Infectious Bovine Viral Diarrhea Virus (Strain NADL) RNA from Stable cDNA Clones: a Cellular Insert Determines NS3 Production and Viral Cytopathogenicity

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Bovine viral diarrhea virus (BVDV), strain NADL, was originally isolated from an animal with fatal mucosal disease. This isolate is cytopathic in cell culture and produces two forms of NS3-containing proteins: uncleaved NS2-3 and mature NS3. For BVDV NADL, the production of NS3, a characteristic of cytopathic BVDV strains, is believed to be a consequence of an in-frame insertion of a 270-nucleotide cellular mRNA sequence (called cIns) in the NS2 coding region. In this study, we constructed a stable full-length cDNA copy of BVDV NADL in a low-copy-number plasmid vector. As assayed by transfection of MDBK cells, uncapped RNAs transcribed from this template were highly infectious (>10⁵ PFU/μg). The recovered virus was similar in plaque morphology, growth properties, polyprotein processing, and cytopathogenicity to the BVDV NADL parent. Deletion of cIns abolished processing at the NS2/NS3 site and produced a virus that was no longer cytopathic for MDBK cells. This deletion did not affect the efficiency of infectious virus production or viral protein production, but it reduced the level of virus-specific RNA synthesis and accumulation. Thus, cIns not only modulates NS3 production but also upregulates RNA replication relative to an isogenic noncytopathic derivative lacking the insert. These results raise the possibility of a linkage between enhanced BVDV NADL RNA replication and virus-induced cytopathogenicity.
cation. For CP9, the sequences encompassing the coding region of C through NS2 have been deleted such that N^{548} is fused directly to NS3 (31). In CP13, two deletions have resulted in the fusion of 13 N^{548} residues and 10 E1 residues to NS3, with the NS3 N terminus truncated by five residues relative to the Ub- and N^{548}-NS3 fusion junctions (14). A CP DI RNA for CSFV in which all sequences between the methionine initiating the open reading frame and NS3 have been deleted has also been identified (18–20).

For two CP strains, CP7 and NADL, the mechanism(s) by which NS3 is produced remains obscure. Both isolates contain insertions in the NS2 region, apparently upstream of the NS2/NS3 (2/3) cleavage site. For CP7, the insertion is a duplicated viral sequence of 27 nucleotides which somehow promotes processing at the 2/3 site, NS3 production, and cytopathogenicity in cell culture (16, 30). In the prototype CP BVDV strain, NADL, the insert is a 270-base portion of a bovine mRNA of unknown function (called cIns [cellular insertion]) that results in an in-frame insertion of 90 amino acid residues.

To investigate the mechanism of NS3 production and cytopathogenicity by BVDV NADL, we constructed a stable, functional cDNA clone for this virus. Using this clone, we have gone on to engineer an isogenic derivative in which cIns has been deleted. Virus production, NS2-3 protein processing, accumulation of virus-specific proteins and RNA, and cytopathogenicity were then assessed. Our results indicate that cIns is necessary for NS3 production and the CP phenotype.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with sodium pyruvate and heat-inactivated 10% horse serum (HS). Cells were maintained at 37°C with 5% CO₂.

The NADL strain of BVDV was obtained from the American Type Culture Collection, plaque purified, and amplified by growth in MDBK cells. For infection of MDBK cells, virus dilutions made in DMEM-HS were adsorbed for 1 h at 37°C, then the inoculum was removed and replaced with fresh DMEM-HS. Cultures were incubated at 37°C for 48 h, or until CE was observed. Virus stocks were prepared by three freeze-thaw cycles of cells in their culture medium and clarified by centrifugation at 1,000 × g for 5 min.

BVDV plaque and focus-forming assays. MDBK cells (70 to 80% confluent) were infected with 10-fold dilutions of virus as described above. Following 1 h of adsorption at 37°C, cells were washed once with DMEM, overlaid with 1.5% low-melting-point (LMP) agarose (Gibco-BRL) in MEM containing 5% HS, and incubated at 37°C. To assay for plaque-forming virus, after 3 days monolayers were fixed with 3.7% formaldehyde for 2 h at room temperature, the agarose was washed two times with PBS, and then incubated with peroxidase-conjugated rabbit anti-bovine immunoglobulin (1:1,000 dilution in PBS; catalog no. A-5295; Sigma Chemical Co.). After 1 h, excess secondary antibody was removed by washing the monolayer twice with PBS, and then fixed with paraformaldehyde (3%) in phosphate-buffered saline (PBS) for 30 min, washed once with PBS, and then incubated with a bovine polyclonal anti-bovine immunoglobulin (1:1,000 dilution in PBS; catalog no. A-5295; Sigma Chemical Co.) for 2 h. Excess secondary antibody was washed out with PBS. Noninfected monolayers were incubated with PBS only. Virus production, NS2-3 protein processing, accumulation of virus-specific proteins and RNA, and cytopathogenicity were then assessed. Our results indicate that cIns is necessary for NS3 production and the CP phenotype.

Radioimmunoprecipitation and SDS-PAGE. Rabbit polyclonal antiserum specific for BVDV NS3 (G90) or bovine anti-BVDV antiserum (used in ref. 49) have been described elsewhere (5, 7). Depending on the antiserum, sodium dodecyl sulfate (SDS) (G90) or Triton X-100 (G94-solubilized) cell lysates were used for immunoprecipitations. Following labeling of MDBK cells, the medium was removed, cells were washed twice with ice-cold PBS, and cell extracts were prepared by lysis (0.5 ml per 35-mm-diameter well) on a Bio-Rad Gene Pulser (1.5 kV, 25 μF, 200 ohm) and frozen in liquid nitrogen. For assay of NS2-3 protein processing at the 2/3 site, NS3 production, and cytopathogenicity by BVDV NADL, we constructed a stable, functional cDNA clone for this virus. Using this clone, we have gone on to engineer an isogenic derivative in which cIns [cellular insertion] has been deleted. Virus production, NS2-3 protein processing, accumulation of virus-specific proteins and RNA, and cytopathogenicity were then assessed. Our results indicate that cIns is necessary for NS3 production and the CP phenotype.

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Immunoprecipitates were washed three times with the same solution and then finally once with TNE lacking Triton X-100. Washed immunoprecipitates were resuspended in Laemmli sample buffer, heated to 85°C for 10 min, and centrifuged at 12,000 \( g \) for 1 min. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on an 8% polyacrylamide gel and visualized by fluorography (15).

**TABLE 1. Oligonucleotides used for construction of pACNR/NADL, pACNR/cIns\(^2\) NADL, and RT-PCR analyses**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>NADL nucleotide position</th>
<th>Sense</th>
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<tbody>
<tr>
<td>183(^a)</td>
<td>1–27</td>
<td>+</td>
</tr>
<tr>
<td>343</td>
<td>4509–4531</td>
<td>+</td>
</tr>
<tr>
<td>344</td>
<td>5810–5835</td>
<td>−</td>
</tr>
<tr>
<td>345</td>
<td>4980–4993/5264–5276(^b)</td>
<td>+</td>
</tr>
<tr>
<td>346</td>
<td>4974–5264/5276(^b)</td>
<td>−</td>
</tr>
<tr>
<td>353</td>
<td>4754–4774</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Consists of a 5’ XbaI restriction enzyme recognition sequence and the T7 promoter, followed by BVDV NADL nucleotides 1 to 27.

\(^b\) Mutations relative to the BVDV NADL sequence are noted in Fig. 1B.

**Analysis of the cIns genetic marker.** Virus (culture media and freeze-thaw lysates) from ACNR/cIns-NADL- and control virus-infected MDBK cells was treated with 2 U of DNase I (RQI; Promega) and 1 \( \mu \)g of RNase A (catalog no. 1119915; Boehringer) for 30 min at 37°C and then used for infection of MDBK cells. Multiple sequential passages were conducted in duplicate, using these conditions. At each passage, RNA was obtained from the infected cells of one sample by using the RNAzol method as instructed by the manufacturer (Tel-Test, Inc.). RNA samples were used for RT-PCR with oligonucleotides 353 and 344 (Table 1). Amplified PCR products were extracted with phenol-chloroform and precipitated with ethanol before restriction enzyme digestion with ApaI or other enzymes. Passaged samples of wild-type (wt) BVDV/NADL and ACNR/NADL were used as controls for the absence of the ApaI site and presence of cIns.

**Western blotting.** SDS-solubilized MDBK cell lysates were separated by SDS-PAGE (10% gel) and transferred to Immobilon P nitrocellulose membranes by using the semidyry Multiphor II Nova blot system (LKB). The membranes were then stained for 90 s with 0.25% (wt/vol) fast green FCF in 10% acetic acid and then destained for 10 min in 10% acetic acid. Nonspecific binding sites were blocked overnight at 4°C with 5% milk in 20 mM Tris-Cl–137 mM NaCl–0.1% Tween 20, pH 7.6 (TBS-T). All following serum dilutions and washing steps were carried out in TBS-T. The membranes were incubated for 1 h at room temperature with primary rabbit polyclonal antisera specific for BVDV NS3 (G40) and E2 (D31) (5, 7) diluted 1/400 each, ensuring antibody saturation (data not shown), followed by a secondary horse radish peroxidase-conjugated goat anti-rabbit serum. Extensive wash steps were performed before primary and second-
ary antibodies and prior to detection with SuperSignal chemiluminescent substrate (Pierce) and exposure to X-ray film.

Northern blotting. Total RNA was extracted from MDBK cells by using TRIZOL reagent (Gibco-BRL). Northern blotting and hybridization was performed essentially as described by Sambrook et al. (28). RNA from 10^6 cells was denatured with glyoxal for 1 h at 50°C, separated by sodium phosphate-buffered 1% agarose gel electrophoresis, and blotted overnight on positively charged nylon membranes (Boehringer Mannheim), using the TurboBlotter system (Schleicher & Schuell) and alkaline transfer buffer (3 M NaCl, 8 mM NaOH). The membranes were then washed with 0.2 M sodium phosphate (pH 7.0), and the RNA was cross-linked by irradiation with a 254-nm light source (Stratalinker UV cross-linker; Stratagene). A 32P-labeled antisense RNA probe hybridizing to nucleotides 5413 to 5648 of the NADL genome was transcribed in vitro from the BamHI-linearized cDNA clone pGEM-3Z(f (+) )NADL ΔIns-Bgl, which was constructed by inserting the 790-bp BglII fragment of pNADLCΔIns-NADL into the BamHI site of pGEM-3Z(f (+)). One microgram of DNA was transcribed with SP6 polymerase in the presence of 0.5 mM each ATP, GTP, and CTP, 12.5 μM UTP, and 3.12 μM [α-32P]UTP (800 Ci/mm; Amersham). After treatment with DNase I, the RNA was purified from unincorporated ribonucleoside triphosphates using a Quick Spin G-50 Sephadex column (Boehringer Mannheim). The membrane was incubated in Hybirdaid hybridization oven at 60°C for 5 h in prehybridization/hybridization solution (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA (pH 7.7)], 5× Denhardt’s reagent, 0.5% SDS, 100 μg of denatured salmon sperm DNA per ml, 50 μg of yeast RNA per ml, 50% formamide), followed by overnight incubation at 60°C in fresh hybridization solution supplemented with 2 × 10⁶ cpm of labeled probe. The blot was then washed at 65°C three times for 30 min each with 1× SSPE-0.5% SDS and once for 30 min with 0.1× SSPE-0.5% SDS. Bands were visualized by X-ray autoradiography and quantified with a Molecular Imager (Bio-Rad Laboratories).

Metabolic labeling of viral RNA. For [32P]orthophosphate incorporation, infected MDBK cells were cultured in phosphate-free DMEM supplemented with 2% heat-inactivated HS. Five hours postinfection, the cells were treated with dactinomycin (2 μg/ml; ICN Pharmaceuticals, Inc.). Total RNA was harvested at 12 and 18 h postinfection, using TRIZOL reagent. RNA from 7 × 10⁶ cells was denatured with glyoxal and separated by agarose gel electrophoresis as described above. The gel was then fixed with methanol and dried, and RNA was visualized and quantified as described above.

RESULTS

Construction of a full-length functional clone of BVDV NADL in low-copy-number plasmid pACNR1180. Initial attempts to assemble stable full-length BVDV NADL cDNA clones in high- or medium-copy-plasmid vectors failed. Finally, low-copy-number vector pACNR1180, which had been used for stable propagation of full-length CSFV cDNA clones (27), was successfully employed. pACNR/NADL contains a T7 promoter, the full-length BVDV NADL cDNA reconstructed from previously sequenced overlapping cDNA clones (6) or RT-PCR products, and a unique 3’ Sse8387I site for production of runoff RNA transcripts (see Materials and Methods) (Fig. 1). T7 polymerase transcription of pACNR/NADL template DNA produced RNA transcripts infectious for MDBK cells, as shown in Table 2. Cap analog was not included in transcription reactions since pestivirus RNAs are believed to be uncapped (4, 19, 27); in fact, capping of in vitro-transcribed CSFV RNA actually reduced specific infectivity for MDBK cells, whereas RNase treatment abolished infectivity of transcribed RNAs. These results establish that infectivity was derived by transcription of RNA from the full-length BVDV cDNA template. Typical virus yields harvested from the culture supernatant and cells (by freeze-thaw cycles) at 36 h were 3 × 10⁷ to 10⁸ PFU/ml. The resulting virus was neutralized by BVDV-specific antiserum, as demonstrated by both plaque and CPE reduction (data not shown).

It should be noted that even in the pACNR1180 backbone, bacterial colonies harboring the full-length NADL cDNA were tiny, appearing on semisolid media only after 18 to 20 h at 37°C. The deleterious effects of long pestivirus cDNAs and full-length clones during propagation in E. coli have been noted previously (21, 27, 34). Since future genetic analyses depended on having a reliable NADL molecular clone for manipulation, we investigated the stability of pACNR/NADL in several bacterial hosts, including E. coli MC1061, ABLE-K, ABLE-C, XL1-Blue, and SURE cells. Plasmid DNA from our initial infectious clone was used to transform each of these strains. We monitored colony size, gross plasmid structure by restriction analysis, and the specific infectivity of transcribed RNAs. Among the host strains analyzed, MC1061, ABLE-K, and ABLE-C yielded heterogeneous mixtures of colony sizes. DNA from the larger colonies often showed evidence of deleted or rearranged sequences and no longer yielded infectious RNA transcripts. In contrast, transformation of XL1-Blue and SURE cells produced relatively uniform populations of small colonies, with no evidence of DNA rearrangement, and yielded transcribed RNAs with consistently high specific infectivities (data not shown). SURE cells proved slightly better (faster colony growth and higher specific infectivity RNA) and were used for all subsequent DNA manipulations.

Comparison of virus derived from pACNR/NADL to parental BVDV NADL. As shown in Fig. 2A, plaques on MDBK cells produced by transfection with RNA transcribed from pACNR/NADL were homogeneous and similar to the BVDV NADL parental virus originally used for cDNA cloning. Similar results were obtained in plaque assays using virus harvested from cells transfected with pACNR/NADL transcript RNA (called ACNR/NADL) or infected with the NADL parent (data not shown). Growth properties of ACNR/NADL and the parent were compared after infection of MDBK cells at both low (0.1 PFU/cell) and high (1.0 PFU/cell) multiplicity of infection (MOI). As is apparent from the experiment shown in Fig. 2B, the kinetics of replication and the yield of infectious virus were similar for ACNR/NADL and the parental virus at both MOIs. The patterns of viral proteins were also compared by metabolic labeling between 20 and 24 h postinfection and immunoprecipitation with a BVDV-specific polyconal antiserum (Fig. 2C). Identical patterns of virus-specific proteins were observed for both ACNR/NADL and the parent. Proteins indicated in Fig. 2C were identified not only by size but also by immuno-reactivity with a panel of region-specific antisera (reference 36.

### Table 2. Specific infectivity of in vitro RNA transcripts generated from pACNR/NADL

<table>
<thead>
<tr>
<th>Material used to transfect</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDBK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA linearized with Sse8387I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Transcription reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>ND</td>
<td>2.3</td>
</tr>
<tr>
<td>DNase during</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNase after</td>
<td>2.5 × 10⁵</td>
<td>2.9 × 10⁵</td>
</tr>
<tr>
<td>RNase after</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Virus recovered³ | 6.7 × 10⁶ | 3.9 × 10⁶ |

³ One microgram of pACNR/NADL linearized with Sse8387I was used for transcription either in the presence or in the absence of DNase I. Following synthesis, some transcription reactions were treated with DNase I or RNase A for 20 min at 37°C. After these treatments, samples were used to electroporate MDBK cells and infectious centers were determined as described in Materials and Methods.

⁴ Data are expressed in PFU per microgram of RNA or input DNA.

⁵ Viral titer harvested 36 h postelectroporation.

⁶ ND, not determined.
and data not shown). Of note was the uncleaved NS2-3 species migrating at 125 kDa and the prominent 80-kDa NS3 cleavage product, which are characteristic of CP BVDV strains. The similar plaque morphology, cytopathogenicity, growth properties, and polyprotein processing patterns of ACNR/NADL and the BVDV NADL parent validated the use of pACNR/NADL for future molecular genetic studies.

Deletion of cIns abrogates processing at the 2/3 site and NS3 production, and produces replication-competent, non-CP BVDV. Genome rearrangements and/or inserted sequences in CP isolates appear to be linked to processing at the 2/3 site, NS3 production, and cytopathogenicity. Although this hypothesis is supported by sequence comparisons of non-CP/CP pairs (17), it has been rigorously tested for only one CP isolate, CP7 (16, 30) (see Discussion). To address this for the NADL strain, we constructed pACNR/cIns\(^2\)NADL in which the 270-base cIns was deleted. At the deletion breakpoint, two silent nucleotide changes were introduced to create a novel \(ApaI\) restriction site, which was used as an additional genetic marker for the deletion mutant (Fig. 1; see also Materials and Methods).

Transfection of MGBK cells with RNA transcripts from linearized pACNR/cIns\(^2\)NADL template DNA did not induce CPE after 5 days at 37°C, and these cells looked similar to mock-transfected control monolayers. At the deletion breakpoint, two silent nucleotide changes were introduced to create a novel \(ApaI\) restriction site, which was used as an additional genetic marker for the deletion mutant (Fig. 1; see also Materials and Methods).

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As shown in Fig. 4, amplification of this region for the NADL parent and ACNR/NADL produced a fragment of 1,082 bp (Fig. 4A) that was resistant to digestion by *Apa*I (Fig. 4B, lanes 5 to 8). In contrast, amplification of both early (passage 1)- and late (passage 4)-passage RNA from ACNR/cIns*2 NADL yielded the expected smaller 812-bp fragment that was susceptible to digestion by *Apa*I (Fig. 4B, lanes 1 to 4).

To examine protein processing in the NS2-3 region, MDBK cells were infected with the NADL parent (ACNR/NADL) or ACNR/cIns* NADL and metabolically radiolabeled, and the NS3-related proteins were immunoprecipitated with an NS3-specific polyclonal rabbit antiserum. As shown in Fig. 5, both NS2-3 and NS3 were present in cells infected with NADL and ACNR/NADL (lanes 2 and 3), whereas only NS2-3 was found in ACNR/cIns* NADL-infected cells (lane 4). NS2-3 produced by ACNR/cIns* NADL migrated faster than that produced by NADL and ACNR/NADL, presumably because of the cIns deletion that shortens NS2-3 by 90 amino acids (~10 kDa).

Parallel comparison of RNA, protein, and virus accumu-
tion over time for ACNR/NADL and ACNR/cIns\textsuperscript{N} NADL revealed significantly higher levels of RNA for the CP virus than for its non-CP derivative (Fig. 6), whereas the analyzed proteins NS2-3 or NS3 and E2 (Fig. 7) accumulated to similar levels. The RNA, proteins, and virus titers shown in Fig. 6 and 7 were obtained in parallel from one single experiment and are representative of three identical experiments repeated independently. For viral RNA, Northern blotting and metabolic labeling yielded similar results (Fig. 6). As quantified by Molecular Imager analysis, the calculated ratio of ACNR/NADL to ACNR/cIns\textsuperscript{N} NADL RNA was 3 (Fig. 6A and B) at 12 h postinfection and 5 (Fig. 6A) or 8 (Fig. 6B) at 18 h postinfection.

These results demonstrate that cIns modulates cleavage at the 2/3 site, NS3 production, and cytopathogenicity but does not have dramatic effects on synthesis of virus-specific proteins or virus yield. Remarkably, however, deletion of cIns resulted in significantly lower levels of viral RNA synthesis and accumulation.
promotes cleavage at the 2/3 junction. In the case of CP7, the nine-residue insertion is located even further upstream of the putative 2/3 cleavage site. In addition to their different locations in NS2, there is no obvious sequence similarity between the NADL and CP7 inserts. Whether they activate a cryptic autoprotease present in the NS2-3 region or change the conformation of NS2-3 so as to render it susceptible to site-specific cleavage by a cellular enzyme remains to be determined (see references 29 and 36 for further discussion). Interestingly, in the absence of any inserted sequences or genome rearrangements, NS3 production occurs in cells infected with CSFV isolates (3, 33).

The strongest correlate of pestivirus cytopathogenicity is NS3 production, which is accomplished by myriad different strategies (17). Two groups have recently demonstrated that cell death induced by CP BVDV infection occurs via apoptosis (13, 37). It is possible that NS3 acts as a direct effector of apoptosis by somehow triggering cell death pathways. This is a plausible hypothesis given the obvious structural differences between NS2-3 and NS3, which could affect subcellular localization and interaction with host cell components, as previously discussed (35). Alternatively, cleavage at the 2/3 site (or NS3 production) could upregulate BVDV RNA replication to a level that is deleterious for host cells. In one model, viral RNA replication complexes might sequester cellular components present in limited quantities and required for maintaining homeostasis. In the case of BVDV NADL, increased numbers of replication complexes would then deplete such host factors to a level which triggers apoptosis. This model is consistent with our results, which demonstrate that RNA replication and accumulation are enhanced in ACNR/NADL-infected cells compared to ACNR/cIns-NADL-infected cells. It will be of interest to examine other isogenic non-CP/CP pairs to determine the generality of this observation and its possible correlation with cytopathogenicity.

In summary, genetic analyses of CP7 (16) and NADL (this report) have established that two distinct insertions in NS2 can regulate processing at the 2/3 site, NS3 production, and cytopathogenicity in cell culture. Such isogenic non-CP/CP pairs should be valuable for additional studies aimed at answering key questions in pestivirus biology. Examples include (i) defining the mechanism(s) of cleavage at the 2/3 site, including the responsible protease(s); (ii) establishing the pathway linking NS3 production to cytopathogenicity; and (iii) testing the hypothesis that CP strains with these insertions are sufficient to cause MD in animals persistently infected with the isogenic non-CP derivative.

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