Characterization of rabies virus nucleocapsids and recombinant nucleocapsid-like structures

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Rabies virus nucleoprotein (N) was produced in insect cells using the baculovirus expression system described by Préhau et al. (Virology 178, 486–497, 1990). The protein was either purified on a CsCl gradient, resulting in a mixture of nucleocapsid-like structures and beaded rings, as observed by electron microscopy, or on a glycerol gradient that resulted in a preparation of the rings only. The rings and nucleocapsid-like structures had the same morphological characteristics as viral nucleocapsids. N in these structures is an 84 Å long and thin molecule that is spaced at around 34 Å along the length of the nucleocapsid, identical in shape and spacing as the nucleoprotein in nucleocapsids of rabies virus and very similar to those of vesicular stomatitis virus. The recombinant nucleocapsids contained RNA with a stoichiometry similar to that found in viral nucleocapsids. The RNA bound in the beaded rings was a subset of the insect cellular RNA. One of the RNA species was partially sequenced and, although a positive identification could not be made, could correspond to a trRNA. With respect to sensitivity to trypsin and RNase digestion, the recombinant and viral nucleocapsids behaved similar. Trypsin cleaved a 17 kDa fragment from the carboxy terminus of N with only a very small effect on the morphology of the nucleocapsids. RNase A completely digested the resident RNA in both viral and recombinant nucleocapsids into fragments of 4–5 nt long, again with no effect on the morphology of the nucleocapsids. Thus, when the RNA is cleaved, the structure must be maintained by protein–protein contacts. Experiments to remove the resident RNA from viral and recombinant rabies virus nucleocapsids failed, whereas the same methods used to eliminate the RNA from vesicular stomatitis virus nucleocapsids was successful.

Introduction

The first step in the infectious cycle of negative-strand RNA viruses after cell penetration is transcription. Since the infected cells do not have a suitable enzyme for this activity, all viruses in this group carry their own RNA-dependent RNA polymerase (Baltimore et al., 1970). The genome of the negative-strand RNA viruses, whether segmented or not, is encapsidated by a virus-specific nucleoprotein (N). The nucleoprotein–RNA complexes form a scaffold for the binding and activities (transcription and replication) of the various polymerase complexes [three polymerase subunits for the influenza viruses and a single polymerase (L) plus a phosphoprotein cofactor (P) for the non-segmented viruses]. At least for influenza virus (Baudin et al., 1994) and for vesicular stomatitis virus (VSV) (Keene et al., 1981; Emerson, 1987; F. Iseni and others, unpublished) one of the functions of the nucleoprotein seems to be the unwinding of RNA secondary structures and the exposure of the nucleotide bases to the solvent, so that the polymerases can functionally interact with the nucleotide bases directly after cell penetration.

In most cases, the viral nucleocapsids are functional in vitro, either by just opening up the virus by detergent and adding NTPs (plus a primer for influenza virus) or after purification of the nucleocapsids (Baltimore et al., 1970; Leppert et al., 1979; Vidal & Kolakofsky, 1989; Honda et al., 1986; Ishihama et al., 1986). For VSV and Sendai virus it has been possible to separately purify the nucleoprotein–RNA complexes and L plus P (either from virus or from recombinant sources) and recover activity after reconstitution in vitro (Emerson & Wagner, 1972; Emerson & Yu, 1975; De & Banerjee, 1984; Masters & Banerjee, 1986; Helfman & Perrault, 1989; Emerson, 1982, 1987; Canter et al., 1993; Horikami et al., 1992; Curran et al., 1992). These reconstitution experiments suggest that the N–RNA complex should be considered as one of the structural
components of the transcription process, rather than the protein plus the RNA separately. However, in vitro transcription by rabies virus is 100–200 times less efficient than that by VSV (Kawai, 1977). One of the reasons for this may be that it has not yet been possible to efficiently remove the viral M protein from the rabies virus nucleocapsids while retaining the presence and activities of P and L. For VSV it has been shown that M protein inhibits viral transcription (Carroll & Wagner, 1979).

The nucleoproteins of VSV and Sendai virus have been isolated and separated from the viral RNA (Blumberg et al., 1984). The purified N protein from VSV has been used to study the specificity of RNA encapsidation (Blumberg et al., 1983; Moyer et al., 1991). The nucleoprotein of influenza virus has also been isolated and used for binding studies with viral RNA (Baudin et al., 1994). However, it has not yet been possible to remove the RNA from rabies virus nucleocapsids while maintaining the nucleocapsid in an RNA-binding competent form (Kouznetzoff, 1997). In order to study RNA specificity and signals for the start of encapsidation, we attempted to obtain RNA-free recombinant protein. In this paper we describe the purification of recombinant rabies virus N expressed in insect cells. Préaud et al. (1990, 1992) have previously shown that this recombinant protein is phosphorylated as is the viral protein. We found that all N is associated with cellular RNA and we have characterized the recombinant nucleocapsid structures. In terms of morphology, biophysical and biochemical characteristics (sensitivity to protease and RNase digestion) the recombinant structures are very similar to the rabies viral nucleocapsids, which themselves are very similar to the nucleocapsids of VSV. However, whereas we were able to repeat the experiments of Blumberg et al. (1983, 1984) in removing the RNA from VSV N protein, we were not able to do this with the rabies virus protein.

Methods

- **Cells and viruses.** BSR cells, cloned from BHK21 (baby hamster kidney) cells were grown in Eagle’s minimal essential medium supplemented with 10% calf serum. The rabies virus Challenge Virus Standard (CVS) strain was grown in BSR cells and purified from the cell supernatant by differential glycerol gradient centrifugation as described (Gaudin et al., 1992).

  The recombinant baculovirus used in this report, AcNPV/N, expressing the entire nucleoprotein gene of CVS rabies virus, was obtained from C. Préaud (Préaud et al., 1990).

- **Purification of nucleocapsid from rabies virus-infected cells.** Rabies virus nucleocapsids were infected BSR cells. Cells (3.5 x 10^9) were harvested 3 days post-infection and collected in 2 ml of hypotonic buffer (50 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA). The cells were lysed and the supernatant was loaded onto a 20–40% CsCl gradient in 150 mM NaCl, 20 mM Tris–HCl pH 7.4 and centrifuged for 16 h at 30000 r.p.m. at 4 °C in an SW41 rotor. Nucleocapsids were recovered by puncture of the tube adjacent to the visible band and were dialysed against the same buffer without the CsCl.

- **Expression and purification of rabies virus N protein from insect cells.** SF21 (Spodoptera frugiperda) cells were cultivated in TC 100 medium supplemented with 5% calf serum. Flasks containing 1 x 10^7 cells were infected with AcNPV at an m.o.i. of 4 and incubated for 4 days. Infected cells were collected by low-speed centrifugation and resuspended in 2 ml of hypotonic buffer. After lysis of the infected cells by freezing and thawing three times, the supernatant was recovered after centrifugation at 12000 g for 15 min at 4 °C. Rabies N protein was then purified in two independent ways. The first purification protocol was by loading the supernatant after cell lysis on a preformed 20–40% CsCl gradient using the same conditions as described above for isolation of viral nucleocapsids. The N-containing band was dialysed against 150 mM NaCl, 20 mM Tris–HCl pH 7.4 and stored at 4 °C. The second way of purifying N was adapted from Préaud et al. (1990). The lysed cell supernatant was loaded on a 5–20% glycerol gradient containing 1 M NaCl and 10 mM Tris–HCl pH 7.4 and centrifuged for 15 h at 32000 r.p.m. in an SW41 rotor. The nucleoprotein was recovered from a slow sedimenting band near the bottom half of the tube and dialysed as described above. For both purification methods the fractions containing N were controlled by 10% SDS–PAGE.

- **RNase and trypsin treatments of viral and recombinant nucleocapsids.** About 100 µg of purified viral nucleocapsids or insect cell-expressed nucleocapsid-like structures were digested with either micrococcal nuclease (20 µg/ml) or RNase A (100 µg/ml) for 30–60 min at 37 °C. The digestion products were extracted by phenol–chloroform and precipitated in 100% ethanol. The products were separated by 8% urea–PAGE and stained with ethidium bromide. Trypsin digestion was done with 150–200 µg nucleocapsids and a final trypsin concentration of 100 µg/ml in 150 mM NaCl, 10 mM Tris–HCl pH 7.5 and incubation for 1 h at 37 °C.

- **Radioactive end-labelling of RNA.** RNAs were purified from recombinant nucleocapsid-like structures or insect cells by phenol–chloroform extraction, 3’ end-labelled with [32P]pCp and T4 RNA Ligase according to England et al. (1980) and analysed by 12% urea–PAGE.

- **RNA sequencing.** After autoradiography, the upper RNA band in Fig. 2, lane 2 was excised, eluted according to Maxam & Gilbert (1977), precipitated with ethanol in the presence of 20 µg glycogen as carrier, dissolved in 10 µl water and sequenced using several ribonucleases. For each sequence lane between 20000 and 50000 c.p.m. were used. Digestion was done with RNase T1 (0–5 U) for G; RNase U2 (0–5 U) for A; RNase PhoM (1 U) for A and U; and B. cereus RNase (1–25 U) for C and U. Incubation was at 55 °C for 15 min in 20 mM citrate buffer pH 4.5, 1 mM EDTA, in the presence of 8 M urea for RNases T1, U2, PhoM and in the absence of urea for B. cereus RNase. The nucleotide ladder was made in 0.5 M NaHCO3 pH 9.5 and heating at 95 °C for 3 min. Analysis of the digests was carried out by electrophoresis on 12% polyacrylamide–8 M urea gel.

- **Electron microscopy (EM).** Samples for electron microscopy, about 0.1 mg/ml, were adsorbed onto the clean side of carbon film on mica and negatively stained with 1% sodium silicotungstate. After air-drying, the samples were immediately placed in the microscope (JEOL 1200 EXII) and pictures were taken under low-dose conditions. Measurements were made from prints with a final magnification of 150–17000 using an ocular eye-piece giving additional magnification of 8 x. The magnification was calibrated with crystals of catalase.

Results

Morphology of viral and recombinant nucleocapsids

When rabies virus N protein produced in insect cells was isolated by centrifugation of the lysed cell supernatant over a
Rabies virus nucleocapsids

Fig. 1. Electron micrographs of rabies virus nucleocapsids and recombinant nucleocapsid-like structures, negatively stained with 1% sodium silicotungstate. The bar represents 500 Å. (A) Recombinant nucleocapsid-like structures isolated by CsCl-gradient equilibrium density centrifugation; (B) Viral nucleocapsids and (C) recombinant nucleocapsid rings isolated by glycerol gradient sedimentation velocity centrifugation.

CsCl gradient, this resulted in a band in the gradient at the same position as that of the band of rabies virus nucleocapsids isolated from infected cells. The material in this band was studied by negative-stain EM and showed nucleocapsid-like structures as well as some rings (Fig. 1 A). For comparison, Fig. 1(B) shows rabies virus nucleocapsids isolated by CsCl gradient centrifugation from lysed infected cells. It can be seen that the nucleocapsids form coils with a rather uniform diameter. The dimensions of the N protein monomers in the viral nucleocapsids and in the recombinant nucleocapsid-like
structures were measured from electron micrographs. Because the nucleocapsids are helically coiled, four dimensions could be reliably measured: 1, the maximal length of the N protein monomer when it is lying with its longest dimension flat on the carbon support film; 2, the height of N when N film shows an imprint of its side; 3, the spacing of the monomers along the coil; and 4, the diameter of the coil. The values of the dimensions are given in Table 1 and suggest that N is a thin, long and thin but sometimes N was clearly seen to have a bi-lobed face, or that N can have two conformations. The width of the N monomer when it is lying with its longest dimension flat on the carbon support film; 2, the height of N when N film shows an imprint of its side; 3, the spacing of the monomers along the coil; and 4, the diameter of the coil. The values of the dimensions are given in Table 1 and suggest that N is a thin, long molecule of about 84 Å long (1 Å = 0.1 nm) and 53 Å high and with a spacing of 34–35 Å. The width of the N monomer was not measured since the value is too close to the resolution of the micrographs, but an estimate from the images would suggest a value between 20–30 Å. Sometimes N was just long and thin but sometimes N was clearly seen to have a bi-lobed appearance, like N of VSV (see Thomas et al., 1985). This could either mean that N has two faces, a featureless and a bi-lobed face, or that N can have two conformations. The diameter of the nucleocapsid coil was found to be rather constant with a value of 220 Å. It is clear from Table 1 that the dimensions of the N monomers and of the coil diameter of the viral and recombinant nucleocapsids are the same. In Table 1 we also give some dimensions of VSV N protein from the literature which show that VSV N and rabies virus N are very similar in overall dimensions although they show only 18% amino acid identity.

When rabies virus N protein was purified according to the modified method of Préhaut et al. (1990), by sedimentation over a salt-containing 5–20% glycerol gradient, most of N was pelleted to the bottom of the tube but another band containing N was found to sediment in the lower half of the gradient as shown in Fig. 5 in Préhaut et al. (1990). This band was well separated from the cellular proteins and its exact position depended on the time of centrifugation. When the material in this band was studied by EM it appeared to consist of little rings with a beaded appearance (Fig. 1C). We measured the size of the monomer in the rings and found it to be the same as the height of the monomers in the nucleocapsids (Table 1), so the rings probably correspond to very short nucleocapsids standing on their sides, with the N monomers standing upright. Although we never analysed the pelleted material, it is likely that the pellet contained the longer nucleocapsid-like structures shown in Fig. 1(A). Co-sedimentation of the recombinant rings and the longer nucleocapsid-like structures in the CsCl gradient suggests that they have the same composition and that the glycerol gradient allowed enrichment of the ring fraction of the material.

### Protein–RNA stoichiometry in recombinant nucleocapsids

The way the rings are purified is very similar to the purification method of encapsidated leader RNA from VSV-infected cells, which were found to sediment with a sedimentation coefficient of 18S on sucrose gradients whereas intact nucleocapsids with a coefficient of 150S were pelleted in the sucrose gradient (Blumberg & Kolakofsky, 1981). This suggests that the recombinant rings studied here contain RNA,

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**Table 1. Dimensions of rabies virus N protein in viral nucleocapsids and in recombinant nucleocapsid-like structures – comparison with literature data on VSV**

Dimensions are in Å (1 Å = 0.1 nm), with the number of measurements in parentheses.

<table>
<thead>
<tr>
<th>Rabies nucleocapsids</th>
<th>Length</th>
<th>Height</th>
<th>N–N spacing</th>
<th>Diameter of coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleocapsids</td>
<td>84±5 Å (68)</td>
<td>53±5 Å (51)</td>
<td>35±3 Å (42)</td>
<td>218±20 Å (40)</td>
</tr>
<tr>
<td>Idem after trypsin treatment</td>
<td>84±4 Å (40)</td>
<td>47±5 Å (53)</td>
<td>33±2 Å (29)</td>
<td>182±20 Å (72)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recombinant nucleocapsid-like structures</th>
<th>Length</th>
<th>Height</th>
<th>N–N spacing</th>
<th>Diameter of coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rings (glycerol)*</td>
<td>–</td>
<td>53±3 Å (42)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nucleocapsid-like (CsCl)†</td>
<td>84±5 Å (44)</td>
<td>53±4 Å (36)</td>
<td>34±1 Å (26)</td>
<td>216±22 Å (44)</td>
</tr>
<tr>
<td>Idem after trypsin treatment</td>
<td>84±3 Å (27)</td>
<td>48±4 Å (51)</td>
<td>34±2 Å (35)</td>
<td>209±28 Å (49)</td>
</tr>
<tr>
<td>Idem after RNase A treatment</td>
<td>86±4 Å (42)</td>
<td>52±3 Å (38)</td>
<td>34±1 Å (28)</td>
<td>240±42 Å (41)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VSV nucleocapsids</th>
<th>Length</th>
<th>Height</th>
<th>N–N spacing</th>
<th>Diameter of coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Literature values</td>
<td>90–100 Å</td>
<td>–</td>
<td>35 Å</td>
<td>–</td>
</tr>
<tr>
<td>Nakai &amp; Howatson (1968)</td>
<td>90 Å</td>
<td>50 Å</td>
<td>33 Å</td>
<td>–</td>
</tr>
<tr>
<td>Thomas et al. (1985)</td>
<td>90 Å</td>
<td>50 Å</td>
<td>33 Å</td>
<td>–</td>
</tr>
</tbody>
</table>

* Recombinant rings isolated by glycerol gradient centrifugation.
† Recombinant nucleocapsids isolated by CsCl gradient centrifugation.
Rabies virus nucleocapsids

Fig. 2. Characterization of the recombinant nucleocapsid rings shown in Fig. 1(C). (A) The RNA in the rings was extracted, radioactively end-labelled and analysed on 12% PAGE and revealed by autoradiography. Lane 1, 87 nt RNA size marker. Lane 2, RNA extracted from ring structures. Lanes 3 and 4, total RNA extracted from infected insect cells (lane 4 is a longer exposure of lane 3). The RNA pattern from infected and non-infected insect cells was the same. (B) number of N monomers per ring as counted from electron micrographs as shown in Fig. 1(C). The monomer count and RNA analysis were done on the same preparation of rings.

in agreement with the fact that the viral nucleocapsids and both the rings and the longer nucleocapsid-like structures were found at the same density in the CsCl gradient. Therefore, we measured the RNA content of the recombinant samples. The UV light absorption spectra of viral nucleocapsids, CsCl gradient-purified recombinant nucleocapsid-like structures and glycerol gradient-purified recombinant rings were very similar and all showed an absorption peak at 260 nm, with a 260/280 nm absorption ratio of 1.22 for the viral material and 1.24 for the recombinant material. This ratio is indicative of a significant RNA content in all samples. For comparison, influenza virus pure nucleoprotein (no RNA) showed a 260/280 nm ratio of 0.59 whereas the viral ribonucleoprotein particles (which contain 20–25 nucleotides per nucleoprotein monomer) had a ratio of 1.55 (Ruigrok & Baudin, 1995). Pure RNA has a ratio of 1.80. RNA was isolated from the recombinant material by phenol–chloroform extraction and analysed on 8% urea–PAGE stained with ethidium bromide. RNA from the CsCl-purified samples ran as a smear on the gel with several smaller bands around 100 nt (see Fig. 5A). However, RNA extracted from the ring fraction and subsequently 3' end-labelled with [32P]pCp ran as a collection of several closely spaced bands. The longest RNA ran with the same mobility as an 87 nt standard RNA and the shorter bands were estimated to be around 80 nt long (Fig. 2A). Upon further exposure of the gel, other minor bands were visible as well. The number of N monomers per ring in the sample that was used for RNA extraction was counted on electron micrographs and found to be 10 ± 1 (Fig. 2B). The determined length of bound RNA and number of N monomers per ring would suggest a stoichiometry of 8 or 9 nucleotides of RNA per N monomer. Obviously, since the recombinant structures are not the ‘natural’ structures, this value depends on the number of non-saturated positions on either the protein or on the RNA. A stoichiometry of 9 nt per N monomer has been calculated for VSV nucleocapsids (Thomas et al., 1985) and, in analogy with the case for VSV, the same stoichiometry has been derived for rabies virus nucleocapsids (Flamand et al., 1993).

When total RNA was isolated from the infected and uninfected insect cells, it appeared that the major RNA components in the cell are also 80–90 nt long, probably consisting of the collection of tRNAs in the cell. However, comparison of total cell RNAs and the bands encapsidated by recombinant N (compare lanes 3 and 2 respectively in Fig. 2A) seems to suggest that recombinant N selects a subset of cellular RNAs.

Sequence of one of the RNAs encapsidated by recombinant rabies virus N

The fact that specific lengths of encapsidated RNAs are found could either mean specific encapsidation of a subset of RNAs or could be due to RNase trimming of overhanging RNA not protected by N, which would suggest non-specific encapsidation. In the latter case it would be possible that a mechanical stabilization of the rings would favour protection of a certain length of RNA. In order to study this, we cut the upper band shown in Fig. 2(A) (lane 2) out of the gel, eluted it and sequenced it using a set of RNases specific for G, A, A+U or C+U (see Fig. 3). From the sequencing reaction it was clear that the upper band contained a unique sequence. The same sequence was found in four independent samples. At some positions the RNases could not cleave the RNA, indicated by X in Fig. 3, possibly because of modification of the nucleotide.
Fig. 3. Sequence analysis of the 87 nt RNA extracted from the nucleocapsid-like rings. Left and right, long and short migration respectively of sequencing gels showing the determined sequence. Capital letters indicate residues which are certain and which were also found in other sequencing gels which are not shown here; lower case indicates that the nucleotide base is probably as indicated but with less certainty; nd stands for not determined since a band showed up in the control lane (Ct), probably meaning a cut in a sub-population of the RNA; X means that the nucleotide showed up in the ladder (L) but was not cut by any of the RNases, possibly meaning that the nucleotide was modified. L, nucleotide ladder; Ct, control of untreated RNA; C/U, A/U, G and A indicate the cuts induced by RNases specific for the respective nucleotides.

Characterization of recombinant nucleocapsids by digestion with trypsin and RNases

The viral and recombinant nucleocapsids were compared in trypsin and RNase digestion experiments. Extensive trypsin digestion of the two types of nucleocapsid resulted in digestion base, which would agree with the RNA being a tRNA. Although we have looked in all available databases, we have not been able to identify the sequence. The lower band in Fig. 2(A), at around 80 nt, is in fact a collection of at least three bands and in agreement with this we could not get a unique sequence from these lower bands (not shown).
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Fig. 4. Trypsin digest of viral and recombinant nucleocapsids. (A) SDS–PAGE of intact and trypsin-digested recombinant and viral nucleocapsids. Lanes 2 and 5, undigested; lanes 3 and 6, digested with trypsin. Lanes 1 and 4 are molecular mass markers. (B, C) Electron micrographs of negatively stained viral (B) and CsCl density gradient isolated recombinant nucleocapsids (C) after trypsin digestion. The bar represents 500 Å.

of N to the same 42 kDa trypsin-resistant fragment (Fig. 4A). For viral nucleocapsids this cleavage occurs at lysine-376 (Kouznetzoff et al., 1998). However, whereas cleavage of the viral nucleocapsids was always complete, the recombinant nucleocapsids were never completely digested since there always remained a small band of undigested material (Fig. 4A, lane 3). The reason for this is not known. The cleaved viral and recombinant nucleocapsids were studied by EM (Fig. 4 B and C respectively) and the dimensions of N were measured (Table 1). The cleavage had no effect on the maximal length or on the spacing of N but the height of N was somewhat smaller. In fact, the distribution of measured values for the height of cleaved N had become broader compared to the distribution of heights of intact N, with a skew towards smaller values (not shown), which could suggest that one end of the long N molecule had become thinner but not the other end. Fig. 4(B) (right panel) also shows a nucleocapsid that was pulled out during preparation for EM and which clearly shows the morphology of the cleaved N monomer.

The RNase sensitivity of the resident RNA in viral and recombinant nucleocapsids was compared by treatment with micrococcal nuclease and RNase A (Fig. 5A). Both types of nucleocapsid were totally resistant to micrococcal nuclease treatment, in contrast to the control with naked U6 RNA which was digested. High concentrations of RNase A (100 µg/ml), however, led to total digestion of the RNA in both types of nucleocapsid. The digested RNA from the recombinant nucleocapsids was extracted with phenol–chloroform, 3’ end-labelled with [32P]pCp and analysed on 15% urea-PAGE. The autoradiograph in Fig. 5(B) shows the result of the cleavage reaction compared with the nucleotide ladder of a 16 nt oligoribonucleotide size marker. The longest protected RNA fragment was 9 nt long but most of the RNA inside the nucleocapsids had been digested to fragments.
Fig. 5. RNase digestion of CsCl-gradient isolated recombinant and viral nucleocapsids. (A) Ethidium bromide-stained PAGE of nucleocapsids digested with micrococcal nuclease or with RNase A as indicated. As a control for digestion of naked RNA we used a preparation of U6 RNA from snRNP. (B) Autoradiogram of a gel showing a 16 nt size marker (left lane) and 3’ labelled RNA extracted from recombinant nucleocapsids extensively treated with 100 µg RNase A/ml, as shown in lane 6 in panel A. (C) Electron micrograph of negatively stained recombinant nucleocapsids after extensive RNase A digestion.

4–7 nt long. The RNase A-digested nucleocapsids were then run over a CsCl gradient; the N-containing fractions had their absorption peak between 260 and 270 nm and an $A_{260}/A_{280}$ ratio of 1.12. This suggests that the nucleocapsids had lost some RNA but not all and the most appealing explanation would be that the protected RNA fragments were still bound to N. The digested nucleocapsids were also analysed by EM (Fig. 5C, Table 1). The size of the N monomers and the spacing
of N along the length of the nucleocapsids showed no differences from the corresponding values of the non-digested recombinant nucleocapsids.

**Attempts to remove the RNA from the recombinant nucleocapsids**

The above experiment shows that RNase digestion is not sufficient to remove the RNA from the nucleocapsids. We tried a number of other methods to remove the RNA. The results in this section are described but the data are not shown in figures since all methods failed to take the RNA off the protein. First, we dialysed the complexes to 7 M Urea and then bound the dialysed complexes to an FPLC Mono-Q column (Bio-Rad) also equilibrated with 7 M Urea but in an otherwise low-salt buffer. The material was eluted with the same urea buffer containing a KCl gradient up to 1 M salt and analysed by SDS–PAGE and spectrophotometry. The N protein-containing fractions still had an absorption spectrum with a peak at 260 nm, indicating the presence of RNA. We performed the method described for purification of influenza nucleoprotein (Ruigrok & Baudin, 1995), consisting of a combined glycerol–CsCl step gradient, also without success.

Fu et al. (1991) have described a purification method of rabies virus recombinant N by affinity chromatography and elution with pH 11 ethanolamine. In a later paper, they describe the use of this purified N in RNA encapsidation experiments (Yang et al., 1998). Since treatment of RNA with high pH may lead to hydrolysis, the described pH 11 elution could have been a way to hydrolyse and remove the RNA from the recombinant nucleocapsids. However, after dialysis of the recombinant nucleocapsids against 0.15 M ethanolamine pH 11 at 4 °C overnight and subsequent purification on a CsCl gradient, the banded material still had its absorption peak between 260–270 nm with a 260/280 nm ratio of 1.18, only slightly less than that of viral nucleocapsids. When the RNA was extracted from the banded material and compared on a gel with the extracted RNA of the starting recombinant material, there was no apparent difference in the size of the RNA bands, i.e. the pH 11 treatment had not apparently resulted in hydrolysis of the RNA and, therefore, the RNA was not removed in the CsCl gradient. In fact, a control experiment we found that overnight dialysis against at pH 11 did not lead to hydrolysis of naked RNA at 4 °C, and to only light hydrolysis at room temperature.

Finally, we applied the method described for taking the RNA off VSV nucleocapsids as described in Blumberg et al. (1983, 1984) using CsCl gradients containing 3 M guanidinium chloride, in parallel on VSV nucleocapsids and on viral and recombinant rabies virus nucleocapsids. For VSV the method worked as described (Blumberg et al., 1984) with a band of VSV protein halfway the gradient and virtually all the RNA in the pellet. For rabies virus nucleocapsids, however, the method did not work. All the CsCl gradient fractions were dialysed against 1 M NaCl as described in Blumberg et al. (1984). Although we could not properly measure the absorption spectrum of the banded material, probably due to aggregation, RNA extraction of all the fractions and analysis on gel (ethidium bromide staining) clearly showed that all the RNA was found in the fractions that contained the N protein, and none was found in the pellet.

**Discussion**

Many groups have studied the expression and polymerization behaviour of VSV and rabies virus N protein in infected cells, in in vitro translation experiments and in baculovirus expression systems. There is general agreement that the phosphoprotein (P) keeps N in a soluble form, able to support replication (Davis et al., 1986; Peluso, 1988; Masters & Banerjee, 1988a, b; Peluso et al., 1988; Howard & Wertz, 1989; Préhau et al., 1992). Most of these authors did not try to determine the RNA content of the produced nucleoprotein and seem to suggest that, in the absence of P, N expressed alone forms self-polymers without RNA. Masters & Banerjee (1988b), however, have shown that N expressed alone does form N–RNA complexes. Using glycerald gradient separation, Préhau et al. (1992) showed that in the absence of co-expressed P, all baculovirus-expressed rabies virus N entered into the gradient at the position where we found the rings, and that there was no soluble protein at the top of the gradient where the soluble cellular proteins are found. Combined with the results shown here, this suggests that all recombinant rabies virus N is bound to RNA with the same stoichiometry as found in viral nucleocapsids and that, at least in the system used here, polymerization accompanies RNA binding.

In virtually all aspects the recombinant nucleocapsid-like structures were indistinguishable from viral nucleocapsids. This means that N alone possesses all the information needed to make a nucleocapsid. Cleavage of the covalent RNA backbone of the nucleocapsid had no effect on the structure of the nucleocapsids so N–N interactions must form a major component of the energy holding nucleocapsids together. Since there do not appear to be major fractions of RNA-free N-monomers or -polymers, it is possible that binding of N to RNA induces a polymerization-competent conformation of N. When our results are extrapolated to the viral infection process where P keeps N in a soluble, RNA-free form, it could be possible that interaction of P with N in the soluble N–P complex masks not only the RNA-binding site on N but also a component of the N–N polymerization interface. Removal of P, possibly through contact with the actively replicating viral polymerase, would allowing binding of N to the RNA and to the neighbouring N on the nascent N–RNA complex.

Recently, Yang et al. (1998) described the sequence specificity of encapsidation of leader RNA by recombinant rabies virus N. The material they used was similar to what we used here and our results with the pH 11 treatment suggest
that the protein used by Yang et al. for RNA binding still contained resident insect RNA. Binding of RNA to RNA containing viral nucleocapsids has also been described by A. Kouznetzoff and co-workers (Kouznetzoff, 1997; Kouznetzoff et al., 1998) who also showed sequence specificity of binding. Both groups were only able to bind about 2% of total RNA to the nucleocapsids. We have performed filter binding experiments with the recombinant rings and rabies virus leader RNA and were able to bind 5–10% of the added RNA to the RNA-containing rings (not shown). We do not know how the incoming RNA binds to the RNA-containing nucleocapsids. Yang et al. (1998) suggest that the fixed incoming RNA is protected against RNase digestion whereas Masters & Banerjee (1988b) have shown that exogenous RNA can bind to RNA-containing VSV nucleocapsids but that the incoming RNA is not protected against RNase digestion like the resident RNA.

We have determined one of the insect RNA sequences that bound to the recombinant N (Fig. 3). Although we do not have the full sequence of the extreme 5' end, it seems to contain some C and G residues but only few A residues. The determined sequence is apparently not similar to the 5' end of the antigenome, the leader sequence that was found to bind specifically to N in a nucleocapsid and, in the case of VSV, was found to bind 10 times better to RNA-free N than other RNAs (Blumberg et al., 1983). It is not rich in A residues, 28% compared to 50–60% in the rabies virus and VSV leader sequences and, in relation with this, contained no poly(A) sequences, which have been found to bind efficiently to rabies virus nucleocapsids (Kouznetzoff, 1997; Yang et al., 1998). Finally, there was no homology with the N mRNA, which is probably one of the most strongly expressed mRNAs in the baculovirus-infected cell. For the moment, we do not know why this sequence was encapsidated by the recombinant N.

It is common knowledge that the RNA inside rhabdovirus nucleocapsids is protected against digestion by RNases. This appeared to be true for treatment with micrococcal nuclease but not for high concentrations of RNase A. Masters & Banerjee (1988b) have already shown that 25 µg/ml RNase A can digest RNA encapsidated by \textit{in vitro} translated VSV N and, depending on the conditions, they found protection of fragments between 18–30 nt long or between 9–18 nt, in agreement with a stoichiometry of 9 nt per N monomer. Here, we treated with 100 µg/ml RNase A and the protected fragments were mainly 4–7 nt long, the longest being 9 nt. This result is also in agreement with the 9 nt/N monomer stoichiometry but further suggests that not all nucleotides that are bound to N are equally protected against RNase A digestion.

Finally, it may seem surprising why rabies virus N is so much more tightly bound to RNA than VSV N. We have not been able to remove the RNA from N other than by drastic denaturation of N, like by heating in the presence of 1% SDS, showing that there are no covalent links between N and the RNA. There may be biological reasons for this difference in stability: the infection process of VSV is much faster in every aspect than that of rabies virus and the infection of and transport in neurons may require a particularly high stability of the rabies virus nucleocapsid. On the other hand, since the rhabdoviral nucleocapsids do not undergo decapsidation during the infection process, there is no reason why N should be able to release the bound RNA through \textit{in vitro} biochemical treatment.

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