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Isolation and Characterisation of the Rabies Virus N°-P Complex Produced in Insect Cells

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When the nucleoprotein (N) of nonsegmented negative-strand RNA viruses is expressed in insect cells, it binds to cellular RNA and forms N-RNA complexes just like viral nucleocapsids. However, in virus-infected cells, N is prevented from binding to cellular RNA because a soluble complex is formed between N and the viral phosphoprotein (P), the N°-P complex. N is only released from this complex for binding to newly made viral or complementary RNA. We coexpressed rabies virus N and P proteins in insect cells and purified the N°-P complex. Characterisation by gel filtration, polyacrylamide gel electrophoresis, analytical ultracentrifugation, native mass spectroscopy, and electron microscopy showed that the complex consists of one N protein plus two P proteins, i.e., an N°-P_2 complex.

INTRODUCTION

The template for transcription and replication of negative-strand RNA viruses is not a naked nucleic acid but an N-RNA structure in which the viral RNA (vRNA) is tightly associated with the viral nucleoprotein (N; 50.7 kDa for rabies virus). Transcription signals encoded in the vRNA are only recognised by the specific viral RNA-dependent RNA polymerase when the vRNA is bound to N (Emerson, 1987; Honda *et al.,* 1988; Iseni *et al.,* 2002).

The nonsegmented negative-strand RNA viruses, similar to the rhabdoviruses and the paramyxoviruses, code for a phosphoprotein (P; 33.6 kDa for rabies virus) that is both a polymerase cofactor and a chaperone for newly produced nucleoprotein that is not yet bound to vRNA. As a polymerase cofactor P binds the polymerase to the N-RNA (Mellon and Emerson, 1978; Horikami *et al.,* 1992; Hamaguchi *et al.,* 1983; Portner *et al.,* 1988; Curran *et al.,* 1994; Smallwood *et al.,* 1994). It is likely that the P protein that acts as a polymerase cofactor is in an oligomeric state (Gao and Lenard, 1995) and it has been suggested that phosphorylation of vesicular stomatitis virus (VSV) P has an influence on its polymerisation state and on its activity in transcription (Gao *et al.,* 1996; Spadafora *et al.,* 1996). Although there is still some discussion about the oligomeric state of rhabdovirus P (Gao and Lenard, 1995; Gao *et al.,* 1996; Gigant *et al.,* 2000), the oligomerisation domain of Sendai virus (paramyxovirus) P was recently crystallised and found to be a tetramer (Tarbouriech *et al.,* 2000a,b).

When N is expressed alone in eukaryotic cells, it binds nonspecifically to cellular RNAs. We have expressed rabies virus, Sendai virus, measles virus, and Marburg virus nucleoproteins alone in insect cells and all N that could be isolated from these cells was found in N-RNA structures (Iseni *et al.,* 1998; Schoehn *et al.,* 2001; Mavrakis *et al.,* 2002; M. Mavrakis, G. Schoehn, and R. W. H. Ruigrok, unpublished results). For recombinant rabies virus N-RNA the stoichiometry of N to RNA was found to be the same as that in viral N-RNA (Iseni *et al.,* 1998). However, in rhabdovirus-infected cells virtually all the N binds only to newly produced viral genomic RNA, but not to viral mRNA nor to cellular RNAs. This is because in infected cells the phosphoprotein binds as a chaperone to the nucleoprotein and prevents binding of N to mRNA or cellular RNA. The phosphoprotein only releases the nucleoprotein for binding to newly formed vRNA (or cRNA), possibly by a signal from the viral RNA-dependent RNA polymerase.

When VSV N and P are coexpressed *in vitro*, a number of complexes containing N and P are formed with different N:P ratios (Davies *et al.,* 1986; Masters and Banerjee, 1988a; Peluso, 1988; Howard and Wertz, 1989). However, of all the different complexes, only the complex with an N:P ratio of 1:1 was able to support viral replication, so this complex seems to be the only complex that keeps N in a soluble state that can then be recruited into newly made viral N-RNA (Masters and Banerjee, 1988b; Peluso,

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1988; Peluso and Moyer, 1988). This soluble form of N is also called N° and the 1:1 complex N°-P. Although there has not yet been an extensive analysis of the structure of the VSV N°-P complex, the sedimentation behaviour in a sucrose gradient combined with a Stokes' radius derived from gel filtration data suggested a molecular weight of the complex of 85 kDa (Peluso and Moyer, 1988). The sedimentation behaviour also suggested an extended or elongated shape of the complex.

In this article we describe the coexpression of rabies virus N and P proteins in insect cells and the purification of the N°-P complex. By analytical centrifugation we could show that there exists only a single N°-P complex. By native mass spectroscopy we were able to measure the exact weight of the complex, which corresponds to one molecule of N plus two molecules of P. This conclusion was confirmed by negative-stain electron microscopy that showed a strong stain excluding spot (probably N) attached to two thin tails (probably two P monomers or a P dimer).

RESULTS

In the first instance we expressed non-His-tagged N plus P in insect cells. After initial purification using an anion exchange column (DEAE Sepharose CL-6B) and ammonium sulfate precipitation, the fractions containing the N and P proteins were run over a gel filtration column (Hiload 16/60 Superdex 200). Initially the bands for N and P were identified by Western blots using anti-N and anti-P monoclonal antibodies (not shown). The elution profile from the Superdex column (Fig. 1A) shows two main peaks at 47 and 65/67 ml of the elution volume. Analysis of the fractions of the first peak (47 ml) by SDS–PAGE showed that they contained more N than P (Fig. 1B) and the absorption spectrum shows an absorbance maximum at 260 nm, indicating the presence of nucleic acid (Fig. 1C left; A260/280 ratio of 1.31). When N is expressed alone in insect cells, the protein binds to cellular RNAs and forms N-RNA rings and longer N-RNA coils (Iseni *et al.,* 1998). When the 47-ml fraction was examined with EM (Fig. 2), we indeed observed the N-RNA rings described before (Iseni *et al.,* 1998; Schoehn *et al.,* 2001). However, in contrast to the N-RNA rings produced during expression of N alone, the N-RNA structures produced during N-P coexpression contained additional material inside or outside of the ring that we interpreted as P protein bound to the N-RNA (Schoehn *et al.,* 2001). The 65/67-ml peak seems to be made up by two separate peaks and contains more P than N. The gel in Fig. 1B (right) shows that the maxima for N and P do not coincide; the maximum for N is around 65 ml and the maximum for P is around 67 ml. The absorption spectrum shows that the sample at 65 ml contained little or no nucleic acid (Fig. 1C, right; A260/280 ratio of 0.71).

We then expressed P alone in insect cells and followed the same purification procedure as described above. P alone eluted with an elution volume of 67 ml (Fig. 3A). Analysis of the fractions of this peak by SDS–PAGE is shown in Fig. 3B. Since we expected that the 65/67-ml peak of the coexpression experiment contained the N°-P complex with a molecular weight at least that of the sum of one N (50.7 kDa) plus one P (33.6 kDa), it is surprising that P alone migrates so closely to the N°-P complex. Therefore, we tested the oligomerisation state of P. Chemical cross-linking with EGS showed that all of the protein was in an oligomeric form (Fig. 3C), as did analysis by analytical centrifugation (not shown). However, neither of the two experimental approaches gave a clear answer on whether the oligomer is a trimer or a tetramer. The results that we obtained were very similar to those published by Gigant *et al.* (2000), who analysed rabies virus P protein produced in *Escherichia coli*. Note that neither the bacterially produced P nor the P analysed here from insect cells is phosphorylated (Préhaud *et al.,* 1992). Thus, for rabies virus P phosphorylation is not required for its oligomerisation in contrast to what was reported for the VSV P protein (Gao and Lenard, 1995). The fact that rabies virus P produced in insect cells is an oligomer could explain why it elutes so close to the N°-P complex from the gel filtration column.

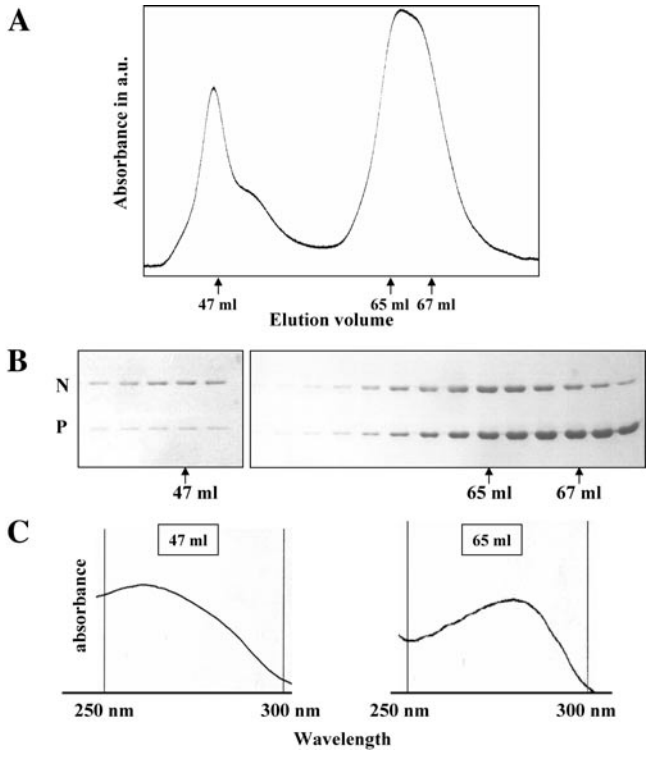

FIG. 1. Separation by gel filtration of complexes formed by coexpression of rabies virus N and P proteins in insect cells. (A) Absorbance signal of the eluate from the Superdex 200 column. (B) SDS–PAGE analysis of these fractions. (C) Absorption spectra from the 47-ml elution volume sample (left, showing high A260/A280 ratio indicating the presence of nucleic acid) and of the 65-ml elution sample (right, showing the absence of nucleic acid).

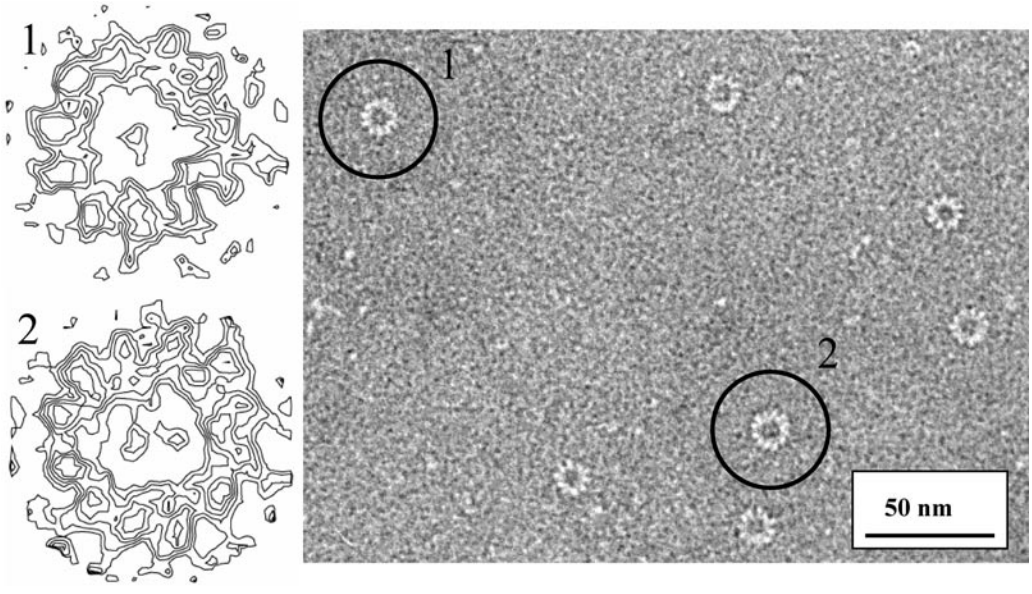

FIG. 2. Negative stain electron micrograph of the sample that eluted after 47 ml from the Superdex 200 column (see Fig. 1). The sample was stained with 1% sodium silicotungstate. Most N-RNA rings have one P-oligomer attached at the inside (see, for example, the particle marked by a ring and the number 1 and its density contour level on the left, there is one blob inside the ring). Some particles (number 2) have two P-oligomers (two blobs of density) inside the ring. The magnification is indicated by the bar that represents 50 nm.

Based on the above results, we propose that the elution profile of the various N-P complexes in Fig. 1A is composed of the elution of at least three different protein complexes: N-RNA with some bound P (peak at 47 ml), N°-P complex (peak at 65 ml), and a P oligomer (peak at 67 ml). In our hands it was not possible to purify oligomeric P away from the N°-P complex, probably because of similarity in size and charge characteristics. For this reason we added an N-terminal His-tag to the N protein and coexpressed N_{His} and P. After the same purification procedure the material around the 65/67-ml peak was applied to a Ni-NTA column that was first washed and then eluted with imidazole. Figure 4A shows the flow-through (Lane 1), the wash (Lane 2), and the eluate (Lanes 3–6). It is clear that P alone was not retained on the column and that the eluted $N°_{His}$-P complex showed equal densities for the two protein components on the Coomassie blue stained gel (Fig. 4A). The absorption spectrum in Fig. 4B showed that this material did not contain nucleic acid (A260/280 ratio of 0.57). It is probably this complex that was shown to support genome replication for VSV (Peluso and Moyer, 1988; Peluso, 1988; Masters and Banerjee, 1988a,b; Howard and Wertz, 1989).

The purified $N°_{His}$-P complex was subsequently analysed by velocity and equilibrium analytical ultracentrifugation. The sedimentation profiles and the theoretical fit to the dcdt+ analysis of the experimental data are shown in Figs. 5A and 5B. We have run several samples at different protein concentrations and we always obtained the same results. The rather symmetrical boundary suggests that there is only one sedimenting species.

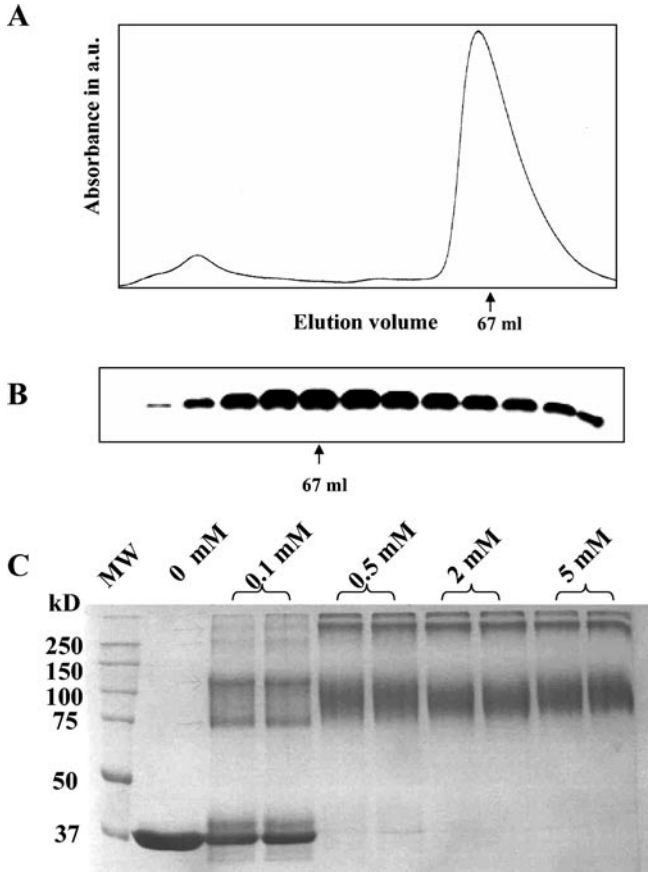

FIG. 3. Characterisation of rabies virus P protein expressed alone in insect cells. (A) Absorbance signal of the eluate from the Superdex 200 column. (B) SDS–PAGE of the same fractions. (C) PAGE of cross-linking of purified P protein with increasing concentrations of ethylene glycol-bis(succinimidyl succinate) (EGS), two independent samples for each EGS concentration.

The s(20,w) value of the sedimenting species was found to be 5.53 ± 0.05 S. When the s value is combined with the Stokes' radius derived from the gel filtration column (53 Å), this leads to a molecular weight of the complex of 128 kDa. Sedimentation equilibrium experiments were performed at three dilutions; one of the profiles is shown in Fig. 5C. All individual profiles yielded molecular weight values between 123 and 132 kDa. These values were independent of the protein concentration and slightly decreased with angular velocity, which is an indication for some heterogeneity in the sample. When all the data were analysed simultaneously, we obtained an average molecular weight of 130 ± 5 kDa. The determined weight is too high for a 1:1 complex of $N°_{His}$-P (87.5 kDa) and could correspond to a $N°_{His}$-P_2 (121 kDa) or to a $(N°_{His})_2$-P complex (141.3 kDa). Based on the densities of the N and P bands from the complex on a blue-stained gel (Fig. 4A), it seems most likely that the complex consists of two P plus one N.

To measure more precisely the molecular weight of the complex, we performed denaturing and native mass spectroscopy. When the sample was analysed after denaturation with 80% formic acid and then diluted with 50% acetonitrile, we could detect a signal at about 33,500 Da, corresponding to the monomer of P, and at 53,800 Da corresponding to the monomer of N_{His}. In the two independent experiments on the native complex we observed particles with a mass of 33,560 Da (monomer of P), 67,120 Da (dimer of P), 53,850 Da (monomer of N_{His}),

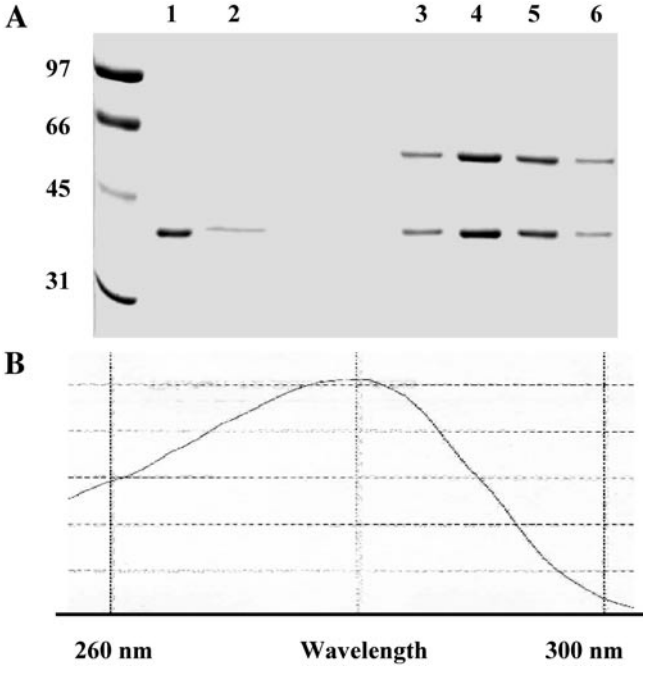

FIG. 4. Separation of P-oligomers from $N°_{His}$-P by Ni-NTA Agarose column chromatography. (A) SDS–PAGE from eluted samples. Lane 1, flow-through. Lane 2, wash. Lanes 3–6, elution with 100 mM imidazole in fractions containing 10 mM EDTA. (B) Absorption spectrum from eluted sample from Lanes 4 and 5 showing the absence of RNA.

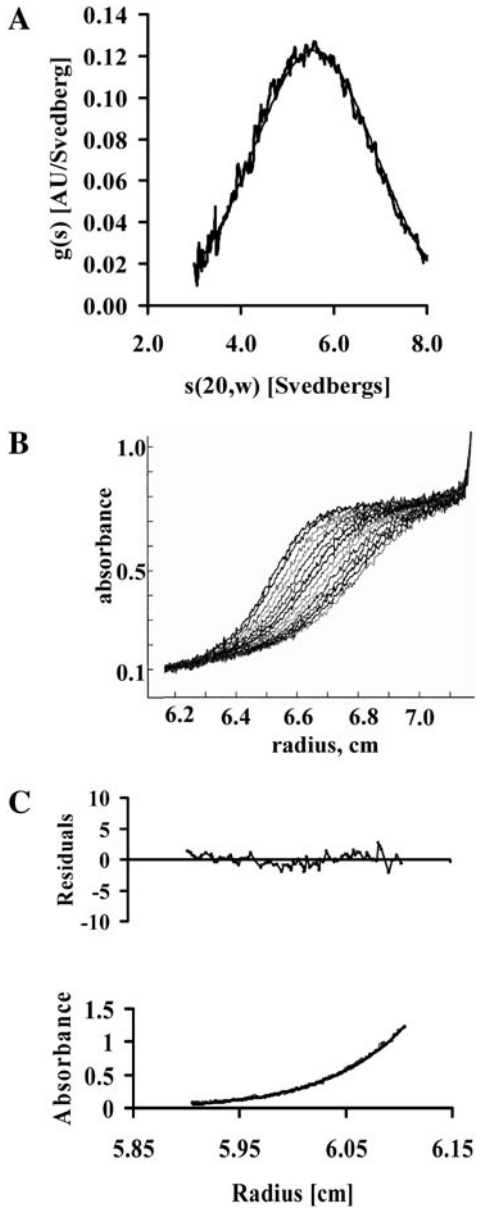

FIG. 5. Characterisation of $N°_{His}$-P by sedimentation analysis. (A) Distribution of sedimentation coefficients calculated from the set of close boundary profiles shown in (B). The g(s) data were derived from 16 scans fitting from s = 2.3 to 7.7 S. The fitting curve resulting from the analysis by dc/dt+ software is shown in a smooth line, whereas the wavy line represents the data points. This plot is for an initial concentration of 0.4 mg/ml and a speed of 42,000 rpm; the fitting model was done with a single species leading to an $s_{20,w}$ = 5.53(±0.05) S. (C) Sedimentation equilibrium experiment on $N°_{His}$-P. The data are presented by open circles and the fitted curves are represented by a solid line. This plot is for an initial concentration of 0.38 mg/mL and a speed of 11,500 rpm; the residuals are plotted above. The baseline that we used was derived experimentally by spinning the sample at 42,000 rpm for 10 h. All three concentrations and all three speeds were fitted simultaneously yielding a MW of 130 kDa.

and, finally, a strong signal at 121,000 Da ($N°_{His}$-P_2 complex) (Fig. 6). There was also a weak signal for a species with a weight of 175 kDa that could correspond to a

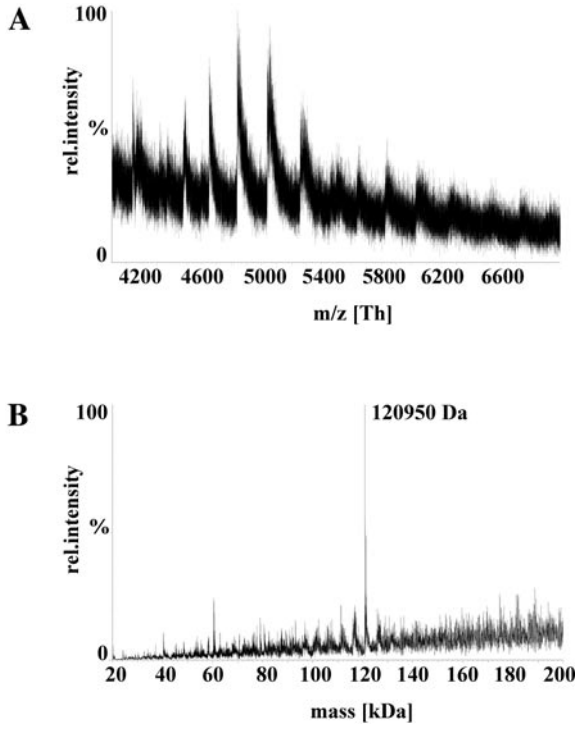

FIG. 6. Mass spectroscopy analysis of the N°-P complex. Magnification of the *m/z* range used for deconvolution (A) and deconvolution (B), showing the presence of a particle with a mass of 121,000 Da.

$(N°_{His})_2$-P_2 complex. The relative concentration of the 175-kDa compound could not be determined nor could we confirm whether it was a N-P complex or a contaminating protein. The conditions that were used for this experiment include high temperature and low salt. We do not know whether the formation of the P_2 and the eventual N_2-P_2 complexes were due to these particular conditions or if they have biological relevance.

The complex was also studied by negative-stain electron microscopy. Using sodium silicotungstate as stain, we found a large variety of small forms, indicating a highly asymmetric object. The particles were analysed and classified in image classes (eigen-images). There were a total of about 80 image classes but some were very poorly populated (only several raw images per class). Figure 7 shows 10 of the most highly populated image classes (each made by averaging approximately 20 raw images). The top row shows image classes with complexes consisting of a strong stain-excluding (white) spot with a lesser stain excluding (less white) tail with various shapes. The bottom row shows complexes with the same bright spot but with the tail split into two parts. The size of the strong stain excluding spot corresponds to the size of the N monomers in the rabies virus N-RNA rings shown in Schoehn *et al.* (2001). The weaker stain excluding tail could then be made up by the two P monomers that either lie on top of each other forming seemingly only a single tail or are (partly) separated, forming a forked structure. This interpretation would be in agreement with the composition of the complex derived from the experiments described above. The images also clearly show the elongated shape of the complex with a length of about 100 Å, which agrees with its large hydrodynamic radius of 53 Å. Within the image classes there are differences between the densities of the tail(s) in the raw images. This could be explained by a certain flexibility of the complex. This flexibility may be the reason we did not succeed in making a reliable 3D reconstruction of the complex. Note that these EM results cannot exclude a certain amount of heterogeneity of the complexes. However, if there were more than one molecular complex, and if these complexes were not flexible but had a defined shape, then the image analysis program would probably have separated them (Schoehn *et al.,* 2000).

DISCUSSION

The P proteins of nonsegmented negative-strand RNA viruses have two functions. The first function is to stabilise the RNA-dependent RNA polymerase (L) and to bind this enzymatic unit to the N-RNA template (Curran *et al.,* 1994; Smallwood *et al.,* 1994; Mellon and Emerson, 1978; Horikami and Moyer, 1995). The P protein with this function is an oligomer: rhabdovirus P forms trimers or tetramers (Gao and Lenard, 1995; Gao *et al.,* 1996; Gigant *et al.,* 2000; this study) and Sendai P forms tetramers (Tarbouriech *et al.,* 2000a,b). VSV P has to be phosphorylated to oligomerise and to be able to bind L (Gao and Lenard, 1995; Takacs and Banerjee, 1995), whereas rabies and Sendai virus P can oligomerise without being phosphorylated (Tarbouriech *et al.,* 2000a; Gigant *et al.,* 2000; this work). Bacterially expressed Sendai virus P (not phosphorylated) can functionally interact with Sendai virus L (Curran *et al.,* 1994). The combined results from these different virus groups suggest that the tri- or tetramerisation of P is essential for the interaction of P with L, whether or not oligomerisation is regulated by phosphorylation.

The second function of P is to keep N in a soluble form (N°) and to prevent it from polymerising onto cellular RNAs, until the moment it can encapsidate newly formed viral or complementary RNA (Peluso and Moyer, 1988; Peluso, 1988; Masters and Banerjee, 1988b; Howard and Wertz, 1989). Phosphorylation of P is not important for the interaction with N° (Takacs and Banerjee, 1995; Gupta and Banerjee, 1997). Although initially multiple complexes between N° and P proteins were identified (Davies *et al.,* 1986; Masters and Banerjee, 1988a; Peluso, 1988; Préhaud *et al.,* 1992), only a single type of complex seemed to be active in supporting the replication (encapsidation) reaction (Peluso and Moyer, 1988; Peluso, 1988). For VSV this complex is thought to be a 1:1 complex between N° and P (Peluso and Moyer, 1988; Peluso, 1988) with an elongated shape and a molecular weight of

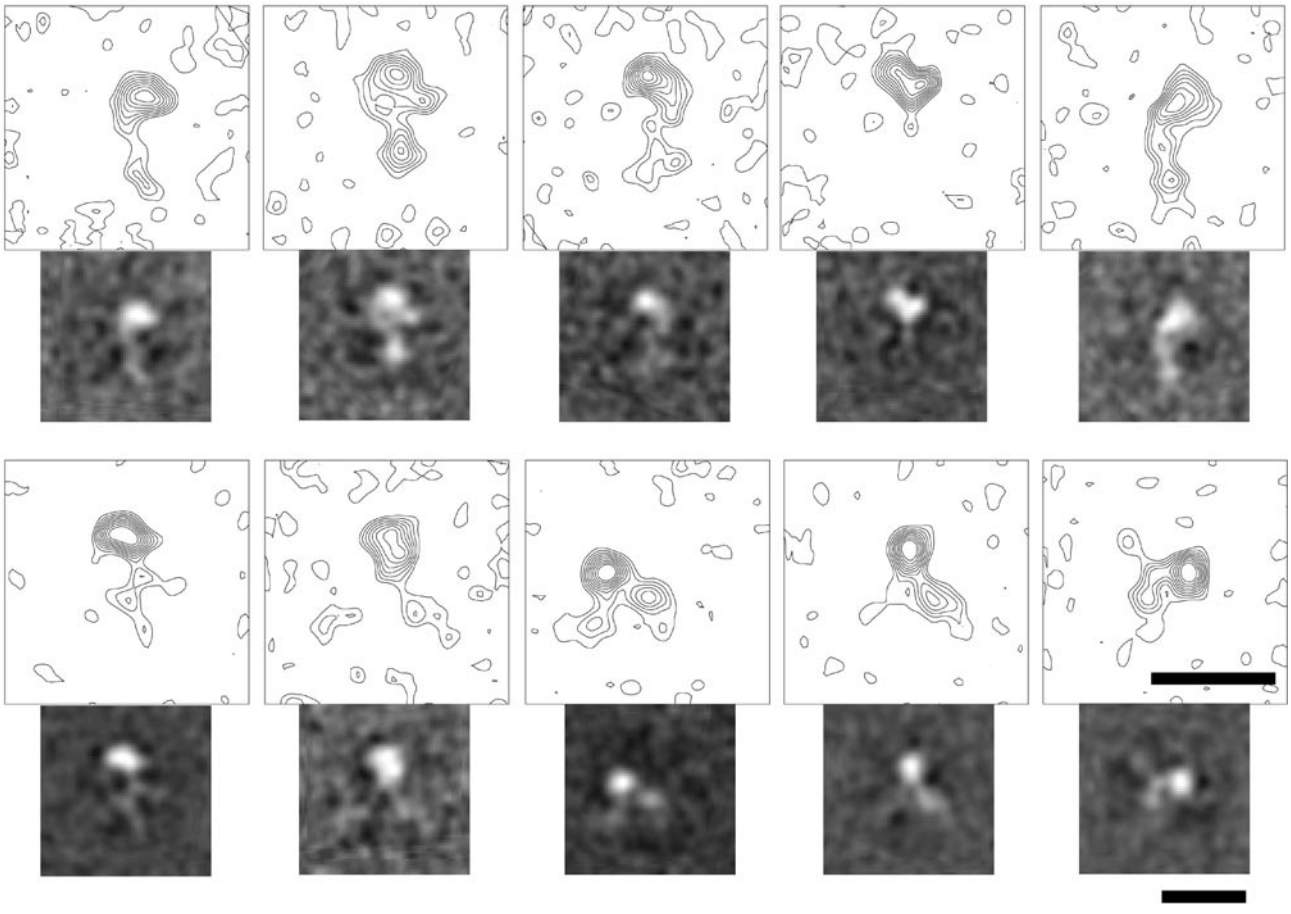

FIG. 7. Electron microscopy of $N°_{His}$-P_2 complex. Ten selected image classes consisting of the averages of approximately 20 raw EM images each. Above each average there is a view with the same number of contour lines showing the level of stain exclusion by the complex. The top row shows images containing a strong stain excluding spot with a single tail, whereas the images in the bottom row show stain excluding spots with a split tail. The scale bars represent 10 nm.

85,000 Da, estimated from sedimentation analysis combined with a Stokes' radius of 53.8 Å. This value falls right between the expected weight for a 1N-1P complex, 72,465 Da, and that for a 1N-2P complex, 97,575 Da. The Stokes' radius of the rabies virus $N°_{His}$-P_2 complex (53 Å) is the same as that of the VSV complex (53.8 Å) (Peluso and Moyer, 1988). We do not know if the stoichiometry of the rabies virus N°-P complex is really different from that of the VSV complex. We expect that a specific molecular complex would be needed to interact with the viral polymerase and that, for a specific virus, only one complex would exist. However, it may be possible that for different viruses different complexes exist. The fact that VSV P cannot oligomerise when it is not phosphorylated whereas rabies P can always oligomerise may be related to this apparent difference between these two types of rhabdovirus.

In the experiments that are described here, we show that the impression of multiple N°-P complexes described in the earlier literature results from the fact that three types of molecular complexes are formed: Small N-RNA rings with some bound P, an $N°$-P_2 complex, and an oligomeric form of P. These three types of complexes overlap in different gradient systems, gel filtration, and other types of separation on charged or hydrophobic columns. This multiplicity of complexes is related to the fact that rhabdovirus P proteins have two sites for interacting with the N proteins, an N-terminal and a C-terminal site (Takacs *et al.,* 1993; Chenik *et al.,* 1994; Fu *et al.,* 1994). This has also been shown for the paramyxovirus P protein where the N-terminal site interacts with N° and the C-terminal site with N-RNA (Curran *et al.,* 1995a,b).

One may wonder why N° needs two P monomers or a P dimer to prevent it to bind to cellular RNA. The exact oligomeric state of rabies virus P is not known and there is no information on the structure of the oligomer. We only know the structure of Sendai virus P that forms tetramers with a rather unusual type of coiled-coil structure (Tarbouriech *et al.,* 2000b). The amount of surface area buried inside the coiled coil is significant and it would be difficult to imagine that free P monomers were folded similar to the monomers in the tetramer. It may be that dimers of P are energetically more favourable for interaction with N° than monomers of P.

Finally, for VSV it is possible to test the biological activity of the N°-P complex in replication in a reconstituted system (Peluso and Moyer, 1988). Unfortunately, such a system does not exist for rabies virus and, therefore, the biological activity of the rabies N°-P complex used in the present work could not be tested. However, the similarity of the gel filtration behaviour of the recombinant rabies virus coexpressed N plus P (three types of complexes) to that of the soluble protein fraction of vesicular stomatitis virus infected cells (compare the results shown Figs. 1A and 1B to the elution profiles for the soluble VSV proteins produced in VSV infected cells in Fig. 2 in Peluso and Moyer, 1988) strongly suggests that the two systems are comparable.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of *Spodoptera frugiperda Sf*21 insect cells were grown at 27–28°C in TC 100 medium (Gibco-BRL, Life Technologies) supplemented with 10% (v/v) foetal calf serum and antibiotics (penicillin/streptomycin). The recombinant baculoviruses, *Autographa californica* nuclear polyhedrosis viruses AcNPVN encoding N (CVS strain) and AcNPVM1 encoding P (CVS strain), were provided by Dr. C. Préhaud (Préhaud *et al.,* 1990, 1992). In these viruses the N gene is under the control of the polyhedrin promoter and the P gene under the control of the p10 promoter.

Baculovirus expressing N protein with an N-terminal His-tag ($N°_{His}$) was produced as follows. The N gene (CVS strain) was amplified by PCR using as template the pCL18 plasmid (Préhaud *et al.,* 1990) and ligated to the pGEM-T Easy vector (Promega) by TA-cloning introducing an *Nco*I site at the N-terminus of the gene. Several clones were sequenced and those having the N-sequence in the proper orientation were digested with *Nco*I and *Not*I and ligated into the pFastBacHTa donor plasmid introducing a 6His-tag and a rTEV protease cleavage site at the N-terminus of N. The Bac-to-Bac Baculovirus Expression System (Gibco-BRL, Life Technologies) was used to generate recombinant baculoviruses coding for His-tagged N. Recombinant viruses were used for infection of *Sf*21 cells at a multiplicity of 5 PFU/cell for expression tests and the virus that gave the best expression profile (AcNPV-His-N) was amplified and titrated by plaque assay. Titers of 2×10^8 PFU/ml were typically obtained.

Expression and purification of N°-P, $N°_{His}$-P, and P homooligomers

*Sf*21 cells were coinfected with AcNPV-His-N and AcNPVM1, both at a multiplicity of 5 PFU/cell. Cells were harvested 52–60 h p.i., pelleted at 350 *g* for 10 min at 4°C, resuspended in hypotonic buffer (50 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA in the presence of the protease inhibitor cocktail complete-EDTA free; Roche Diagnostics), and lysed by three cycles of freezing and thawing (in liquid nitrogen and at 37°C, respectively). Cell debris was pelleted at 12,000 *g* for 15 min at 4°C and the cleared lysate was loaded on a DEAE Sepharose CL-6B (Pharmacia Biotech) column. The proteins were eluted with a continuous 150–300 mM NaCl gradient and fractions analysed by 10% SDS–PAGE. All fractions containing N and P proteins were pooled and precipitated with 32% saturated ammonium sulfate at 4°C and for 30 min. The protein was recovered by centrifugation for 60 min at 4°C and 18,000 *g*, resolubilised in 200 mM NaCl, 20 mM Tris–HCl pH 7.5, 2.5 mM β-mercaptoethanol, and loaded on a HiLoad 16/60 Superdex 200 prep grade (Pharmacia Biotech) column. The complex was eluted at 0.3 ml/min in 200 mM NaCl, 20 mM Tris–HCl pH 7.5, 2.5 mM β-mercaptoethanol. Fractions were analysed by 10% SDS–PAGE and the fractions around 65 ml elution volume (see Results) were pooled and loaded on a Ni-NTA Agarose (QIAGEN) gravity-flow column. The column was washed with 200 mM NaCl, 20 mM Tris–HCl pH 7.5, 2.5 mM β-mercaptoethanol plus 5 mM imidazole and the protein was eluted with the same buffer plus 100 mM imidazole in fractions containing 10 mM EDTA. Fractions were analysed by 10% SDS–PAGE, pooled, concentrated by ultrafiltration (Centrifugal filter devices of Millipore), and used for further analysis.

For the N°-P complex, the same procedure was followed but the nickel-affinity column purification step was omitted. Again the same procedure was followed to purify the P oligomers expressed alone.

Cross-linking of P with ethylene glycolbis(succinimidyl succinate) (EGS)

The protein solution was dialysed extensively against 50 mM HEPES pH 7.5, 150 mM NaCl, and a fresh stock solution of 50 mM EGS in DMSO was prepared prior to use. The cross-linker with a spacer arm length of 16.1 Å was added to the protein sample to a final concentration of 0.1, 0.5, 2, and 5 mM EGS. The reaction mixture was incubated for 1 h on ice, Tris–HCl pH 7.5 buffer was added to a final concentration of 25 mM to quench the reaction and the mixture was incubated for an additional 25 min at room temperature. Samples were analysed by 10% SDS–PAGE. To check reproducibility, duplicate samples were used.

Analytical ultracentrifugation

Measurements of sedimentation velocity were performed with a Beckman Optima XL-I analytical ultracentrifuge and an An-60 Ti analytical rotor (Beckman Instruments). Three concentrations were used (0.4, 0.7, and 1.1 mg/ml in 200 mM NaCl, 20 mM Tris–HCl pH 7.5, 100 mM imidazole, 20 mM EDTA) in double-sector cells with

12-mm Epon centerpieces and sapphire windows. The experiments were done at 42,000 rpm, 20°C, and data were collected at 0.003-cm intervals every 300 s by absorbance measurements at 278 nm for a duration of more than 3 h. The sedimentation profiles were analyzed with the dcdt+ software (V1.13 from J. Philo; http://www.jphilo.mailway.com). For the equilibrium experiments we used three protein concentrations (0.1, 0.18, and 0.38 mg/ml in the same buffer as for the velocity experiments) in a six-channel centrepiece designed for centrifugation of three sample/solvent pairs simultaneously. The measurements were done at 9000, 11,500, and 16,000 rpm at 4°C and for 33 h at each speed to reach equilibrium. The data were collected in the absorbance mode and analysed using Beckman software Origin 4.1.

The partial specific volume, solvent density, and viscosity were calculated with Sednterp software (V1.01 from D. B. Haynes, T. Lane, and J. Philo; http://bbri.harvard.edu/rasmb/rasmb.html), although the contribution of 100 mM imidazole was not taken into account since no tables exist for this compound. The partial specific volume of the $N°_{His}$-P complex was calculated to be 0.7297 ml/g at 20°C and 0.7229 ml/g at 4°C. The solvent density was 1.01061 and 1.01240 g/ml and the viscosity was 1.0458 and 1.6355 cP at 20 and 4°C, respectively.

Native mass spectroscopy

Samples were desalted and taken up in 10 mM $(NH_4)_2CO_3$ pH 6.4 or 25 mM ammonium acetate pH 6.7 at a concentration of 20 pmol/μl. The measurements were carried out on a Q-Tof2 instrument (Micromass, Manchester, U.K.) with syringe injection with the following settings: capillary voltage: 3000 V; cone voltage: 150 V; extractor voltage: 5 V; analyser vacuum: 1.65×10^{-4} bar; desolvation/source temperature: 100°C/120°C; mass range: 2000–7000 *m/z*. We used a Picotip of 30 μm diameter (distal coated, New Objectives, Woburn, MA). For the deconvolution analysis we used the MaxEnt 1 algorithms (optional element of the MassLynx software, Micromass).

Electron microscopy and image analysis

Protein samples at 0.1 mg/ml were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% sodium silicotungstate (pH 7.0). Micrographs were taken under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV and at a nominal magnification of ×60,000.

The images of the N-RNA-P rings as shown in Fig. 2 were not further analysed but the EM negatives of the N°-P complex were digitised on a Zeiss scanner with a pixel size of 14 μm (2.3 Å at the sample scale). One thousand five hundred particles were manually selected from four images and boxed into 128 × 128 pixel boxes using Ximdisp (Crowther *et al.,* 1996). Each image was then interpolated 1.5 times to make the molecule small enough to fit into a 64 × 64 pixel box (3.4 Å per pixel). All particles were band-pass-filtered between 200 and 25 Å without CTF correction and the image densities normalised to the same mean and standard deviation. Processing was done using the SPIDER image-processing package (Frank *et al.,* 1996). The set of images was subjected to multivariate statistical analysis (van Heel and Frank, 1981) to find the characteristic views and then classified in 80 classes. Because the density of the molecules making up the class averages was variable, we did not succeed in calculating a reliable three-dimensional image reconstruction.

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