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Cellular Sequences in Pestivirus Genomes Encoding Gamma-Aminobutyric Acid (A) Receptor-Associated Protein and Golgi-Associated ATPase Enhancer of 16 Kilodaltons

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The presence of cellular protein coding sequences within viral RNA genomes is a unique and particularly interesting feature of cytopathogenic (cp) pestiviruses. Here we report the identification and characterization of two novel cellular sequences in the genomes of cp bovine viral diarrhea virus (BVDV) strains. In BVDV strain CP X604, we detected a duplication of the genomic region encoding NS3, NS4A, and part of NS4B, together with an insertion of sequences that code for cellular gamma-aminobutyric acid (A) receptor-associated protein [GABA(A)-RAP]. Transient-expression studies showed that the GABA(A)-RAP sequence leads to additional processing of the viral polyprotein and thereby to the expression of nonstructural protein NS3. Transfection of bovine cells with RNA transcribed from an infectious cDNA clone revealed that the GABA(A)-RAP-encoding insertion together with the duplicated viral sequences constitutes the genetic basis for the cytopathogenicity of strain CP X604. Surprisingly, molecular analysis of another cp BVDV strain (CP 721) resulted in the identification of a cellular Golgi-associated ATPase enhancer of 16 kDa (GATE-16)-encoding insertion together with duplicated viral sequences. To our knowledge, the genomes of CP X604 and CP 721 are the first viral RNAs found with cellular sequences encoding GABA(A)-RAP and GATE-16, respectively. Interestingly, the two cellular proteins belong to a family of eukaryotic proteins involved in various intracellular trafficking processes. Processing after the C-terminal glycine residue of GABA(A)-RAP and GATE-16 by cellular proteases is essential for covalent attachment to target molecules. Accordingly, it can be assumed that these cellular proteases also recognize the cleavage sites in the context of the respective viral polyproteins and thereby lead to the generation of NS3, the marker protein of cp BVDV.

The genus *Pestivirus* within the family *Flaviviridae* comprises the species *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Classical swine fever virus*, and *Border disease virus* (10, 17). The pestivirus genome consists of a positive-stranded nonpolyadenylated RNA molecule with a size of approximately 12.3 kb which contains one large open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (NTR) (see references 27 and 37 for reviews). This ORF encodes a polyprotein of approximately 3,900 amino acids (aa) which is co- and posttranslationally processed by viral and cellular proteases, leading to the mature viral proteins. The first third of the ORF encodes a nonstructural autoprotease and four structural proteins, while the 3′ part of the RNA genome codes for the other nonstructural proteins (see references 27 and 37 for reviews). Pestiviruses of two biotypes, cytopathogenic (cp) and noncytopathogenic (noncp) viruses, are distinguished by their ability to cause a cytopathic effect in tissue culture (16, 23). One important difference between cp and noncp BVDV is the expression of NS3, which is colinear to the C-terminal part of NS2-3. While NS2-3 is expressed in both cp- and noncp-BVDV-infected cells, NS3 is found exclusively after infection with cp BVDV. Accordingly, NS3 is regarded as the marker protein for cp BVDV strains.

Worldwide, bovine viral diarrhea is one of the most important diseases of cattle, with major economic impact (1). Infections with BVDV can have different consequences, such as abortion, fertility problems, immunosuppression, diarrhea, thrombocytopenia, and, most frequently, inapparent courses (see references 1 and 37 for reviews). In pregnant animals, transplacental infection with noncp BVDV can result in the birth of persistently infected animals with an acquired immunotolerance of the infecting BVDV strain. Such persistently infected animals may come down with mucosal disease (MD), a particularly severe clinical manifestation of BVDV infection. In addition to the persisting noncp virus, cp BVDV can always be isolated from animals with MD (11–13, 27).

Molecular analyses of several BVDV pairs isolated from field cases of MD indicated that the cp viruses can evolve from the respective noncp viruses by RNA recombination (see reference 27 for a review). The mutations identified in the genomes of the cp viruses include insertions of cellular sequences and genomic rearrangements with duplications and deletions of viral sequences (2, 4, 8, 25, 27, 31). The cellular sequences present in the genomes of the cp pestiviruses analyzed so far

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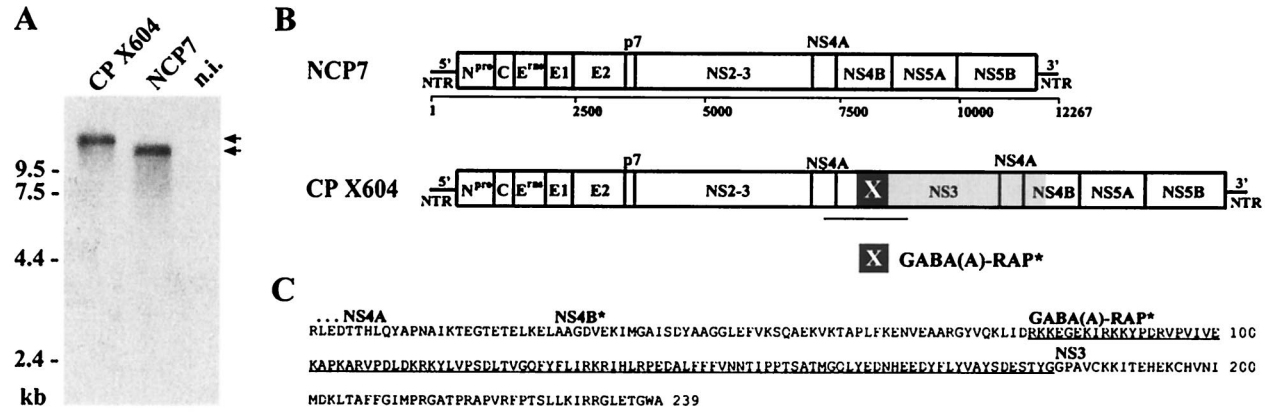

FIG. 1. Analysis of BVDV-1 strain CP X604. (A) Northern blot analysis of total RNA from MDBK cells infected with BVDV CP X604 or NCP7 and from noninfected cells (n.i.). RNA was separated by denaturing agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with a 2.5-kb *Not*I-*Nsi*I fragment from the cDNA clone pCP7-5A (3, 7). Numbers refer to RNA ladder sizes (in kilobases). Migration positions of the viral genomic RNAs are marked with arrows. (B) Genome organizations of the BVDV-1 strains NCP7 and CP X604. For NCP7, the line below the polyprotein demarcates the viral genome, with the scale being in bases (26, 35). The genome of CP X604 contains a duplication of viral sequences encoding NS3, NS4A, and part of NS4B (NS4B*) (grey box) together with a host cell-derived insertion (black box with X). The inserted cellular sequence encodes aa 14 to 116 of GABA(A)-RAP [GABA(A)-RAP*]. The bars are not drawn to scale. The underlined part of the CP X604 genome has been sequenced. (C) Deduced amino acid sequence encoded by part of the CP X604 sequence. The positions of NS4A, NS4B*, GABA(A)-RAP*, and NS3 are indicated. The cellular insertion is underlined.

encode parts of (poly)ubiquitin, the ubiquitin-like proteins SMT3B and NEDD8, ribosomal protein S27a together with an N-terminally truncated ubiquitin, the J domain protein Jiv (previously termed cINS), and light chain 3 (LC3) of microtubule-associated proteins 1A and 1B. Here we report the identification and characterization of two novel cellular sequences within the genomes of two cp BVDV isolates obtained from field cases of MD.

Genome organization of BVDV CP X604. BVDV-1 strain CP X604 was obtained from an animal that died of MD, the fatal form of a BVDV infection. In MDBK cells, this virus causes a cytopathic effect. The cp virus was biologically cloned by plaque purification. Compared to noncp BVDVs, most cp BVDV isolates contain either significantly enlarged or shortened RNA genomes. These size differences are due to large duplications or deletions of viral sequences, respectively (2, 4, 8, 9, 25, 27). To investigate whether similar genomic differences are present in CP X604, a Northern blot analysis was performed. Total RNA from MDBK cells infected with either CP X604 or noncp BVDV strain NCP7 (13, 35) was hybridized with a BVDV-specific cDNA probe (Fig. 1A). As previously reported, the RNA genome of NCP7 is about 12.3 kb in length (26, 35). In contrast, viral genomic RNA with a size of about 15 kb was identified after the infection of bovine cells with CP X604 (Fig. 1A). The detection of the enlarged genome suggests that the cytopathogenicity of CP X604 correlates with a large duplication of viral sequences.

The presence of a large duplicate sequence within the genome of CP X604 was readily confirmed by reverse transcription-PCR (RT-PCR) and subsequent nucleotide sequence analysis of the amplified cDNA fragment. RT-PCR and nucleotide sequencing were carried out as described previously (6, 8). For RT-PCR, primers Ol NS3R (corresponding to nucleotides [nt] 5326 to 5343 of the BVDV strain SD-1 genome, located in the NS3 coding region; antisense) and Ol 7100 (corresponding to nt 7313 to 7335, located in the NS4A coding region; sense) were used (8). To determine the genome organization of CP X604, the obtained sequence was first compared to the genomic sequence of noncp BVDV strain SD-1 (14). The 5′ part of the analyzed CP X604 sequence corresponds to positions 7313 to 7549 of the SD-1 sequence. This region encodes the C-terminal 27 aa of NS4A and the N-terminal 52 aa of NS4B (NS4B*) (Fig. 1B and C). Downstream of this part, a nonviral sequence comprising 309 nt was identified. The remaining nucleotides of the CP X604 sequence are colinear with part of the NS3 gene, starting at position 5153 (SD-1 sequence); this position corresponds to the N terminus of NS3. Taken together, the obtained partial nucleotide sequence and the determined size of the viral genomic RNA indicate that the CP X604 genome contains a duplication of viral sequences encoding NS3, NS4A, and part of NS4B as well as an insertion of a nonviral sequence (Fig. 1B).

Identification of GABA(A)-RAP coding sequences. A data bank search revealed that the nonviral insertion within the genome of CP X604 is 92 to 94% identical to murine and human mRNA sequences encoding aa 14 to 116 of the gamma-aminobutyric acid (A) receptor-associated protein [GABA(A)-RAP]; we refer to this part of GABA(A)-RAP as GABA(A)-RAP*. To our knowledge, this is the first report about the presence of a cellular GABA(A)-RAP coding sequence within a viral genome. The cellular GABA(A)-RAP protein consists of 117 aa and is highly conserved among mammalian species. It has been reported that GABA(A)-RAP interacts with microtubules and plays an important role in intracellular GABA(A) receptor transport and postsynaptic location (32, 39).

The bovine origin of CP X604 suggested that the cellular insertion was obtained from the host species. For identification and characterization of the putative cellular recombination partner, part of the bovine mRNA sequence encoding GABA(A)-RAP was amplified by RT-PCR (using the total RNA from bovine MDBK cells), cloned, and sequenced; the primers used for RT-PCR were deduced from published

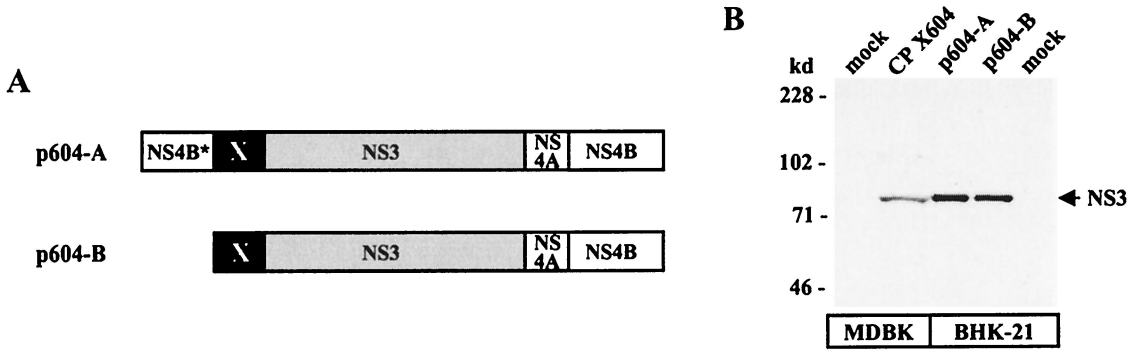

FIG. 2. (A) Schematic representations of the fusion proteins encoded by the constructs p604-A and p604-B used for transient expression. The T7 expression plasmids were based on plasmid pCITE-CP7-NS34AB, which is a derivative of the vector pCITE (Invitrogen, De Schelp, The Netherlands) and encompasses the genomic region encoding NS3, NS4A, and part of NS4B of BVDV strain CP7. To establish a construct for the expression of GABA(A)-RAP*, NS3, NS4A, and part of NS4B, the CP X604-specific cDNA fragment was obtained by RT-PCR with primer Ol X604-INS [5′-TAGTCATGAGGAAAAAGGAAGGAGAAAAG-3′, sense; the underlined nucleotides correspond to the 5′ part of the GABA(A)-RAP* coding sequence], which includes a *Bsp*HI site, and primer Ol X604-AgeIR (5′-CAACCGGTCTCCAGACCCCTCCT-3′, antisense; located in the NS3 coding region), which includes an *Age*I site, and cloned into pCITE-CP7-NS34AB (precut with *Nco*I and *Age*I). This plasmid was termed p604-B. For construction of p604-A, the genomic region encoding NS4B* (N-terminal 52 aa of NS4B), GABA(A)-RAP*, and part of NS3 was generated by RT-PCR with primer Ol X604-NS4B (5′-GAGTCATGAGCGCGGGTGACGTGGAG-3′, sense; the underlined nucleotides encode aa 2 to 6 of NS4B of CP X604), which includes a *Bsp*HI site, and primer Ol X604-AgeIR and cloned into pCITE-CP7-NS34AB (precut with *Nco*I and *Age*I). (B) Immunoblot. MDBK cells infected with CP X604 and BHK-21 cells transfected with p604-A or p604-B were lysed 48 h postinfection and 16 h posttransfection, respectively. The samples were separated by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis under reducing conditions, transferred to nitrocellulose, and incubated with the anti-NS3 monoclonal antibody 8.12.7, kindly provided by E. J. Dubovi (Cornell University, Ithaca, N.Y.). Mock-infected or -transfected cells (mock) served as negative controls. The sizes (in kilodaltons) of marker proteins are indicated on the left. The position of NS3 is indicated by an arrow. In cells infected with CP X604, NS2-3 was clearly visible after longer exposure times (not shown); for expression of NS2-3 and NS3 of CP X604, see also Fig. 3D.

mRNA sequences of mice and humans. Comparative analysis revealed that the obtained bovine sequence comprising 334 nt is >99.5% identical to the respective genomic sequence of CP X604 (data not shown). This result strongly supports the assumption that the cellular insertion identified in the genome of CP X604 was derived from a bovine mRNA encoding GABA(A)-RAP.

GABA(A)-RAP* as processing signal. The cytopathogenicity of BVDV strictly correlates with the expression of NS3, which cannot be detected in cells infected with noncp BVDV (13, 29, 30). Accordingly, NS3 is regarded as the marker protein of cp BVDV. In agreement with the results of previously reported analyses of other cp BVDV strains, infection of bovine cells with cp BVDV strain CP X604 allowed the detection of NS3 in addition to NS2-3 (Fig. 3D).

In the polyprotein encoded by CP X604, the N terminus of NS3 is fused to Gly_{116} of GABA(A)-RAP. Interestingly, according to indirect evidence, GABA(A)-RAP is processed after Gly_{116} by a cellular protease (see below). It was therefore speculated that GABA(A)-RAP leads to an additional processing event in the viral polyprotein of CP X604 and thereby allows the expression of NS3. To test this hypothesis, the genomic regions encoding the N-terminal part of NS4B (NS4B*, with aa 1 to 52), GABA(A)-RAP*, NS3, NS4A, and another N-terminal part of NS4B were transiently expressed in the MVA-T7pol vaccinia virus system (33); this construct was termed p604-A (Fig. 2A). The expression of NS3-specific protein(s) was monitored by immunoblotting with a monoclonal antibody directed against NS3. The expression of p604-A led to the detection of a protein with an apparent molecular mass of about 80 kDa that comigrated with NS3 from CP X604-infected MDBK cells (Fig. 2B). A protein with the same apparent molecular mass was also detected after the expression of construct p604-B, which encodes GABA(A)-RAP*, NS3, NS4A, and part of NS4B (Fig. 2). Taken together, these results demonstrate that GABA(A)-RAP* serves as a processing signal to yield NS3.

Infectious BVDV cDNA clone with the GABA(A)-RAP*-encoding insertion and duplicated NS3 gene. Since only part of the genome of CP X604 was sequenced, the possibility of other changes in addition to the identified insertion and duplication cannot be excluded and may be relevant with regard to viral cytopathogenicity. To investigate whether the GABA(A)-RAP*-encoding insertion together with the identified duplication of viral sequences constitutes the genetic basis of the cytopathogenicity of CP X604, the chimeric cDNA construct p7/X604 was generated. This full-length clone is based on the noncp infectious full-length cDNA clone pNCP7-5A-(AgeI−) and contains the GABA(A)-RAP*-encoding insertion together with a duplication of viral sequences encoding NS3, NS4A, and part of NS4B (Fig. 3A); thus, p7/X604 reflects the genome organization of CP X604.

Two days after transfection of bovine cells with 7/X604 RNA, cell lysis was observed (Fig. 3B). As previously reported, cytolysis was also visible after transfection with CP7-5A RNA while no signs of cytopathic effect were observed in cells transfected with NCP7-5A RNA (2, 9) (Fig. 3B). Furthermore, the growth kinetics of 7/X604, CP7-5A, and NCP7-5A were determined. This analysis revealed that growth of the chimeric virus 7/X604 was slightly retarded in comparison with that of the other two viruses (data not shown). Each of the three viruses reached titers of $>10^5$ 50% tissue culture infective doses/ml.

For further characterization of the recovered recombinant virus 7/X604, a Northern blot analysis was performed.

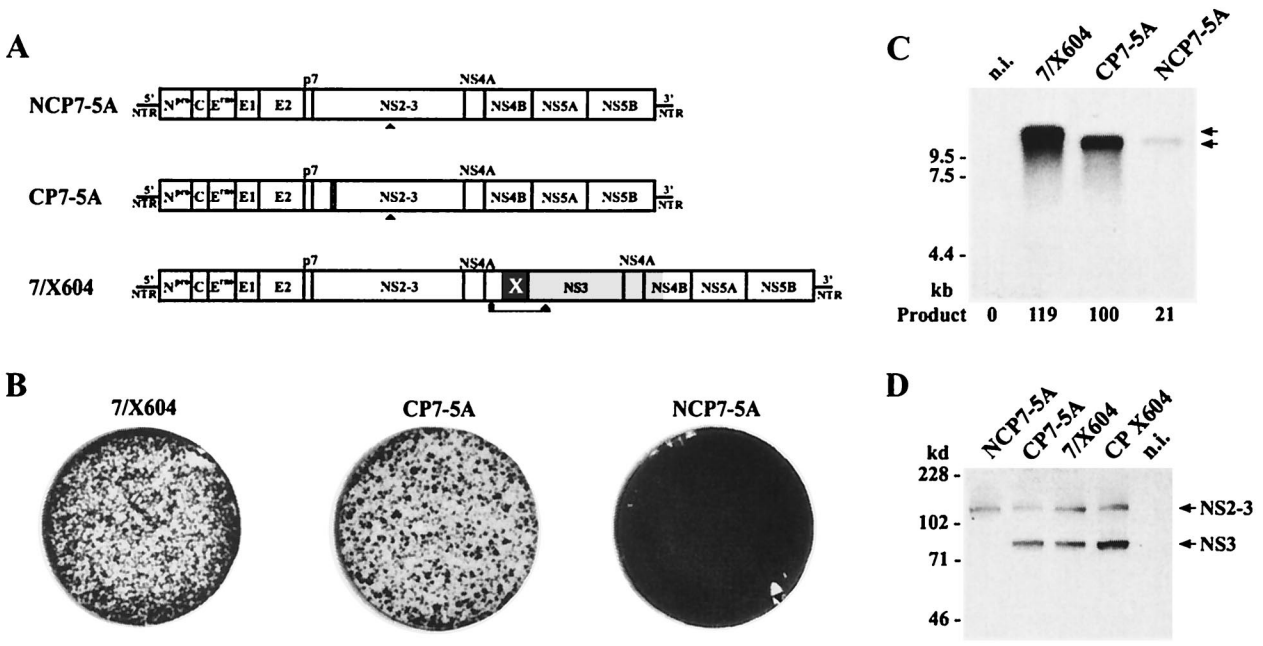

FIG. 3. Transfection experiments with RNA transcribed from infectious clones pNCP7-5A, pCP7-5A, and p7/X604. (A) Genome organizations of noncp BVDV NCP7-5A, cp BVDV CP7-5A, and the chimeric cDNA construct p7/X604, whose structure mirrors the genome structure of CP X604. The BVDV full-length cDNA clones pNCP7-5A and pNCP7-5A-(AgeI−) have been described previously (2, 9). These plasmids are noncp derivatives of the cp BVDV infectious full-length cDNA clone pCP7-5A (7). Construction of p7/X604 was based on pNCP7-5A-(AgeI−), which differs from pNCP7-5A by the absence of the single *Age*I site (nt 5309 to 5314 of the NCP7-5A sequence). A CP X604-specific *Mlu*I-*Age*I fragment was obtained by RT-PCR with primer Ol X604-MluI (5′-TTACGCGTCTGGAGGACTGGAATTCG-3′, sense), which encompasses an *Mlu*I site that corresponds to nt 7434 to 7439 of the NCP7-5A sequence, and primer Ol X604-AgeIR, which encompasses an *Age*I site that corresponds to nt 5309 to 5314 of the NCP7-5A sequence, and cloned into pCR2.1 (Invitrogen). The resulting plasmid was termed pX604-MluI. Next, the *Sac*I (nt 5842 to 5847)-*Mlu*I (nt 7434 to 7439) fragment of pNCP7-5A was cloned into p604-MluI that had been precut with *Sac*I (located in the polylinker) and *Mlu*I. Finally, adding the pNCP7-5A-derived *Age*I-*Sal*I fragment (nt 5310 to 7720) completed the cloning of the *Sac*I-*Sal*I fragment, which was then introduced into pNCP7-5A-(AgeI−) that had been precut with *Sac*I and *Sal*I. For 7/X604, the CP X604-specific part of the genome and the positions of the *Mlu*I site (■) and the *AgeI* site (▲) used for cDNA cloning are indicated by a line below the bar. (B) Crystal violet staining of MDBK cells 2 days after transfection with the indicated RNAs. In vitro synthesis of RNA and transfection of MDBK cells were performed as described previously (7). About 2 μg of RNA was used for each transfection. (C and D) Northern blot (C) and immunoblot (D) analyses of MDBK cells infected with the indicated viruses at a multiplicity of infection of 0.05. Northern blotting and immunoblotting were performed as described in the legends for Fig. 1A and 2B. n.i., noninfected cells. (C) RNAs were extracted 24 h after infection. The intensities of bands were determined with a phosphorimager. The relative amounts of viral genomic RNAs are indicated below the blot (percentages of the value for CP7-5A [100%]). Migration positions of the viral genomic RNAs are marked with arrows.

Genomic RNA exhibiting the expected size of about 15 kb was detected (Fig. 3C). To allow comparison of the amounts of accumulated viral RNAs, MDBK cells were infected with 7/X604, CP7-5A, or NCP7-5A at a multiplicity of infection of 0.05. RNAs were extracted at 24 h after infection and subjected to Northern blot analysis (Fig. 3C); after this incubation time, the titers of infectious virus were similar for all three viruses. The intensities of bands were determined with a phosporimager. After infection of cells with the cp virus 7/X604 or CP7-5A, the amounts of viral RNAs were about five to six times larger than the amount obtained from NCP7-5A-infected cells (Fig. 3C). Similar differences in levels of viral RNA synthesis have recently been reported for other pairs of cp and noncp BVDV strains (9, 24, 38).

In addition, the expression of NS2-3 and NS3 was analyzed by immunoblotting of cells infected with 7/X604, CP X604, CP7-5A, or NCP7-5A. In agreement with the data obtained from the transient-expression study (see above) and previous reports, NS3 was detected in cells infected with the cp viruses CP7-5A, CP X604, and 7/X604 but not in cells infected with NCP7-5A while NS2-3 was found after infection with either cp or noncp viruses (Fig. 3D). Taken together, the results of our analyses demonstrate that the cellular GABA(A)-RAP*-encoding insertion and the duplicated viral sequences constitute the genetic basis for the expression of NS3 and the cytopathogenicity of CP X604.

Analysis of BVDV CP 721. In addition to CP X604, the cp BVDV-1 isolate 721 (5) was characterized at the molecular level; this isolate was also obtained from an animal that died of MD. The cp virus was biologically cloned by three rounds of plaque purification and subsequently termed CP 721. In addition, a noncp virus was obtained by serial dilutions of the third tissue culture passage; the noncp virus was termed NCP 721. Immunoblot analysis revealed the presence of NS2-3 and NS3 in cells infected with CP 721, while only NS2-3 was detected in cells infected with NCP 721(data not shown). According to Northern blot analysis, the genome of CP 721 is about 15 kb long, which is about 2.7 kb longer than the genomes of other noncp BVDV strains, including NCP 721 and NCP7 (Fig. 4A). Molecular cloning of parts of the genome of CP 721 by RT-

FIG. 4. Analysis of BVDV-1 strain CP 721. (A) Northern blot analysis of total RNA from MDBK cells infected with 721 (mixture of cp and noncp virus), NCP 721, or CP 721. Migration positions of the viral genomic RNAs are indicated. (B) Genome organizations of NCP7 and CP 721. The genome of CP 721 contains a duplication of viral sequences encoding part of NS2 (NS2*), NS3, NS4A, and part of NS4B (NS4B*) (grey box), together with an insertion encoding part of GATE-16 (GATE-16*; black box with G). The bars are not drawn to scale. The underlined parts of the CP 721 genome have been sequenced (this study and reference 5). (C) Deduced amino acid sequence of part of the CP 721 sequence. The positions of NS4A, NS4B*, NS2*, GATE-16*, and NS3 are indicated. The cellular insertion is underlined.

PCR (using primer Ol 7100 and primer Ol NS3R) and subsequent analysis of the obtained nucleotide sequence indicated the presence of duplicated viral sequences; these encode the C-terminal 34 aa of NS2 (NS2*), NS3, NS4A, and the N-terminal 110 aa of NS4B (NS4B*) (Fig. 4B and C). In addition, a nonviral insertion comprising 387 nt was found directly upstream of the duplicated NS3 gene.

Identification of GATE-16 coding sequences. A data bank search revealed that the nonviral insertion in the genome of CP 721 is very similar to cellular mRNA sequences encoding part of Golgi-associated ATPase enhancer of 16 kDa (GATE-16). The genome of BVDV CP 721 is the first viral RNA for which a GATE-16 coding sequence has been identified. GATE-16 consists of 117 aa and is highly conserved among mammalian species. It has been reported that GATE-16 functions as a soluble factor involved in intra-Golgi transport (32).

The nucleotide sequence identities between the insertion of CP 721 and cellular GATE-16 coding sequences from rat and human are 93 and 94%, respectively. Since BVDV CP 721 originated from cattle, it could be assumed that the cellular insertion was derived from a bovine mRNA. For further analyses, the bovine mRNA encoding GATE-16 was cloned by RT-PCR and sequenced. The obtained nucleotide sequence of the bovine mRNA encodes the complete GATE-16 protein, consisting of 117 aa. The coding sequence is flanked by 39 nt from the 5′ NTR and 121 nt from the 3′ NTR. A comparative analysis revealed that the cellular sequence identified within the genome of CP 721 is 100% identical to the respective bovine mRNA sequence. The inserted cellular sequence within the genome of CP 721 comprises 387 nt and encodes 129 aa (GATE-16*) (Fig. 4B and C). Remarkably, the 5′-terminal 39 nt encoding the oligopeptide GSESRLRPFFAAA within the viral polyprotein are derived from the 5′ NTR of the cellular mRNA. The following 348 nt encode a truncated GATE-16 lacking 1 aa at the C terminus.

Interestingly, GATE-16 is related to a family of eukaryotic proteins also including GABA(A)-RAP and LC3 of microtubule-associated proteins 1A and 1B as well as proteins from nematodes (*Caenorhabditis elegans*), plants (*Arabidopsis thaliana*), and yeast (*Saccharomyces cerevisiae*) (Fig. 5). The mammalian proteins GATE-16, GABA(A)-RAP, and LC3 are 31 to 61% identical to each other. According to a recent report, GATE-16 and LC3 are processed by cellular proteases after the C-terminal glycine residue (Gly_{116} of GATE-16 or Gly_{120} of LC3) (18) (Fig. 5). If we take into account the similarity between GATE-16, LC3, and GABA(A)-RAP, it appears very likely that GATE-16 functions as a processing signal in the viral polyprotein, leading to the expression of NS3 and the cytopathogenicity of CP 721.

Several studies have concentrated on the molecular characterization of cp and noncp pestiviruses. These analyses resulted in the identification of various alterations in the genomes of the cp viruses, including cellular insertions, large duplications, and deletions of viral sequences as well as point mutations (2, 4, 8, 9, 22, 25, 27, 31). As a unique feature, the majority of cp pestiviruses carry insertions of cellular protein coding sequences. So far, different kinds of cellular sequences have been detected in the genomes of cp pestiviruses; these sequences encode parts of (i) (poly)ubiquitin, ubiquitin together with ribosomal protein S27a, and the ubiquitin-like proteins SMT3B and NEDD8; (ii) LC3; and (iii) the J domain protein Jiv (2, 4, 8, 9, 25, 27, 31). In the present study, we report the identification and characterization of two novel cellular sequences detected in the genomes of the cp BVDV strains CP X604 and CP 721; these host-derived sequences encode parts of the cellular proteins GABA(A)-RAP and GATE-16, respectively. To our knowledge, the genomes of CP X604 and CP 721 are the first viral RNAs with cellular sequences encoding GABA(A)-RAP and GATE-16.

GATE-16 has been described as a soluble factor involved in intra-Golgi transport. This protein can associate with the *N*-ethylmaleimide-sensitive fusion protein (NSF) and with the Golgi soluble NSF attachment protein receptor (SNARE) GOS-28 (32). GATE-16 first stimulates the ATPase activity of NSF, and NSF in turn stimulates the association of GATE-16 with GOS-28. The GATE-16 protein consists of 117 aa and

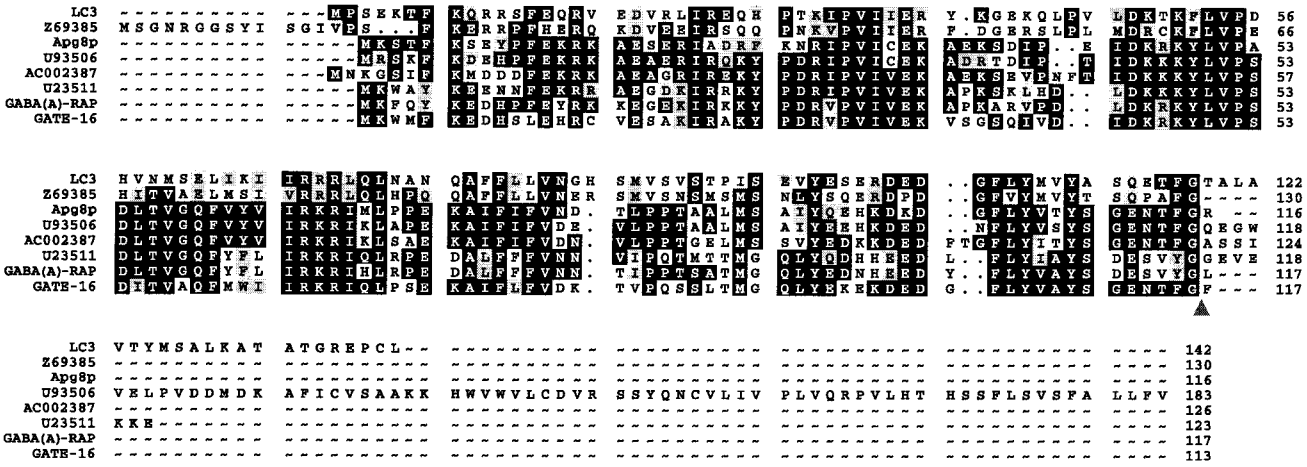

FIG. 5. Alignment of GABA(A)-RAP (GenBank accession no. AV605665), GATE-16 (accession no. AY117147), LC3 (accession no. U05784), and related proteins from *C. elegans* (accession no. Z69385 and U23511), *A. thaliana* (accession no. AC002387), *S. cerevisiae* (Apg8p; accession no. X79489), and *Laccaria bicolor* (accession no. U93506). Black and grey shading indicate identical and similar amino acids, respectively. Note that not all related sequences present in the data banks are included; e.g., several additional homologous sequences are found in *A. thaliana*. The proposed cleavage site after the highly conserved C-terminal glycine residue is marked (▲). Computer analysis of sequence data was performed with HUSAR (Deutsches Krebsforschungszentrum, Heidelberg, Germany), which provides the Genetics Computer Group software package (15).

contains a ubiquitin-like fold decorated by two additional N-terminal helices (28). Interestingly, GATE-16 is related to GABA(A)-RAP, LC3, and several other eukaryotic proteins (Fig. 5). GABA(A)-RAP has been proposed to play a role in intracellular transport and clustering of neurotransmitter receptors by mediating interaction with the cytoskeleton (39). Like GATE-16, GABA(A)-RAP binds to microtubules and interacts with NSF (21, 39). A well-characterized yeast ortholog of GATE-16 and GABA(A)-RAP is the autophagocytosis factor Apg8p (also termed Aut7p) (20). It has been reported that Apg8p is processed by the cysteine protease Apg4p, which leaves a C-terminal glycine residue for covalent conjugation of phosphatidylethanolamine to anchor Apg8p to the membrane prior to autophagosome formation. This conjugation involves an ubiquitination-like enzyme system including activating (Apg7p) and conjugating (Apg3p) enzymes (19, 20). Recently, a human homolog of the yeast activating enzyme Apg7p was found to activate multiple substrates, including GATE-16, GABA(A)-RAP, and LC3 (34). In addition, human homologs of the Apg4 protease have been identified, and proteolytic processing of GATE-16 and LC3 after the C-terminal glycine has been confirmed (18). Interestingly, the C-terminal glycine residue modified in Apg8p corresponds to Gly_{116} of GABA(A)-RAP and GATE-16. This glycine residue is highly conserved among all members of the family (Fig. 5). All these data strongly suggest that Apg8p and its related proteins, including GABA(A)-RAP, GATE-16, and LC3, may undergo similar processing and modification reactions.

The cytopathogenicity of BVDV strictly correlates with the expression of NS3. In the genomes of CP X604 and CP 721, the N terminus of NS3 is fused to Gly_{116} of GABA(A)-RAP and GATE-16. The expression of fusion proteins composed of GABA(A)-RAP∗ (terminating with Gly_{116}) and NS3 demonstrated that the part of GABA(A)-RAP present in the virus-encoded polyprotein of CP X604 is sufficient for the processing and release of NS3 (Fig. 2). Similar to that of the fusion proteins encompassing GABA(A)-RAP∗, the expression of fusion proteins comprising NS3 fused to the C-terminal glycine of LC3 resulted in cleavage between LC3 and NS3 (25). According to these data, the cellular proteases involved in the processing of cellular GABA(A)-RAP and LC3 also recognize the cleavage sites in the context of the respective viral polyproteins and thereby lead to the generation of NS3. Furthermore, transfection experiments with RNA transcribed from the chimeric infectious cDNA clone p7/604 revealed that the insertion of GABA(A)-RAP∗-encoding sequences directly upstream of the NS3 coding region represents the genetic basis of the cytopathogenicity of CP X604 (Fig. 3). Taking into account the similarity between GATE-16, GABA(A)-RAP, and LC3 (Fig. 5), together with our knowledge about the function and biosynthesis of cellular GATE-16, we consider it very likely that GATE-16∗ also functions as a processing signal in the viral polyprotein to yield NS3.

Interestingly, the locations of the inserted GABA(A)-RAP, GATE-16, and LC3 coding sequences within the genomes of BVDV strains CP X604, CP 721, and CP Ja (25) are identical to the positions of ubiquitin coding sequences within pestiviral genomes. In all these cases, the N terminus of NS3 is fused to the C-terminal glycine of the cellular sequence. For viral polyproteins containing ubiquitin and ubiquitin-like proteins, processing is mediated by ubiquitin C-terminal hydrolases (2, 8, 31, 36). The cellular proteases involved in the processing of GATE-16, GABA(A)-RAP, and LC3 are under investigation and need to be further characterized (18).

Molecular analysis of a large number of cp pestiviruses resulted in the identification of various host-derived insertions (2, 4, 8, 9, 25, 27, 31). Apart from Jiv and ribosomal protein S27a, all cellular sequences identified in the genomes of cp pestiviruses belong to two gene families encoding (i) ubiquitin and ubiquitin-like proteins and (ii) LC3, GABA(A)-RAP, and GATE-16 (Table 1). Remarkably, pestiviruses can use several cellular proteases for the generation of NS3. It is fascinating

TABLE 1. Insertion of cellular sequences in cp BVDV strains

Name of insertion	No. of described strains	Location within viral genome	Cellular homolog	Function of cellular homolog	Reference(s)[a]
Ubiquitin	>10	Directly upstream of NS3 gene	Polyubiquitin	Protein degradation, etc.	4, 27
S27a-ubi	1	Directly upstream of NS3 gene	Ubiquitin-S27a	Degradation, ribosomal protein	8, 9
SMT3B[b]	1	Directly upstream of NS3 gene	SUMO-1 (SMT3B)	Nucleocytoplasmic trafficking	31
NEDD8	1	Within NS3 gene	NEDD8 (RUB1)	Regulation of cell cycle	2
LC3	1	Directly upstream of NS3 gene	LC3	Lysosomal, vacuole trafficking	25
GATE-16	1	Directly upstream of NS3 gene	GATE-16	Intra-Golgi transport	This study
GABA(A)-RAP	1	Directly upstream of NS3 gene	GABA(A)-RAP	GABA(A) receptor trafficking	This study
Jiv (cINS)	>10 (also BDV[c])	Within NS2 gene	J domain protein	Chaperone	3, 27

[a] Some references include additional references within the article cited.
[b] SMT3B and NEDD8 are ubiquitin-like proteins.
[c] BDV, border disease virus.

that the emergence of cp pestiviruses during persistent infection can be regarded as an assay system to detect intracellular pathways which involve efficient processing of precursor proteins by cellular proteases.

Nucleotide sequence accession numbers. The nucleotide sequences of parts of the genomes of CP X604 and CP 721 as well as partial bovine mRNA sequences encoding GABA(A)-RAP and GATE-16 have been deposited in GenBank under accession numbers AY117144 to AY117147.

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