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Nucleotide Sequence of Bovine Rotavirus Gene 1 and Expression of the Gene Product in Baculovirus

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The nucleotide sequence of the gene that encodes for the structural viral protein VP1 of bovine rotavirus (RF strain) has been determined. The sequence data indicate that segment 1 contains 3302 bp and is A+T rich (65.3%). The positive strand of segment 1 contains a single open reading frame that extends 1088 codons and possesses 5′- and 3′-terminal untranslated regions of 18 and 20 bp, respectively. The first AUG conforms to the Kozak consensus sequence and if utilized, would yield a protein having a calculated molecular weight of 124,847, very close to the apparent molecular weight of VP1 (M.W. 125,000). The deduced amino acid sequence presents significant similarities with RNA-dependent RNA polymerase of several RNA viruses. VP1 was also synthesized in baculovirus using two transfer vecors: pAC461 and pVL941. Following infection of Sf9 cells with a recombinant baculovirus, a full-length nonfusion protein was synthesised which shares properties with authentic VP1 made in monkey kidney cells. The level of VP1 synthesis was about 10-fold higher when the baculovirus recombinant was derived from the pVL941 transfer vector. In that case, VP1 was expressed in yields approximately equivalent to 10% of the cellular protein. The recombinant protein was immunoprecipitated by hyperimmune serum raised against purified rotavirus. It also was immunogenic; a hyperimmune serum made in guinea pigs reacted with VP1 using immunoprecipitation and Western blot. This serum did not possess neutralization activity.

INTRODUCTION

Rotaviruses, members of a genus of the Reoviridae family, possess a genome containing 11 segments of double-stranded RNA. The 11 segments of genomic RNA code for structural proteins found in viral particles (VP1-8) and for nonstructural (NS53, NS35, NS34, and NS28) proteins found only in infected cells. Nucleotide sequences have been reported (for one or another rotavirus strain) for the RNA segments 4 to 11 (Both *et al.*, 1982, 1983a,b, 1984; Imai *et al.*, 1983; Estes *et al.*, 1984; Kantharidis *et al.*, 1987; Bremont *et al.*, 1987). In this work molecular characterization of the bovine rotavirus RF strain has been continued and we report the complete nucleotide sequence of gene 1 of bovine rotavirus (RF strain) that codes for the internal structural protein VP1.

Three types of rotaviral particles have been described. Complete infectious particles possess a double capsid. Removal of the outer proteins produces a single-shelled capsid that contains four proteins: VP1 (125K), VP2 (90K), VP3 (88K), and VP6 (41K). Treatment of these particles with chaotropic agents removes the major protein VP6 of the single-shelled particles and produces core particles (Bican *et al.*, 1982). Three polypeptides are associated with cores (Liu *et al.*, 1988). VP2 is the major component of cores and has been shown to bind RNA (Boyle and Holmes, 1986). VP1 represents only 2% of the viral protein moiety of the complete virion and probably does not act as a scaffolding protein. As a minor component of single-shelled particles, VP1 could function as part of the transcriptase present in activated particles. VP1 with VP2, VP6, and two nonstructural proteins is also a component of subviral particles containing replicase activity (Patton and Gallegos, 1988). Temperature-sensitive mutants which map to genome segment 1 have RNA-negative phenotypes (Gombold and Ramig, 1987). These observations support a putative enzymatic role for VP1.

No information on the gene 1 structure and its protein product is available to date and we report here its nucleotide sequence. We also inserted a full-length gene 1 cDNA into the genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) adjacent to the strong polyhedrin promoter. In this construct, VP1 was expressed efficiently, and immunologic analysis indicated that the protein possessed native antigenic determinants and was immunogenic. The availability of large amounts of VP1 will help determine intrinsic properties of this protein in the rotavirus replication process.

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Sequence Data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. J04346.

0042-6822/89 $3.00

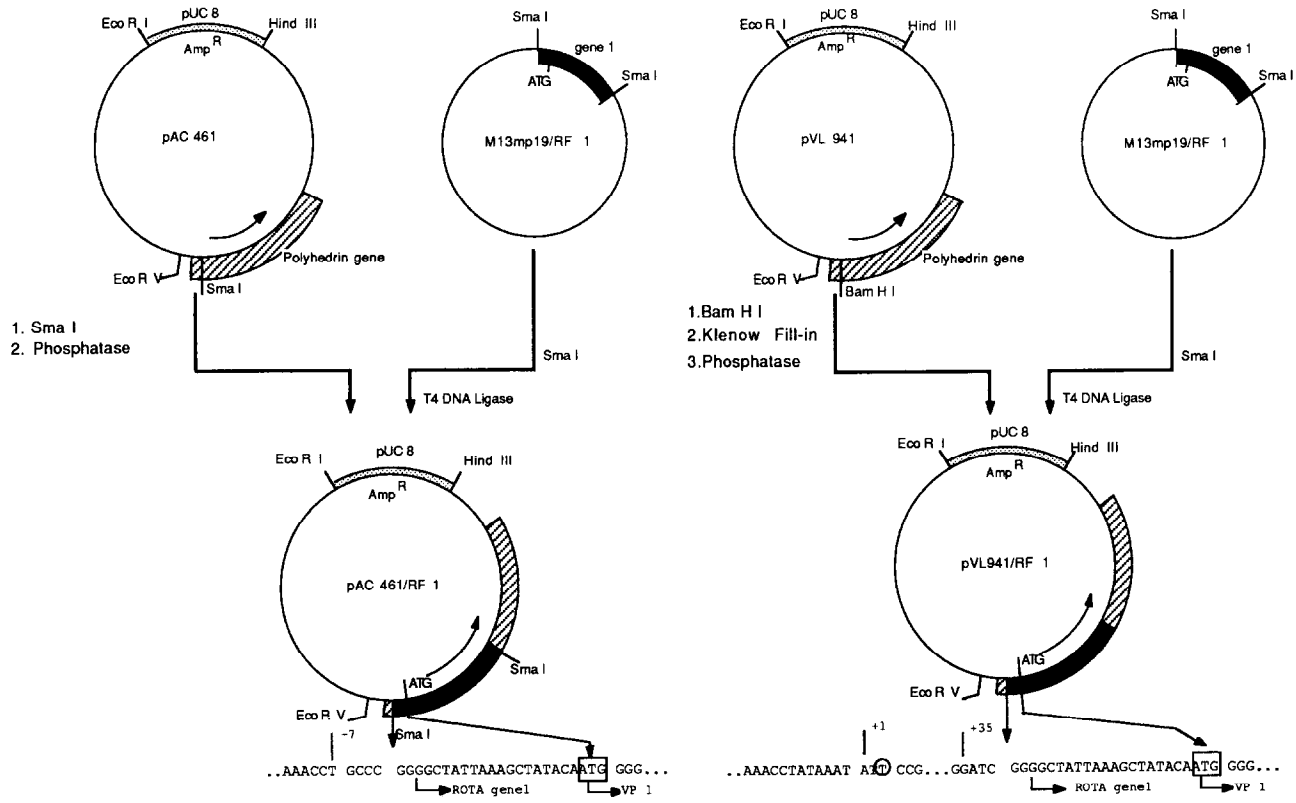

FIG. 1. Construction of baculovirus expression vectors for rotavirus gene 1. The full-length rotavirus gene 1 cDNA clone M13mp19/RF 1 was digested with *Sma*I and cloned either in the *Sma*I site of pAC461 or in the filled in *Bam*HI site of pVL941 by blunt-end ligation. The resultants vectors, pAC461/RF1 and pVL941/RF1, contain the gene 1 coding region fused to the polyhedrin sequence, respectively, at position −7 and +34. The mutated initiation codon of the polyhedrin gene in pVL941 is circled. (■) Rotavirus gene 1 sequence; (□) pUC 8 sequence.

MATERIALS AND METHODS

Viruses and cells

The RF strain of bovine rotavirus was propagated in MA 104 cells, as described previously (L'Haridon and Scherrer, 1976). AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of *Spodoptera frugiperda* cells in Hanks' medium containing 10% fetal bovine serum (FBS) according to the procedures described by Summers and Smith (1987) (Estes *et al.*, 1987).

Synthesis and cloning of cDNA

Genomic dsRNAs were isolated from purified viruses by extraction with phenol and precipitation with ethanol. For cDNA synthesis the genomic RNA was polyadenylated *in vitro* at its 3′-end and first-strand synthesis with reverse transcription was primed with oligo $(dT)_{12-18}$. Cloning in pBR322 has been described previously (Cohen *et al.*, 1984).

DNA manipulations and sequencing

Plasmid DNA manipulations were carried out essentially as summarized by Maniatis *et al.* (1982). Restriction enzymes were purchased from Biolabs (Beverly, MA). T4 DNA ligase, Klenow large fragment of DNA polymerase, and calf intestinal phosphatase were obtained from Boehringer-Mannheim (FRG). The nucleotide sequence was determined using the dideoxy chain termination method of Sanger *et al.* (1977) and the shotgun strategy after subcloning random fragments in M13 phage (Gardner *et al.*, 1981). Three overlapping (partial) clones were sequenced. Each base was read with an average of six independent M13 mp19 subclones. The program "Microgenie" was employed to analyze the sequence data generated by the shotgun cloning strategy (Beckman, France).

Construction of a full-length cDNA clone

Instead of reconstructing a full-length clone from the three partial clones used for sequencing, we obtained a full-length clone by a different strategy: Two oligonucleotides (41-mer and 30-mer) corresponding respectively to 5′- and 3′-end sequences plus the cohesive sequence of *Xma*I restriction endonuclease were synthetized using a "Biosearch 8700" synthetizer. These unphosphorylated oligonucleotides were used to prime the synthesis of cDNA on the plus and the minus

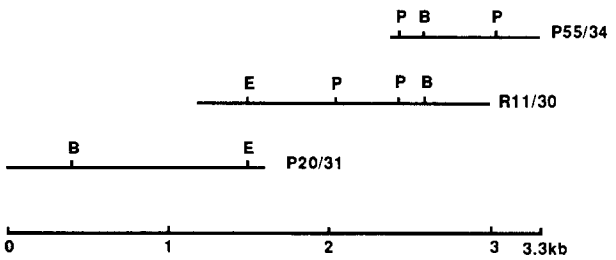

FIG. 2. Diagram showing the positions of the cDNA clones used in obtaining the nucleotide sequence. Positions of some restrictions sites used to orientate the clones are shown. B, *Bam*HI; E, *Eco*RI; P, *Pvu*II.

strands and also to allow ligation in the unique *Xma*I site of M13 mp19 of the reannealed double-stranded cDNA. The first five clear plaques obained appeared to be full-length as the *Sma*I excised insert was about 3300 bp long. One of these clones M13 mp19/RF1A was partially sequenced to verify that the ends of the gene were complete.

Construction of baculovirus recombinants containing bovine rotavirus gene 1

The full-length clone M13 mp19/RF1A was digested with *Sma*I and subcloned either into the *Sma*I site of the baculovirus transfer vector pAC461 or in the Klenow filled-in *Bam*HI site of the transfer vector pVL941 (Fig. 1). After transfection into *Escherichia coli* (strain DH5α), ampicillin-resistant colonies were screened for correct orientation of the gene 1 insert by restriction analysis (digestion by *Eco*RV and *Bgl*II). Baculovirus recombinants were obtained by cotransfecting *S. frugiperda* cells with transfer vector and wild-type AcNPV DNA using the *in situ* phosphate calcium precipitation procedure: approximately 2 μg of recombinant transfer vector and 4 μg of wild-type viral DNA in 750 μl of transfection buffer (25 m*M* HEPES, pH 7.1; 140 m*M* NaCl; 125 m*M* $CaCl_2$) were added to 25-cm^2 flasks seeded with 2.5×10^6 *S. frugiperda* cells and containing 750 μl of Grace's medium supplemented with 10% FBS. Following incubation at 27° for 4 hr, the medium was replaced by 5 ml of fresh Hinks' medium containing 10% FBS, and incubation was continued for 5 days. Thereafter extracellular virus was harvested and titrated by limiting dilution in 96-well microtiter plates containing *S. frugiperda* cell monolayers. The supernatant of wells of the highest dilution of sample found to be positive in a dot blot assay using a gene 1 ^{32}P-labeled probe was titrated again by limiting dilution and the supernatant was plaqued on a monolayer of *S. frugiperda* cells. Virus in polyhedrin-negative plaques was plaque-purified three times and used to propagate virus stocks. For each transfer vector, three independent recombinant viruses designated pAC461/RF1.1 to pAC461/RF1.3 and pVL941/RF1.1 to pVL941/RF1.3 were prepared.

Protein analysis and immunoprecipitation

S. frugiperda cells in 25-cm^2 flasks were infected at a high multiplicity (≥5 PFU/cell) with wild-type AcNPV or with recombinant virus and labeled with [^{35}S]methionine (15 μCi/ml, 1200 Ci/mmol; Amersham) for 2 hr at the indicated time using Grace's medium. Prior to labeling, the cells were incubated for 30 min with Grace's medium. After the labeling period, the medium was removed and the cells were pelleted at 1600 *g* at 4° for 10 min. For analysis by polyacrylamide gel electrophoresis, the cells were lysed by boiling for 10 min in dissociation buffer (2% SDS, 0.5 *M* urea, 10% glycerol, 10% 2-mercaptoethanol, 62.5 m*M* Tris–HCl, 0.01% bromphenol blue, pH 6.8). For immunoprecipitation analysis, the cells were lysed in RIPA buffer (150 m*M* NaCl, 20 m*M* Tris–HCl (pH 7.4), 10 m*M* EDTA, 1% aprotinin, 1% Triton X-100, 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate) and sonicated for 10 sec (Ericson *et al.*, 1983). The sonicate was centrifuged at 13,000 *g* for 10 min. A cytosol fraction of rotavirus-infected Ma104 cells was prepared similarly except that centrifugation was at 100,000 *g* for 1 hr. For immunoprecipitation, purified rabbit anti-rotavirus IgG (raised against cesium chloride-purified bovine rotavirus) were diluted in RIPA buffer and mixed with 100 μl of [^{35}S]methionine-labeled cell extract. Samples were incubated for 1 hr at 37°, protein A–Sepharose (Pharmacia; 80 μl of a 15% suspension in 50 m*M* Tris–HCl (pH 7.5), 10 m*M* EDTA, 0.5 *M* NaCl) was added, and mixing was continued for 1 hr at room temperature. The Sepharose beads were recovered by centrifugation, washed three times with 1 ml of RIPA buffer and once with 10 m*M* Tris–HCl (pH 7.5), and finally boiled for 5 min in 30 μl of SDS electrophoresis dissociation buffer before analysis on SDS–polyacrylamide gels.

Production of antiserum to recombinant VP1

Guinea pigs shown to lack rotavirus antibodies were used for the production of antiserum to recombinant VP1 protein. Each guinea pig was inoculated intramuscularly twice at 2-week intervals as previously described (Estes *et al.*, 1979). The antigen used was a cytosol fraction of infected Sf9 cells harvested 48 hr postinfection. Cells were washed, resuspended in PBS containing 1% NP-40, sonicated, and clarified and the supernatant was mixed with adjuvant for immunization. The guinea pigs were bled 10 days after the third injection.

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5'GGCTATTAAAGCTATACA  ATG GGG AAG TAT AAT CTA ATC TTG TCA GAA TAT TTA TCA    57
                      Met Gly Lys Tyr Asn Leu Ile Leu Ser Glu Tyr Leu Ser  13

 TTT ATA TAT AAC TCA CAA TCC GCA GTT CAA ATT CCA ATA TAC TAT TCT TCC AAT   111
 Phe Ile Tyr Asn Ser Gln Ser Ala Val Gln Ile Pro Ile Tyr Tyr Ser Ser Asn  31

 AGT GAA TTA GAG AAT AGA TGT ATT GAA TTT CAT TCT AAA TGC TTA GAA AAC TCA   165
 Ser Glu Leu Glu Asn Arg Cys Ile Glu Phe His Ser Lys Cys Leu Glu Asn Ser  49

 AAG AAT GGA CTA TCA TTG AAA AAG CTC TTT GTT GAA TAT AGC GAT GTT ATG GAG   219
 Lys Asn Gly Leu Ser Leu Lys Lys Leu Phe Val Glu Tyr Ser Asp Val Met Glu  67

 AAT GCC ACA CTG TTG TCA ATA TTA TCG TAC TCT TAT GAT AAA TAT AAC GCT GTT   273
 Asn Ala Thr Leu Leu Ser Ile Leu Ser Tyr Ser Tyr Asp Lys Tyr Asn Ala Val  85

 GAA AGG AAA TTA GTA AAA TAT GCA AAA GGT AAG CCG CTA GAA GCA GAT TTG ACA   327
 Glu Arg Lys Leu Val Lys Tyr Ala Lys Gly Lys Pro Leu Glu Ala Asp Leu Thr  103

 GTG AAT GAG TTG GAT TAT GAA AAT AAC AAG ATA ACA TCT GAA CTT TTC CCA ACA   381
 Val Asn Glu Leu Asp Tyr Glu Asn Asn Lys Ile Thr Ser Glu Leu Phe Pro Thr  121

 GCA GAG GAA TAT ACT GAT TCA TTG ATG GAT CCA GCA ATT TTA ACT TCA TTA TCA   435
 Ala Glu Glu Tyr Thr Asp Ser Leu Met Asp Pro Ala Ile Leu Thr Ser Leu Ser  139

 TCA AAT TTA AAT GCA GTT ATG TTC TGG TTG GAA AAA CAT GAA AAT GAC GTT GCT   489
 Ser Asn Leu Asn Ala Val Met Phe Trp Leu Glu Lys His Glu Asn Asp Val Ala  157

 GAA AAA CTC AAA ATT TAC AAA AGG AGA TTA GAC TTA TTC ACT ATA GTA GCT TCA   543
 Glu Lys Leu Lys Ile Tyr Lys Arg Arg Leu Asp Leu Phe Thr Ile Val Ala Ser  175

 ACA GTA AAT AAA TAT GGT GTA CCA AGG CAC AAC GCG AAA TAT AGA TAT GAA TAT   597
 Thr Val Asn Lys Tyr Gly Val Pro Arg His Asn Ala Lys Tyr Arg Tyr Glu Tyr  193

 GAA GTA ATG AAA GAT AAG CCG TAC TAC TTG GTA ACA TGG GCA AAT TCT TCA ATT   651
 Glu Val Met Lys Asp Lys Pro Tyr Tyr Leu Val Thr Trp Ala Asn Ser Ser Ile  211

 GAA ATG CTG ATG TCA GTT TTT TCT CAT GAA GAT TAT TTA ATT GCG AGA GAA CTG   705
 Glu Met Leu Met Ser Val Phe Ser His Glu Asp Tyr Leu Ile Ala Arg Glu Leu  229

 ATA GTA CTG TCA TAT TCT AAT AGA TCG ACT CTG GCA AAA CTG GTG TCA TCA CCA   759
 Ile Val Leu Ser Tyr Ser Asn Arg Ser Thr Leu Ala Lys Leu Val Ser Ser Pro  247

 ATG TCA ATT CTG GTA GCT TTA GTG GAT ATA AAC GGA ACA TTT ATT ACG AAT GAA   813
 Met Ser Ile Leu Val Ala Leu Val Asp Ile Asn Gly Thr Phe Ile Thr Asn Glu  265

 GAA TTA GAA CTA GAG TTT TCA AAC AAA TAT GTA CGA GCA ATA GTT CCT GAC CAA   867
 Glu Leu Glu Leu Glu Phe Ser Asn Lys Tyr Val Arg Ala Ile Val Pro Asp Gln  283

 ACA TTT GAT GAA TTA AAA CAA ATG CTT GAC AAT ATG AGA AAA GCT GGG TTA ACT   921
 Thr Phe Asp Glu Leu Lys Gln Met Leu Asp Asn Met Arg Lys Ala Gly Leu Thr  301

 GAC ATA CCT AAG ATG ATA CAG GAC TGG TTG GTC GAT TGC TCT ATT GAA AAA TTT   975
 Asp Ile Pro Lys Met Ile Gln Asp Trp Leu Val Asp Cys Ser Ile Glu Lys Phe  319

 CCA TTG ATG GCT AAA ATA TAT TCG TGG TCA TTT CAC GTC GGA TTC AGG AAA CAG  1029
 Pro Leu Met Ala Lys Ile Tyr Ser Trp Ser Phe His Val Gly Phe Arg Lys Gln  337

 AAA ATG TTG GAC GCC GCA CTA GAT CAA TTG AAA ACT GAG TAT ACA GAA GAT GTA  1083
 Lys Met Leu Asp Ala Ala Leu Asp Gln Leu Lys Thr Glu Tyr Thr Glu Asp Val  355

 GAT GAC GAA ATG TAT CGA GAA TAC ACA ATG CTA ATA AGA GAT GAA GTT GTG AAA  1137
 Asp Asp Glu Met Tyr Arg Glu Tyr Thr Met Leu Ile Arg Asp Glu Val Val Lys  373

 ATG CTT GAG GAA CCA GTA AAG CAT GAT GAC CAT TTG TTA CAG GAT TCT GAA TTG  1191
 Met Leu Glu Glu Pro Val Lys His Asp Asp His Leu Leu Gln Asp Ser Glu Leu  391
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FIG. 3. Sequence of RF bovine rotavirus gene segment 1. The sequence is that of the plus strand (mRNA sense). The predicted amino acid is shown below the gene sequence. Possible glycosylation sites are underlined.

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GCT GGT TTA CTA TCA ATG TCA TCA GCG TCG AAT GGT GAA TCA AGA CAA CTA AAA  1245
Ala Gly Leu Leu Ser Met Ser Ser Ala Ser Asn Gly Glu Ser Arg Gln Leu Lys  409

TTT GGT AGA AAG ACA ATT TTT TCG ACT AAA AAG AAT ATG CAT GTA ATG GAT GAC  1299
Phe Gly Arg Lys Thr Ile Phe Ser Thr Lys Lys Asn Met His Val Met Asp Asp  427

ATG GCT AAT GGA AGA TAC ACA CCA GGC ATA ATA CCA CCA GTG AAT GTC GAT AAA  1353
Met Ala Asn Gly Arg Tyr Thr Pro Gly Ile Ile Pro Pro Val Asn Val Asp Lys  445

CCG ATA CCA TTA GGA AGG AGA GAT GTA CCA GGA AGA CGG ACT AGA ATA ATA TTT  1407
Pro Ile Pro Leu Gly Arg Arg Asp Val Pro Gly Arg Arg Thr Arg Ile Ile Phe  463

ATC TTA CCA TAT GAA TAT TTC ATA GCA CAA CAT GCT GTA GTT GAA AAA ATG CTA  1461
Ile Leu Pro Tyr Glu Tyr Phe Ile Ala Gln His Ala Val Val Glu Lys Met Leu  481

ATT TAC GCG AAA CAT ACT AGA GAA TAT GCT GAA TTC TAC TCA CAG TCA AAT CAG  1515
Ile Tyr Ala Lys His Thr Arg Glu Tyr Ala Glu Phe Tyr Ser Gln Ser Asn Gln  499

TTA TTG TCT TAT GGC GAT GTT ACA CGC TTT TTA TCT AAT AAC TCT ATG GTA CTA  1569
Leu Leu Ser Tyr Gly Asp Val Thr Arg Phe Leu Ser Asn Asn Ser Met Val Leu  517

TAT ACA GAC GTG TCC CAG TGG GAC TCA TCT CAA CAC AAT ACG CAG CCA TTT AGG  1623
Tyr Thr Asp Val Ser Gln Trp Asp Ser Ser Gln His Asn Thr Gln Pro Phe Arg  535

AAA GGG ATA ATT ATG GGA TTG GAC ATG CTA GCC AAT ATG ACT AAT GAT GCT AGA  1677
Lys Gly Ile Ile Met Gly Leu Asp Met Leu Ala Asn Met Thr Asn Asp Ala Arg  553

GTT ATC CAG ACG CTG AAC TTA TAT AAA CAG ACG CAA ATT AAT CTA ATG GAT TCA  1731
Val Ile Gln Thr Leu Asn Leu Tyr Lys Gln Thr Gln Ile Asn Leu Met Asp Ser  571

TAC GTT CAA ATA CCA GAT GGT AAT GTT ATT AAG AAG ATA CAA TAT GGG GCT GTA  1785
Tyr Val Gln Ile Pro Asp Gly Asn Val Ile Lys Lys Ile Gln Tyr Gly Ala Val  589

GCG TCA GGA GAG AAG CAG ACG AAA GCA GCG AAT TCA ATA GCA AAT TTA GCA CTG  1839
Ala Ser Gly Glu Lys Gln Thr Lys Ala Ala Asn Ser Ile Ala Asn Leu Ala Leu  607

ATT AAA ACG GTT TTA TCA CGC ATT TCT AAC AAA TAT TCA TTC GCG ACG AAG ATA  1893
Ile Lys Thr Val Leu Ser Arg Ile Ser Asn Lys Tyr Ser Phe Ala Thr Lys Ile  625

ATA AGA GTT GAC GGA GAT GAC AAT TAC GCA GTA TTG CAG TTC AAT ACA GAA GTA  1947
Ile Arg Val Asp Gly Asp Asp Asn Tyr Ala Val Leu Gln Phe Asn Thr Glu Val  643

ACT GAA CAA ATG GTT CAA GAT GTA TCA AAC GAC GTG AGA GAA ACA TAT GCG CGA  2001
Thr Glu Gln Met Val Gln Asp Val Ser Asn Asp Val Arg Glu Thr Tyr Ala Arg  661

ATG AAT GCT AAA GTT AAA GCC TTA GTA TCT ACA GTG GGA ATA GAA ATA GCT AAA  2055
Met Asn Ala Lys Val Lys Ala Leu Val Ser Thr Val Gly Ile Glu Ile Ala Lys  679

AGG TAT ATT GCA GGT GGG AAA ATA TTC TTT AGG GCT GGA ATA AAT TTA CTG AAT  2109
Arg Tyr Ile Ala Gly Gly Lys Ile Phe Phe Arg Ala Gly Ile Asn Leu Leu Asn  697

AAC GAG AAA AGA GGA CAA AGT ACA CAG TGG GAC CAA GCA GCT GTC CTA TAT TCG  2163
Asn Glu Lys Arg Gly Gln Ser Thr Gln Trp Asp Gln Ala Ala Val Leu Tyr Ser  715

AAC TAT ATT GTG AAT AGA CTT CGA GGA TTT GAA ACT GAC AGA GAG TTC ATT TTA  2217
Asn Tyr Ile Val Asn Arg Leu Arg Gly Phe Glu Thr Asp Arg Glu Phe Ile Leu  733

ACT AAA ATA ATG CAA ATG ACG TCA GTT GCT ATT ACC GGA TCG CTA AGA CTC TTT  2271
Thr Lys Ile Met Gln Met Thr Ser Val Ala Ile Thr Gly Ser Leu Arg Leu Phe  751

CCT TCT GAA CGC GTG TTA ACC ACG AAC TCT ACG TTT AAA GTA TTT GAC TCG GAG  2325
Pro Ser Glu Arg Val Leu Thr Thr Asn Ser Thr Phe Lys Val Phe Asp Ser Glu  769

GAC TTT ATT ATA GAG TAT GGG ACA ACT GAC GAC GAA GTA TAC ATA CAA AGA GCG  2379
Asp Phe Ile Ile Glu Tyr Gly Thr Thr Asp Asp Glu Val Tyr Ile Gln Arg Ala  787
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FIG. 3.—*Continued*

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TTC ATG TCT TTA TCT AGT CAG AAG TCA GGA ATA GCT GAT GAG ATA GCT GCA TCA  2433
Phe Met Ser Leu Ser Ser Gln Lys Ser Gly Ile Ala Asp Glu Ile Ala Ala Ser  805

TCA ACG TTT AAG AAT TAT GTG TCT AGA TTA TCT GGG CAG CTG TTG TTT TCA AAG  2487
Ser Thr Phe Lys Asn Tyr Val Ser Arg Leu Ser Gly Gln Leu Leu Phe Ser Lys  823

AAT AAT ATA GTG TCT AGA GGA ATA GCA TTG ACT GAA AAG GCA AAG TTG AAC TCA  2541
Asn Asn Ile Val Ser Arg Gly Ile Ala Leu Thr Glu Lys Ala Lys Leu Asn Ser  841

TAC GCA CCA ATA TCA CTT GAG AAA AGA CGT GCG CAA ATA TCA GCT TTG CTG ACT  2595
Tyr Ala Pro Ile Ser Leu Glu Lys Arg Arg Ala Gln Ile Ser Ala Leu Leu Thr  859

ATG CTG CAA AAA CCG GTT ACT TTT AAA TCA AGT AAA ATA ACA ATA AAT GAT ATA  2649
Met Leu Gln Lys Pro Val Thr Phe Lys Ser Ser Lys Ile Thr Ile Asn Asp Ile  877

CTT AGA GAT ATA AAG CCA TTT TTC ACT GTA AAC GAA GCA CAT TTA CCG ATA CAA  2703
Leu Arg Asp Ile Lys Pro Phe Phe Thr Val Asn Glu Ala His Leu Pro Ile Gln  895

TAT CAA AAA TTT ATG CCA ACT TTA CCA GAC AAT GTG CAG TAT ATA ATT CAG TGT  2757
Tyr Gln Lys Phe Met Pro Thr Leu Pro Asp Asn Val Gln Tyr Ile Ile Gln Cys  913

ATA GGA TCC AGA ACC TAC CAA ATT GAA GAT GAC GGT TCA AAG TCA GCT ATA TCT  2811
Ile Gly Ser Arg Thr Tyr Gln Ile Glu Asp Asp Gly Ser Lys Ser Ala Ile Ser  931

CGA CTA ATA TCA AAG TAT TCA GTT TAC AAG CCG TCA ATC GAA GAG TTA TAC AAA  2865
Arg Leu Ile Ser Lys Tyr Ser Val Tyr Lys Pro Ser Ile Glu Glu Leu Tyr Lys  949

GTA ATT TCA CTA CAC GAG AAT GAA ATA CAA CTA TAT TTG ATC TCA CTA GGT ATA  2919
Val Ile Ser Leu His Glu Asn Glu Ile Gln Leu Tyr Leu Ile Ser Leu Gly Ile  967

CCG AAA ATA GAC GCT GAT ACG TAC GTC GGA TCG AAA ATT TAT TCT CAA GAT AAA  2973
Pro Lys Ile Asp Ala Asp Thr Tyr Val Gly Ser Lys Ile Tyr Ser Gln Asp Lys  985

TAC AGG ATA TTA GAG TCG TAT GTA TGT AAC TTA TTA TCT ATT AAT TAT GGA TGT  3027
Tyr Arg Ile Leu Glu Ser Tyr Val Cys Asn Leu Leu Ser Ile Asn Tyr Gly Cys 1003

TAT CAA CTA TTC GAC TTT AAT TCA CCA GAT CTA GAA AAA CTG ATC AGA ATA CCG  3081
Tyr Gln Leu Phe Asp Phe Asn Ser Pro Asp Leu Glu Lys Leu Ile Arg Ile Pro 1021

TTT AAA GGA AAA ATA CCA GCT GTC ACT TTT ATA TTG CAT TTA TAC GCT AAG CTA  3135
Phe Lys Gly Lys Ile Pro Ala Val Thr Phe Ile Leu His Leu Tyr Ala Lys Leu 1039

GAA GTT ATA AAT CAT GCC ATC AAA AAT GGC TCA TGG ATA AGT TTA TTC TGT AAC  3189
Glu Val Ile Asn His Ala Ile Lys Asn Gly Ser Trp Ile Ser Leu Phe Cys Asn 1057

TAC CCA AAA TCA GAA ATG ATA AAA TTA TGG AAG AAA ATG TGG AAC ATT ACA TCA  3243
Tyr Pro Lys Ser Glu Met Ile Lys Leu Trp Lys Lys Met Trp Asn Ile Thr Ser 1075

CTA CGT TCA CCG TAT ACC AAT GCA AAC TTC TTT CAA GAT    TAGAGCGCTTAGATGTG 3299
Leu Arg Ser Pro Tyr Thr Asn Ala Asn Phe Phe Gln Asp  1088

ACC                                                                    3302
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FIG. 3.—*Continued*

Electron microscopy

S. frugiperda cells infected at various times with pVL941/RF1.1 were detached from the flask by pipetting, pelleted at low speed, and fixed for 1 hr with 1.6% glutaraldehyde. The cell pellet was washed three times in cacodylate buffer (pH 7.4), then postfixed in 1% osmium tetroxide and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Nucleotide sequence of genomic segment 1

Preliminary sizing of several recombinant plasmids that hybridized with segment 1 in Northern blots demonstrated that none of them could be a full-length clone. However a set of three clones, P20/31, R11/30, and P55/34, that spanned the entire sequence of the gene 1 was identified and used to determined the nu-

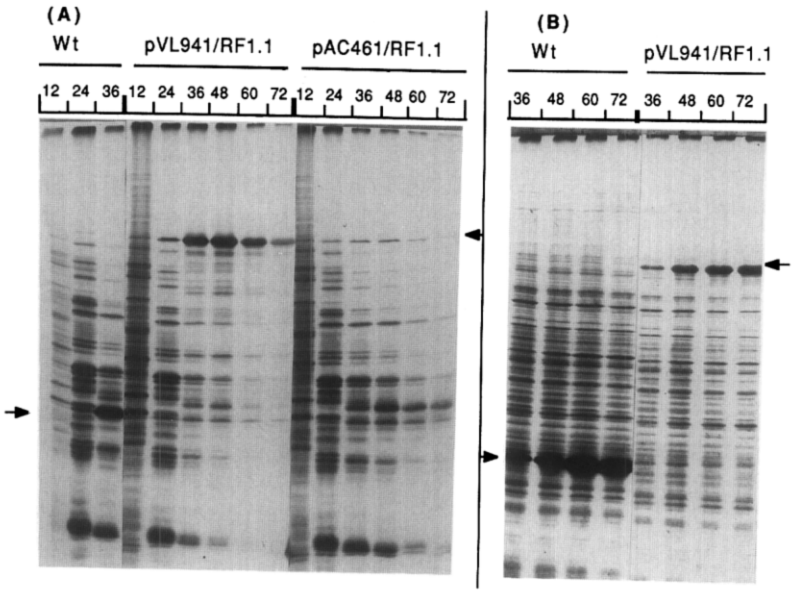

FIG. 4. Expression VP1 in recombinant baculoviruses. *Spodoptera frugiperda* cells were infected with recombinant viruses containing the VP1 gene and derived either from pAC461 or pVL941 transfer vectors. Cells were also infected with wild-type AcNPV (Wt). Proteins were labeled at indicated times (in hours) postinfection for 2 hr with [^{35}S]methionine and analyzed by electrophoresis on a 12% polyacrylamide gel. The gel was either fluorographed (A) or stained with Coomassie blue (B) to demonstrate the accumulation of the expressed proteins. Polyhedrin and rotavirus VP1 are indicated by arrows, respectively, at the left and at the right side of each panel.

cleotide sequence (Fig. 2). The complete nucleotide sequence of the plus strand of segment 1 and the protein it predicts are shown in Fig. 3. Genome segment 1 is 3302 nucleotides long. The 5′-end (5′-GGCUAUU-AAA) and the 3′-end (AUGUGACC-3′) of the gene contain the sequences which are conserved at the ends of all of the rotavirus segments that have been sequenced at this time. The absence of adenine-rich strings of nucleotides at the 3′ noncoding region is consistent with the lack of polyadenylation of rotavirus mRNA. A 5′ noncoding sequence of 18 bp precedes the first AUG codon which initiates an open reading frame of 1088 codons. Reading in any other frame of the coding or on the complementary strand yields numerous stop codons and short open reading frames. No direct evidence is available for the site of translation initiation but the first in-frame AUG is a strong initiation site according to the criteria of Kozak (1984).

The calculated molecular weight of the deduced protein (124,847) corresponds precisely to the apparent molecular as estimated by electrophoresis. There is a preponderance of basic residues (129 Lys + Arg, 15 His) over acidic residues (126 Glu + Asp) indicating a positively charged protein at neutral pH.

Expression of VP1 in *S. frugiperda* cells

Three baculovirus recombinants containing rotavirus gene 1 in position −7 respective to the initiation codon of the polyhedrin (pAC461/RF1.1 to pAC461/RF1.3) and three recombinants containing the rotavirus gene in position +34 (pVL941/RF1.1 to pVL941/RF1.3) were initially identified and tested for their ability to produce rotavirus VP1 after infection of *S. frugiperda* cells. All three recombinants derived from the transfer vector pAC461 expressed equal amounts of VP1. The same observation was obtained with recombinant derived from pVL941. Consequently only pAC461/RF1.1 and pVL941/RF1.1 were used for the subsequent studies. VP1 synthesis in Sf9 cells infected with these two recombinant viruses was compared with that of the proteins of wild-type baculovirus (Fig. 4A). The polyhedrin was absent in recombinant virus-infected cells, and a new band of the expected molecular weight (125,000) was easily identified at 24 hr postinfection. This band migrated the same as the *in vitro* translation product of gene 1 mRNA (data not shown). The kinetics of VP1 expression obtained with each of the recombinant viruses were similar with a peak of synthesis between 36 and 48 hr followed by a gradual decline until 72 hr postinfection. The level of VP1 synthesis by pVL941-derived recombinant was 5 to 10 times greater than from viruses derived from pAC461. Analysis of the stained gels infected with pVL941/RF1.1 recombinant indicated that rotavirus VP1 represent about 10% of the cell proteins and 20% of the amount of the polyhedrin found in Sf9 cells infected with wild-type virus (Fig. 4B). Comparison of the amount of pulse-labeled protein with the total amount of protein in stained gels

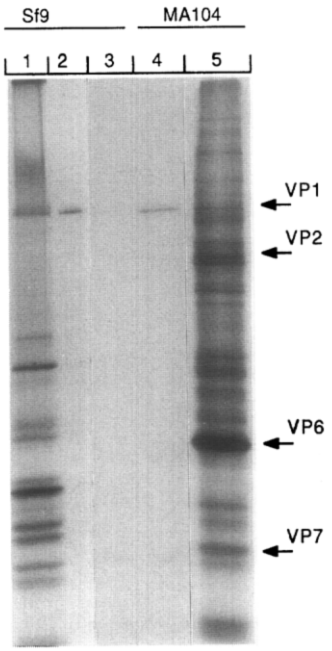

FIG. 5. Immunoreactivity of recombinant VP1. A total cell lysate of *Spodoptera frugiperda* 9 cells infected with pVL941/RF1.1 and labeled with [^{35}S]methionine (16 to 18 hr postinfection) was analyzed by SDS–PAGE (lane 1) in comparison with the immunoprecipitate obtained with either a preimmune serum (lane 3) or a hyperimmune anti-rotavirus serum raised against purified bovine rotavirus (lane 2). [^{35}S]Methionine-labeled polypeptides in rotavirus-infected MA104 cells (lane 5) were immunoprecipitated with guinea pig antiserum made with pVL941/RF1.1-infected *S. frugiperda* cells (lane 4).

showed that of VP1 in Sf9 cells was not degraded as the intensity of the 125K band constantly increased until 3 days postinfection whereas the rate of synthesis decreased after 48 hr.

Immunoreactivity and localization of recombinant VP1

The 125,000 molecular weight protein was identified as authentic VP1 by immunoprecipitation with polyclonal antiserum prepared to cesium chloride gradient-purified bovine rotavirus (Fig. 5). The reaction was specific as the preimmune serum did not react with the Sf9 lysate. An antiserum to gene 1 recombinant-infected Sf9 cells was shown to recognize VP1 in rotavirus-infected Ma104 cell lysate by immunoprecipitation or Western blot. VP1 was not excreted into the supernatant of recombinant virus-infected cells and only 10% of the protein was soluble in RIPA buffer (data not shown). This fairly low percentage led us to examine by electron microscopy *S. frugiperda* cells infected with pVL941/RF1.1 recombinant baculovirus in comparison with wild-type AcNPV-infected cells and uninfected cells. Examination of thin sections at various times postinfection failed to detect specific inclusions in recombinant virus infected cells. However, a significant increase of the electron-dense "spacers" which are found at the border of the fibrous structures formed by the baculovirus protein P10 were seen in these cells (Van der Wilk *et al.,* 1987). The anti-VP1 antiserum also did not possess neutralization activity.

DISCUSSION

Comparison of nucleotide sequences of gene 1 with other published rotavirus genes revealed several short homologous region of statistical significance (data not shown). The deduced amino acid sequence predicts that VP1 is a basic protein with a net positive charge of 10.5 at pH 7.0, assuming glutamic and aspartic acids are each −1 and arginine, lysine, and histidine are +1, +1, and +0.5, respectively at neutral pH. In three places there is an accumulation of basic residues (87–96; 159–166; 451–460), similar to short stretches of basic residues found in histones. The deduced amino acid of VP1 was also analyzed to determine predicted hydropholic and hydrophobic domains. Although the N-terminal region is hydrophobic and the first 18 amino acids could correspond to a signal peptide (polar residue at position 3 and 9 hydrophobic residues in the 18 first residues) according to Perlman and Halvorson (1983). However, these predictions are not supported by evaluation of the translocation of rotavirus proteins made in cell-free systems in the presence of microsomal membranes (Ericson *et al.,* 1983). Although there are nine potential N-type carbohydrate attachment sites there is also no direct evidence that this gene product is glycosylated. The deduced amino acid sequence yields limited information on the secondary structure of the protein. Using the rules proposed by Garnier *et al.* (1978) most of the α-helical regions were found to be confined to the amino-terminal half of the molecule. An examination of the carboxy-terminal region revealed the presence of 8 Ser–Lys or Lys–Ser di-amino acids which have been described as favored phosphorylation sites.

Computer analysis was performed to attempt to identify possible functions of VP1 based on homologies with other proteins. Searches for similarities with other sequences of the NBRF and Genbank databases were performed using several algorithms (Lipman and Pearson, 1985; Kanehisha, 1982). One region, between amino acids 517 and 636, presents a statistically significant homology with consensus sequences that have been established for a number of conserved regions in putative RNA-dependent RNA polymerases (RdRp) from several RNA viruses of eukaryotes by comparison to the known poliovirus polymerase (Pietras *et*

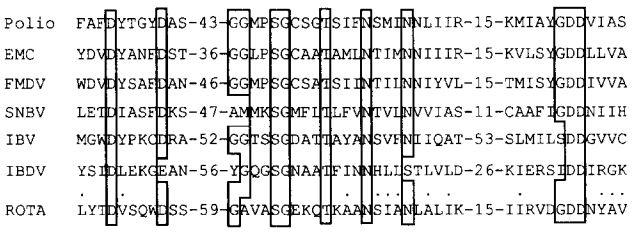

FIG. 6. Comparison between amino acid sequences of VP1 and RNA polymerase of RNA viruses. Identical amino acid are boxed and a dot shows similar amino acids. The numbers in the sequences correspond to the distance in base pairs between conserved elements. Polio, poliovirus; EMC, encephalomyocarditis virus; FMDV, foot and mouth disease virus; SNBV, sindbis virus; IBV, infectious bronchitis virus; IBDV, infectious bursal disease virus; ROTA, rotavirus. The sequences of the other viruses are from Kamer and Argos (1984), Morgan *et al.* (1988), and Boursnell *et al.* (1987).

al., 1988). The amino acid sequence Gly–Asp–Asp (GDD) is present in this region. This sequence is thought to be characteristic of RdRp (Kamer and Argos, 1984). Moreover two regions upstream of the GDD sequence bear a close resemblance to the published alignment (Gorbalenya and Koonin, 1988). The matches present in these three elements are shown in Fig. 6. A fourth consensus sequence usually found 30–50 residues downstream of the thrid element is not present in the VP1 sequence. It should be noted that similar sequences have been found in infectious bursal disease virus whose genome consists also of double-stranded RNA (Gorbalenya and Koonin, 1988; Dobos *et al.*, 1979).

In this study, the construction of two different recombinant viruses indicates that the new vector pVL941 is more efficient than pAC461 for the production of a nonfused protein under the polyhedrin promotor. This result confirms that the level of expression of a foreign gene that replaces the AcNPV polyhedrin gene is related to the preservation of the flanking sequences, upstream and downstream, of the initiating AUG translation codon of the polyhedrin gene (Luckow and Summers, 1988a,b; Matsura *et al.*, 1987). However, it should be noted that another rotavirus gene (segment 6) has been expressed to high level, relative to the level of expression of the polyhedrin, using the transfer vector pAC461 (Estes *et al.*, 1987).

Up to now there is no known function for VP1. The fact that VP1 is an internal protein present in low amount had suggested that it could have an enzymatic (transcriptase or replicase) activity. The sequence data presented here favor this hypothesis and the production of viral proteins in high yield from the baculovirus system offers a new way to analyze the enzymatic role of VP1 by using the monospecific anti-VP1 antiserum either to inhibit transcriptase activity or to test the polymerase activity of the recombinant protein. Experiments to test these hypotheses are in progress.

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Note added in proof. Since submission of the manuscript sequences of reovirus λ_3 and bluetongue virus P have been published. In both sequences a motive similar to the putative polymerase site (fig. 6) could be identified between positions 666-769 and 585-739 respectively for the bluetongue and the reovirus.

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