proteinase K (PK) resistant PrP (PrP-res), the PK-sensitive PrP^{c} tends to bind to PrP-res and then slowly converts to a protease-resistant state like that of the original PrP-res. This reaction has been shown to occur under a variety of conditions. The simplest, most biochemically defined reactions contain mixtures of largely purified PrP^{c} and PrP-res preparations and can be stimulated by chaotropes, detergents and/or chaperone proteins. In an effort to amplify PrP-res substantially in an *in vitro* conversion reaction, the protein misfolding cyclic amplification (PMCA) system has been developed (Saborio, G P et al. 2001). In this system, detergent extracts of TSE-infected brain homogenate are mixed with vast excesses of similar extracts of PrP^{c}-containing normal brain tissue and subjected to repeated cycles of sonication and incubation. More than 30-fold increases in the amount of PrP-res over that provided in the infected brain extract were reported.

To achieve the conversion of the PrP^{D142}-retroparticles, the particles were incubated with the scrapie-infectious agent in form of brain homogenates from scrapie-infected mice (Chandler strain). Particles were incubated overnight at physiological conditions. This setting is achieved by addition of brain homogenates of either infected mouse brain (Ch) to the particles or by addition of brain homogenate from an untreated mouse (HG) as a control. The amounts of brain homogenates were determined by Bradford assay (Biorad). For the

detection of coverted PrP in the retroparticles, PK digestion assays were performed. PK cleaves the pathogenic form of PrP with lower kinetics than the cellular PrP. An important prerequisite for this assay is to apply absolute similar amounts of total protein present in the infectious brain homogenates compared to the solely PrP^{c}-bearing control homogenates. Only when overall amounts of protein are identical, the kinetic of PK is exclusively dependent on the two different PrP forms.

The samples were digested with different concentrations of PK ranging from 1 to 5 µg/ml and then subjected to Western Blot analysis.

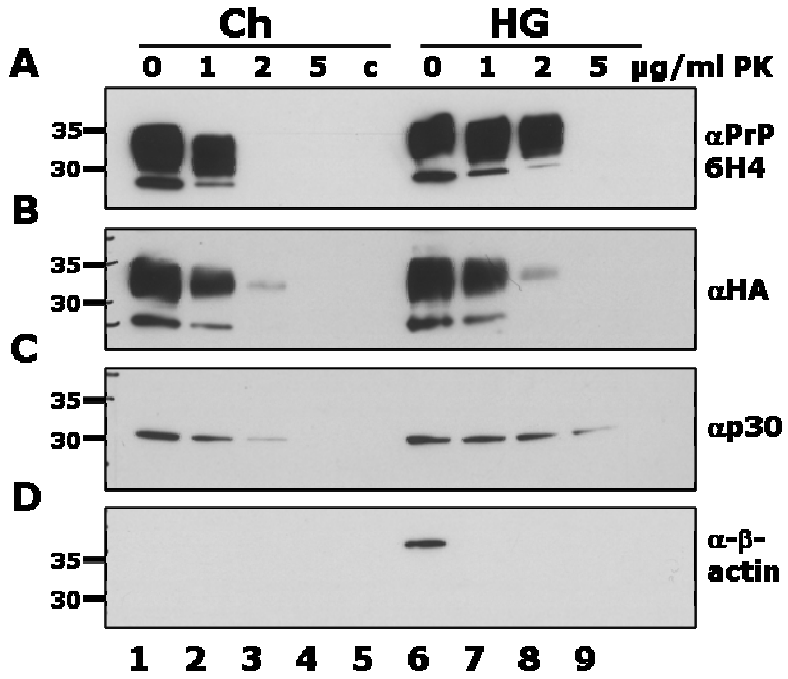


Figure 39 Western Blot analysisof conversion assay. PrP^{D142}–retroparticles particles were incubated with either infected brain homogenates (Ch) (lanes 1-4) to convert PrP content into PrP^{Sc} or uninfected brain homogenates (HG) as a control (lanes 6-9). An additional control, in which Ch was added but particles were absent was included (lane 5). Incubation with 1 µg/ml PK (lanes 2, 7), 2 µg/ml PK (lanes 3, 8), 5 µg/ml PK (lanes 4, 9) or left untreated (lanes 1, 5 and 6) was carried out. Samples were stained by anti-PrP 6H4 (A), anti-HA (B), anti-p30 MLV capsid (C) and anti-β-actin (D).

The differently incubated particles showed distinct titration behaviours. Unexpectedly, the PrP^{D142}-retroparticle sample containing HG was cleaved with a lower kinetic (Figure 39). Therefore staining against the viral capsid was included, to verify that similar amounts of particles were present. This was true for both samples as shown by the presence of equal amounts of the viral capsid protein p30 in the absence of PK (Figure 39 C, compare lanes 1 and 6). Nevertheless, again about half the amounts of PK were sufficient to digest p30 for the HG homogenate than for the Ch homogenate (Figure 39 C, lanes 1-4 to lanes 6-9). This

different titration behaviour was unexpected for p30, which was thought to show equal titration in both samples, when similar amounts of protein are present. Thus, this finding might be explained by different overall protein amounts and led to the assumption that the detection by the Bradford assay revealing 10 µg/ml for both Ch and HG was inappropriate. Therefore, the amounts of brain homogenate proteins were further analyzed by Western Blot analysis using an anti-β-actin antibody (Figure 39 D). The brain homogenate of untreated mouse HG could be stained by anti-β-actin antibody, whereas this band was absent for scrapie-infected mouse brain Ch (Figure 39 D). Thus, the staining revealed that more actin was present in HG homogenate.

As a consequence, the amounts of both homogenates, had to be reevaluated more exactly by further experiments. A Western Blot analysis using anti-β-actin was carried out for various volumes of the homogenate (Figure 40). Densitometric analysis revealed that roughly equal quantities of β-actin were present in 5 µl (lane 4, 6401 pixel) of Ch and 1.5 µl of HG (lane 7, 6344 pixel), indicating a more than twofold difference in the total protein amounts.

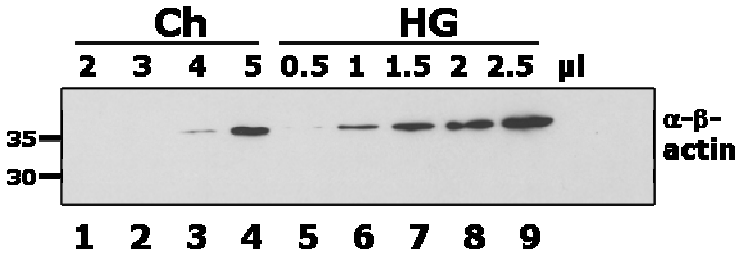


Figure 40 Detection of β-actin in the brain homogenates. Chandler-infected mouse brain homogenate and uninfected mouse brain homogenate was stained by anti-β-actin antibody. Different volumes were loaded ranging from 2-5 µl Ch and 0.5 to 2.5 µl HG. Densities were compared using black tone pixel values (Adobe, Photoshop Software).

However, it has to be considered that in Chandler infected mouse brain, the amount of PrP in the total protein content is higher than in HG, because PrP^{Sc} accumulates in these brains (Brandner, S 2003). Therefore, the determination of β-actin might be inappropriate, as PrP^{Sc} accumulation then influences the percentage of actin in the total protein amount.

Thus, the highly sensitive BCA protein assay (Pierce) was carried out for protein quantification.

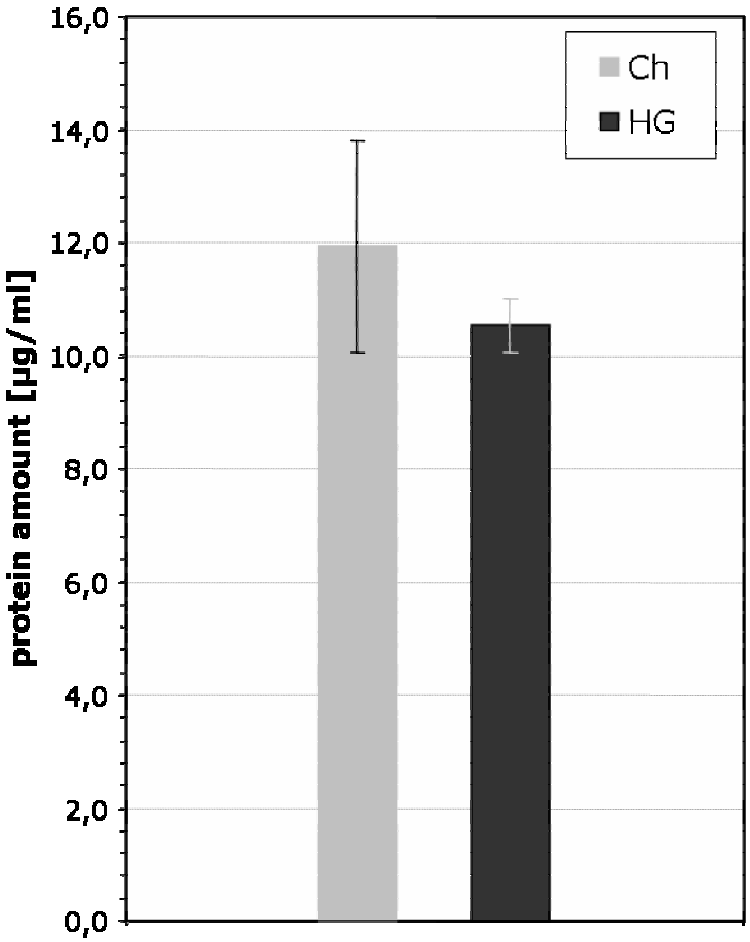


Figure 41 Quantification of total protein amounts in Ch and HG homogenates. Chandler-infected mouse brain (Ch) and uninfected brain homogenate (HG) were measured by the BCA-assay (Pierce) in three different dilutions (1:20, 1:40 and 1:60). BSA-solution (0.025-2µg/ml) was included as a standard. Extinction at 570 nm was measured. Mean values of total nine measurements and the corresponding error bars were determined.

The BCA assay revealed almost equal amounts of total protein present in both homogenates (Figure 41). There was no indication for higher protein levels in the HG homogenate. If at all, a 1.2fold higher amount in Ch was observed. This result was thus in line with previous the Bradford assay measurements.

Thus, these findings indicate that the conversion of PrP^{D142}-retroparticles did not result in altered PK digestion behaviour of PrP in the samples under the chosen conditions. Since in vitro conversions have been carried out under various conditions, as e.g. low pH and addition of detergents, the conversion process might be further adapted. Also, prolonging the incubation times might help to overcome a potentially slower kinetic for conversion of retroparticle-displayed PrP.

IV.2.2 Retroviral infection of scrapie-infected cells

Alternatively to the *in vitro* conversion of PrP^c displayed on the surface of retroparticles, the production of virus particles in scrapie-infected neuroblastoma cells (Race, R E et al. 1987) was considered. Virus particles displaying PrP^c and being released from such cells could present PrP^{Sc} either because of in vivo conversion of PrP^c or because of the incorporation of PrP^{Sc}, present on the cell surface, into their envelopes (Figure 42).

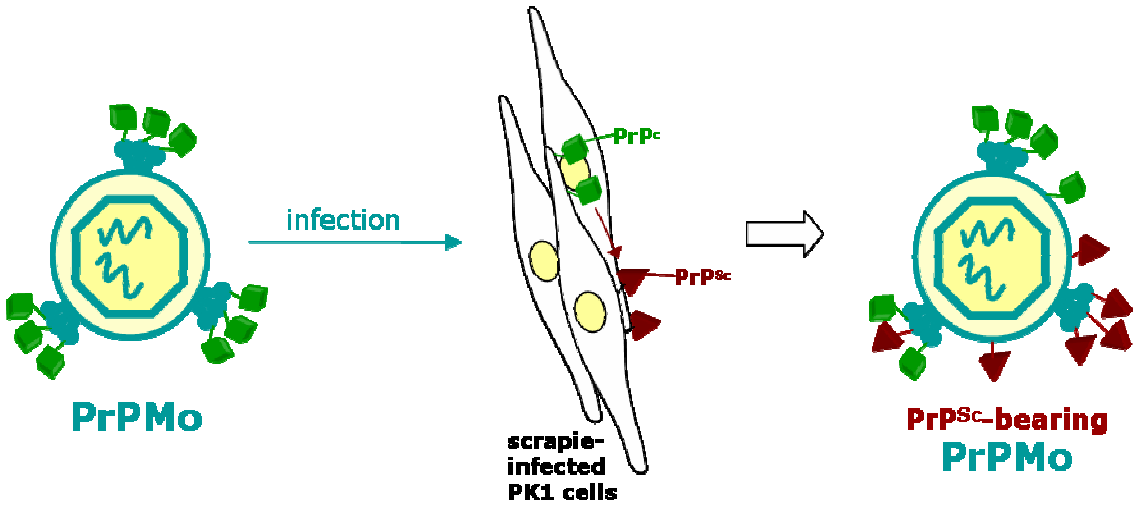


Figure 42 Schematic representation of the infection of scrapie-infected neuroblastoma cells by PrPMo. The replication competent PrPMo[111] is utilized to infect scrapie-infected PK1 cells. Virus particles produced in this cell line might bear the pathogenig form of PrP on the surface either as incorporated PrP^{Sc} or as PrP-Env fusion protein. PrP^c is indicated by green squares. PrP^{Sc} is represented by red triangles. The ecotropic Env protein is represented as blue trimers.

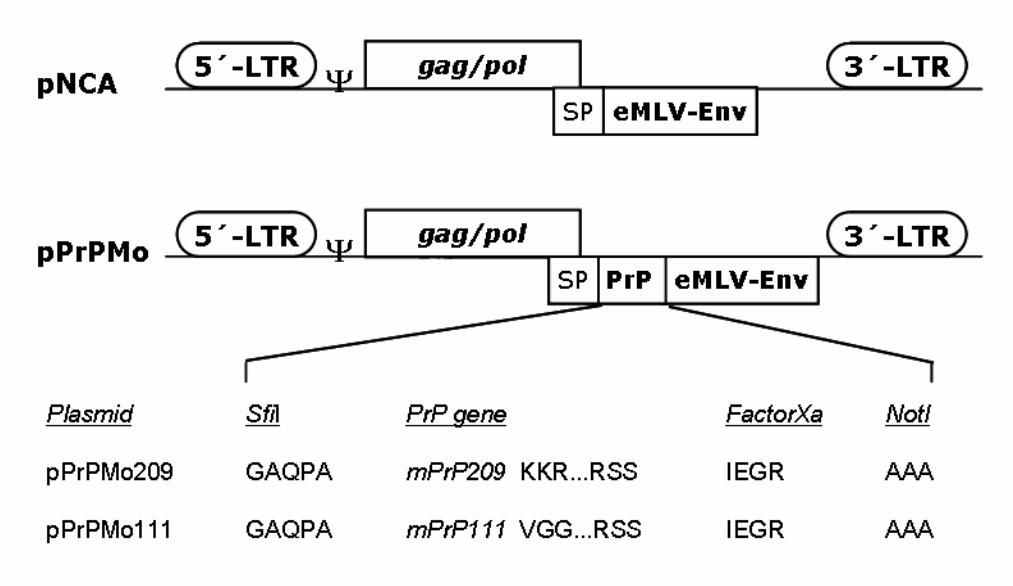
For the infection of scrapie-infected cells an ecotropic Moloney murine leukaemia virus variant (eMLV) had to be used. The ecotropic variant infects mouse cells, only (Kozak, C A et al. 1990). Due to lack of its receptor on the cell surface eMLV can not enter human cells. The ecotropic variants were chosen because of safety aspects of this type of virus. Transfer of this virus to the human population was therefore excluded.

The Prnp gene, or parts thereof, was inserted into the eMLV. For this, the envelope gene, which was proven successful in non-replicative retroparticle display (IV.1.1.4), was chosen. The insertion of Prnp was implemented in order to incorporate PrP into the newly synthesized viruses. Upon replication of such viruses in scrapie-infected cells, the PrP^c fused to viral Env would eventually be converted into the pathogenic form (Figure 42). At least an equilibrium state of the two forms of PrP can be expected for particles which are generated in the

scrapie-infected cell line. In this way, particles containing PrP^{Sc} on top of the viral Env would be harvested from retrovirus- and scrapie-infected cells.

IV.2.2.1.1 Construction

As it was the case for PrP^{E} fusion proteins in the pSALF backbone (IV.1.1.4), the PrP was inserted into the viral Env gene between the virally derived signal peptide and the env coding region (Figure 43). For this purpose, PrP coding sequences for PrP^{209} or PrP^{111} were inserted using *Sfi*I- and *Not*I-containing oligonucleotides (Table 3) as described previously (IV.1.1.4). As parental plasmid pEMo (Appendix, VI.1.9) was used. The generated plasmids were named pPrPMo209 and pPrPMo111, respectively. The corresponding viruses are referred to as $PrPMo^{209}$ and $PrPMo^{111}$.



Plasmid	SfiI	PrP gene		FactorXa	NotI
pPrPMo209	GAQPA	*mPrP209*	KKR...RSS	IEGR	AAA
pPrPMo111	GAQPA	*mPrP111*	VGG...RSS	IEGR	AAA

Figure 43 Schematic representation of the viral expression plasmids. The wt eMLV bearing expression plasmid pNCA is shown in the upper panel. The PrP-bearing pPrPMo plasmids are seen in the lower panel. Both constructs consist of the full viral genome. The PrP coding sequences are inserted into the viral env gene. A factor Xa cleavage site is included in the construct.

IV.2.2.1.2 Generation of PrPMo virus stocks

For production of the virus stocks, HEK-293FT producer cells were transfected with the generated plasmids pPrPMo209 and pPrPMo111 as well as with the wild-type eMLV coding plasmid pNCA (VI.1.8). The virus-containing cell culture supernatants were harvested and analysed by Western Blot for the presence of

the viral Env proteins. The molecular weight of PrPMo^{209} Env was 93.7 kD compared to 83.9 kD of PrPMo^{111} Env and 74.1 kD of wt eEnv.

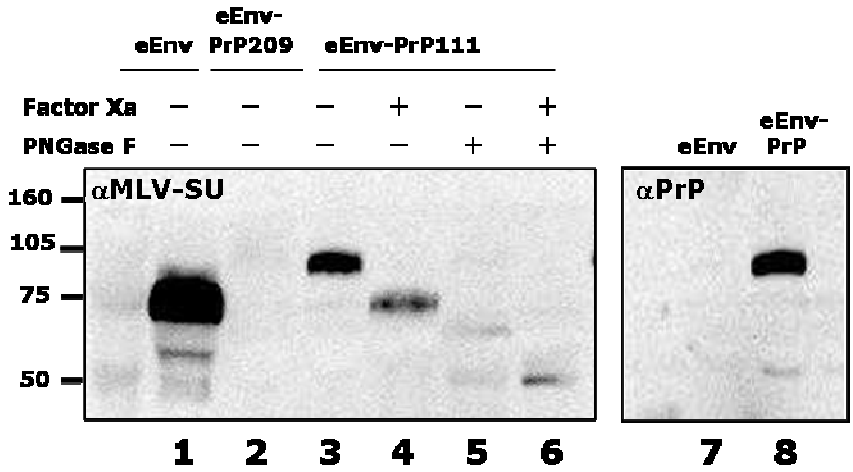


Figure 44 Western Blot analysis of PrPMo virus stocks. HEK-293FT producer cells were transfected with the virus-coding plasmid pNCA (lane 1, 7), PrPMo209 (lane 2) or pPrPMo111 (lanes 3-6, 8), respectively. Supernatants were harvested 2 days post transfection, filtered (0.45 µm filter) and subjected to centrifugation at 13.000 rpm (Eppifuge, Haraeus) for two hours. Loaded volumes corresponded to 0.75 ml (pNCA, lane 1, 7), 6 ml pPrPMo209 (lane 2) and 1 ml pPrPMo111 (lanes 3-6, 8). PrPMo^{111} Env was subjected to factor Xa cleavage (lanes 4, 6) and PNGaseF treatment (lanes 5, 6). Blots were stained by either anti-MLV-SU reactive serum (left panel) or anti-PrP6H4 (right panel).

Virus particles harvested from pNCA-transfected cells were included as a control and contained wt eMLV Env protein, as shown by staining with the anti-MLV-SU reacting antibody (Figure 44, lane 1). No signal was detected, when these particles were stained by anti-PrP 6H4. PrPMo^{111} Env migrated at the expected molecular weight. This protein was either detected by the anti-MLV-SU reacting serum or by the anti-PrP 6H4 antibody (Figure 44, lanes 3, 8). A signal for PrPMo^{209} Env could not be detected, although particles were pelleted from about six times more cell culture supernatants (Figure 44, lane 2).
Upon treatment with factor Xa, PrPMo^{111} Env was cleaved into its PrP and Env part, as confirmed by the appearance of the wt Env band (Figure 44, lane 4). Incubation with a Peptide:N-GlycosidaseF (PNGaseF) led to deglycosylation of PrPMo^{111} Env (Figure 44, lane 5). When both enzymes were added, cleavage and deglycosylation occurred (Figure 44, lane 6).

Thus, this result demonstrated successful production of PrPMo^{111} in transfected HEK-293FT producer cells which was not the case for PrPMo^{209}. Next, the titration of PrPMo^{111} stocks was carried out.

IV.2.2.1.3 Titration of PrPMo

The produced virus stocks were titrated on NIH-3T3 cells using the immune peroxidase assay (IPA) as described in detail in chapter III.3.4. The determination resulted in different titers of the three virus stocks (Table 6).

Table 6 **Titration of virus stocks PrPMo**

virus	wt eMLV	PrPMo111
Positive cells	141	263
positive cells per well	423	789
dilution factor	2×10^4	2×10^2
titer	8.4×10^6	2.3×10^5

The resulting volume used for subsequent experiments varied between 108 µl for PrPMo^{111} and 298 µl for 100fold prediluted wt eMLV to result in a moi of 0.1. In case of PrPMo^{209} revealing no positive infection event even in lowest dilution, the highest possible volume was added to the cells (500 µl).

IV.2.2.1.4 Expression of PrPMo Env post infection

Furthermore the expression of the PrPMo^{111} Env was examined post infection of NIH-3T3 cells carried out by immune fluorescence analysis (Figure 45). The analysis was done by staining with the specific anti-PrP 6H4 antibody and a second antibody labelled with FITC, which was directed against murine IgGs.

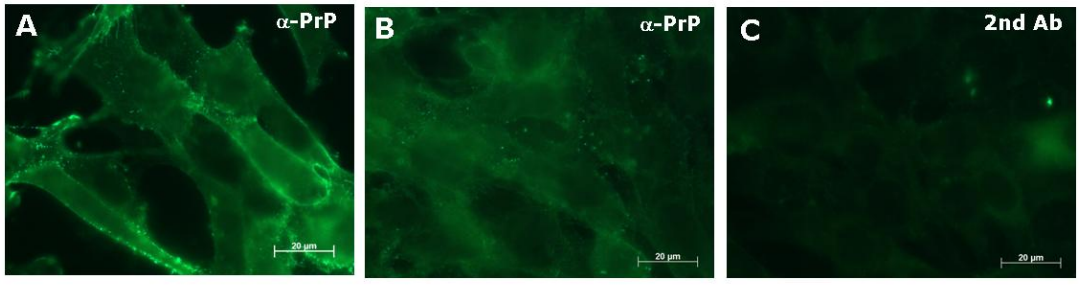


Figure 45 **Cell surface expression of the modified viral envelope protein on NIH-3T3 cells.** NIH-3T3 cells were infected with the modified ecotropic MLV PrPMo111 (A, C) at a moi of 0.1. Cells were analyzed 48h after infection and stained by the anti-PrP 6H4 and a FITC-labelled anti-mouse antibody (A) or by the second antibody, only (C). Uninfected cells (B) were stained in the same way described for (A).

NIH-3T3 cells have a low background level of endogenously expressed PrP^c as confirmed by staining of the cells by the ant-PrP 6H4 antibody i(Figure 45 B). Background binding of the secondary antibody, was rather low (Figure 45 C).
$PrPMo^{111}$ Env was detectable in infected NIH-3T3 cells at a moi of 0.1 using anti-PrP 6H4 antibody (Figure 45 A). Compared to endogenous PrP^c level the signal was slightly increased when cells were infected by $PrPMo^{111}$ (compare Figure 45 A and B).
This result indicated the presence of $PrPMo^{111}$ Env also in virally infected NIH-3T3 cells.

IV.2.2.1.5 Morphology analysis by electron microscopy

To exclude alterations in the morphology of $PrPMo^{111}$ compared to eMLV electron microscopy analysis including immunogold labelling was performed. Transiently transfected HEK-293FT producer cells were therefore examined using ultrathin sections to detect C-type MLV particles. The cell culture supernatants derived from these cells were examined *via* negative contrast. Subsequently they were stained with the anti-PrP 6H4 antibody and a secondary, gold-labelled antibody.
Virus particles were found in different budding states for wt eMLV (**Figure 46** A, B). Furthermore, supernatants contained C-type retrovirus particles in case of wt eMLV (**Figure 46** C), which were not stained by the anti-PrP 6H4 antibody (**Figure 46** G). For $PrPMo^{111}$, budding particles were also found in cell sections to similar extent (**Figure 46** D, E) and the supernatants of these producer cells also revealed C-type MLV particles. The latter contained PrP on their surface as shown by anti-PrP 6H4 specific stain (**Figure 46** F).
Thus, electron microscopy experiments confirmed the successful production of $PrPMo^{111}$ in HEK-293FT producer cells. The virus variant presents PrP on the viral surface without being altered in morphology and shape. Based on this result the question, whether $PrPMo^{111}$ can replicate to same extent as wt eMLV, was adressed next.

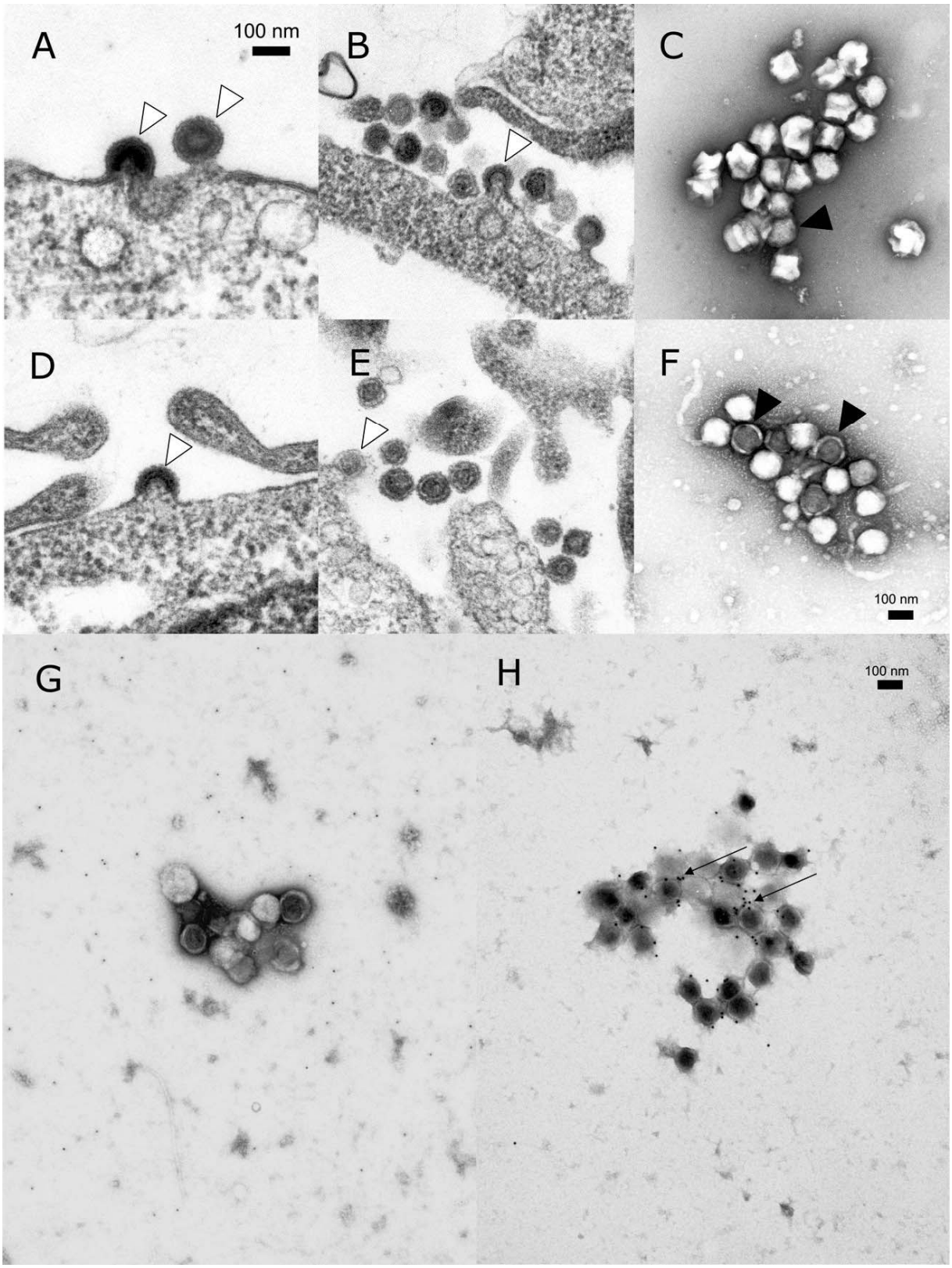


Figure 46 Electron microscopic analysis of PrPMo^{111}. Cell sections of transfected HEK-293FT are shown for both wt eMLV (A, B) and PrPMo^{111} (D, E). Cell culture supernatants are shown for wt eMLV (C, G) and PrPMo111 (F, H). Samples were labelled by anti-PrP 6H4 and immunogold-labelled secondary antibody. Typical C-type retroviruses in budding states are indicates by open arrowheads. Virus particles are indicated by filled arrowheads. Gold particles are indicated by arrows.

IV.2.2.1.6 Replication kinetic of PrPMo

PrPMo^{111} was next tested for its replication kinetics in cell culture. Thus, the kinetic experiment was carried out on NIH-3T3 cells at an moi of 0.1 for both, PrPMo^{111} and wt eMLV. PrPMo^{209} was included in the kinetic study, although due to results from the titration experiment (IV.2.2.1.3) the highest possible volume of this variant was added (500 µl) RT activity (III.4.2) was monitored on day 1, 2, 4, 5, 7 and 8 post infection. Upon this, values were determined in triplica for each sample. Simultaneously, supernatants were subjected to RNA preparation followed by reverse transcription with the EMoseq primer (Table 3). Afterwards, cDNA was analyzed by PCR using the CB6/EMoSeq2 primer pair (Table 3), ensuring that the Prnp gene insertions had not been deleted during viral replication. Expected molecular weights of the PCR fragments were 743 bp of PrPMo^{209} and 446 bp of PrPMo^{111} compared to 98 bp of wt eMLV.

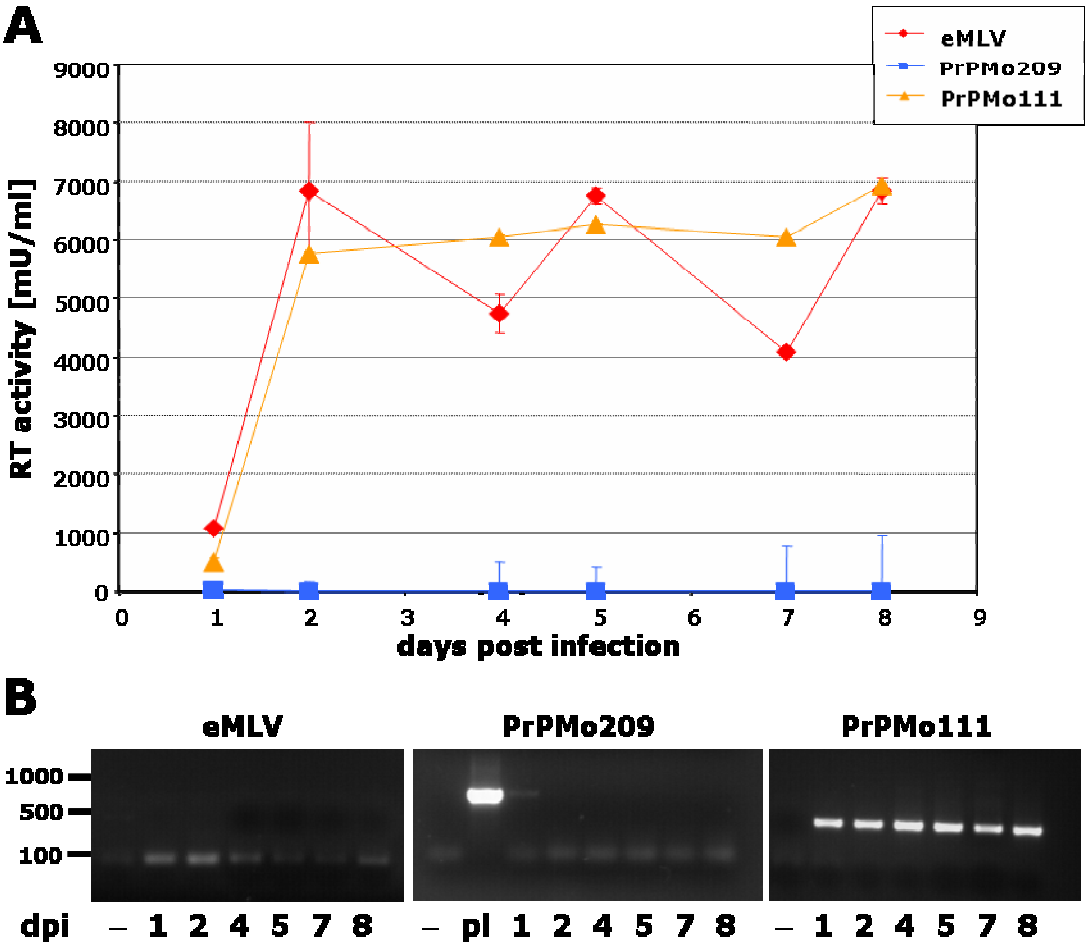


Figure 47 **PrPMo growth kinetics on murine NIH-3T3.** Titrated virus stocks PrPMo^{111} (orange triangles), PrPMo^{209} (blue squares) and wt eMLV (red diamonds) were used for infection of NIH-3T3 cells (A).

Supernatants were tested 1, 2, 4, 5, 7 and 8 days post infection in RT assay to quantify virus. At the same time, samples were subjected to RNA isolation (B) and subsequent reverse transcription/PCR assay using CB6 and EMoSeq2 primers. Non reverse-transcribed RNA samples were included as negative controls (-), pPrPMo209 plasmid (pl) served as positive control. *dpi* days post infection.

The replication kinetic of PrPMo^{209} and PrPMo^{111} showed distinct results. Whereas PrPMo^{111} replicated similar to the wt eMLV, PrPMo^{209} did not replicate. Both PrPMo^{111} and wt eMLV reached high particle numbers on day two post infection and retained constant levels during one week as monitored for 8 days post infection. PrPMo^{209} virus was not detected in NIH-3T3 cell culture supernatants.
Amplification of the corresponding viral env genes showed the expected electrophoretic mobility shifts for PrPMo^{111} and wt eMLV for all time points. In contrast, PrPMo^{209} signals were lost on day two post infection.
These findings indicated that PrPMo^{111} was able to replicate in NIH-3T3 cells roughly to the same extend as wt eMLV. However, PrPMo^{209} was not able to infect and replicate to NIH-3T3 cells. Next it remained to be determined how stable PrPMo^{111} was upon passaging the virus in cell culture. This question was addressed in the following chapter.

IV.2.2.1.7 Genetic stability of PrPMo^{111}

The genetic stability of PrPMo^{111} was addressed *via* passaging of the virus on NIH-3T3 cells (Figure 48). For this purpose, cells were infected with a moi of 0.01 and passaged for 7 days post infection. Supernatants were harvested, 100fold diluted and transferred to naïve NIH-3T3 cells in. Six rounds of passaging were implemented and analysis was done by IPA on day 3 and viral RNA isolation on day 6 post infection of each cycle. Both analysis were done in triplica. For RNA isolation and subsequent PCR analysis the expected molecular weights were 446 bp for PrPMo^{111} and 98 bp for wt eMLV.
For all six passages approximately the same values of virus-infected cells were obtained. Percentage of red stained cells thereby varied from 14-18 % (Figure 48 A-F).
RNA preparations obtained from passage one were free of a PCR signal ensuring lack of plasmid DNA in the virus stock. The PCR analysis performed on plasmid DNA from either pPrPMo111 or pNCA revealed the expected mobility shifts for the amplified sequences.

Signals of all passages of PrPMo^{111} showed intact env sequences including the PrP^{111} insertion (Figure 48 G, lanes 5-22) , which was further verified by sequencing analysis of the amplified sequences (not shown).

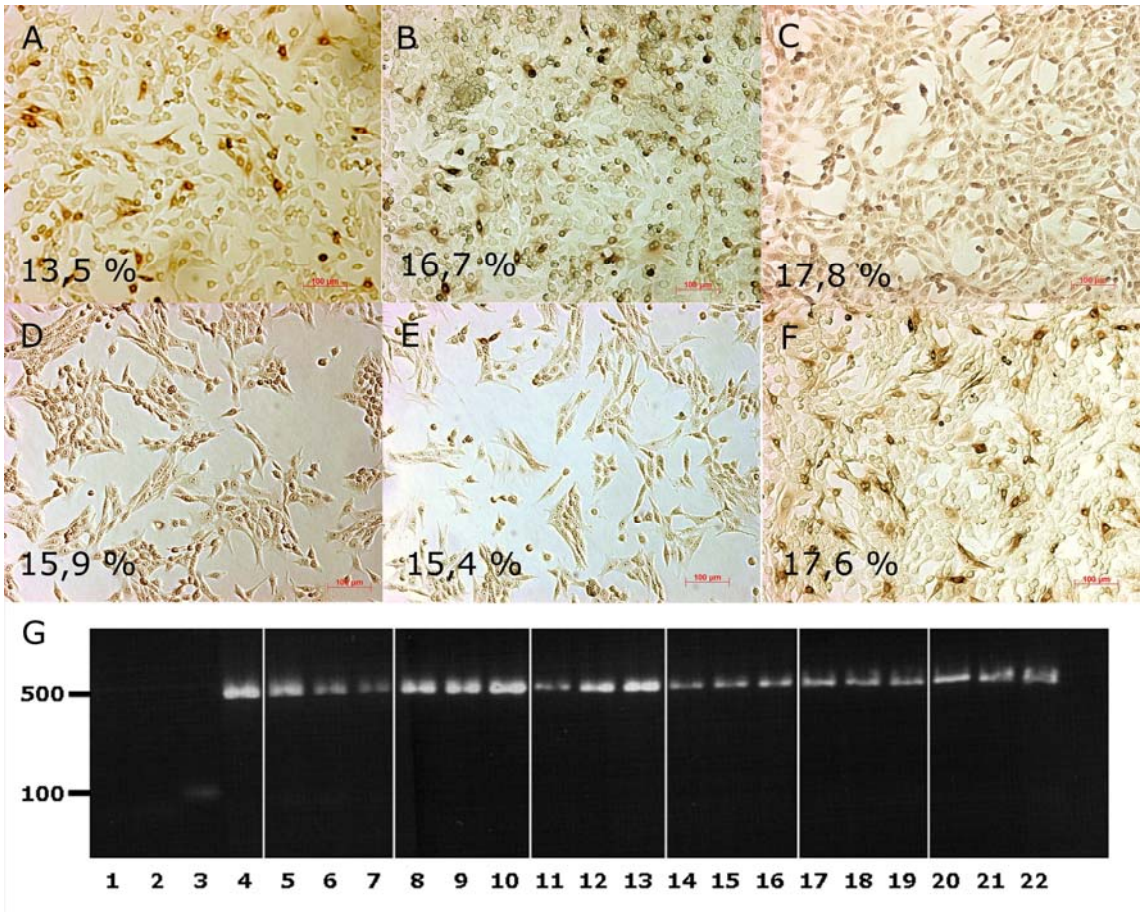


Figure 48 Genetic stability of PrPMo^{111}. The PrPMo^{111} virus stock was used for infection of NIH-3T3 cells at a moi of 0.01. Cells were cultivated for 6 days; supernatants were then diluted 100fold and were subsequently used for the next infection cycle. Immune peroxidase assays were carried out at day 3 post infection of each cycle for passages 1-6 (A-F). Percentage of infected cells is shown as values in the images. Viral supernatants were subjected to RNA preparation at day six post infection of each cycle and subsequent reverse transcription followed by PCR analysis with CB6 and EMoSeq2 primer pair (G) was carried out. PCR control without template (lane 1) and non-reverse-transcribed RNA sample from passage 1 (lane 2) were included during PCR reaction. Plasmids pNCA coding wt eMLV (lane 3) and pPrPMo111 (lane 4) served as positive controls. Each passage was carried out *in triplica*. Passage 1 (lanes 5-6), passage 2 (lanes 8-10), passage 3 (lanes 11-13), passage 4 (lanes 14-16), passage 5 (lanes 17-19), passage 6 (lanes 20-22).

Thus, PrPMo^{111} remained genetically stable during six passages on NIH-3T3 cells, no deletions occured upon replication in these cells throughout the experiment.

IV.2.2.1.8 Host range of PrPMo^{111}

Insertions of foreign proteins have previously been shown to alter the host range of MLV (Cosset, F L, Morling, F J et al. 1995). To exclude, that the *Prnp* gene

influences the host range of PrPMo^{111}, different target cells were tested for virus integration (Figure 49). After infection with a moi of 0.5, the murine cell lines N2a and NIH-3T3 as well as the human cell lines HEK-293FT and A3.01 were examined by PCR analysis using genomic DNA one day post infection.

The cell line A3.01 was especially chosen as a T cell line expressing endogenous human PrP, in contrast to HEK-293FT, which show very low levels of cellular PrP expression. Therewith, the influence of the expression of PrP^{c} on the surface of target cells was considered.

Besides PrPMo^{111} and the wt eMLV infection, also a VSV-G pseudotyped PrPMo^{111} was included in the experiment. This virus was produced by cotransfection of the VSV-G encoding plasmid pM.DG (Appendix, VI.1.10) with pPrPMo111 resulting in production of the virus stock PrPMo^{111}(VSV-G). The stock was titrated on NIH-3T3 cells revealing a titer of 1.3×10^5 IU/ml. VSV-G generally enables cell entry into all tested cells and served as a positive control during the experiment.

A3.01 cells were further subjected to analysis of genomic DNA and cellular RNA on day 2, 4, 6 and 8 post infection to follow-up viral transcription.

The tested cell lines were free of endogenous signals upon analysis of genomic DNA by PCR with the primer pair CB6/EMoSeq2 amplifying the env gene of the eMLV (Figure 49). The PCR analysis was expected to yield a 446 bp signal for PrPMo^{111} as it was observed for the plasmid control (Figure 49 B, upper left panel, lane 2) and a 98 bp signal for wt eMLV. The wt eMLV was restricted to mouse cell lines and showed no integration into human cells when genomic DNA was examined. Genomic DNA of NIH-3T3 cells showed integration of PrPMo^{111} and PrPMo^{111}(VSV-G) viruses. The same result was observed for N2a cells; although the efficiency of infection was reduced compared to NIH-3T3 cells (Figure 49 A, left panel, lanes 3 and 4, respectively). In contrast to that the human cell lines HEK-293FT and A3.01 showed integration of PrPMo^{111}(VSV-G), only. A faint band was also observed for PrPMo^{111} on A3.01 genomic DNA (Figure 49 A, right panel).

This finding indicated, that PrPMo^{111}(VSV-G) and to a minor extend PrPMo^{111} were present in A3.01 cells one day post infection, which was not observed for wt eMLV. However, further analysis showed, that these signals from genomic DNA disappeared from day 2 to day 8 post infection, although the loading control of human glycerolaldehydphosphat dehydrogenase (*h*GAPDH) confirmed that equal amounts of DNA had been used in the samples (Figure 49, upper left panel, lanes 3-6).

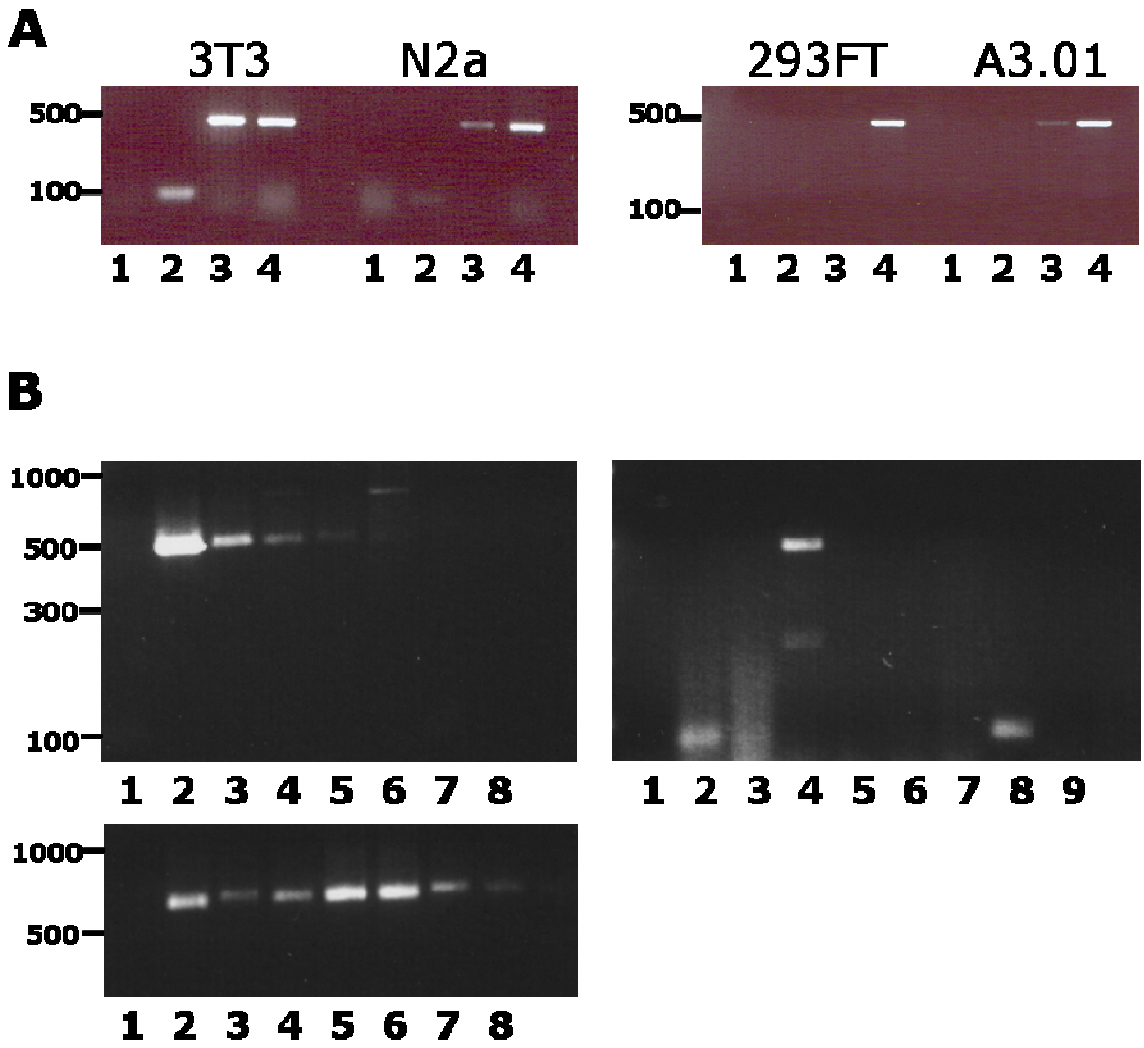


Figure 49 Analysis of the tropism of PrPMo^{111}. A) Cell lines NIH-3T3, N2a, HEK-293FT and A3.01 were infected by either wt eMLV (lane 2), PrPMo111 (lane 3), PrPMo^{111}(VSV-G) (lane 4) or left uninfected (lane 1). PCR analysis was carried out using CB6/EMoSeq2 primer pair detecting viral env genes on genomic DNA prepared on day one post infection. **B)** PrPMo^{111}-infected cell line A3.01 was analyzed on day 2, 4, 6 and 8 post infection using either genomic DNA (left panels, lanes 3-6) or reverse transcribed cellular RNA (right panel, lanes 4-7). As a comparison wt eMLV-infected genomic DNA (left panels, lanes 7, 8) or RNA (right panel, lanes 8, 9) of A3.01 cells was analyzed on day 2 and 4 post-infection. PCR-samples without template (lane 1, each panel), pPrPMo111 plasmid (upper left panel, lane 2), DNA from uninfected A3.01 (lower left panel, lane 2) and non-reverse transcribed RNA of wt eMLV (right panel, lane 2) or PrPMo111 (right panel, lane 3) served as controls. PCR was carried out using CB6/EMoSeq2 primer pair amplifying the viral env gene (upper panels) or hGAPDHforw/rev primer pair detecting the human glycerolealdehydphosphat dehydrogenase (lower panel) as loading control.

In addition to the analysis of genomic DNA, also RNA was isolated from human A3.01 cells. Upon this, RNA isolations of wt eMLV-infected cells showed the corresponding env gene signal, when non-reverse transcribed RNA was loaded (Figure 49 B, right panel lane 2) indicating that the RNA preparation was not

completely free of DNA. Subsequently, reverse transcribed samples showed signals for wt eMLV env as well (Figure 49 B, right panel lane 8). This finding led to the assumption that low levels of pNCA wt eMLV plasmid, present in virus stocks, possibly entered the cells causing background signals of RNA isolates.
Nevertheless, this was not the case for PrPMo^{111} virus stocks (Figure 49 B, right panel lane 3). In this case transcribed RNA was detected in the cells on day 2 post infection, only (Figure 49 B, right panel, lanes 4-7).

Taken together, the results suggested, that PrPMo^{111} did not stably integrate into A3.01 cells. The insertion of the *Prnp* gene into eMLV thus did not extend the tropism of this virus variant to human cells.

IV.2.2.1.9 Infection of scrapie-infected PK1 cells

The scrapie-infected cell line PK1 was infected by either the wt eMLV or PrPMo^{111}. To determine whether these two viruses can enter PK1 cells, genomic DNA and cellular RNA were isolated one day after infection and subsequently analyzed by PCR with the primer pair CB6/EMoSeq2. As a prerequisite for further infection analysis the RT activity of the PK1 cell line in comparison to N2a cells was monitored for 8 days to reveal the presence of endogenous MLV. The RT values were determined in triplica.
For PK1 infection, different moi including 2, 4, 8, 10 and 20 for wt virus and 2, 4, 10 for PrPMo^{111} were chosen. The infected cells were cultivated for 9 days and viral production was followed by RT activity assay in triplica for each value.
For wt eMLV at an moi of 20, cells were (after one freeze and thaw event) recultivated and monitored for additional 20 days.
Analysis of DNA revealed signals for both wt eMLV and PrPMo^{111} in PK1 cells, whereas no endogenous signal was present (Figure 50 A). Also in isolated RNA, presence of PrPMo^{111}-derived RNA was detected (Figure 50 A). PK1 cells lack endogenous MLV in contrast to N2a cells, as shown *via* RT activity assay (Figure 50 B). The N2a cell line showed constantly low levels of MLV particles present in supernatants about 20 mU/ml. This RT activity number corresponds to 6×10^7 virus particles using the equation $n_{CP} = 3 \times 10^9$ [p/units] × RT-value [units] (Pyra, H, Boni, J et al. 1994).

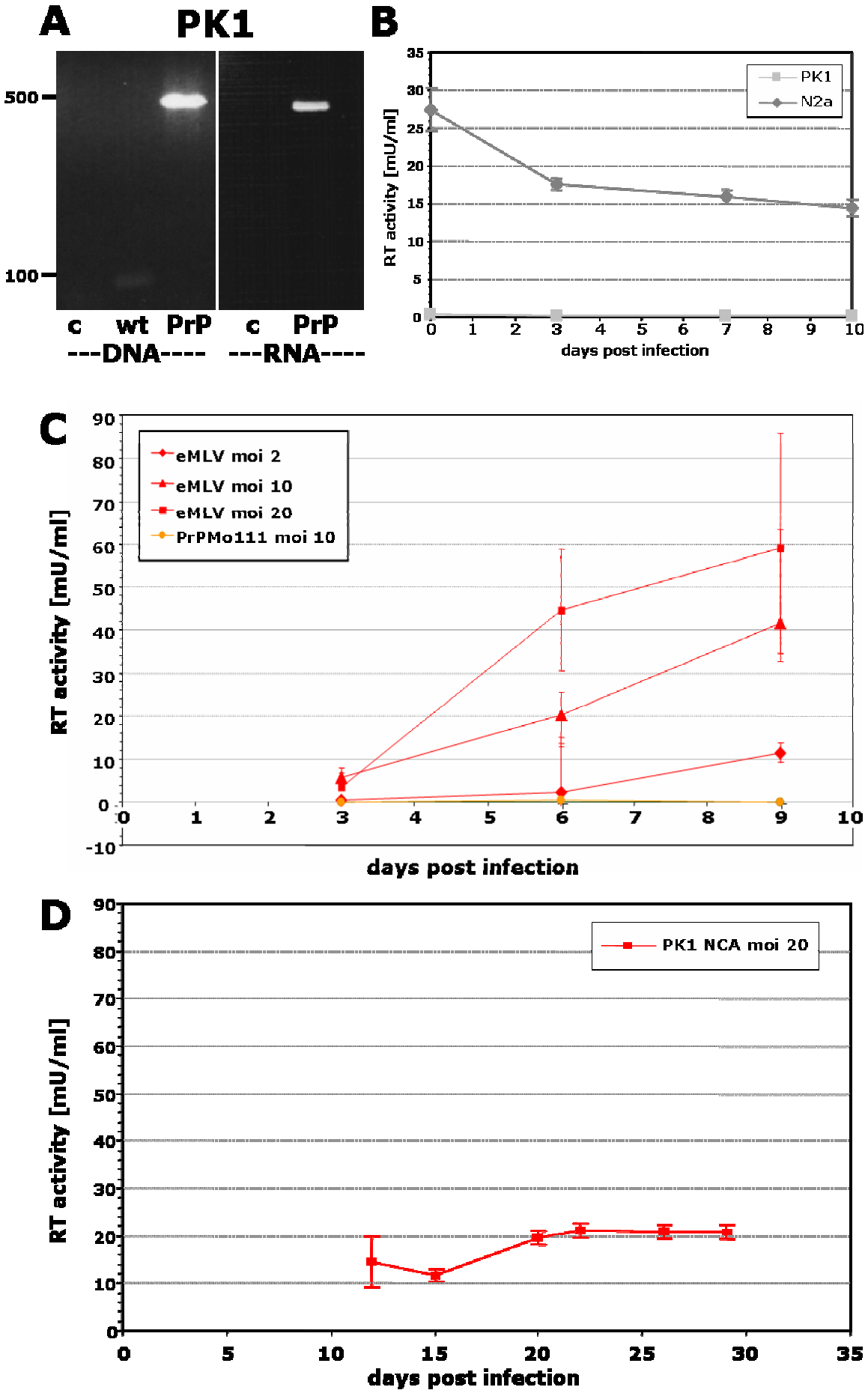

A
PK1
500
100
c
wt
PrP
c
PrP
---DNA----
---RNA----
B
RT activity [mU/ml]
days post infection
PK1
N2a
C
RT activity [mU/ml]
days post infection
eMLV moi 2
eMLV moi 10
eMLV moi 20
PrPMo111 moi 10
D
RT activity [mU/ml]
days post infection
PK1 NCA moi 20

Figure 50 Infection of PK1 cells with wt eMLV and PrPMo^{111}. Scrapie-infected PK1 cells were infected with wt eMLV or PrPMo^{111}. **A)** PCR analysis using CB6/EMoSeq2 was carried out on genomic DNA and cellular RNA isolated one day post infection (moi of 0.4). ***c*** uninfected cells ***wt*** eMLV ***PrP*** PrPMo111 **B)** RT activity test of either uninfected N2a or PK1 cells **C)** RT activity test of PrPMo^{111} or wt eMLV infected PK1 cells using different moi (2, 4, 8, 10, 20). **D)** RT activity test of recultivated wt eMLV-infected PK1 cells (moi 20).

The infection of PK1 cells with PrPMo^{111} did not lead to production of detectable titers independent from the chosen moi (Figure 50 C, orange lines). In contrast to that, infection with wt eMLV resulted in low level particle production. The amount of produced particles correlated well with the corresponding moi (Figure 50 B, red lines). Further analysis of the infected PK1 cells (moi 20) showed that the numbers remained at a low level. Released RT values did not exceed 20 mU/ml and stayed constant for over 20 days (Figure 50 D). As for N2a cells, the particle numbers produced from infected PK1 cells mounted up to 6×10^7.

Taken together, it was not possible to infect PK1 cells with PrPMo^{111}, although the infection with wt eMLV at high mois resulted in low titer production of virus in PK1 cells. Nevertheless, the amount was too low to subsequently analyze the particles for PrP incorporation.

V Discussion

Basically, this thesis has provided insights into the incorporation of the prion protein (PrP) into retroparticles and the connection between prions and retroviruses. A novel vaccine candidate against prion disease has been developed and implications on further improvement are being made as it will be discussed in the last part of this chapter.

V.1 Incorporation into retroviral particles

Retroviral particles were selected as display vehicles based on their known ability to accommodate foreign transmembrane proteins in their envelope membrane. An important prerequisite for incorporation is an efficient cell surface expression thus increasing the likelihood of foreign transmembrane proteins to be present at sites of viral budding.

A system, previously used for cell surface targeting of proteins, consisting of a signal peptide derived from the κ light chain and the platelet derived growth factor receptor (PDGFR) transmembrane domain (Chesnut, J D, Baytan, A R et al. 1996; Douglas, J T, Miller, C R et al. 1999) was used. This approach resulted in efficient cell surface expression of the epidermal growth factor receptor (EGF) molecule (Merten and Buchholz, *unpublished*) as well as of the C-terminal prion protein (PrP) domains (PrP^{111} consisting of aa 121-231 and PrP^{142}, aa 90-231), and consequently in highly efficient particle incorporation.

The incorporation of cellular proteins into retroviruses has previously been shown for human immunodeficiency virus (HIV) and murine leukemia virus (MLV). It was demonstrated that budding by the HIV-derived Gag protein proceeds without significant alteration of the original host protein composition at the cell membrane (Hammarstedt, M and Garoff, H 2004). Upon this, the observation, that the bulk of the host proteins, e.g. actin and clathrin, were passively included into the virus-like Gag particles was made. This result suggested that budding by HIV Gag proceeds without significant alteration of the original host protein composition at the cell membrane. Nevertheless, some proteins were concentrated in the particles, and a few were excluded (the latter were not further identified). The concentrated proteins included cyclophilin A (a peptidyl-prolyl *cis-trans* isomerase, shown to be required for efficient HIV-1 infection) and Tsg-101 (a host protein required for HIV-1 budding, in its absence HIV is arrested at a late stage of its budding process). These were recruited to the

plasma membrane by HIV Gag. The membrane-bound cyclophilin A was concentrated into particles as efficiently as Gag itself, whereas Tsg-101 was concentrated more efficiently. The latter finding is consistent with a role for Tsg-101 in Gag particle release (Hammarstedt, M and Garoff, H 2004).

Also for MLV it was found that most plasma membrane proteins become incorporated into the retrovirus envelope without significant sorting of host proteins (Hammarstedt, M, Wallengren, K et al. 2000). Interestingly, it was furthermore demonstrated that interactions between virion-anchored host molecules and their cognate ligands take place. Such an enhancement of the viral attachment process was found to result in an increase of infectivity of HIV-1. The incorporation of e.g. CD28 and the acquisition of this specific host surface glycoprotein e.g. modulates the virus life cycle (Giguere, J F et al. 2002).

The incorporation of the retroviral Env protein is, in contrast to passively included plasma membrane proteins, a more specific process. At this, the interaction with the retroviral matrix protein MA seems to play a role (Yu, X et al. 1992). For γ-type retroviral Env proteins, an approach using directed evolution implies a role of the so-called R peptide. The corresponding R-peptide cleavage site is processed by the viral protease and seems to be important for the Env incorporation and pseudotyping mechanism (Merten, C A et al. 2005).

Thus, the incorporation of PrP^{c} displayed on either transmembrane domains or on viral Env proteins may also be influenced by such regimens. Similarly to the above mentioned inclusion of cellular proteins, the incorporation of PrP^{D111} and PrP^{D142} into retroviral particles most likely reflected the composition of the producer cell membranes. In comparison to that, the incorporation of the PrP^{c}-carrying Env protein (PrP^{E111}) into particles was found to be substantially lower. Assuming equal well detection with the anti-PrP 6H4 antibody, incorporation rates of of PrP^{E111} were generally lower than those of PrP^{D111} (Figure 51). However, when expressed in context of the replication competent ecotropic MLV (eMLV), PrP Env proteins where more efficiently incorporated, suggesting that the Env protein of ecotropic MLV is more suitable for PrP display than that of amphotropic MLV. Furthermore, incorporation into HIV-derived VLPs with both, PrP^{E111} or PrP^{D111} was about twofold less efficient than for the corresponding MLV-derived VLPs.

The incorporation values in percent were roughly calculated from anti-PrP signals in separate Western Blot analyses.

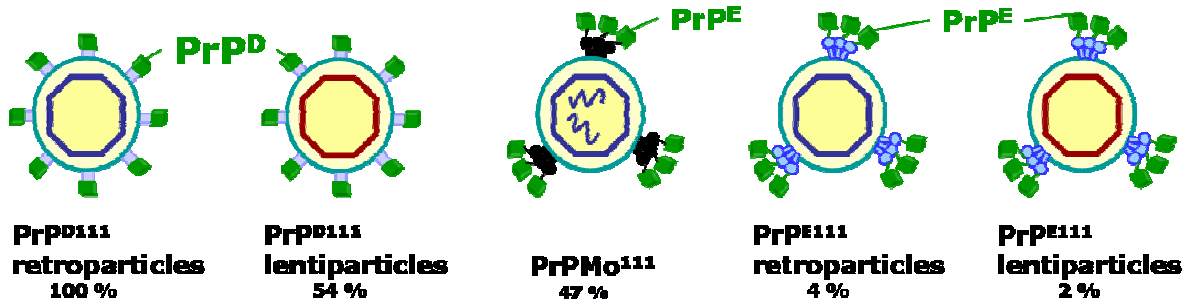


Figure 51 **Comparison of the generated particles.** Display- and Env-based particles are shown from left to right according to their PrP-incorporation capacity. Incorporation rate was roughly calculated from PrP^c signal in Western Blot analysis. Upon this, the amount of particles present was not taken into account and would eventually redirect the resulting efficiencies. However, since all particles were prepared according to the same protocol, the amount of PrP^c present in the particle stocks may be compared without using equal particle amounts as basis. Highest capacity (left hand side), lowest capacity (right hand side). PrP^c is shown as green cubes; different capsids are shown as octagons, *HIV* red octagons, *MLV* blue octagons. *Ecotropic MLV Env* molecules are shown in black, *amphotropic MLV Env* molecules are shown in blue.

The incorporation rates for PrP^{D142} retroparticles even exceeded those of PrP^{D111} retroparticles (130%), thus demonstrating that amino acids 90-120 did not interfere with incorporation into retroparticles. However, in all settings the complete PrP molecule (PrP^{209}, aa 23-231) showed strongly impaired expression and drastically reduced incorporation efficiencies.

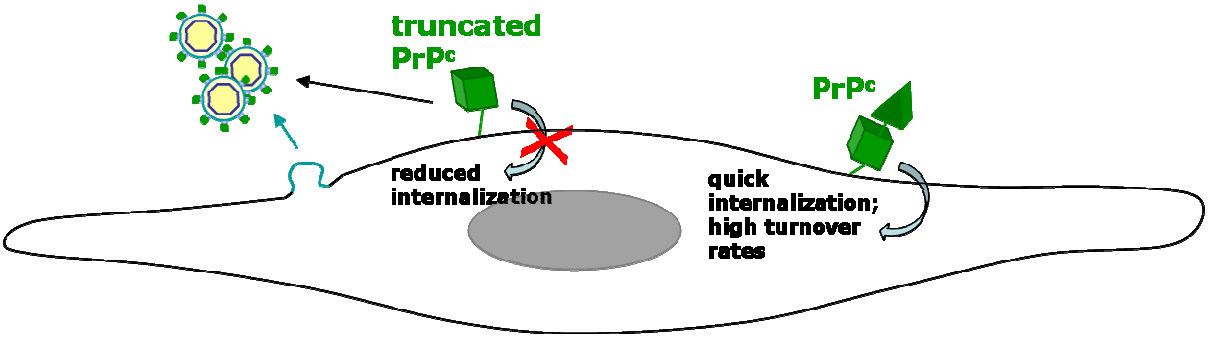


Figure 52 **Hypothesis for incorporation of truncated PrP^c into VLPs.** Truncated PrP forms bearing aa 90-231 or 121-231, respectively, might show reduced internalization and turnover rates compared to wt full-length PrP^c and are therefore incorporated into VLPs with higher efficiency. PrP^c, bearing aa 23-231, contains the flexible N-terminus (triangle) which might contribute to quick internalization and turnover.

This observation might be explained by reduced internalization rates and increased half-life of PrP-truncation mutants (Nunziante, M et al. 2003), in which amino acids 23 to 90 were deleted, might facilitate the incorporation into VLPs. Therefore the hypothesis that the flexible N-terminal arm of PrP^c is responsible for quick internalization and turnover is postulated (Figure 52).

V.2 Prions and retroviruses

V.2.1 PrP influence on cell entry

$PrPMo^{111}$, displaying aa 121-231 of PrP^c on top of the ecotropic Env protein, represents a newly generated virus, that most interestingly propagates to a similar extend as wt eMLV. It was expected that the cell entry properties of MLV were altered upon PrP^c display. In very few cases host range extension of ecotropic MLV to human cells was described, as e.g. the display of a human MHC class I specific single chain variable fragment (scFv), which retargeted an ecotropic MLV to human MHC-positive cells thus extending its host range (Marin, M et al. 1996). Usually the display of foreign polypeptides resulted in host range restriction. Two types of host range restriction can be distinguished. The first one is represented by display of the epidermal growth factor (EGF). The resulting MLV variants replicated freely on EGF receptor-poor cells but did not propagate on EGF receptor-rich cells because they were sequestered by the EGF receptors (Buchholz, C J et al. 1998). As a second possible influence, trimerizing molecules like CD40 ligand have been shown to block retroviral infectivity in general (Morling, F J, Peng, K W et al. 1997).

The insertion of PrP was therefore also expected to alter viral entry into cells. This entry might be abrogated or also facilitated by either PrP-PrP interaction or binding to a putative PrP receptor. Such a putative receptor has been postulated to be the 37-kDa/67-kDa laminin receptor (Gauczynski, S et al. 2001), Heparan sulphate proteoglycan (HSPG) (Horonchik, L et al. 2005) and a few other surface receptors (Lasmezas, C I 2003). Mapping analyses in the yeast two-hybrid system and cell-binding assays identified amino acids (aa) 144-179 as a direct and aa 53-93 as an indirect HSPG-dependent laminin receptor precursor (LRP)-binding site on PrP (Hundt, C et al. 2001). Therefore PrP^{111} would be sufficient at least to bind to the laminin receptor on the cell surface via its direct binding site. Although the laminin receptor is a ubiquitously expressed protein, the expression levels in the herein used cell lines have not been tested. However, PrP^{111} did not show blocking potential, since $PrPMo^{111}$ was replication competent on NIH-3T3 cells. NIH-3T3 cells have low level of PrP^c and might have also low levels of the putative PrP-receptor, so that an influence through receptor binding can not be observed. The human T cell line A3.01, which expresses PrP^c, was therefore also tested for $PrPMo^{111}$ replication capacity. The observation was that $PrPMo^{111}$

entered these cells with higher efficiencies than the wild-type eMLV, but was unable to replicate in these cells. However, MLV has generally a low replication capacity in human primary T cells (Ebeling, S B et al. 2003), so that the A3.01 cell line was not chosen optimally to detect PrP-dependent entry and subsequent virus replication. Contrary, a chimeric eMLV consisting of the amphotropic Env protein has been shown to replicate in human T cell lines Jurkat and A3.01 (Hartl I, unpublished data). Nevertheless, there might be post-entry blocks as Trim5α or APOBEC3G present in A3.01 cells that inhibit ecotropic MLV before the virus integrates into the cellular genome (Bishop, K N et al. 2004; Yap, M W et al. 2004).

Thus, it remains unclear whether PrP^{111} shows either no influence on the host range or can mediate a host range extension on PrP^{c}/PrP^{c}-receptor-positive cells (Figure 53). Further implications would be obtained by introducing the PrP coding sequence into HEK 293FT cells. This cell line was shown to be negative for $PrPMo^{111}$ replication and might show PrP-specific entry and subsequent replication. Upon this, PrP-dependent entry might be competed by use of PrP-specific or PrP receptor blocking antibodies.

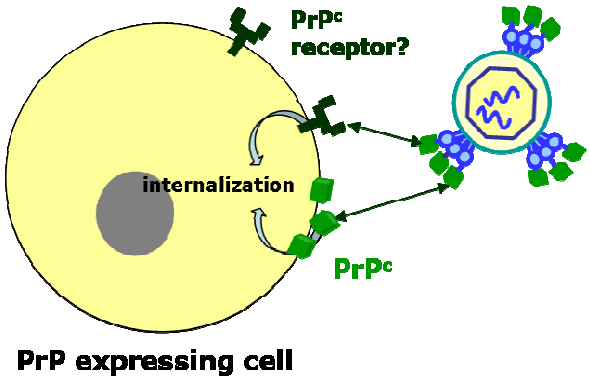


Figure 53 Hypothesis for PrP-mediated cell entry of $PrPMo^{111}$ into human cells. In absence of the MoMLV receptor the generated virus bearing PrP may interact via i) a putative PrP-receptor ii) interaction with PrP^{c} located on the cell surface. Both interactions may lead to increased internalization of the virus and therefore enhanced cell entry events.

V.2.2 PrP influence on replication

In principal, concerning the replication of PrPMo and wild-type ecotropic MLV on different cell lines, the influences of endogenous and transgenic PrP have to be considered as well as the presence of endogenous MLV. The combinations used in this thesis are briefly summarized in Table 7. At this, it seems that both $PrPMo^{111}$

and eMLV does not or only slightly replicate on PrP-positive cells. As it was described in the previous chapter, this low capacity is not due to cell entry inhibition. A possible explanation includes an antiretroviral effect of PrP^c. Such an effect has been proposed for PrP itself as shown by interactions with HIV (Leblanc, P et al. 2004). According to this publication, PrP influences the expression of viral genes. PrP^c was thus shown to inhibit expression of the HIV Env protein and had also an influence on HIV Gag as well as some accessory proteins(Leblanc, P, Baas, D et al. 2004).

Table 7 Influence of PrP on virus replication

Cell line	origin	Endogenous PrP	Endogenous MLV	Virus production (after transfection)	Replication of eMLV	Replication of $PrPMo^{111}$
HEK-293FT	human	low levels	No ✘	Yes ✓	No ✘	No ✘
NIH-3T3	murine	low levels	No ✘	Not determined	Yes ✓	Yes ✓
N2a	murine	moderate levels	Yes ✓	Not determined	Not above endogenous MLV level	Not above endogenous MLV level
PK1	murine	high levels	No ✘	Not determined	Moderate ~	No ✘

The human HEK-293FT cell line expressing low levels of PrP allowed the efficient production of the eMLV and $PrPMo^{111}$ virus stocks after transfection. Spreading for both viruses was not possible, most likely due to their human origin and therefore lack of the receptor for ecotropic MLV. NIH-3T3, expressing low levels of endogenous PrP, showed equal well replication of both eMLV and $PrPMo^{111}$. For the N2a cell line a low levels of endogenous MLV were detectable (by RT assay and IPA) and result in down regulation of the MLV receptor; this represents the most likely explanation for the inability of both $PrPMo^{111}$ and eMLV to spread through this cell line. The PK1 is a subclone of N2a, which was infected with a scrapie strain. Interestingly, the subclone did not show endogenous levels of MLV, which is eventually due to the fact that it was selected to express high levels of PrP^c. Yet, PK1 cells were unable to propagate the infection $PrPMo^{111}$ and revealed only low-level replication of ecotropic wt MLV even, when high moi were used to infect the cells.

In line with this, the expression of the viral Env protein in N2a- and PK1-derived cell lysates and supernatants was not detectable, suggesting that gene expression was impaired in these cells. Thus, these observations can be regarded as evidence for the interference of PrP with MLV-derived genes as it was also described for HIV derived genes. A similar mechanism might contribute to low-

level replication of eMLV in PrP^c expressing cells by influencing the expression of MLV capsid and Env proteins (Figure 54). Whether this influence takes place on level of transcription, translation or assembly has not been determined.

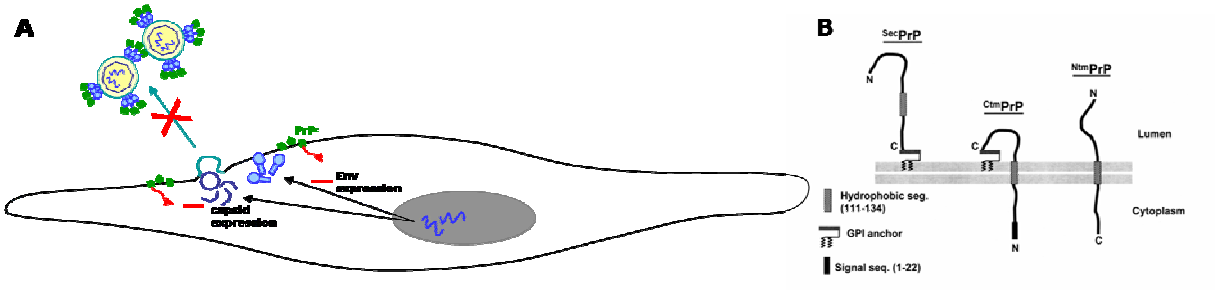


Figure 54 Postulated mechanism of PrP^c influence on the expression of MLV-derived genes. A) Replication of wt eMLV and PrPMo111 viruses might be inhibited by expression of cellular PrP. Levels of viral capsid (octagon) and viral Env protein are reduced resulting in low virus production. **B)** Three topological forms of PrP present in PrP-expressing cells (Harris, D A 2003)

Nevertheless, speculation about the compartment in which such an influence of PrP^c on expression of Env and Gag might take place, it was demonstrated that loss of the GPI anchor of PrP^c anchoring the protein into the membrane resulted in impaired inhibition of HIV (Leblanc, P, Baas, D et al. 2004). Thus, trafficking of PrP^c is critical for this inhibitory effect. Most PrP^c molecules are attached to the outer leaflet of the plasma membrane through a C-terminal glycosyl-phosphatidylinositol anchor (this topology is designated ^{Sec}PrP; see Figure 54). However, when PrP is synthesised in vitro, in transfected cells or in mouse brain, some of the molecules resume a transmembrane orientation (Harris, D A 2003). These species, designated ^{Ntm}PrP and ^{Ctm}PrP, span the lipid bilayer once via a highly conserved hydrophobic region in the centre of the molecule (amino acids 111–134), with either the N-terminus or C-terminus, respectively, on the extracytoplasmic side of the membrane (Figure 54). ^{Ntm}PrP and ^{Ctm}PrP are generated in small amounts (<10% of the total) as part of the normal biosynthesis of wild-type PrP in the endoplasmic reticulum. Thus, an influence of PrPc on viral replication might principally take place through all of these PrP forms in various compartments. In this context, it is notable, that PrP^{111} on top of the viral Env does not show any negative effect on replication, as assayed by replication in NIH-3T3 cells (Table 7). Therefore it can be concluded that the N-terminal part including aa 23-120 is responsible for these properties. This conclusion is further verified by the finding that PrP^{209} display resulted in inhibition of virus production in all tested settings as discussed above (V.1).

PrP^c has also been described to bind nucleic acids resembling the properties of a putative nucleic acid chaperone. Surprisingly, PrP^c possesses all nucleic acid chaperoning properties previously specific to retroviral nucleocapsid proteins, as it was found for NC p7, the nucleocapsid protein of HIV-1 (Derrington, E et al. 2002). Thus PrP^c, like NC p7, chaperones the annealing of tRNA(Lys) to the HIV-1 primer binding site, the initial step of retrovirus replication, the two DNA strand transfers required for production of a complete proviral DNA with LTRs as well as the dimerization of the HIV-1 genomic RNA. The same data were obtained for PrP^c in comparison to feline immunodeficiency virus (FIV) nucleocapsid protein (Moscardini, M et al. 2002). These findings point to a second possible interaction mechanism for PrP^c resulting in virus inhibition. However, the chaperoning property of PrP^c has not been mapped to specific parts of the protein, therefore it remains unclear whether displayed PrP^{111} or PrP^{142} may exhibit such interactions.
Future experiments aiming at the production of MLV in scrapie-infected cells, could focus on NIH-3T3 cells, as recently the scrapie infection of these cells became possible (Vorberg, I et al. 2004). Since wt eMLV and also $PrPMo^{111}$ replicate in these cells, inhibitory effects on virus replication by cellular factors or endogenous retrovirus can be excluded. Thus it should be possible to investigate the above mentioned PrP/MLV interactions directly.

V.3 Towards a vaccine against TSEs

Retroviral PrP-expressing particles represent a highly immunogenic PrP vaccine candidate, which, for the first time, is capable of inducing anti-native PrP^c antibody titers in wild-type mice.

V.3.1 The immune response against VLPs

Virus-like particles (VLPs) have been widely used to generate subunit vaccines (Noad, R and Roy, P 2003; Deml, L, Speth, C et al. 2005). At this, the capacity of VLPs derived from HIV to induce innate immunity by stimulating toll-like receptors (TLR) was shown and cytotoxic T lymphocyte (CTL) response was examined in detail. Since estimated 50-100 major histocompatibility complex (MHC) molecules on the surface of an antigen-presenting cell (APC) are sufficient to trigger CTL response, the uptake of only one VLP bearing 1500-2000 Gag precursor molecules is regarded as sufficient for induction. Moreover, VLPs are lipoprotein spheres and conjugation of proteins with lipids or liposomes has been

previously demonstrated to facilitate their access to the endogenous MHC class I processing pathway. This way of presentation might be further stimulated by viral fusion with APC target membranes. Processing by the MHC class II pathway takes place in addition. (Deml, L, Speth, C et al. 2005)

Immune responses were not only observed for the HIV-derived Gag protein but also for foreign peptides presented in the retroviral context. So far HIV-derived and a few other virally derived genes have been inserted into HIV Gag particles. In all cases strong antibody titers and CTL responses were obtained (Deml, L, Speth, C et al. 2005). The PrP-retroparticles described in this thesis are the first example for self-antigens presented in this system.

V.3.2 Tolerance or auto-reactivity?

In principle VLP vaccination using HIV Gag has extensively been done for virus-derived genes thus presenting foreign protein sequences to the immune system. Contrary, the approach used in this thesis utilizes particles to display antigens of cellular origin and to induce auto-reactive antibody responses for the first time.

Remarkably, a single intravenously (i.v.) immunization with PrP-retroparticles was sufficient to induce native PrP^c binding serum immune globulin (Ig) M and IgG antibodies in *Prnp*$^{o/o}$ mice (PrP gene deficient mice), the latter of which showed at least as strong binding as the monoclonal anti-PrP IgG antibody 6H4. The induction of native PrP^c-specific IgM and IgG upon i.v. immunization with PrP-retroparticles of wild-type mice is remarkable and has not been accomplished in a flurry of previous immunization studies (Hanan, E et al. 2001; Hanan, E, Priola, S A et al. 2001; Souan, L, Tal, Y et al. 2001; Koller, M F, Grau, T et al. 2002; Arbel, M, Lavie, V et al. 2003; Gilch, S, Wopfner, F et al. 2003; Schwarz, A, Kratke, O et al. 2003; Sigurdsson, E M et al. 2003; Rosset, M B, Ballerini, C et al. 2004).

In a recent report, immunization with bacterially expressed recombinant full-length PrP emulsified in complete Freund´s adjuvants (CFA) resulted in the induction of antibodies directed against native PrP^c, only in mice aberrantly expressing transgenic PrP under the control of an oligodendrocyte and Schwann cell specific promoter, whereas wild-type controls and all other PrP transgenic mice tested showed at best serum binding to recombinant PrP coated to plastic (Polymenidou, M, Heppner, F L et al. 2004). The authors concluded that the expression of PrP^c in other than these organs leads to impaired antibody titers

due to tolerance induction. Given the antibody response using PrP-retroparticles in wild-type mice observed in this thesis, this conclusion has to be reconsidered. It is rather likely, that the immunization using bacterially expressed PrP^c is the main determinant for the observed antibody titers and that the use of eukaryotic expressed PrP^c in the context of highly immunogenic retroparticles may circumvent these limitations.

The magnitude of PrP-specific IgG responses upon i.v. PrP-retroparticle immunization inversely correlated with the number of *Prnp* alleles expressed, i.e. highest PrP-specific IgG was induced in $Prnp^{o/o}$ mice, whereas intermediate and lower levels were detected in $Prnp^{o/+}$ (heterozygous) and $Prnp^{+/+}$ (wild-type) mice, respectively. Nevertheless, it is remarkable, that overall similar IgM levels were induced in mice of all three genotypes, especially at early time points. The switch from the IgM to the IgG isotype of PrP^c-specific antibodies is less pronounced in wild type animals when compared to $Prnp^{0/0}$ mice (Nikles, D, Bach, P et al. 2005).

An explanation for the observations from this and other studies would be that immunologic host tolerance is manifested on the T cell level and that T helper determinants may be provided by MLV-derived VLP related antigens. IgM responses of B cells do not depend on T cell help and would therefore remain more or less unaffected (Adelstein, S et al. 1991). On the contrary, the IgG switch takes place in $Prnp^{0/0}$ mice but it is impaired upon introduction of the *Prnp* gene. Nevertheless, PrP-retroparticles seemed to be capable of inducing MLV-specific T cell help accounting for PrP^c-specific IgG production with low affinity in 20-25% of the immunized wt animals (Nikles, D, Bach, P et al. 2005).

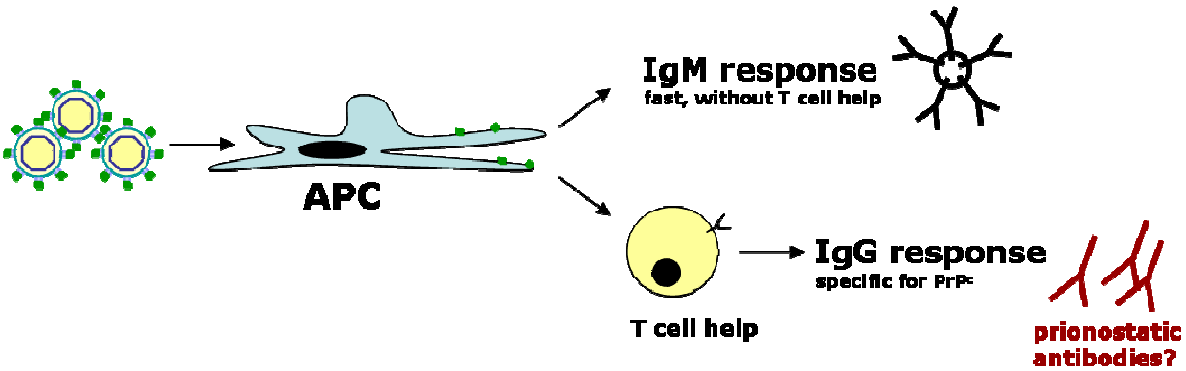


Figure 55 **Immune response against PrP^c-displaying VLPs.** The antigen is processed by antigen-presenting cells (APC) upon presentation via MHC class I or class II pathways. A fast IgM response takes place without T cell help, whereas the switch to specific IgG response needs the activation of $CD4^+$ T cells. PrP^c-specific IgGs might be generated with MLV capsid-specific T cell help rather than with PrP^c-specific help due to tolerance mechanisms.

In conclusion, it is remarkable that host tolerance left enough room for the induction of potentially auto-reactive PrP-specific antibodies. However, this finding is in line with previous observations that PrP-specific B cells can develop in the presence of endogenously expressed PrP in transgenic mice expressing the antibody chain 6H4μ derived from a monoclonal anti-PrP antibody (Heppner, F L, Musahl, C et al. 2001). It remains to be determined whether the induced antibodies belong to weak or efficiently opsonizing (and therefore stable and persistent) antibody titers and whether they have prionostatic potential (Figure 55).

V.3.3 Outlook: Improving the antigen

Two further vaccine candidates were generated in this thesis which might further improve quality and quantity of the immune responses generated so far. Firstly, HIV-derived VLPs are available, for which the induction of strong antibody responses against presented foreign polypeptides in BALB/c mice has been shown (Osterrieder, N et al. 1995; Deml, L et al. 1997). Especially prime/boost experiments will be attractive to maximize the PrP^{c} response. Secondly, a lentiviral vector packaging the Prnp gene was produced to combine protein- and nucleic acid-based vaccination strategies. The potential of such combination-based immunization strategies has not been tested before and remains to be determined.

Furthermore, VLPs presenting PrP as an Env fusion protein have been generated. The viral envelope protein has previously been shown to be highly immunogenic but suffers from the presence of low numbers of molecules on the surface of viral particles with a Gag:Env ratio of approximately 60:1 (Chertova, E et al. 2002).

The Env display might facilitate the development of an oligomeric PrP status, believed to be present in equilibrium with the monomeric form of cellular PrP (Weissmann, C 2004), may be enhanced. The oligomeric or multimeric forms of PrP, resembling the infectious so-called "scrapie isoform" of the protein (PrP^{Sc}), might be an improved tool for immunization approaches representing the more adequate targets in terms of antibody production.

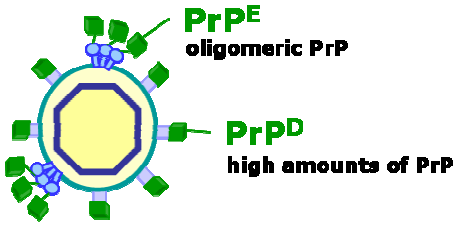


Figure 56 Combination of Env- and Display-system might yield improved immune responses. The cellular form of PrP is either presented on top of the viral Env protein (PrP^{E}) to generate oligomeric PrP or as fusion to PDGFR-TM (PrP^{D}) to obtain high amounts of PrP in the retroparticles. The combined approach may enhance immune response against cellular PrP.

However, since both number and structure play a role in presenting the antigen, a combination of monomeric and multimeric PrP^{c} display might give rise to highest possible titers (Figure 56).

A hallmark of propagation of the infectious agent of TSEs is the conversion of the cellular form of PrP into the pathogenic form PrP^{Sc}. So far, approaches to isolate prion infectivity in vitro are limited to the stable infection of cell cultures. Only recently, the *de novo* synthesis of prions was achieved via conversion of recombinant PrP (Legname, G et al. 2004).

In terms of a more specific immune response, the generation of VLPs displaying the pathogenic isoform of PrP (PrP^{Sc}) will offer advantages. The organism would then directly respond against the pathogen, thus avoiding potential severe side-effects upon an response against cellular PrP. However, the generation of VLPs containing PrP^{Sc} has been and remains an ambitious target.

In this thesis the conversion of PrP^{c} displayed on the surface of viral particles was tempted with a truncated PrP consisting of amino acids 90-231. This shortened form was previously shown to support prion propagation and thus to be sufficient for the conversion of PrP^{c} into PrP^{Sc} (Flechsig, E, Shmerling, D et al. 2000). Thus, the $PrP^{D}142$ particle was in principle thought to be amenable to conversion into PrP^{Sc}, although there might be a negative impact of the altered transmembrane domain. However, difficulties also arise from the fact that the only assay differentiating between the two PrP forms is the proteinase K (PK) digestion assay. The titration of PrP with the enzyme critically depends on the amount of total protein. Since there is only a relative difference between PrP^{c} and PrP^{Sc} upon titration with PK, the conditions for titration are highly sensitive to slight changes. Under the conditions applied here it seems that $PrP^{D}142$ particles were not converted, when they were incubated for 1d at 37°C. However it may well be

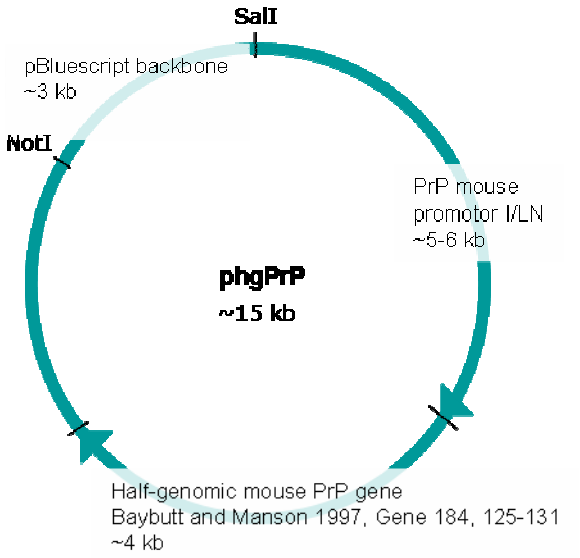
possible to identify conditions that will allow the conversion of PrP^c displayed on retrovirus particles.

Taken together, the PrP-vaccination approach described in this work represents the first successful system inducing PrP-specific antibody responses in wt mice against the native form of the prion protein.

VI Appendix

VI.1 Parental plasmids

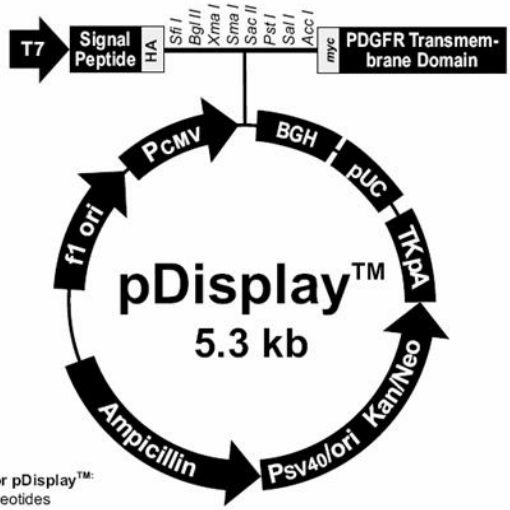
VI.1.1 phgPrP



VI.1.2 pDisplay

Map of pDisplay™

The figure below shows the features of pDisplay™. The complete sequence of pDisplay™ is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 9). Details of the multiple cloning site are shown on page 5.



Comments for pDisplay™:
5325 nucleotides

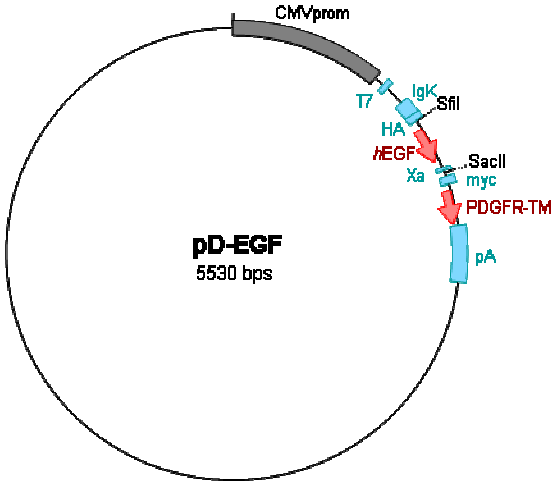
CMV promoter: bases 1-596
T7 promoter: bases 638-657
Murine Ig kappa-chain V-J2-C signal peptide: bases 737-799
Hemagglutinin A epitope: bases 800-826
Multiple Cloning Site: bases 827-873
myc epitope: bases 874-903
PDGFR transmembrane domain: bases 907-1056
Bovine growth hormone polyadenylation signal: bases 1069-1288
pUC origin: bases 1378-2051
Thymidine kinase polyadenylation site: bases 2458-2187
Neomycin/Kanamycin resistance gene: bases 3421-2366
SV40 origin and promoter: bases 3797-3456

Features of pDisplay™

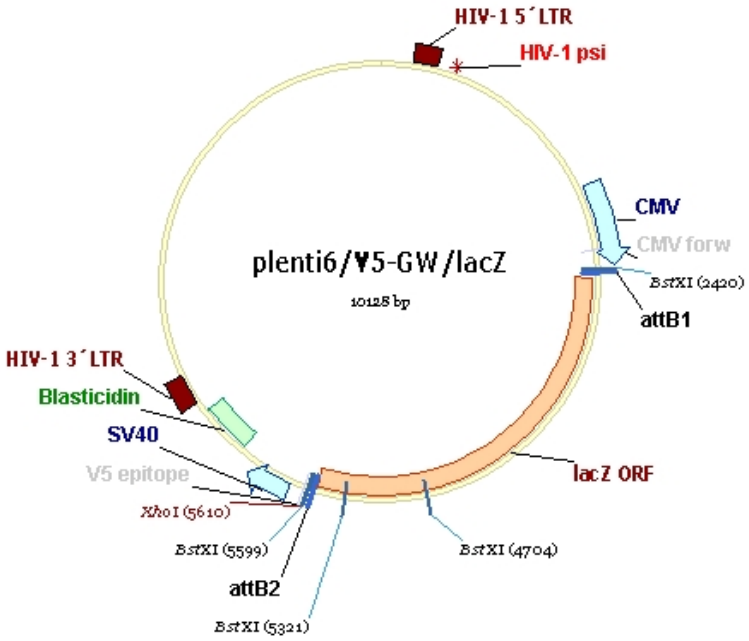
pDisplay™ (5325 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
ATG initiation codon	Permits initiation of translation of the pDisplay™ fusion protein
Murine Ig κ-chain leader sequence	Targets protein to secretory pathway (Coloma *et al.*, 1992)
Hemagglutinin A epitope tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala)	Allows detection of the fusion protein by monoclonal antibody 12CA5 (Kolodziej and Young, 1991; Niman *et al.*, 1983)
Multiple cloning region with eight unique sites	Allows insertion of your gene and facilitates cloning
myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of pDisplay™ fusion protein with the Anti-*myc* Antibodies (Evan *et al.*, 1985)
Platelet-derived growth factor receptor transmembrane domain (PDGFR-TM)	Anchors the fusion protein to the plasma membrane for display (Gronwald *et al.*, 1988)
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
pUC origin	High-copy number replication and growth in *E. coli*
SV40 early promoter and origin	Permits expression of the kanamycin resistance gene for Geneticin® resistance in mammalian cells Allows episomal replication in cells containing SV40 large T antigen
Kanamycin resistance gene	Confers resistance to Geneticin® in mammalian cells
TK polyadenylation signal	Efficient transcription termination and polyadenylation of kanamycin resistance gene mRNA
Ampicillin resistance gene (β-lactamase)	Selection in *E. coli*
f1 origin	Allows rescue of single-stranded DNA

VI.1.3 pD-EGF

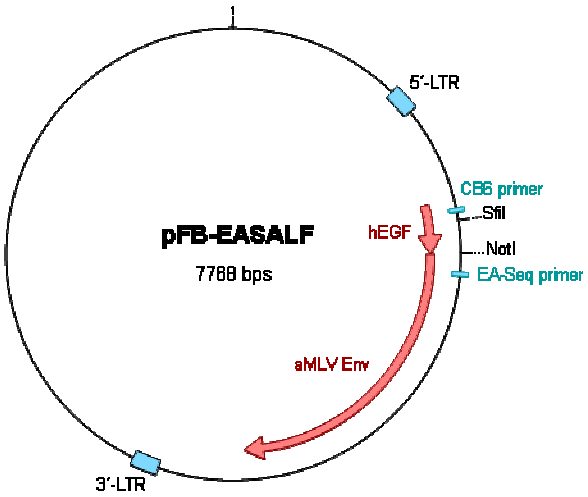


VI.1.4 pLenti6/V5-lacZ



Feature	Benefit
HIV-1 truncated 5'LTR	**Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).**
HIV-1 psi (ψ) packaging signal	**Allows viral packaging (Luciw, 1996).**
HIV-1 Rev response element (RRE)	**Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems et al., 1991; Malim et al., 1989).**
Human CMV promoter	**Permits high-level expression of the gene of interest (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).**
CMV forward priming site	**Permits sequencing of the insert.**
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	**Allows detection of your recombinant protein with the Anti-V5 antibodies (Southern et al., 1991).**
V5(C-term) reverse priming site	**Allows sequencing of the insert.**
SV40 early promoter and origin	**Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen.**
Blasticidin (bsd) resistance gene	**Permits selection of stably transduced mammalian cell lines (Kimura et al., 1994).**
ΔU3/HIV-1 truncated 3'LTR	**Modified 3'LTR that allows viral packaging but self-inactivates the 5'LTR for biosafety purposes (Dull et al., 1998). The element also contains a polyadenylation signal for efficient transcription termination and polyadenylation of mRNA in transduced cells.**

VI.1.5 pFB-EASALF

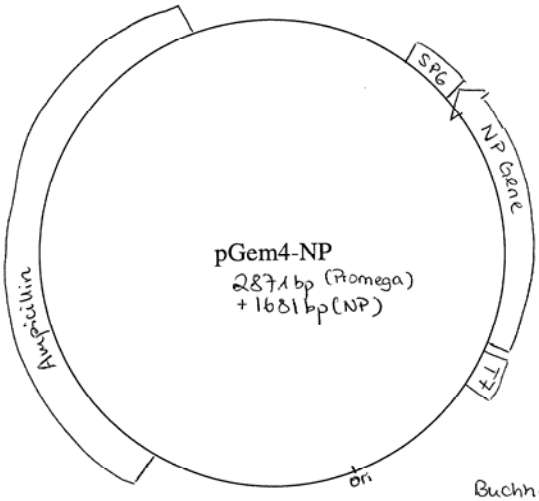


translated sequence

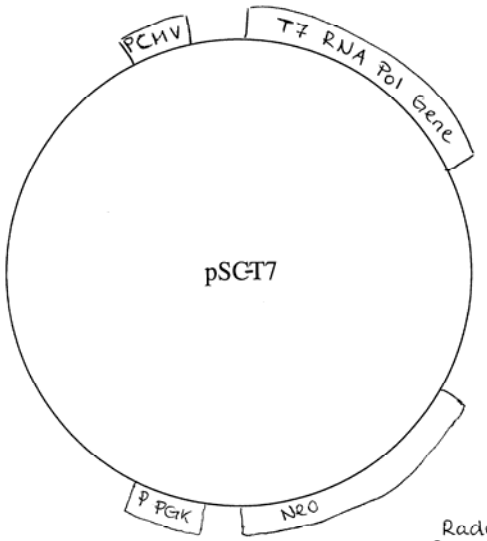
```
                aMLV env
1620  cctctagact gacatggcgc gttcaacgct ctcaaaaccc cttaaaaata aggttaaccc gcgaggcccc
      i  l  -  t  d  m  a  r  s  t  l  s  k  p  l  k  n  k  v  n  p  r  g  p
                                                                    SfiI
1690  ctaatcccct taattcttct gatgctcaga ggggtcagta ctgcttcgcc cggctccagt gcggcccagc
        l  i  p  l  i  l  l  m  l  r  g  v  s  t  a  s  p  g  s  s  a  a  q
              hEGF
1760  cggccatggc caatagtgac tctgaatgtc ccctgtccca cgatgggtac tgcctccatg atggtgtgtg
       p  a  m  a  n  s  d  s  e  c  p  l  s  h  d  g  y  c  l  h  d  g  v

1830  catgtatatt gaagcattgg acaagtatgc atgcaactgt gttgttggct acatcgggga gcgatgtcag
      c  m  y  i  e  a  l  d  k  y  a  c  n  c  v  v  g  y  i  g  e  r  c  q
                                    NotI
1900  taccgagacc tgaagtggtg ggaactgcgc gcggccgca…  aMLV-Env
        y  r  d  l  k  w  w  e  l  r  a  a  a
```

VI.1.6 pGem-NP and pSCT7

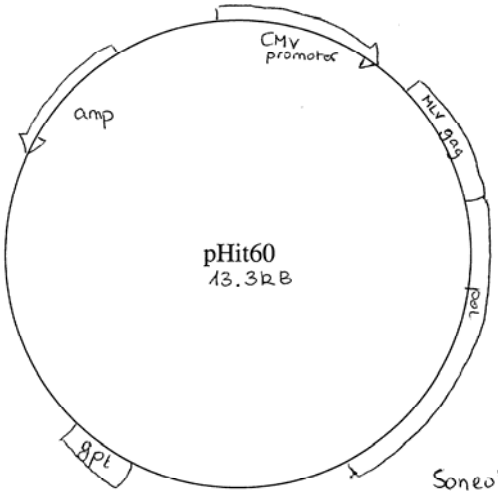


Buchholz et al. 1993
J Virol 67(10), 5803-12

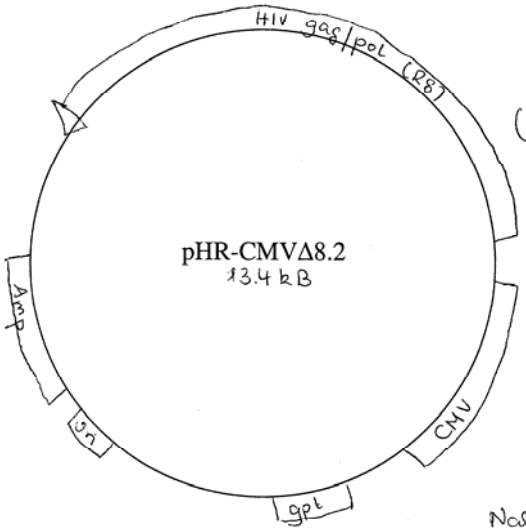


Radecke et al. 1995
EMBO J 14, 5773-5784

VI.1.7 pHit60 and pCMVΔR8.2

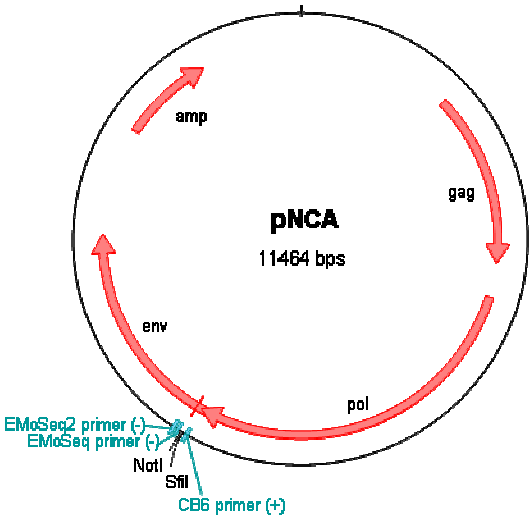


Soneoka et.al. 1995
Nucleic Acids Res 23(4), 628-33

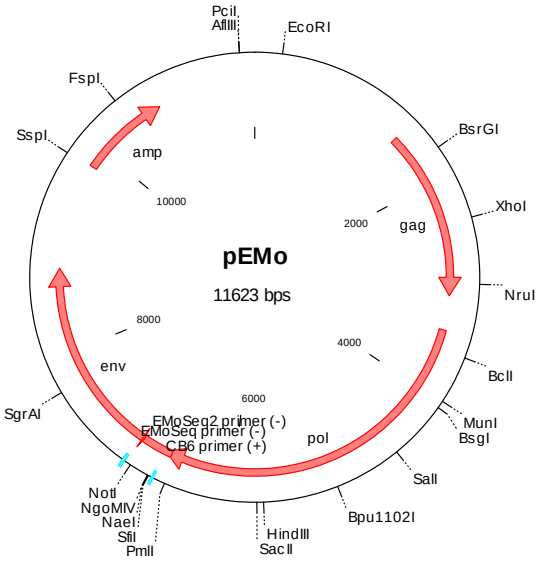


Naldini et. al. 1996
Science 272, 263-267

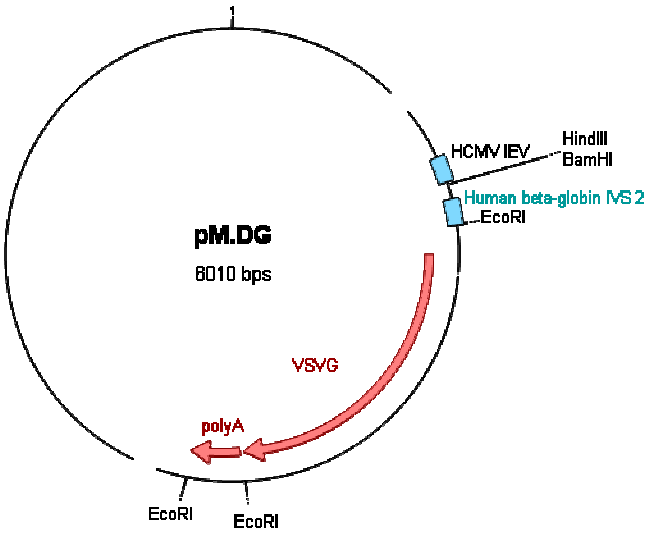
VI.1.8 pNCA



VI.1.9 pEMo

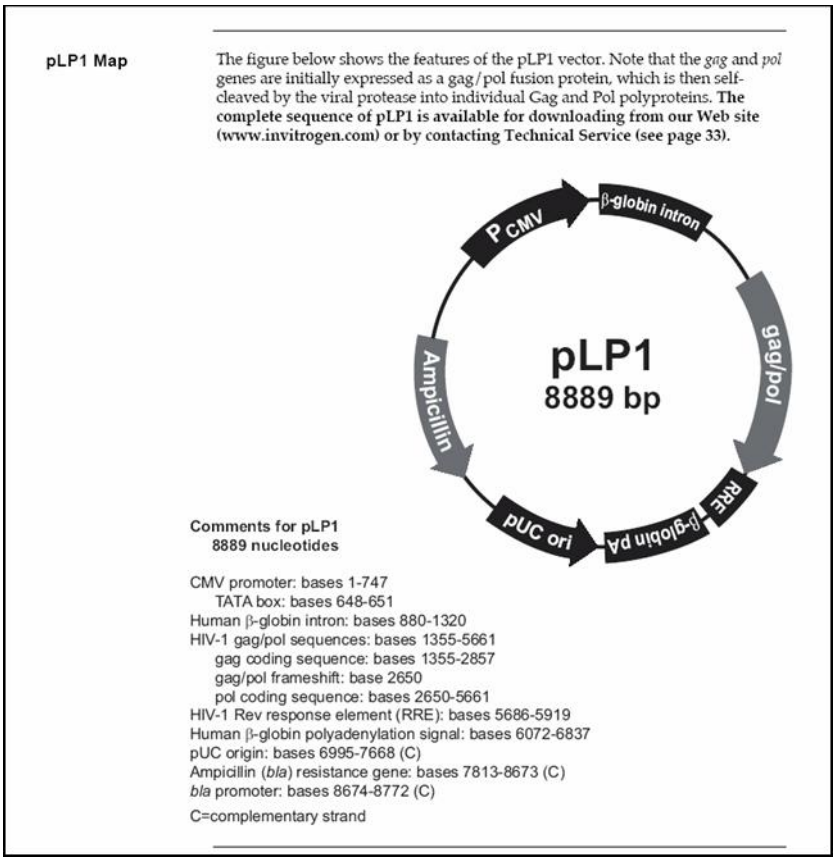


VI.1.10 pm.DG



VI.1.11 Lentipower packaging mix

VI.1.11.1 pLP1



pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is then self-cleaved by the viral protease into individual Gag and Pol polyproteins. **The complete sequence of pLP1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**

Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
gag coding sequence: bases 1355-2857
gag/pol frameshift: base 2650
pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

Features of pLP1

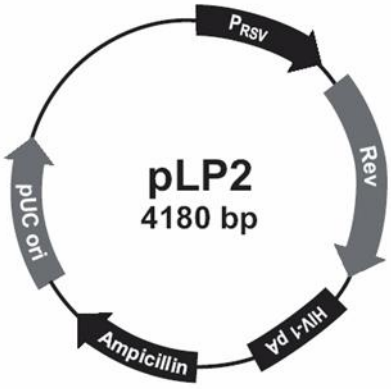
pLP1 (8889 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) promoter	Permits high-level expression of the HIV-1 *gag* and *pol* genes in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the *gag* and *pol* genes in mammalian cells.
HIV-1 *gag* coding sequence	Encodes the viral core proteins required for forming the structure of the lentivirus (Luciw, 1996).
HIV-1 *pol* coding sequence	Encodes the viral replication enzymes required for replication and integration of the lentivirus (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent expression of the *gag* and *pol* genes
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

VI.1.11.2 pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. **The complete sequence of pLP2 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

Features of pLP2

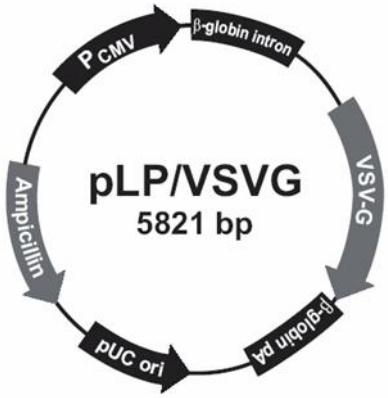
pLP2 (4180 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
RSV enhancer/promoter	Permits high-level expression of the *rev* gene (Gorman *et al.*, 1982).
HIV-1 Rev ORF	Encodes the Rev protein which interacts with the RRE on pLP1 to induce Gag and Pol expression, and on the pLenti6/V5 expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles.
HIV-1 LTR polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

VI.1.11.3 pLP-VSV

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. **The complete sequence of pLP/VSVG is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

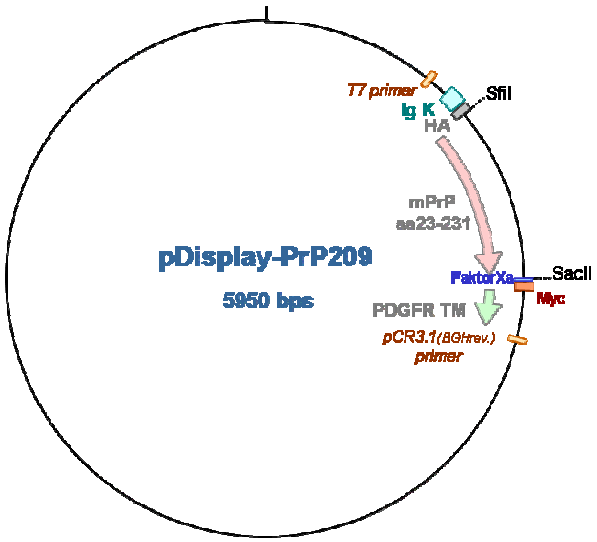
Features of pLP/VSVG

pLP/VSVG (5821 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human CMV promoter	Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the VSV-G gene in mammalian cells.
VSV G glycoprotein (VSV-G)	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

VI.2 Constructed plasmids

VI.2.1 pD-PrP209



translated sequence

HA-Tag SfiI PrP-208

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800  tatccatatg atgttccaga ttatgctggg gcccagccgg ccaaaaagcg gccaaagcct ggagggtgga
       y  p  y   d  v  p   d  y  a  g   a  q  p   a  k  k   r  p  k  p   g  g  w

870  acaccggtgg aagccggtat cccgggcagg gaagccctgg aggcaaccgt tacccacctc agggtggcac
       n  t  g   g  s  r   y  p  g  q   g  s  p   g  g  n   r  y  p  p   q  g  g

940  ctgggggcag ccccacggtg gtggctgggg acaaccccat gggggcagct ggggacaacc tcatggtggt
       t  w  g   q  p  h   g  g  g  w   g  q  p   h  g  g   s  w  g  q   p  h  g  g

1010 agttggggtc agccccatgg cggtggatgg ggccaaggag ggggtaccca taatcagtgg aacaagccca
       s  w  g   q  p  h   g  g  g  w   g  q  g   g  g  t   h  n  q  w   n  k  p

1080 gcaaaccaaa aaccaacctc aagcatgtgg caggggctgc ggcagctggg gcagtagtgg ggggccttgg
       s  k  p   k  t  n   l  k  h  v   a  g  a   a  a  a   g  a  v  v   g  g  l

1150 tggctacatg ctggggagcg ccatgagcag gcccatgatc cattttggca acgactggga ggaccgctac
       g  g  y   m  l  g   s  a  m  s   r  p  m   i  h  f   g  n  d  w   e  d  r  y

1220 taccgtgaaa acatgtaccg ctaccctaac caagtgtact acaggccagt ggatcagtac agcaaccaga
       y  r  e   n  m  y   r  y  p  n   q  v  y   y  r  p   v  d  q  y   s  n  q

1290 acaacttcgt gcacgactgc gtcaatatca ccatcaagca gcacacggtc accaccacca ccaaggggga
       n  n  f   v  h  d   c  v  n  i   t  i  k   q  h  t   v  t  t  t   t  k  g

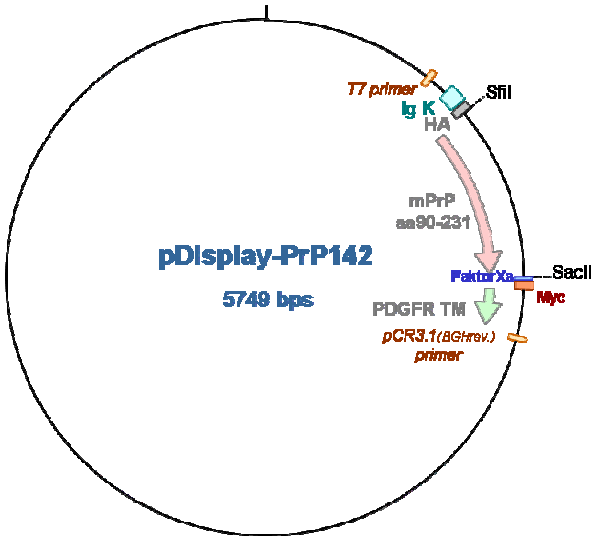
1360 gaacttcacc gagaccgatg tgaagatgat ggagcgcgtg gtggagcaga tgtgcgtcac ccagtaccag
       e  n  f   t  e  t   d  v  k  m   m  e  r   v  v  e   q  m  c  v   t  q  y  q

                    Faktor Xa     SacII
1430 aaggagtccc aggcctatta cgacgggaga agatccagca tcgagggaag gccgcggctg caggtcgacg
       k  e  s   q  a  y   y  d  g  r   r  s  s   i  e  g   r  p  r  l   q  v  d
     myc epitop
1500 aacaaaaact catctcagaa gaggatctga atgctgtggg ccaggacacg caggaggtca tcgtggtgcc
       e  q  k   l  i  s   e  e  d  l   n  a  v   g  q  d   t  q  e  v   i  v  v

1570 acactccttg ccctttaagg tggtggtgat ctcagccatc ctggccctgg tggtgctcac catcatctcc
       p  h  s   l  p  f   k  v  v  v   i  s  a   i  l  a   l  v  v  l   t  i  i  s

1640 cttatcatcc tcatcatgct ttggcagaag aagccacgtt ag
       l  i  i   l  i  m   l  w  q  k   k  p  r   -
```

VI.2.2 pD-PrP142



translated sequence

HA-Tag SfiI PrP-142

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       y  p  y   d  v  p   d  y  a  g   a  q  p   a  q  g   g  g  t  h   n  q  w

870  acaagcccag caaaccaaaa accaacctca agcatgtggc aggggctgcg gcagctgggg cagtagtggg
       n  k  p   s  k  p   k  t  n  l   k  h  v   a  g  a   a  a  g  a   v  v

940  gggccttggt ggctacatgc tggggagcgc catgagcagg cccatgatcc attttggcaa cgactgggag
       g  g  l   g  g  y   m  l  g  s   a  m  s   r  p  m   i  h  f  g   n  d  w  e

1010 gaccgctact accgtgaaaa catgtaccgc taccctaacc aagtgtacta caggccagtg gatcagtaca
       d  r  y   y  r  e   n  m  y  r   y  p  n   q  v  y   y  r  p  v   d  q  y

1080 gcaaccagaa caacttcgtg cacgactgcg tcaatatcac catcaagcag cacacggtca ccaccaccac
       s  n  q   n  n  f   v  h  d  c   v  n  i   t  i  k   q  h  t  v   t  t  t

1150 caaggggggag aacttcaccg agaccgatgt gaagatgatg gagcgcgtgg tggagcagat gtgcgtcacc
       t  k  g   e  n  f   t  e  t  d   v  k  m   m  e  r   v  v  e  q   m  c  v  t
                                Faktor Xa  SacII
1220 cagtaccaga aggagtccca ggcctattac gacgggagaa gatccagcat cgagggaagg ccgcggctgc
       q  y  q   k  e  s   q  a  y  y   d  g  r   r  s  s   i  e  g  r   p  r  l
            myc epitop
1290 aggtcgacga acaaaaactc atctcagaag aggatctgaa tgctgtgggc caggacacgc aggaggtcat
       q  v  d   e  q  k   l  i  s  e   e  d  l   n  a  v   g  q  d  t   q  e  v

1360 cgtggtgcca cactccttgc cctttaaggt ggtggtgatc tcagccatcc tggccctggt ggtgctcacc
       i  v  v   p  h  s   l  p  f  k   v  v  v   i  s  a   i  l  a  l   v  v  l  t

1430 atcatctccc ttatcatcct catcatgctt tggcagaaga agccacgtta g
       i  i  s   l  i  i   l  i  m  l   w  q  k   k  p  r   -
```
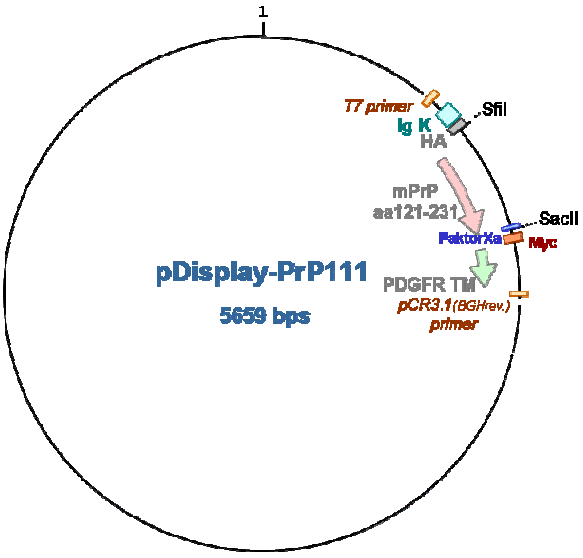

VI.2.3 pD-PrP111



translated sequence

HA-Tag SfiI PrP-110

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800  tatccatatg atgttccaga ttatgctggg gcccagccgg ccaaagtggg gggccttggt ggctacatgc
       y  p  y   d  v  p   d  y  a  g   a  q  p   a  k  v   g  g  l  g   g  y  m

870  tggggagcgc catgagcagg cccatgatcc attttggcaa cgactgggag gaccgctact accgtgaaaa
       l  g  s   a  m  s   r  p  m  i   h  f  g   n  d  w   e  d  r  y   y  r  e

940  catgtaccgc taccctaacc aagtgtacta caggccagtg gatcagtaca gcaaccagaa caacttcgtg
       n  m  y   r  y  p   n  q  v  y   y  r  p   v  d  q   y  s  n  q   n  n  f  v

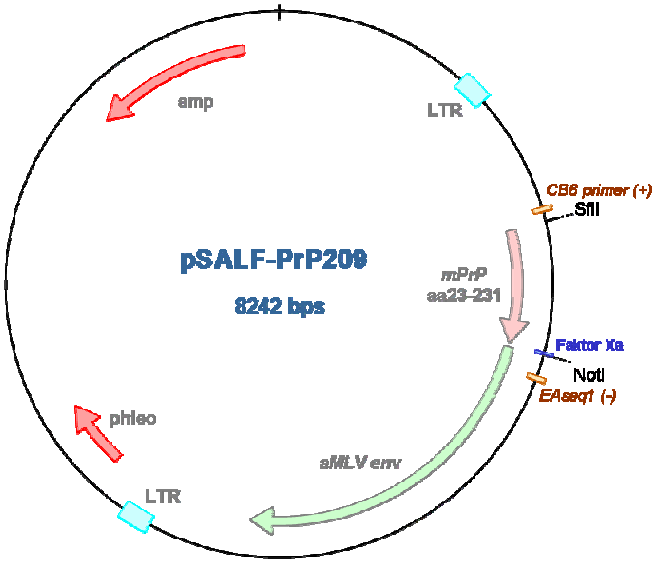
1010 cacgactgcg tcaatatcac catcaagcag cacacggtca ccaccaccac caaggggag aacttcaccg
       h  d  c   v  n  i   t  i  k  q   h  t  v   t  t  t   t  k  g  e   n  f  t

1080 agaccgatgt gaagatgatg gagcgcgtgg tggagcagat gtgcgtcacc cagtaccaga aggagtccca
       e  t  d   v  k  m   m  e  r  v   v  e  q   m  c  v   t  q  y  q   k  e  s
                Faktor Xa          SacII              myc epitop
1150 ggcctattac gacgggagaa gatccagcat cgagggaagg ccgcggctgc aggtcgacga acaaaaactc
       q  a  y   y  d  g   r  r  s  s   i  e  g   r  p  r   l  q  v  d   e  q  k  l
                PDGFR-TM
1220 atctcagaag aggatctgaa tgctgtgggc caggacacgc aggaggtcat cgtggtgcca cactccttgc
       i  s  e   e  d  l   n  a  v  g   q  d  t   q  e  v   i  v  v  p   h  s  l

1290 cctttaaggt ggtggtgatc tcagccatcc tggccctggt ggtgctcacc atcatctccc ttatcatcct
       p  f  k   v  v  v   i  s  a  i   l  a  l   v  v  l   t  i  i  s   l  i  i

1360 catcatgctt tggcagaaga agccacgtta g
       l  i  m   l  w  q   k  k  p  r   -
```

VI.2.4 pSALF-PrP209



translated sequence

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                                                              SfiI
1690  ctaatcccct taattcttct gatgctcaga ggggtcagta ctgcttcgcc cggctccagt gcggcccagc
        l  i  p   l  i  l   l  m  l   r  g  v  s   t  a  s   p  g  s   s  a  a  q
         PrP-208
1760  cggccaaaaa gcggccaaag cctggagggt ggaacaccgg tggaagccgg tatcccgggc agggaagccc
      p  a  k   k  r  p  k   p  g  g   w  n  t   g  g  s  r   y  p  g   q  g  s

1830  tggaggcaac cgttacccac ctcagggtgg cacctggggg cagccccacg gtggtggctg gggacaaccc
      p  g  g   n  r  y  p   p  q  g   g  t  w   g  q  p  h   g  g  g   w  g  q  p

1900  catgggggca gctggggaca acctcatggt ggtagttggg gtcagcccca tggcggtgga tggggccaag
        h  g  g   s  w  g   q  p  h  g   g  s  w   g  q  p   h  g  g  g   w  g  q

1970  gaggggggtac ccataatcag tggaacaagc ccagcaaacc aaaaaccaac ctcaagcatg tggcaggggc
      g  g  g   t  h  n  q   w  n  k   p  s  k   p  k  t  n   l  k  h   v  a  g

2040  tgcggcagct ggggcagtag tgggggggcct tggtggctac atgctgggga gcgccatgag caggcccatg
      a  a  a   a  g  a  v   v  g  g   l  g  g  y   m  l  g   s  a  m   s  r  p  m

2110  atccattttg gcaacgactg ggaggaccgc tactaccgtg aaaacatgta ccgctaccct aaccaagtgt
        i  h  f   g  n  d   w  e  d  r   y  y  r   e  n  m   y  r  y  p   n  q  v

2180  actacaggcc agtggatcag tacagcaacc agaacaactt cgtgcacgac tgcgtcaata tcaccatcaa
      y  y  r   p  v  d  q   y  s  n   q  n  n   f  v  h  d   c  v  n   i  t  i

2250  gcagcacacg gtcaccacca ccaccaaggg ggagaacttc accgagaccg atgtgaagat gatggagcgc
      k  q  h  t   v  t  t   t  t  k   g  e  n  f   t  e  t   d  v  k   m  m  e  r

2320  gtggtggagc agatgtgcgt cacccagtac cagaaggagt cccaggccta ttacgacggg agaagatcca
        v  v  e   q  m  c   v  t  q  y   q  k  e   s  q  a   y  y  d  g   r  r  s
     Faktor Xa          NotI
2390  gcatcgaggg aaggcggccc gcaatggcag agagccccca tcaggtcttt aatgtaacct ggagagtcac
        s  i  e   g  r  a  a   a  m  a   e  s  p   h  q  v  f   n  v  t   w  r  v

2460  caacctgatg actgggcgta ccgccaatgc cacctccctc ctgggaactg tacaagatgc cttcccaaaa
      t  n  l  m   t  g  r   t  a  n   a  t  s  l   l  g  t   v  q  d   a  f  p  k

2530  ttatattttg atctatgtga tctggtcgga gaggagtggg acccttcaga ccaggaaccg tatgtcgggt
        l  y  f   d  l  c   d  l  v  g   e  e  w   d  p  s   d  q  e  p   y  v  g

2600  atggctgcaa gtaccccgca gggagacagc ggacccggac ttttgacttt tacgtgtgcc ctgggcatac
      y  g  c   k  y  p  a   g  r  q   r  t  r   t  f  d  f   y  v  c   p  g  h

2670  cgtaaagtcg gggtgtgggg gaccaggaga gggctactgt ggtaaatggg ggtgtgaaac caccggacag
      t  v  k  s   g  c  g   g  p  g   e  g  y  c   g  k  w   g  c  e   t  t  g  q

2740  gcttactgga agcccacatc atcgtgggac ctaatctccc ttaagcgcgg taacacccca tgggacacgg
        a  y  w   k  p  t   s  s  w  d   l  i  s   l  k  r   g  n  t  p   w  d  t

2810  gatgctctaa agttgcctgt ggcccctgct acgacctctc caaagtatcc aattccttcc aaggggctac
      g  c  s   k  v  a  c   g  p  c   y  d  l   s  k  v  s   n  s  f   q  g  a

2880  tcgaggggggc agatgcaacc ctctagtcct agaattcact gatgcaggaa aaaaggctaa ctgggacggg
      t  r  g  g   r  c  n   p  l  v   l  e  f  t   d  a  g   k  k  a   n  w  d  g
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2950  cccaaatcgt ggggactgag actgtaccgg acaggaacag atcctattac catgttctcc ctgacccggc
        p  k  s  w  g  l  r  l  y  r  t  g  t  d  p  i  t  m  f  s  l  t  r

3020  aggtccttaa tgtgggaccc cgagtcccca tagggcccaa cccagtatta cccgaccaaa gactcccttc
        q  v  l  n  v  g  p  r  v  p  i  g  p  n  p  v  l  p  d  q  r  l  p

3090  ctcaccaata gagattgtac cggctccaca gccacctagc cccctcaata ccagttaccc cccttccact
        s  s  p  i  e  i  v  p  a  p  q  p  p  s  p  l  n  t  s  y  p  p  s  t

3160  accagtacac cctcaacctc ccctacaagt ccaagtgtcc cacagccacc cccaggaact ggagatagac
        t  s  t  p  s  t  s  p  t  s  p  s  v  p  q  p  p  p  g  t  g  d  r

3230  tactagctct agtcaaagga gcctatcagg cgcttaacct caccaatccc gacaagaccc aagaatgttg
        l  l  a  l  v  k  g  a  y  q  a  l  n  l  t  n  p  d  k  t  q  e  c

3300  gctgtgctta gtgtcgggac ctccttatta cgaaggagta gcggtcgtgg gcacttatac caatcattcc
        w  l  c  l  v  s  g  p  p  y  y  e  g  v  a  v  v  g  t  y  t  n  h  s

3370  accgctccgg ccaactgtac ggccacttcc caacataagc ttaccctatc tgaagtgaca ggacagggcc
        t  a  p  a  n  c  t  a  t  s  q  h  k  l  t  l  s  e  v  t  g  q  g

3440  tatgcatggg ggcagtacct aaaactcacc aggccttatg taacaccacc caaagcgccg gctcaggatc
        l  c  m  g  a  v  p  k  t  h  q  a  l  c  n  t  t  q  s  a  g  s  g

3510  ctactacctt gcagcacccg ccggaacaat gtgggcttgc agcactggat tgactccctg cttgtccacc
        s  y  y  l  a  a  p  a  g  t  m  w  a  c  s  t  g  l  t  p  c  l  s  t

3580  acggtgctca atctaaccac agattattgt gtattagttg aactctggcc cagagtaatt taccactccc
        t  v  l  n  l  t  t  d  y  c  v  l  v  e  l  w  p  r  v  i  y  h  s

3650  ccgattatat gtatggtcag cttgaacagc gtaccaaata taaaagagag ccagtatcat tgaccctggc
        p  d  y  m  y  g  q  l  e  q  r  t  k  y  k  r  e  p  v  s  l  t  l

3720  ccttctacta ggaggattaa ccatgggagg gattgcagct ggaataggga cggggaccac tgccttaatt
        a  l  l  l  g  g  l  t  m  g  g  i  a  a  g  i  g  t  g  t  t  a  l  i

3790  aaaacccagc agtttgagca gcttcatgcc gctatccaga cagacctcaa cgaagtcgaa aagtcaatta
        k  t  q  q  f  e  q  l  h  a  a  i  q  t  d  l  n  e  v  e  k  s  i

3860  ccaacctaga aaagtcactg acctcgttgt ctgaagtagt cctacagaac cgcagaggcc tagatttgct
        t  n  l  e  k  s  l  t  s  l  s  e  v  v  l  q  n  r  r  g  l  d  l

3930  attcctaaag gagggaggtc tctgcgcagc cctaaaagaa gaatgttgtt tttatgcaga ccacacgggg
        l  f  l  k  e  g  g  l  c  a  a  l  k  e  e  c  c  f  y  a  d  h  t  g

4000  ctagtgagag acagcatggc caaattaaga gaaaggctta atcagagaca aaaactattt gagacaggcc
        l  v  r  d  s  m  a  k  l  r  e  r  l  n  q  r  q  k  l  f  e  t  g

4070  aaggatggtt cgaagggctg tttaatagat ccccctggtt taccacctta atctccacca tcatgggacc
        q  g  w  f  e  g  l  f  n  r  s  p  w  f  t  t  l  i  s  t  i  m  g

4140  tctaatagta ctcttactga tcttactctt tggaccttgc attctcaatc gattagttca atttgttaaa
        p  l  i  v  l  l  l  i  l  l  f  g  p  c  i  l  n  r  l  v  q  f  v  k

4210  gacaggatct cagtagtcca ggctttagtc ctgactcaac aataccacca gctaaaacca ctagaatacg
        d  r  i  s  v  v  q  a  l  v  l  t  q  q  y  h  q  l  k  p  l  e  y

4280  agccatgata aataaaagat t
        e  p  -  -  i  k  d
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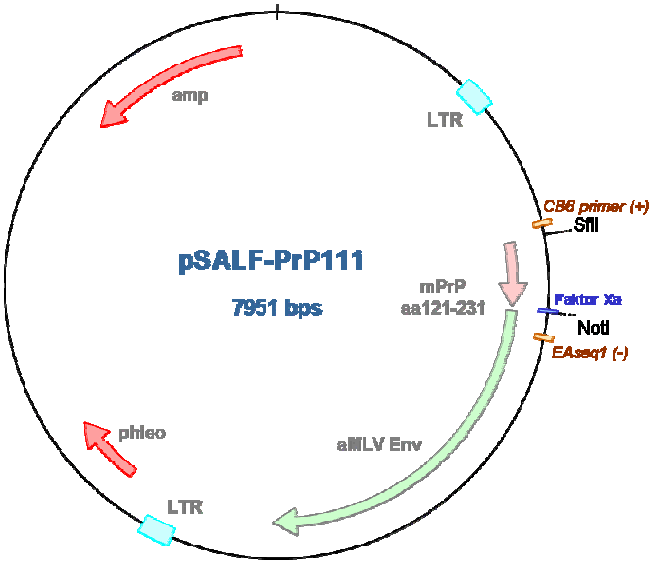
VI.2.5 pSALF-PrP111



translated sequence

aMLV env

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                                    SfiI
1690  ctaatcccct taattcttct gatgctcaga ggggtcagta ctgcttcgcc cggctccagt gcggcccagc
        l  i  p  l  i  l  l  m  l  r  g  v  s  t  a  s  p  g  s  s  a  a  q
       PrP-110
1760  cggccaaagt ggggggcctt ggtggctaca tgctggggag cgccatgagc aggcccatga tccattttgg
       p  a  k  v  g  g  l  g  g  y  m  l  g  s  a  m  s  r  p  m  i  h  f

1830  caacgactgg gaggaccgct actaccgtga aaacatgtac cgctacccta accaagtgta ctacaggcca
      g  n  d  w  e  d  r  y  y  r  e  n  m  y  r  y  p  n  q  v  y  y  r  p

1900  gtggatcagt acagcaacca gaacaacttc gtgcacgact gcgtcaatat caccatcaag cagcacacgg
        v  d  q  y  s  n  q  n  n  f  v  h  d  c  v  n  i  t  i  k  q  h  t

1970  tcaccaccac caccaagggg gagaacttca ccgagaccga tgtgaagatg atggagcgcg tggtggagca
       v  t  t  t  t  k  g  e  n  f  t  e  t  d  v  k  m  m  e  r  v  v  e
                                      Faktor Xa
2040  gatgtgcgtc acccagtacc agaaggagtc ccaggcctat tacgacggga gaagatccag catcgaggga
      q  m  c  v  t  q  y  q  k  e  s  q  a  y  y  d  g  r  r  s  s  i  e  g
          NotI
2110  agggcggccg caatggcaga gagccccat caggtcttta atgtaacctg gagagtcacc aacctgatga
        r  a  a  a  m  a  e  s  p  h  q  v  f  n  v  t  w  r  v  t  n  l  m
… aMLV-Env
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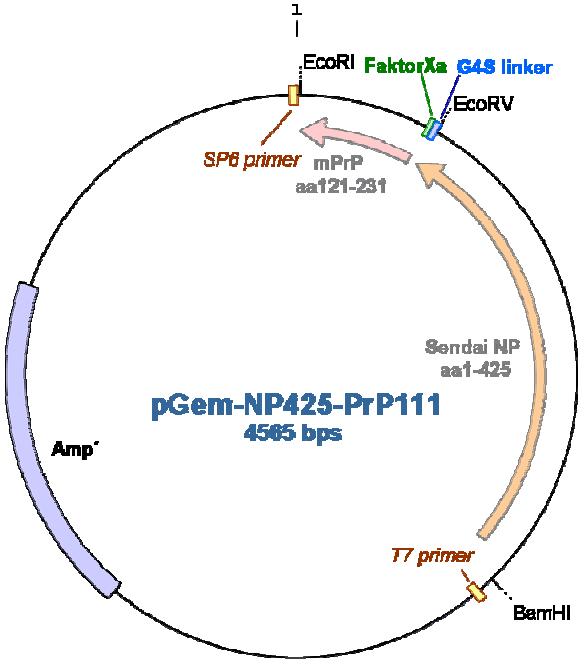
VI.2.6 pGem-NP425-PrP111



sequence

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  71  acgcacatct gctccaccac gcgctccatc atcttcacat cggtctcggt gaagttctcc cccttggtgg
 141  tggtggtgac cgtgtgctgc ttgatggtga tattgacgca gtcgtgcacg aagttgttct ggttgctgta
 211  ctgatccact ggcctgtagt acacttggtt agggtagcgg tacatgtttt cacggtagta gcggtcctcc
 281  cagtcgttgc caaaatggat catgggcctg ctcatggcgc tccccagcat gtagccacca aggcccccca
 351  cccttccctg gattgatcca ccaccgccga tatcggcatt gtctagagct acctcaattg caccaccgcc
 421  tgttggtttg tggtaagcac catccccacc ggacaagttt gccagatgat gtctgagcct ctccttggct
 491  gtatccgtca ctcctaactc atcttccagg gcactgctga tcttcgattc agcatccttt gccacggctt
 561  gtcctagtaa gaacatttcc atatcaaggt atgtcccccc tgtgacgtac tgctgcattg ccttgttctg
 631  tacgacggcg actcccatgg cgtaactcca tagtgcagga taattgcctg gagcaaattc accatgaaca
 701  gggtccttga ggatacagat aaagggagct ctggggcctt ttgacaggta ggtgtctatg aggcttctaa
 771  gcttattaat atcgggcctc aggtttgaca acgttagagc tgccatcttt gtctccaccc catatttaat
 841  agtgttcatg aaggaagcca gccctgcatc tcggatgtag ttcccaacga tctggatgtt cttctctaat
 911  gtggtgagat cagatcttgc agtattcata gtcacaaggg tctcaaccat gagagataca aggctttgct
 981  gagatctcat aaccgagcct atcccctcaa ctgtctcccc agtgaaaatt aaggcacctt tcacggtgcc
1051  gtcttgtctg aacgcctcta acctgttgaa gaacccttttc cttaagccgg cgctgcttgt gatggccttc
1121  accagcacaa tccagacttg gacaattatt gctcctaggc atgcaggata cccataggtt tgaaggagtg
1191  tgtcagggtc tgcagcatcc cgttgaccct ggaagagtgg gctcttgttg accataggtc caaacagcca
1261  ttctgtggtc ctctcatact ccatatctct cgtcttcaca atgaatccgt ctgtcttcgt cctcttaggg
1331  tctttctcta tgttgtagat cacatatttg acatcggcgt ttactccgtt tgttgtcaag tacaattctg
1401  gactactgta agccatggca agcagagaga cgaggaaccc tcctctctga gagtgctgct tatctgtgtc
1471  caatgagtga gctaggaagg tagttgcaat gaataacttg tctgcatcat cagtcacact tgggcctagt
1541  acgaacactg agactgtgct cctctggccg gggataacag cacctcctcc cgacttatta atactttcgc
1611  tcctcctaga gctaaatgta tcgaaggtgc tcaacaaccc ggccatcgtg aactttggca gcaaagcaaa
1681  gggtctggaa cctgctcctc agggtggata ctttgaccct aaggggatcc tctagagtcg acctgcaggc
1751  atgcaagctt ccggtctccc tatagtgagt cgtattaatt tcgataagcc agctgcatta atgaatcggc
1821  caacgcgcgg ggagaggcgg tttgcgtatt gggcgctctt ccgcttcctc gctcactgac tcgctgcgct
1891  cggtcgttcg gctgcggcga gcggtatcag ctcactcaaa ggcggtaata cggttatcca cagaatcagg
1961  ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa aaggccagga accgtaaaaa ggccgcgttg
2031  ctggcgtttt tccataggct ccgccccccct gacgagcatc acaaaaatcg acgctcaagt cagaggtggc
2101  gaaacccgac aggactataa agataccagg cgtttccccc tggaagctcc ctcgtgcgct ctcctgttcc
2171  gaccctgccg cttaccggat acctgtccgc ctttctccct tcgggaagcg tggcgctttc tcatagctca
2241  cgctgtaggt atctcagttc ggtgtaggtc gttcgctcca agctgggctg tgtgcacgaa cccccgttc
2311  agcccgaccg ctgcgcctta tccggtaact atcgtcttga gtccaacccg gtaagacacg acttatcgcc
2381  actggcagca gccactggta acaggattag cagagcgagg tatgtaggcg gtgctacaga gttcttgaag
2451  tggtggccta actacggcta cactagaagg acagtatttg gtatctgcgc tctgctgaag ccagttacct
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2591  caagcagcag attacgcgca gaaaaaaagg atctcaagaa gatcctttga tcttttctac ggggtctgac
2661  gctcagtgga acgaaaactc acgttaaggg attttggtca tgagattatc aaaaaggatc ttcacctaga
2731  tccttttaaa ttaaaaatga agttttaaat caatctaaag tatatatgag taaacttggt ctgacagtta
2801  ccaatgctta atcagtgagg cacctatctc agcgatctgt ctatttcgtt catccatagt tgcctgactc
2871  cccgtcgtgt agataactac gatacgggag ggcttaccat ctggccccag tgctgcaatg ataccgcgag
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3011  tcctgcaact ttatccgcct ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca
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3151  cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatccccc atgttgtgca aaaaagcggt
3221  tagctccttc ggtcctccga tcgttgtcag aagtaagttg gccgcagtgt tatcactcat ggttatggca
3291  gcactgcata attctcttac tgtcatgcca tccgtaagat gcttttctgt gactggtgag tactcaacca
3361  agtcattctg agaatagtgt atgcggcgac cgagttgctc ttgcccggcg tcaatacggg ataataccgc
3431  gccacatagc agaactttaa aagtgctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc
3501  ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg atcttcagca tcttttactt
3571  tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg
3641  gaaatgttga atactcatac tcttcctttt tcaatattat tgaagcattt atcagggtta ttgtctcatg
```

```
3711  agcggataca tatttgaatg tatttagaaa aataaacaaa taggggttcc gcgcacattt ccccgaaaag
3781  tgccacctga cgtctaagaa accattatta tcatgacatt aacctataaa aataggcgta tcacgaggcc
3851  ctttcgtctc gcgcgtttcg gtgatgacgg tgaaaacctc tgacacatgc agctcccgga gacggtcaca
3921  gcttgtctgt aagcggatgc cgggagcaga caagcccgtc agggcgcgtc agcgggtgtt ggcgggtgtc
3991  ggggctggct taactatgcg gcatcagagc agattgtact gagagtgcac catatcgacg ctctccctta
4061  tgcgactcct gcattaggaa gcagcccagt agtaggttga ggccgttgag caccgccgcc gcaaggaatg
4131  gtgcaaggag atggcgccca acagtccccc ggccacgggg cctgccacca tacccacgcc gaaacaagcg
4201  ctcatgagcc cgaagtggcg agcccgatct tccccatcgg tgatgtcggc gatataggcg ccagcaaccg
4271  cacctgtggc gccggtgatg ccggccacga tgcgtccggc gtagaggatc tggctagcga tgaccctgct
4341  gattggttcg ctgaccattt ccggggtgcg gaacggcgtt accagaaact cagaaggttc gtccaaccaa
4411  accgactctg acggcagttt acgagagaga tgatagggtc tgcttcagta agccagatgc tacacaatta
4481  ggcttgtaca tattgtcgtt agaacgcggc tacaattaat acataacctt atgtatcata cacatacgat
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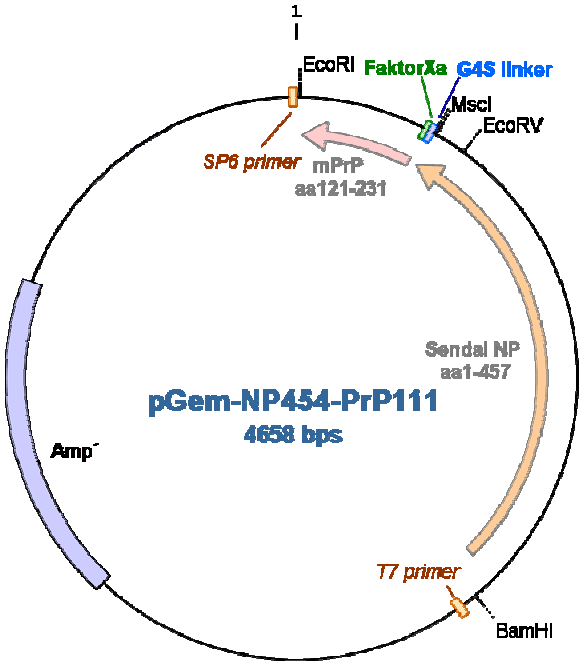
VI.2.7 pGem-NP454-PrP111



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 141  tggtggtgac cgtgtgctgc ttgatggtga tattgacgca gtcgtgcacg aagttgttct ggttgctgta
 211  ctgatccact ggcctgtagt acacttggtt agggtagcgg tacatgtttt cacggtagta gcggtcctcc
 281  cagtcgttgc caaaatggat catgggcctg ctcatggcgc tccccagcat gtagccacca aggccccca
 351  cccttccctc gattgatcca ccaccgccgt ggccaccact cacctgacgt gcccatcttt caccactttc
 421  tccaccccaa cccctagcgt cctggtccgc atgagcttct gtttctaggt cgatatcggc attgtctaga
 491  gctacctcaa ttgcaccacc gcctgttggt ttgtggtaag caccatcccc accggacaag tttgccagat
 561  gatgtctgag cctctccttg gctgtatccg tcactcctaa ctcatcttcc agggcactgc tgatcttcga
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 701  tactgctgca ttgccttgtt ctgtacgacg gcgactccca tggcgtaact ccatagtgca ggataattgc
 771  ctggagcaaa ttcaccatga acagggtcct tgaggataca gataaaggga gctctggggc cttttgacag
 841  gtaggtgtct atgaggcttc taagcttatt aatatcgggc ctcaggtttg acaacgttag agctgccatc
 911  tttgtctcca ccccatattt aatagtgttc atgaaggaag ccagccctgc atctcggatg tagttcccaa
 981  cgatctggat gttcttctct aatgtggtga gatcagatct tgcagtattc atagtcacaa gggtctcaac
1051  catgagagat acaaggcttt gctgagatct cataaccgag cctatcccct caactgtctc cccagtgaaa
1121  attaaggcac ctttcacggt gccgtcttgt ctgaacgcct ctaacctgtt gaagaaccct ttccttaagc
1191  cggcgctgct tgtgatggcc ttcaccagca caatccagac ttggacaatt attgctccta ggcatgcagg
1261  atacccatag gtttgaagga gtgtgtcagg gtctgcagca tcccgttgac cctggaagag tgggctcttg
1331  ttgaccatag gtccaaacag ccattctgtg gtcctctcat actccatatc tctcgtcttc acaatgaatc
1401  cgtctgtctt cgtcctctta gggtctttct ctatgttgta gatcacatat ttgacatcgg cgtttactcc
1471  gtttgttgtc aagtacaatt ctggactact gtaagccatg gcaagcagag agacgaggaa ccctcctctc
1541  tgagagtgct gcttatctgt gtccaatgag tgagctagga aggtagttgc aatgaataac ttgtctgcat
1611  catcagtcac acttgggcct agtacgaaca ctgagactgt gctcctctgg ccggggataa cagcacctcc
1681  tcccgactta ttaatacttt cgctcctcct agagctaaat gtatcgaagg tgctcaacaa cccggccatc
1751  gtgaactttg gcagcaaagc aaagggtctg gaacctgctc ctcagggtgg atactttgac cctaagggga
1821  tcctctagag tcgacctgca ggcatgcaag cttccggtct ccctatagtg agtcgtatta atttcgataa
1891  gccagctgca ttaatgaatc ggccaacgcg cggggagagg cggtttgcgt attgggcgct cttccgcttc
1961  ctcgctcact gactcgctgc gctcggtcgt tcggctgcgg cgagcggtat cagctcactc aaaggcggta
2031  atacggttat ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca
2101  ggaaccgtaa aaaggccgcg ttgctggcgt ttttccatag gctccgcccc cctgacgagc atcacaaaaa
2171  tcgacgctca agtcagaggt ggcgaaaccc gacaggacta taaagatacc aggcgtttcc ccctggaagc
2241  tccctcgtgc gctctcctgt tccgaccctg ccgcttaccg gatacctgtc cgcctttctc ccttcgggaa
```

```
2311  gcgtggcgct ttctcatagc tcacgctgta ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg
2381  ctgtgtgcac gaaccccccg ttcagcccga ccgctgcgcc ttatccggta actatcgtct tgagtccaac
2451  ccggtaagac acgacttatc gccactggca gcagccactg gtaacaggat tagcagagcg aggtatgtag
2521  gcggtgctac agagttcttg aagtggtggc ctaactacgg ctacactaga aggacagtat ttggtatctg
2591  cgctctgctg aagccagtta ccttcggaaa aagagttggt agctcttgat ccggcaaaca aaccaccgct
2661  ggtagcggtg gttttttttgt ttgcaagcag cagattacgc gcagaaaaaa aggatctcaa gaagatcctt
2731  tgatcttttc tacggggtct gacgctcagt ggaacgaaaa ctcacgttaa gggattttgg tcatgagatt
2801  atcaaaaagg atcttcacct agatcctttt aaattaaaaa tgaagtttta aatcaatcta aagtatatat
2871  gagtaaactt ggtctgacag ttaccaatgc ttaatcagtg aggcacctat ctcagcgatc tgtctatttc
2941  gttcatccat agttgcctga ctccccgtcg tgtagataac tacgatacgg gagggcttac catctggccc
3011  cagtgctgca atgataccgc gagacccacg ctcaccggct ccagatttat cagcaataaa ccagccagcc
3081  ggaagggccg agcgcagaag tggtcctgca actttatccg cctccatcca gtctattaat tgttgccggg
3151  aagctagagt aagtagttcg ccagttaata gtttgcgcaa cgttgttgcc attgctacag gcatcgtggt
3221  gtcacgctcg tcgtttggta tggcttcatt cagctccggt tcccaacgat caaggcgagt tacatgatcc
3291  cccatgttgt gcaaaaaagc ggttagctcc ttcggtcctc cgatcgttgt cagaagtaag ttggccgcag
3361  tgttatcact catggttatg gcagcactgc ataattctct tactgtcatg ccatccgtaa gatgcttttc
3431  tgtgactggt gagtactcaa ccaagtcatt ctgagaatag tgtatgcggc gaccgagttg ctcttgcccg
3501  gcgtcaatac gggataatac cgcgccacat agcagaactt taaaagtgct catcattgga aaacgttctt
3571  cggggcgaaa actctcaagg atcttaccgc tgttgagatc cagttcgatg taacccactc gtgcacccaa
3641  ctgatcttca gcatctttta ctttcaccag cgtttctggg tgagcaaaaa caggaaggca aaatgccgca
3711  aaaaagggaa taagggcgac acggaaatgt tgaatactca tactcttcct ttttcaatat tattgaagca
3781  tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag aaaaataaac aaataggggt
3851  tccgcgcaca tttccccgaa aagtgccacc tgacgtctaa gaaaccatta ttatcatgac attaacctat
3921  aaaaataggc gtatcacgag gccctttcgt ctcgcgcgtt tcggtgatga cggtgaaaac ctctgacaca
3991  tgcagctccc ggagacggtc acagcttgtc tgtaagcgga tgccgggagc agacaagccc gtcagggcgc
4061  gtcagcgggt gttggcgggt gtcggggctg gcttaactat gcggcatcag agcagattgt actgagagtg
4131  caccatatcg acgctctccc ttatgcgact cctgcattag gaagcagccc agtagtaggt tgaggccgtt
4201  gagcaccgcc gccgcaagga atggtgcaag gagatggcgc ccaacagtcc cccggccacg gggcctgcca
4271  ccatacccac gccgaaacaa gcgctcatga gcccgaagtg gcgagcccga tcttccccat cggtgatgtc
4341  ggcgatatag gcgccagcaa ccgcacctgt ggcgccggtg atgccggcca cgatgcgtcc ggcgtagagg
4411  atctggctag cgatgaccct gctgattggt tcgctgacca tttccggggt gcggaacggc gttaccagaa
4481  actcagaagg ttcgtccaac caaaccgact ctgacggcag tttacgagag agatgatagg gtctgcttca
4551  gtaagccaga tgctacacaa ttaggcttgt acatattgtc gttagaacgc ggctacaatt aatacataac
4621  cttatgtatc atacacatac gatttaggtg acactata
```

VI.2.8 pGem-NP523-HA

```
   1  gaatacacga attcgagctc ggtacctcaa taatctggaa catcatatgg atacatatgc
  61  tcgaggattc ctcctatccc agctactgct gcggcatcgt catcttcgtc gtgatcgaca
 121  ccgttattgc ggccttcatc tccatgggtt gcagaatcct cttgccgtct ctctgcgagt
 181  ctcatggcta ttcttctctc tatgtctgat acatcctcat cattggtttc ctcctctaac
 241  cgttcagccc catgtagtgt gacaaagtgg ccaccactca cctgacgtgc ccatctttca
 301  ccactttctc caccccaacc cctagcgtcc tggtccgcat gagcttctgt ttctaggtcg
 361  atatcggcat tgtctagagc tacctcaatt gcaccaccgc ctgttggttt gtggtaagca
 421  ccatccccac cggacaagtt tgccagatga tgtctgagcc tctccttggc tgtatccgtc
 481  actcctaact catcttccag ggcactgctg atcttcgatt cagcatcctt tgccacggct
 541  tgtcctagta agaacatttc catatcaagg tatgtccccc ctgtgacgta ctgctgcatt
 601  gccttgttct gtacgacggc gactcccatg gcgtaactcc atagtgcagg ataattgcct
 661  ggagcaaatt caccatgaac agggtccttg aggatacaga taaagggagc tctggggcct
 721  tttgacaggt aggtgtctat gaggcttcta agcttattaa tatcgggcct caggtttgac
 781  aacgttagag ctgccatctt tgtctccacc ccatatttaa tagtgttcat gaaggaagcc
 841  agccctgcat ctcggatgta gttcccaacg atctggatgt tcttctctaa tgtggtgaga
 901  tcagatcttg cagtattcat agtcacaagg gtctcaacca tgagagatac aaggctttgc
 961  tgagatctca taaccgagcc tatcccctca actgtctccc cagtgaaaat taaggcacct
1021  ttcacggtgc cgtcttgtct gaacgcctct aacctgttga agaacccttt ccttaagccg
1081  gcgctgcttg tgatggcctt caccagcaca atccagactt ggacaattat tgctcctagg
1141  catgcaggat acccataggt ttgaaggagt gtgtcagggt ctgcagcatc ccgttgaccc
1201  tggaagagtg ggctcttgtt gaccataggt ccaaacagcc attctgtggt cctctcatac
1261  tccatatctc tcgtcttcac aatgaatccg tctgtcttcg tcctcttagg gtctttctct
1321  atgttgtaga tcacatattt gacatcggcg tttactccgt ttgttgtcaa gtacaattct
1381  ggactactgt aagccatggc aagcagagag acgaggaacc ctcctctctg agagtgctgc
1441  ttatctgtgt ccaatgagtg agctaggaag gtagttgcaa tgaataactt gtctgcatca
1501  tcagtcacac ttgggcctag tacgaacact gagactgtgc tcctctggcc ggggataaca
1561  gcacctcctc ccgacttatt aatactttcg ctcctcctag agctaaatgt atcgaaggtg
1621  ctcaacaacc cggccatcgt gaactttggc agcaaagcaa agggtctgga acctgctcct
1681  cagggtggat actttgaccc taaggggatc ctctagagtc gacctgcagg catgcaagct
1741  tccggtctcc ctatagtgag tcgtattaat ttcgataagc cagctgcatt aatgaatcgg
1801  ccaacgcgcg gggagaggcg gtttgcgtat tgggcgctct tccgcttcct cgctcactga
```

```
1861  ctcgctgcgc tcggtcgttc ggctgcggcg agcggtatca gctcactcaa aggcggtaat
1921  acggttatcc acagaatcag gggataacgc aggaaagaac atgtgagcaa aaggccagca
1981  aaaggccagg aaccgtaaaa aggccgcgtt gctggcgttt ttccataggc tccgcccccc
2041  tgacgagcat cacaaaaatc gacgctcaag tcagaggtgg cgaaacccga caggactata
2101  aagataccag gcgtttcccc ctggaagctc cctcgtgcgc tctcctgttc cgaccctgcc
2161  gcttaccgga tacctgtccg cctttctccc ttcgggaagc gtggcgcttt ctcatagctc
2221  acgctgtagg tatctcagtt cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga
2281  acccccccgtt cagcccgacc gctgcgcctt atccggtaac tatcgtcttg agtccaaccc
2341  ggtaagacac gacttatcgc cactggcagc agccactggt aacaggatta gcagagcgag
2401  gtatgtaggc ggtgctacag agttcttgaa gtggtggcct aactacggct acactagaag
2461  gacagtattt ggtatctgcg ctctgctgaa gccagttacc ttcggaaaaa gagttggtag
2521  ctcttgatcc ggcaaacaaa ccaccgctgg tagcggtggt ttttttgttt gcaagcagca
2581  gattacgcgc agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga
2641  cgctcagtgg aacgaaaact cacgttaagg gattttggtc atgagattat caaaaaggat
2701  cttcacctag atccttttaa attaaaaatg aagtttaaa tcaatctaaa gtatatatga
2761  gtaaacttgg tctgacagtt accaatgctt aatcagtgag gcacctatct cagcgatctg
2821  tctatttcgt tcatccatag ttgcctgact ccccgtcgtg tagataacta cgatacggga
2881  gggcttacca tctggcccca gtgctgcaat gataccgcga gacccacgct caccggctcc
2941  agatttatca gcaataaacc agccagccgg aagggccgag cgcagaagtg gtcctgcaac
3001  tttatccgcc tccatccagt ctattaattg ttgccgggaa gctagagtaa gtagttcgcc
3061  agttaatagt ttgcgcaacg ttgttgccat tgctacaggc atcgtggtgt cacgctcgtc
3121  gtttggtatg gcttcattca gctccggttc ccaacgatca aggcgagtta catgatcccc
3181  catgttgtgc aaaaaagcgg ttagctcctt cggtcctccg atcgttgtca gaagtaagtt
3241  ggccgcagtg ttatcactca tggttatggc agcactgcat aattctctta ctgtcatgcc
3301  atccgtaaga tgcttttctg tgactggtga gtactcaacc aagtcattct gagaatagtg
3361  tatgcggcga ccgagttgct cttgcccggc gtcaatacgg gataataccg cgccacatag
3421  cagaacttta aaagtgctca tcattggaaa acgttcttcg gggcgaaaac tctcaaggat
3481  cttaccgctg ttgagatcca gttcgatgta acccactcgt gcacccaact gatcttcagc
3541  atcttttact ttcaccagcg tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa
3601  aaagggaata agggcgacac ggaaatgttg aatactcata ctcttccttt ttcaatatta
3661  ttgaagcatt tatcagggtt attgtctcat gagcggatac atatttgaat gtatttagaa
3721  aaataaacaa atagggggttc cgcgcacatt tccccgaaaa gtgccacctg acgtctaaga
3781  aaccattatt atcatgacat taacctataa aaataggcgt atcacgaggc cctttcgtct
3841  cgcgcgtttc ggtgatgacg gtgaaaacct ctgacacatg cagctcccgg agacggtcac
3901  agcttgtctg taagcggatg ccgggagcag acaagcccgt cagggcgcgt cagcgggtgt
3961  tggcgggtgt cggggctggc ttaactatgc ggcatcagag cagattgtac tgagagtgca
4021  ccatatcgac gctctccctt atgcgactcc tgcattagga agcagcccag tagtaggttg
4081  aggccgttga gcaccgccgc cgcaaggaat ggtgcaagga gatggcgccc aacagtcccc
4141  cggccacggg gcctgccacc atacccacgc cgaaacaagc gctcatgagc ccgaagtggc
4201  gagcccgatc ttccccatcg gtgatgtcgg cgatataggc gccagcaacc gcacctgtgg
4261  cgccggtgat gccggccacg atgcgtccgg cgtagaggat ctggctagcg atgaccctgc
4321  tgattggttc gctgaccatt tccggggtgc ggaacggcgt taccagaaac tcagaaggtt
4381  cgtccaacca aaccgactct gacggcagtt tacgagagag atgatagggt ctgcttcagt
4441  aagccagatg ctacacaatt aggcttgtac atattgtcgt tagaacgcgg ctacaattaa
4501  tacataacct tatgtatcat acacatacga tttaggtgac actata
```

VI.3 Alignment pDisplay-PrP plasmids

pD-PrP111

Sequencing result using T7 primer

```
pD-PrP110       675 cgagctcggatccactagtaacggccgccagtgtgctggaattcggcttg
seq-pDPrP111      1 ..................................................

pD-PrP110       725 gggatatccaccatggagacagacacactcctgctatgggtactgctgct
seq-pDPrP111     51 ..................................................

pD-PrP110       775 ctgggttccaggttccactggtgactatccatatgatgttccagattatg
seq-pDPrP111    101 ..................................................

pD-PrP110       825 ctggggcccagccggccgtgggggccttggtggctacatgctggggagc
seq-pDPrP111    151 ..................................................
```

```
pD-PrP110       875 gccatgagcaggcccatgatccattttggcaacgactgggaggaccgcta
seq-pDPrP111    201 ..................................................

pD-PrP110       925 ctaccgtgaaaacatgtaccgctaccctaaccaagtgtactacaggccag
seq-pDPrP111    251 ..................................................

pD-PrP110       975 tggatcagtacagcaaccagaacaacttcgtgcacgactgcgtcaatatc
seq-pDPrP111    301 ..................................................

pD-PrP110      1025 accatcaagcagcacacggtcaccaccaccaccaaggggagaacttcac
seq-pDPrP111    351 ..................................................

pD-PrP110      1075 cgagaccgatgtgaagatgatggagcgcgtggtggagcagatgtgcgtca
seq-pDPrP111    401 ..................................................

pD-PrP110      1125 cccagtaccagaaggagtcccaggcctattacgacgggagaagatccagc
seq-pDPrP111    451 ..................................................

pD-PrP110      1175 atcgagggaaggccgcggctgcaggtcgacgaacaaaaactcatctcaga
seq-pDPrP111    501 ..................................................

pD-PrP110      1225 agaggatctgaatgctgtgggccaggacacgcaggaggtcatcgtggtgc
seq-pDPrP111    551 ..................................................

pD-PrP110      1275 cacactccttgccctttaaggtggtggtgatctcagccatcctggccctg
seq-pDPrP111    601 ..................................................

pD-PrP110      1325 gtggtgctcaccatcatctcccttatcatcctcatcatgctttggcagaa
seq-pDPrP111    651 ..................................................

pD-PrP110      1375 gaagccacgttaggcggccgctcgagatcagcctcgactgtgccttctag
seq-pDPrP111    701 ..................................................

pD-PrP110      1425 ttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctgg
seq-pDPrP111    751 ..................................................

pD-PrP110      1475 aaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcg
seq-pDPrP111    801 ...-.................-.........t.......-........

pD-PrP110      1525 cattgtctgagtaggtgtcattctattctgggggggtggggtgg
seq-pDPrP111    848 ..............-......-....ctg..............
```

pD-PrP142

Sequencing result using T7 primer

```
pD-PrP142       697 ggccgccagtgtgctggaattcggcttggggatatccaccatggagacag
seq-pD-PrP14      1 ..................................................

pD-PrP142       747 acacactcctgctatgggtactgctgctctgggttccaggttccactggt
seq-pD-PrP14     51 ..................................................

pD-PrP142       797 gactatccatatgatgttccagattatgctggggcccagccggcccaagg
seq-pD-PrP14    101 ..................................................

pD-PrP142       847 agggggtacccataatcagtggaacaagcccagcaaaccaaaaaccaacc
seq-pD-PrP14    151 ..................................................

pD-PrP142       897 tcaagcatgtggcaggggctgcggcagctggggcagtagtgggggggcctt
seq-pD-PrP14    201 ..................................................
```

```
pD-PrP142       947 ggtggctacatgctggggagcgccatgagcaggcccatgatccattttgg
seq-pD-PrP14    251 ..................................................

pD-PrP142       997 caacgactgggaggaccgctactaccgtgaaaacatgtaccgctacccta
seq-pD-PrP14    301 ..................................................

pD-PrP142      1047 accaagtgtactacaggccagtggatcagtacagcaaccagaacaacttc
seq-pD-PrP14    351 ..................................................

pD-PrP142      1097 gtgcacgactgcgtcaatatcaccatcaagcagcacacggtcaccaccac
seq-pD-PrP14    401 ..................................................

pD-PrP142      1147 caccaaggggagaacttcaccgagaccgatgtgaagatgatggagcgcg
seq-pD-PrP14    451 ..................................................

pD-PrP142      1197 tggtggagcagatgtgcgtcacccagtaccagaaggagtcccaggcctat
seq-pD-PrP14    501 ..................................................

pD-PrP142      1247 tacgacgggagaagatccagcatcgagggaaggccgcggctgcaggtcga
seq-pD-PrP14    551 ..................................................

pD-PrP142      1297 cgaacaaaaactcatctcagaagaggatctgaatgctgtgggccaggaca
seq-pD-PrP14    601 ..................................................

pD-PrP142      1347 cgcaggaggtcatcgtggtgccacactccttgccctttaaggtggtggtg
seq-pD-PrP14    651 ..................................................

pD-PrP142      1397 atctcagccatcctggccctggtggtgctcaccatcatctcccttatcat
seq-pD-PrP14    701 ..................................................

pD-PrP142      1447 cctcatcatgctttggcagaagaagccacgttaggcggccgctcgagatc
seq-pD-PrP14    751 ..................................................

pD-PrP142      1497 agcctcgactgtgccttctagttgccagccatctgttgtttgcccctccc
seq-pD-PrP14    801 ..................................................

pD-PrP142      1547 ccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaa
seq-pD-PrP14    851 ................-......-.............t........

pD-PrP142      1597 taaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattct
seq-pD-PrP14    899 ....-.......-....................-...-..........

pD-PrP142      1647 gggggg
seq-pD-PrP14    945 ......
```

VI.4 Alignement pLenti-DPrP plasmids

VI.5 Alignement pSALF-PrP plasmids

pS-PrP209

Sequencing result using EASeq1 primer

```
pS-PrP209      1703 ttcttctgatgctcagaggggtcagtactgcggcccagccggccaaaaag
seq-pS-PrP20    780 ..................................................
```

```
pS-PrP209      1753 cggccaaagcctggagggtggaacaccggtggaagccggtatcccgggca
seq-pS-PrP20    730 ..................................................

pS-PrP209      1803 gggaagccctggaggcaaccgttacccacctcagggtggcacctgggggc
seq-pS-PrP20    680 ..................................................

pS-PrP209      1853 agccccacggtggtggctggggacaaccccatgggggcagctggggacaa
seq-pS-PrP20    630 ..................................................

pS-PrP209      1903 cctcatggtggtagttggggtcagccccatggcggtggatggggccaagg
seq-pS-PrP20    580 ..................................................

pS-PrP209      1953 agggggtacccataatcagtggaacaagcccagcaaaccaaaaaccaacc
seq-pS-PrP20    530 ..................................................

pS-PrP209      2003 tcaagcatgtggcaggggctgcggcagctggggcagtagtgggggggcctt
seq-pS-PrP20    480 ..................................................

pS-PrP209      2053 ggtggctacatgctggggagcgccatgagcaggcccatgatccattttgg
seq-pS-PrP20    430 ..................................................

pS-PrP209      2103 caacgactgggaggaccgctactaccgtgaaaacatgtaccgctacccta
seq-pS-PrP20    380 ..................................................

pS-PrP209      2153 accaagtgtactacaggccagtggatcagtacagcaaccagaacaacttc
seq-pS-PrP20    330 ..................................................

pS-PrP209      2203 gtgcacgactgcgtcaatatcaccatcaagcagcacacggtcaccaccac
seq-pS-PrP20    280 ..................................................

pS-PrP209      2253 caccaagggggagaacttcaccgagaccgatgtgaagatgatggagcgcg
seq-pS-PrP20    230 ..................................................

pS-PrP209      2303 tggtggagcagatgtgcgtcacccagtaccagaaggagtcccaggcctat
seq-pS-PrP20    180 ..................................................

pS-PrP209      2353 tacgacgggagaagatccagcatcgagggaagggcggccgcaatggcaga
seq-pS-PrP20    130 ..................................................

pS-PrP209      2403 gagcccccatcaggtctttaatgtaacctggagagtcaccaacctgatga
seq-pS-PrP20     80 ..................................................

pS-PrP209      2453 ctgggcgtaccgccaatgccacctccctcc
seq-pS-PrP20     30 ............................
```

pS-PrP111

Sequencing result using EASeq1 primer

```
pS-PrP111      1421 gaccgaccggtggtacctcacccttaccgagtcggcgacacagtgtgggt
seq-pS-PrP11    766 ..................................................

pS-PrP111      1471 ccgccgacaccagactaagaacctagaacctcgctggaaaggaccttaca
seq-pS-PrP11    716 ..................................................

pS-PrP111      1521 cagtcctgctgaccacccccaccgccctcaaagtagacggcatcgcagct
seq-pS-PrP11    666 ..................................................

pS-PrP111      1571 tggatacacgccgcccacgtgaaggctgccgaccccgggggtggaccatc
seq-pS-PrP11    616 ..................................................
```

```
pS-PrP111      1621 ctctagactgacatggcgcgttcaacgctctcaaaaccccttaaaaataa
seq-pS-PrP11    566 ..................................................

pS-PrP111      1671 ggttaacccgcgaggccccctaatcccctttaattcttctgatgctcagag
seq-pS-PrP11    516 ..................................................

pS-PrP111      1721 gggtcagtactgcggcccagccggccgtgggggggccttggtggctacatg
seq-pS-PrP11    466 ..................................................

pS-PrP111      1771 ctggggagcgccatgagcaggcccatgatccattttggcaacgactggga
seq-pS-PrP11    416 ..................................................

pS-PrP111      1821 ggaccgctactaccgtgaaaacatgtaccgctaccctaaccaagtgtact
seq-pS-PrP11    366 ..................................................

pS-PrP111      1871 acaggccagtggatcagtacagcaaccagaacaacttcgtgcacgactgc
seq-pS-PrP11    316 ..................................................

pS-PrP111      1921 gtcaatatcaccatcaagcagcacacggtcaccaccaccaccaaggggga
seq-pS-PrP11    266 ..................................................

pS-PrP111      1971 gaacttcaccgagaccgatgtgaagatgatggagcgcgtggtggagcaga
seq-pS-PrP11    216 ..................................................

pS-PrP111      2021 tgtgcgtcacccagtaccagaaggagtcccaggcctattacgacgggaga
seq-pS-PrP11    166 ..................................................

pS-PrP111      2071 agatccagcatcgagggaagggcggccgcaatggcagagagcccccatca
seq-pS-PrP11    116 ..................................................

pS-PrP111      2121 ggtctttaatgtaacctggagagtcaccaacctgatgactgggcgtaccg
seq-pS-PrP11     66 ..................................................

pS-PrP111      2171 ccaatgccacctccct
seq-pS-PrP11     16 ................
```

VI.6 Alignement pPrPMo plasmids

pPrPMo209

Sequencing result using CB6 primer

```
pPrPMo209      6721 gcggcccagccggccaaaaagcggccaaagcctggagggtggaacaccgg
seq-pPrPMo20      1 ..........t.....................................

pPrPMo209      6771 tggaagccggtatcccgggcagggaagccctggaggcaaccgttacccac
seq-pPrPMo20     51 ..................................................

pPrPMo209      6821 ctcagggtggcacctgggggcagccccacggtggtggctggggacaaccc
seq-pPrPMo20    101 ..................................................

pPrPMo209      6871 catgggggcagctggggacaacctcatggtggtagttggggtcagcccca
seq-pPrPMo20    151 ..................................................

pPrPMo209      6921 tggcggtggatggggccaaggaggggtacccataatcagtggaacaagc
seq-pPrPMo20    201 ..................................................

pPrPMo209      6971 ccagcaaaccaaaaaccaacctcaagcatgtggcaggggctgcggcagct
seq-pPrPMo20    251 ..................................................
```

```
pPrPMo209      7021 ggggcagtagtgggggccttggtggctacatgctggggagcgccatgag
seq-pPrPMo20    301 ..................................................

pPrPMo209      7071 caggcccatgatccattttggcaacgactgggaggaccgctactaccgtg
seq-pPrPMo20    351 ..................................................

pPrPMo209      7121 aaaacatgtaccgctaccctaaccaagtgtactacaggccagtggatcag
seq-pPrPMo20    401 ..................................................

pPrPMo209      7171 tacagcaaccagaacaacttcgtgcacgactgcgtcaatatcaccatcaa
seq-pPrPMo20    451 ..................................................

pPrPMo209      7221 gcagcacacggtcaccaccaccaccaaggggagaacttcaccgagaccg
seq-pPrPMo20    501 ..................................................

pPrPMo209      7271 atg
seq-pPrPMo20    551 ...
```

pPrPMo111

Sequencing result using CB6 primer

```
pPrPMo111      6719 ctgcggcccagccggccgtgggggcccttggtggctacatgctggggagc
seq-pPrPMo11      1 ............c.................................

pPrPMo111      6769 gccatgagcaggcccatgatccattttggcaacgactgggaggaccgcta
seq-pPrPMo11     51 ..................................................

pPrPMo111      6819 ctaccgtgaaaacatgtaccgctaccctaaccaagtgtactacaggccag
seq-pPrPMo11    101 ..................................................

pPrPMo111      6869 tggatcagtacagcaaccagaacaacttcgtgcacgactgcgtcaatatc
seq-pPrPMo11    151 ..................................................

pPrPMo111      6919 accatcaagcagcacacggtcaccaccaccaccaaggggagaacttcac
seq-pPrPMo11    201 ..................................................

pPrPMo111      6969 cgagaccgatgtgaagatgatggagcgcgtggtggagcagatgtgcgtca
seq-pPrPMo11    251 ..................................................

pPrPMo111      7019 cccagtaccagaaggagtcccaggcctattacgacgggagaagatccagc
seq-pPrPMo11    301 ..................................................

pPrPMo111      7069 atcgagggaagggcggccgcagcttcgcccggctccagtcctcatcaagt
seq-pPrPMo11    351 ..................................................

pPrPMo111      7119 ctataatatcacctgggaggtaaccaatggagatcgggagacggtatggg
seq-pPrPMo11    401 ..................................................

pPrPMo111      7169 caacttctggcaaccaccctctgtggacctggtggcctgaccttacccca
seq-pPrPMo11    451 ..................................................

pPrPMo111      7219 gatttatgtatgttagcccaccatggaccatcttattgggggctagaata
seq-pPrPMo11    501 ..................................................

pPrPMo111      7269 tcaatccccttttttcttctcccccggggccccccttgttgctcaggggca
seq-pPrPMo11    551 ..........................................a.......

pPrPMo111      7319 gcagcccaggctgttc
seq-pPrPMo11    601 ................
```

VI.7 Alignement pNP-Gem-PrP plasmids

pGem-NP425-PrP111

Sequencing result using pGem4-Seq primer

```
pGem-NP425-P     11 attctcatgctggatcttctcccgtcgtaataggcctgggactccttctg
seq-pGemNP42    840 ..................................................

pGem-NP425-P     61 gtactgggtgacgcacatctgctccaccacgcgctccatcatcttcacat
seq-pGemNP42    790 ..................................................

pGem-NP425-P    111 cggtctcggtgaagttctccccttggtggtggtggtgaccgtgtgctgc
seq-pGemNP42    740 ..................................................

pGem-NP425-P    161 ttgatggtgatattgacgcagtcgtgcacgaagttgttctggttgctgta
seq-pGemNP42    690 ..................................................

pGem-NP425-P    211 ctgatccactggcctgtagtacacttggttagggtagcggtacatgtttt
seq-pGemNP42    640 ..................................................

pGem-NP425-P    261 cacggtagtagcggtcctcccagtcgttgccaaaatggatcatgggcctg
seq-pGemNP42    590 ..................................................

pGem-NP425-P    311 ctcatggcgctccccagcatgtagccaccaaggccccccaccttccctg
seq-pGemNP42    540 ..................................................

pGem-NP425-P    361 gattgatccaccaccgccgatatcggcattgtctagagctacctcaattg
seq-pGemNP42    490 ..................................................

pGem-NP425-P    411 caccaccgcctgttggtttgtggtaagcaccatccccaccggacaagttt
seq-pGemNP42    440 ..................................................

pGem-NP425-P    461 gccagatgatgtctgagcctctccttggctgtatccgtcactcctaactc
seq-pGemNP42    390 ..................................................

pGem-NP425-P    511 atcttccagggcactgctgatcttcgattcagcatcctttgccacggctt
seq-pGemNP42    340 ..................................................

pGem-NP425-P    561 gtcctagtaagaacatttccatatcaaggtatgtccccctgtgacgtac
seq-pGemNP42    290 ..................................................

pGem-NP425-P    611 tgctgcattgccttgttctgtacgacggcgactcccatggcgtaactcca
seq-pGemNP42    240 ..................................................

pGem-NP425-P    661 tagtgcaggataattgcctggagcaaattcaccatgaacagggtccttga
seq-pGemNP42    190 ..................................................

pGem-NP425-P    711 ggatacagataaagggagctctggggccttttgacaggtaggtgtctatg
seq-pGemNP42    140 ..................................................

pGem-NP425-P    761 aggcttctaagcttattaatatcgggcctcaggtttgacaacgttagagc
seq-pGemNP42     90 ..................................................

pGem-NP425-P    811 tgccatctttgtctccaccccatatttaatagtgttcatg
seq-pGemNP42     40 ....................................
```

pGem-NP455-PrP111

Sequencing result using pGem4-Seq primer

```
pGem-NP455-P     10 aattctcatgctggatcttctcccgtcgtaataggcctgggactccttct
seq-pGemNP45    897 ..................................................

pGem-NP455-P     60 ggtactgggtgacgcacatctgctccaccacgcgctccatcatcttcaca
seq-pGemNP45    847 ..................................................

pGem-NP455-P    110 tcggtctcggtgaagttctccccttggtggtggtggtgaccgtgtgctg
seq-pGemNP45    797 ..................................................

pGem-NP455-P    160 cttgatggtgatattgacgcagtcgtgcacgaagttgttctggttgctgt
seq-pGemNP45    747 ..................................................

pGem-NP455-P    210 actgatccactggcctgtagtacacttggttagggtagcggtacatgttt
seq-pGemNP45    697 ..................................................

pGem-NP455-P    260 tcacggtagtagcggtcctcccagtcgttgccaaaatggatcatgggcct
seq-pGemNP45    647 ..................................................

pGem-NP455-P    310 gctcatggcgctccccagcatgtagccaccaaggccccccaccttccct
seq-pGemNP45    597 ..................................................

pGem-NP455-P    360 cgattgatccaccaccgccgtggccaccactcacctgacgtgcccatctt
seq-pGemNP45    547 ..................................................

pGem-NP455-P    410 tcaccactttctccaccccaacccctagcgtcctggtccgcatgagcttc
seq-pGemNP45    497 ..................................................

pGem-NP455-P    460 tgtttctaggtcgatatcggcattgtctagagctacctcaattgcaccac
seq-pGemNP45    447 ..................................................

pGem-NP455-P    510 cgcctgttggtttgtggtaagcaccatccccaccggacaagtttgccaga
seq-pGemNP45    397 ..........-.......................................

pGem-NP455-P    560 tgatgtctgagcctctccttggctgtatccgtcactcctaactcatcttc
seq-pGemNP45    357 ..................................................

pGem-NP455-P    610 cagggcactgctgatcttcgattcagcatcctttgccacggcttgtccta
seq-pGemNP45    307 ..................................................

pGem-NP455-P    660 gtaagaacatttccatatcaaggtatgtccccctgtgacgtactgctgc
seq-pGemNP45    257 ....a---------.............t..................

pGem-NP455-P    710 attgccttgttctgtacgacggcgactcccatggcgtaactccatagtgc
seq-pGemNP45    216 ..................................................

pGem-NP455-P    760 aggataattgcctggagcaaattcaccatgaacagggtccttgaggatac
seq-pGemNP45    166 ..................-...............................

pGem-NP455-P    810 agataaagggagctctggggccttttgacaggtaggtgtctatgaggctt
seq-pGemNP45    117 ..................................................

pGem-NP455-P    860 ctaagcttattaatatcgggcctcaggtttgacaacgttagagctgccat
seq-pGemNP45     67 ........................-.........................

pGem-NP455-P    910 ctttgtctccaccccatatt
seq-pGemNP45     18 ..............a.....
```

pGem-NP523-HA

Sequencing result using pGem4-Seq primer

```
pGem-NP523-H     50 gatacatatgctcgaggattcctcctatcccagctactgctgcggcatcg
seq-pGem-NP5     76 ..................................................

pGem-NP523-H    100 tcatcttcgtcgtgatcgacaccgttattgcggccttcatctccatgggt
seq-pGem-NP5    126 ..................................................

pGem-NP523-H    150 tgcagaatcctcttgccgtctctctgcgagtctcatggctattcttctct
seq-pGem-NP5    176 ..................................................

pGem-NP523-H    200 ctatgtctgatacatcctcatcattggtttcctcctctaaccgttcagcc
seq-pGem-NP5    226 ..................................................

pGem-NP523-H    250 ccatgtagtgtgacaaagtggccaccactcacctgacgtgcccatctttc
seq-pGem-NP5    276 ..................................................

pGem-NP523-H    300 accactttctccaccccaacccctagcgtcctggtccgcatgagcttctg
seq-pGem-NP5    326 ..................................................

pGem-NP523-H    350 tttctaggtcgatatcggcattgtctagagctacctcaattgcaccaccg
seq-pGem-NP5    376 ..................................................

pGem-NP523-H    400 cctgttggtttgtggtaagcaccatccccaccggacaagtttgccagatg
seq-pGem-NP5    426 ..................................................

pGem-NP523-H    450 atgtctgagcctctccttggctgtatccgtcactcctaactcatcttcca
seq-pGem-NP5    476 ..................................................

pGem-NP523-H    500 gggcactgctgatcttcgattcagcatcctttgccacggcttgtcctagt
seq-pGem-NP5    526 ..................................................

pGem-NP523-H    550 aagaacatttccatatcaaggtatgtccccctgtgacgtactgctgcat
seq-pGem-NP5    576 ..........................t.......................

pGem-NP523-H    600 tgccttgttctgtacgacggcgactcccatggcgtaactccatagtgcag
seq-pGem-NP5    626 ..................................................

pGem-NP523-H    650 gataattgcctggagcaaattcaccatgaacagggtccttgaggatacag
seq-pGem-NP5    676 ..................................................

pGem-NP523-H    700 ataaagggagctctggggccttttgacaggtaggtgtctatgaggcttct
seq-pGem-NP5    726 ..................................................

pGem-NP523-H    750 aagcttattaatatcgggcctcaggtttgacaacgttagagctgccatct
seq-pGem-NP5    776 ..................................................

pGem-NP523-H    800 ttgtctccaccccatatttaatagtgttcatgaaggaagccagccctgca
seq-pGem-NP5    826 ..................................................

pGem-NP523-H    850 tctcggatgtagttcccaacgatctggatgttcttctctaatgtggtgag
seq-pGem-NP5    876 ........................................-.........

pGem-NP523-H    900 atcagatcttgcagtattcatagtc
seq-pGem-NP5    925 .........................
```

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VIII Publication

Nikles, D., P. Bach, et al. (2005). "Circumventing Tolerance to the Prion Protein (PrP): Vaccination with PrP-Displaying Retrovirus Particles Induces Humoral Immune Responses against the Native Form of Cellular PrP." J Virol **79**(7): 4033-42.

IX Abbreviations

aa	amino acid
aMLV	amphotropic murine leukemia virus
Asn	asparagines
bp	base pair
BSE	bovine spongiform encephalopathy
Ch	brain homogenate of Chandler scrapie strain-infected mice
CWD	chronic wasting disease
CJD	Creutzfeldt-Jakob disease
d	days
DNA	desoxyribonucleic acid
EGF	epidermal growth factor
EGF^D	epidermal growth factor fusion protein with platelet derived growth factor receptor transmembrane domain
eMLV	ecotropic murine leukemia virus
FITC	fluorescence isothiocyanate
GPI	glycosyl phosphatidyl inositol
h	hours
HA	immunological tag derived from hemagglutinine A
HG	brain homogenate of unifected mice
HEK-293FT	human embryonic kidney cells, fast growing subclone
Ig	immune globuline
IPA	immune peroxidase assay
µg	microgramm
min	minutes
ml	milliliter
MLV	murine leukaemia virus
mPrP	murine prion protein
mPrP209	murine prion protein containing amino acids 23 to 231
mPrP142	murine prion protein containing amino acids 90 to 231
mPrP111	murine prion protein containing amino acids 121 to 231
moi	multiplicity of infection
myc	immunological tag derived from the human c-myc protooncogene
N2a	murine neuroblastoma cell line

NP	nucleocapsid protein of the Sendai virus
NP-PrP	prion protein fused to the nucleocapsid protein of the Sendai virus
p30	MLV-derived capsid protein
PDGFR	platelet derived growth factor receptor
PDGFR-TM	platelet derived growth factor receptor transmembrane domain
PIPLC	phospholipase C
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PrPMo	PrP-coding MLV-Variant
Prnp	prion protein gene
PrP	prion protein
PrP^{c}	cellular prion protein
PrP^{D}	prion protein fusion protein with platelet derived growth factor receptor transmembrane domain
PrP^{D111}	prion protein aa 121-231 fusion protein with platelet derived growth factor receptor transmembrane domain
PrP^{D142}	prion protein aa 90-231 fusion protein with platelet derived growth factor receptor transmembrane domain
PrP^{D209}	prion protein aa 23-231 fusion protein with platelet derived growth factor receptor transmembrane domain
PrP^{E}	prion protein fusion protein with the murine leukaemia virus derived envelope protein
PrP^{E111}	prion protein aa 121-231 fusion protein with the murine leukaemia virus derived envelope protein
PrP^{E209}	prion protein aa 23-231 fusion protein with the murine leukaemia virus derived envelope protein
PrP-res	proteinase K resistant form of the prion protein
PrP^{Sc}	pathogenic isoform of the prion protein
RT	reverse transcriptase
SDS	sodium dodecyle sulfate
SDS-PAGE	sodium dodecyle sulfate polyacrylamid gelelectrophoresis
sec	seconds
SP	signal peptide
SU	soluble unit of the retroviral envelope protein
SV40	simian virus 40

TM	transmembrane domain of the retroviral envelope protein
TSE	transmissible spongiform encephalopathies
VLP(s)	virus-like particles(s)
Wt	wild-type
Wt-eMLV	wild-type ecotropic murine leukaemia virus
6H4	PrP-specific monoclonal antibody

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XII Eidesstattliche Versicherung

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Dissertation selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass aus Schriften Entlehnungen, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.

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