

Mapping of monoclonal antibody epitopes of the rabies virus P protein

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Thirty-six monoclonal antibodies (MAbs) specific for the rabies virus P phosphoprotein were obtained from mice immunized with recombinant P (PV strain) produced in *E. coli*. All MAbs reacted against the corresponding rabies virus protein by ELISA and by Western blot analysis and revealed the presence of cytoplasmic inclusions in rabies virus infected cells. The epitopes of seven MAbs were mapped by testing their reactivity with protein fragments expressed from deletion mutants in transfected cells. Western blotting, immunoprecipitation and immunofluorescence assays were performed. These MAbs recognized epitopes in different domains of the P protein: 60% were directed against a region lying between residues 83–172 suggesting a major antigenic determinant of the rabies virus P protein in this region. Most of the antigenic sites appeared to be composed of linear epitopes. These MAbs will be useful as tools to dissect structural and functional properties of the rabies virus P protein.

Transcription and replication of rhabdoviruses are mediated by ribonucleoprotein (RNP) complexes that contain genomic RNA tightly wrapped with the nucleoprotein N, the RNA polymerase L protein and the phosphoprotein P (Emerson & Wagner, 1972). For vesicular stomatitis virus (VSV), the best characterized rhabdovirus, the P protein is a multifunctional regulatory protein involved in virus transcription and replication; it associates with the L protein in the polymerase complex and interacts with both soluble and genome-associated N protein (Emerson & Schubert, 1987; Masters & Banerjee, 1988; Takacs & Banerjee, 1995). The P protein has different phosphorylation states, believed to bind with different affinities to the RNP template and which have different transcription activities (Barik & Banerjee, 1992; Gao & Lenard, 1995). Rabies virus and VSV are structurally similar. Thus, by

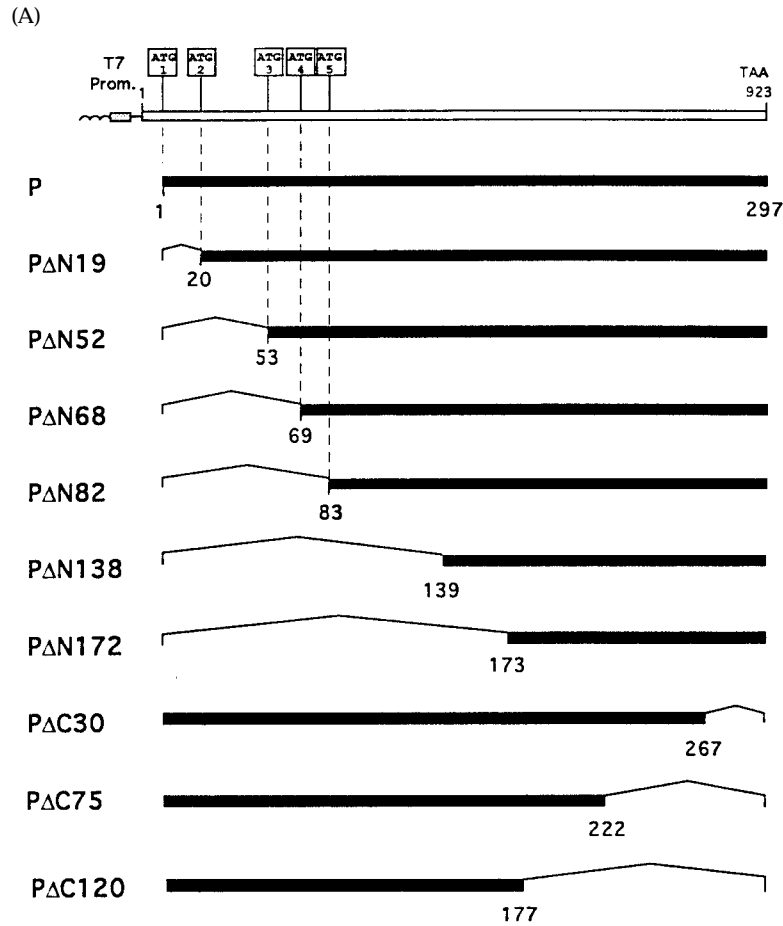
analogy phosphoproteins of rabies virus and VSV may have similar properties. As previously reported for VSV (Takacs *et al.*, 1993), two domains of the rabies virus P protein are involved in the interaction with N protein *in vitro* and *in vivo* (Chenik *et al.*, 1994; Fu *et al.*, 1994). The rabies virus P protein has at least two differently phosphorylated forms (Tuffereau *et al.*, 1985). Four additional proteins (P2, P3, P4 and P5) translated from the P gene have been found in infected cells, in cells transfected with a plasmid encoding the complete P protein and in purified virus (Chenik *et al.*, 1995). Translation of these proteins is initiated from secondary, downstream, in-frame AUG initiation codons by a leaky scanning mechanism.

One approach to characterizing the RNA polymerase complex of rabies virus is to identify domains in each constituent protein involved in function and in interactions between the different subunits. Monoclonal antibodies (MAbs) specific for individual subunits would be powerful tools for structural and biological analyses. However, no panel of antibodies specific for the rabies virus phosphoprotein P was available. We thus prepared anti-P MAbs and we mapped the epitopes recognized by these MAbs using a set of truncated proteins.

The P proteins are present in small amounts in both infected cells and purified virus preparations. To obtain enough protein for preparing MAbs, the P gene (from the PV strain) was placed downstream from the T7 promoter between the cloning sites *Nde*I and *Xho*I in the *E. coli* expression vector pET-22b+ (Novagen). The *Nde*I site (CATATG) overlaps the initiation methionine codon ATG of the P gene. The expressed P protein contains two additional amino acids. Seven histidine residues were tagged at the carboxy-terminal end of the P protein to facilitate purification of the expressed protein. The recombinant plasmid (pET 22-P) was then transferred into *E. coli* BL21(DE3) (Novagen) and expression of the gene induced with IPTG. A polypeptide accumulated which migrated in SDS–polyacrylamide gels with an apparent molecular mass of 42 kDa, indistinguishable from that of rabies virus P protein (not shown). This protein was solubilized in a buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl pH 7.9, 0.1% Triton X-100, 100 µg/ml lysozyme and purified by nickel-affinity chromatography as described by the manufacturer Novagen. It was recognized by a previously available

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(B)

	P	P Δ N19	P Δ N52	P Δ N68	P Δ N82	P Δ N138	P Δ N172	P Δ C30 (a) (b)	P Δ C75 (b)	P Δ C120 (a) (b)	Localization (c)
26G6	+	+	+	+	+	+	+	+/- -	-	- -	222-297
27A5/25C2	+	+	+	+	+	+	+	+/- +	-	- -	222-267
30F2/23H4	+	+	+	+	+	-	-	+ +	+	+ +	83-138
23E2	+	+	-	-	-	-	-	+ +	+	+ +	20-52
25E6	+	-	-	-	-	-	-	+ +	+	+/- +	1-19
A17 ⁺	+	+	+	+	+	-	-	+ +	+	+/- +	83-138

Fig. 1. Schematic representation of the truncated P proteins and reactivity of MAbs to truncated proteins expressed in transfected BSR cells. (A) Black bars represent the protein product of each deleted P gene with amino acid position indicated. The thin angled lines indicate deleted regions. The P gene (CVS strain) under the control of the T7 promoter is shown at the top with a scale in nucleotides. The plasmids encoding the proteins P Δ N19, P Δ N52, P Δ N68, P Δ N82 correspond respectively to pPA2, pPA3, pPA4 and pPA5 described previously (Chenik *et al.*, 1994). The other plasmids encoding P Δ N172, P Δ C30 and P Δ C120 have been described (Chenik *et al.*, 1994, 1995). The plasmid encoding P Δ N138 was obtained by a deletion of 443 bp from the P gene 5' terminus by PCR amplification of the wild-type P gene using two oligonucleotides. The reverse primer GCCTCTAGA(dT)₁₂ and the cloning strategy have been described previously (Chenik *et al.*, 1994). The other oligonucleotide (GCCTCTAGAATGAGGTCTTCGGAGGAT) contains the sequence corresponding to nucleotides 444–459 of the P mRNA. The plasmid encoding the protein P Δ C75 was obtained by a deletion of 222 bp from the P gene 3' terminus by PCR

monoclonal anti-P antibody (A17) (Chenik *et al.*, 1994) in Western blot analysis and was thus authentic P protein (not shown). Spleen lymphocytes from mice immunized with this polypeptide were used to produce hybridomas as previously described (Benmansour *et al.*, 1991). A polyclonal serum was also obtained from these mice. Hybridomas secreting antibodies specific for the P protein were characterized by ELISA using recombinant P protein (from the PV strain) coated on plastic wells and by fluorescent antibody staining of rabies virus infected cells. Eighty-five hybridomas were obtained, each secreting a MAb which recognized the P polypeptide and stained cytoplasmic inclusions in infected cells which are known to contain N, P and L proteins and are probably aggregates of viral nucleocapsids analogous to the Negri bodies observed *in vivo* (Chenik *et al.*, 1994). The specificity of the MAbs was tested by Western blotting; all antibodies recognized the P protein present in purified PV rabies virus although the binding of some was poor (data not shown). Six MAbs out of the 85 failed to bind with the P protein of CVS strain in Western blots (data not shown).

Thirty-six antibodies with the highest affinity for the viral P protein were chosen for further analysis. We first mapped the antigenic determinants recognized by these MAbs. A set of plasmids containing parts of the P gene (from the CVS strain) (Chenik *et al.*, 1994, 1995) (Fig. 1) under the control of the T7 RNA polymerase promoter was used to transfect cells and expressed using the VTF7 recombinant vaccinia virus system (Fuerst *et al.*, 1986). Binding of the MAbs to the truncated proteins P Δ N19, P Δ N82, P Δ N172, P Δ C30 and P Δ C120 produced in transfected cells was analysed by immunofluorescence staining. The MAbs were thereby classified into epitope groups according to their reactivity. Three of the 36 MAbs reacted with the amino terminus (residues 1–19) of the P protein, seven recognized a domain lying between amino acids 20–82, twenty-one recognized an antigenic determinant located in the region 83–172, and five bound to the carboxy terminus (residues 177–297) (data not shown).

We further investigated seven MAbs (26G6, 25C2, 27A5, 30F2, 23H4, 23E2, 25E6) directed against different regions of the P protein. Hybridoma cells producing these MAbs were cloned and ascitic fluids were obtained. They produced antibodies of the IgG class. These MAbs together with MAb A17 mentioned above and the polyclonal anti-P antibody were assayed by Western blotting with the eight truncated proteins (described in Fig. 1). Results are shown in Fig. 2. All eight truncated proteins were recognized by the mouse polyclonal

anti-P serum and had mobilities consistent with their predicted sizes. However, the signals for the P protein derivatives lacking the amino- or the carboxy-terminal half of the molecule (P Δ N172 and P Δ C120 respectively) were weaker than those of other truncated proteins. This may be because large deletions affected the expression efficiency and/or protein folding and stability. Alternatively, removal of a part of the protein may influence or alter the structure adopted by the remaining polypeptide chain. As expected, the polyclonal anti-P antibody detected P and the small polypeptides P2, P3, P4 and P5 (translated from secondary downstream AUG codons) in transfected cells producing the wild-type P protein (Chenik *et al.*, 1995). Reactivity of a MAb with a polypeptide lacking one domain demonstrates that the relevant epitope does not reside within the deleted domain. Inability of a MAb to react with a truncated polypeptide suggests but does not prove that the corresponding epitope is in the deleted region. Therefore, analysing the reactivity of a set of truncated polypeptides should allow an epitope to be mapped. MAb epitopes were thereby mapped to domains of the P protein. MAb 26G6 reacted with all the amino-truncated proteins but it recognized P Δ C30 only poorly and did not bind P Δ C120; the epitope is presumably in the carboxy-terminal part of the protein between amino acids 177–297. MAbs 27A5 (and 25C2) recognized neither P Δ C120 nor P Δ C30. However, an over-exposure of the blot revealed a very weak reactivity with P Δ C30 (not shown) suggesting that those antibodies could bind a region between amino acids 177–297. MAb 30F2 (and MAb 23H4) failed to react with truncated P proteins P Δ N138 and P Δ N172 indicating that they recognize a region between amino acids 83–138. MAb 23E2 reacted only with P Δ N19, P Δ C30 and P Δ C120 suggesting that its epitope is located between amino acids 19–52. Finally, MAb 25E6 was directed against the 19 amino-terminal amino acids: it did not recognize any amino-truncated proteins but was able to recognize the carboxy-truncated proteins P Δ C30 and P Δ C120. MAb A17 has been previously shown to recognize a region between amino acids 69–177 (Chenik *et al.*, 1994). As shown in Fig. 2, this MAb did not detect proteins P Δ N138 and P Δ N172 and thus was probably specific for a region containing amino acids 83–138.

The specificity of the antibodies was also tested by immunoprecipitation using transfected cells expressing truncated proteins. To better define the epitopes recognized by the three MAbs (26G6, 27A5, 25C2) directed against the region 177–297, an additional plasmid encoding a protein lacking 75

using an oligonucleotide (GCCTCTAGAACCATCCCAATATGAG) described previously (Chenik *et al.*, 1994) and a primer (GCCTCTAGATTATATTCTGAAGATCG) complementary to nucleotides 683–696 of the P mRNA. (B) Summary of the reactivity, by Western blot analysis and immunoprecipitation or immunofluorescence experiments, of seven MAbs to truncated proteins expressed in transfected BSR cells. Where the results obtained with the different techniques are consistent, they are reported without distinction. Where the results conflict, the assay is indicated (a, Western blot; b, immunoprecipitation). The asterisk indicates that MAb A17 was isolated from a previous fusion obtained from mice immunized with purified CVS virus (Chenik *et al.*, 1994). Localization (c) is the most probable localization of an epitope taking into account inability to react with one truncated protein while reacting with another.

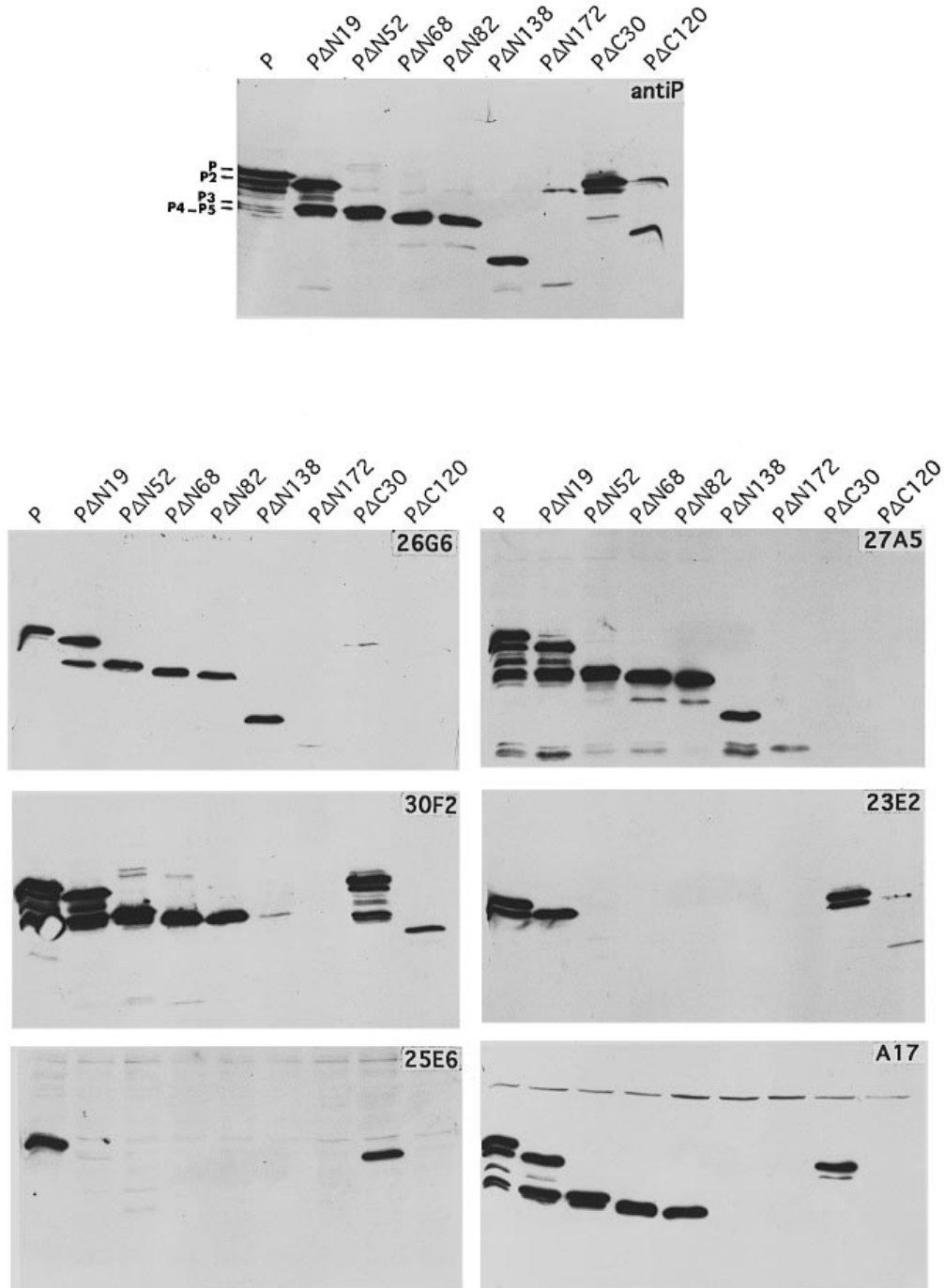


Fig. 2. Mapping P protein epitopes by Western blotting. BSR cells (3×10^6) were infected with the VTF7-3 vaccinia virus (at an m.o.i. of 5 p.f.u. per cell), then transfected with $10 \mu\text{g}$ of plasmid encoding truncated P proteins. Total cell extracts were prepared and protein fragments from 7×10^5 cells were analysed by Western blotting with the MAbs indicated at the top. MAb binding was revealed by peroxidase activity detection with a light-based ECL system (Amersham). Two other MAbs (25C2 and 23H4) were also tested. Their patterns of reactivity were identical to 27A5 and 30F2 respectively. MAb A17, isolated from a previous fusion, has been partially characterized (Chenik *et al.*, 1994). The reactivity obtained with the polyclonal anti-P serum is shown on the top of the figure. The patterns of reactivity of some antibodies are complex because of the presence on the blot of proteins P2, P3, P4 and P5 derived from the P gene (Chenik *et al.*, 1995). Note that PΔN19, PΔN52, PΔN68 and PΔN82 correspond to P2, P3, P4 and P5 respectively.

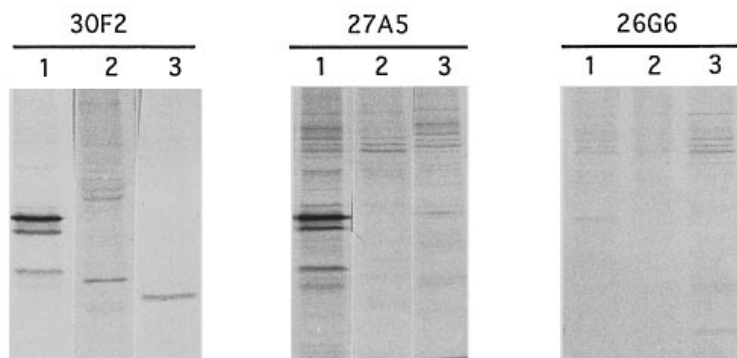


Fig. 3. Mapping antigenic determinants of MAbs 26G6, 27A5 and 30F2 by immunoprecipitation. BSR cells were infected with VTF7-3 and were then transfected with plasmids encoding PAC30 (lanes 1), PAC75 (2) and PAC120 (3) (as described in the legend to Fig. 2). The cells were labelled with [³⁵S]methionine (50 μ Ci/ml) and cell extracts were prepared and immunoprecipitated with 30F2, 27A5 and 26G6 in a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.5% NP40. Immune complexes were collected with protein A-Sepharose and analysed by SDS-PAGE (14% polyacrylamide). MAb 30 F2, which immunoprecipitates PAC30, PAC75 and PAC120, was used as a positive control.

carboxy-terminal amino acids (PAC75) was constructed. For most of the antibodies, the results obtained by immunoprecipitation were consistent with the epitope mapping by Western blot analysis (Figs 1 and 2). However, among the three MAbs (26G6, 27A5, 25C2) which reacted poorly by immunoblot with PAC30, one (MAb 27A5) precipitated PAC30 with great efficiency but not PAC75 and PAC120 (Fig. 3); the epitope probably resided between amino acids 222–267. The difference for 27A5 in immunoblot and immunoprecipitation experiments might be due to its epitope adopting different structures in the conditions of the two experiments. On the other hand, 26G6 reacted very inefficiently with PAC30 in both immunoprecipitation and Western blot assays and did not precipitate either PAC75 or PAC120. Thus, the epitope is presumably lost when the 30 carboxy-terminal amino acids are absent. This suggests that the epitope lies between amino acids 222–267 and probably close to position 267 although additional residues further upstream from position 267 may be involved in the structure of the epitope. The results are summarized in Fig. 1. These MAbs could be classified into five epitope groups with their epitopes: (I) located between residues 1–19, (II) between 20–52, (III) between 83–138, (IV) between 222–267 and (V) between 222–297.

Of the 36 MAbs 60% recognized (in a Western blot) an antigenic determinant located in region 83–172. A17, directed against amino acids 83–138, was the only MAb obtained from mice immunized with purified UV-inactivated virus. This suggests that domain 83–172 contains the major antigenic determinant(s) of the molecule. We cannot exclude that this domain may contain separate or overlapping epitopes. A previous antigenic study (using synthetic peptide analysis) of rabies virus P protein from strain ERA, localized one antigenic site between amino acids 75–98 (Dietzschold *et al.*, 1987). This domain is partially included in the major antigenic site that we identified. Some antibodies recognized epitopes mapping in other parts of the protein. It should be mentioned that no antibody recognized residues between 138–222. These resi-

dues and more precisely amino acids 191–206 have been reported to contain CTL epitopes (Larson *et al.*, 1991). The epitope distribution contrasts with results reported for the P protein of other negative-strand RNA viruses indicating that most of the epitopes are clustered in the carboxy-terminal region for VSV (Williams *et al.*, 1988) and Sendai virus (Vidal *et al.*, 1988) or in the amino-terminal region for respiratory syncytial virus (Garcia *et al.*, 1993). These differences might reflect structural differences between the rabies virus P protein and homologous protein from related viruses. We cannot exclude that these differences could be due to the conformation of the P protein expressed in bacteria.

Six MAbs out of 85 failed to react with the P protein of strain CVS in Western blots. As the deleted P constructs were made from the P gene of strain CVS, it was impossible to map their epitopes. However, in Western blots four MAbs recognized only P and P2 proteins of strain PV and did not recognize P3 and P4 proteins (data not shown). Therefore, these antibodies are directed toward a region located in the amino-terminal part of P between amino acids 20–52. There is one amino acid difference in this region between the two strains: Asp-36 in CVS corresponds to Gln-36 in PV (Poch *et al.*, 1988). Thus, amino acid 36 may be located in the epitope. These antibodies will be good tools to discriminate between these two strains.

Immunofluorescence experiments with transfected cells using various antibodies confirmed the immunoprecipitation results and the diffuse fluorescence staining pattern observed for the complete P protein was also obtained for the truncated P proteins (not shown). As previously shown with antibody A17, PAN52, PAN68 and PAN82 (revealed by MAbs 26G6, 27A5 or 30F2) were detected in the nuclei (Chenik *et al.*, 1995). We had hypothesized that a truncation of at least 52 amino acids from the amino-terminal part of the P protein may result in its ability to enter the nucleus. However MAb 23E2, which bound only the complete P protein in immunoblots and immunoprecipitation analyses, stained both the cytoplasm and the nucleus (not shown). The explanation for this nuclear

localization is thus unclear and may be more complex than previously thought requiring further investigations.

The antigen used for both immunization and ELISA was unphosphorylated P protein. However, most MAbs react with the hyperphosphorylated P form detected in purified virus (not shown) (Chenik *et al.*, 1995); this suggests that phosphorylation does not greatly affect the antigenic sites or that there are no phosphates in the epitopes characterized above. The phosphorylation sites have not been accurately located in the rabies virus P protein: putative sites have been approximately mapped to the central hydrophilic region of the protein (Poch *et al.*, 1988).

Almost all the antibodies reacted with the wild-type P protein in denaturing conditions. Therefore, most of the epitopes did not involve extensive tertiary structures and consisted of linear stretches of amino acids as described for VSV P protein (Williams *et al.*, 1988). This situation is very different from the situation described for rabies virus glycoprotein G, which mostly contains conformational antigenic sites (Benmansour *et al.*, 1991; Lafay *et al.*, 1996). This difference could be due to the conformation of the antigen used for immunization.

P protein has two N protein-binding sites: one is located between amino acids 69–177 and another requires the region between amino acids 268–297 (Chenik *et al.*, 1994). Some antibody epitopes map to domains that have been implicated in this binding. Consequently, we plan to use these antibodies in immunoprecipitation analyses of viral complexes (P–N and P–L) to investigate the interactions within a functional complex.

In conclusion, the MAbs obtained in this study are the first large panel against the rabies virus P protein and will be useful tools to define its structural and functional aspects.

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