Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids

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Scrapie can be transmitted by novel infectious pathogens termed prions. No evidence for a scrapie-specific nucleic acid has been detected to date. To investigate amounts, types and sizes of nucleic acid molecules associated with prions in purified preparations, aliquots were deproteinized, and the nucleic acids analysed by PAGE and silver staining. Digestion with nucleases and exposure to Zn2+ prior to analysis substantially diminished the content of nucleic acids, but did not alter the prion titre indicating that those nucleic acids which were removed are not essential for infectivity. Since a single species of scrapie-specific nucleic acid could not be identified, we explored the unprecedented possibility of scrapie-specific nucleic acids of variable length which are biologically active. If such molecules of variable length exist then they might be hidden within the background smear on silver-stained gels after PAGE. A new procedure designated return refocusing gel electrophoresis (RRGE) was developed to identify heterogeneous nucleic acids in purified prion fractions. The content of variable length nucleic acids was reduced by a factor of 10 by exhaustive Bal 31 exonuclease digestion after dispersion of purified prions into detergent–lipid–protein complexes. For example, a typical sample after Bal 31 digestion contained approximately 4 ng of nucleic acid of variable length and 107 1D50 units of scrapie prion infectivity. Consideration of different models for a hypothetical scrapie-specific nucleic acid suggests that such a molecule would have to be: (i) quite small (<100 nucleotides), (ii) possess a particle-to-infectivity ratio near unity or (iii) heterogeneous in size. Although our results do not eliminate the possibility that prions possess a scrapie-specific nucleic acid of variable length, they narrow considerably the spectrum of features specifying such a candidate molecule.

Introduction

Alper and colleagues first reported the small target size of the scrapie particle and later described its extreme resistance to ultraviolet irradiation (Alper et al., 1966, 1967). Subsequent studies confirmed and extended these observations (Bellinger-Kawahara et al., 1987a, 1988). After procedures for partial purification of scrapie infectivity were developed, convincing data were obtained indicating that a protein is required for scrapie agent infectivity (Prusiner et al., 1981; Prusiner, 1982; Cho, 1983; Lax et al., 1983) and that several procedures which modify or hydrolyse nucleic acids do not alter the scrapie infectivity titre (Diener et al., 1982; McKinley et al., 1983). Because of the proteinaceous character of the scrapie agent as well as its unusual properties, the term 'prion' was introduced (Prusiner, 1982).

A protein of 27K to 30K designated prion protein (PrP) is the major component in fractions highly enriched for scrapie infectivity (Bolton et al., 1982; Prusiner et al., 1982a). The discovery that purified prions aggregated into rod-shaped structures (Prusiner et al., 1982a, 1983) could be dispersed into detergent–lipid–protein complexes (DLPCs) and liposomes offered yet another approach to search for a scrapie-specific nucleic acid, but no evidence for such a polynucleotide could be found (Gabizon et al., 1987, 1988a). The molecular cloning of a PrP cDNA (Chesebro et al., 1985; Oesch et al., 1985) provided an alternative approach to search for a scrapie-specific nucleic acid. Hybridization studies using a PrP cDNA probe in purified prion fractions indicated that a nucleic acid encoding PrP is not required for infectivity (Oesch et al., 1985). Instead, the PrP gene is a single-copy chromosomal gene found in all mammals examined to date (Basler et al., 1986). In infected animals, an abnormal isoform of the prion protein (PrPSc) is found in excess of the cellular form (PrP). Considerable evidence argues that PrPSc is a major and
necessary component of the infectious prion particle (for a review see Prusiner, 1989). A mutation in the PrP gene has been linked to the development of Gerstmann-Sträussler syndrome (GSS) in humans (Hsiao et al., 1989). Recently, transgenic mice expressing the Syrian hamster PrP gene have been used to demonstrate that the sequence of the PrP gene determines the species specificity of infectious prions (Scott et al., 1989).

Intensive searches have consistently failed to produce any virions associated with scrapie or evidence for a scrapie-specific polynucleotide in purified preparations. It has often been proposed that the scrapie agent might harbour a well protected, small nucleic acid molecule and that PrPSc accumulates only as a pathological product of a 'virus' infection (Czub et al., 1986, 1988; Aiken et al., 1989; Sklaviadis et al., 1989; Murdoch et al., 1990). The existence of isolates or 'strains' with different biological properties (Bruce & Dickinson, 1987; Kimberlin et al., 1987) has offered the strongest argument for a scrapie-specific nucleic acid. Some 'strains' appear to breed true (Kimberlin et al., 1987) while others appear to owe their different properties to the distinct prion proteins encoded by the host in which they were last passed (Carlson et al., 1989).

To search for a scrapie-specific nucleic acid by molecular cloning two approaches have been utilized. Firstly, subtractive hybridization of cDNA libraries has failed to identify a putative scrapie-specific nucleic acid (Wietgrefe et al., 1985; Diedrich et al., 1987; Duguid et al., 1988, 1989). Secondly, cloning residual DNA and RNA molecules present in highly purified infectious fractions has not identified a scrapie-specific nucleic acid which should be recovered at high frequency and would also be expected to hybridize with nucleic acids from scrapie-infected tissues (Oesch et al., 1988).

In the studies reported here, we used physicochemical methods to search for a scrapie-specific nucleic acid. In an early attempt using radioiodination, protein contaminants in phenol-extracted fractions of prion preparations prevented efficient labelling of the nucleic acid. Like radioiodination, silver staining detects nucleic acids regardless of structure and has the advantage that it can be applied after PAGE (Follett & Desselberger, 1983; Schumacher et al., 1986), thereby, interference by protein staining can be avoided. If a quantitative approach is applied to detect nucleic acids regardless of structure, it should be possible to identify or to exclude nucleic acids if the sensitivity of the detection method is sufficient. To exclude a nucleic acid, a method capable of detecting less than one nucleic acid molecule per infectious unit is required.

Although micrococcal nuclease (MN) digestion is used in the purification of prion preparations (Prusiner et al., 1982a, 1983), we found that additional procedures were needed to remove contaminating nucleic acids. Since no nucleic acid of uniform length was found, the unprecedented possibility that a scrapie-specific nucleic acid might remain biologically active and yet be of variable length was addressed. A procedure for concentrating heterogeneous nucleic acid molecules was developed, designated return refocusing gel electrophoresis (RRGE). Our most highly purified preparations of prions contain nucleic acid molecules varying in length between 10 and 200 nucleotides (nt). About $10^4$ nt/ID$_{50}$ unit of scrapie infectivity were found.

Although a small scrapie-specific nucleic acid cannot be excluded, our results suggest that if such a molecule exists then it will be of variable length. Alternatively, the nucleic acids identified in our experiments are non-specific and prions are devoid of polynucleotides.

**Methods**

**Chemicals.** All chemicals and solvents were of reagent grade from commercial sources. Lipids were from Avanti. Radionucleotides were from Amersham.

**Enzymes.** Bovine pancreas RNase A and DNase (RNase-free) (Boehringer Mannheim), nuclease Bal 31 (U.S. Biochemicals), MN (Beckman), alkaline phosphatase (Boehringer Mannheim) and spleen phosphodiesterase (Worthington) were used.

**Control and marker nucleic acids.** Escherichia coli tRNA$^{Phe}$ was purchased from Boehringer Mannheim. Single-stranded DNA oligomers, with a length of 16, 20, 35 and 54 nucleotides, respectively, were synthetic oligonucleotides. $A(pA)_{10}$, $A(pA)_{15}$, and heterogeneous oligo(A) used as reference RNA were provided by R. Kapahke (Düsseldorf), the circular potato spindle tuber viroid RNA PSTVd by R. Hecker (Düsseldorf). $d(A)_{2}$ and $d(A)_{8}$ were from Pharmacia.

**Preparation of prion rods.** Prion rods were purified from the brains of scrapie-infected Syrian golden hamsters as previously described (Prusiner et al., 1983). The hamsters (LVG :LaK) were purchased from Charles River Laboratories.

**Bioassays.** Bioassays of scrapie prions were performed in Syrian golden hamsters by an incubation time interval procedure as previously described (Prusiner et al., 1982b). The correlation between inoculated dose and incubation time has been reexamined twice and was found to be quite similar to the published curves (D. Groth, M. Torchia & S. B. Prusiner, unpublished data). The same correlation was found for homogenates, purified rods and DPLCs indicating that a variety of chemical treatments and fractionation procedures did not alter the relationship between inoculated dose and incubation time.

**Procedure I: degradation of nucleic acids in samples of prion rods.** Sucrose gradient fractions were diluted with one volume of TE buffer (10 mM-Tris-acetate pH 7.4, 1 mM-EDTA) and then precipitated with 4 volumes of ethanol. The pellet was resuspended to a protein concentration of 1 mg/ml in 10 mM-Tris-acetate pH 7.4, 100 mM-NaCl, 4 mM-CaCl$_2$ and 12.5 units/ml of MN. The samples were incubated at 37°C for approximately 20 h. Sodium dodecyl sarcosinate (Sarkosyl) to a final concentration of 0.1% and Zn(NO$_3$)$_2$ to a final concentration of 2 mM were added to the samples. Following an incubation period of approximately 20 h at 65°C, EDTA, pH 8.0 was added to a final concentration of 10 mM. Four volumes of ethanol were used to precipitate the samples for 1 h at $-20^\circ$C. The pellet was
resuspended to a final protein concentration of 0.2 mg/ml in 1 mM-Tris-acetate pH 8.0 and the sample was either deproteinized directly by procedure III ('standard' sample) or was treated by procedure II ('Bal 31' and 'MN' samples).

Procedure II: transformation of prion rods into DLPCs and subsequent degradation of nucleic acids with nuclease.

The sample from procedure I was divided into two aliquots.

(i) Bal 31. One aliquot was adjusted to 600 mM-NaCl, 12.5 mM-CaCl2, 12.5 mM-MgCl2, 20 mM-Tris-acetate pH 8.0, 1 mM-EDTA, 2% sodium cholate, 32 units/ml Bal 31, and 0.07 mg/ml RNase A. The detergent/protein mixture was added to alpha-egg lecithin (200 mg/mg protein), which had been dried under nitrogen in glass vials. The solution was vortexed and sonicated for 20 min and incubated overnight at 37 °C.

(ii) MN. The other aliquot was adjusted to 71 mM-Tris-acetate pH 8.6, 4 mM-CaCl2, 1 mM-MgCl2, 2% sodium cholate, 78 units/ml MN, 7 units/ml alkaline phosphatase (AP) and 0.07 mg/ml RNase A. The mixture was treated with egg lecithin as described above and incubated for 2 h at 37 °C. The pH was lowered to 7.0 by the addition of concentrated HCl, 0.28 units/ml spleen phosphodiesterase (PDE) was added and incubation continued at 37 °C overnight.

Both DLPC mixtures Bal 31 and MN, were precipitated with chloroform: methanol (1:2) using nine parts organic to one part DLPC mixture. The pellets were resuspended to a final protein concentration of 0.2 mg/ml in 1 mM-Tris-acetate pH 8.0.

Procedure III: deproteinization of prions. To the samples obtained from either procedure I or II 2% SDS was added and the samples were boiled for 5 min. A proteinase K digestion was performed in 50 mM-Tris-acetate pH 7.5, 2.5 mM-EDTA, 0.1 mM-NaCl and 0.5 mg/ml proteinase K at 37 °C for 3 h. The samples were extracted once with phenol and once with phenol:chloroform (1:1). The aqueous phase was precipitated with four volumes of ethanol and the final pellet was resuspended in double-distilled water.

Nuclease digestion after deproteinization.

Nuclease digestions after deproteinization were carried out with 333 μg/ml RNase A and/or 167 units/ml Dnase I for 2 h at 37 °C in 10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 50 mM-MgCl2. The samples were mixed with loading buffer and analysed with RRGE.

PAGE. Gel electrophoresis was carried out in a Hoefer Scientific SE 600 gel apparatus at 50 °C. Slab gels (14 cm x 16 cm x 1.5 mm) contained 20% polyacrylamide (acrylamide: bisacrylamide, 19:1), 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA, 0.001% TEMED and 0.04% ammonium persulphate.

RRGE. RRGE consists of two PAGE procedures. They were performed in a Hoefer Scientific SE 600 gel apparatus. The various steps of the procedure are depicted in Fig. 2.

The first electrophoresis was a conventional PAGE (Fig. 2a) with the electrophoretic direction from top to bottom. The slab gel (13.5 cm x 14 cm x 1.5 mm) contained 15% polyacrylamide (acrylamide: bisacrylamide, 19:1), 8 mM-urea, 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA, 0.001% TEMED and 0.04% ammonium persulphate. The samples were mixed with loading buffer (178 mM-Tris, 178 mM-boric acid, 5 mM-EDTA, 40% glycerol, 0.006% bromophenol blue, 0.006% xylene cyanol) and incubated for 10 min at 50 °C before being loaded onto the gel. The electrophoresis was carried out at 50 °C at 350 V.

After the first electrophoresis, the gel lanes were either stained or used for subsequent RRGE steps. For RRGE, gel lanes were incubated for 15 min at room temperature in 8 mM-urea, 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA and 1% SDS. They were then cut into four to six segments designated A to E in Fig. 2. Excess liquid was carefully removed from the gel segments with a paper towel. The gel segments were placed at the bottom of the new gel (Fig. 2b) so that their top-to-bottom orientation was the same as during the first electrophoresis. The polyacrylamide gel buffer of the second electrophoresis contained the same components as that of the first plus 1% SDS. Each gel segment was washed three times with 15 ml of the SDS-containing buffer prior to its placement in a gel casting apparatus which was then filled with the new polyacrylamide matrix (16 cm x 14 cm x 1.5 mm).

The second electrophoresis was carried out at 50 °C and 350 V, but its polarity was reversed compared to that of the first. The time of electrophoresis was only 70% that of the first as it had been determined experimentally that after this time all nucleic acids from one gel segment met within one sharp band.

Silver staining. According to Schumacher et al. (1986) the gel was gently shaken three times for 20 min in 10% ethanol, 0.5% acetic acid; 30 min (in the case of RRGE, overnight) in 0.19% AgNO3; three times for 15 s in distilled H2O; 20 min in 1.5% NaOH, 0.009% NaBH4, 0.4% HCHO (37% w/v); 10 min in 5% acetic acid and finally 1 min in H2O.

In the case of RRGE, the gel segments became disconnected from the rest of the gel several times during the staining procedure. Therefore, each gel segment was identified by small cuts.

Estimation of the amount of nucleic acid in RRGE. A dilution series of heterogeneous oligo(A) as a reference RNA with known amounts of nucleic acid was analysed by RRGE. The nucleic acid content of prion samples was estimated by comparing bands of similar intensities in the RRGE of prions and with that of reference nucleic acids.

Results

Search for homogeneous nucleic acids by PAGE

Purified preparations of hamster scrapie prions containing rod-shaped aggregates of PrP 27-30 isolated from a discontinuous sucrose gradient were employed for our studies (Prusiner et al., 1983). The prion rods were precipitated from the sucrose gradient fractions with ethanol and subjected to a nucleic acid degradation protocol using DNase and Zn²⁺ (procedure I) followed by deproteinization (procedure III). After incubation at 37 °C for 24 h with 28 units/ml of DNase I followed by exposure to 2 mM-Zn²⁺, pH 7 at 65 °C for 24 h, no significant change in prion titre was detected. The data from four experiments are listed in Table 1 in order to display the variations inherent in the animal bioassays which are used to measure scrapie infectivity titres. Generally, the extent of variation and the standard errors (0.2 to 0.3 log ID₅₀ units/ml) were the same whether the titres were measured by incubation times or endpoint titrations (Prusiner et al., 1982b). Hence, DNase I digestion, Zn²⁺-catalysed hydrolysis or a combination of the two failed to alter significantly scrapie prion titres as shown in Table 1.

PAGE analysis of the DNase- and Zn²⁺-treated prions showed some background smearing as well as distinct bands migrating near the dye front (Fig. 1a, lane 2). Omission of DNase digestion and Zn²⁺ hydrolysis resulted in a prion fraction with a large number of
Fig. 1. Analysis of nucleic acids in prion rod fractions by 20% PAGE before and after DNase and Zn2+ treatment. (a) Nucleic acids without and with DNase and Zn2+ treatment. Lane 1, prion rods not treated with DNase and Zn2+; lane 2, prion rods treated with DNase and Zn2+. Lane 3, marker nucleic acids: circular potato spindle tuber viroid (PSTVd, 300 pg), tRNA (80 nt, 1 ng), oligo DNA (29 nt, 200 pg), oligo RNA (11 nt, 3 ng), oligo RNA (10 nt, 2 ng). (b) Comparable amounts of control nucleic acids (number of molecules) and prions (ID50 units). Three × 1010 molecules of control nucleic acids and 1.2 × 1010 ID50 units of prions recovered from sucrose gradients were analysed. Lane 1, control nucleic acids: tRNA (80 nt, 1·4 ng), oligo DNA (54 nt, 0·94 ng), oligo DNA (29 nt, 0·5 ng), oligo RNA (11 nt, 0·19 ng), oligo RNA (10 nt, 0·17 ng); lane 2, control nucleic acids as in lane 1 but treated by the deproteinization procedure (procedure III); lane 3, prions treated with DNase and Zn2+ (procedure I) and by deproteinization (procedure III); lane 4, control nucleic acids as in lane 1 added to prions after DNase and Zn2+ treatment but before deproteinization. The bands of 10 and 11 nt are weak in the photographic reproduction but were clearly seen in the original gel.

Table 1. Neither prolonged DNase I digestion nor exposure to Zn2+ ions alters scrapie prion infectivity

<table>
<thead>
<tr>
<th>Experimental conditions*</th>
<th>Scrapie prion infectivity ((\log ID_{50}/ml \pm S.E.))</th>
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<tbody>
<tr>
<td></td>
<td>1†</td>
</tr>
<tr>
<td>Control</td>
<td>8·2 ± 0·2</td>
</tr>
<tr>
<td>DNase I</td>
<td>8·1 ± 0·2</td>
</tr>
<tr>
<td>DNase I + Zn2+</td>
<td>8·7 ± 0·2</td>
</tr>
<tr>
<td>Control</td>
<td>7·9 ± 0·3</td>
</tr>
<tr>
<td>Zn2+</td>
<td>9·1 ± 0·2</td>
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* DNase I digestions (28 units/ml) were performed at 37 °C for 24 h. Exposure to 2 mM-Zn2+ was performed at 65 °C for 24 h.
† Experiment number.

rapidly migrating bands observed on PAGE (Fig. 1a, lane 2) might be either complex Asn-linked oligosaccharides released from PrP 27-30 during proteinase K digestion or non-covalently bound sugar polymers which are copurified with the prion rods.

Quantitative analysis

Since at least one molecule of a specific nucleic acid must be present per infectious unit if it is required for scrapie infectivity, we performed experiments to determine the minimum amounts of nucleic acid detectable as discrete bands of uniform length in polyacrylamide gels by silver staining. As little as 170 pg of nucleic acid could be detected after electrophoresis. This corresponds to 3 × 1010 molecules with a length of 10 nt or 3 × 109 molecules with a length of 100 nt of single-stranded nucleic acid.

Control nucleic acids ranging from 10 to 80 nucleotides were analysed in the same way as infectious scrapie prions by PAGE after boiling in SDS, digesting with proteinase K, extraction with phenol and ethanol precipitation (procedure III). Lane 1 in Fig. 1(b) demonstrates that 3 × 1010 molecules of each of the nucleic acids were readily detected and that they could be recovered using our protocol (lane 2). Similar experiments were performed with PSTVd RNA (359 nt). A single nick in the circular RNA leads to a shift in the migration of the viroid (Sänger et al., 1979). No linear viroid molecules were found after being processed by our protocol, confirming the lack of nucleic acid degradation (data not shown). If the control nucleic acids were hydrolysed with DNase I and Zn2+ (procedure I), no silver-stained bands were detected (data not shown). We collected prion rods to give a total of 3 × 1010 ID50 units. This number of ID50 units corresponds to the number of nucleic acid molecules (10 nt in length) which can be detected by silver staining after PAGE.
Prion rods recovered from sucrose gradient fractions containing $3 \times 10^{10}$ ID$_{50}$ units were incubated with DNase I and Zn$^{2+}$ followed by a PAGE analysis as described above. Silver staining of a sample after PAGE showed complete absence of banding above 20 nt (Fig. 1b, lane 3). As a control, $3 \times 10^{10}$ nucleic acid molecules ranging in length from 10 to 80 nt (see also lanes 1 and 2) were added to the scrapie sample after DNase and Zn$^{2+}$ treatment but before destroying the infectivity by SDS (Fig. 1b, lane 4). All control nucleic acids added to the scrapie prion sample were visible except for those 10 nt and 11 nt in length because they were hidden by the non-nucleic acid molecules purifying with the prions. The prion infectivity after procedure I was determined to be $1.2 \times 10^{10}$ ID$_{50}$. The sample was then subjected to procedure III and PAGE. From the absence of silver-stained bands in our gels we conclude that there are no nucleic acids of uniform length $>25$ nt which are required for infectivity assuming a particle-to-infectivity ratio of unity. Our conclusion is restricted to molecules longer than 25 nt instead of 10 nt since only $1.2 \times 10^{10}$ ID$_{50}$ instead of $3 \times 10^{10}$ ID$_{50}$ was used for the analysis. Furthermore, the imprecision of the bioassay forces us to qualify this conclusion. In our experience, fluctuation in scrapie prion titres of a factor of 10 are common and cannot be considered significant (see Table 1). For example, if the bioassay estimate of scrapie prion infectivity is 10-fold too high, then we could have failed to detect a putative scrapie-specific nucleic acid by PAGE and silver staining.

Since our analysis utilized 20% polyacrylamide gels, we could only quantitatively analyse nucleic acids $<300$ nt in length. There was, however, no indication of larger nucleic acids in the region of the gel very close to the slot where larger nucleic acids should have accumulated (Fig. 1b, lanes 3 and 4). Larger nucleic acids were not assessed specifically because the results of earlier studies made their existence highly unlikely (Alper et al., 1966, 1967; Bellinger-Kawahara et al., 1987a, b; Gabizon et al., 1987).

**Detection of heterogeneous nucleic acids by RRGE**

The possibility remained that prions contain nucleic acid molecules of non-uniform length. In such a case, the nucleic acid would migrate during PAGE as many bands and each band might either be below the threshold for detection or not resolved from neighbouring bands, resulting in a smear of staining. To evaluate this unprecedented but formal possibility, we developed a technique to measure nucleic acid molecules of variable size.

With RRGE, nucleic acids can be separated from other stainable molecules and focused into one sharp band. The heterogeneous nucleic acid can then be detected with a sensitivity close to that of the detection of a homogeneous nucleic acid. RRGE is performed in two steps. After conventional PAGE as a first step, heterogeneous nucleic acids are dispersed over the whole length of the lane (black bands in Fig. 2a). The lane is cut into a
Fig. 3. Sensitivity and selectivity of RRGE. (a) Sensitivity of detection of heterogeneous nucleic acid. A series of oligo(rA) ranging in size from 5 nt to about 100 nt was used as a model for heterogeneous nucleic acid. The increase in sensitivity was estimated with a series of threefold dilutions: lanes 1 and 3, undiluted; lanes 2 and 4, ½ dilution; lane 5, ¼ dilution; lane 6, 1/16 dilution; lane 7, 1/64 dilution; lane 8, 1/128 dilution. The starting amount was 42 ng. The increase in sensitivity of nucleic acid detection was obvious even when only segment B and not the whole gel lane was used. There is a factor of 10 increase in sensitivity. (b) Selectivity of detection of nucleic acids in the presence of peptides. Forty-two ng of oligo(rA) and 1 ng tRNA_Phe were used as a model for a heterogeneous nucleic acid. Lanes 1, 3, 5, 7, 9 and 11 contain RNA; lanes 2, 4, 6, 8, 10 and 12 contain protein. The mixture of peptides was 20 μg of bovine serum albumin which was digested for 2 h at 37 °C with 3 μg proteinase K in 20 μl total volume. After the first electrophoresis, a smear was visible in the nucleic acid lane as well as in the protein lane. Similar staining intensities were obtained from gel segment C from the nucleic acid lane as compared to gel segment B from the protein lane. After the second electrophoresis, the nucleic acid sample yielded well stained refocused bands whereas no bands could be detected for the protein sample after the second electrophoresis except in gel segment A (see text).

To test the RRGE system, a series of oligo(rA) molecules ranging in size from 5 nt to 100 nt were used. A weakly staining region of the gel after the first electrophoresis was found for 42 ng of oligo(rA) but barely detectable staining was observed after a threefold dilution of the sample (Fig. 3a, left panel). After refocusing, the latter sample was readily detected and even a 27-fold diluted sample (1.6 ng) could be detected (right panel). In more recent experiments focusing could be improved and a detection limit of 300 pg for heterogeneous nucleic acids of 10 to 40 nt and of 100 pg for 100 to 200 nt was achieved.

RRGE not only increases the sensitivity of nucleic acid detection, but it also gives information about the size distribution of these molecules. If sections of the first electrophoretic gel were analysed by RRGE (Fig. 3b), a diagonal pattern of stained nucleic acids was observed after RRGE as a consequence of the distance that the nucleic acids migrated in the first electrophoresis. Since both nucleic acids and proteins stain with silver, SDS was added to the refocusing electrophoresis in order to prevent the proteins from being refocused. A mixture of peptides was obtained from 20 μg of bovine serum albumin digested for 2 h with 3 μg proteinase K in a volume of 20 μl. The protein smear is shown in Fig. 3b, lane 2 (1st dimension). After the refocusing (Fig. 3b, 2nd dimension), even the gel segment containing the highest amount of the protein (Fig. 2b, lane 6, segment B) showed no bands. A gel segment containing a comparable amount of heterogeneous nucleic acid (Fig. 3b, lane 7, segment C) exhibited a distinct band after refocusing. Because most peptides electrophoresed in the absence of SDS (1st electrophoresis) are not highly charged compared to nucleic acids, their migration is minimal. That being the case, most of the peptides remain near the origin migrating only a short distance (Fig. 3b, lane 2, segment A). For this reason, a small piece of the gel below the slot was cut and refocused by itself (segment...
A). In the second electrophoresis, the mobility of proteins was increased appreciably due to the addition of SDS which creates a smear shown above gel segment A. Otherwise, essential staining of nucleic acids may be lost under this smear.

**Analysis of prion samples by RRGE**

Purified prions were evaluated by RRGE after incubations with DNase I and Zn$^{2+}$ (Fig. 4). After the first electrophoresis, only a faintly stained smear in gel sections A and B as well as a ladder of silver-stained bands comigrating with oligonucleotides ranging from four to 12 bases were visible. After RRGE, clear bands were obtained from pieces A and B and weaker bands from pieces C to E. The improvement of focusing in comparison to Fig. 3(a) is most obvious from segment B. The total amount of heterogeneous nucleic acid in the prion sample was estimated to be about 20 ng.

The weak signals from pieces D and E after RRGE are evidently not from the ladder, because a comparison with reference standards (see Fig. 3) shows that the ladder should have resulted in much stronger staining after RRGE if it were composed of nucleic acids. Therefore, we conclude that the components forming the ladder were not refocused. This conclusion is in accordance with the finding that the low Mr ladder is composed of sugar.

**Differentiation between RNA and DNA**

From the foregoing results, we concluded that preparations of prion rods digested with DNase I and subsequently incubated with Zn$^{2+}$ overnight at 65 °C still contained detectable nucleic acids, but these nucleic acids were heterogeneous in size. Nuclease digestion studies were carried out prior to RRGE in order to confirm the nucleic acid nature of the bands and to differentiate between RNA and DNA. A prion sample prepared as described above (after procedures I and III) was divided into four aliquots: one was treated with RNase, one with DNase, one with both and one was left undigested as a control. As may be seen from a comparison of Fig. 5(a) with 5(d), virtually all of the stained molecules were nucleic acids because they disappeared after the combined RNase/DNase digestion. In control experiments (data not shown), it was found that the remaining band detected above gel segment B after DNase digestion (Fig. 5b) and RNase/DNase digestion (Fig. 5d) is from the DNase bound near the slot in the first electrophoresis. The DNase migrates more rapidly during the second electrophoresis due to the added SDS, and its position is clearly different from that of the nucleic acid over the same gel piece, which can be seen from RRGE in the absence of nucleases (Fig. 5a, gel segment B). RNA and DNA differed in their size distribution; more RNA was found below 50 nt (Fig. 5b) and more DNA above (Fig. 5c).

**Analysis of prions dispersed into DLPCs**

During the course of this work, procedures were developed for dispersing prion rods into DLPCs and liposomes with retention of infectivity (Gabizon et al., 1987, 1988a, b). We expected that nucleic acids, which were possibly protected against degradation by inclusion in the rod-shaped aggregates, would become accessible to nucleases upon formation of the DLPCs (procedure II).

In control experiments, radioactively labelled double-stranded linear plasmid was added to scrapie prion rods prior to their dispersion into DLPCs. The sample was then treated with nuclease as described for the Bal 31 and MN schemes. Afterwards, the samples were analysed by thin-layer chromatography. Using either of these schemes, the radiolabelled nucleic acids were degraded down to dimers or monomers (data not shown).

A sample of prions was divided after procedure I into three aliquots. One served as a control and was analysed by RRGE without further treatment. The remaining aliquots were transformed into DLPCs and digested according to the Bal 31 and MN schemes. The digested
Fig. 5. Additional verification of the nucleic acid nature of the bands detected with RRGE in prion rod preparations and differentiation between RNA and DNA. A sample of prion rods was divided after DNase and Zn$^{2+}$ treatment and deproteinization into four aliquots and analysed by RRGE without further treatment (a), after DNase digestion (b), after RNase digestion (c) and after RNase and DNase digestion (d). The staining material which had been found in prion rods (a) was clearly destroyed by nucleases (d). DNA was mainly found above a length of about 50 nt (c) and RNA was mainly found below the size of 50 nt (b). The remaining band above gel segment B in (b) and (d) was caused by DNase (see text).

Fig. 6. RRGE of the nucleic acids in prion preparations before and after DLPC formation with subsequent nuclease digestions. A sample of prion rods were DNase- and Zn$^{2+}$-treated (procedure I) and divided into three aliquots. Aliquot (a) was directly deproteinized (standard, procedure III), aliquots (b) and (c) were transformed into DLPC, aliquot (b) was digested by Bal 31 (Bal 31, procedure II), aliquot (c) by MN (MN, procedure II), and both the latter aliquots were deproteinized (procedure III). The amount of nucleic acid was drastically reduced due to the Bal 31/DLPC as well as the MN/DLPC treatment (procedure II). The following sizes and amounts of nucleic acid were estimated. Total sample (a): 43 ng \([\text{gel segment B (65 to 200 nt), 20 ng}; \text{C (64 to 35 nt), 13 ng}; \text{D (34 to 17 nt), 3 ng}; \text{E (16 to 6 nt), 5 ng}; \text{F (5 to 3 nt), 2 ng}\). Total sample (b): 4 ng \([\text{B (200 to 64 nt), 2-5 ng}; \text{C (63 to 27 nt), 1 ng}; \text{D (26 to 12 nt), 0-5 ng}\). Total sample (c): 3 ng \([\text{B (200 to 64 nt), 2 ng}; \text{C (63 to 29 nt), 0-5 ng}; \text{D (28 to 14 nt), 0-5 ng}\]. The corresponding data of the infectivities were for sample (a) $10^{7.7}\ \text{ID}_{50}$, (b) $10^{8.7}\ \text{ID}_{50}$, and (c) $10^{8.3}\ \text{ID}_{50}$ (see also Table 2, experiment 7, values with †).

Correlation of nucleic acids with scrapie infectivity

The scrapie infectivity was monitored at all steps of the preparation. The infectivity data from seven indepen-
Table 2. Scrapie prion infectivity in fractions analysed for nucleic acids

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* Experiment number. Data (log10 IDs0) from independent experiments are listed. If a sample was divided into aliquots all data refer to the amount analysed in one RRGE. Aliquots for bioassay were taken after the indicated preparation steps. For details of the procedures standard, Bal 31 and MN see Methods.

† Scrapie infectivity (ID50 units) used to calculate the ratio of nucleotides per ID50 unit in Table 3.

Discussion

Prions resist inactivation by harsh procedures that specifically hydrolyse or modify nucleic acids. Such procedures render viruses and viroids inactive. These features of prions argue that they are probably devoid of polynucleotides, but such negative results are always vulnerable to the criticism that a putative scrapie-
specific nucleic acid might be so well protected by some unusual structure that its presence is yet to be detected. The existence of scrapie 'strains' or isolates which produce incubation periods of specific length has been used as an argument in favour of a scrapie-specific nucleic acid (Bruce & Dickinson, 1987; Kimberlin et al., 1987). In some cases, it has been shown that scrapie incubation times can be modified by the amino acid sequence of the PrPSc molecule which is encoded by the host (Carlson et al., 1989; Scott et al., 1989).

There are reports of nucleic acids in preparations derived from scrapie or Creutzfeldt-Jakob disease-infected rodent brains (Braig & Diringer, 1985; Dees et al., 1985; German et al., 1985; Narang et al., 1988; Sklaviadis et al., 1989; Murdoch et al., 1990); none of these preparations was highly enriched for scrapie infectivity with respect to cellular protein. In this context, it should be emphasized that our purified preparations enriched 1000-fold for infectivity contained numerous non-specific nucleic acid molecules unless the preparations had been treated exhaustively with nucleases and Zn²⁺.

Our quantitative physicochemical study was carried out based on the consideration that one infectious unit must contain at least one nucleic acid molecule if it is required for infectivity. In order to identify a putative nucleic acid in the scrapie prion, a method was needed which would be able to detect nucleic acids at very low concentrations. The size and sequence of the hypothetical scrapie nucleic acid are unknown, of course. Such a molecule may be DNA or RNA, single- or double-stranded, circular or linear, capped, chemically modified or covalently bound to proteins. In addition, it might be heterogeneous in size. Until recently, virtually all studies on scrapie have utilized animals; this makes it difficult to incorporate radiolabelled nucleotides into the putative scrapie nucleic acid. Perhaps future cell culture systems with higher titres will help to resolve this problem. In the present work, we chose a general method utilizing PAGE combined with silver staining (Sammons et al., 1981; Follett & Desselberger, 1983; Schumacher et al., 1986). This method should detect any nucleic acid. Our experience 170 pg of nucleic acid per gel band was safely detectable. This amount would be contained in 3 × 10⁶ ID₅₀ units of prions if the hypothetical nucleic acid was 100 nt in length and the particle-to-infectivity ratio was one. The sensitivity of silver staining after conventional PAGE decreased substantially if the nucleic acid was heterogeneous in size. To detect heterogeneous nucleic acids, RRGE was developed.

Our initial studies showed that no nucleic acid of homogeneous size could be detected by PAGE and silver staining at one molecule of 25 bases or greater per ID₅₀ unit, but difficulties in recovering and concentrating all of the infectivity found in sucrose gradient zonal rotor fractions prevented us from obtaining a more definitive result. Furthermore, the imprecision in determining ID₅₀ values (Prusiner et al., 1982b) demands that a definitive observation on the absence of a putative nucleic acid must demonstrate considerably less than one molecule per ID₅₀ unit. This imprecision is not the result of the particular bioassay procedure, i.e. incubation time measurement employed in our work, but rather is a consequence of the number of animals used for each bioassay.

Because of the difficulties in recovering the infectivity from sucrose gradient fractions, one may argue that we carried out our analysis on only a small subfraction of the total scrapie infectious units and that we lost the scrapie-specific nucleic acid. However, the semi-quantitative evaluation of the amount and size of nucleic acids is expressed in terms of polynucleotides per ID₅₀ unit and the infectivity values were determined after the loss of much infectivity during recovery from sucrose gradient (see † in Table 2). Thus, our conclusions on the amount, type and size of nucleic acid in purified prion fractions seem warranted.

No homogeneous nucleic acid could be found in fractions containing between 10⁸ and 10¹⁰ ID₅₀ units whereas nearly 10⁶ nt/ID₅₀ unit were found by RRGE. These polynucleotides varied between 10 and 200 bases in length. After dissociating the prion rods into DLPCs, followed by procedures to reduce polynucleotides to mono- and dinucleotides, we found 10⁴ nt/ID₅₀ units. This reduction of the ratio by a factor of 100 demonstrates that the vast majority of the nucleic acid found in our purified prion rod preparations, even after prolonged MN digestion and Zn²⁺ hydrolysis was composed of non-specific contaminants. While the nuclease digestion procedures reduced the content of heterogeneous nucleic acid, they did not substantially alter the titre of scrapie prions dispersed in DLPCs. The heterogeneity of the nucleic acids in prion preparations is in accordance with results from cloning studies, showing that no high-frequency sequence could be identified (Oesch et al., 1988).

How can we interpret the observation of 10⁴ nt/ID₅₀ unit? If the nucleic acids that we detected were related to scrapie infectivity, then one of two alternative paradigms as shown in Fig. 7 would be correct. First, a putative scrapie-specific nucleic acid of uniform length might be hidden amongst an ensemble of background nucleic acid. Such a scrapie-specific nucleic acid would not have been detected by normal PAGE (see Fig. 1b) even if it were present in sufficient amounts. Second, we must consider the possibility of a scrapie-specific polynucleotide which is heterogeneous in length. In Fig. 7 the numbers of nucleic acid molecules per ID₅₀ unit are
search for a scrapie-specific nucleic acid

plotted as a function of their length as estimated from the individual gel sections.

In the case of the homogeneous species paradigm, a significant decrease in the number of nucleic acid molecules per ID$_{50}$ unit was found as the size of the polynucleotide increased. For small nucleic acid molecules (20 nt), about 10 molecules/ID$_{50}$ are estimated. If the scrapie-specific nucleic acid were longer (100 nt), then the particle-to-infectivity ratio would fall below unity.

The heterogeneous nucleic acid paradigm allows for a particle-to-infectivity ratio of about 100 based on our experimental results. We found that the number of nucleic molecules was constant over the range from 13 to 200 nt in length.

Our data suggest one of three possible conclusions. If a scrapie-specific nucleic acid exists, it might be (i) very small (<100 nt), (ii) very efficient, i.e. a low molecule-to-infectivity ratio (one) or (iii) heterogeneous in size. It should be noted that the analysis with normal PAGE was restricted to nucleic acids smaller than 300 nt and those with RRGE smaller than 200 nt. However, no indication for detectable amounts of larger nucleic acids was found in PAGE. The length distribution as determined with RRGE (see Fig. 7a) shows a decrease in the amount of nucleic acids with increasing length. Furthermore, the inactivation and modification studies reported in the literature made the existence of a larger nucleic acid highly unlikely.

Two observations on viroids raise the possibility of heterogeneous genomes in nature. First, circular viroids can be converted within the host cell into linear molecules which retain infectivity by cleavage at several different sites in the molecule (Palukaitis & Zaitlin, 1987). Second, infectious viroids were recovered from plants inoculated with two cDNA clones each encoded by different portions of the viroid sequence (Tabler & Sänger, 1984).

Our assumption of one nucleic acid molecule per infectious unit is extreme considering the situation with animal viruses. In most cases, the particle to infectivity ratio for animal viruses in experimental animals ranges from 10 to 10000 (Davis et al., 1980). But since there are reports of particle to infectivity ratios near unity for animal viruses (Davis et al., 1980; Hundley et al., 1985) we assumed that each infectious unit may contain only one prion-specific nucleic acid molecule.

It is worth noting that the nucleic acids found in prion fractions are remarkably well protected against degradation. After extensive DNase digestion and Zn$^{2+}$ treatment, $10^6$ polymeric nucleotides per ID$_{50}$ unit were detected. Even after DLPC formation and further nuclease treatment, $10^6$ polymeric nucleotides per ID$_{50}$ unit were found, whereas exogenously added control nucleic acids were completely degraded. Our observations imply that nuclease insensitivity alone does not exclude the presence of a nucleic acid within the infectious scrapie particle. Furthermore, our results establish that 90% of the nucleic acid found in preparations of prion rods becomes exposed upon DLPC formation and is readily hydrolysed by nucleases. Whether or not prions contain macromolecules such as PrP$^\text{Sc}$ which bind to specific nucleic acid sequences remains to be established.

It must be stressed that although 90% of the nucleic acid in prion rod preparations could be removed by Bal 31 digestion of DLPCs without loss of titre, we still cannot definitively eliminate the possibility that prions carry a well protected small polynucleotide. Only in future studies with more concentrated fractions, containing higher prion titres than those used in the studies reported here, will it be possible convincingly to exclude a scrapie-specific nucleic acid.

![Fig. 7. Two possible models for hypothetical scrapie-specific nucleic acid molecules. The results shown in Fig. 6(b) and (c) for prions after DLPC formation and treatment with nucleases (procedure II) were averaged for these estimates. The number of polymeric nucleotides was estimated for each gel segment separately and was correlated with the corresponding size range. The number of putative scrapie-specific nucleic acid molecules per infectious unit was based on alternative assumptions. (a) A putative scrapie-specific nucleic acid molecule of uniform length (heavy line) is hidden amongst an ensemble of background nucleic acids. (b) All nucleic acid molecules detected are putative scrapie-specific nucleic acids, although of different size (all heavy lines).](image-url)
We thank Professors U. Desselberger and C. Weissmann for helpful comments and incisive criticisms as well as Ms Ch. Meiering and M. Gerads for skilful technical assistance. This work was supported by research grants from the National Institutes of Health (AG02132 and NS 14669), the Senator Jacob Javits Center of Excellence in Neuroscience (NS22786) and the Fond der Chemischen Industrie, a gift from the Sherman Fairchild Foundation and a travel grant (D.R.) from the Deutsche Forschungsgemeinschaft.

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(Received 22 June 1990; Accepted 26 September 1990)