JOURNAL OF VIROLOGY, June 2004, p. 6271–6281 Vol. 78, No. 12
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.12.6271–6281.2004

RNA Recombination In Vivo in the Absence of Viral Replication

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Received 21 November 2003/Accepted 10 February 2004

To study fundamental aspects of RNA recombination, an in vivo RNA recombination system was established. This system allowed the efficient generation of recombinant cytopathogenic pestiviruses after transfection of synthetic, nonreplicatable, subgenomic transcripts in cells infected with a replicating noncytopathogenic virus. Studies addressing the interplay between RNA recombination and replication revealed that cotransfection of noninfected cells with various pairs of nonreplicatable RNA derivatives also led to the emergence of recombinant viral genomes. Remarkably, homologous and nonhomologous recombination occurred between two overlapping transcripts, each lacking different essential parts of the viral RNA-dependent RNA polymerase (RdRp) gene. Apart from the generally accepted viral replicative copy choice recombination, our results prove the existence of a viral RdRp-independent mechanism of RNA recombination that occurs in vivo. It appears likely that such a mechanism not only contributes to the evolution of RNA viruses but also leads to the generation of recombinant cellular RNAs.

RNA recombination leads to the exchange of genetic information. The recombinant RNA molecules contain covalently linked nucleotide sequences from each parental RNA molecule. RNA recombination plays a significant role in the evolution of animal, plant, and bacterial RNA viruses by generating genetic variation, by removing debilitating or lethal mutations from a replicating genome, and also by generating new viruses (2, 11, 19, 29, 32, 34, 40, 42, 52, 55). Moreover, RNA recombination can contribute to pathogenicity of viral diseases (8, 30, 38). Whereas cellular RNA sequences are joined together by nonreplicative site-specific splicing reactions (1, 20, 47, 51), viral RNA recombination is in many cases not site specific, suggesting different mechanisms for these processes. For viral RNA recombination, two main mechanisms have been proposed: (i) replicative template switching, also known as copy choice recombination, and (ii) nonreplicative breakage and rejoining (2, 32, 42).

It is generally accepted that viral RNA recombination involves a viral RNA-dependent RNA polymerase (RdRp) that eventually jumps, together with the nascent RNA, to a different region of the same template or to another template and continues RNA synthesis (16, 23, 29, 32, 34, 42, 43, 45). Although copy choice recombination during viral RNA synthesis has been extensively studied, only a few studies have supported nonreplicative recombination. Two reports have described viral nonreplicative RNA recombination in a cell-free system composed of the 5′ and 3′ fragments derived from a replicable RNA, purified bacteriophage Qβ replicase, and ribonucleoside triphosphates (14, 15). However, the presence of Qβ replicase required for amplification of the recombinant molecules did not fully exclude a replicative mechanism. Even if we assume that RNA recombination in this in vitro system was actually the result of the suggested nonreplicative transesterification mechanism, it is still unknown whether nonreplicative RNA recombination also occurs in vivo. There is only one study suggesting nonreplicative RNA recombination in vivo. In that study, Gmyl et al. described the generation of recombinant polioviruses after transfection of cells with overlapping 5′ and 3′ RNA fragments of the poliovirus genome (25). The 5′ fragment encompassed a nearly entire 5′ nontranslated region (NTR) of the poliovirus genome harboring all *cis*-active elements for replication and translation, whereas the 3′ fragment contained the large open reading frame encoding the complete poliovirus polyprotein and the 3′ NTR but lacked essential elements in the 5′ NTR. Since the 3′ recombination partner encoded a complete viral RdRp, it could not be excluded that minimal levels of translation led to expression of the viral polymerase promoting replicative copy choice RNA recombination. Thus, the existence of nonreplicative RNA recombination in vivo remains to be proven.

The pestivirus *Bovine viral diarrhea virus* (BVDV) belongs to the family *Flaviviridae*, which also comprises human *Hepatitis C virus*, as well as flaviviruses such as *Yellow fever virus* and *West Nile virus* (27). Pestiviruses possess a positive-sense nonpolyadenylated RNA genome with a length of 12.3 kb and encode one large polyprotein that is co- and posttranslationally processed (36). The single open reading frame is flanked by 5′ and 3′ terminal NTRs encompassing *cis*-acting elements essential for viral transcription, translation, and replication. For BVDV, cytopathogenic (cp) viruses often evolve by nonhomologous RNA recombination in animals persistently infected with noncytopathogenic (noncp) BVDV and are crucial for induction of fatal mucosal disease (5, 8, 38). Molecular analyses of cp pestivirus strains led to detection of genomic alterations including insertions of cellular or viral sequences, as well as deletions and duplications of viral sequences (5, 6, 8, 9, 37, 38, 53). However, these studies contributed only little to our understanding of fundamental aspects of RNA recombination.

We describe here a novel system for studying the mechanism of RNA recombination in vivo. The results of our analyses revealed that pestiviral genomes can be generated by RNA recombination between two replication-incompetent partners. The emergence of recombinant BVDV after transfection of

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cells with overlapping 5′ and 3′ fragments of the BVDV genome, each lacking different essential parts of the viral RdRp gene, unequivocally demonstrates the existence of a viral polymerase-independent mechanism of RNA recombination occurring in vivo.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% horse serum. The cp and noncp BVDV strains CP7 and NCP7 have been described previously (17, 38).

Construction of cDNA clones. The infectious BVDV full-length cDNA clones pCP7-5A and pNCP7-5A have been described previously (5, 7). All nucleotide numberings included here refer to pCP7-5A. For construction of pNCP7-M1, nucleotides 12226 to 12229 (located in the 3′ NTR) were deleted by quick-change PCR (QC-PCR) with a subgenomic cDNA clone encompassing a ClaI (11076)/AatII(12259) fragment (nucleotide positions are indicated in parentheses); this fragment was then cloned into pNCP7-5A precut with ClaI and AatII (see Fig. 4A). For cloning of pNCP7-GAA, the nucleotide sequence GGGGA TGAC (positions 11285 to 11293) encoding the GDD motif was changed by QC-PCR to GGGGCTGCC encoding the amino acids GAA; finally, the XhoI (222)/ClaI(11076) fragment of pNCP7-5A was cloned into the resulting plasmid precut with ClaI and XhoI (see Fig. 6). Plasmid pNCP7-ΔNaeI/SmaI resulted from religation of NaeI(11491)/SmaI(12294)-digested pNCP7-5A (see Fig. 6). pNCP7-ΔClaI/ClaI was generated after introduction of an additional ClaI site at nucleotide 12081 into pNCP7-5A, followed by ClaI digestion and religation (see Fig. 6). For generation of pCP7-11449, an additional SmaI site was introduced by QC-PCR in pNCP7-ΔNaeI/SmaI at position 11449; this silent mutation represents the genetic marker M4 (see Fig. 7C). Finally, the XhoI(222)/ClaI(11076) fragment of pCP7-5A was cloned into the resulting plasmid precut with ClaI and XhoI. For generation of plasmid pCP7-11284, a SmaI site, followed by an FseI site, was introduced at position 11284 by PCR, resulting in a subclone of pNCP7-5A encompassing positions 10756 to 11284; this SmaI site represents the genetic marker M5. The ClaI/FseI fragment of the resulting plasmid was introduced into pCP7-5A precut with ClaI and FseI (located directly downstream of the SmaI site). The cDNA clones pCP7-11284 and pCP7-11449 lack the entire or most of the genomic region downstream of the SmaI site used for linearization (see Fig. 7A).

For construction of p+ubi SGT, the NheI [preceding the SP6 promoter sequence of pCP7-5A)/AgeI(5336)] region of pCP7-5A was replaced by an NheI/AgeI fragment encompassing the T7 promoter sequence, followed by a cDNA sequence derived from BVDV strain CP14; the latter encodes the C-terminal five amino acids of NS2, a C-terminal fragment, as well as two complete monomers of cellular ubiquitin, and the N-terminal part of NS3. For generation of p−ubi SGT, the T7 promoter sequence upstream of the position corresponding to the genomic 5′ terminus was replaced by a singular SrfI site and a reverse T7 promoter sequence was integrated downstream of nucleotide 12294 (corresponding to the 3′ end of the viral genome). The lethal deletion of four nucleotides in p+ubi SGT M1M2 was introduced into +ubi SGT in analogy to the construction of pNCP7-M1 (see above). The genetic marker M3 within the 3′ NTR of p+ubi SGT M2M3 is represented by a nonlethal deletion of nucleotides 12195 to 12198. The genetic marker M2 representing a silent mutation of the EcoRV site at position 11904 was introduced into p+ubi SGT M2M3, p+ubi SGT M1M2, p+SGT-CP7-11201, and p+SGT-CP7-11450 (see Fig. 5 and 7B). To generate plasmids p+SGT-CP7-11201 and p+SGT-CP7-11450, CP7-5A-specific fragments were cloned by PCR with a reverse primer corresponding to nucleotides 12257 to 12274 and a coding primer encompassing a ClaI site followed by a T7 promoter sequence and positions 11201 to 11226 or positions 11450 to 11473 of CP7-5A, respectively; by using these primers, the genetic markers M6 and M7 each representing two silent nucleotide exchanges at positions 11203 and 11206 (M6) and 11452 and 11455 (M7) were introduced (Fig. 7C). After digestion with ClaI and AatII, the resulting fragments were introduced into pNCP7 HindIII/HindIII (lacking nucleotides 1902 to 10435) precut with ClaI and AatII. Further details of the cloning strategies, including the sequences of primers used for plasmid construction, are available on request. All changes introduced into the cDNA clones were controlled by automated nucleotide sequencing (7). After introduction of the genetic markers into the genome of CP7-5A, subsequent experiments showed that M2, M3, M4, M6, and M7 did not impair virus viability, whereas the presence of M5 resulted in almost complete loss of infectivity.

In vitro synthesis of RNA. After complete digestion of plasmid pNCP7-ΔNaeI/SmaI with ClaI and all other plasmids with SmaI, the linearized DNA was extracted with phenol-chloroform and precipitated with ethanol. Depending on the promoter of the cDNA clone, RNA was transcribed by SP6 (Takara) or T7 RNA polymerase (Ambion) by using standard conditions. Photometric quantification of the transcribed RNAs was carried out by using a GeneQuant II photometer (Pharmacia). The quality and the calculated amount of each RNA were controlled by ethidium bromide staining of samples after agarose gel electrophoresis. RNA transcripts used for transfection contained >80% of intact RNA.

Transfection of RNA, isolation of single recombinants, and quantification. For each transfection, the confluent cells from a 10-cm-diameter dish were resuspended in 0.4 ml of phosphate-buffered saline without Ca^{2+} and Mg^{2+} and then mixed with in vitro-transcribed RNA immediately before the pulse (950 μF and 180 V). For electroporation, a GenePulser II (Bio-Rad, Munich, Germany) was used. The electroporated cells were distributed to 48 wells of two 24-well plates immediately posttransfection and adjusted to 1 ml with medium for each well; at 72 to 96 h posttransfection, the cells were checked for cytopathic effect (CPE) by microscopic inspection and, after treatment with trypsin, the resuspended cells of each well were transferred separately to a well of a six-well plate. For detection of noncp BVDV, immunofluorescence (IF) analysis was performed with anti-NS3 monoclonal antibody 8.12.7 (7, 17). For further analyses, supernatants and cellular RNAs were harvested after additional incubation for 20 to 48 h. In control experiments, the transfected cells were seeded on six-well plates immediately posttransfection and checked 24 and 96 h posttransfection by light microscope and IF analysis.

Control experiments. For degradation of the template DNA, transcription reaction mixtures were digested with RNase-free Turbo-DNase I (Ambion), followed by extraction with phenol-chloroform and precipitation with ethanol if indicated. Moreover, transfection of linearized DNA templates alone did not lead to the generation of recombinant genomes. This demonstrates that DNA was not involved in the generation of recombinant genomes. To exclude that recombination during in vitro transcription by SP6 or T7 RNA polymerases led to the emergence of recombinant viral genomes, each transcript was synthesized separately. After transfection of replication-incompetent RNAs into naive cells, replicating recombinant viral genomes did not emerge. With regard to the transcripts CP7-11284, CP7-11449, +SGT-CP7-11201, and +SGT-CP7-11450, removal of the RNA polymerases from the transcription reactions by phenol-chloroform treatment after digestion with DNase I ensured that DNA-dependent RNA polymerases used for in vitro synthesis of transcripts did not contribute to the generation of recombinant genomes. In two independent experiments, cotransfection of the linearized DNA templates pCP7-11449 and p+SGT-CP7-11201 did not result in the emergence of recombinant viruses. Since reverse transcriptases and DNA polymerases can switch templates (28, 33, 57), reverse transcription-PCR (RT-PCR) used for analysis of recombinant genomes could have resulted in the generation of recombinant sequences. For all recombinants the genome lengths calculated after identification of the crossover sites agreed with the genome sizes when directly monitored by Northern blot analysis. Accordingly, there is no evidence that the recombinant sequences described here are due to RT-PCR artifacts. Finally, nucleotide sequence analysis of at least two different cDNA clones for each recombinant genome led to detection of unique crossover sites and confirmed the presence of genetic markers derived from the parental RNA molecules. This proves that the recombinant genomes originated from the respective recombination partners.

RESULTS

Establishment of the in vivo RNA recombination system. To gain insight into the mechanism of RNA recombination, we established an in vivo RNA recombination system. This system uses cells infected with noncp BVDV strain NCP7 that are transfected with a synthetic subgenomic transcript termed +ubi SGT. The latter encodes a C-terminal fragment as well as two complete copies of cellular ubiquitin and the viral nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B of NCP7; it also comprises the 3′ NTR but lacks the entire 5′ NTR with *cis*-acting signals essential for translation and replication, as well as the genomic region encoding the NS proteins N^{pro}, p7, and NS2 and the structural proteins (Fig. 1A). Previous analyses revealed that presence of ubiquitin-coding sequences directly upstream of the NS3-coding gene leads to expression of NS3, the marker protein of cp BVDV strains (53), and to viral cytopathogenicity (8). According to our work-

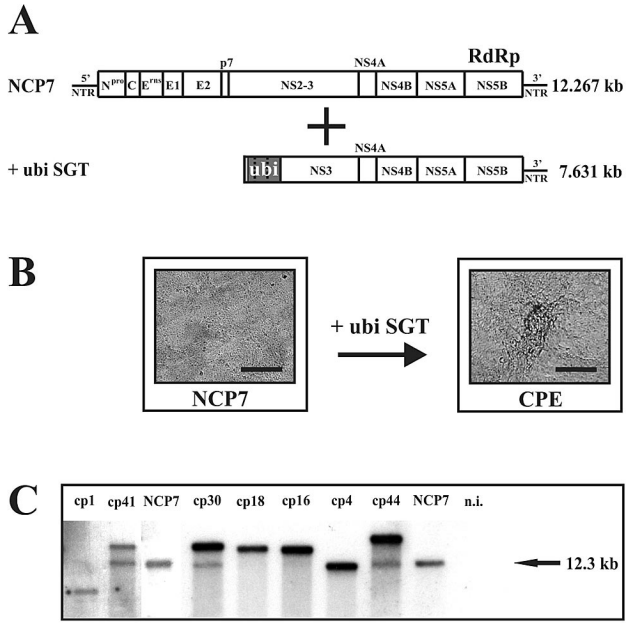


FIG. 1. In vivo RNA recombination system. (A) Schematic representation of the recombination partners. The RNA genome of noncp BVDV strain NCP7 contains one large open reading frame (box) flanked by 5′ and 3′ NTRs. The positions of the viral structural proteins (capsid protein C, envelope proteins E^{rns}, E1, and E2) and nonstructural proteins (N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B) within the viral polyprotein are indicated. The synthetic, replication-incompetent transcript +ubi SGT encodes the C-terminal five amino acids of NS2, a C-terminal fragment, as well as two complete monomers of cellular ubiquitin (ubi; dark gray box), and viral proteins NS3, NS4A, NS4B, NS5A, and NS5B. The transcript +ubi SGT also comprises the 3′ NTR but lacks the entire 5′ NTR and thereby *cis*-acting signals essential for translation and replication. The lengths of the bars are not drawn to scale. The sizes of the NCP7 genome and +ubi SGT are indicated in kilobases on the right. (B) MDBK cells 6 days after infection with NCP7 showing no signs of CPE (left) and NCP7-infected MDBK cells 4 days after transfection with +ubi SGT RNA (right). Note the CPE produced after transfection with +ubi SGT. Scale bar, 100 μm. (C) Northern blot analysis of total RNA from MDBK cells infected with NCP7 and seven independently emerged cp viral genomes (cp1, cp4, cp16, cp18, cp30, cp41, and cp44) and from noninfected (n.i.) MDBK cells. Preparation of RNA, gel electrophoresis, radioactive labeling of the probes, hybridization, and posthybridization washes were performed as described previously (7). Migration position and size (in kilobases) of the viral genomic RNA of NCP7 are indicated on the right. Note the various genomic sizes of the emerged cp viral genomes in comparison to the genomic RNA of NCP7.

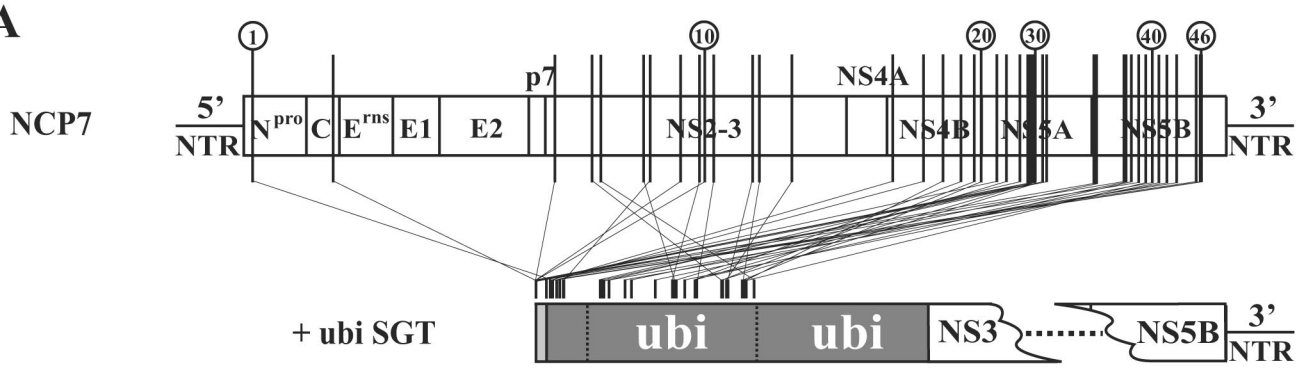
ing hypothesis, the joining of +ubi SGT with 5′-terminal parts of the NCP7 genome should result in the generation of cp viruses and/or subgenomes that can be identified by the appearance of a CPE. To address the possible significance of the polarity of the transfected RNA molecules, transcript −ubi SGT representing the minus strand that corresponds to +ubi SGT was included in these studies. Several independent transfection experiments with noninfected cells demonstrated that +ubi SGT and −ubi SGT are not able to replicate (data not shown). Interestingly, a clear CPE was visible 4 days after transfection of noncp BVDV-infected cells with either 0.3, 1, or 10 μg of +ubi SGT RNA (Fig. 1B) but not after transfection with ca. 10 μg of −ubi SGT RNA (data not shown).

Analysis of recombinants. To obtain several independently emerged cp viral genomes, the experiment including transfection with +ubi SGT was repeated, and the cells were seeded into 48 wells immediately after transfection. In each of four experiments, 10 μg of +ubi SGT RNA was used, and 16 to 28 CPE-producing genomes emerged. Of these, a total of 46 were analyzed. Northern blot assays indicated the presence of viral genomes being in most cases considerably larger or shorter compared to the genome of NCP7 (Fig. 1C). In contrast to the cp genomes, the genome of the noncp virus with a size of 12.3 kb was in many cases hardly visible. This interesting phenomenon will be addressed in future studies.

For further characterization, the RNAs of the 46 independently emerged cp viral genomes were subjected to RT-PCR and nucleotide sequence analyses. For RT-PCR, a reverse primer corresponding to positions 5373 to 5392 of the CP7-5A genome (located in the 5′-terminal region of the NS3 coding gene), and different coding primers corresponding to various locations of the NCP7 genome were used. For each cp viral genome, the appropriate coding primer was selected on the basis of the apparent size of the viral genomic RNA as determined by Northern blot analysis (Fig. 1C; data not shown). To assess whether RT-PCR artifacts contributed to the generation of recombinant cDNA molecules, RNAs prepared from cells infected with BVDV NCP7 and from noninfected cells were included in the RT-PCR analyses for each primer pair used. Taken together, these analyses resulted in the detection of a unique amplification product for each of the 46 cp viral genomes being absent when the RNAs from NCP7 and noninfected cells were used as templates for RT-PCR (data not shown). After being cloned in a bacterial vector, the nucleotide sequences of the RT-PCR products were determined by sequencing at least two different cDNA clones. Depending on the size of the individual amplification products, 0.7 to 2.0 kb were sequenced for each cp viral genome. According to nucleotide sequence analysis, the 46 cp genomes each comprise unique duplications or deletions of viral sequences (Fig. 2). The shortest recombinant genome (R-cp 1) had a calculated length of 8.010 kb, while the longest genome (R-cp 46) comprised 19.332 kb (Fig. 2 and 3); the latter is considerably larger than any pestiviral genome described thus far. For all analyzed recombinant sequences the open reading frame was maintained.

Analysis of the crossover regions revealed that the 5′ region of +ubi SGT was incorporated in 13 of the 46 analyzed recombinants, whereas the remaining recombinants comprised 5′-terminally truncated versions of +ubi SGT still encoding at least one ubiquitin monomer. With respect to the replicating partner (NCP7), the crossover sites were found to be distributed over the whole genome, with a preference for the genomic region encoding the NS proteins (Fig. 2A). Interestingly, 7 of the 46 crossover sites are present within 66 nucleotides of the NS5A coding region. According to the results of the RT-PCR and nucleotide sequence analyses, there is no evidence for the occurrence of multiple crossover events. However, it cannot be excluded that additional deletions and/or duplications are present in the cp recombinant viral genomes outside the analyzed genomic regions. For 23 recombinants, between one nucleotide (R-cp 2 and 11 others) and five nucleotides (R-cp 36) at the recombination junctions are identical for both

A



B

R-cp 1	5`-AAAUGAACUUUUAU AC AAAACAUACAAAC 425 18 AGAGUCCACUCUGC AC CUGGUCCUCCGCC- 3`	R-cp 24	5`-GUAAAUGAAGAUAUU GGGACUAUAAAACCA 9205 73 AAGACCUUGACCGGC AAGACCAUCACCCUG- 3`
R-cp 2	5`-AGAAAAAAGGAAAA G UCAAGAGCAAGAAC 1075 1 G GAUGGGUACUGAGG- 3`	R-cp 25	5`-UAAAUGAqGAUAUU GG GACUAUAAAACCA 9205 1 GG AUGGGUACUGAGG- 3`
R-cp 3	5`-GUGAUCCCACUGUG G UGCCACUAGUCACU 3907 1 G GAUGGGUACUGAGG- 3`	R-cp 26	5`-GAUAUUGGGACUAUA AAACCAGUACUAGGA 9214 1 GGAUGGGUACUGAGG- 3`
R-cp 4	5`-AAUGGCAGAUGGUG UA UAUGGCUUACUUA 4564 218 GCACCCUGUCUGAC UA CAACAUCCAGAAA- 3`	R-cp 27	5`-UACUAGGAGAGAGA G UGGUCACCGACCCA 9235 1 G GAUGGGUACUGAGG- 3`
R-cp 5	5`-CAGCACUUAUAGAAU UAAACUGGUCCAUGG 4676 266 UCCUCCGCCUCAGAG GUGGGAUGCAGAUCU- 3`	R-cp 28	5`-GGAGAGAGAGUGGUC ACCGACCCAGUGGUG 9241 187 AUCUUUGCCGGGAAA CAGCUGGAAGAUGGC- 3`
R-cp 6	5`-GACCCUUCAGGCAG GA AUACAAUGGGUUU 5044 158 AGGGCAUCCCCCCA GA CCAGCAGAGGCUG- 3`	R-cp 29	5`-CCAGUGGUGGAUGUU AACUUACAACCAGAA 9262 1 GGAUGGGUACUGAGG- 3`
R-cp 7	5`-CAACCAAAGUAAAAA UGAUCAUGGUAGGCA 5126 17 AAGAGUCCACUCUGC ACCUGGUCCUCCGCC- 3`	R-cp 30	5`-GGAUGUUAACUUAC A ACCAGAAGUACAAG 9269 222 CCUGUCUGACUACA A CAUCCAGAAAGAGU- 3`
R-cp 8	5`-CUAUUGGUUUGUGAC AGUAUGGGUAGAACU 5431 1 GGAUGGGUACUGAGG- 3`	R-cp 31	5`-CACCCGUAGUCGAA AA AACAGAGCCCAAU 9361 59 UGCAGAUCUUCGUG AA GACCUUGACCGGC- 3`
R-cp 9	5`-AGUGGGAAAGAAUGA GGAAUCCAAACCCAC 5745 160 AGACAAAGAGGGCAU CCCCCCAGACCAGCA- 3`	R-cp 32	5`-UGGGUUGGAUGAA GGA AGAUACCCAGGAC 9419 1 GGA UGGGUACUGAGGA- 3`
R-cp 10	5`-UUAAUGAGUGGUAUC CAAACUGUUUCUAAA 5779 1 GGAUGGGUACUGAGG- 3`	R-cp 33	5`-ACAAAUAAACAGAUG AGCUUGACCCCUUUA 9997 1 GGAUGGGUACUGAGG- 3`
R- cp 11	5`-AAGAUAACCAGCAUG AACAGGGGGGACUUU 5839 187 AUCUUUGCCGGGAAA CAGCUGGAAGAUGGC- 3`	R-cp 34	5`-UUGACCCCUUUAUUU GAGGAGCUAUUGCUA 10015 100 CUGGAGGUGGAGCCC AGUGACACCAUCGAG- 3`
R-cp 12	5`-GCAGUUuGCUGUUAU AGGAAAAAUUCACAG 6192 234 CAACAUCCAGAAAGA GUCCACUCUGCACCU- 3`	R-cp 35	5`-CUAAAAGCUUCCAU G AGGCAAUCAGAGAU 10459 1 G GAUGGGUACUGAGG- 3`
R-cp 13	5`-UCUGAGAGCAUAAGA GUGGUUGCCAUGACU 6226 256 CUGCACCUGGUCCUC CGCCUCAGAGGUGGG- 3`	R-cp 36	5`-GCAAUCAGAGAU AAGAU AGACAAGAAUGA 10476 130 AACGUCAAGGCC AAGAU CCAAGACAAAGA- 3`
R-cp 14	5`-CUGCCAGAUUUAGAU ACAGUUGUUGACACA 6583 232 UACAACAUCCAGAAA GAGUCCACUCUGCAC- 3`	R-cp 37	5`-UUCAUACAAUAGCC CA ACCCAGCCUAAGA 10549 188 UCUUUGCCGGGAAA CA GCUGGAAGAUGGC- 3`
R-cp 15	5`-UGCCCCCGCUUUCA A AGAAAACGUGGAAG 7541 6 GGUACUGAGGAAAG A GUCCACUCUGCACC- 3`	R-cp 38	5`-UUCUAGAGAAGAA GAA UGUUGGAGAAGUA 10645 2 GAUGGGUACUGAG GAA AGAGUCCACUCUG- 3`
R-cp 16	5`-CUGAAACCCAACAGG AAGGAAGGCGAUUCG 7829 11 UGAGGAAAGAGUCCA CUCUGCACCUGGUCC- 3`	R-cp 39	5`-GAGAGAUGUCAGUGA UGAUUGGCAAUCAGG 10773 252 CACUCUGCACCUGGU CCUCCGCCUCAGAGG- 3`
R-cp 17	5`-UUAGUACCACAAUAU ACAAAACUUACCUCU 8018 257 UGCACCUGGUCCUCC GCCUCAGAGGUGGGA- 3`	R-cp 40	5`-UAAAACAAGACUAG CC AUCACUAAAGUAA 10850 63 GAUCUUCGUGAAGA CC UUGACCGGCAAGA- 3`
R-cp 18	5`-CCAGAGAAAAUAAU A AUGGCCCUAUUUGA 8277 187 AUCUUUGCCGGGAA A CAGCUGGAAGAUGG- 3`	R-cp 41	5`-GGAAUGGGAUUUGU U CAAUGAACCAGUAG 10967 171 AGACCAGCAGAGGC U GAUCUUUGCCGGGA- 3`
R-cp 19	5`-UGGGGUUUACUACA A AGGCUGGGAAGCAA 8354 219 CACCCUGUCUGACU A CAUCCAGAAAGAGU- 3`	R-cp 42	5`-CAUAAGAAAUGGAC A AAGGGGUAGUGGCC 11165 90 GACCAUCACCCUGG A GGUGGAGCCCAGUG- 3`
R-cp 20	5`-AAGGGAAGAUAAGGA ACCUGUCUGGGAAUU 8468 251 CCACUCUGCACCUGG UCCUCCGCCUCAGAG- 3`	R-cp 43	5`-UAACAGAGAAGGGGC UGGGAUUAAAGUUUG 11333 8 UACUGAGGAAAGAGU CCAgUCUGCACCUGG- 3`
R-cp 21	5`-ACAAACAACUAUCUA AGAGUAGAAACUAAG 8626 1 GGAUGGGUACUGAGG- 3`	R-cp 44	5`-UUUCACAGCAGCCA G AGACAACUCCAUCA 11674 1 G GAUGGGUACUGAGG- 3`
R-cp 22	5`-UCAACUAUAGAGUCA CAAAGUACUAUGAUG 8765 191 UUGCCGGGAAACAGC UGGAAGAUGGCCGCA- 3`	R-cp 45	5`-AGAUGUAAUAGGUA A GAAUUUGUGUGAAU 11750 18 AGAGUCCACUCUGC A CCUGGUCCUCCGCC- 3`
R-cp 23	5`-CAAUUUAACCAGGUU AAUUGAAUUGGUACA 9108 60 GCAGAUCUUCGUGAA GACCUUGACCGGCAA- 3`	R-cp 46	5`-GUAAGAAUUUGUGU GA AUUAAAAAGGACG 11761 5 GGGUACUGAGGAAA GA GUCCACUCUGCAC- 3`

recombination partners, and therefore the crossover sites cannot be determined unambiguously; two and three identical nucleotides were found for eight and two of these recombinants, respectively (Fig. 2B). The fact that at least one ambiguous nucleotide was found in 50% of the recombination junctions provides evidence for a role of primary sequences in determining recombination sites, because only 25% of junctions would be predicted to show single ambiguous nucleotides if recombination occurred randomly. Moreover, the frequencies of junctions with two to five ambiguous nucleotides were between 3.8 and 22 times higher than expected on the basis of a random model. If we assume a replicative mechanism of RNA recombination, such short regions of sequence identities at the crossover sites suggest that sequence complementarity between the incomplete nascent RNA strand and the acceptor template may guide the RdRp as it switches templates (6, 42, 45). However, the absence of sequence identity at 23 of the analyzed recombination junctions indicates that at least 50% of the recombination sites were selected independent of base pairing between the nascent strand and the acceptor template. Similar to findings with other recombination systems (22, 41, 48), we also found that heteroduplexes between the two recombination partners can form between the sequences in the vicinity of some crossover sites (data not shown). The significance of heteroduplex formation for RNA recombination of pestiviruses will be addressed in a future study.

RNA recombination after transfection with two replication-incompetent RNAs. It was then examined whether RNA recombination occurs after transfection of cells with two synthetic transcripts. First, infectious RNA transcribed from the full-length cDNA clone of BVDV strain NCP7-5A (Fig. 4A) and +ubi SGT RNA were cotransfected, and this actually led to the emergence of a cp recombinant. To study the interplay between RNA recombination and replication, the recombination system was further modified by introduction of genetic alterations into both recombination partners. Various derivatives of both recombination partners were generated, and pairs of the resulting RNAs were cotransfected into noninfected cells.

To analyze whether RNA recombination requires the replication ability of one recombination partner, the genetic marker M1 representing a lethal deletion of four nucleotides was introduced into the 3′ NTR of NCP7-5A (Fig. 4A and B). Transfection with up to 10 μg of RNA derived from the respective construct pNCP7-M1 was repeated several times and did not result in the generation of replicating viruses, demonstrating the noninfectious nature of this RNA (data not shown). After cotransfection of the RNAs derived from pNCP7-M1 and p+ubi SGT, the emergence of 39 autonomously replicating viruses was observed in two independent experiments. In contrast to the experiments described above, the use of two non-

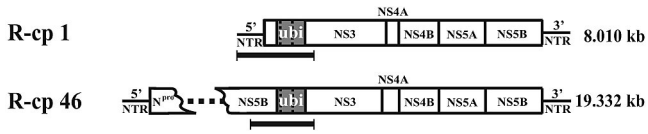


FIG. 3. Genome organization and calculated length of the shortest subgenome (R-cp 1; top) and the largest genome (R-cp 46; bottom) obtained by RNA recombination between NCP7 and +ubi SGT. For the latter recombinant genome, the region between the N^{pro} gene and the 5′-terminal NS5B coding gene is indicated by dots. Both recombinant RNAs contain ubiquitin-coding sequences (gray box) derived from +ubi SGT. The lengths of the bars are not drawn to scale. The underlined parts of the genomes have been sequenced.

infectious RNAs allowed the detection of not only cp viruses but also recombinant noncp viruses identified by IF analysis. Five emerged noncp viruses were further characterized. Northern blot hybridization, RT-PCR, and nucleotide sequence analysis indicated the presence of unique nonhomologous crossovers, resulting in different duplications of viral sequences; all of these recombinants lack ubiquitin-coding sequences (Fig. 4C and D; data not shown). A single crossover site was found for R-ncp 1, whereas two crossover sites were identified for R-ncp 2 and three additional noncp recombinants (Fig. 4C and D; data not shown). For the latter, the 5′ recombination junction between sequences of the 3′ NTR of NCP7-M1 and +ubi SGT resulted in a significantly enlarged 3′ NTR which still comprises almost 4 kb after deletion of viral sequences by a second recombination event. Taken together, our results demonstrate that RNA recombination also occurs between two replication-incompetent RNAs and does not require the functionally intact 3′ NTR of NCP7. Instead, it is assumed that the 3′ NTR of the recombinant genomes derived from +ubi SGT.

3′ NTR of the recombinants originates from +ubi SGT. To determine the origin of the 3′ part of genomes generated by RNA recombination between two recombination partners, each encompassing an intact 3′ NTR, two nonlethal genetic markers were introduced into +ubi SGT: one (M2) in the 3′-terminal part of the RdRp coding gene and the other (M3) into the 3′ NTR (Fig. 5). After transfection of NCP7-infected cells with +ubi SGT M2M3 13 cp recombinants emerged in two independent experiments. Further analyses of three of these recombinants indicated the presence of both markers (data not shown). To investigate whether an intact 3′ NTR of +ubi SGT is obligatory for the generation of recombinant genomes in our system, the lethal deletion of NCP7-M1, together with M2, was introduced into p+ubi SGT (Fig. 5). Then, 5 μg of RNA derived from the resulting construct p+ubi SGT M1M2 was transfected into cells infected with NCP7. In two independent experiments, infectious virus did not emerge. Taken together, the results of our analyses demonstrate that the 3′ NTR of the

FIG. 2. Analysis of crossover sites. (A) The crossover sites of 46 unique recombinants (R-cp 1 to R-cp 46) are schematically displayed by lines between the recombination partners NCP7 (top) and +ubi SGT (bottom); numbering of the recombinants corresponds to numbering of cp viral genomes analyzed by Northern blot (see Fig. 1C; data not shown). For +ubi SGT, the region between NS3 and NS5B is indicated by dots. (B) Nucleotide sequences of the crossover regions of 46 recombinants (R-cp 1 to R-cp 46). The sequences of the recombination partners NCP7 (top) and +ubi SGT (bottom) are indicated. The emerged recombinant sequences are highlighted by gray boxes. Nucleotide sequence differences between the recombinant and parental sequences are indicated by lowercase letters. Numbering refers to the sequences of CP7-5A (top) and +ubi SGT (bottom).

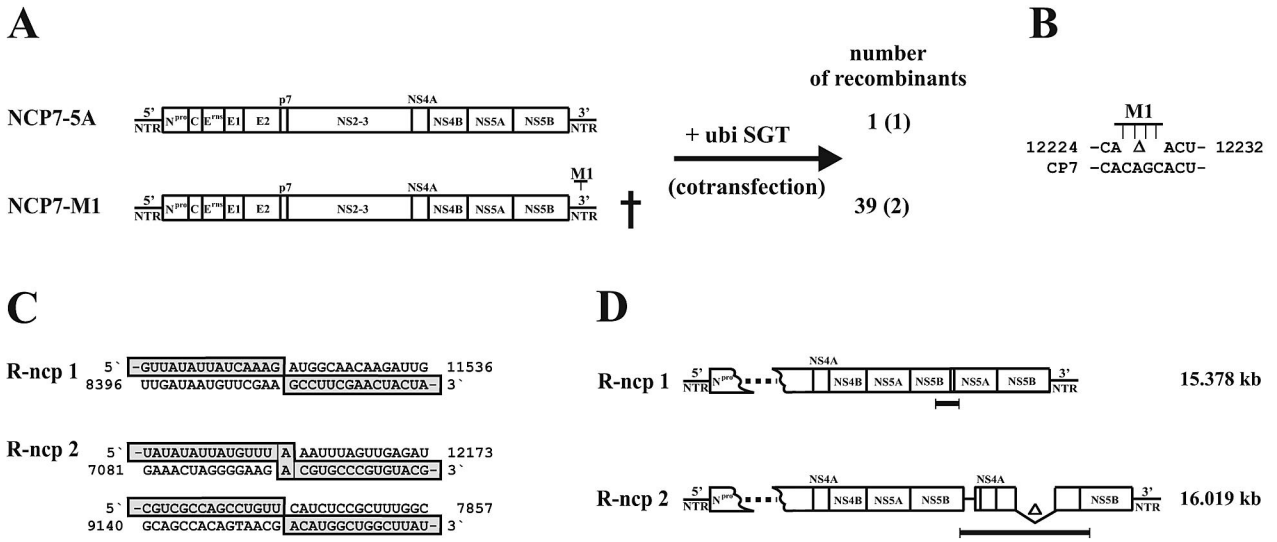


FIG. 4. RNA recombination between two synthetic transcripts. (A) Schematic representation of NCP7-5A and its derivative NCP7-M1. The cross indicates the noninfectious nature of NCP-7-M1. The number of recombinants produced after cotransfection of cells with +ubi SGT (see Fig. 1A) and RNA of NCP7-5A or NCP7-M1 are indicated. Numbers in parentheses show the numbers of independent experiments. (B) Comparison of partial nucleotide sequences of NCP7-M1 (top) encompassing the genetic marker M1 (deletion of four nucleotides within the 3′ NTR) and CP7 (bottom). Numbering refers to the sequence of CP7-5A. (C) Crossover regions of two selected noncp recombinants (R-ncp 1 and R-ncp 2). Both emerged after recombination between the RNAs of NCP7-M1 and +ubi SGT. The respective sequences of the recombination partners NCP7-M1 (top) and +ubi SGT (bottom) and the recombinant sequences (gray boxes) are indicated. Numbering refers to the CP7-5A sequence. Note that a single crossover region was found within the analyzed region of R-ncp 1, whereas two crossover regions are present in the genome of R-ncp 2. (D) Genome organizations and calculated lengths of R-ncp 1 (top) and R-ncp 2 (bottom). The bars are not drawn to scale. The underlined parts of the genomes have been sequenced.

recombinants originates from +ubi SGT. Accordingly, an intact 3′ NTR of +ubi SGT, including the *cis*-active elements required for viral replication, is essential for the generation of replicating recombinants.

RNA recombination does not require the presence of a viral RdRp. It is generally believed that RNA recombination of viruses is the result of a viral polymerase-driven copy choice mechanism. To investigate the significance of the viral RdRp for RNA recombination, several lethal mutations were introduced into the RdRp-coding region of NCP7-5A. In NCP7-GAA, the highly conserved GDD motif, which is known to be part of the active center of viral RdRps, was replaced by the motif GAA (Fig. 6). NCP7-ΔNaeI/SmaI lacks the 3′-terminal half of the RdRp coding gene, together with the entire 3′ NTR, whereas NCP7-ΔClaI/ClaI is characterized by a large internal deletion of the 3′-terminal half of the RdRp gene (Fig. 6). Surprisingly, cotransfection of each of these mutant RNAs, together with +ubi SGT resulted in the generation of cp and noncp recombinants (Fig. 6). Taking into account that +ubi SGT lacks the entire IRES element, which is essential for cap-independent translation, the results of these experiments suggest that RNA recombination does not require the presence of the viral RdRp. However, even in the absence of elements essential for translation, the presence of an intact RdRp-coding gene within the second recombination partner (+ubi SGT) does not fully exclude the possibility that a very low level of translation leads to synthesis of viral RdRp that could promote replicative copy choice RNA recombination.

To further address the question whether RNA recombination can occur in the absence of an active viral RdRp, pairs of subgenomic transcripts lacking different parts of the viral RdRp coding gene were cotransfected. For detection of re-

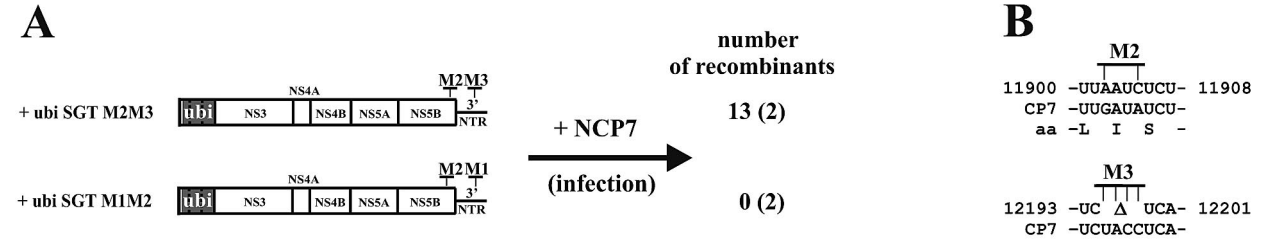


FIG. 5. RNA recombination between derivatives of tubi SGT carrying mutations in the 3′ NTR and the genome of NCP7. (A) Schematic representation of +ubi-SGT M2M3 and +ubi SGT M1M2. The numbers of recombinants produced after transfection of NCP7-infected cells with +ubi SGT M2M3 and +ubi-SGT M1M2 are indicated. Numbers in parentheses show the numbers of independent experiments. (B) Comparisons of partial nucleotide sequences encompassing the genetic markers M2 and M3 with the respective parental CP7 sequence. Numbering refers to the CP7-5A sequence. The deduced amino acid sequence of CP7 is shown below the nucleotide sequence.

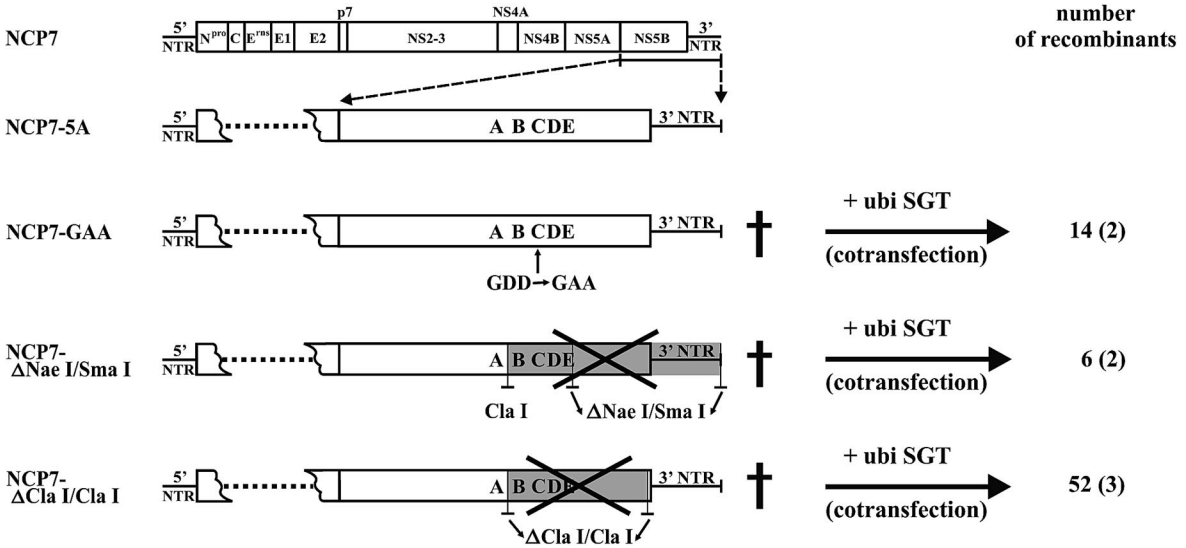


FIG. 6. RdRp mutants of NCP7. Schematic representation of the genomes of NCP7, NCP7-5A and its noninfectious derivatives NCP7-GAA, NCP7-ΔNaeI/SmaI, and NCP7-ΔClaI/ClaI carrying different mutations within the RdRp coding region. Within the enlarged NS5B boxes the positions of five highly conserved domains of the RdRp are indicated by letters A, B, C, D, and E (35). Deleted parts of the RNAs are shaded in gray. Crosses indicate the noninfectious nature of NCP7-GAA, NCP7-ΔNaeI/SmaI, and NCP7-ΔClaI/ClaI. The numbers of recombinants produced after cotransfection of cells with +ubi SGT and the RNAs of NCP7-GAA, NCP7-ΔNaeI/SmaI, and NCP7-ΔClaI/ClaI are indicated. Numbers in parentheses show the numbers of independent experiments.

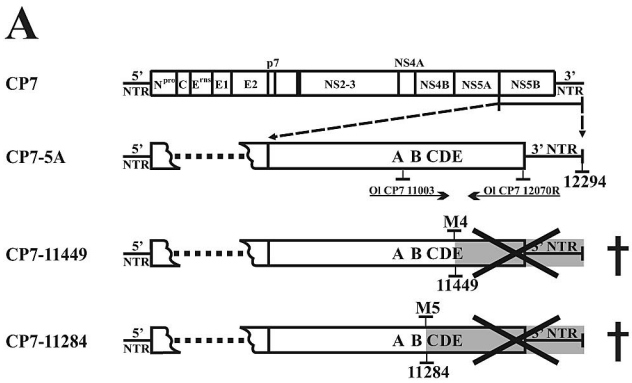
combinant viruses by the appearance of CPE, derivatives of the genomic RNA of cp BVDV strain CP7-5A were used. Transcripts CP7-11449 and CP7-11284 lack the entire 3′ NTR, as well as the genomic region encoding the C-terminal 218 and 273 amino acids of the viral RdRp, respectively (Fig. 7A). It has been reported that C-terminal deletion of 218 amino acids results in complete loss of activity of the pestiviral RdRp (35). RNAs +SGT-CP7-11201 and +SGT-CP7-11450 start at different positions within the 3′-terminal half of the RdRp coding gene and thus also lack essential parts of the RdRp gene (Fig. 7B). Transcripts containing the 5′ or 3′ parts of the viral genome are referred to below as the 5′ or 3′ recombination partner, respectively.

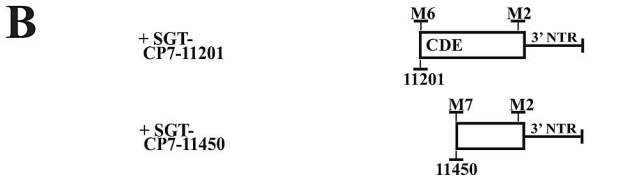
Remarkably, cotransfection of cells with CP7-11449 and +SGT-CP7-11201 that overlap in 249 nucleotides resulted in the generation of 11 recombinant viruses that were obtained in three independent experiments. RT-PCR and nucleotide sequence analyses revealed that 1 recombinant (R1) resulted from a precise homologous reaction, whereas the remaining 10 recombinants (R2 to R11) are due to unique nonhomologous reactions, leading in one case to a deletion of 18 nucleotides and in the other cases to duplications of 21 to 135 nucleotides (Fig. 7D and E and 8A); for all recombinant sequences, the open reading frame was maintained. Cotransfection with partners CP7-11284 and +SGT-CP7-11201, which overlap in 84 nucleotides, yielded one recombinant virus (R12) encompassing a duplication of 3 nucleotides (Fig. 7E and 8B). Since Northern blot analysis of viral genomic RNA molecules comprising ca. 12.3 kb is not appropriate to demonstrate the described small differences in genome length (3 to 135 nucleotides), the respective size alterations are demonstrated by RT-PCR analyses (Fig. 7D; data not shown). All recombinants described above carry the 3′-terminal genetic marker M2 of the respective 3′ recombination partner, whereas the other genetic marker of the 3′ partner (M6) and the genetic markers introduced into the 5′ recombination partner (M4 and M5) were eliminated upon recombination (Fig. 7A to C and 8). This indicated that the obtained recombinants resulted from joining of internal nucleotides of both recombination partners. Moreover, no recombinants emerged after cotransfection with CP7-11449 and +SGT-CP7-11450 (Fig. 7), which hypothetically can be precisely fused to a complete infectious viral genome by joining of the 3′-terminal nucleotide of the 5′ partner to the 5′-terminal nucleotide of the 3′ partner. End-to-end ligation thus did not lead to generation of infectious recombinant RNA molecules. With the exception of the recombinant virus resulting from homologous RNA recombination (R1; Fig. 8A), all recombinants carrying either a deletion or duplications within the RdRp coding gene (R2 to R12; Fig. 8) were restricted in viral replication to various extents (data not shown). RdRps are highly conserved among pestiviruses, and it is therefore remarkable that such structurally altered RNA polymerases still support viral replication.

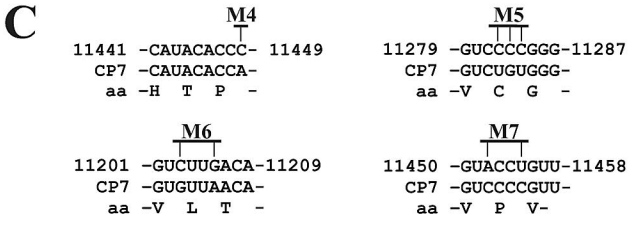
Taken together, since all synthetic RNAs used for cotransfection do not encode a complete and active viral RdRp, the emergence of viruses demonstrates that the formation of infectious recombinant RNA molecules must have occurred prior to the synthesis of an active RdRp. Accordingly, our results prove the existence of RNA recombination in the absence of a viral polymerase.

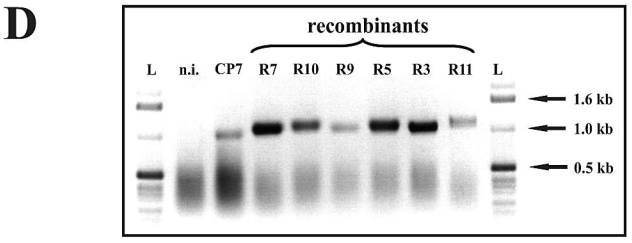
DISCUSSION

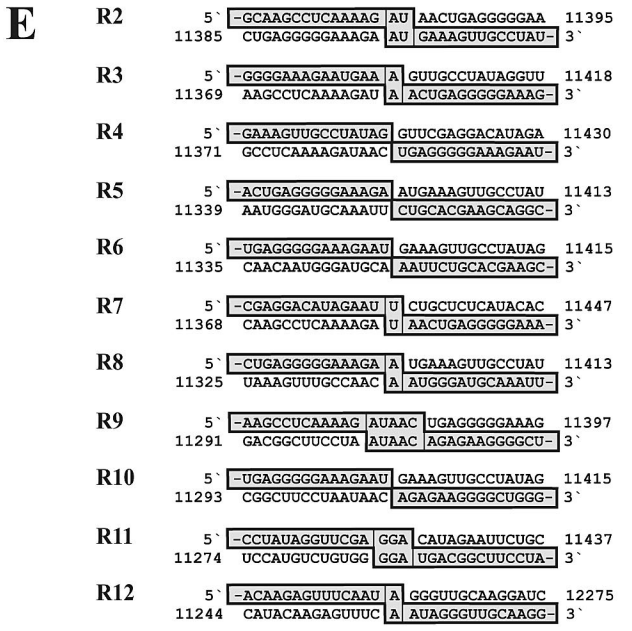
The importance of RNA recombination for the evolution of RNA viruses has been well documented for a large number of RNA viruses from animals, plants, and bacteria (2, 19, 34, 42, 52, 56). We present here the first in vivo system to study fundamental aspects of RNA recombination in pestiviruses.











This system allows the efficient generation of recombinant viruses by RNA recombination between infectious viral genomic RNA and replication-incompetent transcripts, as well as between two replication-incompetent transcripts. To exclude that phenomena other than RNA recombination contributed to the generation of recombinant viral genomes, extensive controls were performed (see Materials and Methods). Taken together, the results of these analyses clearly demonstrate that the recombination events described here are not due to artifacts and actually occurred at the RNA level in vivo.

One main conclusion of the present study is the existence of a viral RdRp-independent mechanism of RNA recombination in vivo. With regard to the mechanism of RNA recombination, it is generally believed that recombinant viral RNA molecules are generated by template-switching of a viral RdRp during RNA synthesis (16, 23, 29, 32, 34, 42, 43, 45, 55). The capability of template-switching has been reported for the RdRps of several positive-strand RNA viruses, including poliovirus and BVDV (4, 31). Thus far, two in vitro and one in vivo study provided evidence for nonreplicative viral RNA recombination (14, 15, 25). In these studies the presence of a viral polymerase or a gene encoding a complete viral polymerase did not, however, rule out a viral RdRp-dependent mechanism. Accordingly, the existence of nonreplicative RNA recombination has

FIG. 7. RNA recombination between noninfectious transcripts lacking different essential parts of the viral RdRp coding gene. (A) Schematic representation of the genome organization of cp BVDV strains CP7, CP7-5A, and its 3′-terminally truncated derivatives CP7-11449 and CP7-11284. Deleted parts of the RNAs are shaded in gray. Crosses indicate the noninfectious nature of both transcripts. The lengths of the CP7-5A genome and the two constructs are indicated by nucleotide numbers below the genomes. The positions of the genetic markers M4 and M5 and the five RdRp domains A to E are indicated. Apart from an insertion of 27 nucleotides (indicated by a thick black line) and a few point mutations within the NS2 region of CP7, (indicated by a thick black line) the genome of CP7-5A is identical to that of NCP7-5A (7). (B) Schematic representation of the 3′ recombination partners +SGT-CP7-11201 and +SGT-CP7-11450, each comprising different 3′ parts of the viral genome. The positions of the 5′-terminal nucleotides of these constructs corresponding to the genome of CP7-5A and the genetic markers M6 and M7 are shown below and above the bars, respectively. The positions of the RdRp domains C, D, and E are indicated. (C) Comparisons of partial nucleotide sequences encompassing the genetic markers M4, M5, M6, and M7 with the respective parental CP7 sequence. The deduced amino acid (aa) sequences of CP7 are shown below the nucleotide sequences. Numbering refers to the CP7-5A sequence. (D) RT-PCR analysis of six selected recombinants (R3, R5, R7, R9, R10, and R11) with RNAs prepared from MDBK cells 6 days after cotransfection with CP7-11449 and +SGT-CP7-11201. The position and orientation of primers OL CP7 11003 and OL CP7 12070R used for RT-PCR are indicated in panel A below the genome of CP7-5A. As controls, RNA from noninfected (n.i.) cells and from cells infected with CP7 were included in the respective analysis. Note the different sizes of the RT-PCR products obtained from the recombinants compared to the one of CP7. L, size standards. (E) Crossover regions of recombinants R2 to R12. The respective sequences of the 5′ recombination partners CP7-11449 (top; R2 to R11) and CP7-11284 (top; R12) and the 3′ recombination partner +SGT CP7-11201 (bottom) are indicated. The emerged recombinant sequences are highlighted by gray boxes. For seven recombinants, one to five nucleotides at the recombination junction are identical to the sequences of both recombination partners and therefore the crossover sites could not be determined unambiguously. Numbering refers to the CP7-5A sequence.

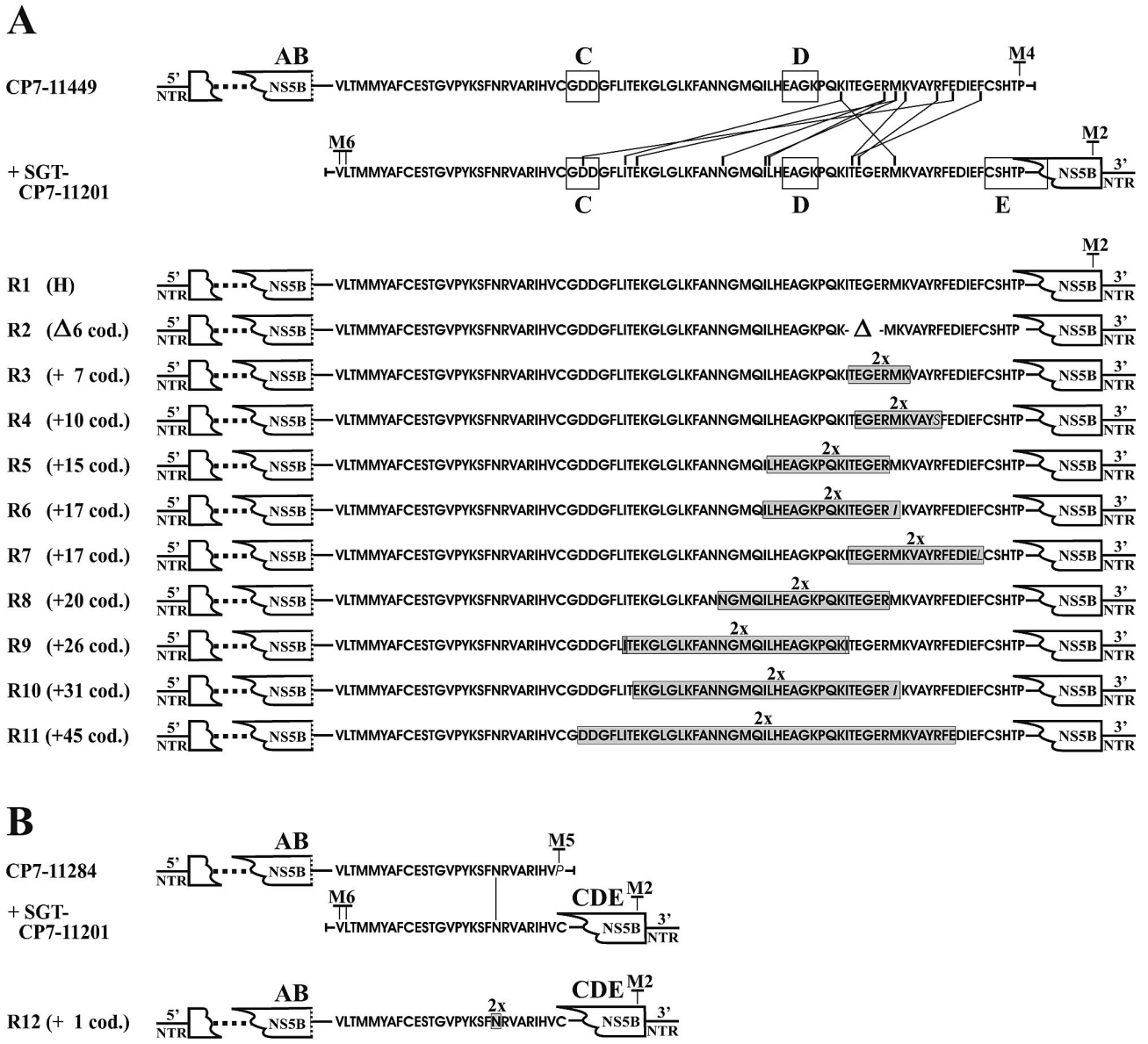


FIG. 8. Amino acid sequences of recombinants obtained after cotransfection with two noninfectious RNAs each lacking different essential parts of the RdRp coding region. (A) Schematic representation of the recombination partners CP7-11449 and +SGT-CP7-11201 and the resulting recombinants R1 to R11. The amino acid sequences corresponding to the overlapping 83 codons are aligned to each other. R1 was generated by precise homologous recombination, whereas R2 to R11 resulted from unique nonhomologous reactions. The crossover sites of the emerged recombinant viruses R2 to R11 are indicated by lines between the sequences of the recombination partners (top). The positions of the genetic markers M2, M4, and M6, as well as the five RdRp domains A, B, C, D, and E, are indicated. Note that domain E comprises 22 amino acids. For R2, the deletion of six codons is highlighted by a triangle. For R3 to R11 the sequences, which are duplicated (2×) are indicated by gray boxes. For R4, R6, R7, and R10 the crossover resulted in change of a single amino acid residue at the junction site; the changed amino acid residues are italicized. Note the presence of M2 and the absence of M4 and M6 for all recombinants. (B) Schematic representation of recombinant R12 (bottom) obtained after cotransfection with RNAs derived from CP7-11284 (top) and +SGT-CP7-11201 (middle). The positions of the RdRp domains A to E and the genetic markers M2, M5, and M6 are indicated. The amino acid sequences correspond to the overlapping 28 codons. Due to the cloning procedure, the C-terminal amino acid encoded by CP7-11284 is proline (italicized) instead of cysteine. The crossover of R12 is indicated by a line between the recombination partners. For R12, the duplicated (2×) asparagine residue (N) is highlighted by a gray box.

not been proven by these studies, and basic aspects of RNA recombination have been the source of controversy in recent years.

We show here that transfection of BVDV NCP7-infected cells with the replication-incompetent plus-strand transcript +ubi SGT resulted in the generation of cp recombinants (Fig. 1 to 3), whereas transfection with >30-fold-higher amounts of the corresponding minus-strand transcript −ubi SGT did not. Theoretically, this failure to generate recombinant viruses may indicate that replicative recombination during plus-strand RNA synthesis and nonreplicative recombination between RNA minus strands do not occur. Alternatively, recombinant

minus-strand RNA molecules were formed, but could not serve as templates for synthesis of genomic plus-strand RNAs. To examine this hypothesis, the full-length minus-strand RNA corresponding to the viral genome of cp BVDV was transfected into cells already transfected with a noncp BVDV replicon (data not shown). Even in the presence of the functionally active viral replication machinery, cp viruses were not generated. This shows that the RdRp of pestiviruses is not able to initiate RNA synthesis in *trans* at the 3′ end of the viral minus-strand RNA. Accordingly, possible recombination events between viral minus strands cannot be detected with our system, which monitors replicating viral genomes. Although our analyses show that the observed recombination events occurred exclusively between plus-strand RNA molecules, it remains an open question whether RNA minus strands participate in recombination. The described recombination events between plus-strand RNA molecules can be due to replicative or nonreplicative mechanism(s). Our experiments with mutants of +ubi SGT carrying genetically modified 3′ ends demonstrated that the 3′ ends of the resulting recombinants were obtained from the +ubi SGT derivatives (Fig. 5). This excludes a double template switch leading to the emergence of recombinants. If we assume a replicative mechanism, the RNA synthesis had to be initiated in *trans* at the 3′ end of +ubi SGT, followed by a template switch to the 5′ recombination partner. Alternatively, RNA recombination might be the result of a nonreplicative mechanism.

Theoretically, recombination partners can be entirely or partially incorporated into the resulting recombinant RNA molecule. The characterization of 46 recombinants obtained after transfection of NCP7-infected cells revealed that the 5′ region of +ubi SGT was entirely present in 13 of 46 recombinant genomes (Fig. 2). In contrast, the analysis of 12 recombinants (R1 to R12) generated after cotransfection of naive cells with two replication-incompetent RNAs showed that all crossovers resulted from joining of internal nucleotides (Fig. 7E and 8). This observation supports the assumption that different mechanisms are involved in the generation of recombinant RNA molecules. Concerning the experiments with a replicating viral genomic RNA as one of the recombination partners, the presence of an active RdRp does not allow to distinguish between replicative template-switching mediated by the viral RdRp and viral RdRp-independent recombination.

Remarkably, transfection experiments with two replication-defective RNAs each lacking essential parts of the viral RdRp coding gene (Fig. 7 and 8) demonstrated that RNA recombination occurred prior to the production of an active polymerase. This proved the existence of viral replication-independent RNA recombination in vivo. In this context it is noteworthy that while the present paper was being prepared, the generation of replicating poliovirus genomes by recombination between poliovirus RNA fragments lacking either 5′- or 3′-terminal parts of the viral RdRp coding gene was reported (26). That study and the results of our analyses both show that viral replication-independent RNA recombination is not restricted to a particular virus system but most likely represents a widespread mechanism of general interest.

The occurrence of RNA recombination in the absence of a viral RdRp may be due to nonreplicative reaction(s). Analysis of the recombinants obtained in the present study revealed that the crossover sites were unique and thus did apparently not result from sequence-specific reactions. This is in contrast to nonreplicative cellular (*trans*-) splicing reactions (1, 10, 20, 21, 51). Furthermore, self-cleavage of the RNA resulting from cryptic ribozyme activity (14, 25), as well as cleavage by ribonucleases, followed by a ligation reaction, can be hypothesized. In addition, other as-yet-unknown nonreplicative mechanisms may have contributed to the viral RNA recombination events described here. Although it is tempting to speculate that the observed viral RdRp-independent recombination is due to a nonreplicative mechanism, the absence of a viral RdRp does not exclude a replicative mechanism mediated by cellular polymerases. Some cellular RdRps that play a significant role in posttranscriptional gene silencing can synthesize RNA with single-stranded RNA templates (3, 44, 49, 50). If we take into account that such polymerases have thus far not been identified in mammals and have never been reported to synthesize large viral genomic RNAs, it appears unlikely that these cellular enzymes are involved in RNA recombination. Interestingly, host DNA-dependent RNA polymerases such as RNA polymerase II exist that replicate RNA genomes of hepatitis delta virus and plant viroids (12, 18, 24, 46). However, respective promoter-like elements have not been identified in pestiviral RNA genomes. It is noteworthy that the analysis of the recombinant sequences generated by viral replication-independent recombination identified one homologous recombinant (Fig. 8A) and revealed the presence of one to five ambiguous nucleotides at most nonhomologous recombination junctions (Fig. 7E and 8). Similar to the analysis of the 46 recombination junctions shown in Fig. 2B (and described in Results), this suggests a role of primary sequences in determining crossover sites by base pairing between a nascent RNA strand and the acceptor template. According to these results, a replicative mechanism mediated by cellular polymerase(s) can be considered. Taken together, it remains an open question whether the viral RdRp-independent mechanism of RNA recombination is due to replicative or nonreplicative reactions. The identification of factors driving viral replication-independent RNA recombination represents an important focus of future work.

Recombination between viral RNA genomes significantly contributes to genetic variation of viruses and provides a mechanism for the repair of viral genomes. Both consequences have been described for a number of viruses (2, 34, 42, 52, 56) and were experimentally demonstrated in the present study. Furthermore, recombination between viral and cellular RNAs has been reported (13, 30, 37, 39, 40, 54). Molecular analyses of pestiviruses revealed that integration of ubiquitin- and other cellular protein-coding sequences into viral genomes is responsible for an additional processing step of the viral polyprotein, cell lysis, and the induction of a lethal disease in cattle (5, 8, 9, 38). This natural selection system, which allowed the identification of a large number of recombinant cp pestiviruses, is mirrored by the recombination system described here. Our finding that viral replication-independent RNA recombination occurs in vivo strongly suggests that not only viral but also cellular RNAs recombine with each other. Such recombination events between cellular RNAs may result in formation of biologically active molecules and might have contributed to the early stages of evolution in a hypothetical RNA world.

ACKNOWLEDGMENTS

We thank M. Orlich for excellent and dedicated experimental work, as well as for stimulating discussions in the initial phase of the project, and S. Widauer for excellent technical assistance.

A.G. was supported by SFB 535 Invasionsmechanismen und Replikationsstrategien von Krankheitserregern (TP B8 RNA-Rekombination bei Pestiviren) from the Deutsche Forschungsgemeinschaft.

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