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# Phosphorylation of the Nucleocapsid Protein of Bovine Coronavirus Expressed with a Recombinant Baculovirus Vector

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**Post-translational modifications of the nucleocapsid protein of bovine coronavirus (Quebec strain) were investigated. Coronavirions were radiolabelled *in vivo* with inorganic [$^{32}$P]orthophosphate and analysed by SDS-PAGE, followed by autoradiography. A single polypeptide with a migration rate of 55 KDa was identified by metabolic phosphate labelling, demonstrating that the nucleocapsid protein of bovine coronavirus was a phosphoprotein. A gene encoding the nucleocapsid protein was inserted immediately downstream from the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis baculovirus. *Spodoptera frugiperda* cells infected with this recombinant baculovirus synthesized a 55 KDa polypeptide, as demonstrated by immunoprecipitation with anti-nucleocapsid monoclonal antibody. The recombinant nucleocapsid protein synthesized in *Spodoptera* cells could also be labelled by [$^{32}$P]orthophosphate. Phosphoamino acid analysis showed that both serine and threonine residues were phosphorylated in authentic, as well as in recombinant nucleocapsid proteins, with a relative phosphorylation ratio of 7:3. Our studies demonstrated that the nucleocapsid protein of bovine coronavirus was a serine and threonine-phosphorylated protein and that *Spodoptera* insect cells were able to properly phosphorylate the relevant foreign proteins.**

Coronaviruses are enveloped animal viruses with a genome of single-stranded, positive sense RNA of 25 to 30 Kb in length (14, 23). During coronavirus infection the viral genome is transcribed, by the early viral RNA-dependent RNA polymerase, into a genome-size negative-stranded RNA. This replicative form of negative-stranded RNA is transcribed by a late polymerase into a full-length genomic RNA (13). The full length genomic RNA is encapsidated by nucleocapsid protein, which is then packaged into a mature virion. Six to nine species of viral specific subgenomic RNAs are additionally synthesized in virusinfected cells. These mRNAs form a nested set with 3′ co-terminal common sequences derived from the 3′ terminus of the genomic RNA, and extend for various distances in the 5′ direction (11). Furthermore, each subgenomic RNA contains the identical leader sequences of approximately 70 nucleotides at the 5′ terminus (10, 31). These leader sequences are derived from the 5′ terminus of the genomic RNA and are joined to the mRNAs by a leader-primed discontinuous transcription (12). Only the 5′ proximal region of each subgenomic RNA is translated (for a review, see ref. 30).

Bovine coronavirus (BCV) is an enteropathogenic coronavirus causing severe scours in newborn calves (19). Bovine coronavirus contains four major structural proteins: spike protein (S), hemmagglutinin/esterase (HE), matrix protein (M) and nucleocapsid protein (N) (5, 9). The spike protein functions in the virus attachment, cell fusion (36), and induction of major BCV neutralizing antibodies (4, 37). The hemagglutinin/esterase is an enzyme hydrolyzing 9-O-acetylated neuraminic acid (24, 35). The matrix protein is believed to determine the site of virus maturation (25). The nucleocapsid protein is associated with the viral RNA, forming a helical ribonucleoprotein structure (18). The N protein of coronavi-

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rus is a phosphoprotein (9, 32) and seems to play different roles in viral pathogenesis and replication, in addition to serving as a structural component of the helical nucleocapsid since anti-N monoclonal antibodies protect mice from lethal infection (21) and inhibit viral transcription *in vitro* (2). A cDNA encoding the nucleocapsid protein was cloned from the Mebus strain of bovine coronavirus, and its nucleotide sequences were determined (16). However, the molecular basis of N protein function has not been well characterized and remains to be investigated further. We reexamined post-translational modifications of the authentic N protein purified from BCV virions, as well as recombinant protein synthesized from insect cells infected with a baculovirus vector.

## MATERIALS AND METHODS

### Cells, Viruses and Antibodies

The Quebec strain of bovine coronavirus (3) was used. Coronavirus was grown at 37℃ on MDBK (Mardin Darby Bovine Kidney) cells with Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (GIBCO). The E2 strain of *Autographa californica* monocapsid nuclear polyhedrosis baculovirus (AcMNPV) was plaque-purified and propagated on *Spodoptera frugiperda* (Sf9, ATCC CRL 1711) insect cells. The cells were maintained at 28℃ with Grace's medium containing 10% serum supplemented with 0.3% yeastolate and 0.3% lactalbumin hydrolysate (Difco). Monoclonal antibody MD8-3, specific for the N protein, and polyclonal anti-BCV rabbit antibody were prepared as described (4).

### DNA Cloning

Restriction endonucleases and DNA-modifying enzymes were purchased from Pharmacia (Montreal, Quebec) and New England Biolabs (Mississauga, Ontario). Standard molecular cloning techniques were employed for subcloning and manipulation of bacterial plasmids (26).

### Construction of Recombinant Baculovirus

The entire nucleocapsid gene of BCV, cloned in *Bam*HI site of pTZ19R (Pharmacia), was subcloned after modification with a Klenow fragment and a linker addition into the *Nhe*I cloning site of baculovirus transfer vector pJVP10/Z (a derivative pf pJVNheI) (supplied by Dr. C. Richardson, Biotechnology Research Institute of the National Research Council of Canada). Approximately $1.0\times10^6$ Sf9 cells on a 35 mm dish were cotransfected with 1 μg of the wild-type viral DNA and 3 μg of the transfer plasmid DNA using a standard calcium phosphate transfection technique (23). The transfection fluid was collected after 3 day, and plated on Sf9 cell monolayers for plaque assay. Plaque assays were performed in 35 mm dishes with 1.5% agarose. After 4 days of incubation 1 m*l* of fresh medium containing 150 μg/m*l* of Bluo-gal (BRL) was added to the agarose overlay. The plaques which developed a blue color were picked with Pasteur pipets and further purified. Purified recombinant viruses were amplified on Sf9 cells and the virus stock, with a titer of approximately $10^8$ PFU/ml, was used in this study.

### Preparation of Radiolabelled Virus and Cell Lysates

A monolayer of MDBK cells was infected with BCV at a multiplicity of infection (m.o.i.) of 10 PFU/cell. Twenty for hours after infection cells were placed in methionine-free MEM containing 80 μCi/m*l* of [$^{35}$S]methionine (specific activity 800 μCi/mmol, Amersham, Oakville, Ontario) for 12 hr. For the preparation of insect cell lysates a monolayer of Sf9 cells was infected with wild-type or recombinant baculoviruses at an m.o.i. of 5~10 PFU/cell. Fourty hour after infection cells were incubated for 1 hour in methionine-free Grace's medium containing 3% dialysed fetal bovine serum, followed by labelling with 100 μCi/m*l* of [$^{35}$S]methionine for 2 hours. Cell monolayers were washed with PBS and lysed in a lysis buffer solution (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 1 mM EDTA), Cell lysates were then cleared by centrifugation at 10,000 rpm in a microcentrifuge for 10 minutes. For the preparation of BCV virions BCV-infected MDBK cells were labelled at 24 hours after infection for 1 hour in phosphate-free MEM containing 120 μCi/m*l* of [$^{32}$P]orthophosphate (ICN Biochemicals Inc.). Culture supernatants were collected and cell debris was cleared. Viruses were then pelleted by centrifugation for 2 hours at 25,000 rpm (SW28, Beckman) and subsequently purified through a 30% to 55% continuous sucrose gradient.

### Radioimmunoprecipitation and SDS-PAGE

Immunoprecipitation was performed as described (37). Samples were incubated with the antibody at room temperature for 2 hours, then 10 mg of Protein A Sepharose beads (Pharmacia) was added. The mixture containing 0.5% SDS was further incubated overnight in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA) at 4℃ with continuous shaking. Immune complexes were washed three times with a RIPA buffer solution containing 0.5% SDS, then dissociated by boiling for 5 minutes in a dissociation buffer solution (10% SDS, 25% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue, 10 mM Tris-HCl, pH 6.8). The immune complexes were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The gels were impre-

gnated with Amplify (Amersham) and dried under a vacuum. The dried gels were then exposed to Du Pont Cronex Lightening-Plus X-ray film at minus 70℃ with an intensifying screen.

**Phosphoamin Acid Analysis**

The $^{32}$P-labeled protein was immunoprecipitated with anti-N monoclonal antibodies and resolved by SDS-PAGE. The protein was localized by exposing the gel to X-ray film. Gel slices, corresponding to the N protein, were excised and the protein was eluted using an electroeluter (IBI Model UEA). After precipitation with 50% TCA the sample was hydrolysed in 6N HCl at 110℃ overnight. The hydrolyzed amino acids were dried in a lyophilizer and redissolved, followed by electrophoresis on thin-layer cellulose chromatographic plates in pH 7.9 buffer (formic acid-acetic acid-water 1:4:100) at 1000 volts for 60 minutes (28). Samples, including the unlabeled phosphoamino acid standards (Sigma), were visualized by staining with ninhydrin solution in n-butanol, and the radiolabeled phosphoamino acids were localized by autoradiography.

## RESULTS

**Phosphorylation of the N Protein *in vivo***

The genomic RNA of bovine coronavirus is capable of encoding four major structural proteins and at least six potential nonstructural proteins (6). Their coding regions on the genome are illustrated in Fig. 1. The gene encoding the nucleocapsid protein of bovine coronavirus is located at the most 3′ region of the genome. We first examined the molecular migration profiles of the structural proteins of BCV virions. BCV virions were radiolabeled *in vivo* with [$^{35}$S] methionine and purified through sucrose gradients. The virion proteins were dissociated with SDS in the presence of a reducing agent and resolved on a polyacrylamide gel followed by autoradiography (Fig. 2, lane 1). The S1 and S2 subunits of the BCV spike protein were co-migrated as an 85 KDa, whereas the HE, N and M proteins were identified as 65 KDa, 55 KDa, and 27 KDa, respectively. The

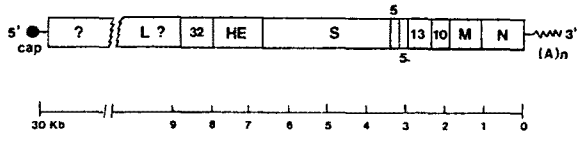


**Fig. 1. Organization of the genomic RNA of bovine coronavirus.**
The reading frames are deduced from the primary sequences of the cDNAs of viral RNA. They are identified as nucleocapsid protein (N), matrix protein (M), spike protein (S), hemagglutinin-esterase protein (HE), and potential nonstructural proteins by size in kilodaltons (6).

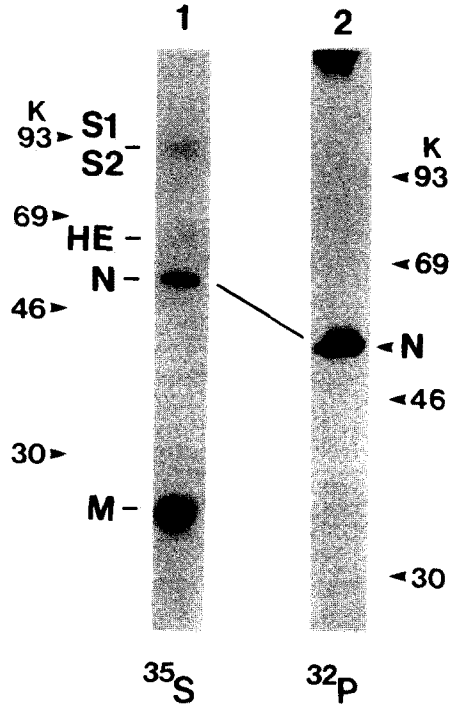


**Fig. 2. Autoradiogram of the structural proteins of BCV radiolabelled *in vivo*.**
BCV virions were grown in MDBK cells and labelled with [$^{35}$S]methionine (lane 1) or with [$^{32}$P]orthophosphate (lane 2). The virus was harvested and purified through sucrose gradients. The purified virions were dissociated with SDS and electrophoresed on 10% polyacrylamide gel in the presence of β-mercaptoethanol followed by autoradiography. Lane 1: structural proteins of BCV virions: S1 and S2 (85 KDa), two subunit proteins of the S spike; HE (65 KDa), hemagglutinin/esterase protein; N (55 KDa), nucleocapsid protein; M (27 KDa), matrix protein. Lane 2: identification of the phosphorylated N protein.

matrix protein and the nucleocapsid protein were the most abundant proteins, judging by the density of the corresponding bands on the autoradiogram.

The molecular mass of the N protein, predicted from the nucleotide sequences (16), was 50 KDa, whereas the N protein prepared from the virions grown in MDBK cells showed a migration rate of 55 K (Fig. 2, lane 1). The apparent difference in molecular weight suggests that post-translational modifications may contribute to the migration differences of the N protein. We, therefore, examined phosphorylation of the N protein. Virions were labeled *in vivo* with [$^{32}$P] phosphate and the total viral proteins were analysed on a gel without immunoprecipitation (Fig. 2, lane 2). Only a 55 KDa protein was identified by metabolic phosphate labeling, indicating that the nucleocapsid protein of BCV, based upon molecular mi-

gration, was post or co-translationally modified with the addition of phosphate.

**Expression of the N Protein in Sf9 Cells**

In order to further examine the nature of the N protein we intended to produce a recombinant protein in a heterologous eukaryotic gene expression system. We chose a baculovirus system, since it has gained wide popularity with a high level of foreign gene expression (17). A baculovirus transfer vector pJVP10/Z contains two very late promoters derived from the P10 and polyhedrine genes of *Autographa californica* baculovirus. The P10 promoter directs the synthesis of β-galactosidase, while the polyhedrine promoter controls the synthesis of the foreign gene product. The insertion of an entire expression cassette containing the *lacZ* gene and the foreign gene into the baculovirus genome allows the convenient identification of the putative recombinant viruses by virtue of the expression of β-galactosidase (34). An entire coding sequence of the BCV N protein was subcloned into *Nhe*I site of pJVP10/Z so that the N gene was placed under the control of the baculovirus polyhedrin promoter (Fig. 3). Recombinant viruses so constructed should be polyhedrine negative, produce foreign gene products, and form blue plaques when β-galactosidase indicator is present in the agarose overlay. Viral plaques developing blue color in the presence of Bluo-gal were picked and recombinant viruses were isolated by several rounds of subsequent plaque purification. Synthesis of the N protein by the recombinant baculovirus was examined by immunoprecipitation and SDS-PAGE of the virus-infected cell extracts (Fig. 4). Sf9 cells were infected with wild-type or recombinant baculoviruses and radiolabelled with [$^{35}$S]methionine at 20 or 40 hours after infection. Polyclonal anti-BCV rabbit antibody immunoprecipitated two species of polypeptides, 55 K and 60 K, from the cells infected with the recombinant baculovirus at 20 hours after infection (Fig. 4, lane 2). In contrast, only 55 K polypeptide was immunoprecipitated at 40 hours after infection (Fig. 4, lane 3). These results suggest that the recombinant baculovirus mediated synthesis of the 60 K polypeptide and this polypeptide was further processed, yielding a mature 55 K polypeptide. The dicrease in molecular migration of the 60 K polypeptide could be due to a partial dephosphorylation by cellular phosphatase activity. Synthesis of the 55 K polypeptide by the recombinant baculovirus suggests that the recom-

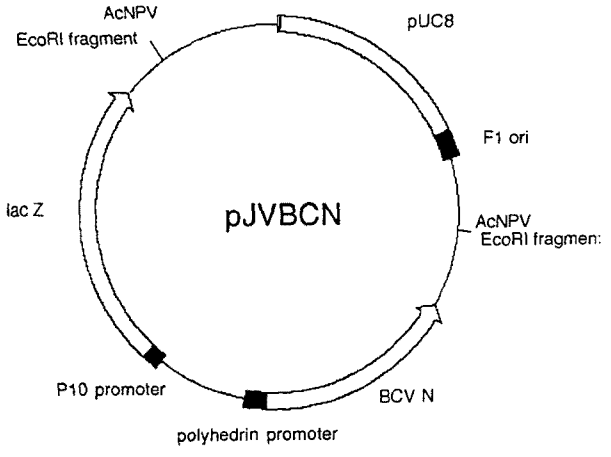


**Fig. 3. Construction of the recombinant baculovirus transfer vector.**
The N gene of BCV was isolated from pTZBCV by digestion with *Bam*HI. The *Bam*HI fragment was blunt-ended with a Klenow fragment and the *Nhe*I linker was attached. The N gene fragment was then ligated into a unique *Nhe*I cloning site of pJVP10/Z in a parellel orientation to the polyhedrine promoter.

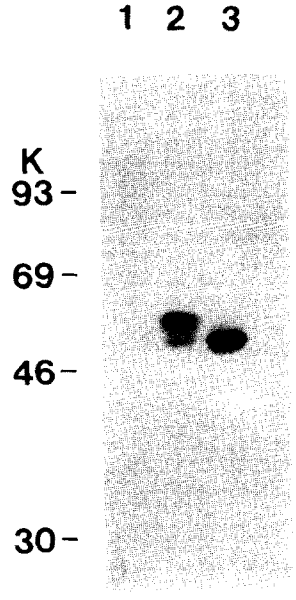


**Fig. 4. Synthesis of the recombinant N protein in Sf9 cells.**
Sf9 cells were infected with recombinant baculovirus and labelled for 2 hours with 100 μCi/ml of [$^{35}$S]methionine. Cell lysates were prepared as described in Materials and Methods and immunoprecipitated with anti-BCV polyclonal rabbit antisera followed by 10% SDS-PAGE. Numbers on the left of the Figure refer to molecular mass in kilodaltons of protein markers (Bio-Rad). Lanes: 1, wild-type AcNPV-infected cell lysates at 40 hours after infection; 2, recombinant virus-infected cell lysates at 20 hours after infection; 3, recombinant virus-infected cell lysates at 40 hours after infection.

binant N polypeptide produced in insect cells may also be phosphorylated, since the molecular weight of the N protein deduced from the nucleotide sequence is 52 K (16).

**Phosphorylation of the Recombinant N Protein**

In order to determine if the recombinant N protein synthesized in insect cells was also phosphorylated, Sf9 cells infected with the recombinant virus were labelled with [$^{32}$P]orthophosphate and the cell lysates were immunoprecipitated by anti-N monoclonal antibody. As shown in Fig. 5 a single species of phosphorylated polypeptide with a molecular weight of 55 K was immunoprecipitated (Fig. 5, lane 2). The 55 K polypeptide synthesized in Sf9 cells was identical in both immunoreactivity and molecular migration to the authentic N protein of the BCV virions (Fig. 5, lane 1), demonstrating that the recombinant protein N synthesized in insect cells was also phosphorylated.

**Identification of Phosphoamino Acids**

To analyze phosphorylated amino acid residues in the N protein $^{32}$P-labelled N protein was prepared from purified BCV virions, or cells infected with the recombinant baculovirus. The radiolabeled protein was hydrolysed in acid to generate O-phosphoamino acid residues and the hydrolysed products were separated by electrophoreis on thin layer chromatographic plates in one dimension. As shown in Fig. 6, radioactivities were comigrated with the O-phosphoserine and O-phosphothreonine standards in both authentic (Fig. 6A) and recombinant N proteins (Fig. 6B). Detectable radioactivity was

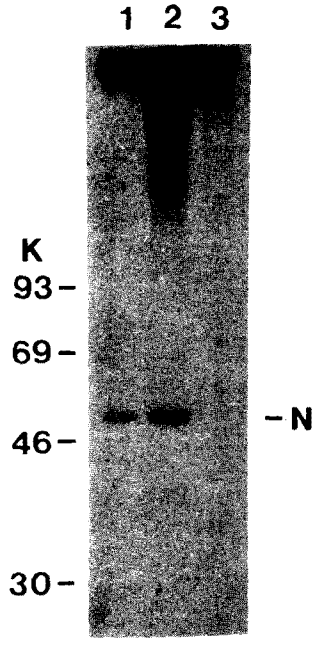


**Fig. 5. Phosphorylation of the recombinant N protein synthesized in Sf9 cells.**
Sf9 cells were infected with wild0type or recombinant baculoviruses. Affter 36 hours of infection cells were radiolabelled with 60 μCi/ml of [$^{32}$P]orthophosphate for 6 hours. Cell lysates were prepared and immunoprecipitated with monoclonal antibody MD8-3 and Sepharose beads coupled with Protein A (Pharmacia). Immune complexes were resolved by SDS-PAGE and the gel was dried and exposed to an X-ray film at minus 70℃. Lanes: 1, $^{32}$P-labelled BCV virions purified from BCV-infected MDBK cells; 2, recombinant baculovirus-infected cell lysates; 3, wild-type baculovirus-infected cell lysate.

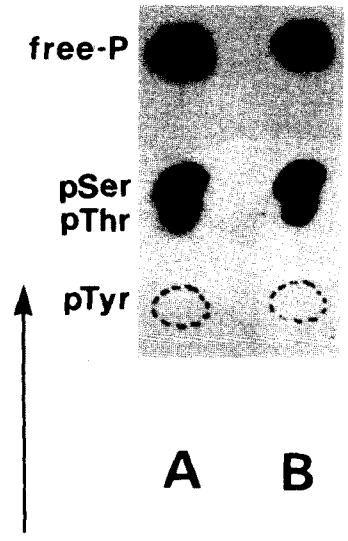


**Fig. 6. Phosphoamino acid analysis of the authentic (A) and recombinant (B) N proteins.**
The N protein resolved on a polyacrylamide gel was localized by wet-gel exposure, excised, and purified. The purified protein was then hydrolysed at 110℃ overnight with HCl. The hydrolysed amino acids were separated by uni-directional electrophoresis (arrow) on a TLC plate. O-phosphoserine, O-phosphothreonine and O-phosphotyrosine were included in the samples as phosphoamino acid standards. Positions of the standard phosphoamino acids were visualized by ninhydrin staining and the radioactive phosphoamino acids were localized by an autoradiography. Dotted circles indicate the migrated position of O-phosphotyrosine standard. Free-P indicates the unicorporated free phosphates: 1, N from BCV virion; 2, recombinant N synthesized from Sf9 cells.

not identified with the O-phosphotyrosine standards. To determine the phosphorylation ratio between the phosphoamino acids the autoradiogram was scanned with a densitometer and the relative ratio was calculated (data not shown). Approximately 70% of the radioactivities measured were associated with phosphoserine, and 30% with phosphothreonine. These results demonstrate that both serine and threonine residues are phosphorylated in BCV N protein with an approximate ratio of 7:3.

## DISCUSSION

We demonstrated and characterized phosphorylation of the N protein of bovine coronavirus. The N protein was the only phosphoprotein found in BCV virion. Since potential glycosylation sites were not found in the N protein (16) only phosphorylation appears to account for differences between the predicted molecular weight (50) and the apparent molecular weight (55 K). Phosphorylation sites of the N protein were both serine and threonine residues and the phosphorylation ratio between the two amino acids was approximately 7:3. Unlike many viral oncogenic proteins tyrosine was not found to be phosphorylated. Previous studies with mouse hepatitis coronavirus (MHV) demonstrated that serine was the residue exclusively phosphorylated (32). It remains to be determined if the additional phosphorylation of the threonine residues in BCV N protein has any significance in the biological functions of the N protein.

Many eukaryotic regulartory proteins have been found to be phosphorylated. Furthermore, the phosphorylation state of these proteins controls their regulatory activities. In viral proteins exact phosphorylation sites have been mapped in many cases and correlated to biological function. In Sendai virus phosphorylation may play a regulartory role in the selection of the negative-stranded RNA genome and in the regulation of overall RNA synthesis (15). Phosphorylation may regulate the switch from transcription to replication in vesicular stomatitis virus (1). Phosphorylation also affects the specific binding of the viral phosphoprotein to the RNA genome in RNA tumor viruses (29). The single-stranded RNA genome of poliovirus is ligated to a virus-specified protein through a phosphodiester linkage between the 5'-phosphate of UMP and the hydroxyl group of tyrosine. Simian virus 40 (SV40) large tumor (T) antigen is a multifunctional regulartory protein which binds to viral DNA (27). Thus, it is of interest to investigate if the BCV N protein specifically binds to the RNA molecule and to investigate the role of phosphorylation in this activity.

Since many eukaryotic genes have been expressed in insect cells using a baculovirus vector, it becomes important how post-translational modifications in insect cells compare with those in mammalian cells. Phosphorylation in insect cells has been relatively less investigated than other modifications. The Kruppel gene product of *Drosophila* (22), the $p40^x$ protein of human T-cell leukemia virus type 1 (8), and human *c-myc* protein (20) have been reported to be phosphorylated in baculovirus-infected insect cells. We demonstrated that the N protein synthesized in insect cells was phosphorylated and the nature of the phosphorylation was similar to that of the authentic N protein. Studies with the SV40 T antigen showed that the phosphorylation pattern expressed in baculovirus was quantitatively different and underphosphorylated (7). Thus, it seems that some phosphorylation occurs in insect, cells, but it needs to be further determined whether phosphorylation occurs in an exact position identical to that in proteins isolated from the natural host cells. Further information regarding the efficiency and accuracy of insect phosphorylation is required.

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