Comparison of RNA from Healthy and Scrapie-infected Hamster Brain

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SUMMARY

Density gradient fractions prepared from healthy or scrapie-infected hamster brain tissue enriched in plasma membrane vesicles were treated with nucleases prior to phenol extraction and ethanol precipitation. The recovered nucleic acids were 3' end-labelled and run on one-dimensional polyacrylamide gels. Autoradiography revealed the presence of low molecular weight RNAs (4S) in both healthy and scrapie samples. Two-dimensional fingerprint analysis indicated that the RNAs isolated from scrapie-infected hamsters contained oligonucleotides that were not present in RNAs isolated from healthy hamsters.

INTRODUCTION

The scrapie agent produces a degenerative disease of the central nervous system in sheep and is similar to the transmissible agents causing kuru and Creutzfeldt-Jakob disease in man. The literature is replete with hypotheses concerning the chemical nature of these unusual neuropathic agents. Their resistance to inactivating procedures has led to speculation that these pathogens might not contain nucleic acid (Alper et al., 1967; Prusiner, 1982), and attempts to demonstrate an agent-specific nucleic acid in infected tissue by 5' end-labelling (Prusiner et al., 1982) or nick translation (Manuelidis & Manuelidis, 1981; T. L. German & R. F. Marsh, unpublished results) have been unsuccessful.

To characterize the physicochemical nature of the scrapie agent, we have developed a purification procedure in which membrane vesicles are separated from a sonicated plasma membrane-enriched suspension by density gradient centrifugation (Marsh et al., 1984). Since scrapie infectivity is known to be resistant to nuclease attack (Millson et al., 1976; McKinley et al., 1981) and susceptible to deproteinization (Cho, 1980; Prusiner et al., 1981a), these studies were undertaken to compare the nucleic acid components of gradient fractions prepared from normal and scrapie-infected hamster brain which had been treated with nuclease prior to phenol extraction.

METHODS

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 (Kimberlin & Marsh, 1975). Infectivity was quantified by incubation interval assay (Prusiner et al., 1981b) after intracerebral inoculation into weanling male outbred hamsters purchased from Harlan Sprague Dawley (Indianapolis, Ind., U.S.A.).

Brain tissue. Five randomly chosen male hamsters showing endstage clinical signs of scrapie, or healthy age-matched 12-week-old controls were killed with chloroform and the brains removed aseptically. Brains were ground in a Ten Broeck glass grinder to a concentration of 10% (w/v) using homogenization buffer (HB) composed of 50 mM-Tris, 100 mM-NaCl, and 1 mM-dithiothreitol adjusted to pH 7-8 using glacial acetic acid. Ten% suspensions were centrifuged at 1500 g at 4 °C for 10 min and the pellets discarded. Supernatants were centrifuged at 3000 g at 4 °C for 30 min and the plasma membrane-enriched pellet (Semancik et al., 1976) resuspended to 10% (w/v) in HB containing 10 mM-EGTA and 2 mM-EDTA. These suspended pellets were vortexed for 1 min, then placed at 4 °C overnight after which they were re-vortexed, sonicated for 2 min with an Ultrasonics model W-10
Sonicator* and fractionated on a 9 ml 10 to 25% linear Nycodenz (Accurate Chemical Corp., Westbury, N.Y., U.S.A.) gradient centrifuged at 35000 r.p.m. at 4°C for 90 min in a Beckman SW41 rotor. Three ml of these sonicated preparations, representing material from approximately three brains per tube were applied to each gradient. After centrifugation, a fraction enriched for large membrane vesicles was collected from the middle of the tube (Marsh et al., 1984).

Preparation of nucleic acid. Gradient fractions of membrane vesicles were dialysed against 50 mM-Tris-acetate pH 7.8, 100 mM-NaCl, 1 mM-dithiothreitol overnight. Five ml fractions were then treated with RNase A (50 μg/ml) in 10 mM-MgCl₂ for 50 min at 37°C followed by proteinase K (50 μg/ml) for 30 min at 37°C and, finally, SDS (0.1%) for 30 min at 37°C. Fractions were extracted with phenol/chloroform/isoamyl alcohol (25:25:1), ethanol-precipitated twice, and labelled with T4 RNA ligase with [5'-32p]pCp (England et al., 1980).

Polyacrylamide gel electrophoresis. Labelled RNA was ethanol-precipitated and resuspended in 40 μl 50 mM-Tris-HCl pH 7.8, 150 mM-NaCl, 1 mM-EDTA. Samples were diluted 1:1 with 178 mM-Tris, 177 mM-boric acid, 5.5 mM-EDTA, 1% SDS, 6 M-urea, and 1% 2-mercaptoethanol, heated (60°C, 5 min) and quench-cooled. RNA was electrophoresed on a 2.5% polyacrylamide-0.5% agarose gel (Peacock & Dingman, 1967) and autoradiographed.

RNA fingerprinting analysis. Scrapie and healthy RNAs were extracted from hamster brain as described above. Following extraction, the RNAs were suspended in 10 mM-Tris-HCl pH 7.5 and precipitated three times with ethanol. The precipitate was dried and resuspended in 20 mM-Tris-HCl pH 7.5, 2 mM-EDTA. Two units RNase T₁ were added, the solutions were heated (100°C, 3 min) and quench-cooled. An additional 3 units of RNase T₁ were added and the samples were digested for 30 min at 37°C. The digested RNA was phenol-extracted, precipitated twice with ethanol, dried and resuspended in 20 mM-Tris-HCl pH 7.5. The RNA samples were then treated with alkaline phosphatase and labelled with [γ-32p]ATP in the presence of polynucleotide kinase (Pedersen & Hazeltine, 1980). Approximately 1 × 10⁶ c.p.m. of each RNA was loaded and electrophoresed in the first dimension on a polyacrylamide gel (11%) containing 6 M-urea and 25 mM-citric acid pH 3.4. The second dimension gel (22%, polyacrylamide, 50 mM-Tris, 50 mM-boric acid, 2 mM-EDTA, pH 8.3) was cast around a 23 cm strip cut from the first dimension gel which contained the xylene cyanol FF and bromophenol blue dye markers and approximately 90% of the 32p activity. The second dimension gels were frozen (-80°C) and autoradiographed.

Computer analysis of RNA fingerprints. The two-dimensional fingerprint array was analysed using programs written by one of us (B.C.M.) for the Univac 1180 mainframe. The programs compare the autoradiograph of one gel to that of another by positionally translating the oligonucleotides of the autoradiograph to be matched with those of the reference autoradiograph until a 'best fit' is established. Matched and orphan oligonucleotides are located and the data are presented on a terminal viewing screen or at a digital plotter. Oligonucleotides that migrated faster than the bromophenol blue dye marker (± the variation in replicate runs) were excluded from the analysis since they cannot be resolved by the fingerprinting method described above.

RESULTS

As shown in Fig. 1, bands of RNA with similar mobilities (approx. 4S) were observed on gels when gradient fractions of either healthy or scrapie-infected materials were used as starting material. The apparent higher yield of RNA from scrapie gradients is reproducible. Bands were not seen by autoradiography when RNA was labelled at the 5' ends using polynucleotide kinase and [γ-32p]ATP following the procedures of Maxam & Gilbert (1977). Failure to label at the 5' end may be due to a high degree of methylation, covalent attachment of a protein at the 5' end similar to the VpG protein of poliovirus, or insufficient amount of material. Bands were not observed if 3' end-labelled material was treated with RNase prior to electrophoresis, confirming that the 3' end-labelled material was RNA.

Two-dimensional RNA fingerprints of healthy and scrapie RNA were prepared as described in Methods and the results are shown in Fig. 2. The 4S RNAs harvested from scrapie-infected or healthy hamsters contained 15 to 25 oligonucleotides in the significant area of the T₁ map. Based on their migration rate in the second dimension gels, these oligonucleotides range between 10 and 15 nucleotides in length. This indicates that these RNAs have a low guanosine content but this interpretation must be tempered by the fact that the RNAs were small (4S) and would therefore be expected to contain fewer guanosine residues than RNA in the 20S to 50S range (Aaronson et al., 1982). Computer analyses of these RNA fingerprints show that 50% of the oligonucleotides present in the scrapie RNA were different from those appearing in healthy RNA.
We expected that the RNA extracted from brain tissue of scrapie-infected hamsters would contain all of the oligonucleotides present in the brain tissues of uninfected hamsters in addition to those oligonucleotides which were unique to the scrapie infection. However, the RNA extracted from uninfected hamster brains had seven unique oligonucleotides when its T₁ map was compared with that of the RNA harvested from scrapie-infected hamster brains (Fig. 2e). This appears to indicate that one result of scrapie infection in hamsters is the alteration of cellular syntheses for certain species of RNA. This observation confirms and extends the work of Bountiff & Hunter (1982) who reported a change in low molecular weight RNA in the brains of scrapie-infected mice and observed that some species were increased while others were decreased with respect to normal animals.

**DISCUSSION**

We report here the reproducible observation that the RNAs isolated from normal or identically fractionated scrapie-infected hamster brain are different with respect to oligonucleotide composition. The role of the RNA of different sequence in scrapie is unknown. It is unlikely that the sequence differences between the healthy and scrapie samples represent an artefact of preparation. These experiments were repeated three times with different groups of healthy and scrapie-infected animals and five pooled brains were used in order to randomize individual differences in animals. In addition, the size of the protected RNA species and the observation that they label on the 3' end preferentially makes it doubtful that they are random hydrolysis products. Since we have repeated these observations using scrapie-infected mouse brains (data not shown), it is apparent that this phenomenon is not peculiar to a single animal species.

Our interpretation of the one-dimensional electrophoresis data is that there are one or more RNA species in both preparations whose molecular weights are too similar to be resolved. The
Fig. 2. Fingerprints of scrapie RNA and healthy RNA are shown in (a) and (b) respectively. (c) Computer schematic showing the location of all the oligonucleotides present in the fingerprint of scrapie RNA (designated ×) and (d) the same information for healthy RNA (designated +). (e) Computer schematic of the oligonucleotides in the significant areas of the T₁ maps that are unique to scrapie RNA (marked ×) and those unique to healthy RNA (marked +). (f) Computer schematic of the oligonucleotides in the significant areas of the T₁ maps which are shared by both scrapie and healthy RNA (designated *). The xylene cyanol FF marker appears in the lower left quadrant of each section; bromophenol blue appears at coordinates (11, 21) in the schematics and on the fingerprints and is also marked.
two-dimensional fingerprint analysis indicates that the RNA in the scrapie-infected sample consists of species whose sequence is different from those seen in healthy material. This interpretation has been substantiated by further work indicating that the bands visualized at 4S in our gel contain more than one RNA species in both scrapie-infected and healthy RNA preparations (C. Dees et al., unpublished results).

Speculation that this RNA may be required for infectivity is intriguing, but the observation of a unique sequence RNA must be carefully evaluated before drawing any conclusions. The finding that scrapie infectivity is reduced substantially by base hydrolysis (Prusiner et al., 1981a) is consistent with the possibility that these pathogens contain an essential RNA component. The possibility exists that RNA sequences are differentially protected by normal or scrapie-infected brain components. Should such differential protection occur in vivo it may be either the cause of the disease or the result of the infection process. In either case, it will be a useful marker for the study of pathogenesis.

The replicative mechanism of the scrapie agent remains to be elucidated. The purification characteristics, nuclease resistance prior to phenol extraction and the size of these molecules are similar to the RNAs of small nuclear and cytoplasmic ribonucleoproteins (snRNPs, scRNPs) found in eukaryotic cells (Lerner & Steitz, 1981). These RNPs may be involved in the metabolism of RNA molecules utilizing RNA–RNA interactions to provide precise recognition for RNA processing (Zieve, 1981; Brunel et al., 1981). If the small RNA which we have characterized is a component of the agent or the result of infection, its low potential for coding capacity suggests a regulatory function. We have proposed a replicative model for the scrapie agent with a role for subgenomic nucleic acid (German & Marsh, 1983). The results reported here, the recent postulation that 'signal' and 'antenna' RNAs may be involved in the regulation of translation (Zimmern, 1982) and the recent demonstration that viroids have structural similarities to transposable elements (Kiefer et al., 1983) are consistent with the possibility that a small protected RNA species is involved in the pathogenesis or aetiology of scrapie. We are presently examining other animal models of unconventional virus diseases to determine whether similar unique sequence RNAs can be identified in infected animals.

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REFERENCES


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