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

J. COHEN,* A. CHARPILIENNE,* S. CHILMONCZYK,* AND M. K. ESTES†

*Station de Virologie et d'ImmunoMoléculaires, INRA, C.R.J. Domaine de Vilvert, 78350 Jouy-en-Josas, France, and †Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030

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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 vectors: pAC461 and pVL941. Following infection of Sf9 cells with a recombinant baculovirus, a full-length nonfusion protein was synthesized 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.

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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 rotavirus 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 polyproteins 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.

1 To whom requests for reprints should be addressed.
Sequence Data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. J04346.
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 Xmal restriction endonuclease were synthesized using a “Biosearch 8700” synthesizer. These unphosphorylated oligonucleotides were used to prime the synthesis of cDNA on the plus and the minus
strands and also to allow ligation in the unique XmaI site of M13 mp19 of the reannealed double-stranded cDNA.  The first five clear plaques obtained appeared to be full-length as the Smal 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 Smal and subcloned either into the Smal site of the baculovirus transfer vector pAC461 or in the Klenow filled-in BamHI 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 EcoRV and BglII).  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 mM HEPES, pH 7.1; 140 mM NaCl; 125 mM CaCl₂) were added to 25-cm² flasks seeded with 2.5 x 10⁶ S. frugiperda cells and containing 750 µl of Grace’s medium supplemented with 10% FBS.  Following incubation at 27°C 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 ³²P-labeled probe was titrated again by limiting dilution and the supernatant was plated on a monolayer of *S. frugiperda* cells.  Virus in polyhedrin-negative plaques was plaque-purified three times and used to purify virus stocks.  For each transfer vector, three independent recombinant viruses designated pAC461/RF1.1 to pAC461/RF1.3 and pVI 941/RF1.1 to pVI 941/RF1.3 were prepared.

Protein analysis and immunoprecipitation

*S. frugiperda* cells in 25-cm² flasks were infected at a high multiplicity (≥5 PFU/cell) with wild-type AcNPV or with recombinant virus and labeled with [³⁵S]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°C 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, 1% glycerol, 10% 2-mercaptoethanol. 62.5 mM Tris–HCl, 0.01% bromphenol blue, pH 6.8).  For immunoprecipitation analysis, the cells were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 10 mM 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 [³⁵S]methionine-labeled cell extract.  Samples were incubated for 1 hr at 37°C, protein A-Sepharose (Pharmacia; 80 µl of a 15% suspension in 50 mM Tris–HCl (pH 7.5), 10 mM 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 mM Tris–HCl (pH 7.5), and then 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.
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 glyclosylation sites are underlined.
RECOMBINANT ROTAVIRUS INTERNAL PROTEIN

Ala Gly Leu Leu Ser Met Ser Ser Ala Ser Asn Gly Glu Ser Arg Gln Leu Lys 1245
Phe Gly Arg Lys Thr Ile Phe Ser Thr Lys Lys Asn Met His Val Met Asp Asp 1299
Met Ala Asn Gly Arg Tyr Thr Pro Gly Ile Ile Pro Ile Pro Val Asn Val Asp lys 1353
Pro Ile Pro Leu Gly Arg Arg Asp Val Pro Gly Arg Thr Arg Ile Ile Phe 1407
Ile Leu Pro Tyr Phe Ile Ala Gln His Ala Val Val Glu Met Leu 1461
Ile Tyr Ala Lys His Thr Arg Glu Tyr Ala Glu Phe Ser Arg Asn Gln 1515
Leu Ser Tyr Asp Val Thr Arg Phe Leu Ser 1569
Leu Ser Tyr Asp Val Ser Gin Thr Ser Gin His Arg Thr Gin Pro Phe Arg 1623
Leu Ser Gly Glu Ile Ile Met Gly Leu Arg Phe Asp Ser Met Leu Ala 1677
Pro Ile Ile Gln Thr Leu Asn Leu Tyr Lys Gln Thr Gin Ile Asn Met 1731
Ile Ile Tyr Ile Tyr Gin Thr Gin Val Ile Lys Lys Ile Gln Tyr Gly Ala Val 1785
Val Ile Gln Thr Leu Asn Leu Tyr Lys Gln Thr Gin Ile Asn Met 1839
Val Ile Gln Thr Leu Asn Leu Tyr Lys Gln Thr Gin Ile Asn Met 1893
Ile Val Leu Ser Arg Ile Ser Asn Lys Tyr Ser Phe Ala Thr Lys Ile 1947
Ile Arg Val Asp Gly Asp Asp Thr Val Ala Val Leu Gin Phe Thr Asn Thr Gly Val 1995
Thr Gly Gin Met Val Ser Gin Thr Ser Gin His Gin Thr Gin Pro Phe Arg 2055
Thr Gly Gin Met Val Ser Gin Thr Ser Gin His Gin Thr Gin Pro Phe Arg 2111
Thr Glu Gin Met Val Ser Gin Thr Ser Gin His Gin Thr Gin Pro Phe Arg 2163
Arg Asp Ile Thr Gin Gin Gin Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin 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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-
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...
showed that 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 hydrophobic 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 first 18 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
either to inhibit transcriptase activity or to test the poly-
tor pAC461 (Estes et al., 1987).

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).

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 promoter. 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, 1984). Moreover two regions upstream of the GDD sequence bear a close resemblance to the published consensus sequences of reovirus X3 and bluetongue virus P. However 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., 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 third 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 both sequences, a motive similar to the putative polymerase site is more efficient than pAC461 for the production of a nonfused protein under the polyhedrin promoter. 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; Matsuura 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 poly-

merase activity of the recombinant protein. Experiments to test these hypotheses are in progress.

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