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# Viral Cytopathogenicity Correlated with Integration of Ubiquitin-Coding Sequences

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The RNA genomes of cytopathogenic bovine viral diarrhea virus (BVDV) isolates contain insertions highly homologous to cellular sequences. For two of them the insert was identified as ubiquitin coding sequence. The genome of BVDV Osloss contains exactly one ubiquitin gene monomer. In the case of BVDV CP1 the cellular insertion comprises one complete ubiquitin gene and part of a second monomer. The host cell-derived element in the CP1 genome is embedded in a large duplication of about 2.4 kb of viral sequences. Cellular insertion and duplication were not found in the genome of NCP1, the noncytopathogenic counterpart of CP1. These results strongly suggest that recombination between viral and cellular RNA is responsible for development of the cytopathogenic viruses, which is linked to pathogenesis of a lethal disease in cattle. 

## INTRODUCTION

Bovine viral diarrhea virus (BVDV) belongs to the genus pestivirus within the family Togaviridae (Westaway *et al.*, 1985). Pestiviruses have a single-stranded RNA genome of positive polarity which is about 12.5 kb in length (Renard *et al.*, 1987; Collett *et al.*, 1988a; Meyers *et al.*, 1989a). Viral gene expression is believed to occur via synthesis of a polyprotein and subsequent proteolytic processing (Collett *et al.*, 1988b,c). With respect to genome organization and strategy of gene expression pestiviruses are more similar to flaviviruses than to togaviruses. It has therefore been proposed to classify the pestiviruses as a new genus of the family Flaviviridae (Collett *et al.*, 1988c).

In tissue culture two BVDV biotypes, cytopathogenic BVDV (cpBVDV) and noncytopathogenic BVDV (noncpBVDV), can be distinguished (Baker, 1987). Both biotypes are involved in pathogenesis of mucosal disease (MD), the most severe clinical manifestation of BVDV infections. A prerequisite for MD is a persistent infection with noncpBVDV. Surprisingly, cpBVDV can always be isolated from MD animals in addition to the persisting noncp virus (Brownlie *et al.*, 1984; Bolin *et al.*, 1985). In contrast to the described antigenic variability of BVDV field isolates the members of such a "pair" of noncpBVDV and cpBVDV are antigenically very closely related (Pocock *et al.*, 1987; Corapi *et al.*, 1988). This observation led to the hypothesis that during pathogenesis of MD a cpBVD virus develops from the noncp virus by acquiring some kind of mutation (Corapi *et al.*, 1988).

Comparison of the genomic sequences of two cpBVDV strains (BVDV Osloss [Renard *et al.*, 1987] and BVDV NADL [Collett *et al.*, 1988a]) led to the identification of small insertions located in a region coding for a nonstructural protein (Collett *et al.*, 1989; Meyers *et al.*, 1989a,b). The insertion of 228 nucleotides identified in the BVDV Osloss genome encodes a complete ubiquitin-like element with only two amino acid exchanges with respect to the ubiquitin sequence conserved in all animals (Meyers *et al.*, 1989b, 1990). The sequence of 270 nucleotides which is inserted in the BVDV NADL genome shows no homology to a ubiquitin gene but is almost identical with another bovine mRNA sequence (Meyers *et al.*, 1990). We therefore proposed as a working hypothesis that recombination between viral and cellular RNA led to the formation of these cpBVDV genomes (Meyers *et al.*, 1989b, 1990).

The mutation responsible for development of cpBVDV from a noncytopathogenic virus might be a recombination process. As a consequence, cytopathogenicity in BVDV would be linked to insertions of host cellular sequences in the viral genomes. Verification of this hypothesis was hampered by the fact that sequence data from noncytopathogenic viruses were not available. In this report molecular characterization of a "pair" of noncpBVDV (NCP1) and cpBVDV (CP1) (Corapi *et al.*, 1988) isolated from one animal suffering from MD is presented for the first time. The resulting data are discussed with respect to the mechanism of RNA recombination, implications for cytopathogenicity of

Sequence data from this article have been deposited with the GenBank Data Library under the following accession numbers: M37793, rpubl sequence; M37794, rpub2 sequence; M37795, BVDV NCP1 sequence; M37796, BVDV CP1 sequence.

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cpBVDV, pathogenesis of mucosal disease, and evolution of RNA viruses.

## MATERIALS AND METHODS

### Materials

Restriction enzymes and modifying enzymes were obtained from Pharmacia-LKB, New England Biolabs, Boehringer-Mannheim, and Life Sciences, Inc. Radioactive compounds were purchased from Amersham–Buchler. Kodak XAR5 X-ray films were used for autoradiography.

### Cells and viruses

MDBK cells and the BVDV strain NADL (Gutekunst and Malmquist, 1963) were obtained from the American Type Culture Collection (Rockville, MD). The BVDV strain Osloss (Renard *et al.,* 1985) was kindly provided by Dr. Liess (Veterinary School, Hannover, FRG). Isolation and serological characterization of BVDV strains NCP1 and CP1 have been described elsewhere (Corapi *et al.,* 1988). Cells were grown in DMEM supplemented with 10% FCS. Cells and virus stocks were tested every 3 to 6 months for the absence of mycoplasma contamination.

### RNA preparation, gel electrophoresis, and Northern hybridization

Preparation of RNA was done as described (Rümenapf *et al.,* 1989). Five micrograms of glyoxylated RNA (Maniatis *et al.,* 1982) was separated in phosphate-buffered 1% agarose gels containing 5.5% formaldehyde, and transferred to Duralon membranes (Stratagene). An RNA ladder (Bethesda Research Laboratories) served as a size standard. Hybridization with probes labeled with $^{32}P$ by nick translation (Rigby *et al.,* 1977) (nick translation kit, Amersham–Buchler) was performed in 0.5 *M* sodium phosphate, pH 6.8, 1 m*M* EDTA, and 7% SDS. Hybridization temperature was 54° for the HCV cDNA probe and 68° for the ubiquitin probe. Posthybridization washes were carried out with 40 m*M* sodium phosphate, pH 6.8, 1 m*M* EDTA, 5% SDS, and 40 m*M* sodium phosphate, pH 6.8, 1 m*M* EDTA, 1% SDS two times each for 30 min at hybridization temperature.

### Oligonucleotides

Oligonucleotides were synthesized on a Biosearch 8700 DNA synthesizer (New Brunswick Scientific) using the phosphoamidite method (Beaucage and Caruthers, 1981). Sequences of the oligonucleotides used for cDNA priming were as follows.

First libraries:
BVDV13: GCCATRTCXCCYTCYTTCAT (6226–6245)
BVDV14: GRCCARTCRTARTTCATYTC (7033–7052)
Second library:
PES9: ACYTCCCAYTTRTCXGT (9079–9095)
ORG345: GATGAGCCTCTGCTGGTCAGGGG-GAATGCCTTCC (5250–5283)

R represents both purine nucleotides, Y both pyrimidine nucleotides, and X all four nucleotides. Numbers indicate the positions of the complementary sequences in the Osloss genome (Renard *et al.,* 1987).

### cDNA synthesis, cloning, and library screening

About 5 $\mu$g of total RNA of MDBK cells infected with either CP1 or NCP1 were heat denatured (5 min, 65°). Annealing of oligonucleotides (0.1 $\mu$g each of BVDV13 and BVDV14 or PES9 and ORG345, first or second cDNA libraries, respectively) was at 37° for 20 min. cDNA synthesis was performed as described (Rümenapf *et al.,* 1989). Cloning in λ ZAPII (Stratagene) and *in vitro* packaging using GIGAPACK GOLD (Stratagene) was performed as recommended by the supplier.

The bovine polyubiquitin-specific cDNA clones were isolated from a commercially available cDNA library (constructed with RNA of MDBK cells, Stratagene).

Screening of libraries by hybridization with radiolabeled DNA probes was done as described by Benton and Davies (1977). Subcloning into Bluescript plasmids by *in vivo* excision was performed as recommended by the supplier (Stratagene).

### Nucleotide sequencing

Exonuclease III and nuclease S1 were used to establish deletion libraries of cDNA clones (Hennikoff, 1987). Dideoxy sequencing (Sanger *et al.,* 1977) of double-stranded DNA templates was carried out using the T7 polymerase sequencing kit (Pharmacia-LKB). Computer analysis of sequence data was performed on a Digital Microvax II using the UWGCG software (Devereux *et al.,* 1984).

### Radioimmunoprecipitation and SDS–PAGE

BVDV-infected MDBK cells ($1.5 \times 10^6$ per 3.5-cm dish) were labeled for 6 hr with 0.5 mCi/ml [$^{35}S$]-methionine/[$^{35}S$]cysteine. Labeling medium contained no cysteine and $\frac{1}{20}$ of normal methionine content. Cell extracts were prepared as described (Rümenapf *et al.,* 1989). For precipitation with the anti-ubiquitin or the anti-peptide antisera extracts were prepared under denaturing conditions (Harlow and Lane, 1988). Extracts were incubated with 5 $\mu$l of undiluted serum. Precipitates were formed with crosslinked *Staphylococcus aureus* (Kessler, 1981), analyzed by 10% SDS–PAGE (Laemmli, 1970), and processed for fluorography using Enhance (New England Nuclear). The anti-peptide an-

tiserum kindly provided by Dr. M. S. Collett was prepared as described by Suzich and Collett (1988).

## RESULTS

### Characterization of the viral genomes by hybridization

As a first step towards molecular analysis of the genomes of the cytopathogenic BVDV strain CP1 and its noncytopathogenic counterpart NCP1 (Corapi *et al.*, 1988) hybridization experiments with RNA of bovine kidney cells (MDBK) infected with different BVDV strains were performed. For detection of the genomic viral RNAs an hog cholera virus (HCV)-derived cDNA fragment encompassing nucleotides 4377 to 6532 of the viral genome was used as a probe (Meyers *et al.*, 1989a). In hybridizations at reduced stringency this DNA molecule recognized all pestivirus genomic RNAs tested so far (data not shown). After hybridization of this probe to RNA of cells infected with four different BVDV strains, respectively, dramatic differences in the electrophoretic mobility of the viral genomic RNAs were detected. While the genomes of BVDV strains Osloss, NADL and NCP1 (Fig. 1A, lanes 1, 2, and 3, respectively) have similar sizes of about 12.5 kb, the genomic RNA of BVDV CP1 is much larger with an estimated size of 14 to 15 kb (Fig. 1A, lane 4).

Northern hybridization experiments with a porcine ubiquitin clone (pCL208, Einspanier *et al.*, 1987) had shown that among five common cpBVDV laboratory strains, which represent independent isolates from different countries, the ubiquitin-coding insertion was specific for the Osloss strain (Meyers *et al.*, 1990). An analogous experiment was conducted with RNA of cells infected with CP1 and NCP1. Surprisingly, a positive signal was obtained with genomic CP1 RNA after stringent hybridization with the ubiquitin probe (Fig. 1B, lane 4). Since the genomic RNA of BVDV NCP1 is not recognized by this DNA fragment (Fig. 1B, lane 3) at least part of the additional RNA in the CP1 genome represents a ubiquitin-like sequence.

The three major additional bands visible in all lanes of Fig. 1B probably represent the host cellular ubiquitin mRNAs since they can also be detected among RNA of noninfected cells (Fig. 1B, lane 5). The corresponding RNA molecules have sizes of about 5.0, 4.0, and 1.4 kb, respectively, and bind to oligo d(T) cellulose (data not shown).

### Genome organization of BVDV NCP1 and CP1

In order to compare the genomes of BVDV NCP1 and CP1 at the level of nucleotide sequences, parts of the viral genomes were cloned as cDNA. First strand synthesis was specifically primed with degenerated oligonucleotides deduced from heptapeptide sequences conserved in all three known pestivirus-encoded polyproteins. As both the BVDV Osloss and NADL insertions are located around nucleotide 5000 of the genomic RNAs (Collett *et al.*, 1989; Meyers *et al.*, 1989a, 1990) a mixture of two oligonucleotides priming at about 6 and 7 kb was used for the construction of the cDNA libraries. Positive phage clones were isolated after screening with the heterologous HCV cDNA probe described above.

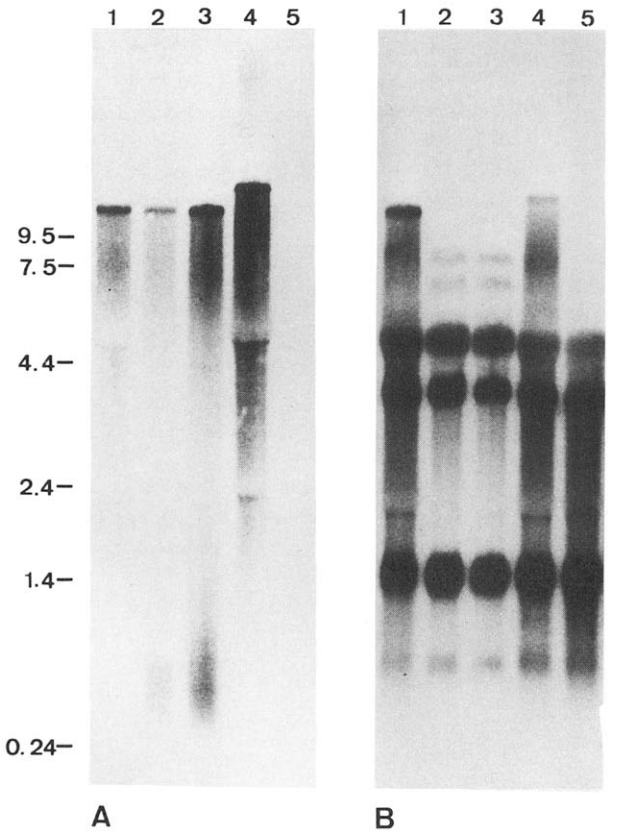


FIG. 1. Northern blot analyses of total RNA from MDBK cells infected with BVDV strains Osloss (lanes 1), NADL (lanes 2), NCP1 (lanes 3), and CP1 (lanes 4), respectively, and noninfected cells (lanes 5). The blots were hybridized with (A) a 2.2-kb *Sal*I fragment derived from the HCV cDNA clone 4.5 (Meyers *et al.*, 1989a) and (B) a 0.2-kb *Bgl*II fragment isolated from the porcine ubiquitin clone pCL208 (Einspanier *et al.*, 1987).

By restriction site mapping and cross hybridization studies the relative positions of the different cDNA fragments were established (Fig. 2). NCP1 represents the first noncytopathogenic BVDV strain which has been characterized at the genome level; a continuous sequence of 3199 nucleotides was determined for the respective clones (Fig. 3A). In contrast to the two published genomic sequences of cpBVDV strains (Osloss [Renard *et al.*, 1987] and NADL [Collett *et al.*, 1988a]) the NCP1 RNA does not contain an insertion in the analyzed genomic region. In this aspect NCP1 is most similar to HCV, which also lacks insertions of cellular sequences (Meyers *et al.*, 1989a). Since the inserts identified in the genomes of BVDV Osloss and NADL

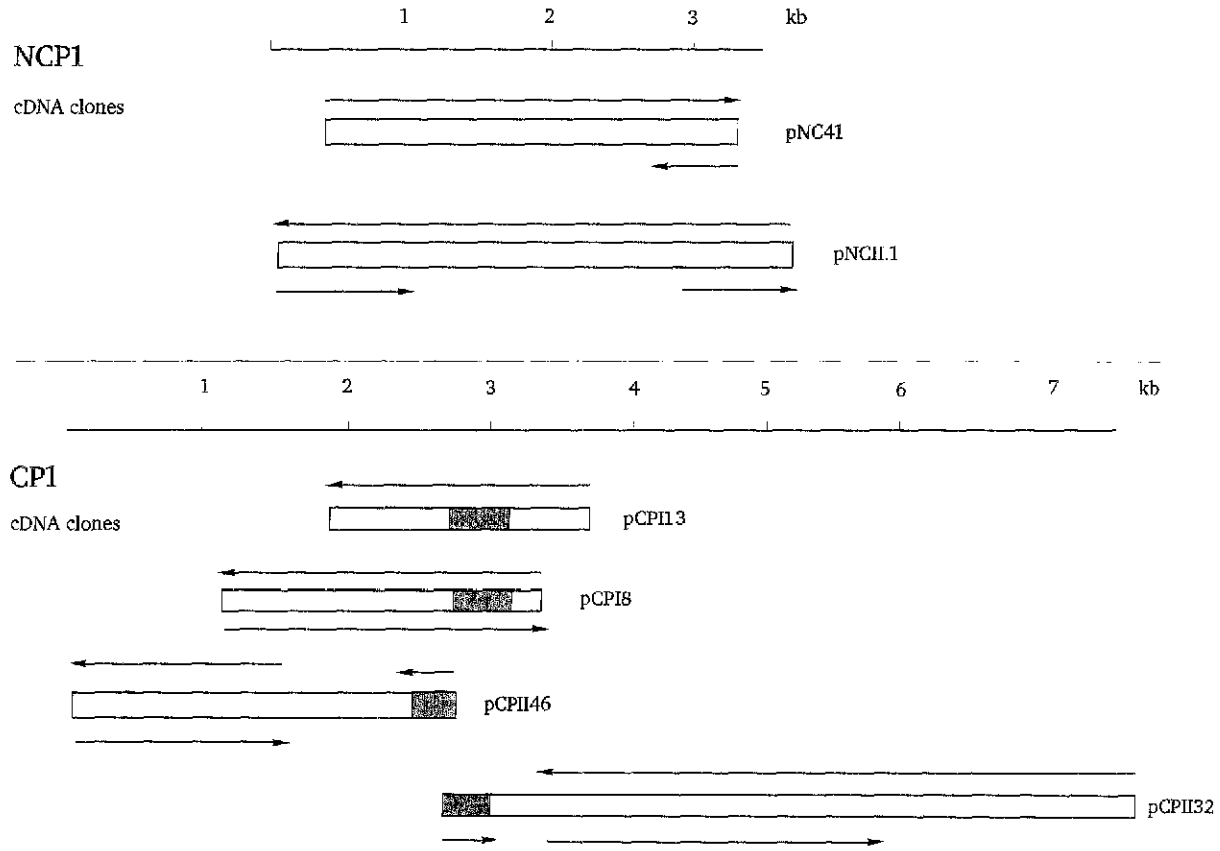


FIG. 2. Schematic drawing indicating the relative positions and sequencing strategies for the BVDV NCP1 and CP1 cDNA clones. For each cDNA fragment the regions which have been sequenced are marked by arrows. Ubiquitin-coding sequences are shown as dark bars.

are small (228 and 270 nucleotides, respectively), their genomic RNAs migrate on agarose gels like that of BVDV NCP1 (Fig. 1).

The genome organization of BVDV CP1 is more complex than that of the other described pestiviruses. Analysis of the sequences of the cDNA clones pCPI8 and pCPI13 (Fig. 2) resulted in identification of 366 nucleotides of ubiquitin-coding sequence. Thus, in addition to a complete ubiquitin gene of 228 nucleotides which is also found in the BVDV Osloss genome (Meyers *et al.*, 1989b), the CP1 genomic RNA contains part of a second ubiquitin-gene monomer truncated at the 5′ end (Fig. 3B).

Sequence analysis revealed that the nucleotides preceding the ubiquitin-coding element in the CP1 genome correspond to a region upstream of nucleotide 7765 in the BVDV Osloss sequence (denoted C in Fig. 4), while the nucleotides following the insertion are homologous to the Osloss sequence downstream of nucleotide 5380 (denoted B in Fig. 4). To further investigate this unusual arrangement of the CP1 genome the nucleotide sequence starting about 3 kb upstream and ending more than 4 kb downstream of the ubiquitin-coding element of CP1 was determined. Parts of the respective clones (pCPII32 and pCPII46 in Fig. 2) were obtained from an additional cDNA library constructed after priming with oligonucleotides complementary either to sequences located around nucleotide 9000 of the BVDV genome or to part of the ubiquitin sequence, respectively. As indicated in Fig. 3B and Fig. 5 both the sequences preceding and following the ubiquitin-coding element in the CP1 genome are found again at another position of the determined sequence (Fig. 5, box 1 versus 3 and box 2 versus 4, respectively). Accordingly, in the genome of CP1 the host cell-derived insertion is embedded in a large duplication of viral sequences which corresponds to nucleotides 5380 to 7763 of the BVDV Osloss sequence (Figs. 4 and 5). Therefore the genomes of NCP1 and CP1 can only be aligned if either the 5′ or the 3′ duplicated element is regarded as part of an insertion (Fig. 5, upper section). The duplication together with the 366 nucleotides of ubiquitin-coding sequence comprises 2750 nucleotides and apparently accounts for the observed differences in genome size (Fig. 1).

## Nucleotide sequence homology between BVDV NCP1 and CP1

According to our working hypothesis the genome of BVDV CP1 has developed from that of NCP1 by inte-

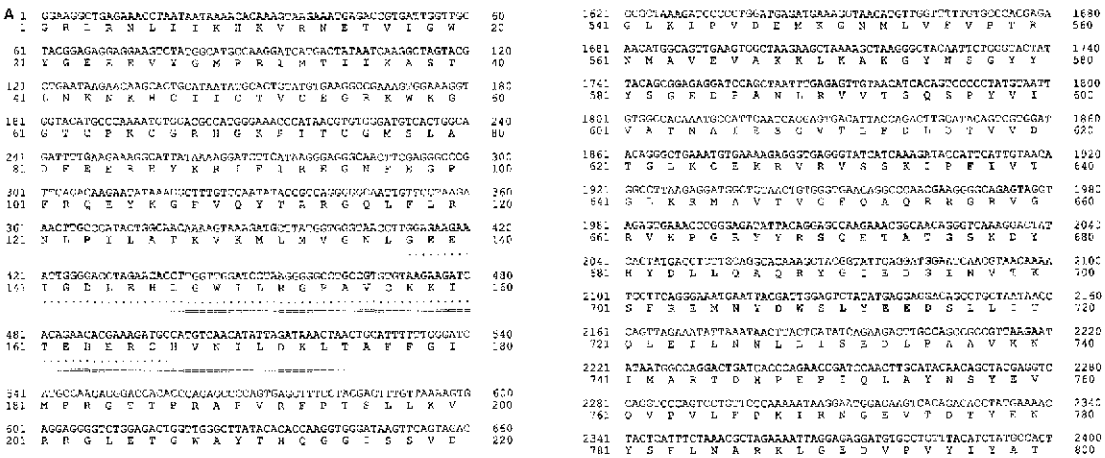

FIG. 3. Nucleotide and deduced amino acid sequences determined from the BVDV NCP1 (A) and CP1 (B) cDNA clones. For both sequences the regions compared in Fig. 5 are underlined (box 1, . . .; box 2, ---; box 3, = = =; and box 4, """"). The ubiquitin-coding region of the CP1 sequence and the methionine representing the aminoterminal end of the complete ubiquitin monomer are marked by boxes. Only sequences determined from both strands of one cDNA fragment or from two independent cDNA clones are shown (see Fig. 2). Comparison of the sequences of clones pCPI8 and pCPI13 resulted in detection of three differences (positions 3531, T or G; 3603, G or A; 3695, T or C, in pCPI8 or pCPI13, respectively). In (B) the sequence of pCPI8 is shown. Because of the difference at nucleotide position 3695, the deduced amino acid sequence also varies (T instead of I).

gration of additional RNA in a recombination process. This theory implies that the sequences of the virus-derived parts of the BVDV CP1 RNA should be almost identical with the respective regions of the NCP1 genome. The nucleotide sequences determined from the CP1- and NCP1-specific cDNA clones were compared with each other and with those published for BVDV Osloss and NADL. As already shown above the NCP1 sequence and in analogy also the Osloss and NADL sequences can be aligned with the CP1 genome in two different ways depending on which of the duplicated sequences of CP1 is regarded as part of the inserted element (see also Fig. 5, upper section). Therefore each of the duplicated regions in the CP1 genome was analyzed separately. The host cell-derived regions of the three cpBVDV genomes were not included. The homologies of 99.6 and 99.7% observed between the NCP1 and the two CP1 sequences demonstrate their extremely close relationship (Table 1). A similarly low number of nucleotide substitutions was found when the 5′ and the 3′ duplicated regions of the CP1 RNA (nucleotides 445 to 2829 versus 3196 to 5580 in Fig. 3B) were compared (Table 1).

## Comparison of the sequences flanking the insertions

The two cpBVDV strains CP1 and Osloss both have integrated ubiquitin-coding sequences into their genomes. Because of their rather low degree of nucleotide homology (Table 1) as well as their differences in

FIG. 3—*Continued*

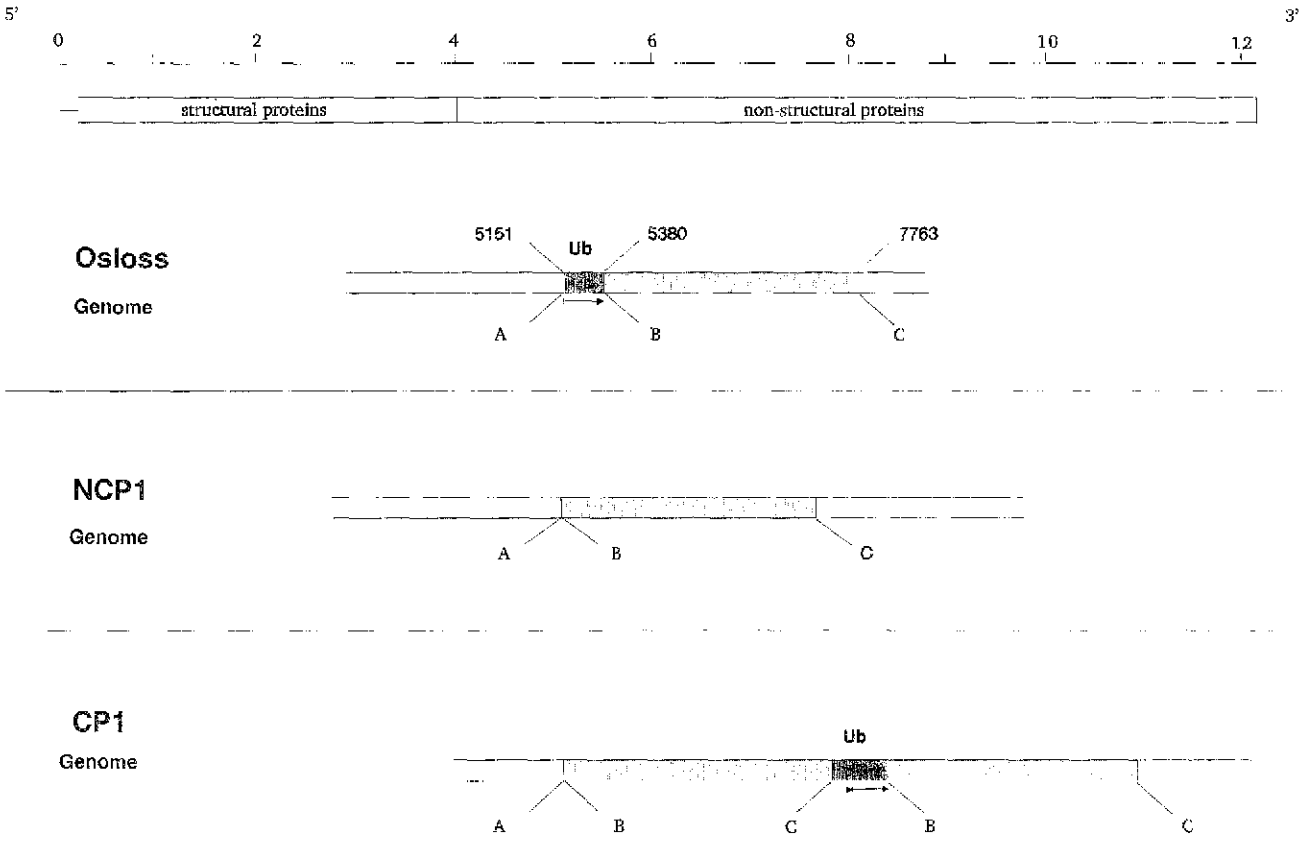


FIG. 4. Schematic drawing demonstrating the genome organization of BVDV strains Osloss, NCP1, and CP1 for the analyzed region. To localize the respective part on the genome a BVDV genomic RNA is indicated on top; numbers refer to the sizes of the RNA in kb. The long open reading frame of the genome is shown as a bar, 5' and 3' noncoding regions as thin lines. Sequences coding for ubiquitin-like elements (Ub) are shown as dark gray bars. Ubiquitin gene monomers or parts thereof are marked by arrows. The region duplicated in the CP1 genome is indicated by light gray bars. The positions of the nucleotides directly preceding and following the ubiquitin element in the Osloss genome are marked by the letters A and B, respectively. In addition the position equivalent to the last nucleotide of the sequence duplicated in the CP1 genome is marked by the letter C. The positions analogous to those denoted A, B, and C in the Osloss genome are marked in the same way for NCP1 and CP1. Numbers refer to the published BVDV Osloss sequence (Renard *et al.*, 1987).

genome organization (Fig. 4) independent recombination events probably led to formation of the two viral genomes. To obtain more information about these putative RNA recombination processes the sequences flanking the insertions in the BVDV CP1 and BVDV Osloss genomes were analyzed. If for CP1 the 5' duplicated element together with the ubiquitin-coding sequence is regarded as insertion (Fig. 5), it would be located at exactly the same genomic position as the Osloss inserted element (indicated by the letters A and B in Fig. 4 and Fig. 5). Accordingly the sequences flanking this tentative insertion in the CP1 genome are highly homologous to those adjacent to the Osloss insertion (for CP1 see Fig. 5, sequences upstream of "A" in box 1 and downstream of "B" in box 3, respectively; for Osloss see Fig. 6A). The second possible CP1 insertion would be located in a completely different genomic region (Fig. 5). It therefore seems more logical to regard the 5' duplicated element together with the ubiquitin-coding region as the sequence which has been integrated in the CP1 genome by the recombination process.

According to the model presented above the 5' ends of the sequences integrated into the genomes of BVDV Osloss and CP1 are different: While the Osloss insertion starts with the ATG of a ubiquitin gene, the insertion in the CP1 genome contains part of a second monomer of this gene preceded by the large duplicated element (Fig. 4 and Fig. 6). When the 5' borders of the ubiquitin-coding elements were compared the respective viral and host cell-derived sequences are not homologous for both viruses (Fig. 6A). The situation is different for the 3' ends: Both the CP1 and the Osloss insertion terminate exactly with the end of a ubiquitin gene monomer even though the Osloss ubiquitin has acquired a mutation which changes the carboxyterminal glycine into a serine residue (Fig. 6B). Thus at the 3' borders both the host cell-derived and the flanking viral sequences are conserved (Fig. 6A), indicating that the respective regions of the viral and

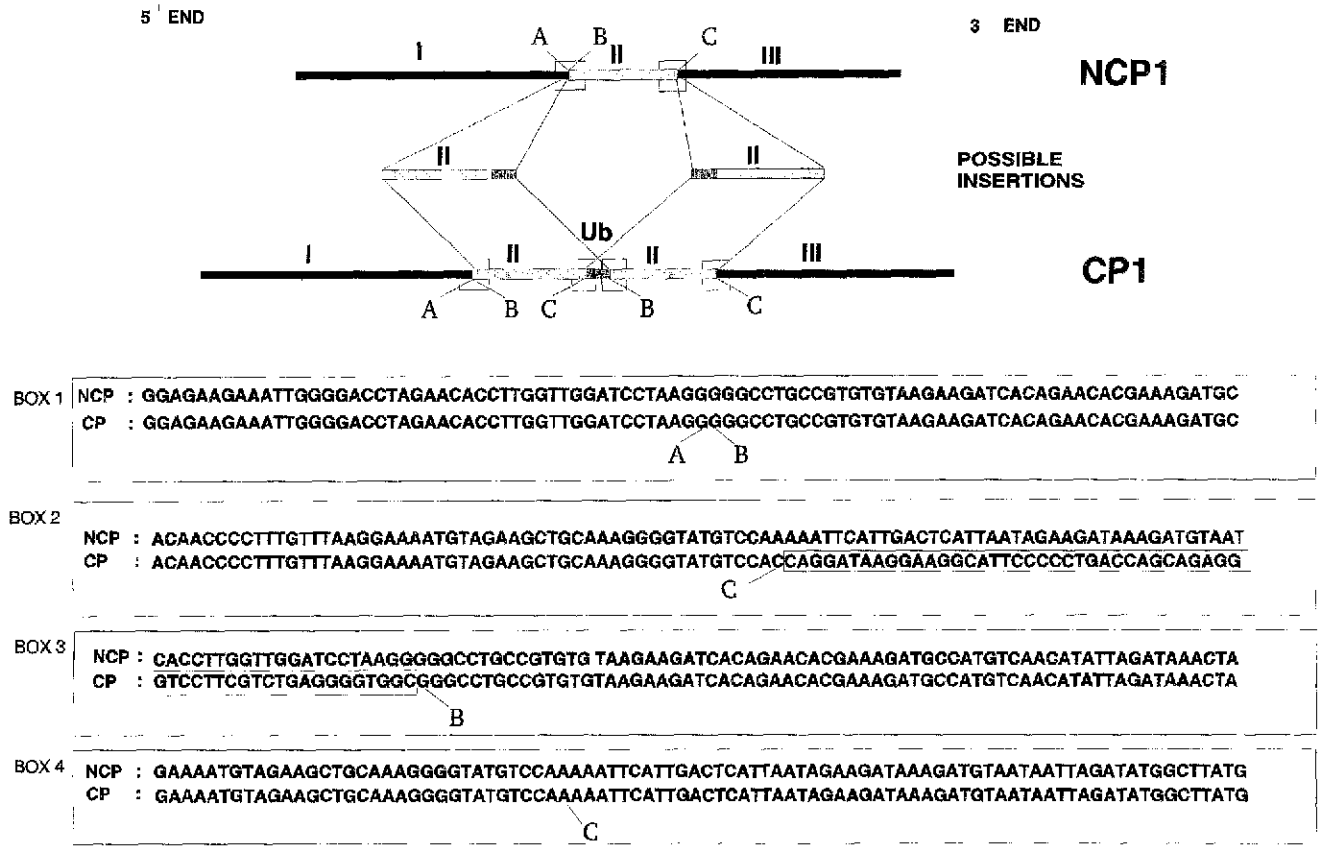


FIG. 5. Sequence comparison of the genomic regions containing the 5' and 3' ends of duplicated elements in the CP1 genome with the respective parts of the NCP1 sequence. The upper section shows schematically the genomic location of the sequences compared in the boxes below. The duplicated elements in the CP1 sequence and the respective unique region of the NCP1 genome are shown as light gray bars denoted "II," while the 5' and 3' flanking regions are denoted "I" and "III," respectively. Depending on which of the duplicated elements in the CP1 genome is regarded as part of the inserted sequence, two different insertions are possible, as indicated in the second lane. A, B, and C denote the same nucleotide positions already shown in Fig. 4 and correspond here to the last nucleotide of the unique sequence (A) and the first (B) and last (C) residue of the duplicated region. The ubiquitin coding sequence (dark gray denoted Ub in the schematic drawing) is marked by a box in the sequence comparison.

host cellular RNAs might play a role in a site-specific recombination process (see discussion).

## Proteins expressed from the recombinant RNA

The region affected by the integration of additional sequences into the genomes of BVDV CP1 and Osloss encodes a nonstructural protein of about 125 kDa (p125) (Collett *et al.*, 1988b). While this protein apparently remains unchanged after infections with noncpBVDV, processing of p125 has been observed in cells infected with cpBVDV strains. A cleavage product of about 80 kDa (p80) has been described as a marker for the cytopathogenic viruses (Purchio *et al.*, 1984; Donis and Dubovi, 1987a,b). Using a monospecific antiserum the second processing product of 54 kDa (p54) could be detected in cells infected with BVDV NADL (Collett *et al.*, 1988b).

Radioimmunoprecipitation assays (RIP) with different anti-pestivirus as well as anti-ubiquitin antisera were employed for investigation of the respective proteins encoded by BVDV NCP1, CP1, and Osloss. With an antiserum directed against a bacterial fusion protein containing part of p80 of BVDV NADL (expression clone B10, Collett *et al.*, 1988b) p125 is precipitated

TABLE 1

NUCLEOTIDE SEQUENCE HOMOLOGY BETWEEN THE 5' AND 3' DUPLICATED ELEMENTS IN THE GENOME OF BVDV CP1 AND THE RESPECTIVE GENOMIC REGIONS OF NCP1, OSLOSS (Os), AND NADL

| | 5'DE | 3'DE |
|---|---|---|
| NCP1 | 99.6% | 99.7% |
| Os | 81.8% | 81.9% |
| NADL | 89.9% | 89.9% |
| 5'DE | 100% | 99.5% |
| 3'DE | 99.5% | 100% |

*Note.* 5' and 3' duplicated elements are denoted 5'DE and 3'DE, respectively.

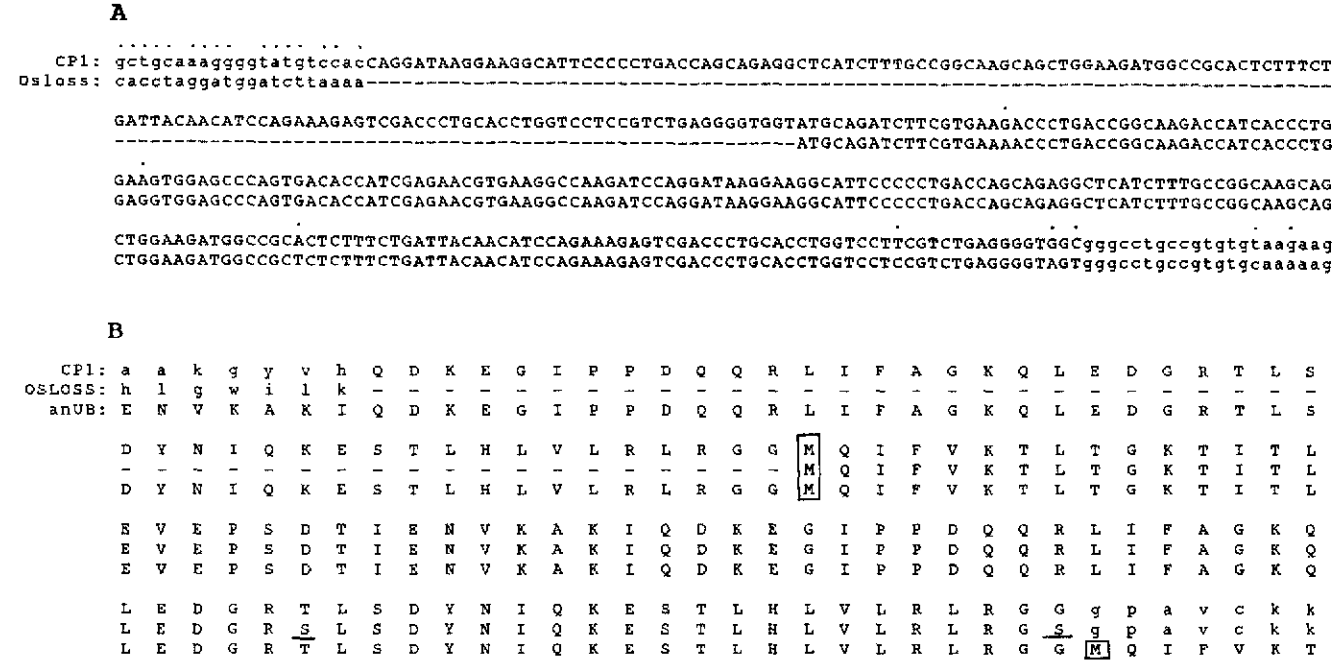


FIG. 6. Sequence comparison of the host cell-derived insertions in the genomes of BVDV CP1 and Osloss (Renard *et al.*, 1987) including the flanking viral sequences. The host cell-derived regions are capitalized while viral parts of the sequence are indicated by lower case letters. Residues missing in the Osloss sequence are indicated by a dashed line. (A) Comparison of the nucleotide sequences, differences are marked by dots. (B) In addition to the CP1 and Osloss amino acid sequences the conserved animal ubiquitin sequence shown as part of a polyubiquitin (denoted "anUB") is included. The first methionine of each ubiquitin monomer is marked by a box. The two exchanges in the Osloss ubiquitin are underlined.

from all BVDV-infected cells (Fig. 7A, lanes 2, 3 and 4). As expected, p80 can only be detected in Osloss and CP1 infected cells (Fig. 7A, lanes 2 and 3, respectively) but not after infection with NCP1 (Fig. 7A, lane 4). In all aspects these data are in agreement with the results reported for cpBVDV and noncpBVDV. Taking into account, however, the major differences in genome organization of the three strains investigated here, these findings were surprising. According to the genetic map determined for BVDV NADL the duplication in the CP1 genome encompasses part of the region coding for p125 and should also be expressed.

Further insight was obtained after RIP with a serum raised against a BVDV NADL-deduced peptide located within p54 of p125 (peptide encompassing amino acids 1335 to 1351 of the published BVDV NADL sequence (Collett *et al.*, 1988a)). As expected precipitation of p125 was observed for all three BVDV strains (Fig. 7B, lanes 3, 6, and 9). In addition a protein with an apparent molecular weight of 41 kDa (p41) was specifically precipitated from extracts of Osloss-infected cells (Fig. 7B, lane 9). This protein most likely is analogous to p54 of BVDV NADL, and thus represents the second product of p125 processing. The differences in size between p54 of BVDV NADL and p41 of BVDV Osloss indicate differences in the location of at least one of the respective processing sites. If the carboxyterminal cleavage site of this protein is conserved for both viruses, p41 of the Osloss strain should contain the ubiquitin sequence. In fact, antibodies directed against ubiquitin specifically reacted with p41, even though they failed to precipitate p125 (Fig. 7B, lanes 7 and 8). p41 therefore represents a stable ubiquitin fusion protein which could thoroughly influence the host cell biology (see Discussion).

A cleavage product equivalent to p41 of Osloss or p54 of NADL could not be detected after infection with CP1 (Fig. 7B, lane 6), indicating that in the respective cells p125 is not cleaved. As, however, p80 is detectable in CP1-infected cells it seems likely that this protein is encoded by the duplicated sequence following the ubiquitin element while the genomic region preceding the cellular insertion in the CP1 genome codes for p125 of this virus (Fig. 8). This would be in accordance with the genetic map published for BVDV NADL (Collett *et al.*, 1988b). Cleavage between adjacent ubiquitin monomers is a standard cellular function responsible for the release of monomeric ubiquitin from the polymeric precursors (Finley *et al.*, 1987; Özkaynak *et al.*, 1987; Rechsteiner, 1987). Since the cellular sequence integrated into the CP1 polyprotein encompasses more than one ubiquitin monomer it also contains the respective protease target site. Accordingly proteolytic processing within the host cell-derived sequence of

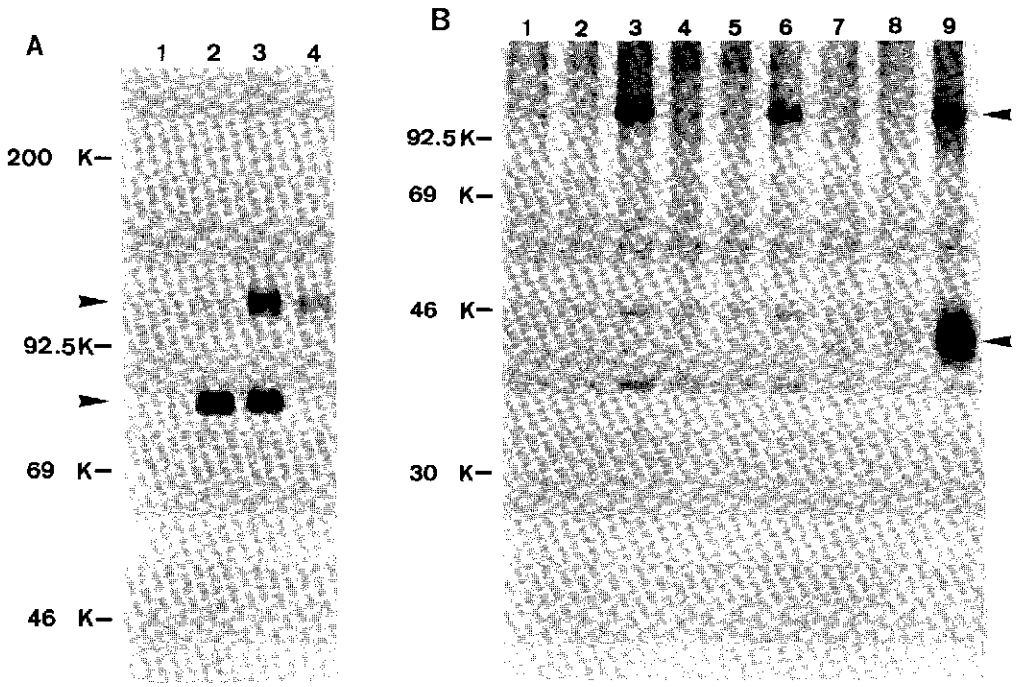


FIG. 7. Immunoprecipitation of proteins extracted from BVDV-infected cells after metabolic labeling with a mixture of [$^{35}$S]cysteine/[$^{35}$S]-methionine. Immunoprecipitates were analyzed by 10% SDS–PAGE. (A) Extracts of MDBK cells infected with strains Osloss (lane 2), CP1 (lane 3), and NCP1 (lane 4) and extracts of noninfected cells (lane 1) were incubated with an antiserum against a bacterial fusion protein containing part of p80 of BVDV NADL (expression clone B10; Collett *et al.*, 1988b). Arrows indicate the positions of p125 and p80. (B) Extracts of cells infected with BVDV NCP1 (lanes 1–3), CP1 (lanes 4–6), or Osloss (lanes 7–9) were incubated with a serum directed against a peptide encompassing amino acids 1335 to 1351 of the polyprotein of BVDV NADL (Collett *et al.*, 1988a) (lanes 3, 6, and 9) or two different antisera raised against yeast ubiquitin (serum 1: lanes 1, 4 and 7; serum 2: lanes 2, 5, and 8). Bands corresponding to p125 and p41 are marked by arrows. The additional bands visible in all lanes were also observed with noninfected cells. The host cellular ubiquitin cannot be detected on this figure, but was clearly visible using another gel system (data not shown).

the CP1 polyprotein could occur, resulting in generation of p125 and p80.

A possible explanation for the failure to precipitate virus-specific proteins from CP1-infected cells with the anti-ubiquitin antisera (Fig. 7B, lanes 4 and 5) is that the respective epitopes are not accessible for the antibodies. This must be the reason why these antisera did not react with the Osloss p125 since the protein contains the target sequences. According to our model on generation of p125 and p80 in BVDV CP1-infected cells different ubiquitin fusion proteins may be generated.

## Search for the putative cellular recombination partner

The identical location of the insertions in the genomes of BVDV Osloss and CP1 could be explained by site-directed recombination reactions between the viral and cellular RNAs based on primary or secondary structure elements. The integration of ubiquitin-coding sequences at a specific position in the BVDV genome could be guided by nucleotide interactions between the two RNA molecules. To investigate this question bovine ubiquitin-specific cDNA clones were analyzed. Two types of ubiquitin mRNAs have been described for eukaryotic cells, one encoding a ubiquitin monomer fused with small ribosomal proteins, and the other one coding for a polyubiquitin (Finley *et al.*, 1987, 1989; Özkaynak *et al.*, 1987; Redmann and Rechsteiner, 1989). The cellular insertion in the BVDV Osloss genome could be derived from both types of ubiquitin mRNAs. The ubiquitin-coding element in the CP1 RNA, however, most likely originates from a polyubiquitin mRNA since more than one monomer of the gene is

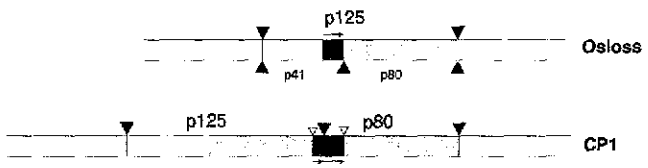


FIG. 8. Schematic drawing indicating the location of p125 and p80 in the deduced polyproteins of BVDV Osloss (Renard *et al.*, 1987) and CP1. Ubiquitin sequences are shown as dark gray bars. The duplicated regions of CP1 and the respective unique part of the Osloss polyprotein are indicated by light gray bars. Closed triangles indicate putative protease cleavage sites; their exact location is not known. Open triangles represent speculative further cleavage sites. Ubiquitin monomers or parts thereof are marked by arrows.

found. We have isolated two types of polyubiquitin-specific cDNA clones from a library constructed with poly(A)$^+$ RNA of MDBK cells. No full-length cDNA fragments were obtained but both types of clones contain several ubiquitin gene monomers and end in a poly(A) tail. The last ubiquitin gene of each clone is followed by a short clone-specific 3′ extension capable of encoding 6 and 1 amino acids (clones rpub1 and rpub2, respectively). Between the stop codons and the poly(A) tails short 3′ nontranslated trailers are found (Fig. 9).

The ubiquitin coding sequences of the rpub1 and rpub2 exhibit no homology to the regions flanking the insertions in the viral genomes. The sequence encoding the carboxyterminal extension of rpub1, however, is capable of hybridizing to the sequence directly upstream of the BVDV Osloss insertion. An analogous, however weaker, interaction is possible for BVDV NCP1. Base pairing between a polyubiquitin mRNA and genomic BVDV RNA might be involved in the mechanism of the proposed recombination reactions (see Discussion).

## DISCUSSION

Pathogenesis of mucosal disease in cattle represents a complex process which is linked to the coexistence of two different biotypes of a virus in one animal (Brownlie *et al.*, 1984; Bolin *et al.*, 1985; Baker, 1987). BVDV CP1 and NCP1 constitute such a pair of cytopathogenic and noncytopathogenic viruses isolated from one animal that had come down with MD (Corapi *et al.*, 1988). Our findings provide substantial evidence that BVDV CP1 represents a mutant of NCP1. It seems very likely that the described dramatic differences between the genomes of both viruses are responsible for their different phenotypes although changes in other genomic regions cannot be fully excluded. Accordingly, the most reasonable explanation for pathogenesis of MD is the generation of a cytopathogenic virus by a recombination process. This has already been proposed after the identification of host cell-derived sequences in the genomes of the cpBVDV strains NADL and Osloss (Meyers *et al.*, 1989b, 1990).

The cytopathogenicity of cpBVDV could be due either to altered viral proteins or to the expression of the inserted cellular sequences. Cleavage of the 125-kDa protein has been proposed as a molecular difference between cpBVDV and noncpBVDV (Donis and Dubovi, 1987a). This model has to be modified since at least in CP1-infected cells the described 80-kDa protein is not generated by cleavage of p125 but is expressed from a duplicated region in the viral genome.

Our data on BVDV CP1 indicate that cytopathogenicity is not dependent on processing of p125 or the presence of a protein like the NADL p54 or the Osloss p41. Thus on the protein level expression of p80 represents the only obvious marker of cpBVDV. Whether this protein is responsible for the cytopathic effect remains to be investigated. However, the observation that all cpBVDV strains analyzed so far express a protein analogous to p80, whether by processing of p125 or by duplication of the respective coding RNA, indicates a linkage between mucosal disease, the cytopathic effect, and this protein. According to sequence comparison studies p80 contains serine protease motifs as well as amino acid sequences previously found in helicases (Gorbalenya *et al.*, 1989a,b). Biochemical studies on the function of this protein will help to understand its putative role for the cytopathic effect of cpBVDV.

Cytopathogenicity of BVDV Osloss could also be due to expression of the nonphysiological ubiquitin fusion protein p41 which may act as an effective competitor in enzymatic reactions of the different ubiquitin pathways. In this context it is important to mention that expression of certain point mutants of ubiquitin in yeast had cytostatic effects on the cells (Butt *et al.*, 1988).

Direct evidence for the molecular basis of cytopathogenicity of cpBVDV could be obtained by analyses involving mutagenesis of the viral genome. However, these experiments require infectious BVDV cDNA which is thus far not available.

In considering the mechanism of the proposed recombination it has to be kept in mind that the respective reactions represent rare events. Mucosal disease develops only sporadically during years of persistent infection although high virus titers are detectable in the animals, indicating enormous virus replication activity (Baker, 1987). Thus, statistically, a large number of replication cycles seems to be necessary for a recombination resulting in integration of additional sequences. In addition only recombinants with the ability to cause MD are selected from a probably much larger number of recombinant viruses. To achieve this selective advantage it appears likely that the location of the possible recombination positions as well as the nature of the cellular reaction partner is restricted. That is probably the reason why all three identified insertions are located in the region coding for p125 within a stretch of less than 200 nucleotides, and two of them are derived from ubiquitin-coding sequences.

Pestiviruses are considered to replicate in the cytoplasm of the host cell, and reverse transcription has not been reported for these viruses. The integration of additional sequences described here for BVDV therefore should have occurred at the RNA level. RNA recombination has been reported for different viruses (for review see King *et al.*, 1987). In addition to homologous reactions between two molecules of viral RNA (Lai *et al.*, 1985; Saunders *et al.*, 1985; Kirkegaard and

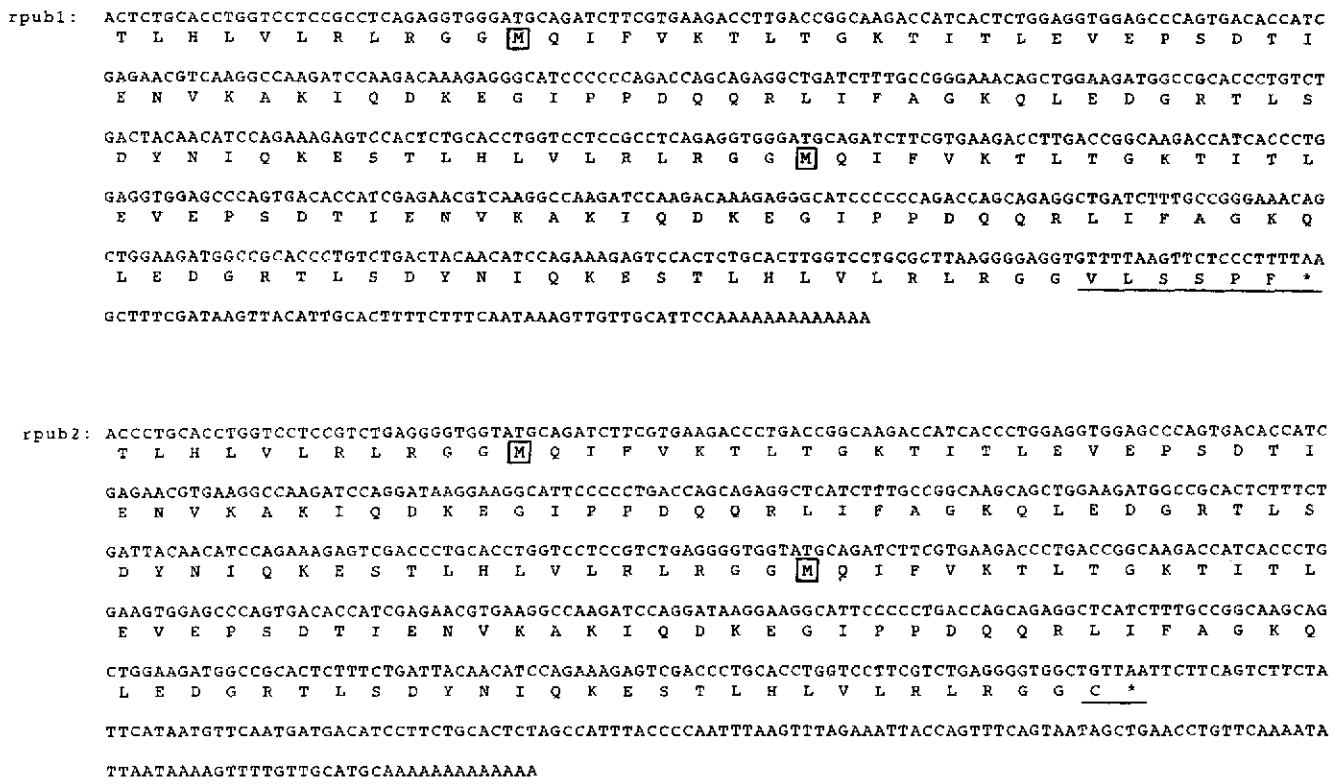


FIG. 9. Partial nucleotide and deduced amino acid sequence of two cDNA clones derived from bovine polyubiquitin mRNAs. The first methionine of each ubiquitin monomer is marked by a box. The carboxyterminal extensions of the primary proteins are underlined.

Baltimore, 1986; Makino *et al.,* 1986) much less frequent heterologous recombinations between viral RNAs and other partners, i.e., tRNA (Monroe and Schlesinger, 1983) or ribosomal RNA (Khatchikian *et al.,* 1989), have been reported. A so-called "copy choice" mechanism has been proposed as the enzymatic basis for both types of reactions (Lazzarini *et al.,* 1981; Kirkegaard and Baltimore, 1986; King *et al.,* 1987; Khatchikian *et al.,* 1989). Accordingly recombination results from a template switch of the viral RNA polymerase during genome replication. Base pairing between the nascent RNA strand and the recombination partner has been proposed to participate in directing recombination reactions (Lazzarini *et al.,* 1981; Lai *et al.,* 1985; Makino *et al.,* 1986; King *et al.,* 1987).

While most RNA recombinations were explained by a single template switch, the integration of host cellular sequences into the genomes of BVDV NADL, Osloss, and CP1 by a copy choice mechanism would represent heterologous reactions requiring two template switches between viral and cellular RNA. As the cellular reaction partner is of positive polarity such a process should occur during viral negative-strand synthesis to result in the observed integration of cellular sequences in coding orientation. Accordingly, the first template switch would determine the 3′ border of the insertion while the second one would be responsible for its 5′-end formation. If the second polymerase jump occurred to a position downstream of the first "crossing-over" point a duplication as observed for CP1 would result (Fig. 10). Switching back to the original position could generate a genome as found for Osloss and NADL. For a "backward switch" as postulated for CP1 less stringent conditions can be imagined as long as the increase in genome size resulting from the duplication does not interfere with virion formation. The conserved location of the 3′ ends of the ubiquitin-coding elements of BVDV Osloss and CP1 as well as the integration of cellular sequences into the genomes of Osloss and NADL without any duplication or deletion argues, however, in favor of specific and directed processes. Base pairing between the nascent viral negative strand and the sequences downstream of a ubiquitin gene monomer in one of the two analyzed bovine polyubiquitin mRNAs (rpub1 or rpub2) should not occur. As already mentioned above the rpub1 mRNA could hybridize to positive-stranded BVDV genomic RNA directly upstream of the first recombination point. A template switch during negative-strand synthesis just at this region of base pairing would lead to formation of the conserved 3′ recombination product (Fig. 11). After copying part of the ubiquitin-coding sequence the polymerase would then execute a second template switch back to the viral RNA (Fig. 10). How-

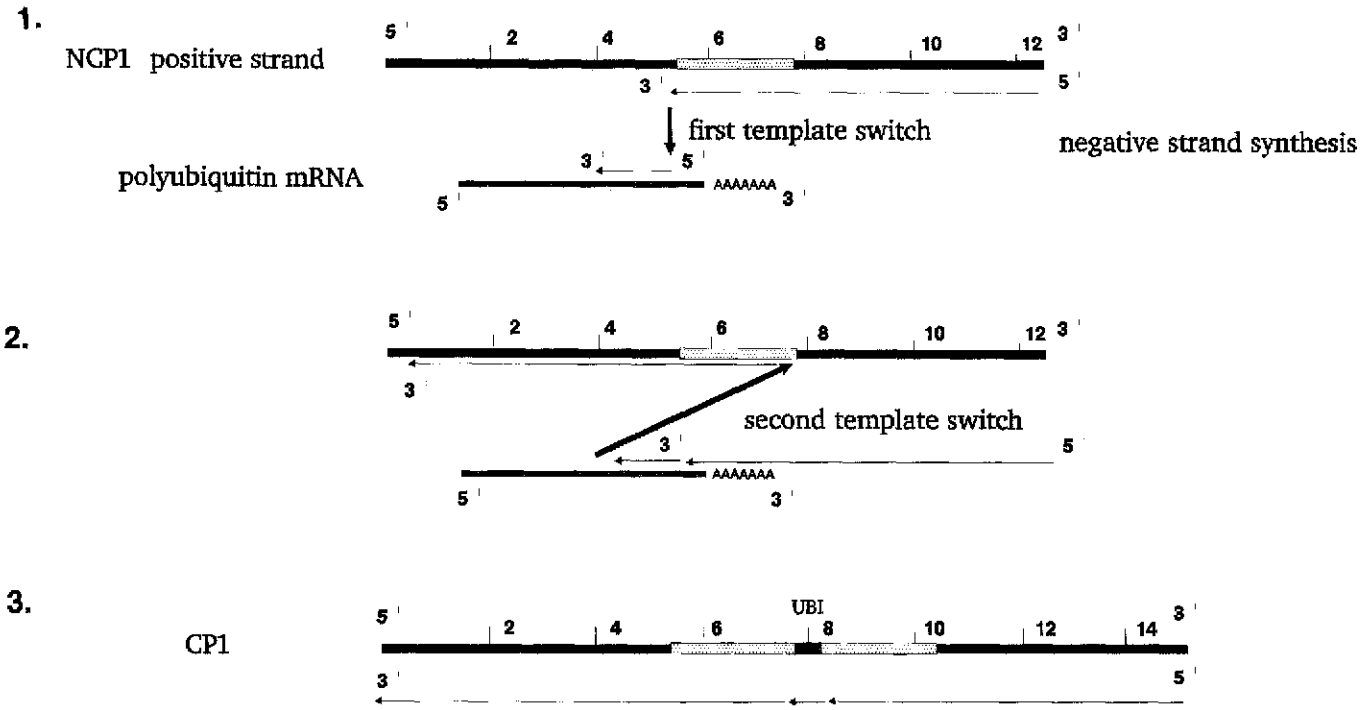


FIG. 10. Model for generation of the genome of BVDV CP1 by a recombination process between genomic NCP1 RNA and polyubiquitin mRNA. RNA of positive polarity is indicated by bars while negative strands are shown as thin lines. Dark gray bars represent ubiquitin-coding sequences. The region of the NCP1 genome which is duplicated in the CP1 RNA is indicated by light gray bars. Numbers along the bars indicate the size of the viral RNA in kilobases.

ever, the codon usage of the ubiquitin sequences which would be integrated by this reaction involving rpub1 is quite different from that found in the respective viral insertions. For the Osloss ubiquitin 26 out of 228 and for the CP1 insertion 43 out of 366 nucleotides are different from the respective sequence in rpub1. In the case of CP1 this would mean that 43 mutations had to be acquired in a stretch of 366 host cell-derived nucleotides (about 12% difference) while in the same time only 0.3% of BVDV-specific nucleotides have been changed with respect to the NCP1 sequence (Table 1). The nucleotide sequences of the ubiquitin-coding elements in the genomes of BVDV Osloss and CP1 exhibit only six differences (Fig. 6A). It therefore seems likely that in both recombinations the same type of ubiquitin mRNA was involved. This low number of differences between the Osloss and CP1 ubiquitin insertions also indicates conservation of the host cell-derived sequences within the viral genomes. Because of this observation recombination between BVDV genomic RNA and rpub1 and subsequent mutation of the ubiquitin-coding sequences at a frequency necessary for the observed differences seems rather unlikely. Therefore a template switch guided by the proposed interaction between BVDV genomic RNA and rpub1 may not be responsible for the observed integration of ubiquitin coding sequences.

While the two insertions containing ubiquitin-coding elements (BVDV strains CP1 and Osloss) are located at exactly the same genomic position, the BVDV NADL insertion is found about 150 nucleotides upstream of the other two and its flanking viral sequences are completely different from those of the Osloss and CP1 insertions (Meyers *et al.*, 1990). In addition no conservation of distinct nucleotide sequence elements like inverted or direct repeats are detectable in the sequences adjacent to the inserted elements nor do computer calculations reveal striking secondary structures in the respective regions of the RNA (data not shown). Thus no obvious recombination motif respon-

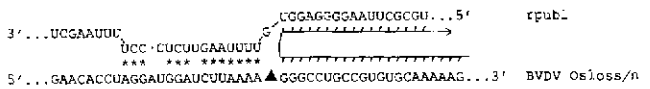


FIG. 11. Possible interaction between complementary nucleotides of a bovine polyubiquitin mRNA (rpub1) and the genomic RNA of BVDV Osloss. The ubiquitin coding insertion has been deleted (position marked by a triangle) to demonstrate the genome of the putative noncytopathogenic ancestor of this virus (denoted BVDV Osloss/n in this drawing). Nucleotide interactions are indicated by stars while missing nucleotides are represented by dots. The bent arrow represents the negative strand created by a hypothetical template switch of the viral RNA polymerase leading to recombination. The nucleotides of both RNA molecules found in the primary recombined genome are marked by small strokes on the bent arrow.

sible for the uptake of the additional sequences into the genomes of BVDV strains CP1, Osloss, and NADL could be identified. Nevertheless, a mechanism based on template switches of the viral RNA polymerase during negative-strand synthesis appears to be the most probable explanation for the observed recombinations.

To our knowledge bovine viral diarrhea virus is the first classical positive-stranded RNA virus for which integration of host cellular protein coding sequences has been demonstrated. Acquisition of new properties from the host cell by recombination has long been proposed as an important force in the evolution of RNA viruses (Steinhauer and Holland, 1987; Strauss and Strauss, 1988). In contrast to the usually slow development achieved by point mutations recombination reactions as observed here for BVDV represent large-scale evolutionary jumps. In the case of BVDV this evolutionary force becomes obvious by the connection of the recombination process with both pathogenesis of mucosal disease and development of cytopathogenicity. Identification of the BVDV recombination was facilitated by the biological selection system "mucosal disease" but analogous processes may of course also happen with other RNA viruses. A recombination between influenza virus RNA and ribosomal RNA which also led to a virus with altered biological properties has been observed (Khatchikian *et al.*, 1989). BVDV pairs isolated from MD animals can serve as model systems for future studies on RNA recombination, virus–host cell interactions, and pathogenesis of virus-induced diseases.

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## REFERENCES

BAKER, J. C. (1987). Bovine viral diarrhea virus: A review. *J. Amer. Vet. Med. Assoc.* **190,** 1449–1458.

BEAUCAGE, S. L., and CARUTHERS, M. H. (1981). Deoxynucleoside phosphoramidites: A new class of key intermediates for deoxynucleotide synthesis. *Tetrahedron Lett.* **22,** 1859–1862.

BENTON, W., and DAVIS, R. (1977). Screening lambda GT recombinant clones by hybridization to single plaques in situ. *Science* **196,** 180–182.

BOLIN, S. R., MCCLURKIN, A. W., CUTLIP, R. C., and CORIA, M. F. (1985). Severe clinical disease induced in cattle persistently infected with noncytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus. *Amer. J. Vet. Res.* **46,** 573–576.

BROWNLIE, J., CLARKE, M. C., and HOWARD, C. J. (1984). Experimental production of fatal mucosal disease in cattle. *Vet. Rec.* **114,** 535–536.

BUTT, T. R., KHAN, M. I., MARSH, J., ECKER, D. J., and CROOKE, S. T. (1988). Ubiquitin-metallothionein fusion protein expression in yeast. *J. Biol. Chem.* **262,** 16,364–16,371.

COLLETT, M. S., LARSON, R., GOLD, C., STRINCK, D., ANDERSON, D. K., and PURCHIO, A. F. (1988a). Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology* **165,** 191–199.

COLLETT, M. S., LARSON, R., BELZER, S. K., and RETZEL, E. (1988b). Proteins encoded by bovine viral diarrhea virus: The genomic organization of a pestivirus. *Virology* **165,** 200–208.

COLLETT, M. S., ANDERSON, D. K., and RETZEL, E. (1988c). Comparisons of the pestivirus bovine viral diarrhoea virus with members of the Flaviviridae. *J. Gen. Virol.* **69,** 2637–2643.

COLLETT, M. S., MOENNIG, V., and HORZINEK, M. C. (1989). Recent advances in pestivirus research. *J. Gen. Virol.* **70,** 253–266.

CORAPI, W. V., DONIS, R. O., and DUBOVI, E. J. (1988). Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. *J. Virol.* **62,** 2823–2827.

DEVEREUX, J., HAEBERLI, P., and SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12(1),** 387–395.

DONIS, R. O., and DUBOVI, E. J. (1987a). Molecular specificity of the antibody responses of cattle naturally and experimentally infected with cytopathic and noncytopathic bovine diarrhea virus biotypes. *Amer. J. Vet. Res.* **48,** 1549–1554.

DONIS, R. O., and DUBOVI, E. J. (1987b). Characterization of bovine viral diarrhea-mucosal disease virus-specific proteins in bovine cells. *J. Gen. Virol.* **68,** 1597–1605.

EINSPANIER, R., SHARMA, H. S., and SCHEIT, K. H. (1987). An mRNA encoding poly-ubiquitin in porcine corpus luteum: Identification by cDNA cloning and sequencing. *DNA* **6,** 395–400.

FINLEY, D., ÖZKAYNAK, E., and VARSHAVSKY, A. (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell* **48,** 1035–1046.

FINLEY, D., BARTEL, B., and VARSHAVSKY, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature (London)* **338,** 394–401.

GORBALENYA, A. E., DONCHENKO, A. P., KOONIN, E. V., and BLINOV, V. M. (1989a). N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res.* **17,** 3889–3897.

GORBALENYA, A. E., KOONIN, E. V., DONCHENKO, A. P., and BLINOV, V. M. (1989b). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* **17,** 4713–4730.

GUTEKUNST, D. E., and MALMQUIST, W. A. (1963). Separation of a soluble antigen and infectious particles of bovine virus diarrhea virus and their relationship to hog cholera. *Canad. J. Comp. Med. Vet. Sci.* **27,** 121–123.

HARLOW, E., and LANE, D. (1988). *In* "Antibodies, A Laboratory Manual," p. 460. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

HENNIKOFF, S. (1987). Unidirectional digestion with exonuclease III in DNA sequence analysis. *In* "Methods in Enzymology" (R. Wu, Ed.), Vol. 155, pp. 156–165. Academic Press, San Diego.

KESSLER, S. W. (1981). Use of protein A-bearing staphylococci for the immunoprecipitation and isolation of antigens from cells. *In* "Methods in Enzymology" (J. J. Langone and H. Van Vunakis, Eds.), Vol. 73, pp. 442–459. Academic Press, New York.

KHATCHIKIAN, D., ORLICH, M., and ROTT, R. (1989). Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. *Nature (London)* **340,** 156–157.

KING, A. M. Q., ORTLEPP, S. A., NEWMAN, J. W. I., and MCCAHON, D. (1987). Genetic recombination in RNA viruses. *In* "The Molecular

Biology of the Positive Stranded RNA Viruses" (D. J. Rowlands, M. A. Mayo, and B. W. J. Mahy, Eds.), pp. 129–152. Academic Press, London.

KIRKEGAARD, K., and BALTIMORE, D. (1986). The mechanism of RNA recombination in poliovirus. *Cell* **47,** 433–443.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227,** 680–685.

LAI, M. M. C., BARIC, R. S., MAKINO, S., KECK, J. G., EGBERT, J., LEIBOWITZ, J. L., and STOHLMAN, S. A. (1985). Recombination between nonsegmented RNA genomes of murine coronaviruses. *J. Virol.* **56,** 449–456.

LAZZARINI, R. A., KEENE, J. D., and SCHUBERT, M. (1981). The origins of defective interfering particles of the negative-strand RNA viruses. *Cell* **26,** 145–154.

MAKINO, S., KECK, J. G., STOHLMAN, S. A., and LAI, M. M. C. (1986). High-frequency RNA recombination of murine coronaviruses. *J. Virol.* **57,** 729–737.

MANIATIS, T., FRITSCH, E. F., and SAMBROOKS, S. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

MEYERS, G., RÜMENAPF, T., and THIEL, H.-J. (1989a). Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology* **171,** 555–567.

MEYERS, G., RÜMENAPF, T., and THIEL, H.-J. (1989b). Ubiquitin in a togavirus. *Nature (London)* **341,** 491.

MEYERS, G., RÜMENAPF, T., and HEINZ-JÜRGEN, T. (1990). Insertion of ubiquitin-coding sequence identified in the RNA genome of a togavirus. *In* "New Aspects of Positive Strand RNA Viruses", (M. A. Brinton and F. X. Heinz, Eds.), pp. 25–29. American Society for Microbiology, Washington DC.

MONROE, S. S., and SCHLESINGER, S. (1983). RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5′ ends. *Proc. Natl. Acad. Sci. USA* **80,** 3279–3283.

ÖZKAYNAK, E., FINLEY, D., SOLOMON, M. S., and VARSHAVSKY, A. (1987). The yeast ubiquitin genes: A family of natural gene fusions. *EMBO J.* **6,** 1429–1439.

POCOCK, D. H., HOWARD, C. J., CLARKE, M. C., and BROWNLIE, J. (1987). Variation in the intracellular polypeptide profiles from different isolates of bovine viral diarrhea virus. *Arch. Virol.* **94,** 43–53.

PURCHIO, A. F., LARSON, R., and COLLETT, M. S. (1984). Characterization of bovine viral diarrhea viral proteins. *J. Virol.* **50,** 666–669.

RECHSTEINER, M. (1987). Ubiquitin-mediated pathways for intracellular proteolysis. *Annu. Rev. Cell Biol.* **3,** 1–30.

REDMANN, K. L., and RECHSTEINER, M. (1989). Identification of the long ubiquitin extension as ribosomal protein S27a. *Nature (London)* **338,** 438–440.

RENARD, A., GUIOT, C., SCHMETZ, D., DAGENAIS, L., PASTORET, P.-P. DINA, D., and MARTIAL, J. A. (1985). Molecular cloning of bovine viral diarrhea viral sequences. *DNA* **4,** 429–438.

RENARD, A., DINO, D., and MARTIAL, J. (1987). Vaccines and diagnostics derived from bovine diarrhea virus. European Patent Application No. 86870095.6. Publication No. 0208672, 14 January 1987.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C., and BERG, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113,** 237–251.

RÜMENAPF, T., MEYERS, G., STARK, R., and THIEL, H.-J. (1989). Hog cholera virus—Characterization of specific antiserum and identification of cDNA clones. *Virology* **171,** 18–27.

SANGER, F., NICKLEN, S., and COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

SAUNDERS, K., KING, A. M. Q., MCCAHON, D., NEWMAN, J. W. I., SLADE, W. R., and FORSS, S. (1985). Recombination and oligonucleotide analysis of guanidine-resistant foot and-mouth disease virus mutants. *J. Virol.* **56,** 921–929.

STEINHAUER, D. A., and HOLLAND, J. J. (1987). Rapid evolution of RNA viruses. *Annu. Rev. Microbiol.* **41,** 409–433.

STRAUSS, J. H., and STRAUSS, E. G. (1988). Evolution of RNA viruses. *Annu. Rev. Microbiol.* **42,** 657–683.

SUZICH, J. A., and COLLETT, M. S. (1988). Rift valley fever virus M segment: Cell-free transcription and translation of virus-complementary RNA. *Virology* **164,** 478–486.

TSIANG, M., MONROE, S. S., and SCHLESINGER, S. (1985). Studies of defective interfering RNAs of Sindbis virus with and without $tRNA^{Asp}$ sequences at their 5′ termini. *J. Virol.* **54,** 38–44.

WESTAWAY, E. G., BRINTON, M. A., GAIDAMOVICH, S. Y. A., HORZINEK, M. C., IGARASHI, A., KÄÄRIÄINEN, L., LVOV, D. K., PORTERFIELD, J. S., RUSSEL, P. K., and TRENT, D. W. (1985). Togaviridae. *Intervirology* **24,** 125–139.