In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein

Mehdi Chenik, Karim Chebli, Yves Gaudin and Danielle Blondel*

Laboratoire de Génétique des Virus, CNRS, 91198 Gif sur Yvette cédex, France

The rabies virus phosphoprotein (P) and nucleoprotein (N) are involved in transcription and replication of the viral genome. Interaction between N and P was studied in vivo in transfected cells expressing both proteins. Co-immunoprecipitation assays revealed that the N-P complex is present in cells expressing both proteins as well as in infected cells. Furthermore, immunostaining showed that coexpression of N and P was sufficient to induce the formation of cytoplasmic inclusions similar to those found in infected cells. In addition, deletion mutant analysis of P was performed to identify the regions of P interacting with N. The results indicate that at least two independent N-binding sites exist on P protein: one is located in the carboxy-terminal part of the protein and another between amino acids 69 and 177. The formation of cytoplasmic inclusions seems to require the presence of both N-binding sites on P protein.

Introduction

Rabies virus is a member of the family Rhabdoviridae. Its genome is a 12 kb negative-stranded RNA which consists of five genes encoding successively the nucleoprotein (N), the phosphoprotein (P; previously referred to as M1 or NS), the matrix protein (M; previously referred to as M2), the glycoprotein (G) and the RNA polymerase (L). The genome organization is functionally similar to that of vesicular stomatitis virus (VSV), the prototype rhabdovirus. Transcription and replication are thought to be similar to those of VSV (Banerjee & Barik, 1992).

In the case of VSV, the active template for both transcription and replication is the viral nucleocapsid, which consists of the genomic RNA tightly wrapped with N (Emerson & Wagner, 1972). During transcription, the polymerase complex (an association of L and P proteins), synthesizes a 47 nucleotide leader RNA and five polyadenylated mRNAs (Testa et al., 1980). The P and L proteins are also required for replication of a full-length sense or antisense RNA. The N protein is also involved in replication: it encapsidates progeny RNA and thereby protects the template from RNase degradation (Masters & Banerjee, 1988a). N protein also modulates the switch from transcription to replication (Blumberg & Kolakofsky, 1987). In addition to being a subunit of the polymerase, the P protein forms complexes with newly synthesized N protein to prevent it from self-aggregating. Thus, the N protein is available to encapsidate the RNA (Davis et al., 1986; Peluso & Moyer, 1984, 1988). It has also been suggested that the N–P association prevents the N protein from binding to non-viral RNA and is thus responsible for the specificity of RNA encapsidation (Masters & Banerjee, 1988a).

However, despite considerable analysis of the VSV RNA polymerase complex, the mechanism of the switch between transcription and replication remains unclear. Furthermore, the interactions between the polymerase complex and the nucleocapsid template have not been thoroughly characterized. Studies of these molecular processes in other rhabdoviruses, such as rabies virus, may provide new insights into these issues. Recently, it has been shown that baculovirus-expressed rabies virus P and N proteins form heterogeneous complexes in insect cells (Fu et al., 1994; Prehaud et al., 1992) which appear to be organized in a regular lattice of hexagonally shaped structures (Pinto et al., 1994).

Here, we discuss the use of a different expression system to express N and P in mammalian cells and show that the proteins associate in vivo. Furthermore, cells containing both proteins display a granular immunofluorescence pattern very similar to that observed in infected cells. We analysed deletion mutants and demonstrated the existence of two N-binding sites on the P protein. One is located at the carboxy terminus of the P protein and the other between amino acids 69 and 177. Immunofluorescence experiments showed that both sites are required for granule formation.

Methods

Cells and virus. BSR cells, a clone of BHK-21 (baby hamster kidney) cells, were grown in Eagle’s MEM supplemented with 10% calf serum. The CVS strain of rabies virus was cultivated and purified as previously described (Gaudin et al., 1992).
Recombinant vaccinia virus (VTF7-3) containing the T7 RNA polymerase gene has been described previously (Fuerst et al., 1986) and was kindly provided by Dr B. Moss (NIH, Bethesda, Md, U.S.A.).

**Antibodies.** Monoclonal antibodies directed against N protein (5D53, 8D2) and against P protein (A17) were produced in BALB/c mice immunized with u.v.-inactivated CVS virus. Cloned hybridomas were transfected using cells expressing N or P protein. The regions of N and P proteins recognized by the different anti-N or anti-P antibodies were defined by immunoprecipitation of in vitro-translated, truncated N or P. The epitopes recognized by 8D2 and 5D53 anti-N antibodies were located in two domains of the N protein (length 451 amino acids) between amino acids 87 and 190, and 400 and 451, respectively (data not shown). The epitope recognized by the A17 anti-P antibody was located between amino acids 69 and 177 (data not shown).

**Plasmid construction**

(i) Preparation of rabies virus mRNA. Confluent cultures of BSR cells were infected with the CVS strain at a m.o.i. of 10 p.f.u./cell. Cells were harvested by scraping into PBS and collected by low-speed centrifugation. RNA was extracted by the guanidinium thiocyanate method (Sambrook et al., 1989), precipitated with ethanol, dissolved in distilled water and stored at –70 °C.

(ii) Synthesis of double-stranded cDNA and cloning of wild-type N and P genes. First-strand DNA copies of P mRNA were specifically synthesized using the oligonucleotide PB (GCCCTCTAGA(dT)12CAT). Oligonucleotide PB consisted of oligo(dT)12 flanked at its 3' end with three nucleotides complementary to the P mRNA sequence preceding the poly(A) tail, and at its 5' end with six nucleotides representing a XbaI restriction site (underlined) plus three additional bases (GCC). The CDNA was amplified by 30 cycles of PCR using the oligonucleotide PA (GCCCTCTAGAACCATCCCATAATGAG), which is identical in sequence to the 5' end of the mRNA, preceded at its 5' end by the six nucleotides of the XbaI site (underlined) plus GCC.

The oligonucleotides NB and NA were used to amplify the cDNA corresponding to the N gene. NB (GCCCTGCGAG(dT)12ATGA) contains at its 3' end four nucleotides complementary to the N mRNA sequence preceding the poly(A) tail and is flanked at its 5' end with six nucleotides representing an XbaI restriction site (underlined) plus GCC. NA (GCCAAGTTACACCCCTACAAATGAG) is identical in sequence to the 5' end of N mRNA and contains a HindIII site (underlined) plus GCC. Oligonucleotides PA and NA contain the initiation codon ATG (bold). The sequences of the oligonucleotides were determined from the N and P gene sequences published by Poch et al. (1988).

The amplified double-stranded cDNAs corresponding to wild-type P and N proteins were digested with XbaI (P gene) or HindIII and PstI (N gene) and were then inserted, under the control of the T7 RNA polymerase promoter, into the corresponding cloning sites of the CD8 expression vector (Aruffo & Seed, 1987) to obtain the plasmids pMCP and pMCN, respectively.

(iii) Construction of deleted P genes. The constructs pPΔc30 and pPΔc120 differed from pMCP by deletions of 90 bp and 360 bp at the 3' terminus of the P gene, respectively. These deletions were introduced by PCR amplification of the wild-type P gene using oligonucleotides PB1, PB2 and PA. PB1 (GCCCTCTAGATTACGGGACCCATCCC) is complementary to nucleotide positions 788 to 801 of the P mRNA. PB2 (GCCCTCTAGATTTAACCTGAGCCACCA) is complementary to positions 518 to 531 of P mRNA. Both oligonucleotides are flanked at their 5' ends with an XbaI site (underlined) plus GCC preceded by a stop codon (bold). The construct pPΔA-n52-c30 was obtained by deletion of 156 bp from the 5' terminus and 90 bp from the 3' terminus of pMCP using the oligonucleotides PA3 and PB1. PA3 (GCCCTCTAGACTGAAGGC ATGAAGC) contains the sequence corresponding to nucleotides 149 to 163 of P mRNA including an in-frame ATG (bold) and the same nine nucleotides described for other constructs.

The amplified double-stranded cDNAs corresponding to the deleted P genes were digested with XbaI and were inserted into the corresponding cloning sites of the CD8 expression vector (Aruffo & Seed, 1987).

**DNA transfection.** Proteins were transiently expressed using a T7 vaccinia virus expression system according to the method of Fuerst et al. (1986). BSR cells were grown in 3.5 cm dishes until about 80% confluency and infected with VTF7-3 at a m.o.i. of 5 p.f.u./cell. After 1 h of adsorption, the cells were transfected with 5 μg of supercoiled plasmid DNA by the calcium phosphate coprecipitation procedure (Parker & Stark, 1979).

**Radiolabelling and immunoprecipitation of viral proteins.** At 24 h after infection or transfection, proteins were labelled with 50 μCi/ml of [35S]methionine (sp. act. > 1000 Ci/mmol; Amersham) for 4 h. Cells were harvested by scraping and lysed in 50 mm-Tris–HCl pH 7.5 and 0.5% NP40. Nuclei were eliminated from the lysate by centrifugation at 10000 r.p.m. for 5 min at 4 °C. The cytoplasmic fractions were incubated overnight at 4 °C with specific murine monoclonal anti-N or anti-P antibodies. Immune complexes were precipitated by adding Protein A-Sepharose (Sigma), washed three times in the lysis buffer and analysed by SDS–PAGE followed by autoradiography.

**Indirect immunofluorescence staining.** At 24 h after transfection, transfected or infected cells were fixed in 4% paraformaldehyde in PBS pH 7.5 for 10 min and permeabilized for 5 min with 0.05% Triton X-100 in PBS. Viral P and N proteins were stained using mouse monoclonal anti-P and anti-N antibodies respectively and the corresponding goat anti-mouse, fluorescein isothiocyanate-conjugated antibody (Cappel).

In vitro transcription and translation. Plasmids were linearized by digestion at a unique site, PstI for N constructs or Dral for P constructs, downstream of the CD8 inserts. Capped runoff transcripts were synthesized using the T7 Microprobe kit (Promega). Template DNA was eliminated by treatment with DNase Q (Promega). The RNA was then translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (sp. act. > 1000 Ci/mmol; Amersham) for 1 h. The proteins were analysed by SDS–PAGE and autoradiography.

**Results**

**Interaction between rabies virus N and P proteins**

In order to study the interaction between N and P proteins we used a transient expression system to obtain a high level of the proteins in BSR cells. Cells were...
infected with the VTF7-3 recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (Fuerst et al., 1986), then transfected with plasmids encoding either N or P protein under the control of the T7 RNA polymerase promoter. In parallel experiments, cells were cotransfected with both plasmids. Proteins were extracted and immunoprecipitated under mild conditions (avoiding treatment with high salt concentration or ionic detergent). Cell extracts were immunoprecipitated with specific mouse monoclonal anti-P (A17) or anti-N (8D2 and 5D53) antibodies. Immunoprecipitates were analysed by SDS-PAGE (12% polyacrylamide). Lane 2, [35S]methionine-labelled proteins from purified virus.

First, two large terminal deletions of P protein (PAΔN172 and PAΔC120; Fig. 2) were tested for their ability to bind to N protein in co-immunoprecipitation assays. Both truncated P proteins formed a complex with N protein since they were present in the immunoprecipitates from cotransfected cell extracts obtained with the anti-N antibody 8D2 (Fig. 3, lanes 5 and 8). The complex PAΔC120–N was also detected in the immunoprecipitates obtained with the anti-P antibody A17 (lanes 3). Although the truncated P proteins appeared to bind to N less efficiently than the wild-type P protein, these results showed that P proteins lacking more than the amino-terminal half or almost all of the carboxy-terminal half of the molecule still interacted with N protein. These results strongly suggest that P protein contains at least two binding sites for N protein. One is located between amino acids 69 and 177, the other between amino acids 173 and 297.

However, when the same experiments were performed using the other anti-N antibody (5D53), no complex PAΔC120–N was immunoprecipitated (Fig. 3, lane 6). As this antibody was able to immunoprecipitate the complex N–P (see Fig. 1), this suggests that the binding of anti-N

![Image](image_url)
Fig. 2. Schematic representation of the truncated P proteins. Dark bars represent the protein product of each deleted P gene with amino acids positions. Lines indicate deleted regions. Diagram of the P gene inserted in the CDM8 vector is shown at the top with a scale in nucleotides.

Fig. 3. Analysis of interaction between N protein and amino- or carboxy-terminal-truncated P proteins by immunoprecipitation. (a) Cells were transfected with plasmid encoding truncated P protein, PAc120 (Tr N) or cotransfected with plasmids encoding the wild-type N protein and PAc120 (Tr N). [35S]Methionine-labelled cell extracts were immunoprecipitated with anti-P A17 or anti-N antibodies 8D2 or 5D53. The positions of M₉ marker proteins are shown on the left (lane 1; ¹⁴C-methylated; Amersham). (b) Cells were transfected with plasmid encoding truncated P protein, PAn172 (Tr PAn172) or cotransfected with wild-type N protein (Tr N) and PAn172. Cell extracts (³⁵S-labelled) were immunoprecipitated with 8D2 or 5D53. The deleted protein PAn172 was not recognized by the anti-P antibody. This protein was therefore synthesized by in vitro transcription and translation of the cDNA (described in Methods) to indicate its migration (lane 11). Immunoprecipitates were analysed by SDS-PAGE (12% polyacrylamide) followed by autoradiography.

Fig. 4. Analysis of interaction between N protein and carboxy-terminal-truncated P protein by immunoprecipitation. Cells were transfected with the plasmid encoding the carboxy-terminal-truncated protein, PAc30 (Tr PAc30) or cotransfected with plasmids encoding the N protein (Tr N) and PAc30. Cell extracts (³⁵S-labelled) were immunoprecipitated with anti-P A17 antibody or anti-N 5D53 antibody. The immunoprecipitates were analysed by SDS-PAGE (12% polyacrylamide) followed by autoradiography.

antibody 5D53 interfered with the binding of PAc120 to N protein.

We took advantage of the properties of this antibody to locate more precisely the carboxy-terminal region of P involved in the association with N, and thus constructed another plasmid encoding truncated P protein, PAc30, by removing the carboxy-terminal 30 amino acids. As expected, PAc30 still interacted with N as demonstrated by co-immunoprecipitation assays performed with anti-P antibody A17 (Fig. 4, lane 2). However, as for PAc120, 5D53 anti-N antibody did not immunoprecipitate the complex N–PAc30 and only precipitated the N protein (Fig. 4, lane 3). This indicated that PAc30 contained only one binding site, between amino acids 1 and 177 (the same as in PAc120) and that the last 30 amino acids of P protein, between 268 and 297, were essential for the carboxy-terminal site to be functional.

To define more precisely the residues between 1 and 177 involved in the association with N, we constructed another deletion mutant, PA–N52-c30, by removing both the carboxy-terminal 30 amino acids (and thus the carboxy-terminal binding site) and also the amino-terminal 52 amino acids (Fig. 2). Although the truncated protein bound less efficiently to the N protein than the wild-type P protein, this protein retained the ability to interact with N (Fig. 5, lane 3). Thus, amino acids 53 to 177 of P protein were sufficient for N–P interaction. Taken together, these results indicated that two independent N-binding sites exist on the P protein: one is located between amino acids 53 and 177, the other is...
Rabies virus N–P interaction

Fig. 5. Analysis of interaction of wild-type N protein with PA-N52-c30 protein. Cells were transfected with the plasmid encoding the wild-type N protein (Tr N) or the truncated protein, PA-N52-c30 (Tr PA-N52-c30) or cotransfected with both plasmids. Cell extracts (3SS-labelled) were immunoprecipitated with anti-P A17 antibody. The immunoprecipitates were analysed by SDS-PAGE (12% polyacrylamide) followed by autoradiography.

located in the carboxy-terminal part of P and requires the region between amino acids 268 and 297 to be functional.

Effect of P truncations on the pattern of immunofluorescence of the N–P complex in cotransfected cells

We investigated the effect of P deletions on the distribution of the complex using immunofluorescence staining. Cells transfected with plasmid encoding wild-type N protein showed diffuse staining throughout the cytoplasm and rare granules (1 to 2 per cell) (Fig. 6a). Similarly, staining in cells expressing wild-type P protein appeared diffuse (Fig. 6b). Cells were also transfected with plasmids encoding wild-type N and P proteins together, and stained for N or P protein with specific monoclonal antibodies. In all cases, the proteins were organized in numerous granules of heterogeneous size giving a range of stained structures from small cytoplasmic clusters to large inclusion bodies, as would be expected in rabies virus-infected cells (Fig. 6c, d).

The P protein deletions (those already used in previous experiments, plus PAN68; Fig. 2) were then analysed for their ability to associate with the N protein in such clusters. As observed for the wild-type P protein, staining patterns of truncated P proteins (PΔc30, PΔc120, PAN68 and PAN172) were diffuse (Fig. 7a and d for PΔc30 and PAN68). Cells cotransfected with plasmids encoding N protein and truncated P proteins showed different immunofluorescence staining patterns depending on the P deletion. In the case of PAN68, staining for N and P proteins (Fig. 6c, d) showed almost the same granular and patchy distribution as the wild-type proteins (Fig. 7b, c). In contrast, staining patterns were diffuse when the other deleted proteins (PΔc30, PΔc120 and PAN172) were coexpressed with N (Fig. 7e, f; shown only for PΔc30). Thus, removal of the amino-terminal 68 amino acids was the only truncation that did not affect granule formation. In contrast, the other deletions abolished cluster assembly although the deleted proteins retained the ability to bind to N protein.

These data suggested that two N-binding regions within the P protein are required simultaneously for granule formation. Therefore, the observation that PAN68 gave the same granular distribution as the wild-type P protein in presence of N protein indicated that the
amino-terminal N-binding site in P is located between amino acids 69 and 177.

**Discussion**

In this paper, we studied the interaction between the rabies virus P and N proteins using an efficient expression system in mammalian cells. Interactions were studied *in vivo* in transfected cells by co-immunoprecipitation of both proteins with an antibody specific for one component and by immunofluorescence experiments. The P protein interacts with the N protein. This confirmed the observation of Prehaud *et al.* (1992) using the baculovirus expression system. N–P complexes have also been described for other negative-stranded RNA viruses including VSV, Sendai virus and measles virus (Emerson & Schubert, 1987; Horikami *et al.*, 1992; Huber *et al.*, 1991; Masters & Banerjee, 1988a). We tried to determine the ratio of each protein present in the complexes. We found that N–P complexes contained two- to threefold more N molecules than P molecules (data not shown). However, as N protein probably exists as different forms in the cell (nucleocapsid-like structure containing cellular RNA, N protein aggregations, soluble N monomers), this stoichiometry probably reflects P binding to multiple N protein species. In the case of VSV, different kinds of complexes having a 1:1 or a 2:1 stoichiometry of N to P have been demonstrated *in vitro* (Masters & Banerjee, 1988b). However, only complexes with a 1:1 molar ratio were observed *in vivo* (Peluso & Moyer, 1988).

Immunofluorescence experiments showed that N and P proteins form granules of heterogeneous size from small aggregates to large cytoplasmic inclusions which seem to be similar to the Negri's bodies observed in rabies virus-infected cells (Negri, 1903). This granule formation required the association of N and P proteins since N and P proteins expressed alone in cells give a diffuse immunostaining pattern. The few small dots observed with anti-N antibodies are most likely due to N aggregation as described for VSV and Sendai virus (Buchholz *et al.*, 1993; Ohno *et al.*, 1985; Sprague *et al.*, 1983). However, at present, we do not know if N and P proteins associate with cellular RNA and assemble into nucleocapsid-like particles as demonstrated for respiratory syncytial virus (Garcia *et al.*, 1993). For two other paramyxoviruses, Sendai virus and measles virus, the individual expression of the N protein is sufficient to induce the formation of nucleocapsid-like structures, which aggregate within the cytoplasm (Buchholz *et al.*, 1993; Fooks *et al.*, 1993; Spehner *et al.*, 1991). Electron microscopy and biochemical studies might clarify this issue for rabies virus.

Using deletion mutant analysis, we identified domains on the P protein responsible for binding to N protein.
Both PAc120 and PAN172, each composed of different halves of the P protein, interacted with N protein. Therefore, P protein carries two binding sites. To map the sites, two monoclonal anti-N antibodies directed against different parts of the N protein were used to co-immunoprecipitate the N–truncated P protein complexes. Anti-N (5D53) directed against the carboxy-terminal region of the N protein failed to recognize the complexes between N protein and carboxy-terminal-truncated P proteins (PAc30 and PAc120) although such complexes were immunoprecipitated by the anti-N antibody (8D2) directed against the amino-terminal half of N. This suggests that the 5D53 epitope is located near the P-binding site on N protein involved in the interaction with PAc120. A similar situation has recently been reported for the N protein of two paramyxoviruses, the human parainfluenza virus type 1 and measles virus: antibody inhibition of P protein binding was used to map the N protein domains involved in P binding during nucleocapsid assembly (Gombart et al., 1993; Ryan et al., 1993). Our findings are consistent with the recent results obtained with VSV and Sendai viruses for which residues near the carboxy-terminal part of the N protein are also required for N–P interaction (Buchholz et al., 1993; Homann et al., 1991; Takacs et al., 1993).

We also studied the effect of the P deletions on the distribution of the N–P complexes in cotransfected cells. Truncation of 68 amino acids from the amino terminus of P did not affect the granular immunofluorescence pattern of N–P complexes. However, other truncated P proteins (PAc30, PAc120 and PAN172) did not form granules despite binding to N protein. Thus, the carboxy-terminal 30 amino acids and a region extending from amino acids 69 to 177 are required simultaneously for granule formation.

Taken together, these data indicate that two independent N-binding sites exist on P protein. One is located in the carboxy-terminal part of P protein and requires the region between amino acids 268 and 297 to be functional, the other is located between amino acids 69 and 177. Both of them are required to form the Negri’s body-like aggregates. Recently Fu et al. (1994), using an in vitro transcription–translation system, have also proposed the existence of two N-binding sites on the P protein. They demonstrated the existence of a carboxy-terminal binding site and suggested that the amino-terminal binding site is located between amino acids 1 and 19. However, we showed that PA-N52-c30 still interacts with N. This difference could be due to the fact that their assays were performed in vitro, in contrast to our experiments which were performed in vivo. Alternatively, we cannot totally exclude the possibility that a third site (located between amino acids 1 and 19) is involved in interaction with the N protein.

A model for N–P interaction is proposed in Fig. 8. These results are in agreement with recent work on VSV demonstrating that two regions of P are important for N–P complex formation in vivo, one of which is in the carboxy-terminal domain (Takacs et al., 1993). This latter region of VSV P protein had already been described to be critical for in vitro interaction with VSV soluble N protein and with the ribonucleocapsid template (Emerson & Wagner, 1972; Gill et al., 1986). The carboxy-terminal regions of the P protein of Sendai virus and measles virus also appear to contain the nucleocapsid attachment site required for N–P interaction (Ryan & Kingsbury, 1988). For rabies virus, it is not yet known whether the same regions of P are important for the association with soluble N and with N bound to the RNA genome.

Studies of the location of P-binding domains on N protein are in progress to delineate the precise interacting domains within these two viral proteins and should provide further information about their roles.

We thank Patrice Coulon for providing monoclonal antibodies. We are grateful to Anne Flamand for constant interest and support, to Françoise Wyers for helpful discussions and to Bruno Blondel, Jean-Michel Rossignol and Marie Christine Tuffereau for critical reading of the manuscript. This work was supported by the CNRS. These results were presented at the IXth International Congress of Virology (Glasgow, U.K., 8th to 13th August, 1993).

References


Fig. 8. Model of the N–P interaction. There are at least two N-binding sites on the P protein. One is located between amino acids 69 and 177 (Ap), another between amino acids 173 and 297 (Bp). These sites interact with the corresponding sites located on the N protein (Bn and An). The binding of anti-N antibody 5D53 (located in the carboxy-terminal part of N) interfered with the binding of P to N protein. Note that An and Bn are not localized.

---

References:


(Received 17 May 1994; Accepted 29 June 1994)