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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 sequences. 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. © 1991 Academic Press, Inc.

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 (Ronard 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., 1988a).

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 cpBVDV 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...
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 XAR6 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 (Romenapf 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 mM 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 mM sodium phosphate, pH 6.8, 1 mM EDTA, 5% SDS, and 40 mM sodium phosphate, pH 6.8, 1 mM 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**: GCCATRCTXCCYTYTCAT (6226–6245)

**BVDV14**: GRCCARTCRTARTCATYTCTC (7033–7052)

Second library:

**PES9**: ACYTCCCCCAYTTRTCXGT (9079–9095)

**ORG345**: GATGAGCCCTCCTGCTGTCAGGGG/GAATGCCTTCC (5250–5263)

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 µg of total RNA of MDBK cells infected with either CP1 or NCP1 were heat denatured (5 min, 65°). Annealing of oligonucleotides (0.1 µg 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 (Romenapf et al., 1989). Cloning in λ ZAPIII (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 × 10$^8$ 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 1/10 of normal methionine content. Cell extracts were prepared as described (Romenapf 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 µl of undiluted serum. Precipitates were formed with crosslinked Staphylococcus aureus (Kessler, 1981), analyzed by 10% SDS–PAGE (Laemmli, 1970), and processed for autoradiography using Enhance (New England Nuclear). The anti-peptide an-
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., 1988; 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.

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...
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 pCP18 and pCP113 (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 7/6b 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-
igration 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 CPI 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
structural proteins

non-structural proteins

Osloss

5161 Ub 5380

Genome

A B C

NCP1

Genome

A B C

CP1

Genome

A Ub B C

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 are 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).

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
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 | 99.5% | 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.
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 and should also be expressed.

Further insight was obtained after RIP with a serum raised against a BVDV NADL-deduced peptide located within p64 of p125 (peptide encompassing amino acids 1335 to 1351 of the published BVDV NADL sequence (Collett et al., 1988)). 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 sequence (Collett et al., 1987) including the flanking viral sequences. The host cell-derived regions are capitalized while viral parts of the 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.

**Raw Text**

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 p64 of p125 (peptide encompassing amino acids 1335 to 1351 of the published BVDV NADL sequence (Collett et al., 1988)). 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 sequence (Collett et al., 1987) including the flanking viral sequences. The host cell-derived regions are capitalized while viral parts of the 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.
FIG. 7. Immunoprecipitation of proteins extracted from BVDV-infected cells after metabolic labeling with a mixture of [35S]cysteine/[35S]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 p80 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; Ozkaynak 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; Boon 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...
UBIQUITIN IN A PESTVIRUS

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, 1988; 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 recombinant 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-
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-
sible for the uptake of the additional sequences into the genomes of BVDV strains CP1, Oslloss, 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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