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Nucleic Acids in Prion Preparations: Unspecific Background or Essential Component?

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Nucleic acids in prion preparations: unspecific background or essential component?

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SUMMARY

As recently published (Kellings *et al. J. gen Vir.* **73**, 1025–1029 (1992)), the analysis of purified scrapie prions by return refocusing gel electrophoresis revealed remaining nucleic acids in the size range up to 1100 nucleotides. The results defined the possible characteristics of a hypothetical scrapie-specific nucleic acid. If homogeneous in size, such a molecule would be less than 80 nucleotides in length at a particle-to-infectivity ratio (P:I) near unity; if heterogeneous, scrapie-specific nucleic acids would have to include molecules smaller than 240 nucleotides. To decrease the amount of nucleic acids, several modifications of the PrP^{Sc} purification scheme were introduced. Instead of sucrose gradient, ultrafiltration was applied as a purification step and nucleic acids were degraded by Benzonase™ after ultrafiltration, but significant reduction of the P:I ratio could not be achieved. To prevent trapping of nucleic acids in prion rods, nuclease (Benzonase™) was added into the tissue homogenate and incubated at 37°C, overnight. The Benzonase treatment revealed no loss of infectivity, but the whole procedure of nucleic acid analysis did not lead to a reduction of the P:I ratio. In another approach the number of nucleic acid degradations steps was reduced to essentially two steps: Zn^{2+} hydrolysis and Benzonase digestion. Higher Zn^{2+} concentrations and prolonged incubation times resulted in a more efficient nucleic acid degradation. The bioassays yielded complete recovery of infectivity. Large-scale preparations for determining the P:I ratio are still underway.

1. INTRODUCTION

Alper and colleagues (Alper *et al.* 1966, 1967) had proposed in the mid 1960s the heretical idea that the causative agent of scrapie does not depend on an intrinsic nucleic acid moiety for replication, but still today it has not been satisfactorily established whether this is indeed the case or not. 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). Physical or chemical evidence for a scrapie-specific nucleic acid has not been found to date. A recent review (Riesner 1991) was devoted to a critical assessment of the different approaches, which have not as yet provided unequivocal evidence for or against a nucleic acid component. Studies with transgenic mice lacking the PrP-gene have unequivocally shown that the protein component PrP is absolutely necessary for infection and pathogenesis, but they could not rule out a nucleic acid component (Büeler *et al.* 1992, 1993). 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).

In the studies reported here, physico-chemical methods were used to search for a scrapie-specific nucleic acid. These studies could not establish a nucleic acid component to infectivity, but they do put constraints on the size of such a component. The particle (nucleic acid molecule)-to-infectivity ratio (P:I) is determined; all nucleic acid molecules with P:I < 1 are excluded as components to infectivity because infectious units exist without such nucleic acids. On the basis of this quantitative evaluation, hypothetical scrapie-specific nucleic acids larger than 80 nucleotides (nt) can be excluded. Further attempts to achieve higher purification of prions and to analyse the nucleic acids smaller than 80 nt will be described.

2. MATERIALS AND METHODS

(*a*) *Prion purification*

Prion rods from infected hamster were purified by sedimentation and treated with DNase and Zn^{2+},

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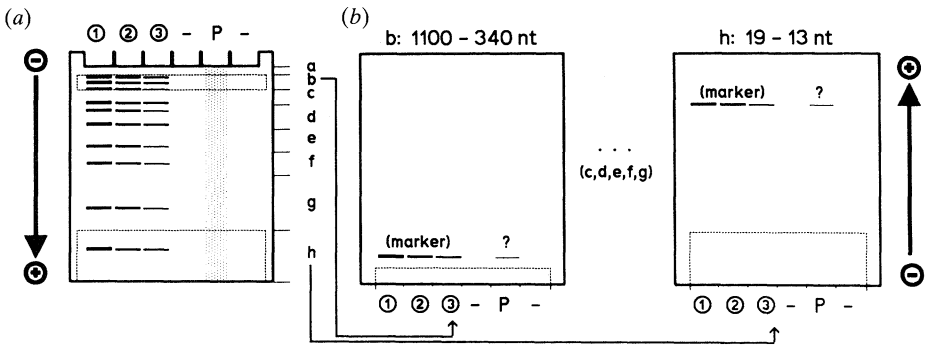

Figure 1. Principle of the return refocusing gel electrophoresis (RRGE). After conventional PAGE (e.g. 100 min, 250 V) heterogeneous nucleic acids are dispersed over the whole length of the lane (lane P in (*a*)). The lane is cut into a few segments (a–h), each corresponding to a well defined range of M_r. The segments are repolymerized into the bottom of new gel matrices (*b*) and a second electrophoresis (250 V) is performed with reversed polarity so that the nucleic acids migrate into the new gel matrix. Because all nucleic acids in a gel segment begin migration from the same position at the beginning of the first PAGE, they meet again after reversal of the polarity of the second electrophoresis if the second run is stopped at a definite time. By adding SDS to the second PAGE, the focusing effect still works for nucleic acids, while other substances such as proteins and polysaccharides remain dispersed. This is a significant advantage since proteins, like nucleic acids, stain with silver. The times of refocusing of different gel segments are chosen to be optimal for the different segments (between 42 and 48 min). The unknown nucleic acid amount (? in (*b*)) of the prion sample is determined by comparison with the nucleic acid markers of known concentrations (markers 1, 2 and 3). Only the two gel segments b and h are given as an example for the refocusing step; gel segment 'a' is not used for refocusing. Figure reproduced from Kellings *et al.* (1992).

treatments which do not reduce infectivity, but do reduce contamination by extraneous host nucleic acids. The prion rods were dispersed into detergent lipid protein complexes (DLPCS) which resulted in retention of infectivity but made nucleic acids, formerly protected in rods, accessible to degradation (Gabizon *et al.* 1987, 1988). DLPCS were digested either with a mixture of DNase, Bal31, and RNase A or a mixture of micrococcal nuclease, alkaline phosphatase, RNase A and phosphodiesterase. After dissociation in SDS and deproteinization by proteinase K treatment and twofold phenol-chloroform-isoamylalcohol (25:24:1) extraction, the preparations were analysed by polyacrylamide gel electrophoresis (PAGE) and return refocusing gel electrophoresis (RRGE), respectively. The details are described elsewhere (Meyer *et al.* 1991; Kellings *et al.* 1992).

(*b*) *Return refocusing gel electrophoresis*

Because heterogeneous nucleic acids would migrate in PAGE as many bands and would be unresolved in a background smear, the method of return refocusing gel electrophoresis (RRGE) was developed. The method is depicted in figure 1, and the details are described in the legend.

3. RESULTS

(*a*) *Analysis of homogeneous nucleic acids by* PAGE

A direct assault on the problem by physico-chemical methods must currently satisfy two basic requirements.

1. It has to be capable of detecting the hypothetical scrapie nucleic acid that might be DNA or RNA, single- or double-stranded, circular or linear, capped, chemically modified or covalently bound to proteins, and homogeneous or heterogeneous in size. It should be emphasized, however, that only conventional nucleic acid chemistry can be considered. Chemically modified nucleic acid, which have not been described as natural compounds so far, and would have unknown chemical properties, will not be considered.

2. It has to be capable of detecting unlabelled nucleic acids in the pg range, calculated as follows: if we take 100 pg as the current limit of nucleic acid detection, 2×10^9 ID_{50} with a hypothetical nucleic acid of 100 nt would contain this amount of nucleic acid, assuming a particle-to-infectivity ratio (P:I) of at least one. A smaller scrapie-specific nucleic acid would require starting with more ID_{50} units, a larger nucleic acid material with fewer ID_{50} units to yield the same amount of the nucleic acid in pg. The requirement for sensitive detection of unlabelled nucleic acids is imposed by the inefficiency of radiolabelling nucleic acids in animals and the low titres of the agent in cell culture.

Polyacrylamide gel electrophoresis (PAGE) combined with silver staining satisfies all of these requirements if the hypothetical nucleic acid is a discrete species. It cannot differentiate, however, between cellular and exogeneous nucleic acids. Those studies have been described in detail by Meyer *et al.* (1991). They showed that the infectious particle does not contain a homogeneous nucleic acid. Within the limits of the bioassay for determination of the infectious units applied, it could be concluded that a homogeneous nucleic acid species larger than 25 nucleotides in length would have been detected.

(*b*) *Analysis 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 as many bands during

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PAGE 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. The estimation of nucleic acid content of the prion sample was carried out by comparison with the marker bands. Control nucleic acids, which were added to the DLPC fraction and treated as described above, were degraded to mono- and di-nucleotides.

Altogether seven independent prion samples were analysed by the procedure described above, with slight modifications. Before the deproteinization/nucleic acid extraction step the infectivities of these preparations were log ID_{50} between 7.8 and 8.7. These infectivities were used *in toto* for one gel electrophoretic analysis. The scrapie infectivity was monitored by an incubation time interval procedure (Prusiner *et al.* 1982) at all steps of the preparation. Separation of the prion rods from the sucrose used for gradient centrifugation resulted in some experiments in a loss of infectivity of 1–3 orders of magnitude, probably as a result of aggregation and denaturation. It is worth noting that the dispersion of ethanol-precipitated prion rods into DLPCs frequently increased the titer more than tenfold.

The yield of nucleic acid after deproteinization was estimated quantitatively by several radiolabelled nucleic acids and amounted in all cases to approximately 90% (Kellings *et al.* 1992).

(*c*) *Ratio of nucleic acids molecules per infectious unit*

Based on the amount of nucleic acid estimated from RRGE and the titres of the prion fractions prior to boiling in SDS, the ratio of nucleic acid molecules to ID_{50} units (P:I) was calculated. If the nucleic acids detected were related to scrapie infectivity, one of two alternative paradigms would be correct. First, a putative scrapie-specific nucleic acid of uniform length might be hidden among an ensemble of background nucleic acid (cf. insert in figure 2). Such a scrapie-specific nucleic acid would not have been detected by conventional PAGE, even if it were present in sufficient amounts. Second, one must consider the possibility of a scrapie-specific polynucleotide which is heterogeneous in length. In figure 2 the numbers of nucleic acid molecules per ID_{50} unit are plotted as a function of their length as estimated from the individual gel sections. In this plot, the calculation was based upon the first paradigm, i.e. a well-defined scrapie-specific nucleic acid among the heterogeneous background nucleic acids. Data from published experiments (Kellings *et al.* 1992) are presented in the plot.

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 to several hundred molecules per ID_{50} unit were estimated. If the scrapie-specific nucleic acid were longer (more than 76 nt), the particle-to-infectivity ratio would fall below unity and continues to drop several orders below unity. Thus, one can safely conclude that a unique nucleic acid of this size range cannot be scrapie-specific. The straight line in figure 2 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.

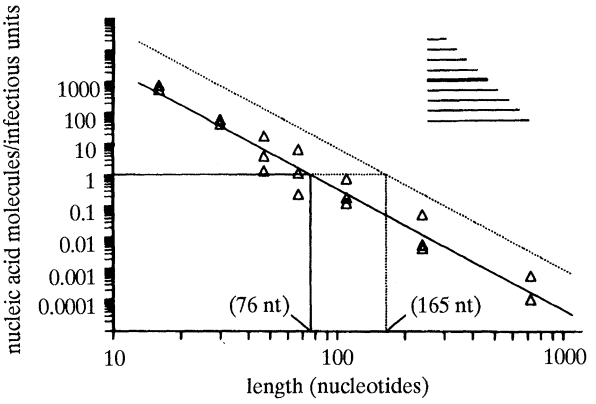

Figure 2. Relationship of P:I ratio to average length of nucleic acid species from determinations on seven independent prion samples. The relationship is linear over the size range of 10–1100 nucleotides with an intercept of about 80 nt for a P:I of unity. Only small nucleic acids of less than 80 nt have P:I > 1. Data (open triangles) were taken from Kellings *et al.* (1992). The relationships were calculated as follows, using fragment e as an example: this fragment contains 450 pg of nucleic acids in the size range of 54–79 nt in one experiment. Assuming a continuous distribution of different sizes, the 26 species in this class will have an average molecular mass of 22×10^3 and there will be:

$$450 \times 10^{-12}\ \text{g} \times \frac{6 \times 10^{23}\ \text{mol}}{26 \times 22 \times 10^3\ \text{g}} = 4.7 \times 10^8\ \text{mol}$$

of a particular size in this ensemble. As the starting sample contained $10^{8.7}$ (5×10^8) ID_{50}, the P:I is approximately 1 for a hypothetical discrete scrapie-specific nucleic acid in the ensemble. Figure modified from Riesner (1991).

A discussion of the maximum error (Kellings *et al.* 1992) assumed one log ID_{50} lower than measured and twofold higher nucleic acid content; this estimation shifts P:I ratio of unity from 76 nt to 165 nt. 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 (cf. Meyer *et al.* 1992); for comparison, 10^4–10^5 PrP molecules are necessary for one infectious unit (Prusiner *et al.* 1982).

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 done after nuclease digestion. One might discuss several cases of heterogeneity, e.g. that all molecules of sizes between 200 nt and 300 nt could act as scrapie genome. It is more interesting, however, to discuss an extreme hypotheti-

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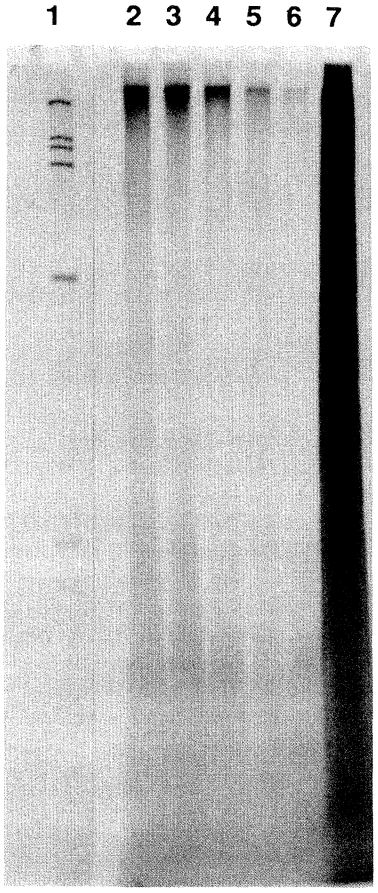

Figure 3. Nucleic acid degradation by Zn^{2+}-hydrolysis. Two hundred micrograms of prion material after ultrafiltration and centrifugation were incubated with Zn^{2+} at 65°C for 24 h, phenol-chloroform extracted, deproteinized (cf. Kellings *et al.* 1992), analysed by 5% polyacrylamide gel electrophoresis, and stained with silver. Lane 1 contains a nucleic acid length marker (1075, 515, 465, 396, 201, 75 and 65 nt), and lane 7 a prion sample without Zn^{2+} as control. Zn^{2+} concentrations in the samples were: lane 2, 4 mM; lane 3, 8 mM; lane 4, 12 mM; lane 5, 16 mM, and lane 6, 20 mM.

cal situation, i.e. total heterogeneity. In that case it was estimated (Kellings *et al.* 1992) that all nucleic acid molecules from 239 nt to infinite lengths, which were detected in prion samples, add up to a P:I ratio of unity. Consequently, even in this unprecedented case, nucleic acid molecules as small as 239 nt act as scrapie genome. Possible functional relevance of the remaining nucleic acids in prions will be discussed below.

(*d*) *Approaches for further purification of prions*

Besides a hypothetical function of the remaining nucleic acid the relationship shown in figure 2 might simply reflect the decreasing efficiency of nuclease degradation with decreasing size of the nucleic acid in combination with the known protective effects of PrP. In this case, the nucleic acids would be mere impurities of cellular nucleic acid in the highly purified preparations without any functional relevance for scrapie infectivity. This possibility would be confirmed experimentally, however, if one could succeed in higher purification of infectious prion material. For this purpose several new purification procedures were applied.

(i) *Replacement of sucrose gradient centrifugation by ultrafiltration*

Sucrose gradient centrifugation for recovery of prions and either EtOH-precipitation or high speed centrifugation had led to irreproducible losses in infectivity. Therefore, prions were separated from components which copurify after the differential centrifugation steps (pellet P3 according to the original preparation scheme (Prusiner 1982)), by ultrafiltration. Bioassays showed that the infectivity could be recovered completely within the limits of accuracy of the bioassay. Thus, all future experiments were carried out applying ultrafiltration.

(ii) *Modifications in nucleic acid degradation procedures*

Different attempts were carried out to further suppress the amount of nucleic acids in the purified prion samples. It was assumed that the residual nucleic acids are of cellular origin and are entrapped in prion rods as a result of the combined treatment of proteinase K and detergent, and partially but not completely released during DLPC formation. It can be inferred from these considerations that the amount of nucleic acids in prion preparations might be lowered only, if degradation of nucleic acids is carried out before formation of prion protein aggregates. Consequently, the Benzonase as a rather strong DNA and RNA degrading enzyme was added to the supernatant after the first centrifugation of the brain homogenate. Infectivity was not lost after this treatment. However, when these samples were used in the purification and RRGE analysis was carried out as in the earlier experiments, no reduction in nucleic acid content but even higher amounts were detected. One has to conclude, that degradation of large nucleic acids in an early stage disfavours strongly the effective purification of prions.

As an alternative, nucleic acid degradation was carried out by adding high concentrations of Zn^{2+}-ions (up to 40 mM) to prion samples after ultrafiltration and incubating the samples for times as long as 72 h at 65°C. Complete recovery of infectivity was obtained. On an analytical scale the nucleic acids in prion samples were more effectively degraded due to the presence of high concentration of Zn^{2+} as compared to Benzonase treatment alone, as judged by conventional PAGE analysis. This result is demonstrated in figure 3.

To avoid losses of infectivity during EtOH precipitations which was a major problem in our earlier work, all precipitation steps were omitted, or replaced by high speed centrifugation, respectively. A combination of ultrafiltration, high speed centrifugations and extensive Zn^{2+}-hydrolysis and avoiding EtOH-precipitation led to a significant reduction of residual nucleic acids after RRGE analysis. The amounts of nucleic acids would lead, however, to firm conclusions only, if they could be related to the infectivity data, which are not yet available. Therefore, we can report here only our present purification protocol, and have

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to leave new results on the amount of residual nucleic acids for a future publication.

4. DISCUSSION

Despite this and many other studies (cf. review by Riesner 1991), the question of whether scrapie infectivity depends on a nucleic acid component or not remains to be settled. No essential nucleic acid has been isolated, but neither have all been excluded. The argument that the physical, chemical and enzymic treatments applied to infectious material constitute evidence against a nucleic acid component is greatly weakened by the demonstration that small nucleic acids (up to several hundred nucleotides) in prion preparations withstand these treatments; and by the tacit assumption in these arguments that a hypothetical scrapie nucleic acid will have properties similar to nucleic acids free in solution, in contact with viral proteins or components of cellular extracts. This assumption may well be invalid as PrP clearly differs from viral and cellular proteins with respect to solubility, resistance against proteinase K and tendency to self-aggregate.

Our approach to exclude a nucleic acid component to infectivity by largely structure-independent methods for detection and quantitation has led to the firm conclusion that a viral nucleic acid of conventional size (e.g. a few thousand nucleotides) is definitely absent, and has defined constraints on acceptable models about smaller nucleic acids. At present the discussion has to rely still on the results of figure 2, whereas the quantitative results from more harsh degradation procedures are not yet available.

1. The hypothetical scrapie-specific nucleic acid might be a well-defined molecular species hidden in the smear of heterogeneous nucleic acids which represent preparative impurities of the sample. In that case, the 'genome' must be about 76 nt or smaller, as longer molecules were not present in concentrations above one molecule per infectious unit. If we assume an order of magnitude error in the bioassay for determining the infectious units and a twofold underestimation of nucleic acid, the limit would be 165 instead of 76 nucleotides chain length. The one sure conclusion is that larger nucleic acids can be excluded. These smaller molecules are none the less of uncertain functional significance since their co-purification with infectivity does not infer that they are essential for infectivity. Speculations about possible functional modes of a small scrapie-specific nucleic acid were outlined in a recent review (Riesner 1991).

2. The scrapie-specific nucleic acid itself might be heterogeneous in size. In this case, more of the detected nucleic molecules would serve as candidate 'genomes' and all the molecules detected in a particular size range have to be counted. In this model, the limit size of the ensemble is no more than 240 nucleotides in a P:I of one. Some observations on viroids raise the possibility that a heterogeneous ensemble of nucleic acids might be capable of information storage and replicative functions. For example, cadang-cadang viroid molecules have been described with variable lengths from 246 to 574 nucleotides, and all were infectious (Haseloff *et al.* 1982). Circular viroids could be converted within the host cell into linear molecules which retain infectivity by cleavage at several different sites in the molecule (Palukaitis & Zaitlin 1987). Infectious viroids could be recovered also from those plants inoculated with two cDNA clones each encoded by different portions of the viroid sequence (Tabler *et al.* 1984).

3. The relationship shown in figure 2 might simply reflect the decreasing efficiency of nuclease degradation with decreasing size of the nucleic acid in combination with the known protective effects of PrP. In this case, the nucleic acids would be mere impurities from cellular origin in the highly purified preparations without any functional relevance for scrapie infectivity. At present, this possibility appears to us most probable, particularly if all other information on the scrapie infectious agent, for example also that from experiments with transgenic animals is included. Our recent experiments in which even more harsh nucleic acid degradation procedures were applied, are directed to the question of a reduction of the P:I ratio, and possibly to rule out completely a nucleic acid component to scrapie infectivity.

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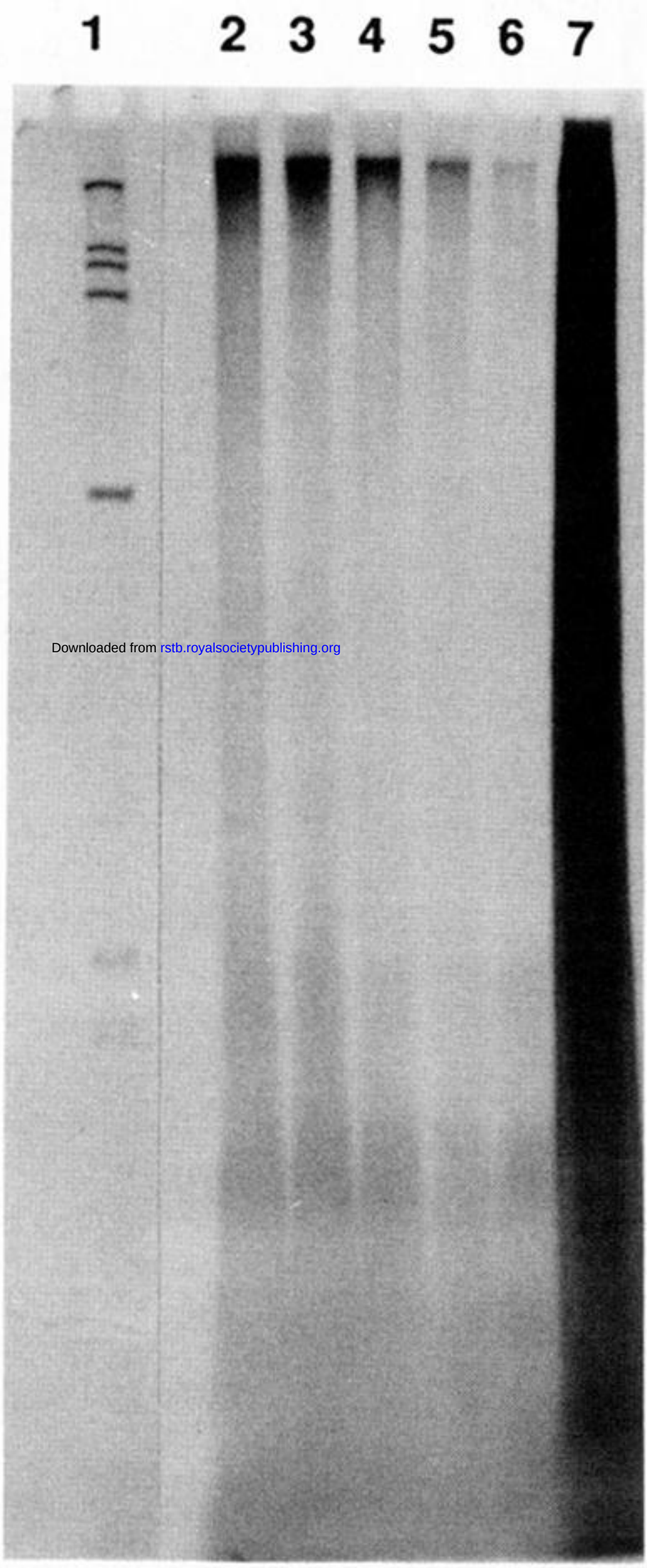

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