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Evidence of Mitochondrial Involvement in Scrapie Infection

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Two cDNA libraries were constructed from brain membrane and cytoskeletal preparations purified from scrapie-infected hamster brains. Four recombinants strongly preferential to the scrapie cytoskeletal preparation were identified by the differential hybridization of 7,000 recombinants. These clones were not, however, preferential to total nucleic acids extracted from scrapie-infected hamster brains. DNA sequence analysis revealed all four clones to have significant sequence similarities to the mouse mitochondrial genome. This correlation led us to consider a mitochondrial association with scrapie infectivity. Brain mitochondria were purified by sucrose gradient density centrifugation and found to contain high infectivity. Removal of mitochondrial outer membranes by osmotic shock or digitonin treatment resulted in no detectable loss of titer.

Scrapie is a transmissible, degenerative neurological disease. It occurs naturally in sheep and goats and has been experimentally transmitted to hamsters, mice, and rats. Despite intense research efforts, the cause of the disease remains obscure. A nucleic acid(s) responsible for infectivity has yet to be identified.

The characterization of scrapie infectivity in hamster brain membranes indicates that the transmissible agent exists primarily as an insoluble macromolecular complex (36, 46). Fractionation of cytoskeletal preparations by nondenaturing conditions has further shown that a large proportion of this membrane-associated infectivity copurifies with an insoluble cytoskeletal protein of astrocytic glial cells, glial fibrillary acidic protein (GFAP) (53).

Two distinct factors have been shown to influence scrapie infection, the host genome and the infectious agent itself. Twenty years ago, the role of the host genome in scrapie infection was demonstrated by the discovery of the scrapie incubation (Sinc) gene in mice (17). By use of the ME7 strain of the scrapie agent, two nondominant alleles, *s7* and *p7*, were identified. Mice homozygous for the *s7* allele had a much shorter incubation period than those homozygous for the *p7* allele. The Sinc gene has since been found to influence incubation time in every known strain of mouse scrapie (16).

It is also well documented that the infectious agent itself plays a separate and distinct role in determining the nature of a scrapie infection. Over 15 different strains of the scrapie agent have been identified and characterized (4, 16–18). The majority of the scrapie strains produce incubation patterns similar to that of the ME7 strain. A few strains of the agent, however, have been documented to produce an opposite pattern, with *p7* homozygous mice having shorter incubation times than mice homozygous for the *s7* allele (14–16). Further evidence of the scrapie agent being an entity independent of the host genome includes mutation of the agent (4, 28) and interspecies transmission (7, 23).

The prion hypothesis has dominated the scrapie field for many years. There is substantial evidence that the prion gene product has an impact on scrapie infection or the disease process or both (33, 40, 42, 43). This prion protein (PrP) is a host-encoded gene product which is expressed at similar levels in uninfected and infected tissues (9, 38). PrP is closely linked to the Sinc gene in the mouse (27), and recently a correlation has been made with respect to PrP primary sequence and the alleles for short and long scrapie incubation period (55). Codon 108 encodes leucine in short-incubation-period mice while encoding phenylalanine in long-incubation-period mice. Thus, it would appear the prion gene product is the major host-encoded component affecting scrapie infectivity.

Consequently, even though many data have been generated about the influence of the host factor (PrP) on scrapie infection, little is known about the second and most critical factor, the agent itself. In this article, we present evidence indicating a possible mitochondrial involvement in scrapie infection.

MATERIALS AND METHODS

Membrane preparations. Brains from 30 endstage scrapie-infected male hamsters were homogenized to 10% (wt/vol) in buffer A (20 mM Tris hydrochloride, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, pH 7.8) with a Ten Broeck glass grinder. After clarification at 500 × *g* for 10 min, the supernatant was centrifuged at 1,000 × *g* for 30 min to obtain a plasma membrane-enriched pellet (46). Nucleic acids were extracted from this suspended pellet to prepare the brain membrane (BrM) library. This pellet was also used as the starting material to prepare cytoskeletal extracts.

Cytoskeletal extracts. Membrane pellets were suspended to 10% (vol/vol) in buffer B (10 mM Tris hydrochloride, 6 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 1 mM EGTA, pH 7.8) and then extracted with 1% Nonidet P-40 at 5°C for 2 h. The cytoskeletal fraction was pelleted at 12,000 × *g* for 15 min and washed twice to remove residual detergent. The washed pellets were resuspended to 10% in buffer B without $MgCl_2$ and rotated overnight at 5°C.

After centrifugation for 20 min at 12,000 × *g*, the pellet was resuspended to 10% in buffer B without $MgCl_2$ and then extracted with 4% *N*-lauroylsarcosine for 2 h at 5°C, followed by clarification at 20,000 × *g* for 30 min. Samples (5 ml) of this supernatant were separated on 7-ml 20 to 40% Nycodenz gradients [in buffer A containing 0.4 M $(NH_4)_2SO_4$] which were centrifuged at 100,000 × *g* for 16 h. Gradients were fractionated from the bottom, and densities were measured before the fractions were dialyzed for 48 to 72 h at 5°C against buffer B. A fraction (Gp) having a density of 1.226 g/ml was found to have the highest scrapie infectivity ($10^{8.5}$ 50% lethal doses [LD_{50}] per ml) and contained 150 μg of protein per ml as measured by the dye-binding method

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of Bradford (3) and 30 to 40 μg of nucleic acid per ml as measured by A_{260}.

Nucleic acid extraction. Gp and BrM preparations were adjusted to contain 0.5% sodium dodecyl sulfate (SDS), 100 mM NaCl, and 25 mM EDTA and were extracted with an equal volume of phenol. The samples were then extracted with phenol-chloroform (1:1), followed by a chloroform extraction and ethanol precipitation.

Nucleic acid slot blots. Nucleic acid (400 ng) was dissolved in 1× slot buffer (5 mM methyl mercury hydroxide, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and bound to nitrocellulose filters with a slot-blot manifold (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the instructions of the manufacturer. The filters were then baked at 80°C in vacuo, prehybridized, and hybridized as described by Maniatis et al. (30).

Postnuclear nucleic acid isolation. Postnuclear nucleic acids were isolated by homogenizing brain tissue in buffer A. The homogenate was centrifuged at 500 × *g* for 10 min to pellet nuclei and large cell fragments. The supernatant was then adjusted to a final concentration of 0.5% SDS, 100 mM NaCl, and 25 mM EDTA and extracted once with an equal volume of phenol, twice with an equal volume of phenol-chloroform (1:1), and finally with an equal volume of chloroform prior to ethanol precipitation.

Construction of cDNA libraries. cDNA libraries were constructed by a modification of standard techniques (30) to ensure incorporation of small (less than 100-nucleotide) cDNAs into the plasmid vector. First-strand cDNAs were synthesized by priming with an N_6 oligonucleotide (Pharmacia, Inc., Piscataway, N.J.) at a final concentration of 100 μg/ml. Second-strand synthesis and S1 digestion were performed as described by Maniatis et al. (30). The double-stranded cDNA was blunt ligated into the *Sma*I site of the pUC9 plasmid and transformed into TB1 cells. The integrity of the libraries was determined by insert size, variability, and the ability to hybridize to the first-strand cDNA probe.

Differential hybridization. First-strand cDNA probes were prepared by the random priming of cytoskeletal nucleic acid by a modification of the first-strand cDNA synthesis protocol. Nucleic acid (1 to 2 μg) was denatured and random primed as described above in 1× reverse transcription buffer (100 mM Tris hydrochloride [pH 8.3], 140 mM KCl, 2.5 mM $MgCl_2$) in the presence of 1 mM dATP, 1 mM dTTP, 1 mM dGTP, and 100 μCi of [^{32}P]dCTP. Avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 1 U/ml and incubated at 37°C for 1 h. RNA was selectively degraded by the addition of NaOH to a final concentration of 50 mM and incubated at 68°C for 1 h. Ammonium acetate was then added to a final concentration of 2 M, and the probe was ethanol precipitated in the presence of 50 μg of yeast RNA.

Hybridization of the colony lifts was performed as described by Maniatis et al. (30). Autoradiography was performed with Kodak XAR film at −80°C with an intensifying screen.

DNA sequence analysis. Hamster insert DNA was cleaved out of the pUC9 vector with the restriction endonucleases *Hin*dIII and *Eco*RI and recloned into pGEM1 plasmid (Promega Biotec, Madison, Wis.). Dideoxy sequencing of double-stranded plasmid was performed with DNA primers complementary to the T7 and SP6 RNA polymerase promoters (Promega Biotec). A computer search of the hamster sequences against GenBank DNA sequences was performed with software provided by the University of Wisconsin Genetics Computer Group (13).

Agent and bioassay. The scrapie agent used in these studies was serially passaged in outbred hamsters after adaptation from the Chandler strain of mouse scrapie as previously described (26). Infectivity was determined by the method of incubation time interval assay (41) by intracerebral inoculation of weanling outbred male hamsters (Harlan Sprague-Dawley Indianapolis, Ind.) and had a standard error of 0.5 $\log_{10}$ LD_{50}.

Mitochondrial purification. Hamster brains were homogenized to 10% (wt/vol) in 10 mM Tris hydrochloride–1 mM EGTA–0.32 M sucrose (pH 7.8) with a Ten Broeck glass grinder having a 1-mm clearance. Nuclei and large fragments were removed by centrifugation at 700 × *g* for 10 min followed by centrifugation of the supernatant at 40,000 × *g* for 20 min to pellet synaptosomes, mitochondria, and large membrane vesicles. After resuspension and mild vortexing of the pellets in 9 volumes of the same buffer, these elements were separated by centrifugation (120,000 × *g* for 1 h) in 0.8 M/1.2 M/1.5 M discontinuous sucrose gradients by the method of Gray and Whittaker (22).

Removal of mitochondrial outer membrane. Mitochondria recovered at the 1.5 M interface were carefully equilibrated to 0.32 M sucrose and treated with digitonin at a concentration of 1 mg/mg of mitochondrial protein to remove the outer membrane (54). The outer mitochondrial membrane was also removed by hypotonic swelling (29) by incubating purified mitochondria in 10 mM Tris hydrochloride (pH 7.8)–1 mM EGTA for 30 min at 4°C before adding an equal volume of 2 M sucrose in the same buffer. After 10 min, the suspensions were sonicated for 30 s with a sonicator (model W-10 S; Heat Systems Ultrasonics). Mitoplasts prepared by both procedures were recovered at the 1.5 M interface after centrifugation in discontinuous sucrose gradients.

SAF purification. Scrapie-associated fibrils (SAF) were prepared as described by Merz et al. (35). Hamster brains were homogenized to 10% (wt/vol) in 20 mM Tris-acetate–2 mM EDTA–0.32 M sucrose, pH 7.8. After centrifugation at 800 × *g* for 10 min, the supernatant was decanted and further centrifuged at 13,000 × *g* for 30 min. The pellets were resuspended to 10% (vol/vol) in the same buffer and extracted with 2% *N*-lauroylsarcosine. The extracts were then separated on discontinuous 1.2 M/2 M sucrose gradients, and material at the interface was collected.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed as described by O'Farrell (39).

Silver staining. The procedures followed for silver staining of the protein gels were a combination of methods used by Merrill et al. (34), Oakley et al. (37), and Gooderham (21). After electrophoresis, the gels were placed in a fixing solution (50% methanol–10% acetic acid) overnight in the dark. The gels were then washed with double-distilled water for 2 h with constant agitation and allowed to soak for 90 min in 50% methanol. Two 10-min double-distilled-water washes followed. The proteins were stained in a 0.8% silver nitrate–1.4% ammonium hydroxide–7.6% sodium hydroxide solution for 20 min with rapid agitation. Two 15-min double-distilled-water washes followed. Development was initiated with 0.005% citric acid–0.18% formaldehyde followed by an 18% acetic acid solution.

Mitochondrial F1 ATPase. Bovine heart F1 ATPase was a gift from S. Schuster. Hamster brain F1 ATPase was prepared by the chloroform extraction method of Ragan et al. (44).

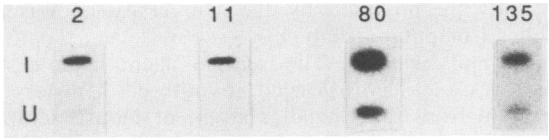

FIG. 1. Slot-blot hybridizations of the four scrapie BrM and cytoskeletal preferential clones hybridized to total nucleic acid (400 ng) isolated from cytoskeletal preparations from scrapie-infected (I) or uninfected (U) tissue. Lanes: 2, pBrM2; 11, pBrM11; 80, pGp80; 135, pGp135.

Antibody sources. Antiserum to bovine heart mitochondrial F1 ATPase was a gift from S. Schuster. PrP monoclonal antibody was provided by R. Kascsak. R. Rubenstein provided antisera against SAF protease-resistant proteins.

Western blot (immunoblot) analysis. The application of protein to nitrocellulose with a slot-blot manifold (Bethesda Research Laboratories) was performed according to the instructions of the manufacturer. Protein samples fractionated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose electrophoretically with the TE22 Mini-Transfer system (Hoefer Scientific Instruments, San Francisco, Calif.). Transfer time was 1 h at 250 mA.

Treatment of the filters was similar to that of Towbin et al. (51) with the following modifications. Western blots were incubated with primary antiserum in 1% bovine serum albumin–20 mM Tris hydrochloride–500 mM NaCl (1% bovine serum albumin–saline) for 2 h at 24°C. The filters were then washed in TTBS (20 mM Tris hydrochloride, 500 mM NaCL, 0.5% Tween 20, pH 7.5) for 20 min. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) was used as the secondary antibody. Incubation was for 1 h at 24°C, followed by two 10-min TTBS washes. The peroxidase reaction product was visualized by soaking the filter in 0.3% (wt/vol) horseradish peroxidase color developer (Bio-Rad) in methanol–0.06% (vol/vol) H_2O_2–saline (pH 7.5) for 20 to 30 min.

RESULTS

Differential hybridization. Two cDNA libraries were constructed for this study. One was synthesized from hamster BrM nucleic acid (BrM), and a second was synthesized from nucleic acids isolated from cytoskeletal preparations (Gp). The titer of this Nycodenz-fractionated cytoskeletal preparation was 10^8 LD_{50}/ml. Approximately 2,000 recombinants from the BrM library and 5,000 from the Gp library were screened by differential hybridization. The clones were initially challenged with a first-strand probe synthesized from nucleic acids isolated from the high-titer cytoskeletal preparation. Following autoradiography, the initial probe was stripped and the filters were rehybridized with first-strand probe produced from nucleic acids isolated from cytoskeletal fractions prepared from uninfected hamster brains. Comparison of the autoradiograms and subsequent rescreening identified four recombinants (pBrM2, pBrM11, pGp80, and pGp135) that appeared strongly preferential to the first-strand cDNA probes synthesized from scrapie-infected preparations.

Slot-blot hybridizations of nucleic acids isolated from the infected and uninfected cytoskeletal preparations were probed with the DNA inserts isolated from the four preferential clones. All produced strong signals in the scrapie samples while hybridizing weakly to cytoskeletal preparations from uninfected hamsters (Fig. 1). Thus, all four clones have significant sequence homology to nucleic acid species relatively abundant in the infectious cytoskeletal preparations and are present at very low levels in similar preparations from uninfected hamsters. Hybridization of the cloned inserts to hamster brain total nucleic acids, however, produced similar signals between the scrapie-infected and uninfected samples (data not presented). Thus, none of the clones were found to be scrapie preferential to total nucleic acids, but rather the cytoskeleton preparations from scrapie-infected hamsters appear to be greatly enriched in nucleic acids homologous to the four recombinants.

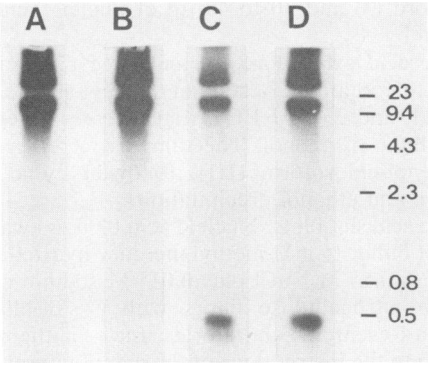

FIG. 2. Southern blot analysis of cytoplasmic DNA isolated from scrapie-infected hamster brains. The filters were probed with pBrM2 insert (lane A), pBrM11 insert (lane B), pGp80 insert (lane C), or pGp135 insert (lane D). Positions of the molecular weight markers (in kilobase pairs) are given to the right.

Mitochondrial origin of clones. DNase 1 and RNase A sensitivity studies of nuclear and cytoplasmic nucleic acids indicated that all four recombinants had homology to cytoplasmic DNA. Hybridization of the clones to Southern blots of cytoplasmic DNA produced identical high-molecular-weight bands with all four probes (Fig. 2). The largest species was greater than 23 kilobases (kb) long, while the second largest band was approximately 16 kb in size. In addition, two of the clones, pGp135 and pGp80, produced strong signals to low-molecular-weight nucleic acids (approximately 500 nucleotides long).

The identity of the four clones was also examined by DNA sequence analysis. Comparison of the sequences with the GenBank database indicated that the clones contained strong homology to the mouse mitochondrial genome (Fig. 3). pBrM2 exhibited a 79% homology to the mouse mitochondrial 12S rRNA region, pBrM11 was 77% homologous to the mitochondrial ATPase 6 gene, and both pGp135 and pGp80 had strong homology to the displacement loop region of the mouse mitochondrial genome. Comparison of the pGp80 sequence with the mouse mitochondrial genome revealed excellent homology (72%) to the 5′ end of the displacement loop region, while pGp135 was 88% homologous to the 3′ end of the displacement loop.

The strong homology of the clones to the mouse mitochondrial genome substantiates the Southern blot data. The 16-kb band produced in the hybridization of Southern blots with each of the clones corresponds in size to the intact mitochondrial genome. The higher-molecular-weight species (greater than 23 kb) are undoubtedly catenated forms of the mitochondrial genome (8). The additional smaller band produced when pGp80 and pGp135 were used as probes is of similar size to the mouse displacement loop transcript, a single-stranded DNA of 550 to 670 nucleotides (20).

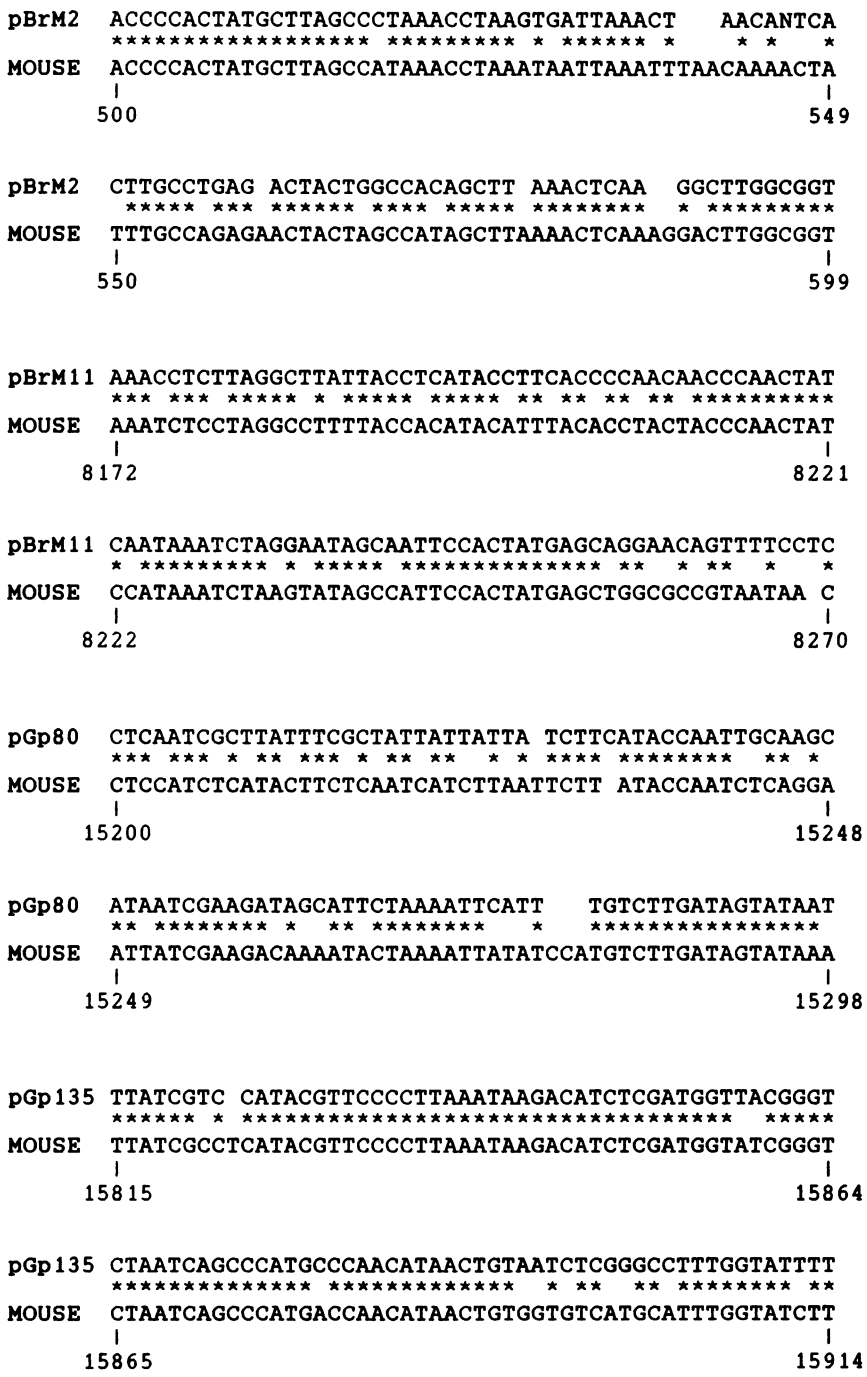

FIG. 3. DNA sequence homology of the four scrapie BrM and cytoskeletal preferential clones to the mouse mitochondrial genome. Numbers refer to the mouse mitochondrial sequence (2). Spaces have been introduced to optimize homology. Asterisks indicate homologous nucleotides between the hamster and mouse sequences.

Mitochondrial purification. We followed established homogenization and fractionation procedures for purifying mitochondria and mitoplasts from brain tissue (29). Differential centrifugation of brain homogenates separated the crude mitochondrial-cytoskeletal fraction from the nuclear fraction (Fig. 4). Purified mitochondria were then obtained by banding on sucrose gradients. Mitochondrial outer membranes were removed by treatment with digitonin (54) or by osmotic stress (29), and the resulting mitoplasts were banded on a second sucrose gradient.

Standard O'Farrell gels (39) were used to monitor the purity of our mitochondrial preparations. Proteins with isoelectric points between 4 and 7 and molecular weights between 15 and 200 were separated and visualized by silver staining. The purity of the mitochondrial samples was determined by the lack of actin, tubulin, and GFAP, as well as the presence of the beta subunit of mitochondrial F1 ATPase as the predominant protein.

The location of actin, tubulin, and GFAP were confirmed by Western blot analysis. Beta ATPase was identified by its

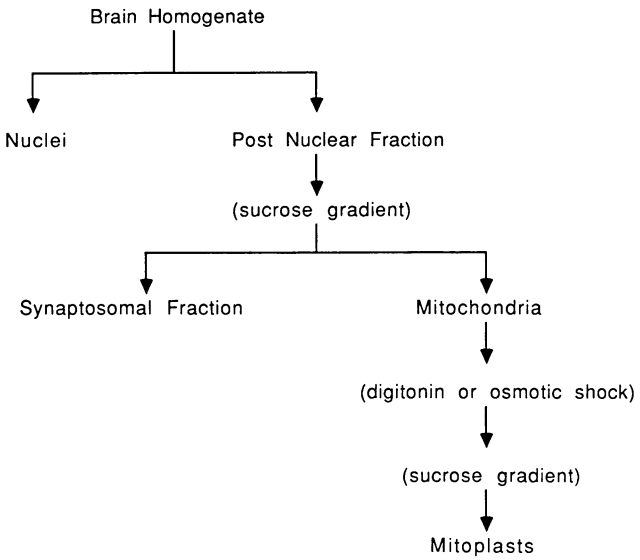

FIG. 4. Separation of hamster brain homogenate into mitochondrial, mitoplast, and synaptosomal fractions.

size, 50 kilodaltons (kDa), and reaction to F1 ATPase antibody. Actin, tubulin, and GFAP, easily identifiable in crude hamster brain homogenates (Fig. 5A), were greatly reduced in mitochondrial fractions from the first sucrose gradient (Fig. 5B) and not identifiable in mitoplast fractions from the second sucrose gradient (Fig. 5C). Beta ATPase increased in abundance as the mitochondria were purified.

Mitochondrial infectivity. We investigated the level of infectivity copurifying with mitochondria during the course of a scrapie infection. At 2-week intervals during the preclinical and clinical course of disease, five hamsters which had been inoculated intracerebrally with $10^{7.4}$ LD_{50} of scrapie infectivity were sacrificed and their brains were fractionated as described above. These preparations were then tested for infectivity by intracerebral inoculation. Figure 6 shows the results of three trials comparing infectivity of 5% suspensions of crude brain homogenates with that of mitochondria and mitoplast fractions. Purified mitochondria contain a high titer (10^8 LD_{50}/ml of a 5% suspension) of infectivity by the onset of clinical signs of disease at 8 weeks after inoculation. Removal of the outer membrane by either digitonin treatment or osmotic shock resulted in no loss of titer. As seen in previous studies on time course infectivity (5), the high titers measured early in the incubation period represent the input inoculum since the scrapie agent has no detectable eclipse period (25).

Fractionation of PrP. Numerous studies have shown an association between scrapie infectivity and the presence of the PrP. Given the high infectivity of our mitochondrial samples, it was essential to determine whether our mitochondrial preparations contained detectable levels of the PrP. PrP is typically purified from the cytoskeletal fraction—the fraction we separated on a sucrose gradient into synaptosomal and mitochondrial fractions. Synaptosomal and mitoplast proteins were tested for the presence of PrP by Western blot analysis. When the blots were probed with the PrP monoclonal antibody, an intense smear in the 33- to 35-kDa region was observed in the synaptosomal lane (Fig. 7). This pattern is typical of PrP and was identical to the SAF control. Barely visible in the mitoplast lane (containing $10^{8.5}$ LD_{50}/ml) are two bands of 27 and 33 kDa. Thus, the vast majority of the prion antibody-reacting proteins are not present in our mitoplast sample but separate into the synaptosomal fraction.

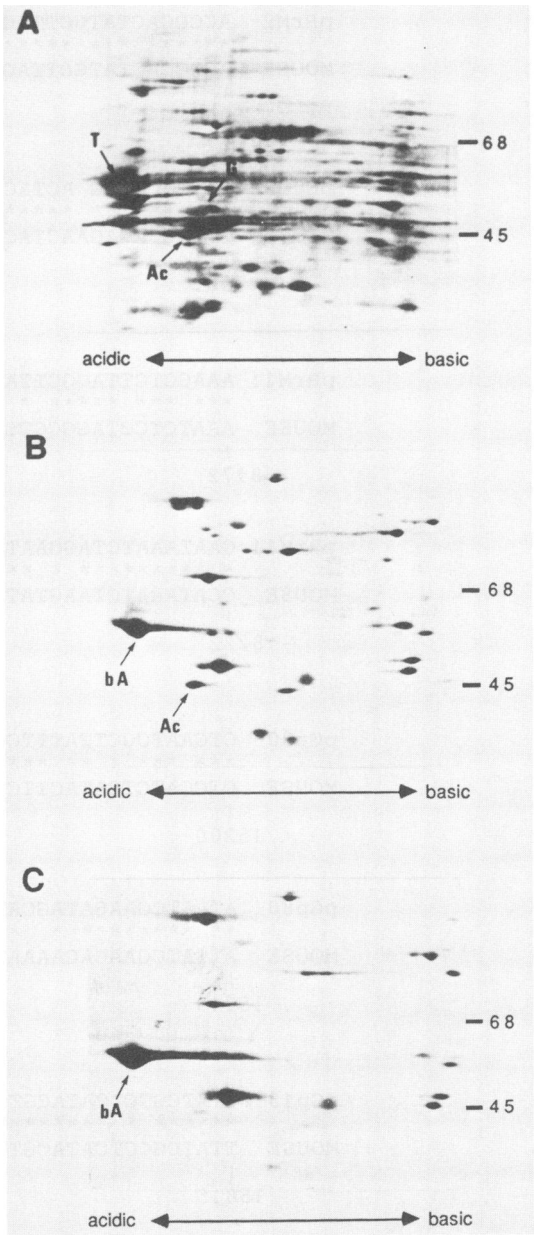

FIG. 5. Two-dimensional gel electrophoresis of proteins extracted from (A) crude homogenate, (B) mitochondria purified from single discontinuous gradient, and (C) mitoplasts prepared from purified mitochondria and rebanded on a second discontinuous sucrose gradient. The positions of actin (Ac), tubulin (T), mitochondrial F1 beta ATPase (bA), and GFAP (G) are indicated by arrows. Positions of molecular weight standards ($M_r \times 10^3$) are given at the right.

DISCUSSION

The objective of these investigations was to attempt to identify and characterize a scrapie-specific nucleic acid. The differential screening of two cDNA libraries consistently identified mitochondrial sequences as being a major nucleic

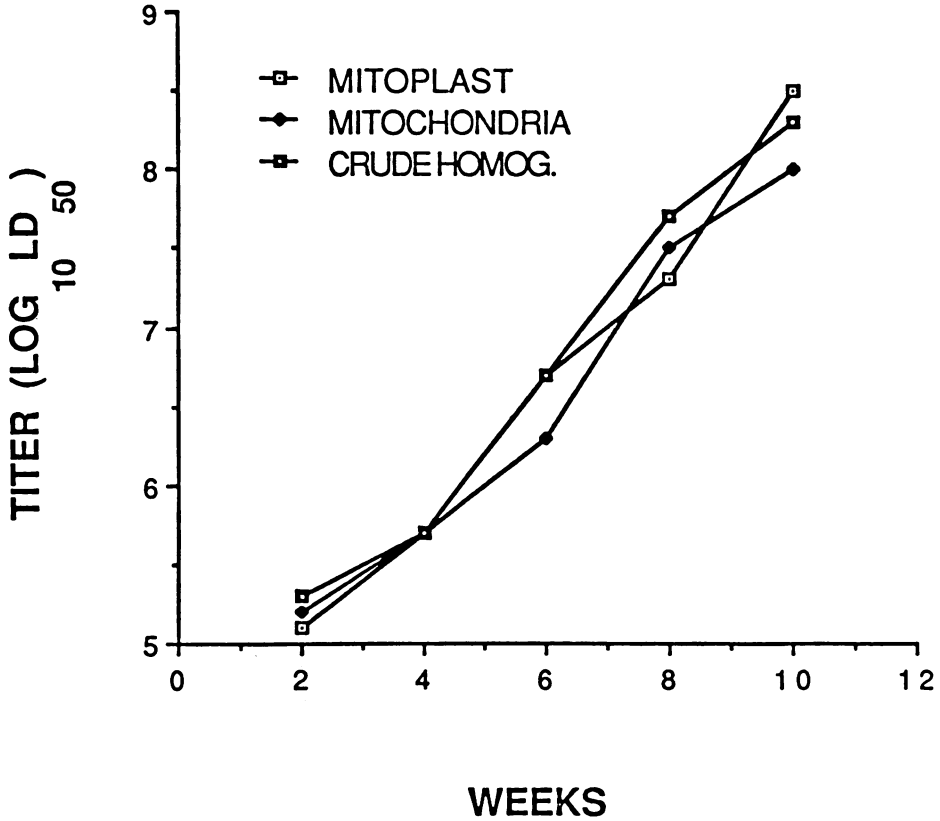

FIG. 6. Infectivity of 5% suspensions of crude brain homogenates, purified mitochondria, and mitoplasts at 2-week intervals during the preclinical and clinical (onset at 8 weeks) infection. The titers represent the mean values of three trials and are expressed as $\log_{10}$ LD_{50} per milliliter as measured by incubation interval assay (standard error = 0.5 $\log_{10}$ LD_{50}).

acid difference between scrapie-infected and uninfected cytoskeletal preparations.

Our laboratory has previously reported a unqiue 4.3S RNA associated with scrapie-infected hamster BrM vesicles (12), and we hoped to extend this finding by amplifying this nucleic acid in cDNA libraries constructed from purified preparations. Scrapie-specific recombinants have not yet been detected, nor have any scrapie-preferential clones smaller than 500 nucleotides in length been identified. As

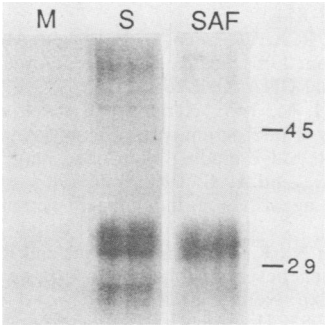

FIG. 7. Immunoblots of mitoplast (lane M), synaptosomal (lane S), and SAF (lane SAF) proteins. Samples of 60 μg of mitoplast and synaptosomal proteins and 25 μg of SAF proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose, and reacted with PrP 27-30 monoclonal antibody. Positions of molecular weight standards ($M_r \times 10^3$) are given at the right.

care was taken during the construction of these libraries for the inclusion of small cDNAs (less than 100 nucleotides), either an insufficient number of clones have been screened or the differential hybridization technique has failed to identify the 4.3S nucleic acid. It is possible that this RNA may have extensive homology to host nucleic acids or that its low abundance resulted in an undetectable signal when first-strand cDNAs were used as a probe.

Our cytoskeletal preparations from scrapie-infected hamsters were found to be greatly enriched for mitochondrial nucleic acids. These findings may reflect cellular or subcellular changes or both in brain tissue resulting from scrapie infection. Neuronal degeneration, microvacuolation of the neuropil, and proliferation of reactive astrocytes are prominant pathologic features which have been well documented (19, 32). Such cellular changes could result in the differences we observed in the increased association of mitochondrial nucleic acids with detergent-insoluble membrane fractions.

We found that purified mitochondria contain a high level of scrapie infectivity which was not reduced by removal of the mitochondrial outer membrane by osmotic swelling or by treatment with digitonin. These results suggest that infectivity is associated with the inner membrane or matrix or copurifies with mitochondria and mitoplasts in a manner that makes them inseparable by techniques of rate gradient centrifugation.

In recent years, scrapie research has centered on two very similar infectious preparations, SAF and prions. Both preparations are characterized by rodlike or fibrillike structures consisting primarily of a single protein, PrP 27-30. One group

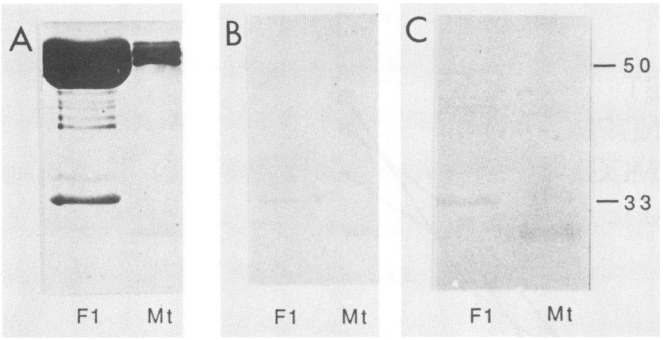

FIG. 8. Western blot analysis of (A) bovine heart F1 ATPase (lane F1) and mitoplast proteins (lane Mt) reacted with bovine heart F1 ATPase antibody; (B) bovine heart F1 ATPase (lane F1) and mitoplast proteins (lane Mt) reacted with SAF polyclonal antibody; and (C) hamster brain F1 ATPase (lane F1) and mitoplast proteins (lane Mt) reacted with PrP monoclonal antibody. Positions of molecular weight standards ($M_r \times 10^3$) are given at the right.

has maintained that PrP 27-30, the PrP, is the infectious agent (33, 40, 42, 43). Others have presented evidence that the PrP is not essential for infectivity and have concluded that some other factor present in the preparations is responsible for infectivity (31, 45, 50). Our data clearly support the latter interpretation.

Three lines of evidence indicate that the faint 27- and 33-kDa bands identified in the mitoplast samples by Western blotting with PrP antibody (Fig. 7) are not the result of a reaction with the PrP. First, the reaction in the mitoplast samples with the prion antibody produces distinct bands, quite different from PrP proteins, which form a smear. Second, the mitoplast samples were not protease treated. In the absence of protease, PrP has a molecular size of 33 to 35 kDa (1). A predominant band of 27 kDa would not be expected. Third, the 27- and 33-kDa bands cross-react with bovine heart mitochondrial F1 ATPase antibody. We initially included this antibody as a control to monitor our mitochondrial preparations. Mitochondrial proteins and purified bovine heart F1 ATPase samples were loaded in duplicate on a SDS-12% polyacrylamide gel and analyzed by the Western blot procedure. One filter was reacted with the bovine F1 ATPase antibody, while the second filter was reacted with the PrP polyclonal antibody. As expected, the ATPase antibody produced a strong reaction with alpha (53 kDa) and beta (50 kDa) F1 ATPase in both the bovine heart F1 and hamster brain mitoplast lanes (Fig. 8A). Gamma ATPase (33 kDa) was easily identifiable in the bovine F1 lane. In the hamster mitoplast lane, two bands (33 and 27 kDa) were produced. As indicated above, the prion antibody reacted with our mitoplast samples to produce bands of 27 and 33 kDa (Fig. 8B). Surprisingly, we found the prion monoclonal antibody to react with the bovine heart F1 ATPase sample (Fig. 8C), producing a band identical in size (33 kDa) to bovine gamma ATPase as well as the 27-kDa band. The patterns produced by the two antibodies in the 29- to 35-kDa regions of the blots were virtually identical. Thus, the signal we observed between the prion antibodies and our mitochondrial proteins is most likely due to cross-reactivity of the antibody to components of the mitochondrial F1 ATPase complex. The 33-kDa mitoplast band is very likely mitochondrial F1 gamma ATPase. The identity of the 27-kDa protein remains to be determined. For the reasons stated above, it does not appear to be PrP. Other investigators have also observed cross-reactivity between prion antibodies and a 33-kDa protein. Turk et al. (52) recently identified a 33-kDa triplet which was determined not to be the PrP and which reacted with a number of prion antibodies.

There have been several reports of mitochondrial involvement in degenerative diseases. Human mitochondrial myopathies, though rare, have been described (6, 10, 11), and mitochondrial DNA deletions have recently been associated with them (24, 56). Interestingly, a form of parkinsonism associated with drug abuse has been demonstrated to result from the disruption of a mitochondrial enzyme, NADH dehydrogenase (49). Two studies have associated mitochondrial abnormalities with Alzheimer's disease. Activity of the mitochondrial enzyme pyruvate dehydrogenase was found to be significantly diminished (47), and in a separate study, there were reduced respiration levels in purified mitochondria from Alzheimer's disease brains (48).

The association of scrapie infectivity with mitochondria is compatible with the well-documented resistance of scrapie infectivity to various physical and chemical treatments which inactivate most viruses and also with association of scrapie infectivity with BrM fractions. While the latter may not be obvious, submitochondrial particles containing vesiculated mitochondrial inner membrane are widely dispersed in subcellular brain fractions by homogenization or detergent extraction.

We believe that the most important findings in these studies are that mitoplasts have high scrapie infectivity and appear to contain very little PrP. We are continuing with studies to characterize the nature of mitochondrial infectivity and concentrating our search for a scrapie-specific nucleic acid on this organelle. We are also using the cloned mitochondrial probes developed in these stuides to attempt to detect nucleic acids in samples purified for PrP.

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