Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocusing gel electrophoresis

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Although increasingly unlikely, the possibility of a scrapie-specific nucleic acid carried by infectious prion particles is still unresolved. Return refocusing gel electrophoresis was developed to detect homogeneous and heterogeneous nucleic acids extracted from highly purified scrapie prion preparations. This method was improved with respect to the size range from 13 to 1100 nucleotides (nt) over which analyses could be performed. The yield of nucleic acid, particularly of small DNA oligonucleotides and polyadenylated RNA, was determined after deproteinization and two-phase extraction. Despite extensive nuclease digestions some small polynucleotides remained. Although a scrapie-specific nucleic acid cannot be excluded, the results further define the possible characteristics of a hypothetical molecule. If homogeneous in size, such a molecule would be < 80 nt in length at a particle-to-infectivity ratio near unity, if heterogeneous, scrapie-specific nucleic acids would have to include molecules smaller than 240 nt.

A wealth of data indicate that a protein, designated prion protein (PrP), is required for scrapie infectivity. Protease treatment and protein denaturing agents affect infectivity, whereas procedures that modify or hydrolyse nucleic acids do not alter infectivity (Prusiner, 1982). In spite of many attempts, no physical or chemical evidence for a scrapie-specific nucleic acid has been found to date. The existence of multiple 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. The failure to explain such strain variation in terms of molecular variation in PrP continues to stimulate the search for a scrapie-specific nucleic acid (Prusiner, 1991; Weissmann, 1991).

A recent search for a putative scrapie genome in purified prion preparations revealed a paucity of nucleic acids (Meyer et al., 1991). Purified fractions were extensively treated with nucleases and Zn²⁺ ions, dispersed into detergent–lipid–protein complexes (DLPC), treated again with nucleases followed by deproteinization, and extracted for nucleic acids. Nucleic acids were analysed by PAGE and detected by silver staining. No well defined molecular species of nucleic acid could be detected. The use of return refocusing gel electrophoresis (RRGE) has made it possible to analyse nucleic acids which are heterogeneous in length with the same sensitivity after silver staining as is possible for nucleic acids of homogeneous length. Based on the consideration that a hypothetical scrapie-specific nucleic acid has to be present with at least one copy per infectious unit (ID₅₀), our results suggest that such a nucleic acid would have to be quite small [< 100 nucleotides (nt)], possess a particle-to-infectivity (P/I) ratio near unity or be heterogeneous in size. Since such experiments have been restricted to analyses of nucleic acids smaller than 200 nt and the conclusions were based on semiquantitative estimates of nucleic acids, we modified the procedures to extend the size range of nucleic acids investigated, and improved our quantification of nucleic acid molecules. The inclusion of larger nucleic acid sizes into our experimental analysis was necessary as genomic segments of conventional viruses and viroids are larger than 200 nt; some recent reports (Aiken et al., 1990; Akowitz et al., 1990; Narang, 1990) have suggested larger scrapie-specific nucleic acids.

RRGE was described earlier (Meyer et al., 1991) and the improvements in RRGE are outlined briefly in the legend of Fig. 1. We found that a 9% polyacrylamide gel matrix (acrylamide:bisacrylamide, 19:1) and a Tris-acetate–EDTA buffer (8 mM-Tris, 4 mM-sodium acetate and 0.4 mM-EDTA pH 8.4) was the most suitable for analysis of nucleic acids from 13 to 1100 nt in length. Since the time of refocusing depends slightly upon the
Fig. 1. Scheme of the improved RRGE. The RRGE consists of a separation and a refocusing gel electrophoresis. The separation run (100 min, 250 V) is equivalent to a normal PAGE (a). The nucleic acid from a prion sample yields an undetectable smear (P in a). The gel is cut into different segments (a to h). These are repolymerized at the bottom of new gel matrices (b). The refocusing run (250 V) is carried out in the opposite direction and in presence of SDS. The times of refocusing of different gel segments are given in Table 1. All nucleic acids which were contained in one gel segment are concentrated into one band, thereby increasing the sensitivity of detection. The unknown nucleic acid amount (in b) of the prion sample is determined by comparison with described markers of known concentrations (Table 1, markers 1, 2 and 3). Only two gel segments, b and h, are given as an example for the refocusing run; gel segment a is not used for refocusing, but silver-stained after the separation run.

Table 1. RRGE analysis of prion preparation CAM8

<table>
<thead>
<tr>
<th>Gel segment</th>
<th>Size range (nt)</th>
<th>Time of refocusing (min)*</th>
<th>Nucleic acid marker (pg)†</th>
<th>Prion sample (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>&gt; 1100</td>
<td>No refocusing</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>b</td>
<td>1100–340</td>
<td>48</td>
<td>860 260 25</td>
<td>75</td>
</tr>
<tr>
<td>c</td>
<td>339–141</td>
<td>44</td>
<td>220 20 50</td>
<td>50</td>
</tr>
<tr>
<td>d</td>
<td>140–80</td>
<td>42</td>
<td>400 200 100</td>
<td>300</td>
</tr>
<tr>
<td>e</td>
<td>79–54</td>
<td>42</td>
<td>450 215 100</td>
<td>450</td>
</tr>
<tr>
<td>f</td>
<td>53–40</td>
<td>42</td>
<td>415 205 160</td>
<td>600</td>
</tr>
<tr>
<td>g</td>
<td>39–20</td>
<td>42</td>
<td>2100 1200 600</td>
<td>8000</td>
</tr>
<tr>
<td>h</td>
<td>19–13</td>
<td>42</td>
<td>2100 1200 600</td>
<td>20000</td>
</tr>
</tbody>
</table>

* Taken after 100 min in the separation run.
† The marker nucleic acids consisted of purified restriction fragments from pTZ18U digested with HindIII (1075, 517, 465, 396, 201, 75, 65, 44 and 22 nt), PSTVd fragment (192 nt), pTZ18U digested with Sau3A (105 nt), pTZ18U digested with Alul (64, 63 and 49 nt) and synthesized oligomeric DNA (28 and 16 nt). These nucleic acids were mixed in ratios such that the markers designated 1, 2 and 3 contained the concentrations as listed for the size range. The same markers were applied to the RRGE of Fig. 2.
‡ ND, Not detectable.

Preparation of purified prion fractions for RRGE was similar to that used for previous studies (Meyer et al., 1991). In order to improve the recovery of prion infectivity during the collection of prion rods from sucrose gradient fractions, ultracentrifugation steps (2 × 4 h, 100000 g) were substituted for the ethanol precipitation previously used. Furthermore, DNase digestion of the rods was omitted because the material was treated with Bal 31, micrococcal nuclease and RNase A after DLPC formation. Three independent prion preparations (CAM6, CAM7 and CAM8) were analysed, CAM6 for nucleic acid sizes from 40 to 1100 nt, and CAM7 and CAM8 over a wider range from 13 to 1100 nt. Before the deproteinization/nucleic acid extraction step the infectivities of these preparations were log ID50 8.3 (CAM6), 8.5 (CAM7) and 8.7 (CAM8). The bioassays were carried out with 10% of one sample and the remaining 90%, for which the ID50 values were calculated, were used in toto for one gel electrophoretic analysis.

The yield of nucleic acid after deproteinization was estimated quantitatively by three radiolabelled model nucleic acids: an RNA transcript of 154 nt, the same RNA transcript with a polyadenylated tail of 80 to 100 nt and a short DNA oligomer of 28 nt. No significant loss was observed during the phenol and phenol:chloroform extractions with the RNA transcripts. The yield of the DNA oligomer was, however, only 50%; therefore, the phenol extraction of former prion preparations was replaced by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction which permitted approximately
Fig. 2. RRGE of a prion sample. The refocused gel segments b to h are shown. Each gel segment consists of six lanes: sonicated calf thymus DNA (ctDNA), 85 ng (total); nucleic acid markers 1, 2, 3 (Table 1); empty; prion sample CAM8 (lanes 1 to 6, respectively). The estimation of the nucleic acid content of the prion sample was carried out on the original gels. The intensity as well as the width of a band were considered for comparison between prion and marker bands. Bands from adjacent gel segments were also included in the comparison. The sonicated ctDNA was used as an internal control of refocusing and not for quantification. The silver-stained gel segment a showed no nucleic acid or protein signal after the separation run (not shown).

90% recovery (data not shown). The quantitative determination of the recovery shows that the evaluation as carried out by Meyer et al. (1991) was correct for nucleic acids in the larger size range (64 to 200 nt), whereas the number of molecules per ID$_{50}$ given for smaller sizes needs correction by a factor of about two. This correction does not affect the basic conclusion of that work.

The results of experiments with prion preparation CAM8 are given in Table 1 and Fig. 2. Gel segment a (> 1100 nt) was also silver-stained, but no stained band or smear could be detected after the electrophoretic separation in any of the prion preparations (data not shown). If a scrapie-specific nucleic acid larger than 1100 nt exists, a log ID$_{50}$ titre of 8.5 (CAM7) would necessitate at least $3.2 \times 10^9$ molecules corresponding to about 200 pg, an amount which would have been detected easily in gel segment a.

From the amounts of nucleic acids as estimated from gel segments b to h (Fig. 2) and from the infectivity of the prion samples before deproteinization, the P/I ratio was determined. This calculation is based on the assumption of a scrapie-specific nucleic acid molecule which is hidden in the heterogeneous background nucleic acid. Fig. 3(a) is a double-logarithmic plot of P/I ratio as a function of the length of the hypothetical nucleic acid molecule. For the size range from 60 to 200 nt, the results from earlier measurements (Meyer et al., 1991) were confirmed, but more nucleic acid molecules of <60 nt were found than previously reported. Besides the twofold increase due to the more effective extraction as mentioned above, we have to conclude that the omission of DNase digestion of prion rods yields more undigested nucleic acids in this size range and thus results in higher P/I ratios. Amounts of nucleic acids above 200 nt were determined by RRGE for the first time. Since the P/I ratio continues to drop several orders of magnitude below unity, nucleic acids of this size range cannot be scrapie-specific. The straight line (Fig. 3) is an interpolation of the experimental data by linear regression in order to determine an average nucleic acid size at a P/I ratio of 1.

Fig. 3(a) also contains an estimation of the maximum error: it was assumed that the correct titre was one log ID$_{50}$ lower than the measured one (see Meyer et al., 1991) and that the nucleic acid content was twofold higher than estimated from RRGE. This calculation shifts the P/I ratio of unity from 76 nt to 165 nt (Fig. 3, dotted line). It is worth noting that the limit of one nucleic acid molecule per ID$_{50}$ (at a P/I ratio of 1) is an extreme assumption (see Meyer et al., 1991), for comparison $10^4$ to $10^5$ PrP molecules are necessary for one infectious unit (Prusiner et al., 1982, 1983).

If heterogeneous scrapie-specific nucleic acids were assumed, all molecules of a certain heterogeneity class would have to be added up to account for the corresponding P/I ratio. Such heterogeneity would be an intrinsic property of the scrapie genome and not an artefact from nuclease digestion, because the bioassays yielding the ID$_{50}$ value were carried out after nuclease digestion. In Fig. 3(b) the P/I ratio is plotted against the length of hypothetical heterogeneous scrapie-specific
Particle-to-infectivity ratio (P/I); dependence upon the length of the hypothetical scrapie-specific nucleic acid. In (a) a distinct molecular species among the heterogeneous background nucleic acids was assumed. The experimental values from three independent prion preparations (CAM6, CAM7 and CAM8) were evaluated. The straight line is an interpolation of the experimental data by linear regression in order to determine an average nucleic acid size at P/I = 1. The abcissa denotes the average of the size range of a gel segment (see Table 1). The interpolating line is shown for the whole experimentally covered range of 13 to 1000 nt. The dotted lines represent the maximum error, i.e. one log ID_{50} for the bioassay and a factor of two in the nucleic acid content. This calculation shifts the P/I ratio of unity from 76 nt to 165 nt. In (b) heterogeneous scrapie-specific nucleic acids were assumed. The same experimental data as in (a) were evaluated. P/I values for heterogeneity classes (H = 10, 100, 1000) are plotted against the minimum length of the class. H denotes the number of heterogeneous molecules in one class and, because of the continuous size distribution, the maximum difference in length. For example, P/I = 1 at 209 nt for H = 100 means that all molecules between 209 nt and 308 nt add up to a P/I ratio of one. In the case of nearly total heterogeneity, i.e. H = 1000, nucleic acid molecules smaller than 240 nt are needed for a P/I ratio of at least unity.

Although detectable amounts of nucleic acids were still found in highly purified prion preparations, it appears at present inappropriate to discuss their possible functional relevance; on the contrary, more rigorous procedures for degrading nucleic acids are needed to decide whether the small or heterogeneous nucleic acids described in this work are essential for scrapie infectivity or are non-specific background.

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References


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