

Genome Instability in BVDV: An Examination of the Sequence and Structural Influences on RNA Recombination

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Received February 11, 1998; returned to author for revision March 3, 1998; accepted April 30, 1998

The cytopathogenic biotype of the pestivirus, bovine viral diarrhea virus, is frequently a product of nonhomologous recombination in the region of the genome encoding the viral NS2-NS3 proteins. The possibility that sequences or structures in this region contributed to a hotspot for RNA recombination was examined. A PCR-based strategy was used to examine viral genomic RNA isolated from tissue samples of cattle persistently infected with the noncytopathogenic biotype of the virus. Analysis of two different regions of the viral genome revealed that recombination was not restricted to particular sequences. Alignment of the genomic sequences undergoing recombination and examination of the predicted secondary structures of the participating RNAs revealed that the dissociation of partial, newly synthesized negative strand RNAs from the positive strand template occurred at many different sites on the molecule. Similarly, it appeared that the reassociation of the RNA polymerase complex with a second positive strand template was frequently influenced by short regions of homology between the nascent RNA strand and open secondary structures in the template molecule. © 1998 Academic Press

INTRODUCTION

RNA recombination is a common event in the life cycle of many different viruses and is a major mechanism for the evolution of RNA virus genomes. Depending on the level of sequence similarity between the recombining RNAs, the process can be classified as homologous or nonhomologous (Lai, 1992). Where homology between the donor and acceptor templates is extensive, homologous recombination can occur at high frequency (Banner and Lai, 1991; Lai, 1992; Nagy and Bujarski, 1995). It is also possible for nonhomologous recombination to occur between unrelated RNA molecules or between nonidentical sequences on closely related RNA templates (Lai, 1992; Li and Ball, 1993; White and Morris, 1994). The mechanism of RNA recombination is generally thought to involve a copy choice or template switching event between a donor template on which RNA synthesis is initiated and a second acceptor template on which synthesis continues (Lai, 1992), although a breakage and rejoining mechanism has been postulated for some *in vitro* recombination events (Chetverin *et al.*, 1997). Both homologous and nonhomologous recombination can have profound effects on virus growth, virulence, antigenicity, and evolution (Khatchikian *et al.*, 1989; Banner and Lai, 1991; Charini *et al.*, 1994; Jia *et al.*, 1994).

The role of nonhomologous RNA recombination in the generation of cytopathogenic strains of bovine viral diarrhea virus (BVDV) and the consequences for the complex pathogenesis of mucosal disease have been well documented (Meyers *et al.*, 1989, 1991, 1992; Tautz *et al.*, 1994; Meyers and Thiel, 1996). While acute infection of cattle with the noncytopathogenic biotype of the pestivirus (BVDVnc) is manifest in a wide variety of clinical syndromes, it is only the fatal syndrome known as mucosal disease which is inevitably precipitated by cytopathogenic strains of BVDV (BVDVc) (Brownlie *et al.*, 1984).

The vast majority of circulating BVDV strains are noncytopathogenic (BVDVnc), and a typical genome is a positive sense, single-stranded RNA of approximately 12.5 kb in size. Immune-mediated clearance of acute infections occurs within 10 to 14 days postinfection in immunocompetent cattle. Infection of a pregnant dam by BVDVnc during the first trimester can result in the infection of a fetus prior to immunocompetence and the establishment of fetal tolerance of the virus. The consequence of this is a lifelong persistent infection with BVDVnc and only these animals are susceptible to mucosal disease. The BVDVc strain which induces mucosal disease must be antigenically "homologous" to the persisting BVDVnc, and nonhomologous recombination of the BVDVnc genome with other viral or cellular RNAs is believed to generate the BVDVc strain within the persistently infected animal.

In many cases, the genomic RNA of BVDVc strains is longer than the 12.5-kb BVDVnc genome, although smaller defective genomes have also been described (Meyers *et al.*, 1992; Tautz *et al.*, 1994). The recombination events required to produce a BVDVc genome are

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varied but they all result in the expression of the viral NS3 protein as an unfused, 80-kDa product. In contrast, in BVDVnc strains the same primary sequence is translated as part of the 125-kDa, NS2-3 fusion protein. BVDVc genomes incorporating cellular mRNAs or viral proteases have been described (reviewed in Meyers and Thiel, 1996), but the consequence is always the co- or posttranslational generation of the NS3 protein (Purchio *et al.*, 1984; Pocock *et al.*, 1987; Donis and Dubovi, 1987; Meyers *et al.*, 1991; Greiser-Wilke *et al.*, 1993). Although the functional role of NS3 in cytopathogenicity is still being elucidated, the association of phenotypic changes in viral growth and virulence with the insertion of cellular RNA sequences has been reported for other viral systems, including influenza and polio viruses (Khatchikian *et al.*, 1989; Charini *et al.*, 1994).

It is believed that a template-switching mechanism is responsible for the genome rearrangements observed in BVDVc strains and, for those viruses incorporating cellular RNA insertions, this must have occurred during synthesis of the viral negative strand RNA. This is also consistent with data on recombination of other virus genomes. The number of positive strand genomes available as potential acceptor templates would be significantly in excess during the replication of positive strand RNA viruses. Therefore, the number of recombinants where the positive strand genome acts as the acceptor template is greater and a higher frequency of recombination is observed during negative strand synthesis (Jarvis and Kirkegaard, 1992; Li and Ball, 1993). Analyses of the viral and nonviral RNAs participating in the recombination events leading to the generation of BVDVc strains has failed to identify any conserved sequence motifs around the recombined sequences in the RNA partners, although the site of dissociation from the primary template (the sequences encoding the N-terminus of the NS3 protein) is conserved in most cases (Meyers *et al.*, 1992; Meyers and Thiel, 1996). This consistent association of the N-terminus of the (unfused) NS3 as the target for nonhomologous recombination has stimulated speculation that this region of the genome is a recombinogenic hotspot (Meyers and Thiel, 1996). Previous reports of RNA recombination hotspots in Coronavirus genomes have subsequently been shown to be the products of selection for replicative fitness following numerous random recombination events (Banner and Lai, 1991). Therefore it is important to ascertain whether the observations concerning recombination in BVDVc genomes are a result of targeted recombination in the NS3 region or subsequent *in vivo* selection of a subpopulation of randomly recombined viruses.

The aim of this study was to address the question of whether the 5'-end of the NS3 coding region of BVDV was a hotspot for genome recombination. A PCR-based strategy was used to detect nonhomologous recombination (see Fig. 1), to examine the possibility that such

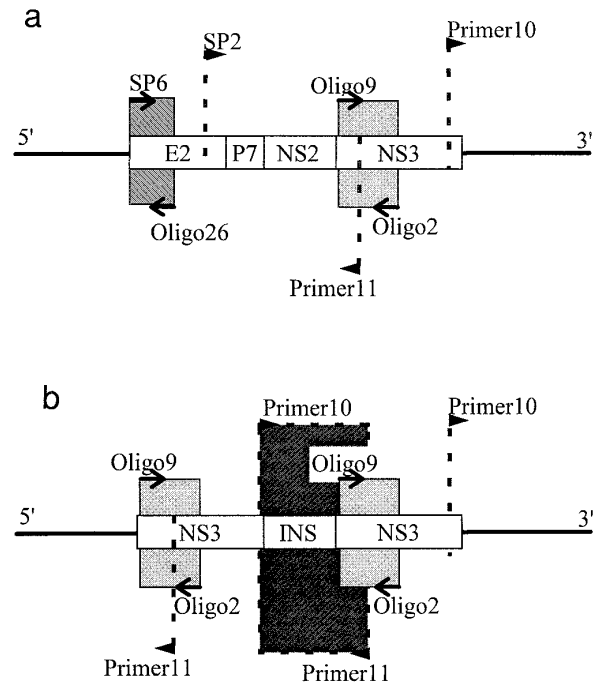


FIG. 1. Schematic representation of the genome location of the PCR primers used in this study, primer SP6 + oligo26, primer pair 9 (comprising oligo26 + SP2), primer pair 2 (comprising oligo9 + oligo2), and primer pair 6 (comprising primer10 + primer11). The shaded boxes represent amplicons generated on (a) a standard BVDVnc genome and (b) on a genome with duplication of NS3 sequences.

events occurred but were previously undetected in other parts of the BVDV genome (encoding the major glycoprotein, E2), and to analyze the sequences participating in the recombination process.

RESULTS

The serum and tissue samples derived from the persistently infected animals used in this study all contained BVD viral RNA as judged by the strong positive control amplification product (amplicon) obtained following RT-PCR with primer pair 2 as expected. Samples of RNA extracted from a BVDV-negative animal or PCR amplifications containing water in the place of cDNA were consistently negative in these analyses.

When primer pair 6 was used on total RNA samples extracted from BVDV-infected animals, many weak amplicons, ranging in size between 100 and more than 1400 basepairs, were obtained. This indicated that targets containing duplications of the NS3 coding region were present in the sample, probably at low levels compared to the nonrearranged genomes detected with primer pair 2. The size distribution of the primer 6 amplicons suggested that many different recombination events were occurring (Fig. 2). Similar amplicons were detected in samples from lymph node and cerebellum, indicating that the phenomenon was not restricted by tissue type.

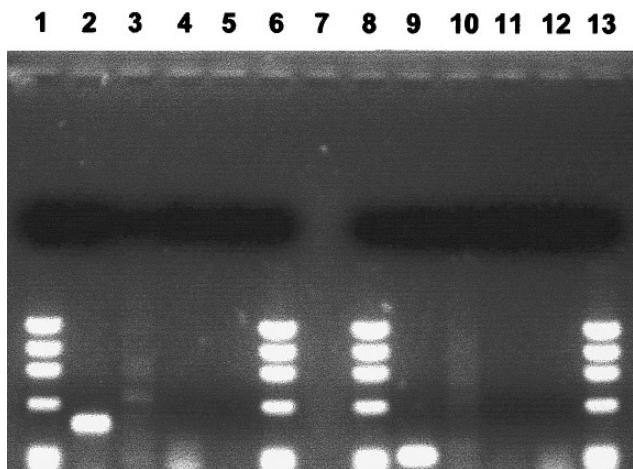


FIG. 2. Photograph showing the amplimers generated with various PCR primer pairs on total RNA samples extracted from tissues of BVDV-infected animal 1280 or BVDV-free animal 1154. Lane 1, PhiX174 *Hae*III markers; lane 2, primer pair 2 (NS3 positive control) amplifying 450 bp in total RNA extracted from a BVDV-infected animal; lane 3, primer pair 6 (NS3 duplication) amplifying multiple bands in total RNA extracted from a BVDV-infected animal; lane 4, primer pair 6 (NS3 duplication) amplifying total RNA extracted from a BVDV-free calf; lane 5, primer pair 6 with no template (negative control); lane 6, PhiX174 *Hae*III markers; lane 7, empty; lane 8, PhiX174 *Hae*III markers; lane 9, primer SP6 + oligo26 (E2 positive control) amplifying 295 bp in total RNA extracted from a BVDV-infected animal; lane 10, primer pair 9 (E2 duplications) amplifying multiple bands in total RNA extracted from a BVDV-infected animal; lane 11, primer pair 9 (E2 duplications) amplifying total RNA extracted from a BVDV-free calf; lane 12, primer pair 9 with no template (negative control); lane 13, PhiX174 *Hae*III markers.

The specificity of these primers was confirmed when all negative controls, including total RNA samples from the serum or lymph nodes of a BVDV-free animal, were negative when amplified with the same primer combination. The use of primer pair 6 on total RNA extracted from tissue culture cells infected with a cytopathogenic virus known to have a duplication of the NS3 gene gave a single discrete band corresponding in size to the known insert sequence in this strain (Meyers et al., 1992).

A similar strategy was used to analyze a different region of the BVDV genome. Primer pair 9 was used to detect rearrangements in the E2 coding region and produced a similar range of PCR fragments from tissue samples containing BVDV RNA. Again, the size distribution of the amplimers generated indicated that many different rearranged genomes were present in the sample (Fig. 2).

RNA extracted from the serum of infected animals was also analyzed with the same primer pairs. The positive control primer pair 2 readily amplified the specific PCR product but, initially, the combinations of primer pair 6 and primer pair 9 failed to detect recombined genomes in the serum samples. When increased amounts of RNA were analyzed, very low levels of multiple band products were obtained.

The PCR products generated by primer pairs 6 and 9 were shotgun cloned and individual recombinant plasmids sequenced. The data generated confirmed that all the amplimers were the products of viral genomes which had undergone at least one recombination event. Several clones had multiple recombination events resulting in the insertion of fragments of non-contiguous viral RNA or, in one case, nonviral RNA, between the two copies of the NS3 or E2 genes. Although each of the clones analyzed was unique, the same patterns of recombination were observed in each of the three persistently infected animals examined, occurred in samples from both lymph node and cerebellum, and were found to affect both the NS3 and the E2 regions of the viral genome. The results obtained from the analysis of NS3-rearranged clones derived from lymph node samples of animal 1279 are shown schematically in Fig. 3. Two clones were also found to have small deletions of 8 and 11 bp in addition to the recombinations (data not shown).

Detailed analysis of the sequences of the 5' and 3' RNAs involved in the rearrangements revealed that, in some cases, between one and five bases could not be unambiguously assigned to one side of the recombined RNA or the other, but formed a short region of homology between the donor and acceptor RNAs. In other clones no homology was detected at the recombination site and in a single clone a nontemplated base seems to have been added to the sequence (Fig. 4). Analysis of the crossover sequences was performed using sequences obtained from cDNA generated from cloned Pe515nc virus. Translation of the protein coding sequences of these clones indicates that, in the majority of cases, a frameshift at the site of recombination would cause premature termination of the viral polyprotein, almost certainly resulting in the production of a nonviable genome.

In order to ascertain the possible mechanism directing these recombination events, the secondary structures of the participating RNAs were predicted using the program MFOLD and both the optimal and several suboptimal predictions were visualized using the SQUIGGLES option of the program PLOTFOLD. The sites of recombination, including any regions of homology, were located on the predicted structures adopted by the positive strand RNAs for both the primary donor template and the second acceptor template as indicated in Fig. 5. The predicted sites of dissociation of the RNA polymerase complex from the primary, donor template are not consistently associated with a particular secondary structure motif (Fig. 5a). In contrast, the sites of reassociation of the nascent RNA with the acceptor template are frequently regions which are predicted to be in loops or bulges without significant basepairing interactions (Fig. 5b).

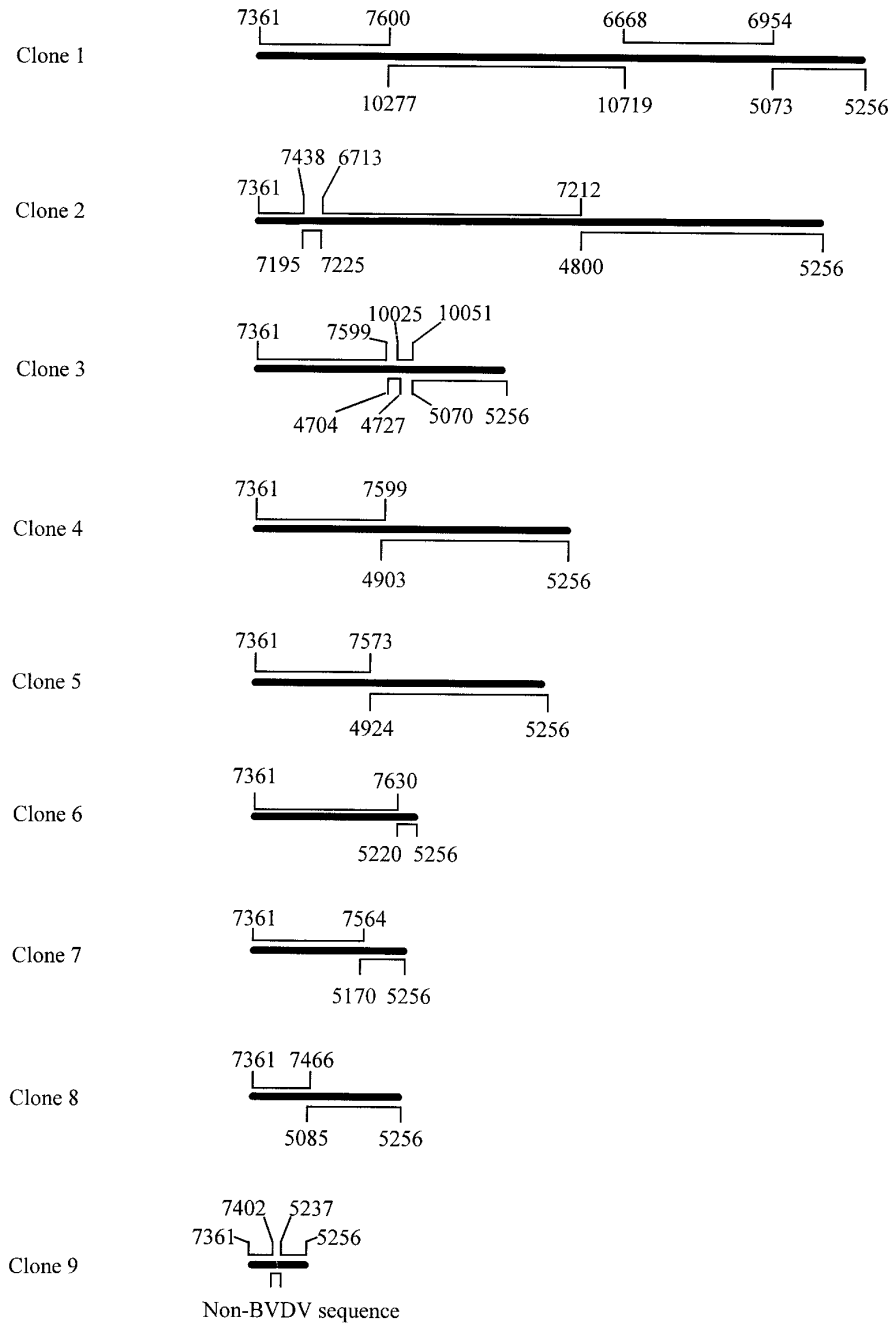


FIG. 3. Schematic representation of some of the clones derived from duplications of the NS3 genes of BVDV as detected in total RNA samples extracted from the lymph node of animal 1279. The figures indicate the genome locations of the different RNA fragments with respect to strain SD1 (Deng and Brock, 1992).

DISCUSSION

RNA virus recombination has been described for many different animal, plant, and bacterial viruses. Homologous recombination mediated by template switching is known to occur with a high efficiency where the donor and acceptor template RNAs have regions of perfect homology greater than 15 bases in length (Nagy and Bujarski, 1995). Where levels of homology are greater than this, the sites of recombination are less influenced

by RNA secondary structure and occur randomly throughout the homologous sequences (Banner and Lai, 1991; Nagy and Bujarski, 1995). The RNA recombination events previously described for BVDV strains or leading to the generation of cytopathogenic strains cannot be considered to be homologous.

Previous accounts of genome rearrangement in BVDV have all described nonhomologous recombination events and, with few exceptions, most have been di-

	Pe515 CTTGTGGGGAACAC	Pe515 ATGAGAAGAGAGA	Pe515 GATCCA ACTTGCT
Clone 1	(i) CTTGTGGGAACTG	(ii) ATGAGAAGAGTAG	(iii) GATCCAACTAAAG
	Pe515 CCCCTGGGAACTG	Pe515 AGGGGTAGAGTAG	Pe515 CTGGCA ACTAAAG
	Pe515 ATCTCGGATTATGT	Pe515 TGCTCATAGCTT	Pe515 AGCTGAGA ATGCC
Clone 2	(i) ATCTCGGATTGTCT	(ii) TGCTCACAGCAA	(iii) AGCTGTTAATAAA
	Pe515 TGGCCGGATTGTCT	Pe515 AGGAAACAGCAA	Pe515 TGACTATA ATAAA
	Pe515 GTGGGGAACACATA	Pe515 AAAACACAAAGTA	Pe515 AATTGGCA CAGGG
Clone 3	(i) GTGAGGAACCTAAT	(ii) AAAACACATGGCA	(iii) AATTGGCAACTAA
	Pe515 CTGAGGAACCTAAT	Pe515 GGGACACATGGCA	Pe515 TACTGGCA ACTAA
	Pe515 TATGGCTTGTGGGGAACACA		
Clone 4	TATGGCTTGTGGGAAACCCA		
	Pe515 CGGACGCCATGGGAAACCCA		
	Pe515 AAGAAAAGATGTAATAAT		
Clone 5	AAGAAAAGATGTCATTAG		
	Pe515 ACATGTGGGATGTCATTAG		
	Pe515 AAAAGCATAGCTGCAAGACTG		
Clone 6	AAAAGCATAGCTGCATTTTTC		
	Pe515 GACAAGTTAACTGCATTTTTC		
	Pe515 TTAATTGAAGAAAAGATG		
Clone 7	TTAATTGAAGATCACAGAA		
	Pe515 GTGCAAGAAGATCACAGAA		
	Pe515 GAATTCGTAAAATCTCAAG		
Clone 8	GAATTCGTAAAATGCTCAT		
	Pe515 CTAAAGTAAAATGCTCAT		

FIG. 4. Sequence alignment of the acceptor (upper), donor (lower), and recombinant (center) RNA templates as identified in the clones presented in Fig. 3. The sequences shown in boldface are the donor or acceptor RNAs which are likely to have contributed to the final recombinant. Vertical lines indicate homology between the recombinant and either the donor or acceptor RNAs; bold vertical lines indicate a short region of homology between the donor and the acceptor RNAs which may have facilitated recombination.

rected to exactly the same base position on the genome, that encoding the N-terminus of the NS3 protein. The frequent incorporation of cellular mRNA sequences at this point also indicates that, at least in those BVDVc strains with cellular insertions, the template switching event occurred during the synthesis of the virus negative

strand RNA. This observation led to speculation that this genome location was a favored site for recombination. The homology between the G/AGCCC sequence at the 3'-end of the negative strand at this point of template switching and the extreme 3'-terminus of the positive strand RNA was noted and the possibility of this being a

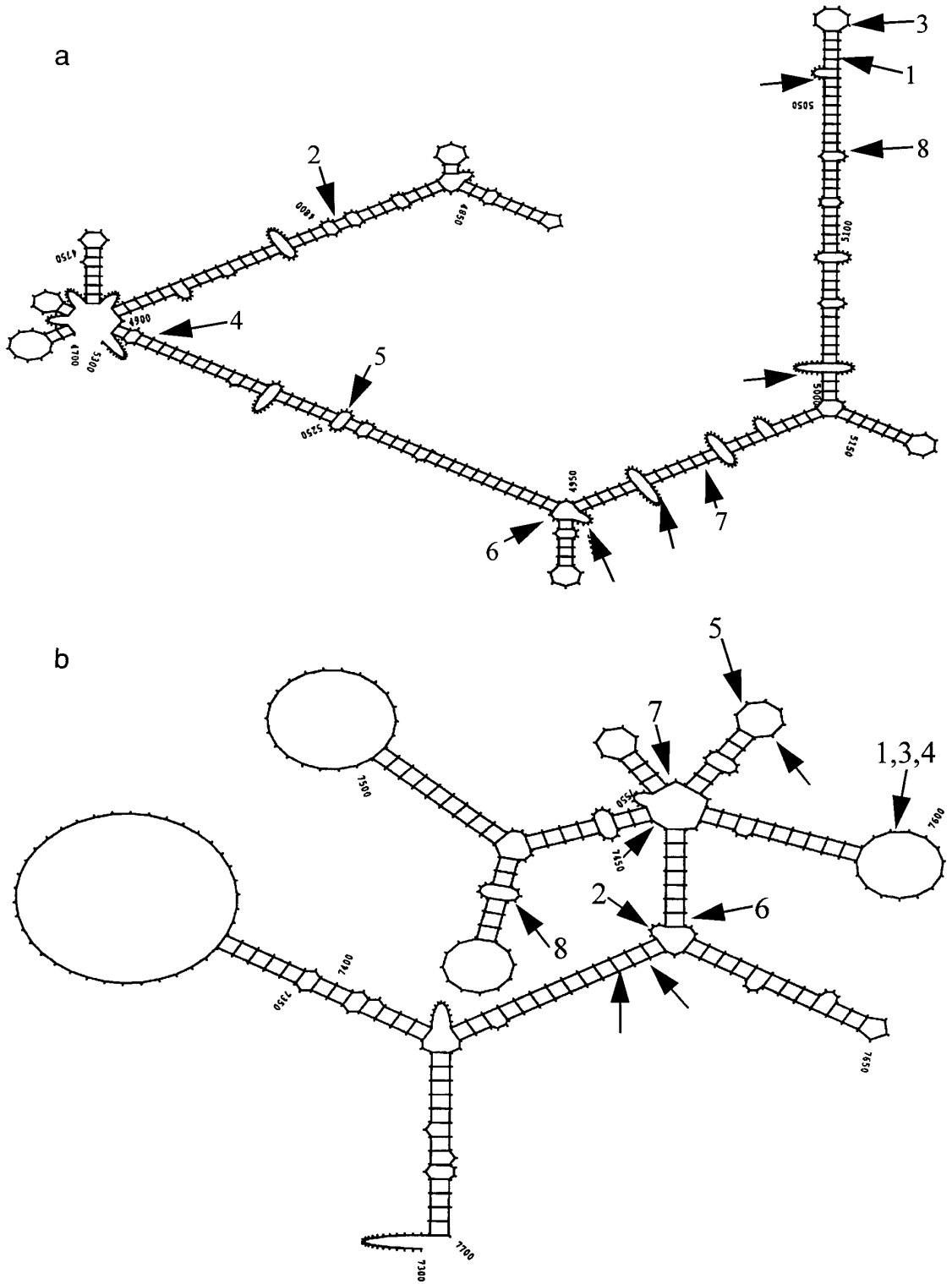


FIG. 5. Predicted secondary structure (using the programs MFOLD and PLOT FOLD/SQUIGGLES) of (a) the donor (SD1 sequences 4700–5300) and (b) acceptor (SD1 sequences 7300–7700) RNAs involved in genome recombination for some of the clones observed. Individual sites of recombination are indicated by arrows on each figure. Numbered arrows correspond to first sites of recombination of clones described in Figs. 3 and 4 (other sites were examined with similar results but cannot be represented on these models). (a) The sites of dissociation are frequently, but not consistently, associated with regions which are predicted to be small bulges. (b) The sites of reinitiation are frequently regions of the acceptor RNA molecule predicted to be loops or junctions at the bases of multiple stem structures.

signal for dissociation of the RNA polymerase from the template was raised (Meyers and Thiel, 1996). An alternative scenario is that, in a population of viruses undergoing random recombination, individual recombinants targeting this location are preferentially selected because of an effect on their *in vivo* replicative fitness. The majority of recombined BVDV genomes analyzed to date have been cytopathogenic and these BVDVc strains are consistently associated with fatal mucosal disease in cattle. The production of the unfused NS3 protein exclusively in BVDVc strains (non-cytopathogenic strains of BVDV express the same primary amino acid sequence as part of the NS2-3 fusion protein) and their ability to induce fatal disease may suggest that only those recombinants which generate the same functional N-terminus of the NS3 protein will give rise to a BVDVc strain. This phenotypic selection for viruses with the ability to induce fatal disease has been a major bias in selection procedures as recombined viruses which have little or no effect on the health of persistently infected cattle have not been a focus of research interest.

The results of the current study would support the contention that selection of viral recombinants which have genome rearrangements affecting the expression of the NS3 protein has been biased by the dramatic biological properties of cytopathogenic viruses. The evidence obtained has indicated that RNA recombination can readily be detected in viral RNA extracted from BVDV-infected tissue samples, and to a lesser extent, in serum samples from infected animals. Similar results were obtained independently of the tissue (lymph node or cerebellum) or animal (1263, 1279, 1280) used for extraction of the RNA and occurred in both the NS3- and the E2-coding regions of the genome. Although the methods used were not designed to quantitate the RNA species involved, the relative ease with which amplimers could be generated with, for example, primer pair 2 (positive control), compared to pairs 6 or 9 (designed to identify recombined genomes), indicates that recombinants were present at lower levels, particularly in serum-derived RNA samples where evidence of recombined genomes could only be detected if an improved RNA extraction protocol was used. (The amounts of "normal" viral RNA in tissue- and serum-derived RNA samples used for the RT-PCR were adjusted to produce similar amounts of amplification product with the positive control primers.) The specificity of the reaction was confirmed when the same primer pairs failed to produce amplimers when used on total RNA samples derived from noninfected cattle and produced a single defined product when used to amplify total RNA extracted from cells infected with the cytopathogenic Pe515 strain of BVDV, known to have a duplication of the NS3 gene. No fainter bands were detected in this reaction, indi-

cating that, when an appropriate template was present, these primers bound efficiently and did not give a range of nonspecific reaction products. Similarly, the vast excess of normal genomes available for amplification by the positive control primers (primer pair 2 or SP6 + oligo26) accounts for the failure to detect genome recombinants in these reactions. The optimization of PCR amplifications is such that, under normal circumstances, conditions are chosen to minimize unfavorable amplifications. The recombined genomes detected by the use of primer pairs 6 and 9 was only possible because a "normal" template for amplification does not exist to out-compete the amplification of recombinants.

When the amplimers from the NS3 duplications were sequenced it became apparent that recombination could occur at a range of different base locations within the NS3 region and there was no evidence for clustering of recombination at the position corresponding to the N-terminus of the NS3 coding sequence. This does not exclude the possibility that the motif G/AGCCC is a signal for the dissociation of the RNA polymerase complex (Meyers and Thiel, 1996) but confirms that dissociation can and does occur at many other regions of the viral genome. This property may reflect other features of the BVDV RNA polymerase, such as inherent processivity, misincorporation of nucleotides, or the influence of RNA secondary structure.

The mechanisms of nonhomologous recombination, where the donor and acceptor templates have maximally 4 to 5 nucleotides in common at the site of recombination, is thought to be influenced to a greater degree by RNA secondary structure than by sequence (Hajjou *et al.*, 1996). The progress of the RNA polymerase along the primary donor template is paused, either as an inherent property of the processivity of the enzyme or because of stable structures such as strong hairpins, and then dissociates. The complex subsequently associates with a new acceptor template (or at a different position on the same template) and RNA synthesis is reinitiated. The reassociation of the RNA polymerase complex with the new template could be guided by secondary structure, as many different RNA binding proteins are known to interact with the loop or loop-bulge motifs of hairpin or cloverleaf structures in RNA. The exact point of reinitiation of RNA synthesis may then be influenced by short sequence homologies between the nascent RNA strand and the exposed loops in the new template. This may account for the frequent observation of very limited homology between the donor and the acceptor templates. The results of the current analysis are in agreement with previous reports and failed to identify any consistent primary sequence motifs at the site of RNA recombination. No attempt has been made to physically characterize the structures of the RNAs described in the

current study, but computer predictions of RNA secondary structure have been undertaken. While such predictions are less reliable than biochemical or physical methods for structure determination, they would support the possible role of basepairing between the 3'-end of the nascent RNA (following dissociation from the primary template) and unpaired sequences (loops or bulges) in the new, acceptor template, in directing the site for reinitiation of RNA synthesis. Using these predictions, analysis of 15 different recombinants failed to identify consistent secondary structures associated with the point at which dissociation of the nascent strand occurred (see Fig. 5). In contrast, the majority of sites at which synthesis reinitiated were in regions of the molecule where the template was predicted to be unpaired. However, clones were observed with no homology at all at the site of reinitiation, confirming that even limited hybridization of the nascent strand to the new template is not essential.

Primer pair 9 was used to analyze duplications involving RNA sequences in the E2 region of the BVDV genome. This region of the genome had not previously been implicated in recombination events but the PCR-based strategy used readily detected recombinants in RNA samples extracted from tissues. Sequence analysis of these recombinants revealed structures similar to those described for the NS3 duplications (Figs. 3, 4, and 5) with the positions of reassociation of the nascent strand and the template being in regions of the RNA predicted to be unpaired (data not shown).

These results suggest that template switching may be a common event in the intracellular replication of BVDV genomic RNA. The identification of multiple recombination events in single cloned isolates (Fig. 3, clones 1, 2, and 3) was unexpected because of the relatively small size of the amplicons but recombined BVDVc genomes have been described on numerous occasions (see Meyers and Thiel, 1996). As the sequences analyzed in this study are not limited by a requirement to produce viable virus or selected for biological fitness, it is possible that these observations are a more accurate reflection of the real frequency of RNA recombination. However, the observation of multiple recombination events in a single virus genome is not without precedent as the BVDVc isolates represented by CP13 DI, TGAC, or IIIC are also the product of multiple template-switching events and give rise to viable cytopathogenic viruses (Meyers and Thiel, 1996). If such recombination is a property of replicating RNA molecules and influenced by local RNA secondary structures then, in a high proportion of cases, the products of these recombination events are likely to result in the generation of nonviable genomes. Analysis of the effects of the recombination events described in this work on the predicted open reading frames suggests that in many cases there is a frameshift that would result in the introduction of an in-frame stop codon and prema-

ture termination of the viral polyprotein. In those cases where a stop codon is not encountered in the amplicon sequence, there is no evidence to indicate whether the recombined genomes would have been viable. Given the essential roles of both the E2 and the NS3 proteins in the viral life cycle (major viral glycoprotein and protease/helicase involved in polyprotein processing, respectively), it is highly likely that the gross changes in the protein sequences caused by the recombination and fusion events described would result in nonviability. The available evidence suggests that there are limited circumstances where gross genome recombinations are tolerated and must in some way enhance virus viability, as is observed for the known BVDVc strains, but these individual recombinants must be selected from a much larger population of nonviable viruses generated by random recombination.

Non-homologous recombination provides the possibility of rapid evolution of the viral genome and has previously been documented in several different viral systems, including BVDV. The results presented in this paper confirm that recombination is a frequent event for viruses replicating in the persistently infected animal and, at the level of the participating RNAs, is not restricted to a hotspot encoding the N-terminus of the NS3 protein. However, the generation of cytopathogenic viruses which replicate to a high level and cause fatal disease is a very powerful phenotypic bias of the biological selection procedures.

METHODS

Primer design

The primers used were based on consensus BVDV sequences and, where available, the known sequences of the Pe515nc strain. Primer pair 2 (comprising oligo9 + oligo2) was used as a positive control to amplify a 450-bp region of the NS3 gene in all viral samples while SP6 + oligo26 confirmed the integrity of RNAs encoding the E2 protein. Other primers were designed with a "back-to-back" arrangement, specifically to detect genome duplications. These primers would hybridize to any BVDV genome, but a PCR amplification product would only be generated where nonhomologous recombination had resulted in duplications of viral coding sequences as previously described only for BVDVc strains (see Fig. 1). The primer pair 6 (comprising primer10 + primer11) was targeted to the NS3 coding region while a second primer pair, oligo26 + SP2, was targeted to sequences in the E2 coding region. A BVDVnc genome containing no duplication of the viral NS3 or E2 genes would not be expected to give a PCR product with either of these primer pairs. The relative positions of these primers on the BVDV genome is shown schematically (not to scale) in Fig. 1.

TABLE 1

Genome Positions (BVDV NADL) of the Primers Used in This Study

Pair no.	Primer no.	NADL base no.	Target	Function
2	Oligo 2	5890–5910	NS3	Positive control
2	Oligo 9	5467–5450	NS3	
6	Primer 10	7630–7651	NS3	NS3 duplications
6	Primer 11	5526–5507	NS3	
	SP6	2405–2425	E2	Positive control (with oligo26)
9	Oligo 26	2700–2678	E2	E2 duplications
9	SP2	3149–3175	E2	

Primer sequences

The primers used were based on the NADL genome sequences as detailed in Table 1.

RNA extraction

Serum and lymph node biopsies were taken from two animals (1263 and 1279) and postmortem tissues taken from a third animal, 1280, all persistently infected with the BVDV strain Pe515nc. Serum and tissues were also taken from a BVDV-negative cow (1154) as a control. The BVDV-infected animals remained healthy for at least 2 years following sampling. Total RNA was extracted from 10- μ m cryosections of prescapular lymph node or cerebellum and from 500- μ l serum samples, using two similar methods, namely that of Chomczynski and Sacchi (1987) or the Biotecx Laboratories RNAzol B reagent (800 μ l used according to the manufacturer's instructions). After ethanol precipitation the RNA was resuspended in 20 μ l of DEPC-treated water.

RT-PCR

RNA was reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Gibco/BRL) in a final volume of 20 μ l according to the manufacturer's instructions. The RT was inactivated by heating at 70°C for 10 min. PCR, using *Taq* polymerase (Promega), was performed according to standard procedures using 2 μ l RT reaction product with 50 pmol of each primer in a final volume of 50 μ l. The target was amplified with 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension time of 7 min at 72°C.

Following amplification, 10 μ l of the PCR product was examined by agarose gel electrophoresis. Successfully amplified PCR products were shotgun cloned into pGEM-T (Promega) and transformed into *Escherichia coli* XL1 Blue (Stratagene).

Sequence analysis

Plasmid DNA from individual cloned PCR products was purified (using the Qiagen Spin Plasmid kit) prior to

sequencing on a Pharmacia ALF Express sequencer using labeled primers and a Thermosequenase cycle sequencing kit (Amersham). Both strands of each clone were sequenced completely (forward and reverse) until an unambiguous, clone consensus sequence was achieved. DNA sequence data were analyzed using the GCG package of programs (Devereux *et al.*, 1984) at the SEQNET facility. The possible role of repeated sequences or RNA secondary structure in the recombination events was investigated using a range of programs in this package.

ACKNOWLEDGMENTS

We are grateful to Ms. N. J. Archer and the compound staff at the Institute for Animal Health at Compton for the care of the animals used in this study.

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