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Rearrangement of Viral Sequences in Cytopathogenic Pestiviruses

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Two cytopathogenic isolates of bovine viral diarrhea virus (cpBVDV) have been analyzed. For both viruses two regions of their genomic RNAs were found to be duplicated and rearranged. The viral genomes contain a small duplicated element (SD) derived from the genomic 5’ end far downstream of its original context. This sequence is followed by a larger duplication which encompasses the region coding for the protein p80(LD), a molecular marker for cpBVDV. The SD element codes for the viral protease p20. In the case of the viruses analyzed here the aminoterminus of p80 is generated by autoproteolytic removal of the preceding SD-encoded protease. For one of the cpBVDV isolates a specific fusion protein (~28) could be identified which is composed of p20 and part of p10, another viral protein. Molecular characterization of the respective noncytopathogenic counterpart revealed that duplication and rearrangement of sequences as well as the expression of p28 and p80 are specific for the cytopathogenic virus. © 1992 Academic Press, Inc.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) represents a small enveloped RNA virus that belongs to the genus pestivirus within the family Flaviviridae (Wengler, 1991). Other members of the genus are hog cholera virus (HCV) and border disease virus (BDV) of sheep. The single-stranded RNA genome of pestiviruses has a size of about 12.5 kb and possesses positive polarity (Renard et al., 1987; Collett et al., 1988a; Meyers et al., 1989a). Viral gene expression occurs via synthesis of a polyprotein and subsequent proteolytic processing (Collett et al., 1988b,c; Meyers et al., 1989a).

BVDV is the causative agent of a variety of syndromes in cattle among which the most severe is mucosal disease (MD) with almost 100% lethality (for review: Baker, 1987). Interestingly, a pair of viruses belonging to two different biotypes of BVDV can always be isolated from animals which come down with MD. These two biotypes are distinguished by their ability to replicate in tissue culture cells with or without cytopathic effect (cpBVDV or noncpBVDV, respectively). Elaborate studies revealed that a prerequisite for development of MD is an intrauterine infection with noncpBVDV (Brownlie et al., 1984; Bolin et al., 1985). In a second step generation of cpBVDV occurs, which was assumed to be due to a mutation in the viral genome (Pocock et al., 1987; Corapi et al., 1988).

At the molecular level the only difference between cpBVDV and noncpBVDV known until recently was the presence of an 80-kDa protein (p80) in cells infected with cytopathogenic viruses. This protein is colinear with the carboxyterminal part of a polypeptide of 125 kDa (p125) detectable in cells infected with either cpBVDV or noncpBVDV (Purchio et al., 1984; Pocock et al., 1987; Corapi et al., 1988; Collett et al., 1988b). Surprisingly, for two cpBVDV strains host cell-derived insertions were detected within the genomic region coding for p125 (Meyers et al., 1989b, 1990). One of the inserted sequences codes for a complete ubiquitin-like protein while the function of the cellular homologue of the other insertion is still unknown (Meyers et al., 1989b, 1990). We proposed that recombination between cellular and viral RNA led to the formation of these cpBVDV genomes and that the insertion of cellular elements is linked to generation of p80 within the infected cells (Meyers et al., 1990).

To investigate directly the difference between a cytopathogenic virus and its noncytopathogenic counterpart we analyzed the genome of a pair of cpBVDV (CP1) and noncpBVDV (NCP1) isolated from one MD animal. For CP1 a ubiquitin-coding element which is embedded in a large duplication of viral sequences encompassing the p80-coding region was identified. In contrast, the genome of NCP1 does not contain either insertion or duplication (Meyers et al., 1991). According to the results of these studies, one possible mutation leading to cytopathogenic BVDV is a recombin-
tion process between cellular and viral RNA. In this report molecular characterization of two additional pairs of cpBVDV and noncpBVDV is reported which adds a new aspect to our understanding of generation of cytopathogenic BVDV.

MATERIALS AND METHODS

Materials

Restriction enzymes and modifying enzymes were from Pharmacia-LKB (Freiburg, FRG), New England Biolabs (Schwalbach, FRG), Boehringer-Mannheim (Mannheim, FRG), and Life Sciences, Inc. (USA). AmpliTaq DNA polymerase was obtained from Cetus-Perkin–Elmer (Langen, FRG). Radioactive compounds were purchased from Amersham–Buchler (Braunschweig, FRG). 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., 1987) was kindly provided by Dr. Liess (Veterinary School, Hannover, FRG). Isolation and serological characterization of BVDV strains NCPl, CP1, NCP6, and CP6 have been described by Corapi et al. (1988); for strains Pe515CP and Pe515NCP, see Brownlie et al. (1984). Cells were grown in DMEM supplemented with 10% fetal calf serum and were infected with BVDV as layer cells at 1.5 × 10⁶ per 3.5-cm dish. Cells and virus stocks were tested every 6 to 12 months for the absence of mycoplasma contamination.

RNA preparation, gel electrophoresis and Northern hybridization

Preparation of RNA was done as described (Rumenapf et al., 1989). Five microgramsms of glyoxylated RNA (Maniatis et al., 1989) was separated in phosphate-buffered 1% agarose gels containing 5.5% formaldehyde. An RNA ladder (Bethesda Research Laboratories, FRG) served as a size standard. For Northern hybridization the RNA was transferred to Duralon-UV membranes (Stratagene, Heidelberg, FRG). Membranes were hybridized to the NCPl cDNA probe labeled with ³²P by nick translation (Rigby et al., 1977) (nick translation kit, Amersham–Buchler, Braunschweig, FRG) in 0.5 M sodium phosphate, pH 6.8, 1 mM EDTA, and 7% SDS at 54°C. Posthybridization washes were carried out with 40 mM sodium phosphate, pH 6.8, 1 mM EDTA and 5% SDS, and 40 mM sodium phosphate, pH 6.0, 1 mM EDTA, and 1% SDS two times each for 30 min at hybridization temperature.

Oligonucleotides

Oligonucleotides were synthesized on a Biosearch 8700 DNA synthesizer (New Brunswick Scientific, FRG) using the phosphoamidite method (Beaucage and Caruthers, 1981). Oligonucleotides BVDV 13, BVDV 14, and PES 9 are described elsewhere (Meyers et al., 1991). OIBVD32: AAATCTCTGCTGTACATCGCACATG OIBVD33: GCATCCATCATNCCRTGRAT

cDNA synthesis, cloning, and library screening

Synthesis of double-stranded cDNA, cloning in λ ZAPII (Stratagene, Heidelberg, FRG), and establishment and screening of the phage library using the NCPl cDNA probe was done as described before (Moyoro et al., 1991). Subcloning of cDNA fragments into pBluescript plasmids by in vivo excision was performed as recommended by the supplier (Stratagene, Heidelberg, FRG).

Polymerase chain reaction

First strand cDNA was synthesized as described (Rumenapf et al., 1989) using oligonucleotide OIBVDV33 as primer. For RNA hydrolysis 1 volume of 0.3 M NaOH and ½ volumes of 0.03 M EDTA were added and the mixture incubated at 95°C for 5 minutes. After neutralization with ½ volume of 1 M Tris/HCl (pH 8.0) the volume was adjusted to 100 μl and the solution was passed through a Sephadex G50 spin column (Maniatis et al., 1989). 1% of the single stranded cDNA served as template for the polymerase chain reaction (PCR). Buffer conditions for PCR were as described by Jeffreys et al. (1988) except that the 50 μl reaction mixture contained 10% dimethylsulfoxide and 50 pMol of primers OIBVDV32 and OIBVDV33. Samples were overlaid with 75 μl of paraffin and cycled 30 times for 100 s at 94°C, 100 s at 50°C and 200 s at 72°C in a thermocycler TPS (Landgraf, Hannover, FRG). After purification by chloroform extraction and preparative agarose gel electrophoresis the PCR product was cloned blunt end into the Smal site of pBluescript SK-(Stratagene, Heidelberg, FRG) according to standard procedures (Maniatis et al., 1989).

Nucleotide sequencing

Exonuclease III and S1 were used to establish deletion libraries of cDNA clones (Henikoff, 1987). Di-deoxy sequencing (Sanger et al., 1977) of double-stranded DNA templates was carried out using the T7
polymerase sequencing kit (Pharmacia-LKB). All sequences shown in the figures have been determined from two complementary strands. Computer analysis of sequence data was performed on a Digital Microvax II using the UWGCG software (Devereux et al., 1984).

Preparation of antisera against bacterial fusion proteins or synthetic peptides

For preparation of the anti D1, anti-K1, anti-L1, and antiP sera HCV-derived cDNA fragments coding for amino acids 102–292 (anti-D1), 2555–2766 (anti-K1), 2766–2985 (anti-L1), and 2290–2555 (anti-P1) of the HCV polyprotein (Meyers et al., 1989a) were subcloned into the expression vector pEX34 using standard procedures (Maniatis et al., 1989). pEX34 is identical to the previously described plasmid pEX31 (Strebel et al., 1986), except for a deleted PstI site in the ampicillin resistance gene; expression and enrichment of bacterial fusion proteins were performed basically as described previously (Strebel et al., 1986). Fusion proteins were further purified by preparative 3D-S-PAGE and, after electroelution, injected subcutaneously into rabbits with Freund adjuvant (complete for basic immunization, incomplete for booster injections). Preparation of anti-G1 (Rumenapf et al., 1991) and anti-A3 (Thiel et al., 1991) has been reported before.

Peptide HCV-Pep14 was synthesized on a Milligen 9050 PepSynthesizer (Millipore) by using the Fmoc-polyamide method. For generation of antibodies the peptide was coupled to Key Hole Limpet hemocyanin using glutaraldehyde as crosslinker (Harlow and Lane, 1988). Immunization of rabbits was carried out as described above with an equivalent of 1 mg of peptide per injection.

The antiserum against the peptide corresponding to amino acids 1335–1351 of the BVDV NADL ORF has been described before (Collett et al., 1991).

Radioimmunoprecipitation and SDS–PAGE

BVDV-infected MDBK cells (1.5 × 10⁶ per 3.5-cm dish) were labeled for 6 hr with 0.5 mCi/ml [³⁵S]methionine/[³⁵S]cysteine. Labeling medium contained no cysteine and 1/25 of normal methionine content. Cell extracts were prepared under denaturing conditions (Harlow and Lane, 1988). Extracts were incubated with 5 μl of undiluted serum. Precipitates were formed with crosslinked Staphylococcus aureus (Kessler, 1981), analyzed by SDS–PAGE, and processed for fluorography using Enhance (New England Nuclear, Boston). Competition for anti-peptide antisera was done with 100 μg of peptide per RIP sample.

RESULTS

Hybridization studies

Characterization of the first BVDV pair isolated from one animal with MD revealed a striking difference between the genome sizes of the cytopathogenic virus CP1 and the noncytopathogenic virus NCP1 which was clearly detectable on Northern blots (Meyers et al., 1991). The analysis of the viruses belonging to pairs Pe515 (Pe515CP and Pe515NCP) and No. 6 (CP6 and NCP6) was also started with Northern hybridization experiments (Fig. 1). The cDNA insert from clone pNCII.1 (Meyers et al., 1991) was used as a probe which was hybridized against total RNA from bovine kidney (MDBK) cells infected with different virus strains. The migration rate determined for the genome of the cytopathogenic member of each virus pair was considerably slower than that of the noncytopathogenic counterpart (Fig. 1). Among the cpBVDV strains analyzed here CP6 apparently possesses the largest genome while the RNA from Pe515CP has about the same size as that of the CP1 genome (Fig. 1).
According to additional hybridization experiments with a ubiquitin probe the viruses from pairs No. 6 and Pe515 did not contain ubiquitin-coding sequences in their genomes while those from BVDV Osloss and CP1 were clearly recognized (data not shown). Therefore the putative recombination leading to the genomes of BVDV CP6 and Pe515CP did not imply integration of ubiquitin-coding sequences.

**Analysis of BVDV strains Pe515CP and Pe515NCP**

For elucidation of the genome structure of BVDV Pe515CP and Pe515NCP cDNA cloning and sequencing was employed. Since all insertions identified so far in BVDV genomic RNAs were located around nucleotide 5400, construction of the cDNA library was performed with primers specific for the regions at 6 and 7 kb (Meyers et al., 1991). The library screening was done with the same probe as used for the blot in Fig. 1. In order to investigate the genomic localization of the respective cDNA fragment, BVDV-specific cDNA clones were first analyzed by determination of the terminal sequences (Fig. 2). With respect to the published BVDV genomic sequences the 5' ends of clones pCB32 and pCB38 corresponded to positions around 7662 and 5529, while the 3' ends were homologous to sequences upstream of nucleotides 9814 and 6166 (pCB32 and pCB38, respectively). Accordingly, the expected sizes of the cDNA inserts in clones pCB32 and pCB38 are 2.15 and 0.64 kb, respectively. This finding already indicated the presence of sequence rearrangements in the genome of Pe515CP since the observed length of the cloned cDNA fragments was about 4.9 kb (pCB32) and 3.4 kb (pCB38) (Fig. 2). To determine the genome organization of Pe515CP additional nucleotide sequencing of both pCB32 and pCB38 was employed (indicated by arrows in Fig. 2). The 5' end of the sequence shown in Fig. 3 corresponds to position 7089 of the published BVDV NADL genomic sequence (Collett et al., 1988a). Up to nucleotide 636 the determined Pe515CP sequence is colinear with the published sequences. Then 462 residues follow which do not show any homology to the respective BVDV genomic region. The third part of the sequence corresponds to the region following position 5423 of the BVDV genome (strain NADL) (Fig. 3A). In general this genome organization is reminiscent of the one described for BVDV CP1 (Meyers et al., 1991) with an insertion flanked by a duplicated viral sequence encompassing about 2.5 kb downstream of position 5423 (termed LD) (Fig. 5). For BVDV CP1 the inserted element of 366 residues was a host cell-derived sequence coding for ubiquitin. Surprisingly, the 462 nucleotides present in the genome of Pe515CP (termed SD) are not of cellular origin but are homologous to the sequence located at position 428–889 of the BVDV NADL genome (Figs. 3A and 5). Accordingly, the genome of BVDV Pe515CP represents the product of a recombination process which probably involved only viral sequences. The differences in genome sizes observed in the Northern blot experiments (Fig. 1) suggested that the duplication and rearrangement of sequences identified for Pe515CP are not present in the RNA of Pe515NCP. To investigate this directly a cDNA library of Pe515NCP was established using a primer complementary to a sequence located at about 9 kb in a standard BVDV genome (Pes9). Priming in this region should provide cDNA clones encompassing the two recombination positions identified for Pe515CP (for nucleotide positions see Fig. 3A). After screening the library with the pNCPII.1 probe clone pNCB3 was isolated which had a size of about 3.9 kb (Fig. 2). Determination of the terminal sequences allowed the positioning of the cDNA insert between nucleotides 4886 and 9048 with respect to the BVDV NADL genome. Fig. 3B shows the complete nucleotide sequence of clone pNCB3. Insertions or discontinuances
FIG. 3. Nucleotide and deduced amino acid sequences determined from the BUVV Pe515CP cDNA clones pUC32 and pUC38 (A), the Pe515NCP cDNA clone pNCB3 (B), and the Pe515CP PCR clone pCBP6 (C). The residues flanking the Pe515 5' recombination position are marked by the letters c' and D' and those flanking the 3' recombination position by E and B. In (C) also the location of the CP6 recombination positions is indicated (D: 5' recombination position; E: 3' recombination position). To show the homology of the Pe515CP LD and SD regions to different parts of a standard BVDV genome the corresponding sequences from the BVDV NADL genome (numbers as published by Collett et al., 1988a) have been integrated in (A).
RECOMBINATION OF PESTIVIRAL RNAs

EB

CTATGGGTCATGAGCTG; G;;GCCTGCCGT&ACAAGAAGATCACAG=ChTGAAAGATGi

LWVMSC GPAVHKKITERERC

CTATGGGTCACAAGTTGC TCAGACACGAAAGAAGAGGGAGCAACA

(BVDV-NADL)

CTTGGGTGGATCCTAAGG GGGCCTGCCGTGTGTAAGAAGATCACAGAGCAC

(BVDV-NADL)

CATGTCAACATACTAGACAAGTTAACTGCATTTTTCGGGA

HVNILDKLTXPAVHKK'TEH

CCCAGAGCCCCAGTGAGGTTCCCCACGAGTTTGTTAAAAG

PRAPVRFPTS LLKVRRGLET

GGTTGGGCTTATACGCAACCAAGTGGAATGTTGCTGCACTAACAACCCGGCAGA

380

400

420

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460

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3660

3680

3700

3720

FIG. 3—Continued
FIG. 3—Continued
RECOMBINATION OF PESTIVIRAL RNAs

FIG. 3—Continued
FIG. 3—Continued
TABLE 1

HOMOLOGY BETWEEN THE REGIONS FLANKING THE SD ELEMENT IN THE BVDV Pe515CP NUCLEOTIDE SEQUENCE (NUCLEOTIDES 1 TO 636 AND 1099 TO 1740 IN FIG. 3A) AND THE CORRESPONDING SEQUENCES FROM OTHER BVDV GENOMES

| Pe515NCP | Osloss | NADL | NCP1 | CP1 | CP6 |
|----------|--------|------|------|-----|-----|
| Pe515CP  | 99%    | 02%  | 09%  | 92% | 01% |

The genome of BVDV CP6

The Northern blot analysis indicated that the CP6 genomic RNA is larger than that of any pestivirus analyzed so far (Fig. 1). On the account of the molecular characterization of BVDV CP1 and Pe515CP it was expected that a duplication of viral sequences was at least in part responsible for this increase in genome size. Following the strategy outlined above cDNA cloning was performed. The terminal sequence of the insert from plasmid pCP6/10 corresponded to positions 438 and 10861 of the BVDV NADL sequence and those from plasmid pCP6/N to 6877 and 8026 (Fig. 2). Since the inserts of the two cDNA clones had sizes of 5.8 and 5.3 kb (pCP6/10 and pCP6/N, respectively) this finding strongly indicated rearranged viral sequences. For further investigation, part of pCP6/10 and pCP6/N was sequenced (Figs. 2 and 4). The first 1188 determined nucleotides are colinear with the BVDV NADL sequence from 7869 to 9057. The following 507 residues again represent a sequence derived from the 5' part of a standard pestivirus genomic. The 5' end of this element corresponds to position 383 of the NADL genome, thereby starting 3 nucleotides upstream of the viral translational initiation codon. Interestingly, the 3' end of this inserted element is exactly the same as that of the Pe515CP SD sequence (corresponds to nucleotide 089 in the NADL genome) (Figs. 3A, 4, and 5). For both viruses the region downstream of this 5'-derived element is homologous to the sequence starting with nucleotide 5423 of the NADL genome.

For Pe515CP the large duplicated element encompasses 2304 nucleotides thus being 81 residues shorter than that of BVDV CP1. The respective element in the CP6 genome, however, has a size of 3635 nucleotides since it corresponds to nucleotides 5423 to 9057 of the BVDV NADL genome. Because of this large duplication together with the SD element of 507 nucleotides the CP6 genome is 4142 nucleotides longer than that of a noncytopathogenic BVDV strain.

Recombination-induced changes at the protein level

As a marker characteristic for infection with cpBVDV a protein of 80 kDa (p80) has been described (Purchio...
FIG. 4. Nucleotide and deduced amino acid sequence of part of the BVDV CP6 genome. The 5' and 3' recombination positions are marked by the letters C'/D and E/B, respectively. Corresponding parts of the BVDV NADL nucleotide sequence are shown in the lines below the CP6 amino acid sequence.
FIG. 4—Continued
Pestivirus genome

Fig. 5. Schematic representation of the genome organization of the analyzed BVDV isolates. The upper bar shows a scheme of a complete pestivirus genome with indication of the various sites involved in the recombination reactions identified for the cpBVDV genomes shown below (letters below the bar). The numbers give the positions of these sites with respect to the published BVDV NADL genome. p20-coding sequences are indicated by chess board bars, while streaked bars represent the region coding for the structural proteins. The LD regions duplicated in the genomes of Pe515CP and CP6 are shown as dotted bars. (A) Last residue preceding the position of the ubiquitin insertion in the BVDV Osloss genome. (B) First nucleotide of all LD regions and also first residue following the ubiquitin insertion in the BVDV Osloss genome. (C) Last residue of the Pe515CP LD region. (C') Last residue of the CP6 LD region. (D) First residue of the CP6 SD region. (D') First residue of the Pe515CP SD region. (E) Last residue of both the CP6 and Pe515CP SD regions.

Pair Pe515

Pair #6

NCP

CP

As a consequence of recombination reactions the genomes of BVDV CP1, Pe515CP, and CP6 contain duplicated and rearranged sequences. In all these cases the genomic region coding for p125 is affected. However, the changes identified at the genome level seem not to result in expression of aberrant p125, since the respective protein from the cpBVD viruses perfectly comigrates with that from the noncp viruses (Fig. 6A and Meyers et al., 1991). Accordingly, the 5' recombination point should be located downstream of the carboxyterminal processing site of p125 thereby allowing expression of this protein from the sequences upstream of the SD element. In this case aberrant protein(s) could be translated from the region downstream of the p125 gene. To investigate this hypothesis for BVDV Pe515CP RIP with different antisera, specific for the respective sequences, was performed. In addition, antibodies were included which are directed against the protein encoded by the SD-specific genomic region.

The SD region in the Pe515CP genome corresponds to codons 15–168 of the viral ORF. Therefore antisera directed against the first 15 amino acids of the pestiviral polyprotein (antiG1) and against residues 102–292 (antiD1) were employed (Thiel et al., 1991). AntiG1 was shown to precipitate a protein of about 20 kDa from BVDV NADL-infected cells (p20), which represents the first protein of the BVDV ORF (Thiel et al., 1991). For antiD1 precipitation of two proteins, namely p20 and the pestiviral core protein p41, has been reported (Thiel et al., 1991). In addition to these proteins antiG1 and anti D1 precipitated a protein of 28 kDa...
Fig. 6. Immunoprecipitation of proteins extracted from BVDV-infected cells after metabolic labeling with a mixture of [35S]cysteine/ [35S]methionine. Immunoprecipitates were analyzed by SDS-PAGE (10% for A, 7.5 to 20% for B, and 12% for C). For designation of antisera see Fig. 8. (A) Extracts from MDBK cells infected with BVDV Pe515NCP (lanes 1, 2), Pe515CP (lanes 3, 4), NCP6 (lanes 5, 6), and CP6 (lanes 7, 8) were incubated with a serum directed against a peptide encompassing amino acids 1335 to 1351 of the polyprotein of BVDV NADL (Collett et al., 1991) (lanes 2, 4, 6, 8) or with the antiA3 serum (Thiel et al., 1991) (lanes 1, 3, 5, 7). (B) Extracts from MDBK cells infected with BVDV Pe515CP were incubated with the following rabbit antisera: anti-G1 (lane 1), anti-D1 (lane 2), rabbit preimmune serum (lane 3), anti-L1 (lane 4), anti-K1 (lane 5), and anti-P1 (lane 6). The bands corresponding to BVDV proteins ~10, ~14, ~20, and p30 are indicated. The fusion protein p28 is marked by an open triangle. (C) To show the specificity of p28 for the cp virus of pair Pe515 extracts from cells infected with Pe515CP (lanes 1, 2, 3, 4) or Pe515NCP (lanes 5, 6, 7, 8) were incubated with anti-HCVpep14 (lanes 1, 2, 7, 8), anti-G1 (lanes 3 and 5), and anti-D1 (lanes 4 and 6). For lanes 2 and 8 100 μg of peptide HCVpep14 were added as competitor to the immunoprecipitation reaction. The position of proteins p10, p14, and p20 is indicated. p28 is marked by a triangle.

(p28) from extracts of BVDV Pe515CP-infected cells (Fig. 6B, lane 1 and 2, respectively). For further investigation three antisera specific for the region following p125 in the pestiviral polyprotein were used; they were prepared against bacterial fusion proteins containing amino acids 2290 to 2555 (antiP1) or 2555 to 2766 (antiK1) or 2766 to 2985 (antiL1) of the HCV ORF. For BVDV NADL it has been reported that downstream of the p125 gene a protein of 10 kDa (p10) is encoded while for the following nearly 1 kb no translation prod-
uct except for a short-lived precursor of 42 kDa was found (Collett et al., 1991). A stable polypeptide of 30 kDa (p30) could, however, be demonstrated for HCV and different BVDV strains with antiP1 and antiK1 (Stark and Thiel, unpublished). By using these antisera, p30 was also precipitated from extracts of Pe515CP-infected cells (Fig. 6B, lanes 5 and 6). The antiP1 serum also recognized p10 since the bacterial fusion protein employed for generation of this antiserum overlaps the respective region of the polyprotein (Fig. 6B, lane 6). Importantly, the antiP1 serum precipitated an additional antigen which comigrated with the 28-kDa protein described above and presumably represents the same polypeptide (Fig. 6B, lane 6). Since p28 reacted with antiG1 and antiD1 as well as with antiP1 but not with antiL1 or antiK1 (Fig. 6B, lanes 1, 2, 6, 4, 5, respectively) this polypeptide most likely represents a fusion protein containing sequences from p20 as well as p10.

According to the hypothesis presented above p28 should be specific for Pe515CP. To investigate this more closely, RIP with extracts from cells infected with either the cytopathogenic virus (Fig. 6C, lanes 1–4) or the noncytopathogenic virus (Fig. 6C, lanes 5–8) was performed. In addition to antiG1 (lanes 3 and 5) and antiD1 (lanes 4 and 6) a serum specific for p10 (antiHCVPep14, prepared against a peptide corresponding to amino acids 2309–2325 of the HCV ORF) was employed. While p20, p14, and p10 can easily be demonstrated for both viruses p28 is only precipitated from extracts of Pe515CP-infected cells. In conclusion, the rearranged genome of Pe515CP leads to expression of at least two proteins which are not found in cells infected with Pe515NCP, namely p80 and p28.

The carboxyterminal end of the Pe515CP fusion protein is generated by autoproteolytic cleavage

The SD sequences integrated between the LD elements in the genomes of BVDV Pe515CP and CP6 are derived from a region of the viral RNA which has been shown to code for p20, the first protein of the BVDV ORF (Collett et al., 1988b; Thiel et al., 1991). Because of the rapid liberation of this polypeptide during translation which can also be observed in vitro, it has been proposed, that p20 represents an autoprotease (Wiskerchen et al., 1991; Thiel et al., 1991). Recently experimental evidence has been obtained for this theory (Wiskerchen et al., 1991; Stark et al., unpublished). The sequence tryptophane-valine (position 164/165) has been proposed as the cleavage recognition site of this enzyme (Wiskerchen et al., 1991). Since the conserved 3’ end of the SD elements maps to codon 168 of the BVDV ORF the putative cleavage signal is also duplicated and transferred to the downstream position. Accordingly, for BVDV Pe515CP and CP6 the aminoterminal of p80 could be generated in an autoproteolytic cleavage reaction executed by the preceding p20 protease. For BVDV CP6 the SD sequence covers the complete p20-coding region. In the case of Pe515CP, however, the aminoterminal 14 amino acids of the protease molecule are missing in the downstream SD element. To analyze whether this aminoterminally truncated enzyme is active, in vitro studies were performed. Clone pCR32 was linearized with HindIII and used as template for in vitro transcription. The synthesized RNA contains an AUG suitable for translation initiation at position 94–96 (position 598–600 in Fig. 3A). In order to provide a control for the cleavage reaction, clone pCBP6 which contains the 1-kb PCR fragment encompassing the complete p20-coding region from the 5’ part of the Pe515CP genome was also used for in vitro transcription. Translation of the resulting RNA should start at the original initiation codon of the viral ORF (position 23–25 in Fig. 3C). For analysis of the in vitro translation products, RIP with different antisera was performed. Translation of the pCBP6-derived RNA gave the following results: A full-length product of 38 kDa could not be identified (Fig. 7). Instead, an aminoterminal cleavage product of about 20 kDa was precipitated with antiG1 and antiD1 (Fig. 7, lanes 1 and 2,
**DISCUSSION**

The generation of cytopathogenic BVDV within a calf persistently infected with noncytopathogenic virus results in development of mucosal disease and death of the animal (Brownlie et al., 1984; Bolin et al., 1985). The discovery of host cell-derived insertions in the genomes of two cpBVDV strains (Osloss and NADL) led us to propose a recombination as mechanism for development of cpBVDV (Meyers et al., 1989b, 1990). Clear evidence for this model was gained by analysis of a BVDV pair consisting of the viruses CP1 and NCP1. The genome of CP1 contains a ubiquitin-coding sequence which is flanked by a large duplication of viral sequences. Neither insertion nor duplication are present in the respective part of the NCP1 genome. However, the two viruses exhibit 99.6% of sequence identity within corresponding regions of their genomes (Meyers et al., 1991).

The data presented in this report broaden the proposed model. In the case of BVDV Pe515CP and CP6 no host cell-derived insertion could be identified. However, elaborate duplication and rearrangement of viral sequences were found for both genomes. The analysis of the noncp virus (Pe515NCP) revealed that neither duplication nor rearrangement of sequences are present, and thus supported the linkage between recombination and establishment of the cytopathogenic phenotype. Accordingly, generation of cpBVDV is not restricted to recombination between cellular and viral sequences but can also be achieved by mere rearrangement of viral sequences.

Until now recombination-induced changes at the genome level have been identified for 6 cpBVDV strains. Three of these viruses contain ubiquitin-coding insertions, namely BVDV Osloss, CP1, and CP14 (manuscript in preparation for the latter), while the NADL strain has integrated another cellular sequence. The other two, Pe515CP and CP6, have been generated in a recombination process involving only viral sequences. On the basis of PCR analyses it has been found that the genomes of several cpBVDV strains including Pe515CP do not contain insertions within the p125 gene (DeMoerlooze et al., 1990). For Pe515CP, this finding describes only one feature of the respective genomic region. The viral RNA contains a p125 gene without insertion but nevertheless it represents the product of a recombination which affected p125-coding sequences. Since part of the p125-coding region has been duplicated the respective rearrangement in the Pe515CP RNA could not be detected by a simple PCR assay.

The situation is different for some cpBVDV laboratory strains, like Singer, Oregon, and Danmark. None of these strains has a genome size indicating duplications (G. Meyers, unpublished). The PCR analysis revealed that the region around position 5400 of the BVDV Singer genome does not contain an insertion
The same result was obtained when the nucleotides adjacent to the recombination site are marked with the letters B and E according to the nomenclature listed in legend of Fig. 5. Sequences 5' to the recombination site are shown in capital letters, nucleotides 3' of this position in lower case.

![Fig. 9. Comparison of the sequences flanking the 3' recombination site (vertical line) of different cpBVDV isolates. The residues adjacent to the recombination site are marked with the letters B and E according to the nomenclature listed in legend of Fig. 5. Sequences 5' to the recombination site are shown in capital letters, nucleotides 3' of this position in lower case.](image)

(DeMoerlooze et al., 1990). After conventional cDNA cloning and sequencing of about 2 kb derived from the respective genomic region the same result was obtained for the strains Oregon and Danmark (G. Meyers, unpublished). More elaborate cloning and sequencing is necessary to identify the genetic basis for generation of p80 and the establishment of a cytopathogenic phenotype by these viruses.

Recombination at the RNA level has been observed for several RNA viruses. Template switching of the viral polymerase during replication or transcription has been proposed as a mechanism for these reactions (Lazzarini et al., 1981; Lai et al., 1985; Kirkegaard and Baltimore, 1986; Makino et al., 1986; King et al., 1987; and Khatchikian et al., 1989). Based on this hypothesis a model for the generation of the cpBVDV genomic RNAs has been put forward which includes two subsequent switches (Meyers et al., 1991). Since the cellular mRNAs which serve as templates for integration of, e.g., ubiquitin-coding sequences, are present in the "sense" orientation in the cell and in the viral genome, the recombination should occur during synthesis of negative strand viral RNA. Interestingly, the 3' recombination position was the same for the genomes of CP1 and Osloss and it was obvious to look for sequences responsible for this site specificity. Two different bovine polyubiquitin mRNAs were analyzed but no homology or complementarity between the sequences flanking the putative recombination positions in the viral and cellular RNAs could be identified (Meyers et al., 1991). The same result was obtained when the nucleotides adjacent to the SD and LD regions of BVDV Pe515CP and CP6 were analyzed. Surprisingly, sequence comparison revealed identical 3' recombination positions regardless whether viruses with ubiquitin-coding insertions or duplication of p20- and p80-coding sequences were looked at (Fig. 9). It therefore has to be considered that the site-specific recombination observed for these viruses might be a result of functional pressure rather than being guided by specific sequences. This functional pressure could be based on the requirement of a given aminoterminus for the cpBVDV-specific protein p80. Accordingly, development of a cytopathogenic virus in conjunction with outbreak of MD would represent the final step with regard to selection for the correct recombinant. Studies on coronaviruses tend to support this view since selection for certain recombination hotspots is preceded by random RNA recombination (Banner and Lai, 1991).

The genome rearrangements which have been identified for the different cpBVDV strains result in changes of virus-encoded proteins. One of these changes represents the expression of aberrant fusion proteins composed of (i) cellular and viral sequences (NADL p54 and Osloss p41) or (ii) sequences from two viral polypeptides derived from different regions of the genome (Pe515CP) (Fig. 8). For BVDV CP6 a fusion protein could so far not be demonstrated. This might either be due to a technical problem or to rapid degradation of the respective product which according to the genome organization of CP6 should be composed of p20 sequences and a small part of p58, the second last polypeptide encoded by the pestiviral ORF (Collett et al., 1991). Further investigation is needed to clarify this point.

The other important change at the protein level distinguishing cpBVDV from noncpBVDV is the expression of p80 in addition to p125. While the above-mentioned fusion proteins show a high degree of variability concerning nature and size, not only the mere presence of p80 in cpBVDV-infected cells but also its size is strictly conserved (Purchio et al., 1984; Donis and Dubovi, 1987a; Greiser-Wilke et al., 1992; Meyers et al., unpublished). A prerequisite for generation of p80 is the introduction of a protease cleavage site at the aminoterminus of this protein. Based on our current knowledge two basically different ways can be distinguished. One possibility is represented by the Osloss strain where the signal for cleavage is provided by a cellular ubiquitin sequence inserted into the p125 region of the viral polyprotein. Ubiquitin itself is synthesized within eucaryotic cells in form of fusion proteins which are subsequently cleaved at the carboxyterminal end of the ubiquitin moiety (Finley et al., 1987, 1989; Özkanayk et al., 1987; Redmann and Rechsteiner, 1989, Rechsteiner, 1987). Accordingly, the insertion of ubiquitin into p125 introduces a signal for processing by a cellular protease (manuscript in preparation).

The second way to generate p80 requires duplication of the respective coding region. In addition a processing signal is placed in front of the duplicated sequence. In the case of CP1 ubiquitin provides again the signal for cleavage by a cellular protease (manuscript in preparation). For Pe515CP and CP6 a processing

| CP1    | CGCTCTGAGGGGTGCCG | gggacctggcgtgtgtgc | B |
|--------|------------------|---------------------|---|
| Osloss | CGCTCTGAGGGGTAGTC | gggacctggcgtgtgc   | E |
| Pe515CP| TGGGTATGAGCTGCG  | gggacctggcgtgcac   | B |
| CP6    | TGGGTATGAGCTGCG  | gggacctggcgtgtgtgc | E |

![Table](image)
site already present in the virus serves this purpose. Interestingly, insertion of the p20-coding region also transfers the protease responsible for the cleavage to its point of action. The integration of both protease and cleavage site might be necessary because of the lack of in trans action of the p20 protein. This would be in accordance with the hypothesis that p20 can only act as an autoprotease (Wiskerchen et al., 1991).

Both alternatives outlined above would allow identical aminoterminal ends of the different p80 proteins since the insertion point for the ubiquitin gene in the Osloss RNA and the 5′ end of the 1D region in the LD/SD type of genomes correspond to the same genomic position. Even though further investigation is necessary to fully understand the generation of p80, one has to keep in mind that expression of this protein is strictly correlated with the cytopathogenic phenotype and the development of MD. p80 therefore represents the prime candidate for the agent responsible for killing the infected cells while the different fusion proteins might be only byproducts resulting from the recombination. According to sequence comparison studies p80 contains protease as well as helicase motifs (Gorbaleanya et al., 1989a,b). Experimental evidence for the protease function of p80 has recently been obtained (Wiskerchen et al., 1991; Tautz et al., in preparation). If p80 turns out to be required for the cytopathogenic effect it will be interesting to determine why p125, having the same enzymatic domains, does not exhibit this effect.

The BVDV/MD system offers the opportunity to study RNA virus evolution in action. Development of MD appears to be obligatorily linked to generation of cpBVDV. The required recombination process leads to expression of novel proteins, especially p80. The analysis of additional virus pairs will provide insights into the different possibilities to generate viruses with a given phenotype.

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