CHAPTER 3

Expression of the nucleo- and phosphoproteins in an eukaryotic and prokaryotic expression system

3.1 Introduction

Due to their essential roles in virus replication and transcription, the N and P proteins of rabies viruses have been studied in some detail during recent years. These studies have been facilitated by the ability to express the respective genes in a variety of different eukaryotic, mammalian and prokaryotic expression systems. An important finding emanating from these studies was the fact that both the N and P proteins are phosphorylated by cellular kinases (Anzai *et al.*, 1997; Gupta *et al.*, 2000). Although the phosphorylated amino acid residues have been identified (Anzai *et al.*, 1998; Chenik *et al.*, 2000), knowledge regarding the functional significance of phosphorylation of these proteins is still limited.

The rabies virus N and P proteins have been cloned and expressed by means of recombinant baculoviruses (Fu *et al.*, 1991, 1994; Iseni *et al.*, 1998; Prehaud *et al.*, 1990, 1992; Reid-Sanden *et al.*, 1990), as vaccinia virus recombinants (Chenik *et al.*, 1994) and in *E. coli* (Goto *et al.*, 1995; Gupta *et al.*, 1997, 2000; Takamatsu *et al.*, 1998; Gigant *et al.*, 2000). A general problem encountered in expressing the N protein is the high level of insolubility of the expressed protein, irrespective of the expression system used. In the case of the N protein of VSV, the protein has been reported to rapidly aggregate into insoluble high-molecular weight complexes (Sprague *et al.*, 1983). Using *E. coli* as an expression host, Das and Banerjee (1993) have reported the expressed N protein of VSV to be insoluble, but it could be solubilized in a 1 M NaCl solution. Several groups (Fu *et al.*, 1994; Chenik *et al.*, 1994; Gupta and Banerjee, 1997; and Prehaud *et al.*, 1992) have reported that co-expression of the rabies virus N and P proteins led to complex formation which maintains the N protein in a soluble form.

Since the primary aim of this study was to investigate the functional significance of rabies virus N and P protein phosphorylation, these proteins would have to be expressed to high levels, as soluble proteins, and preferentially in phosphorylated and unphosphorylated forms. Two different expression systems were subsequently chosen

based on their ability or inability to perform post-translational modifications such as phosphorylation.

The first system selected for expression of the rabies virus N and P genes was the eukaryotic baculovirus expression system. The baculovirus expression vector system can express high levels of heterologous genes, and in most cases the recombinant proteins are processed, modified and targeted to their appropriate cellular locations where they are functionally similar to the authentic protein (Galarza *et al.*, 1992; Theron *et al.*, 1996). The successful expression of proteins relies on cloning the heterologous gene under the transcriptional control of the promoters of two dispensable baculovirus gene products, p10 and polyhedrin. These proteins are expressed to very high levels in baculovirus-infected cells and their replacement by almost any heterologous gene result in expression of the recombinant genes to similarly high levels. The construction of recombinant baculoviruses have been greatly facilitated by the introduction of the BAC-to-BAC™ baculovirus expression system (Life Technologies, BRL Gibco) in which foreign genetic material is introduced into the viral genome within *E. coli* cells (Luckow *et al.*, 1993). Recombinant Bacmids are obtained following transposition of a mini-Tn7 element from the donor plasmid to the mini-*att*Tn7 attachment site on the Bacmid DNA. The Tn7 transposition functions are provided *in trans* by a helper plasmid. The mini-Tn7 contains an expression cassette consisting of a gentamycin gene, baculovirus-specific promoters, an extensive multiple cloning site, and an SV40 poly(A) signal. Subsequently, only recombinant Bacmid DNA is introduced into insect cell culture.

The expression of large amounts of protein from a cloned gene introduced into *E. coli*, has been of great value in the purification, localization and functional analysis of proteins. Additional advantages of expressing genes in *E. coli* are that it utilizes general laboratory protocols, and is much cheaper than eukaryotic tissue culture systems. Although prokaryotic cells do not possess the appropriate machinery required to perform post-transcriptional modification of expressed heterologous proteins, they do, however, facilitate studies regarding the significance or function of such modifications present in the appropriately modified proteins. We have elected to express the rabies virus N and P

genes as fusion proteins in *E. coli* using the pGEX plasmid expression vectors (Smith and Johnson, 1989). Each of these plasmids contain the IPTG-inducible *tac* promoter which directs the high level synthesis of heterologous proteins as fusions with the C-terminus of Sj26, a 27.5 kDa glutathione *S*-transferase (GST) protein encoded by the parasitic helminth *Schistosoma japonicum*. In the absence of IPTG, the plasmid-encoded $lacI^q$ allele is efficient in repressing transcription from the *tac* promoter, thus preventing the accumulation of foreign proteins which may lead to growth retardation and cell death. Furthermore, the recombinant fusion proteins can easily be purified from crude bacterial lysates by glutathione affinity chromatography under non-denaturing conditions.

Therefore, the aims of this part of the study were to express the full-length N and P genes of a viverrid rabies virus strain, singularly and in combination, in insect cells by means of recombinant baculoviruses, and secondly to express the full-length N and P genes in *E. coli*, as recombinant GST fusion proteins.

3.2 Materials and Methods

3.2.1 Construction of recombinant baculoviruses

3.2.1.1 Construction of the recombinant Bacmid donor vectors (pFastBac1 and pFastBac™DUAL)

- *Cloning of the full-length rabies virus N and P genes into the pFastBac1 donor vector*

To enable cloning of the N gene into pFastBac1, both the recombinant NpGEM and the pFastBac1 plasmid DNA were restricted with BamH1 and EcoR1. The 1.4 kb full-length N gene and restricted pFastBac1 vector DNA were purified from an agarose gel, ligated overnight and then transformed into competent *E. coli* JM109 cells. Similarly, the 0.9 kb P gene was recovered from the recombinant PpGEM plasmid following EcoR1 digestion and then cloned into a EcoR1-digested, dephosphorylated pFastBac1 vector. The vector DNA was dephosphorylated by treatment with 0.5 U Alkaline Phosphatase (Roche) for 15 min at 37°C according to the manufacturer's instructions. Recombinant transformants were selected from LB-agar plates supplemented with ampicillin (100 µg/ml) after overnight incubation at 37°C, and further characterized by restriction digestion following

plasmid DNA extraction. These recombinant plasmids were designated NpFastBac and PpFastBac, respectively.

- *Cloning of the full-length N and P genes into the pFastBac™DUAL donor plasmid*

For co-expression of the full-length N and P genes, the donor plasmid pFastBac™DUAL was utilized. The full-length N gene was recloned into pFastBac™DUAL using the procedures described above. To allow for directional cloning of the P gene into pFastBac™DUAL, the full-length P gene was recovered from PpGEM by EcoR1 digestion and then recloned into an EcoR1-linearised, dephosphorylated pBluescript SKII (-) phagemid vector. Following orientation determination of the cloned P gene by BamH1 digestion, the P gene was recovered by Kpn1 and Sma1 digestion and finally cloned into the identically cut recombinant NpFastBac™DUAL plasmid. The resultant recombinant plasmid was designated N+PpFastBac™DUAL.

3.2.1.2 Transformation of competent *E. coli* DH10BAC™ cells

Competent *E. coli* DH10BAC™ cells, containing the Bacmid genome as well as a helper plasmid, were prepared as previously described (Section 2.2.8) and then transformed with the recombinant donor plasmids as follows. Competent DH10BAC™ cells (100 µl) were mixed with 100 ng recombinant donor plasmid in glass transformation tubes and incubated on ice for 30 min. The cells were heat-shocked for 45 s at 42°C, and chilled on ice for 2 min. Following addition of 900 µl of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose; final pH 7.0), the transformation mixtures were incubated for 4 h at 37°C with agitation. The transformed cells were selected by plating the cells onto LB-agar plates containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracyclin (10 µg/ml), IPTG (100 µg/ml) and X-gal (40 µg/ml). The plates were incubated at 37°C for 24 h after which white colonies were selected and restreaked onto fresh plates in order to verify the phenotype. Recombinant transformants were subsequently selected and inoculated into 5 ml of LB-broth containing antibiotics (50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracyclin) and incubated at 37°C for 16 h with shaking.

3.2.2 Analysis of recombinant Bacmid DNA

The cells from 1.5 ml of the overnight cultures were harvested by centrifugation at 15 000 rpm for 1 min in microfuge tubes. The bacterial cell pellets were suspended in 300 µl of Solution I (15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A), and after addition 300 µl of Solution II (0.2 N NaOH, 1% (w/v) SDS), the tubes were incubated at room temperature for 5 min. This was followed by the addition of 300 µl of 3 M potassium acetate (pH 5.5) and incubation on ice for 10 min. The precipitate consisting of protein and *E. coli* genomic DNA was removed by centrifugation at 15 000 rpm for 10 min. The Bacmid DNA was precipitated from the supernatant by the addition of 800 µl isopropanol on ice for 10 min. The precipitated Bacmid DNA was collected by centrifugation at 15 000 rpm for 15 min, washed with 70% ethanol and air-dried for 10 min at room temperature. The Bacmid DNA was suspended in 40 µl 1 × TE and stored at –20°C until needed.

- *Analysis of recombinant Bacmid DNA*

A PCR reaction was performed in order to verify transposition of the full-length N and P genes into the Bacmid DNA. The recombinant Bacmid DNA was diluted 10-fold after which 1 µl of the DNA was used as template in the PCR reactions. The PCR was carried out as described in Section 2.2.5, using 10 pMol of JJ1 and PHF for the N and P genes respectively in combination with 25 pMol M13 reverse primer. The presence of both the N and P genes in Bacmid DNA transposed with the P+NpFastBacDual donor plasmid was investigated by using, a multiplex PCR, in which three primers, the M13 reverse primer, the N_3 and the PHF primers were used in one PCR reaction. Conditions for the multiplex PCR were as described in Section 2.2.5.

3.2.3 Transfection of *Spodoptera frugiperda* cells with recombinant Bacmid DNA

Spodoptera frugiperda (*Sf*9) cells were used for the transfection and propagation of the wild-type and recombinant baculoviruses using Grace's insect medium (Highveld Biological) containing 10% fetal calf serum (FCS). Cell concentrations of viable cells were determined using a haemacytometer and staining with trypan blue.

The *Sf*9 cells were transfected with the purified recombinant Bacmid DNA using Cellfectin™ reagent (Life Technologies), according to the instructions of the manufacturer. *Sf*9 cells were seeded in 35 mm diameter wells (1.2×10^6 cells/well) and following attachment of the cells for 1 h at room temperature, were rinsed twice with Grace's medium, without FCS. A further 1 ml of FCS-free medium was then added to the cells. The recombinant Bacmid DNA (5 µg) was diluted with 100 µl Grace's medium without antibiotic and FCS. In a separate tube, 6 µl of the Cellfectin™ reagent was diluted to 100 µl with Grace's medium without antibiotics and FCS. Just prior to transfection, the two solutions were mixed and added dropwise to the monolayers of *Sf* 9 cells. After an incubation period of at least 5 h at 28°C under humid conditions, the transfection mixtures were removed and replaced by 2 ml Grace's medium supplemented with 10% FCS and antibiotics. The cells were then incubated at 28°C for 4 days, after which the supernatants were recovered and used in titration assays to investigate the presence of recombinant baculoviruses. Cells transfected with Bacmid DNA or mock-infected cells were included as controls.

3.2.4 Virus titration and plaque purification

In order to obtain purified single plaques or to determine the virus titre, a method based on the procedures described by Brown and Faulkner (1977), Possee and Howard (1987) and Kitts *et al.* (1990) was used. Serial dilutions (1.0×10^{-1} to 1.0×10^{-9}) of the transfection supernatants were prepared in Grace's medium. *Sf*9 cells were seeded in 35 mm diameter wells at a density of 1.5×10^6 cells/well and after adsorption for 1 h at room temperature, the medium was replaced with 1 ml of the serial dilutions. The virus particles were left to infect the cells for 2 h after which the virus dilutions were removed and the cells gently overlaid with sterile 3% (w/v) low melting temperature agarose at 37°C, diluted 1:1 in Grace's medium. The tissue culture dishes were incubated at 28°C for 4 days in a humid environment. The cells were then stained with 2 ml Neutral Red (100 µg/ml in Grace's medium) for 5 h at 28°C. The liquid overlay was removed and the dishes were incubated overnight before screening the plaques. Putative recombinant translucent plaques were plucked as an agarose plug with a pipette tip, transferred to 1 ml

Grace's medium in Eppendorf tubes, vortexed vigorously and stored at 4°C (*ca.* 10^4 pfu/plug). Following one round of plaque purification, high titre viral stocks were subsequently prepared from single plaques by infecting monolayer cultures at a MOI of 0.01 to 0.1 and harvesting the virus-containing supernatants at 96 h post-infection. The recombinant baculoviruses were stored at 4°C after filter-sterilization of viral stocks and virus titres of the prepared virus stocks was determined as described above.

3.2.5 Expression of the N and P proteins by recombinant baculoviruses

Monolayers of *Sf*9 cells in 75 cm^3 tissue culture flasks (1.0×10^7 cells/flask) were mock-infected or infected with wild-type Bacmid or recombinant Bacmid baculovirus recombinants at a MOI of 10 and incubated at 28°C. The cells were harvested from the bottom of the flasks at 72 h post-infection and collected by low speed centrifugation (2 000 rpm for 10 min) and washed in phosphate-buffered saline (1 × PBS). The cell pellets were resuspended in 0.01 M STE (1 mM Tris (pH 7.4), 0.1mM EDTA, 10 mM NaCl) and incubated on ice for 10 min. Subcellular fractions were then prepared according to the procedures described by Huismans *et al.* (1987). The cell suspensions were homogenized by 15 strokes with a Dounce homogenizer, the nuclei were removed by centrifugation at 1 000 rpm for 1 min. The supernatants (cytoplasmic or S10 fractions) were recovered and an equal volume 2 × protein solvent buffer (PSB) (50 mM Tris (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) glycerol) was added to the respective samples. The samples were heated for 5 min in boiling water and sonicated for 1 min prior to analysis by 12% SDS-PAGE.

3.2.6 Cloning of the full-length rabies virus N and P genes into bacterial pGEX expression vectors

- *Construction of recombinant pGEX expression vectors*

To enable cloning of the rabies virus N gene into pGEX-1, both the recombinant NpGEM and pGEX-1 plasmids were restricted with BamH1 and EcoR1. The 1.4 kb full-length N gene and restricted pGEX-1 vector DNA were purified from the agarose gel, ligated overnight and then transformed into competent *E. coli* JM109 cells. Similarly, the 0.9 kb P-gene was recovered from the recombinant PpGEM plasmid following EcoR1 digestion

and then cloned into a EcoR1-digested, dephosphorylated pGEX-1 vector. Recombinant transformants were selected from LB-agar plates supplemented with ampicillin (100 µg/ml) after overnight incubation at 37°C, and further characterized by restriction digestion following plasmid DNA extraction. The orientation of the cloned P gene was determined by BamH1 restriction mapping. The recombinant plasmids were designated NpGEX and PpGEX, respectively.

3.2.7 Expression of N and P proteins as fusion proteins in *E. coli*

Individual colonies of *E. coli* JM109 transformed with recombinant pGEX-1 and parental pGEX-1 plasmids were inoculated into 1 ml LB-broth supplemented with ampicillin and cultured overnight at 37°C for 16 h. The overnight cultures were diluted 1:10 in 10 ml of fresh medium and incubated for 2 h at 37°C. To each culture, IPTG was added to a final concentration of 1 mM in order to induce expression of the recombinant fusion proteins (Smith and Johnson, 1989). After a further 6 h of culturing, the cells were harvested by centrifugation at 4 000 rpm for 5 min at 4°C in a Sorvall® RC-5B Refrigerated Superspeed centrifuge (Du Pont Instruments). A aliquot (10 µl) of the cell lysates were added to an equal volume of 2 × PSB, boiled for 5 min and after sonication analyzed by SDS-PAGE.

3.2.8 Purification of soluble N and P fusion proteins

- *Solubilization of induced proteins*

To increase the solubility of the expressed recombinant fusion proteins, the pellet of each 10 ml-induced culture was resuspended in 1 ml ice-cold Mix 1 (20% (w/v) sucrose, 100 mM Tris-HCl (pH8), 10 mM EDTA) and incubated on ice for 1 h, after which 10 mM DTT, 1% (v/v) Tween-20, 1% (v/v) Triton X-100 and 1 × PBS (final concentrations) was added. The suspensions were either incubated on ice for 90 min or overnight at 4°C. The suspensions were further homogenized by using two pulses of 20 s each of a probe Ultrasonic Homogenizer (Cole-Palmer Instruments). Following centrifugation at 4 000 rpm for 3 min, the supernatants containing the soluble proteins were recovered and stored at –20°C, until later use.

- *Glutathione agarose affinity chromatography*

The recovered supernatants were purified by affinity chromatography with glutathione agarose (Sigma-Aldrich) as described by Smith and Johnson (1988). The glutathione agarose (0.087 g) was pre-swollen in 7 ml MT-PBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4; pH 7.3), washed twice with MT-PBS and stored at 4°C as a 50% (v/v) solution. The fusion proteins were purified by mixing 200 µl of the prepared glutathione agarose with 250 µl of the P-GST or 400 µl of the N-GST cleared lysates, respectively. To facilitate proper mixing, the reactions were diluted to 800 µl in MT-PBS, and incubated at room temperature for 40 min on an orbital shaker. After adsorption, the beads were collected by brief centrifugation at 2 000 rpm for 2 min and washed 3 times with 1 ml MT-PBS each. In the case of N-GST protein purification, this process was repeated twice prior to elution of the fusion protein. The fusion proteins were eluted from the agarose beads by competition with free glutathione in two 2 min washes with 1 bead volume of MT-PBS, followed by 1 wash with 1 bead volume of 5 mM reduced glutathione, in 50 mM Tris-HCl (pH 8.0), at a final pH of 7.5. The proteins were analyzed by SDS-PAGE and Western blot analysis.

3.2.9 Detection of the expressed proteins

- *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Protein samples were analyzed by electrophoresis in the discontinuous gel system as described by Laemmli (1970). The 12% separating gel (1.5 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 12 % (w/v) acrylamide) and 5% stacking gel (1.0 M Tris-HCl (pH6.8)l, 0.1% (w/v) SDS, 5% (w/v) acrylamide) were polymerized by the addition of 0.001% TEMED and 0.1% ammonium persulfate. Electrophoresis was performed in a Hoefer Mighty Small™ electrophoresis unit at a constant voltage of 10 V/cm gel in 1 × TGS buffer (25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS). After electrophoresis, the gels were stained for 10 min with a Coomassie Brilliant Blue solution (prepared in 50% (v/v) methanol and 10% (v/v) acetic acid) and then counter-stained for 1 h in a solution containing 25% (v/v) methanol and 10% (v/v) glacial acetic acid.

- *Western blot analysis* [1]

After SDS-PAGE, the gel and a Hybond-C nitrocellulose membrane (Life Technologies, Inc.), cut to the same size as the gel, were equilibrated for 15-30 min in transfer buffer (0.2 M Tris, 1 M Glycine). The proteins were transferred to the membrane at 28 V, 120 mA for 90 min using the TE Series Transfor Electrophoresis unit (Hoefer Scientific Instruments). Following transfer, the gel was recovered and stained with Coomassie Brilliant Blue to determine the efficiency of the transfer process. The membrane was rinsed in 1 × TBS (20 mM Tris, 137 mM NaCl, 1 M HCl; pH 7.6) for 5 min and a non-specific binding sites were blocked by immersing the membrane in blocking solution (1 × TBS containing 1% (w/v) fat-free milkpowder) for 30 min at room temperature on an orbital shaker. The primary antibody was added to the membrane and incubated with gentle agitation for 1 h at room temperature. The unbound primary antibodies were removed by first rinsing the membrane in washing buffer (0.05% (v/v) Tween-20 in 1 × TBS), and then by washing the membrane once for 10 min and twice for 5 min each in washing buffer. The secondary antibody was added to the membrane and incubated at room temperature for 1 h with gentle agitation. The membrane was washed as described above. Calorimetric detection of immunoreactive proteins was performed by immersing the membrane in ice-cold peroxidase enzyme substrate (60 mg 4-chloro-1-naftol in 20 ml ice-cold methanol, mixed with 100 ml 1 × TBS containing 60 µl H_2O_2). Once the bands become visible, the membrane was rinsed with water and air-dried.

Primary antibodies used in the analysis included a rabies virus-specific serum (diluted 1:100 in TBS) as well as monoclonal and polyclonal anti-GST antibodies (Santa Cruz Biotechnology, Inc.). The anti-GST antibodies were both diluted 1:1000 in 1 × TBS prior to use. For detection of the bound primary antibodies, horseradish peroxidase-conjugated Protein A (Sigma-Aldrich) was used as the secondary antibody and diluted 1:1000 in 1 × TBS.

[1] The Rabies Unit at the Onderstepoort Veterinary Research Institute, kindly donated the rabies virus serum used in this analysis.

3.3 Results

3.3.1 Expression and co-expression of the rabies virus N and P proteins in insect cells using the BAC-to-BAC™ baculovirus expression system

- *Construction of donor plasmids containing full-length N and P genes*

Full-length copies of the N and P genes were obtained by restriction enzyme digestion of recombinant pGEM®-T Easy vectors (Chapter 2). The N gene was recovered from NpGEM by digestion with both EcoR1 and BamH1 to allow for directional cloning into similarly digested pFastBac1 and pFastBac™DUAL vectors. The P gene was recovered with EcoR1 restriction enzyme digestion from PpGEM for cloning into linearised and dephosphorylated pFastBac1 vector. The resultant recombinant plasmids were selected and characterized by agarose and restriction enzyme analysis. The results are shown in Figures 3.1 and 3.2, respectively. Digestion of the recombinant NpFastBac and NpFastBac™DUAL plasmids with BamH1 and EcoR1 resulted in excision of a 1.4 kb fragment, corresponding in size to the full-length N gene (Fig. 3.1, lanes 6 and 8, respectively). EcoR1 digestion of recombinant PpFastBac1 plasmids excised a DNA fragment of *ca.* 900 bp, corresponding with the expected size of the full-length P-gene (Fig 3.2, lane 5). The recombinant PpFastBac plasmids were also digested with BamH1, which recognizes one site in the vector and cuts asymmetrically in the P gene 300 nt from the 5' end, in order to verify the transcriptional orientation of the cloned insert (not shown).

For construction of the N+PpFastBacDUAL vector the P gene was first cloned into pBluescript SKII (-) to facilitate directional cloning of the P gene. The full-length P gene was recovered from PpGEM by restriction with SmaI and KpnI and cloned into pBluescript SKII (-). The P gene was subsequently recloned into the SmaI and KpnI sites of the recombinant NpFastBacDUAL plasmid to generate the P+NpFastBac™DUAL donor plasmid. Recombinant P+NpFastBac™DUAL plasmids were screened by digestion with EcoR1 which resulted in digestion products of *ca.* 0.9 kb, 1.7 kb and 5.0 kb (Fig 3.2). The sizes of these fragments corresponded with the expected sizes of the P

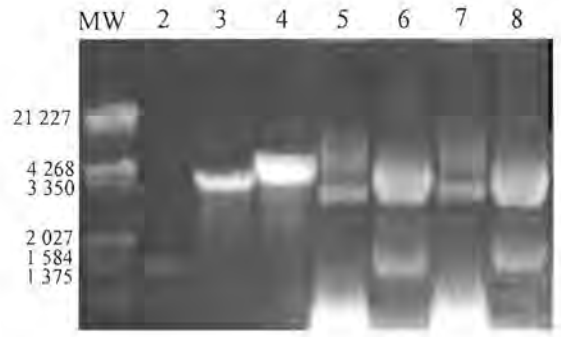

Fig. 3.1 Agarose gel electrophoretic analysis of the recombinant NpFastBac and NpFastBacDUAL donor plasmids. Lane MW, Molecular weight marker; Lane 2, purified N gene amplicon; Lane 3, pFastBac1 plasmid restricted with BamH1 and EcoR1; Lane 4, pFastBacDUAL plasmid digested with BamH1 and EcoR1; Lane 5, uncut recombinant NpFastBac; Lane 6, recombinant NpFastBac digested with BamH1 and EcoR1; Lane 7, uncut recombinant NpFastBacDUAL; Lane 8, recombinant NpFastBacDUAL digested with BamH1 and EcoR1. The sizes of molecular weight marker, phage Lambda restricted with EcoR1 and HindIII, are indicated to the left of the figure in bp.

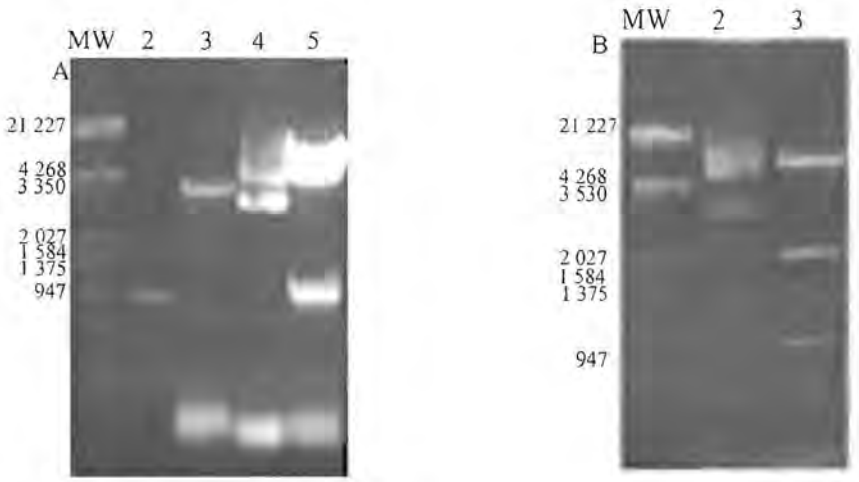

Fig. 3.2 Agarose gel electrophoretic analysis of (A) recombinant pFastBac and (B) P+NpFastBacDUAL donor plamids. (A) Lane MW, Molecular weight marker; Lane 2, purified P gene amplicon; Lane 3, pFastBac1 digested with EcoR1; Lane 4, uncut recombinant PpFastBac; Lane 5, recombinant PpFastBac digested with EcoR1. (B) Lanes: MW, Molecular weight marker; 2, uncut N+PpFastBacDUAL; 3, recombinant N+PpFastBacDUAL digested with EcoR1. The sizes of molecular weight marker, phage Lambda digested with EcoR1 and HindIII, are indicated on the left of the figure.

gene, the N gene together with 300 bp of flanking sequences, and the remainder of the pFastBac™DUAL vector.

- *Construction of recombinant baculoviruses*

Recombinant donor plasmids NpFastBac, PpFastBac and P+NpFastBacDual were used for transposition to the Bacmid DNA in *E. coli* DH10BAC™ cells. During the site-specific transposition of the recombinant donor plasmids, the mini-Tn7 cassette is inserted into the mini-*att*Tn7 attachment site on the Bacmid, thereby disrupting expression of the lacZα peptide. Consequently, white colonies containing the recombinant Bacmid could be distinguished from blue colonies (harboring the non-recombinant Bacmid), when plated in the presence of X-gal and IPTG. The high molecular weight recombinant Bacmid DNA was isolated from *E. coli* DH10BAC™ cells, and the presence of the rabies virus N and P genes in the Bacmid DNA was investigated by a PCR approach. The primers used in this assay were the gene-specific primers, JJ1 and PHF, that anneals to the 5' end of the N and P genes respectively (Table 2.1), and the universal M13 reverse primer that anneals on the mini-*att*Tn7 site within the *lacZα* gene of the Bacmid DNA. By making use of recombinant Bacmid DNA transposed with the NpFastBac donor plasmid as template for the PCR with primers JJ1 and M13, a single discreet band of the expected size (2 kb) was obtained. The presence of the P gene in the recombinant Bacmid DNA was indicated by an amplicon of 1.5 kb following PCR amplification. A multiplex PCR protocol was used to verify the presence of both the N and the P genes in the N+PpFastBacDual transposed Bacmid DNA. The N_3 primer that anneals to the 3' end of the N gene and the PHF primer that anneals to the 5' end of the P gene were used in combination with the M13 reverse primer in one PCR reaction, resulting in two amplicons of 3.2 kb and 1.5 kb, respectively. The controls (no template and Bacmid transposed with pFastBac) were amplified using the M13 forward and reverse primers, and resulted in no amplification and a 2.3 kb amplicon respectively.

The recombinant Bacmid DNA was subsequently used to transfect *S. frugiperda* cells using Cellfectin™ reagent. Progeny viruses resulting from each transfection were obtained by plaque assay of the recovered supernatant fluids. Following plaque

purification, single plaques were selected and used in the preparation of recombinant viral stocks of which the titres were determined by plaque assays. These recombinant baculoviruses were designated BacN, BacP and BacN+P, respectively.

- *Analysis of proteins synthesized in infected S. frugiperda cells*

To investigate whether the rabies virus N and/or P proteins were expressed in *Sf9* cells by the baculovirus recombinants, monolayers of *Sf9* cells were mock-infected and infected at a MOI of 10 with the wild-type and respective recombinant baculoviruses. The cells were harvested at 96 h post-infection and subcellular cytoplasmic and particulate fractions were prepared, as described under Materials and Methods (Section 3.2.5). The fractions were then subjected to SDS-PAGE. Analysis of the Coomassie-stained gels indicated that no unique recombinant proteins were visible following comparison of the recombinant baculovirus-infected cells to the control mock- or wild-type infected cells (results not shown). The presence of rabies virus N and P proteins could however be indicated by Western blot using a rabies virus-specific serum as the primary antibody (Fig. 3.4). The results indicated that the serum reacted with a protein of *ca.* 55 kDa in size, which was present in the particulate fraction of cells infected with the BacN baculovirus recombinant (Fig.3.4, lane 2). No proteins that reacted with the rabies virus antiserum could be identified in the cytoplasmic fractions (Fig. 3.4, lane 6). The antiserum also reacted strongly with a 36 kDa protein present in the cytoplasmic and particulate fractions prepared from BacP-infected cells (Fig. 3.4, lanes 3 and 7). In the case of the recombinant baculovirus BacN+P, the virus-specific serum reacted with both the 36 kDa and 55 kDa proteins present in the particulate fraction prepared from the infected cells (Fig. 3.4, lane 4). However, only the 30 kDa protein reacted with the serum in the corresponding cytoplasmic fraction (Fig. 3.4, lane 8).

It could be concluded that the rabies virus N and P proteins were indeed expressed by the baculovirus recombinants, albeit in very low amounts as the proteins could not be observed in the Coomassie-stained gels. Furthermore, the N protein appears to be insoluble as it could only be observed in the particulate fraction prepared from the

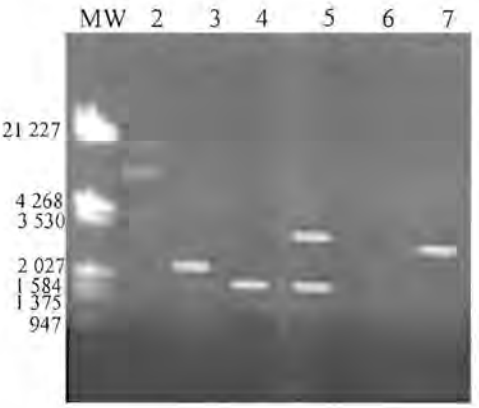

Fig. 3.3 Agarose gel electrophoresis of PCR amplicons, in order to verify the presence of rabies virus N and P genes in the recombinant Bacmid DNA. Lanes: MW, Molecular weight marker; 2, purified Bacmid DNA; 3, N gene in reombinant Bacmid DNA amplified with primers JJ1 and M13; 4, P gene in recombinant Bacmid DNA amplified with primers PHF and M13; 5, Multiplex PCR of the N and P genes in the recombinant Bacmid DNA transposed with the P+NpFastBac DUAL vector; 6, no template DNA control; 7, Bacmid DNA with transposed pFastBac1 plasmid amplified with the pUC forward and reverse primers. The sizes of molecular weight marker, phage Lambda restricted with EcoR1 and Hind III, are indicated to the left of the figure in bp.

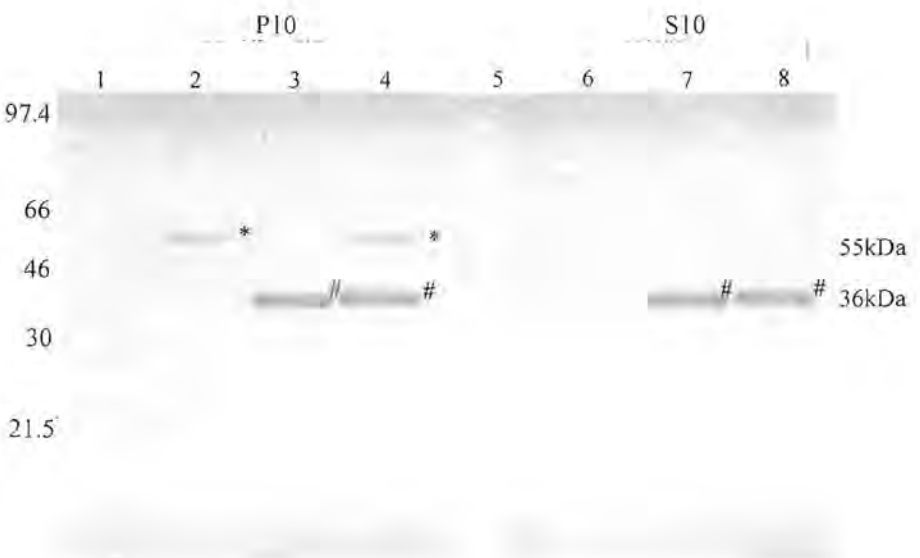

Fig. 3.4 Western blot analysis of recombinant baculovirus-expressed rabies virus N and P proteins using rabies virus-specific serum as primary antibody. Lane 1, Mock-infected *Sf*9 cells; Lanes 2 and 6, BacN-infected cells; Lanes 3 and 7, BacP-infected cells; Lanes 4 and 8, BacN+P-infected cells; Lane 5, Wild-type baculovirus-infected cells. P10 refers to particulate fraction prepared from the infected cells, while S10 refers to the corresponding cytoplasmic fraction. Sizes of the protein molecular weight marker is indicated to the left of the figure in kDa. The * indicates the recombinant N protein, and # indicates the recombinant P protein expressed by the recombinant baculoviruses.

recombinant baculovirus infected cells. The assays used to analyze the co-expression of the N protein with the P protein were not sensitive enough to indicate whether complex formation did occur.

3.3.2 Expression of the rabies virus N and P proteins in *E. coli*

- *Cloning of the full-length N and P genes into the pGEX-1 expression vectors*

In order to express the N and the P genes in *E. coli*, the respective genes were cloned into the pGEX-1 expression vector (Smith and Johnson, 1988), thereby positioning the respective genes in the correct reading frame for translation of N-GST and P-GST fusion proteins. The full-length N gene was recovered from the NpGEM clone (Chapter 2) by restriction with EcoR1 and BamH1. The gene was agarose gel-purified and then directionally cloned into the EcoR1 and BamH1 sites of the pGEX-1 vector. The full-length P gene was recovered from the PpGEM clone (Chapter 2) by restriction with EcoR1, agarose gel-purified and then cloned into the dephosphorylated EcoR1 site of the pGEX-1 vector. Transformants were selected and cultured overnight at 37°C after which the extracted plasmids were subjected to restriction enzyme analysis. Restriction of the recombinant NpGEX plasmids with BamH1 and EcoR1 (Fig. 3.5A, lane 5) and restriction of recombinant PpGEX plasmids with EcoR1 (Fig. 3.5B, lane 3) indicated that the N and P gene-specific inserts were of full-length. The correct transcriptional orientation of the cloned P gene was verified by further restriction mapping with BamH1 (not shown).

- *Expression of recombinant GST fusion proteins*

For bacterial expression, overnight cultures of *E. coli* JM109 cells containing either the parental or recombinant pGEX-1 vectors were diluted and grown in the presence of IPTG. After incubation at 37°C, the cells were harvested and protein samples were prepared for analysis by SDS-PAGE as described in Section 3.2.9. Analysis of the Coomassie-stained gel (Fig. 3.6) indicated that *E. coli* cells transformed with pGEX-1 only produced the 27.5 kDa GST protein. Induced cultures of *E. coli* containing the recombinant NpGEX clone, indicated the synthesis of a polypeptide of *ca.* 82 kDa, while *E. coli* containing the recombinant PpGEX clone indicated the synthesis of a 57 kDa

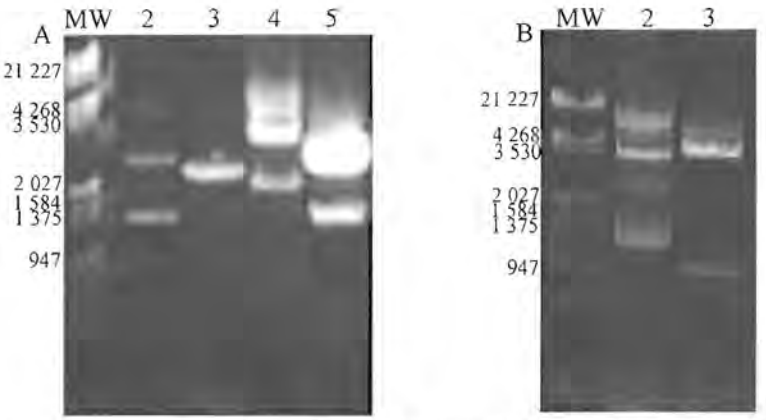

Fig. 3.5 Agarose gel electrophoretic analysis of the recombinant (A) NpGEX and (B) PpGEX plasmids. (A) Lanes: MW, Molecular weight marker; 2, uncut pGEX-1; 3, pGEX -1 digested with BamH1 and EcoR1; 4, uncut recombinant NpGEX; 5, recombinant NpGEX digested with BamH1 and EcoR1. (B) Lanes: MW, Molecular weight marker; 2, recombinant PpGEX (uncut); 3, recombinant PpGEX digested with EcoR1. Sizes of the molecular weight marker phage, Lambda digested with EcoR1 and Hind III are indicated on the left of the figure in bp.

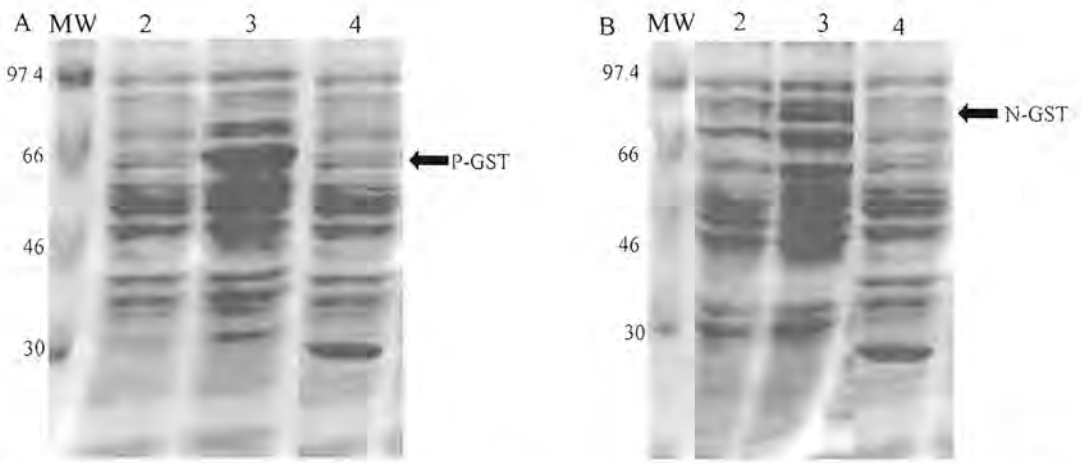

Fig. 3.6 Coomassie-stained SDS-PAGE gel of the *E. coli*-expressed rabies virus (A) P-GST and (B) N-GST fusion proteins. (A) Lanes: MW, Rainbow molecular weight marker (in kDa); 2, IPTG-induced *E. coli* culture; 3, IPTG-induced *E. coli* culture transformed with PpGEX; 4, IPTG-induced *E. coli* culture transformed with parental pGEX; (B) Lanes: MW, Rainbow molecular weight marker (in kDa); 2, IPTG-induced *E. coli* culture; 3, IPTG-induced *E. coli* culture transformed with NpGEX; 4, IPTG-induced *E. coli* culture transformed with parental pGEX. The position of the P and N fusion proteins are indicated by the arrows.

protein. In both cases, the sizes of these unique proteins corresponded with the predicted sizes of the N-GST and P-GST proteins. A duplicate, but unstained SDS-PAGE gel was subjected to Western blotting using a polyclonal anti-GST antibody. The antibody reacted with the overproduced 82 and 57 kDa polypeptides as well as the control 27.5 kDa GST protein (Fig. 3.7A). To verify that the respective fusion proteins included the rabies virus N and P proteins, Western blot analysis was performed using a rabies virus-specific serum (Fig. 3.7B). The antiserum reacted strongly with only the overproduced 82 kDa and 60 kDa proteins, indicating the specific expression of the rabies virus N and P proteins as GST fusion proteins.

Western blot analysis using the anti-GST and rabies virus serum, also indicated that the 82 kDa N-GST protein as well as proteins migrating slightly faster than the N-GST fusion protein, reacted with the antisera (Fig. 3.7A and B, lanes 5). Since no cross-reaction of the antisera with host cell proteins were observed, it was concluded that these smaller proteins represented degraded derivatives of the 82 kDa N-GST protein. Similar degraded derivatives of the 57 kDa P-GST fusion protein was also occasionally observed. Such instability of expression products appears to be a common phenomenon in *E. coli* (Marston, 1988).

To determine whether the expressed viverrid rabies virus N-GST and P-GST fusion were present in a soluble form, the GST-fusion proteins were purified from the bacterial extracts by glutathione affinity chromatography and the yields and purity were assessed by SDS-PAGE (Fig. 3.8). Although high concentrations of purified control GST and P-GST fusion proteins could be obtained, the concentration of the purified N-GST fusion protein was much lower. This may have been due to the majority of N-GST protein being expressed in an insoluble form and could thus not be purified under the conditions used. Nevertheless, by expressing the N gene as a GST fusion protein in *E. coli*, a source of soluble N protein was established which could be used in subsequent investigations.

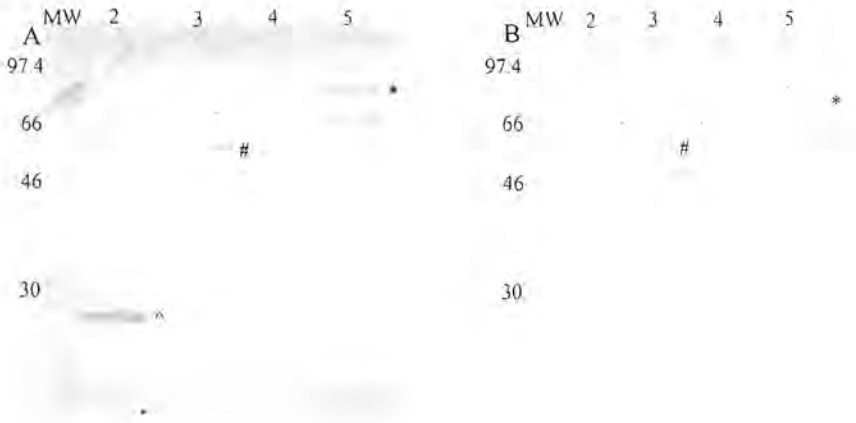

Fig. 3.7 Western blot analysis of recombinant rabies virus N and P fusion proteins using (A) anti-GST antibody and (B) anti-rabies virus serum as primary antibody. Lanes: MW, Rainbow molecular weight marker (in kDa); 2, IPTG-induced *E. coli* culture transformed with pGEX-1; 3, IPTG-induced *E. coli* culture transformed with PpGEX; 4, IPTG-induced *E.coli* culture; 5, IPTG-induced *E. coli* culture transformed with NpGEX. The * indicate the N-GST, the # indicate the P-GST and ^ indicate the GST.

Fig. 3.8 Coomassie-stained SDS-PAGE gel of purified GST fusion proteins following glutathione affinity chromatography. Lane MW, Rainbow molecular weight marker (in kDa); Lanes 2, 3, and 4, purified N-GST protein; Lanes 5 & 6, purified P-GST protein; Lanes 7 & 8, purified GST protein.

3.4 Discussion

We have been able to express the N and P proteins of a South African rabies virus strain in both an eukaryotic and a prokaryotic expression system. Expression of the rabies virus proteins in a BAC-to-BAC™ baculovirus expression system enabled us to express recombinant rabies virus N and P proteins individually or in combination. The baculovirus expression system has been used successfully to express a wide variety of genes. In the case of rabies virus, both the N and P proteins have been expressed in the baculovirus system (Fu *et al.*, 1991, 1994; Iseni *et al.*, 1998; Prehaud *et al.*, 1990, 1992; Reid-Sanden *et al.*, 1990). The synthesis of N and P proteins in cell culture has made significant contributions to the understanding of the function of the individual proteins. The co-expression of these proteins have also been used for studying complex formation between the N and the P proteins (Fu *et al.*, 1994; Chenik *et al.*, 1994), the maturation of the N protein (Kawai *et al.*, 1999) and specificity of RNA encapsidation (Yang *et al.*, 1998).

In this chapter, the N and P proteins of a viverrid rabies virus isolate were expressed in the BAC-to-BAC™ baculovirus expression system. This system offers the advantage of characterizing the viral DNA prior to its introduction into insect cells and thus only modified recombinant baculoviral DNA is introduced into insect cell cultures. Recombinant baculoviruses expressing the N or P genes were subsequently constructed. *S. frugiperda* cells were infected with the respective recombinant baculoviruses after which subcellular extracts were prepared and subjected to SDS-PAGE analysis. It was found that the proteins were expressed to very low levels, as they could not be detected in Coomassie-stained gels. However, proteins corresponding in size to the N and P proteins reacted strongly with an anti-rabies virus-specific serum in Western blot analysis. From the obtained results (Fig. 3.4), it could be concluded that the P protein was expressed in a soluble form, but the expressed N protein was insoluble as it could only be detected in the particulate fraction of infected cells. It has been previously reported that co-expression of the rabies virus N and P proteins maintains the N protein in a soluble form (Yang *et al.*, 1998). To obtain soluble N protein, a baculovirus recombinant capable of co-expressing the viverrid rabies virus N and P proteins was also constructed. The use of a single

recombinant baculovirus for the simultaneous expression of these genes offers certain advantages over co-infection of insect cells with the previously constructed N and P baculovirus recombinants. By co-expressing both proteins in the same cell, the proteins can interact *in vivo* to form N-P protein complexes whereas by co-infection of the cells, not all the cells may be infected by both individual recombinant baculoviruses. It is therefore to be expected that these interactions will be inefficient and the yield of N-P protein complexes may be low. Under the centrifugation conditions used during this study the proteins pelleted and complex formation could not be observed. Optimized centrifugation conditions, sucrose gradient centrifugation, or co-immunoprecipitation would have given more definite answers regarding complex formation between these dual expressed proteins as well as the solubility of the N protein as part of the N-P complex.

The results obtained indicated that although both proteins were being synthesized in the recombinant baculovirus-infected cells, complex formation between the N and P proteins could not be observed. Consequently, the N protein was insoluble and could not be recovered for the cytoplasmic subcellular fraction. The rabies virus genes were placed under the control of the polyhedrin and p10 promoters, since both these promoters are very late promoters these two proteins will be expressed at the same time (O'Reilly *et al.*, 1992).

A further complication that was encountered during co-expression of the rabies virus N and P genes, was the apparent instability of the constructed baculovirus recombinant, in spite of several plaque purifications. First round infection of the insect cells with the recombinant baculovirus did result in expression of both the N and P proteins. However, use of the virus-containing supernatant to re-infect *Sf*9 monolayers resulted in only the P protein being expressed. Such instability with regards to heterologous protein expression using this system has also been noted during the expression of Bluetongue virus and African horsesickness virus gene products (Maree, 2001).

To overcome the problems associated with the BAC-to-BAC™ baculovirus expression system, the use of *E. coli* as an alternative expression host was investigated. The rabies virus N (Goto *et al.*, 1995) as well as VSV N and P proteins (Das and Banerjee, 1993; Gupta and Banerjee, 1997) has previously been expressed in *E. coli*. Although the solubility of the rabies virus N protein when expressed in *E. coli* was not investigated by Goto *et al.* (1995), Das and Banerjee (1993) reported that the bacterial-expressed N protein of VSV was insoluble. The protein could, however, be solubilized in 1 M NaCl. In a subsequent report, Gupta and Banerjee (1997) co-expressed the VSV N and P proteins in *E. coli* and found that the resulting N-P protein complexes were soluble. In order to obtain soluble rabies virus N and P protein, the use of a pGEX expression system was investigated since it has been reported that the expressed heterologous fusion proteins are predominantly soluble (Wang and Zang, 1997; Berg *et al.*, 1998). The N and P genes of a typical viverrid rabies virus were cloned into pGEX-1 after which crude cell extracts of the selected recombinants were analyzed by SDS-PAGE. The respective proteins were synthesized as GST fusion proteins and found to be expressed to high levels. Their identity was subsequently confirmed by Western blot analysis using both anti-GST and rabies virus-specific antisera (Fig 3.7). The rabies virus P-GST fusion protein was found to be soluble and could easily be purified to high levels from bacterial extracts by glutathione affinity chromatography. In contrast, the bacterial-expressed N-GST fusion protein remained largely insoluble. Different approaches were therefore investigated in order to obtain soluble N-GST fusion proteins. These included varying the growth parameters such as lowering the growth temperature to between 20-30°C, inducing protein expression for a shorter period of time and also at a higher cell density for a shorter period of time. Treatment of the bacterial cell lysates with varying concentrations of N-laurylsarcosine was also investigated (Frangioni and Neel, 1993). None of these approaches appeared to be adequate as only a small amount of the soluble N-GST fusion protein could be recovered. However, a higher level of soluble protein could be obtained by treatment of the bacterial cell extracts in a solution containing DTT, Tween-20 and Triton-X100 (Theron *et al.*, 1996). This resulted in soluble N-GST fusion protein, which could subsequently be purified using glutathione affinity chromatography.

In conclusion, the results obtained in this part of the investigation indicated that the viverrid rabies virus N and P genes could be expressed in insect cells infected with the constructed recombinant baculoviruses, and confirmed the integrity of the cloned rabies virus genes. However, the insolubility of the expressed N proteins hampered further use of the recombinant protein. The synthesis of the N and P proteins as GST fusion proteins in *E. coli* provided a source of soluble proteins which were subsequently used for ssRNA- and protein-protein binding studies of which the results will be detailed in the following chapter.

CHAPTER 4

Role of rabies virus nucleoprotein phosphorylation during interaction with single stranded RNA and the phosphoprotein

4.1 Introduction

Rabies virions contain two kinds of phosphorylated proteins, namely the nucleoprotein and phosphoprotein. The nucleoprotein is a 55 kDa RNA-binding protein and represents *ca.* 30% of all virion proteins (Kawai, 1977). The N protein binds to the single-stranded viral genomic RNA *via* the phosphate-sugar backbone of the RNA and exposes the nucleotide bases to the RNA polymerase during transcription and replication (Emerson, 1987; Iseni *et al.*, 2000; Keene *et al.*, 1981; Klumpp *et al.*, 1997). The 36 kDa phosphoprotein is present in the virions as well as in infected cells. Although being present abundantly in the cell, the P protein constitutes less than 6% of the virion (Kawai, 1977). In addition to being a non-catalytic subunit of the viral polymerase, the P protein forms complexes with nascent N protein to prevent it from self-aggregating (Emerson and Schubert, 1987; Bell *et al.*, 1984). It has been suggested that this N-P protein association ensures that N protein is available for encapsidation of the viral genomic RNA and is also responsible for conferring specificity to the RNA encapsidation, thereby preventing binding to non-specific RNAs (Yang *et al.*, 1998).

In the case of VSV, only the P protein is phosphorylated (Sokol and Clark, 1973) and its role during viral replication and transcription has been studied extensively. The VSV P protein has been reported to comprise at least two different phosphorylated components, a hypophosphorylated P1 and a hyperphosphorylated P2 form (Barik and Banerjee, 1991). The interconversion between these two forms of the P protein is a result of phosphorylation and dephosphorylation of phosphate groups which may regulate its functions (Takacs *et al.*, 1992; Barik and Banerjee, 1992b; Chen *et al.*, 1997). The VSV P protein is phosphorylated by cellular casein kinase II (CK II) and a viral L protein-associated kinase (Chen *et al.*, 1997) at two sites located in the N-terminal half and C-terminus of the protein (Barik and Banerjee, 1992a; Chattopadhyay and Banerjee, 1987b). Phosphorylation in both these domains is essential for transcription, but not for replication of the viral genome. Similar to the VSV P protein, the rabies virus P protein also appears to exist in heterogeneous forms (Takamatsu *et al.*, 1998). By making use of an anti-P antibody, a 40 kDa protein (p40) was detected in the virion, but not in the infected cell. The p40 is more phosphorylated than the 37 kDa component of the P

protein (Chenik *et al.*, 1995; Tuffereau *et al.*, 1985). Although the p40 protein is continuously produced in the cell, most of these proteins are immediately dephosphorylated, resulting in p40 becoming almost absent in the cell (Takamatsu *et al.*, 1998). The kinases responsible for rabies virus P protein phosphorylation, namely protein kinase C (PKC) and a novel rabies virus protein kinase (RVPK), as well as the phosphorylation sites, have been identified (Gupta *et al.*, 2000). The RVPK appears to be packaged with the virions and phosphorylation of unphosphorylated *E. coli*-expressed P proteins by this kinase resulted in an alteration of the protein mobility in SDS-PAGE. The protein migrates as a 40 kDa protein, in contrast to phosphorylation with PKC which does not change the mobility of the 37 kDa P protein during electrophoresis (Gupta *et al.*, 2000). Functions of the rabies virus P protein are also thought to be regulated by phosphorylation and dephosphorylation of the protein.

In contrast to the rabies virus P protein, phosphorylation of the N protein has not been well characterized. Although both serine and threonine have been identified as phospho-amino acid residues (Anzai *et al.*, 1997; Yang *et al.*, 1999), the kinase(s) responsible for N protein phosphorylation is not known. In addition, information regarding the role of N protein phosphorylation in regulating its functions is limited. Such investigations have been largely restricted due to an inability to obtain sufficient amounts of soluble N protein for functional studies. Recently, however, using a reverse-genetics system to express a rabies virus minigenome, Yang *et al.* (1999) have however shown that unphosphorylated N protein encapsidated more leader RNA than phosphorylated N protein. In addition, the lack of N protein phosphorylation resulted in a decrease of both viral transcription and replication.

The objectives of this part of the study were to investigate the influence of N protein phosphorylation on its ssRNA-binding activity and on its ability to interact with the P protein.

4.2 Materials and Methods

4.2.1 Expression of the rabies virus P protein in *E. coli* using the pPROEX™HT prokaryotic expression system

- *Cloning of the P gene into pPROEX™HT expression vector*

The rabies virus P gene was cloned into the prokaryotic pPROEX™HTc vector by making use of the procedures described in Section 2.2. The full-length P gene was recovered from the recombinant PpGEM vector by EcoR1 excision and ligated into the EcoR1-linearised, dephosphorylated pPROEX™ vector. Following transformation of competent *E. coli* JM109 cells and plating of the cells onto LB agar plates supplemented with ampicillin (100 µg/ml), recombinant transformants were selected for further analysis. Plasmid DNA was isolated by the alkaline lysis method (Section 2.2.9) and analyzed by agarose gel electrophoresis and EcoR1 restriction enzyme digestion. The translational orientation of the cloned inserts was determined by BamH1 restriction mapping. A clone containing the full-length P gene, in the correct translational orientation, was selected and designated PpPROEX.

- *Expression of the P fusion protein*

Overnight *E. coli* JM109 cells transformed with the parental PpPROEX™ HTc and recombinant PpPROEX™ HTc vectors were diluted 1:10 in 10 ml of fresh medium and grown for 2 h at 37°C. Expression of the recombinant P-His fusion protein was induced by the addition of 1 mM IPTG after which incubation was continued for a further 5-6 h. The cells were harvested at 4 000 rpm for 5 min at 4°C in a Sorvall® RC-5B Refrigerated Superspeed centrifuge (Du Pont Instruments) using a SS-34 rotor. The cells were resuspended in 4 volumes of lysis buffer (50 mM Tris (pH 8.5 at 4°C), 5 mM 2-mercaptoethanol), sonicated and debris pelleted by centrifugation at 2 000 rpm for 1 min. The crude cell lysates were either stored at -20°C until needed or analyzed by SDS-PAGE and immunoblotting (Section 3.2.9).

4.2.2 Radiolabeling of the rabies virus fusion proteins synthesized in *E. coli*

Bacterial proteins were labeled *in vivo* with [^{35}S]methionine as described by Barik and Banerjee (1991). Briefly, single colonies of *E. coli* cells transformed with either PpGEX (Chapter 3), PpPROEX or parental plasmids were grown in 10 ml low-phosphate glucose-peptone medium (1.2% (w/v) Tris-base, 0.75% (w/v) peptone, 0.3% (w/v) NaCl, 0.01 mM $MgSO_4.7H_2O$, 0.001 mM $CaCl_2$, 0.3% (w/v) glucose). The pH of the medium was adjusted to 7.1 before addition of Ca^{2+} and Mg^{2+}. Simultaneous with the induction of the respective cultures with IPTG, 30 µCi of [^{35}S]methionine (1 mCi/ml, 1000 Ci/mmol; Amersham) was added per ml of culture. Following harvest, the cells were washed once with 1 x PBS and the proteins were analyzed by SDS-PAGE and autoradiography.

4.2.3 *In vitro* phosphorylation of the *E. coli*-expressed fusion proteins

A method for phosphorylating the bacterial-expressed proteins was developed based on the procedures described by Ogita *et al.* (1991), Gupta *et al.* (2000) and Kikkawa *et al.* (1982). The cells from IPTG-induced cultures of *E. coli* transformed with NpGEX, PpGEX, PpPROEX and parental plasmids were harvested by centrifugation and lysed as previously described (Section 4.2.1). The phosphorylation reaction mixtures (250 µl) contained 0.5 to 1 µg of the different fusion proteins, 5 µCi of [γ^{32}P]ATP in a kinase buffer (50 mM Tris (pH8), 1 mM DTT, 2 mM $MgCl_2$, 50 µM ATP) and 0.2 U of protein kinase C enzyme (PKC; Roche). The PKC activity was started with the addition of 200 µM $CaCl_2$, 8 µg 3-sn-Phosphatidyl-L-serine (Fluka) per ml, and 0.8 µg of 1,2-Dioleoyl-sn-glycerol (Fluka) per ml. The Phosphatidylserine and diacylglycerol, stored separately as chloroform solutions at -20°C, were mixed first in chloroform, dried, suspended in 20 mM Tris (pH 7.5) and sonicated. The reaction mixture was incubated at 30°C for 1 h and the reactions were terminated by the addition of ETDA (12 mM final concentration). The reaction mixtures or immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

4.2.4 Immune-precipitation of GST fusion proteins

The bacterial-expressed rabies virus N- and P-GST fusion proteins were immunoprecipitated using a monoclonal anti-GST antibody (Santa Cruz Biotechnology,

Inc.). The samples were diluted in 500 µl in 1 × PBS. After addition of 1 µl of the anti-GST antibody, the reactions were incubated at room temperature for 90 min with gentle shaking. Then 40 µl of a 10% (w/v) suspension of insoluble Protein A (Sigma-Aldrich) was added, and incubation continued for 2 h. The immune-complexes were pelleted by centrifugation at 2 000 rpm for 1 min, and washed twice by resuspension in 40 µl 1 × PBS. The precipitated proteins were resuspended in 15 µl 1 × PBS and an equal volume of 2 × PSB was added. After boiling for 5 min, the samples were subjected to SDS-PAGE. For radiolabeled proteins, the gels were dried and autoradiographed. In the case of unlabeled proteins, the gels were further analyzed by Western blot using a rabies virus-specific antiserum as previously described (Section 3.2.9).

4.2.5 RNA-binding analysis

The ssRNA-binding ability of purified, *in vitro* phosphorylated and unphosphorylated N-GST and P-GST fusion proteins were investigated by means of a poly(U)- and poly(A)-Sepharose binding assay as previously described by Theron *et al.* (1994). Three hundred µl of the N-GST or 100 µl of the P-GST fusion proteins were each incubated with 10 mg poly(U)- or Poly(A)-Sepharose in 300 µl STE-TX buffer at NaCl concentrations between 0.01 M and 0.4 M NaCl, as indicated in Figs. 4.5 and 4.6. After allowing the proteins to bind to the respective Sepharose substrates for 30 min at room temperature with gentle agitation, the Sepharose was collected by centrifugation at 2 000 rpm for 1 min and washed twice with 300 µl of the corresponding binding buffer. The supernatant fractions were pooled, and the proteins precipitated with 4 volumes of acetone at – 70°C for 1 h and then pelleted by centrifugation at 18 000 rpm for 30 min at 4°C in a Beckmann SW50.1 rotor. The bound protein (Sepharose pellets) and unbound proteins (precipitated supernatants) were resuspended in 35 µl 0.01 M STE-TX and analyzed by SDS-PAGE. As a control, the affinity of the GST protein for poly(U)- and poly(A)-Sepharose was also assayed in 0.015 M STE-TX buffer. The RNA-binding was quantified by scanning the Coomassie-stained protein bands representing the bound and unbound fractions. The bound protein fraction was expressed as a fraction of the total protein recovered, bound and unbound.

4.2.6 Protein-protein binding analysis

[^{35}S]methionine-labeled cell lysates containing *in vitro* phosphorylated or unphosphorylated N-GST and P-His fusion proteins were used in a modified version of the GST pull-down assay described by Ansari *et al.* (1997). Crude N-GST lysates (400 µl) were incubated with 200 µl glutathione agarose for 30 min at room temperature with gentle agitation. Following brief centrifugation, the pellets were washed twice with MT-PBS to remove unbound N-GST fusion proteins. The pellet was gently resuspended in 300 µl of the binding buffer (50 mM Tris (pH7.4), 150 mM NaCl, 5mM EDTA) after which 300 µl of a P-His cell lysate was added. The mixture was incubated for 2 h at 4°C. Following incubation, the agarose-bound complexes were collected by low-speed centrifugation at 2 000 rpm for 1 min, washed twice with 300 µl binding buffer and then resuspended in 40 µl 1 × PSB. The recovered agarose-immobilized protein-complexes were subjected to SDS-PAGE, followed by autoradiography of the dried gel. As a control, the affinity of the P-His protein for glutathione agarose was also assayed. Quantification of protein complexes was performed by scanning of the radiolabeled bands representing the bound N and P proteins. The peaks were determined and expressed as the ratio of P bound to N.

4.3 Results

4.3.1 Cloning and expression of the rabies virus P protein in *E. coli* with the pPROEX™ HT prokaryotic expression system

In order to investigate the role of protein phosphorylation on rabies virus N and P protein interaction, the P protein was expressed as a His fusion protein in *E. coli* by making use of the pPROEX™ HT prokaryotic expression system. The use of a different affinity tag to GST was aimed at preventing the non-specific binding of the P-His fusion protein to glutathione agarose, thereby ensuring that the agarose-immobilized protein complex were the result of N and P protein interaction. The pPROEX™ HT vector contains a *trc* promoter and *lacI*^{q} gene that enables inducible expression of the cloned P gene with IPTG, thus resulting in a fusion protein with a histidine affinity tag at the amino terminus.

To enable expression of the rabies virus P protein in *E. coli*, the P gene was recovered from PpGEM by EcoR1 excision and recloned into an EcoR1-digested and dephosphorylated PpPROEX™ HTc vector. Following plasmid extraction, the DNA was characterized by restriction enzyme digestion (Fig. 4.1). EcoR1 restriction of selected plasmid DNAs resulted in the recovery of an *ca.* 900 bp DNA fragment, corresponding in size to the full-length P gene. Following restriction mapping with BamH1, a clone containing the P gene-specific insert in the correct translational orientation was selected for further use and designated as PpPROEX. For bacterial expression, overnight cultures of *E. coli* JM109 cells containing either parental or recombinant pPROEX vectors were diluted and grown in the presence of IPTG, as described under Materials and Methods (Section 4.2.1). Protein samples prepared from the cultures were subsequently analyzed by SDS-PAGE. The results indicated the presence of a unique protein band of 36 kDa in the extracts prepared from *E. coli* cells containing PpPROEX (Fig. 4.2A). No similar band was observed in the control cellular extracts. The size of the unique protein corresponds with the predicted size of the rabies virus P protein. To confirm the rabies virus origin of the induced protein, a Western blot was performed by making use of rabies virus serum as the primary antibody. The antibody reacted strongly with both the 36 kDa P-His fusion protein as well as the control 82 kDa N-GST fusion protein (Fig. 4.2B).

4.3.2 *In vitro* phosphorylation of the recombinant *E. coli*-expressed rabies virus N and P fusion proteins

In order to investigate the role of phosphorylation in the interaction of rabies virus N protein with ssRNA and the P protein, a source of phosphorylated bacterial-expressed rabies virus proteins was required. Since bacterial kinases are generally specific for their natural substrates (Barik and Banerjee, 1991), various exogenous kinase sources were investigated for their ability to phosphorylate the bacterial-expressed rabies virus fusion proteins. The use of uninfected *S. frugiperda* and BHK-21 cytoplasmic extracts for this purpose was investigated. However, incubation of the bacterial-expressed rabies virus fusion proteins with these extracts in the presence of [γ-^{32}P]ATP did not result in labeling of the respective fusion proteins (results not shown).

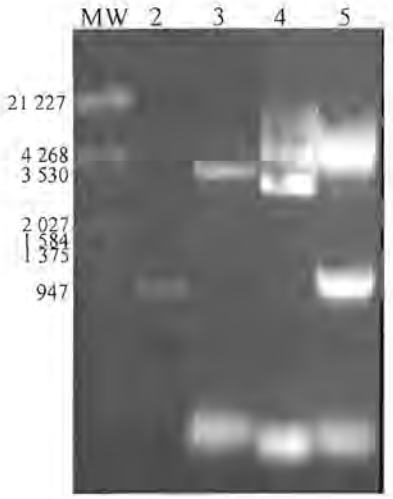

Fig. 4.1 Ethidium bromide stained 1% agarose gel, showing the construction of the recombinant PpPROEX Hc expression vector. Lane MW, Molecular weight marker; Lane 2, purifed P gene amplicon; Lane 3, EcoR1-linearized pPROEX Hc; Lane 4, uncut recombinant PpPROEX; Lane 5, EcoR1 digested PpPROEX. The sizes of molecular weight marker, phage Lambda DNA digested with EcoR1 and HindIII, are indicated to the left of the figure (in bp).

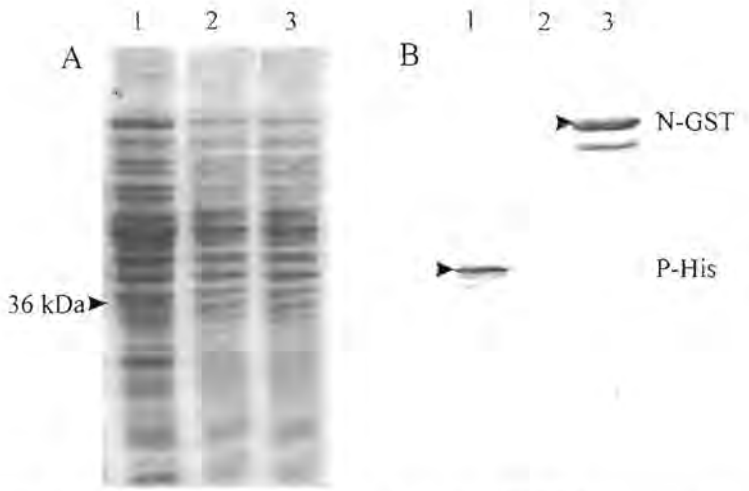

Fig. 4.2 Expression of the P protein in *E. coli* using the prokaryotic pPROEX expression system. (A) SDS-PAGE, and (B) Western blot with anti-rabies virus serum. (A) Lanes: 1, IPTG-induced cell cultures of *E. coli* transformed with PpPROEX Hc; 2, IPTG-induced *E. coli* transformed with pPROEX Hc; 3, unduced *E.coli*. (B) Lanes: 1, IPTG-induced *E. coli* cultures transformed with PpPROEX H; 2, IPTG-induced *E. coli* culture; 3, IPTG-induced cell-cultures of *E. coli* transformed with recombinant NpGEX. The arrows indicate the position of the 36 kDa P-His and the 80 kDa fusion protein.

Since both the rabies virus N and P proteins were found to contain the consensus sites for potential phosphorylation by protein kinase C (Chapter 2), an assay was developed whereby these proteins could be phosphorylated *in vitro* by making use of a commercially available phospholipid-dependent Protein Kinase C. Each phosphorylation reaction consisted of *E. coli* lysates containing either the N-GST, P-GST or P-His fusion proteins and [γ-^{32}P]ATP in the presence or absence of PKC enzyme together with its required activators. As controls, induced *E. coli* JM109 cells transformed with the parental pGEX-1 or pPROEX vector were included in the assay. Reaction mixtures containing N-GST, P-GST and the control GST protein were first immunoprecipitated with an anti-GST antibody prior to analysis by SDS-PAGE and autoradiography. Analysis of the autoradiographs indicated that neither the GST (Fig. 4.4A, lane 3) nor the histidine (Fig 4.4C, lane 3) affinity tags were phosphorylated when the respective extracts were incubated with [γ-^{32}P]ATP and the kinase enzyme. Furthermore, no incorporation of the radiolabel by the respective fusion proteins were observed in reaction mixtures from which the kinase enzyme was omitted (Fig. 4.4, lanes 2). However, the respective rabies virus fusion proteins were strongly labeled when incubated in the presence of both the PKC enzyme and [γ-^{32}P]ATP (Fig. 4.4, lanes 1). From these results it could be concluded that the rabies virus N and P proteins lacks autophosphorylation activity and, more importantly, that both the proteins could be *in vitro* phosphorylated by protein kinase C and that the kinase exhibits a specificity for both the N and P proteins.

4.3.3 Effect of phosphorylation on the ssRNA-binding activity of the rabies virus N protein

In the previous section it was shown that the bacterial-expressed rabies virus N and P proteins were unphosphorylated, but could be phosphorylated *in vitro* by protein kinase C. These fusion proteins could therefore be used to investigate the influence of phosphorylation on its ssRNA-binding ability. The ability of the unphosphorylated *E. coli*-expressed N-GST and P-GST fusion proteins to bind to ssRNA was therefore investigated before and after *in vitro* phosphorylation of the respective proteins by means

of poly(U)- and poly(A)-Sepharose affinity assays. In these assays, the purified fusion proteins (as described in Section 3.2.8) were allowed to interact with the respective Sepharose substrates in the presence of increasing concentrations of NaCl and the step-wise increase in salt concentration was used to measure changes in the degree of protein-RNA interaction. The purified GST protein served as a control in these assays. The control GST protein did not bind to either poly(U)- or poly(A)-Sepharose (results not shown). As expected, the P-GST fusion protein also did not bind to the respective substrates, irrespective of it being phosphorylated or not (results not shown). This corresponds with the lack of RNA-binding activity of the rabies virus P protein described in the literature (Yang *et al.*, 1999). The results obtained from the affinity binding assays of unphosphorylated and phosphorylated N-GST protein to poly(A)- and poly(U)-Sepharose are indicated in Fig. 4.5. The results indicated that *in vitro* phosphorylated and unphosphorylated N protein could bind to both poly(U)- and poly(A)-Sepharose. At low salt concentrations (0.025 to 0.1 M STE-TX) more of the phosphorylated N protein remained bound to poly(A)-Sepharose compared to poly(U)-Sepharose, but at higher salt concentrations (0.2 to 0.3 M STE-TX) more of the phosphorylated N protein remained bound to the poly(U)-Sepharose. Similarly, at salt concentrations ranging from 0.025 M to 0.2 M STE-TX, more of the unphosphorylated N protein remained bound to poly(A)-Sepharose compared to poly(U)-Sepharose. At salt concentrations between 0.25 and 0.4 M STE-TX, no binding of the unphosphorylated N protein to poly(A)-Sepharose was observed, although the unphosphorylated N protein still bound to the poly(U)-Sepharose.

In the case of the poly(U)-Sepharose affinity assays (Fig 4.6A), slightly less of the unphosphorylated N bound to the poly(U)-Sepharose at low salt concentrations (0.01 to 0.1 M) compared to the phosphorylated N protein. The average difference in protein bound was less than 1.2 %. At higher salt concentrations of 0.15 M to 0.3 M STE-TX, there was a clear difference in the capacity of phosphorylated and unphosphorylated N protein to bind to the poly(U)-Sepharose. At these salt concentrations, more of the phosphorylated N protein remained bound to the poly(U)-Sepharose compared to the unphosphorylated N protein. The most drastic difference was observed at 0.15 M STE-TX with almost 6-fold more of the phosphorylated N protein being bound compared to

the unphosphorylated N protein. Similarly, throughout a wide range of salt concentrations, more of the phosphorylated N protein remained bound to poly(A)-Sepharose compared to the unphosphorylated N protein (Fig. 4.6B). At a salt concentration of 0.15 M STE-TX, very little difference in the capacity of phosphorylated and unphosphorylated N proteins to bind to poly(A)-Sepharose was observed. However, at salt concentrations of 0.25 M STE-TX and higher, none of the unphosphorylated N protein remained bound. At the same salt concentrations, the phosphorylated N protein remained bound to the poly(A)-Sepharose, albeit at reduced capacity, and no binding was observed at the highest salt concentration investigated (0.4 M STE-TX).

4.3.4 Protein-protein binding assay

The ability of phosphorylated and unphosphorylated rabies virus N and P proteins to associate with each other were also investigated. The bacterial-expressed N-GST and P-His fusion proteins were metabolically labeled with [^{35}S]methionine during induction of proteins synthesis (Fig. 4.3). The [^{35}S]methionine-labeled GST or N-GST fusion proteins were bound to glutathione agarose, as described in Section 3.2.8, and then incubated with similarly labeled P-His bacterial lysates. After incubation, the unbound material was removed by several washing steps. Finally, the material bound to the glutathione agarose was analyzed by SDS-PAGE and autoradiography. The interaction between the N-GST and P-His proteins were determined by measuring the amount of N-GST and P-His in each interaction and expressing the results as the ratio of P bound to N (Fig. 4.7). The ratio of unphosphorylated P bound to unphosphorylated N is 2.07, while the ratio of phosphorylated P bound to unphosphorylated N, phosphorylated N bound to phosphorylated P is 0.48 and 0.45 respectively. In the case of phosphorylated N bound to unphosphorylated P the ratio is 1. The results from the control GST assay indicated that the P-His protein did not bind to the GST protein (results not shown).

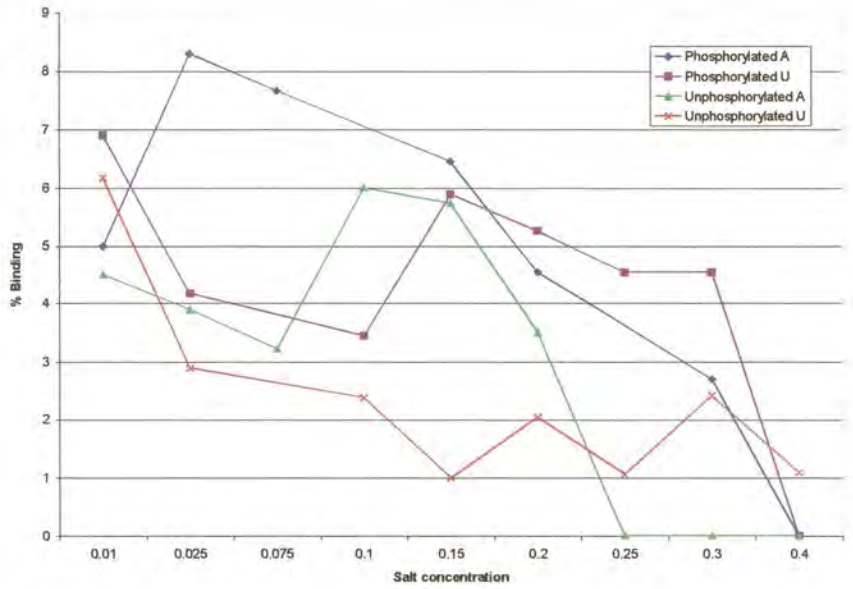

Fig. 4.5 Affinity of unphosphorylated and phosphorylated N-GST for poly(A) and poly(U) ssRNA. The N-GST was expressed in *E. coli*, purified, with or without phosphorylation and assayed for poly(A)- and poly(U)-Sepharose-binding ability over a range of different ionic strengths, as described in Materials and Methods. Phosphorylated N-GST binding to poly(A)-Sepharose is indicated in blue, phosphorylated N-GST binding to poly(U)-Sepharose is indicated in pink, unphosphorylated N-GST binding to poly(A)-Sepharose is indicated in green and unphosphorylated N-GST binding to poly(U)-Sepharose is indicated in red.

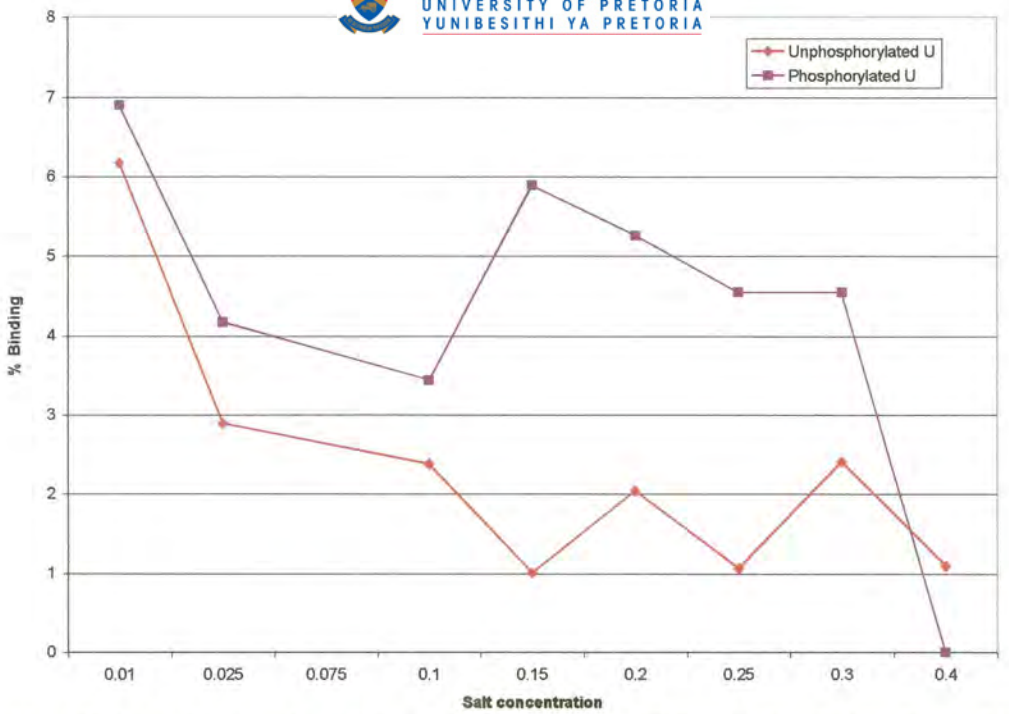

Fig. 4.6(A) Affinity of phosphorylated and unphosphorylated N-GST for poly(U) ssRNA. The N-GST was expressed in *E. coli*, purified, phosphorylated or not and assayed for poly(U)-Sepharose-binding ability over a range of different ionic strengths, as described in Materials and Methods. Phosphorylated N-GST is indicated in red and unphosphorylated N-GST is indicated in pink.

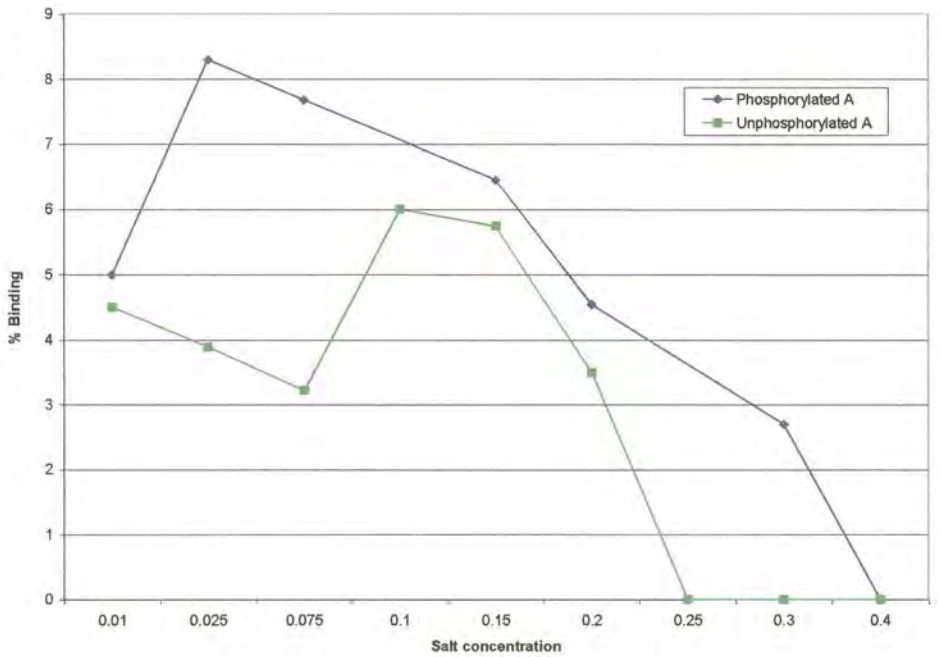

Fig. 4.6(B) Affinity of phosphorylated and unphosphorylated N-GST for poly(A) ssRNA. The N-GST was expressed in *E. coli*, purified, phosphorylated or not and assayed for poly(A)-Sepharose-binding ability over a range of different ionic strengths, as described in Materials and Methods. Phosphorylated N-GST is indicated in blue and unphosphorylated N-GST is indicated in green.

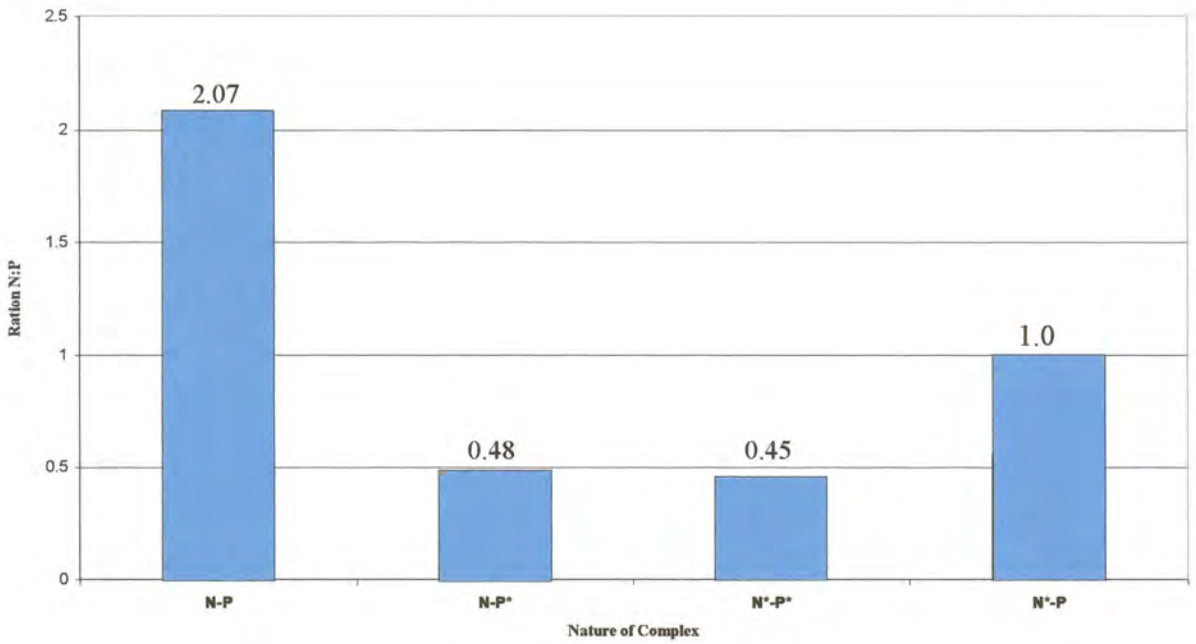

Fig. 4.7 Graphical presentation of the complexes formed between rabies virus N and P protein, with various combinations of phosphorylated and unphosphorylated N and P, respectively. The N:P ratio is given in the bar chart, with actual value given on top of the bar. The * indicate the protein that is phosphorylated.

4.4 Discussion

The importance of phosphorylation of the rabies virus N protein for its interaction with ssRNA and with the rabies virus P protein was studied *in vitro* using bacterial-expressed fusion proteins. For this purpose phosphorylated versions of the unphosphorylated bacterial-expressed rabies virus N and P proteins were required. Initially, the use of eukaryotic cell extracts as sources of kinases for phosphorylation of the N and P fusion proteins were investigated. These attempts at phosphorylating the bacterial-expressed proteins were, however, unsuccessful. Similarly, Gupta *et al.* (1995) also could not phosphorylate *E. coli*-expressed P protein *in vitro* by using a BHK cell extract and Prehaud *et al.* (1992) found that expression of the P protein in insect cells did not result in phosphorylation of the protein. It would thus appear that phosphorylation of the rabies virus proteins requires a specific protein kinase, which may be absent from the cell extracts used above.

Analysis of the rabies virus N and P amino acid sequences indicated the presence of consensus recognition sites for protein kinase C (PKC) and casein kinase II (CKII) (Chapter 2). Recently, Gupta *et al.* (2000) demonstrated that the rabies virus P protein could be phosphorylated by PKC and a second unique kinase, rabies virus protein kinase (RVPK), which is selectively packaged in the mature virion. The respective kinases were shown to phosphorylate the P protein at the C- and N terminal, respectively. In the case of P protein phosphorylation by PKC, four isomers (α, β, γ, ζ) of the enzyme were capable of phosphorylating the protein, but the γ isomer was found to be preferred to the other isomers (Gupta *et al.*, 2000). In contrast to the rabies virus P protein, the kinase(s) responsible for N protein phosphorylation has not been characterized. By making use of a commercially available preparation of PKC, bacterial-expressed rabies virus N and P fusion proteins could both be specifically phosphorylated. This also constitutes the first evidence for phosphorylation of the rabies virus N protein by PKC.

The N protein of rabies virus is a RNA-binding protein that encapsidates both positive and negative sense RNA, thereby protecting it from nuclease digestion and keeping it available for transcription and replication (Emerson, 1987; Iseni *et al.*, 2000; Keene *et al.*,

1981; Klumpp *et al.*, 1997). The rabies virus N protein binds to an A-rich encapsidation site in the leader RNA with the following sequence: 5'-AAGAAAAAACA-3' (Yang *et al.*, 1998). To investigate whether the N protein-RNA interaction may be the result of the A-rich nature of the RNA substrate, the bacterial-expressed *in vitro* phosphorylated and unphosphorylated N-GST fusion proteins were subjected to RNA-binding affinity assays using two different Sepharose substrates, poly(U)- and poly(A)-Sepharose. The percentages ssRNA-binding obtained in this study are very low. The binding experiments done by other groups (Yang *et al.*, 1998, 1999; Kawai *et al.*, 1999) did not quantify the binding results and only concluded whether binding occurred or not. The conditions under which the binding experiments during this study was done, is not optimal, but the fact that P-GST do not bind to the ssRNA template, while binding do occur with the N-GST indicate that the results reflect the binding characteristics of the rabies virus N protein. The results obtained indicated that the unphosphorylated N protein had an affinity for both poly(U)- and poly(A)-Sepharose. In the case of *in vitro* phosphorylated N-GST protein, the protein bound preferentially to poly(A)-Sepharose at salt concentrations lower than 0.15 M NaCl, and to poly(U)-Sepharose at salt concentrations higher than 0.15 M NaCl. At physiological salt concentrations (0.15 M), the phosphorylated N-GST protein bound equally well to both the ssRNA substrates. From these results it can be concluded that the N protein binds nonspecifically to ssRNA. These results are in agreement with those reported by Yang *et al.* (1999). The rabies virus N protein was reported to bind non-specifically to viral and non-viral cellular RNA in the absence of P protein. Simultaneous addition of the N and P proteins to leader RNA resulted in the specific binding of viral RNA. It would thus appear that the specificity in N protein-virus RNA binding is conferred by the P protein.

It has previously been reported that unphosphorylated N protein encapsidates more leader RNA than the phosphorylated N protein, but that N protein phosphorylation increases viral transcription and replication (Yang *et al.*, 1999). Protein phosphorylation was prevented by mutating Ser-389 to an alanine amino acid, this amino acid forms part of a Casein Kinase II recognition site (Fig. 2.4A). Newly synthesized N proteins became associated with the P protein prior to it being phosphorylated and is only then able to

encapsidate viral RNA (Kawai *et al.*, 1999). The encapsidation-associated conformational change is a prerequisite step for N protein to be phosphorylated at Ser-389 (Kawai *et al.*, 1999). To investigate the role of Protein Kinase C specific phosphorylation on the ssRNA-binding activity of the rabies virus N protein, *in vitro* phosphorylated and unphosphorylated bacterial-expressed N-GST were used in similar RNA-binding assays as above. It was found that throughout a wide range of different ionic strengths the unphosphorylated N-GST protein preparations were less effective in binding ssRNA, compared to the phosphorylated N-GST preparations. The control GST and P-GST protein preparations did not display an affinity for the ssRNA, but it can be that the 27.5 kDa fusion part of the N-GST protein changes the conformation to such an extent that the unphosphorylated protein is less effective during its binding to ssRNA. From these results it could be concluded that specific phosphorylation by Protein Kinase C changes the conformation of the rabies virus N protein to such a degree that it enhances its ability to bind to ssRNA.

The role of phosphorylation on the ability of rabies virus N and P proteins to associate with each other during complex formation was also investigated. *In vivo* [^{35}S]methionine-labeled N-GST and P-His fusion proteins were phosphorylated *in vitro* or left unphosphorylated and then mixed in different combinations to allow complexes to form. In the case of both the N and P proteins being unphosphorylated, N and P proteins interact to form complexes with a 2:1 stoichiometry. This is similar to the N:P ratios found in rabies virus virions (Fu *et al.*, 1994; Wunner, 1991) and also in VSV infected cells (Davis *et al.*, 1986; Howard *et al.*, 1987). Phosphorylation of the P protein complexed with either phosphorylated or unphosphorylated N protein resulted in a stoichiometry of 1:2 of N to P protein. In the event of unphosphorylated P protein complexed to the phosphorylated N protein, a N:P ratio of 1:1 was found. The association of newly synthesized unphosphorylated N protein with the P protein is required for phosphorylation of the N protein at Ser-389 (located within a CKII recognition site) where after encapsidation of viral RNA occurs (Kawai *et al.*, 1999). It would thus seem that both the N and P proteins must be unphosphorylated in order to correctly interact with each other. Phosphorylation of either N or P protein may alter the

protein conformation due to a charge difference thereby preventing the proteins to form complexes with a 2:1 stoichiometry.

CHAPTER 5

Concluding Remarks

The nucleoprotein of rabies virus, the prototype virus of the *Lyssavirus* genus, plays an important role during virus transcription and replication. It is capable of binding to the viral RNA genome *via* the phosphate-sugar backbone thereby exposing the nucleotide bases to the RNA polymerase and simultaneously protecting it from nuclease digestion (Emerson, 1987; Iseni *et al.*, 2000; Klumpp *et al.*, 1997). It also forms complexes with the phosphoprotein which maintains the N protein in a soluble form. In contrast to vesicular stomatitis virus (VSV), the prototype model for the *Rhabdoviridae* virus family, the rabies virus N protein is phosphorylated. Although much research regarding the kinases responsible for P protein phosphorylation as well as the identification and localization of the phosphorylated amino acid residues have been performed (Gupta *et al.*, 2000), no information regarding the kinase(s) responsible for phosphorylation of the N protein is available. Neither has the functional role that phosphorylation may play in N protein function been extensively investigated.

Towards investigating the functional significance of N protein phosphorylation, cDNA copies of the N and P genes, respectively, of a South African viverrid rabies virus isolate was cloned and sequenced. The nucleotide sequences displayed high degrees of homology when compared to the cognate sequences of other rabies virus strains. Important functional domains such as the ssRNA-binding domain and P protein-binding site on the N protein as well as the domain responsible for P protein binding to the L protein was found to be highly conserved amongst all the proteins included in the analysis. Analysis of the N and P amino acid sequences furthermore indicated the presence of several potential kinase recognition sites for protein kinase C and casein kinase II. Previously identified phosphorylated amino acid residues in the rabies virus P protein (Ser-162, Ser-210 and Ser-271; Gupta *et al.*, 2000), were found to be conserved in the P protein of the South African rabies virus strain and was located within protein kinase C recognition sites. The rabies virus N protein has been reported to be phosphorylated at Ser-389 and Thr-375 (Yang *et al.*, 1998; Kawai *et al.*, 1999) and these sites were also conserved in the South African rabies virus strain.

The rabies virus N and P proteins were subsequently expressed in a eukaryotic expression system. Expression of the rabies virus N and P proteins in the eukaryotic BAC-to-BAC™ baculovirus expression system resulted in very low levels of expression and the expressed proteins could only be detected following Western blot analysis using a rabies virus-specific serum. Whereas the recombinant baculovirus-expressed P protein was found to be soluble, the recombinant baculovirus-expressed N protein was insoluble and could only be detected in the particulate fraction prepared from the infected cells. Previous reports have indicated that co-expression of the rabies virus N and P proteins maintain the N protein in a soluble form (Yang *et al.*, 1999). A baculovirus capable of co-expressing the rabies virus N and P proteins was subsequently constructed. Although both proteins were synthesized in the recombinant baculovirus-infected cells, soluble N-P protein complexes could not be observed. This may have been due to the low expression levels obtained.

To overcome the low levels of protein synthesis obtained in the baculovirus expression system, the rabies virus N and P proteins were expressed in a prokaryotic pGEX expression system as GST fusion proteins. The respective proteins were successfully expressed as soluble fusion proteins that could be purified from bacterial lysates by glutathione affinity chromatography. The availability of purified unphosphorylated soluble N-GST allowed for further investigations regarding the functional significance of N protein phosphorylation. To obtain phosphorylated versions of the rabies virus N and P proteins, an *in vitro* phosphorylation assay was developed by making use of a commercially available phospholipid-dependent protein kinase C. It was found that protein kinase C phosphorylated both the rabies virus N and P proteins specifically. The *in vitro* phosphorylated proteins were subsequently used to investigate the influence of phosphorylation on the ssRNA-binding ability of the N protein. Poly(U)- and poly(A)-Sepharose binding assays, performed in the presence of increasing salt concentrations, indicated that throughout a wide range of salt concentrations, phosphorylated N-GST protein displayed a higher affinity for ssRNA than unphosphorylated N-GST. As expected, the P protein did not bind to the ssRNA. Yang *et al.* (1999) reported that phosphorylation of the N protein resulted in encapsidation of less RNA. In their

investigtion, the unphosphorylated N protein was obtained following mutation of Ser-389 to alanine, thereby preventing phosphorylation of the protein by casein kinase II. In this investigation, a commercially available protein kinase C enzyme was used to phosphorylate the N protein *in vitro*. It is known what the effect of phosphorylation by casein kinase II, if any, on the ssRNA-binding ability of the N protein is. This aspect of N protein phosphorylation should be further investigated.

Since the encapsidation initiation site on the virus genome has been mapped to an A-rich region in the leader RNA, it was investigated whether specificity in N protein-RNA interaction may be the result of the A-rich nature of the RNA substrate. The bacterial-expressed *in vitro* phosphorylated N-GST fusion protein, however, did not display a higher affinity for the A-rich ssRNA substrate as both poly(U)- and poly(A)-Sepharose was bound equally well at physiological salt concentration of 0.15 M. These findings are in agreement with those of Iseni *et al.* (1998) and Yang *et al.* (1999) who reported that the rabies virus N protein binds non-specifically to RNA in the absence of P protein. Previous reports have indicated that when preformed N-P protein complexes were used in the binding studies, the ability of the N protein to bind to the viral RNA was reduced. However, when the N and P proteins were simultaneously added to leader RNA, the N protein specifically bound to the viral RNA. It would thus appear that the specificity in N protein-virus RNA binding is conferred by binding of the P protein to the N protein.

The influence of rabies virus N and P protein phosphorylation on complex formation between these two proteins was furthermore investigated. Different combinations of *in vitro* phosphorylated N-GST and P-His fusion proteins were allowed to interact and the ratio of N:P was determined. A 2:1 stoichiometry of N to P in *in vitro* protein complexes could be obtained when both the N and P fusion proteins were unphosphorylated. This corresponds to the 2:1 ratio of N to P observed in purified rabies virions (Fu *et al.*, 1994; Wunner, 1991). However, phosphorylation of the P protein resulted in *in vitro* protein complexes having a N to P ratio of 1:2. However, phosphorylated N protein bound to unphosphorylated P protein to form complexes with a ratio of 1:1. These results suggest that the N and P proteins interact with each other inside the virus before phosphorylation

to form N:P complexes of the correct stoichiometry. Phosphorylation of either the N and/or the P protein resulted in *in vitro* complexes with different stoichiometries compared to that found in the rabies virions. This is consistent with the model proposed by Kawai *et al.* (1999) in which newly synthesized unphosphorylated N protein associates with the P protein before being phosphorylated at Ser-389 (located within a CKII recognition site).

Historically rabies virus research has focused on the epidemiology of the virus and characterization of the different proteins to determine their role in the virulence and antigenicity of the virus. In the last decade the focus has shifted towards understanding the role of the viral proteins during the process of transcription and replication. The identification of the kinases involved in phosphorylation of the phosphoprotein (Gupta *et al.*, 2000) and reverse genetics approaches (Yang *et al.*, 1999), will enable researchers to elucidate the mechanisms involved in controlling the replication and transcription processes. Future research should center on the role of the P protein in conferring specificity to the encapsidation of viral RNA, a similar approach to the one used for investigating the protein-protein interactions could be adopted, but by allowing the different complexes to bind to different virus and non-virus RNA. The role of N protein phosphorylation by both casein kinase II and protein kinase C can be investigated by making cocktails of these two kinases. This will indicate the effect of differentially phosphorylated rabies virus N protein and N-P complexes on ssRNA binding. By investigating the functional significance of phosphorylation of the N and P proteins during RNA-binding and protein complex formation, new aspects of the control process may be revealed.