Conservation of the cellular gene encoding the scrapie prion protein

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ABSTRACT

The major protein, PrP 27-30, in purified preparations of hamster scrapie prions is encoded within the genome of the experimental host. DNA sequences related to a PrP cDNA clone can be detected in a wide variety of organisms under relatively stringent conditions where the only signal generated by hamster or mouse DNA corresponds to the *PrP* gene. Three hosts for scrapie, goat, sheep and rat gave strong hybridization signals. In addition, three invertebrate DNAs reacted with the PrP probe, in the order nematode ~ *Drosophila* >> yeast. Thus, the sequences detected in goat, sheep, rat, nematode, *Drosophila* and possibly yeast DNA may arise from authentic *PrP* genes. This evolutionary conservation is consistent with the notion that PrP proteins participate in essential cellular processes.

INTRODUCTION

Scrapie is a transmissible, degenerative neurological disease. Extensive purification of the scrapie agent or prion passaged in hamsters has revealed that a protease-resistant protein, designated PrP 27-30, purifies with, and is required for infectivity (1). The sequence of 15 N-terminal residues of PrP 27-30 was determined (2), allowing the identification of the cognate cDNA clone from a library of recombinants generated from the RNA of infected animals (3).

Use of this cDNA clone revealed that PrP 27-30 is encoded by a single cellular gene in the hamster. The mRNA is expressed in normal and scrapie-infected brain at similar levels and at lower levels in the other organs of healthy animals. An antigenically related, but protease-sensitive protein, designated PrP 33-35^C, was detected in uninfected cells. In addition, PrP-related sequences were detected in mouse and human DNA (3). We concluded that PrP 33-35^C proteins may feature in the metabolism of the normal cells, and a fraction of these molecules are converted by an unknown mechanism to the protease-resistant scrapie form, PrP 33-35^{Sc}, during the course of infection.

In this paper we have extended our phylogenetic survey to examine *PrP*

gene sequences in experimentally attractive species, e.g., species which exhibit strain variation in scrapie susceptibility and species which lend themselves to the techniques of surrogate genetics.

MATERIALS AND METHODS

PrP Probe Fragments

The cDNA insert was excised from pHaPrP (3) by cleavage with Bam HI followed by preparative agarose gel electrophoresis. The recovered fragment was digested with Taq I, which cleaves the cDNA at nucleotide 909. The digest was phenol-extracted and precipitated and ligated to the plasmid pUC8 (4) which had been digested with Bam HI and Acc I and dephosphorylated. The ligation mix was transformed into *E. coli* TB1 [an $r_k^- m_k^+$ derivative of JM83 (4)] and plated onto ampicillin plates in the presence of 5-bromo-4-chloro-3-indolyl-B-D-galactoside ("x-gal"). White colonies, which correspond to subcloning of the 5' or 3' half of the cDNA, were grown in liquid culture and used to prepare plasmid DNA. An isolate which contained a Sau I site (diagnostic for a clone containing the 5' Bam HI-Taq I cDNA fragment) was grown in mass culture to prepare plasmid DNA. Restriction mapping with a variety of endonuclease confirmed the identity of this subclone. The probe fragment was liberated from this subclone by digestion with Sau I and Pst I (which cleaves in the polylinker immediately 3' to the hybrid Taq I/Acc I site).

A 900 base-pair Eco RI fragment derived from a mouse PrP cDNA subclone [kindly provided by Bruce Chesebro (5)] was prepared by agarose gel electrophoresis. Coordinates of these probe fragments are detailed in Figure 1. At present, only a partial sequence has been published for the mouse PrP cDNA fragment. Within the area available for comparison with the hamster sequence, there is 84% homology at the nucleotide level.

Southern Transfer Analysis

Procedures for preparation of DNA, restriction and endonuclease digestion, gel electrophoresis and transfer, and probe preparation are as described previously (3). All gel loadings represent 10 μ g of DNA. However, amendments to the hybridization procedure are detailed below. The hybridization solution contained 30% formamide, 0.05 M HEPES, pH 7.4, 0.3% sodium dodecyl sulfate (SDS), 0.5 mg/ml heparin [Sigma, sodium salt grade II (6)] and 3 x SSC. One x SSC is 0.15 M NaCl, 0.015 sodium citrate. Filters were hybridized in plastic pouches at 42°C with approximately 70 μ l of liquid per cm² of the filter, which is shaken at 110 rpm. Posthybridization washes

were also performed with constant shaking. Filters were autoradiographed at -70°C using Kodak XAR-5 film and Dupont "Cronex" intensifying screens. Exposure times are detailed in the Figure Legends.

RESULTS

Hybridization Specificity of Hamster and Mouse PrP cDNA Probes

Previous Southern transfer (7) analyses using the hamster PrP cDNA clone indicated that related sequences occurred in mouse and human DNA (3). However, we revised two aspects of our experimental protocol prior to extending our phylogenetic survey. Firstly, we used a more specific hybridization probe. It is a subfragment of the hamster PrP cDNA clone extending from nucleotides 120 to 909. Use of this probe fragment eliminates the possibility of spurious hybridization signals arising through homology with the dG:dC tract or the 3' untranslated region. The fragment encompasses 84% of the open reading frame of pHaPrP and includes 179 nucleotides beyond the putative translation termination codon (Figure 1). Secondly, we deduced empirically the least stringent posthybridization wash conditions such that the only signal arising from hamster DNA derives from the PrP gene itself.

These experiments are presented in Figure 2. Using wash conditions of $0.40 \times \text{SSC}$ (Figure 2, lane 1) or $0.20 \times \text{SSC}$ (Figure 2, lane 2) at 55°C , we can detect multiple PrP-related restriction fragments in hamster DNA. However, when the stringency is increased to $0.15 \times \text{SSC}$ (at the same temperature) only a single 3.1 kb Eco RI fragment is detected (Figure 2, lane 3). Molecular cloning and sequencing has demonstrated that this fragment encompasses the entire pHaPrP cDNA clone (3) (C. Basler, B. Oesch, M. Scott, D. Westaway, M. McKinley, S. Prusiner and C. Weissmann, personal communication).

Figure 1. Coordinates of PrP cDNA hybridization probes. The PrP cDNA clone, pHaPrP (3), is shown. The open reading frame (ORF) is indicated as an open box and the 3' untranslated region as a solid line. The hamster probe fragment is shown underneath. The approximate coordinates of a mouse PrP cDNA probe fragment (5) are also shown. This fragment was only partially sequenced and its exact relationship to the total mouse PrP cDNA sequence remains to be established.

Figure 2. Southern transfer analysis of Eco RI digested hamster and mouse DNA with PrP probes. Lanes 1, 2, 3 and 5, hamster DNA; lanes 4 and 6, mouse (C3H) DNA. Lanes 1-4 were analyzed with hamster PrP probe, lanes 5 and 6 with mouse PrP probe. Filters were washed after hybridization in 0.4 x SSC, 0.1% SDS (lane 1), 0.2 x SSC, 0.1% SDS (lane 2), and 0.15 x SSC, 0.1% SDS (lanes 3-6). All washes were performed at 55°C. Exposure time for all samples was 30 hr. Sizes are indicated in kilobases.

Analysis of mouse DNA under the latter conditions reveals a 2.3 kb Eco RI fragment, in accord with our previous results (3). The availability of a murine PrP cDNA clone (5) (Figure 1) allowed us to confirm that this 2.3 kb fragment corresponded to the mouse PrP gene. As anticipated, the mouse PrP probe detected the same fragments as the hamster PrP probe in mouse and hamster DNA (Figure 2, lanes 5 and 6, respectively).

PrP-Related Sequences in Mammalian DNA

We have previously proposed that PrP 27-30 is the major component of the scrapie prion (1). It follows that all species susceptible to scrapie infection must contain PrP gene sequences. We, therefore, performed Southern transfer analyses on DNA from sheep and goats, the natural hosts for scrapie (8, 9). We also analyzed DNA from the rat, as this is another experimental

Figure 3. Detection of PrP-related DNA sequences in mammals susceptible to scrapie. Lane 1, DNA from the rat-2 cell-line; lane 2, Nubian goat DNA; lane 3, mouse (I/LnJ) DNA; lane 4, mouse (NZW) DNA; lane 5, Suffolk sheep DNA. All samples were digested with Eco RI and were hybridized with the hamster cDNA probe. Exposure time was 4 days. Positions of size-markers are indicated in kilobases.

host (10). All of these species contain PrP-related sequences (Figure 3, lanes 1, 2 and 5).

In addition, we analyzed DNA from two strains of mice that exhibit short (NZW) or long (I/LnJ) incubation periods when injected with the same isolate of scrapie prions (11). Both NZW (Figure 3, lane 3) and I/LnJ (Figure 3, lane 4) DNAs contain the same Eco RI fragment. The variation in band intensity reflects slightly different DNA loadings, and is not significant. Parallel analyses with Bam HI (data not shown) reveal similar patterns of hybridization. Therefore, strain differences in scrapie susceptibility do not appear to arise from gross structural arrangements of PrP sequences.

PrP Gene Sequences in Lower Organisms

We have sought PrP-related genes in organisms that particularly lend themselves to biochemical or genetic analysis. Southern transfer analyses were performed on *Xenopus laevis*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* DNA, using the hamster PrP cDNA probe

Figure 4. Detection of PrP-related DNA sequences in lower organisms. Lane 1, *Xenopus laevis* DNA; lanes 2 and 4, *Drosophila melanogaster* DNA (Schneider cell-line); lanes 3 and 5, *Caenorhabditis elegans* DNA; lanes 6 and 8, mouse DNA; lanes 7 and 9, yeast DNA. All DNA were digested with Eco RI. All filters were washed at 55°C in 0.15 x SSC, 0.1% SDS except Lanes 4 and 5 which were washed in 0.20 x SSC, 0.1% SDS at 55°C. Lanes 1-7 were analyzed with hamster PrP cDNA, lanes 8 and 9 with mouse PrP cDNA. Exposure times: lanes 1-5, 28 hr; lanes 6-9, 7 days. Sizes are indicated in kilobases. The number of genome equivalents loaded are, relative to mouse (= 1.0), *Xenopus laevis* = 0.11, *Caenorhabditis elegans* = 29, *Drosophila melanogaster* = 13.6 and *Saccharomyces cerevisiae* = 230 (18, 19).

and posthybridization wash conditions described above. *Xenopus laevis* DNA (Figure 4, lane 1) does not yield a hybridization signal. This observation is unaltered by 10-fold increased exposure times and less stringent wash conditions (not shown). *Drosophila* and nematode DNA contain PrP-related sequences (Figure 4, lanes 2 and 3) of 5.6 and 2.5 kb, respectively. It is noteworthy that under slightly less stringent wash conditions (0.2 x SSC, 0.1% SDS, 55°C), both of these DNAs exhibit additional PrP-related restriction fragments (Figure 4, lanes 4 and 5). These results are compatible with the possibility that families of PrP-related sequences are present in these organisms.

Yeast DNA exhibits 3.1 and 2.1 kb restriction fragments related to the hamster PrP cDNA (Figure 4, lane 7). However, a 6-fold longer exposure time was needed to obtain a signal comparable to that seen in Figure 4, panel A. The position of another hybridizing fragment, visible in longer exposures, is indicated by an arrow (Figure 4, lane 7). Because of the extreme phylogenetic distance between hamster and yeast, we also performed an analysis with the mouse PrP cDNA probe (Figure 1) (5). The rationale for using a second probe is that diverged species which harbor a related protein will exhibit most DNA sequence homology in the first and second nucleotide positions of codons within the gene. Adventitious sequence homology is not expected to follow this pattern. Hence, if probes derived from two diverged gene sequences give a congruent pattern of hybridization in a third species, then this is consistent with the presence of an authentic gene sequence. In fact, restriction fragments of 3.1 and 2.1 kb are also detected with the mouse probe (Figure 4, lane 9). Samples of mouse DNA processed in parallel and autoradiographed for the same length of time are included for comparative purposes (Figure 4, lanes 6 and 8).

DISCUSSION

We have searched for PrP-related sequences in experimentally tractable organisms using filter hybridization at reduced stringency. We have attempted to exclude spurious hybridization signals in two ways. First, hybridization probes used for these analyses were predominantly derived from the protein coding portions of PrP cDNA clones (Figure 1). Second, mouse and hamster genomic DNA was used as an internal control for the fidelity of hybridization. With the exception of the goat, two independent samples of DNA from each organism were analyzed (not shown). In each case, the independent DNAs yielded the same pattern of hybridization.

Under conditions which exclude hybridization with sequences other than the authentic hamster and mouse PrP genes, we can detect PrP nucleotide sequences in rat, goat, and sheep DNA. It is therefore likely that all mammals susceptible to scrapie contain PrP genes. These results are compatible with the preëminent role assigned to PrP 27-30 in scrapie pathogenesis (1).

Our results suggest that all mammals may have PrP-related sequences and raise the question of how many other prion diseases may exist. Besides the related sequences reported here, dogs, cats and apparently rabbits also have PrP-related sequences (D. Kingsbury, personal communication). These results force us to ask whether all or most mammals may develop prion diseases many

of which have not yet been recognized. In addition to scrapie in sheep and goats, prions appear to cause transmissible encephalopathy in mink as well as chronic wasting disease in mule deer and elk (12). In humans, prions have been implicated in the transmissible encephalopathies, Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome and kuru, based on radiation inactivation studies and the demonstration of protease-resistant proteins antigenically related to PrP (13-17). The recent isolation and sequencing of a human PrP cDNA clone (H. A. Kretzschmar, L. E. Stowring, D. Westaway, W. H. Stubblebine, S. B. Prusiner and S. J. DeArmond, in preparation) has revealed 90% amino acid homology to hamster PrP, in accord with the sequence conservation proposed in this paper.

Identical hybridization conditions reveal PrP-related sequences in several invertebrate DNAs. The signal intensity was in the order: nematode > *Drosophila* >> yeast (Figure 4). Because yeast DNA yielded a weak hybridization signal with hamster PrP probe, we also performed an analysis with a mouse PrP cDNA probe (5). The two probes which exhibit sequence variation at the third position of several amino acid codons [-16% divergence (3, 5)] produce partially congruent hybridization patterns. These data suggest that the sequences detected in yeast may encode PrP-related proteins. However, a variable in these analyses is the number of genome equivalents of DNA loaded for different organisms. The smaller a species' genome, the more equivalents are loaded when DNAs are normalized by weight. As the relative genome sizes of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* are in the ratio 80:180:1 (18, 19), a better representation of PrP signal intensity may be nematode ~ *Drosophila* >> yeast.

It is noteworthy that our conditions used to detect invertebrate PrP sequences are considerably more stringent than those used to retrieve the homologues of vertebrate proto-oncogenes from lower organisms. For example, homologues of the oncogene v-Ha-ras were detected in yeast (20) and *Drosophila* (21) using 2 x SSC 55°C posthybridization wash. A 2 x SSC, 55°C wash was also used in the isolation of the *Drosophila* homologue of v-erb-B (22). Finally, the *Drosophila* homologue of v-myb was detected using 4 x SSC, 50°C wash (23).

Screening a panel of 200,000 *Drosophila* genomic clones has yielded two apparently overlapping recombinant phages which hybridize under the exact conditions described above. Furthermore, one of these recombinants contains a 5.3 kb PrP-related Eco RI fragment, in good agreement with our figure of 5.6 kb (Figure 4, lane 2) (D. Westaway, unpublished observations). These

results are compatible with a single-copy PrP sequence in the *Drosophila* genome. If sequencing reveals this as an authentic gene, we will test for allelism with *Drosophila* behavioral mutants. Some of these mutants exhibit vacuolation of the brain (24-26), as is seen during the course of scrapie in the hamster (27).

Overall we conclude that authentic *PrP* genes are likely to occur in all organisms with a nervous system, and possibly in lower eukaryotes; our failure to detect PrP sequences in *Xenopus* may reflect the extremely large DNA content of the amphibian genome (18). Conservation of PrP DNA sequences is consistent with the notion that PrP proteins serve an essential biological function. Characterization and manipulation of *PrP* genes in organisms amenable to reversed genetics will allow a definitive testing of this hypothesis.

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