MOLECULAR CHARACTERIZATION AND HUMORAL IMMUNITY OF BOVINE ROTAVIRUS VP4, VP6 and VP7

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by

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ABSTRACT

The studies described in this thesis include the production and characterization of three bovine rotavirus structural proteins, VP4, VP6 and VP7, which are very important in eliciting protective immune responses in convalescent animals. To understand these proteins at a molecular level, and the role of individual proteins in immunity, rotavirus VP4, VP6 and VP7 proteins were expressed by recombinant vaccinia virus and baculovirus. The evaluation of the immunogenicity of the recombinant proteins may aid in the development of more effective subunit vaccines to prevent and control rotavirus infection.

The recombinant forms of VP4, VP6 and VP7 expressed by recombinant vaccinia virus and baculovirus were analyzed by immunoprecipitations and western blotting. These *in vitro* characterizations revealed that the different recombinant forms of VP4 are similar in size and antigenicity to authentic BRV VP4. I have shown that: (1) the recombinant VP4 protein produced in mammalian cells or insect cells had the same molecular weight as the authentic protein; (2) the trypsin cleavage patterns of recombinant VP4 were similar to the pattern produced by trypsin treatment of authentic VP4, and; (3) recombinant VP4 was stable in BSC-1 and Sf9 cells, while recombinant VP4 expressed by baculovirus was not stable during post-translational processing. The reason for this instability has been found. There is a proteolytic agent in infected Sf9 cells which cleaves the VP4 protein.

The characterization of recombinant VP6 demonstrated that its configuration closely mimicked the authentic VP6 protein. It was shown that: (1) the recombinant VP6 protein produced in mammalian cells or

insect cells had the same molecular weight and antigenicity as authentic BRV VP6; (2) recombinant VP6 is not expressed on the cell surface but in the cytoplasm of infected cells, and; (3) recombinant VP6 protein could be assembled into single shelled particles *in vitro* in a fashion identical to authentic VP6 protein.

Characterization of recombinant VP7 produced by vaccinia virus revealed that it was similar to authentic VP7. However, VP7 expressed by recombinant baculovirus was similar to the native form of the primary translation product but differed in its post-translational modification. The differences are: (1) the molecular weight of VP7 expressed by recombinant baculovirus was lower than the molecular weight of authentic VP7 or VP7 expressed by recombinant vaccinia virus, and; (2) VP7 expressed by recombinant baculovirus was secreted into the medium, which is different from VP7 expressed by rotavirus and recombinant vaccinia virus. However. when tunicamycin was used to inhibit glycosylation, the VP7 expressed by recombinant baculovirus was of the same molecular weight as VP7 expressed by rotavirus or recombinant vaccinia virus and none were secreted into the medium. This also suggested that VP7 expressed by recombinant vaccinia virus and baculovirus were decorated with N-linked oligosaccharides, as is authentic VP7.

The antigenicity of recombinant VP4, VP6 and VP7 and their ability to induce protective immune responses were analyzed by immunizing mice with live recombinant vaccinia virus or recombinant proteins produced by baculovirus mixed with adjuvant. ELISA results indicate that antibodies raised in mice which recognize rotavirus after two immunizations. Antibodies in the milk were also analyzed by ELISA. The results indicate that vaccination elicits high lacteal Ab titers. We also examined whether

antibodies raised in vaccinated mice reacted with individual rotavirus proteins. The ELISA and immunoprecipitation results indicate that antibody induced by the recombinant viruses were specific to the BRV protein expressed by the specific vaccine viruses.

Protection of neonates from rotavirus infection was tested by challenging mice suckling from vaccinated dams. The results indicate that protection from rotavirus diarrhea occurred in neonates nursed by dams immunized with recombinant vaccinia virus expressing VP7, a mixture of recombinant vaccinia viruses expressing VP4, VP6 and VP7, recombinant baculovirus expressing VP7 and a mixture of recombinant baculoviruses expressing VP4, VP6 and VP7, while protection was not conferred to offspring suckling from dams immunized with recombinant baculovirus expressing VP6, wild type vaccinia virus, placebo and sentinel.

In the vaccinia virus system, two promoters were used, one was a vaccinia virus 7.5 K gene promoter, the other was a vaccinia virus consensus late gene promoter. The results indicate that the consensus late gene promoter expresses significantly higher levels of protein than the 7.5 K promoter. However, the increase in the level of protein expression is dependent on the gene being expressed. For example, the consensus late gene promoter produced > 16 times the quantity of VP6 protein when compared to that produced by the 7.5 K promoter, while gene 4 (VP4) and 8 (VP7) expressed only 4-8 times the quantity of protein. Thus, both the gene and the promoter appear to influence the level of expression.

In conclusion, these studies characterized recombinant BRV VP4, VP6 and VP7 proteins expressed by vaccinia virus and baculovirus and defined the immunogenicity of these proteins in dam vaccination studies.

These studies may lead to the development of more effective vaccines to deliver rotavirus antigens and to prevent rotavirus infection.

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LIST OF ABBREVIATIONS

Ab	Antibody
ABTS	2, 2'-Amino-di-(3-ethylbenzthiazoline Sulphonate)
AcMNPV	Autographa Californica Nuclear Polyhedrosis Virus
ATI	A-type Inclusions
BRV	Bovine Rotavirus
BSA	Bovine Serum Albumin
BSC-1	African Green Monkey Kidney Cell Line
BudR	5-Bromo, 2'-Deoxyuridine
cDNA	Complementary Deoxyribonucleic Acid
CF	Complement-Fixation Test
CPE	Cytopathogenic Effect
CTL	Cytotoxic T Lymphocyte
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
ds RNA	Double-Stranded RNA
E. coli	Escherichia coli
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FCA	Freunds Complete Adjuvant
FICA	Freunds Incomplete Adjuvant
GSA	Group Specific Antigen
GTP	Guanosine Triphosphate

HBSS	Hank's Balanced Salt Solution
HRV	Human Rotavirus
ID	Immunodiffusion
IEM	Immunoelectron Microscopy
IL-2	Interleukin-2
LMTK$^-$	Thymidine Kinase Negative Murine Cell line
NGS	Normal Goat Serum
NK cell	Natural Killer Cell
mRNA	Messenger Ribonucleic Acid
MA 104	Rhesus Monkey Kidney Cell Line
MAb	Monoclonal Antibody
$Man_3GlcNAc_2$	$Mannose_3N$-$acetylglucosamine_2$
MEM	Minimal Essential Medium
MOI	Multiplicity of Infection
MW	Molecular Weight
Occ$^-$	Occlusion Negative Virus
Occ$^+$	Occlusion Positive Virus
OD 405 nm	Optical Density at 405 nm
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Bufferd Saline
PFU	Plaque Forming Unit
rBV-VP4	Recombinant Baculovirus Expressing VP4
rBV-VP6	Recombinant Baculovirus Expressing VP6
rBV-VP7	Recombinant Baculovirus Expressing VP7
RER	Rough Endoplasmic Reticulum
rVV-VP4	Recombinant Vaccinia Virus Expressing VP4

rVV-VP6	Recombinant Vaccinia Virus Expressing VP6
rVV-VP7	Recombinant Vaccinia Virus Expressing VP7
RNA	Ribonucleic Acid
RT	Room Temperature
SA11	Simian Rotavirus 11
SDS	Sodium Dodecyl Sulfate
Sf9 cell	Spodoptera Frugiperda Cell Line
ssRNA	Single Stranded RNA
SV 40	Simian Virus 40
TK	Thymidine Kinase
TK-143	Human Thymidine Kinase Negative Cell Line
ts	Temperature Sensitive
TSA	Type Specific Antigen
VIDO	Veterinary Infectious Disease Organization
VV	Vaccinia Virus
wt	Wild-type

1. LITERATURE REVIEW

1.1 Rotavirus

1.1.1 Introduction

Rotaviruses were first identified and associated with calf scours in 1969 (Mebus et al., 1969). Four years later, a similar virus was found in acute neonatal gastroenteritis in children (Bishop et al., 1973). Since the time of these initial reports, rotaviruses have been recognized as the most important cause of acute neonatal gastroenteritis in human and animals (Flewett and Woode, 1978; Estes et al., 1983; Holmes, 1983; Cukor and Blacklow, 1984; Flewett and Babiuk, 1984; Kapikian and Chanock, 1985; Ho et al., 1988). In addition to gaining a better appreciation of the importance of these viruses in enteric infection during the ensuing 21 years, studies on the biochemistry, biology, and the molecular and antigenic properties of rotaviruses have resulted in a relatively comprehensive understanding of these important pathogens.

Rotaviruses are members of the reoviridae family. They are classified serologically into groups (or serogroups), containing viruses that share cross-reacting antigens detectable by serologic tests such as immunofluorescence, ELISA, and immunoelectron microscopy. Six distinct groups (A to F) of viruses have been described (Pedley et al, 1983; Nakata et al, 1986; Bridger, 1987). Members from groups A, B, and C have been found in both humans and animals; groups D, E, and F have been found only in animals (Bridger, 1987). The complete rotavirus particle is approximately 76.5 nm in diameter with a double-layed icosahedral protein capsid composed of an outer layer, an inner layer and a core (Newman et

al., 1975; Rodger et al., 1975). The arrangement of these proteins to form viral particles results in a structure which resembles a wheel, with short spikes and a well-defined rim. Single shelled (ss) particles, lacking the outer coat proteins, are initially assembled around the core, which contains 11 segments of double-stranded RNA (ds RNA). The ss particles also contain the virion-associated transcriptase (Cohen, 1977; Mason et al., 1980). Since the rotavirus genomic double-stranded RNAs are capped (Imai et al.,1983), the ss particles are also assumed to contain the other activities associated with modification of mRNA transcripts (i.e., 7-methyl G and 2'-O-methyl G methylases and guanylate transferase activities, as well as the nucleotide triphosphohydrolases). Following the initial production of the ss particles, maturation and addition of the outer shell proteins occur in the lumen of the rough endoplasmic reticulum (ER) of infected cells (Estes et al, 1983). Consequently, the assembly of rotaviruses involves the transport of viral components across the membrane of the ER, a process that involves a membrane budding event in which partially assembled virus particles are enveloped in a transient membranous vesicle, and then mature particles are liberated from infected cells by cell lysis (Bellamy and Both, 1990).

1.1.2 Protein-coding assignments of the rotavirus genome segments

The correlation of rotavirus polypeptides with the genes coding for them is important for two reason. First, if *in vitro* translation systems are employed, both primary gene products and polypeptides which are post-translationally derived can be identified. Second, if recombinant DNA technology is to be used in the production of vaccines, it is imperative that the gene encoding the important antigen is identified so that the correct

genetic information is cloned (Babiuk et al., 1985).

Several independent methods have been used to produce and assign proteins to individual segments of rotavirus double-stranded RNA (Both et al., 1983; Mason et al., 1983; Greenberg et al., 1983; Ramig and Fields, 1983; McCrae and McCorquodale, 1982). The ability to isolate individual rotavirus genes combined with appropriate *in vitro* transcription and translation systems (Both et al., 1983; Mason et al., 1983) has been the major method of assigning gene products to specific rotavirus genomic fragments. These studies were further supported by genetic recombination studies using different rotavirus strains (Greenberg et al., 1983). This genetic approach was possible because rotaviruses have the ability to undergo genetic recombination at a high rate when more than one strain is used to infect target cells (Ramig and Fields, 1983).

A summary of the gene-coding assignments and known structural and nonstructural proteins encoded in each of the 11 genome segments is presented in Table 1. The consensus is that the protein products VP1 to VP4, VP6 and VP7 (encoded by genomic segments 1 through 4, 6, and 9 respectively) are structural proteins found in virus particles. The rotavirus ss particle is composed of four structural proteins: VP1, VP2, VP3 and VP6; the latter being the major component of the inner capsid. The outer shell of the virion is composed of two proteins: VP4 and VP7. Two proteins are generated by cleavage of VP4, namely VP5 and VP8. The remaining five genome segments code for nonstructural proteins found in infected cells, but not in mature particles. One of them, NS28 is a glycoprotein. cDNA clones are now available for all 11 of the segments, and all but gene segment 3 have been sequenced (Both et al., 1982; 1984 a; Elleman et al., 1983; Estes et al., 1984; Lopez and Arias, 1987; Nishikawa and Gorziglia,

Table 1. Rotavirus Genome RNA Segments and Protein Products

Genomic segment	Length (base pairs)	Proteins	Mol wt of nascent polypeptide (no. of amino acids)	Mature protein Modification	Approx % (by wt) of virion protein	Comments/function
1	3302	VP1	124,847 (1088)		2	Inner-core protein exhibit homology with RNA polymerases
2	2687	VP2	102,431 (880)	Myristilated	15	Inner-core protein, RNA binding; leucine zipper (aa 536 to 559 and 665 to 686)
3	2591	VP3	98,142 (835)		0.5	Inner-core protein
4	2362	VP4	86,775 (776)	Cleaved VP5 and VP8	1.5	Surface protein, hemagglutinin, protease-enhanced infectivity, neutralization antigen, virulence, putative fusion region (aa 384 to 401)
5	1611	NS53	58,654 (491)			Possible metal binding domain
6	1356	VP6	44,816 (397)	Myristilated	51	Inner capsid protein, trimer, hydrophobic subgroup antigen, required for transcription
7	1104	NS34	36,072 (312)			RNA binding
8	1059	NS35	36,128 (317)			Role in RNA replication?
9	1062	VP7	37,198 (326)	Cleaved signal sequence, high-mannose glycosylation and trimming	30	Rough endoplasmic reticulum (RER) integral membrane glycoprotein, cell attachment protein, neutralization antigen, two hydrophobic NH_2-terminal regions, bicistronic gene? putative Ca^{2+}-binding site (aa 127-157)
10	751	NS28	20,309 (175)	High-mannose glycosylation and trimming		Nonstructural RER transmembrane glycoprotein, two hydrophobic NH_2-terminal regions, role in morphogenesis, putative Ca^{2+}-binding site
11	667	NS26	21,520 (198)	Probably phosphorylated		Nonstructural

Modified from Bellamy and Both, 1990; Estes and Cohen,1989.

1988; Powell et al., 1988; Rushlow et al., 1988; Ernst and Duhl, 1989). These cloned gene copies have enhanced our understanding of the function of some of these gene products (Estes et al., 1983; Estes and Cohen, 1989; Bellamy and Both, 1990).

1.1.3 Rotavirus structural proteins

The protein composition of rotavirus particles was first demonstrated in 1975 (Newman et al., 1975; Rodger et al., 1975). Since then, many studies have described the rotavirus structural polypeptides. These studies analyzed the polypeptides of several rotavirus species obtained from tissue culture infected cells. The following review will attempt to summarize the salient features of these studies.

1.1.3.1 Outer capsid proteins

VP4 is the outer capsid protein encoded by gene segment 4 and has a molecular weight of 84-88 kDa. Recently, it has been shown by cryoelectron microscopy that VP4 forms spike-like structures on the surface of the virus (Prasad et al., 1988; 1990; Yeager et al., 1990). VP4 is a nonglycosylated protein, and constitutes 1.5% of the virion protein (Estes and Cohen, 1989). Although VP4 is a minor component of the outer capsid, it has a number of very important biological functions. (1) It induces antibodies capable of neutralizing virus infectivity and therefore is implicated in playing a role in virus attachment (Hoshino et al., 1985; Matsui et al., 1989). (2) It has been shown to bind to and hemagglutinate erythrocytes (Kalica et al., 1983; Mason et al., 1983). (3) It has been shown to be an important determinant of virulence and growth in cell culture (Greenberg et al., 1983 a; Offit et al., 1986a). Following moderate digestion with trypsin, VP4 is cleaved to two

smaller polypeptides of 60 and 28 kDa, termed VP5 and VP8, respectively (Estes et al., 1981). Both fragments remain associated with the virion after cleavage. Proteolytic cleavage, which is also believed to occur in the intestinal lumen during infection, strongly enhances rotavirus infectivity in tissue culture, probably by enhancing virus penetration and uncoating (Estes et al., 1981; Fukuhara et al., 1988 and Kaljot et al., 1988). In the prototype rotavirus, SA11, the enhancement of infectivity by trypsin is due to the specific cleavage of VP4 at two arginines within the following sequence: NTRNIVPVSIVSR*NIVYTR*AQPNQDIVVSKTS. Due to the two potential cleavage sites (arginine at 241 and 247), VP5 can be produced by cleavage at either site with the latter position being preferred [Fig. 1 (Lopez et al., 1985)]. The mechanism of activation of infectivity following cleavage is not clear. However, it has been proposed that cleavage activates an early step in genomic RNA replication. Since cleavage of VP4 significantly affects biological activity of the virus, this site is highly conserved among rotaviruses of different serotypes (Kalica et al., 1983; Mason et al., 1983). Studies designed to localize the hemagglutinin have clearly indicated that this function is associated with the VP8 fragment (Mackow et al., 1989; Flore et al., 1991). In addition to being able to inhibit hemagglutination, antibodies directed to either VP8 or VP5 have been demonstrated to neutralize the virus *in vitro* (Greenberg et al., 1983 a; Shaw et al., 1986; Taniguchi et al., 1987; Dyall-Smith et al., 1986; Mackow et al., 1988), and passively protect mice from rotavirus illness *in vivo* (Offit et al., 1986 b, c; Matsui et al., 1989). In animal rotaviruses, VP4 is composed of 776 amino acids (aa). In contrast, human rotaviruses contains 775 aa (Kantharidis et al., 1987; Gorziglia et al., 1988). The difference between the size of the human and animal rotavirus VP4 proteins is that the human

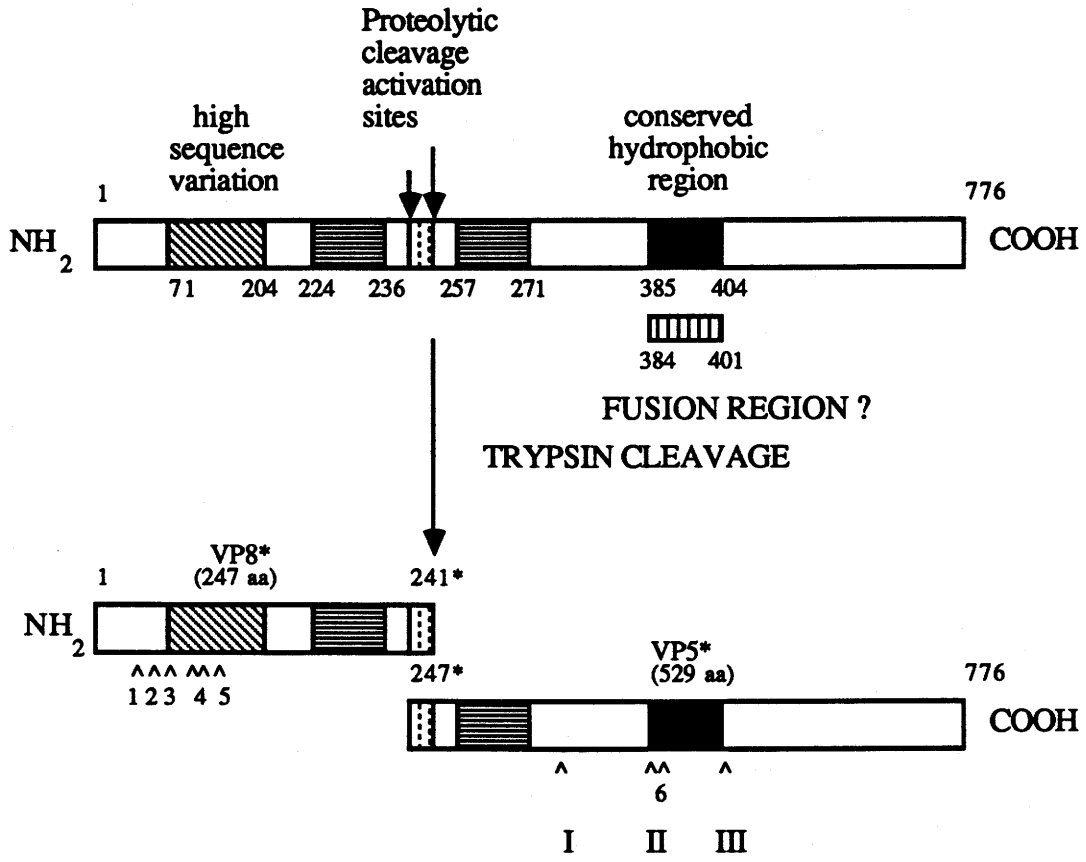


Fig. 1. Features of the Outer Capsid Protein VP4. Schematic of structural and antigenic properties of VP4 based on the analyses of nucleotide sequences of different virus strains and escape mutants selected with neutralizing MAbs. Symbols represent: ▤ , regions of sequence conservation among different virus strains; ▧ , region of greatest sequence variation in VP8*; ▥ , potential fusion region; ⇊ , sites of cleavage by trypsin. The locations of neutralization epitopes defined by Mackow et al. (1988) are shown by ^ and are numbed from 1 to 6; the epitopes defined by Taniguchi et al. (1988 a) are indicated by ^ and numbered with a Roman numerals (I to III). The peptide possibly removed by trypsin cleavage at aa 241- 247 is shown (⊡). The numbering shown here for VP4 is based on a protein of 776 aa for animal rotavirus strains. VP4 of human rotavirus strains contains 775 aa, lacking an amino acid at residue 136 (from Estes and Cohen, 1989).

strains are lacking one amino acid at residue 136, which is present in the animal strains (Kantharidis et al., 1987); therefore the VP8 of human strains is one amino acid shorter.

VP7 is the second most abundant capsid protein, and the major component of the outer capsid. It was the first protein shown to be able to induce neutralizing antibodies and is the principal antigen by which rotavirus serotypes are defined (Kapikian et al., 1976; Woode et al., 1976; Mathan et al., 1977; Schoub et al., 1977). It is encoded by genome segment 8 of C486 and UK bovine virus, genome segment 9 of SA11 and genome segment 7 of rhesus rotavirus. VP7 is initially synthesized as a precursor protein with a molecular weight of 37 kDa. It is subsequently cleaved to remove a small 1.5 kDa peptide, followed by the addition and processing (trimming) of N-linked high-mannose residues at one or two glycosylation sites (depending on the virus strain) to yield a mature protein of 38 kDa (Ericson et al., 1983). VP7 is the only structural polypeptide which is glycosylated.

Early investigations regarding the carbohydrates attached to VP7 were carried out by labeling the protein with [^{3}H]glucosamine (Arias et al., 1982; Ericson et al., 1983). These studies showed that VP7 carbohydrates were sensitive to digestion with endoglycosidase H, indicating that they were of the high-mannose type and contained only N-linked high-mannose oligosaccharide residues. The studies also demonstrated that oligosaccharides are added cotranslationally as this protein is inserted into the membrane of the ER. VP7 has clearly been shown to be an integral membrane protein with a luminal orientation whose oligosaccharides are modified by trimming in the ER (Ericson et al., 1983; Kabcenell and Atkinson, 1985).

Analysis of the nucleotide sequence of VP7 from six human serotypes and two animal serotypes has shown an open reading frame capable of encoding 326 amino acids (Kozak, 1986), and the existence of two tandem NH_2-terminal hydrophobic domains within the first 50 amino acids, which act as a signal sequence to direct VP7 to the ER (Gunn et al., 1985). Each hydrophobic domain is preceded by an inframe ATG codon. The first ATG is "weak" and the second one has the preferred consensus sequence for initiation of protein synthesis (Poruchynsky et al., 1985). The hydrophobic regions have been referred to as the H1 and H2 domains (Fig 2) (Whitfeld et al., 1987). This correlates well with the observation that VP7 has a cleavable signal peptide (Ericson et al., 1983), but raises the question as to which region actually functions as the signal sequence and, therefore, where cleavage occurs. These questions were addressed by introducing mutations into the VP7 gene (Poruchynsky et al., 1985; Whitfeld et al., 1987). Mutations which resulted in a deletion of the regions coding for the first, the second, or both hydrophobic domains were constructed. These genes were then transiently expressed in COS cells using a simian virus 40 (SV40) expression vector. The presence of signal peptide function was monitored by determining whether the resulting protein became glycosylated. This work revealed that, in the absence of both hydrophobic regions, glycosylation did not occur. However, the presence of either of the hydrophobic domains was sufficient to permit glycosylation; that is, signal peptide function was present in both the H1 and H2 domains (Whitfeld et al., 1987). Other work demonstrated that the presence of the second hydrophobic domain alone was sufficient to permit the glycosylation and processing of VP7 (Stirzaker et al., 1987; Poruchynsky and Atkinson, 1988). Stirzaker et al. (1987) constructed modified forms of the VP7 gene in which

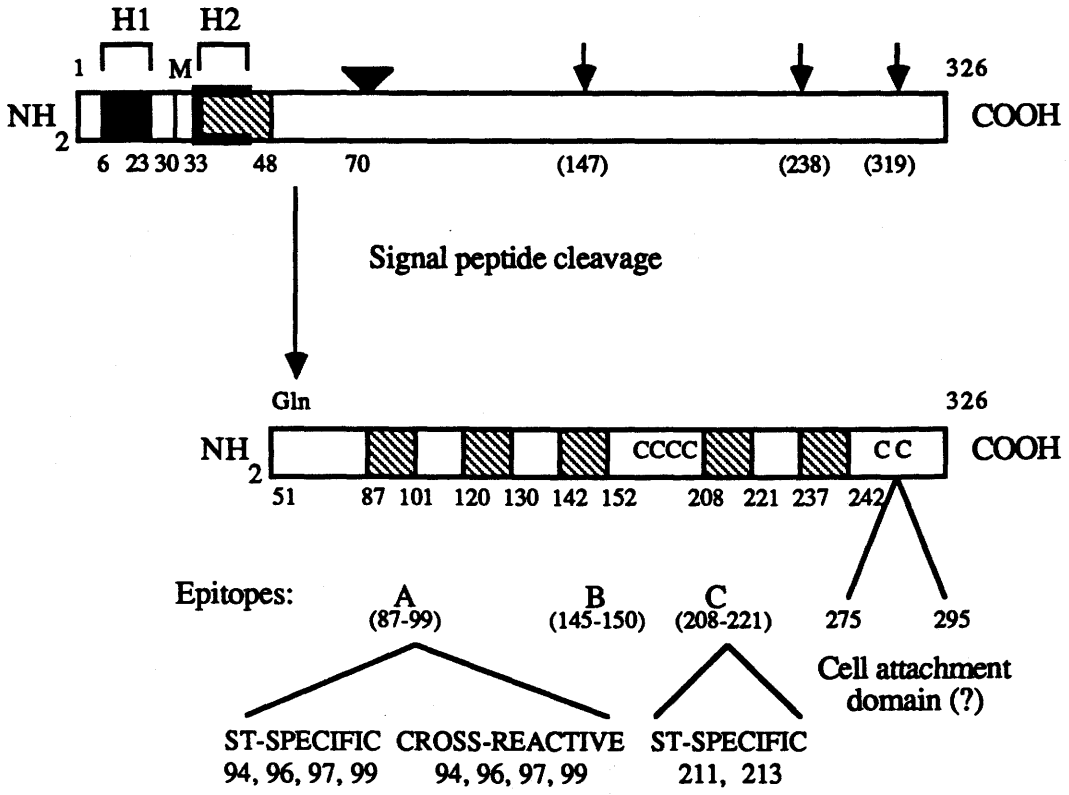


Fig. 2. Features of the Outer Capsid Protein VP7. The figure shows a schematic of structural and antigenic properties of VP7 based on the analyses of nucleotide sequences of different virus strains and escape mutants selected with neutralizing MAbs. The location of the two amino-terminal conserved hydrophobic regions (■), the second in-phase methionine and the preferred initiation codon (M), the one glycosylation site known to be used in SA11 (▼), additional potential glycosylation sites found in other virus strains (↓), conserved cysteine residues (C), and the one known site of signal peptide cleavage are shown. The lower diagram illustrates the regions of sequence variation (▨), the proposed cell attachment domain (aa 275 to 295), and the locations of amino acid changes in escape mutants selected following reactivity with serotype-specific and cross-reactive neutralizing MAbs (from Estes and Cohen, 1989).

initiation occurred specifically at either the first or second ATG codons. The mutated genes were then transcribed and translated *in vitro* in a reticulocyte lysate system in the presence of canine pancreatic microsomes. The sizes of the processed products in each case were identical and therefore independent of whether translation began at the first or second ATG. This finding strongly suggests that cleavage occurs downstream from the second signal peptide, irrespective of whether one or both hydrophobic domains are present. A likely cleavage site is between ala-50 and gln-51. Amino-terminal sequence analysis identified gln-51 as the amino-terminal residue present in complete virions of at least some molecules of VP7. gln-51 is conserved in all strains (Stirzaker et al., 1987). von Heijne (1986) constructed a mutated VP7 gene to determine whether the cleavage site was between ala-50 and gln-51. Depending on whether both hydrophobic domains or just one (i.e., the H2) was present, changing ala-50 to valine completely prevented cleavage, or resulted in another site being utilized, providing indirect evidence that gln-51 was the amino-terminal residue of mature VP7.

In vitro studies (Kabcenell and Atkinson, 1985; Poruchynsky et al., 1985; Stirzaker et al., 1987; Poruchynsky and Atkinson, 1988) indicate that VP7 is directed to the endoplasmic reticulum (ER) by a cleavable signal sequence within the first 50 amino acids and then processed to remove the two most prominent hydrophobic regions (H1 and H2) of the protein. Furthermore, VP7, translated in the presence of microsomes becomes resistant to digestion with trypsin, indicating that it is translocated completely into the lumen of the ER (Kabcenell and Atkinson, 1985; Stirzaker et al., 1987). In an *in vitro* translation system supplemented with dog pancreas microsomes, VP7 remained membrane bound after high salt

treatment and release of the microsomal contents at alkaline pH by sodium carbonate treatment. By this criterion it was concluded that VP7 becomes an integral membrane protein after entering the ER (Kabcenell and Atkinson, 1985). This membrane-bound form of VP7 can be distinguished by immunological and biochemical techniques from the polypeptide incorporated into virus particles, indicating that there are two pools of the protein present in the cell during infection (Kabcenell et al., 1988). Based on the kinetics of the processing of oligosaccharides attached to virus- versus membrane-bound VP7, it was suggested that the latter is the precursor of the former. Considering the mode of virus replication this would be expected.

Studies with bovine and human rotaviruses indicate that VP7 is one of the cell attachment proteins (Matsuno and Inouye, 1983; Sabara et al., 1985; Fukuhara et al., 1988). Initial studies showed that a polyclonal antibody, directed against VP7, blocked the attachment of radiolabeled virus to MA104 cells (Matsuno and Inouye, 1983). Later studies reported that bovine rotavirus VP7 was capable of binding to permissive cells and that monoclonal antibodies to VP7 could inhibit attachment of bovine rotavirus to these cells (Sabara et al., 1985). These results were substantiated by Fukuhara et al. (1988) using the human rotavirus KUN strain (HRV-KUN) to infect MA104 cells. Fukuhara demonstrated that a cell lysate prepared from MA104 cells infected with HRV-KUN contained a 35 kDa protein capable of binding to MA104 cells. The binding of the 35 kDa protein was inhibited by the addition of antiviral serum or VP7-specific monoclonal antibodies. In studies designed to identify and characterize the domain on VP7 responsible for binding to cells, Sabara et al (1985) used cyanogen bromide to cleave the VP7 molecule into various sized fragments and tested

the ability of the fragments to interact with host cells. Using this approach, the region was localized to a 14 K fragment of VP7. Further characterization of the 14 K peptide led to the identification of aa 275 to 295 as the major attachment domain. Evidence for aa 275-295 being a major attachment domain came from studies showing that the peptide was able to block virus attachment to permissive cells. This peptide was also reported to induce protection against virus challenge in a murine passive protection model (Frenchick et al., 1988). MAbs to this peptide also blocked attachment (Frenchick et al., 1988; Sabara et al., 1985). The 14K fragment of VP7 was reported to be an immunodominant domain on VP7 capable of inducing neutralizing antibodies (Sabara et al., 1985). MAbs that reacted with the 14K fragment and the peptide containing aa 275 to 295 also reacted with other serotypes, suggesting that this region may be conserved among rotaviruses (Sabara et al., 1985; Frenchick et al., 1988).

Protein conformation maintained by disulfide bonds has been shown to be important in maintaining the reactivity of the major glycoprotein (via neutralizing epitopes on VP7) with cell surface receptors. This was first suggested by the inability to produce hyperimmune antiserum with neutralizing activity against VP7 purified from denaturing polyacrylamide gels (Bastardo et al., 1981; Estes and Graham, 1985; Sabara et al., 1985). This was further supported by the inability of many virus neutralizing MAbs to react with denatured VP7 in Western immunoblots or by immunoprecipitation (Heath et al., 1986; Coulson, 1987; Taniguchi et al., 1985, 1987; Gerna et al., 1988) and the failure of linear synthetic peptides derived from different regions of VP7 to elicit neutralizing antibodies (Gunn et al., 1985). Although, surprisingly, the 14 K peptide that blocked binding did not contain any disulfide bonds, disulfide bridging was essential for

binding (Frenchick et al., 1988). Based on these somewhat contradictory results one can not definitely assume that glycosylation and secondary structure are essential for binding of virus to cells and antibody.

Antigenic sites on the rotavirus VP7 glycoprotein have been identified by studying nucleic acid differences between serotypes and by using mutant viruses selected for resistance to virus neutralizing (serotype-specific) monoclonal antibodies. Six regions were localized on the basis of the divergence of nucleic acid sequences between viruses with different serotypes. However, only five of these are apparently present in mature VP7, and only three of these regions (A, B, and C), located at aa 87 to 101, 142 to 152, and 108 to 221, respectively, have been confirmed to be involved in virus neutralization. These conclusions were forthcoming from studies which mapped the sequence changes in neutralization escape mutants (Dyall-Smith et al., 1986; Mackow et al., 1988 a; Taniguchi et al., 1988). Region C appears to be the most important. A mutation in region C caused a 10-fold increase in resistance to neutralization by polyclonal antiserum. These results, taken together with competition binding studies of neutralizing MAbs, indicate that the three-dimensional folding of the native protein of region A and C are actually in close proximity on the viral surface. A similar conclusion was drawn from the observation that the same MAb was capable of selecting mutations in both the A and C regions. It was also concluded that region C was the immunodominant epitope on the virus because mutations at amino acid 211 greatly reduced the reactivity of SA11 variants to high titer neutralizing hyperimmune antibody (Dyall-Smith et al., 1986). On the basis of these results, region C has been suggested to be an immunodominant antigenic site.

The study of viruses with different numbers of glycosylation sites

(from zero to two) on VP7 is beginning to show that glycosylation can modulate the biologic and antigenic properties of VP7. The observation that an SA11 variant with a nonglycosylated VP7 is infectious and possesses the biologic properties of hemagglutination and serotype specificity, indicates that glycosylation is not essential for these functions (Petrie et al., 1982). These studies were confirmed by the observation that VP7 peptide could attach to host cells and block rotavirus infectivity. Selection and characterization of neutralization escape mutants of this nonglycosylated variant of SA11 found that some mutants possess an amino acid substitution at residue 238 of VP7, whereas mutants of wild-type SA11 (selected with the same antibody) contained an amino acid substitution at residue 211 (in the C antigenic region). In both cases, the mutations produced new potential glycosylation sites, and these were found to be used. These mutations led to gross antigenic changes, which were found to be reversible upon removal of the attached carbohydrate (Caust et al., 1987). Andrew et al. (1987) also postulated that the presence of carbohydrate at aa 146 in serotype 2 viruses, blocked cross-reactivity in immunoblots with an anti-SA11 VP7 antibody that reacted with serotype 1, 3, and 4 viruses. These results show that carbohydrates can have an important role in affecting the exposure of antigenic determinants on VP7. However, this is not a universal phenomenon, since the addition of a new carbohydrate side chain at residue 99 in another neutralization escape mutant did not dramatically affect antibody binding to either the A or C region (Mackow et al., 1988a).

1.1.3.2 Inner capsid and core proteins

VP6, the nucleocapsid protein, is the major structural protein of the

virus, comprises 80% of the protein in single shelled particles (Bican et al., 1982), and forms the ring-like morphological subunits of the inner capsid (Novo and Esparza, 1981). It is encoded by genome segment 6. The nucleotide sequence has been determined for a variety of VP6 genes (Both et al., 1984; Estes et al., 1984; Hofer et al., 1987; Gorziglia et al., 1988 a). VP6 contains a common rotavirus group antigen, since VP6 specific monoclonal antibodies react with all mammalian rotaviruses, and polyclonal serum raised against a single rotavirus type can detect most other mammalian rotavirus strains (Greenberg et al., 1983 a). It is very immunogenic and antigenic, and it is the most frequently targeted protein in diagnostic assays to detect virus particles. Whether VP6 plays a role in inducing protective immunity remains unclear.

Biochemical characterization of VP6 revealed that it is composed of trimeric units in both the virus particle and in infected cells, with the subunits being linked by non-covalent interactions. These trimeric units complex further, by disulphide bridges into larger units which may represent the hexameric structures observed by electron microscopy (Gorziglia et al., 1985; Sabara et al., 1987). Three-dimensional structure studies of single-shelled particles have shown trimers to be present on the surface of these particles (Prasad et al., 1988). Trimerization and formation of tubules is an intrinsic property of this protein, since VP6 synthesized in the absence of other viral proteins forms such structures (Estes et al., 1987). *In vitro*, purified VP6 will also form tubules and virus-like particles at low pH (Ready and Sabara, 1987).

Biochemical and immunological approaches have been used to determine whether VP6 performs specific biologic functions during virus replication. Particles with only the inner shell proteins (single-shelled

particles) have transcriptase activity and are able to synthesize viral mRNA *in vitro* (Cohen, 1977; Mason et al., 1983 a; Helmberger-Jones and Patton, 1986). Transcription of the genome is dependent on the presence of VP6, since removal of VP6 from single-shelled particles with chaotropic agents results in a loss of transciptase activity. Reconstitution of the core particles with purified VP6 restores their ability to synthesize mRNA; and addition of excess VP6 to single-shelled particles inhibits transcription (Bican et al., 1982; Sandino et al., 1986). These experiments indicate that VP6 is required for polymerase activity, but they do not directly prove that VP6 itself is involved in transcription. VP6 may merely be important as a structural component to maintain the proper conformation or organization of the viral core structure or transcriptional complex composed of one or more of the core proteins. Recently, Mansell and Patton (1990) used two temperature sensitive (ts) mutants, of simian rotavirus SA11, to determine whether VP2 and VP6 are essential components of enzymatically active replicase and transcriptase. The results demonstrated that VP2, but not VP6 is an essential component of active replicase, whereas VP6 seems to be only an essential component of transcriptase. VP6 is myristilated, and this modification may be important for formation of virus particles or for targeting single-shelled particles to the ER membrane (Clark and Desselberger, 1988).

VP1 is encoded by genome segment 1 in all rotavirus isolates, and it is one of three proteins (VP1, VP2, and VP3) that make up the rotavirus core particle. VP1 represents only 2% of the viral protein of the complete virion. The deduced amino acid sequence of VP1 of a bovine rotavirus strain indicates that this protein migrates in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to its calculated molecular weight of

124,847 and that it is a relatively hydrophobic and a slightly basic protein (Cohen et al., 1989). As a minor component of single-shelled particles, VP1 could function as part of the transcriptase present in activated particles (Fukuhara et al., 1989). Temperature-sensitive mutants which map to the genome segment 1, have RNA-negative phenotypes (Gombold and Ramig, 1987). One region, between amino acids 517 and 636, contains significant homology with consensus sequences that have been established for a number of putative RNA-dependent RNA polymerases present in RNA viruses of eukaryotes (Pietras et al., 1988; Cohen et al., 1989). These observations support a putative enzymatic role for VP1.

VP2, encoded by genome segment 2, is a most abundant structural protein composing about 90% of the core (Bican et al., 1982; Liu et al., 1988). It is the third most abundant protein in double-shelled particles. It has been shown that VP2 binds nucleic acids in a non-sequence-specific manner (Boyle and Holmes, 1986). Furthermore, nucleotide sequence analysis indicates that VP2 possesses two motifs present in various nucleic acid-binding proteins. Two "leucine zippers" are present between amino acids 536 and 686 and could be implicated in dimerization of VP2 and enhancement of binding to nucleic acid (Landschulz et al., 1988; Kumar et al., 1989). The region between amino acids 65 and 120 is predicted (Garnier et al., 1978) to have an alpha helix-turn-alpha helix secondary structure, a motif found in several DNA-binding proteins. Another sequence between amino acids 53 and 81 could permit the dimerization of VP2. This sequence, predicted to be an alpha helix, contains five acidic amino acids and five lysines which are spaced exactly seven amino acids apart and hence could be viewed as being repeated every two turns in the alpha helix. Their arrangement of oppositely charged amino acids might also allow

dimerization of VP2 via alignment of parallel helices (Mitchell and Both, 1990). It has been suggested that VP2 may be a nucleocapsid protein which is bound tightly to the RNA segments. It has also been suggested that this protein is necessary to induce additional bending of the double-stranded RNA segments, to allow packaging in the capsid (Kapahnke et al., 1986). Its apparent molecular weight of 94 K calculated from its mobility in SDS-PAGE differs from the molecular weight of 102,431 deduced from its nucleotide sequence. VP2 has been found to be myristilated, and although the functional significance of this is unknown, such modifications in other viral systems have been found on scaffolding proteins important for the formation of virus particles (Clark and Desselberger, 1988).

VP3, encoded by genome segment 3, is a minor structural protein. It was described only recently because it proved difficult to resolve from other viral proteins by PAGE (Liu et al., 1988). This protein is also poorly translated in vitro and is synthesized at low levels in infected cells. VP3 can be detected in early replication complexes (Gallegos and Patton, 1989) and is a structural polypeptide found in the central core of the virus. Analysis of the deduced amino acid sequence indicates that it is a basic protein that contains multiple repeats of amino acids. VP3 has sequence homology with the RNA polymerases of other RNA viruses (Liu and Estes, 1989), has GTP-binding activity and is responsible for the capping of viral transcripts (Fukuhara et al., 1989). Therefore, it has been suggested that this protein is involved in RNA replication (Liu et al., 1988).

1.1.4 Rotavirus nonstructural proteins

The rotavirus nonstructural proteins are found in infected cells but not in mature particles. There are a total of five nonstructural proteins in

infected cells, which are designated as NS, followed by their molecular weight (i.e., NS53, NS35, NS34, NS28 and NS26).

NS53 is a nonstructural protein encoded by genome segment 5. The gene was cloned from SA11 and the bovine RF strain and sequenced (Bremont et al., 1987; Mitchell and Both, 1990 a). Very little is known about the function of the protein. It is only expressed at very low levels early in infection (Ericson et al., 1982; Johnson and McCrae, 1989) and its cellular location has not been determined. The sequence predicts that NS53 is a basic protein with a net positive charge of 9 at pH 7.0 (Bremont et al., 1987). The basic nature of NS53 might help in binding to the nucleic acid. NS53 has been successfully expressed in *E. coli* , which will facilitate the study of intrinsic biochemical and functional properties of this protein in the rotavirus replication process (Arias et al., 1987; McCrae and McCorquodale, 1987; Bremont et al., 1987).

NS35 is encoded by genome segment 7, 8, or 9, depending on the virus strain. It is a basic protein on the basis of the deduced amino acid sequence and is a nonglycosylated protein which is found in relative abundance in lysates of rotavirus-infected cells (Petrie et al., 1984). It appears to be relatively immunogenic, since antibodies against it are found in both hyperimmune and postinfection sera (Svenson et al., 1986). This protein has been localized in association with viroplasms in infected cells by using immunocytology (Petrie et al., 1984). The function of NS35 in rotavirus replication is unknown, although one study has suggested that it may be an important component of the rotavirus replicase particle (Patton and Gallegos, 1988). The presence of a helix-turn-helix motif between residues 160 and 218 in this protein is consistent with possible interactions with nucleic acid. Recent study also found that NS35 and not VP7 binds to the

surfaces of MA104 cells and murine enterocytes but did not confirm that NS35 functioned as the rotavirus attachment protein (Bass et al., 1990).

NS34 is encoded by SA11 genome segment 7 and is a slightly acidic protein (Both et al., 1984a). In infected cell extracts, NS34 was detected as an RNA-binding protein. NS34 bound both single- and double-stranded RNA equally well. Binding did not appear to be nucleotide sequence specific (Boyle and Holmes, 1986). NS34 has also been found in RNA replicase particles. These studies suggest that NS34 is involved in the replicase activity (Helmberger and Patton, 1986; Patton and Gallegos, 1988).

NS28 is the product of gene 10 (Both et al., 1983 a; Baybutt and McCrae, 1984; Okada, et al., 1984) and is a nonstructural protein of 175 amino acids containing three hydrophobic domains, H1, H2, and H3, near the N terminus (Both et al., 1983 a). H1 has two sites where N-linked high-mannose oligosacchrides are added (Both et al., 1983 a). H2 is the internal uncleaved signal and membrane anchor sequence (Bergmann et al., 1989). H3 remains exposed on the cytoplasmic side of the membrane, leaving approximately 131 amino acids available for receptor-ligand interaction. The mature glycoprotein is a integral membrane protein of the ER (Ericson et al., 1983; Kabcenell and Atkinson, 1985) and is thought to have at least one membrane-spanning domain (Chan et al., 1988; Bergmann et al., 1989). Studies of the topograghy of NS28 indicate that the amino terminus of the molecule is maintained in the membrane and the carboxy terminus extends into the cytoplasm of infected cells (Chan et al., 1988; Bergmann et al., 1989).

Since NS28 accumulates in the rough endoplasmic reticulum (RER) lumen, when virus-infected cells are grown in the presence of tunicamycin (Holmes, 1983; Petrie et al., 1983), it was proposed that it functions as a

scaffolding protein involved in virion morphogenesis This enveloped intermediate stage has been hypothesized to result from the budding of subviral particles (possibly ss particles) through the RER membrane (Estes et al., 1983; Holmes et al., 1983). Glycosylation of the rotavirus nonstructural glycoprotein NS28 was recognized to be important in the budding and maturation process when enveloped particles were shown to accumulate in tunicamycin-treated cells infected with a variant of SA 11 that codes for nonglycosylated VP7 (Estes et al., 1982; Petrie et al., 1983). NS28 acts as a receptor for the rotavirus core and mediates their budding into the lumen of the ER (Petrie et al.,1983). Au et al. (1988) demonstrated that membranes prepared either from SA11 virus infected cells, or from Sf9 insect cells infected with recombinant baculovirus expressing NS28, were able to bind rotavirus core. Membranes not incorporating NS28 did not possess receptor activity. Specificity of the interaction of rotavirus cores with the ER membrane demonstrate that NS28 acts as the receptor. This inference is supported by the topology of the protein in the membrane: NS28 is 175 amino acids in length and most of the protein is displayed to the cytoplasmic side of the membrane (Chan et al.,1988; Bergmann et al.,1989) and therefore is available for interaction with the ligand.

NS26 was identified as a product of genome segment 11. The main features conserved in this protein are the unusually high serine content and the presence of clusters of charged amino acids. Although the primary translation product was identified, its designation as a structural or nonstructural protein has remained controversial. For example, the gene segment 11 protein was tentatively assigned as a nonstructural glycoprotein (Arias et al., 1982), a structural protein of the inner virus shell (McCrae and McCorquodale, 1982), a minor outer capsid protein (Mason et

al., 1983), or a minor neutralizing antigen (Matsuno et al., 1980). Recently, NS26 was expressed in Sf9 insect cells-infected with recombinant baculovirus. The results indicate that NS26 is a nonstructural protein which is not associated with double-shelled virus particles (Welch et al., 1989).

NS26 has an apparent molecular mass of 28 kDa seen on polyacrylamide gels, which differs from that of the primary translation product obtained in a cell-free translation system (26 kDa), suggesting that it undergoes post-translational modification, which is phosphorylation (Welch et al., 1989). However, removal of the phosphate group did not change the electrophoretic mobility of the protein, thus the nature of the main modification responsible for the observed molecular weight shift remains unknown. Gonzalez and Burrone (1991) indicated that NS26 is processed to a 28 K polypeptide by the addition of O-linked monosaccharide residues of N-acetylglucosamine. This study helps to explain the reason for the mobility shift on polyacrylamide gels.

1.1.5 Immunity to rotavirus infection

Immunologic studies of the rotavirus group have indicated the existence of two major antigenic components in the native virion. One is recognized as the group specific antigen (GSA) which is located in the inner capsid and can be demonstrated by a complement-fixation test (CF), immunodiffusion (ID) and immunoelectron microscopy [IEM; (Flewett and Woode, 1978)]. VP6 is considered to be the protein responsible for GSA. The other major antigen, recognized as the type specific antigen (TSA), is located on VP7 which is the major outer capsid protein. TSA is defined by plaque reduction or fluorescent-focus reduction neutralization assays using

antisera to purified virus particles prepared in hyperimmunized animals (Kapikian et al., 1976; Woode et al., 1976; Mathan et al., 1977; Schoub et al., 1977).

A considerable amount of information is available regarding the rotavirus proteins that stimulate protective immunity. Furthermore, the role of antibody in the intestinal lumen of infected animals has clearly been shown to provide protection against rotavirus infection and disease. In experimental studies, rotavirus infection of cattle, lambs and piglets has successfully been prevented by feeding neonates virus neutralizing antibody. In contrast, systemic administration of antibody was less effective in preventing diarrhea. Thus rotavirus illness, in calves and lambs can occur in the presence of circulating rotavirus antibody, whereas rotavirus antibody in the lumen of the small intestine provides protection (Woode et al., 1975; Snodgrass and Wells, 1976).

Cross-protection with heterologous rotavirus serotypes or strains has also been shown in passive protection studies (Snodgrass and Wells,1976; Gaul et al.,1982; Snodgrass, 1982; Snodgrass et al., 1982; Torres and Lin, 1986; Bridger and Oldham, 1987; Donald et al., 1987; Woode et al., 1987; Hoshino et al., 1988). However, the limited availability and cost of conducting large animal studies has hampered extensive studies in these species. To overcome these limitations, individuals have focused on developing small laboratory animal models. Offit et al. (1985) developed a passive rotavirus protection model in mice. In this model, they first studied the relative roles of passively acquired circulating and intestinal rotavirus-specific antibodies in protecting suckling mice against diarrhea induced by SA11 virus challenge. The result indicated that passively acquired gastrointestinal antibody, but not circulating antibodies, protect suckling

mice against diarrhea induced by challenge with SA11 virus (Offit et al., 1985). These studies paralleled the protection observed in large animals, demonstrating the relevance of the murine model to study immunity to rotavirus infection. Subsequently, they demonstrated that passive protection was mediated by monoclonal antibodies directed against VP4, VP7 and partially to VP6. They also showed that protection of both homoserotypic or heteroserotypic rotaviruses could be partially produced. The results also demonstrated that maternal antibody-mediated protection against rotavirus challenge is dependent on the serotype of the virus, titer of antibody, and that immune responses to both outer capsid proteins (VP4 and VP7) are primarily involved in inducing protection from diarrheal disease (Offit et al., 1985 a, 1986 c; Matsui et al., 1989).

Recently, serologic and mucosal immune responses to rotavirus infection of rabbits has also been examined (Conner et al., 1991). Serologic and mucosal antibody responses were initially detected at 1 week postinoculation. Following challenge, rabbits were protected from subsequent infection. All serologic and mucosal immune responses persisted at high levels for at least 204 days postinoculation. Based on these results, it is accepted that the antibodies in the intestinal lumen are necessary to afford protection against rotavirus. Since diarrhea, especially in animal species, occurs during the first few weeks of life, it is often difficult to induce adequate active immunity prior to their first exposure to the pathogen. Consequently, one of the most productive strategies for control of enteric viral infections involves vaccination of the mothers, who transfer sufficiently high titers of colostral antibodies that the young animals are passively protected during the time of maximum sensitivity to infection (Saif, 1985). This approach has been especially successful in

calves (Snodgrass et al., 1980; Saif et al., 1984; Beards and Desselberger, 1989). Since the virus is ubiquitous in cattle, vaccination with live or inactivated vaccines boosts the immune response in naturally primed animals. As a result of multiple exposure and/or vaccination, heterotypic lactogenic immune responses generally develop (Brussow et al., 1988; Snodgrass et al., 1984).

To support the studies conducted with monoclonal antibody and to confirm the role of the individual proteins in inducing protection, a number of groups have cloned the individual rotavirus gene into various expression systems. This approach has been feasible since many of the rotavirus genes have been sequenced (Both et al., 1983; Elleman et al., 1983; Arias et al., 1984; Dyall-Smith and Holmes, 1984; Richardson et al., 1984; Glass et al., 1985). Some structural and nonstractural proteins have also been expressed. Animals immunized with VP4 or VP7 developed neutralizing antibodies, whereas those immunized with VP6 did not express virus neutralizing activity (Andrew et al., 1987; Estes, et al., 1987; Francavilla et al., 1987; Brussow et al., 1990; Mackow et al., 1990; Poncet et al., 1990).

To localize the regions on rotavirus proteins VP4 and VP7 that may be involved in inducing protective immunity, mice were immunized with synthetic peptides corresponding to regions of VP4 and VP7 (Ijaz et al., 1991). A synthetic peptide derived from the conserved trypsin cleavage site on VP4 provided excellent protection in the murine passive protection model. A second peptide from VP7 (aa 275-295) only provided partial protection in the same model.

There are also a few studies which have examined the host's cellular immune response to rotavirus infection. These studies have demonstrated that rotavirus-infected cells, but not uninfected cells, stimulated peripheral

blood cells to produce a cytokine that enhanced NK cell activity to rotavirus infected cells (Kohl et al., 1983). In addition, rotavirus specific cytotoxic T lymphocytes have been shown to kill target cells infected with different human or animal rotavirus serotypes (Offit et al., 1988, 1991). These studies demonstrated that rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface following rotavirus infection. Regardless of whether challenge was oral or via parenteral inoculation, specific CTL cytotoxic activity was shown to be greatest at 6 days after inoculation (Offit and Dudzik, 1989).

More recently, Dharakul, et al (1991), reported that mice immunized with VP1, VP4, VP6, and VP7 proteins expressed by recombinant baculovirus did induce $CD8^+$ T lymphocytes that mediated clearance of chronic rotavirus infections.

1.2 Expression of Viral Proteins in Eukaryotic Systems

1.2.1 Introduction

Although viral proteins can be produced very efficiently in prokaryotic expression systems, glycosylation and folding of the protein is dramatically different from that produced in eukaryotic systems. As a result of these differences, the proteins are often poorly immunogenic. Thus, most studies have focused on expressing viral genes in eukaryotic systems. At present, most cloned genes are introduced into eukaryotic cells by using mechanical microinjection, liposomes, transfection with recombinant plasmid and infection with recombinant virus. For a number of reasons many of nonviral systems are limited in efficiency (Gruss and Khoury, 1982). In contrast, infection of cells with recombinant viruses containing foreign genes can be a much more efficient way of introducing DNA into some cells. With virus infection, it is possible to ensure that each recipient cell has many copies of the foreign gene, and since the viral genome includes strong promoters, it is possible to ensure efficient expression of foreign genes inserted into the viral DNA. As a result, the amount of gene products, both RNA and protein, are often higher and therefore more accessible to study (Watson et al., 1983).

A variety of virus-derived vector systems including polyoma virus (Gluzman, 1981; Okayama and Berg, 1983; Reddy et al., 1985), papilloma virus (Sarver et al., 1981), picornaviruses (Burke et al., 1988), retrovirus (Mann et al., 1983; Watanabe and Temin, 1983), adenovirus (Johnson et al., 1988), herpes simplex virus (Post and Roziman, 1981; Spaete and Frenkel, 1982), vaccinia virus (Mackett et al., 1982; Panicali and Paoletti, 1982) and baculovirus (Summers and Smith, 1985) have been developed. The choice of

a particular viral vector depends on the specific experimental goals. The small and intermediate sized viruses, including polyoma, papilloma, adenovirus and retroviruses offer simplicity of vector construction and relatively high-level expression. As a trade-off, however, these viruses have a limited host range and/or can accommodate little extra genetic information before becoming defective in replication (Rigby, 1983; Subramani and Southern, 1983). The large DNA viruses, such as vaccinia virus and herpes virus, are intrinsically more difficult to engineer genetically than are the viruses listed above, but they have the potential of retaining complete infectivity in a wide range of cell types and have a greater capacity for the introduction of foreign DNA (Mackett et al., 1985).

In this thesis, the use vaccinia virus and baculovirus as expression vectors will be discussed in the following section.

1.2.2 Vaccinia virus as an expression vector

1.2.2.1 Introduction

Vaccinia virus (VV) has been the most commonly used vector for introducing foreign genes into animal cells (Mackett et al., 1985). Its significant advantages as a eukaryotic cloning vector are: (1) VV is relatively safe to work with. Vaccine strains are available which do not cause serious diseases in animals or humans. Furthermore, studies designed to identify virulence genes have proven that avirulent VV can be developed. Addition of a cytokine gene, such as IL2, can further reduce the virulence of vaccinia virus. (Moss, 1973). (2) VV has a very broad host range, allowing it to be used to infect cells from a variety of species (Franke and Hruby , 1985). (3) Both the VV virion and its genomic DNA are quite large. This allows large and /or multiple foreign genes to be inserted into

the VV genome. About 25,000 base pairs of foreign DNA can be incorporated into the vaccinia genome without a deleterious effect on virus replication (Panicali et al., 1983; Smith and Moss, 1983; Rice et al., 1985). Furthermore, large segments of the vaccinia virus genome can be deleted to provide additional capacity for inserting genes (Moss et al., 1981; Panicali et al., 1981). (4) VV is not tumorogenic since it replicates within the cytoplasm of infected cells and does not integrate into host cell genome. (5) VV genes, or inserted foreign genes, are transcribed by viral enzymes using unique VV regulatory signals, as opposed to the host cell's nuclear transcription apparatus. This allows one to avoid potential problems due to inefficient processing, aberrant splicing, or transport when genes are expressed within the nuclear compartment of infected cells. (6) Since VV replicates within mammalian cells, foreign animal virus proteins which are expressed by recombinant VV are modified and transported in a manner analogous to the native situation. (7) Since VV replicates in common tissue culture cells or laboratory animals, production of live recombinant viral vaccines is relatively inexpensive, allowing this methodology to be applied to diseases of veterinary importance (Hruby, 1988).

1.2.2.2 Biology of vaccinia virus

Vaccinia virus is the prototype of the orthopoxvirus genera of the poxvirus family. Poxviruses, of which vaccinia virus is by far the most thoroughly characterized member, are among the largest of all animal viruses. The genome of vaccinia virus consists of a long linear double-stranded DNA molecule of approximately 187,000 base pairs (Geshelin et al., 1974). The double-stranded genome is characterized as having inverted

terminal repeats. The genome is cross-linked at the termini by a covalently closed, single-stranded DNA loop (Tattersall et al., 1976; Baroudy et al., 1982). Isolated virus DNA is not infectious, consistent with the presence of virus-specific enzymes within the virus particle that are necessary for transcription of virus genomic DNA (Baroudy et al., 1982).

After entry into a cell, the viral transcription system is activated and approximately 100 early genes are expressed. Early gene expression occurs within the first 6 hours. The early viral proteins synthesized include a DNA polymerase and other factors needed for replication of the vaccinia genome. The late genes are transcribed only after the commencement, 6 hours post-infection, of viral DNA synthesis. Once the late genes are transcribed, there is a decline in early gene expression. Most late genes contain a conserved TAAAT element. The encoded mRNA sequence starts within three consecutive A residues present in a conserved TAAAT element and the third A most usually represents first nucleotide of a late gene initiation codon (Berthelot et al., 1986; Rosel et al., 1986; Weir and Moss, 1987; Lee-Chen and Niles, 1988). In contrast to early and late genes, a gene encoding a mRNA that is translated into a protein with a molecular weight of 7.5 K is expressed constitutively during infection. The promoter of this gene is called the 7.5 K promoter which contains both early and late signals (Mackett et al., 1982a; Smith et al., 1983; Davison and Moss, 1989).

The products of the late genes include the majority of structural proteins which are assembled into virus particles within the cytoplasm. Some virions remain intracellular, while others become wrapped in Golgi membranes and are extruded through the plasma membrane with an additional envelope. Both the intracellular and extracellular forms of vaccinia virus are infectious (Moss and Flexner, 1989).

1.2.2.3 Expression of foreign genes in recombinant vaccinia virus

The efficient expression of foreign genes in vaccinia virus is produced by a two-stage process. First, recombinant DNA techniques are used to construct a plasmid (insertion vector) that contains the foreign gene linked to a vaccinia virus promoter. Secondly, the insertion of the gene into the vaccinia virus genome occurs by homologous recombination between the virus genome and plasmid *in vivo* . A more detailed description of these steps is presented below.

1.2.2.3.1 Design of vaccinia insertion vectors

The sequences of several vaccinia virus genes and their promoters have been determined (Weir and Moss, 1983). This information has allowed the construction of several plasmid insertion vectors which can be used to insert and express foreign genes in vaccinia virus. These vectors contain VV sequences, including a defined vaccinia promoter with the transcriptional but not translational initiation site. Immediately downstream from the RNA start site of the vaccinia promoter, multiple unique restriction endonuclease sites are positioned for insertion of foreign protein-coding sequences. It is important that the DNA between the transcriptional start site and the downstream restriction sites does not contain any translation initiation sites. This must be then provided by the foreign gene, ensuring that fusion polypeptides with unpredictable properties are not produced, and problems with improper reading frames are avoided. The foreign gene should provide its own translation termination site as well so that authentic polypeptides are produced. Foreign DNA has been inserted into several loci within the vaccinia virus genome that are non-essential for virus replication in tissue culture

(Mackett et al., 1982; Panicali and Paoletti, 1982). The site of foreign gene insertion is determined by the flanking vaccinia virus DNA present in the insertion vector. The non-essential site often chosen is the thymidine kinase (tk) gene, since loss of tk activity through insertion of foreign DNA can be positively selected. Several insertion vectors that are generally applicable for the expression of any continuous protein-coding sequences in vaccinia have been constructed using this approach. Using these vectors, only one cloning step is usually required to produce a plasmid that can be used directly to insert genes into the viral genome (Smith et al., 1984).

1.2.2.3.2 Construction of recombinant vaccinia virus

Three properties of vaccinia virus are important when considering the construction of recombinant viruses. First, the double-stranded DNA genome is very large so that in vitro manipulation of the whole genomic DNA is extremely difficult. Second, isolated virus DNA is not infectious, so that recombinant virus will not be formed following introduction of genomic DNA to cultured cells. Third, vaccinia virus possesses an RNA polymerase that recognizes its own transcriptional control signals, but which will not recognize eukaryotic RNA polymerase II promoters (Smith et al., 1984).

The basic strategy involved in the construction of the recombinant vaccinia virus initially described was an extension of the marker rescue technique applied to this virus. Sam and Dumbell (1981) used temperature-sensitive markers to demonstrate the transfer of endogenous genetic markers into infectious vaccinia virus. Nakano et al. (1982) also demonstrated marker rescue by reinserting unique vaccinia DNA sequences into vaccinia deletion mutants. Marker rescue has subsequently been used to localize the genes encoding thymidine kinase (Weir et al.,

1982), DNA polymerase (Jones and Moss, 1984; Traktman et al., 1984), rifampicin resistance (Tartaglia and Paoletti, 1985), various temperature-sensitive genetics markers (Condit et al., 1983; Ensinger and Rovinsky, 1983; Drillien and Spehner, 1983), and sequences specifying host range (Gillard et al., 1985). Extensions of these basic protocols have allowed for the insertion of foreign genes into viable vaccinia virus. First, foreign genes of interest are isolated and modified at their termini to allow insertion into recombinant plasmids such that they are downstream of an efficient VV transcriptional promoter and flanked on both sides by vaccinia DNA sequences. These recombinant plasmids are introduced into the cytoplasm of VV-infected cells. The VV DNA sequences in the plasmids are thought to allow homologous recombination which results in insertion of the passenger foreign DNA into the viral genome but does not disrupt the flow of essential viral genetic information (Hruby, 1988; Paoletti, 1990). Thus, the foreign gene is now flanked by normally continuous DNA sequences of the vaccinia virus genome. The rescuing virus goes through its normal replication cycle, including penetration into the cell, uncoating, transcription, DNA replication, and maturation into progeny virus. At a frequency of approximately 0.1%, a replicating DNA molecule from the input virus comes in contact with the chimeric donor plasmid such that the vaccinia sequences flanking the foreign gene undergo homologous recombination with identical sequences on the vaccinia genome. This causes the foreign DNA to be inserted into an intact recombinant vaccinia genome which can in turn be incorporated into infectious recombinant vaccinia virus.

Recombinant virus can be identified by a number of strategies. Depending on the strategy used, individual recombinants are recovered by

plaque purification, and analyzed for their ability to express the gene product encoded by the inserted DNA sequences (Nakano et al., 1982; Mackett et al., 1984; Rice et al., 1985).

Since only about one in a thousand virus particles produced by marker rescue is a recombinant, efficient selection procedures are very important. Commonly, the nonessential vaccinia thymidine kinase (TK) gene is used as the flanking sequences. When the foreign gene is inserted by reciprocal recombination into the TK locus, the TK gene is inactivated. Selection of TK^- virus is achieved by plaquing the virus in TK^- cells in the presence of 5-bromodeoxyuridine. Phosphorylation of this nucleoside analogue and consequent lethal incorporation into viral DNA occurs only in cells infected with TK^+ parental virus. Depending on the efficiency of transfection and recombination, up to 95% of the plaques are recombinants, and the rest are spontaneous TK^- mutants. Once a recombinant is identified, it can be isolated, purified, and grown to high titers to be used for subsequent analyses.

The development of vaccinia virus for cloning and expressing foreign genes is a technological advance that occurred in 1982. Since that time, protocols have been developed and refined to facilitate the rapid construction and selection of vaccinia virus recombinants that express foreign genes. The expression of foreign proteins in tissue culture cells infected with the recombinant vaccinia viruses has been detected by several methods including black plaque assays (Johnson et al., 1986), immunostaining (Mackett and Smith, 1986), radioimmunoassay (Mackett et al., 1985) and western blotting (Mackett and Smith, 1986). Furthermore, animals vaccinated with virus recombinants can produce humoral antibody and cell-mediated immune responses against the foreign protein.

These immunological responses have been shown to protect vaccinated animals against challenge with hepatitis B, influenza, herpes simplex virus, rabies, malaria and rinderpest (Smith et al., 1983, 1983 a; Wiktor et al., 1984; Cremer et al., 1985)

1.2.3 Baculovirus as an expression vector

1.2.3.1 Introduction

The use of baculovirus as an expression vector is similar to vaccinia virus in which recombination occurs between the virus genome and homologous sequence within an insertion plasmid. It was developed, and has been used as vector, for the expression of a wide variety of foreign genes since 1983 (Smith et al., 1983, 1983a; Summers and Smith, 1985; Summers and Smith, 1987). Several features of baculovirus make it a highly attractive expression vector. These features include: (1) the very abundant expression of recombinant proteins; (2) a large viral genome (130 kbp), which can accommodate large segments of foreign DNA; (3) the baculovirus polyhedrin gene is nonessential for viral reproduction, it is a late gene with a strong promoter, and its gene product or its absence is easily detected by light microscopy; (4) baculovirus is not pathogenic to vertebrates and their promoters are inactive in mammalian cells (Carbonell et al., 1985), which gives them a possible advantage over other systems when expressing oncogenes or potentially toxic proteins, and; (5) many of the protein modifications, processing, and transport systems present in higher eukaryotic expression systems are functionally similar in insect cells. This makes the foreign protein produced in baculovirus antigenically and immunologicly similar to their authentic counterparts. Therefore, the baculovirus polyhedrin gene is an ideal candidates for a

foreign gene insertion site and its promoter may be used to drive the high-level expression of the inserted gene. These features make baculovirus a very attractive expression vector.

1.2.3.2 Biology of baculovirus

Autographa californica nuclear polyhedrosis virus (AcMNPV) is the prototype virus of the family Baculoviridae. The genome of AcMNPV consists of a single molecule of circular supercoiled double-stranded DNA of 130 kb in length (Smith and Summers, 1978, 1978a). Virus particles enter the cell by the process of adsorptive endocytosis or fusion. Uncoated nucleocapsids are transported into the nucleus where their DNA is released. Transcription and DNA replication occur within the nucleus. The virus undergoes a primary round of replication with progeny nucleocapsids observed as early as 8 hours post-infection (p.i.). Replication is followed by viral assembly in the nucleus of infected cells. Two phenotypes of viral progeny are generated in the infectious cycle: nonoccluded form of the virus (NOV) and occluded viruses (OV). Progeny NOV is released from the cell by budding within 10 hours p.i. Polyhedrin protein can be detected 12 hours p.i., but OV are not detected until 18-24 hours p.i. NOV reaches a maximum level between 36-48 hours p.i. but OV continues to accumulate for 4-5 days until the lysis of infected cells (Luckow and Summers, 1988).

The polyhedrin gene of AcMNPV has been mapped and sequenced (Hooft van Iddekinge et al., 1983). This gene has been shown to be nonessential for infection and replication of the virus (Smith and Summers, 1983). Therefore, replacement of the polyhedrin gene with a foreign gene results in the production of occlusion negative (Occ^-)

phenotype which form clear plaques that are distinctly different from opaque those of wild-type, occlusion positive (Occ^+) viruses. These distinctive plaque morphologies provide a simple visual screen for identifying the recombinant viruses. Since the nonessential nature and high levels of expression of the polyhedrin gene, foreign genes are often cloned behind the polyhedrin gene promoter in recombinant baculovirus expression vectors (Pennock et al., 1984; Smith et al., 1983 a).

1.2.3.3 Expression of foreign genes in insect cells by baculovirus vectors

To produce a recombinant virus that expresses the gene of interest, the gene is first cloned into an insertion vector. These vectors contain sequences from AcMNPV including the promoter of the polyhedrin gene and varying amounts of 5' and 3' viral DNA flanking the polyhedrin gene. The desired foreign gene sequences in the recombinant plasmid can be transferred to wild-type AcMNPV by homologous recombination within a cell cotransfected with both the plasmid and wild-type virus DNAs. The foreign gene is inserted into the viral genome and the polyhedrin gene is excised *in vivo* during replication. Recombinant virus lacks the polyhedrin gene, which is replaced by the gene of interest and whose expression is under the control of the polyhedrin promoter. Recombinant viruses that do not produce polyhedral occlusion bodies are isolated by plaque purification. Recombinant virus can then be cultured either in the established cell line or in silkworm larva to isolate large quantities of the gene product (Maeda, et al., 1985).

Foreign proteins produced in baculovirus infected cells undergo many of the posttranslational modifications, including glycosylation (Smith et al.,1983a, 1985), phosphorylation (Miyamoto et al., 1985), correct signal

peptide cleavage (Smith et al., 1983a), and the removal of introns by proper splicing (Jeang et al., 1987). Therefore, baculoviruses are a valuable tool for *in vitro* production of biologically active proteins. Furthermore, they are useful vectors for high-level expression of eukaryotic genes. The success in constructing vectors that produce biologically active protein products using relatively easy and rapid technology makes this vector system a popular one from a research perspective and as a result, many genes have been produced in baculovirus systems to date (Estes et al., 1987; Hu et al., 1987; Inumaru and Roy, 1987; Inumaru et al., 1987; Lanford et al., 1987; Madisen et al., 1987; Rice et al., 1987; Thomsen et al., 1987).

2. OBJECTIVES

Since rotavirus is a major cause of acute neonatal gastroenteritis in human and animals, it is very important to develop effective vaccines against this important pathogen. In order to accomplish this, characterization of the major structural proteins of this virus is required.

The objectives of this thesis are:

(1) To characterize BRV VP4, VP6, and VP7 proteins at the molecular level.

(2) To express the VP4, VP6 and VP7 genes via recombinant vaccinia virus gene delivery using an early and a late homologous promoter.

(3) To compare the levels of production and antigenic authenticity of the vaccinia virus derived proteins, to identical gene products produced by recombinant baculovirus in insect cells and to the authentic proteins produced by BRV.

(4) To study humoral immunity induced by the different recombinant proteins in a mouse challenge model.

These studies may lead to, the use of vaccinia virus as a vaccine vector to deliver rotavirus antigens, and a more effective vaccine capable of preventing rotavirus infection.

3. MATERIALS AND METHODS

3.1 Reagents and Media

All enzymes for DNA manipulations and protein A sepharose were purchased from Pharmacia LKB Biotechnology AB, Dorval, Quebec, Canada. Other chemicals used for DNA manipulations were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Cell culture reagents were purchased from GIBCO/BRL, Life Technologies Inc., Burlington, Ontario, Canada. Avidin-biotin immunoperoxidase staining kits, antisera and other reagents for immunochemical assays, were purchased from Dimension Laboratories Inc. Mississauga, Ontario, Canada. Immun-blot assay kits, protein standards and chemicals for protein gels were purchased from Bio-Rad Laboratories, Mississauga, Ontario, Canada. Trypsin was purchased from Difco Laboratories, Detroit Michigan. L-[^{35}S] methionine was purchased from Amersham Canada Limited Corporation, Oakville, Ontario, Canada.

3.2 Cells and Viruses

Five continuous cell lines were employed in this thesis: MA104, a rhesus monkey kidney cell line; BSC-1, an African green monkey kidney cell line; TK$^-$143, a human thymidine kinase negative cell line; LMTK$^-$, a murine cell line; and Sf9, an insect cell line derived from Spodoptera frugiperda (fall army worm) larvae. The MA104, BSC-1 and TK$^-$143 cell lines were maintained in Eagle's minimal essential medium (MEM), supplemented with 10% fetal bovine serum (FBS). LMTK$^-$ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. Sf9 cells were grown in Grace's medium containing 10% FBS

+ 0.3 % yeastolate and 0.3 % lactalbumin hydrolysate.

Bovine rotavirus (BRV) C486 was grown in MA104 cells. Immediately prior to virus infection, the cell monolayers were washed twice with MEM to remove residual serum. The washed monolayers were then infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (PFU)/cell. The virus was allowed to adsorb to cells for one hour at 37°C in a 5% CO_2 atmosphere (standard conditions). After virus adsorption, the virus inoculum was removed and replaced with MEM supplemented with 2% FBS. The infected monolayers were maintained at 37°C until extensive cytopathogenic effect (CPE) was observed. Before the cells were harvested they were treated with trypsin (10 μg/ml) and then frozen and thawed three times. The supernatant was kept at -70°C as stock virus. Wild-type (wt) vaccinia virus (VV), strain WR, was propagated on freshly confluent monolayer of BSC-1 cells supplemented with 2% FBS. Baculovirus, Autographa californica nuclear polyhedrosis virus (AcMNPV), was grown in Sf9 cells. Subconfluent monolayers of Sf9 cells were grown in Grace's medium supplemented with 10% FBS + 0.3 % yeastolate and 0.3 % lactalbumin hydrolysate. Following infection with baculovirus at an MOI of 1, the cells were incubated at 28°C for 1 h. After virus adsorption, the inoculum was removed and replaced with fresh Grace's medium supplemented with 2% FBS + 0.3 % yeastolate and 0.3 % lactalbumin hydrolysate. The infected cells were incubated at 28°C for 3 days. Extracellular virus was collected by centrifugation at 1000xg for 15 min., then the supernatant was stored at 4°C.

3.3 Plasmids and Methods for DNA Manipulation

Insertion plasmids pGS20 (Mackett et al., 1984) and pSLAVE were

generously provided by Dr. T. Zamb (VIDO). Rotavirus gene 4, 6 and 8 cDNA clones were generously provided by Dr. M. Parker (VIDO). All plasmids were grown and maintained in *Escherichia coli* strain MC 1066 according to standard methods (Maniatis et al., 1982). Plasmid DNA was prepared from chloramphenicol-amplified cultures of bacteria by alkaline lysis, cesium chloride-ethidium bromide equilibrium density gradient centrifugation and dialysis (Maniatis et al., 1982). Purified plasmids were stored at -20°C until use.

3.4 Construction and Selection of Recombinant Vaccinia Viruses

A newly confluent monolayer (one 75 cm^2 flask per transfection) of BSC-1 cells was infected with vaccinia virus, strain WR, at 0.05 MOI in a minimum amount of MEM. After one and a half hours at 37°C in an atmosphere of 5% CO_2, the infected cell monolayer was fed with MEM containing 10% FBS and incubated for another 2.5 h. The cells were then trypsinized and washed once with MEM containing 10% FBS, followed by two successive washes in ice-cold HBSS buffer (GIBCO). The cells were resuspended in ice-cold HBSS buffer (1.5 ml per 75 cm^2 flask) and transferred to a Bio Rad electroporation cuvette (Gene Pulser™ cuvette) on ice. 10 µg of the appropriate insertion plasmid in 100 µl of sterile water was then added to the cuvette. The cuvette was then gently inverted to mix the contents. Prior to plasmid addition, each was linearized by restriction endonuclease digestion, precipitated with two volumes of 95% ethanol, washed twice with 70% ethanol, dried and resuspended in sterile water. After plasmid addition, the cuvettes were left on ice for 10 min. Electroporation was performed at 200 v, 500 uF using a BioRad Gene Pulser system. After electroporation, the cuvettes were left on ice for an additional

10 min. The cells were then transferred to a 25 cm^2 flask, fed with 10% FBS supplemented MEM, and incubated at 37°C in a 5% CO_2 atmosphere until complete CPE was achieved (normally 3-4 d). The flasks was then frozen (at -70°C) and thawed three times to liberate virus from transfected cells. The virus arising from transfections was stored at -70°C.

Putative vaccinia recombinants were selected by plaquing virus (Mackett et al., 1985) arising from the transfection on TK^-143 cells, under a 1.5% agarose overlay (Sea plaque) supplemented with 5% FBS, 1X MEM and 25 µg/ml of 5-bromo, 2'-deoxyuridine (BUdR). Recombinants expressing either BRV VP4 or VP7 were identified by direct black plaque assays (below) of the TK^- plaques produced under BUdR selection. Recombinant virus expressing BRV VP6 were identified by immunoperoxidase staining (below) of TK^-143 cells infected with putative recombinant virus isolated following BUdR selection. Recombinant viruses expressing the appropriate BRV genes were purified to homogeneity by 3 successive cycles of plaque purification on TK^-143 cells under BUdR. Finally, individual plaques were amplified by growth on BSC-1 cells. Stock virus was stored at -70°C.

3.5 Black Plaque Assay

Virus arising from the vaccinia transfections were serially diluted and plated onto TK^-143 cells and incubated under standard conditions for 1.5 h. The virus inoculum was removed and replaced with 1.25% methycellulose supplemented with 5% FBS, 1X MEM and 25 µg/ml BUdR. The infected monolayers were incubated under standard conditions for 4-5 d to allow plaques to develop. The overlay was then removed by aspiration. Monolayers were then washed two times with HBSS. Blocking solution, consisting of 3% normal goat serum (NGS) in HBSS, was added and the

plates were incubated at room temperature (rt) with gentle gyrotary shaking for 1 h. Blocking solution was then removed by aspiration, and replaced with polyclonal anti-rotavirus rabbit serum (1:200 dilution in HBSS +1% NGS). The plates were incubated at rt for 1 h. with gentle shaking. The plates were washed twice with HBSS+ 1% NGS, followed by the addition of biotinylated goat anti-rabbit serum (1:2,000 dilution in HBSS+ 1% NGS). The plates were then incubated for 1 h. at rt with gentle shaking. The plates were washed twice with HBSS+ 1% NGS followed by addition of streptavidin-peroxidase complex (1:4,000 dilution in HBSS + 1% NGS) and incubated at rt with shaking for 45 min. The plates were washed as before. Peroxidase substrate, which consisted of 1 mg / ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB) in a buffer containing 50 mM Tris hydrochloride (pH 7.5), 0.01% hydrogen peroxide and 1.7 mM $NiCl_2$ was then added and the plates were allowed to develop with gentle shaking. Colour development normally took 1-5min. Following colour development, the substrate was removed by aspiration and the monolayers were washed two times with HBSS. Positively stained infected cells were recovered by scraping them with a sterile Eppendorf tip into 1 ml of MEM. The recovered cells were broken by three cycles of freezing (-70°C) and thawing to liberate virus. The liberated virus was plated on freshly confluent BSC-1 cells (one 25 cm^2 flask per plaque isolate) to generate an infection stock. Following complete CPE of the BSC-1 infected cells, stock virus was stored at -70°C.

3.6 Immunocytochemistry

BSC-1 cells were seeded onto glass chamber slides (Lab-Tek, tissue culture chamber, Nunc, Inc.) and grown to confluency. The slides were washed once with MEM and then infected with the appropriate vaccinia

recombinants, or with wt vaccinia virus as a negative control. MA104 cells were grown on slides to support the growth of BRV, which was used as a positive control. All cell types were infected with 0.5 MOI. Following a 1 h. incubation under standerd conditions, the inoculum was removed and replaced with MEM containing 2% FBS. The infected cells were then incubated under standard conditions for an additional 18-24 h. The slides were washed three times with phosphate-bufferd saline (PBS), fixed with absolute methanol at -20°C for 15 min. PBS consists of 38.4 mM $NaH_2PO_4.H_2O$, 61.2 mM $Na_2HPO_4.7H_2O$, 145 mM NaCl pH7.2. Following fixation, the slides were washed with PBS for 30 minutes at room temperature. Blocking solution, consisting of 3% horse serum in PBS was added for 30 minutes at room temperature. The blocking solution was removed and replaced with the appropriate monoclonal Ab diluted 1: 1,000 in PBS and incubated for an additional 1 h. at room temperature. The slides were then washed with PBS for 30 minutes. Biotinylated goat anti-mouse serum (1: 2,000 diluted in PBS) was added to slides which were incubated at room temperature for 1 hour. The slides were washed with PBS for 30 minutes. Avidin-biotin conjugated peroxidase complex (1: 1,000 dilution in PBS containing 0.05% Tween 20) was added and incubated for 45 minutes at room temperature. The slides were washed with PBS for 30 minutes. DAB substrate as above was added to the washed slides. Colour development was stopped by rinsing the slides in tap water. The slides were then dried and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA) before microscopic examination and/or photography.

3.7 Radioimmunoprecipitation Assay

In order to prepare [^{35}S] methionine-labeled protein, MA104, BSC-1

and Sf9 cells were infected with rotavirus, wt or recombinant vaccinia viruses, and wt or recombinant baculoviruses respectively, each at a MOI of 5. After adsorption of virus for 1 hour, MEM supplemented with 2% FBS was added to MA104 or BSC-1 cells for a 6-8 hour incubation under standard conditions. The media was then removed by aspiration and replaced with methionine-free MEM containing 2% FBS. The flasks were incubated under standard conditions for an additional 1 h. The media was then removed and replaced with fresh methionine free MEM containing 2% FBS and 25 μCi of L-[^{35}S] methionine per ml. Following label addition, the infected cells were incubated under standard conditions for 10-12 h.

Grace's medium containing 2% FBS + 0.3 % yeastolate and 0.3 % lactalbumin hydrolysate was added to Sf9 cells which were incubated for 40 hours at 28°C. The medium was removed by aspiration, and methionine-free Grace's medium containing 2% FBS + 0.3% yeastolate and 0.3 % lactalbumin hydrolysate was added, followed by a 1 h. incubation at 28°C. The medium was withdrawn and replaced with fresh methionine-free Grace's medium supplemented with 2% FBS, 0.3 % yeastolate and 0.3 % lactalbumin hydrolysate and 25 μCi of L-[^{35}S] methionine per ml. Following addition of label, flasks were incubated for an additional 10-12 h. at 28°C.

For glycosylation inhibition studies, tunicamycin was added (to a final concentration of 2 μg / ml), in all media added to cells after the virus adsorption step and until cells were harvested.

Radiolabelled cells were harvested by scraping, washed with PBS and resuspended in modified RIPA buffer [50 mM Tris hydrochloride (pH 8.0), 150 mM sodium chloride, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride]. After a 15 min. incubation on ice, the cell suspension was sonicated with a stepped microtip at 50% duty

cycle, power setting of 1 (Sonics Materials Co), and then centrifuged at 75,000 xg for 1 hour at 4°C. The supernatants were collected and mixed with either VP6-specific MAb or rabbit polyclonal anti-rotavirus Ab added to a final dilution of 1:40 or 1:20, respectively. The samples were incubated for 16 hours at 4°C on a rocking platform. Protein A sepharose beads were swelled with modified RIPA buffer at a concentration of 100mg per ml for 1 hour at 4°C on a rocking platform, followed by the addition of rabbit anti-mouse Ig G (heavy and light chains) to a final concentration of 800 μg per ml. The beads were incubated for an additional 16 h. After incubation, unbound rabbit Ig G was removed from the coated beads by 3 successive washes with modified RIPA buffer. Approximately 10 mg of coated beads were added to each of the VP6 samples. For polyclonal rabbit serum, protein A sepharose beads were swelled with modified RIPA buffer (as above) and added directly to the samples containing polyclonal anti-rotavirus Ab. All samples were incubated for 3 hours at 4°C on a rocking platform and then washed four times with modified RIPA buffer. The samples were suspended in electrophoresis sample buffer [62 mM Tris hydrochloride (pH6.8), 2% SDS, 5%2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue] and heated at 95°C for 5 min. Samples were separated by electrophoresis in SDS-10% polyacrylamide gels, stained with Coomassie brilliant blue R-250, soaked with 1M sodium salicylate for 30 min. and then dried. The dried gel was exposed to Kodak X-O-Mat AR diagnostic film at -70°C for autoradiography. Molecular weight standards were included in each gel.

3.8 Western Blot Assay

Virus infected cells were harvested and washed once with PBS. The

cell pellets were suspended in sample buffer (as above) and heated at 95°C for 5 min. The samples were separated by 10% SDS-polyacrylamide gel eletrophoresis. A biotinylated protein standard (BioRad) was included for relative molecular weight determinations. Separated proteins were transferred by electroblotting to 0.45 nm nitrocellulose at constant current of 300 mA for 2 hours using a BioRad Electroblotting Apparatus (Mini Trans-Blot Electrophoretic Transfer Cell). The transfer buffer consisted of 20 mM Tris, 150 mM glycine pH 8.0, 20% (v/v) methanol.

Following the transfer of proteins, the nitrocellulose sheets were incubated in blocking solution, containing 3% gelatin in TBS (20 mM Tris-HCl, 500 mM NaCl pH 7.5) on a rocking platform for 1 hour at room temperature. The nitrocellulose membranes were then washed with TTBS (TBS supplemented with 0.05% Tween 20) at room temperature for 10 min. with gentle agitation. MAb (1: 1,000 dilution) or polyclonal rabbit anti-rotavirus serum (1: 100 dilution) in TTBS containing 1% gelatin was added to the membranes which were incubated as above for an additional 2 h. After two TTBS washes, the membranes were incubated with goat anti-mouse or goat anti-rabbit peroxidase conjugates (1:3,000 dilution) in TTBS containing 1% gelatin, plus avidin-peroxidase conjugate (1: 3,000) for 1 h. at room temperature. The filters were washed twice with TTBS followed by one wash with TBS (as above). Immunoreactive bands were visualized by the addition of the colour reagent (4-chloro-1-naphthol/hydrogen peroxide). The developed blots were washed with double distilled water twice for 10 min. to stop colour development. The sheets were dried and stored between dark paper to protect them from light.

3.9 Enzyme-Linked Immunosorbent Assay (ELISA)

96-well round bottom microtiter plates (Immulon II Dynatech Laboratories Inc, Alexandria, VA, USA) were coated with assorted antigens described below. For comparison of protein levels produced by different recombinant viruses, plates were coated with supernatants from cell lysates containing rotavirus protein in doubling dilutions beginning at 1: 500 in coating buffer (0.05 M sodium carbonate and sodium bicarbonate pH 9.6). For titrations of anti-rotavirus Ab induced by the different recombinant viruses, plates were coated with 0.5 μg of partially purified rotavirus per well, diluted in coating buffer, and incubated at 4°C overnight. The plates were then washed with distilled water six times, the non-specific protein binding sites were blocked by addition of 1% bovine serum albumin (BSA) in PBS for 1 h. at room temperature. The plates were washed six times (as above) followed by the addition of polyclonal anti-rotavirus Ab (1: 1,000 dilution) , VP6 specific MAb (1:1,000 dilution) in PBS with 0.05% Tween 20. After incubation for 1 hour at room temperature, the plates were washed six times with distilled water. Biotinylated species-specific second Ab (1:5,000 diluted in PBS containing 0.05% Tween 20) was added and incubated for an additional 1 h. The plates were washed 6 times with distilled water. Horseradish peroxidase-conjugated streptavidin (1: 4,000 diluted in PBS containing 0.05% Tween 20) was then added and incubated for 1 hour at room temperature, followed by washing as above. Finally, the substrate consisting of 1 mg per ml ABTS [2,2'-amino-di-(3-ethylbenzthiazoline sulphonate)] and 0.015% hydrogen peroxide in 0.1M citric acid, pH 4.0, was added at room temperature. The reaction was stopped after 10 minutes by addition of sodium dodecyl sulphate (SDS) to a final concentration of 5% and the adsorbance (A 405 nm) of each well was

read using a microplate reader (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

3.10 Purification of Rotavirus VP6 from Recombinant Vaccinia Virus and Baculovirus

BSC-1 cells were infected with recombinant vaccinia virus expressing VP6 at an MOI of 5. Similarly, Sf9 cells were infected with recombinant baculovirus expressing VP6 at an MOI of 5. After complete CPE was achieved, the cells were collected and washed once with PBS. Lysis buffer (2mM $NaHCO_3$ pH 7.4, 0.05% Triton X 100, 1% aprotinin) was then added to the infected cells. The cells were incubated on ice for 2 hours with occasional agitation. The cell suspension was sonicated with a tapered microtip, 50% duty cycle, at a power setting of 5, and spun at 1,000 xg for 20 minutes. Supernatants were collected and put into 30 ml of 0.01 M glycine pH 4.0 and 0.02% sodium azide. The mixtures were stirred overnight at room temperature. The samples were then dialysed against 0.01M citrate buffer pH 4.0 containing 0.02% sodium azide overnight at 4°C The samples were centrifuged at 75,000 xg for 30 minutes at 15°C. The pellets were resuspended in 0.01M sodium borate buffer pH 9.0 and an equal volume of 4M LiCl in 0.01M sodium borate pH 9.0 was then added. The samples were vortexed and quickly stored at -70°C. After 4-5 days, the samples were centrifuged at 75,000 xg for 4 h. at 25°C. Supernatants were dialysed against 0.01 M citrate buffer pH 4.0, containing 0.02 % sodium azide, for two days with a total of 3 buffer changes. Samples were then dialyzed in 0.01M citrate pH5.0, containing 0.02% sodium azide, for two days with three changes. Protein concentration was determined by a Bio-Rad protein assay. VP6 purified in this fashion was used for electron

microscope and PAGE gel analyses as well as an antigen for mouse immunizations.

3.11 Preparation of Rotavirus VP4 and VP7 Produced by Recombinant Baculovirus

VP4 preparation: Sf9 cells were infected with recombinant baculovirus expressing the BRV VP4 gene at an MOI of 5. After 72 h., the cells were harvested by centrifugation at 1,000 xg for 20 min. and resuspended in lysis buffer (as above). The cells were incubated on ice for 15 min. and then sonicated with a tapered microtip, 50% duty cycle, at a power setting of 5. The supernatant was collected by centrifugation at 75,000 xg for 1 h. at 4°C. The protein concentration of the supernatant was determined by a Bio-Rad protein assay. The supernatant was stored at -70°C until use.

VP7 preparation: Sf9 cells were infected with recombinant baculovirus expressing the BRV VP7 gene at an MOI of 5. After 72 h. the culture supernatant was collected by centrifugation at 1,000 xg for 20 min. to remove cells. The clarified supernatant was lyophilyzed and stored at -70°C until use.

3.12 Purification of Recombinant Vaccinia Viruses

LMTK$^-$ cells were inoculated with recombinant vaccinia virus at an MOI of 5. After incubation under standard conditions for 1.5 h., MEM containing 2% FBS and 25 μg per ml of BUdR was added to the infected cells. The cells were then incubated under standard conditions until extensive CPE was observed. The infected cells were then subjected to three cycles of freezing (-70°C) and thawing. Supernatants were collected by

centrifugation at 1,000 xg for 20 minutes. Cell pellets were sonicated in 5 ml of medium with a tapered microtip, 50% duty cycle, at power setting of 5, and then centrifuged at 1,000 xg for 20 minutes to remove cell debris. Both supernatants were layered onto 36% (w/v) sucrose in TNE buffer (10 mMTris-HCl, 100mM NaCl, 1mM EDTA) pH 9.0, and centrifuged at 25,000 xg in a SW 28 rotor (Beckman) for 80 minutes at 4°C. Virus pellets were suspended in PBS and centrifuged at 25,000 xg for 80 minutes at 4°C. Viruses were resuspended in PBS, titrated and stored at -70°C until use.

3.13 Inactivation of Bovine Rotaviruses

The supernatant from bovine rotavirus infected MA-104 cells was harvested and the cell debris was pelleted by centrifugation at 1,000 xg for 20 min. The virus in the resulting supernatant was pelleted through a 40% (w/v) sucrose cushion at 75,000 xg in a Beckman SW 28 rotor for 3 hours. The virus pellet was then resuspended in 1-4 ml of PBS pH 7.2 to yield partially purified virus.

The purified virus was mixed with formaldehyde to a final concentration of 0.16% (v/v) and incubated at 37°C overnight with shaking. Sodium metabisulfate at a final concentration of 0.12% (v/v) was added to neutralize the formaldehyde. The virus solution was dialyzed overnight against PBS at 4°C and then tested for infectivity. The killed viruses were stored at -70°C until use.

3.14 Mouse Immunization and BRV Challenge

SPF rotavirus-free mice (CD1) were purchased from Charles River Breeding Laboratories, Wilmington, MA, USA. In order to ensure that they were not previously exposed to rotavirus, each mouse was bled from

its coccygeal vein upon arrival. The sera were tested for the presence of rotavirus antibodies using ELISA, and all mice were found to be seronegative. Animals were housed in HPA-filtered isolation units to keep them pathogen-free throughout the experiment. Maintenance, handling, immunization and blood sample collection was conducted with the help of Ms G. Mcleod, and the other Animal Services Staff of the Veterinary Infectious Disease Organization.

The mouse protection study was designed as follows. Each experimental group consisted of seven mature female mice immunized with the indicated antigen, prior to BRV challenge.

Group 1: received 100 µl of solution containing 50 µl of PBS and 50 µl of adjuvant [first immunization with Freunds complete adjuvant (FCA); second with Freunds incomplete adjuvant (FICA)] by intramuscular immunization of the hind limb to serve as a placebo control group.

Group 2: received vaccinia virus expressing the BRV VP4 gene.

Group 3: received vaccinia virus expressing the BRV VP6 gene.

Group 4: received vaccinia virus expressing the BRV VP7 gene.

Group 5: received a combination of the vaccinia viruses used to immunized groups 2-4.

Group 6: received TK^- vaccinia virus, derived from the WR wt strain, as a vaccinia virus control group.

Groups 2-6 were immunized with $2x10^6$ PFU of virus administered by intraperitoneal injection in 100 µl of PBS, with the exception of group 5 which received $2x10^6$ PFU of each recombinant virus.

Group 7: received inactivated BRV.

Group 8: received partially purified VP4 produced by recombinant baculovirus.

Group 9: received purified and assembled VP6 particles produced by recombinant baculovirus.

Group 10: received partially purified VP7 produced by recombinant baculovirus.

Group 11: received a mixture of VP4+VP6+VP7 produced by recombinant baculoviruses.

Groups 7-10 were immunized with 50 μg of protein formulated with adjuvant (first immunization with FCA; second with FICA), in final volume of 100 μl, administered by intramuscular injection of the hind leg.

Group 11 was immunized with 50 μg of each protein formulated with ajuvant as above, in final volume of 100 μl, administered by intramuscular injection of the hind leg.

Group 12: were not immunized and served as sentinel controls.

All animals were vaccinated on day zero, and received a second identical immunization on day 21. Eight days after the second immunization, individual blood samples were taken and resulting sera were titrated for BRV antibody. Vaccinated dams were then bred. Seven days after littering, individual blood samples from the dams of each group were collected. The above sera were used for, rotavirus antibody titrations, and specificity of antibody responses measured by immunoprecipitation of BRV infected cell extracts.

Seven day old neonates were challenged with bovine rotavirus. The

pups were removed from their dams at least 1 hour before challenge to ensure their stomachs were empty. $1x10^6$ PFU of BRV C486 strain in 100 µl of PBS were given to the pups by oral administration using a soft flexible tube. The pups were kept from their dams for at least 1 hour after challenge. After that, the pups were returned to their respective dams and scored for clinical disease symptoms at 8, 24, 48 and 72 h. post challenge.

The scoring system is described below.

0 degree: Tissues (ie bedding) are clean. When pups are squeezed, no feces are expressed or if expressed, look a normal-dark color and not runny.

1 degree: Tissues are clean. When pups are squeezed, expressed feces are bright yellow and pasty in consistency.

2 degree: Bums are usually clean. Tissues are usually soiled, more with solids than liquids. Feces are expressed easily.

3 degree: Bums are dirty and feces are dried around the anal cavity. Tissues can be quite soiled, mostly with liquid. On palpation, feces are easily expressed and quite liquid. If tissues are clean, there could be blockage of the anal cavity, then feces will not appear as easily when the pups are squeezed. Mucus may be evident in the feces.

4. RESULTS

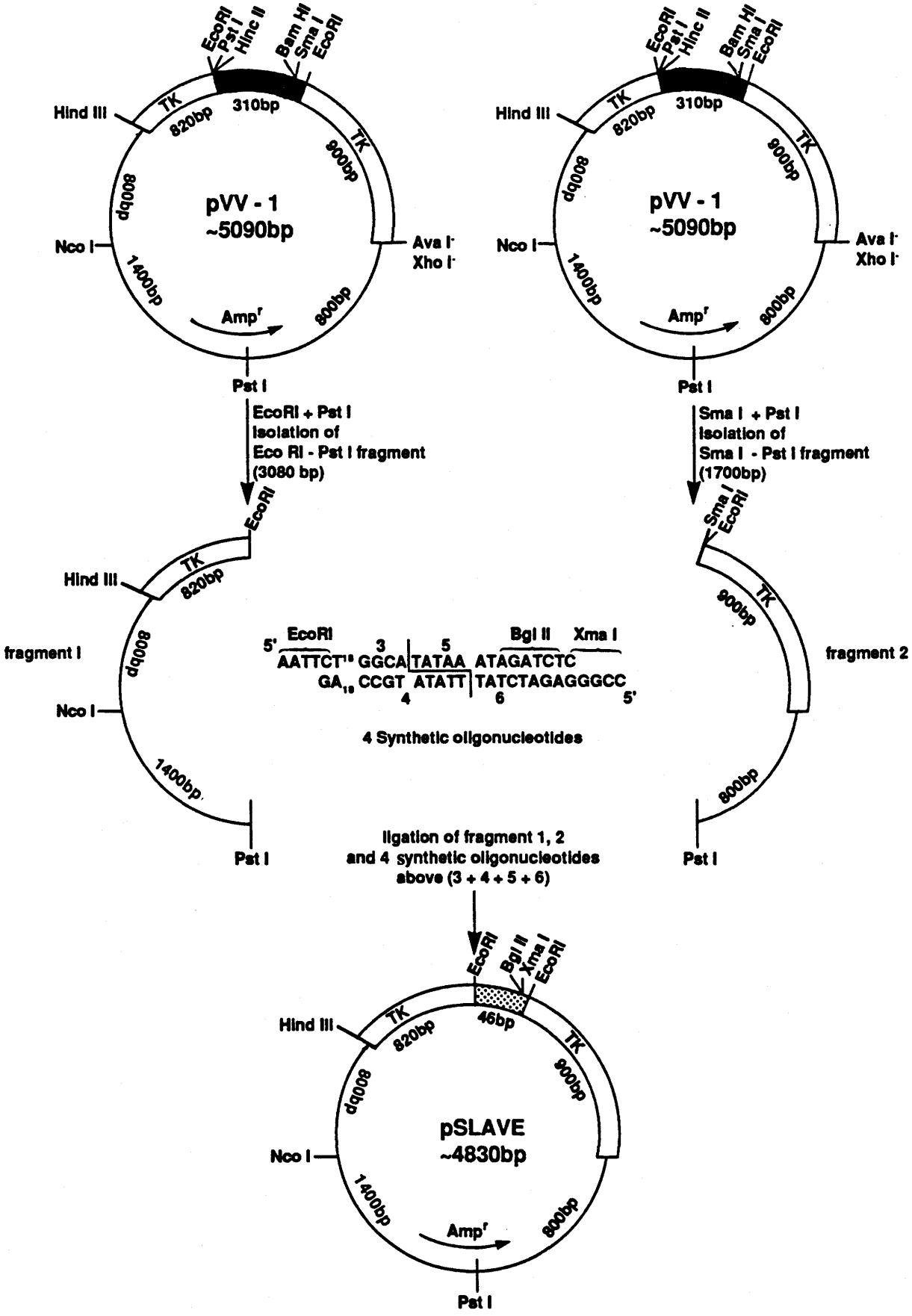
4.1 Construction of Recombinant Plasmids and Recombinant Vaccinia Viruses

The plasmid pSLAVE (Fig. 3) was made by Dr. T. Zamb. It was made from plasmid pVV-1, a derivative of pGS 20 (Mackett et al., 1984). A 3020 bp EcoRI-PstI and a 1700 bp Sma I-PstI fragments were isolated from pVV-1 by standard methods (Maniatis et al.,1982). These two fragments and four synthetic oligonucleotides (which constituted a consensus vaccinia virus late gene promoter) were ligated together to form the plasmid pSLAVE (Fig. 3). The consensus late gene promoter in pSLAVE replaced the vaccinia 7.5 K gene promoter in pVV-1, also present in pGS 20 (Fig. 4). A 2,200 bp Xho I-Ava I fragment was excised from pGS 20 to produce pVV-1.

The intact coding sequences of BRV genes 4, 6 and 8 were excised with Bam HI digestion of pTZ 19 R cDNA clones (Pharmacia LKB Biotechnology) and were inserted into the Bgl II cloning site of pSLAVE (Fig. 5). Each Bam HI fragment was inserted into the Bam HI cloning site present in pGS 20, as well (Fig. 6). Proper orientation of the inserts was established by mapping asymmetric restriction endonuclease sites within the inserts. In both cases, the promoter-gene cassettes are flanked by vaccinia virus TK gene sequences. Recombinant plasmids were CsCl gradient purified prior to cotransfection.

Linearized recombinant insertion plasmid containing the rotavirus genes were used to transfect cells previously infected with the WR strain of vaccinia virus to permit homologous recombination between the flanking TK gene sequences on the insertion plasmids and the TK gene within the

Fig. 3. Construction of the Vaccinia Virus Insertion Vector pSLAVE. pSLAVE was constructed from elements of pVV-1 plus synthetic oligonucleotides representing a consensus vaccinia virus late gene promoter. The pVV-1 vector was derived from pGS 20 by digestion with Xho I and Ava I, treatment with Klenow enzyme to blunt asymmetric ends followed by ligation, causing the deletion of a 2,200 bp Xho I-Ava I fragment. A 3,080 bp Eco RI-Pst I fragment and a 1,700 bp Sma I-Pst I fragment from pVV-1 and 4 synthetic oligonucleotides representing the consensus promoter were ligated together to form pSLAVE. Stippled box is the late gene promoter. The black box is the vaccinia virus 7.5 K gene promoter (from pGS 20) which is replaced by the consensus late gene promoter in pSLAVE. TK gene sequences of vaccinia virus are represented by the opened boxes.


EcoRI
Pst I
Hinc II
Bam HI
Sma I
EcoRI
Hind III
TK
820bp
310bp
TK
900bp
pVV - 1
~5090bp
dq008
Nco I
1400bp
Amp^r
800bp
Ava I
Xho I
Pst I
EcoRI + Pst I
Isolation of
Eco RI - Pst I fragment
(3080 bp)
Sma I + Pst I
Isolation of
Sma I - Pst I fragment
(1700bp)
fragment I
fragment 2
5' EcoRI
AATTCT
GA
3
GGCA
TATAA
ATAGATCTC
TATCTAGAGGGCC
4
5
6
Bgl II
Xma I
5'
4 Synthetic oligonucleotides
ligation of fragment 1, 2
and 4 synthetic oligonucleotides
above (3 + 4 + 5 + 6)
46bp
pSLAVE
~4830bp

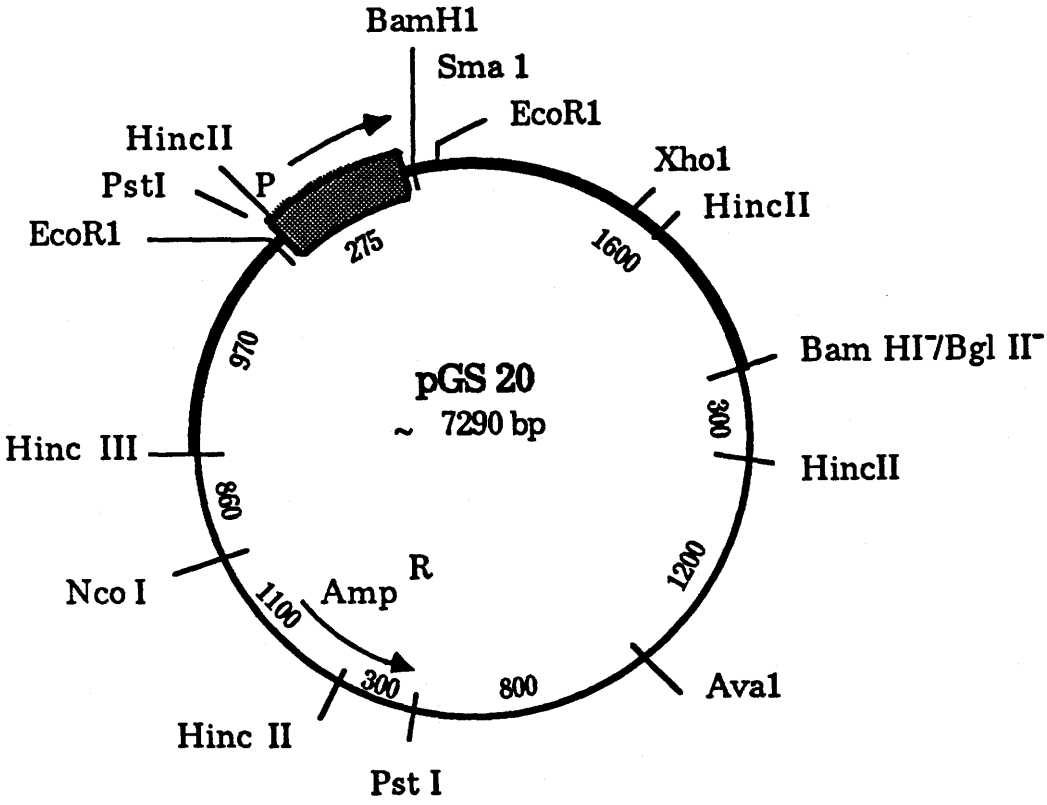


Fig. 4. **Plasmid pGS-20.** Thickened lines indicates vaccinia virus sequences derived from the Hind III J fragment of vaccinia. P indicates the promoter from the 7.5 K gene (275 bp Hinc II-Rsa I fragment sequenced in Venkatesan et al., 1981) inserted into the Eco RI site of the vaccinia TK gene. The Eco RI site of pBR 328 (thin line) has been deleted. The sequences derived from Hind III-J to the 3' side of the TK gene ending at the Bgl II site has been ligated to the Bam HI site of pBR 328, thus destroying both sites.

Fig. 5. **Construction of Vaccinia Virus Insertion Vector Containing Rotavirus Genes under the Control of the Consensus Late Gene Promoter.** The VP4, VP6 and VP7 genes were recovered by Bam HI digestion of the pTZ 19R clones carrying cDNA copies of the BRV genes. The Bam HI fragments were inserted into the Bgl II cloning site of pSLAVE. Proper gene orientation was established by mapping the asymmetric restriction endonuclease site within the individual gene coding sequences to sites within the pSLAVE backbone. The start and stop codons of the rotavirus genes were included and the direction of transcription was indicated by the arrows. Symbols: open box, vaccinia virus TK flanking sequences; hatched box, rotavirus 4, 6 and 8 genes; stippled box, consensus late gene promoter; solid line, bacterial plasmid DNA; Ba, Bam HI.

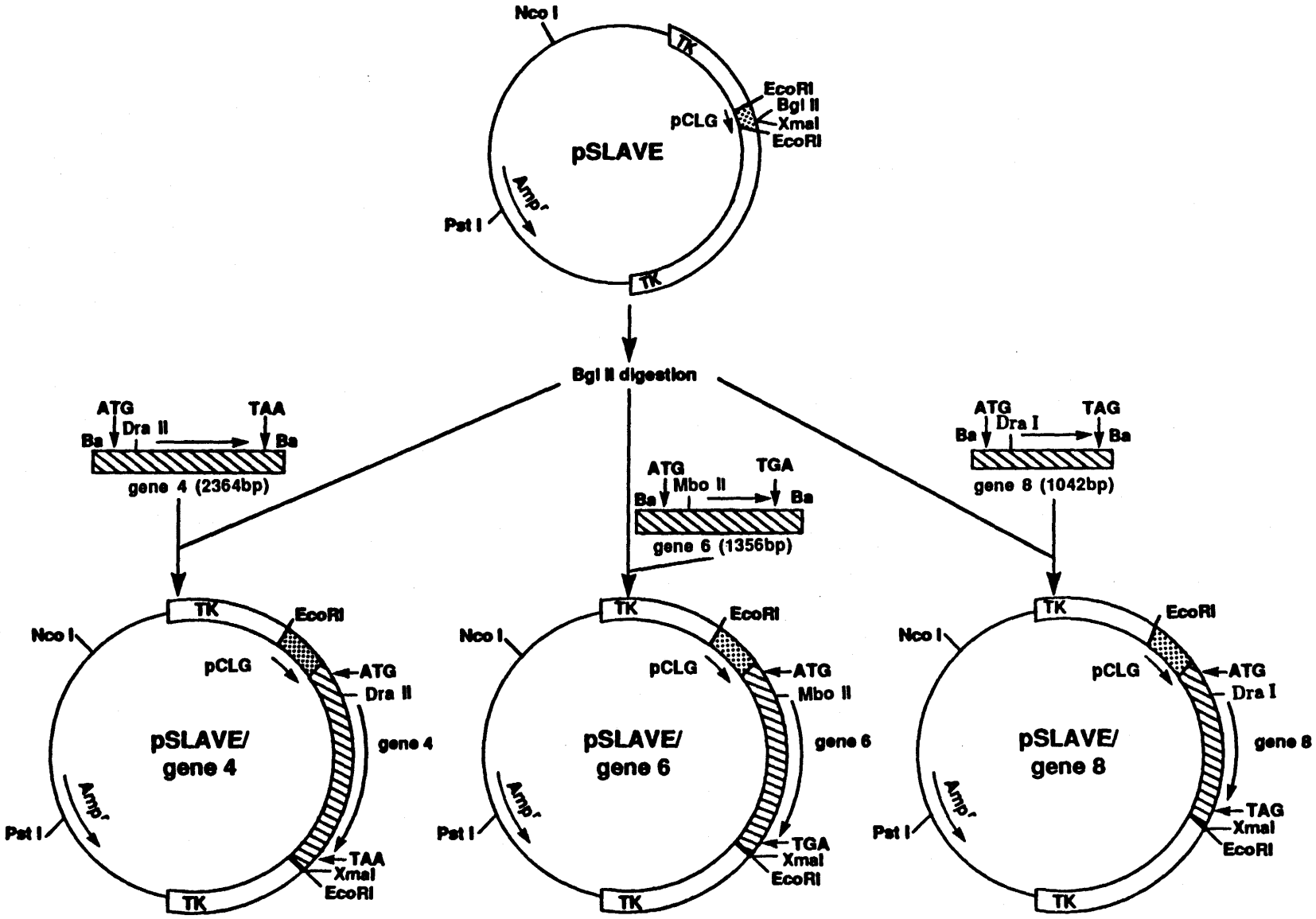
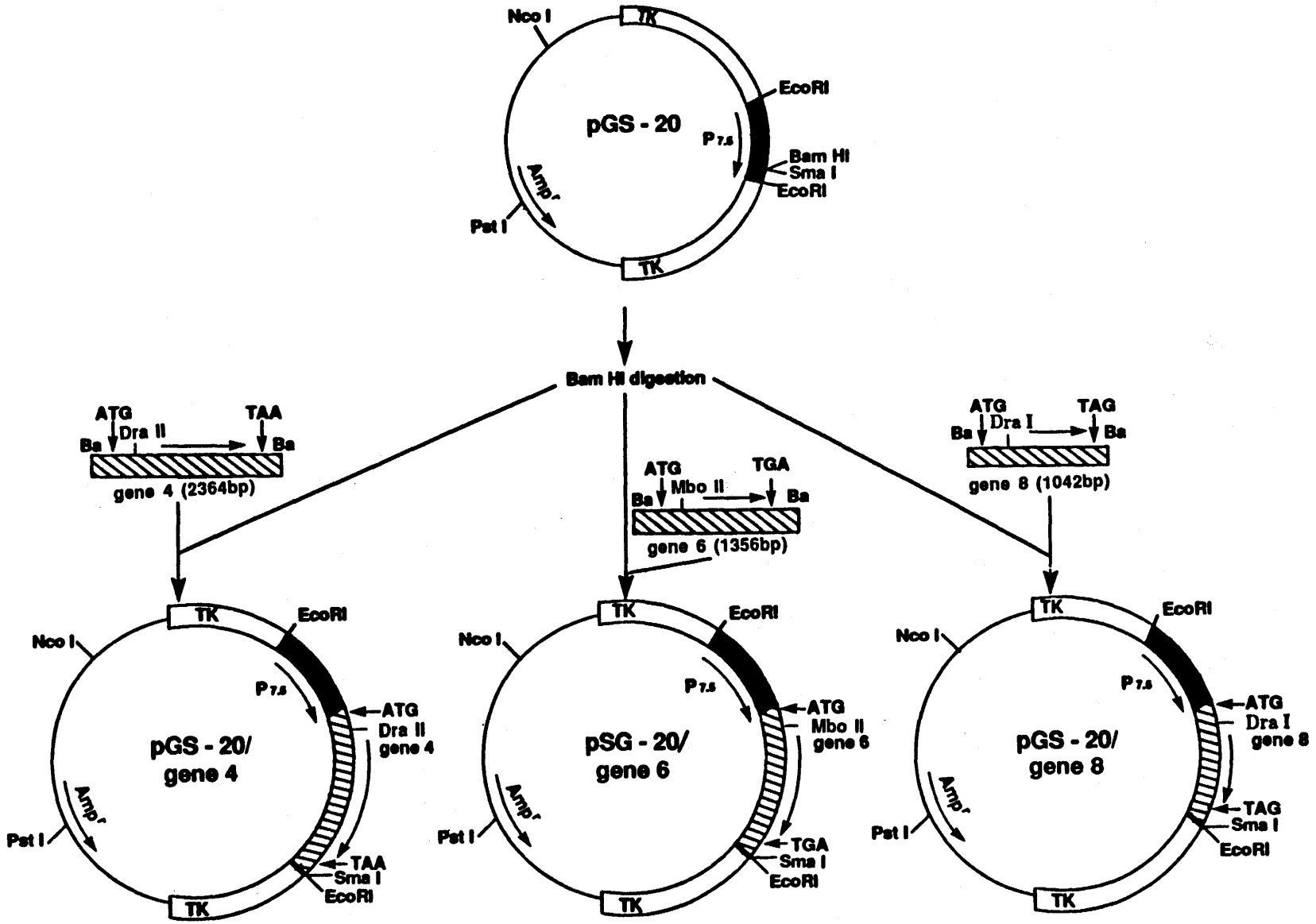

Nco I
TK
EcoRI
Bgl II
pCLG
XmaI
EcoRI
pSLAVE
Amp'
Pst I
TK
Bgl II digestion
ATG
Dra II
TAA
Ba
Ba
gene 4 (2364bp)
ATG
Mbo II
TGA
Ba
Ba
gene 6 (1356bp)
ATG
Dra I
TAG
Ba
Ba
gene 8 (1042bp)
Nco I
TK
EcoRI
pCLG
ATG
Dra II
pSLAVE/
gene 4
gene 4
Amp'
Pst I
TAA
XmaI
EcoRI
TK
Nco I
TK
EcoRI
pCLG
ATG
Mbo II
pSLAVE/
gene 6
gene 6
Amp'
Pst I
TGA
XmaI
EcoRI
TK
Nco I
TK
EcoRI
pCLG
ATG
Dra I
pSLAVE/
gene 8
gene 8
Amp'
Pst I
TAG
XmaI
EcoRI
TK

Fig. 6. **Construction of Vaccinia Virus Insertion Vector Containing Rotavirus Genes under the Control of the 7.5 K Gene Promoter.** The BRV VP4, VP6 and VP7 genes were recovered as Bam HI fragments from pTZ 19R clones and inserted into the Bam HI cloning site of pGS 20. The start and stop codons of rotavirus genes are indicated and the direction of gene trascription is shown by the arrow. Symbols: open box, vaccinia virus TK flanking sequences; black box, vaccinia virus 7.5 K gene promoter; hatched box, rotavirus 4, 6 and 8 genes; solid line, bacterial plasmid DNA; Ba, Bam HI.


Nco I
TK
EcoRI
pGS - 20
P7.5
Bam HI
Sma I
EcoRI
Amp'
Pst I
TK
Bam HI digestion
ATG
Dra II
TAA
Ba
Ba
gene 4 (2364bp)
ATG
Mbo II
TGA
Ba
Ba
gene 6 (1356bp)
ATG
Dra I
TAG
Ba
Ba
gene 8 (1042bp)
pGS - 20/
gene 4
ATG
Dra II
gene 4
TAA
pSG - 20/
gene 6
ATG
Mbo II
gene 6
TGA
pGS - 20/
gene 8
ATG
Dra I
gene 8
TAG

virus genome. Putative recombinant viruses were then selected as TK$^-$ plaques grown on TK$^-$143 cells in the presence of BUdR.

4.2 Selection of Recombinant Vaccinia Virus Expressing Rotavirus VP4, VP6 and VP7

Rotavirus gene expression by the TK$^-$ vaccinia virus was determine by either black plaque assay or by immunocytochemical staining. Recombinant vaccinia virus expressing either VP4 or VP7 were detected by the black plaque assay. Because cells were not fixed prior to staining, this assay only detects protein present on surfaces of infected cells (Fig. 7). Cells infected with wt VV served as a negative control (Fig. 7, A and B). Negatively staining plaques are seen in wells A and B, indicating that wt VV does not produce proteins that are cross-reactive with polyclonal anti-rotavirus antiserum used in this assay. Fig. 7, C and D are cells infected with recombinant vaccinia virus expressing the BRV VP4 gene under control of the 7.5K promoter (rVV-VP4 7.5) in C or the consensus late gene promoter (rVV-VP4 SLAVE) in D. Positively staining plaques were seen in both wells indicated that both recombinants produced VP4. Fig. 7, E and F are cells infected with recombinant vaccinia expressing the BRV VP7 gene under the control of the 7.5 K promoter (rVV-VP7 7.5) in E and the consensus late gene promoter (rVV-VP7 SLAVE) in F. The result is similar to that observed in Fig. 7, C and D. In contrast, VP6 could not be detected by the black plaque assay. Since VP6 was not expressed on the surface of infected cells, cytochemical staining of fixed cells infected with putative recombinant vaccinia was used to detect VP6 production. The results are shown in Fig. 8. Cells infected with wt VV (Fig. 8A) served as a negative control, and BRV infected MA104 cells (Fig. 8B) serve as a positive

Fig. 7. **Black Plaque Assay for Detection of VP4 and VP7 Proteins in Cells Infected with Recombinant Vaccinia Virus.** TK$^-$ 143 cells were infected with: wt VV (A and B); rVV-VP4 7.5 (C); rVV-VP4 SLAVE (D); rVV-VP7 7.5 (E); and rVV-VP7 SLAVE (F). The infected monolayers were then subjected to the black plaque assay as described in methods using polyclonal anti-rotavirus rabbit serum.

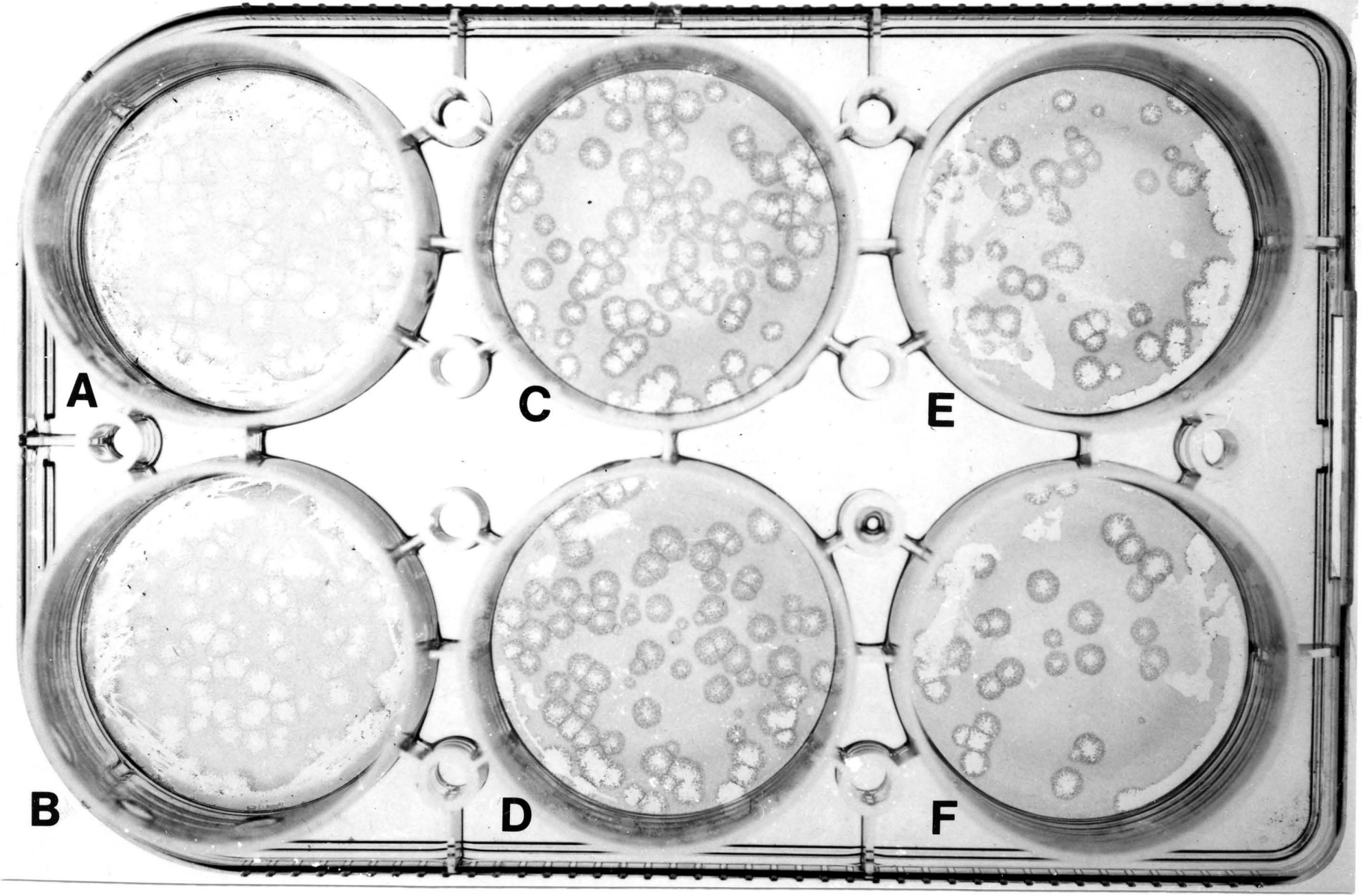
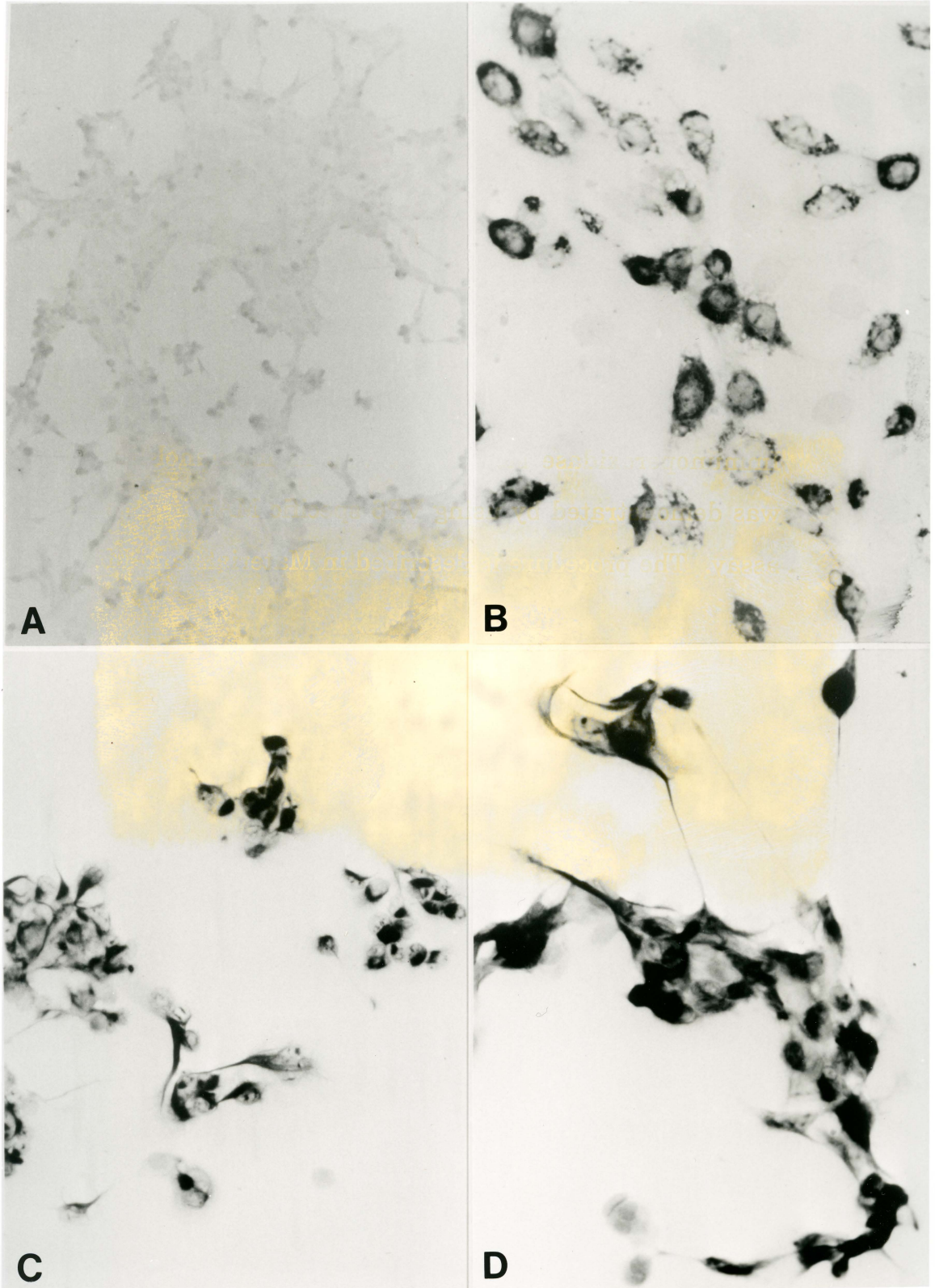

A
C
E
B
D
F

Fig. 8. Immunoperoxidase Staining for Detection of VP6 in Recombinant Vaccinia Virus Infected Cells. BSC-1 cells were infected with: wt VV (A), rVV-VP6 7.5 (C), and rVV-VP6 SLAVE (D). MA104 cells were infected with BRV (B). Immunoperoxidase staining of VP6 in methanol-fixed cells was demonstrated by using VP6 specific MAb in an indirect assay. The procedure is described in Materials and Methods.


A
B
C
D

control. The cells infected with recombinant vaccinia containing the VP6 gene under control of 7.5 K gene promoter (rVV-VP6 7.5) are shown in Fig. 8C, or under the control of the consensus late gene promoter (rVV-VP6 SLAVE) in Fig. 8D, stained positively indicating expression of rotavirus antigens. Once it was established that recombinant viruses were producing rotavirus antigens, the respective viruses were purified to homogeneity by three successive cycles of plaque purification. Finally, individual plaques were amplified by growth on BSC-1 cells.

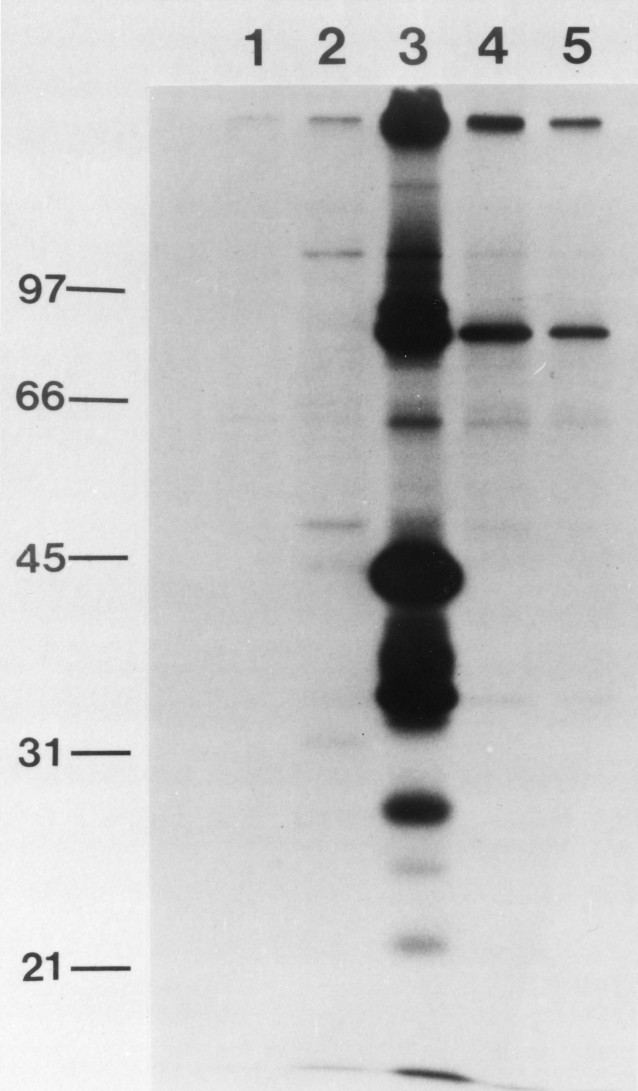
4.3 Evaluation of Authenticity of Rotavirus VP4, VP6 and VP7 Expressed by Recombinant Vaccinia Virus and Baculovirus

BSC-1 cells were infected with wt VV, rVV-VP4, rVV-VP6 or rVV-VP7 and labeled with L-[^{35}S] methionine. The radiolabeled proteins were immunoprecipitated with rabbit polyclonal anti-rotavirus serum or with monoclonal Ab against VP6 and characterized by SDS-PAGE. MA104 cells infected with BRV were included as a control. In order to compare the authenticity of the rotavirus proteins produced by recombinant vaccinia virus to the same proteins produced by the insect viruses, Sf9 insect cells were infected with recombinant baculovirus expressing either VP4, VP6 or VP7 (rBV-VP4, -VP6 or -VP7) and then labeled with L-[^{35}S] methionine. Recombinant baculoviruses expressing VP4, VP6 and VP7 were provided by Dr. M. Parker (VIDO, Saskatoon). In addition to immunoprecipitation, western blotting and ELISA were used for these comparisons.

4.3.1 Evaluation of authenticity of VP4 expressed by recombinant vaccinia virus

Rabbit polyclonal anti-rotavirus serum precipitated VP4 from BSC-1 cells infected with rVV-VP4 SLAVE and rVV-VP4 7.5. The results show that the rVV-VP4 produced by either rVV-VP4 SLAVE or rVV-VP4 7.5 comigrated exactly with the authentic molecule produced in BRV infected MA104 cells. In each case the apparent molecular weight of VP4 is approximately 84 K (Fig. 9). The VP4 band produced under the control of the consensus late gene promoter is more intense than the band produced by the 7.5 K gene promoter, suggesting a difference in the relative strengths of these promoters. Polyclonal anti-rotavirus rabbit serum precipitated VP4 from Sf9 cells infected with rBV-VP4 (Fig. 10), as well. However, full

Fig. 9. Expression of Rotavirus VP4 by Recombinant Vaccinia Virus. BSC-1 cells were infected with: mock virus (lane 1); wt VV (lane 2); rVV-VP4 SLAVE (lane 4); and rVV-VP4 7.5 (lane 5). MA104 cells were infected with BRV (lane 3) as a positive control. Infected cells were labeled with L-[^{35}S] methionine from 8 to 18 hours postinfection. Immune precipitates were prepared from the cell lysates by using rabbit polyclonal anti-rotavirus serum and the products were analyzed by electrophoresis through a 10% SDS-PAGE gel. Molecular weight markers are on the left with sizes in kilodaltons.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4
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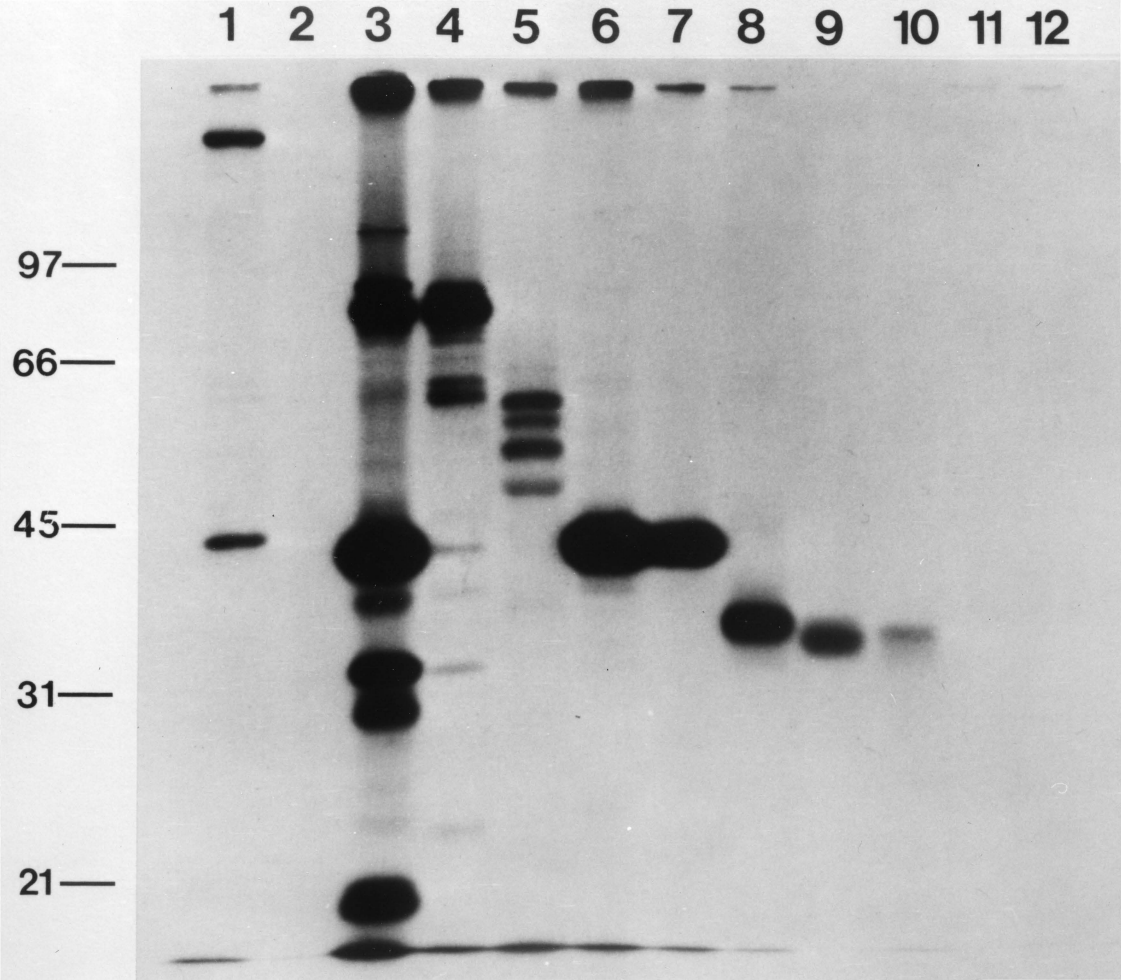
97
66
45
31
21

length VP4 was not precipitated from recombinant baculovirus infected cells. Only low molecular weight bands were observed (lane 5). In order to investigate whether there may be an agent which cleaves VP4 during the immunoprecipitation process, another immunoprecipitation assay was designed. In this case supernatants from BRV and rVV-VP4 SLAVE cell lysates were mixed with the supernatants from cell lysates infected with rBV-VP4, which was then immunoprecipitated. Also, to study whether the agents cleaved specially or non-specificlly, trypsin at a final concentration of 10 μg/ml was added to BRV, rVV-VP4 SLAVE and rBV-VP4 infected cell extracts. Trypsin cleaves VP4 to VP 5 (60 kDa) and VP8 (28 kDa) which enhances infectivity of rotavirus (Clark et al., 1981; Espejo et al., 1981; Estes et al.,1981). The supernatants were incubated at 37°C for 2 hours and then immunoprecipitated. The results are shown in Fig. 11. VP4 produced by rBV-VP4 is shown in lane 4, in panel B. VP4 produced by rVV-VP4 (lane 3, in panel B) treated with extracts from rBV-VP4 cell lysates was degraded into low molecular weight bands. VP4 produced by BRV was treated with supernatants from cell lysates infected with rBV-VP4 (lane 7, in panel A). Most of authenticVP4 also was degraded into low molecular weight bands, since very little full-length VP4 remains when compared to cells infected with rotavirus without any treatment (panel A, lane 5). Trypsin cleavage of BRV-VP4 (panel A, lane 6), rVV-VP4 SLAVE (panel B, lane 2) or rBV-VP4 (panel B, lane 5) yields a band with a MW characteristic of VP8 plus a number of additional low MW bands. A band characteristic of VP5 was not observed. These results strongly suggest that there were agents produced by baculovirus infected cells which cleave VP4 during the immuno-precipitation process. In order to confirm that VP4 was degraded during immuno-precipitation rather than as a result of unexpected translation

Fig.10. **Expression of Rotavirus VP4, VP6 and VP7 by Recombinant Vaccinia Virus and Baculovirus.** BSC-1 cells were infected with: mock virus (lane 1); wt VV (lane 2); rVV-VP4 SLAVE (lane 4); rVV-VP6 SLAVE (lane 6); and rVV-VP7 SLAVE (lane 8). Sf9 cells were infected with: mock virus (lane 11); wt BV (lane 12); rBV-VP4 (lane 5); rBV-VP6 (lane 7); rBV-VP7 (lanes 9 and 10). MA104 cells were infected with BRV (lane 3) as a positive control. BSC-1 and MA104 cells were labeled with L-[^{35}S] methionine from 8 to 18 hours postinfection. Sf9 cells were labeled with L-[^{35}S] methionine from 40 to 50 hours postinfection. Immune precipitates were prepared from cell lysates, except the sample in lane 9 which was prepared from culture medium of rBV-VP7 infected Sf9 cells. All immune precipitations were conducted using rabbit polyclonal anti-rotavirus serum. All samples were analyzed by electro phoresis through a 10% SDS-PAGE gel. Molecular weights are given on the left with sizes in kilodaltons.


1 2 3 4 5 6 7 8 9 10 11 12
97
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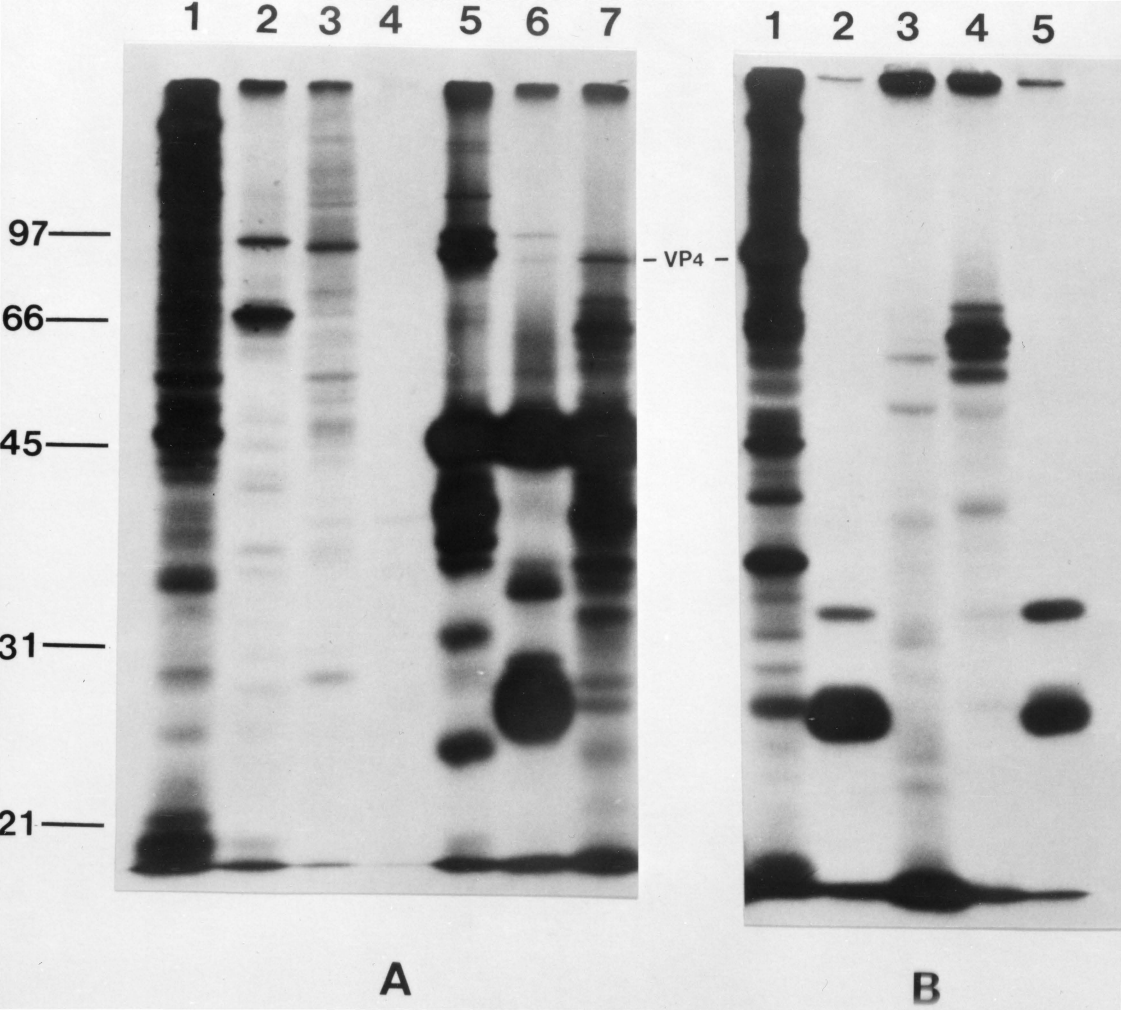
31
21

Fig.11. Analysis of VP4 Produced by Rotavirus, Recombinant Vaccinia Virus and Baculovirus by Immunoprecipitation. BSC-1 cells were infected with: mock virus (panel A, lane 1); wt VV (panel A, lane 2); rVV-VP4 SLAVE (panel B, lanes 1, 2 and 3). MA104 cells were infected with BRV (panel A, lanes 5, 6 and 7). Sf9 cells were infected with: mock virus (panel A, lane 3); wt BV (panel A, lane 4); rBV-VP4 (panel B, lanes 4 and 5). BSC-1 cells and MA104 cells were labeled with L-[35S] methionine from 8 to 18 hour postinfection. Sf9 cells were labeled with L-[35S] methionine from 40 to 50 hours postinfection. Trypsin was added to the supernatants from cell lysates infected with: BRV (panel A, lane 6); rVV-VP4 (panel B, lane 2); and rBV-VP4 (panel B, lane 5). Trypsin treated samples were incubated at 37°C for 2 hours, prior to immunoprecipitation. Supernatants from cell lysates infected with BRV (panel A, lane 7) and rVV-VP4 SLAVE (panel B, lane 3) were mixed with the supernatant from non-isotopically labelled cell lysates infected with rBV-VP4. All samples (panel A and panel B) were immune precipitated with rabbit polyclonal anti-rotavirus serum and resolved on a 10% SDS-PAGE gel. Molecular weights are given on the left with sizes in kilodaltons.


1 2 3 4 5 6 7
97
66
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31
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VP4
A
1 2 3 4 5
B

initiation or termination, western blotting analysis (Fig. 12) was employed. BSC-1 cells were infected with wt VV, rVV-VP4 SLAVE and rVV-VP4 7.5. MA104 cells were infected with BRV. Sf9 cells were infected with wt BV and rBV-VP4. Cells were harvested after complete CPE was observed and washed with PBS. The cell pellets were resuspended in electrophoresis sample buffer and heated at 95°C for 5 minutes. The samples were run on SDS-PAGE, transferred to nitrocellulose membranes and then processed by the immunoblot method as described in materials and methods. The results clearly show that the VP4 band expressed by recombinant baculovirus has the same molecular weight as that produced by recombinant vaccinia virus, which corresponds exactly with the MW of VP4 produced by BRV. This result supports the contention that degradation of VP4 observed in Fig. 11 occured during the immunoprecipitation process. The VP4 band produced by rVV-VP4 7.5 is very weak. In contrast, the intensity of the bands produced by rVV-VP4 SLAVE and rBV-VP4 are almost identical, implying the relative strengths of the different promoters used.

In order to further compare the relative amounts of VP4 produced under the control of the different promoters, an ELISA was performed. Microplates were coated with two-fold serial dilutions (starting with 1:500) of supernatants from cells infected with wt VV, rVV-VP4 (SLAVE or 7.5), wt BV and rBV-VP4. Rabbit polyclonal anti-rotavirus serum was added, followed by peroxidase conjugated goat anti-rabbit serum. ABTS substrate was then added to develop the colour reaction. Fig. 13 shows that the relative amounts of VP4 produced by recombinant baculovirus were greater than that produced by either vaccinia virus promoter. The amount of VP4 expressed by rVV-VP4 under the control of 7.5 K gene promoter is

Fig.12. Western Blot Analysis of Rotavirus VP4 Expressed by Recombinant Vaccinia Virus and Baculovirus. BSC-1 cells were infected with: wt VV (lane 6); rVV-VP4 SLAVE (lane 3) and rVV-VP4 7.5 (lane 2). Sf9 cells were infected with: wt BV(lane 5); and rBV-VP4 (lane 1). MA104 cells were infected with BRV (lane 4) as a positive control. The cells were harvested after complete CPE was achieved and then resuspended in PAGE sample buffer. The samples and biotinylated protein molecular weight markers (lane 7) were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose. The proteins were treated with rabbit polyclonal anti-rotavirus serum, followed by adding peroxidase conjugated goat anti-rabbit serum plus avidin-peroxidase conjugate. Molecular weights are given on the right, with sizes in kilodaltons.

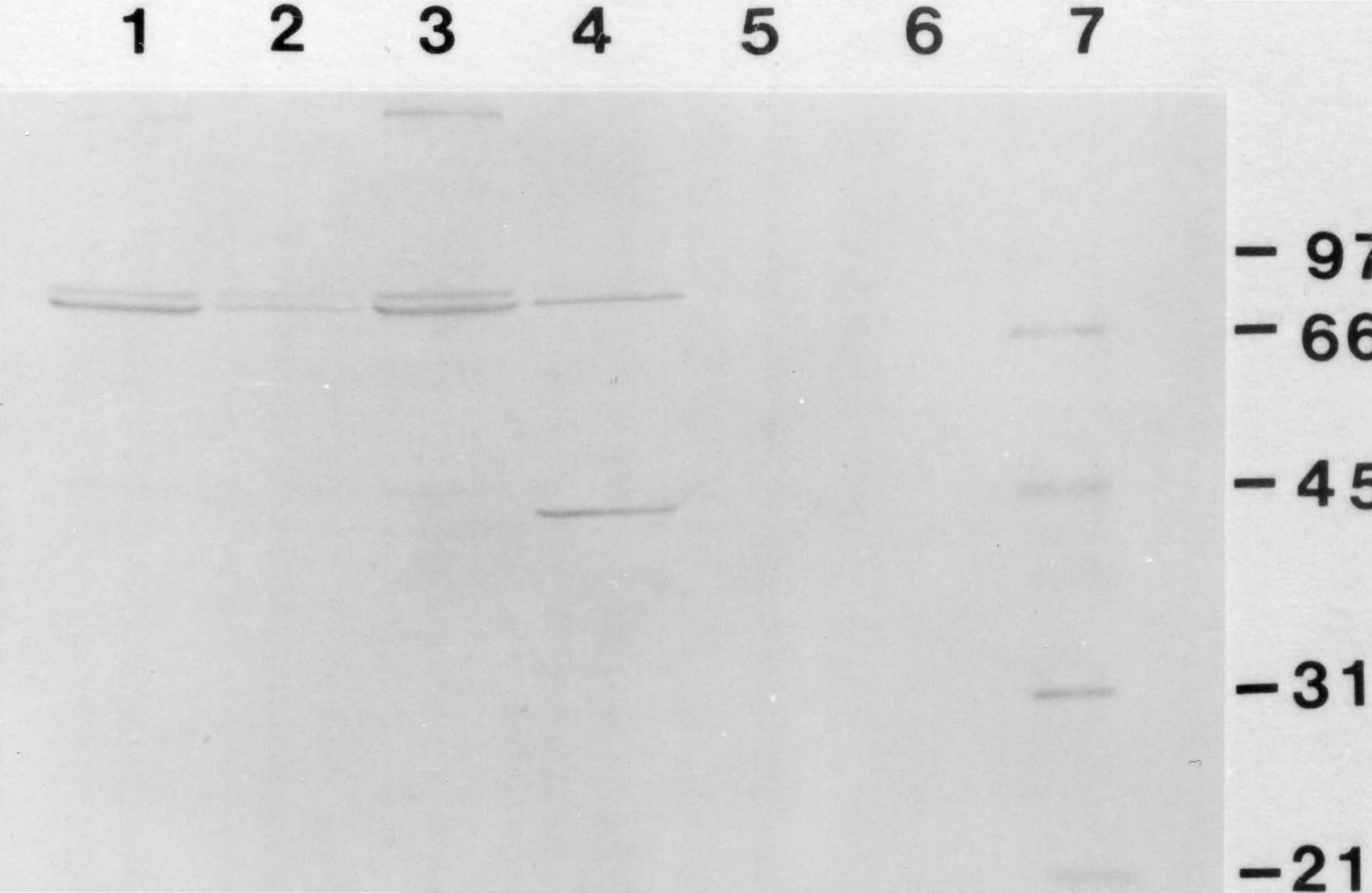
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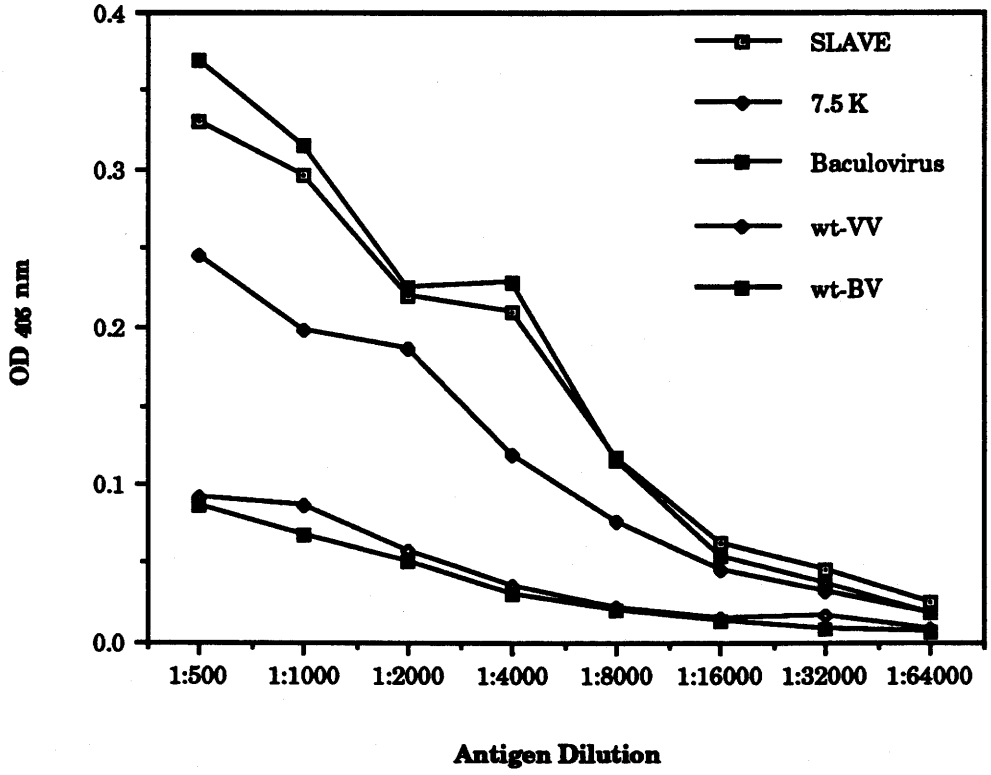
Fig.13. Comparison of Rotavirus VP4 Expressed by Recombinant Virus under the Control of Different Promoters Using ELISA. BSC-1 cells were infected with: wt VV; rVV-VP4 SLAVE and rVV-VP4 7.5. Sf9 cells were infected with: wt BV and rBV-VP4. The supernatants from infected cell lysates were diluted to 1:500 in coating buffer, then two-fold serial dilution thereafter were placed in 96 well microtiter plates. Indirect ELISA was performed using rabbit polyclonal anti-rotavirus serum. Symbols: —▫—, represents rVV-VP4 SLAVE; —◆— , represents rVV-VP4 7.5; —■— , represents rBV-VP4; —◆— , represents wt VV; and —■— , represents wt BV.


0.4
0.3
0.2
0.1
0.0
OD 405 nm
SLAVE
7.5 K
Baculovirus
wt-VV
wt-BV
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
Antigen Dilution

approximately 4X lower than the amount produced by the consensus late gene promoter. Cells infected with wt VV and wt BV did not produced any rotavirus VP4 as expected.

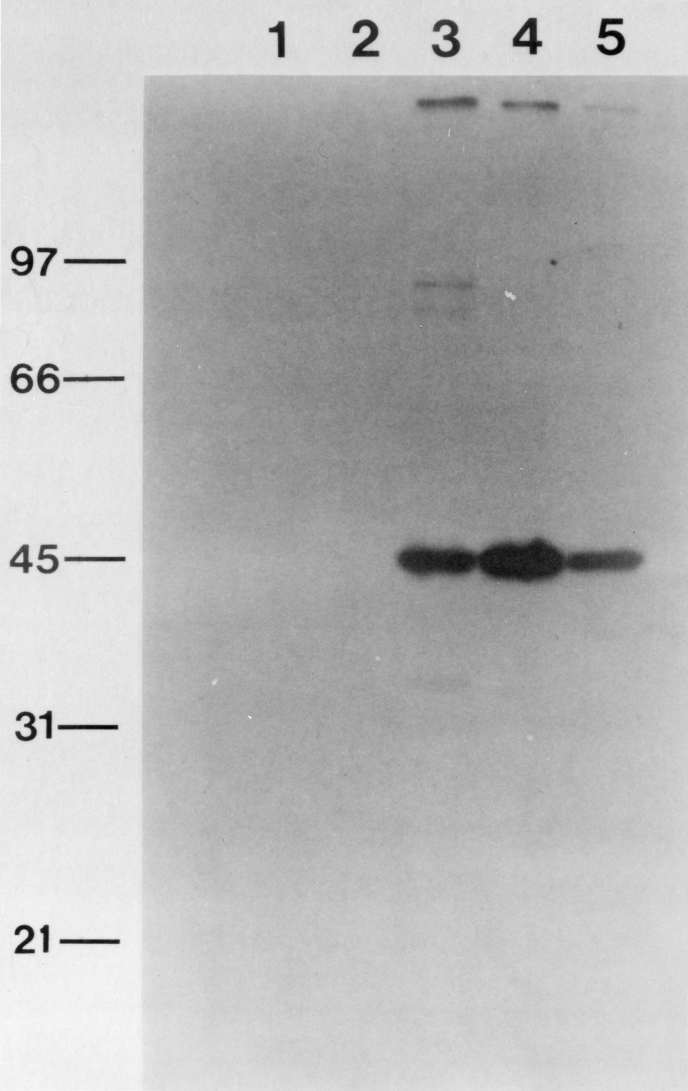
4.3.2 Immune reactivity of VP6 expressed by recombinant vaccinia virus and baculovirus

Monoclonal antibody specific for VP6 was used to precipitate VP6 protein from cells infected with BRV, wt VV or rVV-VP6 under control of the consensus late gene and the 7.5 K gene promoters (Fig. 14). The results show that VP6 from BSC-1 cells infected with rVV-VP6 under control of the two different promoters comigrated with authentic BRV VP6. As was observed previously with VP4, the VP6 band produced by the consensus late gene promoter is the most intense, followed by VP6 expressed by rotavirus and then the recombinant vaccinia virus expressing the VP6 gene under the control of the 7.5 K gene promoter.

The result shown in Fig. 8 demonstrate that VP6 (lane 6) produced by rVV-VP6 SLAVE has the same migration pattern as VP6 (lane 7) expressed by recombinant baculovirus. Western blotting (Fig. 15) also demonstrated that VP6 expressed by rVV-VP6 SLAVE produced a much more intense band than the one produced by rVV-VP6 7.5 and is of similar intensity to the VP6 band produced by rotavirus and recombinant baculovirus. In order to compare the relative amounts of VP6 produced by each virus an ELISA assay was performed. Supernatants from cells infected with wt VV, rVV-VP6 (SLAVE or 7.5 K), wt BV and rBV-VP6 were serially diluted and used to coat microtiterplates. MAb specific to VP6 was added, followed by peroxidase conjugated goat anti-mouse serum.

ABTS substrate was used to develop the colour reaction. The results

Fig.14. Expression of Rotavirus VP6 by Recombinant Vaccinia Virus. BSC-1 cells were infected with: mock virus (lane 1); wt VV (lane 2); rVV-VP6 SLAVE (lane 4) and rVV-VP6 7.5 (lane 5). MA104 cells were infected with BRV (lane 3) as a positive control. The cells were labeled with L-[^{35}S] methionine from 8 to 18 hours postinfection. Immune precipitates were prepared from the cell lysates by using MAbs specific to VP6 and the products were analyzed by electrophoresis through a 10% SDS-PAGE gel. Molecular weights are given on the left, with sizes in kilodaltons.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66
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31
21

Fig.15. Western Blot Assay of Rotavirus VP6 Expressed by Recombinant Vaccinia Virus and Baculovirus. BSC-1 cells were infected with: wt VV (lane 2); rVV-VP6 SLAVE (lane 5) and rVV-VP6 7.5 (lane 6). Sf9 cells were infected with: wt BV (lane 3) and rBV-VP6 (lane 7). MA104 cells were infected with BRV (lane 4) as a positive control. The cells were harvested after complete CPE was achieved and resuspened directly in PAGE sample buffer. The samples and biotinylated protein molecular weight markers (lane 1) were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose. The proteins were reacted with BRV VP6 specific MAbs, followed by adding peroxidase conjugated goat anti-mouse serum plus avidin-peroxidase conjugate. Molecular weights are given on the left, with sizes in kilodaltons.

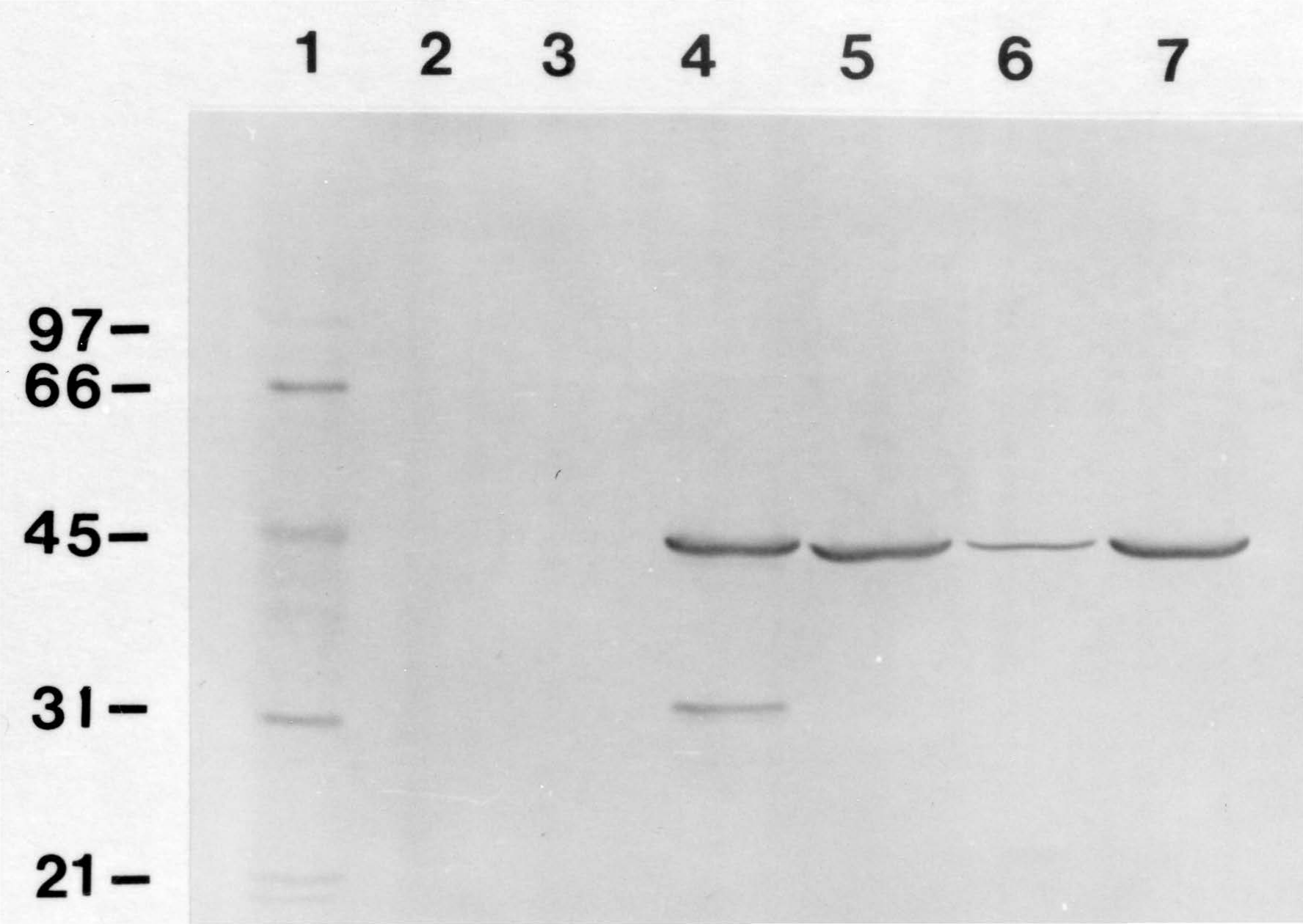
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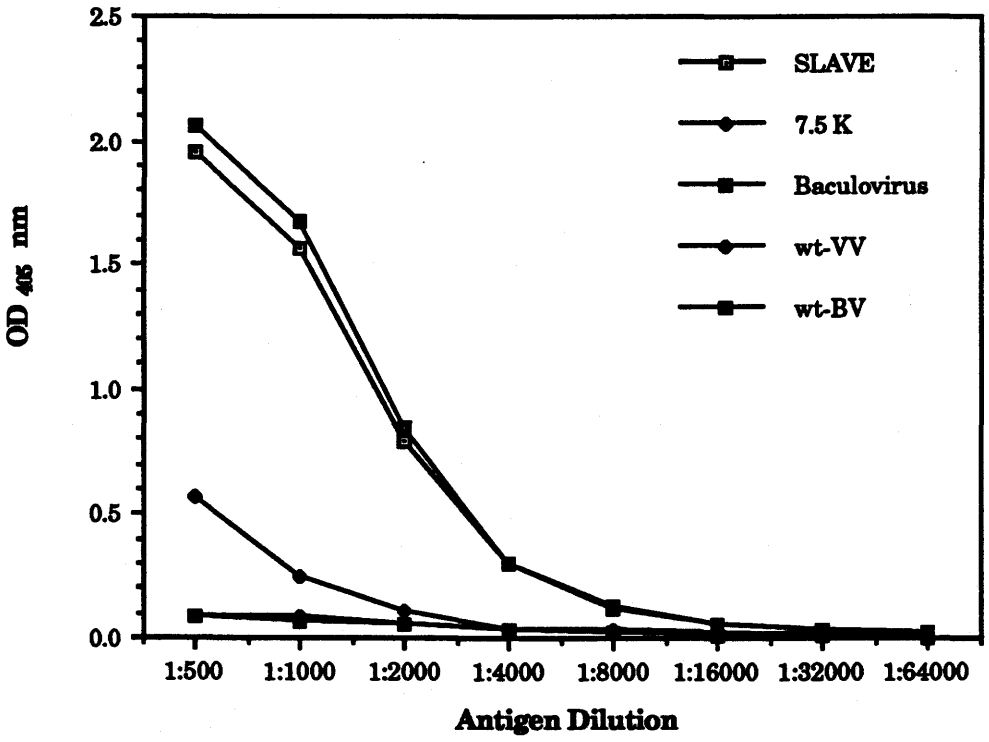
Fig.16. Comparison of Rotavirus VP6 Expressed by Different Recombinant Viruses Using ELISA. BSC-1 cells were infected with: wt VV; rVV-VP6 SLAVE and rVV-VP6 7.5. Sf9 cells were infected with: wt BV and rBV-VP6. The supernatants from cell lysates infected with the above viruses were diluted to 1:500 in coating buffer, then two-fold serial dilutions thereafter were placed in 96 well microtiter plates. Indirect ELISA was performed using BRV VP6 specific MAbs. Symbols: —▫— , represents rVV-VP6 SLAVE; —◆—, represents rVV-VP6 7.5;—■—, represents rBV-VP6; —◆— , represents wt VV; and —■— , represents wt BV.

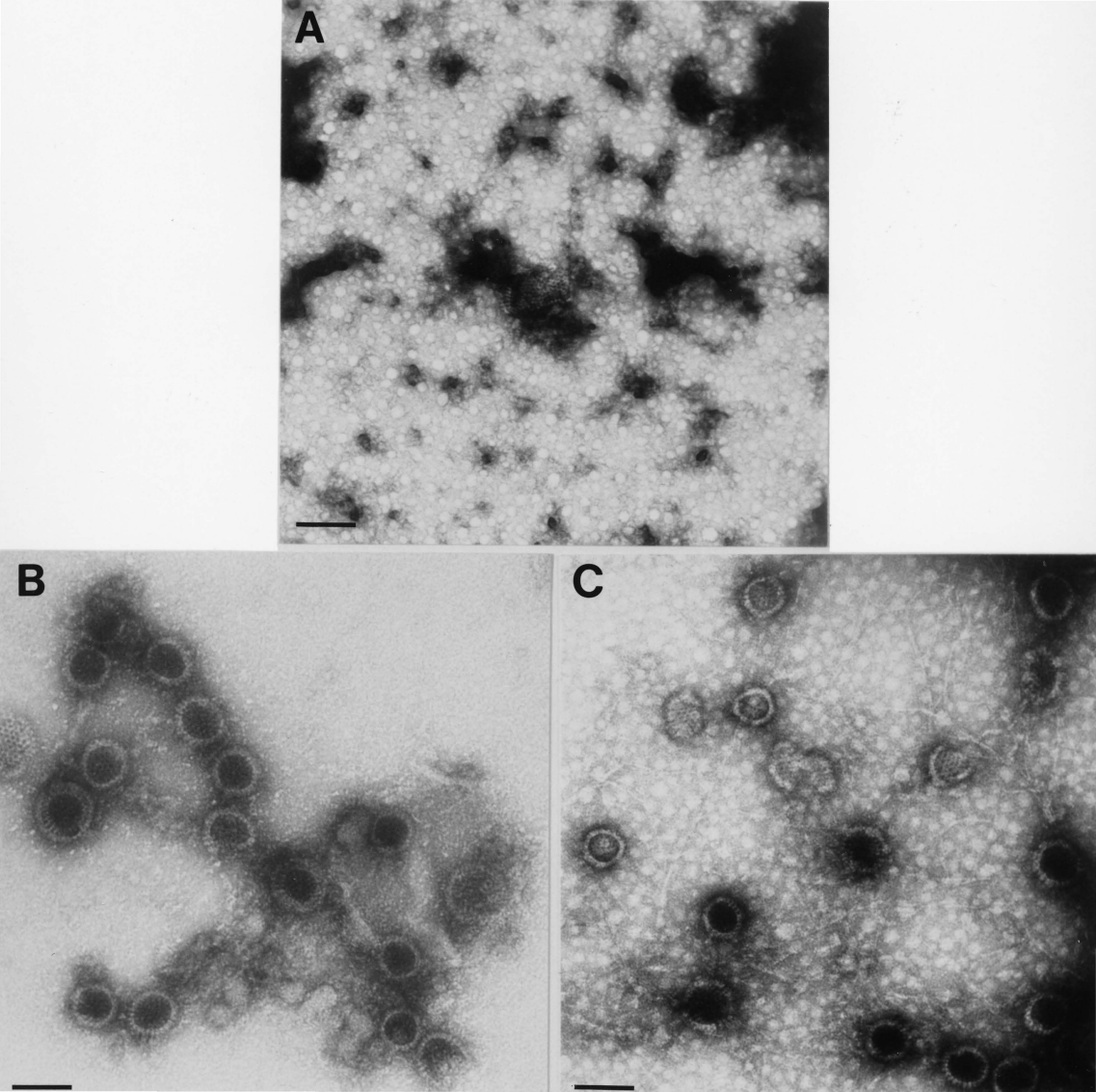

2.5
2.0
1.5
1.0
0.5
0.0
OD 405 nm
SLAVE
7.5 K
Baculovirus
wt-VV
wt-BV
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
Antigen Dilution

shown on Fig. 16 indicate that the highest level of VP6 was produced by rVV-VP6 under control of the consensus late gene promoter. The level of VP6 expressed by rBV-VP6 is almost identical to that of VP6 expressed by rVV-VP6 SLAVE. In contrast, the level of VP6 produced by rVV-VP6 7.5 is much lower than VP6 produced by the other recombinant viruses. Wt VV and wt BV infected cell extracts were used as negative controls.

4.3.3 Production of VP6 spheres

Although immune reactivity of proteins is a partial indication of authenticity, the ability of VP6 to self-assemble into single shelled spherical particles would be further confirmation. To test this possibility, *in vitro* , assembly of recombinant VP6 into core like particle was investigated. It was previously reported that VP6 purified from bovine rotavirus can be assembled into single shelled particles (Ready et al., 1987). Based upon these findings, BSC-1 cells were infected with recombinant vaccinia virus expressing VP6 under the control of the consensus late gene promoter or the 7.5 K gene promoter. In a parallel experiment, Sf9 cells were infected with recombinant baculovirus expressing VP6. Cells were harvested after complete CPE, and washed with PBS. The infected cells were then lysed by the addition of lysis buffer followed by sonication. Cell debris was removed by low speed centrifugation. VP6 was then purified from these supernatants as described in Materials and Methods and examined with the aid of an electron microscope. Fig. 17 shows that single shelled particles were produced from cultures infected with rVV-VP6 SLAVE and rBV-VP6 (Fig. 17 B and C), but not by rVV-VP6 7.5 (Fig. 17 A). The absence of assemble particles in Fig. 17 A was probably due to the low concentration of VP6 in rVV-VP6 7.5 infected cells. In order to confirm that the particles

Fig.17. Electron Micrographs of VP6 Particles. Panel A is a preparation from cells infected with rVV-VP6 7.5; Panel B is a preparation from cells infected with rVV-VP6 SLAVE; Panel C is a preparation from cells infected with rBV-VP6. Bar = 100 nm. The samples were prepared as described in Materials and Methods.


A
B
C

were composed of VP6, SDS-PAGE and western blotting analysis were performed. The VP6 particles derived from rVV-VP6 SLAVE, 7.5, rBV-VP6, and partially purified BRV were separated by SDS-PAGE. The VP6 particles derived from rVV-VP6 7.5 were loaded on gel 3X volume mroe than others. The crude cell lysates from cells infected with BRV, rVV-VP6 SLAVE and 7.5, and rBV-VP6 were also separated by the same above SDS-PAGE. The results were analyzed by Coomassie blue staining (Fig. 18) and western blotting (Fig. 19). The bands at 45 K MW in Fig. 18 correspond to molecular weight of VP6. Confirmation that the particles were made up of VP6 was provided by western immunoblot analyses using MAb specific for VP6. The MAb reacted strongly with a 45 K protein in lanes from 5 to 12 (Fig 19). Partially purified authentic VP6 from BRV infected cells was included as positive control (Fig. 19, lane 12) and the band that it produced comigrated with the recombinant forms of VP6 that were successfully assembled into spheres (lanes 9 and 11). No reaction was detected with mock-infected or wild-type infected cells (Fig. 19, lanes 1 to 4).

4.3.4 Analysis of VP7 expressed by recombinant vaccinia virus and baculovirus

Rabbit polyclonal anti-rotavirus Ab precipitated VP7 from BSC-1 cells infected with rVV-VP7 SLAVE and rVV-VP7 7.5 in both the absence or presence of tunicamycin. The VP7 bands produced by either recombinant correspond to the VP7 produced by BRV (Fig. 20). It was previously suggested (Sabara et al., 1982; Petrie et al., 1983; Suzuki et al., 1984) that VP7 contained only N-linked oligosaccharides, since tunicamycin is a specific inhibitor of N-linked glycosylation. The results in Fig. 20 also show a more intense band of VP7 (both in tunicamycin treated and non-treated)

Fig.18. Coomassie Blue Staining of Rotavirus VP6 Expressed by Recombinant Vaccinia Virus and Baculovirus. BSC-1 cells were infected with: mock virus (lane 1); wt VV (lane 2); rVV-VP6 SLAVE (lane 6); and rVV-VP6 7.5 (lane 7). Sf9 cells were infected with: mock virus (lane 3); wt BV (lane 4); and rBV-VP6 (lane 8). MA104 cells were infected with BRV (lane 5). Lane 9 represents VP6 particles purified from cells infected with rVV-VP6 SLAVE; Lane 10 represents VP6 particles purified from cells infected with rVV-VP6 7.5; Lane 11 represents VP6 particles purified from cells infected with rBV-VP6; Lane 12 is partially purified rotavirus from MA104 infected cells. All samples were separated on 10% SDS-PAGE gel and stained with coomassie brilliant blue (R-250). Molecular weights are given on the left, with sizes in kilodaltons.

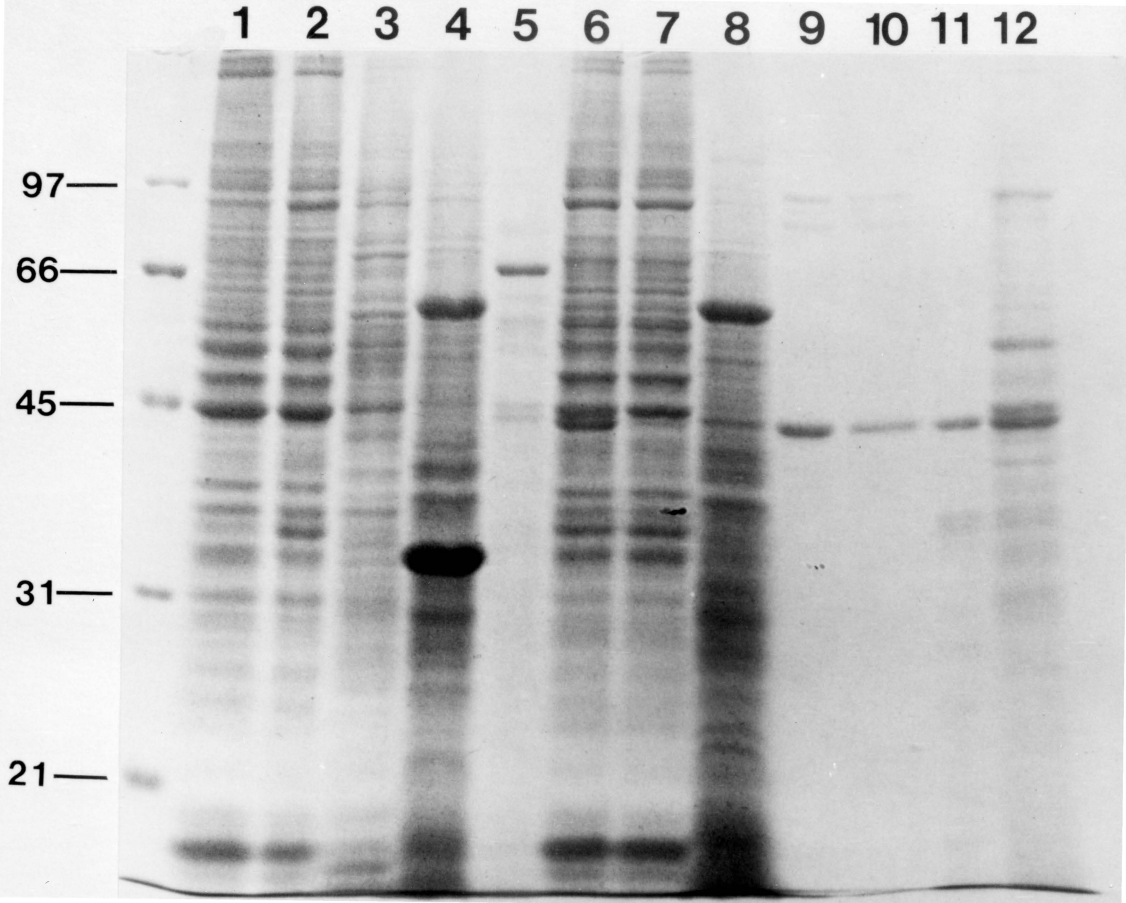

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Fig.19. Western Blot Analysis of VP6 Produced by Recombinant Vaccinia Virus and Baculovirus. BSC-1 cells were infected with: mock virus (lane 1); wt VV (lane 2); rVV-VP6 SLAVE (lane 6); and rVV-VP6 7.5 (lane 7). Sf9 cells were infected with: mock virus (lane 3); wt BV (lane 4); and rBV-VP6 (lane 8). MA104 cells were infected with BRV (lane 5). Lane 9 is VP6 particles purified from cells infected with rVV-VP6 SLAVE; lane 10 is VP6 particles purified from cells infected with rVV-VP6 7.5; lane 11 is VP6 particles purified from cells infected with rBV-VP6; lane 12 is partially purified BRV from infected MA104 cells. All samples and biotinylated protein molecular weight markers were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose. The proteins were reacted with VP6 specific MAbs, followed by adding peroxidase conjugated goat anti-mouse serum. Molecular weights are given on the left, with sizes in kilodaltons.

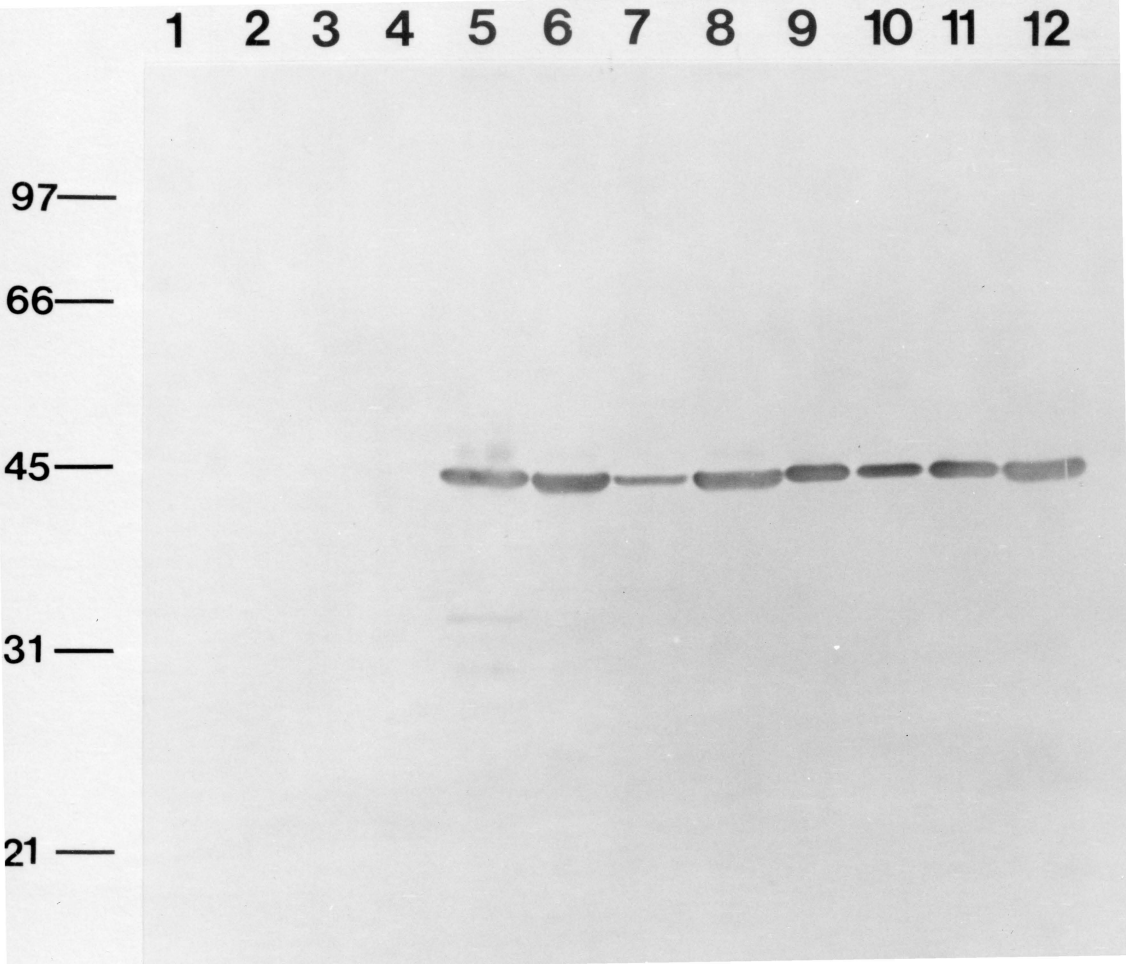
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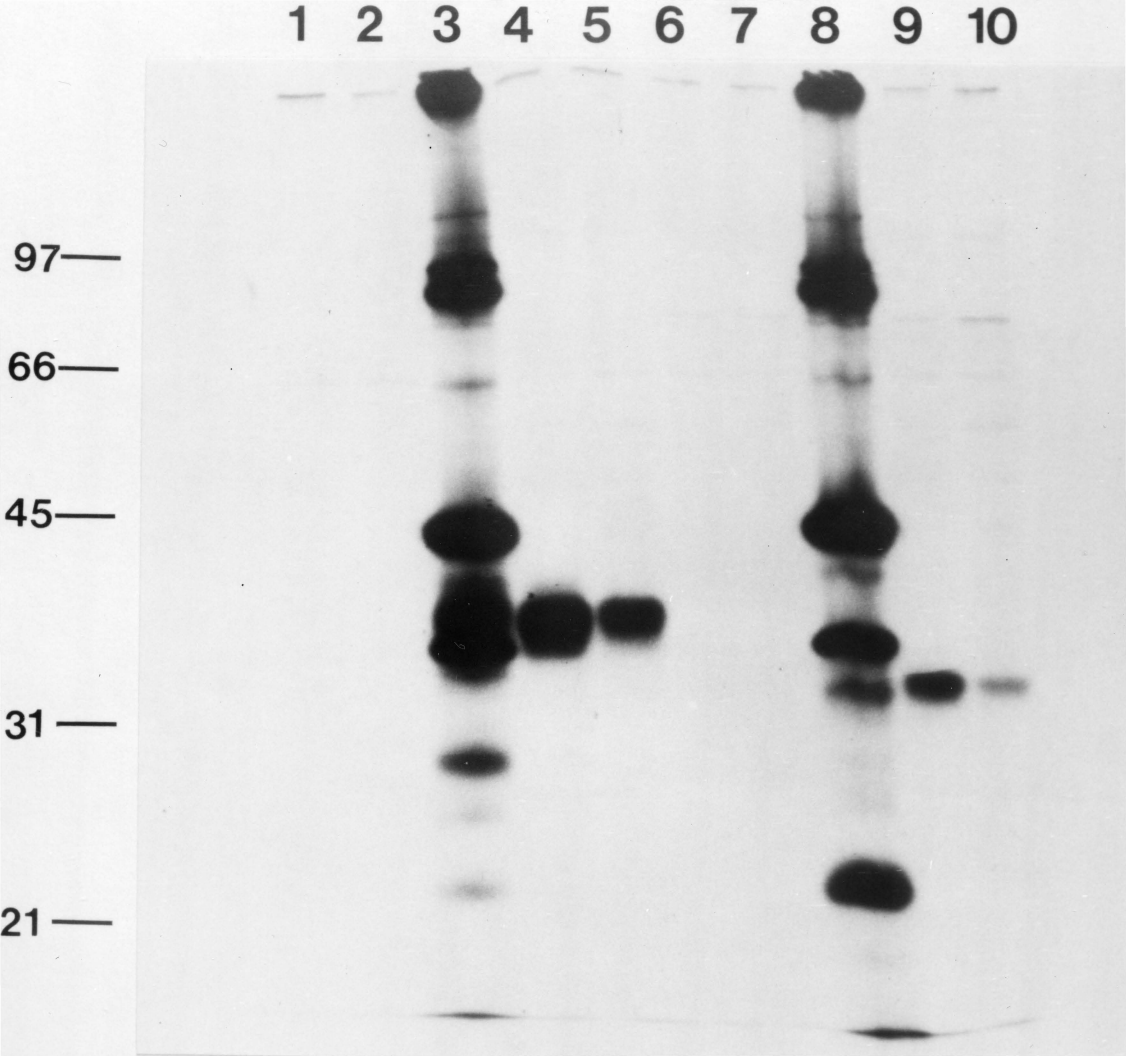
Fig. 20. Expression of Rotavirus VP7 by Recombinant Vaccinia Virus. BSC-1 cells were infected with: mock virus (lanes 1 and 6); wt VV (lanes 2 and 7); rVV-VP7 SLAVE (lanes 4 and 9); and rVV-VP7 7.5 (lanes 5 and 10). MA104 cells were infected with BRV (lanes 3 and 8) as a positive control. Samples in lane 6 to 10 are from tunicamycin treated cells. The cells were labeled with L-[^{35}S] methionine from 8 to 18 hours postinfection. Immune precipitates were prepared from the cell lysates by using rabbit polyclonal anti-rotavirus serum and the products were separated on a 10% SDS-PAGE gel. Molecular weights are given on the left, with sizes in kilodaltons.


1 2 3 4 5 6 7 8 9 10
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21

produced by rVV-VP7 SLAVE than that produced by rVV-VP7 7.5. From the data presented in Fig. 10 and 21 it is clear that VP7 expressed by recombinant baculovirus has a lower molecular weight in both medium and cells than VP7 expressed by recombinant vaccinia virus or authentic VP7 produced by BRV (Fig. 10, lanes 9 and 10; Fig. 21, lanes 7 and 8). When tunicamycin was used to block glycosylation, the VP7 produced by rBV-VP7 had the same molecular weight as VP7 expressed by recombinant vaccinia virus (Fig. 21, lanes 14 and 16). This indicates that VP7 is glycosylated in a different manner in insect cells than in mammalian cells (i.e. BSC-1 cells). When tunicamycin was added to rBV-VP7 infected cells, VP7 was not secreted to growth medium, since there is no band visible in Fig. 21, lane 15. This indicates that blocking glycosylation of VP7 also prevents its secretion.

ELISA was performed to compare the relative amounts of VP7 produced by rVV-VP7 under the control of different promoters. For recombinant baculovirus the medium and cells were collected separately. For recombinant vaccinia virus infections only cells were examined. Infected cells were collected and lysed with lysis buffer, then sonicated and centrifuged to remove cell debris. The supernatant from cell lysates and the medium into which VP7 was secreted were placed into 96 well round bottom microplates and incubated at 4°C overnight. Rabbit polyclonal anti-rotavirus serum was applied, followed by peroxidase conjugated goat anti-rabbit serum. Finally, ABTS substrate was added to develop the colour reaction. The results are shown on Fig. 22. The highest concentration of VP7 was found in the medium of rBV-VP7 infected cells, followed by extracts of rVV-VP7-SLAVE infected cells. These results confirm that most of the VP7 produced by rBV-VP7 was secreted. rVV-VP7 under

Fig. 21. Expression of Rotavirus VP7 by Recombinant Vaccinia Virus and Baculovirus. BSC-1 cells were infected with: mock virus (lanes 1 and 9); wt VV (lanes 2 and 10); and rVV-VP7 SLAVE (lanes 6 and 14). Sf9 cells were infected with: mock virus (lanes 3 and 11); wt BV (lanes 4 and 12); and rBV-VP7 (lanes 7, 8, 15 and 16). MA104 cells were infected with BRV (lanes 5 and 13) as a positive control. Samples in lanes 9 to 16 were derived from tunicamycin treated cells. BSC-1 and MA104 cells were labeled with L-[^{35}S] methionine from 8 to 18 hours postinfection. Sf9 cells were labeled with L-[^{35}S] methionine from 40-50 hours postinfection. Immune precipitates were prepared from cell lysates by using rabbit polyclonal anti-rotavirus serum, except the samples in lanes 7 and 15 which were prepared from culture medium of rBV-VP7 infected Sf9 cells. All products were separated on a 10% SDS-PAGE gel. Molecular weights are given on the left, with sizes in kilodaltons.

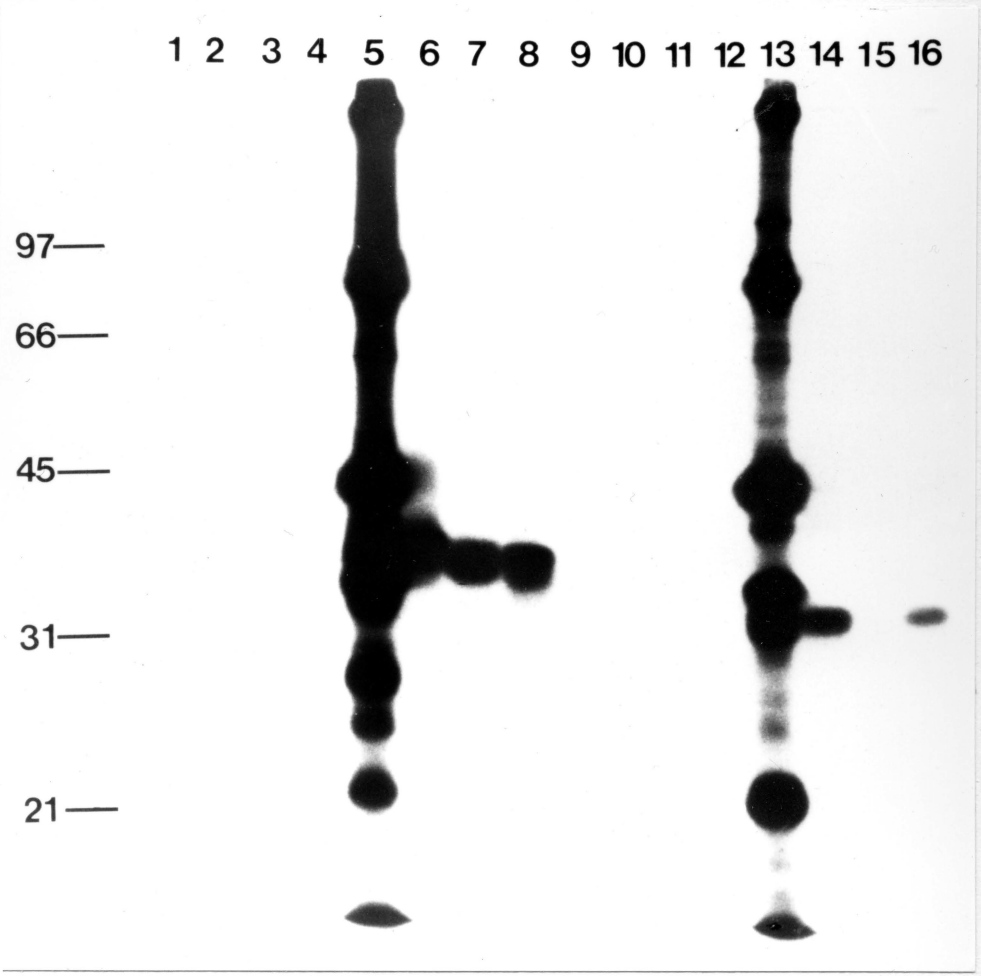
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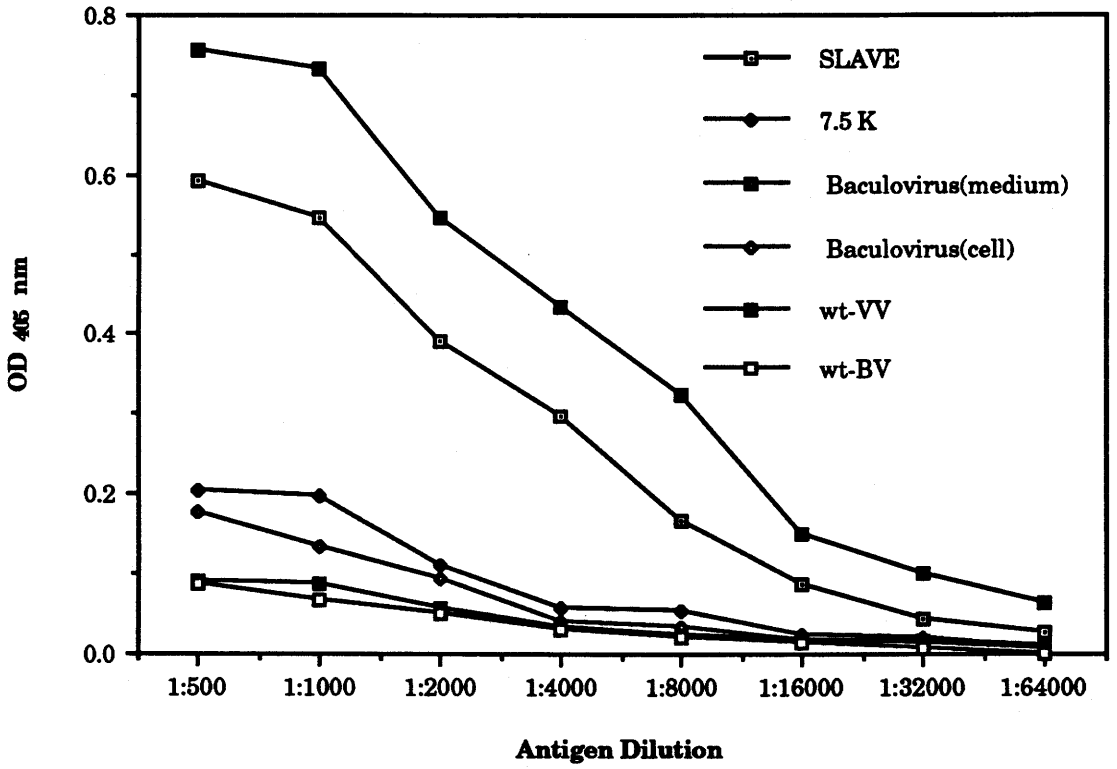
Fig. 22. Comparison of Rotavirus VP7 Expressed by Recombinant Vaccinia Virus and Baculovirus by ELISA. BSC-1 cells were infected with: wt VV; rVV-VP7 SLAVE and rVV-VP7 7.5. Sf9 cells were infected with wt BV and rBV-VP7. The supernatants from cell lysates infected with the above viruses were collected. All samples were diluted 1:500 in coating buffer, then it, and serial two-fold dilutions thereafter, were placed in 96 well microtiterplates. An indirect ELISA was performed by using rabbit polyclonal anti-rotavirus serum. Symbols: —▫— , represents rVV-VP7 SLAVE; —◆— , represents rVV-VP7 7.5; —■— , represents rBV-VP7 medium; —◆— , represents rBV-VP7 cell lysate; —■— , represents wt VV; and —□— , represents wt BV.


0.8
0.6
0.4
0.2
0.0
OD 405 nm
SLAVE
7.5 K
Baculovirus(medium)
Baculovirus(cell)
wt-VV
wt-BV
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
Antigen Dilution

control of the 7.5 K gene promoter produces approximatelly 8X lower levels of VP7 than the consensus late gene promoter.

4.4 Evaluation of Humoral Immunity in Mice Immunized with Recombinant Vaccinia Virus and Baculovirus Expressing Bovine Rotavirus Antigens

Analysis of Ab produced to recombinant rotavirus antigens in vaccinated mice was done by ELISA and immunoprecipitation assays. Mice were immunized intraperitoneally with wt VV, rVV SLAVE expressing VP4, VP6 or VP7, or intramuscularly with inactivated BRV, or rVP4, rVP6 or rVP7 produced by recombinant baculovirus. The inactivated BRV and baculovirus derived BRV proteins were formulated in Freunds complete adjuvant (FCA) prior to immunization. Three weeks after the first immunization, mice were reimmunized intraperitoneally with wtVV, rVV SLAVE expressing VP4, VP6 or VP7, or intramuscularly with the inactivated BRV and baculovirus derived BRV proteins in Freunds incomplete adjuvant (FICA). Sera were obtained from vaccinated mice 8 and 90 days after the second immunization to determine the levels of rotavirus antibodies. All mice that were immunized with the recombinant vaccinia viruses, inactivated bovine rotavirus and subunit proteins from recombinant baculovirus developed a detectable response to whole or to the respective bovine rotavirus antigens. The ELISA results show the ability of the mouse sera to recognize VP4, VP6, and VP7 in intact rotavirus (Table 2) or recombinant rotavirus proteins produced by rVV or rBV (Table 3, 4, and 5). Preimmune sera did not possess antibody against rotavirus (data not shown). The microtiter plates were coated with 0.5 μg per well of partially purified rotavirus to test titers against whole rotavirus. Table 2 shows

antibody titers against intact rotavirus induced in mice by: rVV-VP4, -VP6, and -VP7; VP4, VP6, and VP7 produced by rBV; mixtures of rVV-VP4, -VP6 and -VP7; mixtures of VP4, VP6, and VP7 produced by rBV, and; inactivated bovine rotavirus. In order to further determine the titers elicited by the recombinants to the individual rotavirus proteins, the microtiter plates were coated with cell lysates from cells infected with rVV-VP4, VP6 or VP7, or rBV-VP4, VP6 or VP7, diluted at 1:500 (Table 3, 4, and 5). For example, the microtiter plates were coated with rBV-VP4 infected cell lysates to test titer elicited by rVV-VP4, while a second set of plates were coated with rVV-VP4 infected cell lysates to test titer elicited by rBV-VP4. The same procedure was used to determine Ab titers specificly against VP6 or VP7. These assays also demonstrate that the anti-BRV antibody response in mice was due to immunization and not due to accidental infection. The results in Table 3, 4, and 5 clearly demonstrated that the antibodies were specific to the immunizing antigens.

Since it is known that mice transfer antibody into the milk which can passively protect neonates from infection, milk antibody levels of vaccinated dams were also assessed (Table 6). To obtain sufficient quantities of milk, it was isolated from the stomachs of 7 day old mice suckling from the vaccinated dams. The results presented in Table 6 indicate that the dams were successfully immunized and anti-rotavirus antibody was secreted in the colostrum and milk. The mouse sera were also used to immunoprecipitate rotavirus antigens from a [^{35}S] methionine-labeled BRV infected cell lysate. Analysis of representative sera from vaccinated mice are shown in Fig. 23. Rabbit polyclonal anti-rotavirus antibody was used as a positive control (lane 1). The sera from mice immunized with placebo (lane 2) and wt VV (lane 3) did not react with any rotavirus antigens. Sera

Table 2. Serologic Responses of Mice Immunized with Recombinant Vaccinia Virus[a] and Baculovirus[b]

Inoculation route	Vaccination groups	No. of mice	ELISA titers[c] at indicated day after second immunization	
			8	90
Intraperitoneally	wt VV	5	<20	<20
	rVV-VP4 SLAVE	7	1:2560	1:2560
	rVV-VP6 SLAVE	7	1:2560	1:2560
	rVV-VP7 SLAVE	7	1:1280	1:1280
	rVV-VP4+VP6+VP7 SLAVE	7	1:2560	1:1280
Intramuscularly	Placebo	10	<20	<20
	Sentinel	5	<20	<20
	rBV-VP4	7	1:1280	1:1280
	rBV-VP6	7	1:1280	1:1280
	rBV-VP7	7	1:640	1:640
	rBV-VP4+VP6+VP7	7	1:2560	1:1280
	Inactivated Rotavirus	5	1:2560	1:2560

a. Mice were immunized intraperitoneally with $2x10^6$ PFU of recombinant vaccinia virus or wild-type vaccina virus.

b. Mice were immunized intramuscularlly with 50 μg of protein from recombinant baculovirus infected cells plus adjuvant.

c. Mean ELISA Ab titers versus 0.5 μg/well of partially purified rotavirus.

Table 3. Specificity of Serologic Responses to the BRV VP4 Protein

Inoculation route	Vaccination groups	No. of mice	ELISA titers[a] at indicated day after second immunization	
			8	90
Intraperitoneally	rVV-VP4 SLAVE	7	1:2560	1:2560
	rVV-VP6 SLAVE	7	<20	<20
	rVV-VP7 SLAVE	7	<20	<20
	rVV-VP4+VP6+VP7 SLAVE	7	1:1280	1:1280
	wt VV	5	<20	<20
Intramuscularly	rBV-VP4	7	1:1280	1:1280
	rBV-VP6	7	<20	<20
	rBV-VP7	7	<20	<20
	rBV-VP4+VP6+VP7	7	1:640	1:640
	Inactivated Rotavirus	7	1:640	1:640
	Placebo	10	<20	<20
	Sentinel	5	<20	<20

a. Mean ELISA Ab titers using plates coated with: 1. rBV-VP4 infected cell lysates for testing Ab titers of mice immunized with rVV-VP4 or; 2. rVV-VP4 infected cell lysates for testing Ab titers of mice immunized with VP4 produced by rBV-VP4.

Table 4. Specificity of Serologic Responses to the BRV VP6 Protein

Inoculation route	Vaccination groups	No. of mice	ELISA titers[a] at indicated day after second immunization	
			8	90
Intraperitoneally	rVV-VP4 SLAVE	7	<20	<20
	rVV-VP6 SLAVE	7	1:2560	1:2560
	rVV-VP7 SLAVE	7	<20	<20
	rVV-VP4+VP6+VP7SLAVE	7	1:1280	1:1280
	wt VV	5	<20	<20
Intramuscularly	rBV-VP4	7	<20	<20
	rBV-VP6	7	1:2560	1:2560
	rBV-VP7	7	<20	<20
	rBV-VP4+VP6+VP7	7	1:1280	1:1280
	Inactivated Rotavirus	7	1:1280	1:1280
	Placebo	10	<20	<20
	Sentinel	5	<20	<20

a Mean ELISA Ab titers using plates coated with: 1. rBV-VP6 infected cell lysates for testing Ab titers of mice immunized with rVV-VP6 or; 2. rVV-VP6 infected cell lysate for testing Ab titers of mice immunized with VP6 produced by rBV-VP6.

Table 5. Specificity of Serologic Responses to the BRV VP7 Protein

Inoculation route	Vaccination groups	No. of mice	ELISA titers[a] at indicated day after second immunization	
			8	90
Intraperitoneally	rVV-VP4 SLAVE	7	<20	<20
	rVV-VP6 SLAVE	7	<20	<20
	rVV-VP7 SLAVE	7	1:640	1:640
	rVV-VP4+VP6+VP7 SLAVE	7	1:640	1:640
	wt VV	5	<20	<20
Intramuscularly	rBV-VP4	7	<20	<20
	rBV-VP6	7	<20	<20
	rBV-VP7		1:640	1:640
	rBV-VP4+VP6+VP7	7	1:640	1:640
	Inactivated Rotavirus	7	1:320	1:320
	Placebo	10	<20	<20
	Sentinel	5	<20	<20

a. Mean ELISA Ab titers using plates coated with: 1. rBV-VP7 infected cell lysates for testing Ab titers of mice immunized with rVV-VP7 or; 2. rVV-VP7 infected cell lysate for testing Ab titers of mice immunized with VP7 produced by rBV-VP7.

from sentinal mice (lane 4) also did not react with rotavirus antigens indicating that an accidental rotavirus infection did not occur during the experiment. Sera from mice immunized with rVV-VP4 (lane 5) and rBV-VP4 (lane 6) are as effective as the rabbit polyclonal antibody in reacting with BRV VP4. Sera from mice immunized with rVV-VP6 (lane 7) and rBV-VP6 (lane 8) specifically reacted with rotavirus VP6. Sera from mice immunized with rVV-VP7 (lane 9) and rBV-VP7 (lane 10) reacted with rotavirus VP7, as expected. Sera from mice immunized with mixed rVV-VP4, -VP6 and -VP7 (lane 11) and rBV-VP4, -VP6 and -VP7 (lane 12) reacted with rotavirus VP4, VP6 and VP7. Sera from mice immunized with inactivated rotavirus reacted with all of the rotavirus proteins. These results demonstrated that sera from mice immunized with recombinant vaccinia virus and baculovirus developed specific antibody responses to the appropriate vaccine antigen.

Table 6. Ab Titers in Milk of Dams Immunized with Recombinant Vaccinia Virus[a] or Baculovirus[b]

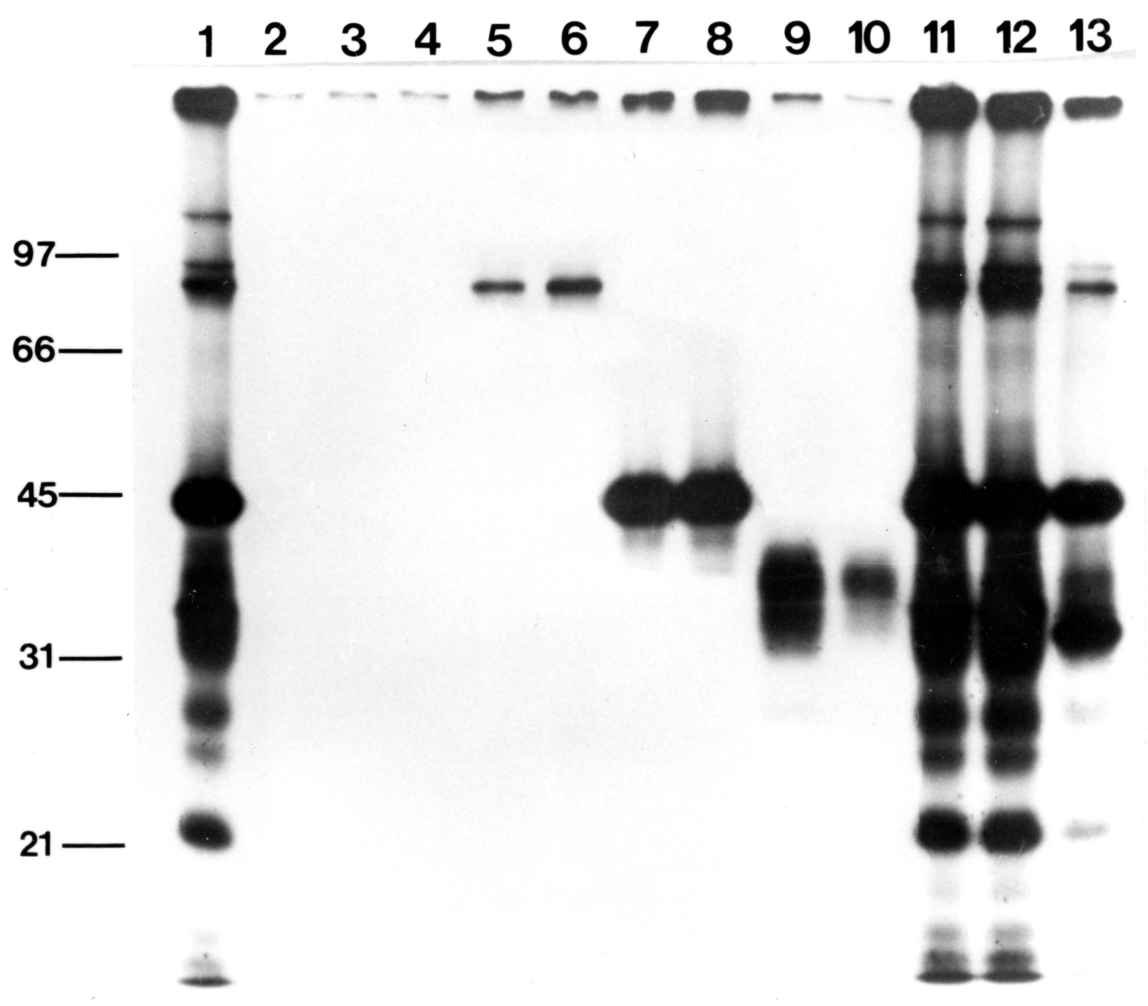
Dam vaccinated groups	Number of baby mice	Ab titers[c] in milk
Placebo	10	< 20
Sentinel	5	< 20
wt VV	5	< 20
rVV-VP4 SLAVE	7	1:640
rVV-VP6 SLAVE	7	1:2560
rVV-VP7 SLAVE	7	1:1280
rVV-VP4+VP6+VP7 SLAVE	7	1:640
rBV-VP4	7	1:640
rBV-VP6	7	1:1280
rBV-VP7	7	1:1280
rBV-VP4+VP6+VP7	7	1:1280
Inactivated Rotavirus	7	1:1280

a. Dams were immunized intraperitoneally with $2x10^6$ PFU of recombinant vaccinia virus or wild-type vaccina virus.

b. Dams were immunized intramuscularlly with 50 μg of total protein from cells infected with recombinant baculovirus plus adjuvant (first immunization with FCA, second with FICA)

c. Indirect ELISA Ab titers using 0.5 μg/well of partially purified rotavirus as the capture antigen.

Fig. 23. Immunoprecipitation Assays of Sera from Vaccinated Mice. Sera were collected from vaccinated dams 8 days following the second immunization. Lysates were prepared from L-[^{35}S] methionine-labeled MA104 cells infected with BRV. Rabbit polyclonal anti-rotavirus serum was used as a positive control (lane 1). The sera from mice immunized with placebo (lane 2), wt VV (lane 3) and sentinel mice (lane 4) did not react with rotavirus proteins. The sera from mice immunized with rVV-VP4 SLAVE (lane 5) and rBV-VP4 (lane 6) react with rotavirus VP4. The sera from mice immunized with rVV-VP6 SLAVE (lane 7) and rBV-VP6 (lane 8) react specifically with rotavirus VP6. The sera from mice immunized with rVV-VP7 SLAVE (lane 9) and rBV-VP7 (lane 10) react with rotavirus VP7. The sera from mice immunized with a mixture of rVV-VP4, -VP6 and -VP7 (lane 11) and rBV-VP4, -VP6 and -VP7 (lane 12) reacted with rotavirus VP4, VP6 and VP7. These responses were identical to the positive control. Lane 13 is the BRV proteins that react with sera from mice immunized with inactivated rotavirus.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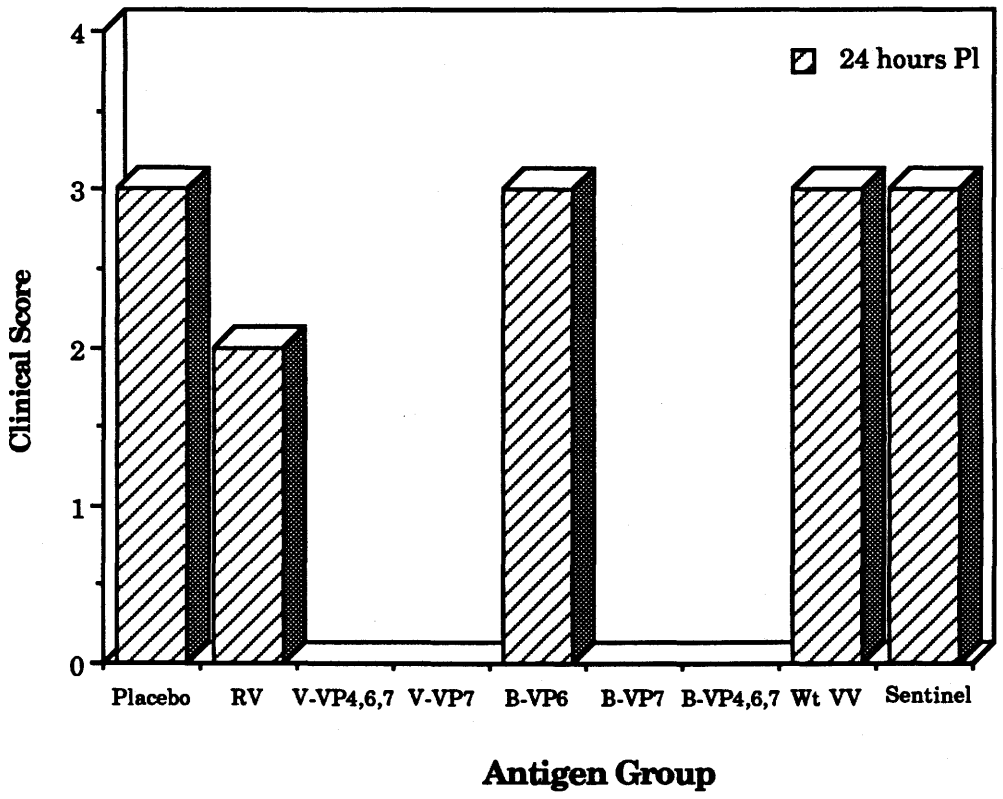
4.5 Protective Responses to BRV Challenge Conferred to Suckling Mice by Dam Vaccination

To determine if colostrum-milk antibodies could protect neonatal mice from rotavirus challenge, dams were immunized as previously described and then their pups were challenged orally at 7 days of age with $1x10^6$ PFU of bovine rotaviruses in 100 µl. Clinical scoring was done at 8, 24, 48 and 72 hours after challenge.

Neonates nursed by dams immunized with rVV-VP7, a mixture of rVV-VP+VP6+VP7, rBV-VP7 or a mixture of rBV-VP4+VP6+VP7 produced by recombinant baculovirus did not develop diarrhea when challenge with rotavirus. Neonates nursed by dams immunized with killed rotavirus developed a mild diarrhea. Neonates nursed by dams immunized with placebo, wild-type vaccinia virus and recombinant vaccinia virus expressing VP6, and sentinel dams developed diarrhea (Fig. 24). Since baby mice from dams immunized with rVV-VP4, -VP6 and rBV-VP4 died, a challenge experiment was not performed in these groups. Therefore, we can speculate that Ab induced by rVV-VP7 and rBV-VP7 can protect neonates from rotavirus infection, while Ab induced by rBV-VP6 is not protective. Ab induced by killed rotavirus could only partially protect neonates from rotavirus infection. Since VP7 epitopes are conformation dependent, inactivation of BRV may have denatured the protein sufficiently to reduce its ability to provide complete protection from virus challenge. Therefore, neonates nursed by dams immunized with killed rotavirus were not completely protected from rotavirus challenge.

Fig. 24. Passive Protection of Suckling Neonates from BRV Challenge. Dams were immunized twice with recombinant vaccinia virus or recombinant rotavirus proteins produced by baculovirus and then their pups were challenged orally at 7 days of age with $1x10^6$ PFU of bovine rotavirus in 100 µl. Clinical scoring was done at 8, 24, 48 and 72 hours after challenge. This figure is based on clinical scoring at 24 hours. Scores from zero to three were assigned to challenged animals as follows: 0 represents the normal condition; 1-3 represent diarrhea of progressive severity. Neonates nursed by dams immunized with rVV-VP7, rVV-VP4+VP6+VP7, rBV-VP7 and rBV-VP4+VP6+VP7 were protected from diarrhea. Neonates nursed by dams immunized with inactivated rotavirus had mild diarrhea, while neonates nursed by dams immunized with placebo, wt VV and rBV-VP6 and sentinal dams had diarrhea.


24 hours Pl
Clinical Score
4
3
2
1
0
Placebo
RV
V-VP4,6,7
V-VP7
B-VP6
B-VP7
B-VP4,6,7
Wt VV
Sentinel
Antigen Group

5. DISCUSSION AND CONCLUSIONS

5.1 Expression of Rotavirus VP4, VP6 and VP7 by Recombinant Virus Vectors

Rotaviruses are the major pathogens that cause life-threatening diarrhea in young children and animals. There is a need to understand rotavirus structural proteins at the molecular level, since the immune responses induced by them are essential for protection from disease. In order to accomplish this, I produced individual BRV proteins using recombinant vectors in sufficient quantity and purity for further analysis. Vaccinia virus and baculovirus were used because these two expression systems provide an opportunity for the effective production of BRV proteins. Furthermore, they may also prove to be excellent expression systems for the production of candidate vaccine antigens. The studies described in this thesis address these two key issues.

To aid in a better understanding of the the role of individual proteins in immunity, rotavirus VP4, VP6 and VP7 protein were expressed by vaccinia virus and baculovirus. Developing vaccinia-rotavirus recombinants required the construction of an insertion vector which was used to cotransfect vaccinia virus infected cells. This vector contained a bacterial origin of replication for growth and amplification of the plasmid in a bacterial host, a bacterial drug resistant marker for identification and cloning of recombinant plasmids in a bacterial host, a defined vaccinia virus promoter and multiple unique restriction enzyme sites engineered downstream from the vaccinia promoter. These above elements were previously shown to be required for efficient expression of foreign genes in vaccinia virus (Mackett et al.,1984). To help distinguish recombinant

viruses from wild-type vaccinia virus, the rotavirus genes were inserted into the coding region of the TK gene. Since insertion of rotavirus genes within the TK gene, destroys TK enzyme activity, recombinant viruses could be grown on TK$^-$ cells in the presence of BUdR to provide a means for their selection (Mackett et al., 1982; Smith et al., 1983). Under this condition, only TK$^-$ viruses form plaques, thereby making their selection highly efficient (Falkner and Moss, 1990). Using this strategy, the majority of the TK$^-$ plaques isolated were true recombinants, while the others (always a minority) were spontaneous TK$^-$ mutants. Since spontaneous TK$^-$ mutants could not express rotavirus proteins they could be distinguished from true recombinants by a black plaque assay or immunoperoxidase staining.

The procedure to construct recombinant baculovirus was similar to that used for recombinant vaccinia virus. Following insertion of the rotavirus gene into the transfer vector, these constructs were cotransfected with authentic wild-type AcMNPV DNA into Sf9 cells. Plaques showing no evidence of occlusion bodies were picked and titrated on Sf9 cells to obtain recombinant, polyhedrin-negative viruses. Following a second or third plaque purification, high-titer stock recombinant viruses were prepared by two consecutive passes of the recombinant viruses. Two cycles of plaque purification were usually sufficient to yield a homogeneous recombinant population. Previous studies also indicated that one plaque purification step was not sufficient to yield a uniform virus population (Mackett et al.,1984).

5.1.1 Analysis of promoter strength

A strong promoter used to produce high yields of foreign protein in

novel expression vector systems offers new ways to study viral protein function and to develop effective vaccines. Therefore, scientists have focused on isolating strong promoters compatible with virus vehicles to produce foreign proteins. Initial studies in vaccinia virus involved the use of early promoters and the 7.5 K promoter. Subsequently, gene sequences of some late promoters, such as 28 K, 11 K and ATI, have been analysed and used for expression of foreign genes (Weir and Moss, 1984; Falkner and Moss, 1988; Patel et al., 1988). The results indicated that much higher levels of expression may be achieved by the late promoters than the early promoters (Falkner and Moss, 1988; Patel et al., 1988). Recently, a synthetic late promoter, based on a detailed analysis of vaccinia late promoters, was used to produce a much higher level of foreign proteins than any of the native late promoters (Davison and Moss, 1989a, 1991). In the present study, a synthetic late promoter was designed and used in parallel with the 7.5 K promoter. This study indicates that the synthetic late promoter expresses significantly higher levels of protein than the 7.5 K promoter. However, the increased level of protein expression by the synthetic late promoter over the 7.5 K promoter is dependent on the gene being expressed. For example, the consensus late gene promoter produced > 16 times the quantity of VP6 protein when compared to that produced by the 7.5 K promoter, while the level of expression for genes 4 and 8 were 4-8 times greater for the consensus late gene promoter when compared to the 7.5 K gene promoter. Thus, both the gene and the promoter appear to influence the level of expression.

High levels of protein expression by the consensus late gene promoter allowed investigation of the *in vitro* assembly of VP6 into inner capsid particles. VP6 protein expressed by the 7.5 K gene promoter did not

assemble into virus particles *in vitro*. In contrast, VP6 expressed by the consensus late gene promoter could be assembled into viral particles quite easily. These results suggest that the concentration of protein influences its ability to interact with other molecules and assemble into virus-like particles. This conclusion is supported by the observation that the protein produced by either the consensus late gene promoter or the 7.5 K gene promoter was indistinguishable. Protein produced by both promoters had the same molecular weight, reacted with polyclonal and monoclonal antibodies in an identical manner and induced antibodies *in vivo* . Thus, the only observed difference between the protein produced by these two promoters was the quantity.

In addition to the quantity of protein produced, the kinetics of rotavirus protein synthesis will also depend on the vaccinia virus promoter chosen. If early transcriptional promoters are used, expression occurs within the first 6 hours after infection (Mackett et al., 1982 a; Weir and Moss, 1984). The 7.5 K gene promoter is both an early and late promoter, so that it leads to continuous expression for 1-2 days following infection, depending on the conditions (Mackett et al., 1982 a; Smith et al., 1983). In contrast, the consensus late gene promoter does not start to transcribe until 6 hour after infection and continuos until the infection is completed. Furthermore, if one wants to study immune responses to individual proteins *in vitro* , it may be better to use the early promoter so that assays can be concluded before extensive cytopathotogy and intracellular release of molecules occurs (i.e in cytotoxic T cell response assays).

The consensus late gene promoter contains a highly conserved TAAAT element within which transcription initiates. Information for this synthetic promoter was derived from Davison and Moss (1989a). Their

result indicated that the TAAAT motif of late promoters was essential for expression. All nucleotide substitutions of the A residues in the TAAAT element led to a drastic decrease in promoter activity, wheres certain substitutions of T residues had a lesser effect. The nucleotides surrounding the TAAAT element, upstream and downstream, also affected the level of expression. Nucleotide substitutions upstream or downstream from TAAAT element resulted in either increased or decreased promoter activity depending on optimal substitution of nucleotides. In our study, we used this information to design a strong late promoter for high-level expression of rotavirus genes in vaccinia virus.

5.1.2 Expression of rotavirus VP4 protein by recombinant vaccinia virus and baculovirus

In any attempt to develop vaccines, it is important to produce protein as close to the authentic molecule as possible. Thus, the first series of experiments were designed to determine the similarity in structure, function, antigenicity, and immunogenicity of recombinant VP4 when compared to the authentic viral protein. Previous studies using monoclonal antibodies suggested that conformational epitopes were crucial in inducing protection from infection (Shaw et al., 1986; Mackow et al., 1988; Taniguchi et al., 1988). Thus, the authenticity of the recombinant protein was considered to be extremely important. Results from immuno-precipitation and western blotting indicate that recombinant VP4 produced by rVV-VP4 or rBV-VP4 had the same molecular weight as VP4 produced by BRV (Fig. 9 and 12). Secondly, following trypsin cleavage, the recombinant protein and native protein were cleaved at the same position, therefore, same cleavage bands were obtained (Fig. 11). The recombinant

VP4 proteins also possess serological specificity similar to that of authentic VP4 as demonstrated by their ability to react with polyclonal antibodies raised against BRV VP4. Unfortunately, I did not have access to a battery of monoclonal antibodies which recognized the important VP4 epitopes. Therefore I can not definitively state that all epitopes were appropriately expressed by the recombinant protein. However, the observation that the protein reacted with polyclonal antibodies and induced antibody in mice suggested that, at least, some of the important epitopes were preserved. Thus, all evidence indicates that the expression of VP4 protein by recombinant vaccinia virus and baculovirus resulted in proteins that were similar to native VP4.

Recombinant VP4 produced by rVV-VP4 infected BSC-1 and rBV-VP4 infected Sf9 cells was stable as shown in western blotting assays (Fig. 12). However, during immunoprecipitation VP4 expressed by recombinant baculovirus appeared to be degraded as evidenced by a series of lower molecular weight proteins not observed in western blots (Fig. 12). These results suggest that Sf9 infected cells contain a proteolytic enzyme that cleave rotavirus VP4 during the process of immunoprecipitation. To test this hypothesis, VP4 protein produced by rotavirus or recombinant vaccinia virus was incubated with lysates from recombinant baculovirus infected cells (Fig. 11). The results indicated that baculovirus infected cells contain proteolytic enzymes that can degrade rotavirus VP4. This feature may therefore make it difficult to prepare a vaccine from recombinant baculovirus extracts, since in the process of purification VP4 will be degraded. In contrast, recombinant vaccinia virus produced VP4 in BSC-1 was not degraded. This observation suggests that vaccinia virus produced VP4 could be useful as a subunit vaccine for controlling rotavirus

infections. Furthermore, since vaccinia virus can replicate in many mammalian cells, it could be used as a live vaccine. Therefore, vaccinia virus appears to be better than baculovirus for a number of reasons, especially since the yields of VP4 in vaccinia virus, using the late consensus promoter, were comparable to baculovirus.

5.1.3 Expression of rotavirus VP6 by recombinant vaccinia virus and baculovirus

As described above for VP4, VP6 expressed by recombinant vaccinia virus and baculovirus also retains authenticity. Several properties of the expressed recombinant VP6 protein are noteworthy. First, VP6 is not expressed on the cell surface. This was supported by the absence of surface staining as seen in black plaque assays. These studies indicate that expression in vaccinia or baculovirus does not alter the processing and transport of VP6, since it also is not expressed on the surface of cells during rotavirus infection (Estes and Cohen, 1989). Second, VP6 was probably expressed in its native conformation. This statement is based on the fact that electron microscopic analysis revealed that recombinant VP6 assembled into single-shelled particles (Fig. 17). This assembly was spontaneous and occurred under the same conditions that permitted native VP6 assembly (Ready and Sabara, 1987). This characteristic was also described by Estes, et al., in 1987. This further indicates that particle formation is an intrinsic property of this protein, since interaction with other viral proteins or with preformed subviral structures or cores was not required to elicit particles. Furthermore, it has been reported that co-expression of VP6 and VP7 by baculovirus in Sf9 cells resulted in the production of smooth particles resembling double-shelled virus (Sabara et

al., 1991). All of these results suggest that VP6 can be expressed *in vitro* and retain most, it not all, of its biological properties.

Recently, bluetongue virus (BTV) core-like and double-shelled virus-like particles have been synthesized *in vitro* by co-infecting cells with recombinant baculovirus expressing the two major structural core proteins (VP3 and VP7) and outer capsid proteins (VP2 and VP5). Co-expression of two major core proteins (VP3 and VP7) in insect cells resulted in spontaneous formation of core-like particles of the same size, appearance, and stoichiometric arrangement of VP3 to VP7 as in authentic BTV core particles. If cells were co-infected with the VP2-VP5 recombinant, plus the VP3-VP7 recombinant, empty double shelled virus-like particles were assembled. The interaction of these proteins was not dependent on the presence of BTV double-stranded RNA or the minor core proteins. In addition, BTV non-structural proteins were not required to either assist or direct the assembly of these empty particles (French and Roy, 1990; French et al., 1990). These results clearly indicate that the process of virus assembly is spontaneous and is not directed by a series of integrated biological steps *in vivo*.

Previous studies have indicated that parvovirus and poliovirus could also be assembled into virus-like particles (Urakawa et al., 1989; Brown et al., 1991). However, there are no reports that viral proteins expressed by vaccinia virus can assemble into core- or virus-like particles. In the present studies, using a consensus late gene promoter to produce high levels of rotaviral proteins, assembly of VP6 into virus-like particles was achieved. This result indicates that the production of viral proteins in high yields from vaccinia virus offers a new way to study viral protein function and for producing vaccines. This system should prove to be better than the

baculovirus system, since insect cells decorate viral proteins somewhat differently than mammalian cells. These subtle differences may play a role in reducing immunogenicity of viral antigens or altering other subtle protein interactions.

Recently, rotavirus VP6 protein was expressed by a baculovirus vector and assembled into spheres which were used as an immunological carrier for the delivery of a number of antigens, including viral peptides and peptide hormones. In every case, the humoral immune response to the antigen was greater than that produced by more traditional carrier proteins (Redmond et al., 1991). Hence, the use of VP6 spheres as a carrier for inducing immune responses to synthetic peptides appears to be very attractive for future vaccine development. This delivery system will become even more important as crucial epitopes are identified for pathogens for which no vaccines are presently available. Therefore, large amounts of VP6 produced by recombinant viruses may be important for ensuring the development of cost-effective vaccines.

5.1.4 Expression of rotavirus VP7 by recombinant vaccinia virus and baculovirus

VP7 expressed by recombinant vaccinia virus was similar to the native form of VP7 expressed in BRV infected MA104 cells. However, VP7 expressed by recombinant baculovirus was only similar to the authentic form of VP7 with respect to the primary translation product but differed in its post-translational modifications. When tunicamycin was used to inhibit glycosylation, the VP7 protein was of the same mobility regardless of whether it was produced by baculovirus, rotavirus or vaccinia virus (Fig 21). However, in the absence of tunicamycin differences were observed.

First, the molecular weight of VP7 expressed by recombinant baculovirus was lower than authentic VP7 and VP7 expressed by recombinant vaccinia virus. Since VP7 is a glycoprotein, the differences in molecular weights were probably due to difference in glycosylation. The difference in glycosylation pattens between mammalian cells and insect cells is that mammalian cells extensively modify the core oligosaccharide in terminal glycosylation events involving the transfer of glucosamine-galactose and sialic acid residues to form complex oligosaccharides. Insect cells appear to lack galactose and sialic acid transferases (Butters and Hughes, 1981; Butters et al., 1981) and trim the oligosaccharide to a central core of mannose$_3$N-acetylglucosamine$_2$ ($Man_3GlcNAc_2$) (Hsieh and Robbins, 1984). Second, VP7 expressed by recombinant baculovirus was secreted into the infected cell medium. This is different from VP7 expressed by rotavirus and recombinant vaccinia virus. It is well known that native rotavirus VP7 is a membrane-associated protein of the ER, thus the protein is retained in the ER. Only when deletions affecting the second hydrophobic domain (mutants a.a. 42-61, 43-61,47-61) were present, was VP7 secreted into the supernatant, apparently via the normal secretory pathway, including passage through the Golgi apparatus (Poruchynsky et al., 1985; Poruchynsky and Atkinson, 1988). If a deletion of 15 amino acids (a.a. 47-61) was introduced into recombinant vaccinia virus expressing VP7, VP7 was secreted into the medium of vaccinia virus-VP7 infected cells (Andrew, M. E., et al., 1987). As expected, since the VP7 produced in vaccinia virus in this study did not contain deletions, the protein was not secreted. The mechanism which caused to be VP7 secreted into the medium of recombinant baculovirus infected cells is unclear.

In order to study whether N-linked or O-linked glycosylation of

recombinant VP7 occurred, recombinant viruses were cultured in the presence of tunicamycin. Tunicamycin, a specific inhibitor of N-linked glycosylation, has been used extensively to probe viral glycoprotein function (Leavitt et al., 1977; Garoff and Schwarz, 1978; Cash et al., 1980; Peake et al., 1982; Sabara et al., 1982). In the presence of tunicamycin (2ug/ml), glycosylation of VP7 was completely inhibited (Fig. 20 and 21), suggesting that VP7 proteins expressed by recombinant vaccinia virus and baculovirus contain N-linked oligosaccharides, since tunicamycin does not necessarily inhibit O-linked glycosylation. These studies support previous reports indicating that native VP7 also contained only N-linked oligosaccharides (Sabara et al., 1982; Petrie et al., 1983; Suzuki et al., 1984).

Although VP7 protein is glycosylated, carbohydrate decoration is not essential for protein function. Previous reports indicated that glycosylation of VP7 did not appear to play a role in the reactivity of rotavirus with antibodies and with cell surface receptors (Petrie et al., 1982; 1983). A simian rotavirus variant lacking any detectable carbohydrate residues is capable of infecting cells *in vitro*. In addition, it appears that the lack of sugar residues also did not influence glycoprotein reactivity with neutralizing antibodies. In the present study, even though glycosylation of VP7 expressed by recombinant baculovirus was different from native rotavirus and recombinant vaccinia virus, this difference did not significantly alter its immunogenicity. This was demonstrated by its ability to react with antibody produced against native VP7, as well as its ability to protect neonates from challenge with rotavirus (discussed in section 5.3).

5.2 Analysis of Immunogenicity

Rotaviruses are the single most important etiologic agents of severe

dehydrating infantile gastroenteritis in infants and young animals, and the recognition of the profound nature of this disease has focused research on disease prevention and control. At present, there is no effective vaccine which can prevent rotavirus infection. Vaccines that are presently being tested include attenuated heterologous vaccines and reassortant vaccines containing genes from different species. The experimental attenuated vaccine does induce a significant homotypic antibody response but a poor heterotypic response. At present, three candidate vaccines derived from animals, RIT4237 (bovine, serotype 6), RRV-1 (rhesus monkey, serotype 3), and WC3 (bovine, serotype 6) are being tested in the field. These trials have shown that heterotypic vaccines derived from animal rotavirus strains confer inconsistent protection (Clark et al., 1986; De Mol et al., 1986; Flores et al., 1987; Hanlon et al., 1987; Vesikari et al., 1984; 1985; 1987). Efforts to develop attenuated strains of human rotavirus have been hampered by the poor growth characteristics of human strains and difficulty in determining the pathogenic potential of the different strains (Kapikian et al., 1986). To overcome the problem of *in vitro* growth, human rotavirus has been cocultivated with less fastidious animal rotavirus strains in tissue culture to produce reassortant virus. Single gene substitutions have been prepared for human serotype 1, 2, 3, and 4 with RRV or the UK bovine strain (Midthun et al., 1985; 1986). However, these reassortants are not proving to be much more effective than heterologous vaccines. Therefore, the development of a effective vaccine is crucial if society hopes to reduce the millions of deaths annually from rotavirus diarrhea.

The development of subunit vaccines appear to be a promising way to prevent rotavirus infections. In this study, rotavirus proteins were expressed by recombinant vaccinia virus and baculovirus to provide a safe

and efficient means of generating immunogens for the development of a subunit vaccine. First, the antigenicity and immunogenicity of recombinant rotavirus proteins were examined by immunizing mice with recombinant vaccinia virus or baculovirus produced proteins mixed with adjuvant. Rotavirus specific Ab responses were detectable after two immunizations. The results demonstrate that recombinant-expressed rotavirus proteins can elicit a rotavirus specific immune response in mice and the antigenicity of rotavirus proteins is retained by recombinant proteins and does not seem to be dependent on the presence of rotavirus infection. These studies provide a basis for development of a subunit or a virus vector vaccine.

Once it was demonstrated that mice could recognize the recombinant rotavirus proteins, studies were designed to see whether these proteins could be of value as a vaccine. To achieve this goal a murine challenge model was used to study protection of neonates following rotavirus infection. Since neonates are often exposed to enteric viruses before they have sufficient time to develop active immunity, they are highly susceptible to infection unless passively immunized before exposure to these pathogens. For these reasons, the mouse is a useful model to examine the serologic and protective ability of a vaccine.

In the protection-challenge experiments, the mouse model was used primarily for economical reasons. Neonates were challenged at 7 days of age, the time when mouse enterocytes have the highest number of rotavirus specific receptors, thereby making them most susceptible to infection (Riepenhoff-Talty et al., 1983). Moreover, a previous report by Ijaz et al., (1987) demonstrated that the highest titer of anti-rotavirus neutralizing antibodies were present at 7 to 9 days postparturition in mice. Therefore,

the 7 day old neonates were used as my challenge model.

In this study, neonates from representative groups were challenged to determine whether they could be protected or not. The results indicate that protection from rotavirus diarrhea occurred in neonates nursed by dams immunized with rVV-VP7, rVV-VP(4+6+7), rBV-VP7 and rBV-VP (4+6+7), while protection was not conferred in dams immunized with rBV-VP6. These observations confirm previous reports regarding humoral immunity induced by recombinant rotavirus proteins (Carlos et al., 1986; Andrew et al., 1987; Francavilla et al., 1987; McCrae and McCorquodale., 1987). Antibodies induced by recombinant vaccinia virus expressing the SA11 VP7 gene was able to recognize and neutralize SA11 (Andrew et al., 1987). VP7 synthesized in *Escherichia coli* also induce neutralizing antibodies (Carlos et al., 1986; Francavilla et al., 1987; McCrae and McCorquodale., 1987). In spite of some conflicting results indicating that VP6 induces neutralizing antibodies (Sonza et al., 1984), our results in the protection-challenge experiments, as well as those of others, regardless of whether recombinant vaccinia virus (Poncet et al., 1990) or baculovirus expressing VP6 (Estes et al., 1987), were used, indicated that no virus neutralizing activity could be induced by VP6. VP4 was also previously shown (Estes and Cohen, 1989) to be a very effective immunogen, by inducing neutralizing antibodies. Due to technical problems we could not confirm or refute these previous observations.

Local immunity has been shown to play a major role in protection from rotavirus diarrhea in many species (Snodgrass et al., 1982; Offit and Clark, 1985a; McNulty and Logan, 1987). In contrast, the presence of high titers of passively acquired rotavirus neutralizing antibodies in the sera of suckling mice was not capable of protecting neonates against rotavirus

induced gastroenteritis (Offit and Clark, 1985). Hence, passively acquired antibodies from colostrum and milk in the neonatal gut are very important in preventing rotavirus diarrhea. Local immunity against rotavirus can perform two functions. First, the presence of antibody in the intestine will protect the neonate from disease by neutralizing the virus. Since protection is mediated by neutralizing virus infectivity, the degree of protection is dependent on the concentration of anti-rotavirus antibodies in the intestine. Secondly, since the virus infectivity is neutralized by these antibodies, it results in the reduction of infectious rotavirus in feces, therefore, reducing infection of cohorts (Saif and Smith, 1983).

To demonstrate that protection of mice occurred as a result of antibodies in the milk, the levels of lacteal antibody were determined. The results demonstrate that there were significant antibodies against rotavirus in the milk. These results confirm passive transfer of protective colostral antibody from an immunized dam to suckling mice. This passive Ab transfer provides effective protection of neonates from challenge with rotavirus. Hence, immunization of dams is an effective method for providing protection from rotavirus diarrhea. Indeed this is the method presently used to protect animals from rotavirus infection (Estes and Cohen, 1989). The present study showing that recombinant protein can induce neutralizing Ab and protect neonates from infection, suggests that a subunit vaccine is a promising way to protect neonates from rotavirus diarrhea especially in species such as cattle and pigs where the majority of antibodies acquired by the neonate is via the milk.

One goal of these studies was to develop a subunit vaccine by first understanding the structural proteins of rotavirus at the molecular level. Both the vaccinia virus and baculovirus expression systems yielded

rotaviral proteins which effectively induced immune responses in mice. Each of these two systems has advantages for use in the development of a subunit vaccine suitable for humans and animals. Recombinant vaccinia virus also offers the advantages of a live virus vaccine without the danger of reversion to the virulent form of the virus. Unlike vaccinia virus, baculovirus cannot infect mammalian cells, so a vaccine developed from recombinant baculovirus offers the advantages of a traditional inactivated vaccine, without the potential risk of incomplete inactivation. Furthermore, production of such a vaccine could be accomplished safely under minimal containment conditions. Therefore, these two systems can be used in the development of different vaccine types depending on specific purposes.

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