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Presence of Mitochondrial D-Loop DNA in Scrapie-Infected Brain Preparations Enriched for the Prion Protein

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The prion preparation has, in recent years, been the focal point of scrapie research. The inability to identify agent-specific nucleic acids in this sample has led to the formulation of the infectious protein or prion hypothesis. In this study, we analyzed three different prion protein-enriched preparations and found all to contain significant amounts of mitochondrial nucleic acid. Southern blot analyses indicated that they are enriched for a specific component of the mitochondrial genome, the single-stranded displacement loop fragment. Our results suggest that if mitochondrial nucleic acids are involved in scrapie infection, it is the displacement loop fragment that is specifically responsible.

Scrapie is an infectious neurological disorder of sheep and goats. It belongs to a family of related diseases known as subacute spongiform encephalopathies, a group which includes three human disorders, Creutzfeldt-Jakob disease, kuru, and Gerstmann-Strassler syndrome. Etiologic agents responsible for these diseases have not been identified.

In recent years, scrapie research has focused on a single, highly infectious brain fraction. The predominance of a single glycoprotein and the failure to identify significant amounts of nucleic acid in the preparation have led some investigators to propose that the glycoprotein itself is the infectious agent and have referred to it as the prion protein (proteinaceous infectious protein or PrP) (19). Other investigators have, however, concluded that the PrP is not the infectious agent and that some other as yet unidentified component is responsible for the infection (13, 20–22). PrP has also been found to be the major macromolecule associated with scrapie-associated fibrils (SAF) (14). Prepared in a manner similar to that for the prion preparation, SAF also contain high infectivity. PrP has a native molecular mass of 33 to 35 kilodaltons, is host encoded, and is expressed at similar levels in infected and uninfected tissues (6, 18). In addition to being expressed in the brain, PrP is also synthesized in heart, lung, and spleen tissues (18, 20). Scrapie-specific posttranslational modifications of PrP, although proposed (2, 23, 24), have yet to be demonstrated.

We have recently shown that mitochondria and mitoplasts purified from scrapie-infected hamster brains possess high infectivity (1). If mitochondria are directly involved in scrapie infection, one would expect to find mitochondrial nucleic acid in PrP-enriched fractions.

MATERIALS AND METHODS

Prion-enriched preparations. The Zn^{2+}-hydrolyzed, DNase I-, micrococcal nuclease-treated prion preparation was a gift of S. Prusiner (University of California, San Francisco). A prion preparation was also produced by the method of Hilmert and Diringer (11). SAF were prepared by the method of Merz et al. (15).

Nucleic acid extraction. The PrP-enriched fractions were treated with digitonin (Sigma Chemical Co.) at a concentration of 1.2 mg of digitonin per mg of protein and incubated on ice for 15 min. The samples were then digested with proteinase K (0.5 μg of proteinase K per μl of sample) for 20 min at 37°C, adjusted to 0.4% sodium dodecyl sulfate (SDS), and incubated for 10 min at 37°C. They were then phenol-extracted prior to ethanol precipitation.

Slot blot analysis. Nucleic acid samples were hydroxide treated by incubating the samples at 68°C for 1 h at a final NaOH concentration of 0.3 M. The samples were then adjusted to a final concentration of 1 M ammonium acetate prior to application to the nitrocellulose membrane. The samples were bound to nitrocellulose filters by using a slot blot manifold (Bethesda Research Laboratories, Inc.) 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. (12). Final wash conditions were 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 68°C.

Southern blot analysis. Nucleic acid samples were size fractionated on 1.2% agarose gels and transferred to nitrocellulose by the Southern blot technique (12). DNA probes were prepared by agarose gel isolation of plasmid DNA inserts followed by radiolabeling with [^{32}P]dCTP, using the random priming procedure described by the manufacturer (Bethesda Research Laboratories). Final wash conditions were 1× SSC–0.1% SDS at 68°C. RNA probes were prepared by using T7 and SP6 polymerases (Promega) according to the instructions of the manufacturer. Filters were hybridized as described above. Final wash conditions were 0.1× SSPE (0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA [pH 7.7])–1% SDS at 68°C.

RESULTS

We examined three PrP-enriched preparations for the presence of mitochondrial nucleic acids: (i) a prion preparation produced by the method of Hilmert and Diringer (11), (ii) a prion preparation that was Zn^{2+} hydrolyzed and DNase I and micrococcal nuclease treated (generously provided by S. Prusiner), and (iii) SAF prepared by the method of Merz et al. (15).

One hundred microliters of the nuclease-treated, Zn^{2+}-hydrolyzed prion preparation, having a titer of 10^9 50% lethal doses (LD_{50}) per ml, was treated with digitonin, digested with proteinase K, and phenol extracted. Half of the aqueous phase was ethanol precipitated, while 5 μg of tRNA was added to the other half of the sample prior to ethanol precipitation to ensure quantitative recovery of any nucleic

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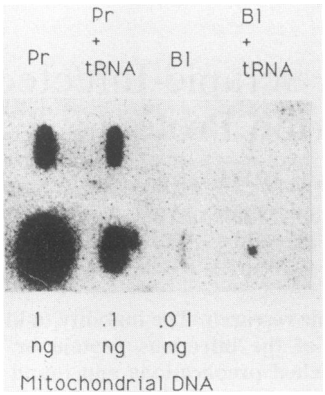

FIG. 1. Slot blot analysis of nucleic acids isolated from the nuclease/Zn^{2+}-treated prion preparation. Nucleic acids purified from the Zn^{2+}-hydrolyzed nuclease-treated samples were ethanol precipitated in the presence (Pr + tRNA) and absence (Pr) of tRNA carrier and applied to nitrocellulose by using a slot blot manifold. Bl and Bl + tRNA refer to the controls (water only, no prion sample) extracted at the same time in an identical manner to that used for the prion samples. Mitochondrial DNA dilutions (1, 0.1, and 0.01 ng) were also included as controls.

acids in the preparation. A control containing 100 μl of water was treated in an identical manner to detect any nucleic acid contamination that might have occurred during the nucleic acid purification.

Both prion samples (with or without carrier tRNA) and controls were suspended in 50 μl of H_2O. Then, 4 μl of each sample was applied to a nitrocellulose filter using a slot blot manifold. In addition, dilute samples of DNA isolated from sucrose gradient purified mitochondria were included to facilitate quantitation. The probe used in the hybridization was pGp135 (a plasmid construct containing hamster mitochondrial displacement loop [D-loop] sequence; 1). Both prion samples reacted with the mitochondrial DNA probe, whereas neither of the controls produced a detectable signal (Fig. 1). Comparison of the signals produced by the PrP samples with those of the mitochondrial DNA controls indicated that approximately 75 pg of mitochondrial nucleic acid hybridizing material was present in the 4-μl sample. Thus, from 100 μl of prion preparation (starting sample), we were able to detect approximately 1.8 ng of mitochondrial nucleic acid-hybridizing material.

Two other PrP-enriched samples were also analyzed. A prion preparation was prepared by the method of Hilmert and Diringer (11) and contained a titer of 10^9 LD_{50} per ml. In addition, SAF containing a titer of 10^8 LD_{50} per ml were also examined. Five hundred microliters of each sample was treated with digitonin and proteinase K and phenol extracted as described above. Because of the larger sample size, carrier tRNA was omitted. Similar to the nuclease/Zn^{2+}-treated prion preparation, slot blot analysis demonstrated the presence of mitochondrial nucleic acids in both preparations (data not presented). Southern blot analysis using a hamster D-loop probe (pGp135) showed that the size of the hybridizing material was localized in a single band of approximately 450 nucleotides (nt) (Fig. 2). Significantly, unlike the mitochondrial DNA control, neither the SAF or prion preparation produced a signal in the 16-kilobase region, indicating the absence of the mitochondrial genome in the two fractions.

The 450-nt band was present in the PrP and SAF samples and appeared to be identical in size to the single-stranded

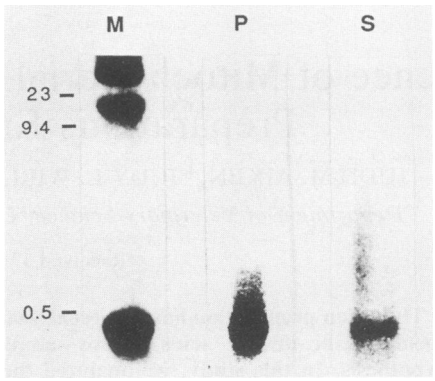

FIG. 2. Southern blot analysis of nucleic acid isolated from a prion preparation (P), SAF (S), and hamster brain mitochondria (M). pGp135, a hamster D-loop clone, was used as a probe. Molecular size markers (in kilobase pairs) are given at the left.

DNA component of the mitochondrial genome, the D-loop fragment (Fig. 2). We obtained confirmation that the hybridizing band in the PrP-enriched samples was indeed the D-loop fragment through the use of strand-specific RNA probes. Radiolabeled RNA probes were synthesized from SP6 and T7 RNA polymerase promoters from a mitochondrial D-loop clone (pGp135). These strand-specific probes were hybridized to hamster mitochondrial DNA as well as to nucleic acids isolated from the SAF sample. From our sequence data of pGp135 (1; J. L. Williamson, unpublished data), the T7 polymerase would be expected to synthesize an RNA complementary to the D-loop fragment. As expected, only the probe synthesized from the T7 promoter hybridized to the 450-nt band (Fig. 3), indicating that this fragment was single stranded.

Mammalian D-loop fragments range in size from 450 to 700 nt (3). In the mouse, the predominant D-loop fragments are 555 to 625 nt in length (10). On our nondenaturing agarose gels, the D-loop fragment migrates at approximately 450 nt. We have observed variability in its size under certain electrophoretic conditions (J. M. Aiken and J. L. Williamson, unpublished observations), most likely because of the fragment being single stranded. The PrP and SAF D-loop-

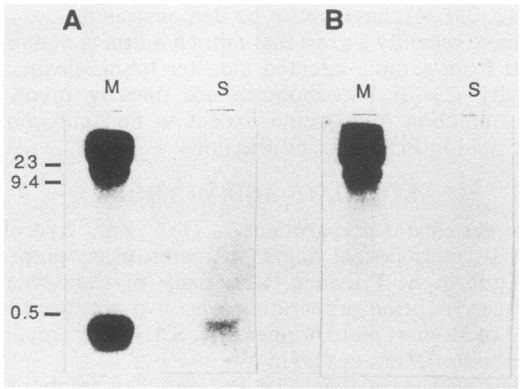

FIG. 3. Southern blot analysis of nucleic acid isolated from hamster brain mitochondria (M) and SAF (S). Single-stranded RNA probes (pGp135) complementary to the hamster mitochondrial D-loop fragment (A) and in the same sense as the D-loop (B) were used as probes. Molecular size markers (in kilobase pairs) are given at the left.

hybridizing bands always comigrate with the hamster mitochondrial D-loop fragment.

DISCUSSION

The resistance of the scrapie agent to treatments that degrade nucleic acids has suggested to some investigators that a nucleic acid-free pathogen is the causative agent (19). In this study, we demonstrated the presence of a specific component of the mitochondrial genome, the D-loop fragment, in highly infectious prion preparations. Mitochondrial DNA is a common contaminant of almost every subcellular fraction, thus the presence of mitochondrial sequences in the prion preparations may simply be a consequence of its abundance. On the other hand, the D-loop fragment has many qualities that make it an attractive candidate for the nucleic acid component of the scrapie agent.

The 16-kb mammalian mitochondrial genome contains 2 rRNA genes, 22 tRNA genes, and 13 protein-coding sequences. Also present is an unusual triple-stranded region known as the D-loop. Essential to the proper functioning of the mitochondrion, the D-loop region contains the origin for heavy-strand DNA replication (7) as well as the two promoters from which the mitochondrial polycistronic mRNAs are transcribed (8). A small single-stranded DNA strand (the D-loop fragment) maintains this novel triple-stranded structure by displacing the parental heavy-strand mitochondrial DNA.

We have recently demonstrated that mitochondria purified from scrapie-infected hamster brains contain high infectivity (1). Removal of the mitochondrial outer membrane to produce mitoplasts resulted in no loss of infectivity. In this study, we analyzed highly infectious prion preparations for the presence of mitochondrial nucleic acids. We found these prion preparations to contain the small single-stranded D-loop fragment. Quantitation of D-loop-hybridizing material in the Zn^{2+}-treated prion preparation indicated that 10^{10} D-loop fragments are present in 1 ml of the 10^9 LD_{50} per ml preparation.

Because of the detergent extraction and centrifugation conditions used to produce the PrP-enriched preparations, intact mitochondria would not be expected to be present in the fraction. The presence of D-loop sequences as the primary mitochondrial genome component identified in the preparation would support this interpretation. Our data suggests that an altered D-loop sequence may be the transmissible agent. Presumably, such D-loop fragments could disrupt the normal functioning of the mitochondrion and are self-replicated when the mitochondrion genome replicates. In the prion preparation, we are finding D-loop fragments in a protected (nuclease-resistant) form. Such small particles could be the product of cell death or brain homogenization. Thus the agent may exist in at least two forms, (i) as part of an intact mitochondrion or (ii) as a subviral particle in which D-loop fragments are complexed with various brain proteins. Such properties are consistent with the extreme size diversity associated with the agent (from 40S to 400S) and with radiation target size data indicating a very small nucleic acid component in the range of the D-loop fragment.

Genetic linkage studies (4, 5), as well as prion gene variability (25), suggest that the PrP gene is identical to the scrapie incubation (Sinc) gene characterized more than 20 years ago (9). The nuclear-encoded Sinc gene product (PrP) could have a considerable influence on mitochondria since proper mitochondrial functioning results from the interaction of both the nuclear and mitochondrial genomes. Mitochondrial replication and respiratory functions are regulated by nuclear-encoded proteins, while components of the ATP-synthase complex are encoded by the mitochondrial genome. Recently, a human mitochondrial myopathy was found to be the result of a nuclear-encoded gene (26). Correlated with the inheritance of this gene were multiple deletions of mitochondrial DNA in the D-loop region. The function of PrP has yet to be determined; however, a direct or indirect involvement of the PrP gene product in scrapie infection would not be incompatible with a mitochondrial basis for the disease.

Our ability to identify mitochondrial nucleic acids in PrP-enriched fractions was enhanced by the use of specific nucleic acid probes. Other studies have indicated associations of nucleic acids with scrapie infectious preparations. Sklaviadis et al. (22) fractionated Creutzfeldt-Jakob disease agent and found nucleic acid-protein complexes to migrate with infectivity. DNA has been identified in abnormal tubulofilaments containing inner cores of SAF (16). Hamster DNA repetitive sequences have been recovered from PrP-enriched preparations (17). Thus, it is possible that other specific probes may identify additional nucleic acids in the preparation. The presence of mitochondrial D-loop fragments in highly infectious samples could, therefore, be a fortuitous finding or may argue that it plays an essential role in scrapie infection. We are concentrating our efforts in a search for abnormal D-loop sequences unique to scrapie-infected hamster brains.

These studies have demonstrated that PrP-enriched preparations contain mitochondrial nucleic acids and strengthen the hypothesis that nucleic acids are directly involved in scrapie infection. Thus, the notion of a nucleic acid-free pathogen remains controversial.

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