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In vitro expression in eukaryotic cells of a prion protein gene cloned from scrapie-infected mouse brain

(biosynthesis/secretion/immunofluorescent labeling/proteinase K sensitivity/infectivity)

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ABSTRACT **It has been proposed that the causative agent of scrapie represents a class of infectious particle that is devoid of nucleic acid and that an altered form of the endogenous prion protein (PrP) is the agent. However, it has been difficult to exclude the possibility that PrP purified from scrapie tissues might be contaminated with a more conventional viral agent. To obtain PrP uncontaminated by scrapie-infected tissues, PrP cDNA cloned from a scrapie-infected mouse brain was expressed in mouse C127 cells *in vitro*. mRNA and protein encoded by the cloned PrP gene were identified. The expressed PrP polypeptides appeared to be glycosylated and were released from the cell surface into the medium. Homogenates of the cells expressing the cloned PrP gene were inoculated into susceptible mice but failed to induce clinical signs of scrapie. Thus, either PrP is not the transmissible agent of scrapie or the expressed PrP requires additional modification to be infectious.**

Identification of the infectious agents causing scrapie, kuru, Creutzfeldt–Jakob, and related degenerative neuropathological disorders has been a baffling problem in biology. Among the unusual characteristics of these agents is their resistance to treatments deleterious to nucleic acids (1–5). Furthermore, no agent-specific nucleic acids have been identified in agent preparations. At the same time, studies with scrapie have indicated that protein is a vital component of the agent (3). These and other factors have led to proposals that the scrapie agent represents a class of infectious agent that is solely proteinaceous and thus devoid of genetic material (1, 3, 6, 7). The discovery of a particular protein, named prion protein (PrP) (8–10) or scrapie-associated fibril protein (11), as the major protein component of fractions enriched in scrapie infectivity elicited the further proposal that this protein, or an aggregate thereof, is the scrapie infectious agent (8, 11, 12). However, subsequent studies have shown that PrP is a normal endogenous protein of brain and probably other tissues (13–18) and that scrapie infection results in the accumulation of a modified form of PrP that is partially proteinase K-resistant (10, 11, 15–18). It remains unclear whether the scrapie-associated modification of PrP is secondary to scrapie disease or whether it represents a structural change that is sufficient to initiate and transmit scrapie pathogenesis. Because of the uncertainty over the role of PrP in scrapie induction, we decided to express the mouse PrP cDNA derived from scrapie-infected brain in eukaryotic cells to test directly whether this PrP product was capable of transmitting the scrapie disease. The results presented here indicate that the cloned PrP cDNA was expressed in mouse tissue culture cells; however, extracts of these cells failed to cause scrapie when inoculated into susceptible mice.

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MATERIALS AND METHODS

RNA Gel Blot Analysis. Poly(A)$^+$ RNA was isolated from transformed C127 cell cultures, electrophoresed in duplicate lanes (6 μg per lane) on a 6% (vol/vol) formaldehyde/1% agarose gel, blotted onto an Amersham Hybond-N membrane, and baked as described (14), except for the substitution of the Hybond membrane for nitrocellulose. The membrane was prehybridized for 4 hr at 55°C in a solution of 3× SSPE (14), 0.1% $NaDodSO_4$, 1× Denhardt's solution (14), and sheared and freshly boiled salmon sperm DNA at 0.05 mg/ml. The membrane was cut and the halves were hybridized at 55°C for 24 hr in the prehybridization buffer containing freshly labeled 1.3-kilobase (kb) PrP cDNA inserts or simian virus 40 (SV40) DNA probes. After rinsing with 2× SSPE plus 0.1% $NaDodSO_4$, the blots were washed in the same buffer for 1 hr and then in 0.1× SSPE plus 0.1% $NaDodSO_4$ for 30 min at 55°C. The blots were exposed for autoradiography at −70°C with a DuPont Lightning Plus intensifying screen. The SV40 probe was the *Bam*HI–*Bgl* II restriction fragment from p341-1 (19). The PrP and SV40 probes were labeled with ^{32}P by using a New England Nuclear nick-translation kit (to specific activities, 7×10^7 dpm/μg and 2×10^7 dpm/μg, respectively), boiled 5 min, and used at a concentration of 14 ng/ml in the hybridization buffer.

Metabolic Labeling of PrP. Cell cultures were grown to confluency in 24-well tissue culture plates (Linbro) or 25-cm^2 flasks, preincubated at 37°C for 45 min with methionine-free minimal essential medium containing 1% dialyzed fetal calf serum and labeled in the same medium containing [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq). The cells were then rinsed with a phosphate-buffered balanced salt solution (pH 7.2) and lysed in ice-cold lysing buffer (LB; 50 mM Tris·HCl at pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% $NaDodSO_4$, 1% Nonidet P-40, 0.1% gelatin, and 0.1% NaN_3). The lysates were precleared by incubating overnight at 4°C with 2 μl of normal rabbit serum per 2.5×10^5 cell-equivalents and by two cycles of mixing with protein A-Sepharose (Pharmacia; 1.5 mg/μl of serum) at 4°C for 30 min and of microcentrifuging to remove the Sepharose. The anti-PrP peptide serum 783 (2 μl per 2.5×10^5 cell-equivalent) was incubated overnight at 4°C with the lysates, and immune complexes were removed with a single cycle of protein A-Sepharose precipitation. The Sepharose beads were washed three times in LB, and bound proteins were eluted by boiling in $NaDodSO_4$/PAGE sample buffer (20). Labeled proteins were analyzed by $NaDodSO_4$/PAGE and fluorography with EN^{3}HANCE (New England Nuclear) as described by the manufacturer. To obtain the anti-PrP peptide serum 783, a PrP peptide fragment,

CGQGGGTHNQWNKPSK-COOH,

Abbreviations: PrP, prion protein; BPV, bovine papilloma virus; SV40, simian virus 40.

representing the N-terminal portion of the PrP 27–30 sequence (13), except for an additional terminal cystine residue, was synthesized, coupled by the N terminus to keyhole limpet hemocyanin, and used to immunize rabbits as described (21). This antiserum not only was effective in PrP immunoprecipitation, as shown in the present study, but also was found to recognize proteinase K-treated PrP 27-30 from brain and spleen tissue of scrapie-infected mice and hamsters on immunoblots.

RESULTS AND DISCUSSION

To express the PrP gene from scrapie-infected mouse brain in a mouse cell line, a plasmid vector containing the sequence of the entire PrP open reading frame was constructed (Fig. 1). In the vector, the PrP gene fragment was located downstream from the mouse metallothionine promoter and upstream from SV40 sequences that included a polyadenylylation site. These flanking sequences provided transcription initiation and termination signals, respectively, since such signals were likely to be missing from the PrP cDNA fragment. A portion of the bovine papilloma virus (BPV) genome was also inserted into the plasmid to enable it to transform the host cell and replicate stably as an episome. The final PrP-BPV construction was transfected into mouse C127 cells as described (24), and morphologically transformed colonies were visible after ≈14 days. These were picked and expanded for further analysis. Southern blot analysis of DNA from these cells indicated that the PrP and BPV inserts remained intact and were present in an episomal form. As a control, a similar construct lacking the PrP insert and the SV40 sequences was made and control C127 transformants obtained with this BPV construct were also analyzed.

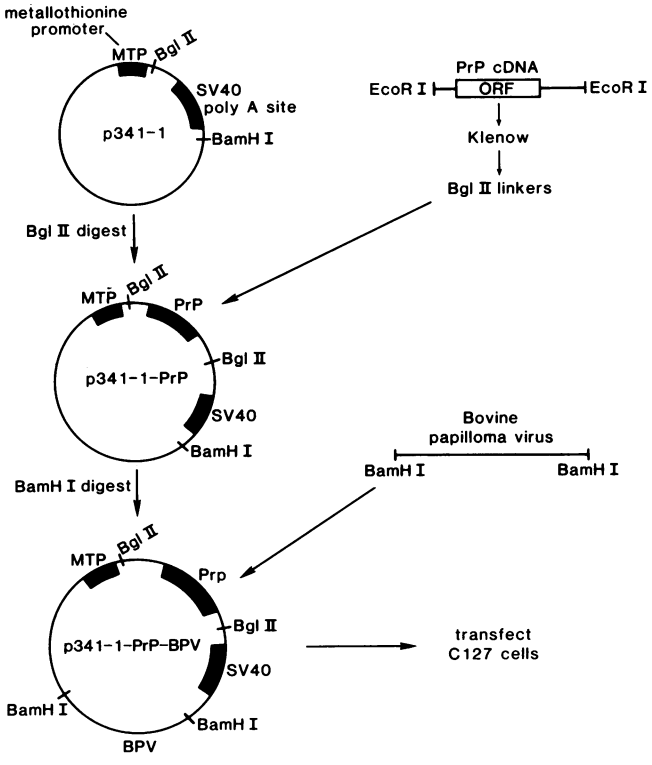

FIG. 1. Construction of the PrP expression vector. The 1.3-kb insert of mouse PrP clone 7 (22) was isolated by *Eco*RI digestion followed by low-melting-point agarose gel electrophoresis and excision of the appropriate fragment. After purification on an Elutip ion-exchange column (Schleicher & Schuell), the ends were blunted by the Klenow fragment of DNA polymerase I and *Bgl* II linkers were added. This fragment was digested with *Bgl* II and inserted into the *Bgl* II site of p341-1, a plasmid kindly provided by Peter Howley (National Cancer Institute, Bethesda, MD) (19). After transformation into JM109 cells, appropriate recombinants with the correct orientation were selected, DNA was cut with *Bam*HI, and the entire BPV genome, derived from *Bam*HI digestion of p142-6 (23) was inserted. Appropriate recombinants were again selected, and the plasmid DNA was purified for transfection into mouse C127 cells.

PrP mRNA encoded by the PrP–BPV plasmid was detected in a cloned line (2P) of the transformed C127 cells by RNA gel blot analysis (Fig. 2). RNA of ≈2.3 kb hybridizing to the PrP probe was identified in the 2P cells and in control cells transformed with a BPV plasmid lacking the PrP gene. This RNA was similar in molecular size to the PrP mRNA expressed in numerous mouse tissues and cell types (13, 14, 25), and its presence in the control cells indicated that the endogenous PrP gene was expressed in these cells. In addition to the endogenous PrP mRNA, PrP RNA species specific to the 2P cells were observed at ≈1.8, ≈2.6, and ≈3.8 kb, indicating that transcripts containing the PrP sequence were being synthesized from the plasmid. A similar pattern was observed with a probe made from an SV40 sequence containing the polyadenylylation site immediately downstream from the PrP insert in the expression plasmid. Thus, these transcripts were encoded by the plasmid. The SV40 probe also hybridized to a 2.3-kb RNA that was near the expected 2.2-kb size of an RNA transcript from the metallothionine promoter (19) to the SV40 polyadenylylation site. It is possible that transcription could initiate within the inserted PrP sequence as well as in the metallothionine promoter, resulting in different-sized transcripts containing the PrP and SV40 sequences. In addition, transcription may terminate at sites other than the SV40 polyadenylylation site. For instance, the larger PrP–SV40 mRNAs may be the result of transcription passing through the SV40 sequence to termination sites within the BPV sequence downstream. No SV40 poly(A)$^+$ RNA sequences were observed in the control cells since the BPV plasmid used to transform the cell did not contain SV40 sequences.

To demonstrate that PrP protein was being synthesized from plasmid-encoded PrP mRNAs, 2P cells were metabolically labeled with [^{35}S]methionine, and cell lysates were

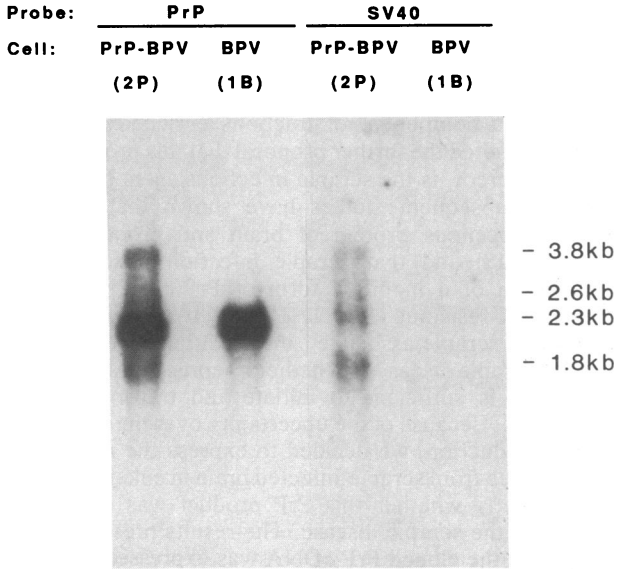

FIG. 2. Expression of mRNA encoded by the mouse brain PrP gene in C127 cells. Duplicate RNA gel blots of poly(A)$^+$ RNA from C127 cells transformed with the PrP–BPV plasmid (clone 2P) or the control BPV plasmid (clone 1B) were hybridized with ^{32}P-labeled 1.3-kb PrP cDNA insert or SV40 DNA probes and autoradiographed for 1 week.

immunoprecipitated with a rabbit anti-PrP peptide serum. After labeling for 15 min, discrete bands at 37, 33, 31, 29, 27, and 23 kDa were observed (Fig. 3). After 1 hr, these PrP bands became more heavily labeled, and an additional band spanning apparent molecular sizes of 35–41 kDa appeared. Competitive inhibition of the immunoprecipitation of these bands by the PrP peptide demonstrated that these proteins contained the specific PrP peptide antigenic determinant. PrP polypeptides with apparent molecular sizes of 31, 27, and 23 kDa were also specifically immunoprecipitated from the control cells and thus appeared to be encoded by the endogenous PrP gene. The overall labeling of these forms was less by a factor of 10–20 than in the 2P cells. This quantitative difference in PrP expression combined with appearance of PrP forms with distinct molecular sizes indicated that PrP forms encoded by the PrP–BPV plasmid were being expressed in the 2P cells. There appeared to be a profound difference in the rates of translation of the PrP mRNAs encoded by the endogenous PrP gene and the PrP–BPV plasmid since, although the plasmid-derived PrP mRNAs were much less abundant than the endogenous PrP mRNAs (Fig. 2), the reverse was true of their respective translation products. This suggested that, if the scrapie brain and C127 PrP mRNAs were identical initially, removal or replacement of certain portions of the original noncoding regions with metallothionine promoter at the 5′ end or with SV40 sequences at the 3′ end might have greatly enhanced their translational activity.

Since multiple molecular size forms of PrP were identified with the 15-min and 1-hr [^{35}S]methionine labeling of the PrP–BPV cells, a pulse–chase labeling experiment was performed to determine whether the bands observed were intermediates or final products in the biosynthesis of PrP (Fig. 4). During 1- and 3-hr chase periods, label progressively decreased in the discrete PrP bands at 23, 31, 33, and 37 kDa and shifted primarily to the broad 35- to 41-kDa band and to a minor band at 18 kDa. This suggested that at least some of the bands that lost ^{35}S were precursors of the 35- to 41-kDa band and that the latter represented the final product(s) of PrP biosynthesis in the C127 cells. The apparent microheterogeneity of the 35- to 41-kDa PrP suggested that this product, like PrP isolated from brain (26), was modified by the addition of glycosyl or of other nonpeptide residues. After 17 hr of chase, most of the labeled PrP had disappeared from the cell. Concurrent with the loss of labeled cellular PrP was the appearance of labeled 35- to 41-kDa PrP in the chase medium. This indicated that PrP was targeted as a secretory protein or as a cell surface protein that was released over the course of time. The PrP released from the cell was either soluble in aqueous medium and no longer membrane bound or associated with small particles not pelleted by centrifugation at 12,000 × *g* for 15 min. The latter criterion excluded the association of PrP with dislodged cells or the free nuclei and plasma membrane ghosts of disrupted cells. Membrane immunofluorescence studies with the anti-PrP peptide serum on live cells indicated that PrP was bound to the surface of the 2P and the control C127 cells (data not shown). The PrP specificity of the surface labeling was demonstrated by competitive inhibition with the PrP peptide antigen. Treatment of the cells with trypsin prior to incubation with anti-PrP eliminated the labeling, confirming that the PrP detected was exposed to the cell surface. Thus, 2P cells appeared to synthesize plasma membrane-bound and secreted forms of PrP.

Since it has been proposed that PrP is the transmissible agent of scrapie, we tested the possibility that expression of the PrP gene from scrapie-infected brain in 2P cells generated the infectious agent. 2P cell homogenates were prepared and inoculated into mice in a manner similar to that used for scrapie-infected tissue culture cells (27). As of 335 days after

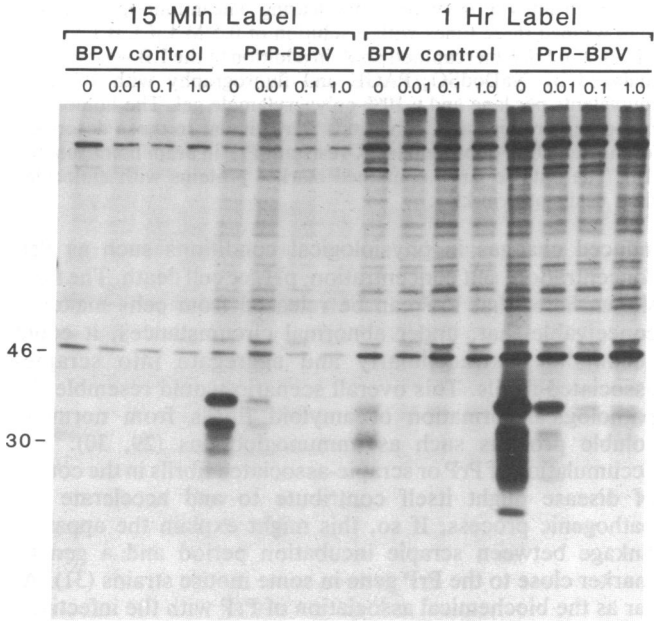

FIG. 3. Expression of the mouse brain PrP gene in C127 cells. Clones 2P and 1B, transformed with the PrP–BPV and BPV plasmids, respectively, were metabolically labeled for 15 min or 1 hr with [^{35}S]methionine. PrP was immunoprecipitated from lysates of the cells with a rabbit anti-PrP peptide serum 783. To distinguish PrP from nonspecifically precipitated proteins, various amounts of unlabeled PrP peptide antigen (0, 0.01, 0.1, and 1.0 μg) were preincubated with the antiserum for 30 min at room temperature prior to immunoprecipitation to eliminate the PrP-specific interactions. Immunoprecipitates representing 2.5 × 10^5 cell-equivalents were analyzed by NaDodSO$_4$/PAGE and fluorography by using 8.5% polyacrylamide gels. The positions of molecular size marker proteins are designated in kDa.

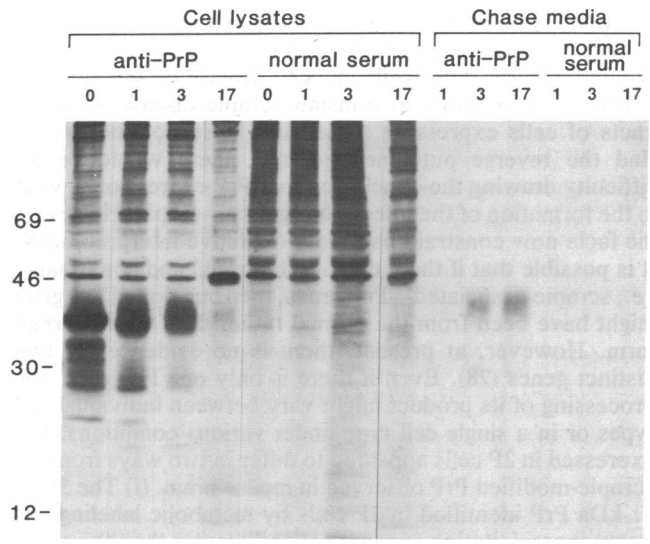

FIG. 4. Pulse–chase metabolic labeling of PrP expressed in PrP–BPV-transformed (2P) C127 cells. Cells were metabolically labeled for 1 hr. Following the labeling period, the cells were rinsed and chased with complete minimum essential medium containing 10% (vol/vol) fetal calf serum for the designated periods of time (in hr). After collecting the chase medium, the cells were rinsed and lysed. The chase medium was microcentrifuged at 12,000 × *g* for 15 min and concentrated LB was added to the supernatant to make a final 1× concentration. The cell lysates (2.5 × 10^5 cell-equivalents) and the chase medium (from 5 × 10^5 cells) were immunoprecipitated with either anti-PrP 783 or normal rabbit serum and analyzed by NaDodSO$_4$/PAGE and fluorography on 8.5% polyacrylamide gels. The positions of molecular size marker proteins are designated in kDa.

injection, none of the mice exhibited symptoms of scrapie (Table 1). In contrast, high dilutions of control scrapie-infected tissue culture cells induced disease by this time.

Thus, our attempts to transmit scrapie disease with extracts of cells expressing cloned PrP were not successful. Had the reverse outcome occurred, there would be no difficulty drawing the conclusion that PrP expression is vital to the formation of the infectious agent of scrapie. However, the facts now constrain us to less definitive interpretations. It is possible that if there exist both normal and abnormal—i.e., scrapie-associated—PrP genes, then our cloned PrP gene might have been from the normal rather than the abnormal form. However, at present, there is no evidence for two distinct genes (28). Even if there is only one PrP gene, the processing of its product might vary between individual cell types or in a single cell type under various conditions. PrP expressed in 2P cells appeared to differ in two ways from the scrapie-modified PrP observed in mouse brain. (*i*) The 35- to 41-kDa PrP identified in 2P cells by metabolic labeling and immunoprecipitation appeared slightly larger than the major 33- to 35-kDa form of PrP detected by immunoblot in normal and scrapie-infected mouse brain (16). It is unclear whether this represents a true dissimilarity between the total PrP populations or is merely a technical artifact due to the different techniques used to detect PrP. (*ii*) Whereas some PrP in scrapie-infected brain preparations is partially resistant to proteinase K digestion (10), the PrP forms labeled in the 2P cells were completely digested (Fig. 5). In this respect, the 2P cell PrP resembled the normal PrP of brain tissue (18). Proteinase K sensitivity may be a function of the biosynthetic processing or the environment of PrP. Since the partially resistant form of PrP has been found only in extracts of diseased tissues, its formation may require conditions that exist *in vivo* but not *in vitro* in tissue culture cells.

The lack of proteinase K-resistant PrP in 2P cells might account for the lack of induction of clinical scrapie. However, there is an alternative explanation, namely, that no form of PrP is the infectious scrapie agent. Rather, PrP is a normal endogenous protein that may accumulate in the partially protease-resistant form in brain and perhaps other tissues secondary to the scrapie pathogenic process. Although partial proteinase K resistance has been used as the "hallmark" of scrapie-modified PrP in the brain, there is no evidence that this characteristic reflects an alteration in the covalent structure of PrP. The resistance to proteolysis may be evidence of denaturation and refolding of endogenous PrP that permits aggregation into insoluble proteinase K-resistant fibrils. Such denaturation might be caused by scrapie-induced changes in physiological conditions such as PrP concentration, salt concentration, pH, or cell death. The fact, shown here, that PrP can be released from cells makes it conceivable that, under abnormal circumstances, it could accumulate extracellularly and aggregate into scrapie-associated fibrils. This overall scenario would resemble the pathological formation of amyloid fibrils from normally soluble proteins such as immunoglobulins (29, 30). The accumulation of PrP or scrapie-associated fibrils in the course of disease might itself contribute to and accelerate the pathogenic process. If so, this might explain the apparent linkage between scrapie incubation period and a genetic marker close to the PrP gene in some mouse strains (31). As far as the biochemical association of PrP with the infectivity is concerned, one can only speculate that aggregated PrP might bind nonspecifically to the infectious agent since PrP was found by differential centrifugation to bind to cellular components of various sizes (32). Perhaps this might occur to such an extent as to alter the true biophysical properties of the agent, serving to confuse the issue further.

Table 1. Lack of transmission of scrapie to mice by C127 cells expressing cloned mouse PrP

Cell-equivalents inoculated	Scrapie-positive mice/total mice	
	PrP–BPV C127	Scrapie-infected neuroblastoma
2×10^6	0/10	10/10
2×10^5	0/10	10/10
2×10^4	0/10	10/10
2×10^3	0/10	9/9
2×10^2	NT*	7/9
2×10^1	NT	0/10

Data shown were as of 335 days after inoculation. Mice inoculated with 2×10^3 or more scrapie-infected neuroblastoma cell-equivalents were dead by 210 days after inoculation. RML mice were inoculated intracerebrally with C127 cells transformed with the PrP–BPV expression plasmid (clone 2P) and scrapie-infected mouse neuroblastoma cells. The neuroblastoma cells were from a culture that, after infection with scrapie *in vitro*, replicated the agent through >40 passages (27).
*NT, not tested.

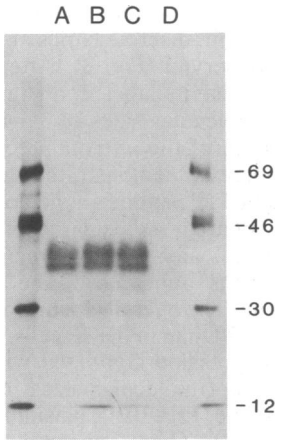

FIG. 5. Proteinase K sensitivity of metabolically labeled PrP in clone 2P cells. Cells were labeled for 4 hr, released from the flask with phosphate-buffered saline containing 2 mM EDTA, and pelleted. A 10% (vol/vol) homogenate was prepared in a solution of 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM KCl, and 1% sarcosyl by Dounce homogenization and passage through a 25-gauge needle. Control portions were left on ice (lane A), treated with proteinase K for 10 min at room temperature only after the addition of the proteinase K inhibitor phenylmethylsulfonyl fluoride to control for subsequent interference of immunoprecipitation by proteinase K (lane B), or incubated at 37°C without proteinase K to test for digestion by endogenous proteases (lane C). The remaining portion (lane D) was treated with proteinase K for 30 min at 37°C. To terminate the digestion, phenylmethylsulfonyl fluoride was added and the mixture was incubated at room temperature for 10 min. The final concentrations of proteinase K and phenylmethylsulfonyl fluoride in samples B and D were 50 μg/ml and 1 mM, respectively. All the samples were then diluted 1:20 with LB and immunoprecipitated with anti-PrP 783 without preclearing. The immunoprecipitates were washed three times with a solution of 0.5 M LiCl, 0.1 M Tris (pH 9), and 1% 2-mercaptoethanol, in addition to the LB washes, and analyzed by $NaDodSO_4$/PAGE and fluorography with 10^6 cell-equivalents per lane and a 10% polyacrylamide gel. The homogenization and proteinase K conditions were similar to those described for the detection of proteinase K-resistant PrP in brain homogenates (16). The outside lanes contained marker proteins with molecular sizes designated in kDa.

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