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

ERNESTO MENDEZ,† NICOLAS RUGGLI, MARC S. COLLETT, AND CHARLES M. RICE

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093, and ViroPharma Incorporated, Exton, Pennsylvania 19341

Received 31 July 1997/Accepted 10 February 1998

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 (>10^6 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 N980 is fused directly to NS3 (31). In CP13, two deletions have resulted in the fusion of 13 N980 residues and 10 E1 residues to NS3, with the NS3 N terminus truncated by five residue floats relative to the Ub- and N980-NS3 fusion junctions (14). A CP DI RNA for CSFV in which all sequences between the methionine initializing 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 NS2/NS3 cleavage site, and cytotoxicity in cell culture (16, 30). In the P13 strain, which is from a bovine prototype CP BDV strain, NADL, the insert is a 270-base portion of a bovine mRNA of unknown function (called cns [cellular insertion]) that results in an in-frame insertion of 90 amino acid residues.

To investigate the mechanism of NS3 production and cytopathogenicity by BDV 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 cns has been deleted. Virus production, NS2-3 protein processing, accumulation of virus-specific proteins and RNA, and cytotoxicity were then assessed. Our results indicate that cns 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. MDBK cells (70 to 80% confluent) were used to infect cells as described above. Samples were harvested at 72 to 96 hpi for plaque and focus-forming assays. MDBK cells were propagated in Dulbecco’s modified medium (DME) supplemented with sodium pyruvate and heated to 75°C for 10 min, and clarified by centrifugation at 12,000 rpm for 10 min. Cells were washed two times with PBS, incubated with peroxidase-conjugated rabbit anti-bovine immunoglobulin (1/1,000 dilution in PBS; catalog no. A-5295; Sigma Chemical Co.). After fixation with formaldehyde, agarose plugs were removed, and the monolayers were stained with crystal violet (25).

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 washed two times with PBS, incubated with peroxidase-conjugated rabbit anti-bovine immunoglobulin (1/1,000 dilution in PBS; catalog no. A-5295; Sigma Chemical Co.). After fixation with formaldehyde, agarose plugs were removed, and the monolayers were stained with crystal violet (25). Foci produced by non-CP BVDV were visualized by immunostaining. After fixation with formaldehyde, agarose plugs were removed, and cells were permeabilized with Triton X-100 (0.25% in phosphate-buffered saline [PBS]) for 10 min, washed once with PBS, and then incubated with a bovine polyclonal antiserum specific to the Ub- and Npro-NS3 fusion junctions (14). A CP DI RNA for CSFV in which all sequences between the methionine initializing the open reading frame and NS3 have been deleted is also described elsewhere (7, 31). Depending on the particular experiment, samples were diluted further and plated in multiple wells or tissue culture dishes. An infectious center assay (12), with slight modifications, was performed. Tenfold dilutions of electroporated MDMH cells (in DMEM-HS) were plated in 2 ml (35-mm-diameter well) on monolayers of MDMH cells grown to 50 to 60% confluency. To permit recovery and attachment of the electroporated cells, plates were incubated for 4 h at 37°C, after which the medium was replaced with a 1.5% LMP agarose overlay as described above. Plates were incubated for 3 days at 37°C, and infectious centers were visualized and counted by staining for plaques or foci as described above.

Radioimmunoprecipitation and SDS-PAGE. Rabbit polyclonal antisera specific for BDV NS3 (G40) or bovine anti-BDV antiserum (cag49) have been described elsewhere (5, 7). Depending on the antiserum, sodium dodecyl sulfate (SDS) (G40)– or Triton X-100 (cag49)–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) with either 0.5% SDS or 0.5% Triton X-100 in TNE (50 mM Tris-Cl [pH 7.5], 1 mM EDTA, 0.15 mM NaCl, 20 μg of phenylmethylsulfonyl fluoride). SDS-solubilized lysates were sheared, heated to 75°C for 10 min, and the cleared lysate was passed through a 0.22-μm pore-size filter. Triton X-100-solubilized lysates were also clarified. Clarified lysates were diluted 1:5 in TNE containing 0.5% Triton X-100, 2.5 μg (G40) or 5 μl (cag49) of antiserum was added, and then the mixture was incubated overnight at 4°C with rocking at 200 rpm. Washed three times with TNE, Triton X-100, and 0.1% Triton X-100, was added, and incubation was continued for 2 h at 4°C.
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).

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 mg 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 (TelTest, 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 semidyrid 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 horseradish peroxidase-conjugated goat anti-rabbit serum. Extensive wash steps were performed before primary and second-

---

**TABLE 1. Oligonucleotides used for construction of pACNR/NADL, pACNR/cIns-NADL, and RT-PCR analyses**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>NADL nucleotide position</th>
<th>Sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>183a</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>4980–4993/5264–5276b</td>
<td>+</td>
</tr>
<tr>
<td>346</td>
<td>4754–4774</td>
<td>+</td>
</tr>
<tr>
<td>353</td>
<td>4974–5264–5276b</td>
<td>–</td>
</tr>
<tr>
<td>353</td>
<td>4974–5264–5276b</td>
<td>–</td>
</tr>
</tbody>
</table>

* Consists of a 5’ XhoI restriction enzyme recognition sequence and the T7 promoter, followed by BVDV NADL nucleotides 1 to 27.

* Mutations relative to the BVDV NADL sequence are noted in Fig. 1B.

**FIG. 1.** Diagram of plasmid pACNR/NADL sequences surrounding transcription initiation and runoff sites, and engineered pACNR/NADL derivatives. (A) pACNR/NADL (15,016 bp) with the BVDV cDNA insert and the positions of BVDV-encoded polyprotein cleavage products are indicated. The Npro autoproteinase (checkered box), the cellular sequence insert (cIns; solid box), and serine protease domain (hatched box) are highlighted. Also shown are restriction sites used for subsequent constructions (positions are given in the NADL nucleotide sequence) and production of runoff RNA transcripts (Sse8387I). Sequences shown below include the T7 promoter (lowercase, underlined), the T7 transcription start site (arrow), the 5’- and 3’-terminal BVDV cDNA sequences (positive sense; uppercase), and the Sse8387I runoff site (shaded). (B) Structures of RNAs transcribed from pACNR/NADL (top) and pACNR/cIns-NADL (below). 5’ and 3’ NTRs are indicated by lines, and polyprotein cleavage products are represented by boxes. Processing sites for Npro (curved arrow), signal peptidase (solid diamonds), the serine protease (double arrows), and unidentified proteases (question marks) are also shown. For the cIns deletion mutant, the parental (upper staggered sequences) and mutant (below) nucleotide and amino acid sequences at the deletion breakpoints are shown. Silent nucleotide changes (underlined) were used to create a novel ApaI restriction site (shaded) to facilitate plasmid constructions and to serve as a convenient marker for distinguishing between each mutant and the parent.
ary antibodies and prior to detection with SuperSignal chemiluminescent sub-
strate (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 10^5 cells was denatured with glyoxal for 1 h at 50°C, separated by sodium phosphate-buffered 1% agarose gel electrophoresis, and blotted overnight onto 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 (Stratallinker 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-3Zf(+)NADLΔIns-Bgl, which was constructed by inserting the 790-bp ByII fragment of pNADLΔIns-NADL into the BamHI site of pGEM-3Zf(+) (2). One microgram of DNA was transcribed with SP6 polymerase in the presence of 0.5 mM each ATP, GTP, and CTP, 12.5 mM UTP, and 3.12 mM[^32P]UTP (800 Ci/ml; 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 a Hybaid 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, 5% formamide), followed by overnight incubation at 60°C in fresh hybridization solution supplemented with 2 × 10^5 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[^14C]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) for 1 h prior to addition of[^14C]orthophosphate (200 µCi/ml; ICN Pharmaceuticals, Inc.). Total RNA was harvested at 12 and 18 h postinfection, using TRIZOL reagent. RNA from 7 × 10^6 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 about 10-fold (27). Optimized electroporation conditions yielded >10^5 PFU/µg of RNA transcript. Template DNA alone was not infectious, but intact template was required during transcription since DNase treatment abolished infectivity. After transcription, treatment with DNase had no effect 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 × 10^7 to 10^8 PFU/ml. The resulting virus was neutralized by BVDV-specific antisera, 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 cDNAs 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-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.

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

<table>
<thead>
<tr>
<th>Material used to transfect</th>
<th>Ext 1</th>
<th>Ext 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACNR/NADL cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA linearized with Sse8387I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNA transcribed from</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sse8387I-linearized cDNA clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACNR/NADL</td>
<td>Yield&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Virus recovered&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Complete transcription</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>DNase during transcription</td>
<td>2.3 × 10^5</td>
<td>6.7 × 10^6</td>
</tr>
<tr>
<td>RNase after transcription</td>
<td>2.5 × 10^5</td>
<td>3.9 × 10^6</td>
</tr>
<tr>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> Data are expressed in PFU per microgram of RNA or input DNA.

<sup>c</sup> Viral titer harvested 36 h postelectroporation.

<sup>d</sup> ND, not determined.

--

on November 7, 2017 by guest
http://jvi.asm.org/ Downloaded from

Downloaded from http://jvi.asm.org/ Downloaded from
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 $\text{ApaI}$ restriction site, which was used as an additional genetic marker for the deletion mutant (Fig. 1; see also Materials and Methods).

Transfection of MDBK 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 $\text{ApaI}$ restriction site, which was used as an additional genetic marker for the deletion mutant (Fig. 1; see also Materials and Methods).

Transfection of MDBK 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. In contrast, RNA transcribed from pACNR/NADL induced CPE after 24 h (data not shown). We could, however, readily detect ACNR/cIns$^-$NADL replication by immunostaining of foci by using a polyclonal anti-BVDV antiserum (Fig. 3A). Using an infectious center assay for electroporated MDBK cells (see Materials and Methods) and this immunostaining protocol, we determined that RNA transcripts from pACNR/cIns$^-$NADL had a specific infectivity approaching that of pACNR/NADL ($\sim 8 \times 10^6$ focus-forming units [FFU] per µg of RNA). Low- and high-multiplicity infection comparisons of ACNR/cIns$^-$NADL and ACNR/NADL (Fig. 3B) revealed similar growth kinetics and virus yields, with the non-CP derivative showing slightly faster replication and higher cumulative virus titers, which approached $10^7$ FFU/ml. As seen in Fig. 3C, ACNR/cIns$^-$NADL did not induce CPE in cultures even at 50 h postinfection, when peak titers were reached, in contrast to ACNR/NADL and wt BVDV NADL, which had caused dramatic CPE by this time.

To confirm the genomic structure of ACNR/cIns$^-$NADL, we serially passaged the virus in MDBK cells, each time incubating the resulting virus with RNase and DNase to avoid carryover of input transcript RNA and plasmid template DNA. Total cellular RNA, isolated at each passage, was used for amplification of a NS2-3 subregion that included the cIns locus, and the resulting fragments were analyzed by agarose gel electrophoresis, either with or without digestion with $\text{ApaI}$.
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 ApaI (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 ApaI (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\(^2\)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\(^2\)NADL-infected cells (lane 4). NS2-3 produced by ACNR/cIns\(^2\)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 accumula-
in significantly lower levels of viral RNA synthesis and accumulation over time for ACNR/NADL and ACNR/cIns 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) as well as the virus titers (Fig. 6 and 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 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.

In this work, we succeeded in constructing a functional BVDV NADL cDNA clone in a low-copy-number plasmid. Full-length RNAs transcribed by T7 polymerase from this cDNA template have the authentic viral 5′- and 3′-terminal sequences, are highly infectious for MDBK cells (>10^5 PFU/μg), and yield a virus that has properties similar to those of the BVDV NADL parent. This plasmid clone is stable when propagated in the SURE strain of E. coli. Recently, assembly of a full-length BVDV NADL clone in a high-copy-number plasmid was reported by another group (34). Although infectious RNA could be transcribed from this template, the authors noted problems with plasmid transformation efficiency and stability and the production of full-length RNA transcripts. Some of these difficulties mimic our earlier unsuccessful attempts to construct such clones in high- or medium-copy-number plasmids. These problems were alleviated when the pACYC177 backbone was used. This plasmid had been used successfully for several other full-length pestivirus cDNAs (16, 19, 27). The reason(s) for the observed toxic effects of pestivirus cDNAs in some E. coli strains remains to be determined, but similar problems have also been encountered for several members of the flavivirus genus (22, 25).

The creation of a functional BVDV NADL cDNA clone allowed us to directly test the role of cIns in NS3 production and cytopathogenicity. Deletion of the 270-base cIns element produced a viable non-CP virus, in which detectable cleavage at the 2/3 site and NS3 production were abolished. Similar results were recently reported for CP BVDV strain CP7, which contains a 27-base duplication of viral sequences in the NS2 region (30). Using a vaccinia virus transient expression assay, deletion of this 27-base sequence eliminated cleavage at the 2/3 site (30). Further studies demonstrated that an isogenic derivative lacking this insertion was non-CP (16).

The mechanism(s) by which cIns (NADL) or the 27-base (CP7) insertions in NS2 promote cleavage at the 2/3 site is unknown. Although the NS3 N terminus has yet to be precisely determined for these strains, based on the similar apparent molecular masses of pestivirus NS3 proteins (10, 11, 30) and the conserved Ub-NS3, Npro-NS3, and Met-NS3 junctions observed for other CP isolates, Gly-1590 (SD-1 numbering [8, 9]) is the likely NS3 N-terminal residue. For NADL, this would imply that the 90-amino-acid cIns insertion, located 53 residues upstream of this Gly residue, somehow...
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 insertions. 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.

ACKNOWLEDGMENTS

We thank Carol Read for expert technical assistance. We are also grateful to many colleagues for helpful discussions during the course of this work and to M. Scott McBride, Tina Myers, and Karen Reed for critical reading of the manuscript.

E.M. was supported by a fellowship from the Human Frontiers of Science Program Organization and by the Universidad Nacional Autónoma de México. N.R. was supported by fellowships from the Swiss National Science Foundation and from the Swiss Foundation for Biomedical Stipends (SSMBS).

REFERENCES


