An in vitro Flaviviridae replicase system capable of authentic RNA replication

J.E. Tomassini, a,* E. Boots, a L. Gan, b P. Graham, a V. Munshi, a B. Wolanski, a J.F. Fay, a K. Getty, a and R. LaFemina a

a Department of Biological Chemistry, Merck Research Laboratories, West Point, PA 19486, USA
b Department of Molecular Biology, Scripps Research Institute, La Jolla, CA 92037, USA

Received 16 January 2003; returned to author for revision 19 February 2003; accepted 28 March 2003

Abstract

We have established an in vitro replication system for bovine viral diarrhea virus (BVDV), a surrogate for the closely-related hepatitis C virus. In an in vitro reaction, BVDV replication complexes synthesize vRNA and replicative form (RF) and replicative intermediate (RI) RNAs. Kinetic and heparin trapping experiments demonstrate the recycling of RF and RI products and the initiation of vRNA synthesis in this system. Consistent with this, quantitative hybridization reveals the asymmetric synthesis of positive and negative strand RNA products. These findings support the notion that RF serves as a template and RI as a precursor in the synthesis of vRNA. Furthermore, the antiviral activity of an NS5B inhibitor was similar in BVDV replicase and infectivity assays. Together, these results indicate that the in vitro activity of BVDV replicase complexes recapitulates RNA replication that occurs in infected cells, providing a system in which to study both mechanisms and inhibitors of Flaviviridae replication.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Bovine viral diarrhea virus (BVDV); Replication complexes; Flaviviridae

Introduction

Bovine viral diarrhea virus (BVDV) is a positive strand RNA virus and one of the best studied pestiviruses within the Flaviviridae family (Lindenbach and Rice, 2001). It represents an attractive model system to characterize the replication of the closely related hepacivirus, hepatitis C virus (HCV), a major cause of hepatitis and chronic liver disease for which an infectious cell culture system does not exist (Lindenbach and Rice, 2001). The viral genomes of BVDV and HCV encode single polyproteins, which are processed cotranslationally by viral and cellular proteases, yielding several functionally conserved gene products. These are the nucleocapsid protein, C, three virion glycoproteins E1, E2, and E, and several genes encoding enzymatic functions and structural components of the replication complex, including NS2-NS3 (helicase/protease)-NS4A-NS4B-NS5A-NS5B (polymerase)-COOH. Additionally, cis-acting elements located within the nontranslated regions which regulate the translation and replication of HCV and BVDV can be functionally substituted, further suggesting that the viruses share similar replication strategies (Frolov et al., 1998). Thus, comparative studies will aid in the search for antiviral agents.

The replication of BVDV has been characterized in infected cells and is predicted to be similar to that of other positive strand RNA viruses which occurs in membrane-associated complexes localized to the endoplasmic reticulum (Gong et al., 1996, 1998). Replication is postulated to proceed first through the synthesis of a negative strand copy of genomic RNA which produces a double-stranded (ds) replicative form (RF) containing the input positive strand RNA. The resulting RF then serves as a template for the synthesis of numerous copies of positive strand RNA, which comprises a structure known as the replicative inter-
mediate (RI) containing associated nascent plus strand molecules, and supports the asymmetric replication of viral RNA (Racaniello, 2001).

The replication of positive strand RNA viruses has also been studied in vitro using crude replication complexes (CRCs), which catalyze the synthesis of viral RNA in infected cells. Although the precise composition of replication complexes is unknown, complexes isolated from infected cells contain replicative intermediate RNA, newly synthesized vRNA, and several viral proteins including an RNA-dependent RNA polymerase, helicase, and proteinases (Bienz et al., 1992; Egger et al., 1996; Grassman et al., 1999; Westaway et al., 1999). When reconstituted in vitro reactions, the complexes synthesize full-length vRNA, RI, and RF products, resembling those identified in infected cells (Chu and Westaway, 1987, 1992; Grun and Brinton, 1986; Warrilow et al., 2000). Additionally, strand-specific, protein-primed (VPg) initiation of RNA synthesis was demonstrated for poliovirus replication complexes and more recently picornavirus replication has been elucidated in an in vitro reaction, in which RNA synthesis is de novo initiated by VPg (3B), following its nucleotidylation by the 3D polymerase (Paul et al., 2000; Reider et al., 2000; Takeda et al., 1986; Takegami et al., 1983). Replication complexes have also been isolated for the West Nile, Kunjin, and BVDV viruses (Chu and Westaway, 1987; Grun and Brinton, 1986; Warrilow et al., 2000) and while RNA products similar to those observed in infected cells are synthesized, the mechanisms involved in the RNA replication of these viruses are still largely unknown.

Detailed biochemical analysis of the polymerase activity of positive strand RNA viruses has been performed using recombinant polymerases (Behrens et al., 1996; Carroll et al., 2000; Lai et al., 1999; Neufeld et al., 1991; Zhong et al., 1998). However, these studies have indicated that the in vitro activity of the recombinant polymerases is characteristically different from replication observed in vivo or in isolated complexes. For example, reactions containing only the 3D polymerase of poliovirus, expressed and purified from either bacterial or insect cells, are capable of elongating template and/or primed template pairs; however, the enzyme will copy templates of heterologous, nonviral sequence (Arnold et al., 1999). Similarly, the NS5B polymerases of BVDV and HCV have been shown to synthesize RNA in vitro from heterologous templates, although a mode of de novo initiation has been demonstrated for both enzymes (Hong et al., 2001; Kao et al., 1999; Luo et al., 2000; Ranjith-Kumar et al., 2002). Therefore, it appears that either additional viral replicative proteins are required or a membrane environment and/or cellular factor(s) is involved in conferring specificity to RNA replication.

As a means to elucidate BVDV RNA replication, we have isolated fractions containing replication complexes from infected cells. When reconstituted in an in vitro system, optimal for the synthesis of genomic-length vRNA, RF, and RI products, RNA replication characteristic of that observed during viral infection is exemplified (Gong et al., 1996). Kinetic studies demonstrate the sequential synthesis and recycling of RF and RI products, suggesting that these RNAs functionally serve as template and precursor, respectively, in vRNA synthesis. In line with this, heparin trapping experiments and quantitative hybridization of replicase products reveal that this system is capable of the initiation of RNA synthesis and the asymmetric synthesis of positive and negative strand RNAs. Notably, the inhibition of BVDV replicase by a small molecule with a potency similar to that observed in viral infection indicates that the activity of the isolated complexes directly reflects the catalysis of RNA synthesis in infected cells. This system will prove useful in delineating the mechanisms and inhibition of RNA replication of BVDV and other members of the Flaviviridae family.

Results

Isolation and assay of BVDV replication complexes

To study the in vitro replication of BVDV RNA, subcellular fractions containing replication complexes were isolated from MDBK cells infected with the NADL strain, using methods similar to those previously described for other positive strand viruses (Grun and Brinton, 1986; Takegami et al., 1983). Following fractionation, the transcriptional activity of the various subcellular fractions was assayed in a reaction containing [32P]CTP, nucleotide triphosphates (NTPs), actinomycin D (act D), and an ATP-regenerating system. The resulting RNA products synthesized in the presence of CRC and perinuclear supernatant (PNS) fractions isolated from infected, but not from equivalent fractions of mock infected cells, specifically hybridized to cDNA probes representing the structural and nonstructural (NS2/3 and NS4/5) regions of the BVDV genome (Fig. 1A). Enrichments of 4.4- and 1.6-fold in replicase activity and recoveries of 63 and 26% of total activity were obtained in the CRC and PNS fractions, respectively, with little activity remaining in the postcytoplasmic extract (Fig. 1A and Table 1). A greater specific activity was routinely observed in the CRC fraction relative to the PNS fraction and was subsequently pursued as the enzyme source for RNA synthesis (Table 1).

The RNA transcripts present in the hybridizable fractions migrated with a mobility of less than or equal to the size of full-length vRNA (12.5 kb) on totally denaturing agarose gels (Fig. 1B). Larger molecular weight products, ca. 24 kb in size, were also present and were deduced to be dsRI/RF products by denaturation and RNase sensitivity studies (data not shown). As shown in Fig. 1B and C, the reaction was linear during a 90-min period and optimal activity was obtained at 35°C in the presence of >500 μM concentrations of A and GTP. Synthesis was detected only in the presence of all four NTPs and not in the presence of each
single, radiolabeled nucleotide, indicating that terminal transferase activity does not contribute to the reaction (data not shown).

Protein content of the isolated BVDV replication complexes

Western blot analysis revealed that the nonstructural proteins (NS2, NS3, NS4A, NS4B, and NS5B) are present in the CRC and PNS fractions isolated from BVDV infected, but not from equivalent fractions of mock infected cells. An antibody was not available to test for NS5A. As shown in Fig. 2, the fully processed forms of the nonstructural proteins were detected. When probed with the anti-NS3 antibody, in addition to the NS3 protein (71 kDa), presumably full-length and NS2-3 precursors >220 and 125 kDa, respectively, were observed. Probing with anti-NS2 resulted in a strong detection of NS2 but minimal detection of the 125-kDa NS2-3 precursor, potentially due to poorer recognition of the uncleaved C terminal region with this antibody. The reason for the doublet which reproducibly appears when probed with anti-NS4A antibody is unclear and may be attributed to aberrant cleavage and/or proteolytic degradation. Cellular proteins were also observed in...
stained protein gels of replicase complexes (data not shown).

**Polarities of the RNA products synthesized by BVDV replication complexes**

The polarities of the endogenous complex RNA and the products synthesized were determined by a ribonuclease protection assay (RPA). The specificity of the RPA and copy numbers were determined from positive and negative strand transcripts assayed in parallel in varying amounts (data not shown). As shown in Fig. 3A and in Table 2, a 9-fold greater amount of positive than negative strand RNA was detected in the isolated CRCs, referred to as time zero (\( t_0 \)), which was estimated to be approximately 6500 copies of positive strand and 750 copies of negative strand RNA per cell equivalent (ceq). Following synthesis for 60 min, a 4.7-fold increase in positive strand and a 2-fold increase in negative strand RNA was observed which corresponds to 24,520 and 809 copies synthesized per cell equivalent, respectively, indicating that amplification of both strands occurred. The total amount of product RNA quantitated per reaction, ca. \( 3.16 \times 10^{10} \) copies or 216 ng of RNA, is comparable that detected by TCA precipitation (Table 1).

Increases in positive and negative strand RNA products were also detected by Northern blot analysis (Fig. 3B). When probed with antisense 5′ and 3′ NTR probes, increases of 4.4- and 2.9-fold were observed for positive strand RNA, respectively, and increases of 1.5- and 2.8-fold were observed for negative strand RNA when probed with sense 5′ and 3′ NTR probes (Fig. 3C). Note that higher amounts of RNA were detected using probes that recognized the 5′ ends of each RNA. Similarly, RT-mediated PCR of replicase products using 5′ NTR primers and quantitation following Southern blot transfer indicated that positive and negative strand RNAs were present in the CRC fraction at time 0, and at 60 min increases of 6-fold in positive strand and 2-fold in negative strand RNA were observed (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (ng/( \mu )g/h)a</th>
<th>Relative activityb</th>
<th>Total (ng) product</th>
<th>% Total activityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>2.5</td>
<td>1.0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cytoplasmic extract</td>
<td>3.1</td>
<td>1.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CRC</td>
<td>11.0</td>
<td>4.4</td>
<td>321</td>
<td>63</td>
</tr>
<tr>
<td>Postcytoplasmic extract</td>
<td>1.9</td>
<td>0.8</td>
<td>58</td>
<td>11</td>
</tr>
<tr>
<td>PNS</td>
<td>4.1</td>
<td>1.6</td>
<td>129</td>
<td>26</td>
</tr>
</tbody>
</table>

a Expressed as nanograms of TCA precipitable product RNA per microgram of total protein from a 50-\( \mu \)l reaction containing \( 1.25 \times 10^6 \) ceq of each fraction.

b Compared to total cell homogenate.

c Total in recovered fractions.

### Synthesis of RI, RF, and vRNA products by BVDV replication complexes

To delineate the strandedness of the RNA products, a partially denaturing gel system was used, in which only single-stranded (ss) RNAs are denatured and the replication products separate as RI, vRNA, and RF on 3% polyacrylamide (PAGE)–7 M urea gels (Chu and Westaway, 1985). The identities of these RNA species were verified on the basis of LiCl separation and RNase sensitivities wherein mainly dsRNA (RI and RF) was detected in the supernatant fraction of LiCl precipitated replicase products, while an enrichment of ss vRNA was observed in the precipitate (Fig. 4). Correspondingly, RNase A/T1 digestion of the RNA products under high salt conditions resulted in preferential degradation of ss vRNA and partially ssRI, and all products were totally degraded under low salt conditions.

### Kinetics and sequence of RNA product synthesis

The sequence of the synthesis of RNA products was determined by continuous labeling in the in vitro replicase reaction. As shown in Fig. 5A, the greatest labeling occurred first in the RI product, followed by RF and vRNA (Fig. 5A). Although the labeling of RF was linear throughout the reaction time course, the labeling of RI was initially linear but then plateaued and slightly decreased (Fig. 5B). A peak of vRNA which was routinely observed at 5 min

![Fig. 2. Western blot analysis of BVDV CRC and PNS fractions. CRC and PNS fractions (1.25 \( \times 10^6 \) ceq) isolated from mock and BVDV infected cells were electrophoresed on 10% Bis-Tris Nupage (Novex) gels in MES or Mops buffer then transferred to nitrocellulose and Western blotted with the indicated anti-peptide polyclonal sera as described under Materials and methods. Arrows indicate protein positions.](image)
disappeared at 10 min, but then reappeared at 20 min and linearly increased thereafter. As the labeling of RI decreased, the labeling of vRNA increased, indicating the release of vRNA from the RI. Notably, the increase in RF labeling correlated with an increase in vRNA and a decrease in RI product, suggesting the recycling of RI to RF.

To further determine whether the initiation of RNA synthesis and template recycling occur in this system, pulse labeling of RNA products was performed at the indicated times as outlined in the legend to Fig. 5C. In this way, RNA transcripts are elongated in the absence of label, and then RNA synthesis is pulsed for short periods with labeled nucleotide. As shown in Fig. 5C and D, apart from its absence at 10 and 60 min, vRNA was synthesized throughout the reaction, indicating that following elongation and release of nascent transcripts, initiation likely occurs. Consistent with this, the labeling of RI decreased only slightly over time, whereas RF decreased significantly and, by 60 min, the ratio of RI to RF labeling was 1:1, compared to ratios of 0.3–0.5:1 observed earlier in the reaction. This pattern of early labeling of RI followed by a slow decrease is similar to that seen in the continuous labeling time course (Fig. 5B). However, the pulse labeling of RF decreased in contrast to the linear increase observed during the time course labeling, indicating that the increase in RF labeling is attributed at least in part to the recycling of RF from RI.
the compound had an IC50 of 80 nM when measured by in situ RPA in the absence of cytotoxicity and was also active in an NS5b assay with an IC50 of 800 nM (Fig. 7B).

Discussion

In an effort to elucidate the mechanisms of BVDV RNA replication, we have investigated the transcriptional activity of replication complexes isolated from infected cells. Although the in vitro properties of BVDV replicase have been previously reported (Warrilow et al., 2000), we herein present an extensive study using an in vitro system optimized for the synthesis of vRNA, RI, and RF products, in which the sequence and recycling of RNA products, the initiation of RNA synthesis, and the asymmetric synthesis of positive and negative strand RNA products are demonstrated.

Similar to the replication complexes isolated from membranes of cells infected with positive strand RNA viruses, subcellular fractions containing BVDV complexes are found to contain the viral nonstructural proteins (NS2–5B). Positive and negative strand BVDV RNAs are present in the isolated complexes in a ratio of 9:1, respectively, comparable to the 10:1 asymmetry observed in infected cells at 12 to 18 h pi (Gong et al., 1996, 1998). Approximately 6500 copies of positive strand and 750 copies of negative strand RNA are detected in the complex per cell equivalent isolated in the CRC fraction, which is roughly 50–60% of the total viral RNA (ca. 1.5–2.0 × 10^4 copies) in an infected cell (Gong et al., 1996, and R. LaFemina, unpublished observations). Upon cell fractionation, 63 and 26% of the replicase activity is recovered in the CRC and PNS fractions, respectively, and thus the majority of vRNA in infected cells can be accounted for in these membranous fractions (Fig. 1, Table 1).

When reconstituted in an in vitro reaction, fractions containing BVDV replication complexes synthesize vRNA, RI, and RF products, reminiscent of those observed in infected cells (Gong et al., 1996). Despite the presence of cellular proteins in the complexes, replicase activity is specific and distinct from that of terminal transferase and/or other cellular enzymes, since RNA synthesis occurs only in reactions containing replicase isolated from infected cells in the presence of all four NTPs. Unlike the previously reported in vitro system (Warrilow et al., 2000), the synthesis of ss vRNA is robust and reproducible, likely attributed to differences in reaction conditions. Optimal transcription of vRNA, RI, and RF products is observed at 35°C, in the presence of KCl (25–50 mM), Mg^{2+} (2–5 mM), and high concentrations (>500 μM) of the nucleotides ATP and GTP. The importance of GTP has been implicated previously in the de novo initiation of RNA synthesis by BVDV NS5B polymerase (Kao et al., 1999; Ranjunith-Kumar et al., 2002) and, correspondingly, RNA synthesis is not observed in this system at <80 μM of GTP (data not shown). The synthesis of full-length vRNA is ATP-dependent. In the
absence of an ATP regenerating system and <80 μM of ATP, RI and RF but not vRNA products are visible on partially denaturing urea gels, which is furthermore consistent with the inhibition of vRNA synthesis by nonhydrolyzable analogs of ATP (data not shown) and implies that ATPase and/or helicase unwinding functions may contribute to replicase activity (Grassman et al., 1999). Replicase activity is membrane-dependent as inclusion of NaDOC (0.1–2%) results in a reduction of vRNA but not RI/RF synthesis and sensitivity of endogenous RNA template to nucleases (data not shown). Similarly, NaDOC blocks initiation of vRNA but not elongation of RI and RF RNA by membrane fractions containing poliovirus replication complexes (Egger et al., 1996).

The asymmetry of BVDV RNAs observed in the isolated complex is conserved in the in vitro reaction. Routinely, a 4.7-fold increase in the amount of positive strand and a 2-fold increase in negative strand RNA are detected during a 60-min reaction by quantitative RPA. The probes used to quantitate product RNA hybridize to the 3′ ends of full-length BVDV RNAs and hence may underestimate the actual product yield. Indeed, a greater amount of RNA

---

Fig. 5. Time course and pulse chase labeling of replicase reaction. (A) RNA products were purified at the indicated times following synthesis by CRC replicase (6.25 × 10⁵ ceq) isolated from mock and BVDV infected cells in the presence of [³²P]CTP. RNA was electrophoresed on a 3% PAGE–7 M urea gel. Positions of RI, vRNA (12.5 kb), and RF are indicated. (B) RNA products on the gel were quantitated by phosphorimager analysis and plotted per time. (C) RNA products were purified from a reaction which was initiated with unlabeled NTPs and then pulsed by the addition of [³²P]CTP at 5, 10, 20, 30, 40 and 60 min for a 20-min period. Transcripts were electrophoresed on a 3% PAGE–7 M urea gel and autoradiographed. (D) RNA products on the gel were quantitated by phosphorimager analysis and plotted per time.
products are detected by Northern blot using probes which hybridize to the 5' ends of the positive and negative strand RNAs relative to probes which recognize the 3' ends, signifying that short transcripts are also initiated (Fig. 3B and C). Approximately 24,500 and 800 copies of positive and negative strand RNA products are synthesized, respectively, during a 60-min reaction, which is reasonable considering that six to seven nascent strands of RNA can be made per BVDV RI molecule in infected cells (Gong et al., 1998) and that recycling and reinitiation from the RI appear to take place in this system.

Kinetic experiments demonstrate the sequential synthesis of product RNAs and the recycling of RF and RI as template and precursor, respectively, during the in vitro synthesis of BVDV vRNA. Initially, the greatest product labeling occurs in the RI as nascent vRNA transcripts are elongated, and then plateaus concomitant with the release of vRNA from the intermediate and the generation of RF product. A significant accumulation of dsRF occurs which can be attributed to either the synthesis of negative strand RNA or the recycling of RF from RI as a dead-end product containing transcripts that are not displaced from the negative strand template. However, the limited amount of negative strand RNA synthesis which occurs in this system indicates that minimal dsRF can be formed by complementary synthesis. On the other hand, RF cannot be solely a dead-end product, since pulse labeling indicates the continuous synthesis of RI and vRNA throughout the reaction, whereas RF decreases significantly over time. Consistent with this, the ratio of pulse labeled RI to RF at 60 min is 2- to 3-fold larger than

![Fig. 6. Effect of heparin on BVDV replicase activity. RNA products were synthesized at the indicated times in the in vitro BVDV replicase assay (6.25 × 10^5 ceq) in the absence or presence of added heparin (2 mg/ml). (A) RNA products were electrophoresed on a 3% PAGE–7 M urea gel. (B) RNA products on the gel were quantitated by phosphorimager analysis and plotted per time.](image_url)
that observed earlier in the reaction. Taken together, our results indicate that RI is maintained in equilibrium as a precursor for additional rounds of vRNA synthesis, supported by a partial recycling of RF as a template. However, the greater linear rate of RF labeling compared to that of RI and the overall decrease in pulse labeling of all products over time indicates that a limited amount of initiation occurs in this system, in line with the modest increases in RNA synthesis that are measured by RPA, Northern, and PCR analyses. These observations are somewhat similar to those reported for the replication of Kunjin Flavivirus RNA (Chu and Westaway, 1985, 1987).

The potential relevance of the in vitro replicase system to in vivo replication is additionally provided by the finding that a triazinoindole compound, previously reported to inhibit BVDV replication, inhibits the in vitro synthesis of RNA by BVDV replicase complexes with a potency similar to that observed in infected cells (Baginski et al., 2000). This is a striking result given that the previously reported IC₅₀ for recombinant BVDV NS5b polymerase was 10-fold less than that obtained in infected cells (Baginski et al., 2000). We likewise observe that the inhibition of BVDV NS5b polymerase activity is 10-fold less compared to that of a single cycle infectivity assay. The equivalent sensitivities demonstrated by the replicase and viral infectivity assays indicate that the antiviral effect is attributed to the inhibition of RNA synthesis catalyzed by replication complexes in infected cells and that the replicase activity is an in vitro correlate of replication in cells. Additionally, the more pronounced effect of the inhibitor upon replicase in comparison to NS5B activity may reflect fundamental differences in the function of NS5b in the context of membrane complexes and/or the mode of action of the inhibitor.

Overall, BVDV replicase complexes isolated from infected cells exhibit properties of RNA synthesis which are hallmarks for the positive strand RNA viruses, typified by the asymmetric synthesis of product RNAs, the recycling of replicative intermediates, and the initiation of RNA synthesis. Clearly, more work is necessary to establish the functional contributions of the individual nonstructural proteins to replicative activity and the mechanism(s) involved in the initiation of RNA synthesis. Nonetheless, the in vitro system described here offers the propensity to study replicase activity isolated in its native state from infected cells which will aid in mechanistic studies and in the characterization of inhibitors of Flaviviridae replication.

Materials and methods

Compound 1, 3-[(2-dipropylamino)ethyl]thio]-5h-[1,2,4]-triazino[5,6-b]indole, was synthesized according to that previously described (Gladych et al., 1972).
Isolation of CRC and PNS fractions from infected MDBK cells

Cells (MDBK) at 85% confluency were infected with or without BVDV (NADL) at MOIs ranging from 0.1 to 2.0 and incubated at 37°C, 5% CO₂, until 75% CPE was observed in monolayers. Routinely, cells were infected at an MOI of 0.1 for 48 hr and replicate complexes were isolated by modification of previously described procedures (Grun and Brinton, 1986; Takegami et al., 1983). Briefly, cells were scraped and washed in cold phosphate-buffered saline, pelleted by centrifugation at 800g for 5–10 min at 4°C, and then resuspended in ice-cold hypotonic lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 15% glycerol) at 2.5 × 10⁵ cell equivalent/μl. A 2.3 mg/ml total protein by Bradford assay (Bio-Rad, Inc.), and stored at −70°C. The perinuclear supernatant was obtained by shearing of nuclei resuspended in buffer [10 mM Tris–Cl, pH 8.0, 10 mM NaCl] at 1.25 × 10⁶ ceq/μl, six times each in 20- and 26-gage needles, and then clarified at 1000g for 10 min, 4°C, and stored at −70°C.

Replicase assay

Indicated amounts (3.1 × 10⁵–1.25 × 10⁶ ceq) of CRC or PNS fractions were incubated in a reaction containing 50 mM Hepes, pH 8.0, 3.5 mM magnesium acetate, 10 mM DTT, 50 mM KCl, and 1 mM each of A, G, and UTP, 10 μM CTP, and 10 μCi 32P-CTP [3000 Ci/mMole], RNasin, 2.5 mM phosphoenol pyruvate, 0.7 units pyruvate kinase, and 40 μg/ml act D at 35°C for the indicated times. RNA was phenol extracted, ethanol precipitated, and then heat denatured in glyoxal sample buffer (Ambion, Inc.) at 55°C for 45 min and electrophoresed on 0.7% agarose–glyoxal denaturing gels or was partially denatured in 1X sample buffer and electrophoresed on 3% PAGE–7 M urea gels as described previously (Chu and Westaway, 1985). Alternatively, purified RNA products were hybridized to cDNA probes immobilized on a nitrocellulose filter and quantitated by phosphorimager analysis or TCA precipitated on glass filters and quantitated by liquid scintillation counting. Compound 1, DMSO, or heparin was added for 5 min to the enzyme reaction prior to initiation with NTPs when indicated. Inhibitory concentration 50% (IC₅₀) was determined as a percentage of the DMSO control by fitting the data to a four-parameter fit function using Kaleidagraph software (Synergy Software, Reading, PA).

RNA protection assay

RNA products synthesized in reactions containing CRC replicase (1.25 × 10⁶ ceq) isolated from mock or BVDV infected MDBK cells in the absence of [³²P]-CTP at 0 or 60 min were purified by phenol extraction and ethanol precipitation and then subjected to RNA protection using an RPA kit II, according to the manufacturer’s protocol (Ambion, Inc.). Probes were prepared by runoff transcription in the presence of [³²P]-UTP from the 5’ NTR for the sense probe and from the 3’ NTR for the antisense probe, gel purified, and hybridized to RNA overnight at 37°C. Hybrids were digested with RNase A/T1 for 30 min at 37°C. Following precipitation, the products were electrophoresed on 5% PAGE–8 M urea gels and quantitated by phosphorimager analysis.

Western blot analysis

Proteins were electrophoresed on 10% Bis–Tris Nupage (Novex) gels in MES or Mops buffer and then transferred onto PVDF membranes. Membranes were blocked in PBS containing 1.5% Tween 200 and 3% BSA and then incubated with appropriate antisera diluted in wash buffer, PBS containing 0.1% Tween 20 for 1–2 h at RT, washed extensively, and then incubated with HRP-conjugated antibody provided with ECL (Amersham) kit reagents. Blots were processed with ECL kit reagents and autoradiographed. Anti-peptide antisera were generated in rabbits by N-terminal cysteine coupling to KLH (Zymed, Inc.): anti-NS2 (No. 1541–1560), anti-NS3 (No. 2296–2309), anti-NS4A (No. 2397–2422), anti-NS4B (2546–2562); anti-NS5B (No. 3837–3849).

Northern blot analysis

RNA was purified by phenol extraction and ethanol precipitation from CRC replicase reactions (1.25 × 10⁶ ceq) at various times and then electrophoresed on 0.7% agarose–glyoxal denaturing gels. RNA was transferred onto nylon membranes, blotted, and probed according to the manufacturer’s protocol using the Northern Max-Gly kit (Ambion, Inc.) with [³²P]-labeled NS5b sense or antisense RNA probes (1–2 × 10⁵ cpm/ml). Blots were quantitated by phosphorimager analysis.

LiCl precipitation and RNase sensitivity assays

LiCl precipitation and RNase sensitivity assays were performed as previously described (Behrens, et al., 1996; Chu and Westaway, 1985). Briefly, purified replicate products were treated with 2 M LiCl overnight at 4°C and then pelleted at 17,000g. Precipitates were resuspended in a volume equivalent to the supernatant fraction and the RNA was ethanol precipitated from both fractions and then electrophoresed on a 3% PAGE–7 M urea gel as described.
previously (Chu and Westaway, 1985). Where indicated, products were digested with RNase A/T1 in high salt (500 mM NaCl) or low salt (50 mM NaCl) in 10 mM Tris–Cl, pH 7.5, 5 mM EDTA) for 20 min at 37°C and ethanol precipitated prior to electrophoresis (Behrens et al., 1996).

**BVDV infectivity assay**

Compound or DMSO was added to MDBK cells growing in 96-well cytostar plates at 80% confluence and were then infected at an MOI of 0.3 with the NADL strain for 24 h. Medium was removed and cells were fixed in formalin, permeabilized with 0.25% Triton X-100 in PBS for 20 min at room temperature, and then hybridized overnight at 50°C with an anti-sense, 33P-labeled probe (1 × 106 cpm), transcribed from the BVDV 3’ NTR. Plates were washed and then treated with RNase A (20 μg/ml) in RNase A buffer at room temperature for 30 min and then washed in 0.25 × SSC/well at 65°C/20 min and counted in a Top-Count (Packard, Inc.).

**BVDV NS5b polymerase assay**

BVDV NS5B protein was purified according to the method described previously (Carroll et al., 2000) from Sf21 cell pellets following expression of full-length BVDV NS5B protein (NADL) in a Baculovirus vector. RNA-dependent polymerase activity was assayed in a total volume of 50 μL and was measured as the incorporation of [α-32P]GTP using the RNA template (t500) as described. Compound or DMSO were added to a reaction mix containing enzyme and template was added and then initiated by the addition of a mixture of NTPs (1 μM GTP, 500 μM ATP, UTP, CTP) and incubated at 37°C. Reactions were quenched 20 μL of 0.5 M EDTA, and product formation was determined by DE-81 filter binding (Whatman) as previously described (Carroll et al., 2000).

**References**


