Expression and characterization of bovine rotavirus proteins in the baculovirus system

Young Soo Lyoo
Iowa State University

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Expression and characterization of bovine rotavirus proteins
in the baculovirus system

by

Young Soo Lyoo

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirement for the Degree of
DOCTOR OF PHILOSOPHY

Department: Microbiology. Immunology and Preventive Medicine
Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Major

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1995
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<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> Matrix Nuclear Polyhedrosis Virus</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>[a^{32P}]dCTP</td>
<td>alpha phosphorous-32 deoxycytosine triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BEI</td>
<td>2-Bromoethylamine</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BRV</td>
<td>bovine rotavirus</td>
</tr>
<tr>
<td>BTI-TN-5B1-4</td>
<td>insect cell line derived from <em>Trichoplusia ni</em>, egg homogenate</td>
</tr>
<tr>
<td>CDCD</td>
<td>colostrum deprived cesarean derived</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMI</td>
<td>cell mediated immunity</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DPI</td>
<td>days post inoculation</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FFU</td>
<td>fluorescent focus units</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>Hi-Five</td>
<td><em>Trichoplusia ni-</em> derived BTI-TN-5B1-4 cells</td>
</tr>
<tr>
<td>IFA</td>
<td>indirect immunofluorescence assay</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl b-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>MA-104</td>
<td>rhesus monkey kidney cells</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>Mr</td>
<td>molecular mass</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>μCi</td>
<td>micro Curies</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mU</td>
<td>milliunits</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NSP</td>
<td>non-structural protein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide(s)</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>p.i.</td>
<td>post infection</td>
</tr>
<tr>
<td>PTA</td>
<td>phosphotungstic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minutes</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugipedia ovary cell line</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded ribonucleic acid</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride-sodium citrate buffer</td>
</tr>
<tr>
<td>TAE</td>
<td>tris[hydroxymethyl aminomethane]-acetate-ethylenediamine tetraacetic acid buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TCID</td>
<td>tissue culture infectious dose</td>
</tr>
<tr>
<td>TE</td>
<td>tris[hydroxymethyl aminomethane]ethylenediamine tetraacetic acid buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris[hydroxymethyl aminomethane]</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris[hydroxymethyl aminomethane]-hydrochloric acid</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>VN</td>
<td>virus neutralization</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle(s)</td>
</tr>
<tr>
<td>VMRI</td>
<td>strain of bovine rotavirus</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
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GENERAL INTRODUCTION

Dissertation Organization

This dissertation is composed of two separate manuscripts. Each manuscript is complete in itself. The Ph.D. candidate, Young S. Lyoo, is the senior author and principal investigator for both manuscripts. The first manuscript describes expression of recombinant proteins VP2, VP4, VP6 and VP7 of bovine rotavirus, in a baculovirus system and preliminary characterization of the recombinant proteins. Rotavirus specific recombinant proteins were detected by immunoassays using polyclonal antiserum to the VMRI strain of bovine rotavirus. Kinetics of protein expression and characteristics of recombinant proteins were determined by radioimmunoprecipitation. Antigenicity of recombinant proteins was determined by inoculation of guinea pigs and the immune response of guinea pigs was evaluated by indirect immunofluorescence and virus neutralization tests.

The second manuscript reports on the effect of removal of terminal noncoding sequences on expression of VP7 in a baculovirus expression system. Removal of both the 3' end and 5' end noncoding sequences upstream and downstream of the open reading frame increased VP7 expression.

In addition, a General Introduction and a current review of the literature related to this research work is included before the manuscripts, as well as a General Conclusion section, Literature Cited section listing references cited in the General Introduction and literature Review, and Acknowledgment section, after the manuscripts.
Introduction

Rotaviruses are a common cause of gastroenteritis in neonates of animals and man (Flewett and Woode, 1978; Estes et al., 1983; Paul and Stevenson, 1992; Paul and Lyoo, 1993). They were first isolated in the U.S. from calves with diarrhea by Mebus et al., (1969), and now have been identified in most animal species of veterinary importance including cattle, swine, sheep, horses, dogs, cats, chickens and turkeys (Flewett and Woode, 1978; Estes et al., 1983; Estes and Cohen, 1989; Paul and Stevenson, 1992. Paul and Lyoo, 1993). Rotavirus infection in humans was first described in 1973 by electron microscopy of samples isolated from the duodenum, detecting the infectious agent of acute gastro-intestinal infection of hospitalized young children (Bishop et al., 1973). Infections with rotavirus are common in most of these species and often may be subclinical. Virus strain, dosage, environmental factors, and involvement of other secondary pathogens influence the severity of disease and the outcome of infection. Rotaviruses infect the small intestine via fecal-oral route, and the rapidity of spread is aided by inhalation after aerosol formation (Sattar and Ijaz, 1987). Rotaviruses survive well in the environment and thus provide an ample opportunity of exposure to susceptible host animals. Rotavirus survival on contaminated human hands, environmental surfaces (Ansari et al., 1988), tap water (Sattar et al., 1984a, b), sewage (Hejkal et al., 1984), and virus resistance to chemical disinfectants (Sattar et al., 1983) are well understood.

Protection of neonates against rotaviruses is complicated by extensive antigenic variability among rotaviruses, and resistance to environmental factors. Seven antigenically distinct groups (A-G) have been identified which further contain serotypes. Within the group A rotaviruses, which were the first to be identified and are the best characterized, are large number of serotypes based on the two surface proteins VP4 and
VP7. The VP4 and VP7 of group A rotaviruses are important in serotype determination and in inducing neutralizing antibodies and protective immunity (Dyall-Smith et al., 1986; Hoshino et al., 1985, 1988; Hardy et al., 1992). Multiple serotypes of group A rotaviruses based on glycoprotein VP7 (designated as G types) and based on VP4 (P types) have been identified. There are at least 14 G types and 12 P types identified so far and this trend will possibly continue in the future. Groups B and C rotaviruses, which have recently been characterized, have been shown to possess VP4 and VP7 equivalents (Chen et al., 1990; Bremont et al., 1992; Qian et al., 1991). The induction of immunity is further complicated by the fact that circulating antibodies may not provide solid protection against rotavirus infection. Antibodies, preferably IgA, need to bathe the villus epithelium of the gastrointestinal tract for them to be protective against rotavirus infection. Therefore, the most successful approaches in inducing protective immunity have utilized the stimulation of lactogenic immunity in dams so that the antibodies in milk protect the intestinal tract of the suckling animals from rotaviral infection. Active immunity, especially mucosal immunity, protects older animals from re-exposure to rotavirus infection of a homologous serotype. Vaccines are commercially available for rotavirus infections in cattle and pigs. In addition, genetic reassortant viruses have been tested as potential vaccine candidates. Current development of recombinant protein expression technology allowed us to characterize the recombinant rotavirus proteins expressed in baculovirus.

Our hypothesis was that recombinant proteins expressed in baculovirus would be authentic viral proteins as in rotavirus and they would induce neutralization antibodies to rotavirus. Our belief in part was based on studies showing that the recombinant proteins expressed in baculovirus are similar to native proteins because the insect cell system mimics similar machinery of protein processing to that of mammalian cells. Because one
of the major neutralization proteins, VP7, is a glycosylated protein. The choice of this eukaryotic expression system has an advantage over the prokaryotic expression system in post translational modification of recombinant protein. We also expected the recombinant proteins of VP4 and VP7 would retain their ability to induce neutralizing antibodies in experimental animals. Expression of VP2 and VP6 provided an opportunity to investigate the assembly of virus-like particles.
LITERATURE REVIEW

The History of Rotavirus

It has been 25 years since rotavirus was recognized as a causative agent of gastroenteritis in calves and now rotavirus has been isolated from a variety of species of animals and in humans. Extensive information is available on pathogenesis, modes of virus transmission, diagnosis of rotaviral infection, and molecular genetics of rotavirus. However, rotavirus infection still remains an important health problem in animals and in humans. Vaccines are available in a few species, however their effectiveness is limited. Mechanisms of mucosal immunity to rotavirus and other enteric pathogens needs better clarification. Even though rotavirus immunogens have been identified (Paul and Lyoo, 1993) their role in protection against calf diarrhea is poorly understood. Serotypic diversity further complicates prevention and control of rotavirus associated gastroenteritis.

Rotavirus was first isolated from calves with diarrhea by Mebus et al. (1969), and subsequently this virus was identified in most animal species (Table 1) of veterinary importance including cattle, pigs, sheep, horses, dogs, cats, chickens and turkeys (Flewett and Woode, 1978; Estes et al., 1983; Snodgrass et al., 1984; Estes and Cohen, 1989; Paul and Stevenson, 1992). Rotavirus infection in human was first described in 1973 by electron microscopy of intestinal contents of hospitalized young children with acute gastro-intestinal infection (Bishop et al., 1973).

Rotaviruses

Rotaviruses belong to the genus rotavirus under the family Reoviridae. The name is derived from the Latin word rota (wheel) which illustrates the appearance of virus particles by electron microscopy. Rotavirus particles are nonenveloped, are
Table 1. Known serogroups and RNA electrophoretic patterns of rotaviruses affecting animals and human.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Electrophoretic Pattern of RNA segments</th>
<th>Animal Species Affected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4:2:3:2</td>
<td>Cattle (NCDV), Pigs, Sheep, Foals, Dogs, Cats; Rabbits; Man</td>
<td>Mebus et al., 1969; See review by Flewett and Woode, 1978; Estes et al., 1983; Paul &amp; Stevenson, 1992</td>
</tr>
<tr>
<td></td>
<td>5:1:3:2</td>
<td>Avian</td>
<td>Todd et al., 1980</td>
</tr>
<tr>
<td>B</td>
<td>4:2:2:3</td>
<td>Pigs</td>
<td>Pedley et al., 1983; Theil et al., 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cattle; Man</td>
<td>Snodgrass et al., 1984; Chen et al., 1985</td>
</tr>
<tr>
<td>C</td>
<td>4:3:2:2</td>
<td>Pigs</td>
<td>Saif et al., 1980; Bohl et al., 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cattle; Man</td>
<td>Tsunenitsu et al., 1991; Rodger et al., 1982</td>
</tr>
<tr>
<td>D</td>
<td>5:2:2:2</td>
<td>Chicken</td>
<td>McNulty et al., 1981; McNulty et al., 1984; Bridger, 1987; Snodgrass et al., 1984</td>
</tr>
<tr>
<td>E</td>
<td>4:2:2:3</td>
<td>Pigs</td>
<td>Pedley et al., 1986</td>
</tr>
<tr>
<td>F</td>
<td>4:3:2:2</td>
<td>Chickens and Turkeys</td>
<td>McNulty et al., 1984; Bridger, 1987</td>
</tr>
<tr>
<td>G</td>
<td>4:2:2:3</td>
<td>Chickens</td>
<td>McNulty et al., 1984; Bridger, 1987</td>
</tr>
</tbody>
</table>

* Adapted from Paul and Lyoo (1994)
triple-layered and measure approximately 75 nm in diameter by electron microscopic examination. Recent evidence based on high resolution cryoelectron microscopy (Prasad et al., 1988, 1990; Anthony et al., 1991) suggest that rotaviral particles have 60 surface spikes about 10 nm long with a globular end structure. Rotaviral particles with surface spikes measure approximately 100 nm in diameter. The single capsid viral particles measure 60 nm and the core particles are 52 nm in diameter. Viral cores contain the viral transcriptase and a genome consisting of 11 segments of double stranded RNA. Each RNA segment starts with a 5' guanidine followed by highly conserved sequences that are part of the 5' non-translational sequences, an open reading frame coding for each protein, another set of 3' non-coding sequences and finally a 3'-terminal cytosine (Estes and Cohen, 1989).

Only triple-layered particles are infectious in susceptible animals and cell cultures. The virus enters into susceptible cells by direct cell membrane penetration, not by endocytosis (Kaljot et al., 1988). But there is controversy regarding the proteins involved in target cell binding and cell entry. The VP4, VP7 and NS35 are believed to possess target cell binding proteins (Sabara et al., 1985; Bass et al., 1990, 1991).

Only the triple-layered (previously called double-shelled) viral particles are infectious (Zeng et al., 1994). Rotaviruses are difficult to adapt for growth in cell cultures with conventional virus isolation methods in the presence of serum as a supplement. They require pretreatment with proteolytic enzymes such as trypsin and pancreatin (Theil and Bohl, 1980; Terrett and Saif, 1987). Cell lines commonly used for rotavirus propagation include the monkey kidney cell line MA-104 and CV-1, the human liver cell line HepG2, and the human colon cell line CaCo-2 (Kitamoto et al., 1991).

Rotaviruses are classified into antigenically distinct serogroups by immunofluorescence, immune electron microscopy, or enzyme linked immunosorbent
assay (ELISA). Seven serogroups have been established. Rotaviruses within a serogroup, regardless of species of origin, share a common group antigen. The electrophoretic patterns of rotaviruses belonging to separate groups are also distinctive and can tentatively be used to classify rotaviruses. The genomic RNA segments cluster into four regions I to IV. Mammalian group A rotaviruses have a 4:2:3:2 pattern in regions I, II, III, and IV. Avian group A rotavirus has a 5:1:3:2 pattern. This pattern is 4:2:2:3 in group B and E mammalian rotaviruses, 4:3:2:2 in group C rotavirus and 5:2:2:2 in avian group D rotaviruses (McNulty et al., 1981). Species from which these rotaviruses have thus far been isolated is shown in Table 1. Rotaviruses within serogroup A are further divided into subgroups and serotypes. Two subgroups, I and II, are well established. There is evidence that there may be additional serogroups of rotaviruses which lack these subgroup antigens. Serotyping of rotaviruses is cumbersome and sometimes confusing as the two surface proteins, VP4 and VP7, both contribute to virus neutralization and serotype specificity. The VP4 in older literature was referred to as VP3 until a new name was proposed by Liu et al. (1988). The genes coding for these two proteins segregate independently and classical virus neutralization tests using hyperimmune antisera to rotavirus primarily determine VP7 types. A new classification has been proposed (Estes and Cohen, 1989) which takes into account both surface proteins. Rotavirus types based on VP4 have been designated as P types, whereas types based on VP7 have been referred to as G types. Monoclonal antibodies and nucleic acid probes have been successfully used to differentiate rotavirus G and P types (Johnson et al., 1990; Zaberezhny et al., 1991). A summary of rotaviruses with the major G and P types detected in animals is presented in Table 3.

Limited data are available as to the predominant species of rotaviruses prevalent in diarrheic neonates of most species. In cattle, rotaviruses with G6 are most common and
two additional serotypes designated serotypes 8 and 10 have been reported (Taniguchi et al., 1991b; Lu et al., 1994). Snodgrass et al. (1990) using MAb-based serotype-specific ELISA found that 66% of 162 samples were of G6 serotype, 7% were G10 and the others were untypable. Using Northern blot hybridization or dot blot hybridization, rotaviruses with P5 and G6 were the most common in diarrheic calves (Brooks et al., 1989; Mummidi et al., 1992). One report indicates that serotype 1 (T449) is also present in bovine (Blackhall et al., 1992) but significance of this serotype in gastroenteritis and its relative prevalence in cattle is not known. Recent data indicates that rotaviruses with additional G and P types may be present in calves (Mummidi et al., 1992). In horses, the G3 serotype is most predominant (Browning et al., 1992; Hardy et al., 1991). Out of 70 equine rotavirus isolates tested 63% were serotype G3, 4% were G13, and 33% were untypable (Browning et al., 1992). Hardy et al. (1991) found all of the isolates they analyzed were of G3 serotype. In pigs, rotaviruses with G4 and G5 predominate (Zaberezhny et al., 1991; Paul et al., 1992a).

Group A rotaviruses are the best characterized group of rotaviruses. There are at least 11 proteins coded in rotavirions (Estes and Cohen, 1989), six of which are major structural proteins which are VP1, VP2, VP3, VP4, VP6, and VP7 (Table 2). Among these proteins, VP6 is the most abundant protein making up 51% of the virion, forms the inner capsid and is required for viral transcription. All viruses within a serogroup share this antigen regardless of species of origin. The major group and subgroup antigens are encoded in the VP6 protein. VP1 is a viral polymerase and resides in the subcore of the virion. This protein is slightly basic and has a GDD motif which is important for RNA dependent RNA polymerase activity (Mattion et al., 1994). VP2 has a nucleic acid binding activity and is present in the core with a portion of it sticking through the inner capsid. VP2 and VP6 contribute to subgroup specificity. VP3 is encoded by gene
segment 3 and is present in core particles as a subcore protein with approximately 0.5% of virion weight. The protein is basic and possesses guanyltransferase activity. VP4 is a spike protein which possesses neutralization and fusion activities (Mattion et al., 1994). VP7 is heavily glycosylated and possesses neutralization activity (Table 3). More detailed information on the characteristics of VP2, VP4, VP6, and VP7 follow in order to provide better understanding of this dissertation.

VP2

The VP2 is encoded by gene segment 2 (2690 bp) of rotavirus. It is the most abundant protein found in rotavirus core particles, and is the third abundant viral protein in triple-layered particles. The core of the virion is formed predominantly by this VP2, which forms the innermost layer and surrounds the viral genome, VP1 and VP3. Rotavirus particles contain 200 molecules of VP2 (Mattion et al., 1994) which are partially exposed on single shelled particles. This protein is highly immunogenic and serum antibodies to VP2 are an evidence of prior infection. The VP2 protein is rich in proline (>3.6%) and most of the proline residues are conserved among various serotypes of rotaviruses. There are two perfect heptad repeats of leucine present in the RF strain of bovine rotavirus (aa 536-557 and aa 665-686) and simian rotavirus strain SA11 (aa 538-560, and aa 667-689) which are suggestive of leucine zipper (Labbe et al., 1994; Mattion et al., 1994). It was hypothesized that these two motifs are important in protein-nucleic acid interaction. The VP2 is a structural protein which has been shown to possess nucleic acid binding activity with a preference for ssRNA over dsRNA. Its binding activity is not sequence dependent.
Table 2. Genome size and selected characteristics of group A rotavirus*.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Number of base pairs</th>
<th>Length of noncoding sequence**</th>
<th>Protein products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3302</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>2690</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>2591</td>
<td>49</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>2362</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>1611</td>
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<tr>
<td>9</td>
<td>1062</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>751</td>
<td>41</td>
<td>182</td>
</tr>
<tr>
<td>11</td>
<td>667</td>
<td>20</td>
<td>49</td>
</tr>
</tbody>
</table>

* Adapted from Mattion et al., (1994) for group A simian rotavirus SA11
** Number of 5' noncoding sequences is up to the first AUG start codon; number of 3' noncoding sequences does not include the termination codon.
*** Nomenclature for non-structural proteins (NSP) is adapted from recent papers (Mattion et al., 1994).
Table 3. Structural proteins of group A rotavirus and their characteristics*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nascent polypeptide molecular mass (number amino acids)</th>
<th>Mature protein modified</th>
<th>No. of molecules per virion (% w/v)**</th>
<th>Location and Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>125 kDa (1088)</td>
<td></td>
<td>(2)</td>
<td>Subcore protein; GDD motif, polymerase activity</td>
</tr>
<tr>
<td>VP2</td>
<td>102.4 kDa (880)</td>
<td>Myristoylated</td>
<td>200 (15)</td>
<td>Core protein; RNA binding; Leucine zipper</td>
</tr>
<tr>
<td>VP3</td>
<td>98.1 kDa (835)</td>
<td></td>
<td>(0.5)</td>
<td>Subcore protein; guanylytransferase</td>
</tr>
<tr>
<td>VP4</td>
<td>86.7 kDa (776)</td>
<td>Cleaved</td>
<td>120 (1.5)</td>
<td>Spike protein; dimer; HA; P-type antigen; attachment protein; virulence</td>
</tr>
<tr>
<td>VP6</td>
<td>44.8 kDa (397)</td>
<td>Myristoylated</td>
<td>780 (51)</td>
<td>Subgroup antigen trimer; inner capsid protein</td>
</tr>
<tr>
<td>VP7</td>
<td>37.3 kDa (326)</td>
<td>Glycosylation</td>
<td>780 (30)</td>
<td>Neutralization antigen; surface glycprotein; G-type antigen; Ca++ binding</td>
</tr>
</tbody>
</table>

* Adapted from Mattion et al. (1994) based on data generated for simian group A rotavirus strain SA11

** % by weight of virion protein
The VP4 is a minor surface protein comprising 1.5% of the virion. Based on cryoelectron microscopy, VP4 is present in a dimeric form making up 60 surface spikes (Prasad et al., 1988). It is nonglycosylated and is a hemagglutinin in many virus strains. It has many important biological functions, e.g. virulence, restriction of virus growth in cell cultures, virus neutralization, and hemagglutination (Estes and Cohen, 1989). The VP4 is very important in homotypic protection as well as heterotypic immunity (Paul and Lyoo, 1993). The VP4 is coded in gene segment 4 (2362 bp) has 776 amino acid residues and is sensitive to cleavage by proteolytic enzymes. Trypsin cleaves VP4 at arginine residue 241 or 247 resulting in two fragments: VP5 and VP8. The VP8 fragment is 247 amino acids in length and represents approximately the amino-terminal one-third portion of VP4. Five type-specific sites are located in VP8, with a majority of these located in the hypervariable region between amino acids 71 and 204 (Larralde and Gorziglia, 1992). The VP5 fragment is 529 amino acids long and has 3 major neutralization epitopes. Epitope I is a linear epitope located at amino acid residue 306. Epitope II is a conformational epitope located at amino acid residues 392 and 439 and epitope III is located at amino acid residue 434 (Taniguchi et al., 1989). A cross reactive epitope has been localized at amino acid residue 393. Also VP4 is the primary immunogen that stimulates neutralization antibodies following natural infection with rotavirus (Ward et al., 1988). Immunization of mice with recombinant VP4 produced in a baculovirus expression system induced a high level of neutralizing antibodies to the homologous serotype as well as a low level of a cross reactive neutralization response to heterologous serotypes (Mackow et al., 1990). Heterotypic neutralization epitopes have been located on VP5, the carboxy terminal protein of VP4 after cleavage with a proteolytic enzyme such as trypsin. Neutralization epitopes of VP4 have been mapped
using synthetic peptides (Arias et al., 1989). Amino acid residues 232-255 emulsified with keyhole limpet hemocyanin as an immunological carrier produced neutralizing antibodies to VP4. This synthetic peptide also provided protection equal to that obtained with whole bovine rotavirus immunization (Arias et al., 1989).

**VP6**

The VP6 is encoded by genome segment 6 (1362 bp) and is the major viral structural protein in the virus particles comprising 51% of virion. The protein is located on the double layered particles with trimeric structure. The intermediate layer of rotavirus particles are formed by this protein. High resolution electron cryomicroscopy has shown that VP6 is composed of 260 trimeric columns and is stabilized by noncovalent bonds (Prasad et al., 1988, Yeager et al., 1990). A proportion of the VP6 protein in the virion is about 60% in triple-layered particles with a high percentage of predicted β structures and a low percentage of α helix. VP6 is myristoylated, despite the absence of amino-terminal sequences which are associated with N-myristoylation and is rich in proline (5.2%) (Clark and Desselberger, 1988). VP6 is the primary antigen used in most of the diagnostic tests. Serologically, subgroup antigens have been found in VP6 which have served as epidemiological tools as subgroup I has primarily been associated with human rotavirus strains and subgroup II with animal rotavirus strains. The role of VP6 in protection from rotavirus associated gastroenteritis is not clear however, partial protection in offspring of mice immunized with recombinant VP6 has been reported (Redmond et al., 1993).
VP7

VP7 is the major neutralization glycoprotein which plays an important role in serotype specific virus neutralization (Greenberg et al., 1983a). The outer capsid of the virion is made up of 780 molecules of VP7 (Prasad et al., 1988). The VP7 is coded in gene segment 7, 8 or 9 depending upon the virus strain. VP7 gene is 1062 bases long and the number of amino acid residues coded by the open reading frame is 326. After cleavage of the signal peptide, the mature cleaved protein contains 276 amino acids. The VP7 is glycosylated with high concentration of mannose, is the major surface protein and comprises 30% of the virion. It has a molecular mass of 37 kDa. The VP7 is the major neutralization antigen of the rotavirus and it is believed to function as a cell attachment protein (Estes and Cohen, 1989; Mattion et al, 1994). Nucleotide sequence analysis has identified six regions on VP7 which are highly conserved among different strains of the same serotype but are divergent among different serotypes (Glass et al., 1985; Green et al., 1987). Three of these regions encode for neutralization epitopes A, B and C as shown by studies using escape mutants. Region A is located at amino acid residues 87-101, region B at amino acid residues 145-152, and region C is located at amino acid residues 211-223 (Dyall-Smith et al., 1986). Regions A and C are located in close proximity after protein folding and region C appears to be the most immunodominant antigenic site on VP7. The recombinant VP7 protein and synthetic peptide (275-295) of VP7 has been shown to induce neutralizing antibodies as well as protection in animals against rotavirus infection (Arias et al, 1986; Francavilla et al, 1987; Bishop, 1993; Dormitzer et al, 1994).

Viral proteins involved in virus neutralization of group A rotaviruses are protease sensitive protein VP4 and glycoprotein VP7. These two proteins are also important for virus classification and serotyping of group A rotaviruses. VP4 and VP7 equivalents
have also been identified in groups B and C rotaviruses by nucleotide sequence analysis (Chen et al., 1990; Bremont et al., 1992; Qian et al., 1991). Surface proteins VP4 and VP7 are the major immunogens of rotavirus (Paul and Lyoo, 1993). Both VP4 and VP7 have been shown to elicit neutralization antibodies to rotavirus. Studies on reassortant viruses containing either the VP4 or VP7 genes of the parent virus and other genes from an unrelated serotype demonstrated that the neutralization specificities of these genes segregate independently (Hoshino et al., 1985; Offit et al. 1986). Hyperimmune monospecific antisera to VP4 and VP7 neutralized rotavirus (Bastardo et al., 1981). Additionally, monoclonal antibodies to these proteins have been shown to neutralize rotavirus and the neutralizing monoclonal antibodies to VP4 and VP7 have been shown to be protective against rotavirus infections (Greenberg et al., 1983a, 1983b; Sabara et al., 1985; Matsui et al., 1989).

Immunity to Rotavirus Infections

Animals develop an active immunity to rotavirus infection following natural or experimental exposure. Immunity is usually serotype specific (Murakami et al., 1986; Snodgrass et al., 1991), however, a low level of heterotypic immunity may also be observed (Woode et al., 1987). Humoral, mucosal and cellular immunity have been demonstrated in natural host and experimental animal models following infection (Hung et al., 1985; Dharakul et al., 1991). Mucosal immunity is of prime importance in protection against rotavirus infections as protection is usually correlated with the presence of antibodies in secretions (Woode et al., 1983). Neutralizing antibodies in the intestinal tract have been correlated with protection from infection with rotavirus. Secretory IgA is more effective in protection than IgG and IgM because IgA is more resistant to proteolytic degradation in the intestinal tract due to a carbohydrate-rich secretory
component and is the predominant immunoglobulin in secretions, including milk, in most animal species (Grimwood et al., 1988). Mucosal immunity is generally best induced by oral immunization and circulating antibodies are usually not protective (Offit and Clark, 1985).

Immunity in neonates may also be passively acquired through colostrum and/or milk. This has been referred to as lactogenic immunity and is the only viable approach in protection of animals during early life. Again, IgA is the predominant immunoglobulin in milk and is most efficiently induced by oral immunization. In cattle, the major immunoglobulin in milk is IgG1 whose concentration increases in milk following maternal immunization. Efficacy of passive immunity has also been demonstrated by feeding dairy calves colostrum from cows vaccinated with rotavirus (Acres et al., 1978; Snodgrass et al., 1982a, 1982b; Tsunemitsu et al., 1989). This data indicates that vaccination of pregnant cow results in high level of antibodies in colostrum. Susceptible calves fed antibody enriched colostrum are protected from rotavirus associated gastroenteritis.

Cellular immunity has also been shown to play an important role in viral clearance. Offit and Dudzik (1989) demonstrated cytotoxic T cell activity in mice 7 days after rotavirus infection. This cellular cytotoxic activity was specific for rotavirus and was the major histocompatibility complex-restricted. The CD8+ T lymphocytes have also been shown to play an important role in virus clearance in a severe combined immunodeficiency (SCID) mice model (Dharakul et al., 1990). SCID mice inoculated early in life with a murine rotavirus strain EDIM (Epidemic Diarrhea in Infant Mice) became persistently infected (Riepenhoff-Talty et al., 1987) and chronically shed virus without overt clinical signs of gastroenteritis. Transfer of splenic cells or intraepithelial lymphocytes transiently eliminated chronic virus shedding demonstrating the role of T
lymphocytes in rotavirus clearance. Natural killer cell activity has also been demonstrated for chicken intraepithelial lymphocytes against target cells infected with chicken rotavirus (Myers and Schat, 1990).

The immune response to specific rotavirus proteins is generally proportional to their concentration on the rotavirion, but is influenced by the assay used. By conventional ELISA, the majority of the immune response detected is directed to the major structural protein VP6 and therefore ELISA results do not correspond to resistance of animals to rotavirus infection. Rotavirus specific IgA levels correspond with resistance to rotavirus infection. There may be a correlation between protection and neutralization antibody titers. In one study, neutralization titers of 1:128 to a homologous virus correlated with resistance in infants to the same serotype, whereas, lower titers were not protective (Chiba et al., 1986). The neutralizing antibodies are directed to the surface proteins VP4 and VP7 and can be measured by epitope blocking assays using VP4 and VP7 specific neutralizing monoclonal antibodies. The immune response is mainly homotypically directed to the type specific epitopes on both VP4 and VP7 after primary infection, and is against both type and cross reactive epitopes on the two surface proteins after secondary infection or multiple immunizations (Snodgrass et al., 1991). The immune response to different subunits of VP4 has also been investigated in infants vaccinated with rhesus rotavirus. It was found that the immune response to VP4 correlated with responses detected by a plaque reduction test and that the VP8 subunit was more immunogenic than the VP5 subunit of VP4 (Padilla-Noriega et al., 1992).

Tremendous effort has been made to develop an effective vaccine to prevent rotavirus gastroenteritis in animals and humans. Development of an effective vaccine is a high priority in controlling rotavirus gastroenteritis in reducing economic losses and in
keeping children healthy (Kapikian et al., 1989; Daum et al., 1991). Rotavirus vaccines are currently commercially available for cattle and pigs and are expected to be available for other species in the near future. In cattle, a modified live virus vaccine is commercially available but does not provide complete protection. There are two possible explanations for limited efficacy of currently available vaccines. Serotype diversity possibly plays a role for the limited protection in that the vaccine virus strain does not induce protective immunity against heterologous strains. The other possible reason is that the amount of immunogens in vaccine preparation might not be sufficient to induce protective immunity.

Current trends and approaches in developing an effective rotavirus vaccine are diverse. Reassortant rotaviruses with a VP4 from one serotype and VP7 from a different rotavirus serotype have shown to protect against a challenge with both parent viruses (Hoshino et al., 1985, 1988). Hoshino et al. (1988) produced a reassortant rotavirus 11-1 (P7: G4) which contained the VP7 gene from the Gottfried strain (P6: G4) and the VP7 and other genes from the OSU strain (P7: G5). Oral administration to gnotobiotic pigs with the reassortant rotavirus 11-1 induced high levels of neutralizing antibody titers for both OSU and Gottfried and also protected the pigs from infection with both parent virus strains. Similar experiments have been conducted in a mouse model (Offit et al., 1986) in which reassortant rotavirus S-4 containing gene 4 from simian rotavirus SA11 and the rest of the genes from bovine rotavirus NCDV induced neutralization antibodies for both SA-11 and NCDV. In addition to dual neutralization antibody production, mice immunized with this reassortant virus were protected against rotavirus gastroenteritis from both parent rotavirus strains. These studies suggest that heterogeneity among rotaviruses can in part be overcome by employing reassortant rotaviruses encompassing the spectrum of rotavirus serotypes prevalent in a particular animal species. They also
could be useful in making vaccines against strains that grow poorly in cell cultures by incorporating the VP7 genes from such strains in cell culture adapted rotaviruses.

Animal rotavirus strains such as a bovine strain WC3 and rhesus rotavirus strain RRV have been tested for vaccines in infants (Clark et al., 1986; Berstein et al., 1990). This approach has been termed the Jennerian approach and has some potential in development of a vaccine for the prevention of rotavirus gastroenteritis (Kapikian, 1994).

The recombinant approach is also being explored for vaccine development. Rotaviral proteins have been expressed using prokaryotic and eukaryotic expression vectors with varying results (Francavilla et al., 1987; Sabara et al., 1991). VP7 expressed in vaccinia virus and recombinant vaccinia virus containing SA11 rotavirus VP7 induced serotype specific neutralizing antibodies in rabbits (Andrew et al., 1987). In contrast VP7 expressed in \textit{E. coli} was not consistent in inducing virus neutralization antibodies (Arias et al., 1986; Francavilla et al., 1987). VP4 has also been successfully expressed in baculovirus (Nishikawa et al., 1989; Mackow et al., 1989) and the recombinant VP4 protein similar to that of the parent virus, reacted with monoclonal antibodies directed to major neutralization epitopes, and induced neutralizing antibodies in mice (Nishikawa et al., 1989; Mackow et al., 1990).

**Baculovirus as an Expression Vector**

Recent biotechnological developments in virology have provided a better understanding of the viral genome structure and characterization of the function(s) of proteins including their role in immunity and protection. The baculovirus expression system has been quite useful. Baculoviruses are commonly found in insects and 'baculo' refers to the rod-shaped capsids of the viral particles. Viral replication occurs in the nucleus of the infected insect cell. An extracellular form of the virus is produced early in
infection by migration of the progeny virus from the nucleus to the cytoplasm and buds out of the cell membrane into the cellular surface. This mechanism results in cell-to-cell spread as well as secondary infection in the organism. During late infection, viral particles are embedded in a protein crystal (polyhedrin). Such crystals have been referred to as occlusions. The genome of the *Autographa California* nuclear polyhedrosis virus (AcMNPV) is approximately 130 Kb and is a covalently closed double stranded DNA molecule with a superhelical configuration (O'Reilly et al., 1992). The helper independent baculovirus is one of the most popular eukaryotic expression systems. It is unique from bacterial and yeast systems in that foreign genes are expressed under the transcriptional control of the very strong baculovirus polyhedrin gene promoter and recombinant proteins are produced in insect cells during lytic infection with a DNA viral vector (O'Reilly et al., 1992; Luckow and Summers, 1988a, 1989; Maeda, 1989). Recently, a variety of expression vectors have been developed and used to express foreign genes (Luckow and Summers, 1988b). This system has been used to express many viral, mammalian, plant and prokaryotic genes with relatively abundant amounts in a functionally authentic form (see Table 4). Genomic DNAs containing genes without introns or cDNA clones are presently recommended for expression in baculovirus. A number of factors affect the level of protein expression. Removal of the 5’ non-translated DNA sequence is also recommended for optimal expression of a foreign gene (Luckow and Summers, 1988a). Insect cell lines also differ in the amount of protein expression and *Trichoplusia ni*,-derived BTI-TN-5B1-4 (Hi-Five) was one of the very efficient cell lines for a high level of protein expression (Davis et al., 1993). The baculovirus expression system allows virus-like particle assembly when the proper combination of viral protein(s) is expressed in insect cells. Norwalk virus, rotavirus, rabbit hemorrhagic disease virus and parvovirus were able to produce virus-like particle
formation in the baculovirus expression system (Jiang et al., 1992; Labbe et al., 1991; Laurent et al., 1994). Recombinant proteins expressed in the baculovirus system are listed in Table 4.
Table 4. Recombinant proteins expressed in the baculovirus system and their selected characteristics.

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Cellular location</th>
<th>Protein modification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Membrane</td>
<td>Glycosylated</td>
<td>Janssen et al., 1988</td>
</tr>
<tr>
<td>Bovine eye protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodopsin receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 (T4) (hydrophilic</td>
<td>Secreted</td>
<td>Glycosylated</td>
<td>Hussey et al., 1988</td>
</tr>
<tr>
<td>extracellular element)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dengue envelope</td>
<td>Plasma membrane</td>
<td>Glycosylated</td>
<td>Zhang et al., 1988</td>
</tr>
<tr>
<td>glycoprotein (E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dengue NS1</td>
<td>Secreted</td>
<td>Glycosylated</td>
<td>Zhang et al., 1988</td>
</tr>
<tr>
<td>Endogenous human</td>
<td>Secreted</td>
<td></td>
<td>Lebacq-Verheyden et al., 1988</td>
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<tr>
<td>gastrin-releasing</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>peptide precursor</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Lumen, RER</td>
<td>Glycosylated</td>
<td>Lanford et al., 1988</td>
</tr>
<tr>
<td>surface antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B virus</td>
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<td>Glycosylated</td>
<td>Kang et al., 1987</td>
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<td>Hepatitis B virus</td>
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<td>HIV gp160</td>
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<td>interferon</td>
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<td></td>
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Table 4. (Continued).

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<th>Recombinant protein</th>
<th>Cellular location</th>
<th>Protein modification</th>
<th>Reference</th>
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<tbody>
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<td>Human insulin receptor cytoplasmic domain</td>
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<td>PT phlebovirus NS and N protein</td>
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<td>Phosphorylated</td>
<td>Overton et al., 1987</td>
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<td>Sindbis virus capsid</td>
<td>Cytosol</td>
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<tr>
<td>Hepatitis B virus core antigen</td>
<td>Nucleus</td>
<td></td>
<td>Lanford et al., 1987</td>
</tr>
<tr>
<td>HTLV-I p40x</td>
<td>Nucleus</td>
<td></td>
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</tr>
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<td>Nucleus</td>
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<td>Cytosol</td>
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<tr>
<td>Hantann virus capsid</td>
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<td>Schmaljohn et al., 1988</td>
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<td>Hepatitis A virus capsid</td>
<td>Nucleus</td>
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<td>Harmon et al., 1988</td>
</tr>
<tr>
<td>Rotavirus VP7</td>
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<td>Redmond et al., 1993</td>
</tr>
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<td>Bovine rotavirus VP1</td>
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* If blank, no protein modification has been reported.
EXPRESSION AND CHARACTERIZATION OF RECOMBINANT PROTEINS VP2, VP4, VP6, AND VP7 OF BOVINE ROTAVIRUS IN INSECT CELLS

A paper to be submitted to the Journal of General Virology

Young S. Lyoo, Prem S. Paul, and Alexei D. Zaberezhny

Abstract

The VP2, VP4, VP6 and VP7 genes of bovine rotavirus were cloned and expressed in insect cells using the baculovirus expression system, and were then characterized. The recombinant proteins were detected in the cytoplasm of insect cells infected with recombinant baculovirus by indirect immunofluorescence. Molecular mass of the recombinant proteins VP2, VP4, VP6 and VP7 was similar to that of wild type rotavirus by radioimmunoprecipitation. Three additional minor bands were detected in insect cells infected with recombinant baculovirus Bac. pVL/VP4 containing the VP4 gene of bovine rotavirus. Two bands of 60 kDa and 28 kDa were presumably VP5 and VP8. The thirs band of approximately 25 kDa was possibly due to the VP4 cleavage at amino acid 231 (arginine) suggesting that the putative cleavage site at amino acid 231 was active. The ability of recombinant proteins to inhibit virus binding to cells was examined singly or in combination. Recombinant proteins VP4 and VP7 but not VP2 and VP6 inhibited the binding of rotavirus to MA-104 cells. The antigenicity of the recombinant proteins was then tested in guinea pigs. All four recombinant proteins induced antibodies to rotavirus proteins as detected by indirect immunofluorescence test. Recombinant viruses Bac.pVL/VP4 and Bac.pVL/VP7.D induced the highest levels of virus neutralization antibodies, whereas Bac.pVL/VP6 induced low levels of virus
neutralization antibodies. In contrast, recombinant virus Bac.pB/VP2 did not induce any virus neutralizing antibodies. This study showed that rotavirus proteins expressed in insect cells using the baculovirus expression system were authentic and may have a potential as a vaccine for rotavirus associated gastroenteritis in calves.

Introduction

Bovine rotavirus (BRV), a member of the family *Reoviridae* and its genome is composed of 11 double stranded RNA segments. Electron microscopic studies revealed that the virus is triple layered and is approximately 70 nm in diameter (Estes, 1990). The virus was first isolated in the U.S. from calves with diarrhea by Mebus et al. (1969) and has been shown to be associated with gastroenteritis in calves, usually occurring at 1-3 weeks of age (Bridger and Woode, 1975). Gastroenteritis has also been reproduced experimentally with BRV in gnotobiotic, colostrum-deprived, and conventional calves (Bridger and Woode, 1975; Tzipori, 1981; Flewett and Woode, 1978). Modified live virus vaccine has been developed which appears to be effective experimentally in gnotobiotic calves in protection against rotavirus gastroenteritis (DeLeeuw et al., 1985; Saif et al., 1990). Vaccination of dams has been a successful approach in delivering passive immunity to calves when evaluated under experimental and field conditions (Saif et al., 1984). Vaccine prepared from the NCDV strain of BRV is commercially available. It appears to be effective in most situations, however, vaccine breaks have been observed with increased frequency. The authors believe that this in part is due to serotypic diversity among bovine rotavirus isolates and the immunity to rotavirus is primarily serotype specific. Serotypes in rotavirus are determined by two surface proteins VP4 and VP7. Rotavirus types based on VP4 and VP7 are referred to as P and G types.
respectively. The VP4 is protease sensitive outer capsid protein encoded by gene 4 and is present as a dimer in the rotavirus virion. The VP7 is a glycoprotein encoded by gene 7, 8 or 9 depending on strains, is high in mannose and is a major neutralization antigen. Recent work has shown that there are three VP4 types and three VP7 types of bovine rotavirus. Although detailed studies on their relative prevalence is not known, rotavirus with P5 and G6 appear to be the most common types in calves. Some evidence suggests that there are additional serotypes present in the field.

Both VP4 and VP7 induce neutralizing antibodies and are important potentially in protection. Antibodies in animals naturally infected with rotavirus are primarily against VP4, however, antibodies in animals immunized with inactivated virus are generally directed against VP7. Furthermore, VP4 is only a minor protein in rotavirus (Paul and Lyoo, 1993). Therefore, our hypothesis is that recombinant VP4 will be a good candidate for a vaccine. Some studies have been done in the production of bovine rotavirus VP4 and VP7 by recombinant DNA technology with mixed results. The VP7 gene of the NCDV strain of BRV has been expressed in E. coli but the recombinant proteins did not induce neutralization activity (Fracavilla et al., 1987). Consistent results on ability of recombinant proteins to induce virus neutralizing antibodies have not been observed regardless of expression of recombinant proteins in prokaryotic or eukaryotic systems (Arias et al., 1986; Fracavilla et al., 1987; Redmond et al., 1993). It has been speculated that correct glycosylation is essential for the production of virus neutralization antibodies in VP7 recombinant proteins (Arias et al., 1986; Francavilla et al., 1987).

The VP4 gene has also been expressed in E. coli and baculovirus (Arias et al., 1987; Mackow et al., 1989). The recombinant proteins expressed in these systems retained functional and antigenic properties (Mackow et al., 1989; Redmond et al., 1993) and were recognized by VP4-specific neutralizing monoclonal antibodies (Arias et al.,
1987; Mackow et al., 1989). Combination of VP4 and VP7 recombinant proteins were suggested for vaccine composition for effective protection against the rotavirus infection (Redmond et al., 1993). Limited information on role of VP6 in immunity is available. In one study, offsprings of mice immunized with VP6 were partially protected from a rotavirus challenge (Redmond et al., 1993).

This study was undertaken to express rotavirus proteins VP2, VP4, VP6, and VP7 from the same strain of the BRV to better characterize their role in virus binding and immunity. Expressed recombinant proteins were characterized and their ability to produce antibodies in guinea pigs was tested. Blocking of rotavirus infection with these recombinant proteins was also examined. These studies showed that proteins expressed in baculovirus expression system are authentic and antigenic.

Materials and Methods

**Virus and cells**

The VMRI strain of bovine rotavirus (Paul et al., 1988a) was used as a parent virus for the cloning and expression experiments. The plaque purified virus was propagated in the monkey kidney cell line (MA-104) in the presence of 0.5 μg of crystal trypsin (Type IX, Sigma St. Louis, MO) to improve virus replication (Theil and Bohl, 1980; Paul et al 1988a). The MA-104 cells were washed three times with serum free DMEM (Gibco-BRL, Gaithersburg, MD) and inoculated with the VMRI strain of bovine rotavirus at 0.1 multiplicity of infection (MOI). The virus-inoculated MA-104 cells were incubated at 37°C for 60 minutes in a CO₂ incubator with occasional rocking to increase attachment of the virus to cells. After incubation for 60 minutes, the viral inoculum was
removed and the cells were washed once with serum free DMEM to remove residual viral inoculum. Then fresh DMEM containing 0.5 μg of trypsin was added as a maintenance medium. The cells were incubated for three days in a CO2 incubator at 37°C. Rotavirus was extracted with 'Genetron 113' and purified on cesium chloride density gradient by modification of the method described by Cohen et al. (1977). The infected cell culture materials were frozen and thawed three times, and the virus was then pelleted by high speed centrifugation for 90 minutes at 100,000 Xg (Beckman). The viral pellet was extracted in Genetron (1,1,2-Trichloro-1,2,2-trifluoroethane, Johnson Matthey Electronics, MA) and virus in the aqueous phase was collected after low speed centrifugation for 20 minutes at 3000 rpm. To pellet virus particles, collected supernatant was centrifuged for 90 minutes at 25,000 rpm and the pellets were resuspended in 500 μl of sterile deionized water. The resuspended sample was layered on a continuous cesium chloride density gradient (1.2-1.6 gm/ml) for further purification. After a 4 hour centrifugation at 100,000 Xg, the virus bands were collected and their density was measured. To remove the cesium chloride the sample was dialyzed against PBS at 4°C overnight. The virus sample was treated with EDTA at a final concentration of 50 mM for 2 hour at 37°C and core particles were pelleted through a 30% sucrose cushion by centrifugation at 25,000 rpm for 60 minutes. The pellet was resuspended with 100 μl of sterile water and stored at -80°C for the in vitro transcription of viral mRNA.

*Autographa californica* matrix nuclear polyhedrosis virus (AcMNPV) and *Trichoplusia ni*,-derived BTI-TN-5B1-4 cells (Hi-Five) were used for production of recombinant baculovirus and protein expression studies. For the construction of the transfer vector, commercial baculovirus expression system (MAXBAC™ version 1.4, Invitrogen corporation, San Diego, CA) was used. The linearized wild type baculovirus DNA or wild type baculovirus with lethal deletion was used for transfection experiments.
(Invitrogen corporation, San Diego, CA or Pharmingen San Diego, CA). For the growth of insect cells, we used serum free Excell-400 (JRH Biosciences, Lenexa, KS) insect cell culture medium supplemented with antimycotic and antibiotics. Insect cells were detached from the surface by scraping with a soft rubber policeman and carefully resuspended by gentle pipetting without generating foam.

**Antiserum production**

Hyperimmune serum to the VMRI strain of bovine rotavirus was prepared by immunizing guinea pigs using a cesium chloride-purified VMRI strain of BRV following protocols described previously (Bastardo et al., 1981). The VMRI strain of bovine rotavirus (p18) was propagated in MA-104 cells in the presence of 0.5 μg of trypsin (Type IX, Sigma St. Louis, MO). The cell culture materials were harvested when CPE reached 80% and virus was pelleted by centrifugation at 4°C for 90 minutes at 25,000 rpm using SW 28 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellets were resuspended with Tris.HCl buffer (pH 7.4) and an equal volume of Genetron-113 was added. This mixture was homogenized in the vertis mixer for 3 minutes twice and the aqueous phase was collected after centrifugation at 2000 rpm for 20 minutes. The virus containing aqueous phase was centrifuged to pellet the virus at 4°C for 90 minutes at 25,000 rpm. The pellets were resuspended with a small volume of resuspension buffer. The cesium chloride gradient ranging from a density of 1.1 to 1.5 gm/ml was formed using a gradient former (Hoffer Scientific Instruments, San Francisco, CA) and resuspended pellets were layered on the top of the gradient. To prevent evaporation, 1 ml of mineral oil was layered on the top of the virus sample. After centrifugation at 25,000 rpm for 60 minutes, the virus containing bands were visualized by reflecting light over the tube in the dark and three bands consisting of empty, double and single capsid
virus particles were collected. The virus in three bands was pooled and dialyzed extensively against PBS then inactivated by treatment with 4 mM of bromoethyleneimine overnight at 37°C. The inactivated rotavirus was mixed with Freund's complete adjuvant (FCA, Sigma, St. Louis, MO) and inoculated into rotavirus free guinea pigs. Three weeks after the first immunization, the guinea pigs were given immunogen with Freund's incomplete adjuvant (FICA) as a booster injection to enhance the immune response. Antiserum was collected at the time of first immunization and at 3 weeks after immunization to detect the presence of antibodies against the homologous strain of rotavirus. Final bleeding was carried out three weeks later and serum samples were separated from blood then inactivated with heat at 56°C for 30 minutes. Antibody titers were determined and serum samples were stored at -20°C.

_Virus purification and core particle preparation_

The VMRI strain of BRV was inoculated onto monolayers of MA-104 cells in the presence of 0.5 µg/ml of trypsin and cells were incubated for 3 days or until CPE reached around 80%. Cell cultures were frozen and thawed three times to disrupt virus-infected cells to release virus particles. The culture material was centrifuged at 25,000 rpm for 90 minutes at 4°C and pellets were collected in sterile new tubes. The pellets were mixed with an equal volume of Genetron-113 (1,1,2-Trichloro-1,2,2-trifluoroethane, Johnson Matthey Electronics, MA) and extracted using the vortex mixer for 10 minutes. The extracted material was centrifuged for 20 minutes at 3000 rpm (Sorvall RC5B refrigerated superspeed centrifuge, DuPont Instruments) and the aqueous phase was carefully decanted in sterile new tube. To pellet virus particles, virus containing aqueous phase was centrifuged for 90 minutes at 25,000 rpm and pellets were resuspended with 500 µl of sterile deionized water. The resuspended sample was
layered on the continuous CsCl gradient with a starting density of 1.2 to 1.4 mg/ml and gradients were centrifuged at 25,000 rpm for 2 hours. The virus bands were collected and their density was determined by weighing 25 μl into capillary tubes. Samples were dialyzed against PBS at 4°C overnight. The dialyzed virus sample was treated with EDTA at a final concentration of 50 mM for 2 hours at 37°C and core particles were pelleted through a 30% sucrose cushion at 25,000 rpm in a SW 28 rotor for 60 minutes. The pellet was resuspended with 100 μl of sterile water and stored at -80°C.

In vitro transcription of rotavirus mRNA

The viral mRNA was transcribed *in vitro* from purified VMRI core particles containing RNA dependent RNA polymerase. The reaction mixture was prepared in 2X transcription buffer (100 mM Tris-HCl, 0.5 mM NaOAc, 30 mM MgOAc, pH 8.0) containing ATP (8 mM), CTP (2.5 mM), GTP (2.5 mM), UTP (2.5 mM), S-adenosylmethionine (0.5 mM), RNase inhibitor, and purified viral cores. The tube containing the reaction mixture was incubated for 6 hours at 42°C and newly synthesized mRNA was extracted with 400 μl of a phenol/chloroform mixture. The supernatant was mixed with equal volume of 4 M LiCl and incubated at 4°C overnight. To pellet ssRNA from LiCl mixture, the tube was centrifuged for 15 minutes at 4°C and the pellet was washed once with 100% ethanol followed by washing with 70% ethanol to remove salt residues. The pellets were dried under vacuum and resuspended in 100 μl of DEPC-treated sterile water and stored at -80°C.

First strand cDNA synthesis

The first strand cDNA was synthesized using AMV reverse transcriptase using mRNAs from VMRI strain of BRV as a template. For the first strand cDNA synthesis,
Table 1. Primer sequences for PCR amplification of genes VP2, VP4, VP6 and VP7.*

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Gene specificity and Position</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP2 5' end</td>
<td>CCGGCCTAGGCAAGGGCTCAATGGCG</td>
<td>Tian et al., 1990.</td>
</tr>
<tr>
<td>2</td>
<td>VP2 3' end</td>
<td>ACAACTAGTTCATGATGGCG</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>VP4 5' end</td>
<td>CCGCGCGCGCGGCGTATAAAATGGCTTCGCTC</td>
<td>Nishikawa and Gorziglia, 1988.</td>
</tr>
<tr>
<td>4</td>
<td>VP4 3' end</td>
<td>CCGCGCGCGCGGGTCACAACCTGAGC</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>VP4 5' end</td>
<td>GGGAATCCGGCTATAAAATGGCTTCGCTC</td>
<td>Mumunidi et al., 1995.</td>
</tr>
<tr>
<td>6</td>
<td>VP4 3' end</td>
<td>GGGAATTCGGTCACAACCTGAGC</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>VP6 5' end</td>
<td>GGTCGGATCCCTCTGACTAGC</td>
<td>Cohen et al., 1984.</td>
</tr>
<tr>
<td>8</td>
<td>VP6 3' end</td>
<td>CGGGATCCGGCTTTAAACGAA</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>VP7 5' end</td>
<td>CCGCGCGCGCGGGCTTTAAAGAGAGAATTTC</td>
<td>Gorziglia et al., 1986.</td>
</tr>
<tr>
<td>10</td>
<td>VP7 3' end</td>
<td>CCGCGCGCGCGGGTACATCATACTAATCTCTA</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>VP7 5' end</td>
<td>GGGAATCCGGCTTTAAAGAGAGAATTTC</td>
<td>Mumunidi et al., 1995.</td>
</tr>
<tr>
<td>12</td>
<td>VP7 5' internal</td>
<td>GGGAATCCGATAATAATATGGTATTGAA</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>VP7 3' internal</td>
<td>GGGAATTCGGTCACATCATACTAATCC</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>VP7 3' end</td>
<td>GGGAATTCGTTATGATGGACGTAAATCC</td>
<td></td>
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* Sequences for restriction sites are shown in italics with underlines, and bold indicate start codon in primers.
we used primers specific to the 3' end of BRV genes 2, 4, 6, and 9 (Table 1) which encode viral proteins VP2, VP4, VP6, and VP7, respectively. Microcentrifuge tubes and water used for mRNA transcription were treated with DEPC and autoclaved to eliminate RNase contamination. Viral mRNAs were resuspended in distilled water and divided into 11.5 μl aliquots in four 0.5 ml microcentrifuge tubes for cDNA synthesis of VP2, VP4, VP6, and VP7 genes. Tubes containing mRNA were heated at 65°C for 10 minutes to remove the secondary structure followed by incubation at room temperature for 2 minutes. Reaction mixture was prepared by adding 1.0 μl of RNase inhibitor, 4.0 μl of 100 mM dNTPs, 1.0 μl of 80 mM sodium pyrophosphate, 0.5 μl of AMV reverse transcriptase and a gene-specific primer. Primers used for cDNA synthesis are shown in Table 1. These primers did not alter any sequences in the ORF of the corresponding genes. The tubes were gently tapped to mix the solution then spun briefly to collect the reaction mixture. Tubes were placed in a 42°C water bath for 60 minutes followed by incubation at 95°C for 2 minutes to denature the RNA-cDNA hybrids. To increase efficiency of cDNA synthesis, a second round of cDNA synthesis was carried out by adding an additional 0.5 μl of reverse transcriptase and tubes were incubated for an additional 60 minutes at 42°C following heating at 95°C for 3 minutes. The reaction was stopped by placing the tubes on ice. After completion of cDNA synthesis, the final product was extracted with phenol/chloroform and concentrated by precipitation with ethanol and sodium acetate. The pellets were redissolved in 10 mM Tris.HCl buffer containing 1 mM EDTA, pH 8.0. The first strand cDNAs were used as templates for PCR amplification of VP2, VP4, VP6, and VP7 genes.
PCR amplification of bovine rotavirus genes

The VP2, VP4, VP6, and VP7 genes of VMRI strain of bovine rotavirus were amplified from cDNAs by polymerase chain reaction (PCR) using gene-specific primers and thermostable Taq-1 DNA polymerase (Invitrogen, San Diego, CA). Sequence of primer sets used for gene amplification is shown in Table 1. Reaction mixtures containing cDNA template, dNTPs (0.2 mM/each, Pharmacia, Piscataway, NJ), primers (2 μM), MgCl2 (3 mM, Pharmacia, Piscataway, NJ), Taq-1 DNA polymerase (2.5 U) and 10X PCR reaction buffer (500 mM KCl, 100 mM Tris.HCl and 1 mg/ml gelatin, pH 8.4) were prepared in a laminar flow hood with sterile negative pressure conditions to prevent nonspecific DNA amplification by contamination. The contents in the tube were mixed by tapping and the tubes were centrifuged briefly to collect the reaction mixture on the bottom of the tube. Fifty μl of mineral oil (Sigma, St. Louis, MO) was overlayed to prevent evaporation during amplification. PCR amplification was conducted in thermocycler (Coy, MI) with the first cycle of denaturation for 90 seconds at 94°C, primer annealing for 2 minutes at 48-54°C (depending on amplified gene) and primer extension for 5 minutes at 72°C. Thirty amplification cycles were performed with denaturation at 94°C for 1 minute, primer annealing at 48°C for 90 seconds, and primer extension at 72°C for 2 minutes. The last cycle was the same except the extension time was increased to 10 minutes. The amplification products were analyzed in 1% agarose (FMC) gel by electrophoresis at 100 volts for 1 hour and the DNA bands were visualized by staining with ethidium bromide. DNA bands were cut out from the gels, purified using charged glass milk (Geneclean Kit, Bio101, La Jolla, CA), and used for cloning and sequencing experiments.
Molecular cloning and sequencing

The PCR amplified cDNAs for VP2, VP4, VP6 and VP7 genes of bovine rotavirus were inserted into multi-cloning sites of phagemid vector pKS⁺. To enhance the ligation efficiency of vector and insert, the vector DNA was dephosphorylated with calf intestinal phosphatase (Sigma, St. Louis, MO) and PCR amplified viral DNA was phosphorylated using protein kinase (Sigma, St. Louis, MO). The PCR amplified rotavirus cDNA and the vector DNA were ligated using T4 DNA ligase (Promega, Madison, WI). Colonies containing rotavirus cDNA inserts were selected by antibiotic resistance and color selection on LB agar containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG). Positive colonies were picked and plasmid DNA was isolated by alkaline extraction method (Sambrook et al., 1989). Plasmid DNA was then cut with an appropriate restriction enzyme, electrophoresed in a 1% agarose gel and examined for the presence of insert.

Sequencing was carried out by using Sanger's dideoxy method with Taq-1 polymerase reaction using an automated sequencer at the Nucleic acid facility, Iowa State University. Initial sequencing was performed using universal and reverse primers and additional internal primers were designed for complete sequencing. Sequencing data were analyzed using computer software programs MacVector (IBI, New Haven, CT) and GeneWorks (IntelliGenetics, Inc., Mountain View, CA). Information obtained by sequence analysis was used for the construction of transfer vectors.

Construction of recombinant baculovirus transfer vectors with rotavirus gene inserts

1. Construction of baculovirus transfer vector containing VMRI VP2 gene insert
To construct the baculovirus transfer vector pB/VMRI.VP2, the cloned cDNA for VP2 gene was cut out of plasmid pKS+/VP2 with Nhe I and Spe I restriction endonucleases and separated by preparative agarose gel electrophoresis. The cDNA for VP2 gene was purified with glass milk (Gene clean Kit, Bio 101, La Jolla, CA) and used for ligation with the baculovirus transfer vector pBlueBac DNA (Fig. 1). Purified DNA was treated with Klenow and protein kinase for blunt end ligation. Baculovirus transfer vector pBlueBac was cut with restriction enzyme and purified by column chromatography using Sepharose CL-4B column. Purified DNA was precipitated in ethanol and treated with enzymes to make a blunt end for the ligation. BRV VP2 gene and the transfer vector were ligated using T4 DNA ligase at 16°C overnight and the ligation mixture was used for transformation of competent E. coli cells. The E. coli (INV-1 α F) colonies containing rotavirus inserts were confirmed by analysis of miniprep DNA with Pst I restriction enzyme. After selection of positive colonies the orientation of the VP2 gene insert in the vector was determined by restriction analysis and recombinant clones with correct VP2 gene orientation were selected. The selected clones were inoculated into 150 ml of LB broth containing 50 μg/ml of ampicillin to inhibit growth of undesired organisms. The DNA for transfection was prepared by using a maxiprep protocol (QIAGEN, Hilden, Germany) and the concentration of DNA was determined by spectrophotometer (Perkin-Elmer, Norwalk, CT).

2. Construction of the baculovirus transfer vector containing VP4 gene of bovine rotavirus

The VP4 gene of the VMRI strain of BRV was amplified using gene specific primers from the first strand cDNA template. For the construction of transfer vector (Fig. 2), two primers with built-in restriction sites were used. The 5'-end primer with a
Fig 1. Construction of recombinant baculovirus transfer vector pB/VMRI.VP2 containing VP2 gene from VMRI strain of BRV showing noncoding sequences of 16 bp at the 5'-end and 28 bp at the 3'-end ([A]). Selected features of rotavirus VP2 are shown in ([B]).

Abbreviations: pH: polyhedrin promoter; MCS: multi-cloning site; CH: charged helix; HTH: helix turn helix; LZ: leucine zipper; P1 kinase homology: putative nucleic acid binding motifs
[A]  

pPH  
MCS  
pBlueBac  

VMRI VP2 Gene (2687 bp)  
Nhe I Start  
16 bp  
Stop Spe I  
28 bp  

[B]  

VP2  

53 77 102 154 536 557 665 686
17 bp 2662 bp

CH  BASIC  LZ  LZ

66 121 64 76
HTH
P1 kinase homology
Fig. 2. Construction of recombinant baculovirus transfer vector pVL/VMRI.VP4 containing VP4 gene of VMRI strain of BRV ([A]). Selected features of rotavirus VP4 gene are shown under ([B]). The sequence of 9 bp at 5'-end and 25 bp at 3'-end represents noncoding sequences in the VP4 gene of the VMRI strain of BRV. The closed triangle at the amino acid position 306 represents epitope I, and at amino acid 392-439 represent epitope II. Synthetic peptide corresponding to amino acids 232-255 are known to induce neutralization antibodies. Putative proteolytic enzyme cleavage sites are located at amino acid residues 241, 246 and 247. An additional cleavage site was detected at aa 231. Abbreviations: B: Bam HI restriction cleavage sequence; E: Eco RI restriction cleavage sequence; pPH: polyhedrin promoter; MCS: multi-cloning site; V: variable region; C: constant region; Cy: conserved cysteine; Pr: conserved proline; HR: heptad repeat
[A] pVL1393

VMRI VP4 Gene (2362 bp)

B Start

9 bp

Stop E

25 bp

pVL/VMRI.VP4

[B] VP4

10 bp

71 204 224 236 257 271 384 404 494 554 580 610 776

2337 bp

V Cy 241 Cy Pr

C C 246 247

71 204

232 255

high sequence variation

Fusion domain

392 439

HR V

Cy
built-in Bam HI restriction endonuclease cleavage site was CCGGATCCGCTAT AAAATGGGCTTC GCTC and the 3'-end primer with Eco RI restriction endonuclease cleavage site was CCGGAAATTC GGTCACAACCTCT AGACACTA. The two restriction sites used corresponded to the available restriction sites in baculovirus transfer vector pVL 1393 (Fig 2). The PCR amplified VP4 gene products were digested with Bam HI and Eco RI and then purified using sepharose CL-4B column. Baculovirus transfer vector was cut with Bam HI and Eco RI then purified as described for the PCR amplified VP4 gene DNA. Recombinant E. coli colonies containing rotavirus VP4 gene inserts were selected by restriction analysis of plasmid DNA. Three colonies were selected and DNA from three recombinant plasmids was purified by passing through columns (Qiagen, Hilden, Germany) and used for transfection. Concentration of each DNA was measured by spectrophotometry at 260 nm (Sambrook et al., 1989).

3. Construction of a baculovirus transfer vector containing the VP6 gene from the VMRI strain of bovine-rotavirus

The recombinant baculovirus transfer vector pVL/VP6 containing the VP6 gene of the VMRI strain of BRV was constructed using the VP6 gene cloned in plasmid (Fig. 3). The VMRI VP6 cDNA insert was cut out of plasmid DNA with Bam HI and isolated by preparative agarose gel electrophoresis. DNA was purified using glass milk (Gene clean Kit, Bio 101, La Jolla, CA) following manufacturer's instructions. The isolated VP6 gene DNA was precipitated in ethanol and ligated into baculovirus transfer vector pVL 1393 at the Bam HI site. The direction of the VP6 gene insert in baculovirus transfer vector was determined by restriction analysis. Clones with correct orientation
Fig. 3. Construction of recombinant baculovirus transfer vector pVL/VMRI.VP6 containing VMRI VP6 gene ([A]) and selected features of VP6 gene of group A rotavirus ([B]). The sequence of 23 bp at the 5'-end and 139 bp at the 3'-end represent the non-coding sequences of the VP6 gene of VMRI strain of BRV. Abbreviations: B: Bam HI; pPH: polyhedrin promoter; MCS: multi-cloning site; Hy: hydrophilic region; Dashed line: hydrophobic regions; ssp: single shelled particle.
[A] pPH MCS pVL1393

VMRI VP6 Gene (1356 bp)

B Start 23 bp Stop B 139 bp

pPH

VMRI VP6 Gene

pVL/VMRI.VP6

[B] VP6

24 bp 1217 bp

20

90 105 150

370 397

Hy

105 Trimerization domain 328

1 Conserved in A & C 140 251 ssp assembly domain 397

353 Conserved in A & C 394
Fig. 4. Construction of recombinant baculovirus transfer vector pVL/VMRI VP7 containing VP7 gene of VMRI strain of bovine rotavirus (A), and selected features of group A rotavirus VP7 gene (B). The 48 bp at the 5'-end and 35 bp at the 3'-end represent non-coding sequences of the VP7 gene of the VMRI strain of BRV.

Abbreviations: pH: polyhedrin promoter; MCS: multi-cloning site; H1, H2: two amino-terminal hydrophobic signals; AQ: signal is cleaved between alanine (A) and glutamic acid (Q); C: cysteine; A, B and C: three major antigenic regions; (G): glycosylation site; CA: putative cell attachment domain
VMRI VP7 Gene (1063 bp)

[B]

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>1 30 33 48 124 155 192 231 278 309 326</td>
</tr>
<tr>
<td>49 bp</td>
</tr>
</tbody>
</table>

[A]

pUL1393

VMRI VP7 Gene

pVL/VMRI,VP7

M | AQ(G) | C | C | C | C | C | C | C |
<table>
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were selected and DNA samples for transfection were prepared by passing through QIAGEN column. Concentration of DNA was measured by spectrophotometry.

4. Construction of the baculovirus transfer vectors containing VP7 gene of bovine rotavirus

The VP7 gene of the VMRI strain of BRV was amplified by PCR using a gene specific primer set with built-in restriction sites. The amplified DNA product was purified by spin columns (Linkers 6 Quick spin column Sepharose CL-6B, Boehringer Mannheim Biochemicals, Indianapolis, IN) to remove protein and oligos followed by ethanol precipitation. As shown in Fig. 4 the purified PCR products were inserted into a baculovirus transfer vector pVL1393 and clones containing inserts were identified by restriction analysis. DNA was prepared from selected clones and concentration was measured by spectrophotometry.

Transfection and detection of expressed recombinant viral proteins by IFA

To generate recombinant baculovirus containing bovine rotavirus genes, insect cells were transfected with DNA from wild type baculovirus AcMNPV and a recombinant transfer vector. For the generation of a recombinant baculovirus Bac.pB/VP2, 500 ng of pB/VMRI VP2 DNA was mixed with 1 μg of linearized AcMNPV DNA (Invitrogen, San Diego, CA) and lipofectin (Gibco-BRL, Gaithersburg, MD) solution. For other experiments, baculovirus DNA with a lethal deletion (PharMingen, San Diego, CA) was used to avoid generation of occlusion positive wild type baculovirus. For these experiments, 500 ng of each recombinant transfer vector DNA containing bovine rotavirus VP4 (pVL/VMRI.VP4), VP6 (pVL/VMRI.VP6), or VP7 (pVL/VMRI.VP7) genes and 50 ng of wild type baculovirus DNA were mixed into lipofection (Gibco-BRL, Gaithersburg, MD) solution in separate tubes containing
different genes. The plasmid DNA and baculovirus DNA mixtures were incubated for 15 minutes at room temperature. Meanwhile, the insect cells were plated in 6-well cell culture clusters at a concentration of $3 \times 10^5$ cells/ml and the plates were placed in a laminar flow hood at room temperature for 60 minutes to allow the cells to attach onto the plastic surface. After incubation for 60 minutes at room temperature, the cell culture medium (Excell-400, JRH Bio Sciences, Lenexa, KS) was replaced and a DNA/lipofection mixture was added drop by drop. For efficient transfection, cells were placed at room temperature for 4-6 hours depending on the condition of the cells. The transfection material was removed and 3 ml of fresh insect cell culture medium (Excell-400) was added. The cell culture plates were incubated at 27°C for 72 hours.

**Plaque purification of recombinant viruses**

Plaque purification was performed to isolate pure clones of recombinant baculoviruses. Insect cells infected with recombinant virus were harvested and culture materials were clarified by low speed centrifugation at 2000 rpm for 20 minutes at 4°C. Supernatants were collected in sterile snap cap tubes (Falcon, Lincoln Park, NJ) and serially diluted ten fold from $10^{-1}$ to $10^{-7}$. Diluted inocula were added onto insect cells and incubated for 60 minutes at room temperature to allow infection of insect cells with the recombinant virus. After a 60 minute incubation, the inocula were removed and 3 ml of 1.25% Sea Plaque agarose (FMC, Rockland, ME) prepared in Excell-400 insect cell culture medium was added. After solidification of agarose, 3 ml of insect cell culture medium Excell-400 was added on agarose to prevent drying during further incubation period. Cells were incubated for 72 hours at 27°C without a CO2 supply. Occlusion negative isolated virus plaques were picked using sterile Pasteur pipettes and diluted in 0.5 ml of insect cell culture medium. Recombinant virus containing tubes were stored at
4°C overnight to release virus particles into the media and then centrifuged at 2000 rpm for 10 minutes at 4°C. This purification step was repeated three times to ensure clonal population. The plaque purified recombinant baculoviruses were designated as Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 containing VP2, VP4, VP6 and VP7 genes, respectively. After three rounds of purification, supernatants containing recombinant baculovirus were collected and stored at -80°C.

Detection of rotaviral genes in recombinant baculovirus by dot blot hybridization assay

The presence of a rotavirus gene in recombinant baculovirus was determined by a dot hybridization assay using radiolabeled probes. To extract the nucleic acid, insect cells were frozen and thawed once and clarified by low speed centrifugation. Recombinant baculoviruses were pelleted from supernatant by centrifugation at 37,000 rpm for 90 minutes using SW 41 rotor and viral DNA was extracted with phenol/chloroform. The aqueous phase was collected and DNA was precipitated with ethanol at -70°C and collected by centrifugation. DNA in pellets was resuspended and nucleic acid was denatured by boiling for 5 minutes followed by chilling on ice immediately to avoid renaturation. Insect cells infected with wild type baculovirus, treated similar to those infected with the recombinant baculovirus, were used as a negative control. Recombinant phagemid DNA containing bovine rotavirus VP2, VP4, VP6, or VP7 genes of bovine rotavirus were applied on the same membrane and used as positive controls. The phagemid vector pKS+ DNA without an insert was also added on the same membrane as a negative control.

Denatured nucleic acid solutions were applied on a nylon membrane (Bio-Rad Laboratories, Hercules, CA) using a 96-well manifold (Bio-Rad Laboratories, Hercules, CA) and baked for 2 hours at 80°C to immobilize nucleic acids. Membranes were
prehybridized for 4 hours at 65°C in prehybridization solution containing 5X Denhardt's solution, 6X SSC and denatured salmon testes DNA. Fifty ng each of PCR amplified cDNA corresponding to VP2, VP4, VP6, and VP7 genes of bovine rotavirus were labeled in separate reaction mixture with [α-32P]dCTP (ICN Biochemicals, Costa Mesa, CA) in the presence of a random hexamer and a Klenow fragment of DNA polymerase I (Amersham Corporation, Arlington Heights, IL). Radiolabeled DNA was passed through the column (Linkers 6 Quick spin column Sepharose CL-6B, Boerringher Mannheim, Indianapolis, IN) to remove unincorporated [α-32P]dCTP, and just prior to hybridization boiled for 5 minutes at 100°C and placed on ice. Radiolabeled probes were added into each hybridization roller bottle containing the membrane and hybridized overnight in a hybridization chamber at 65°C (Hybaid, Valley Park, MO) with a rolling speed of 10 rpm. To remove unincorporated radiolabeled probes, the membranes were washed three times in 1X SSC-0.1% SDS, three times in 0.1X SSC-0.1% SDS and three times with sterile water at 65°C. An autoradiograph was made by exposing X-ray film (Kodak, Rochester, NY) to the hybridization membrane.

Detection for the presence of rotaviral genes in recombinant virus by PCR amplification

To confirm the presence of rotavirus genes in recombinant baculoviruses, PCR was performed using rotavirus gene specific primers. Insect cells were infected with wild type baculovirus or one of the recombinant baculoviruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7. Total nucleic acid was isolated and a rotavirus specific gene was amplified using gene-specific primer sets for VP2, VP4, VP6 and VP7 (Table 1). The PCR amplified product was analyzed by agarose electrophoresis. The PCR conditions were as described in the previous section.
Detection of rotavirus proteins by indirect immunofluorescent antibody test

Indirect immunofluorescent antibody tests were performed to detect rotavirus specific recombinant proteins in insect cells infected with recombinant baculovirus. Cells infected with the wild type baculovirus or mock infected cells were used as controls. Monolayers of insect cells were infected with recombinant baculovirus, incubated at 27°C for 72 hours after infection at which time they were fixed with cold methanol at room temperature for 15 minutes. Guinea pig antiserum to VMRI strain of bovine rotavirus, which had been tested for rotavirus specificity, was used to detect rotaviral antigens. Cells were incubated with serum for 60 minutes at 37°C at which time they were washed three times with PBS to remove unbound antibodies. FITC-labeled anti-guinea pig antibodies (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) were applied and incubated for 30 minutes at 37°C. The cells were rinsed three times with PBS, and examined under fluorescent microscope. Photographs were taken using Olympus BH-2 UV microscope with an automatic exposure control unit.

Titration of recombinant baculoviruses

Titers of recombinant baculoviruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 were determined in BTI-TN-5B1-4 cells by indirect immunofluorescent assay. Briefly, insect cells were split into 96-well tissue culture plates (Corning Incorporated, Corning, NY) and left undisturbed to allow attachment of the cells to the plastic surface. After 60 minutes incubation, the medium was removed and 10-fold dilutions of recombinant virus were added into 4 wells per sample. Cells were incubated for 60 minutes and medium was decanted. Cells were further incubated for 72 hours at 27°C then fixed with cold methanol for 15 minutes. Cells were reacted with hyperimmune guinea pig anti-VMRI rotavirus hyperimmune serum in an indirect
immunofluorescent test. Reciprocal of the highest dilution that gave positive
immunofluorescence was considered as the virus titer and the virus titer was expressed as
fluorescent focus units (FFU) per ml.

**Kinetics of recombinant rotavirus protein expression in insect cells**

To test the kinetics of recombinant protein expression, insect cells were infected
with recombinant baculovirus Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, or
Bac.pVL/VP7. The insect cells were labeled with $^{35}$S-methionine/cysteine at 24, 48, 72
and 96 hours post infection and immunoprecipitated with anti-VMRI hyperimmune
serum. Insect cells prepared in 24-well tissue culture plates were inoculated with wild
type or recombinant baculovirus and metabolically labeled with 200 μCi of $^{35}$S-
methionine/cysteine. Supernatant and cells were collected separately at each time point to
examine the presence of recombinant proteins in cells and secreted proteins in culture
medium. The tubes containing supernatant were centrifuged for 2 minutes in
microcentrifuge and the pellets were discarded to eliminate cell contamination. The
supernatant was mixed with an equal volume of lysis buffer and vortex mixed for 2
minutes, then placed on ice for 3 minutes. Cells were lysed with 0.5 ml of lysis buffer
and processed as described for the supernatant. Radioimmunoprecipitation was carried
out as described under the previous section. Gels were dried under vacuum and X-ray
film was exposed to it. Images on X-ray film were traced on a transparency film and
rotavirus specific bands in gel were located by overlaying the transparency film on dried
gel. The bands were cut and radioactivity was measured using liquid scintillation
counter.
**Virus-like particle (VLP) formation**

To determine the combination of rotavirus proteins that were necessary for virus-like particle (VLP) formation, insect cells were infected with different combinations of recombinant baculoviruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7. Mock infected and wild type baculovirus infected insect cells were used as controls. Insect cells infected with recombinant baculovirus were collected at 6 days post inoculation and stored at 5°C overnight. Cell culture materials were centrifuged at 25,000 rpm for 6 hours at 5°C and supernatants were decanted. Pellets were resuspended with 50 mM TE buffer and extracted with Genetron (1,1,2-Trichloro-1,2,2-trifluoroethane, Johnson Matthey Electronics, MA) for 15 minutes on a vortex mixer. The virus and Genetron mixture was centrifuged at 5,000 rpm for 30 minutes at 5°C. The virus containing supernatants were collected into new sterile tubes for each sample and examined by immune electronmicroscopy. Ten ml of the supernatant was mixed with 50 μl of polyclonal guinea pig anti-VMRI hyperimmune serum and incubated overnight at 5°C. To pellet the virus and antibody complexes, samples were centrifuged at 35,000 rpm for 90 minutes in a SW 41 rotor at 5°C. The supernatants were discarded and 500 μl of sterile water was added into each tube to resuspend pellets. After overnight incubation, pellets were transferred to 1.5 ml microcentrifuge tubes and centrifuged for 5 seconds to remove large size debris. Clear supernatants were stained with 2% potassium tungstic acid (PTA) and sprayed on grids using a nebulizer and examined under an electron microscope (Hitachi 500, Japan).

**Radioimmunoprecipitation**

Radioimmunoprecipitation was carried out to identify the expressed recombinant protein and to determine its molecular mass. Insect cells were inoculated with wild type
baculovirus, recombinant baculovirus Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, or Bac.pVL/VP7. Three days after inoculation, methionine free insect cell culture medium (Gibco-BRL, Gaithersburg, MD) was added for 2 hours followed by addition of 200 μCi of 35S-methionine (ICN, Costa Mesa, CA). Cells were labeled for 2 hours at 28°C.

Guinea pig anti-VMRI serum was pre-treated with cold cell lysate from insect cells infected with wild type baculovirus for 1 hour at room temperature to eliminate non-specific binding. To prepare cold lysate, insect cells were infected with the wild type baculovirus and incubated at 27°C for 72 hours. After removal of supernatant from flask, 2.5 ml of lysis buffer (1% NP40, 50 mM NaCl, 50 mM Tris/HCl, 0.5 μg/ml Leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml Pepstatin A, pH 8.0) was added into each flask and flasks were incubated at room temperature for 5 minutes. The lysed cells were collected into 1.5 ml microcentrifuge tubes and vortex mixed for 3 minutes then incubated for 3 minutes on ice. Cell debris was removed by centrifugation and supernatant was saved as a cold lysate at -80°C. Hot lysate was prepared as described previously (O'Reily et al., 1992). For radioimmunoprecipitation, 50 μl of the serum treated with cold lysate was added into 100 μl of hot lysate and the lysate-serum mixture was incubated overnight at 4°C to form an immune complex. To capture the immune complex, 100 μl of CL-4B bound protein A (Sigma, St. Louis, MO) was added and mixed by rotating for 1 hour at room temperature. Protein-A-bound immune complexes were washed three times with lysis buffer and an additional three times with sterile water. Samples were mixed with 50 μl of sample buffer then boiled for 5 minutes just before analysis by gel electrophoresis. An X-ray film was exposed to the dried gel and results were analyzed.
Target receptor blocking assay using recombinant proteins

To identify rotavirus protein(s) which bind to target cells, recombinant proteins were used to block target receptors on MA-104 cells. Lysates of insect cells, wild type baculovirus or recombinant baculovirus Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, or Bac.pVL/VP7 were prepared as described previously (Bass et al. 1991). To determine saturation point for receptor blocking, 10-fold dilutions of lysates of insect cells infected with recombinant baculovirus Bac.pVL/VP4, and Bac.pVL/VP7 were added onto MA-104 cells.

Monolayers of MA-104 cells at 65-70 passage level were grown in 24-well tissue culture plates and washed three times with serum-free DMEM. Cells in two control wells were trypsinized and the number of cells were counted. Concentration of MA-104 cells was 1X10⁵ cells/well. One ml lysate of cells infected with different recombinant baculovirus was added into each well. The tissue culture clusters were further incubated for 2 hours at 37°C and washed gently three times with serum-free DMEM. Cells were inoculated with 100 FFU of the VMRI strain of BRV at passage 19. Cells were incubated for 60 minutes at 37°C and washed three times to remove the virus inoculum. Maintenance medium containing 0.5 μg/ml of trypsin was added and incubated for 18-20 hours at 37°C. The cells were fixed with cold methanol and the number of rotavirus specific foci were determined by the indirect immunofluorescent test. Rotavirus specific foci were counted three times from duplicate wells infected with 100 FFU and the data reported as an arithmetic mean of two experiments.

To examine the effect of trypsin treatment on receptor binding, recombinant baculoviruses were treated with 5 μg/ml of trypsin and their ability to block receptor binding was measured.
Preparation of immunogen

For the preparation of the immunogen, recombinant baculoviruses were propagated in insect cells individually or in combination. Briefly, insect cells were detached by a rubber policeman and plated at a cell concentration of $2.5 \times 10^5$ cells/ml. Cells were allowed to attach to the surface of culture vessel for 1 hour at which time the culture medium was removed and replaced with recombinant baculovirus at a MOI of 10. After one hour, the medium was removed and fresh insect cell culture medium was added and cells were further incubated at $27^\circ C$. When CPE reached 80%, usually in about 4 to 5 days post infection, the cells were frozen and thawed three times and used as immunogen. One ml of immunogen was emulsified with 10% of Freund's incomplete adjuvant (Sigma, St. Louis, MO) in the presence of RAS (MPL+TDM+CWS Emulsion, RIBI ImmunoChem Research Inc, Hamilton, MT) to enhance immunogenicity of recombinant proteins.

Antigenicity of recombinant proteins in guinea pigs

Guinea pigs were inoculated with lysates of insect cells infected with recombinant baculovirus Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, or Bac.pVL/VP7. Preimmune serum samples were collected from guinea pigs and tested for antibodies to rotavirus. Recombinant viruses were propagated singly or in combination, on insect cells. Virus preparations were inactivated with 4 mM bromoethyleneimine (BEI) for 1 hour at $37^\circ C$. Sodium thiosulfate was added at a final concentration of 40 mM to neutralize BEI before immunization. Inactivated VMRI strain of bovine rotavirus grown in MA-104 cells, uninfected BTI-TN-5B1-4 cell culture material and BEI-inactivated wild type baculovirus AcMNPV grown in BTI-TN-5B1-4 cells were also prepared as control antigens. Antigens were emulsified with adjuvant and inoculated into guinea pigs by intramuscular
and subcutaneous routes. Guinea pigs were anesthetized with acepromazine and ketamine mixture and blood sample was collected from each individual guinea pig one day before immunization. Serum samples were tested for the presence of antibody to rotavirus by indirect immunofluorescence. Blood samples were collected at 3 weeks after the first immunization and the guinea pigs were given a booster injection. Three weeks later, final blood samples were collected and serum samples were stored at -20°C.

**Detection of immune response**

To detect the immune response to recombinant rotavirus proteins, serum samples were tested for antibodies by an indirect immunofluorescent assay and a virus serum neutralization test using the VMRI strain of BRV. Serum samples were heat inactivated for 30 minutes at 56°C, before tests. For the indirect immunofluorescent test, serial dilutions of serum samples were applied onto rotavirus-infected and methanol-fixed MA104 cells. FITC-labeled secondary antibody was then added to examine rotavirus specific fluorescence with fluorescence microscope. For virus serum neutralization test, cells were grown in 96-well plates and infected with the VMRI strain of BRV. Serially diluted serum samples were mixed with 200 TCID50/ml of VMRI strain of BRV and incubated for 60 minutes at 37°C. The serum and virus mixture were inoculated onto monolayers of MA-104 cells and incubated at 37°C. After 60 minutes of incubation, inocula were aspirated and cell monolayers were washed once with serum-free DMEM. Serum-free DMEM containing 0.5 μg/ml of trypsin was added into each well and cells were further incubated for 24 hours at 37°C. The cells were fixed with cold methanol for 15 minutes at room temperature and then stained to examine rotavirus specific immunofluorescence. Reciprocal of the last dilution of serum that neutralized virus i.e. gave negative fluorescence was considered as a virus serum neutralization titer.
Results

Cloning of rotavirus genes and construction of transfer vectors

The cDNAs for VP2, VP4, VP6, and VP7 genes of the VMRI strain of BRV were synthesized by reverse transcription, amplified by PCR using gene-specific primers and cloned into phagemid vector pKS+. The recombinant colonies were identified by color selection and recombinant plasmids were examined for the presence of inserts by restriction endonuclease analysis. As expected, the size of cDNA inserts for VP2, VP4, VP6, and VP7 genes was 2.69, 2.36, 1.36, and 1.06 kb, respectively (Fig. 5). The cDNA from recombinant plasmids containing VP2 gene was released and subcloned into baculovirus transfer vector pBlueBac as illustrated in Fig. 1. The cDNA from the recombinant plasmid with the VP6 insert was released and subcloned into baculovirus transfer vector pVL1393 (Fig. 3). The PCR amplified cDNAs of VP4 and VP7 genes were cloned into a baculovirus transfer vector pVL1393 (Fig. 2 and 4). The presence of the inserts in baculovirus transfer vectors was confirmed by restriction endonuclease digestion and size of released some fragments were different from original inserts due to the restriction enzymes used (Fig. 6).

Production of recombinant baculovirus containing VP2, VP4, VP6 and VP7 genes of bovine rotavirus

Recombinant baculoviruses were prepared by co-transfection of insect cells with DNA from wild type baculovirus and recombinant plasmids. Occlusion negative plaques were picked, and purified by three rounds of plaque purification. Presence of cDNA inserts in baculovirus was confirmed by dot blot hybridization and PCR (Fig. 8 and 9).
Fig. 5. Cloning of bovine rotavirus VP2, VP4, VP6, and VP7 genes into phagemid vector pKS+. Genes were released using appropriate restriction endonuclease. DNA from VMRI VP2 gene of 2.7 kb (lane 2), VP4 gene of 2.4 kb (lane 4), VP6 gene of 1.3 kb (lane 6) and VP7 gene of 1.06 kb (lane 9) were separated on 1% agarose gel electrophoresis at 100 volt, stained with ethidium bromide, and visualized under ultra violet light.
Fig. 6. Recombinant plasmids containing rotavirus genes VP2, VP4, VP6 and VP7 cloned in baculovirus transfer vector. Insert DNAs were released from recombinant plasmid with restriction endonucleases. The cDNAs from recombinant plasmids pB/VMRI VP2 digested with restriction enzyme Pst I (lane 2), pVL/VMRI VP4 digested with restriction enzymes Bam HI and Eco RI (lane 4), pVL/VMRI VP6 digested with restriction enzyme Bam HI (lane 6), pVL/VMRI VP7 digested with restriction enzymes Bam HI and Eco RI (lane 9), and 1 kb size marker (Lane L) were analyzed by gel electrophoresis.
Fig. 7. Insect cells BTI-TN-5B1-4 infected with recombinant baculoviruses Bac.pB/VP2 (2), Bac.pVL/VP4 (4), Bac.pVL/VP6 (6), Bac.pVL/VP7 (7), wild type baculovirus AcMNPV (W) or insect cell control (H). Cytopathic effect of recombinant virus Bac.pVL/VP4 showed extensive fusion activity and Bac.pVL/VP7 recombinant showed moderate fusion activity. Recombinant baculoviruses Bac.pB/VP2 and Bac.pVL/6 did not show any fusion activity in insect cells. The occlusion positive wild type baculovirus showed polyhedra in insect cells.
Recombinant baculoviruses were purified by three rounds of plaque purification and positive clones were selected for the presence of occlusion negative CPE (Fig. 7). Further confirmation was done by indirect immunofluorescence by staining with hyper-immune serum to the VMRI strain of BRV (Fig. 10).

Detection of rotavirus specific nucleic acid in recombinant baculoviruses

Nucleic acid from recombinant baculoviruses Bac.pVL/VP2, Bac.pVL/VP4, Bac.pVL/VP6 and Bac.pVL/VP7 were hybridized with probes derived from their corresponding genes. The VP2 gene probe only reacted with nucleic acid from insect cells infected with Bac.pB/VP2 but not with nucleic acid from cells infected with wild type baculovirus or other recombinant baculoviruses. Similarly, VP4, VP6 and VP7 genes specifically hybridized to nucleic acid from cells infected with recombinant baculovirus Bac.pVL/VP4, Bac.pVL/VP6 and Bac.pVL/VP7, respectively. As a control pKS+ vectors containing VP2, VP4, VP6 and VP7 genes showed strong positive signals with corresponding gene probe, but the probes did not react with vector without insert (Fig. 8).

Characterization of recombinant baculovirus and proteins expressed in insect cells

All recombinant baculoviruses were cytolytic and plaques were occlusion negative. Based on cytopathic effects in insect cells recombinant baculoviruses Bac.pB/VP2 and Bac.pVL/VP6 did not possess any fusion activities in the insect cells infected with those recombinant baculoviruses. Insect cells infected with recombinant virus Bac.pVL/VP4 showed extensive cell fusion activity (Fig. 7). The recombinant virus Bac.pVL/VP7 also showed weak fusion activity in insect cells which was not expected from the VP7 proteins. But there is no clear evidence which protein(s) are
Fig. 8. Dot hybridization of nucleic acids from insect cells infected with recombinant baculovirus with α^{32}P[dCTP] labeled gene specific probes of bovine rotavirus. Nucleic acid from insect cells infected with Bac.pB/VP2 (2A and 2a), Bac.pVL/VP4 (4A and 4a), Bac.pVL/VP6 (6A and 6a), Bac.pVL/VP7 (7A and 7a), wild type baculovirus AcMNPV (WA and Wa), phagemid vector pKS^+ (PA), and phagemid vector (Pa) with corresponding gene insert were applied on nylon membrane and hybridized. Probes used were from VP2 gene (R2), VP4 gene (R4), VP6 gene (R6) or VP7 gene (R7).
Fig. 8. (Continued).
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Fig. 8. (Continued).
Table 2. Titers of recombinant viruses and occlusion positive baculovirus AcMNPV.

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<td>$10^{8.5}$ FFU/ml</td>
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<tr>
<td>Bac.pVL/VP7</td>
<td>$10^{8.0}$ FFU/ml</td>
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<td>$10^{8.5}$ TCID&lt;sub&gt;50&lt;/sub&gt;/ml</td>
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<tr>
<td>VMRI</td>
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* All recombinant baculoviruses were titrated in insect cells, whereas VMRI strain of bovine rotavirus was titrated in MA-104 cells by indirect immunofluorescence.

responsible for receptor binding. Rotavirus specific inserts in recombinant baculoviruses were confirmed by PCR amplification using specific primer sets to the corresponding genes. Inserts of 2.7 kb, 2.36 kb, 1.36 and 1.1 kb DNA were amplified from insect cells infected with recombinant baculoviruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6 and Bac.pVL/VP7, respectively (Fig. 9).

Titers of recombinant viruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 were determined in insect cells BTI-TN-5B1-4 (Table 2). As a control, wild type baculovirus AcMNPV was also titrated under the same conditions. Titers of these recombinant viruses are shown in Table 2 and were titrated by an indirect immunofluorescent assay using guinea pig hyperimmune serum to the VMRI strain of BRV. The wild type baculovirus titer was determined by CPE with formation of typical polyhedron in insect cells (Fig. 7). Recombinant virus titers were $10^{7.5}$ FFU/ml for Bac.pB/VP2, $10^{8.0}$ FFU/ml for Bac.pVL/VP4, $10^{8.5}$ FFU/ml for Bac.pVL/VP6 and
Fig. 9. PCR amplification of bovine rotavirus VP2, VP4, VP6 and VP7 genes from insect cells infected with recombinant baculovirus Bac.pB/VP2 (lane 2), Bac.pVL/VP4 (lane 4), Bac.pVL/VP6 (lane 6), Bac.pVL/VP7 (lane 9), and wild type baculovirus AcMNPV (lane W). Lane L contains 1 kb size markers. The VP2, VP4, VP6 or VP7 gene-specific primers (Table 1) were used for PCR amplification.
10^8.0 FFU/ml for Bac.pVL/VP7, respectively. There was no significant difference in virus titers among recombinant viruses. As a control, the titer of wild type baculovirus was determined by the presence of occlusion positive CPE in insect cells. As shown in Table 2 the wild type baculovirus titer was 10^8.5 TCID50/ml by CPE, and the titer of VMRI strain of BRV in MA-104 cells was 10^6.0 FFU/ml using an indirect immunofluorescent test (Fig. 10). The titers of the recombinant viruses were slightly lower than that of the wild type baculovirus. Recombinant virus titers in insect cells were about 2 log 10 higher than that of the VMRI strain of BRV in MA-104 cells.

**Characteristics of recombinant proteins**

Radioimmunoprecipitation was carried out to characterize expressed recombinant proteins. The molecular mass of the recombinant protein VP2 expressed in insect cells infected with recombinant virus Bac.pBA/VP2 was 102.4 kDa. An additional slower migrating minor band of approximately 115 kDa was observed (Fig. 11-R2).

Recombinant baculovirus Bac.pVL/VP4 gave a heavy precipitate of an 87 kDa polypeptide band and additional weak bands which migrated faster than the VP4 recombinant protein and had a molecular mass of 60 kDa and 28 kDa. These bands presumably are VP5 and VP8, the cleaved products of VP4. This cleavage might occur due to the presence of cellular proteolytic enzymes. The molecular mass of VP8 corresponded to that of the NCDV VP8 protein which is located on the amino terminal portion of VP4. Based on sequence analysis, the putative cleavage sites were located at amino acid sites 241 and 246 (Fig 12). Additional minor band 8a was also detected which is possibly generated by cleavage at amino acid residue 231 which is a putative cleavage site in VP4 of VMRI strain BRV (Mummidi et al. 1995). These results are supported by the predicted amino acid sequences of VMRI VP4 and partial amino acid
Fig. 10. Indirect immunofluorescent antibody test to detect bovine rotavirus antigen in insect cells infected with recombinant baculovirus Bac.pB/VP2 (panel 2), Bac.pVL/VP4 (panel 4), Bac.pVL/VP6 (panel 6), Bac.pVL/VP7 (panel 7), wild type baculovirus AcMNPV (W) or insect cell control (H). VMRI strain of BRV in MA-104 cell (V) or MA-104 cell control (M) were also tested by IFA. Anti-bovine rotavirus polyvalent guinea pig sera and FITC labeled anti-guinea pig Igs were used to stain for rotavirus proteins.
Fig. 11. Radioimmunoprecipitation of $^{35}$S-labeled proteins in cell lysates of insect cells infected with wild type baculovirus (lane W) or recombinant baculovirus Bac.pB/VP2 (lane 2), Bac.pVL/VP4 (lane 4), Bac.pVL/VP6 (lane 6), or Bac.pVL/VP7 (lane 7) with antisem to rotavirus. Recombinant baculoviruses were labeled with 200 μCi of $^{35}$S-methionine/cysteine for 2 hours at 72 hour post infection. Rotavirus specific recombinant proteins were precipitated with hyperimmune serum to VMRI strain of bovine rotavirus and electrophoresed. All recombinant proteins detected in insect cells infected with recombinant baculovirus were similar to that of original rotavirus proteins.
Fig. 11. (Continued).
Fig. 11. (Continued).
Fig. 11. (Continued).
37.3 KDa
Fig. 12. Partial amino acid sequence of VMRI VP4 showing location of putative proteolytic cleavage sites at amino acid residues 241 and 246 in VMRI strain of rotavirus (bold). In VP4 of VMRI strain there is one additional arginine at amino acid residue 247 (italic) and an extra putative cleavage site at amino acid residue 231 (bold and italic).
sequences which correspond to this region as shown in Fig. 11-R4. Recombinant protein VP6 had a molecular mass of 60 kDa similar to that of the prototype bovine rotavirus VP6 and was the most abundant protein expressed in the insect cell system (Fig. 11-R6). The recombinant protein VP7 showed a heavy precipitate of a diffuse band of approximately 37 kDa in size. This diffuse band indicates that the recombinant protein expressed in insect cells is glycosylated (Fig. 11-R7). Details of these results are described in a later section.

Kinetics of recombinant protein expression

To determine the kinetics of recombinant protein expression, insect cells were infected with recombinant baculovirus and proteins in cell lysates were immunoprecipitated with anti-VMRI hyperimmune serum at 24, 48, 72 and 96 hours post infection. At 96 hours, because of extensive CPE, there were not enough cells remaining for RIP, therefore data was not reliable at this time point. Radioactivity in rotavirus specific bands was counted and values are shown in Fig. 13. These results indicate recombinant protein expression was highest at 72 hours post infection and declined at 96 hours post infection possibly due to the extensive CPE in recombinant virus infected insect cells (Fig. 13). The recombinant protein expression in insect cells infected with Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 infected insect cells was highest at 72 hours post infection (Fig. 13).

Antigenicity of recombinant proteins for guinea pigs

Immune response of guinea pigs to recombinant proteins was determined by the indirect immunofluorescence and virus neutralization tests. Weak immune response was detected by indirect immunofluorescence at 3 weeks after the first immunization.
Fig. 13. Kinetics of recombinant protein expression in insect cell were determined by radioimmunoprecipitation. Insect cells were infected with recombinant viruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, or Bac.pVL/VP7. Proteins were labeled with $^{35}$S-methionine/cysteine and immunoprecipitated with hyper-immune serum to VMRI strain of bovine rotavirus and electrophoresed. Rotavirus specific bands were cut from the gel by superimposing X-ray film on the dried gel. Radioactivity in each band was counted in a liquid scintillation counter. Mean values of radioactivity was plotted against time.
CPM (Log10)

- Supernatant
- Cells

Hours post infection with recombinant baculovirus Bac.pB/VP2

CPM (Log10)

- Supernatant
- Cells

Hours post infection with recombinant baculovirus Bac.pVL/VP4
Fig. 13. (Continued).
Hours post infection with recombinant baculovirus Bac.pVL/VP6

Hours post infection with recombinant baculovirus Bac.pVL/VP7
Serum from animals immunized with Bac.pB/VP2, Bac.pVL/VP4, and Bac.pVL/VP7 had titers of 20. The antibody titer to Bac.pVL/VP6 was higher than the others (1:320 - 1:640). In contrast, serum antibody titers of guinea pigs immunized with the VMRI strain of BRV was 1:40 - 1:80. Guinea pigs immunized with the wild type baculovirus AcMNPV remained seronegative. Antibody titers increased following the booster injection. The IFA titers in these animals were 1:640 - 1:1280, 1:2560 - 1:5120, 1:1280 - 1:2560, 1:640 - 1:1280, 1:640 - 1:1280 for Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, Bac.pVL/VP7, and BRV VMRI, respectively. Guinea pigs inoculated with wild type baculovirus remained seronegative (Fig. 14). Serum samples were also tested for rotavirus specific neutralization antibodies. Preimmune serum samples were negative for neutralization antibodies. The virus neutralization antibodies were detected in serum of animals immunized with recombinant baculovirus Bac.pVL/VP4, Bac.pVL/VP7, or VMRI strain of BRV immunized animals. Virus neutralization titers ranged from 1:4 - 1:16 at 3 weeks after immunization. Sera from guinea pigs inoculated with Bac.pB/VP2, Bac.pVL/VP6, or wild type baculovirus were negative for virus neutralization antibodies at 3 weeks after the first immunization. Virus neutralization titers increased 3 weeks after booster injection and ranged from 64 to 128 in guinea pigs immunized with Bac.pVL/VP4, Bac.pVL/VP7. Titers in guinea pigs immunized with VMRI strain of BRV ranged from 32 to 128. Low titers of virus neutralization activity was also detected in animals immunized with Bac.pVL/VP6. Three weeks after the second immunization guinea pigs immunized with recombinant baculovirus Bac.pB/VP2 and wild type baculovirus AcMNPV remained seronegative (Fig. 15). The statistical analysis of the data by Duncan's multiple range test showed that the virus neutralization titers of guinea pigs immunized with recombinant baculovirus Bac.pVL/VP4.
Fig. 14. Indirect immunofluorescent antibody titers of serum samples from guinea pigs immunized with recombinant baculovirus. Guinea pigs were given booster injection at 3 weeks after the initial immunization. The blood samples were collected before immunization and at 3 and 6 weeks post injection. The data were analyzed using one-way ANOVA with repeated measures. P<0.0001
Fig. 15. Virus neutralization titers of serum samples from guinea pigs immunized with recombinant baculovirus. VMRI strain of bovine rotavirus or wild type baculovirus are plotted vs time (weeks) after initial immunization. Two hundred FFU of bovine rotavirus was mixed with serum samples and incubated for 60 minutes. Unneutralized virus was titrated in MA-104 cells. The data were analyzed using one-way ANOVA with repeated measures. P<0.0001
Bac.pVL/VP7 and the VMRI strain of BRV were significantly different from those immunized with Bac.pB/VP2, Bac.pVL/VP6, or AcMNPV (P<0.05).

**VLP formation**

Insect cells were infected with recombinant baculovirus and cultures were examined for virus-like particles. Infection with recombinant baculovirus Bac.pB/VP2 generated sub-core like particles (Fig. 16). The size of these subcore particles ranged from 35-45 nm in diameter. Rotavirus-like particles without cores were formed in cells infected with the four recombinant viruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 (Fig. 16). The VLPs shown in these figures were purified and incubated with anti-VMRI hyperimmune serum followed by negative staining and examined under electron microscope. These empty particles were similar to the rotavirus particles purified from a tissue culture grown VMRI strain of BRV (Fig. 16). A long tubular structure approximately 70 nm in diameter was also observed (Fig. 16). VLPs or tubular structures were not detected in cells infected with recombinant viruses Bac.pVL/VP4, and Bac.pVL/VP7. This indicates that for a VLP formation, recombinant proteins expressed from Bac.pB/VP2, and Bac.pVL/VP6 need to be included. The size of the VLPs was 70-75 nm in diameter which is similar to the rotavirus particle. The inside diameter of an empty virus-like particle was 42-46 nm which was equivalent to that of the sub-core particles (Fig. 16). The empty hollow structure was icosahedral in shape.

**Inhibition of receptor binding by recombinant proteins**

To identify the receptor binding protein(s) of the rotavirus, lysates of insect cells infected with recombinant baculovirus were incubated with MA-104 cells followed by
Fig. 16. Electronmicrographs of rotavirus propagated in mammalian cells MA-104 (A), virus-like particles (VLP) observed in insect cells infected with recombinant baculovirus Bac.pB/VP2 (B), and cells co-infected with recombinant baculovirus Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6 and Bac.pVL/VP7 with sizes approximately 70-75 nm in diameter (C and D). A tubular structure was also observed and the diameter of tubular structure was similar to the size of VLPs (C). The inside diameter of empty VLPs with icosahedral symmetry was 42-46 nm in diameter (D).
Fig. 16. (Continued).
Fig. 16. (Continued).
Fig. 16. (Continued).
Table 3. Rotavirus receptor blocking assay in MA-104 cells by using recombinant viruses.

<table>
<thead>
<tr>
<th>Recombinant Baculovirus</th>
<th>Number of Foci</th>
<th>Percentage of Blocking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac.pB/VP2</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td>Bac.pVL/VP4</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>Bac.pVL/VP6</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>Bac.pVL/VP7</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>Bac.pB/VP2+Bac.pVL/VP6</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>Bac.pVL/VP4+Bac.pVL/VP7</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>Bac.pB/VP2+Bac.pVL/VP4+Bac.pVL/VP6</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td>Bac.pB/VP2+Bac.pVL/VP6+Bac.pVL/VP7</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>VP2+Bac.pVL/VP4+Bac.pVL/VP6+Bac.pVL/VP7</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>VLPs@</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>AcMNPV</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>BTI-TN-5B1-4</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

MA-104 cells were preincubated with recombinant baculovirus cell lysates for 2 hours at 37°C then 100 FFU of VMRI strain of BRV was added and cells incubated for 18 hours. Number of fluorescent foci were counted after indirect immunofluorescent staining using guinea pig anti-VMRI antiserum and FITC-labeled anti-guinea pig IgG. Numbers represent arithmetic means of foci counted from each treatment.

+: recombinant viruses co-infected into insect cells.

@: Virus-like particles.

1: wild type baculovirus.

2: Trichoplusia ni egg cell homogenates cell line.
Table 4. Effect of trypsin treatment of recombinant proteins on blocking of rotavirus binding to MA-104 cells.

<table>
<thead>
<tr>
<th>Recombinant Baculovirus</th>
<th>Number of Foci</th>
<th>Percentage of Blocking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac.pVL/VP4</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>Bac.pVL/VP4+Bac.pVL/VP7</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>Bac.pB/VP2+Bac.pVL/VP4+Bac.pVL/VP6</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>Bac.pB/VP2+Bac.pVL/VP4+Bac.pVL/VP6+Bac.pVL/VP7</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>AcMNPV¹</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>BTI-TN-5B1-4²</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Recombinant protein VP4 individually or in combination with other proteins was treated with 10 μg/ml of type IX trypsin (Sigma, St. Louis, MO) at 37°C for 60 minutes. MA-104 cells were preincubated with trypsin treated recombinant protein(s) followed by infection with 100 FFU of VMRI strain of BRV. Number of fluorescent foci were counted after indirect immunofluorescent staining using guinea pig anti-VMRI antiserum and FITC-labeled anti-guinea pig IgG.

+: recombinant viruses co-infected into insect cells.
1: wild type baculovirus.
2: Trichoplusia ni egg cell homogenates cell line.
Fig. 17. Blocking of rotavirus receptors in MA-104 cells using recombinant proteins expressed in baculovirus. Recombinant virus Bac.pVL/VP4, Bac.pVL/VP7, and wild type baculovirus AcMNPV were used to block rotavirus receptors in MA-104 cells followed by infection with 100 FFU of VMRI strain of BRV. Rotavirus specific fluorescent foci were counted by IFA at 20 hours after rotavirus infection.
addition of 100 FFU of rotavirus. The ability of recombinant proteins to block binding of rotavirus to MA-104 cells was measured. As shown in Table 3, baculovirus expressed rotavirus proteins VP4 and VP7 independently blocked infection of MA-104 cells with the VMRI strain of BRV. But VP2 and VP6 did not inhibit the rotavirus infection of MA-104 cells (Table 3). As shown in table 4, trypsin treatment did not effect the blocking of receptors by recombinant proteins in MA-104 cells. Recombinant baculoviruses Bac.pVL/VP4 and Bac.pVL/VP7 with titer of $10^{8.0}$ TCID$_{50}$/ml were able to block greater than 90% of rotavirus receptors in $10^5$ cells/ml of MA-104 cells (Fig. 17). The numbers representing fluorescent foci from 100 FFU of VMRI strain of BRV infected MA-104 cells. Purified VLPs most effectively blocked rotavirus receptors on MA-104 cells. VLPs concentrated one hundred times from supernatant containing recombinant baculovirus at approximately $10^{8.0}$ FFU/ml were diluted 10 fold and added to MA-104 cell ($10^{5.0}$ cells/ml). The saturation point was found to be $10^{8.0}$ FFU/ml for $10^{5.0}$ cells/ml (Fig. 17). A thousand fold diluted VLPs were able to block about 50% of the rotavirus receptors in MA-104 cells. These results indicate that MA-104 cells have as many as $10^3 - 10^3.5$ receptors per cell. Cell lysate prepared from insect cells infected with recombinant baculovirus singly or in combination did not influence virus blocking on MA-104 cells (Table 3). Results of blocking of virus infectivity on MA-104 cells with recombinant baculovirus cell lysates or purified virus-like particles were similar.

Discussion

Recombinant rotavirus proteins VP2, VP4, VP6 and VP7 were successfully expressed in baculovirus expression system. We were able to detect rotavirus specific antigens in cells infected with recombinant baculovirus containing these genes by indirect
immunofluorescence and radioimmunoprecipitation. Reactivity of antisera was specific to rotavirus as the antisera to rotavirus did not react with uninfected cells or cells infected with wild type baculovirus. Similarly, preimmune sera also did not react with insect cells infected with recombinant baculoviruses. Characterization of these recombinant proteins by radioimmunoprecipitation further showed that VP2, VP4, VP6 and VP7 were successfully expressed in insect cells.

Recombinant proteins expressed in the baculovirus system appeared authentic and were similar to that of rotavirus proteins. The molecular mass of recombinant protein VP2 expressed in insect cells was approximately 102 kDa which is similar to that of the VP2 protein in group A rotavirus (Mattion et al., 1994). A slower migrating weak band was detected by RIP with lysates of cells infected with Bac.pB/VP2. This band possibly represents a heavy glycosylation product which is common in baculovirus expressed proteins. Sequence analysis shows that there are two potential N-glycosylation sites at amino acid residues 590 and 845 in VP2 of the bovine rotavirus (Tian et al., 1990).

The molecular mass of 88 kDa of recombinant protein VP4 was also similar to that observed in rotavirus (Mattion et al., 1994) and it was the most abundant protein detected, although three minor bands were also observed. There were three minor bands observed by RIP in insect cells infected with recombinant baculovirus Bac.pVL/VP4. The two bands with Mr. of 60 and 28 kDa which are possibly represent VP5 and VP8. The VP8 has shown to possess an hemagglutinin epitope in rotavirus (Fiore et al., 1991). The VP5* is a 60 kDa protein which is located on the carboxy terminal side of VP4 (Mattion et al., 1994). The third minor band designated VP8a, is possibly generated due to the presence of an additional trypsin cleavage site found at amino acid residue 231 in the VMRI strain of BRV. This study that the predicted trypsin
cleavage site at amino acid residue 231 may be active. The abundance of VP4 protein expressed in insect cells was unexpected as in previous RIP studies of rotavirus grown in mammalian cells (Estes, 1990). In this study, the baculovirus expressed recombinant VP4 protein showed very clearly an advantage in protein expression over the mammalian cell system. Using the recombinant protein expression in insect cells, we have successfully demonstrated that the arginine at aa 231 of the VP4 is an active cleavage site for cellular proteolytic enzymes and insect cells have the ability to process recombinant rotavirus protein in the same manner as mammalian cells. An additional putative proteolytic enzyme cleavage site at aa 231 found in the VP4 gene sequence was confirmed as a proteolytic enzyme cleavage site.

The molecular mass of 45 kDa recombinant protein VP6 was also similar to that in rotavirus and propagated in mammalian (Mattion et al., 1994). It was the most abundantly expressed protein amongst VP2, VP4, VP6 and VP7 in insect cells (data not shown). The mechanism for higher expression of recombinant protein VP6 over the other rotavirus genes in the insect cell system is not understood. The VP6 protein is also the most abundant viral protein in the rotavirus virion which is composed of over 60% of viral proteins.

The recombinant VP7 also appeared authentic as its molecular mass was 38 kDa which is similar to that of native rotavirus protein. The recombinant protein VP7 shown in Fig. 11 indicates that the protein expressed in baculovirus is post translationally modified and shows the advantage of the eukaryotic expression system over prokaryotic expression. Based on similarity in molecular mass and glycosylation in VP7, we expect that the recombinant VP7 will have biological activities similar to that of VP7.

Kinetic studies of recombinant protein expression allowed us to estimate an optimal time point for harvesting recombinant virus to get a maximum yield of
recombinant proteins. In addition, this study provide information on secretion of recombinant protein into the culture medium from recombinant virus infected cells. Previous reports indicate that the expression of rotavirus recombinant proteins in insect cells peaked at 48 hours after infection and low yields of VP7 protein expression was attributed to the secretion of the protein into the culture media (Redmond et al., 1993). They also reported that VP7 protein expression peaked at 24 hours post infection in Sf9 cells and 96 hours post infection in the supernatant of the recombinant virus infected insect cells. But in this study the highest expression of recombinant protein VP7 was at 72 hours after infection in Hi-Five cells. Recombinant proteins were not secreted into the culture medium from any of recombinant viruses during the early stage of infection. We were able to detect recombinant proteins in medium during the late stages of infection but it coincided with extensive CPE in the recombinant virus infected insect cells rather than protein secretion into medium. Lack of protein secretion in the medium is common for proteins expressed in insect cell system (Wickham et al., 1992).

Two different types of CPE were observed in recombinant virus infected insect cells provided some functional characteristics of rotavirus proteins. One type of CPE was cell rounding followed by cell lysis and the other type was fusion of the insect cells followed by cell lysis. Recombinant baculovirus containing genes, VP4 or VP7 produced cell fusion. The fusion activity was greatest with recombinant baculovirus Bac.pVL/VP4, which suggesting that it is the primary protein responsible for fusion. These finding is in agreement with those reported previously (Estes et al., 1989; Mattion et al., 1994). The recombinant virus VP7 also showed weak fusion activity in insect cells which was unexpected. This activity may indicate that VP7 is a putative cell receptor binding protein in mammalian cells. Competition assay confirmed that VP7 does play a role in rotavirus binding to cells. In a receptor blocking assay using
recombinant viruses, both VP4 and VP7 blocked rotavirus infection in MA-104 cells, but VP2 and VP6 recombinant viruses did not block infection. But there is no clear evidence which protein(s) are responsible for receptor binding. Since only triple layered particles are infectious it may be possible that both VP4 and VP7 proteins are responsible for receptor binding because these two proteins comprise the outer layer of the virion.

Current research results indicate that baculovirus expressed viral proteins self assemble into virus-like particles and are immunogenic in experimental animals (French and Roy, 1990; Luo et al., 1990; Sabara et al., 1991; Loudon and Roy, 1991; Jiang et al., 1992; Laurent et al., 1994; Prasad et al., 1994). In this study we have successfully produced virus-like particles by using combinations of recombinant viruses expressing rotavirus proteins. Single infection of insect cells with Bac.pB/VP2 produced sub-core particles and the size of these particles was similar to that of BRV subcores as reported previously (Labbe et al., 1991; Zeng et al., 1994). Co-infection of insect cells with recombinant viruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 formed rotavirus like particles, without the core structures containing VP1. VP3 and segments of rotavirus nucleic acid. Morphology and size of the empty particles was similar to that of rotavirus particles purified from a tissue culture grown VMRI strain of BRV. Similar results with rotavirus capsid proteins expressed in insect cells has been reported (Crawford et al., 1994). Additionally the tubular structures have been observed in insect cells infected with recombinant baculoviruses. Recombinant viruses containing VP4 and VP7 genes singly or in combination did not form any VLPs or tubular structures. This indicates that VP2 and VP6 are required for VLP formation. The size of VLPs was 70-75 nm in diameter which is similar to the rotavirus particles purified from mammalian cell cultures or virus samples processed from fecal materials (Prasad et al., 1988; Yeager et al., 1990). The empty center holes were 42-46 nm in diameter which is
equivalent to core particles. Icosahedral symmetry of the empty hollow structures indicate that protein assembly in baculovirus expressed recombinant protein is similar to the rotavirus assembly process in mammalian cells and infected animals. This protein expression technology is useful for investigating viral protein assembly in individual protein levels and will provide detailed information on virus replication at the assembly stage, and on the requirement of viral proteins for this process.

Immune response to a recombinant virus in experimental animals is an important step for the evaluation of potential application to animals as a preventive vaccine. Authenticity of recombinant protein, and the amount of recombinant proteins expressed in an insect system affect the immune response. The recombinant proteins expressed in baculovirus were immunogenic in guinea pigs. The immune response was specific to rotavirus as guinea pigs immunized with wild type baculovirus were seronegative. Evaluation of sera by virus neutralization test showed that VP4 and VP7 contain the major neutralization epitopes as only not VP2 induced VN antibodies. Ability of VP4 and VP7 proteins to elicit high level of rotavirus specific virus neutralization antibodies has been controversial. Nishikawa et al. (1989) were able to induce neutralization antibodies with baculovirus expressed VP4 protein, however Redmond et al. (1993) were unable to induce neutralization antibodies in animals with recombinant baculovirus containing VP7. The reason for this discrepancy is not clear. Similarly inconsistent results were obtained with recombinant proteins expressed in a prokaryotic system. Recombinant protein expressed in prokaryotic system did not induce any neutralization antibodies whereas in another study neutralization antibodies were induced (Arias et al. 1986; Francavilla et al. 1987).

In addition to VP4 and VP7, VP6 also induced neutralizing antibodies although titers induced with recombinant VP6 were low. This observation was
surprising and new. The VP6 has shown to provide partial protection in mice. (Redmond et al., 1993). The results of this study show that ability of recombinant VP6 to induce neutralizing antibody provide in part explanation of protection observed by Redmond et al. (1993).

There has been a controversy as to which rotavirus protein is responsible for binding to culture cells. Both VP4 and an outer capsid glycoprotein have been proposed to play a role in binding to receptors on rotavirus susceptible cells (Sahara et al., 1985; Ruggeri and Greenberg, 1991). In another study (Bass et al., 1990) a nonstructural rotavirus protein suggested as the major protein responsible for binding to target cells (Bass et al., 1990). Because of the controversy surrounding the role of different proteins in binding with the receptor, we examined the role of recombinant VP4 and VP7 in cells. Our results indicate that VP4 and VP7 proteins are important for the cell binding. In this study, we have clearly shown that both VP4 and VP7 are the major proteins responsible for binding to MA-104 cells. Baculovirus expressed recombinant rotavirus proteins VP4 and VP7 independently blocked target binding of the VMRI strain of BRV to MA-104 cells, resulting in no virus infection in the cells. The virus neutralization test results also support this hypothesis (Table 16). Resistance of receptor binding activity to trypsin treatment as observed by Kaljot et al. (1988) further supported our results that VP4 and VP7 are the major proteins responsible for virus binding to cells. The number of rotavirus receptors on MA-104 cells was shown to be $10^3$-10$^{3.5}$ receptors per cells for the VMRI strain of BRV. This estimate is lower than that of 10$^{5.0}$/cell reported by Kaljot (1990). This difference in results may be due to the different passage level of susceptible cells and/or the different strain and passage levels of rotavirus.

This study has shown that recombinant proteins expressed in insect cells using baculovirus expression system are similar to that of native rotavirus proteins and have
potential as vaccine for rotavirus gastroenteritis. This technology has special role in understanding functional characteristics of rotavirus proteins and development of vaccine for noncultivable rotavirus.

Acknowledgments

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References


INCREASED EXPRESSION OF BOVINE ROTAVIRUS PROTEIN VP7 IN INSECT CELLS BY REMOVAL OF TERMINAL NONCODING SEQUENCES

A paper to be submitted to the Journal of General Virology
Young S. Lyoo and Prem S. Paul

Abstract

The glycosylated capsid protein, VP7, of rotavirus has previously been expressed in eukaryotic and prokaryotic expression systems including insect cells using baculovirus as a vector, however, expression levels have been poor. To increase the expression of VP7, we have investigated the effect of removing terminal non-coding sequences, upstream and downstream of the VP7 open reading frame (ORF), on its expression in insect cells. The VP7 gene from the VMRI strain of bovine rotavirus was amplified by polymerase chain reaction and cloned. Four constructs were generated in a baculovirus transfer vector pVL1393 to be expressed as nonfusion proteins under the control of a baculovirus polyhedrin promoter. The construct pVL/VP7.A contained a full sequence of the bovine rotavirus VMRI VP7 gene including the noncoding sequences, construct pVL/VP7.B contained the VP7 gene in which the noncoding region at 3' end was deleted, construct pVL/VP7.C was composed of the VP7 gene in which the 5' noncoding sequence was deleted, and construct pVL/VP7.D bears the VP7 gene in which noncoding sequences upstream and downstream of the VP7 open reading frame were deleted. Recombinant DNA from these four constructs was used to generate recombinant baculovirus and the expression of VP7 with each recombinant baculovirus was evaluated. Rotavirus VP7 mRNA was detected in insect cells infected with any of the four recombinant baculoviruses. Insect cells infected with recombinant baculovirus Bac.pVL/VP7.D had the highest amount of transcriptional activity. Level of protein
expression by radioimmunoprecipitation was highest with recombinant baculovirus Bac.pVL/VP7.D and was at least ten fold more than that with the other three recombinant baculoviruses. These results show that removal of non-coding sequences at both the 5' and 3' ends increased expression of recombinant protein in this system and expressed protein appeared authentic as the molecular mass and modification of proteins appeared similar to that of rotavirus.

Introduction

Rotavirus belongs to the genus rotavirus in the family Reoviridae and is an important pathogen of gastroenteritis in infants, young children and animals (Estes et al. 1983; Paul and Lyoo, 1993). Rotavirus particles are non-enveloped, triple-layered (previously called double-shelled capsid) and measure approximately 75 nm in diameter (Estes and Cohen, 1989; Zeng et al. 1994). Recent evidence based on high resolution cryoelectron microscopy (Prasad et al. 1988; Anthony et al. 1991) suggests that rotavirus particles have 60 surface spikes, about 10 nm long with a globular end structure, in the outer capsid. Viral cores contain the viral transcriptase and a genome consisting of 11 segments of double stranded RNA. Each RNA segment starts with a 5' guanidine followed by conserved sequences that are part of the 5' non-coding translational sequences, an open reading frame coding for each protein product, and another set of 3' non-coding sequences which end with a 3'-terminal cytosine (Estes and Cohen, 1989).

Rotaviruses have two major surface proteins, VP4 and VP7, which are believed to be the major immunogens of rotavirus (Matsui et al. 1990; Paul and Lyoo, 1993) as both induce neutralization antibodies, and are believed to be important in providing protection. Because of their role in neutralization, both VP4 and VP7 have been used to
type rotaviruses. Rotavirus types based on a protease sensitive protein, VP4, have been designated as P types, whereas types based on glycoprotein VP7 have been referred to as G types. The VP7 is the major neutralization glycoprotein which plays an important role in serotype specific virus neutralization and cell attachment (Greenberg et al. 1983; Hoshino et al. 1985; Sabara et al. 1985; Blackhall et al. 1992). The VP7 protein was clearly demonstrated as the dominant immunogen among the rotavirus proteins in infants using bovine rotavirus as a vaccine and in experimental animals inoculated with inactivated rotavirus (Ward et al. 1990). VP7 is glycosylated with a high concentration of mannose and is comprised of 326 amino acids. After cleavage of the signal peptide, the mature cleaved protein contains 276 amino acids with a molecular mass of 38 kDa. Nucleotide sequence analysis has identified six regions on VP7 which are highly conserved among different rotavirus strains of the same serotype, but are divergent among different serotypes (Pedly et al. 1983; Glass et al. 1985; Green et al. 1987). Three of these regions encode for neutralization epitopes A, B and C as shown by studies using escape mutants. Region A is located at amino acid residues 87-101, region B at amino acid residues 145-152, and region C is located at amino acid residues 211-223 (Dyall-Smith et al. 1986). Using recombinant VP7 protein and a synthetic peptide (aa 275-295), VP7 has been shown to produce neutralization antibodies and provide passive protection in mice against rotavirus infection (Matsui et al. 1989). Passive immunity in calves is considered to be very important in protection against rotavirus infection (Snodgrass, 1982). Recombinant VP7 offers a potentially effective vaccine for rotavirus associated gastroenteritis in calves.

In a preliminary study, we attempted expression of VP7 of a bovine rotavirus in insect cells, using the baculovirus expression system. We were successful in its expression, however expression was poor. Therefore, in order to increase VP7
expression the effect of removal of noncoding sequences was examined. Our results show that expression of VP7 is increased following removal of noncoding sequences. The expressed recombinant protein appears to be authentic as it is glycosylated, and has a molecular mass similar to that of native VP7, it also reacts with antisera to bovine rotavirus.

Materials and Methods

*Virus and cells*

The VMRI strain of bovine rotavirus with a super-short electrophoretic pattern (Paul et al. 1988; Matsui et al. 1990) was used as a parent virus for this experiment. This strain has a P5 and a G6 type. The plaque purified virus was propagated on monolayers of monkey kidney cells (MA-104) in the presence of 0.5 μg/ml of crystal trypsin (Type IX, Sigma St. Louis. MO) to improve virus replication. The MA-104 cells were washed three times with serum free Dullbeco’s minimal essential medium (DMEM, Gibco-BRL. Gaithersburg, MD) and inoculated with the VMRI strain of bovine rotavirus at a multiplicity of infection (MOI) of 0.1. The virus-inoculated MA-104 cells were incubated at 37°C in a CO₂ incubator for 60 minutes with occasional rocking to increase the virus infection. After a 60 minute incubation, the viral inoculum was removed and the cells were washed once with serum free DMEM to remove residual viral inoculum. then fresh DMEM containing 0.5 μg/ml of trypsin as maintenance media was added. The cells were incubated for three days in a CO₂ incubator at 37°C. The infected cell cultures were frozen and thawed three times, and the virus was then pelleted by high speed centrifugation for 90 minutes at 100,000 Xg. The viral pellet was extracted in 1,1,2-
Trichloro-1,2,2-trifluoroethane (Genetron, Johnson Matthey Electronics, MA) and virus in the aqueous phase was collected after low speed centrifugation for 20 minutes at 3000 rpm. To pellet virus particles, supernatant was centrifuged for 90 minutes at 25,000 rpm and the pellets were resuspended in 500 µl of sterile deionized water. The resuspended sample was layered on a continuous cesium chloride density gradient (1.2 - 1.4 mg/ml) for further purification. After a 4 hour centrifugation, the virus bands were collected and their density was measured and dialyzed against PBS at 4°C overnight. An aseptically collected dialyzed virus sample was treated with EDTA at a final concentration of 50 mM for 2 hour at 37°C and core particles were pelleted through a 30% sucrose cushion at 25,000 rpm for 60 minutes. The pellet was resuspended with 100 µl of sterile water and stored at -80°C for the \textit{in vitro} transcription of viral mRNA.

\textit{In vitro} mRNA transcription and first strand cDNA synthesis

The viral mRNA was transcribed \textit{in vitro} by using virus containing RNA dependent RNA polymerase from purified rotavirus core particles as described previously (Flores et al. 1982). The reaction mixtures were prepared in 2X transcription buffer (100 mM of Tris-HCl, 0.5 mM of NaOAc, 30 mM of MgOAc, pH 8.0) containing ATP (8 mM), CTP (2.5 mM), GTP (2.5 mM), UTP (2.5 mM), S-adenosyl-methionine (0.5 mM), RNase inhibitor, and the purified viral cores. The reaction mixture was incubated for 6 hours at 42°C and newly synthesized mRNA was precipitated with lithium chloride. The first strand cDNA was synthesized with a cDNA cycle kit (Invitrogen, San Diego, CA) using mRNA as a template. VP7 gene specific primers and avian myeloblastosis virus (AMV) reverse transcriptase (Invitrogen, San Diego, CA). After completion of the cDNA synthesis the final product was extracted with phenol/chloroform, concentrated by ethanol precipitation and redissolved in 10 mM
Tris-HCl buffer (1 mM EDTA, pH 8.0). The first strand cDNA was used as a template for PCR amplification of the bovine rotavirus VP7 gene and construction of vectors for expression.

PCR amplification of bovine rotavirus genes

For the amplification of the full length VMRI VP7 gene, or the VP7 gene without part or all of the non-coding sequences, primers were designed based on the VP7 gene sequence of bovine rotavirus strain VMRI (Fig. 1). Restriction endonuclease cleavage sites and additional guanidines were added to facilitate the construction of the transfer vector and for protection of the restriction sites. Primers used in this experiment were P1 as a 5' terminal primer (GGG GAT CC G GCT TTA AAA GAG AGA ATT T), P2 as a 5' internal primer with tataaat sequence (GGGGGA TCC tataaatAT G TA TGG TAT TGA A), P3 as a 3' terminal primer (GGG AAT TC G GTC ACA TCA TAC TAA TCC), and P4 as a 3' internal primer (GGG AAT TC T TAT TTG ATT GAC GTA ATC C). These primers were used for the amplification of full or part of the genome without the non-coding regions in a PCR reaction. Briefly, the VP7 gene of bovine rotavirus isolate VMRI was amplified using first strand cDNA as a template in a PCR reaction with Taq-1 DNA polymerase (Invitrogen, San Diego, CA), and various sets of primers. Reaction mixtures containing cDNA template, dNTPs (0.2 mM/each, Pharmacia), primers (2 μM), MgCl2 (3 mM, Pharmacia) and 10X reaction buffer (Invitrogen, San Diego, CA) were prepared in an ultraviolet light sterilized vertical laminar flow hood to prevent nonspecific DNA amplification from contamination. The reaction mixture was overlaid with 50 μl of mineral oil to prevent evaporation during the PCR reaction. PCR amplification was conducted in a thermocycler (Coy, MI). The first cycle consisted of 90 seconds for denaturation at 94°C, 2 minutes for annealing at 48°C and 5 minutes for...
extension at 72°C. It was then followed by 30 cycles of amplification with denaturation at 94°C for 1 minute, annealing at 48°C for 2 minutes, and primer extension at 72°C for 2 minutes. During the last cycle, the extension time was changed to 10 minutes while the other conditions remained the same. The amplification products were analyzed by electrophoresis in a 1% agarose gel (FMC Bioproducts, Rockland, ME) followed by ethidium bromide staining. A DNA band of 1.06 kb was observed for the full length VP7 gene with other bands migrating slightly faster than 1.06 kb. The DNA samples were purified using a sephacyl column (Boehringer Mannheim Biochemicals, Indianapolis, IN) and purified DNAs were used for cloning and sequencing experiments.

**Molecular cloning and construction of baculovirus transfer vectors**

To determine the nucleotide sequence of the VP7 gene, PCR-amplified double stranded cDNA was cloned in an E. coli phagemid vector. A full length VMRI VP7 gene was inserted into phagemid vector pKS+. After transformation into DH5α cells, white colonies were selected on agar plates in the presence of ampicillin, X-gal and IPTG. The nucleotide sequence was determined by the Sanger dideoxy chain termination method (Hindley, 1983) using an automated DNA sequencer (Applied Biosystem, Foster city, CA). Three clones were sequenced (Mummidi et al. 1995, submitted for publication). The sequence was analyzed and translated into amino acids using a computer program (Geneworks, Intelli Genetics Inc., Mountain View, CA).

To construct baculovirus transfer vectors, PCR amplified DNA samples were digested with BamHI and EcoRI and purified by using a CL-4B sephacyl column (Boehringer Mannheim, Indianapolis, IN, size exclusion >271 base pair). The baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA) was digested with BamHI and EcoRI, and purified by using a CL-4B sephacyl column for the ligation
using T4 ligase. Tubes containing the ligation mixture were incubated at 5°C overnight and transformed into competent cells of *E. coli* strain DH5α. Colonies of *E. coli* containing recombinant plasmids were analyzed for the presence of inserts by restriction endonuclease digestion with Bam HI and Eco RI of DNA, followed by electrophoresis in agarose. Selected colonies were cultured on agar plates and single colonies were inoculated into 150 ml of LB broth to prepare the DNA samples for transfection to generate recombinant viruses. DNA was purified by passing through Qiagen columns (Qiagen, Hilden, Germany) following manufacturer's instructions and quantitated by spectrophotometry at 260 nm. Constructs prepared from these ligation were derived from PCR products using various primers: construct A with P1 and P2, construct B with P1 and P3, construct C with P2 and P4, and construct D with P2 and P3 sets of primers. The P3 primer was designed upstream of the stop codon and the stop codon was altered from TAG to TGA. Therefore, the recombinant protein product is expected to be 11 amino acids shorter than that of the full length VMRl VP7 gene product.

*Production of recombinant baculovirus by homologous recombination*

The DNA from recombinant baculovirus transfer vector pVL1393 containing the VP7 gene and wild type baculovirus were used for transfection of insect cells to produce recombinant virus. A 50 μl mixture composed of 50 ng of linearized baculovirus AcMNPV DNA and 1.5 μg DNA from different constructs containing VMRI VP7 gene, were mixed with 50 μl of lipofectin (Gibco-BRL, Gaithersburg, MD) and incubated at room temperature for 15 minutes. Meanwhile, Hi-Five (BTI-TN-5B1-4; Invitrogen, San Diego, CA) insect cells were prepared in 6-well cell culture plates, at a cell concentration of 3X10^5/ml. and placed on a flat surface for 60 minutes at 27°C to allow the cells to attach to the culture vessel. After 60 minutes of incubation, new insect cell culture
medium was added and the DNA/lipofectin mixture was added drop by drop. For efficient transfection, cells were left undisturbed in a vertical laminar flow hood for 6 hours at room temperature. The DNA solution used for transfection was removed by aspiration after 6 hours incubation and 3 ml of fresh insect cell culture medium was added. Transfected cells were further incubated for 72 hours at 27°C to allow for the homologous recombination and replication of recombinant virus.

**Plaque purification of recombinant viruses**

To isolate pure clones, recombinant baculovirus containing the bovine rotavirus VMRI VP7 gene were plaque purified. The insect cell cultures were harvested at 72 hour after transfection and transfected cell cultures were clarified by low speed centrifugation at 4°C for 20 minutes at 2,000 rpm and clear supernatants were collected in sterile snap cap tubes. The supernatants were serially diluted 10 fold to inoculate the insect cells. Cells were incubated for 60 minutes at room temperature to allow infection of insect cells with the recombinant virus. After 60 minutes, the inoculum was removed and 3 ml of 1.25% agarose in Excell-400 insect cell culture medium was added onto the insect cells. After solidification of agarose, 3 ml of insect cell culture medium was added to provide enough moisture during further incubation period and cells were incubated for 72 hours at 27°C without CO2 supply. Occlusion-negative isolated virus plaques were picked using sterile Pasteur pipettes and diluted into 0.5 ml of insect cell culture medium. Tubes containing recombinant virus were stored at 4°C overnight to release virus particles into the media and were then centrifuged at 2,000 rpm for 10 minutes at 4°C. This entire purification step was repeated three times to ensure clonal population. After three rounds of purification, the supernatant containing recombinant virus was stored at -80°C until the next experiment.
Detection of rotaviral genome in recombinant baculovirus infected insect cells by dot blot hybridization assay

Rotavirus cDNA generated by PCR amplification was labeled with $^{32}\text{P}$-dCTP (ICN Biochemical, Costa Mesa, CA) using a random primer extension method (Amersham, Arlington Height, IL) to confirm the presence of rotavirus genes in recombinant baculovirus. Hi Five cells infected with recombinant baculovirus were frozen and thawed once and clarified by low speed centrifugation. The supernatants were then pelleted at 37,000 rpm for 90 minutes and viral DNA was extracted with phenol/chloroform. The aqueous phase was collected and precipitated in ethanol and boiled for 5 minutes to denature the nucleic acids. Hi-Five cells infected with wild type baculovirus were prepared by the same manner and used as a control. Plasmid vectors containing the bovine rotavirus VP7 gene were used as positive controls and the pKS plasmid itself as a negative control. Denatured nucleic acids were applied on a nylon membrane (Zeta probe, Bio-Rad, Hercules, CA) and baked for 2 hours at 80°C to immobilize the nucleic acids. For the probe preparation, PCR amplified bovine rotavirus VP7 gene was labeled with $\alpha^{32}\text{P}$-dCTP for 4 hours at room temperature using random primers multi-labeling kit (Amersham, Arlington Height, IL). A radio-labeled DNA probe was passed through Sepharose columns (Boehringer Mannheim, Linkers 6 Quick spin column Sepharose CL-6B) and boiled for 5 minutes at 100°C before hybridization. The baked membrane was prehybridized for 4 hours at 65°C with a prehybridization solution (5X SSC, 5X Denhardt's solution, and salmon testes DNA), and hybridized overnight with denatured probes. The hybridization reaction was carried out at 65°C without formamide. After hybridization, the membranes were extensively washed with washing buffer containing 0.1% SDS solution. First two washes were with 2X SSC.
followed by two washes with 1X SSC, two washes with 0.1X SSC and final wash with sterile water. The membrane was exposed on X-ray film (Fuji RX film, Japan) for 4 hours at room temperature prior to developing.

Detection of rotavirus antigens by immunofluorescent test

Indirect immunofluorescence test was used to detect rotavirus specific recombinant proteins in insect cells infected with recombinant baculovirus. The Hi-Five cells infected with recombinant or wild type baculovirus or mock infected cells were fixed with cold methanol in 24-well cell culture plates at 72 hours after infection and examined for viral antigens. An appropriate dilution of guinea pig anti-rotavirus polyclonal antibody was added to the fixed cell monolayers and cells were incubated for 60 minutes at 37°C. The cell monolayers were washed to remove unincorporated antibodies. An optimum dilution of fluorescein isothiocyanate labeled anti-guinea pig antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and cells further incubated for 30 minutes at 37°C. After washing three times with PBS, cells on tissue culture plate were examined for fluorescence under the fluorescent microscope.

Detection of mRNA by dot blot hybridization

To compare relative transcriptional activity of each construct, total mRNA was isolated from cells infected with recombinant baculovirus at 28-30 hours after infection. RNA samples were isolated by using a total RNA isolation kit (Stratagene, La Jolla, CA) from 75 cm² flasks of cells infected with recombinant baculovirus Bac.pVL/VP7.A, Bac.pVL/VP7.B, Bac.pVL/VP7.C, and Bac.pVL/VP7.D containing VP7.A, VP7.B, VP7.C and VP7.D construct, respectively, according to manufacturer's instructions. Isolated RNAs were denatured by boiling and applied on a nylon membrane. The
membrane was baked for 2 hours at 80°C and prehybridized with a prehybridization solution (5X SSC, 6X Denhardt's solution, and salmon testes DNA) for 4 hours at 65°C. The PCR amplified VMRI VP7 gene probe was labeled with ^32P-dCTP and was used to hybridize with nucleic acid at 65°C overnight. The membrane was extensively washed twice with 2X SSC (0.1% SDS), two times with 1X SSC (0.1% SDS), two times with 0.1X SSC (0.1% SDS) and once with sterile water. The X-ray film was exposed to the membrane overnight at -70°C before developing. To quantitate the intensity of the signal in the dots, densitometric analysis employing an image analysis system (Zeiss SEM-IPS, Zeiss-Kontron, IBAS version 2.00) was employed. Photographs were placed on a copy stand and viewed with reflected light, while films were transilluminated using a ChromaPro 45 light box. Images were captured with a Kodak XC-77 black and white video camera equipped with a Contax 60 mm macro lens. Areas to be measured were determined interactively and the mean gray value for that area was also measured. Area and grey values were multiplied to generate real values for each dot. Two values from each dot of the samples were calculated to obtain an average value for the sample.

Radioimmunoprecipitation

Radioimmunoprecipitation was carried out for each different recombinant virus to determine molecular mass of recombinant rotavirus proteins in insect cells. The insect cells were infected with the recombinant or wild type baculovirus at a MOI of 0.1. Two days later methionine-free medium (Gibco-BRL, Gaithersburg, MD) was added to cells for 2 hours and then 35S-methionine/cysteine (ICN Biochemicals, Costa Mesa, CA) containing medium was added, and labeled for 2 hours at 27°C. The anti-VMRI serum was incubated for 1 hour at room temperature with cold lysates from Hi-Five cells.
infected with wild-type baculovirus AcMNPV to eliminate antibodies to wild type baculovirus and/or insect cells. The cold lysate was prepared from AcMNPV-infected Hi-Five cells as follows. The Hi-Five cells in 75 cm² tissue culture flask (Corning, NY) were infected with AcMNPV and incubated for 72 hours at 27°C. After removal of supernatant from the flask, 2.5 ml of lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-HCl, 0.5 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonfonylfluoride (PMSF), and 1 μg/ml pepstatin A, pH 8.0) was added into each flask and placed at room temperature for 5 minutes. The lysed cells were collected into 1.5 ml microfuge tubes and vortexed for 3 minutes then incubated for 3 minutes on ice. Cell debris was removed by centrifugation for 1 minute in a microcentrifuge and the supernatant was saved as cold lysate at -80°C. Radiolabeled (hot) cell lysates were prepared as described above and 50 μl of the cold lysate-treated serum was added into 100 μl of hot lysate. The mixture was incubated overnight at 4°C to form an immune complex. To capture the immune complex, 100 μl of CL-4B bound protein-A (SCA, Sigma, St. Louis, MO) was added and rotated for 1 hour at room temperature. Protein-A-bound immune complexes were washed three times with lysis buffer followed by three washes with sterile water. Samples were mixed with 50 μl of sample loading buffer [4% SDS, 125 mM Tris-HCl, pH6.7, 30% glycerol (v/v), 0.002% bromphenolblue (w/v); 2% β-mercaptoethanol (v/v)] then boiled for 5 minutes just before running on a gel. An X-ray film (Fuji RX film, Japan) was exposed to the dried gel. The image analysis method used for mRNA quantitation was used to quantitate the intensity of the bands except that the rotavirus-specific bands were measured instead of dots.
Post translational modification of the recombinant protein

Recombinant proteins were cleaved with endopeptidase F/N-glycosidase mixture (Boehringer Mannheim Biochemicals, Indianapolis, IN) and endopeptidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) to determine glycosylation of the proteins expressed in the baculovirus system. The insect cells infected with recombinant baculovirus were labeled at 72 hours with \(^{35}\)S-methionine/cysteine (ICN Biochem., Costa Mesa, CA) for 2 hours post infection. Cell lysates were then prepared as described under the radioimmunoprecipitation procedure. Metabolically labeled recombinant proteins were separated from protein-A-antibody complex by boiling, using an elution buffer (50 mM Tris/Cl, 0.5 % SDS, and 0.1 M β-mercaptoethanol, pH 6.8), followed by centrifugation. The eluted proteins were treated with 250 mU of endopeptidase F/N-glycosidase F mixture (5 μl 40 mM sodium phosphate, pH 7.2, 4% (v/v) NP40, 20 mM EDTA buffer, 20 μg PMSF, and 10 μl sample) and 5 mU of endopeptidase H (5 μl 200 mM sodium citrate, pH 5.5 buffer, 20 μg PMSF, and 10 μl sample) in separate tubes overnight at 37°C. Endopeptidase-treated samples were boiled for 5 minutes and separated on SDS-PAGE and X-ray film (Fuji RX film, Japan) was exposed to the dried gels.

Results

Molecular Cloning and analysis of VP7 gene

The VP7 gene of the bovine rotavirus VMRI strain was cloned in the pKS\(^{+}\) plasmid vector and inserts in three recombinant clones were sequenced. The complete sequence of VP7 of VMRI strain is presented elsewhere (Mummidi et al. manuscript
submitted for publication). Terminal sequence and location of four primers P1-P4 are shown in Fig. 1. There was one large open reading frame with the start position at 49 and termination at 1026 (Fig. 1). All of the modifications of VP7 for expression were carried out using primers based on the sequence information as depicted in Fig 1.

**Generation of Recombinant Baculovirus**

To optimize expression of the recombinant VP7 protein, four constructs of the VP7 gene of bovine rotavirus strain VMRJ were generated using primers P1, P2, P3 and P4 (Fig. 1) by PCR as depicted in Fig. 2. The PCR product VP7.A contained the full VP7 gene sequence with BamHI and EcoRI restriction sites and was 1083 base pairs in length. The PCR product VP7.B was 983 base pairs in length in which non-coding sequence downstream of the stop codon of VP7 was removed. The PCR product VP7.C was 1038 base pairs in length, was missing non-coding sequence upstream of the VP7 start codon but contained an additional TAAATA sequence. The fourth PCR product VP7.D was 941 bases long and was missing the non-coding region upstream and downstream of VP7 open reading frame. These PCR amplified DNAs were cloned into a baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA). Three clones of each construct were selected and DNAs purified and quantitated. The pure DNAs from recombinant plasmids were isolated and used for transfection of Hi-Five insect cells along with baculovirus DNA. Occlusion negative plaques containing inserts were picked, purified, and the presence of recombinant virus confirmed by dot-blot hybridization. Stocks of recombinant baculovirus were then prepared. Progeny of occlusion-negative plaques were positive by dot blot hybridization for the presence of rotavirus VP7 gene (Fig. 3). Fig. 4 shows the cytopathic effect induced by progeny of occlusion negative recombinant virus and occlusion positive wild type baculovirus. The
Fig 1. Partial nucleotide sequence of VMRI VP7 gene at termini showing positions of 4 primers P1, P2, P3 and P4 used for PCR for elimination of noncoding regions upstream and downstream of the VP7 ORF. Bold characters indicate start or stop codon, and italics are primer sequences without oligonucleotide for restriction endonuclease recognition sites.
Fig 2. Construction of VMRI VP7 genes in baculovirus transfer vector pVL1393 and short arrow inside circle indicate direction of translation. (MCS: Multi cloning site, PH: polyhedrin promoter). Recombinant baculovirus transfer vector with full VP7 gene (pVL/VP7.A) deletion in 3'-end (pVL/VP7.B), deletion in 5'-end (pVL/VP7.C) and deletion in both 5' and 3' ends were constructed. Abbreviations: MCS= multiple cloning site, PH= polyhedrin promoter.
VP7 gene probe hybridized with nucleic acids isolated from insect cells infected with recombinant baculovirus but not with nucleic acid from wild type baculovirus (Fig. 3). Similarly, baculovirus transfer vector pVL1393 DNA were applied on the same hybridization reaction but no signal was detected.

Quantitation of mRNA

Relative concentration of VP7-specific mRNA transcribed in cells infected with four recombinant baculoviruses was determined by dot blot hybridization. Total mRNA from insect cells was hybridized with the VP7 gene probe prepared from VMRI strain of rotavirus and signals were compared by image analysis for relative concentration. Messenger RNA was detected in cultures infected with any of the four recombinant baculoviruses (Fig. 6). In contrast, VP7 mRNA was not detected in uninfected cells or cells infected with the wild type baculovirus (Fig. 4). Level of mRNA with recombinant baculovirus Bac.pVL/VP7.A, Bac.pVL/VP7.B, and Bac.pVL/VP7.C was similar (Table 1). In contrast, the level of mRNA with recombinant baculovirus Bac.pVL/VP7.D was over two times more than that detected in Bac.pVL/VP7.A, Bac.pVL/VP7.B, or Bac.pVL/VP7.C. This result indicated that removal of both the 5' and 3' non-translational sequences increases transcription of VP7 of bovine rotavirus in the insect cell system.

Quantitation and partial characterization of recombinant proteins

To test the authenticity of expressed VP7 protein, reactivity of recombinant protein with polyclonal anti-rotavirus serum in the indirect immunofluorescence and radioimmunoprecipitation tests was determined. Antiserum to the VMRI strain of bovine
Fig. 3. Identification of VMRI VP7 gene insert in recombinant baculovirus by dot blot hybridization using $^{32}$P-labeled PCR-derived VMRI VP7 gene probe. Nucleic acid was extracted from partially purified recombinant viruses and DNA concentration measured by spectrophotometer. Nucleic acid from recombinant baculovirus Bac.pVL/VP7.A (A), Bac.pVL/VP7.B (B), Bac.pVL/VP7.C (C), Bac.pVL/VP7.D (D), wild type baculovirus (W), and Hi-Five insect cells (H) was applied on nylon membrane using 96-well manifold and hybridized with VMRI VP7 gene probe.
Fig. 4. Cytopathic effect of recombinant baculovirus Bac.pVL/VP7.A (A), Bac.pVL/VP7.B (B), Bac.pVL/VP7.C (C) or Bac.pVL/VP7.D (D) and occlusion positive wild type baculovirus in insect cells (W). CPE was photographed at 72 hour post infection.
Fig. 5. Staining of insect cells infected with recombinant viruses Bac.pVL/VP7.A (A), Bac.pVL/VP7.B (B), Bac.pVL/VP7.C (C), and Bac.pVL/VP7.D (D) by indirect immunofluorescence with guinea pig antiserum to VMRI strain of bovine rotavirus. MA-104 cells infected with VMRI strain of bovine rotavirus were used as control (V).
rotavirus reacted with cells infected with recombinant baculovirus containing the VMRI VP7 gene (Fig. 5). Immunofluorescence was cytoplasmic and specific to rotavirus. Immunofluorescence was not detected in uninfected cells and cells infected with the wild-type baculovirus. For radioimmunoprecipitation, insect cells were infected with recombinant baculoviruses Bac.pVL/VP7.A, Bac.pVL/VP7.B, Bac.pVL/VP7.C, Bac.pVL/VP7.D or wild-type baculovirus and cell lysates from these were immunoprecipitated with anti-VMRI rotavirus serum and analyzed by SDS-PAGE (Fig. 7). A protein band was observed in cells infected with any of the four recombinant baculoviruses containing VP7 gene insert. Protein of similar size was not detected in uninfected cells or cells infected with the wild-type baculovirus. The molecular mass of the recombinant protein was approximately 37.3 kDa which is similar to the 38 kDa reported for rotavirus (Fig. 8). The protein band was diffuse which suggested that the protein was glycosylated. Recombinant baculovirus Bac.pVL/VP7.D, which had both 5' and 3' deletion, had thickest precipitation band suggesting that the recombinant protein expression in this construct was more abundant than that in the others. Expression of recombinant proteins in cells infected with recombinant baculovirus containing VP7 gene was compared by image analysis of RIP bands. The VP7 expression in cells infected with the recombinant baculovirus Bac.pVL/VP7.D was approximately 10 fold more than that in cells infected with recombinant baculovirus Bac.pVL/VP7.B or Bac.pVL/VP7.C and about 40 times more than that in cells infected with Bac.pVL/VP7.A (Table 2).

To determine whether VP7 protein expressed in insect cells was glycosylated, proteins were radiolabeled, treated with endopeptidases to cleave the glycosylated side chains and analyzed. Endopeptidase F/N-glycosidase F mixture and endopeptidase H effectively cleaved recombinant protein VP7 after overnight incubation at 37°C. Cleaved products migrated faster than that for uncleaved protein (Fig. 9). This result indicated
Table 1. Comparison of mRNA levels in insect cells infected with wild type or recombinant baculoviruses.

<table>
<thead>
<tr>
<th>Baculovirus*</th>
<th>Geometric mean concentration of VP7 mRNA as a density of hybridization signal ± S.D**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac.pVL/VP7.A</td>
<td>2,720.54 ± 149.5</td>
</tr>
<tr>
<td>Bac.pVL/VP7.C</td>
<td>2,680.00 ± 160.87</td>
</tr>
<tr>
<td>Bac.pVL/VP7.D</td>
<td>5,526.22 ± 93.94</td>
</tr>
<tr>
<td>AcMNPV</td>
<td>424.71 ± 24.09</td>
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</tbody>
</table>

*AcMNPV= wild type baculovirus; Bac.pVL/VP7.A= recombinant baculovirus with full VP7 gene; Bac.pVL/VP7.B= VP7 gene with deletion in 3'-end; Bac.pVL/VP7.C= VP7 gene with deletion in 5'-end; Bac.pVL/VP7.D= VP7 gene with deletion in 5' and 3' ends.

** Geometric mean values with standard deviation of dot hybridization signals generated by image analysis. Total mRNA from cells infected with virus were hybridized with rotavirus VP7 gene. Signal in dot quantitated by image analysis. The data was analyzed statistically using Friedman rank sums test and Bac.pVL/VP7.D showed significant increase to other constructs (P<0.042). There is no significant difference among recombinant transfer vector constructs Bac.pVL/VP7.A, Bac.pVL/VP7.B and Bac.pVL/VP7.C.
Table 2. Comparison of VP7 expressed in cells infected with recombinant baculovirus containing VP7 insert of varying length.

<table>
<thead>
<tr>
<th>Baculovirus*</th>
<th>Geometric mean relative concentration of VP7 as a density of radioimmunoprecipitation band ± **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac.pVL/VP7.A</td>
<td>595.50 ± 36.23</td>
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<tr>
<td>Bac.pVL/VP7.B</td>
<td>1,842.51 ± 174.68</td>
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<tr>
<td>Bac.pVL/VP7.C</td>
<td>1,908.92 ± 209.92</td>
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<tr>
<td>Bac.pVL/VP7.D</td>
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<tr>
<td>AcMNPV</td>
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</tbody>
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*AcMNPV= wild type; Bac.pVL/VP7.A= recombinant baculovirus with full VP7 gene; Bac.pVL/VP7.B= VP7 gene with deletion in 3'-end; Bac.pVL/VP7.C= VP7 gene with deletion in 5'-end; Bac.pVL/VP7.D= VP7 gene with deletion in 5' and 3' ends. Rotavirus specific VP7 protein band following immunoprecipitation of cell lysates with wild type or recombinant baculovirus were quantitated by image analysis.

** Geometric mean values with standard deviation of radioimmunoprecipitation band signals generated by image analysis. The data was analyzed statistically using Friedman rank sums test and Bac.pVL/VP7.D showed significant increase to other constructs (P<0.004).
Fig. 6. Dot blot hybridization of total mRNA isolated from insect cells infected with recombinant baculovirus Bac.pVL/VP7.A (A), Bac.pVL/VP7.B (B), Bac.pVL/VP7.C (C), Bac.pVL/VP7.D (D) or wild type baculovirus AcMNPV (W) with $^{32}$P-labeled VMRI VP7 gene specific probe.
Fig. 8. Radioimmunoprecipitation of recombinant protein expressed in insect cells infected with recombinant baculovirus Bac.pVL/VP7.D. Proteins were labeled with $^{35}$S-met/cys and immunoprecipitated with anti-VMRI serum. Molecular mass of recombinant protein was 37.3 kDa.
37.3 KDa
Fig. 9. Radioimmunoprecipitation of recombinant VP7 protein with anti-VMRI rotavirus serum following endopeptidase treatment. The protein was treated with Endo-H (lane H) or Endo-F (lane F). Endopeptidase treated VP7 migrated faster than untreated VP7 indicating that recombinant VP7 protein is glycosylated. The Mr of untreated VP7 (closed triangle) was 37.3 kDa.
that the recombinant protein VP7 expressed in baculovirus was effectively glycosylated and side chains could be removed by treatment with endopeptidases.

Discussion

A number of different prokaryotic and eukaryotic vectors have been used to express viral proteins. Eukaryotic vectors have an advantage over prokaryotic vectors in post-translational modification such as protein folding which appears to be important in immunogenicity of certain recombinant proteins (Francavilla et al. 1987; Redmond. 1993). The helper independent baculovirus is one of the most popular eukaryotic expression systems for expression of viral proteins for their characterization and use as diagnostics and vaccines. In this expression system, foreign genes are expressed under the transcriptional control of the very strong and late baculovirus polyhedrin gene promoter (Summers and Smith. 1989: O'Reilly et al. 1992). The baculovirus system has been used to express many viral, mammalian, plant and prokaryotic genes in an abundant amount and in a functionally authentic form (Kang, 1988: Smith et al. 1983; Luckow et al. 1988a, 1989: Davis et al. 1992). Expression levels of recombinant proteins do vary in this expression system. Factors that influence protein expression levels in baculovirus are not completely understood. Some of the factors that are believed to influence the expression levels include the insect cell line, gene expressed, distance between polyhedrin promoter, and start site of foreign gene. Insect cell lines TNI-TN-5B1-4 (Hi-Five) and BTI TnM have been reported to be superior to Sf21 and Sf9 in protein expression (Wickham et al. 1992). In a preliminary experiment, we also compared expression of bovine rotavirus VP6 in Sf9 and Hi-Five cells. The expressed protein levels in Hi-Five cells was much greater than that in Sf9 cells. Therefore, in this
experiment we used Hi-Five cells grown in insect cell culture medium Excell 400 without serum supplement for maximum expression. Other factors that are expected to contribute to the recombinant protein expression are the properties of a given gene, RNA stability, and protein processing, transport targeting and stability. But these have not been studied in detail (Luckow et al. 1988). To optimize gene expression in the baculovirus system, it has been recommended that genes be used without introns. Removal of 5' noncoding sequences longer than 30 bp, removal of 3' untranslated sequence which may destabilize mRNA and addition of TAAG which may specify transcriptional initiation in an eukaryotic system is also recommended for optimal gene expression (O'Reilly et al. 1992). Sequences proximal to the polyhedrin promoter can also have a dramatic influence on the level of expression in the insect cell system (Luckow et al. 1988a; Kang et al. 1988).

Several reovirus and rotavirus proteins including VP7 have been successfully expressed and characterized using baculovirus as an expression system (Nishikawa, 1989; Labbe, 1991; Loudon, 1991). These recombinant proteins were evaluated as potential vaccines in experimental animals to prevent rotavirus gastroenteritis (Redmond et al. 1993; Bishop, 1993). Bovine rotavirus C486 VP7 recombinant protein was expressed in insect cells using a pAcYM1 as a transfer vector. In this report seven nucleotides from the 5' end and 27 nucleotides from the 3' end were removed by restriction enzyme digestion of the cloned cDNA (Sabara et al. 1989). Expression of rotavirus proteins in insect cells peaked at 48 hours after infection, and low yields of VP7 protein expression was attributed to the secretion of the protein into the culture medium (Redmond et al. 1993). The VP7 protein expression peaked at 24 hours post infection in Sf9 cells and 96 hours post infection in the supernatant of the recombinant virus infected insect cells. In this experiment, recombinant protein VP7 expression peaked at 72 hours
post infection in Hi-Five cells and recombinant protein was released into the culture medium after extensive cytopathic effect (data not shown). Simultaneous expression of VP6 and VP7 formed double-shelled like particles in insect cells which indicates that VP6 is required for the virus-like particle formation (Sabara et al. 1991).

In this study, expression using the full VP7 gene was poor. Therefore, we examined the effect of deleting non-coding regions upstream and downstream of the VP7 gene ORF in its expression. Removal of either the 5' or 3' non-coding region increased protein expression two fold over that with the full gene. Removal of both the 5' and 3' end had a major impact on the level of mRNA transcription and protein expression. Removal of the 5' non-translated DNA sequences allowed the VP7 gene location close to the polyhedrin promoter as well as eliminated the sequence with high GC content upstream of the VP7 gene start codon. Deletion of the 3' end allows elimination of AT rich sequences which possibly destabilize mRNA transcript. Also the sequence added upstream of ATG might increase transcriptional initiation activity. After these alterations of the VP7 gene we expected more mRNA transcription and protein expression than when using the full length VP7 gene. Messenger RNA transcription was highest in Bac.pVL/VP7.D which has deletions both at 5' and 3' ends. Deletion of both the 5' and 3' non-translational sequences increased expression of recombinant protein but undeleted or a single deletion at 5' or 3' end deletion did not alter expression of VP7 proteins in this insect cell system. We conclude that deletion of noncoding sequences at both the 5' and 3' ends of bovine rotavirus VP7 gene was an important requirement for optimal expression of the VP7 protein in insect cells. Based on all data obtained from this experiment, we could improve recombinant protein expression in baculovirus system by deletion of non-coding sequences.
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References


GENERAL CONCLUSIONS

The first paper describes expression and characterization of bovine rotavirus proteins in the baculovirus system. Bovine rotavirus VP2, VP4, VP6 and VP7 of the VMRI strain were cloned in phagemid vector pKS+ and the size of these clones were found to be 2.67, 2.36, 1.36 and 1.06 kb for VP2, VP4, VP6 and VP7, respectively. These sizes were comparable to that of the corresponding genes of other bovine rotavirus. Sequencing data of these cloned genes were used for designing primers for PCR amplification and insertion of these genes into the baculovirus transfer vectors.

We then expressed recombinant rotavirus proteins VP2, VP4, VP6 and VP7 using baculovirus vectors. Bovine rotavirus specific antigens were detected from cells infected with recombinant baculoviruses by indirect immunofluorescence using hyperimmune serum to the VMRI strain of BRV. All of the recombinant proteins expressed in insect cells showed widespread cytoplasmic fluorescence by IFA. The molecular mass of these recombinant proteins was measured by radioimmunoprecipitation using hyperimmune serum to the VMRI strain of BRV. Antigenicity of these recombinant proteins were evaluated in guinea pigs.

There was no significant difference in the titers of the different recombinant baculoviruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6 and Bac.pVL/VP7. But recombinant virus titers in insect cells were much higher than the titers of the VMRI strain of BRV grown in MA-104 cells. The titer of the VMRI strain grown in MA-104 cell culture was $10^6.0$ FFU/ml by an indirect immunofluorescent test using hyperimmune serum to the VMRI strain of BRV, raised in guinea pigs. Recombinant virus titers determined by an indirect immunofluorescent test using the same antiserum in insect cells were $10^7.5$ FFU/ml for recombinant baculovirus Bac.pB/VP2, $10^8.0$ FFU/ml for
Bac.pVL/VP4. $10^{8.5}$ FFU/ml for Bac.pVL/VP6 and $10^{8.0}$ FFU/ml for Bac.pVL/VP7, respectively. The titers of the recombinant baculoviruses were comparable to that of the wild type baculovirus AcMNPV, in the same insect cell system.

Molecular mass of the recombinant proteins expressed in insect cells were identified by radioimmunoprecipitation. The molecular mass of recombinant protein VP2 expressed in insect cells was approximately 102 kDa which is similar to that of the VP2 protein in BRV. Another slower migrating protein was also detected in the cells infected with recombinant baculovirus Bac.pB/VP2. This band possibly represent a heavy glycosylation product which is common in the baculovirus expressed proteins. Three cleavage products, VP5, VP8, and VP8a were detected in cells expressing recombinant protein VP4. The VP8 product has been shown to possess hemagglutinin activity with an approximate molecular mass of 28 kDa (Fiore et al., 1991). The VP5 is about a 60 kDa protein which is on the carboxy terminal side of the VP4 protein (Mattion et al., 1994). In this study, we also observed a faster migrating protein VP8a which is believed to be due to the cleavage VP4 at amino acid residue 231. In this expression system, the majority of the VP4 recombinant protein expressed in the insect cell system was intact. This abundant amount of expressed VP4 might be an advantage in the immunization of animals. It was reported that in the RIP of a rotavirus grown in mammalian cells, the VP4 protein was very faint and the VP5 and the VP8 proteins were almost undetectable (Estes, 1990). In this study, the baculovirus expressed recombinant protein VP4 clearly showed an advantage in protein expression over the mammalian cell culture system.

Using a recombinant protein expression in insect cells, we have successfully demonstrated that the arginine at amino acid residue 231 of the VP4 protein is an active cleavage site for cellular proteolytic enzymes, and insect cells have the ability to process recombinant rotavirus protein in the same manner as mammalian cells. An additional
putative proteolytic enzyme cleavage site at amino acid residue 231 found in the VP4 gene sequence was confirmed as a proteolytic enzyme cleavage site. During expression of the recombinant protein we observed that VP6 was most abundantly expressed in insect cells by IFA and coomassie staining of the protein by SDS-PAGE (data not shown). We do not understand the mechanisms for higher expression of recombinant protein VP6, over the other rotavirus proteins in the insect cells system. Since the VP6 protein is the most abundant viral protein in the rotavirus virion which is composed of over 60% of the viral proteins, we speculate that the VP6 gene has some inherent property that affects its expression. Deletions of noncoding sequences at the 5' and 3' ends of the VP7 gene increased recombinant protein expression. A diffuse band in the VP7 RIP indicates that the protein expressed in baculovirus is post translationally modified and shows an advantage of an eukaryotic expression system over a prokaryotic expression. Since proteins expressed in baculovirus had a similar molecular mass to that of rotavirus and glycosylation of VP7, we expect that biological activities of recombinant proteins to be similar to those of rotavirus.

We determined the kinetics of recombinant rotavirus protein expression in insect cells by radioimmunoprecipitation. The highest expression of VP6 was at 72 hour post infection. Recombinant proteins were not secreted into the culture medium from any of the recombinant viruses during the early stages of infection. We were able to detect recombinant proteins in the medium during the late stages of infection but it was due to the extensive cytopathic effect of recombinant virus infected insect cells rather than protein secretion. This is not unusual for proteins expressed in an insect cell system to show a lack of protein secretion in the medium (Wickham et al. 1992).

Two different types of CPE were observed in recombinant virus infected insect cells. One type was cell rounding followed by lysis and the other was fusion of the
insect cells followed by lysis. Based on the CPE in the recombinant virus infected cells VP4 and VP7 showed cell fusion activities. Especially, insect cells infected with recombinant baculovirus expressing VP4 fused together significantly, indicating that recombinant protein VP4 expressed in insect cells possess fusion activity that is similar to that of the native viral protein VP4. The recombinant virus VP7 also showed weak fusion activity in insect cells which was unexpected. This activity may indicate that VP7 is a putative cell receptor binding protein in mammalian cells.

Current research results indicate that baculovirus expressed viral proteins self assemble into virus-like particles and these are immunogenic in experimental animals (French and Roy, 1990; Luo et al., 1990; Sabara et al., 1991; Loudon and Roy, 1991; Jiang et al., 1992; Laurent et al., 1994; Prasad et al., 1994). This data may suggest that the baculovirus expression system has an advantage over other expression systems such as E. coli. In this experiment we have successfully produced virus-like particles by using combinations of recombinant viruses expressing rotavirus proteins. Infection of insect cells with Bac.pBAT'2 produced sub-core particles and the size of these particles was similar to that of BRV subcores. The sub-core particles expressed in insect cells were similar to that of previous reports (Labbe et al., 1991; Zeng et al., 1994). Co-infection of insect cells with recombinant viruses Bac.pB/VP2, Bac.pVL/VP4, Bac.pVL/VP6, and Bac.pVL/VP7 produced rotavirus like particles in insect cells without core structures containing nucleic acid and proteins VP1 and VP3.

Morphology and size of the recombinant empty particles was 70-75 nm in diameter which similar to that of rotavirus particles purified from tissue culture grown VMRI strain of BRV. Crawford et al., (1994) had similar result with rotavirus capsid proteins expressed in insect cells. Also there were tubular structures in insect cells infected with recombinant baculovirus. This indicates that the empty virus particles bud
out from primary tubular structures. The results of our data suggests that VP2 and VP6 are required for VLP formation.

Evaluation of antigenicity of recombinant proteins in guinea pigs showed that VP2, VP4, VP6 and VP7 are antigenic, however only VP4, VP6 and VP7 possess ability to induce neutralizing antibodies. The VP4 and VP7 appear to be major immunogen as reported previously (Paul and Lyoo, 1993) Ability of VP6 to produce low level of neutralizing antibodies was surprising and needs further investigation especially in the light of findings of Redmond et al. (1993) that VP6 can provide partial immunity to rotavirus associated gastroenteritis.

There has been a controversy as to which rotavirus protein is responsible for binding to culture cells. Both VP4 and an outer capsid glycoprotein have been proposed to play a role in binding to receptors on rotavirus susceptible cells (Sabara et al., 1985; Ruggeri and Greenberg, 1991). In another study (Bass et al., 1990) a nonstructural rotavirus protein suggested as the major protein responsible for binding to target cells (Bass et al., 1990). Because of the controversy surrounding the role of different proteins in binding with the receptor, we examined the role of recombinant VP4 and VP7 in cells. Our results indicate that VP4 and VP7 proteins are important for the cell binding. In this study, we have clearly shown that both VP4 and VP7 are the major proteins responsible for binding to MA-104 cells. Baculovirus expressed recombinant rotavirus proteins VP4 and VP7 independently blocked target binding of the VMRI strain of BRV to MA-104 cells, resulting in no virus infection in the cells. The virus neutralization test results also support this hypothesis (Table 16). Resistance of receptor binding activity to trypsin treatment as observed by Kaljot et al. (1988) further supported our results that VP4 and VP7 are the major proteins responsible for virus binding to cells. The number of rotavirus receptors on MA-104 cells was shown to be $10^3$-$10^3.5$ receptors per cells for
the VMRI strain of BRV. This estimate is lower than that of 10^5.0/cell reported by Kaljot (1990). This difference in results may be due to the different passage level of susceptible cells and/or the different strain and passage levels of rotavirus.

The second paper describes that recombinant protein VP7 expression is increased by modification of sequences upstream and downstream of the ORF. In a preliminary experiment, we also compared expression of bovine rotavirus VP6 in Sf9 and Hi-Five cells. The expressed protein levels in Hi-Five cells was much greater than that in Sf9 cells (data not shown). Therefore, in this experiment we used Hi-Five cells grown in insect cell culture medium Excell 400 without serum supplement for maximum expression. Other factors that are expected to contribute to the recombinant protein expression are the properties of a given gene, RNA stability, and protein processing, transport targeting and stability. But these have not been studied in detail (Luckow et al., 1988). To optimize gene expression in the baculovirus system, it has been recommended that genes be used without introns. Removal of the 5' noncoding sequences, if longer than 30 bp, 3' untranslated sequence which may destabilize mRNA and the presence of TAAG which may specify transcriptional initiation in an eukaryotic system are also recommended for optimal gene expression (O'Reilly et al., 1992). Sequences proximal to the polyhedrin promoter can also have a dramatic influence on the level of protein expression in the insect cell system (Luckow et al., 1988a; Kang et al., 1988).

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5' end and 27 bp from the 3' end were removed by restriction enzyme digestion of the cloned cDNA (Sabara et al. 1989). Expression of rotavirus proteins in insect cells peaked at 48 hours after infection, and low yields of VP7 protein expression was attributed to the secretion of the protein into the culture medium (Redmond et al. 1993). The VP7 protein expression peaked at 24 hours post infection in Sf9 cells and 96 hours post infection in the supernatant of the recombinant virus infected insect cells. In this experiment, recombinant protein VP7 peaked at 72 hours post infection in Hi-Five cells and recombinant protein was released into the culture medium after extensive cytopathic effect (data not shown). Simultaneous expression of VP6 and VP7 formed double-shelled like particles in insect cells which indicates that VP6 is required for the virus-like particle formation (Sabara et al., 1991).

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but no deletion or a single deletion at the 5' or 3' end did not alter expression of VP7 proteins in this insect cell system. We conclude that deletion of noncoding sequences at both the 5' and 3' ends of the bovine rotavirus VP7 gene was an important requirement for increased expression of the VP7 protein in insect cells.

Rotaviruses of G type 6 and P type 1 are most prevalent in calves with gastroenteritis (Hardy et al., 1992). This simplicity may allow a feasible approach in developing an effective vaccine which can be beneficial to the cattle industry in the prevention of rotavirus associated gastroenteritis in calves. Even though the serological diversity of bovine rotavirus is limited, prevention of rotavirus infection in calves has proven to be a difficult problem and remains to be solved. We believe the recombinant proteins VP4 and VP7 offer a valuable tool for development of an effective vaccine for the prevention and control of rotavirus associated gastroenteritis.
LITERATURE CITED


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