

RABIES VIRUS N, P AND RNA INTERACTIONS IN VIVO AND MAPPING THE
FUNCTIONAL DOMAINS OF N IN N-P AND N-N INTERACTIONS

by

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(Under the Direction of Zhen Fu)

ABSTRACT

Rabies virus (RV) nucleoprotein (N) is a multifunctional protein that plays a central role in viral RNA transcription and replication. Its functions require interactions with itself and with other viral components. *In vitro* studies indicated that rabies virus phosphoprotein (P), by interacting with the nucleoprotein (N), confers the specificity of genomic RNA encapsidation by N. In this study, the interactions among N, P, and the RNA was examined in virus-infected cells as well as in transfected cells. When N is expressed alone, it binds to non-specific RNA, particularly the N mRNA. When N and P are co-expressed, the N and P form N-P complexes that do not bind to any RNA. When the N and P are co-expressed together with the mini-genomic RNA, the N-P complexes preferentially bind to the mini-genomic RNA. This demonstrated that indeed RV P, by binding to N, confers the specificity of genomic RNA encapsidation by N *in vivo*. Furthermore, we investigated the role of N phosphorylation in the N, P, and RNA interactions. It was found that only the N that bound to RNA was phosphorylated while the N in the N-P complex prior to RNA encapsidation was not, suggesting that RV P, by binding to nascent N, prevents the immediate phosphorylation of the *de novo*-synthesized N. However, mutation at the phosphorylation site of the N did not alter the pattern of N-P and N-RNA interactions, indicating that N phosphorylation *per se* does not play a direct role in the N-P

interaction and RNA encapsidation. Nevertheless, the fact that N is not phosphorylated prior to RNA encapsidation may suggest that RV P, by binding to N, keeps the N in a unique conformation for specific encapsidation of the genomic RNA. In order to understand the detailed N-P and N-N interactions, a series of terminal and internal deletion mutants of the N were constructed and attempts were made to map the domains on N that are involved in these interactions. It was determined that a central region of the N from amino acids 150 to 285 and a C-terminal region of 360-420 are important for the N-P interactions. Deletion of these regions abolished its ability to bind to P. For self aggregation, amino acids 25 to 30 are required because deletion of the first 25 amino acids did not affect the N-N interactions. However, deletion of the first 30 amino acids completely abolished the ability of N to self-aggregate. However, none of these mutants supported the transcription and/or replication of the minigenomic RNA. Together these data suggest that although different domains on the N are involved in N-N and N-P interactions, the full-length N is absolutely required for optimal viral transcription and replication.

INDEX WORDS: Rabies virus, Nucleoprotein, Phosphoprotein, RNA.

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B. S., Central China Agricultural University, China, 1992

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

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August 2004

DEDICATION

To my wife, Mingfang Li, for all her support and encouragement to make this work possible. To my children Grace and Tommy, they give me the best times after work.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Zhen Fu, for giving me the opportunity to finish my degree. His guidance and effort will be always appreciated.

I also want to thank the members of my committee, Dr. Michael Adams, Dr. Massimo Palmarini, Dr. Bruce Seal, and Dr. Bi-Cheng Wang for their best suggestion in my research and contribution to my professional development.

I would also thank my lab colleagues, Chakravarthy Chennareddy, Vikas Dhingra, Jiuhua Duan, Xiaofeng Guo, Xiaojun Lei, Ellen (Xiaqing) Li, Qiang Li, Luciana Sarmento, Zhiwei Wang, and Xianfu Wu. This wonderful group provided with me all the techniques help and best discussions which greatly helped my research work.

I extend my most sincere appreciation to the faculty, staff and students of the Department of Pathology. Special thanks to Amanda Crawford, Mia Jackson and Marcia Phillips, their supportive help will be always remembered.

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CHAPTER 1

INTRODUCTION

Despite extensive investigation in the past 100 years, rabies remains a public health threat around the globe (2). Although there are some rabies-free countries and islands, such as Japan, New Zealand, Greece, Portugal, Chile, and Barbados, rabies is prevalent in all the continental regions of Asia, Africa, Europe and America (2, 4). Among human infections, rabies is believed to be the tenth most common cause of death (13). Once clinical symptoms occur, the disease is almost invariably fatal. It is estimated that rabies causes 50,000 deaths each year. Asia accounts for more than 90% of all rabies fatalities with India alone reporting 30,000 deaths per year (5, 6). Because reporting is often incomplete, these numbers may be an underestimate.

Rabies, or hydrophobia (fear of water), is known as a disease that affects all warm-blooded animals including domestic animals such as dogs and cats as well as wild animals such as raccoons, skunks, bats, wolves, and foxes also ruminants, cows and deer (4, 12). Rabies can also infect human through the bite of rabid animals. Rabies is a classic zoonosis, which means that it is an illness that is passed directly from animal to animal and from animal to human.

The causative agent of rabies is the rabies virus, a lyssavirus (5). At the site of bite, rabies virus may or may not replicate in the muscle cells before entering the nervous system (sensory axons of peripheral nerves or motor terminals) through the neuromuscular junction (1, 9, and 10). Rabies virus spreads by retrograde fast axon transport until it reaches the spinal cord (14, 15). It is at this point that first specific symptoms of the disease occur - paraesthesia and pain at

the wound site. Encephalitis occurs as the virus spreads to and replicates to high titers in the brain. After replication in the brain, virus then spreads along peripheral nerves to other parts of the body, e.g. salivary glands where there is resultant shedding of the virus in saliva (6, 7, and 9). Early prodromal symptoms of rabies in humans are nonspecific, consisting of fever, chills, fatigue, headache, and general malaise (13, 16). As the disease progresses, neurological symptoms appear and may include insomnia, anxiety, confusion, slight or partial paralysis, excitation, hallucinations, agitation, hypersalivation, difficulty swallowing, and hydrophobia. Death usually occurs within days of the onset of symptoms (5, 6).

Human rabies can be prevented by post-exposure treatment using a combination of vaccine and rabies immunoglobulin. Rabies control can be carried out by mass vaccination of domestic pet animals. However, rabies remains endemic in most parts of the world because there is a huge global rabies reservoirs, in both domestic and wildlife animals (3, 8). It is estimated that around 6 million people worldwide receive post exposure treatment, which cause an economic burden exceeding \$1 billion annually (2). Therefore, it is imperative that extensive research on rabies and rabies virus continues, particularly in understanding the virus transcription and replication, virus-host interactions, which can constitute a foundation for more effective therapeutic intervention in clinical rabies.

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CHAPTER 2

LITERATURE REVIEW

Part 1: The History of Rabies

The word *rabies* is derived from the Latin *rabere*, meaning “to rage or to rave”. The Greek word *lyssa* means “madness” for rabies. For thousands of years, the image of the mad dogs has been terrifying humans. The first documentation of mad dog can be found as early as 2,300 B.C. in the code of *Eshunna*, "If a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death, then the owner shall pay two-thirds of a min (40 shekels) of silver. If it bites a slave and causes his death, he shall pay 15 shekels of silver" (77).

In 4th century B.C, Aristotle erroneously classified rabies as a disease of animals, he wrote briefly when describing disease of dogs, “Rabies drives the animal mad, and any animal whatever, excepting man, will take the disease if bitten by a mad dog so afflicted; the disease is fatal to the dog itself and to any animal it may bite, man excepted” (77). The Roman writer Cordamus demonstrated increasingly concrete understanding of rabies by suggesting that a "poison" in the saliva causes the hydrophobic condition. His contemporary, the Roman doctor Celsus, described the clinical symptom of rabies as “engendering an altered mind set in the patient, causing his/her torture by thirst and simultaneous invincible repulsion toward water” (77). He also prescribed a treatment by “excising bitten tissue, cauterizing the wound by hot iron, and ducking the victim into a pool” (77). This treatment remained the general accepted remedy

against hydrophobia until the appearance of Pasteur's vaccine in later 1800s. During the Renaissance, Italian physician Girolamo Fracastoro described a clinical case of rabies infection in humans. His vivid descriptions of the patient 'shrinking from water and all liquids, flinging himself hither and thither...like a madman' hold true through the present day (77). Over subsequent centuries, there was little progress in knowledge of how to prevent and cure rabies disease. In 1880's, microcopy has already been used to study pathogenic agents, and the bacillus of anthrax had been discovered. However, virus was still unknown and invisible at that time. In France, rabbit began its rapid rise to a position as the animal model in rabies experiment. Louis Pasteur bypassed the established methods of culture and attenuation and turned to what he knew to the natural habitat of the elusive invisible virus: the spinal cord of his laboratory rabbits. Working with his colleagues, he obtained the necessary proof of the neurotropic character of the virus and found that the agent was present not only in the saliva of the rabid animal but also throughout its nervous system. By inoculation of street virus directly into dogs and then serially passed through rabbits until the length of the incubation period was shortened to a final limit of 6 to 7 day, Louis Pasteur finally obtained a fixed virus on which a rabies vaccine was developed (77, 118).

Part 2: Rabies Virion Structure

Rabies virus is the prototype species of the genus *Lyssavirus* in the family *Rhabdoviridae* (92). The family *Rhabdoviridae* together with the family *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae* constitute the order of *Mononegavirales*, because all members of the order are RNA viruses that contain non-segmented, negative-stranded RNA genome (79). The

Rhabdoviridae family includes at least three genera of animal viruses, *Lyssavirus*, *Ephemerovirus*, and *Vesiculovirus*. The genus *Lyssavirus* can be grouped into seven genotypes: rabies virus, lagos bat, Mokola virus, Duvenhage virus, European bat virus types 1 and 2, and Australian bat lyssavirus (11). Rabies virus is highly neurotropic in the infected host, invariably causing a fatal encephalomyelitis (85). The other six are rabies-related lyssaviruses, reflecting the genetic diversity of viruses that share with rabies virus (118). All lyssaviruses share many biologic and physiochemical features as well as amino acid sequence characteristics that classify them with other rhabdoviruses. These include the bullet-shaped morphology, helical nucleocapsid and structural proteins of the virus. The five structural proteins of the virion include a nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase or large protein (L).

Under electron microscopy, the standard infectious rabies virions are bullet-shaped particles with average length of 180 nm (130-250 nm) and diameter of 75 nm (60-110 nm) (26, 55). Other shorter, often cone-shaped virions co-produced with standard virions are physically different, these virions are much shorter (60 to 80 nm) than the complete virion and have a viral genome of about half the size of normal rabies RNA but with all of the structural proteins of standard virions. The short virions are non-infectious, defective particles that sometimes become the dominant particles in the infected cell by interfering with production of standard infectious virus, thus the defective particles are known as defective-interfering (DI) particles.

The virions are composed of an external envelope derived from the cell membrane and an internal ribonucleoprotein (RNP) core. The single viral glycoprotein (G) spans the membrane once and forms an array of trimeric spikes that extend from the viral envelope (92). At the center of the viral particle is the RNP core. The RNA is negative-stranded, non-segmented RNA,

which implies that naked genomic RNA is not infectious (100). The genomic RNA is in a tight complex with nucleocapsid (N) proteins that are arranged like beads on a string along the RNA. This ribonucleoprotein complex is coiled into a tightly packed helix within the virion. The viral RNA-dependent RNA polymerase, composed of the catalytic larger protein (L) and noncatalytic phosphoprotein (P) proteins, is associated with the RNP core. The matrix (M) proteins are inside the viral envelope between the envelope and the RNP core.

The number of molecules comprising the RNP core has been estimated. In one report, there are 1,325 molecules of N, 691 molecules of P, and 72 molecules of L (35), while in the other, there are 1,800 molecules of N, 950 molecules of P, and 25 molecules of L (72). Nevertheless, the 2:1 ratio of N and P appears to be constant, the same ratio has also been reported in other non-segmented negative-stranded RNA viruses such as vesicular stomatitis virus (107) and Sendai virus (45).

Part 3: Rabies Virus Genome and Encoded Proteins

Rabies virus genome is 11, 928 (PV strain) or 11, 932 nucleotides (SAD B19 strain) long (21, 111). At the 3' end of the genome is a non-coding leader (le) sequence (58 nucleotides). Immediately downstream of the Le sequence, in sequential order, are the sequences coding for the five structural proteins 3'-N-P-M-G-L-5' followed by a noncoding trailer (Tr) sequence (last 70nt) at the 5' end (109, 110). Each of the structural genes is separated by short intergenic sequences (2 or 5nt). The intergenic region between G and L is a long stretch of 423 nucleotides, but does not encode any protein, thus it may be a remnant of an ancient gene (21).

The 3' Le sequence serves a multifunctional purpose in rabies virus as it does in other nonsegmented negative RNA viruses. Within the 3' terminal Le sequence, a specific cis-acting signal functions as a promoter for template recognition by the viral RNA dependent RNA polymerase. This promoter initiates genomic RNA transcription and replication (13, 21, 113, and 114). Within the Le and Tr sequence of the rabies virus RNA genome, there is a high level of sequence complementarity. The promoter sequence in the Le and Tr provide a common function in transcription and replication (118). The Le RNA also provides specific cis-acting signals for RNA encapsidation by N in the N-P soluble form (10, 119).

Nucleocapsid Protein (N)

Rabies virus nucleocapsid protein serves the critical function of tightly packaging the RNA genome into an RNase-resistant core that is the template for both transcription and replication (10, 101). The N contains 450 amino acids and has a calculated molecular weight of ~52 kDa. N is the major component of RNP core. About 1,300 molecules of N are required to encapsidate the ~12kb RNA of rabies virus with each N encapsidating about 9 nucleotides (58, 105). There is little sequence homology between N proteins of different genera in the rhabdovirus family, but the overall structure of the N protein seems to be similar (23). Within the lyssavirus genus, the amino acid sequence of N is highly conserved, particularly within genotypes although a relatively high degree of genetic diversity within short regions was found in the N gene between the genotypes (11, 21, and 64). The high level of conservation, particularly within specific regions in the N, may reflect its important functions. For example, N is specific and absolutely required for N-specific encapsidation of genomic RNA and protection of

the RNA template from ribonuclease activity (99). Another example is its role in regulating RNA transcription and modulating viral RNA transcription and replication by promoting read-through of the termination signals (88, 119, and 120).

Phosphoprotein (P)

P contains 297 amino acids and is expressed in a variety of phosphorylated forms (50). Rhabdo- and paramyxo-virus P presents both as homotrimer (24, 40) and in complex with other proteins (16, 38). Rabies virus P is a multifunctional protein. It is a key component of the virion-associated RNA polymerase complex and also interacts with N in the process of RNA encapsidation (119). In the process of RNA encapsidation, P acts as a chaperone of soluble nascent N, preventing the N from self-aggregation and nonspecific binding to cellular RNA. The P in N-P complexes specifically directs N encapsidation of the viral RNA (16, 38, 47, and 69). As a subunit of the RNA polymerase (P-L) complex, the P plays a pivotal role as a cofactor in transcription and replication of the viral genome (16, 18, and 38). The P serves both to stabilize the L protein (24) and to place the polymerase complex on the RNA template (82).

P protein exists in several forms due to either the extent of phosphorylation or the RNA polymerase initiation at alternative translation initiation codons (18, 21, and 38). The hyperphosphorylated and hypophosphorylated are the two dominant forms which exist both in rabies virions and virus-infected cells. These two forms migrate with different mobilities in SDS-PAGE (50, 116). The most hydrophilic and conserved region of P is located in the central part between amino acids 139 and 170, which is the region most of phosphorylation predicted (17). However, in VSV, the most hydrophilic region is found in the N terminal part of P between

amino acids 35 and 106 and most of the phosphate residues are found in this region (8, 54). Rabies virus P is phosphorylated in the N-terminal portion by two distinct types of protein kinases, rabies virus protein kinase (RVPK) and protein kinase C. RVPK phosphorylates recombinant P at serine 63 and serine 64 and alters its mobility in SDS-PAGE to move more slowly, as a protein of 40 kDa (50); phosphorylation of P by the PKC isoforms, dominated by PKC γ activity, did not alter the mobility of P in SDS-PAGE (50).

Matrix Protein (M)

The M of rabies virus is the smallest and most abundant protein in the virion. It contains 202 amino acids (25 kDa) (21, 108). M protein is very basic and somewhat hydrophobic, there are no domains of sufficient length to span a lipid bilayer and it is translated on free cytoplasmic ribosomes (23). The M forms a sheath around the RNP core in virion assembly, producing the skeleton structure of the virion. M is also a multifunctional protein that interacts with viral proteins and protein components of cellular membranes. The M protein functions include condensation of helical nucleocapsid cores into tight coils, association with membrane bilayers, and involvement in the cytopathogenesis of virus-infected cells (35).

There are many charged amino acids and proline residues at the N-terminal region of the rabies virus M (115), which is similar to the M of VSV and paramyxoviruses (72). The N terminus of rabies virus M plays a critical role in the regulation of RNA transcription (115). As shown in VSV, M is a potent inhibitor of RNA transcription, shutting down transcription of cellular genes of the infected cell by inhibiting host RNA polymerase (78, 105). However, this

function has not been reported in rabies virus M. The central portion of M contains a hydrophobic domain (residues 89-107) that probably interacts with membrane lipids (14, 108).

M protein has been proposed to act as a bridge between the viral envelope and the nucleocapsid (58). When M binds to nucleocapsid, it condenses the nucleocapsid core into a tightly coiled, helical RNP-M protein complex. Approximately 1,200 to 1,500 copies of M molecules bind to rabies virus RNP core. At the same time M binds to the RNP structure, it mediates binding of the viral core structure to the host membrane at the marginal region of the cytoplasm, where it initiates rabies virus budding from the cell plasma membrane (45). The M gives the virion its characteristic bullet-like shape, regardless of whether its location is within the RNP core or on the external surface of the core (20).

Glycoprotein (G)

The mature rabies virus G is 505 amino acids (~65 kDa) type I membrane glycoprotein. Rabies virus G is synthesized as a precursor of 524 amino acids, from which an N-terminal signal peptide of 19 amino acids is cleaved off after the protein is inserted into the endoplasmic reticulum (ER) (118). The G, which forms the trimeric spikes extending from the virus surface, is the only surface proteins of the virus. The hydrophobic domain of 22 amino acids is anchored in the viral envelope. The cytoplasmic domain of the G interacts with M of the skeleton particle to complete the virion assembly. The ectodomain of the G is responsible for virus interaction with its cellular receptors. Rabies virus G is a fusion protein that mediates virus entry into host cells. The G spike fuses in a low-pH-dependent process with the endosomal membranes after

virus is internalized (36). During fusion process, the G protein goes through significant conformational change in a low pH-dependent manner (4).

Mutations in the rabies virus G play a critical role in viral pathogenesis. A single amino acid substitution from the wildtype Arg-333 (or Lys-333) to glutamine (Gln), isoleucine (Ile), glycine (Gly), methionine (Met), or serine (Ser) significantly reduces the virulence of rabies virus when inoculated intracerebrally into adult immunocompetent mice (66, 74). It is remarkable that this single amino acid substitution can affect the rate of virus spread from cell to cell (83) as well as the neuronal pathway that the virus takes to reach the CNS (63, 101). Rabies virus G is also of major importance immunologically for the induction of the host immune response against virus infection. The G induces conformational and linear epitope-specific VNA and stimulates helper as well as cytotoxic T-cell activities. At least eight antigenic sites have been located on the ectodomain of the G of different virus strains (17).

Large Protein (L)

The L is the largest protein in the rabies virus particle. It contains 2124 and 2127 amino acids in the PV and SAD-B19 strains of rabies virus, respectively (21). The L is the catalytic component of the polymerase complex and responsible for the majority of enzymatic activities of transcription, replication, mRNA capping, methylation and polyadenylation (4).

Comparisons of L sequences from different Mononegavirales viruses to other RNA dependent RNA polymerases and DNA polymerases have helped to map the functional domains (56, 95, and 99). Four motifs labeled A through D, in the central part of the rabies virus L, between residues 530 and 1,177 and between residues 532 and 1,201 in VSV L, represent

regions of highest similarity (56, 95, and 99). These motifs, which are thought to constitute the polymerase module, maintain the same linear arrangement and location in all viral RNA-dependent RNA and DNA polymerase (30, 99). Among the conserved sequences in these four motifs is the tri-amino acid core sequence GDN in motif C, which is extensively conserved in all non-segmented negative-strand RNA viruses (99). A recent study has shown that not only the GDN core sequence but also specific amino acids downstream from the core sequence are crucial for the maintenance of polymerase activity (30).

Par 4: Rabies Virus Transcription and Replication

The sequence of events in rabies virus infectious cycle can be divided into three phases (118). The early phase includes virus attachment to receptors on susceptible host cells, entry via endocytosis, fusion with endosomal membranes, uncoating of virus particles, and liberation of the helical RNP to the cytosol. The middle phase includes transcription and replication of the viral genome, and the late phase includes virus assembly and egress from the infected cell.

Adsorption: Rabies virus infection is initiated by attachment of virus to a receptor on the host cell surface. The receptor for rabies virus has been controversial, and recent evidence indicates that several different receptors can be used. The first of such receptor candidates is nicotinic acetylcholine receptor (AChR). The AChR and rabies virus can be found at the same neuromuscular junctions *in situ* (12, 66). Further support for the hypothesis that the AChR may be biologically important in rabies virus infection *in vivo* comes from studies with an anti-idiotypic antibody and virus overlay binding assay (43, 51). Infection of cell lines that lack AChR, however, indicated that other molecules must also be used by rabies virus for entry (91).

Recently, the neural cell adhesion molecule (NCAM) (106) and the low-affinity neurotrophin receptor p75NTR (111) have been reported as rabies virus receptors.

Entry and Uncoating: After binding to its cellular receptor, rabies virus is internalized by receptor-mediated endocytosis or clathrin-coated pits (73, 74). After internalization, the viral glycoprotein mediates fusion of the viral envelope with the endosomal membrane at the low pH environment (74, 115). The threshold pH for fusion activation for rabies virus is about pH 6.3 and involves a series of specific and discrete conformational changes in G (45, 46). Prior to virus binding to the cellular receptor, glycoprotein on the virion surface is in its initial native state. After virus attachment and internalization, glycoprotein is activated and exposes its hydrophobic domain to interact with the hydrophobic endosomal membrane. Once entered the low pH endosomal compartment, the glycoprotein is activated and exposes its fusion domain to interact with the participating membrane (45).

Genomic RNA transcription: Transcription of the viral genomic RNA occurs in the cytoplasm of the infected cell once the RNP core is released from endosome. The transcription process is carried out on the RNP complex by the virion-associated RNA polymerase complex (L-P) and is independent of host-cell functions (2). The virion-associated polymerase complex initiates transcription at the 3' end of the genomic RNA, where the polymerase first synthesizes a small 55 nucleotide RNA called the leader. The leader RNA is neither capped nor polyadenylated (20, 67), in contrast to the mRNA transcripts (20). Each of the five mRNAs encoding the viral proteins is then synthesized in the order it appears from the 3' end of the genome (1, 2). At each intergenic junction, however, the polymerase pauses before continuing the down-stream mRNA transcription process, and an estimated 20-30% of the polymerase complexes that reach the gene junction dissociate from the nucleocapsid (32). As a result, fewer

polymerase molecules remain associated with the genome RNA-N template after each gene junction to resume the transcription process (32). Thus the number of mRNAs synthesized from the remaining genes downstream in the genome gradually decrease in proportion to the number of polymerases that fall off. This phenomenon of self-regulating viral gene expression is a form of “localized” attenuation (60).

Each of the rabies virus N, M, G, and L genes encodes only one protein (450 amino acids for N, 202 amino acids for M, 524 amino acids for G, and 2, 142 amino acids for L) from a single ORF except the P gene. In addition to the full-length P (297 amino acids), three internal initiation sites on the P ORF are used to synthesize three smaller proteins, truncation of the first 19 amino acids (278 amino acids), the first 52 amino acids (242 amino acids), or the first 58 amino acids (249 amino acids). These P truncated form have been found in purified virions, in virus infected cells, and in cells transfected with a plasmid encoding the complete P sequence (17). It is thought that a leaky scanning mechanism is responsible for translation of P gene at the internal in-frame start codons, the functions of these P forms are unknown (17)

Genome replication: As soon as nascent soluble N protein is produced in the cytoplasm, it encapsidates the leader RNA (39, 53, and 119). Encapsidation of the leader RNA prevents termination of leader RNA transcription at the Leader-N gene junction. As a result, transcription is switched to replication to produce a full length antigenomic RNA (5, 10, 119, and 120). This antitermination model is widely accepted to explain negative strand RNA virus replication because immediately after virus entry, transcription would be favored over replication, at later times, when the concentration of N increases, replication would be favored (92). However, recent evidence from vesicular stomatitis virus shows that transcriptase and replicase are two different complexes. The two-polymerase model arose from studying P mutants that were

defective in transcription but able to support efficient replication of VSV DI RNAs (25, 87). The P mutants either had substitutions in the conserved C-terminal basic residues and could bind N protein but did not interact efficiently with L (25), or were defective in domain I phosphorylation (87). Because phosphorylation is required for P oligomerization, transcription activation, and interaction with L, the transcriptase might be composed of an L-P₃ complex, whereas the replicase might be composed of an L-(N-P) complex in which P is not phosphorylated. Regulation of P phosphorylation and ongoing viral protein synthesis would therefore affect the formation of the two different complexes at different times after infection (92).

Encapsidation of the genomic and antigenomic RNA by N occurs at the same time during replication. The 3' terminal cis-acting signal in the genome acts as nucleation signal for the initial encapsidation (7). Once the initial encapsidation occurs, encapsidation of the genomic and antigenomic RNA is believed to advance rapidly, independent of the viral RNA sequence (5). This fits well with the antitermination model for the switch from transcription to replication. The RNA encapsidation signal must reside in the 5'-Tr of the genome and the antigenome (leader) because the leader RNA and progeny genomes are the only virus-specific products that are encapsidated (1, 10, and 67). For rabies virus, leader RNA encapsidation may also be regulated by the phosphorylation status of N protein. Unlike the VSV N, which is not phosphorylated, rabies N is phosphorylated on serine residue 389, and the unphosphorylated form of N protein binds more tightly to leader RNA *in vitro* (120).

During infection, the two full length genomic and antigenomic RNAs are produced in disproportionate amounts. The ratio of genome to antigenome is 49:1 (34). The bias for the excessive production of genomic RNA over antigenomic RNA in the rabies virus infected cells is attributed to the activity of their cis-acting sequence (34). In rabies and vesicular stomatitis virus,

the 3' end of the genomic and antigenomic RNA were important for polymerase binding, because the 3'-terminal 20 nucleotides of the antigenomic RNA serves as a specific enhance for replication (68).

Assembly and Budding: The process of virus assembly can be divided into three distinct phases (44): (1) encapsidation of newly synthesized RNA by N, (2) simultaneous condensation of the RNP core by matrix protein, and (3) particle envelopment and release. The process of virus assembly begins as soon as the viral progeny RNA is encapsidated. When N binds to genomic RNA phosphate-sugar backbone, the RNA becomes fully protected from degradation by cellular ribonucleases (58, 65). After encapsidation, the RNP complex associate with M, the M plays critical role in virus assembly and budding. M binds to RNP and condenses the RNP core from the outside, a step that is sufficient to initiate virus budding (71, 80). Condensation of RNP core inhibits the transcription activity of RNA polymerase. The M then localizes the RNP core at the cellular membrane, where the nascent G protein is concentrated and M is able to interact with G (81, 97). In mature rabies virion, M lies between the lipid membrane and the RNP core, this is different to the VSV model which suggested the M presences inside the RNP core. It was shown that M still binds to the RNP coil when VSV envelope was stripped off the virion (6). In rabies, M covering and condensation is thought to play an important role in virion morphogenesis (81). If M is missing from rabies particles, the budded particle will contain uncondensed RNP core (81). M deficient rabies virus causes increased cell-cell fusion and enhance cell death, however, wild type rabies virus cause relatively minor cytopathic effect to infected cells. In addition to the role of condensation RNP core, M can also down regulate RNA polymerase activity (19, 27, 37, and 59), this is perhaps to preserve the potential of remaining

active RNA polymerase complex in the RNP core to resume its activity when progeny virus infects a second cell (33).

In the final stages of virus assembly, the mature virions acquire their lipid bilayer envelope during budding through the host cell membrane. Virus budding is observed both in extraneural tissue cells *in vivo* and *in vitro* tissue culture system (61, 84, and 110). Occasionally, virion maturation occurs intracellularly by budding through the cytoplasmic ER or Golgi apparatus, this will involve the Golgi membranes (49, 76). If budding occurs at a site in the cell membrane where the nascent rabies virus trans-membrane glycoprotein is also targeted, then infectious virions will be produced. The mechanism by which M mediates the budding of virus from the cell membrane appears to be associated with a proline-rich (PPPY, PPxY or PV) domain located at residues 35-38 within the highly conserved 14-amino-acid sequence near the N terminus of the rabies virus M (67). A corresponding proline-rich motif is found in the M of VSV (92), as well as in the M of Ebola and Marburg viruses (25). The PY motif is very similar to the late budding domain identified in viral proteins such as the Gag proteins p2b of Rous sarcoma virus (87) and the p6 Gag protein in human immunodeficiency virus (1), both of which are associated with virus budding. The unique function of the PY motif is that it interacts with a WW domain, 38-40 amino acids long with two highly conserved tryptophans spaced 20-22 amino acids apart, found in a wide range of cellular proteins. Some of the WW domain-containing proteins are involved in cytoskeletal formation, whereas others are involved in signal transduction and gene regulation (10). It is therefore likely that the rabies virus M involves cellular proteins in the release of rabies virions from the cell (68). Although the exocytotic release of virus is enhanced greatly by the interaction of the RNP-M complex with the envelope G (68), increased virion production as a result of direct interaction of the cytoplasmic domain of the

G and the viral RNP-M core suggests that a concerted action of both core and spike proteins is necessary for efficient recovery of virions. Interaction of G with M is essential for stabilization of the G trimers on the virion surface and for efficient budding of rabies virus (80, 81, and 70).

Part 5: Production of Defective Interfering Particles

In RNA virus, defective interfering (DI) RNAs are shortened forms of viral genomes that have generally lost all essential viral genes for movement, replication, and encapsidation. DI RNAs require the presence of a helper virus to provide *trans*-acting factors necessary for replication and often multiply and accumulate at the expense of the helper virus from which they originated. Interference with the helper virus frequently results in remarkable symptom attenuation (52, 89, and 93).

Part 6 : Rabies Virus Protein Interactions

After attachment of rabies virus to the surface of a cell, the RNP complex of rabies virus is released into the cytoplasm via receptor-mediated endocytosis (73, 74). RNA synthesis occurs entirely in the cytoplasm (36). In primary transcription, the virion-associated polymerase responds to specific signals in the 3' end of the genomic RNA to transcribe six discrete RNAs: a 55-nucleotide leader RNA (Le⁺), which is neither capped nor polyadenylated, and 5 mRNAs that are capped at the 5' end and polyadenylated at the 3' end. As soon as the viral protein is translated, genome replication starts. During replication, the RdRP initiates at the extreme 3' end of the genome, ignores all the signals for production of discrete monocistronic mRNAs and

instead synthesizes a full-length complementary antigenome. In turn, the antigenomic RNA serves as template for synthesis of full-length progeny genomes (9). What determines the RdRP switch from transcription to replication is poorly understood, it was proposed that the availability of soluble N protein to encapsidate the nascent genomic and antigenomic RNA is a key factor (5, 112, 117, and 119). The viral N protein is thought to modulate transcription and replication by its ability to bind to the nascent leader RNA and simultaneously promote read-through of the termination signal and initiate nucleocapsid assembly on the nascent RNA chain (9).

N-P interactions: In rabies virus infected cells, nascent N is quickly consumed in the formation of N-N and N-P complexes and assembled into nascent viral RNP complexes. The immediate interaction of nascent N with P serves to prevent self-aggregation of N and keeps N in a soluble state until its specific encapsidation of viral genomic RNA (16, 38, 75, and 119). Using the method of deletion mutant analysis, two independent regions were found on rabies virus P that binds to N in both in vitro and in vivo (16, 38). One region is located between amino acids 268 and 297 of P and another in the N-terminal portion of the protein between amino acids 69 and 177 (16, 38). In Sendai virus and VSV, similar regions on P were found to be important for N binding (33, 48, and 94).

P-L interactions: Rabies virus P protein is also a co-factor of L, P is required to bind to L to produce a fully active RNA-dependent RNA polymerase (18). The P subunit in the P-L complex has a major binding site for L protein within the first 19 amino acids of P (18). This is in agreement with the model that suggests that the L binding site resides in the negatively charged N terminus of the VSV P, although another region also has been shown to contribute to L binding (103, 118). The N and L binding sites on P do not overlapping, this correlates very well with the dual functionality of the P protein: P can interact simultaneously with both L and N

proteins to act as a transcription factor when binding to the L protein and as a replication factor when binding to the N protein (18). Unlike the P of VSV, the rabies virus P does not appear to be required for L stabilization. The P itself can form trimers or tetramers and the oligomers are necessary for binding to L (41, 47 and 98). Like the P of Sendai virus, oligomerization of rabies virus P does not require phosphorylation nor is the N-terminal domain (first 52 amino acids) necessary for oligomerization or binding to the N-RNA template (24, 47, and 104). This is in contrast to the P of VSV, which requires phosphorylation for oligomer formation to be fully active and necessary for binding both to L protein and to the RNA template (41).

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CHAPTER 3

THE INTERACTION AMONG RABIES VIRUS NUCLEOPROTEIN, PHOSPHOPROTEIN, AND THE GENOMIC RNA IN VIRUS INFECTED CELLS AS COMPARED TO TRANSFECTED CELLS: THE ROLE OF THE NUCLEOPROTEIN IN GENOMIC RNA ENCAPSIDATION¹

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ABSTRACT

It was previously reported that rabies virus (RV) phosphoprotein (P), by interacting with the nucleoprotein (N), confers the specificity of genomic RNA encapsidation by N *in vitro*. Herein we report the interactions among N, P, and the viral genomic RNA in virus-infected as well as in transfected cells. When N is expressed alone, it binds non-specific RNA, also its mRNA. When N and P are co-expressed, the N and P form N-P complexes that do not bind to any non-specific RNA. When the N and P are co-expressed together with the minigenomic RNA, the N-P complexes preferentially bind the minigenomic RNA. This demonstrates that indeed RV P, by binding to N, confers the specificity of genomic RNA encapsidation by N *in vivo*. Furthermore, we investigated the role of N phosphorylation in the N, P, and RNA interactions. Only the N that bound to RNA was phosphorylated while the N in the N-P complex prior to RNA binding was not, suggesting that RV P, by binding to nascent N, prevents the immediate phosphorylation of the *de novo*-synthesized N. However, mutation at the phosphorylation site of N did not alter the pattern of N-P and N-RNA interactions, indicating that N phosphorylation *per se* does not play a direct role in the N-P interaction and RNA binding. Nevertheless, the fact that N is not phosphorylated prior to RNA binding may suggest that RV P, by binding to N, keeps the N in a unique conformation for specific encapsidation of the genomic RNA.

INTRODUCTION

The genomic RNA of rabies virus (RV), like other non-segmented negative-stranded RNA viruses, is tightly encapsidated by the nucleoprotein (N) and this N-RNA complex, together with the phosphoprotein (P) and the RNA-dependent RNA polymerase (L), forms the ribonucleoprotein (RNP) complex (1, 21). The complex interactions involving each of these components within the RNP lead to the initiation and regulation of viral RNA transcription and replication (3, 26, and 29). It has been known that vesicular stomatitis virus (VSV) N plays important roles in the switch from RNA transcription to replication by encapsidating the *de novo*-synthesized viral genomic RNA (2, 5, 18, and 26). The P of VSV, in addition to being a co-factor for the RNA-dependent RNA polymerase (2), plays a vital role in the RNA replication process by forming a complex with VSV N keeping it in a suitable form for nascent RNA encapsidation (2, 5, and 26). Furthermore, it is known from *in vitro* studies that VSV N alone is capable of binding to any RNA (5). It is the P that, by first binding to N, confers the specificity of N encapsidation of genomic RNA (2, 3). Studies of RV N-P and N-RNA interactions *in vitro* also revealed that N alone can bind to any RNA although it preferentially encapsidates RV-specific leader RNA (30). When RV N and P were added simultaneously into the *in vitro* binding reaction, it does not increase the affinity of N binding to the genomic RNA. However, it dramatically reduces the ability of N to bind non-specific RNA, indicating that indeed P, by binding to N, confers the specificity of N for genomic RNA encapsidation by eliminating the non-specific binding to RNA (30). We postulate that these *in vitro* observations reflect the situation in the RV-infected cells. When rabies virus N is expressed on its own in insect cells, it binds to cellular RNA to form nucleocapsid-like structures (14). In contrast, when N is expressed

in virus-infected cells, only genomic RNA is encapsidated, especially in purified virions (29). However, the detailed interactions between N, P, and genomic RNA in the process of RNA encapsidation *in vivo* have not been reported. Furthermore, the mechanism by which the P, by binding to N, confers the specific N encapsidation of genomic RNA is not understood.

RV N is phosphorylated (24) at the serine at position 389 (7). Recently, we have identified cellular casein kinase II as the enzyme that phosphorylates rabies virus N (23). Phosphorylation of RV N plays a regulatory role in the process of viral RNA transcription and replication since mutation of the phosphorylated serine in N leads to a reduction of both viral transcription and replication (22). Because dephosphorylated and unphosphorylated N binds more strongly to RNA than the phosphorylated N (31), we propose that N phosphorylation plays a role in the process of viral RNA transcription and replication via modulation of RNA encapsidation. Phosphorylated N is detected in the nucleocapsids of virus-infected cells, but not in the free N (mostly in the N-P complex) (15). This may indicate that the newly synthesized N is not immediately phosphorylated but rather is associated first with P, which suggests that RV N phosphorylation occurs in the processes of or after RNA binding.

We investigated RV N, P and RNA interactions *in vivo* by expressing these components either individually or in combination in insect and mammalian cells. Our results show that when RV N is expressed alone *in vivo*, it can bind to any RNA, also its N mRNA. When N and P are co-expressed *in vivo*, the N and P form N-P complexes that do not bind to non-specific RNA, even the N mRNA. In the presence of RV (mini-)genomic RNA, the N-P complex binds to genomic RNA (specific RNA binding). Furthermore, the N in the N-P complex is not phosphorylated prior to encapsidation of the genomic RNA, suggesting that rabies virus P, by binding to N, prevents the N from being phosphorylated.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies. BSR cells (a clone of BHK) were grown in Dulbecco's minimal essential medium and insect cell line (Hi-5) was cultured in Insect Express medium (BioWhittaker, Walkersville, MD). RV strain L16 was prepared as described (27) and recombinant baculoviruses expressing RV N (BRN) and P (BRP) were prepared as described (8, 9). Recombinant vaccinia virus expressing bacterial T7 RNA polymerase (vTF7-3) was prepared as described previously (10). Plasmids for expression of N (pRN), mutant N with serine at 389 changed to alanine (pRN-SA), and P (pRP) were constructed previously in our laboratory (9, 31). RV minigenome (pSDI-CAT) was obtained from K. Conzelmann (6). Monoclonal antibody to RV N (MAb 802-2) was obtained from CDC (12) and anti-P polyclonal antibody was prepared in rabbits (9).

Protein expression in insect cells. Insect cells were infected with BRN, BRP, or in combination at a multiplicity of infection (moi) of 1 plaque-forming unit (pfu) per cell as described previously (8, 9). Eight days after infection, cells were harvested by centrifugation and disrupted in a Dounce homogenizer. After removing nuclei and cell debris by low-speed centrifugation, the supernatants were used to purify N or N-RNA complexes (25).

Protein expression in mammalian cells. Transfection and labeling of BSR cells were performed as described previously (27). Briefly, BSR cells in 100 mm Petri dish were infected with recombinant vaccinia virus (vTF7-3) at a moi of 1 pfu per cell. One hour after virus infection, the cells were transfected with 7.5 μ g pRN alone, 7.5 μ g pRN and 3.75 μ g pRP, 7.5 μ g pRN and 3.75 μ g pRP plus 3.0 μ g pSDI-CAT, or pRN and pSDI-CAT. Alternatively, pRN was replaced by pRN-SA in the transfection. Twelve hours after transfection, the cells were washed

with PBS and incubated with methionine-free medium for one hour. Then the cells were labeled with [35 S]-methionine (Amersham Pharmacia Biotech) for eight hours. Cells were harvested and processed as above.

Purification of viral RNP, recombinant N, and B-RNP. Viral RNP was purified as described (22) from virus-infected BSR cells. Briefly, BSR cells were infected with RV strain L16 as described (27). At 5 days post infection (pi), the cells were harvested by centrifugation and lysed with deionized H₂O. After removing the cell debris and nuclei, the supernatants were added to a 40% (W/V) CsCl cushion made in NTE buffer (0.2 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and then centrifuged in a SW41 rotor at 37,000 rpm overnight. Viral RNP banded in the cushion was collected and dialyzed. Recombinant N was purified from insect cells by affinity chromatography as described (8). B-RNP was purified the same way as for viral RNP by centrifuging in the CsCl cushion. To further separate N, P, and N-P complex from N-RNA complex, discontinuous CsCl gradient centrifugation was performed as described (25) with minor modification. Briefly, 2 ml of the protein samples prepared from insect or mammalian cells were loaded onto a discontinuous CsCl gradient (20-40%) made in NTE buffer (0.2 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). After centrifugation in an SW41 rotor at 37,000 rpm overnight, the gradient was fractionated from top to bottom in 1 ml fraction for the first four fractions and then 0.5 ml for the rest. Each of the fractions was dialyzed against NTE buffer before SDS-PAGE and Western-blot analysis.

***In vitro* RNA binding and gel shift assay:** RV leader RNA was transcribed and labeled as described previously (31). RNA binding was performed as the following: recombinant RV N (8) or B-RNP purified from insect cells was allowed to react with *in vitro* transcribed RV leader RNA (10^6 cpm per reaction). The RNA-protein mixtures were subjected to digestion with 2U

Micrococcal nuclease and 2 μ g RNase A at 37°C for 30 min. The reaction products were analyzed by electrophoresis on a 4% polyacrylamide gel containing 5% glycerol. Finally, the gel was dried and exposed to X-ray film.

SDS-PAGE and Western-blotting. Dialyzed samples were subjected to electrophoresis on 12% polyacrylamide-10% SDS gel and the proteins separated on the gel were electroblotted onto positively charged nylon membrane (Osmonic). Membranes were blocked with 5% nonfat dry milk at room temperature for 1 hr and then incubated with anti-N or anti-P antibodies. After washing three times with PBS, the membrane was incubated with biotinylated secondary antibody. Finally, the proteins on the membrane were detected with DAB substrate (Vector Lab) according to manufacture's protocol.

Immunoprecipitation and autoradiography. Samples prepared from mammalian cells (radio-labeled) were diluted three times with NTE buffer and incubated with anti-N or anti-P antibodies overnight at 4°C. Protein A-Sepharose was then added and the reaction was incubated for a further 2 h at 4°C. The immune complexes were precipitated and washed three times in NTE buffer and analyzed by SDS-PAGE followed by autoradiography (25).

RNA extraction, Northern blot, and dot-blot hybridization. RNA extraction was performed either from purified viral RNP, B-RNP, B-N, different fractions of the CsCl gradient, or from immunoprecipitated preparations by using Trizol reagent (Life technologies). RNA samples were subjected to electrophoresis on a 1% agarose gel and blotted onto membrane for Northern blot hybridization (8). Alternatively, RNA was directly dot-blotted to nylon membrane and fixed by UV cross-linking. Genomic RNA, genomic analog or N mRNA was detected by hybridization with digoxigenin-labeled N- or CAT-specific probes as described (27).

Phosphorylation of RV N protein. To study the status of N phosphorylation in infected or transfected cells, BSR cells were labeled with [^{32}P] phosphoric acid (Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (27, 31). Cells were harvested and subjected to immunoprecipitation with anti-N antibodies followed by electrophoresis on 12% polyacrylamide /10% SDS gel and autoradiography (27).

RESULTS

RV N expressed in insect cells exists as RNA-free form and RNA-bound complexes.

We showed previously that RV N purified from insect cells (termed B-N) is capable of binding to RNA *in vitro* (30). However, Iseni et al. (14) reported that RV N expressed in insect cells already bound to RNA, particularly cellular tRNA. This raised the question as to whether the RNA-containing N is still capable of further encapsidating RNA *in vitro* (14, 23). To investigate if RV N purified by affinity chromatography (B-N) is also associated with RNA, we subjected affinity purified N to centrifugation in a 40% CsCl cushion. As a control, viral RNP was prepared from RV-infected BSR cells in a 40% CsCl cushion. A band was formed in the affinity-purified N cushion at the same position as the RNP band from virus-infected cells. The banded material (termed B-RNP) was removed and the refractive index measured. The refractive index of the B-RNP is 1.3665, very similar to that from the viral RNP (1.3655), which corresponds to a density of 1.345 and 1.329, respectively. These data indicated that the affinity purified N from insect cells contained RNA and formed nucleocapsid-like complex.

To analyze whether the recombinant N expressed in insect cells exists in forms other than N-RNA complex, we collected 9 fractions from the cushion. An aliquot from each of the fractions (fractions 5, 6, 7, and 8 contained B-RNP) was analyzed by SDS-PAGE followed by Coomassie blue staining. As shown in Fig. 3.1A, N was found in every fraction. To analyze the RNA content, we pooled the B-RNP portion (fractions 5, 6, 7, and 8) and the rest of the cushion (fractions 1-4 and 9), RNA was extracted from the two pools and subjected to electrophoresis. As shown in Fig. 3.1C, many RNA species were observed ranging from about 100 to 800 nucleotides in the B-RNP portion (lane 2), but not from the rest of the cushion (lane 3). These data indicated that although N was purified to near homogeneity by affinity chromatography (8), both RNA free N (about 40% of total N) and RNA-bound N (B-RNP, about 60% of total N) were found.

RV N expressed in insect cells binds to N mRNA. The RV N expressed in insect cells binds to cellular RNA, particularly tRNA (14). However, our previous *in vitro* studies demonstrated that RV N binds more strongly to N mRNA than to other non-specific and non-viral RNA (30). To investigate if RV N expressed in insect cells also bound to N mRNA, RNA was prepared from each of the fractions as shown in Fig. 3.1A and subjected to Northern blot hybridization using RV N-specific cDNA probe (27). As presented in Fig. 1B, N RNA was detected only in the B-RNP fractions (fractions 5, 6, 7, and 8), but not in other fractions of the cushion, further indicating that N expressed in insect cells existed as both RNA-free and RNA-bound forms. In addition, RNA preparations were also made from equal amount of B-RNP, B-N (before CsCl cushion), or viral RNP and subjected to Northern blot hybridization using RV N-specific cDNA probe (27). As shown in Fig. 3.2A, N RNA was detected in all samples with the most intense RNA band from viral RNP and the least intensive band from B-N. The N RNA in

the RNP sample represents genomic RNA and the N RNA in the B-RNP and B-N represents N mRNA. These data indicate that at least part of the insect cell expressed recombinant N binds to N mRNA. To test the ability of B-RNP to bind to RNA, the same amount of B-RNP and B-N (by weight) was used to bind *in vitro*-synthesized leader RNA in a binding assay as described previously (30). As shown in Fig. 3.2B, B-N bound 15 times more RNA than the B-RNP as measured by densitometry. These results indicate that B-RNP already bound RNA in the insect cells and as expected, its ability to further bind to *in vitro*-synthesized RNA was dramatically reduced.

RV N and P form complexes that prevent N from binding to RNA in insect cells.

Previous *in vitro* studies have demonstrated that the P can prevent N from binding to non-specific RNA, particularly when N and P are simultaneously added into the binding reaction (3, 30). These observations have led to the conclusion that it is the P that confers the specificity of genomic RNA encapsidation by N (2). However, few *in vivo* studies have been completed to confirm these *in vitro* observations. We previously demonstrated that RV N and P expressed in insect cells can interact with each other to form N-P complexes (9). RV N also interacts with RNA to form N-RNA complex in the insect cells (Iseni et al., 1998 and this study). Thus the insect cells expressing RV N and P can be used as a model system to study the detailed N, P and RNA interactions. To this end, RV N and P were expressed either individually or in combination using recombinant baculoviruses (8, 9). RV-infected cells were used as a control. The cells were harvested and the nuclei removed. The soluble portion was subjected to discontinuous CsCl gradient (20-40%) centrifugation and the gradient was fractionated. Each of the fractions was subjected to SDS-PAGE analysis followed by Western blotting using either anti-N or anti-P antibodies. The results are presented in Fig. 3.3A. In rabies virus-infected BSR cells, both N and

P were detected in fractions 11 to 15, and again in fractions 17 and 18, indicating that most of the N forms complexes with P in the infected cells. A small portion of P was detected in fractions 7 to 10, suggesting that a portion of the P exists as monomers or homo-oligomers before binding to N. In the insect cells that express the N alone (B-RN), most of the N was detected in fractions 16-18, near the bottom of the gradient. A minor portion of the N was detected in the upper fractions 10 to 14. In the insect cells that express P alone, all of the P was detected in the upper fractions 5 to 12 and no P was detected in the bottom fractions. In the insect cells that express both the N and P, most of the N was shifted from the bottom fractions 16-18 to the upper fractions 8-14. The P was detected in the upper fractions 5-14, mostly in fractions where N was also detected. However, no P was detected in the bottom fractions (16-17) although some N was detected in these fractions.

To determine if the N in any of these fractions was associated with RNA, each of the fractions was subjected to RNA extraction and the RNA was spotted onto membrane for dot blot hybridization with labeled N-specific cDNA probe. As shown in Fig. 3.3B, RNA was only detected in the bottom fractions, particularly where N was also abundant, indicating that RNA-bound N sediments into the bottom fractions just like viral RNP. The protein distribution profile presented in Fig. 3.3A and the detection of RNA (Fig. 3.3B) indicate the following. 1) In the infected cells, RV N interacts with P to form the N-P complexes that encapsidates genomic RNA, thus sedimenting to the bottom fractions of the gradient. Some N-P complexes are in the upper fractions that have not yet encapsidated genomic RNA. 2) In the insect cells that express N alone, most of the N binds non-specific RNA, forming a nucleocapsid-like structure (B-RNP). B-RNP sediments to the bottom fractions of the CsCl gradient as viral RNP do. 3) P alone does not bind to RNA and remains in the upper fractions. 4) In insect cells that express both the N and P,

most of the N binds to P to form N-P complexes, which do not bind to any RNA. All these data suggest that N by itself can bind to any RNA. However, when P is co-expressed with N in the insect cells, P binds to N and prevents it from encapsidating non-specific RNA.

RV P protein confers the specificity of genomic RNA encapsidation *in vivo*. Although the studies in insect cells suggest that P, by binding to N, prevents the N from binding non-specific RNA, they did not indicate whether the N-P complex is able to selectively encapsidate genomic RNA because no genomic RNA was expressed in the insect cells. To overcome this omission, we employed the RV minigenomic system as described by Conzelmann and Schnell (6). In this system, cells were transfected with RV N (pRN) alone or in combination with P (pRP). Alternatively, RV N alone or N and P were expressed together with the minigenomic RNA (pSDI-CAT). The proteins expressed were metabolically labeled with [³⁵S] methionine. The cells were harvested and processed as described for the insect cells. After centrifugation on the discontinuous CsCl gradient (20-40%), fractions were collected and the N and P immunoprecipitated by using anti-N and/or anti-P antibodies. The immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography. As shown in Fig. 3.4A, the N and P protein profiles in BSR cells transfected with pRN and pRN plus pRP were similar to those in insect cells expressing N and N plus P, respectively. Most of the N in the cells expressing N alone were detected in the bottom fractions 16-18. In contrast, most of the N in the cells expressing both N and P shifted to the upper fractions 6-15 and presumably formed N-P complexes. A minor portion of the N was detected in the bottom fractions 16-17, however, very little or no P was detected in the bottom fractions. In cells expressing rabies virus N, P, and the minigenomic RNA from plasmids, a majority of the N was detected in the bottom fractions 16-18, although there was some N and P complex formation detected in the upper fractions 6-15. In

addition, P was clearly detectable in the bottom fractions 16-18. Thus, the protein profiles in transfected cells expressing N, P and genomic RNA showed similar patterns as those in cells infected with RV (see Fig. 3.3A). When N and the minigenomic RNA were co-expressed in the same cells, most of the N was detected in the bottom fractions 16-18, just like in cells expressing the N alone.

To investigate if the N or the N-P complexes expressed in mammalian cells bound to RNA as demonstrated in insect cells, gradient fractions were collected into three pools, pool 1 (fractions 1-4), pool 2 (fractions 5-14), and pool 3 (fractions 15-18). RNA was extracted from each of the pools, dot-blotted, and hybridized with two probes. One was the RV N-specific cDNA probe that detects N mRNA. The other is the CAT-specific cDNA probe that hybridizes with CAT RNA which is transcribed from the pSDI-CAT (minigenome). As shown in Fig. 3.4B, RNA was only detected in fractions 15-18, similar to what was found in insect cells. No RNA was detected in the top fractions, further confirming that the N-P complex in the top fractions did not bind to any RNA. In cells expressing rabies virus N, P, and the minigenomic RNA, minigenomic RNA was preferentially encapsidated, indicating the specificity of the N-P complex in encapsidation of mini-genomic RNA. In cells expressing rabies virus N and the minigenomic RNA, minigenomic RAN was preferentially encapsidated, indicating the preferentially binding of rabies virus genomic RNA over non-specific RNA as has been reported previously (30).

RV P, by binding to N, prevents N from being phosphorylated. RV N is phosphorylated (24) and its phosphorylation plays a regulatory role in viral RNA transcription and replication (27, 31). Furthermore, Kawai et al. (15) reported that phosphorylated N is detected only in the nucleocapsid, but not in the free N pool, suggesting that N phosphorylation may occur in the process of genomic RNA encapsidation. To investigate this possibility, we

examined N phosphorylation before and after RNA binding. BSR cells were either infected with RV or transfected with plasmids expressing RV N, N and P, N and P plus minigenomic RNA, or N and minigenomic RNA. Proteins were then labeled *in vivo* with [^{32}P] phosphoric acid (27, 30) and cells were harvested for processing. Proteins were separated by CsCl gradient centrifugation followed by fractionation. The proteins from each of the fractions were subjected to immunoprecipitation with anti-N antibodies and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3.5, phosphorylated N was only detected in the bottom fractions 16-18 from all the cells whether infected with the virus or transfected and expressing N, N and P, N and P plus minigenomic RNA, or N and minigenomic RNA. In the cells expressing N and P, very little phosphorylated N was detected and very little N was found in the bottom fractions as shown in Fig. 3.4A. The N in the top fractions, where it is bound to P (see Fig. 3.3A), was not phosphorylated (Fig. 3.5). These data indicate that RV N is not phosphorylated when it binds to P before RNA binding. It is phosphorylated either in the process of or after RNA binding. These studies suggest that P, by binding to N, prevents the N from being phosphorylated, thus facilitating the encapsidation of genomic RNA.

Mutant N (mutation of the phosphorylated Serine to Alanine) interacted with P and RNA in the same way as the native N: Since RV N phosphorylation occurs during the process of or after RNA binding, we investigated if mutant N with the phosphorylated serine changed to alanine (31) can alter the patterns of N-P and N-RNA interactions. BSR cells were transfected and proteins were processed in the same manner as described above except that the pRN was replaced by pRN-SA. As shown in Fig. 3.6A, the mutant N and P distribution profiles were similar to those in cells expressing the non-mutated N and P (see Fig. 3.4). Furthermore, only the mutant N in the bottom fractions 16-18 contained RNA (Fig. 3.6B). Thus, the unphosphorylated

(mutant) N interacted with P and RNA in the same way as the native N in the process of RNA binding.

DISCUSSION

The nucleoprotein (N) of non-segmented negative-stranded RNA viruses plays an important role in the switching from viral transcription to replication by encapsidating the nascent genomic RNA (2, 5, and 26). Although only the genomic RNA is encapsidated by N and packaged into virions (2, 29), it was reported that the N is capable of binding non-specifically to any RNA *in vitro* (2, 5, and 30). It is found that the phosphoprotein (P), by binding to N, confers the specificity of genomic RNA encapsidation by N (2, 6, and 31). We present *in vivo* evidence (in both insect and mammalian cells) using the RV model that indeed it is the P, by binding to N, which prevents the N from binding to non-specific RNA, thus conferring specific encapsidation of genomic RNA. When N is expressed alone, it binds to non-specific RNA to form nucleocapsid-like structures. When N is co-expressed with P in transfected cells, it forms N-P complex, which does not bind to any RNA. When N, P, and genomic RNA (in this case, RV minigenome) are expressed simultaneously in transfected cells (*in vivo*), the N binds to P to form N-P complexes that encapsidate the minigenomic RNA.

RV N has been expressed in insect cells (8, 20) and different methods have been used to purify the N from these cells (8, 14, and 20). Using antibody affinity chromatography, we have purified the N to near homogeneity (8) and the purified N was capable of interacting with P (9) and binding RNA *in vitro* (31). When using glycerol gradient sedimentation velocity centrifugation, Iseni et al (1998) purified the RV N expressed in insect cells and found that the

recombinant N forms ring-like structures. Further analysis (14, 20) indicated that the ring-like structures contained 9 to 11 N monomers which bind to RNA of 80-90 nucleotides. We also found that the recombinant N expressed in insect cells bound to low molecular weight RNA. This indicates that the recombinant N-bound RNA formed nucleocapsid-like structures (we termed it B-RNP) in the insect cells. These studies also raised the question as to how the recombinant N purified by antibody affinity chromatography (8) was still capable of encapsidating RNA *in vitro* (14, 23). To address this, we further analyzed N by centrifugation in a 40% CsCl cushion and found that the recombinant N expressed in insect cells existed as both RNA-free and RNA-bound (B-RNP) forms. *In vitro* binding assays revealed that the ability of RNA-bound N (e.g. B-RNP) to bind to *in vitro*-synthesized RNA decreased 15-fold when compared to the free N (B-N) as measured by density chromatography. These results indicate that the recombinant N in the B-RNP is no longer capable of encapsidating RNA. The RNA-binding activity of the affinity-purified N (31) is not due to the removal of RNA from the B-RNP by alkaline pH during the purification process as suggested by Iseni et al (14). Rather a portion of the N purified by affinity chromatography (9) exists as RNA-free N and thus is capable of encapsidating *in vitro*-synthesized RNA (31). Furthermore, RNA-free and RNA-bound N were always found in cells expressing the N alone (see Figs. 3, 4 and 6) no matter whether the N was purified by centrifugation or affinity chromatograph.

By sequencing the RNA associated with the recombinant N, Iseni et al (14) identified cellular tRNA, suggesting that RV N expressed in insect cells bound to cellular RNA. It is not surprising since we (31) have shown that RV N is capable of binding to non-specific RNA *in vitro*. However, our *in vitro* binding assay showed that RV N binds more strongly to N mRNA than to other non-specific and non-viral RNA (31). It could thus be assumed that in the insect

cells RV N would preferentially bind to N mRNA over other RNA species. To this end, we prepared RNA samples from viral RNP and B-RNP for Northern blot hybridization using N sequence-specific cDNA probe. It was found that the RNA extracted from B-RNP hybridized with the labeled N-specific probe. Furthermore, the RNA isolated from the nucleocapsid-like structures in mammalian cells that expressed the N alone also hybridized with the N-specific probe. All these data indicate that RV N bound to N mRNA in addition to cellular tRNA and other RNA species. Green et al. (11) also detected N mRNA bound by VSV N that was expressed in bacteria, further indicating that the N of VSV as with RV N is capable of encapsidating N mRNA when expressed alone.

The P in RV as in other mononegavirales viruses plays multiple roles during virus replication cycle. In addition to being the co-factor for the RNA-dependent RNA polymerase (L), P interacts immediately with *de-novo* synthesized N (2, 26). The N-P interaction has many facets in the process of viral transcription and replication. P, by binding to N, not only keeps the N soluble for RNA binding (2, 29), it confers the specific encapsidation of genomic RNA (3). Previous *in vitro* studies have amply demonstrated the latter function of P (2, 26, and 31). However, few *in vivo* studies have been conducted to confirm those *in vitro* studies. In one report, Spehner et al. (25) expressed Measles virus N and P either individually or in combination using recombinant vaccinia virus. They reported similar findings to what we have observed for RV N and P in the present paper. When the N is expressed alone, most of N binds to non-specific RNA and forms nucleocapsid-like structures. When the N is co-expressed with P, the N interacts with P to form N-P complexes. The N-P complex formation prevents the N from encapsidating non-specific RNA. However, Spehner et al (25) did not express genomic RNA in their system. When we expressed the N, P, and the minigenomic RNA together, we observed that the N-P

complexes preferentially encapsidated minigenomic RNA, and sedimented to the bottom of the CsCl gradient just like the N encapsidating genomic RNA in virus-infected cells. All these data demonstrate that although N by itself is capable of encapsidating any RNA species to form a nucleocapsid-like structure, the P, by binding to N, eliminates the binding of non-specific RNA. Small amounts of N and RNA are always detected in the bottom fractions of density gradient from cells expressing N and P. However, little or no P was detected in these fractions. This could be due to some of the N that has not yet formed a complex with P, thus still capable of binding non-specifically to different types of RNA. Furthermore, non-specific binding of N RNA was also detected in cells expressing N, P, and minigenomic RNA. This is also due to the fact that not all the N bound to P immediately after synthesis and thus a portion of the free N bound to non-specific RNA. This condition not only exists in cells expressing N, P, and minigenomic RNA, but also in virus-infected cells. Binding of mRNA by N has been reported in RV-infected cells as mRNP (29). Together these studies confirm previous *in vitro* studies that P, by binding to N, confers the specific encapsidation of genomic RNA by eliminating the non-specific RNA binding (31).

In addition to the functions reported previously, we found another function for P, at least in the RV model. RV P, by binding to N, prevents the N from becoming phosphorylated. RV N is phosphorylated in virus-infected cells (7, 15, and 24) as well as when N is expressed alone in insect cells (19) and in mammalian cells (31). Previous studies in our laboratory demonstrated that N phosphorylation plays a regulatory role in the process of RV transcription and replication (27, 30). Since unphosphorylated or dephosphorylated N binds to RNA more strongly than the phosphorylated N, we assumed that RV N phosphorylation may be involved in the RNA binding process. In the present study, we examined the phosphorylation status of the RV N in virus-

infected cells as well as in cells expressing N alone, N and P, or N and P plus minigenome. We found that the N is not phosphorylated before it encapsidates RNA, particularly when the N is coupled with P. Only the RNA-bound N is phosphorylated. These findings are in agreement with those reported by Kawai et al. (15) that phosphorylated N is detected only in the nucleocapsid purified from the virus-infected cells, whereas N in free-form or in the N-P complex is not phosphorylated. Together this indicates that N is phosphorylated either during the process of or immediately after RNA binding. The fact that only the RNA-bound N is phosphorylated suggests that N phosphorylation *per se* is not important in the process of RNA binding. However, these data indicate that P, by binding to N, prevents the N from being phosphorylated.

Previously we showed that dephosphorylated or unphosphorylated N has higher affinity for RNA than does phosphorylated N (31). Thus it is advantageous for the N not to be phosphorylated before encapsidating RNA. Now, we have shown definitively that RV N is not phosphorylated before RNA binding regardless of whether the N is expressed alone or in the virus-infected cells. During the process of, or after, RNA binding, the N may go through conformational changes that enable the N to be phosphorylated. We have hypothesized that following phosphorylation, the charge repulsion between the negatively charged phosphoserine of the N and the negatively charged genomic RNA may weaken the interaction between N and RNA, thus facilitating the initiation of next round viral RNA transcription and replication (27). Thus, although N phosphorylation may not be important in the RNA binding process, it plays a role in subsequent replication cycle.

Another interpretation for the fact that the N is not phosphorylated in the N-P complexes may be that RV N in the N-P complexes prior to RNA binding is in a unique conformation. In such a conformation, the N cannot be phosphorylated by the kinase possibly because the

phosphorylation site is not exposed. Furthermore, we hypothesize that the N in such a unique conformation can only encapsidate the genomic RNA. However, the mechanisms by which the P, by binding to N, keeps the N in a unique conformation for specific encapsidation of genomic RNA are not clear. Recently, it was shown that the ratio between N and P in the N-P complex before RNA binding is 1:2 (17), namely two Ps binding to one N. However, it has been reported that the N: P ratio is 2:1 in the purified virions (29). These observations suggest that the mode of N-P interaction before RNA binding is different from that after RNA binding.

Like the N, RV P is also phosphorylated (24). The role of P phosphorylation in the process of viral transcription and replication has been investigated extensively in other negative-stranded RNA viruses, for example VSV (13). However, the role of P phosphorylation in the RNA binding process has not been studied. Although the role of P phosphorylation is not directly assessed in the present study, it is unlikely that it plays a role. This is because that P is first phosphorylated by L-associated kinase before it is further phosphorylated by cellular kinase (4, 19). Our observation that the N-P interaction in the process of RNA binding is similar in cells infected with the virus (P is phosphorylated, data not shown) and in cells expressing N, P, and mini-genomic RNA (P is not phosphorylated, data not shown).

Based on all the available data, we propose the following model (Fig. 3.7) to explain the detailed N, P, and RNA interactions and N phosphorylation. When the N is expressed alone, it binds to any nearby RNA (Fig. 3.7A). During the process of N-RNA interaction, the RNA-bound N goes through conformational change and thus is phosphorylated. When the N is co-expressed with P, the P binds N to form the N-P complex, which eliminates any non-specific RNA binding that might occur with “free” N. Because no RNA has been bound, the N has not gone through conformational changes and thus stays unphosphorylated (Fig. 3.7B). When the N, P, and

genomic RNA are expressed together, the N can encapsidate the genomic RNA and thus is phosphorylated (Fig. 3.7C). Although the mechanisms by which RV N and P interact with each other to eliminate the binding of non-specific RNA are not understood, we offer the following explanations. It is possible that P binds to N and keeps the N in a unique conformation. Due to steric hindrance, the N in such a conformation cannot bind to non-specific RNA, but can still encapsidate genomic RNA. Because the N is in such a conformation, it cannot be phosphorylated. Alternatively, there are two RNA binding sites on the N, one of which binds to genomic RNA and the other to non-specific RNA. The P occupies the non-specific RNA binding site on the N and leaves the other site that can only bind to the genomic RNA. It is also possible that RV P and RNA share the same binding site. Due to some unknown characteristics, the genomic RNA, not the non-specific RNA, can displace the P, thus encapsidating the genomic RNA. Because the phosphorylation site (389) is close to the putative RNA-binding domain (residues 289 to 352) (16), P on this particular site might prevent the N from phosphorylation. After displacing the P and encapsidating RNA, the N goes through conformational changes, thus the N becomes phosphorylated. Clearly, further studies are needed to investigate the dynamic interactions between N and P during the RNA binding process, which could shed light on the mechanisms by which the P, by binding to N, keeps the N in the unique conformation that can only encapsidate the genomic RNA.

ACKNOWLEDGEMENTS

The authors express their thanks to Dr. William H. Wunner at the Wistar Institute for critically reading the manuscript and Dr. Charles E. Rupprecht at the CDC for supplying anti-N monoclonal antibody 802-2. This work was supported by Public Health Service grant AI-51560 (Z.F.F.) from the National Institute of Allergy and Infectious Diseases and a grant from Ft. Dodge Animal Health Laboratories.

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FIGURE LEGENDS

Fig. 3.1 Recombinant N expressed in insect cells exists as both RNA-free N and RNA-bound N. Recombinant RV N expressed in insect cells and purified by affinity chromatography (Fu et al., 1991) were subjected to centrifugation on a 40% CsCl cushion. The cushion was then fractionated into 9 fractions (fractions 5 to 8 contained the banded material, B-RNP). Aliquots from each of the fractions (numbered from top to bottom) were analyzed by SDS-PAGE and Coomassie blue staining (A). MW, molecular weight marker. RNA was extracted from each of the fractions, subjected to electrophoresis, and then hybridized with N cDNA probe (B). RNA was also extracted from B-RNP (fractions 5 to 8) or from other fractions (1-4, and 9) and subjected to electrophoresis on a 2% agarose gel (C). Lane 1, molecular weight marker, lane 2, RNA extracted from B-RNP (fractions 5-8), lane 3, RNA extracted from other fractions.

Fig. 3.2 Recombinant N binds to N mRNA. Viral RNP, recombinant B-N, and B-RNP (equal amount by weight) were subjected to RNA extraction, electrophoresis on 1% agarose gel, and hybridized with N sequence-specific cDNA probe (A). B-RNP and B-N (equal amount by weight) were allowed to encapsidate *in vitro*-synthesized leader RNA L-70 (Yang et al., 1998). After RNase digestion, the N-RNA complex was analyzed by a gel shift assay on a 4% polyacrylamide gel (B).

Fig. 3.3 RV N and P expressed in insect cells. Insect cells infected with either B-RN, B-RP individually, or in combination, and cells were harvested. Soluble proteins were subjected to CsCl gradient (20-40%) centrifugation. After gradient fractionation, protein in each fraction was subjected to SDS-PAGE and Western blotting using either anti-N or anti-P antibodies (A). BSR

cells infected with RV strain L16 were processed as controls. RNA was also prepared from each of the fractions and subjected to electrophoresis and dot-blot hybridization using RV N sequence-specific cDNA probe (B).

Fig. 3.4 RV N, P, and minigenomic RNA expressed in mammalian cells. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN, pRN and pRP, pRN and pRP plus pSDI-CAT, or pRN and pSDI-CAT. Cells were labeled with [S^{35}] methionine and harvested at 48 h after transfection. Proteins were solublized for immunoprecipitation with anti-N or anti-P antibodies and the immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography (A). RNA was prepared from pooled fractions and subjected to electrophoresis and dot-blot hybridization using N- or CAT-specific cDNA probe (B). Pool 1 (fractions 1-4), Pool 2 (fractions 5-14), and Pool 3 (fractions 15-18).

Fig. 3.5 Phosphorylation of RV N in infected and transfected cells. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN, pRN and pRP, pRN and pRP plus pSDI-CAT, or pRN and pSDI-CAT. Cells were incubated with [P^{32}] phosphate and harvested at 48 h after transfection. Alternatively, cells were infected with RV strain L16 and incubated with [P^{32}] phosphate. P^{32} -labeled proteins were solublized for immunoprecipitation with anti-N antibodies and the immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography.

Fig. 3.6 The effects of mutant N on RNA binding. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN-SA, pRN-SA and pRP, pRN-SA and pRP plus pSDI-CAT, or pRN-SA and pSDI-CAT. Cells were labeled with [S^{35}] methionine and harvested at 48 h after transfection. Proteins were solublized for immunoprecipitation with anti-N or anti-P antibodies and the immunoprecipitated proteins were

subjected to SDS-PAGE followed by autoradiography (A). RNA was prepared from pooled fractions and subjected to electrophoresis and dot-blot hybridization using N- or CAT-specific cDNA probe (B). Pool 1 (fractions 1-4), Pool 2 (fractions 5-14), and Pool 3 (fractions 15-18).

Fig. 3.7 Proposed model of N, P, and RNA interactions and N phosphorylation.

When N is expressed alone, it binds to any RNA and thus is phosphorylated (A). When N is co-expressed with P, P binds to N to form N-P complexes, which eliminates the non-specific RNA binding. Because no RNA has been bound, the N stays unphosphorylated (B). When N, P, and genomic RNA are expressed together, N and P form N-P complexes that encapsidate the genomic RNA and the N becomes phosphorylated (C).

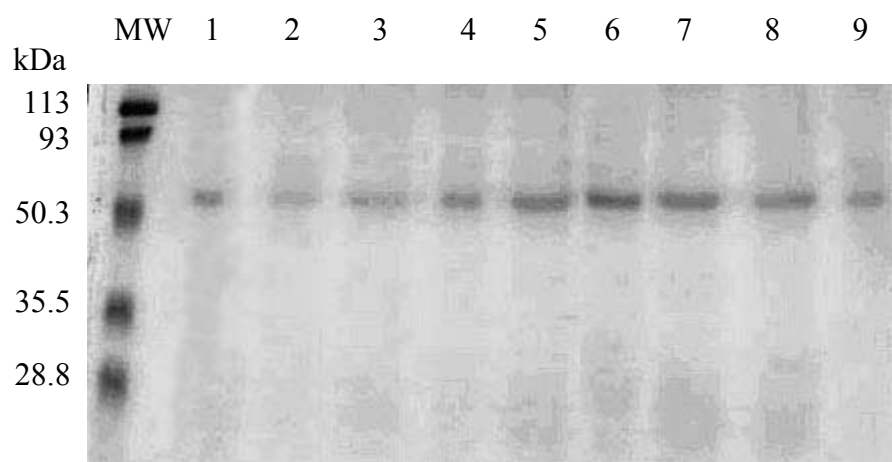
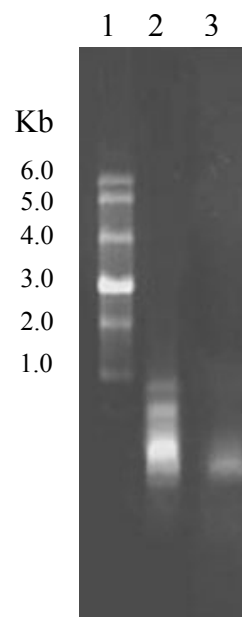
Fig. 3.1**A****B****C**

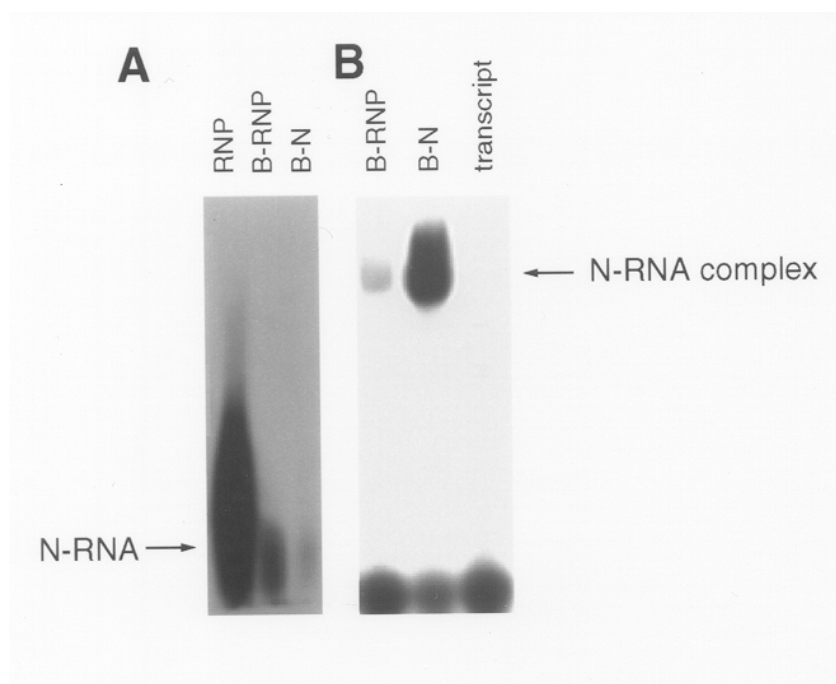
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Fig. 3.3

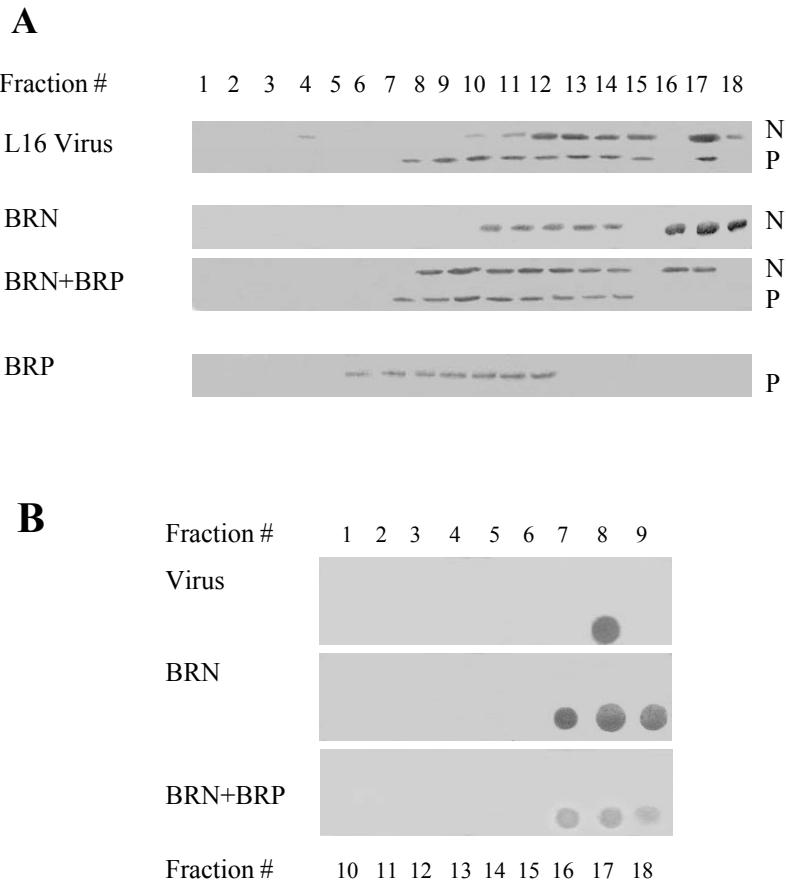


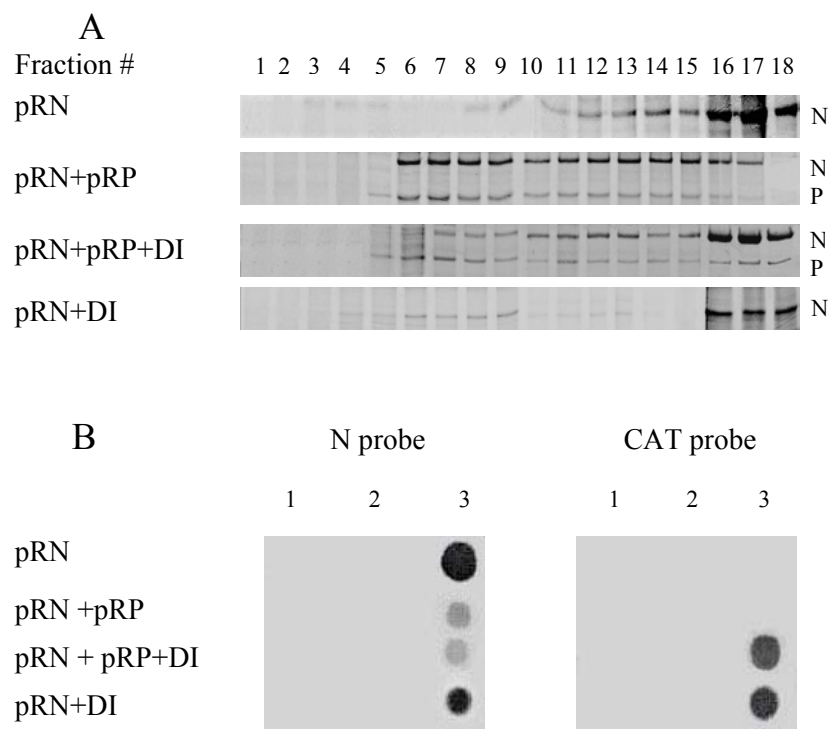
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Fig. 3.5

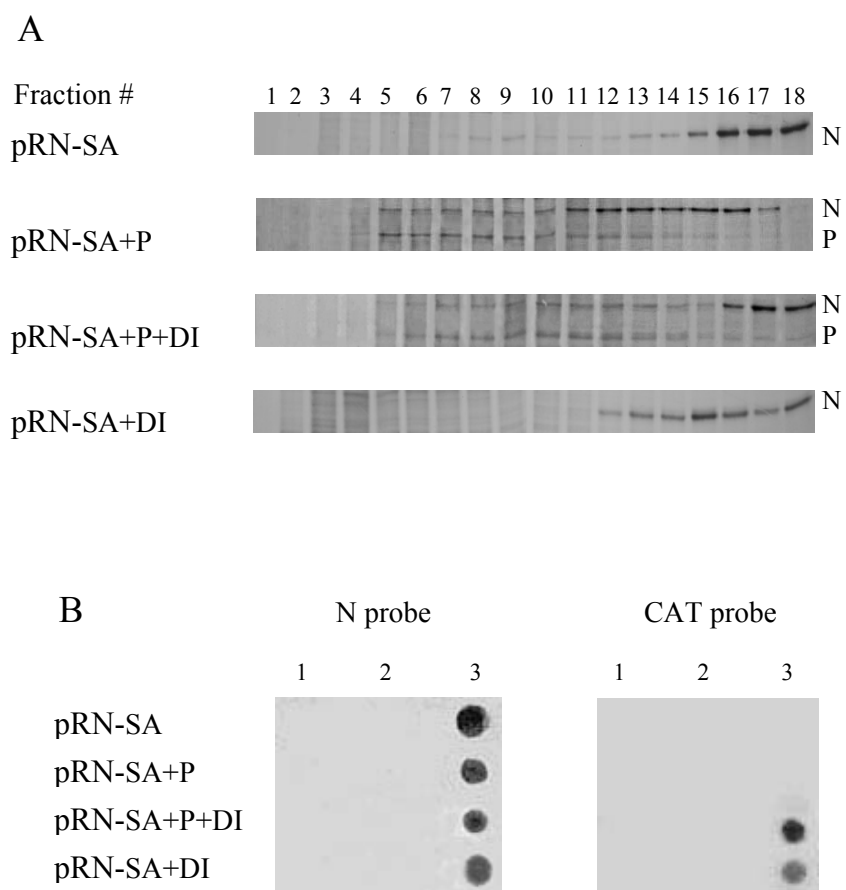
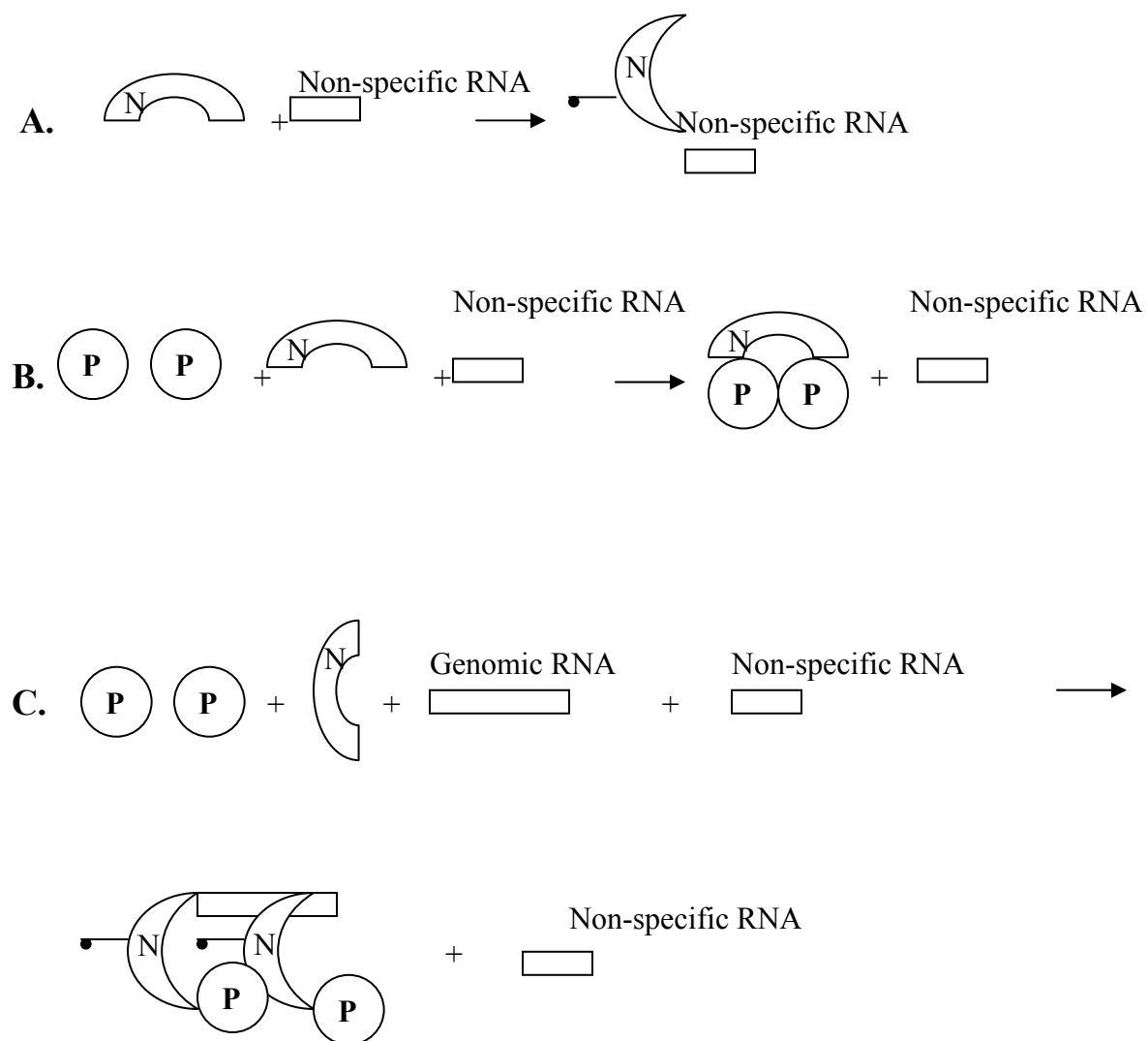
Fig. 3.6

Fig. 3.7



( phosphate group)

CHAPTER 4

MAPPING THE DOMAINS ON RABIES VIRUS NUCLEOPROTEIN REQUIRED FOR N-P AND N-N INTERACTIONS AND THEIR FUNCTIONS IN VIRAL RNA TRANSCRIPTION AND REPLICATION¹

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ABSTRACT

Rabies virus nucleoprotein (N) is a multifunctional protein that plays a central role in viral RNA transcription and replication. Its functions require interactions with itself and with other viral components. N encapsidates genomic RNA, thus forming the N-RNA template. During the RNA encapsidation process, the N also interacts with other N molecules to self-assemble into nucleocapsid particles. N also interacts with P to form a N-P complex that is important for the specific RNA encapsidation. In order to understand the detailed N-N, N-P, and N-RNA interactions, a series of terminal and internal deletion mutants of the N were constructed and attempts were made to map the domains on the N that are involved in these interactions. It was discovered that a central region of the N from amino acids 150 to 285 and a C-terminal region of 360-420 of N protein are important for the N-P interactions. Deletion of these regions abolished its ability to bind to P. For self aggregation, it appeared that amino acids 25 to 30 are required because deletion of the first 25 amino acids did not affect the N-N interactions. However, deletion of the first 30 amino acids completely abolished the ability of the N to self-aggregate. Also, none of these mutants supported transcription and/or replication of the minigenomic RNA. These data suggest that although different domains on the N are involved in N-N and N-P interactions, the full-length N is absolutely required for optimal viral transcription and genome replication.

INTRODUCTION

Rabies virus (RV), a member of the *Lyssavirus* of the family *Rhabdoviridae*, is an enveloped virus containing a single negative (-) strand RNA genome. The viral genomic RNA is tightly encapsidated with the nucleocapsid protein (N) and this encapsidation protects the RNA from ribonuclease digestion (24). Two other viral proteins, the phosphoprotein (P) and large protein (L) also associate with the nucleocapsid and this ribonucleoprotein (RNP) complex serves as template for both transcription and replication (2, 26, and 28). The rabies virus P protein has been extensively studied, *in vitro* transcription-translation study showed that there are two binding regions on P which interact with N, one is located in the first 19 amino acids of P, another is located at the C-terminal of P between amino acids 250 to 297 (8). *In vivo* study showed that the existence of two N protein binding site on the P protein: one is located between amino acids 69-177 and another is at the C-terminal region 268-297 (5).

Rabies virus N is a multifunctional protein. It can bind to P to form N-P complex and this complex keeps the N in a soluble form to facilitate its specific encapsidation of genomic RNA (14, 15, 16 and 29). N is a RNA binding protein and in virus infected cells, N binds specifically to genomic or antigenomic RNA (3, 20, and 29). However, N can bind to nonspecific RNA when expressed in insect cells (11, 13, and 22). N can also forms self-aggregations when expressed in insect cells (22). Thus, N can self-aggregate, interacts with P, and binds to RNA. However, relatively little is known for rabies virus nucleoprotein interaction domains among N-P and N-N interactions.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies. BSR cells, a clone of BHK-21(baby hamster kidney) cells, were grown in Dulbecco's minimal essential medium supplemented with 10% fetus bovine serum. Recombinant vaccinia virus expressing bacterial T7 RNA polymerase (vTF7-3) was prepared as described previously (9). Plasmids for expression of N (pRN) and P (pRP) were constructed previously in our laboratory (8, 29). Rabies virus minigenome (pSDI-CAT) was obtained from K. Conzelmann (6). Mouse monoclonal antibody (clone 12CA5) Anti-HA was purchased from Roche Applied Science. Mouse anti-rabies virus N polyclonal antibody was made in rabbits.

HA tag cloning. The 27-nucleotide HA (influenza A virus haemagglutinin) sequence was cloned between the T7 promoter and the N terminus of N and P gene in the plasmids pRN and pRP via an adaptor-duplex (Roche). Briefly, two complementary single strands of DNA oligonucleotides with 5' phosphate group encoding the HA tag were synthesized. The two oligonucleotides HAF and HAR were annealed together (HAF: 5'pCATGTTACCCATACGAC-GTCCCAGACTACGCTT 3'; HAR: 3'*CATGGTAC*ATGGGTATGCTGCAGGGTCTGATG-CGAAGATCp5'. HA sequence were underlined, the italic sequences were partial *KpnI* and *XbaI* sites) and linked into the pRN and pRP plasmid (cut with *KpnI* and *XbaI* and dephosphorylated). The plasmids were sequenced to confirm their correctness. However, the pRP^{HA} is not in-frame after insertion the HA tag. A single adenine nucleotide was inserted before the initial codon of P gene to keep it in-frame by site-directed mutagenesis using Quick Change Site-Directed Mutagenesis Kit (Stratagen) with primers 5'CGCTTCTAGAGGGGAAA*ACATGAGCAAG-ATCTTTGTC3' and 3'GCGAAGATCTCCCCTT*TTGTACTCGTTCTAG-AAACAG5' (*

inserted nucleotide) and the plasmid was confirmed by sequencing. The tagged plasmids were designated as pRN^{HA} and pRP^{HA}.

N mutants' construction. The N deletion mutants were constructed by PCR cloning according to method described previously (29). Table 1 shows the primers used to construct the deletion mutants of N, briefly, each pair of primers were used to produce required mutants by PCR, using plasmid pRN as template. The PCR products were cloned into pGEM-3Z vector. Table 2 lists all the constructed N mutants.

Western-blotting. BSR cells in six wells plate were infected with vvT7 virus for 1 hr, and then transfected with 1.0 µg of either pRN^{HA} or pRP^{HA}. Twelve hrs after transfection, cells were lysed with SDS lysis buffer and subjected to SDS-PAGE. Proteins were transferred to positively charged PVDF membrane (Roche). Membranes were blocked with blocking reagent (Roche) at room temperature for 1 hr and then incubated with anti-HA-Peroxidase (clone 12AC5, Roche). After washing four times with PBS, the membrane was incubated with chemiluminescent substrate and exposed to Lumi-Film (Roche).

***In vitro* protein interaction.** *In vitro* protein interactions were performed according to the manufacture's instructions using T_NT T7 Quick Coupled Transcription/Translation kit (Promega). Briefly, 0.5 µg of pRP^{HA} and 0.5 µg of one of the N mutants were added into 40µl of TNT T7 Quick Master Mix, then mixed with 2 µl of [³⁵S]-methionine (Amersham Pharmacia Biotech). After 90 min incubation at 30 °C, 800 µl of RIPA buffer (150 mM NaCl, 50 mM Tris.Cl pH 7.5, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF) was added to the reaction. The mixture was then immunoprecipitated with Anti-HA monoclonal antibody and subjected to SDS-PAGE and autoradiography analysis.

***In vivo* protein interaction.** Transfection and labeling of BSR cells were performed as described previously (Wu et al., 2002). Briefly, BSR cells were infected with recombinant vaccinia virus (vTF7-3) at a moi of 1 pfu per cell. One hour after virus infection, the cells were transfected either with wild type pRN or one of the N mutants together with pRP^{HA}. Twelve hours after transfection, the cells were washed with PBS and incubated with methionine-free medium for one hour. Then the cells were labeled with [³⁵S]-methionine for eight hours. Cells were lysed with RIPA buffer and the supernatant was immunoprecipitated with anti-HA monoclonal antibody or anti-N polyclonal antibody and subjected to SDS-PAGE and radiography analysis.

Sedimentation of N-N complex. N-N complex sedimentation was performed as described (1, 4). Briefly, BSR cells were infected with recombinant vaccinia virus (vTF7-3) at a moi of 1 pfu per cell. One hour after virus infection, the cells were transfected either with wild type pRN or one of the N mutants. Twelve hours after transfection, the cells were washed with PBS and incubated with methionine-free medium for one hour. Then the cells were labeled with [³⁵S]-methionine for eight hours. Cells were lysed with RIPA buffer and the supernatant was subjected to ultra-speed centrifugation in 30% glycerol (in 10mM HEPES buffer, pH 7.5) at 50,000 rpm for 90 minutes. The pellets were resuspended in RIPA buffer and immunoprecipitated by anti-N polyclonal antibody. The pull-down pellets were washed and subjected to SDS-PAGE and radiography analysis (1, 4).

CAT-ELISA assay. BSR cells in six wells plate were infected with vvT7 virus at a moi of 1 pfu per cell for 1 hr, and then transfected with 1.2 µg of wild type pRN or one of the N mutants together with 0.6 µg of each of the pRP, pRL, and pSDI-CAT plasmids. After 48 hrs transfection, cells were harvested and washed with PBS buffer. CAT-ELISA assay was

performed according to manufacturer's instructions (Roche). Briefly, cells were lysed with 500 µl of lysis buffer and total protein in the supernatant was quantified using Bradford Protein Concentration Determination Method with a series concentration of BSA protein as a standard. 50 µg of protein from each sample was then added to the coated wells and the ELISA test was performed according to the manufacturer's instructions (Roche), the plate was read on an ELISA machine. The absolute amount of CAT enzyme in each sample was calculated according to the BSA standard. Each of the tests was repeated three times.

RESULTS

HA tagged N and P protein interact with each other. To characterize the functional domains on N that interact with P, we developed an assay for measuring N–P complex formation with the use of HA-tagged P protein (P^{HA}). The 27-nucleotide HA (influenza A virus haemagglutinin) sequence was cloned into the *Kpn I* and *Xba I* sites between the T7 promoter and the N terminus of N and P genes in the plasmids pRN and pRP via an adaptor-duplex. Initially Western blotting analysis was performed to make sure that the HA-tagged proteins can be recognized by the anti-HA antibodies. As shown in Fig. 4.1A, HA-tagged N and P are efficiently expressed in BSR cells and recognized by anti-HA antibodies. Subsequently, HA-tagged N and P were expressed either individually or in combination and labeled with [35 S]-methionine. After immunoprecipitation with anti-HA antibodies, the immunoprecipitated proteins were analyzed by SDS-PAGE. As illustrated in Fig. 4.1B, anti-HA antibodies precipitated the HA-tagged N and P, respectively. When the HA-tagged N was expressed together with untagged P, the P is co-immunoprecipitated with the HA-tagged N. Vice versa, the

untagged N was also co-immunoprecipitated with the HA-tagged P. These results indicate that HA tag does not affect the N-P complex formation, thus facilitating the study of N-P interactions.

Construction and expression of N deletion mutants. To study the detailed N-P interactions and map the domains on N that are involved in N-P, N-N, and N-RNA interactions, we made a series of N-terminal, C-terminal, and internal deletion mutants of N. To ensure that each of these N mutants were expressed, we transfected BSR cells with each of these mutants. Wild type N was also expressed in BSR cells as a positive control. Transfected cells were labeled with [³⁵S]-methionine and the harvested cells were processed for immunoprecipitation with anti-N polyclonal antibodies. As shown in Fig. 4.2, wild type N and all the N-terminal deletion mutants were efficiently expressed and immunoprecipitated with anti-N antibodies (Fig. 4.2). Likewise, all the C-terminal and the internal deletion mutants were also expressed and immunoprecipitated with anti-N antibodies (Data not shown). The only mutant N that was not recognized by the anti-N antibodies is the mutant NΔ376.

Mapping the domains on N involved in N-P interactions *in vitro*. Previous studies showed that truncations of 45 amino acids from either the N or the C-terminus of N abolished the N-P interaction *in vitro* (29). To further define the interactive domains on the N, we made more N and C-terminal deletion mutations by truncating 5, 10, 15, 20, 25, 30, 35, and 40 amino acids from either terminus. Each of the N mutants was synthesized and labeled together with the HA-tagged P in the T7 Quick coupled transcription/translation system. After immunoprecipitation with anti-HA antibodies, the possible N-P complexes were analyzed by SDS-PAGE. As shown in Fig. 4.3A, deletion up to 25 amino acids from the N-terminus of the N did not affect its binding to P. However, deletion of more than 25 amino acids abolish its ability to bind to P. N

mutant with deletion of 5 amino acid from the C-terminus bound to the HA-tagged P as efficiently as the wild type N. Deletion of more than 5 amino acids reduced dramatically its ability to interact with P. Together these results showed that the N-terminal amino acids 25 to 30 and the C-terminal amino acids 5 to 10 are important for N-P interactions.

Different mode of interaction between N and P *in vivo*. To study if the mode of N-P interaction *in vitro* is similar to that *in vivo*, N or each of the N mutants was expressed together with HA-tagged P in BSR cells. Proteins were labeled with [³⁵S]-methionine and immunoprecipitated with anti-HA antibodies. As shown in Fig. 4.4A, N mutants with deletion of up to 150 amino acids from the N-terminus were co-immunoprecipitated with the HA-tagged P. N mutants with deletion of more than 150 amino acids from the N-terminus are not co-immunoprecipitated with the P. N mutants with deletion of up to 165 amino acids from the C-terminus were also co-immunoprecipitated with the HA-tagged P (Fig. 4.4B). N mutants with deletion of more than 165 amino acids from the C-terminus are not co-immunoprecipitated with the P. All these results suggest that a central region from amino acids 150 to 285 on N protein is important for P binding. These results also indicate that the mode of N-P interaction *in vivo* is different from that *in vitro*. Since N-P interaction during the process of viral transcription and replication occurs *in vivo*, our subsequent experiments were conducted under the *in vivo* conditions in cultured BSR cells.

Mapping the detailed interactive domain on the N involved in N-P interactions with internally deleted N mutants: To further map the domains on N that are involved in N-P interactions, we made a series of internally deleted mutants. This was carried out for each construct by deleting 30 amino acids. Each of these N mutants was expressed in BSR cells with HA-tagged P, labeled with [³⁵S]-methionine, and immunoprecipitated with anti-HA antibodies.

As shown in Fig. 4.4C, all of the internally deleted N mutants were co-immunoprecipitated with P except for two constructs with deletion of amino acids 361-390 and 391-420, which suggests that the region between residues of 361-420 is important for P binding.

Mapping the domains involved in N-N self-aggregation. One of the characteristic features of the N protein of the negative-strand RNA viruses, including rabies virus N, is that it self-assembles into the nucleocapsid-like particles when the proteins are expressed in the absence of other viral proteins (1, 7, 22, and 23). To map the domains on rabies virus N involved in N self-aggregation, the N-terminal, C-terminal, and the internal deletion mutants were expressed in BSR cells and labeled with [³⁵S]-Methionine. The cells were harvested and cytoplasmic extracts were centrifuged at 50,000 rpm for 90 minutes in 30% glycerol cushion. The pellets were resuspended in RIPA buffer and immunoprecipitated by anti-N polyclonal antibody and analyzed by SDS-PAGE and radiography analysis. As shown in Fig. 4.5, wild type N, NΔ5 to N Δ25 formed multimeric aggregates in the cushion since these can be immunoprecipitated with anti-N antibodies. All other N deletion mutants including deletion of more than 25 amino acids from the N-terminus and all the C-terminal and internal deletion mutants did not self-aggregate since these mutants were not immunoprecipitated from the pellet of the glycerol cushion (data not shown). These data show that apart from the N-terminal 25 amino acids, the rest of the N is important for self-aggregation.

Analysis of the ability of N mutants to support transcription of the minigenomic RNA. To evaluate the ability of the N mutants to support minigenomic transcription, BSR cells were infected with VVT7 virus and transfected with plasmids pSDI-CAT, pRP, pRL, and pRN (or each of the N mutants). Cells were harvested 48 h post-transcription, CAT enzyme activities in the cell lysate were analyzed by CAT-ELISA assay, each of these experiments was repeated

three times and the data are presented in Fig. 4.6. The ability of all the N mutants to support the minigenomic transcription was dramatically reduced by more than 90% except for mutants NΔ5 and CΔ5. CAT activities were least than 5% for both N- and C-terminal mutants when more than 15 amino acids were deleted and for all the internal mutants. These results indicated that the full length N protein is important for viral transcription and replication.

DISCUSSION

Like any other negative-stranded, non-segmented RNA viruses, rabies virus N and P interacts with each other to play important roles in the process of RNA transcription and replication (3, 30). The N protein, by encapsidating the *de novo*-synthesized genomic RNA, switches the virus from the mode of transcription to that of replication (14, 29). However, the N can bind to any RNA when it is synthesized alone (10, 14). It has been demonstrated that the P protein, by binding to N, confers the specific encapsidation of genomic RNA by N (2, 14). Thus, the N-P interaction is vital to support viral transcription and replication. Previously the domains on P that are involved in binding to N have been mapped to the N- and the C-terminal regions (5, 8). In the present study, we mapped the domains on the N that are involved in the N-P interaction and in the N self-aggregation.

Previous *in vitro* investigations demonstrated that deletion of 45 or more amino acids from each terminus of the N abolished its ability to bind to P (29). To define precisely the interactive domains on the N that bind to P, we made a series of N-terminal, C-terminal, and internal deletion mutants of the N. Initially, we attempted to map the domains on N *in vitro* and found that the N-terminal 25 to 30 and the C-terminal 5 to 10 amino acids are important for N-P interactions. These results also explain why it was found previously that deletion of 45 amino

acids from either terminus abolished its ability to bind to P (29). Since the N-P interaction in the process of rabies virus transcription and replication occurs in the infected cells, the N-P interaction was also studied in the transfected cells. It was found that N mutants with deletion of up to 150 amino acids from the N-terminus and 165 amino acids from the C-terminus were still co-immunoprecipitated with the HA-tagged P, indicating that the central region between residues 150 and 285 is important for N-P interaction. The difference between the *in vitro* and *in vivo* data may indicate that the mode of N-P interaction *in vitro* is different from that *in vivo*. This difference may be due to the involvement of cellular proteins. However, what and how cellular proteins are involved in the N-P interaction are not known at the moment and deserve further investigation.

In addition, we have also made internally deleted N mutants for mapping the domains on N that may be involved in binding to P. Only those N mutants with deletion of amino acids between 360 and 420 failed to interact with P, indicating that there is another domain involved in binding to P. Thus there are at least two domains on the N that can interact with P. One is located in C-terminal region between amino acids 360 and 420. Recently, it has been reported that the N^o-P complex between RNA encapsidation contains one molecule of N and two molecules of P by native mass spectroscopy and negative-stain electron microscopy (16), indicating the existence of two P-binding regions on N. Furthermore, two similar regions are also found to be important for N-P interactions in Sendai virus (SeV) and bovine respiratory syncytial virus (BRSV). In SeV, two non-contiguous regions, amino acids 4-188 and 304-373 of N protein, are required for the formation of soluble N-P complex (1). In BRSV, there are also two regions on N found to be important for P interaction: a central portion of 244-290 amino acids and a C-terminal portion of 338-364 amino acids (12).

One of the characteristic features of the N protein from the negative-strand RNA viruses, including rabies virus N, is that it self-aggregates into the nucleocapsid-like particles when the proteins are expressed in the absence of other viral proteins (1, 7, 22, and 23). These nucleocapsid-like structures sediment through glycerol gradient and band like authentic nucleocapsids on CsCl gradients (1, 4, 11, and 22). This feature has been used to map the N oligomerization site(s) on the N of many negative-stranded RNA viruses. We used this same sedimentation method to map rabies virus N-N interaction sites using this N-terminal, C-terminal, and the internal deletion mutants. It was found that the whole length of N protein might be important for its self aggregation except the first 25 amino acids. Only N mutants with deletion of the first 25 amino acids self-aggregated and all the other N mutants failed to do so. Our results for rabies virus N are different from those of other negative-stranded RNA viruses. The Measles virus N-N interaction site has been mapped to the central portion of the N from amino acids 189 to 373 (1). In Sendai virus, N-N interactions have been identified, including residues 114-129 and 258-357 (17, 18). More recently, Sendai virus N protein between residues 362 and 370 has been found to be important for N-N interactions (19).

Because the most important role of the N is to support viral transcription and replication, we evaluated the function of the N mutants in the minigenomic system (6). In this minigenomic system, only the 3' leader region and the 5' trailer region from the virus are included and the report gene CAT was inserted in between. When rabies virus N, P, and L proteins are provide in trans, the minigenomic RNA can be transcribed and replicated. CAT-ELISA assay was used to detect the amount of CAT enzyme present in the cell lysate, which is proportional to the level of viral transcription and replication. Our results show that deletion of five amino acids from either the N- or the C-terminus reduced the CAT activity by almost 90% when compared with the full-

length N. Almost no CAT activity was detected in cells transfected with N mutants with deletion of more than 15 residues from the N-terminus or 30 residues from the C-terminus. Likewise, no CAT activity was detected in cells transfected with internally deleted N mutants. Together these data indicate that the intact N is required for optimal level of viral transcription and replication. Similar results were observed in BRSV (12). However, these authors showed no or less reduction in CAT activity when BRSV N mutants with deletion or replacement of single amino acid in the terminal 5 residues were used. Thus these studies may suggest that the terminal amino acids are important for the N to support viral transcription and replication, possibly by maintaining the overall conformation of the N.

In summary, we have identified two domains on the rabies virus N that are involved in binding to P and this could explain why two N and one P are required to form the N-P complex (16). Furthermore, we also attempted to map the N self-aggregation sites and we found that only the first 25 amino acids are dispensable. Deletion of more than 25 amino acids abolished the ability of N to self-aggregate. Finally we investigate the ability of all the N mutants to support viral transcription and replication in the minigenomic system, it was found that deletion of any amino acids reduced the level of transcription and replication by more than 90%, suggesting that the intact N is required for optimal level of viral transcription and replication.

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FIGURE LEGENDS

Fig. 4.1 HA tagged N and P protein can be efficiently expressed and co-immunoprecipitated with anti-HA MAb. (A). Western blotting analysis of HA tagged N and P protein expressed in BSR cells. Lane 1: Molecular weight marker; Lane 2: HA tagged N protein; Lane 3: HA tagged P protein; Lane 4: mock transfection. (B). Immunoprecipitation analysis of HA tagged N and P protein expressed in BSR cells with Anti-HA MAb. Lane 1: HA tagged N protein alone; Lane 2: HA tagged N protein with untagged P protein; Lane 3: HA tagged P protein with untagged N protein; Lane 4: HA tagged P protein alone; Lane 5: mock transfection.

Fig. 4.2 N deletion mutants' expression in BSR cells. Wild type and mutated N mutants were expressed in BSR cells and immunoprecipitated with anti-N polyclonal antibody. Lane 1: wild type N; Lane 2-15: N terminal deletion mutants (NΔ5, NΔ10, NΔ15, NΔ20, NΔ25, NΔ30, NΔ35, NΔ40, NΔ45, NΔ72, NΔ126, NΔ150, NΔ284, and NΔ376).

Fig. 4.3 *In vitro* N and P interactions. HA tagged P protein and one of the N mutated proteins were *in vitro* co-synthesized to study their interactions. (A). N terminal mutants interact with P^{HA}. Lane 1. Wild type N; Lane 2-10: N terminal mutants (NΔ5, NΔ10, NΔ15, NΔ20, NΔ25, NΔ30, NΔ35, NΔ40, And NΔ45); Lane 11: P^{HA} alone. (B). C terminal mutants interact with P^{HA}. Lane 1. Wild type N; Lane 2-10: C terminal mutants (CΔ5, CΔ10, CΔ15, CΔ20, CΔ25, CΔ30, CΔ35, CΔ40, And CΔ45); Lane 11: P^{HA} alone.

Fig. 4.4 *In vivo* N and P interactions. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN^{HA} and one of the N mutants. Cells were labeled with [S³⁵]-methionine and harvested 20 hrs after transfection. Cells were lysed and supernatants were immunoprecipitated with anti-HA MAb and subjected to SDS-PAGE followed

by autoradiography. (A). N terminal mutants interact with P^{HA}. Lane 1: wild type N; Lane 2-15: N terminal deletion mutants (NΔ5, NΔ10, NΔ15, NΔ20, NΔ25, NΔ30, NΔ35, NΔ40, NΔ45, NΔ72, NΔ126, NΔ150, NΔ284, and NΔ376); Lane 16: mock transfection. (B). C terminal mutants interact with P^{HA}. Lane 1: wild type N; Lane 2-14: C terminal deletion mutants (CΔ5, CΔ10, CΔ15, CΔ20, CΔ25, CΔ30, CΔ35, CΔ40, CΔ45, CΔ74, CΔ125, CΔ165, and CΔ297); Lane 15: mock transfection. (C). Internal mutants interact with P^{HA}. Lane 1: wild type N; Lane 2-16: Internal deletion mutants (IΔ11-30, IΔ31-60, IΔ61-90, IΔ91-120, IΔ121-150, IΔ151-180, IΔ181-210, IΔ211-240, IΔ241-270, IΔ271-300, IΔ301-330, IΔ331-360, IΔ361-390, IΔ391-420, and IΔ421-440); Lane 17: mock transfection

Fig. 4.5 *In vivo* N and N interactions. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with either wild type pRN or one of the mutants. Cells were labeled with [S³⁵]-methionine and harvested 20 hrs after transfection. Cells were lysed and supernatants were centrifuged at 50, 000 rpm for 90 minutes, pellets were immunoprecipitated with anti-N polyclonal antibody and subjected to SDS-PAGE followed by autoradiography. Lane 1: wild type N; Lane 2-11: N terminal deletion mutants (NΔ5, NΔ10, NΔ15, NΔ20, NΔ25, NΔ30, NΔ35, NΔ40, NΔ45, NΔ72, and NΔ126).

Fig. 4.6 CAT-ELISA assays. BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with wild type pRN or one of the N mutants together with each of the pRP, pRL, and pSDI-CAT plasmids. CAT enzyme in the cell lysate was detected by CAT-ELISA assay. CAT activity of wild type N was set as 100%. CAT activities of each of the mutants were calculated comparing the wild type N. A. CAT activity of N terminal mutants. 1-14: NΔ5, NΔ10, NΔ15, NΔ20, NΔ25, NΔ30, NΔ35, NΔ40, NΔ45, NΔ72, NΔ126, NΔ150, NΔ284, and NΔ376. B. CAT activity of N terminal mutants. 1-13: CΔ5, CΔ10, CΔ15, CΔ20,

CΔ25, CΔ30, CΔ35, CΔ40, CΔ45, CΔ74, CΔ125, CΔ165, and CΔ297. C. CAT activity of N terminal mutants. 1-15: IΔ11-30, IΔ31-60, IΔ61-90, IΔ91-120, IΔ121-150, IΔ151-180, IΔ181-210, IΔ211-240, IΔ241-270, IΔ271-300, IΔ301-330, IΔ331-360, IΔ361-390, IΔ391-420, IΔ421-440.

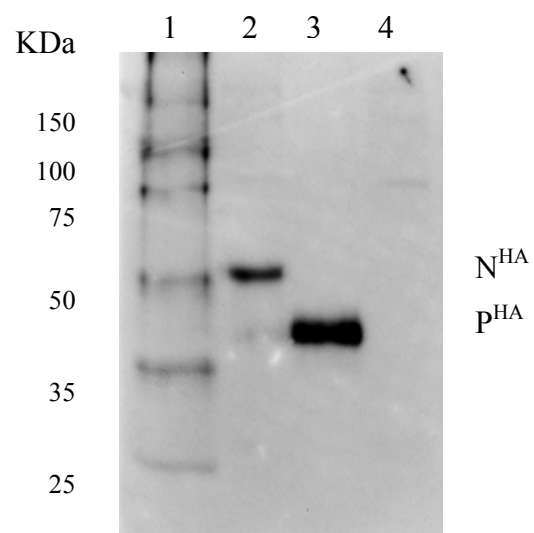
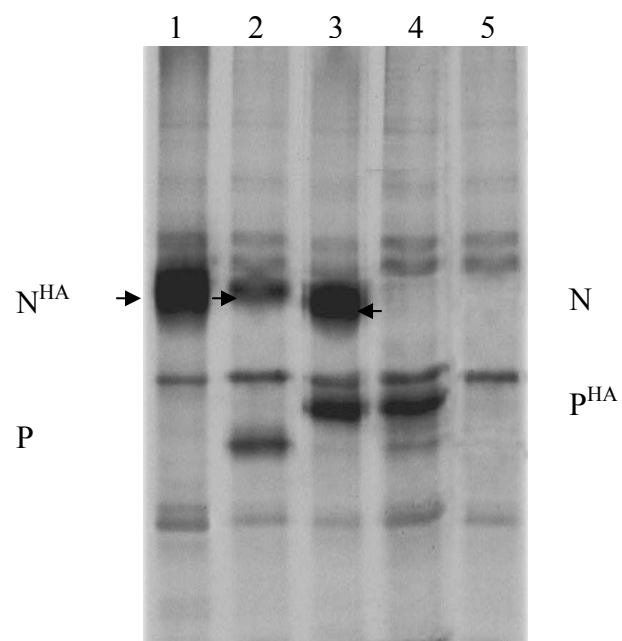
Fig. 4.1**A****B**

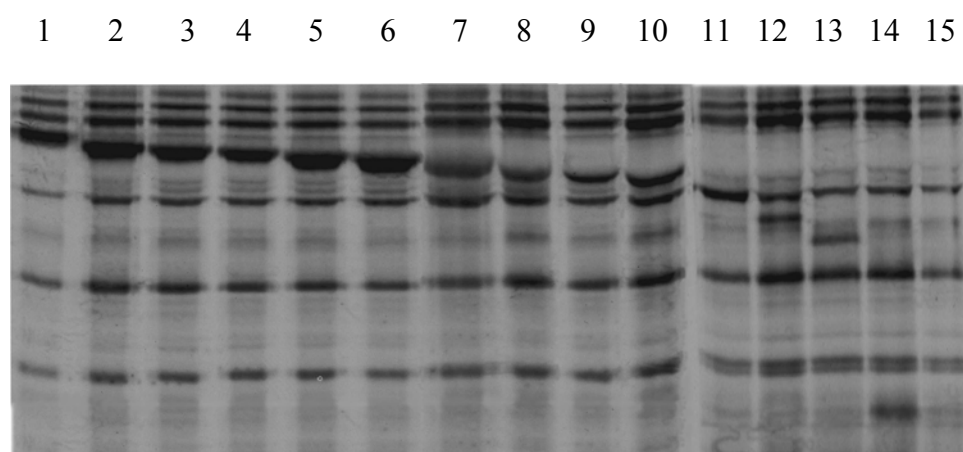
Fig. 4.2

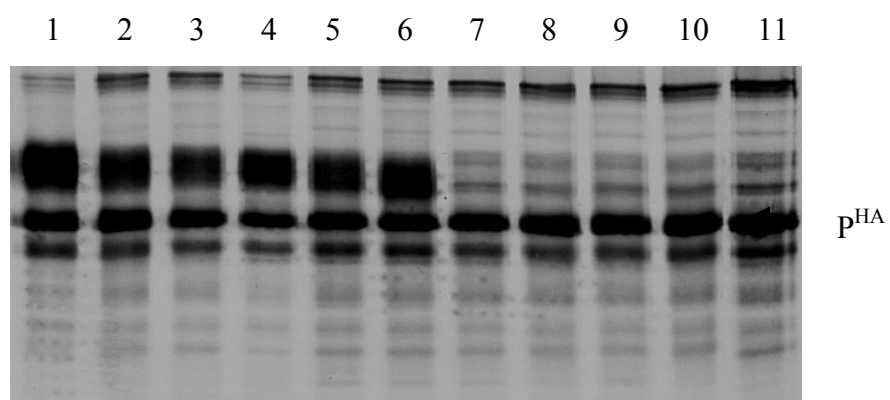
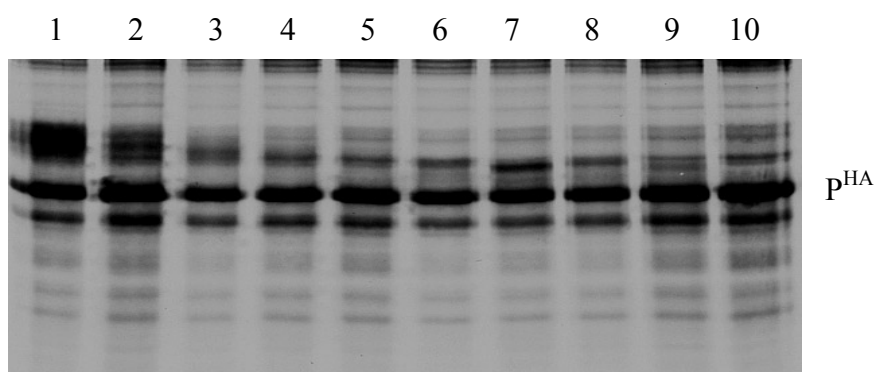
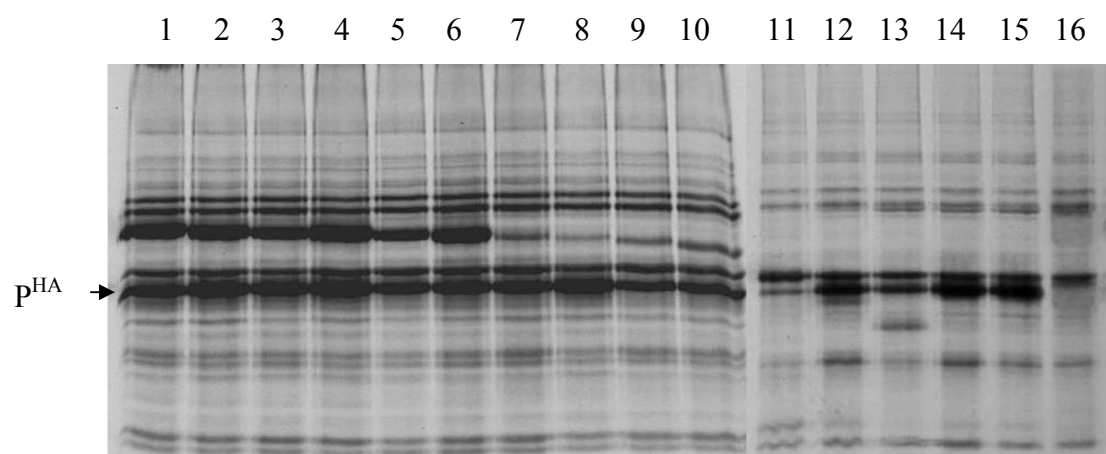
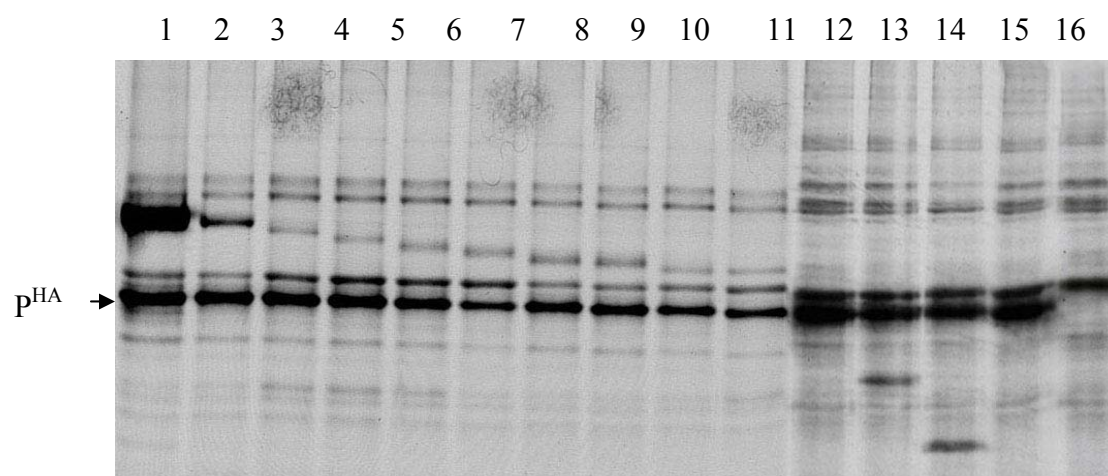
Fig. 4.3**A.****B.**

Fig. 4.4**A.****B.**

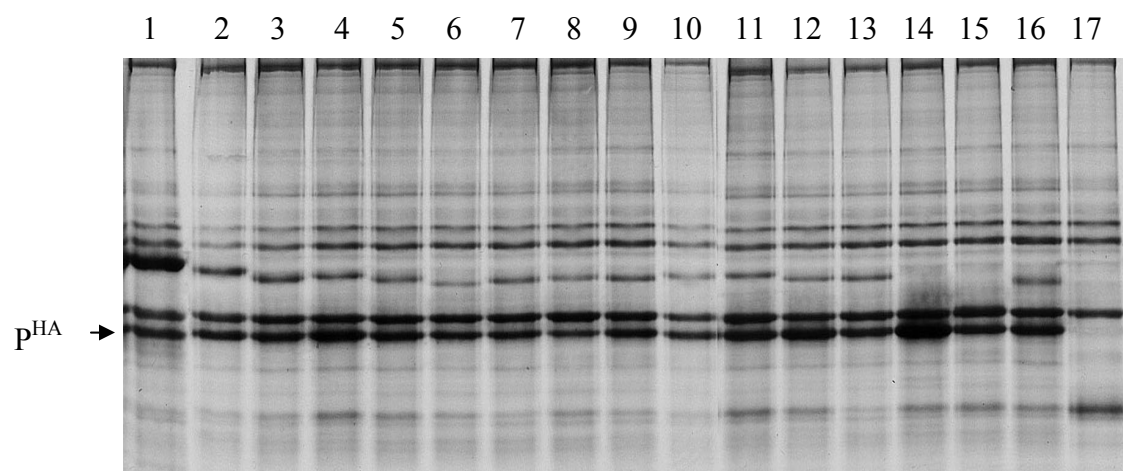
C.

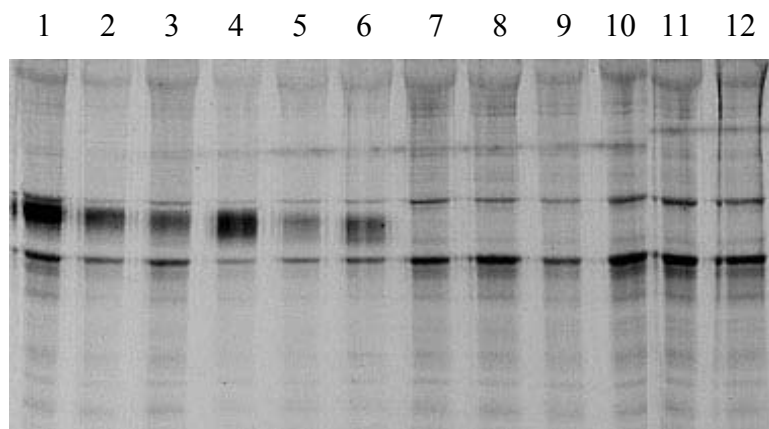
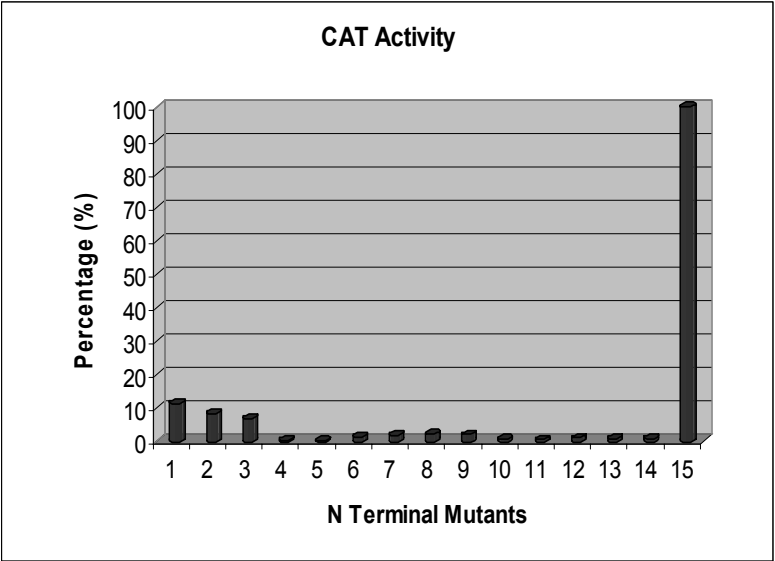
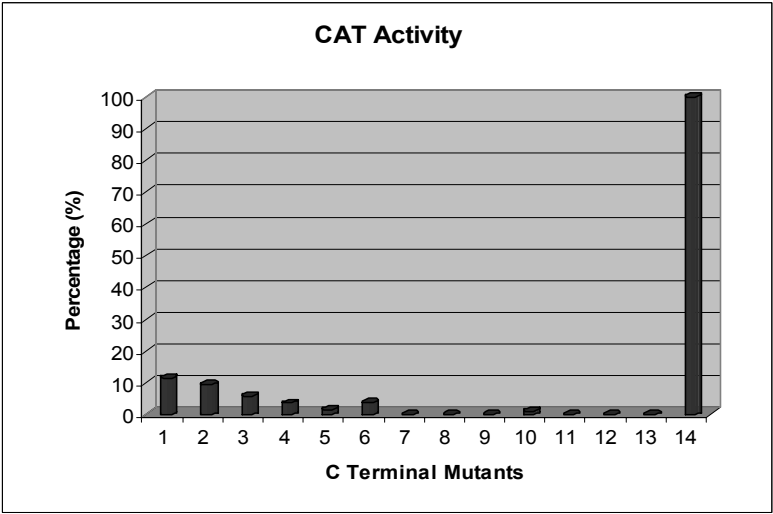
Fig. 4.5

Fig. 4.6

A.



B.



C.

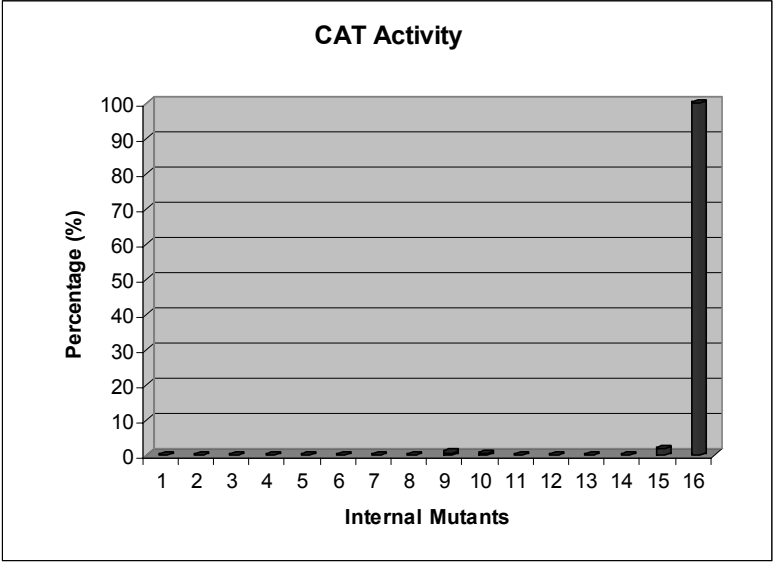


Table 1. Mutation primers

Deletions	Forward primers	Reverse primers
NΔ5	5'aactctagaaatgattgtattca3'	5'cgctgcagcttcttatgagtc3'
NΔ10	5'aactctagaaatgaataatcag3'	5'cgctgcagcttcttatgagtc3'
NΔ15	5'aactctagaaatgtctttgaag3'	5'cgctgcagcttcttatgagtc3'
NΔ20	5'aactctagaaatgattatcgtg3'	5'cgctgcagcttcttatgagtc3'
NΔ25	5'aactctagaaatgtatgattac3'	5'cgctgcagcttcttatgagtc3'
NΔ30	5'aactctagaaatgcctgccat3'	5'cgctgcagcttcttatgagtc3'
NΔ35	5'aactctagaaatgttgaaaaa3'	5'cgctgcagcttcttatgagtc3'
NΔ40	5'aactctagaaatgataacccta3'	5'cgctgcagcttcttatgagtc3'
NΔ45	5'aactctagaaatggctcccg3'	5'cgctgcagcttcttatgagtc3'
NΔ72	5'aactctagaaatgattaatgc3'	5'cgctgcagcttcttatgagtc3'
NΔ126	5'ctgtctagaggcatggaact3'	5'cgctgcagcttcttatgagtc3'
NΔ150	5'aactctagaaatgagaaaata3'	5'cgctgcagcttcttatgagtc3'
NΔ284	5'aactctagaaatgttttgattaa3'	5'cgctgcagcttcttatgagtc3'
NΔ376	5'actgacgtagcactggcag3'	5'cgctgcagcttcttatgagtc3'
CΔ5	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttatgtcttg3'
CΔ10	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttatcgccga3'
CΔ15	5'ccttctagagcaatggatgc3'	5'cagtctgcagttatggacgggcttgatgattgg3'
CΔ20	5'ccttctagagcaatggatgc3'	5'cagtctgcagttaattggaactgactgagac3'
CΔ25	5'ccttctagagcaatggatgc3'	5'cagtctgcagttagacatatctccgtatgtg3'
CΔ30	5'ccttctagagcaatggatgc3'	5'cagtctgcagttagttagatctcttttagtcg3'
CΔ35	5'ccttctagagcaatggatgc3'	5'cagtctgcagttatcgacctccattcatcatg3'
CΔ40	5'ccttctagagcaatggatgc3'	5'cagtctgcagttacatgattcagatataaacagc3'
CΔ45	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttaaacag3'
CΔ74	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttaaacag3'
CΔ125	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttataggatc3'
CΔ165	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttaataaga3'
CΔ297	5'ccttctagagcaatggatgc3'	5'cgctgcagcgagtttaatatatttt3'
IΔ11-30	5'gactttgaatacaatcttgtcggcta3'	5'cctgccatcaaagatttgaa3'
IΔ31-60	5'gtactgtactgatattgatccacg3'	5'agcggccgcaaacttaact3'
IΔ61-90	5'catgcctgacaaaactgac3'	5'agctatggaattgtgattgcacg3'
IΔ91-120	5'ggcctcagcttccggacatg3'	5'gctctgacaggaggcatgg3'
IΔ121-150	5'ccaattccctctacatcag3'	5'agcaaaatatccgggcaaaacac3'
IΔ151-180	5'caacctatacagactcaagag3'	5'atcgtggaacaccatactc3'
IΔ181-210	5'tttaacaaaaggggctgtcctc3'	5'gacatgttttctcccg3'
IΔ211-240	5'ataggtccggccaaaatctg3'	5'tcatttactgggttcataaaac3'
IΔ241-270	5'taccagtctgaacagcttc3'	5'agaatgtttgagccagggc3'
IΔ271-300	5'tcttatctcttctcaaatgc3'	5'tcatcaaatgctgttggtcac3'
IΔ301-330	5'ataaggagatttccactcaag3'	5'gctgcatgtgctcctcatg3'
IΔ331-360	5'aataaccgttgcathtagggatc3'	5'agagatgagaaagaacttcaag3'
IΔ361-390	5'gaagaatcttttcaaatgtccc3'	5'gacgaggactacttttcagg3'
IΔ391-420	5'gtcagagttgacagttcc3'	5'atacggagatatgtctcagtc3'
IΔ421-440	5'gtgagatctcttagtcgac3'	5'tttctaacaagacatatcagtgac3'

Table 2. Deletion mutants of the rabies virus N protein used in this study.

N terminal deletions	C terminal deletions	Internal deletions
NΔ5	CΔ5	IΔ11-30
NΔ10	CΔ10	IΔ31-60
NΔ15	CΔ15	IΔ61-90
NΔ20	CΔ20	IΔ91-120
NΔ25	CΔ25	IΔ121-150
NΔ30	CΔ30	IΔ151-180
NΔ35	CΔ35	IΔ181-210
NΔ40	CΔ40	IΔ211-240
NΔ45	CΔ45	IΔ241-270
NΔ72	CΔ72	IΔ271-300
NΔ126	CΔ125	IΔ301-330
NΔ150	CΔ165	IΔ331-360
NΔ284	CΔ297	IΔ361-390
NΔ376		IΔ391-420
		IΔ421-440

CHAPTER 5

DISSCUSIONS AND CONCLUSIONS

Part 1: N, P and RNA interaction *in vivo*

Completing *in vitro* investigation demonstrated that the Rabies Virus (RV) N is capable of binding non-specifically to any RNA (4, 17, and 20). It was also found that the phosphoprotein (P), by binding to N, confers the specificity of genomic RNA encapsidation by N *in vitro* (2, 4, and 20). However, few *in vivo* studies with cultured cells have been completed to confirm those *in vitro* data. In one report, Spehner et al. (16) expressed Measles virus N and P proteins either individually or in combination using recombinant vaccinia virus. When the N is expressed alone, most of the N binds to non-specific RNA and forms nucleocapsid-like structures. When the N is co-expressed with P, the N interacts with P to form N-P complexes. The N-P complex formation prevents the N from encapsidating non-specific RNA. However, Spehner et al (16) did not express the genomic RNA during N-P complex formation.

In the present study, *in vivo* evidence was presented to demonstrate that the specificity of genomic RNA encapsidation by rabies virus N is incurred by co-expressing of P. When the N is expressed alone in insect cells, it binds to non-specific RNA to form nucleocapsid-like structures. When N is co-expressed with P in transfected cells, it forms N-P complex, which does not bind to any RNA. When N, P, and genomic RNA are expressed simultaneously in transfected cells, the N binds to P and the N-P complex bound to RNA, particularly the minigenomic RNA,

and sedimented to the bottom of the CsCl gradient, just like the N encapsidating genomic RNA in virus-infected cells. All these data demonstrate that although N by itself is capable of encapsidating any RNA species to form nucleocapsid-like structure, the P, by binding to N, eliminates the binding of non-specific RNA. Although N mRNA is also detected in the nucleocapsid-like structures, this could be due to some of the N that has yet formed a complex with P, thus binding to RNA non-specifically. Binding of mRNA by N has also been reported in RV-infected cells (18).

In addition to the above functions of P, we found another function for the P, at least in the RV model. RV P, by binding to N, prevents N from becoming phosphorylated. RV N is phosphorylated in virus-infected cells (5, 7, and 15) as well as in insect cells when expressed alone (13). Since unphosphorylated or dephosphorylated N binds to RNA more strongly than the phosphorylated N (19, 21), we assumed that rabies virus N phosphorylation may be involved in the RNA binding process. We found that the N is not phosphorylated before it binds to RNA, particularly when the N is coupled with P. We also determined that only the RNA-bound N is phosphorylated. This means that N phosphorylation is intimately associated with the RNA binding process and N is phosphorylated either during the process of or immediately after RNA binding.

The fact that N is not phosphorylated when coupled with P before RNA binding may suggest that rabies virus P, by binding to N, keeps the N in a unique configuration. In such a configuration, N cannot be phosphorylated by kinases. Recently, it was demonstrated that the ratio between N and P in the N-P complex before RNA binding is 1:2 (9). However, it was also reported that the N: P ratio is 2:1 in the purified virions (18). These observations suggest that the mode of N-P interaction before RNA binding is different from that after RNA binding.

Therefore, we hypothesize that the N in such a unique configuration can only encapsidate the genomic RNA. However, the mechanisms by which the P, by binding to N, prevents N from being phosphorylated and maintains N in a unique configuration for specific encapsidation of genomic RNA are not clear.

Part 2: Mapping N functional Domains

In order to map the N functional domains, a series of N-terminal, C-terminal and internal deletion mutants of the N were constructed. Initially we attempted to map the domains in N that are involved in N-P interactions in the *in vitro* system and was found that amino acids 25 to 30 in the N-terminus and residues 5 to 10 in the C-terminus of the N are important in binding to P, which explains why in previous studies, deletion of 45 amino acids from either terminus abolished the ability of N to bind to P (20). Since N-P interaction occurs in the virus-infected cells, we also attempted to map the domains *in vivo* by co-expressing each of the N mutants with the HA-tagged P protein in mammalian cells. It was found that N mutants with deletion of 150 amino acids from the N-terminus and 165 amino acids from the C-terminus were still able to bind to P, suggesting that the central region between residues 150-285 is important for N-P interactions. Using a series of internally deleted N mutants, it was found that only N mutants with deletion of amino acids from 360 to 390 and from 390 to 420 failed to interact with P, indicating that the C-terminal region 360-420 is also important for binding to P. Thus, there are at least two binding sites on the N that can interact with P. This is not surprising because it was recently reported that one N molecule is coupled with two P proteins in the N-P complex prior to RNA binding (9). The discrepancies between the *in vitro* and *in vivo* data lead us to hypothesize

that cellular factors may be involved in the N-P interactions *in vivo*. Nevertheless, our *in vivo* data for mapping the domains on N that are involved in N-P interactions are similar to those reported for other negative-stranded RNA viruses (3, 8).

Rabies virus N also self-aggregates and this is important for assembly of the nucleocapsid particles (3, 8, and 16). Thus we also attempted to map the N-N interactive domains by using gradient sedimentation as described for other negative-stranded RNA viruses (3, 6, and 16). Our data demonstrated that the first 25 amino acids appears to be dispensable for N-N interactions. However, the remainder of the N is absolutely required for the N to self-aggregate. It is possible that self-aggregation of N requires a certain conformation and deletion of the first 25 amino acids do not alter that conformation. Our N-N interactive regions, thus, are different from those reported for Measles virus and Sendai virus. In Measles virus, the N-N interaction site is located at the central portion of the N protein from amino acids 189 to 373 (3). Whereas for Sendai virus, N-N interactions domains were identified as located between residues 114-129, 258-357 (10, 11), and 362-370 (12).

To investigate if the N-P and N-N binding domains are important for the process of viral transcription and replication, each of the N mutants was assayed for its ability to support minigenome transcription. The transcriptive activities were reduced by 90% when five amino acids from each terminus were deleted and the activities were reduced more as more amino acids were deleted from the termini. There was almost no transcriptive activity in cells transfected with the internally deleted N mutants. This data suggests that the intact N may be required for optimal viral transcription and replication. Similar results were also obtained in BRSV (8). However, in that study, deletion or replacement of a single residue for the first five amino acids did not alter

the transcriptive activity, indicating that the terminal amino acids are important for the N to support viral transcription and replication.

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