Expression of Recombinant Parvovirus NS1 Protein by a Baculovirus and Application to Serologic Testing of Rodents

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A recombinant baculovirus containing the NS1 gene of minute virus of mice was constructed. Optimal expression of the recombinant NS1 protein (rNS1) was achieved by infecting *Trichoplusia ni* High Five cells at a multiplicity of 10 and incubating them for 72 h postinfection. An enzyme-linked immunosorbent assay (ELISA) with rNS1 as the antigen was evaluated for serologic testing of laboratory rodents. The rNS1 ELISA proved to be a more sensitive method for the detection of antibodies to recently recognized rodent parvovirus species (mouse orphan parvovirus and rat orphan parvovirus) and prototypic parvovirus species (minute virus of mice, Kilham's rat virus, and H-1) than were conventional parvovirus ELISAs that use whole parvovirus virions.

In rodents, three prototypic parvovirus species have been extensively characterized: minute virus of mice (MVM), which infects mice, and Kilham's rat virus (KRV) and H-1, which infect rats (11). Natural infections with these agents are nearly always subclinical, although clinical disease has been reported in rats with KRV infections (9, 16). Clinical signs associated with KRV infection include fetal resorption in dams; runting, ataxia, cerebellar hypoplasia and jaundice in suckling rats; and sudden death, scrotal cyanosis, and dehydration in juvenile rats (9, 16).

Recently, investigators have identified in rodents a number of new parvovirus isolates that are distinct from the prototypic species (3, 14, 15, 22). At present, this group of viruses has been designated orphan parvoviruses until characterization of these viruses can be completed and an appropriate nomenclature can be established. This group of viruses includes variants of a mouse orphan parvovirus (MOPV) and a hamster isolate designated hamster orphan parvovirus (HOPV). Molecular characterization and sequence analysis of the genomes of three of these newly recognized parvoviruses revealed a genetic organization very similar to that of the prototypic species; the viruses are composed of a 5-kb single-stranded DNA genome that encodes four viral proteins analogous to those found in prototypic species (1, 3). Like prototypic parvovirus infections, infections with these newly recognized orphan parvoviruses are asymptomatic in mice and rats.

Infections with rodent parvoviruses appear to be widespread. Infections may compromise biomedical research that uses infected animals since rodent parvovirus infections have been shown to alter *in vitro* and *in vivo* lymphocyte activities, cause oncosuppression, and induce interferon production (4, 6, 7, 12, 17, 20). In addition, tissues from infected rodents can contaminate and insidiously persist in established mammalian cell lines, tumor cells, and transplantable tumors (2, 10, 13, 15, 18, 19), which may alter or invalidate the results of research investigations. To limit the deleterious effects of parvovirus infections on biomedical research, infected rodents need to be identified. Since infections are generally asymptomatic, serol-

ogy is the primary diagnostic method for the detection of parvovirus infections in laboratory rodents. The most common method for serologic evaluation is the enzyme-linked immunosorbent assay (ELISA), which uses intact virions as the antigen; however, currently used conventional ELISAs that use prototypic parvovirus virions lack sensitivity in detecting antibodies to these novel orphan parvoviruses because of the limited antigenic cross-reactivity between these newly identified parvovirus isolates and the prototypic parvoviruses.

The goal of the project described here was to develop a sensitive serologic assay that could be used to identify rodents infected with any of the prototypic or orphan parvovirus species. The nucleotide sequences of three orphan parvoviruses (MOPV1, MOPV2, and HOPV) were determined in other studies in our laboratory (3).

Construction of recombinant baculovirus. MVM ATCC VR-663 was propagated in murine A9_{2L} fibroblasts obtained from P. Tattersall (New Haven, Conn.). A 3,228-bp *Bam*HI-*Bgl*II fragment from the MVM genome initiating at nucleotide (nt) 225 and continuing to nt 3453 was cloned into a pVL941 baculovirus plasmid vector to construct the recombinant plasmid NS1-pVL941 (Fig. 1) (5). This fragment encompassed the NS1 ATG translation start codon located at nt 261 and the UAA terminator at nt 2277. The coding sequence of the NS1 gene was positioned immediately downstream of the polyhedrin promoter and in the appropriate reading frame to allow synthesis of the NS1 protein. To facilitate purification of the recombinant protein, a 54-bp linker (a kind gift of R. Roeder, Rockefeller University) containing a six-residue histidine binding site was inserted at the MVM *Nco*I site at nt 261 immediately upstream of the translation start site of the NS1 gene to create the recombinant construct (His)₆-NS1-pVL941. The predicted fusion protein expressed by this construct retains the complete NS1 protein and an additional 15 amino acids on the amino terminus encoded by the histidine linker. The peptide sequence of the amino terminus of the fusion protein is as follows (MVM sequences starting at amino acid 1 are underlined): MGGHHHHHHGGIEGRMAGNAY. . .

The (His)₆-NS1-pVL941 construct was cotransfected with linear wild-type baculovirus, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), into Sf-9 *Spodoptera frugiperda* insect cells (Invitrogen Corporation, San Diego, Calif.) by cationic liposome-mediated transfection (Transfection

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FIG. 1. Schematic representation of MVM and expression vectors encoding NS1 gene. (A) MVM genome showing the position of restriction sites and the NS1 gene on the MVM genome. (B) Recombinant baculovirus plasmid construct showing the NS1 gene inserted downstream of the promoter for the baculovirus polyhedrin gene (Pph). (C) Recombinant baculovirus plasmid with the (His)₆ linker inserted at the 5' terminus of the NS1 coding region.

Module, Invitrogen Corp.). Insect cells were maintained in Excell 401 medium (JRH Biosciences, Lenexa, Kan.) supplemented with 10% Serum Plus (JRH Biosciences). Plaques containing recombinant viruses were identified by selecting non-occluded plaques.

PCR amplification. PCR was carried out in a total volume of 100 μ l containing 1 μ l of viral DNA, 1 μ M (each) NS1 primer (NS1-1458; 5'-ACC AGC CAG CAC AGG CAA ATC TAT-3'; NS1-1791, 5'-CAT TCT GTC TCT GAT TGG TTG AGT-3'), 200 μ M (each) deoxynucleoside triphosphate (dCTP, dGTP, dATP, dTTP), PCR buffer (10 mM Tris HCl, 1.5 mM

MgCl₂, 50 mM KCl [pH 8.3]), and 5.0 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). Samples were heated at 94°C for 5 min; this was followed by 30 cycles consisting of denaturation (94°C, 1 min), primer annealing (37°C, 1 min), and extension (72°C, 2 min). PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Optimization of recombinant protein expression. To determine the conditions for maximum expression of recombinant protein, a series of experiments were performed. In all experiments, insect cells were seeded into 12-well culture plates, infected with the (His)₆-NS1-pVL941 recombinant baculovirus construct, and incubated at 37°C. At given periods of time postinoculation, cells were harvested and were resuspended in extraction buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.5], 5 mM KCl, 7.5 mM MgCl₂, 0.1 mM dithiothreitol) containing the following protease inhibitors: 50 μ g of antipain-dihydrochloride per ml, 2 μ g of aprotinin per ml, 40 μ g of bestatin per ml, 100 μ g of chymostatin per ml, 10 μ g of E-64 per ml, 0.5 μ g of leupeptin per ml, 100 μ g of Pefabloc SC per ml, 0.7 μ g of pepstatin per ml, and 330 μ g of phosphoramidon (Boehringer Mannheim) per ml. Cells were disrupted by five 1-min cycles of sonication on ice and were incubated for 30 min on ice in the presence of 0.3 M KCl. Cell debris was removed by centrifugation at 20,000 $\times$ g for 10 min at 4°C, and identical amounts of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide gel and evaluated for the expression of recombinant NS1 (rNS1) by Western blot (immunoblot) analysis with rabbit anti-NS1 polyclonal antibody (8). Wells containing insect cells infected with wild-type AcMNPV virus served as controls.

ELISAs and HAI assays. For the rNS1 ELISA, microtiter plates (Pro-Bind Assay Plate; Falcon, Lincoln Park, N.J.) were coated with 4.0 μ g of cell lysate per ml diluted in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3.1 mM NaN₃ [pH 9.6]) at 4°C for 48 h. Antigen was removed and the wells were blocked with 0.5% nonfat dry milk in phosphate-buffered saline (PBS)-Tween 20 (140 mM NaCl, 15 mM NaH₂PO₄, 11 mM Na₂HPO₄, 2.7 mM KCl, 3.1 mM NaN₃, 0.05% Tween 20 [pH 7.4]) for 30 min at 37°C. Serum samples were diluted 1:100 in PBS-Tween 20, 100 μ l was added to coated wells, and the plates were incubated for 2 h at 37°C. The wells were then washed three times with plate wash solution (154 mM NaCl, 0.05% Tween 20, 0.1% thimerosal), and a 1:7,000 dilution of alkaline phosphatase-conjugated affinity-purified goat anti-mouse immunoglobulin G (IgG) or alkaline phosphatase-conjugated affinity-purified anti-rat IgG and IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) in PBS-Tween 20 was added as the secondary antibody. After a 2-h incubation at 37°C, the plates were washed five times and 100 μ l of *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) dissolved at 1 μ g/ml in substrate solution (2.1 mM MgCl₂, 28 mM Na₂CO₃, 23 mM NaHCO₃ [pH 9.8]) was added to each well. Following incubation at room temperature for 1 h, the reaction was terminated by the addition of 100 μ l of 3 M NaOH to each well, and the A_{405} of the reaction solution was measured. All assays were performed in duplicate. The average of the absorbance values from the wild-type AcMNPV-coated wells was subtracted from the average of the absorbance values of the rNS1-containing wells to yield the final absorbance value. Cut-off values were established by evaluating 50 negative serum samples. Sera with ELISA absorbance values greater than the mean plus 2 standard deviations of the absorbance of known negative sera were considered positive.

The MVM ELISA and MVM hemagglutination inhibition (HAI) assay were performed by using modifications of previ-

ously reported methods (23). The MOPV HAI assay was analogous to the previously described MVM HAI assay except that MOPV1-infected CTLL-2 lymphocytes (ATCC TIB 214) were used as the antigen and mouse erythrocytes were used instead of guinea pig erythrocytes.

Comparison of the deduced amino acid sequences of viral proteins among orphan and prototypic parvoviruses (MVM, KRV, and H-1) revealed greater than 91% homology among the NS1 proteins, whereas protein homologies for the other viral proteins (NS2, VP1, and VP2) were lower. On the basis of this finding, the NS1 protein was selected as a potential diagnostic antigen for the detection of parvovirus antibodies in rodents. To produce sufficient quantities of NS1 for diagnostic testing, the NS1 gene was cloned into a baculovirus expression system. Twelve putative recombinant virus clones were selected, plaque purified, and evaluated by PCR analysis to assess whether clones possessed the NS1 insert. Of the 12 clones analyzed, 4 yielded the expected 334-bp PCR product indicating that NS1 sequences were inserted within the baculovirus vector (data not shown).

To confirm that recombinant clones expressed the NS1 protein, lysates were prepared from the four PCR-positive clones and were subjected to Western blot analysis. Blots were probed with rabbit polyclonal anti-NS1 produced against *Escherichia coli*-expressed recombinant NS1 (8). Lysates from each of the evaluated clones exhibited an immunoreactive band with a molecular mass of 83 kDa, which is consistent with the size of native NS1 protein. No immunoreactive peptides were detected in controls with lysates from uninfected insect cells and insect cells infected with the wild-type baculovirus. One of the four clones was selected for subsequent experiments.

Expression of rNS1 was optimized by varying the insect cell line, the multiplicity of infection (MOI), and the time of incubation after the inoculation of insect cells with the recombinant baculovirus. Several insect cell lines have been used for the production of proteins by baculovirus expression systems: Sf-9 and Sf-21 of *S. frugiperda* and the High Five cell line from *Trichoplusia ni* (Invitrogen Corporation). To determine which cell line produced the maximum amount of rNS1, each insect cell line was seeded into 12-well culture plates, infected with recombinant (His)₆-NS1-pVL941 virus at an MOI of 10, and incubated for 72 h. Blots revealed a more intense staining pattern with lysates from infected High Five cells than was observed with either Sf-9 or Sf-21 cells (Fig. 2). The immunoreactive band migrating at 83 kDa was consistent with the size of intact NS1 protein; lower-molecular-mass bands likely represented degradation products of rNS1. The addition of protease inhibitors to the extraction buffer was required to yield intact 83-kDa product, but even high levels of multiple inhibitors did not prevent the degradation of a portion of the recombinant protein to lower-molecular-mass products.

To further optimize the production of rNS1, various infection doses and insect cell seeding concentrations were evaluated by Western blot analysis for rNS1 synthesis in High Five cells infected with the recombinant (His)₆-NS1-pVL941 virus. Four wells were seeded with High Five cells at each of three seeding concentrations (2.5×10^5 , 5×10^5 , and 1×10^6). At each seeding concentration, individual wells were infected at MOIs of 5, 10, 20, and 50. At 72 h postinfection, cell lysates were prepared and examined for rNS1 expression as described above. Evaluation of the results indicated that maximum production of rNS1 was achieved at an insect cell seeding concentration of 5×10^5 cells with an MOI of 5 or 10 (Fig. 3). No antibody-reactive bands were visualized in lanes with protein lysates from uninfected insect cells or cells infected with wild-type AcMNPV.

FIG. 2. Western blot analysis of lysates from three insect cell lines probed with anti-NS1 antibody. The arrow depicts the molecular mass of the intact, 83-kDa NS1 protein.

To determine the incubation time postinfection that yielded the largest amount of rNS1, insect cells were infected with the recombinant baculovirus by using conditions determined to be optimal in the previous experiments. Individual wells were harvested at 18, 24, 36, 48, and 72 h postinfection, and rNS1 expression was assessed by Western blot analysis. The results indicated that the 72-h time point yielded the largest amount of rNS1 protein (Fig. 4). Immunoreactive peptides of less than 83 kDa in size were present at all time points evaluated.

An ELISA was developed by using the rNS1 as the antigen to detect parvovirus infections in rodents. To compare the developed rNS1 ELISA with the conventional MVM ELISA, 227 mouse serum specimens were obtained from the University of Missouri Research Animal Diagnostic and Investigational Laboratory (UM RADIL; Columbia, Mo.). The serum samples tested represented a diverse population of rodents from numerous colonies in the United States and from international sources. Each serum sample was tested on the rNS1 and MVM ELISAs. Mouse sera that were positive by the MVM ELISA or the rNS1 ELISA were retested by MVM and MOPV HAI assays to confirm the presence of antibody and distinguish the specific parvovirus involved. The rNS1 ELISA detected a larger number of HAI assay-confirmed MOPV- and MVM-positive serum samples than the MVM ELISA (Table 1). The rNS1 ELISA provided a 93.1% sensitivity in detecting MOPV-positive serum samples, whereas the MVM ELISA had only a 26.4% sensitivity. For MVM antibody detection, the rNS1 ELISA provided a sensitivity of 95.8%, whereas the MVM ELISA yielded a sensitivity of 81.9%. The specificities of the rNS1 and MVM ELISAs were 96.0 and 95.6%, respectively. Recognizing that the preponderance of antigen in conventional MVM ELISAs is capsid protein and that the deduced capsid sequences of MOPV and MVM have limited homologies, the limited sensitivity of the MVM ELISA in detecting antibodies to MOPV was not surprising. However, it was surprising that the rNS1 ELISA showed greater sensitivity than the conventional MVM ELISA in detecting antibodies to MVM. This may be attributed to the high level of expression of

FIG. 3. Western blot analysis showing expression of rNS1 under various conditions. High Five insect cells were seeded at three different concentrations (2.5×10^5 , 5×10^5 , and 1×10^6 per well), incubated overnight, and infected at an MOI of 5, 10, 20, or 50 on the following day. Cell lysates were harvested at 72 h postinfection for Western blot analysis. Blots were probed with anti-NS1 antibody.

recombinant protein in the baculovirus system. Thus, the amount of viral protein that adhered to the wells in the rNS1 ELISAs may exceed the amount of viral protein that typically adhered to the wells in the conventional MVM ELISA, which uses semipurified MVM-infected mammalian cell preparations to coat the wells of the ELISA plates. ELISAs with unpurified rNS1 yielded high signal-to-noise ratios, indicating that purification of the baculovirus-expressed protein was not necessary. Therefore, although the six-residue histidine tag was present in

the recombinant virus, it was not used for the preparation of rNS1 ELISA antigen in the present study.

The rNS1 assay was also used to evaluate rat serum samples for parvovirus antibodies. Rat sera were obtained from UM RADIL, and each serum sample was evaluated by KRV, H-1, and rNS1 ELISAs. KRV and H-1 ELISAs were performed essentially as described previously (21, 23). Sera that were positive were retested by KRV- and H-1-specific HAI assays to confirm antibody reactivity and determine whether KRV or H-1 parvoviruses induced the antibody response. Evaluation of 167 rat serum samples for KRV antibodies revealed 57 positive samples by the rNS1 ELISA but only 46 positive samples by the conventional KRV ELISA (Table 2). The rNS1 ELISA provided a sensitivity of 98.3%, whereas the conventional KRV ELISA exhibited a sensitivity of only 79.3%. Since H-1 infections occur infrequently in rodent populations, fewer antibody-positive serum samples were evaluated for H-1 antibodies. Calculation of the sensitivities of the H-1 and rNS1 ELISAs in detecting H-1 infection indicated that both assays provided 100% sensitivity. However, the rNS1 ELISA yielded much higher absorbance values than the conventional H-1 ELISA. For example, absorbance values for the rNS1 ELISA ranged from 0.30 to 2.5, whereas the identical sera tested in the conventional H-1 ELISA had absorbance values ranging from 0.1 to 0.4, suggesting that the sensitivity of the rNS1 ELISA may

FIG. 4. Western blot analysis depicting expression of rNS1 in High Five cells at various times postinfection. High Five cells were seeded and infected by using the seeding concentration and MOI determined to be optimal in the previous experiment. Cells were harvested at 18, 24, 36, 48, and 72 h postinfection and were evaluated by Western blot analysis with anti-NS1 antibody.

TABLE 1. Comparison of MVM and rNS1 ELISAs for detection of MVM and MOPV in mouse sera

Immune status ^a	No. of serum samples tested	No. of serum samples			
		MVM ELISA		rNS1 ELISA	
		Positive	Negative	Positive	Negative
MVM	72	59	13	69	3
MOPV	87	23	64	81	6
Negative	68	3	65	1	67

^a Immune status was confirmed by HAI assays for MVM and MOPV.

TABLE 2. Comparison of KRV and H-1 ELISAs with rNS1 ELISA for detection of KRV and H-1 antibodies in rat sera

Immune status ^a	No. of serum samples tested	No. of serum samples			
		KRV and H-1 ELISAs ^b		rNS1 ELISA	
		Positive	Negative	Positive	Negative
KRV	58	46	12	57	1
H-1	13	13	0	13	0
Negative	96	0	96	0	96

^a Immune status was confirmed by HAI assays for KRV and H-1.

^b Sera were tested by KRV and H-1 ELISAs.

be higher than that of the H-1 ELISA. The specificities of both the conventional (KRV and H-1 ELISAs) and the rNS1 ELISAs were 100%.

Speculation about the existence of MOPV in mice was initially based on the finding of a low percentage of serum samples that were positive by the MVM ELISA but negative by MVM HAI assay testing, suggesting the presence of a virus that was antigenically similar to but distinct from MVM. Virus isolation and DNA sequencing subsequently confirmed the existence of MOPVs (1, 3, 17). A similar phenomenon has been recognized in rat colonies; i.e., serum samples from some colonies yield a low percentage of KRV ELISA-positive results but uniformly negative results in KRV and H-1 HAI assays. On the basis of these findings, it is thought that an as yet unrecognized rat orphan parvovirus (ROPV) exists, although in vitro cultivation of a ROPV isolate has not been successful. To determine whether the rNS1 ELISA can be used to detect antibodies to ROPVs, rat sera were obtained via UM RADIL from adult rats in colonies that were suspected of being infected with ROPV on the basis of previous serologic testing. Of the 55 serum samples tested, 54 were positive by the rNS1 ELISA, while only 29 of the 55 serum samples were positive by the KRV ELISA. In other studies in our laboratory, tissues from rNS1 ELISA-positive rats have been amplified with parvovirus-specific primers. Comparison of an amplified 307-bp sequence within the NS-1 gene with the analogous region in the MOPV genome revealed a 97% homology (1, 3), indicating the presence of a closely related but distinct virus. Taken together, these results provide additional evidence of the existence of ROPV and support the premise that the rNS1 ELISA can be used to identify ROPV-infected rats.

The results obtained in the present study indicate that an ELISA based on NS1 produced by a recombinant baculovirus in insect cells can be used as a sensitive and specific method for the identification of rodents infected with prototypic and recently recognized orphan parvovirus species.

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