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Properties of the bovine viral diarrhoea virus replicase in extracts of infected MDBK cells

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Summary. An assay for the bovine viral diarrhoea virus (BVDV) replicase was developed using extracts from BVDV-infected cells. The replicase activity was maximal approximately 8 h post-infection as measured by the generation of a genomic length radiolabelled RNA. Using a semi-denaturing gel system, three virus-specific in vitro radiolabelled nascent RNA species were identified. A fast-migrating RNA was demonstrated to be the double-stranded replicative form (RF). A second form was shown to be a partially single-stranded/partially double-stranded RNA, characteristic of the replicative intermediate (RI). A third form, which was often undetectable, migrated between the RF and RI and was probably genomic viral RNA. The optimal replicase activity was dependent on 5–10 mM Mg^{2+} and although it was also active in 1–2 mM Mn^{2+} it was inhibited at higher concentrations. The optimum KCl concentration for labelling of the RI and RF were different, suggestive of at least two distinct replicase activities. These results are supportive of a semi-conservative model of BVDV RNA replication.

Introduction

Bovine viral diarrhoea virus (BVDV) is a member of the genus *Pestivirus* of the family *Flaviviridae* and causes acute and persistent infections in cattle. It is closely related to the hepatitis C viruses (HCV) (reviewed in [22]). BVDV is an enveloped virus with a 12.5 kb plus-sense RNA genome [7, 9] which encodes a single long open reading frame. The resulting polyprotein is processed by host and virus-encoded proteases [1, 8, 24, 25]. Cap-independent translation is mediated by an internal ribosome entry site (IRES) located in the 5′ untranslated region (5′UTR) [21]. The gene order in the noncytopathic biotype genome is 5′ N^{pro}-C-E_0-E_1-E_2-p7-NS2/3-NS4A-NS4B-NS5A-NS5B 3′ [8]. Strains of the cytopathic biotype

can be distinguished by genomic rearrangements which result in processing of the NS2/3 polypeptide into mature NS2 and NS3 [14, 19, 20].

Recombinant NS5B of BVDV has been shown to have RNA-dependent RNA polymerase activity [27] and is capable of de novo initiation on a synthetic oligoribonucleotide corresponding to the 3′ terminus of the BVDV minus-strand [15] while NS3 has been demonstrated to have protease and helicase activity [23]. Both proteins are likely to be virus-encoded components of the replication complex. Two 5′ terminal hairpin structures in the 5′UTR of BVDV [11] and 126 nucleotides of the N^{pro}-coding region proximal to the 5′UTR [2] contain important cis-acting sequences for replication, as does the 3′UTR [3].

Plus- and minus-sense viral RNAs were detected by Northern blot hybridisation analysis of BVDV-infected cells [12]. The ratio of plus:minus-sense RNA increased from 2:1 at 4 h pi to 10:1 at 12 h pi and later time points. Three virus-specific RNAs were radiolabelled in vivo [12] and identified as the double-stranded replicative form (RF), the partially single-stranded/double-stranded replicative intermediate (RI) and single-stranded genomic RNA (vRNA). After purification by sucrose gradient centrifugation, the RI form was shown to have approximately 6 nascent plus-strands [13].

In this study, we prepared extracts of BVDV-infected cells that were active for the viral replicase and characterised this activity using fully and semi-denaturing gel electrophoresis of the reaction products. An updated model for pestivirus RNA replication is proposed based on these data.

Materials and methods

Virus strain and cells

In this study, the experiments were performed using Madin-Darby bovine kidney cells (MDBK). The cells were routinely passaged in Dulbecco's modified Eagles medium (DMEM; Life Technologies) containing 10% pestivirus-free adult bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin sulphate. To infect the cells, stock BVDV (noncytopathic Trangie strain) in DMEM (without adult bovine serum) was added to MDBK cells at a multiplicity of infection (MOI) of 5, then adsorbed for 1 h at 37 °C. The inoculum was then replaced with fresh medium and the cells were incubated at 37 °C (t = 0 pi). At 3 h prior to harvest, 3 μg/ml actinomycin D (AMD; Roche) was added to the medium.

Preparation of cell extracts

The replicase reaction was a modification of a published procedure [4]. Briefly, cells were harvested at 24 h pi unless indicated otherwise, washed three times in PBS, then resuspended to approximately 4×10^7 cells/ml in homogenisation buffer (10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 10% glycerol) and lysed by passage through a 26 gauge needle 40 times. The homogenate was centrifuged at 1000 **g** for 10 min at 4 °C. The pellet was resuspended in a volume corresponding to 4×10^7 cell-equivalents/ml, then stored in aliquots at −80 °C until use. The extract was active for at least 12 months.

Replicase reactions

Unless otherwise indicated, the standard in vitro replicase reactions contained 50 mM Tris-HCl, pH 7.5 or 8.0; 0.5 or 1 mM each of ATP, CTP and UTP; 10 μM GTP; 10 mM magnesium

acetate; 10 mM dithiothreitol; 50 μg/ml AMD; 5 mM phosphoenopyruvate (Roche); 30 U/ml pyruvate kinase (Roche); 100 U/ml RNasin (Promega); 10 μCi [α-^{32}P]GTP (3000 Ci/mmole) (ICN); and 50 μl of cell extract in a 100 μl reaction volume. The reactions were incubated for 2 h at 30 °C or 37 °C (see Results) and were terminated by the addition of SDS to 0.1%. The reaction products were extracted in phenol:chloroform (1:1) then chloroform, and ethanol precipitated. In vitro radiolabelled RNAs were fractionated in LiCl [6] and digested with 1 μg/ml RNase A for 1 h in reactions containing high salt (1×SSA: 0.15 M NaCl, 0.015 M sodium acetate, pH 7.0) or low salt (0.01×SSA) as described [4, 13].

Two gel systems were used in this work. For fully-denaturing agarose gel electrophoresis, the dried nucleic acid pellet was redissolved in denaturing solution (50% formamide; 2.2 M formaldehyde; 0.02 M MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA), heated at 65 °C for 15 min, then loading buffer was added (5% glycerol, 9 mM Tris-borate, 0.2 mM EDTA, pH 8.0, 0.025% bromophenol blue) and the sample subjected to electrophoresis on a 2.2 M formaldehyde-1% agarose gel in electrophoresis buffer (0.02 M MOPS, pH 7.0, 8 mM sodium acetate and 1 mM EDTA).

For semi-denaturing polyacrylamide gel electrophoresis, the nucleic acid pellet was dissolved in gel-loading buffer (7 M urea; 5% glycerol, 9 mM Tris-borate, 0.2 mM EDTA, pH 8.0, 0.025% bromophenol blue), heated at 37 °C for 5 min then subjected to electrophoresis on a 7 M urea-3% polyacrylamide gel containing TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) as described [4]. Gels were dried and exposed to X-ray film. β-emissions were quantified on a phosphorimager (Molecular Dynamics).

Results

Extracts prepared from MDBK cells were assayed for in vitro BVDV replicase activity. MDBK cells were infected with BVDV at an MOI of 5 and extracts were prepared at various time points pi. The products of the replicase reaction were then analysed using denaturing agarose gels (Fig. 1A, B). A single radiolabelled RNA product was derived from BVDV-infected – but not uninfected – cells. This RNA co-migrated with a full-length in vitro transcript of BVDV RNA (data not shown). Radiolabelling of the product of the in vitro assay was detected from 5 h pi onwards, was maximal at 8 h pi and decreased with time but was still present at 72 h pi. No virus-specific RNAs smaller than the discrete product were visible.

The replicase reaction products were also analysed on a semi-denaturing gel system. Using this system, which is more informative than fully denaturing gels, RF is the fastest-migrating, followed by vRNA and then RI, which barely enters the gel [5]. Two bands were labelled prominently by comparison with the uninfected controls (Fig. 1C, lane 1). To characterise these, the purified reaction products were fractionated in 2 M LiCl. After fractionation, the fast-migrating band remained in the LiCl soluble fraction, consistent with the double-stranded nature of the RF (Fig. 1C, lane 8), whereas the slow-migrating form was present in the insoluble fraction (Fig. 1C, lane 5), consistent with the partially single-stranded/double-stranded nature of the RI [6]. A small amount of the fast migrating form was present in the insoluble fraction and may have been carried over during the precipitation (Fig. 1C, lane 5).

With the exception of a small residue in the unfractionated material (Fig. 1C, lane 4). the slow- and fast-migrating forms were both sensitive to RNase A digestion in low salt (Fig. 1C, lanes 4, 7 and 10). After digestion in high salt,

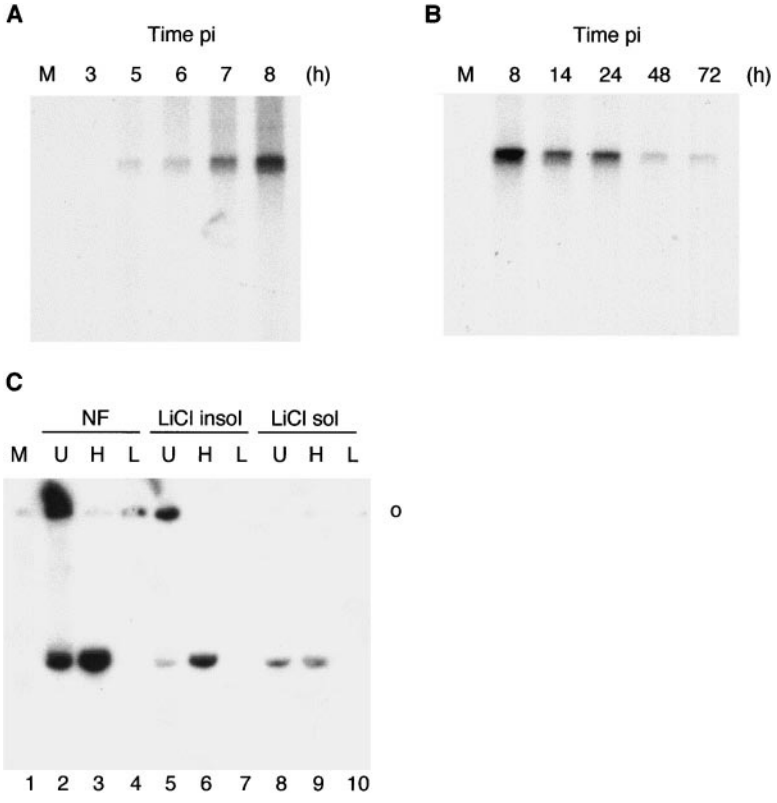

Fig. 1. Synthesis and characterisation of BVDV-specific RNA in vitro. MDBK cells were infected and extracts prepared at **A** 3, 5, 6, 7 and 8 h pi, **B** 8, 14, 24, 48 and 72 h pi, assayed for replicase activity under standard conditions at 30 °C and analysed by 2.2 M formaldehyde-1% agarose gel electrophoresis. **C** Purified in vitro replicase reaction products prepared under standard conditions at 37 °C were LiCl fractionated, digested with RNase A in high or low salt and separated by 7 M urea-3% PAGE. The origin of electrophoresis is indicated (o). *M* Mock-infected cells; *U* undigested; *H* high salt; *L* low salt. *NF* no LiCl fractionation; *LiCl insol*. LiCl insoluble fraction; *LiCl sol*. LiCl soluble fraction

the slow-migrating band in both the unfractionated (Fig. 1C, lane 3) and LiCl-insoluble (Fig. 1C, lane 6) material disappeared with a concomitant increase in the signal of the fast-migrating form whereas the fast-migrating form was resistant to digestion with RNase A in high salt (Fig. 1C, lane 9). The properties of the slow-migrating form indicated that it consists of partially single-stranded/double-stranded RNA, consistent with the previously identified replicative intermediate (RI) [12]. Likewise, the LiCl-soluble nature of the fast-migrating form, and its resistance to digestion with RNase A in high salt, indicates that this represents the double-stranded replicative form (RF). The reaction product which migrated between RF and RI (Fig. 2B) was not detected reproducibly and, consequently, it was not characterised. However, it has the same electrophoretic migration properties as the single-stranded genomic RNA of Kunjin virus, and is, therefore, most likely to be vRNA.

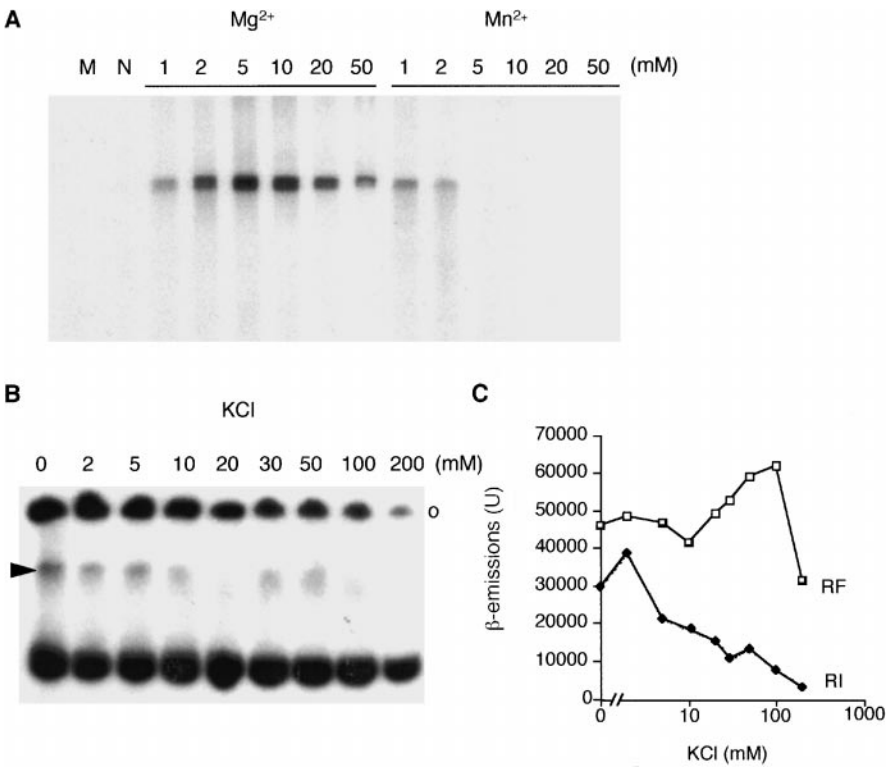

Fig. 2. Effect of variation of the concentration of exogenous reaction components of BVDV in vitro replicase activity. **A** The effect of metal ions. The reactions were performed under standard conditions at 30 °C with $MgCl_2$ or $MnCl_2$ replacing magnesium acetate at the indicated concentrations. The reaction products were analysed by 2.2 M formaldehyde-1% agarose gel electrophoresis. *M* Mock-infected cells; *N* no metal ions. **B** The effect of KCl. The reactions were performed under standard conditions at 37 °C and the reaction products analysed by 7 M urea-3% PAGE. The origin of electrophresis is shown (o). The concentrations of KCl were varied as indicated. The uncharacterised in vitro replicase reaction product is indicated by an arrow. **C** Graphical representation of β-emissions (arbitary units) of RF (□) and RI (◆) from B

The exogenous components of the replicase assay were then characterised. The replicase was Mg^{2+}-dependent and increasing concentrations of $MgCl_2$ to 5–10 mM resulted in higher replicase activity. $MgCl_2$ concentrations which were higher than 10 mM were inhibitory (Fig. 2A). The replicase was also active in 1 and 2 mM Mn^{2+} but no activity was detectable at higher concentrations of this ion (Fig. 2A). The replicase activity was optimal at pH 7.0–7.5 and 30 °C (data not shown). It was not clear if this was due to improved incorporation of radiolabel or a reduction in endogenous RNase activity at 30 °C relative to 37 °C. Radiolabelling of the RI was maximal in 2 mM KCl, and declined thereafter (Fig. 2B,C). The RF was more tolerant of KCl over a broader range of concentrations, and labelling increased with increasing KCl concentration up to 100 mM KCl, but declined at a concentration of 200 mM. Thus, there are at least two distinct activities associated with the in vitro replicase assay.

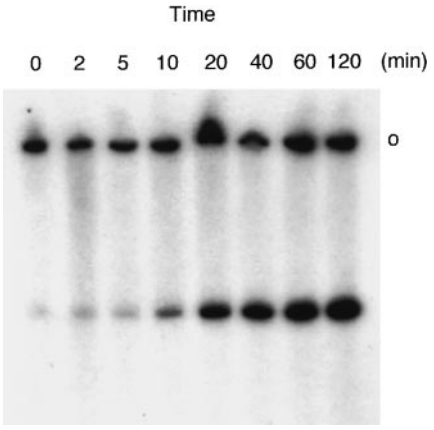

Fig. 3. Labelling of the BVDV in vitro replicase assay products. Extracts of MDBK cells were assayed for in vitro replicase activity in a reaction modified for optimal RF radiolabelling (standard reaction with 5 mM magnesium acetate and 100 mM KCl). The reaction was incubated at 30 ° and terminated at the times indicated, followed by analysis of the reaction products by 7 M urea-3% PAGE. The origin of electrophoresis is indicated (o)

Under conditions optimised for labelling of the RF (5 mM magnesium acetate, 100 mM KCl) the RI was labelled strongly almost immediately (Fig. 3). Trace levels of label were also incorporated into the RF at the early time points; however, the RF only became strongly labelled around 10 min and increased over the course of the reaction. The sequential nature of labelling indicates that the labelled RI is likely to be a precursor to the labelled RF.

Discussion

In this study we have demonstrated a virus-induced replicase activity in BVDV-infected MDBK cells. Incorporation of radiolabel, indicative of BVDV replicase activity, was Mg^{2+}-dependent, as is the RNA-dependent RNA polymerase of Kunjin virus [5]. Recombinant BVDV [16] and HCV [10, 17, 26] NS5B activities are optimal at 5–10 mM $MgCl_2$, more active if $MnCl_2$ is substituted for $MgCl_2$ at the same concentration [10, 16, 17], and inhibited by the monovalent salts NaCl [16] or KCl [17, 26]. Since concentrations of $MnCl_2$ greater than 2 mM inhibited the BVDV replicase, and assuming that the HCV and BVDV replicases function in a similar manner, these results suggest that other host or virus-encoded protein(s) may be associated with the BVDV replicase that are capable of altering its reaction properties. Alternatively, these differences may reflect opposing requirements for the activity of purified recombinant NS5B on a synthetic primer-template compared with the replicase on an authentic viral RNA template.

An updated model based on that proposed previously for Kunjin virus [4] and BVDV [12], and incorporating our new work, is shown in Fig. 4. According to this model, incorporation of radiolabel into the RF occurs either by minus-strand synthesis on a plus-strand template (Fig. 4, steps 5–6) or by completion of RNA elongation on a RI on which no new replication complexes have initiated (Fig. 4B, step 2). Since the rate of minus-stand synthesis is negligible at the point when the infected cells were harvested [12], the RF could only incorporate radiolabel during synthesis of plus-strand i.e. by semiconservative asymmetric replication (Fig. 4, steps 2–4). The possibility that the RF and RI were labelled by a virus-induced terminal transferase was considered unlikely because radiolabelling would then

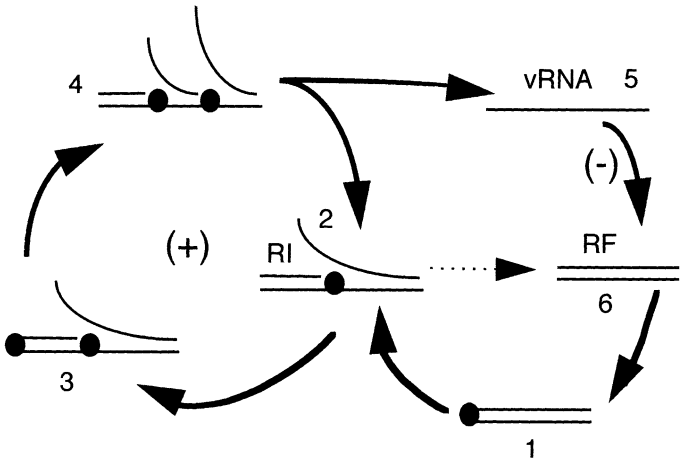

Fig. 4. Model of genomic RNA (vRNA) synthesis in vivo. Initiation of a plus-strand replication complex (dark circle) (*1*). Elongation by the replicase displaces the plus-strand from the RI template (*2*). Continued elongation of the nascent strand and re-initiation of the plus-strand replication complex (*3*). Elongation is completed (*4*) resulting in dissociation of the vRNA (*5*) from the template, and the RI is recycled. RF (*6*) is a product of the reaction when either vRNA is used as a template for minus-strand synthesis or when no new replication initiation complexes on the RI during synthesis of the last nascent strand (dotted arrow)

be expected to demonstrate similar kinetics, and this was not the case. In addition, [α-^{32}P]GTP is not a suitable substrate for end-labelling activities in mammalian cells [18].

The differential labelling of RF and RI suggest that they were labelled, at least in part, by separate activities. These activities may be (1) the initiation of a plus-strand replication complex on RF or RI templates (Fig. 4, steps 1 and 3, respectively) and (2) RNA elongation by the replicase (Fig. 4, steps 2–4). The accumulation of radiolabelled RF may result from a reduced rate of initiation over the course of the reaction. Alternatively, the replicase may pause at the template terminus on completion of elongation. Hence, the differential labelling of RI and RF may be due to inhibition of nascent strand release (Fig. 4, step 4). In this case, initiation fails to occur in vitro and the accumulation of the RF (Fig. 3) indicates that it may be a stable product of RNA synthesis. If this occurs in vivo, this may contribute to the persistent nature of BVDV infection.

The above results are consistent with previous in vivo labelling of BVDV-infected cells and are further supportive of a semi-conservative model for the synthesis of pestivirus plus-strand pestivirus RNA. This work may be relevant to the replication of HCV, for which a reliable cell culture system is lacking. The development of a model for BVDV replication will help in our understanding of HCV replication.

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