Persistence of Bovine Viral Diarrhea Virus Is Determined by a Cellular Cofactor of a Viral Autoprotease

T. Lackner,[†] A. Müller,[†] M. König, H.-J. Thiel, and N. Tautz*

Institut für Virologie (Fachbereich Veterinärmedizin), Justus-Liebig-Universität Gießen, Gießen, Germany

Received 4 March 2005/Accepted 27 April 2005

Polypeptide processing control is a crucial step in the life cycle of positive-strand RNA viruses. Recently, a vital autoprotease generating an essential viral replication factor was identified in such a virus, namely, the pestivirus bovine viral diarrhea virus. Surprisingly, the activity of this protease, which resides in nonstructural protein 2 (NS2), diminishes early after infection, resulting in the limitation of viral RNA replication. Here, we describe that a cellular chaperone termed Jiv (J-domain protein interacting with viral protein) acts as a cofactor of the NS2 protease. Consumption of the intracellular Jiv pool is responsible for temporal regulation of protease activity: overexpression of Jiv interfered with regulation and correlated with increased accumulation of viral RNA; downregulation of the cellular Jiv level accelerated the decline of protease activity and reduced intracellular viral RNA levels and virion production. Accordingly, the amount of a cellular protein controls pestiviral replication by limiting the generation of active viral protease molecules and replication complexes. Importantly, this unique mechanism of replication control is essential for maintenance of the noncytopathogenic phenotype of the virus and thereby for its ability to establish persistent infections. These results add an entirely novel aspect to the understanding of the molecular basis of viral persistence.

The *Flaviviridae* family comprises the genera *Flavivirus*, *Pestivirus*, and *Hepacivirus*; the latter includes the human pathogen *Hepatitis C virus* (HCV) (13). With an estimated 200 million cases of chronic infections worldwide, HCV is a major cause of liver cirrhosis and hepatocellular carcinoma. Due to their close relationship, pestiviruses, especially bovine viral diarrhea virus (BVDV), represent a widely used surrogate system for HCV.

The single-stranded RNA genome of BVDV is of positive polarity and has a length of 12.3 kb. Gene expression occurs via translation of one polypeptide which is processed by cellular and viral proteases giving rise to 12 mature proteins (18). Processing of nonstructural protein 2-3 (NS2-3) into NS2 and NS3 is exerted by a recently characterized viral cysteine autoprotease located in NS2 (17). Interestingly, this enzyme is distantly related to the HCV NS2-3 protease which mediates the analogous cleavage in the HCV polypeptide (11, 14, 18). The NS4A-dependent chymotrypsin-like serine protease in NS3 catalyzes four processing events in the viral polypeptide (29, 32, 33). Moreover, NS3 has helicase and NTPase activity (28, 31). The enzymatic functions of NS3 are essential for viral RNA replication which is accomplished by a replication complex (replicase) containing NS3 and four other NS proteins including NS5B, the viral RNA-dependent RNA polymerase (34), as essential constituents (3, 12). The NS2 protease-mediated cleavage of NS2-3 is essential for replication of BVDV, since its cleavage product, NS3, cannot be functionally replaced by NS2-3 in the viral replicase (17). Uncleaved NS2-3

plays a crucial but so-far-undefined role in the generation of infectious progeny virus (1).

In tissue culture cells, BVDV and other pestiviruses appear in two biotypes: the replication of noncytopathogenic (noncp) strains of BVDV does not induce obvious changes in cell morphology and viability, whereas cytopathogenic (cp) isolates cause the death of the infected cell (21). Only the noncp BVDV strains have the remarkable capability to establish life-long persistent infections, which is of crucial importance for the maintenance of BVDV in cattle populations worldwide. In this context, it is noteworthy that specifically in noncp BVDV-infected cells, a unique temporal regulation of NS2-3 processing was observed (17). While cleavage is almost complete in the first hours of infection, its efficiency decreases below the detection level as early as 9 h after infection. The resulting decrease in NS3 release correlates with a severe downregulation of viral RNA replication. cp BVDV mutants emerge from noncp ancestors in the course of persistent infection and cause progression to lethal disease. These cp BVDV strains have lost the ability to downregulate the generation of NS3 during infection and are characterized by a dramatically upregulated RNA synthesis and their inability to establish persistent infections (17–19, 21). These findings emphasize the biological significance of NS2-3 cleavage regulation in biotype control and viral persistence. The subject of this study was the molecular basis for the unique temporal regulation of the NS2 autoprotease in noncp BVDV-infected cells. One possible mechanism was a limited amount of a cellular factor required for NS2-3 cleavage. With respect to this hypothesis, it was intriguing that overexpression of a cellular chaperone of the J-domain family strongly stimulated NS2-3 processing in noncp BVDV-infected cells, leading to a change in the viral biotype from noncp to cp (22, 25). Moreover, in BHK-21 cells, noncp BVDV-derived NS2-3 displayed significant cleavage only upon coexpression of this chaperone (25). Since NS2 and the chaperone form a

* Corresponding author. Mailing address: Institut für Virologie (FB Veterinärmedizin), Justus Liebig Universität Gießen, Frankfurter Str. 107, 35392 Gießen, Germany. Phone: 49 641 99 3 83 94. Fax: 49 641 99 3 83 59. E-mail: Norbert.Tautz@vetmed.uni-giessen.de.

[†] T.L. and A.M. contributed equally to this work.

complex, the latter was termed "J-domain protein interacting with viral protein" (Jiv); its human ortholog, HDJ3, has been recently described (6). For interaction with NS2 as well as for NS2-3 cleavage induction, a 90-amino-acid (aa) fragment of Jiv, termed Jiv90, was found to be sufficient (25). The mechanism by which Jiv or Jiv90 stimulates NS2-3 cleavage as well as the significance of the intracellular Jiv level for viral replication were studied in this report. A central question was the role of Jiv/Jiv90 in NS2-3 cleavage.

The data obtained strongly suggest that cellular Jiv acts as an activating cofactor of the viral NS2 autoprotease which in turn regulates viral RNA replication. A direct correlation between alterations in the intracellular Jiv level and the efficiency of NS2-3 cleavage as well as viral RNA replication could be demonstrated. The observed limitation of viral RNA replication by the level of a cellular chaperone represents a unique regulatory mechanism which is crucial for the ability of this virus to establish lifelong persistent infections.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells, bovine kidney fibroblast cell line PT, and BHK-21 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were maintained at 37°C and 5% CO₂. PT cells were kindly provided by R. Riebe (Friedrich-Loeffler-Institut, Riems, Germany). Cell line MDBK_{tet-on}Jiv, which allows an inducible overexpression of Jiv, has been published previously (25). Vaccinia virus modified virus Ankara-T7pol (27) was generously provided by G. Sutter (GSF, Oberschleißheim, Germany). BVDV strains NCP8 and NCP7 were described previously (8).

Antibodies and antisera. The anti-Flag tag and anti-glutathione-S-transferase (GST) monoclonal antibody (MAb) were purchased from Sigma-Aldrich (Taufkirchen, Germany). For the detection of NS3, mouse MAb 8.12.7 (7) was used. Secondary species-specific antibodies were purchased from Dianova (Hamburg, Germany).

Expression plasmids. pCITE (Novagen, Madison, Wis.) encompasses the internal ribosomal entry site of encephalomyocarditis virus downstream of the T7 RNA polymerase promoter. The following constructs are based on pCITE and code for the indicated amino acids (the amino acid positions refer to BVDV strain SD-1 [9]): pflag-NS2-4A (MDYKDDDDKL followed by aa 1137 to 2336 of BVDV NCP7), pflag-NS2 (MDYKDDDDKL followed by aa 1137 to 1589 of BVDV NCP7), and pGST-Jiv90 (based on pCITE and codes for GST followed by aa 533 to 622 of bovine Jiv [Jiv90]). Truncations of Jiv90 were generated by PCR and code for GST and the indicated amino acids of the bovine Jiv protein: pGSTJiv90ΔN10 (aa 543 to 622), pGSTJiv90ΔN20 (aa 553 to 622), pGSTJiv90ΔN30 (aa 563 to 622), pGSTJiv90ΔN40 (aa 573 to 622), pGSTJiv90ΔN50 (aa 583 to 622), pGSTJiv90ΔN60 (aa 593 to 622), pGSTJiv90ΔC5 (aa 543 to 617), pGSTJiv90ΔC10 (aa 543 to 612), pGSTJiv90ΔC20 (aa 543 to 602), pGSTJiv90ΔC30 (aa 543 to 592), pGSTJiv90ΔC40 (aa 543 to 582), pGSTJiv90ΔC50 (aa 543 to 572), pGSTJiv90ΔC60 (aa 543 to 562), and pGSTJiv90ΔN10/ΔC5 (aa 543 to 617). Mutations within Jiv or NS2 were introduced by PCR or the QuikChange method (Stratagene, Heidelberg, Germany). All constructs were verified by DNA sequencing.

Metabolic labeling of transiently expressed proteins. For transient expression of proteins, the T7-vaccinia virus system was used. A total of 10⁶ BHK-21 cells per 3.5-cm tissue culture dish were infected with vaccinia virus modified virus Ankara-T7pol at a multiplicity of infection of 5 in 1 ml culture medium lacking fetal calf serum for 1 h at 37°C. For transfection of plasmid DNA (3 μg), Superfect was applied (QIAGEN, Hilden, Germany). Two hours posttransfection of plasmid DNA, cells were incubated for 30 min with Dulbecco's modified Eagle's medium without cysteine and methionine (label medium) at 37°C prior to the addition of 1 ml of label medium containing 70 μCi of [³⁵S]methionine- [³⁵S]cysteine (³⁵S-ProMix; Amersham Biosciences, Freiburg, Germany) per 3.5-cm tissue culture dish; protein expression was allowed to proceed for 2 h at 37°C. Cells were lysed in radioimmunoprecipitation (RIP) assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] NP-40, 1% [wt/vol] deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], and 0.5 mM PeablocSC [Merck, Darmstadt, Germany]).

Metabolic labeling of proteins in BVDV-infected cells. BVDV infection with noncp BVDV strain NCP7 was performed in a 3.5-cm tissue culture dish at a multiplicity of infection of 10 for 1 h at 37°C. Prior to metabolic labeling, cells were incubated for 1 h with label medium at 37°C prior to the addition of 1 ml of label medium containing 300 μCi of [³⁵S]methionine- [³⁵S]cysteine (³⁵S-ProMix; Amersham Biosciences, Freiburg, Germany). Protein expression was allowed to proceed for 1 h at 37°C. Cells were lysed in RIPA buffer.

RIP and SDS-polyacrylamide gel electrophoresis (PAGE). Protein A-Sepharose (Sigma-Aldrich, Taufkirchen, Germany) and RIPA buffer were used for RIP. In order to apply the antibodies in excess amounts, they were titrated against the highest amount of antigen used throughout the study. Proteins were separated in polyacrylamide-Tricine gels with 8 or 10% polyacrylamide (also see reference 25).

Quantification of NS2-3 cleavage efficiencies. Radioactivity of proteins in the dried SDS-PAGE gels was determined by phosphorimaging. Protein amounts were calculated under consideration of the numbers of methionine and cysteine residues in the proteins. Cleavage efficiency was calculated as the quotient of the signals of an NS2-3 cleavage product and this signal plus the signal of the precursor [product/(product + substrate)].

Generation of Jiv knockdown cells. Bovine kidney fibroblast cell line PT_{tet-on}, which stably expresses the reverse tetracycline transcriptional transactivator protein of the Tet-on system (10), was established as described previously for cell line MDBK_{tet-on} (25). Due to the low endogenous expression level of Jiv in all bovine cell lines tested so far, this protein could be detected by neither Western blot nor RIP (25); accordingly, the effectiveness of Jiv-specific hairpin RNAs which were constitutively expressed by a pSUPER (5) derivative and further processed into siRNAs targeting the Jiv mRNA were tested by Western blot in BHK-21 cells overexpressing Jiv (data not shown). Based on these tests, pSUPER-Jiv1 was selected and cotransfected with pEF-Pac (25) into PT_{tet-on} cells; cell clones were selected in the presence of 5 μg/ml puromycin. In addition, pSUPER-Jiv1 encoded the green fluorescent protein under the control of the tetracycline-regulated promoter to ease the identification of stably transfected cells. The sequence inserted downstream of the H1 promoter between sites for BglIII and HindIII was GATCCCCGTGGCTCGACTCTTGACATTCAAGAGATGGTCAAGAGTCGAGCCACTTTTGGAAA (Jiv-specific sense and reverse small interfering RNA [siRNA] sequences are in boldface type). The relative amount of Jiv mRNA in PT "Jiv knockdown" cell clones and parental cells was compared by quantitative real-time reverse transcription-PCR (RT-PCR) analysis using an ABI Prism 7000 apparatus (Applied Biosystems). Cell line PT-Jiv-kd#8.7, which was used in this study, is termed PT-Jiv-kd and displayed a reduction of the Jiv mRNA amount by about 75 to 85% in several independent tests and stably maintained this status over several passages (data not shown).

Generation of PT-Jiv-kd-rescue cells. Silent mutations were introduced into pTRE-Jiv (25) coding for Jiv under the control of a tetracycline-inducible promoter at positions 1477 (T→G), 1480 (A→C), and 1483 (C→G) of the published Jiv sequence (25) to render the transcribed RNAs resistant against the Jiv-specific siRNA expressed in cell line PT-Jiv-kd#8.7. When compared to authentic Jiv mRNA, the recombinant Jiv mRNA lacked a short upstream open reading frame in the 5' nontranslated region; accordingly, the recombinant Jiv mRNA should be translated with a significantly higher efficiency (25). Two micrograms of this plasmid was cotransfected with 50 ng of plasmid pTK-Hyg (Clontech, Palo Alto, Calif.) to confer hygromycin resistance to the transfected cells. Stable cell clones were selected and grown in medium containing 0.5 mg/ml hygromycin B. Further passaging of the cells was performed in the absence of hygromycin. Jiv expression was verified in the obtained cell clones upon induction with 10 μM doxycycline (Dox) by indirect immunofluorescence using a Jiv-specific rabbit antiserum directed against recombinant Jiv90 expressed in *Escherichia coli* and a secondary cyanogen-3-labeled antibody (data not shown).

Quantitative real-time RT-PCR. Total cellular RNA was prepared using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). For comparative quantification of the cellular Jiv mRNA, 500 ng of the RNA from different cell lines was subjected to real-time RT-PCR analysis. After reverse transcription using the reverse primer Jiv02R (GCCAAGAGAAGATCCAGGTGG) and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany), the quantitative real-time PCR was run using the AbiPrism7000 Sequence Detection system (Applied Biosystems, Branchburg, N.J.) in the "Absolute Quantification" modus with TaqMan Universal PCR Master mix (Applied Biosystems, Branchburg, N.J.) without MgCl₂ in combination with the Jiv-specific probe JivTaq01 (VIC-TGACCGGCTAGGCTGGAGGGGATAAA-6-carboxy-tetramethylrhodamine) and the primer pair Jiv01 (GGCGGTTTCTGGTAGGATTG)-Jiv02R. Cycling conditions were 1 cycle (2 min at 50°C and 10 min at 95°C) followed by 40 cycles (15 s at 95°C and 1 min at 60°C).

For comparative quantification of viral genomic RNA in BVDV-infected cells,

the BVDV-1-specific probe pvtq01 (6-carboxyfluorescein-ACAGTCTGATAG GATGCTGCAGAGGCC-6-carboxytetramethylrhodamine) and the primer pair pv02 (GTGGACGAGGGCATGCC)-pv03R (TCCATGTGCCATGTACA GCAG) were used under the conditions described above. The relative mRNA amounts in comparison to control cells were in both cases calculated using the following formula: % mRNA = $1/(2^{\Delta\Delta Ct}) \times 100$.

BVDV growth curves and virus titration. Infection with noncp BVDV strain NCP8 was carried out as described previously for 1 h at 37°C (25). End-point titration was performed in four replicates on MDBK cells. Virus detection occurred after 72 h at 37°C by indirect immunofluorescence analysis using MAb 8.12.7 directed against NS3 of BVDV (7) and a secondary cyanogen-3-labeled antibody as described previously (25).

RESULTS

Induction of NS2-3 cleavage requires stoichiometric amounts of Jiv. To gain insight into the function of Jiv/Jiv90 in NS2-3 processing, the molar ratio of Jiv90 and NS2-3 required for optimal cleavage induction was determined. The N-terminal half of NS2 is highly hydrophobic and is essential for the activity of the NS2 protease (17); this property is probably the reason that we have not yet been able to establish an NS2-3 cleavage assay with purified components or by *in vitro* translation. Therefore, in our studies, we applied the T7-vaccinia virus system (27) for transient expression of NS2-3 as well as Jiv or its fragment, Jiv90, in BHK-21 cells. It has already been shown that noncp BVDV-derived NS2-3 is cleaved only upon coexpression of Jiv or Jiv90 in this system (25). Accordingly, a noncp BVDV-derived NS2-3-NS4A polypeptide fragment with an N-terminal Flag tag (Flag-NS2-4A) and Jiv90 fused to the C terminus of GST-Jiv90 were coexpressed from separate plasmids in BHK-21 cells. Together with a constant amount of plasmid DNA encoding Flag-NS2-4A, increasing molar ratios of the GST-Jiv90-coding plasmid were transfected with final ratios ranging from 0.05:1 to 2:1. Metabolically labeled Flag-NS2-3 and Flag-NS2 were isolated by RIP using a Flag-specific antibody (anti-Flag MAb) (Fig. 1A). Following SDS-PAGE, the proteins were quantified by PhosphorImager analysis, and the absolute ratio [NS2/(NS2-3 + NS2)] was calculated (Fig. 1B). According to this analysis, optimal NS2-3 cleavage required the transfection of a 1:1 molar ratio of pGST-Jiv90 and pflag-NS2-4A. This indicates that not catalytic but stoichiometric amounts of Jiv are required for optimal NS2-3 cleavage.

As observed previously, GST-Jiv90 was coprecipitated with Flag-NS2/Flag-NS2-3 (Fig. 1A) (25). SDS-PAGE and quantitative PhosphorImager analysis revealed a molar ratio of about 1:1 between the proteolytically released Flag-NS2 and GST-Jiv90 in the precipitated complexes (Fig. 1C). The molar ratio between coprecipitated GST-Jiv90 and Flag-NS2 remained unchanged when GST-Jiv90 and Flag-NS2-3 were expressed in ratios between 0.05:1 and 2:1. This result supports the assumption that each GST-Jiv90 molecule which induces the cleavage of one Flag-NS2-3 molecule remains bound to the released Flag-NS2.

The identity of only one amino acid of Jiv90 is critical for cleavage induction. To characterize the minimal part of Jiv90 required for NS2-3 cleavage induction, plasmids that encoded N- or C-terminally truncated derivatives of Jiv90 fused to the C terminus of GST were established. Upon coexpression of these proteins with Flag-NS2-4A in BHK-21 cells, NS2-3 cleavage induction was monitored by RIP analysis. This study revealed that Jiv90 can be truncated N-terminally by 10 or C-terminally

FIG. 1. Influence of the molar ratio of GST-Jiv90 and Flag-NS2-3 on NS2-3 cleavage efficiency. (A) BHK-21 cells were cotransfected with a constant amount of pflag-NS2-4A and increasing amounts of pGST-Jiv90 in the molar ratios indicated above lanes 2 to 8; molar ratios of plasmids were calculated according to their molecular masses. After metabolic labeling of the transfected cells, cell lysates were immunoprecipitated with anti-Flag MAb and further analyzed by SDS-PAGE and autoradiography. After transfection of increasing amounts of pGST-Jiv90, an about proportional increase in GST-Jiv90 was observed (data not shown). (B) Signals of Flag-NS2-3 and Flag-NS2 in the gel shown in panel A were quantified by phosphorimaging, and NS2-3 cleavage efficiency was calculated (see Materials and Methods). (C) The signals of Flag-NS2 and GST-Jiv90 in the gel shown in panel A were quantified by phosphorimaging, and protein amounts were calculated, taking into account the relative number of cysteine and methionine residues in both proteins (see also B); the highest value was set to 1.

FIG. 2. NS2-3 cleavage induction by GST-Jiv90 and its truncated derivatives. Flag-NS2-4A was transiently expressed without GST-Jiv90 (lane 1), together with GST-Jiv90 (lane 2), or together with truncations of Jiv90 as GST fusion proteins (lanes 3 to 7) in BHK-21 cells; T7-vaccinia virus-infected cells served as a control (lane 8). The size of the N- and/or C-terminal truncation of Jiv90 is indicated above lanes 3 to 7. After metabolic labeling of the transfected cells, cell lysates were immunoprecipitated with anti-Flag MAb and further analyzed by SDS-PAGE and autoradiography. wt, wild type.

by 5 amino acids without losing its ability to induce NS2-3 cleavage (Fig. 2). Quantitative analysis demonstrated cleavage induction efficiency at the wild-type level when the C-terminal 5 amino acids of Jiv90 were deleted, while deletion of the N-terminal 10 amino acids of Jiv90 reduced cleavage induction to about 50% (data not shown). Interestingly, simultaneous truncation of Jiv90 by 10 N-terminal and 5 C-terminal amino acids interfered with NS2-3 cleavage induction (Fig. 2). Accordingly, a 75-amino-acid region of Jiv is essential but not sufficient for NS2-3 cleavage induction.

To define the properties of this 75-amino-acid region of Jiv, an alanine scan was performed. Coexpression of the resulting Jiv90-GST mutants with NS2-3 revealed that few exchanges had a significant negative effect on NS2-3 cleavage induction and only the exchange of tryptophan at position 39 by alanine (W39A) abolished this process (Fig. 3A). Further analyses revealed that tryptophan 39 could be functionally replaced by phenylalanine (86% cleavage) and tyrosine (78% cleavage) but not by arginine, glutamic acid, or proline; interestingly, a replacement by histidine also rescued cleavage induction but only very inefficiently (3%) (Fig. 3B and C); the latter finding suggests that the imidazole group of histidine can substitute, at least to a limited degree, the aromatic side chain required at this position. The finding that only one amino acid with an aromatic side chain is indispensable for the ability of Jiv90 to induce NS2-3 cleavage makes it highly unlikely that Jiv90 is itself a protease.

A 20-amino-acid peptide of Jiv is essential but not sufficient for NS2 binding. Interestingly, all Jiv mutants generated for

the alanine scan were coprecipitated with Flag-NS2, including W39A (Fig. 3B). Moreover, also, Jiv90 derivatives with deletions of either the 50 N-terminal or 30 C-terminal amino acids still coprecipitated with Flag-NS2, while Jiv90 derivatives with larger truncations did not (Fig. 4); an analogous Jiv-binding profile was observed for Flag-NS2-3 encompassing mutations that interfered with NS2-3 cleavage (data not shown). When expressed as a C-terminal fusion to GST, a Jiv fragment encompassing amino acids 41 to 60 of Jiv90 still interacted with fragments of Flag-NS2 but not with the entire Flag-NS2 or Flag-NS2-3 (data not shown and our unpublished results). This Jiv-derived peptide was not capable of inducing NS2-3 cleavage (data not shown; Fig. 2).

Jiv-induced NS2-3 cleavage depends on the activity of the cysteine protease in NS2. The data obtained so far did not support a proteolytic function of Jiv90 itself. Therefore, the most likely role for Jiv in NS2-3 cleavage was that of a cofactor activating the pestiviral NS2 protease. For the activity of the NS2 cysteine protease, the two proposed catalytic residues, H1447 and C1512, are essential (17). Each of these residues was replaced by alanine individually in the Flag-NS2-4A polypeptide fragment of noncp BVDV. Changes of residues C1476 and H1483 to alanine, which were previously found to not be critical for protease activity, served as controls. Following coexpression of wild-type and mutant Flag-NS2-4A with GST-Jiv90 in BHK-21 cells, NS2-3 processing was monitored by RIP analysis (Fig. 5). Mutation of the proposed catalytic amino acids H1447 and C1512 strongly interfered with NS2-3 cleavage induction by GST-Jiv90, while cleavage of NS2-3 encompassing the control mutations was unaltered.

Interestingly, none of the above-mentioned amino acid substitutions in NS2 interfered with coprecipitation between NS2 and GST-Jiv90 (Fig. 5). Accordingly, the loss of NS2-3 cleavage observed for mutant C1512A or H1447A does not correlate with an abrogation of the Jiv90/NS2 interaction.

In conclusion, these experiments show that Jiv-induced cleavage of noncp BVDV NS2-3 depends on the activity of the NS2 protease. Thus, the most obvious interpretation of the data outlined so far is a function of Jiv as a cofactor activating the NS2 protease.

The Jiv level determines the kinetics of NS2-3 cleavage. We reported recently that the activity of the NS2 autoprotease after infection with noncp BVDV already diminishes to background levels after 9 h (17). The molecular basis for this observation was unknown. It was intriguing, however, that Jiv was found to be present in bovine culture cells only in very small amounts (25). If the decrease of NS2-3 processing during noncp BVDV infection is caused by a limiting endogenous Jiv pool in the infected cell, an increase of the intracellular level of this chaperone should interfere with regulation of NS2-3 cleavage.

To test this hypothesis, Jiv overexpression was induced in MDBK_{tet-on}Jiv cells (25) 16 h prior to infection with noncp BVDV. These cells as well as MDBK control cells infected in parallel were metabolically labeled for 1-h periods with [³⁵S]methionine and [³⁵S]cysteine. NS3 and NS2-3 were isolated from these cells by RIP and were further analyzed by autoradiography and phosphorimaging (Fig. 6A). For MDBK control cells, NS3 could not be detected when labeling occurred between 8 and 9 h postinfection or later. In contrast,

FIG. 3. Effect of amino acid exchanges in Jiv90 on NS2-3 cleavage efficiency. (A) Alanine scan. Flag-NS2-4A was transiently expressed without GST-Jiv90, together with the GST-Jiv90 wild type (wt) or together with GST-Jiv90 mutants in BHK-21 cells. The positions of the substitutions to alanine within Jiv90 are indicated at the bottom of the graph. After metabolic labeling of the transfected cells, cell lysates were immunoprecipitated with anti-Flag MAb and further analyzed by SDS-PAGE. The signals of Flag-NS2-3 and Flag-NS2 were quantified by phosphorimaging. NS2-3 cleavage efficiency was calculated (see Materials and Methods); NS2-3 cleavage efficiency using GST-Jiv90 wt was set to 100%. (B) Effect of single amino acid exchanges at position 39 of Jiv90 on NS2-3 cleavage efficiency. Tryptophan at position 39 of Jiv90 was replaced by the amino acids indicated above lanes 3 to 9. After metabolic labeling of cells transfected with pflag-NS2-4A and GST-Jiv90 wt or mutants, cell lysates were immunoprecipitated with anti-Flag MAb and further analyzed by SDS-PAGE and autoradiography. A low-level NS2-3 cleavage induction by GST-Jiv90 mutant W39H was reproducibly detected in independent experiments. (C) Quantification of Flag-NS2-3 and Flag-NS2 shown in panel B by phosphorimaging. NS2-3 cleavage efficiency was calculated as described in Materials and Methods; the highest value was set to 100%. (D) Summary of the data obtained in the Jiv90 mutagenesis study. Numbers indicate the position within the amino acid sequence of Jiv90. White background, N- or C-terminal amino acid blocks of which one at a time is dispensable for NS2-3 cleavage induction; grey background, amino acids which can be replaced by alanine without a complete loss of NS2-3 cleavage induction; black background, aromatic residue at position 39 of Jiv90 which is essential for NS2-3 cleavage induction; underlining, minimal part of Jiv90 which is essential but not sufficient for a stable interaction with pestiviral NS2 (Fig. 4).

when the Jiv-overexpressing MDBK_{tet-on}Jiv cells were monitored from 5 to 20 h postinfection, NS3 was efficiently generated at all time points (Fig. 6A). The amount of uncleaved NS2-3 steadily increased up to 12 h postinfection, indicating continuous viral replication; the quantity of NS3 increased up to 9 h postinfection and reached a stable level thereafter (Fig. 6A). Taking into account that stoichiometric quantities of Jiv are required for the induction of NS2-3 cleavage (Fig. 1), these observations are in agreement with the assumption that the steady-state level of Jiv produced in MDBK_{tet-on}Jiv cells allows

the cleavage of a given number of NS2-3 molecules per cell and hour.

To further test the effect of the endogenous Jiv level on temporal regulation of NS2-3 cleavage in noncp BVDV-infected cells, Jiv knockdown cell lines were established. These cells constitutively expressed a small hairpin transcript which was further processed into an siRNA targeting the Jiv mRNA. The PT “Jiv knockdown” (PT-Jiv-kd) cell clone selected for this study displayed 75 to 80% less Jiv mRNA than the parental PT_{tet-on} cells (for details, see Materials and Methods). These

FIG. 4. Binding of truncated GST-Jiv90 derivatives to NS2. Flag-NS2 was transiently expressed without GST-Jiv90 (lane 1) or together with truncated Jiv90 derivatives fused to the C terminus of GST (lanes 2 to 13) in BHK-21 cells. The size of the N- or C-terminal truncation of Jiv90 is indicated above lanes 2 to 13. After metabolic labeling of the transfected cells, cell lysates were immunoprecipitated with anti-Flag MAb (α -flag) (upper part) or anti-GST MAb (α -GST) (lower part) and further analyzed by SDS-PAGE and autoradiography.

cells were infected with noncp BVDV and metabolically labeled for 1-h periods with [35 S]methionine and [35 S]cysteine; PT_{tet-on} cells infected in parallel served as control. The relative amounts of NS3 and NS2-3 in these cells were determined by RIP analysis (Fig. 6B). The obtained data revealed that down-regulation of NS2-3 cleavage efficiency in cell line PT-Jiv-kd

FIG. 5. Influence of single amino acid mutations in the NS2 protease on NS2-3 cleavage efficiency in the presence of GST-Jiv90. The Flag-NS2-4A wild type (wt) (lane 2) or mutants (lanes 3 to 6) were expressed together with GST-Jiv90 in BHK-21 cells. Mutations within NS2 are indicated above lanes 3 to 6. After metabolic labeling of the transfected cells, cell lysates were immunoprecipitated with anti-Flag MAb and further analyzed by SDS-PAGE and autoradiography.

FIG. 6. Effect of the Jiv level on the kinetics of NS2-3 processing in noncp BVDV-infected cells. Cells were metabolically labeled for 1-h periods postinfection (p.i.), as indicated above the lanes. The cell lysates were immunoprecipitated with an anti-NS3 MAb, which also recognizes NS2-3, and were further analyzed by SDS-PAGE and autoradiography. (A) Effect of Jiv overexpression. MDBK cells (left part) or MDBK_{tet-on}Jiv cells induced with 10 μ M Dox for 16 h prior to infection (right part) were infected with noncp BVDV at a multiplicity of infection of 10. (B) Effect of Jiv knockdown. PT_{tet-on} cells (left part) or PT-Jiv-kd cells with a reduced Jiv level (right part) were infected with noncp BVDV at a multiplicity of infection of 10.

already occurs at 6 to 7 h postinfection and thus about 2 h earlier than in PT_{tet-on} control cells. In BVDV-infected cells, a direct correlation between the efficiencies of NS3 generation and viral RNA replication/protein synthesis has been observed (17). In accordance with these findings, viral protein synthesis was severely reduced in PT-Jiv-kd cells compared to PT_{tet-on} control cells (compare NS2-3 amounts in Fig. 6B).

According to this study, the kinetics of NS2-3 cleavage in noncp BVDV-infected cells were determined by the endogenous Jiv level. In conclusion, these data strongly support a model whereby the limiting endogenous amount of Jiv represents the molecular basis for the observed temporal regulation of NS2-3 cleavage in noncp BVDV-infected cells.

The intracellular level of Jiv balances pestiviral replication.

As described above, the endogenous Jiv level determines the kinetics of intracellular NS2-3 processing by the NS2 protease in noncp BVDV-infected cells. Since this protease has been shown to regulate pestiviral RNA replication via generation of NS3 (17), it was tempting to speculate that the intracellular Jiv level has a significant impact on the efficiency of pestiviral replication.

To address the significance of the Jiv level on pestiviral replication, MDBK_{tet-on}Jiv cells either not induced or induced for 16 h were infected with noncp BVDV; MDBK cells infected in parallel served as controls. Total cellular RNA was prepared at different time points postinfection, and the

FIG. 7. Effect of the Jiv level on viral replication. (A and B) Effect of the Jiv level on intracellular viral RNA amounts in noncp BVDV-infected cells. Cells were infected with noncp BVDV at a multiplicity of infection of 5. Total cellular RNA was prepared at indicated times postinfection, and viral RNA was quantified by quantitative real-time RT-PCR (see Materials and Methods). The graph shows mean values from three independent experiments. The amount of BVDV RNA measured at 12 h postinfection in the control cells MDBK (A) or PT_{tet-on} (B) was set to 1. (A) Overexpression of Jiv. Quantification of intracellular viral RNA isolated at different time points postinfection from MDBK cells or MDBK_{tet-on}Jiv cells which were either not induced or induced by 10 μ M Dox for 16 h prior to infection. (B) Jiv knockdown. Quantification of intracellular viral RNA isolated at the indicated time points postinfection from cell lines PT_{tet-on}, PT-Jiv-kd, and PT-Jiv-kd-rescue is shown. (C) Growth kinetics for noncp BVDV in PT-Jiv-kd cells compared to the parental PT_{tet-on} cells and to PT-Jiv-kd-rescue cells. The graph shows the mean values from three independent experiments. Cells were infected at a multiplicity of infec-

amount of viral genomic RNA was determined (Fig. 7A). At all time points, the amount of viral RNA was elevated by at least a factor of 10 in the Jiv-overexpressing MDBK_{tet-on}Jiv cells compared to MDBK cells. The noninduced MDBK_{tet-on}Jiv cells also showed a significant but less dramatic increase in intracellular viral RNA in comparison to MDBK cells; the latter finding is most likely due to the known leaky regulation of the Tet-on system. In contrast to the levels of intracellular viral RNA, the titer of infectious progeny virus harvested 48 h postinfection from the culture supernatants did not differ significantly between these cells (data not shown). This indicates that the amount of intracellular viral RNA does not limit the generation of infectious progeny virus in this system.

According to these findings, an upregulation of the intracellular Jiv level correlates with an increased efficiency of pestiviral RNA replication. In a second step, we tested the effect of the lowered endogenous Jiv level in the established Jiv knock-down cell line PT-Jiv-kd on the replication of BVDV. It was recently shown that expression of small hairpin RNAs can induce the innate immune system (4, 26), which could have a significant impact on the interpretation of the results obtained here. As a control, we therefore reestablished low-level Jiv expression in cell line PT-Jiv-kd expressing the Jiv-specific siRNAs; a recovery of viral replication in these cells would prove that the observed effects are specifically due to the intracellular Jiv level. Thus, regulated transcription of a Jiv-coding mRNA encompassing silent mutations in the Jiv-siRNA binding site was established in PT-Jiv-kd cells by applying the Tet-on system (10). A quantitative real-time RT-PCR revealed that these cells already displayed Jiv-mRNA levels higher than those in cell line PT-Jiv-kd in the noninduced state, which is most likely due to the leakiness of the Tet-on system (data not shown). Therefore, noninduced PT-Jiv-kd-rescue cells were used as a control in our experiments to mimic as closely as possible the low endogenous Jiv expression in bovine fibroblasts. The presence of the Jiv-specific siRNA in PT-Jiv-kd and PT-Jiv-kd-rescue cells was verified by Northern blot (data not shown).

To establish the effect of a decreased intracellular Jiv level on BVDV replication, we infected cell lines PT-Jiv-kd and noninduced PT-Jiv-kd-rescue as well as the parental PT_{tet-on} cells with noncp BVDV. At different time points postinfection, total cellular RNA was prepared and viral genomic RNA was quantified (Fig. 7B). The amounts of intracellular viral RNA were significantly reduced in PT-Jiv-kd cells at all time points. At 24 h postinfection, when the quantity of noncp BVDV RNA peaked in the parental PT_{tet-on} cells, viral RNA was decreased by about a factor of 12 in PT-Jiv-kd cells. In the noninduced PT-Jiv-kd-rescue cells, the amount of intracellular viral RNA was similar to that in PT_{tet-on} cells. The latter result verified that the dramatic effect of the Jiv-specific siRNA on pestiviral RNA replication in the PT-Jiv-kd cells is indeed specifically due to the downregulation of the endogenous Jiv level.

Significantly, in cell line PT-Jiv-kd, also the titer of newly

tion of 5, and the culture supernatants were harvested at the indicated time points postinfection. Virus titers are given as log 50% tissue culture infective dose per milliliter.

generated infectious progeny virus was reduced by up to about 97% ($1.5 \log_{10}$) (Fig. 7C). This effect was pestivirus specific, since the replication of rabies virus was not altered in cell line PT-Jiv-kd compared to PT_{tet-on} cells (data not shown). In PT-Jiv-kd-rescue cells, the amounts of viral RNA as well as the titer of newly generated progeny virus reached the levels observed for the parental PT_{tet-on} cells (Fig. 7C).

In conclusion, the data presented demonstrate that the endogenous level of the cellular host factor Jiv balances NS2-3 cleavage, RNA replication, and virus production of noncp strains of BVDV.

DISCUSSION

Gene expression of the large group of viruses with positive-strand RNA genomes involves translation of polyproteins. Proteolytic processing of these precursors is subtly regulated and highly critical for viral replication. Perturbations of this process as a rule interfere with viral replication (16). Along this line, the pestiviral NS2 autoprotease is vital for pestiviral RNA replication by generating NS3, an essential component of the viral replicase (17). Interestingly, the cellular chaperone Jiv forms a complex with pestiviral NS2 and stimulates NS2-3 cleavage (25); the role of Jiv in NS2-3 processing was one topic of this study. Several lines of evidence argue against a proteolytic activity of Jiv itself. First, the minimal fragment of Jiv able to induce NS2-3 cleavage is only 80 amino acids long (amino acids 11 to 90 of Jiv90) and would therefore represent the shortest protease active in *trans*. Second, in this peptide, not even the identity of a single amino acid is absolutely essential for the induction of cleavage; only an aromatic residue has to reside at position 39. This finding excludes for this Jiv peptide the existence of an amino acid motif essential for cleavage induction, a typical characteristic of all known classes of proteases. Third, Jiv can be efficiently coprecipitated with NS2, and much more than just catalytic amounts of Jiv are required for optimal induction of NS2-3 cleavage. The fact that proteases usually do not form stable complexes with their substrates together with the required amounts of Jiv strongly favor another role of this protein, namely, to function as a protease cofactor. In agreement with this hypothesis, mutations that have been shown to abolish the activity of the NS2 autoprotease (17) were also detrimental for the cleavage of noncp BVDV-derived NS2-3 in the presence of Jiv. Taken together, the results obtained strongly imply that the cellular chaperone Jiv is not a protease itself but represents a cofactor required for the activation of the pestiviral NS2 autoprotease. Future detailed studies on the interaction between these proteins together with structural data are required to elucidate the molecular basis of NS2 protease activation by Jiv.

Jiv is a member of the J-domain protein family which represents a heterogeneous group of chaperones characterized by a 70-amino-acid-long so-called J domain. Binding of substrate as well as of the J-domain protein to Hsp70 dramatically stimulates the protein folding activity of the latter. Cellular J-domain proteins have already been implicated in the replication of viruses. For example, the assembly of the replication complex of hepatitis B virus was found to require the J-domain protein Hdj1 as well as Hsp70 (2, 15). The positive-strand RNA virus brome mosaic virus depends on the assistance of

cellular chaperones including the J-domain protein Ydj1 for the formation of its replication complex (30). In contrast to these systems utilizing authentic chaperones, we observed that a small fragment of Jiv (Jiv90) was sufficient to assist in pestiviral replication. Since Jiv90 does not encompass the J-domain, a crucial determinant for Hsp70 binding, it is questionable whether members of the Hsp70 family of chaperones are essentially involved in the activation of the NS2 protease.

Probably the most intriguing aspect of the pestiviral NS2 protease is its temporal downregulation leading to a severe restriction of viral RNA replication late in noncp BVDV infection. The data obtained in this study fit into a model whereby each NS2-3 molecule recruits one Jiv molecule to activate its NS2 protease for autocleavage. This assumption is supported by the amounts of Jiv required for optimal NS2-3 cleavage and the fact that the released NS2 and Jiv could be coprecipitated in a complex in which both proteins were detected at a ratio of 1:1.

In noncp BVDV-infected cells, the synthesis rate of viral NS2-3 is much higher than the one of Jiv (25). Therefore, the pool of Jiv molecules present at the time of infection most likely is the major determinant for the number of NS2-3 molecules that will undergo autoprocessing in a noncp BVDV-infected cell. Moreover, a Jiv molecule which has induced one NS2-3 cleavage reaction seems to remain bound to NS2 (Fig. 1) and is most likely not available for further reactions. This is indicated by the results shown in Fig. 6A (left). In noncp BVDV-infected bovine fibroblasts, NS2-3 is translated in the presence of endogenous Jiv in the first 8 h postinfection. When cells were labeled between 8 and 9 h (or later), new (labeled) NS2-3 is produced in those cells; in this time window, only NS2-3 but not NS3 is detected by RIP. This suggests that Jiv molecules present in those cells are not capable of inducing further cleavages; accordingly, recycling of Jiv for cleavage induction does not take place. At the moment, we cannot distinguish whether this is due to the stable binding of Jiv to NS2 (strongly favored by the data presented in Fig. 1 and previously published work on Jiv/NS2 interaction [25]) or a shortened half-life of Jiv bound to NS2.

In naive bovine fibroblasts, detectable NS2-3 cleavage is restricted to the first 9 h postinfection (17; this study). We demonstrate here that in cells with a decreased endogenous Jiv level, NS2-3 cleavage disappears as soon as 7 h postinfection. Overexpression of Jiv led to an impairment of cleavage regulation, and a stable steady-state level of NS2-3 cleavage was observed as soon as saturating amounts of NS2-3 were available in the infected cell. Accordingly, alterations in the intracellular Jiv level strictly correlated with changes in viral RNA replication efficiency, underlining the biological significance of NS2 protease regulation by Jiv. Our data strongly suggest that the intracellular Jiv level restricts the replication of noncp BVDV by limiting the generation of NS3 and thereby the number of active replicase complexes formed in the infected cell.

Several viral proteases, like the NS3 proteases of HCV, pestiviruses, and flaviviruses, depend in their activity on cofactors, namely, NS4A of HCV and pestiviruses or NS2B of flaviviruses (18). However, in contrast to Jiv, these cofactors are virus-encoded peptides. An advantage of a cellular cofactor could be a tight coupling of viral replication to its host cell

environment and a possible determination of viral tissue tropism. Our data suggest that Jiv is an important and possibly essential host factor for noncp BVDV and other pestiviruses (this report; A. Müller and N. Tautz, unpublished data). Downregulation of the Jiv level in host cells of the virus led to a significant decrease in viral RNA replication and progeny virus production. It appears likely that replication of noncp BVDV will not occur in cells with very low or no Jiv expression. Preliminary real-time RT-PCR experiments using MDKB and PT cells did not reveal a correlation between noncp BVDV infection and changes in the cellular Jiv mRNA level. Only very limited data are presently available with respect to the expression of Jiv in different bovine cell types (23); accordingly, the significance of Jiv expression for tissue tropism of the virus in the host has yet to be established. However, our experiments clearly show that the low endogenous amount of Jiv present in bovine cells limits RNA replication of noncp BVDV; when this limitation is disturbed, e.g., in cell lines overexpressing Jiv, viral replication is strongly upregulated (this report), which correlates with a switch of the viral phenotype from noncp to cp (25). Another intriguing aspect in this context is the presence of Jiv-coding sequences in the genomes of several cp pestiviruses (21, 22). These viruses have acquired cell-derived Jiv-coding sequences by RNA recombination. Expression of the cell-derived protease cofactor Jiv from the viral genome deregulates NS3 generation, renders these mutant viruses cytopathogenic, and correlates with the progression from persistent infection to lethal disease in cattle (19, 21, 22). In the BVDV system, the maintenance of the noncp biotype is of high significance, since only noncp strains can establish lifelong persistent infections; such animals continuously shed the virus and represent its major reservoir. In contrast, cp BVDV strains have lost the ability to persist in the animal and from the epidemiological point of view may be regarded as dead-end products (20). Accumulating evidence suggests that the regulation of NS2-3 processing is the key factor for the biotype control of BVDV. While NS2-3 cleavage is strictly downregulated early after infection in noncp BVDV-infected cells, all cp BVDV isolates have lost this capacity (17, 18, 20, 21). Only one cp BVDV-derived mutant selected in cell culture by Qu and coworkers (24) displayed a deregulated NS2-3 cleavage in spite of its noncp biotype, but it is unknown yet whether this virus is capable of establishing persistent infection in the animal.

According to the data presented in this study, limiting amounts of the cellular cofactor Jiv represent the molecular basis for the temporal downregulation of NS2-3 cleavage and viral RNA replication in noncp BVDV-infected cells. This regulation is crucial for maintenance of the noncp biotype of this virus which in turn is essential for its capacity to establish persistent infections. The unique limitation of viral RNA replication by a low abundant cellular protein serving as a cofactor of a viral protease is thus part of a strategy which allows the virus to establish lifelong persistent infections. The results obtained in this study provide an entirely novel aspect to the understanding of the molecular basis of viral persistence in the host.

ACKNOWLEDGMENTS

We thank S. Jacobi for excellent technical assistance.

This study was supported by SFB 535 "Invasionsmechanismen und Replikationsstrategien von Krankheitserregern" (T.L.) and Gradui-

ertenkolleg 455 "Molekulare Veterinärmedizin" (A.M.) of the Deutsche Forschungsgemeinschaft.

REFERENCES

- Agapov, E. V., C. L. Murray, I. Frolov, L. Qu, T. M. Myers, and C. M. Rice. 2004. Uncleaved NS2-3 is required for production of infectious bovine viral diarrhoea virus. *J. Virol.* **78**:2414–2425.
- Beck, J., and M. Nassal. 2003. Efficient Hsp90-independent in vitro activation by Hsc70 and Hsp40 of duck hepatitis B virus reverse transcriptase, an assumed Hsp90 client protein. *J. Biol. Chem.* **278**:36128–36138.
- Behrens, S.-E., C. W. Grassmann, H.-J. Thiel, G. Meyers, and N. Tautz. 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. *J. Virol.* **72**:2364–2372.
- Bridge, A. J., S. Pebernard, A. Ducraux, A. L. Nicoulaz, and R. Iggo. 2003. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**:263–264.
- Brummelkamp, T. R., R. Bernards, and R. Agami. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550–553.
- Chen, J., Y. Huang, H. Wu, X. Ni, H. Cheng, J. Fan, S. Gu, X. Gu, G. Cao, K. Ying, Y. Mao, Y. Lu, and Y. Xie. 2003. Molecular cloning and characterization of a novel human J-domain protein gene (HDJ3) from the fetal brain. *J. Hum. Genet.* **48**:217–221.
- Corapi, W. V., R. O. Donis, and E. J. Dubovi. 1990. Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhoea virus. *Am. J. Vet. Res.* **51**:1388–1394.
- Corapi, W. V., R. O. Donis, and E. J. Dubovi. 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhoea infections. *J. Virol.* **62**:2823–2827.
- Deng, R., and K. V. Brock. 1992. Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathogenic bovine viral diarrhoea virus strain SD-1. *Virology* **191**:867–879.
- Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard. 1995. Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**:1766–1769.
- Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. Rice. 1993. A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. USA* **90**:10583–10587.
- Grassmann, C. W., O. Isken, N. Tautz, and S. E. Behrens. 2001. Genetic analysis of the pestivirus nonstructural coding region: defects in the NS5A unit can be complemented in *trans*. *J. Virol.* **75**:7791–7802.
- Heinz, F. X., M. S. Collett, R. H. Purcell, E. A. Gould, C. R. Howard, M. Houghton, J. M. Moormann, C. M. Rice, and H.-J. Thiel. 2000. Family Flaviviridae, p. 859–878. In R. V. M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), *Virus taxonomy*. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
- Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, K. Kimura, and K. Shimotohno. 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **90**:10733–10737.
- Hu, J., D. O. Toft, and C. Seeger. 1997. Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J.* **16**:59–68.
- Krausslich, H. G., and E. Wimmer. 1988. Viral proteinases. *Annu. Rev. Biochem.* **57**:701–754.
- Lackner, T., A. Müller, A. Pankraz, P. Becher, H.-J. Thiel, A. E. Gorbalenya, and N. Tautz. 2004. Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an RNA virus. *J. Virol.* **78**:10765–10775.
- Lindenbach, B. D., and C. M. Rice. 2001. Flaviviridae: the viruses and their replication, p. 991–1042. In D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.
- Mendez, E., N. Ruggli, M. S. Collett, and C. M. Rice. 1998. Infectious bovine viral diarrhoea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. *J. Virol.* **72**:4737–4745.
- Meyers, G., N. Tautz, and H.-J. Thiel. 1995. Host genes in viral genomes, p. 91–102. In A. J. Gibbs, C. H. Calisher, and F. Garcia-Arenal (ed.), *Molecular basis of virus evolution*. Cambridge University Press, Cambridge, United Kingdom.
- Meyers, G., and H.-J. Thiel. 1996. Molecular characterization of pestiviruses. *Adv. Virus Res.* **47**:53–117.
- Müller, A., G. Rinck, H.-J. Thiel, and N. Tautz. 2003. Cell-derived sequences located in the structural genes of a cytopathogenic pestivirus. *J. Virol.* **77**:10663–10669.
- Neill, J. D., and J. F. Ridpath. 2001. Recombination with a cellular mRNA encoding a novel DnaJ protein results in biotype conversion in genotype 2 bovine viral diarrhoea viruses. *Virus Res.* **79**:59–69.
- Qu, L., L. K. McMullan, and C. M. Rice. 2001. Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. *J. Virol.* **75**:10651–10662.

25. Rinck, G., C. Birghan, T. Harada, G. Meyers, H.-J. Thiel, and N. Tautz. 2001. A cellular J-domain protein modulates polyprotein processing and cytopathogenicity of a pestivirus. *J. Virol.* **75**:9470–9482.
26. Sledz, C. A., M. Holko, M. J. de Veer, R. H. Silverman, and B. R. Williams. 2003. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**:834–839.
27. Sutter, G., M. Ohlmann, and V. Erffle. 1995. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Lett.* **371**:9–12.
28. Tamura, J. K., P. Warrener, and M. S. Collett. 1993. RNA-stimulated NTPase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus. *Virology* **193**:1–10.
29. Tautz, N., A. Kaiser, and H.-J. Thiel. 2000. NS3 serine protease of bovine viral diarrhea virus: characterization of active site residues, NS4A cofactor domain, and protease-cofactor interactions. *Virology* **273**:351–363.
30. Tomita, Y., T. Mizuno, J. Diez, S. Naito, P. Ahlquist, and M. Ishikawa. 2003. Mutation of host DnaJ homolog inhibits brome mosaic virus negative-strand RNA synthesis. *J. Virol.* **77**:2990–2997.
31. Warrener, P., and M. S. Collett. 1995. Pestivirus NS3 (p80) protein possesses RNA helicase activity. *J. Virol.* **69**:1720–1726.
32. Wiskerchen, M., and M. S. Collett. 1991. Pestivirus gene expression: protein p80 of bovine viral diarrhea virus is a proteinase involved in polyprotein processing. *Virology* **184**:341–350.
33. Xu, J., E. Mendez, P. R. Caron, C. Lin, M. A. Murcko, M. S. Collett, and C. M. Rice. 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. *J. Virol.* **71**:5312–5322.
34. Zhong, W., L. L. Gutshall, and A. M. Del Vecchio. 1998. Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. *J. Virol.* **72**:9365–9369.