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Conserved Determinants for Membrane Association of Nonstructural Protein 5A from Hepatitis C Virus and Related Viruses▽

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Nonstructural protein 5A (NS5A) is a membrane-associated essential component of the hepatitis C virus (HCV) replication complex. An N-terminal amphipathic alpha helix mediates in-plane membrane association of HCV NS5A and at the same time is likely involved in specific protein-protein interactions required for the assembly of a functional replication complex. The aim of this study was to identify the determinants for membrane association of NS5A from the related GB viruses and pestiviruses. Although primary amino acid sequences differed considerably, putative membrane anchor domains with amphipathic features were predicted in the N-terminal domains of NS5A proteins from these viruses. Confocal laser scanning microscopy, as well as membrane flotation analyses, demonstrated that NS5As from GB virus B (GBV-B), GBV-C, and bovine viral diarrhea virus, the prototype pestivirus, display membrane association characteristics very similar to those of HCV NS5A. The N-terminal 27 to 33 amino acid residues of these NS5A proteins were sufficient for membrane association. Circular dichroism analyses confirmed the capacity of these segments to fold into alpha helices upon association with lipid-like molecules. Despite structural conservation, only very limited exchanges with sequences from related viruses were tolerated in the context of functional HCV RNA replication, suggesting virus-specific interactions of these segments. In conclusion, membrane association of NS5A by an N-terminal amphipathic alpha helix is a feature shared by HCV and related members of the family *Flaviviridae*. This observation points to conserved roles of the N-terminal amphipathic alpha helices of NS5A in replication complex formation.

The family *Flaviviridae* comprises the genera *Flavivirus*, *Hepacivirus*, and *Pestivirus*, as well as the as-yet-unassigned GB virus A (GBV-A), GBV-B, and GBV-C. These enveloped positive-strand RNA viruses express their structural and nonstructural proteins via translation of a single long open reading frame (30). *Hepatitis C virus* (HCV) is the sole member of the genus *Hepacivirus* and a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, affecting an estimated 3% of the world's population (44). GB viruses and pestiviruses are more closely related to HCV than the classical flaviviruses. The availability of efficient cell culture systems and animal models makes these related viruses attractive in vitro and in vivo models for HCV. GBV-B, the closest relative of HCV, was recovered from a tamarin inoculated with blood from a surgeon suffering from acute hepatitis (16, 54) and causes acute and chronic hepatitis in New World monkeys, which are believed to represent the natural hosts (10). Subsequent attempts to isolate GBV-B sequences from humans have failed. By contrast, GBV-C persistently infects humans. However, the pathogenic relevance of GBV-C, if any, remains unclear. It does not cause hepatitis but has been associated with prolonged survival in human immunodeficiency virus-infected individuals (62–64). Bovine viral diarrhea virus (BVDV) is the prototype pestivirus. Based on their behavior in cell culture, BVDV isolates can be divided into noncytopathogenic and cytopathogenic strains, resulting in subclinical persistent infection or lethal mucosal disease, respectively (30).

HCV nonstructural protein 5A (NS5A) is a phosphorylated zinc metalloprotein (57) and an essential component of the HCV replication complex (reviewed in reference 37). Interestingly, its phosphorylation state was found to modulate HCV RNA replication (4, 22, 43). The crystal structure of domain I, comprising the N-terminal one-third of NS5A, has recently been solved, revealing a novel protein fold, a zinc coordination motif, and a dimerization interface (58). Modeling of the NS5A dimer at the membrane surface allowed the proposal of an RNA-binding groove exposed to the cytosol (58). A recent study has confirmed the RNA-binding properties of HCV NS5A (27). Thus, NS5A may be implicated in the regulation of HCV replication by binding and coordinating the different fates of the viral RNA during translation, replication, and packaging (reviewed in reference 37). However, numerous additional functions have been postulated for this enigmatic protein (reviewed in reference 31), and the definitive function of NS5A remains elusive.

Formation of a membrane-associated replication complex composed of viral proteins, replicating RNA, and altered host cell membranes is a hallmark of all positive-strand RNA vi-

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TABLE 1. Primers used in this study

Name	Restriction site	Nucleotide sequence[a]
GBV-B-5AN33fwd-1		5′ CCTTAAAGCTGGAGTTCAGAGCATGGTTAACGGTGGTGGTGTGAGCAAGGGCGAGGAGCTGTTCACCG 3′
GBV-B-5AN33fwd-2		5′ GGCAGTATGTGTGCAATTTCTTTGTGATTTGCTTTAATGTCCTTAAAGCTGGAGTTCAGAGCATGGTTAACGGTGGTGG 3′
GBV-B-5AN33fwd-3	EcoRI	5′ GAGAATTCACC**ATG**GGCCTCATTGCTTGGGGTCTAGAGATTTGGCAGTATGTGTGCAATTTCTTTGTGATTTGCTTTAATG 3′
GBV-C-5AN27fwd-1		5′ AGACTCCGGGCCCTCTGCCCTGTGGTGGGTGGTGGTGTGAGCAAGGGCGAGGAGCTGTTCACCG 3′
GBV-C-5AN27fwd-2		5′ TGGGTGATGCGCCAGGTGCGCATGGTGATGTCTAGACTCCGGGCCCTCTGCCCTGTGGTGGGTGGTGG 3′
GBV-C-5AN27fwd-3	EcoRI	5′ GAGAATTCACC**ATG**TACGTCTGGGATCTGTGGGAGTGGGTGATGCGCCAGGTGCGCATGGTGATGTC 3′
BVDV-5AN28fwd-1		5′ TTGAAAAAAATAGTCTTGGGGTGGGCTGGTGGTGGTGTGAGCAAGGGCGAGGAGCTGTTCACCG 3′
BVDV-5AN28fwd-2		5′ TTGATCTACAGCCTACATAAACAGATAAATAGAGGCTTGAAAAAAATAGTCTTGGGGTGGGCTGGTGGTGG 3′
BVDV-5AN28fwd-3	EcoRI	5′ GAGAATTCACC**ATG**TCTGGGAATTATGTCCTGGATTTGATCTACAGCCTACATAAACAGATAAATAGAGGC 3′
GFP + 600-rev		5′ CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCC 3′
GFP + 250-rev		5′ TATTAACGCTTACAATTTACGCCTTAAGATAC 3′
GFP-XbaIrev	XbaI	5′ GCTCTAGA**TTA**CTTGTACAGCTCGTCCATGCCGAG 3′
GFP-600fwd		5′ AAATGGCCCGCCTGGCTGAC 3′
GFP-250fwd		5′ TCTCCACCCCATTGACGTC 3′
GFP-EcoRIfwd	EcoRI	5′ GAGAATTCACC**ATG**GTGAGCAAGGGCGAGGAGCTGTTCACCGGG 3′
GBV-B-5AN33rev-1		5′ GCCAAATCTCGAGACCCCAAGCAATGAGGCCACCACCACCCTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCG 3′
GBV-B-5AN33rev-2		5′ AGGACATTAAAGCAAATCACAAAGAAATTGCACACATACTGCCAAATCTCGAGACCCCAAGCAATGAGGCCACCACCACC 3′
GBV-B-5AN33rev-3	XbaI	5′ GCTCTAGA**TTA**GTTAACCATGCTCTGAACTCCAGCTTTAAGGACATTAAAGCAAATCACAAAGAAATTGCACACATAC 3′
GBV-C-5AN27rev-1		5′ TCACCCACTCCCACAGATCCCAGACGTAACCACCACCCTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCG 3′
GBV-C-5AN27rev-2		5′ TCTGGACATCACCATGCGCACCTGGCGCATCACCCACTCCCACAGATCCCAGACGTAACCACCACC 3′
GBV-C-5AN27rev-3	XbaI	5′ GCTCTAGA**TTA**CACCACAGGGCAGAGGGCCCGGAGTCTGGACATCACCATGCGCACCTGGCGC 3′
BVDV-5AN28rev-1		5′ GATCAAATCCAGGACATAATTCCCAGAACCACCACCCTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCG 3′
BVDV-5AN28rev-2		5′ GCCTCTATTTATCTGTTTATGTAGGCTGTAGATCAAATCCAGGACATAATTCCCAGAACCACCACC 3′
BVDV-5AN28rev-3	XbaI	5′ GCTCTAGA**TTA**AGCCCACCCCAAGACTATTTTTTTCAAGCCTCTATTTATCTGTTTATGTAGGCTGTA 3′
GBV-B-5Afwd	EcoRI	5′ GAGAATTCACC**ATG**GGCCTCATTGCTTGGGGTC 3′
myc-GBV-B-5Afwd	EcoRI	5′ GCGAATTCACC**ATG**GAACAGAAGCTAATCTCGGAGGAAGATCTGGGATCGGGCCTCATTGCTTGGGGTCTAGAGATTTGGC 3′
GBV-B-5AΔN33fwd	EcoRI	5′ GAGAATTCACC**ATG**ATTCCTGGTTGTCCTTTC 3′
GBV-B-5A-HArev	XhoI	5′ TGCCTCGAG**CTA**GACAGCATAGTCAGGTACGTCATATGGATACGATCCGCACGAAAACTCACTCTTTCCCAACTTCTTTTTTG 3′
GBV-C-5Afwd	EcoRI	5′ GAGAATTCACC**ATG**TACGTCTGGGACTTG 3′
GBV-C-5AΔN27fwd	EcoRI	5′ GAGAATTCACC**ATG**TCATTACCCTTATGG 3′
GBV-C-5A-FLAGrev	XbaI	5′ GCTCTAGA**CTA**GACCTTGTCATCGTCGTCCTTGTAGTCCGATCCAGCCAAGGTCTCTTGCCTAGCCTCACACTTGTCACATTC 3′
GBV-C-5A-HArev	XbaI	5′ GCTCTAGACAGCATAGTCAGGTACGTCATATGGATACGATCCAGCCAAGGTCTCTTGCCTAGCCTCACACTTGTCACATTC 3′
BVDV-5Afwd	EcoRI	5′ GAGAATTCACC**ATG**TCTGGGAATTATGTCCTGG 3′
BVDV-5AΔN28fwd	EcoRI	5′ GAGAATTCACC**ATG**CCCGCACCATTTAGTTGCGAC 3′
BVDV-5A-HArev	XbaI	5′ GCTCTAGA**CTA**CAGCATAGTCAGGTACGTCATATGGATACGATCCCAGCTTCATGGTATAGGTTCTTGCACCCACTTCCTTC 3′
GBV-B8-13fwd		5′ CCGGCTCGTGGCTAAGAGATATTTGGCAGTATGTGTGCACGGTGTTGACTGATTTCAAGACCTG 3′
GBV-B8-13rev		5′ GAAATCAGTCAACACCGTGCACACATACTGCCAAATATCTCTTAGCCACGAGCCGGAGCATGG 3′
GBV-B4-13fwd		5′ GGCTCGTGGGGTCTAGAGATTTGGCAGTATGTGTGCACGGTGTTGACTGATTTCAAGACCTGGCTCC 3′
GBV-B4-13rev		5′ CAACACCGTGCACACATACTGCCAAATCTCTAGACCCCACGAGCCGGAGCATGGCGTGGAGCAGTC 3′
GBV-B4-35fwd-1		5′ CTTAAAGCTGGAGTTCAGAGCATGGTTAACATTCCTGGTGTCCCCTTCTTCTCATGTCAACGTGGGTACAAGGG 3′
GBV-B4-35fwd-2		5′ GTATGTGTGCAATTTCTTTGTGATTTGCTTTAATGTCCTTAAAGCTGGAGTTCAGAGCATGGTTAACATTCCTG 3′
GBV-B4-35rev-1		5′ CACAAAGAAATTGCACACATACTGCCAAATCTCTAGACCCCACGAGCCGGAGCATGGCGTGGAGCAGTCCTC 3′
GBV-B4-35rev-2		5′ CATGCTCTGAACTCCAGCTTTAAGGACATTAAAGCAAATCACAAAGAAATTGCACACATACTGCCAAATCTC 3′
GBV-C8-13fwd		5′ GGCTCGTGGCTAAGAGATTTGTGGGAGTGGATCATGACGGTGTTGACTGATTTCAAGACCTGG 3′
GBV-C8-13rev		5′ CTTGAAATCAGTCAACACCGTCATGATCCACTCCCACAAATCTCTTAGCCACGAGCCGGAGCATGG 3′
GBV-C4-16fwd		5′ GTACGTCTGGGACTTGTGGGAGTGGATCATGCGTCAAGTGACTGATTTCAAGACCTGGCTCCAGTCCAAGCTCC 3′
GBV-C4-16rev		5′ CTTGACGCATGATCCACTCCCACAAGTCCCAGACGTACGAGCCGGAGCATGGCGTGGAGCAGTCCTCGTTGATCC 3′
GBV-C4-29fwd-1		5′ GCGCATGGTGATGGCCAGACTTCGGGCCCTCTGCCCCCGATTGCCGGGAGTCCCCTTCTTCTCATGTCAACGTGG 3′
GBV-C4-29fwd-2		5′ CGTCTGGGACTTGTGGGAGTGGATCATGCGTCAAGTGCGCATGGTGATGGCCAGACTTCGGGCCCTCTGCC 3′
GBV-C4-29rev-1		5′ CTTGACGCATGATCCACTCCCACAAGTCCCAGACGTACGAGCCGGAGCATGGCGTGGAGCAGTCCTCGTTGATCC 3′
GBV-C4-29rev-2		5′ GGGCAGAGGGCCCGAAGTCTGGCCATCACCATGCGCACTTGACGCATGATCCACTCCCACAAGTCCCAGACGTA 3′
BVDV8-12fwd		5′ CCGGCTCGTGGCTAAGAGATGTCCTGGATTTGATCTGCACGGTGTTGACTGATTTCAAGACCTG 3′

BVDV8-12rev		5′ GAAATCAGTCAACACCGTGCAGATCAAATCCAGGACATCTCTTAGCCACCGAGCCGGAGCATGGCG 3′
BVDV8-13fwd		5′ CCGGCTCGTGGCTAAGAGATGTCCTGGATTTGATCTACACGGTGTTGACTGATTTCAAGACCTGGC 3′
BVDV8-13rev		5′ GAAATCAGTCAACACCGTGTAGATCAAATCCAGGACATCTCTTAGCCACCGAGCCGGAGCATGGC 3′
BVDV4-32fwd-1		5′ GAGGCTTGAAAAAAATAGTCTTGGGGTGGGCTCCCCGGAGTCCCCTTCTTCATGTCAACGTGGGTACAAGGGAG 3′
BVDV4-32fwd-2		5′ CCTGGATTTGATCTACAGCCTACATAAACAGATAAATAGAGGCTTGAAAAAAATAGTCTTGGGGTGGGCTCCCG 3′
BVDV4-32rev-1		5′ GTAGGCTGTAGATCAAATCCAGGACATAATTCCCAGAGCATGGCGTGGAGCAGTCCTCGTTGATCCACTGGTG 3′
BVDV4-32rev-2		5′ CAAGACTATTTTTTTTCAAGCCTCTATTTATCTGTTTTATGTAGGCTGTAGATCAAATCCAGGACATAATTCCCAG 3′
3596fwd		5′ TGCTGTATAGGCTGGGAGCC 3′
4923rev	MluI	5′ GGTGTACGCGTTAATGGGG 3′

[a] Restriction enzyme recognition sites are underlined, and start and stop codons are in boldface.

ruses investigated thus far (reviewed in references 1, 32, and 49). A specific membrane alteration, designated membranous web, was found to harbor the HCV replication complex (17, 23). In addition, the determinants for membrane association of the nonstructural proteins involved in HCV RNA replication have been mapped (reviewed in reference 41). In this context, we and others have shown that an N-terminal amphipathic alpha helix mediates the membrane association of HCV NS5A (8, 19). Targeting to the endoplasmic reticulum (ER) or an ER-derived modified compartment occurred via a posttranslational mechanism (8). Analysis of the three-dimensional structure of the membrane segment by nuclear magnetic resonance (NMR) spectroscopy revealed an amphipathic alpha helix that is embedded in plane into the cytosolic leaflet of the membrane bilayer (46). Systematic mutational analyses demonstrated that the exact positioning of the conserved residues exposed to the cytosol on the hydrophobic side of the helix is essential for HCV RNA replication independent of membrane association. Based on these observations, we have proposed that the HCV NS5A membrane anchor domain constitutes a platform that is involved in specific protein-protein interactions essential for the assembly of a functional replication complex (46).

The aim of this study was to identify the determinants for membrane association of NS5A from the related GB viruses and pestiviruses. We demonstrate that membrane association of NS5A by an N-terminal amphipathic alpha helix is a feature shared by HCV and these related members of the family *Flaviviridae*. Despite structural conservation, only very limited exchanges with sequences from these related viruses were functionally tolerated in a subgenomic HCV replicon, suggesting virus-specific interactions of these segments. This conservation and the virus-specific functional differences may be exploited in the search for viral and/or cellular factors involved in HCV RNA replication and the development and evaluation of novel antiviral strategies.

MATERIALS AND METHODS

Sequence analyses and structure predictions. Sequence analyses were performed using facilities available at the Institut de Biologie et Chimie des Protéines Network Protein Sequence Analysis website (NPSA) (http://npsa-pbil.ibcp.fr) (14). Protein secondary structures were deduced from a large set of prediction methods available at the NPSA website, including DSC, HNNC, SIMPA96, SOPM, GOR4, PHD, and Predator. NS5A sequences from GBV-A, GBV-B, GBV-C, BVDV, classical swine fever virus (CSFV), and other pestiviruses, as well as NS5 sequences from flaviviruses, were retrieved from the UniProt protein database (5) using the homology search program BLAST (2) or FASTA (45). HCV NS5A sequences were retrieved from the European HCV Database (http://euhcvdb.ibcp.fr/) (15) using BLAST. Multiple-sequence alignments were performed with CLUSTAL W (61) using default parameters. The repertoire of residues at each amino acid position and their frequencies observed in natural sequence variants were computed by using a program developed in our laboratory (F. Dorkeld, C. Combet, F. Penin, and G. Deléage, unpublished data).

Peptide synthesis and circular-dichroism (CD) analyses. Peptides GBV-C $NS5A_{1–27}$ (YVWDLWEWVMRQVRMVMSRLRALCPVV) and BVDV $NS5A_{1–28}$ (SGNYVLDLIYSLHKQINRGLKKIVLGWA) were synthesized using the stepwise solid-phase method of Merrifield employing Fmoc (*N*-9-fluorenylmethoxycarbonyl) chemistry and were purified to homogeneity by reverse-phase high-pressure liquid chromatography. The purities of both peptides were greater than 95%, as determined by reverse-phase high-pressure liquid chromatography and electrospray mass spectroscopy.

CD spectra were recorded on a CD6 dichrograph from Jobin Yvon calibrated with (1*S*)-(+)-10-camphorsulfonic acid. Measurements were carried out at room temperature using a 0.1-cm-path-length quartz cuvette, with peptide concentra-

tions ranging from 40 to 50 μM. Spectra were recorded in the 190- to 250-nm wavelength range with a 0.5-nm increment and a 2-s integration time. The spectra were processed with CD6 software, baseline corrected, and smoothed using a third-order least-square polynomial fit. Spectral units were transformed into molar ellipticity per residue using peptide concentrations determined by measurements of UV light absorbance at 280 nm and molar extinction coefficients of 1,536 and 5,600 $M^{-1} \cdot cm^{-1}$ for tyrosine and tryptophan, respectively. The alpha helix content was estimated at 222 nm using the empirical equation of Chen et al. (12), as detailed previously (35), with a theoretical molar ellipticity per residue of −35740 and −35870 degrees $\cdot$ cm^2 $\cdot$ $dmol^{-1}$ for 100% helical conformation of the GBV-C $NS5A_{1–27}$ and BVDV $NS5A_{1–28}$ peptides, respectively.

Plasmids. NS5A fragments from GBV-B, GBV-C, and BVDV were amplified by PCR from functional cDNA clones kindly provided by Jens Bukh (National Institutes of Health, Bethesda, MD) (11), Jack Stapleton (University of Iowa, Iowa City, IA) (65), and Sven-Erik Behrens (Fox Chase Cancer Center, Philadelphia, PA) (56), respectively. The beginnings and ends of the NS5A sequences of these related viruses had been determined experimentally in the cases of GBV-C (6) and BVDV (55, 66) or by sequence comparison in the case of GBV-B (51). Plasmids generated by ligation into pcDNA3.1(+) (Invitrogen, La Jolla, CA) allowed both eukaryotic expression from a cytomegalovirus promoter and in vitro transcription from a T7 RNA polymerase promoter. Constructs derived from pUHD10-3 (24) allowed inducible expression from a tetracycline-controlled-transactivator-dependent promoter.

For amplification of GBV-B NS5A sequences, primer pairs GBV-B-5Afwd/GBV-B-5A-HArev, GBV-B-5AΔN33fwd/GBV-B-5A-HArev, and myc-GBV-B-5Afwd/GBV-B-5A-HArev were used (Table 1). PCR fragments were digested with EcoRI and XhoI and cloned into pcDNA3.1(+) to yield plasmids pCMV-GBV-B-5A-HA, pCMV-GBV-B-5AΔN33-HA, and pCMV-myc-GBV-B-5A-HA, respectively. pCMV-GBV-B-5A-HA allows the expression of GBV-B-NS5A with a C-terminal hemagglutinin (HA) epitope tag. In pCMV-myc-GBV-B-5A-HA, an additional c-*myc* epitope tag is fused to the N terminus. pCMV-GBV-B-5AΔN33-HA allows the expression of GBV-B NS5A with a deletion of the N-terminal 33 amino acids and a C-terminal HA tag.

NS5A fragments of GBV-C were generated by PCR using primer pairs GBV-C-5Afwd/GBV-C-5A-HArev, GBV-C-5Afwd/GBV-C-5A-FLAGrev, GBV-C-5AΔN27fwd/GBV-C-5A-HArev, and GBV-C-5AΔN27fwd/GBV-C-5A-FLAGrev (Table 1) to yield plasmids pCMV-GBV-C-5A-HA, pCMV-GBV-C-5A-FLAG, pCMV-GBV-C-5AΔN27-HA, and pCMV-GBV-C-5AΔN27-FLAG, respectively. These constructs allow the expression of full-length GBV-C NS5A or of a protein with deletion of the N-terminal 27 amino acids and a C-terminal HA or FLAG tag.

BVDV NS5A fragments were PCR amplified using primer pairs BVDV-5Afwd/BVDV-5A-HArev and BVDV-5AΔN28fwd/BVDV-5A-HArev (Table 1). The PCR products were cloned into pcDNA3.1(+) and pUHD10-3 (24) via EcoRI and XbaI restriction sites, yielding plasmids pCMV-BVDV-5A-HA, pCMV-BVDV-5AΔN28-HA, pUHD-BVDV-5A-HA, and pUHD-BVDV-5AΔN28-HA, respectively. These constructs allow the expression of full-length BVDV NS5A or of a C-terminally HA-tagged deletion mutant lacking the N-terminal 28 amino acids.

The N-terminal 33 amino acids of GBV-B were fused to the N terminus of the green fluorescent protein (GFP) by three successive PCR amplification steps using pEGFP-N1 (Invitrogen) as a template and the primer pairs GBV-B-5AN33fwd-1/GFP + 600rev, GBV-B-5AN33fwd-2/GFP + 250rev, and GBV-B-5AN33fwd-3/GFP-XbaIrev (Table 1). The product of the third PCR was digested with EcoRI and XbaI, followed by ligation into pcDNA3.1(+) to yield pCMV-GBV-B-5AN33-GFP. Primer pairs GFP-600fwd/GBV-B-5AN33rev-1, GFP-250fwd/GBV-B-5AN33rev-2, and GFP-EcoRIfwd/GBV-B-5AN33rev-3 (Table 1) were employed to fuse the N-terminal 33 amino acids of GBV-B NS5A to the C terminus of GFP by the same strategy. The resulting plasmid was named pCMV-GFP-GBV-B-5AN33. Fusions of the N-terminal 27 and 28 amino acids of GBV-C and BVDV NS5A to the N and C termini of GFP were prepared accordingly using forward primers GBV-C-5AN27fwd-1, GBV-C-5AN27fwd-2, GBV-C-5AN27fwd-3, BVDV-5AN28fwd-1, BVDV-5AN28fwd-2, and BVDV-5AN28fwd-3 and the reverse primers GBV-C-5AN27rev-1, GBV-C-5AN27rev-2, GBV-C-5AN27rev-3, BVDV-5AN28rev-1, BVDV-5AN28rev-2, and BVDV-5AN28rev-3 (Table 1). The resulting constructs will be referred to as pCMV-GBV-C-5AN27-GFP, pCMV-BVDV-5AN28-GFP, pCMV-GFP-GBV-C-5AN27, and pCMV-GFP-BVDV-5AN28.

Replicon constructs were derived from pCon1/SG-Neo(I)/AflII, a subgenomic genotype 1b replicon harboring an adaptive change in NS5A (S2204I) (39) (kindly provided by Charles M. Rice, The Rockefeller University, New York, NY), using overlap extension PCR. Each chimeric sequence was introduced into complementary forward and reverse primers (Table 1). Fragments were amplified from pCon1/SG-Neo(I)/AflII using forward primers with the flanking reverse primer 4923rev and reverse primers with the flanking forward primer 3596fwd. Two forward and reverse primers each were used in two sequential PCRs to exchange the entire HCV NS5A alpha helix with the corresponding segments from GBV-B, GBV-C, and BVDV (Table 1). PCRs yielded two fragments for each construct, with an overlapping region comprising the exchanged sequence. The fragments were connected in a final PCR using flanking primers 3596fwd and 4923rev. The amplification products were subcloned into pCon1/SG-Neo(I)/AflII via SspI and MluI to yield constructs pCon1/SG-Neo(I)/AflII/5A-GBV-B8-13 (GBV-B 8-13), pCon1/SG-Neo(I)/AflII/5A-GBV-B4-13 (GBV-B 4-13), pCon1/SG-Neo(I)/AflII/5A-GBV-B4-35 (GBV-B 4-35), pCon1/SG-Neo(I)/AflII/5A-GBV-C8-13 (GBV-C 8-13), pCon1/SG-Neo(I)/AflII/5A-GBV-C4-16 (GBV-C 4-16), pCon1/SG-Neo(I)/AflII/5A-GBV-C4-29 (GBV-C 4-29), pCon1/SG-Neo(I)/AflII/5A-BVDV8-12 (BVDV 8-12), pCon1/SG-Neo(I)/AflII/5A-BVDV8-13 (BVDV 8-13), and pCon1/SG-Neo(I)/AflII/5A-BVDV4-32 (BVDV 4-32). A replicon construct with substitutions inactivating the NS5B RNA-dependent RNA polymerase, pCon1/SG-Neo(pol^-)/GFP-FLAGI.1 (39) (kindly provided by Charles M. Rice), was used as a negative control.

For analyses of polyprotein processing and subcellular localization of the chimeric constructs, replicon SfiI fragments were subcloned into the T7 RNA polymerase-driven expression construct pTM-NS3-3′ (28) (kindly provided by Ralf Bartenschlager, University of Heidelberg, Heidelberg, Germany).

All plasmids were verified by DNA sequencing.

Cell lines. Tetracycline-regulated cell lines were generated as described previously (38, 40). In brief, the constitutively tetracycline-controlled-transactivator-expressing U-2 OS human osteosarcoma-derived founder cell line UTA-6 (20) was cotransfected with pUHD-BVDV-5A-HA or pUHD-BVDV-5AΔN28-HA and pBabepuro (42), followed by selection with G418 and puromycin. Antibiotic-double-resistant clones were pooled and subjected to immunofluorescence microscopy and membrane flotation analyses 48 h following tetracycline withdrawal.

Cell line Huh-7.5 (7), a highly permissive, alpha interferon-cured Huh-7 derivative was kindly provided by Charles M. Rice (The Rockefeller University, New York, NY).

Huh-7 cells constitutively expressing the T7 RNA polymerase (Huh7-T7-IZ cells) were kindly provided by Thomas Pietschmann and Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany).

Immunofluorescence and confocal laser scanning microscopy. Immunofluorescence staining was performed as described previously (38, 40). In brief, cells grown as monolayers on glass coverslips were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, blocked with phosphate-buffered saline containing 3% bovine serum albumin and 0.05% saponin, and incubated with primary antibodies in phosphate-buffered saline containing 3% bovine serum albumin and 0.05% saponin. Bound primary antibody was revealed with Alexa-488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR), Cy3-conjugated goat anti-rabbit (Jackson Laboratories, West Grove, PA), or fluorescein isothiocyanate-conjugated sheep anti-rabbit (Roche Diagnostics, Indianapolis, IN) antibody. The coverslips were mounted in SlowFade (Molecular Probes) and examined with a Zeiss Axiovert fluorescence microscope equipped with the Spot RT Slider digital camera device (Diagnostic Instruments, Sterling Heights, MI). Confocal laser scanning microscopy was performed using a Zeiss LSM 510 microscope. Images were processed with Adobe Photoshop 7.0 software.

Immunoblot analyses. Immunoblot analyses were performed as described previously (38, 40).

Antibodies. Polyclonal anti-HA serum Y-11 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal antibody (MAb) M2 against the FLAG epitope from Sigma (Saint Louis, MO). Peroxidase-labeled anti-HA high-affinity rat MAb 3F10 was purchased from Roche Diagnostics. MAb JL-8 against GFP was obtained from Clontech (Palo Alto, CA). MAbs G1/296 against p63 (53), 9E10 against the c-*myc* epitope (21), and 11H against HCV NS5A (8) were kindly provided by Hans-Peter Hauri (University of Basel, Basel, Switzerland), Winfried Wels (Georg-Speyer-Haus Institute for Biomedical Research, Frankfurt, Germany), and Jan Albert Hellings (bioMérieux, Boxtel, The Netherlands), respectively.

Membrane flotation assays. Hypotonic lysis and equilibrium centrifugation through Nycodenz gradients were performed as described previously (33, 36, 46). Seven fractions of 200 μl each were collected from the top and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses.

In vitro transcription and RNA electroporation. Plasmids were linearized with ScaI, and in vitro transcription was performed essentially as described previously (7). Transcripts were purified using the RNeasy Mini Kit (QIAGEN, Hilden,

Germany) with an on-column DNase treatment using the RNase-Free DNase Set (QIAGEN).

Huh-7.5 cells were transfected with in vitro-transcribed RNA by electroporation essentially as described previously (7). In brief, RNA transcripts (1 μg) were mixed with 0.4 ml of washed cells containing 6×10^6 cells in a 2-mm-gap cuvette and immediately pulsed (820 V; 99-μs pulse length; five pulses at 1-s intervals) using a BTX ECM 830 square-wave electroporation system (Genetronics, San Diego, CA). The cells were seeded into 100-mm-diameter dishes at 6×10^5, 6×10^4, and 6×10^3 cells per dish, together with cells transfected with pCon1/SG-Neo(pol$^-$)/GFP-FLAGl.1 RNA transcripts so that the total cell number was maintained at 6×10^5 cells per dish. Seventy-two hours after the plating, selection was started with 700 μg/ml G418. Three weeks later, drug-resistant colonies were fixed with 7% formaldehyde, followed by staining with 1.25% crystal violet in 25% ethanol to facilitate colony counting.

RESULTS

Amphipathic alpha helices are present at the N termini of NS5As from GB and pestiviruses. We recently reported that the N terminus of HCV NS5A comprises a unique membrane anchor domain that is folded into an amphipathic alpha helix embedded in plane into the cytoplasmic leaflet of the membrane bilayer (8, 46). We also reported that the N terminus of BVDV NS5A as well includes an amphipathic alpha-helix sharing similar membrane interaction features (50). No obvious amino acid sequence similarities between HCV and GB viruses or pestiviruses were observed in this region. However, as illustrated in Fig. 1, secondary-structure predictions performed using various methods revealed the presence of putative alpha helices in the N-terminal NS5A sequences of HCV and GBV-A, -B, and -C, as well as BVDV and CSFV. To compare the physicochemical properties and the structural constraints of these segments, we assembled the repertoires of amino acids observed at each sequence position from sequence analyses of reported variants of the corresponding viruses (Fig. 1). To highlight the conservation of the physicochemical features of each sequence position despite the variability of the residues observed, each position was classified as hydrophobic, hydrophilic, or variable, according to the nature of the residues observed at the position (see the legend to Fig. 1 for details). Comparison of the resulting hydropathic patterns in the predicted or, in the cases of HCV (46) and BVDV (50), experimentally determined alpha helices revealed common hydrophobic and hydrophilic positions for all viruses. A relatively long stretch of six residues in the N-terminal portion (positions 8 to 13 in relation to the HCV sequence) comprises five hydrophobic positions exhibiting mainly large aliphatic (Leu, Ile, and Val) or aromatic (Trp and Tyr) residues surrounding the acidic position 10 (except for GBV-B, where a glutamine is observed). In addition, single common positions were observed in the C-terminal portion of the helices. These corresponded to hydrophobic positions 19, 23, and 27, exhibiting mainly large aliphatic residues, and hydrophilic position 24, predominantly occupied by basic residues. Helical-wheel projections of the most commonly reported amino acid sequence of each virus are depicted in Fig. 1. These projections clearly demonstrate the amphipathic nature of the N-terminal NS5A alpha-helical segments: the conserved hydrophobic residues, corresponding to amino acids 8, 12, 19, and 23 of HCV, are located within the center of the hydrophobic side of the alpha helix, while the conserved acidic residue is found in the center of the hydrophilic side (position 10 in HCV). In addition, tryptophan and tyrosine residues are found at the predicted interface between the hydrophilic and hydrophobic sides of the helix. This observation argues in favor of an interaction in plane of the membrane interface for these putative membrane anchor domains, as tryptophan and tyrosine are located preferentially at the phospholipid interface rather than within the hydrophobic core of the membrane (25, 46, 48, 52, 67). The remaining amino acid residues, which are not common for the different viruses, also contribute to the amphipathic nature of the alpha helices.

In conclusion, despite the poor amino acid sequence similarities between the NS5A N-terminal sequences of HCV, GB viruses, and pestiviruses, a common amphipathic alpha-helical pattern is predicted, which suggests similar functions for this structural determinant in all these viruses.

Structure predictions were validated experimentally for GBV-C and BVDV. To this end, peptides consisting of the N-terminal 27 and 28 amino acids of NS5A from GBV-C and BVDV, designated GBV-C $NS5A_{1-27}$ and BVDV $NS5A_{1-28}$, respectively, were chemically synthesized (Fig. 2). A recent analysis of BVDV $NS5A_{1-28}$ by NMR and molecular-dynamics simulations using a water-octanol interface allowed us to determine its three-dimensional structure and the positioning of its amphipathic alpha helix at the hydrophobic-hydrophilic phospholipid interface (50). The CD experiments reported here (Fig. 2) allowed us to corroborate our previous findings and constitute a reference for the analysis of the GBV-C $NS5A_{1-27}$ peptide in various membrane-mimetic environments. These CD experiments also allowed us to probe the helix folding and stability dependency to the polar heads of detergents used to mimic the phospholipid polar-head features at the membrane interface.

CD spectra of GBV-C $NS5A_{1-27}$ revealed this peptide to be quite soluble in water at pH 4.0 and to exhibit a spectrum typical of an alpha helix, with a maximum around 192 nm and two minima around 208 and 222 nm (Fig. 2A). Addition of trifluoroethanol (TFE) up to 50%, which stabilizes the folding of peptide sequences with an intrinsic propensity to adopt an alpha-helical structure (9, 34), had almost no effect on the shape and the amplitude of the CD spectra (Fig. 2A). These features are very reminiscent of the previously reported characteristics of an HCV $NS5A_{1-31}$ peptide (8) and suggest that the GBV-C $NS5A_{1-27}$ peptide forms micelle-like oligomers that interact by hydrophobic interactions. In contrast, at neutral pH in water, the amplitude of the CD signal strongly decreased and the spectral shape indicated a mixture of poorly defined conformations, including alpha helix structures, suggesting some aggregation. No such aggregation was observed at neutral pH in the presence of the detergents used here to mimic the membrane environment, since the CD spectra showed roughly the same shape and amplitude as in 50% TFE (Fig. 2B). This behavior is in agreement with a strong propensity of the peptide to bind to lipids. A helical content of 80 to 86% was estimated at 222 nm in water at acidic pH, in 50% TFE, and in the various detergents irrespective of the pH, indicating that about 22 of the 27 amino acids of GBV-C $NS5A_{1-27}$ adopt an alpha-helical fold. This is perfectly in line with the secondary-structure prediction shown in Fig. 1.

In contrast to GBV-C $NS5A_{1-27}$, BVDV $NS5A_{1-28}$ was poorly soluble in water and gave a complex CD spectrum at pH

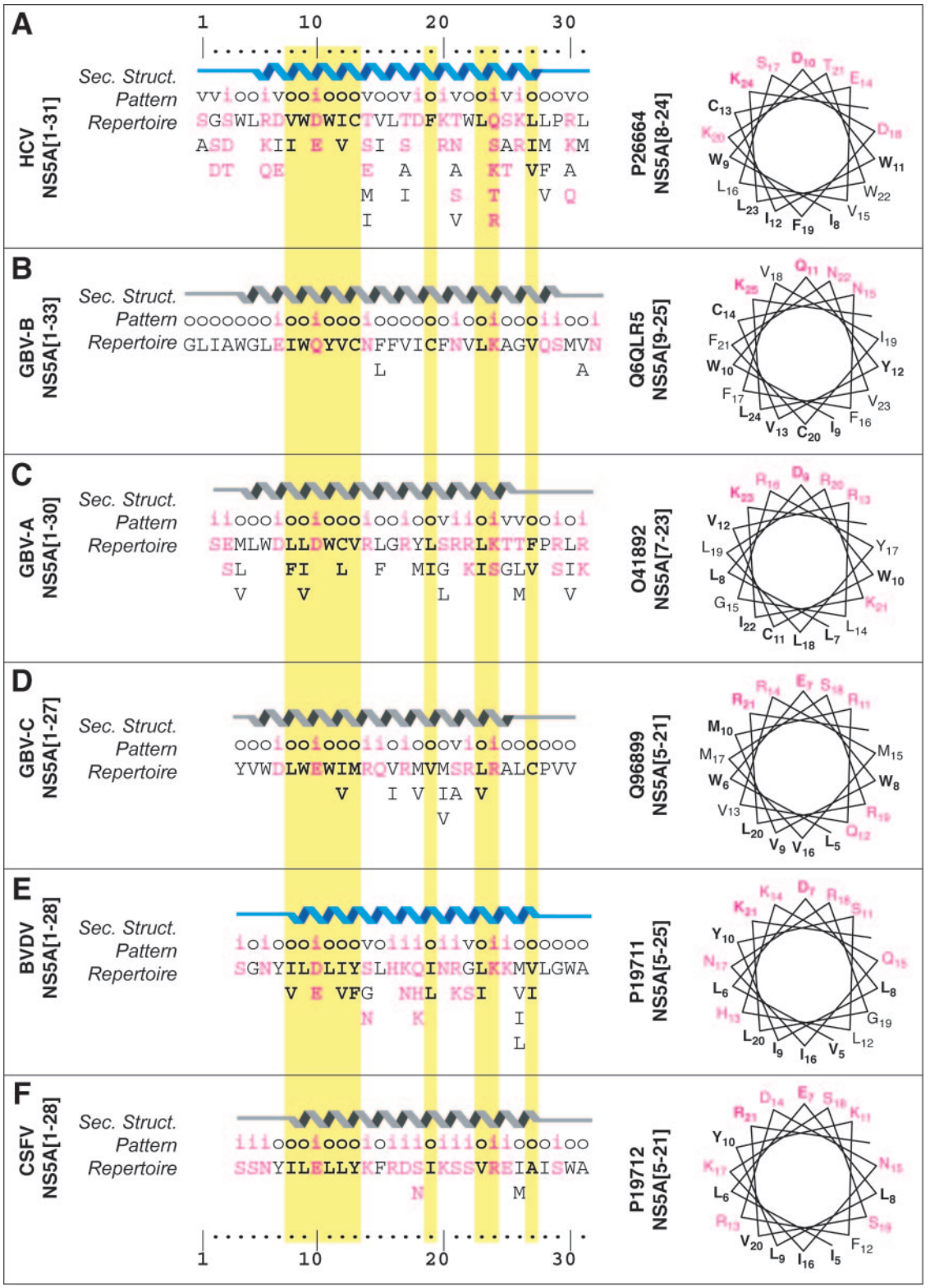

FIG. 1. Amino acid sequence analyses and secondary-structure predictions of the N-terminal segments of NS5A proteins from HCV and related viruses. Sequence analyses and amino acid repertoires of the N-terminal segments of NS5A from (A) HCV, (B) GBV-B, (C) GBV-A, (D) GBV-C, (E) BVDV, and (F) CSFV are shown on the left. The HCV NS5A sequence was used as a reference for amino acid position numbering. The sequence variability observed in natural variants for each virus is presented as the repertoire of amino acids at each position, in decreasing order of observed frequency, from top to bottom. Repertoires were deduced from multiple-sequence alignments, including 116, 3, 3, 15, 16, and 8 sequences for HCV, GBV-B, GBV-A, GBV-C, BVDV, and CSFV, respectively. The least frequently observed residues at each position were not reported, i.e., less than two times for GBV-C, BVDV, and CSFV and less than 2% for HCV, as they might have been due to

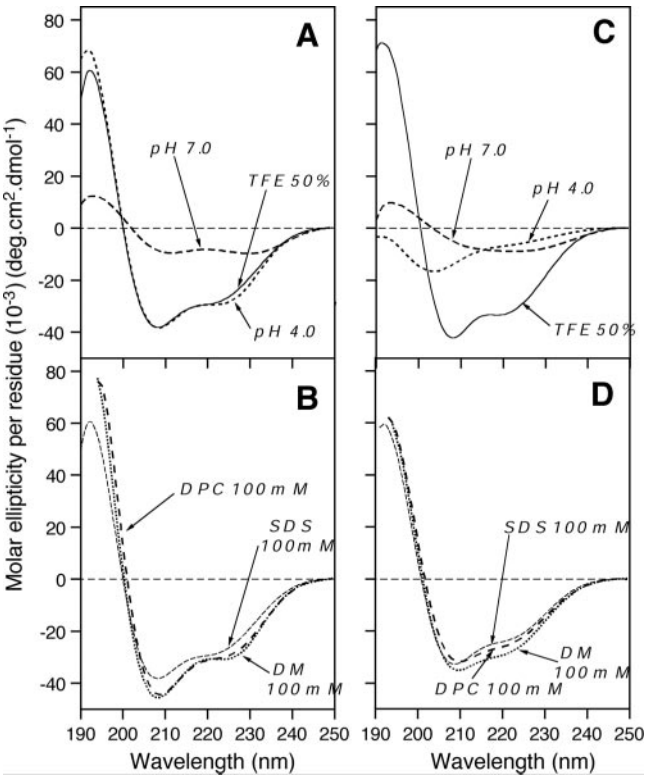

FIG. 2. CD analyses of synthetic peptides GBV-C $NS5A_{1-27}$ and BVDV $NS5A_{1-28}$. CD spectra of (A and B) GBV-C $NS5A_{1-27}$ and (C and D) BVDV $NS5A_{1-28}$ were recorded in water (pH 4.0) or 10 mM sodium phosphate buffer, pH 7.0, complemented with either 50% TFE (solid line) in panels A and C or the following detergents in panels B and D: 100 mM SDS (dashed line), 100 mM dodecyl phosphocholine (DPC) (large dashed line), and 100 mM *n*-dodecyl β-D-maltoside (DM) (dotted line). deg, degrees.

4.0 (Fig. 2C), with a large negative band around 200 nm and a shoulder at about 220 nm, indicating a mixture of random coil structure with some poorly defined secondary structures. At pH 7.0, the positive band around 195 nm and the large negative band in the 210- to 235-nm range indicated some poorly defined secondary structures, suggesting aggregation. The presence of 50% TFE, as well as of various detergents, clearly stabilized an alpha-helical fold of the peptide (Fig. 2C and D). Quantitatively, a helical content ranging from 66 to 78% was estimated in the presence of the various detergents, indicating that 19 to 22 amino acids adopt an alpha-helical fold. This helix content is in accordance with our recent NMR structure analysis of this peptide in SDS micelles (50). The higher helical content of 90% estimated in 50% TFE is due to an improved stabilization of the flexible region in this solvent (50).

For both GBV-C $NS5A_{1-27}$ and BVDV $NS5A_{1-28}$, the amplitude and shape variations observed in the various detergents could be attributed to differences of peptide interactions with the polar heads of the different detergents used here, which exhibit the same dodecyl hydrophobic tail. For GBV-C $NS5A_{1-27}$, the higher helical contents detected in *n*-dodecyl β-D-maltoside and dodecyl phosphocholine compared to negatively charged SDS denote a greater stabilization of the helical fold in neutral and zwitterionic environments (Fig. 2B and D). Similar behavior was observed for BVDV $NS5A_{1-28}$, although the helical-content differences were smaller. This suggests that both peptides might interact with specific phospholipids in the membrane. In contrast, the lack of spectral-shape variation for both peptides at various pHs in the presence of these detergents indicates that the folding of the peptides, as well as their interactions with lipids, is not sensitive to pH variation (data not shown).

In conclusion, GBV-C $NS5A_{1-27}$ and BVDV $NS5A_{1-28}$ display a high propensity to adopt an alpha-helical structure upon association with lipid-like molecules. These experimental data, together with the structure predictions (Fig. 1), demonstrate that the presence of an N-terminal amphipathic alpha helix is a feature shared by NS5A proteins from hepaciviruses, GB viruses, and pestiviruses. By contrast, sequence analyses did not reveal similar structural determinants in the NS5 proteins from members of the genus *Flavivirus* (data not shown).

The N-terminal 33, 27, and 28 amino acids serve as membrane anchors of NS5A proteins from GBV-B, GBV-C, and BVDV, respectively. Systematic sequence analyses and structure predictions, followed by CD analyses of synthetic peptides, identified amphipathic alpha helices at the N termini of NS5A from GBV-B, GBV-C, and BVDV. To assess whether these domains could mediate membrane association, we fused the N-terminal 33, 27, and 28 amino acids of NS5A from GBV-B, GBV-C, and BVDV, respectively, in frame to either the N or C terminus of GFP. The constructs were transfected into U-2 OS human osteosarcoma cells, followed by confocal laser scanning microscopy. Representative results are shown in Fig. 3. In contrast to GFP which is diffusely distributed within the cytoplasm and nucleus (data not shown), the fusion constructs showed a fluorescence pattern that included the nuclear membrane, was pronounced in the perinuclear region, and extended in a reticular fashion throughout the cytoplasm. No staining of the nucleus or plasma membrane was observed. This pattern is indistinguishable from the one previously described for GFP fusion proteins comprising the N-terminal 30

PCR and/or sequencing errors and/or sequencing of defective viruses. However, all variations were considered for GBV-B and GBV-A, since only three sequences were reported for each of these viruses. Hydrophobic (F, I, W, Y, L, V, M, P, C, A, and G) and hydrophilic (T, S, K, Q, N, H, E, D, and R) amino acids are color coded in black and pink, respectively (classification based on the Eisenberg hydrophobicity scale [18]). The hydropathic consensus patterns deduced from repertoires are letter coded as follows: i, hydrophilic positions; o, hydrophobic positions; v, variable positions (i.e., positions where both hydrophilic or hydrophobic residues were observed). Conserved hydrophilic and hydrophobic positions in all virus sequences are in boldface characters and are highlighted in yellow. Above each repertoire, experimentally determined (46, 50) or predicted alpha helices are shown in blue and gray, respectively. The corresponding helical-wheel projections are shown on the right. Note that only the helical segments common to all viruses and including the conserved hydrophilic and hydrophobic positions were reported. The corresponding segment and the SwissProt-TrEMBL ID are indicated for each virus prototype sequence.

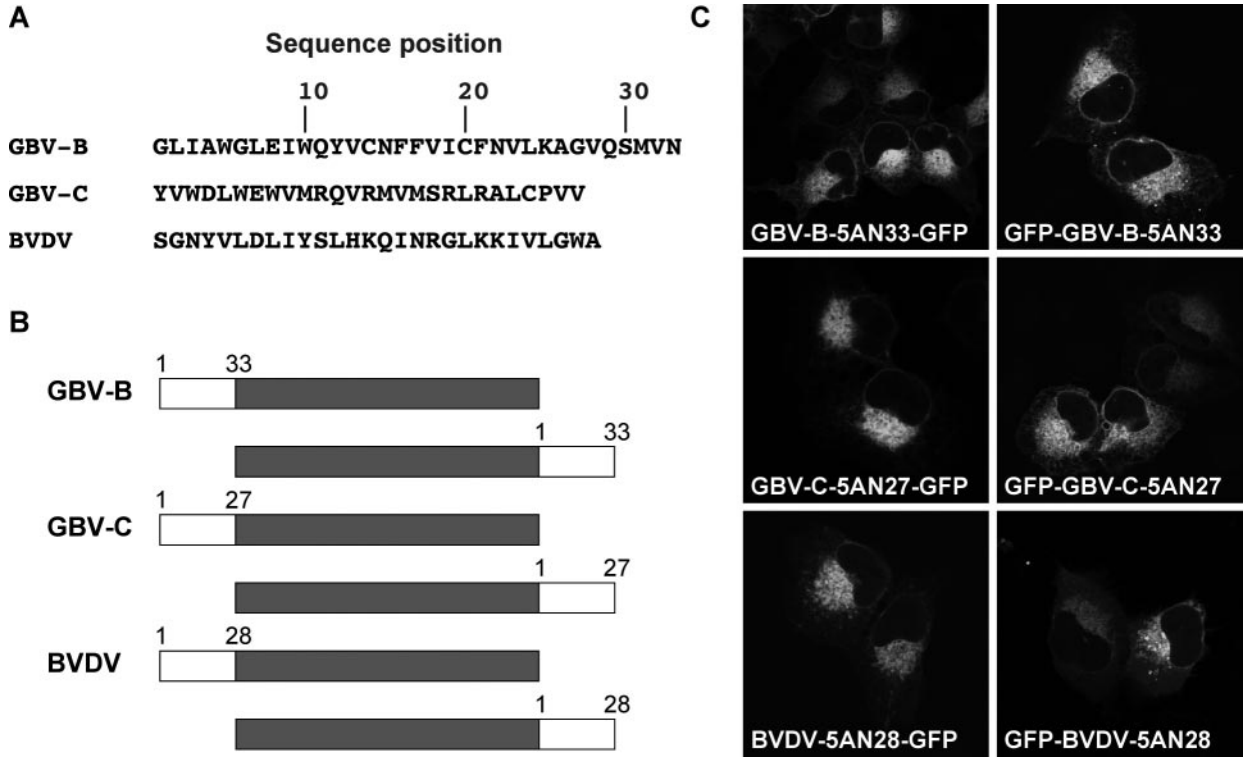

FIG. 3. The N-terminal 27 to 33 amino acids of GBV-B, GBV-C, and BVDV NS5A proteins are sufficient to mediate membrane association. (A) Amino acid sequences of the GBV-B (Q6QLR5), GBV-C (Q96899), and BVDV (P19711) isolates used in this study. (B) The N-terminal 27 to 33 amino acids of GBV-B, GBV-C, and BVDV NS5A proteins were fused to either the N or C terminus of GFP. (C) GFP fusion constructs were expressed in U-2 OS cells and examined by confocal laser scanning microscopy, as described in Materials and Methods.

amino acid residues of HCV NS5A (8), indicating that the N-terminal domains of GBV-B, GBV-C, and BVDV contain sufficient information for targeting of a heterologous protein to the ER membrane. Interestingly, fusion of the putative membrane anchor domains to the C terminus of GFP resulted in the same fluorescence pattern, suggesting that these segments do not function as classical signal sequences but mediate post-translational targeting to the ER membrane. Similar to HCV NS5A, this was confirmed by in vitro transcription-translation analyses performed in the presence or absence of microscomal membranes (data not shown).

Membrane flotation analyses were performed to confirm and extend these observations. To this end, U-2 OS cells transfected with the different expression constructs were subjected to hypotonic lysis, followed by equilibrium centrifugation of cell lysates in 37.5 to 5% Nycodenz gradients. Under these conditions, membrane proteins float to the upper, low-density fractions, while soluble proteins remain in the lower, high-density fractions (Fig. 4A). As shown in Fig. 4B, p63, an integral ER membrane protein (53), floated to the upper fractions. Disruption of membranes by 1% Triton X-100 resulted in a shift into the lower fractions. Constructs comprising the N-terminal segments of NS5A from GBV-B, GBV-C, and BVDV fused to GFP floated to the upper, low-density fractions, confirming the membrane association observed by fluorescence microscopy. A proportion of the fusion proteins could be observed in the high-density fractions, which likely reflected some degree of aggregation. However, the fluorescence pattern and the membrane flotation data clearly demonstrate that the N-terminal 33, 27, and 28 amino acids of NS5A from GBV-B, GBV-C, and BVDV are sufficient to mediate membrane association.

Membrane association of NS5A from GBV-B, GBV-C, and BVDV. The data presented above demonstrate that, in analogy to HCV, NS5A proteins from GBV-B, GBV-C, and BVDV possess membrane anchor domains within the N-terminal 27 to 33 amino acids. To confirm this observation and to investigate whether further determinants are required for proper subcellular localization, we generated epitope-tagged full-length and N-terminally truncated NS5A expression constructs. Tagged GBV-B and GBV-C constructs were transiently transfected into U-2 OS cells and analyzed by immunofluorescence, as well as membrane flotation analyses. Because of rather low protein yields after transient transfection, pools of stable cell lines inducibly expressing NS5A were established in the case of BVDV. As shown in the left column of Fig. 5, the staining pattern of C-terminally tagged NS5A proteins from GBV-C and BVDV was similar to that of the GFP fusion constructs (Fig. 3) and to that previously described for HCV NS5A (8). Unexpectedly, immunofluorescent staining of the C-terminally tagged GBV-B NS5A construct revealed in most cells a diffuse pattern (data not shown), conflicting with the data obtained with the GFP fusion proteins described above. Membrane flotation experiments performed with this construct demonstrated a smaller product in the high-density fractions, suggesting the presence of a degradation product with a deletion of

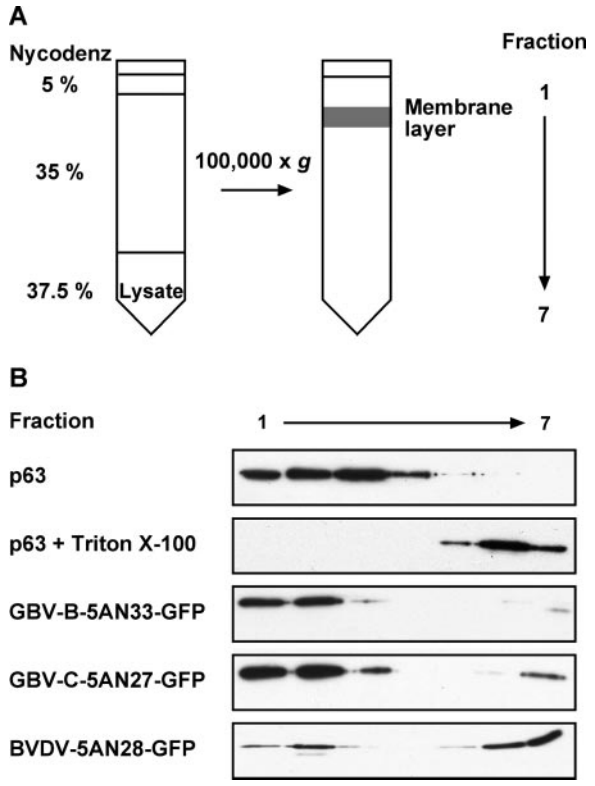

FIG. 4. Membrane flotation analyses of GFP fusion constructs. (A) Schematic diagram of the membrane flotation procedure. Following hypotonic lysis, postnuclear lysates were loaded at the bottom of a 37.5 to 5% Nycodenz gradient. During equilibrium centrifugation for 20 h at 100,000 × *g*, membranes float to the upper, low-density fractions. Seven fractions of equal volume were collected from the top and analyzed by immunoblotting. (B) Lysates of U-2 OS cells transfected with pCMV-GBV-B-5AN33-GFP, pCMV-GBV-C-5AN27-GFP, and pCMV-BVDV-5AN28-GFP were analyzed. p63 was included as a control. Flotation of this integral ER membrane protein was abolished by treatment of the membranes with 1% Triton X-100 for 20 min at 4°C. MAbs JL-8 against GFP and G1/296 against p63 were used for immunoblotting.

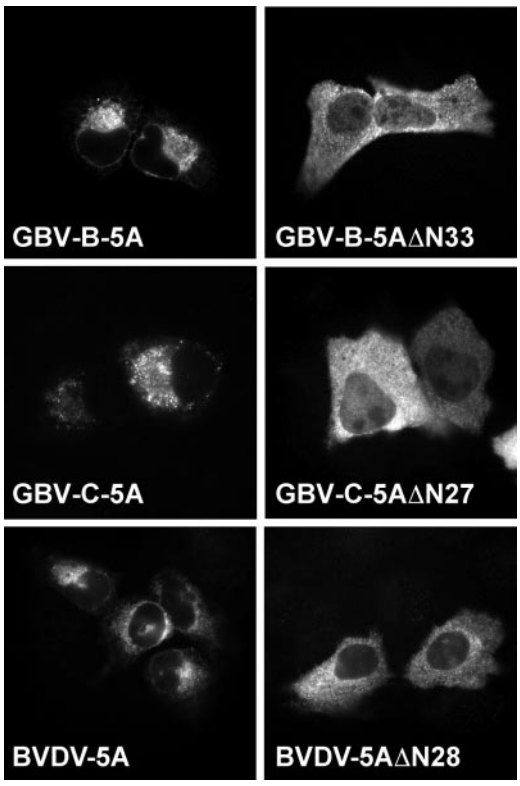

FIG. 5. Subcellular distributions of full-length and N-terminally truncated NS5A proteins from GBV-B, GBV-C, and BVDV. U-2 OS cells were transfected with constructs pCMV-myc-GBV-B-5A-HA (GBV-B-5A), pCMV-GBV-B-5AΔN33-HA (GBV-B-5AΔN33), pCMV-GBV-C-5A-FLAG (GBV-C-5A), and pCMV-GBV-C-5AΔN27-FLAG (GBV-C-5AΔN27) and fixed 48 h later. Pools of cell lines inducibly expressing constructs pUHD-BVDV-5A-HA (BVDV-5A) and pUHD-BVDV-5AΔN28-HA (BVDV-5AΔN28) were analyzed 48 h following tetracycline withdrawal. Proteins were visualized with a polyclonal anti-HA antibody or MAb 9E10 directed against the c-*myc* epitope tag, followed by detection with secondary antibodies, as described in Materials and Methods.

the N-terminal putative membrane anchor domain (data not shown). Therefore, we generated a construct with an additional N-terminal c-*myc* epitope tag. Staining of this construct with a MAb directed against the c-*myc* tag showed the expected subcellular distribution typical of an ER-associated protein (Fig. 5, upper left image). Deletion of the N-terminal 33, 27, and 28 amino acid residues, respectively, of GBV-B, GBV-C, and BVDV led to loss of membrane association by these proteins and a diffuse distribution in the cell (Fig. 5, right column). In contrast to HCV NS5A with a deletion of its N-terminal segment (8), no accumulation in the nuclei was found. In this context, sequence analyses using PredictNLS (http://rostlab.org/cgi/var/nair/resonline.pl) (13) revealed a nuclear localization signal only in HCV NS5A, but not in the NS5A proteins from GB viruses and pestiviruses.

To confirm the immunofluorescence data, full-length and truncated NS5A proteins were expressed in U-2 OS cells and subjected to membrane flotation analyses. As shown in Fig. 6, full-length proteins were found almost exclusively in the upper, low-density fractions while deletion of the N-terminal membrane anchor domains resulted in a shift into the lower, high-density fractions.

Taken together, the immunofluorescence and membrane flotation data demonstrate that, very similarly to HCV, the N-terminal segments are responsible for subcellular targeting and membrane association of NS5A proteins from GB viruses and pestiviruses.

Analyses of chimeric replicon constructs. A panel of chimeric replicons was constructed to investigate whether the N-terminal amphipathic alpha helix of GBV-B, GBV-C, or BVDV NS5A could functionally replace the corresponding segment of HCV NS5A. As illustrated in Fig. 7, segments from GBV-B, GBV-C, and BVDV NS5A proteins of different lengths were introduced into HCV NS5A. The entire N-terminal amphipathic alpha helix was replaced in constructs GBV-B 4-35, GBV-C 4-29, and BVDV 4-32. The first 3 amino acids of HCV NS5A were left unchanged in constructs GBV-B 4-35 and GBV-C 4-29 to maintain proper cleavage at the NS4B/NS5A site. Less extensive exchanges were performed in constructs GBV-B 4-13 and GBV-C 4-16, and only the highly conserved stretch of 6 amino acids from the N-terminal portion of the helix (positions 8 to 13 in relation to the HCV sequence)

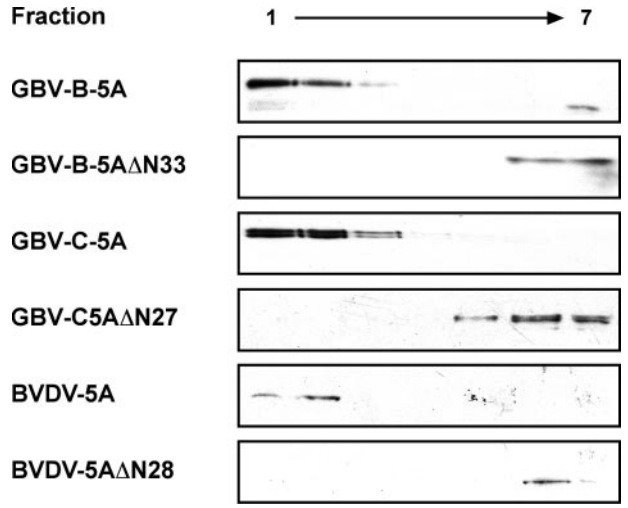

FIG. 6. Membrane flotation analyses of full-length and N-terminally truncated NS5A proteins. U-2 OS cells were transfected with pCMV-GBV-B-5A-HA (GBV-B-5A), pCMV-GBV-B-5AΔN33-HA (GBV-B-5AΔN33), pCMV-GBV-C-5A-FLAG (GBV-C-5A), and pCMV-GBV-C-5AΔN27-FLAG (GBV-C-5AΔN27), followed by hypotonic lysis and membrane flotation analyses 48 h posttransfection. Cell pools stably expressing pUHD-BVDV-5A-HA (BVDV-5A) or pUHD-BVDV-5AΔN28-HA (BVDV-5AΔN28) were derepressed by tetracycline withdrawal for 48 h. A horseradish peroxidase-labeled anti-HA antibody and MAb M2 against the FLAG tag were used for immunoblotting.

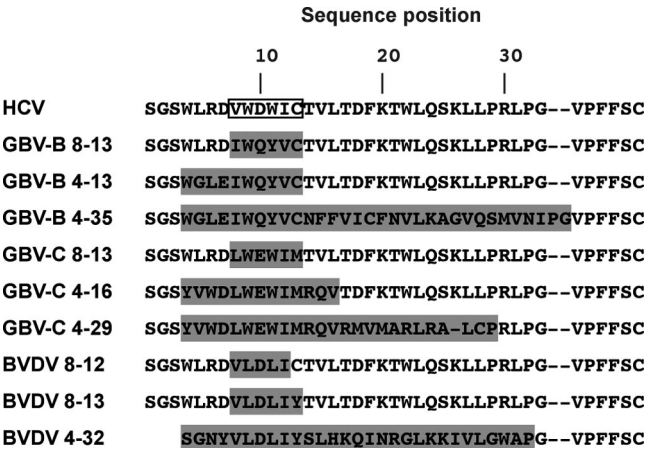

FIG. 7. Chimeric HCV replicons. The highly conserved 6-amino-acid stretch in the N-terminal portion of the amphipathic alpha helices (Fig. 1) is boxed. Substituted amino acid residues are highlighted in gray.

(see above) was replaced in constructs GBV-B 8-13, GBV-C 8-13, and BVDV 8-13. Finally, construct BVDV 8-12 was prepared to maintain the cysteine residue that is absolutely conserved at HCV NS5A position 13. RNA was in vitro transcribed from these constructs and electroporated into Huh-7.5 cells, followed by G418 selection. As shown in Fig. 8A, replication of construct GBV-C 8-13 was only moderately impaired compared to the parental HCV replicon. This construct carried the most conservative changes with respect to the HCV sequence (valine to leucine at position 8, aspartate to glutamate at position 10, and cysteine to methionine at position 13) and maintained the absolutely conserved tryptophan residues at positions 9 and 11. We had previously shown that cysteine 13 is not essential for HCV RNA replication (46). Construct GBV-B 8-13, which carried the second most conservative changes but did not maintain the tryptophan at position 11, yielded only very few colonies at the highest cell density (Fig. 8A). All other constructs did not yield any viable clones, as illustrated in an exemplary manner for GBV-C 4-29 (Fig. 8A). These results indicate that, despite structural conservation, only very limited changes were tolerated in the context of functional HCV RNA replication.

To exclude a defect in the polyprotein processing or membrane association of the chimeric NS5A proteins, all constructs were subcloned into a T7 RNA polymerase promoter-driven expression construct comprising the entire HCV nonstructural region and analyzed in Huh-7 cells that constitutively expressed T7 RNA polymerase (Huh7-T7-IZ cells). As shown in Fig. 8B, NS5A was correctly processed in all constructs. In addition, immunofluorescence microscopy revealed that all constructs were associated with membranes (Fig. 8C).

DISCUSSION

In this study, we demonstrated that in-plane membrane association of NS5A by an N-terminal amphipathic alpha helix is a feature shared by hepaciviruses, GB viruses, and pestiviruses. Similar to our earlier observations with HCV NS5A (8), the N-terminal 27 to 33 amino acids were sufficient to correctly target and anchor to the membrane the NS5A proteins from GBV-B, GBV-C, and BVDV. In addition, systematic sequence analyses and structure predictions allowed us to extend these observations to GBV-A and CSFV, another member of the genus *Pestivirus*.

The observation that very similar membrane anchor domains with highly ordered structures can be found in these related viruses strongly supports the hypothesis that these protein domains share a common function that goes beyond subcellular targeting to membranes. Specific interactions with viral or host cell factors are of particular interest in this context. The primary amino acid sequences of the N-terminal segments of NS5A from GBV-C and BVDV diverge significantly from the HCV sequence. However, detailed analyses of the physicochemical properties of amino acid residues observed at the different positions identified strikingly similar motifs. The three-dimensional structure of the BVDV NS5A membrane anchor domain was recently resolved by NMR (50). In addition, the presence of an amphipathic alpha helix upon association with lipid-like molecules within the N-terminal 27 and 28 amino acids of NS5A proteins from GBV-C and BVDV, respectively, could be validated by CD analyses of synthetic peptides representing these segments in membrane-mimetic environments. These observations indicate that the structural characteristics of these segments, rather than their primary amino acid sequences, are conserved features of these membrane anchors.

Interestingly, it was recently shown that a zinc-binding motif similar to the one characterized in HCV NS5A (57, 58) is conserved in BVDV NS5A (59). In addition, HCV and BVDV NS5As seem to be phosphorylated by the same or similar cellular kinase(s) (47). Finally, NS5As from HCV and BVDV

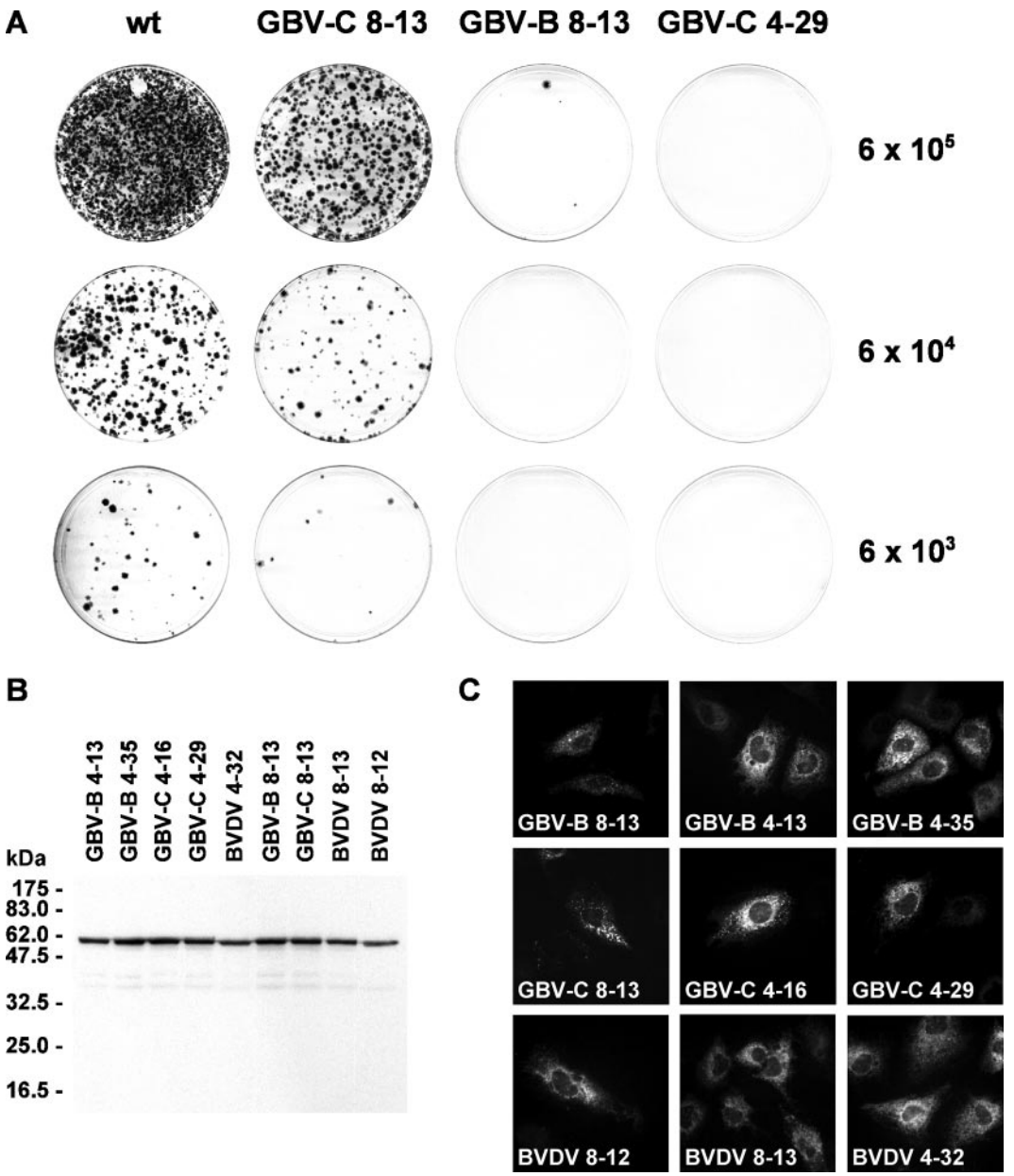

FIG. 8. RNA replication, polyprotein processing and subcellular localization of chimeric constructs. (A) RNA was in vitro transcribed from the indicated replicon constructs and electroporated into Huh-7.5 cells, followed by plating on 100-mm-diameter dishes at 6×10^5, 6×10^4, and 6×10^3 cells per dish and G418 selection, as described in Materials and Methods. G418-resistant colonies were stained with crystal violet after 3 weeks. GBV-C 4-29 is representative of all other constructs, as well as the control construct pCon1/SG-Neo(pol$^-$)/GFP-FLAGI.1, which did not yield any viable clones. wt, wild type. (B) Chimeric constructs in the context of the entire nonstructural-protein region were expressed from a T7 promoter-driven expression construct in Huh7-T7-IZ cells. Cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis, followed by immunoblotting using MAb 11H against HCV NS5A. (C) Huh7-T7-IZ cells transfected as in panel B were analyzed by immunofluorescence microscopy using MAb 11H.

are the only viral replication complex components that can be complemented in *trans* (3, 26). Collectively, structural and functional evidence suggests that the NS5A proteins from HCV and related viruses serve similar functions in the viral life cycle. Determining this function is one of the most active areas in HCV research today.

A panel of chimeric HCV replicons was constructed to investigate whether the N-terminal amphipathic alpha helices of GBV-B, GBV-C, or BVDV NS5A could be exchanged as modules and functionally replace the corresponding segment of HCV NS5A. Interestingly, despite structural conservation, only very limited exchanges were tolerated in this context. The polyprotein processing and membrane association of these chimeras were unaffected. However, only the most conservative chimera, GBV-C 8-13, replicated efficiently. This construct carried changes from valine to leucine at position 8, aspartate

to glutamate at position 10, and cysteine to methionine at position 13 but maintained the absolutely conserved tryptophan residues at positions 9 and 11. We had previously shown that cysteine 13 is not essential for HCV RNA replication (46). Construct GBV-B 8-13, which carried the second most conservative changes but did not maintain the tryptophan at position 11, yielded only very few colonies. The other constructs did not yield any viable clones.

These results point to an essential role of tryptophan residues 9 and 11 in HCV NS5A. The essential role of these tryptophan residues is highlighted by construct BVDV 8-12. The severe replication defect of this construct can be attributed entirely to these tryptophan residues. Tryptophan residues 9 and 11 could be involved in specific interactions with other viral or cellular membrane proteins. Alternatively (or in addition), mutations of these residues may affect the relative positioning of the amphipathic alpha helix at the membrane interface and, in consequence, the accessibility of the putative interaction site for its partner. In support of this idea, ongoing molecular-dynamics studies indicate subtle differences in the depths of membrane embedding of the N-terminal amphipathic alpha helices of NS5A proteins from HCV and BVDV (N. Sapay and F. Penin, unpublished data).

A recent study tested the modularity of an N-terminal amphipathic alpha helix conserved in picornavirus 2C proteins and HCV NS5A (60). Similar to our findings in HCV and related viruses, analyses of chimeric polioviruses in which this segment was replaced by N-terminal sequences of 2C proteins from other picornaviruses or from HCV NS5A suggested that exchangeability may be restricted due to requirements for specific interactions with other components involved in viral replication.

In conclusion, our results demonstrate that in-plane membrane association of NS5A by an N-terminal amphipathic alpha helix is a conserved feature of hepaciviruses, GB viruses, and pestiviruses. This observation supports the potential relevance of GB viruses and pestiviruses as model systems for HCV and points to conserved roles of the N-terminal amphipathic alpha helices of NS5A in replication complex formation. This conservation and virus-specific differences may be exploited in the search for viral and/or cellular factors involved in HCV RNA replication and in the development and evaluation of novel antiviral strategies. Indeed, several groups have recently shown that the membrane anchor domains of NS5A and NS5B as peptides can interfere with membrane association (19, 29) and HCV RNA replication (T. Pietschmann, V. Brass, D. Moradpour, and R. Bartenschlager, unpublished data).

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