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A mutational analysis of the N-terminal protease of bovine viral diarrhea virus

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Abstract : The uncaped genomic RNA of bovine viral diarrhea virus (BVDV) initiates translation by recruitment of eukaryotic translation initiation factors at the internal ribosome entry site (IRES). N-terminal protease (N^{pro}) is the first translation product of the open reading frame (ORF). By using the vaccinia virus SP6 RNA polymerase transient expression system, we showed previously that deletion of N^{pro} region reduced translation by 21%. To better understand the biological significance of N^{pro} for translation, we carried out a mutational analysis of the N^{pro} region of BVDV cloned in the intercistronic region of a bicistronic reporter plasmid. We constructed a bicistronic expression vector in which the entire 5 UTR and the mutated N^{pro} region (Δ 386-901, Δ 415-901 and Δ 657-901) was cloned between two reporter genes, chloramphenicol acetyltransferase (CAT) and luciferase (LUC). *In vivo* translation analyses showed that N^{pro} region was dispensible for efficient translation. The results indicate that the N^{pro} region is not essential for BVDV RNA translation and the 3' boundary of BVDV IRES is expanded into N^{pro} region, suggesting that N^{pro} may not play a major role in BVDV replication.

Key words : Bovine viral diarrhea virus, internal ribosome entry segment, N-terminal protease.

Introduction

Bovine viral diarrhea virus (BVDV) is grouped in the genus pestivirus of the family Flaviviridae. The genus pestivirus comprises three members, BVDV of cattle, classical swine fever virus (CSFV, formerly termed hog cholera virus) of swine and border disease virus (BDV) of sheep[1,2]. BVDV can be divided into two biotypes, cytopathic BVDV and noncytopathic BVDV, based on the cytopathogenicity in cell culture 3. BVDV is the important pathogen of a variety of syndromes in cattle. The appearance of mucosal disease, a severe clinical syndrome, occurs only in persistently infected individuals when they are superinfected with cytopathic BVDV[3,4].

The genome of BVDV consists of an RNA of 12.5kb,

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which encodes a single polyprotein of nearly 4000 codons[5-8], that flanked by 5' untranslated region (UTR) and 3' UTR. BVDV genomic RNA does not posses the typical inverted methylguanosine cap at the 5' end and the adenine homopolymer at the 3' end[6]. The cap structure plays an important role in translation initiation of cellular mRNAs through the interaction with eIF-4F, which binds to the cap structure through the cap binding protein eIF-4E[9]. Poole *et al*[10] demonstrated the presence of an internal ribosome entry segment (IRES) element within the BVDV 5' UTR by translation in cell free systems as well as *in vivo*[10]. An element spanning nucleotide 1-394 was shown to suffice for basal translation, but the import of viral sequences extending 3' of the initiator AUG on translation initiation efficiency was not examined.

We have previously reported that 5' boundary of the IRES residues at the 5' end of the polyprotein open reading frame (ORF) because removal of the N-terminal protease (N^{pro}) region reduced translation by 21%[11]. This protein of 168 amino acids in encoded at the 5' end of the ORF, undergoing a cotranslational self-cleavage of its C-terminus to release the N-terminus of the capsid protein[12]. However, the biological significance of N^{pro} is still unknown for trnaslation or replication of BVDV.

To understand the biological significance of N^{pro} in BVDV translation that determine efficient IRES-mediated trnaslation initiation, we carried out a mutational analysis of the N^{pro} region of BVDV inserted between two cistrons encoding reporter enzymes. Translation of N^{pro} deletion mutants *in vivo* revealed that a N^{pro} region and 4 amino acids of capsid coding sequences (Δ 386-901) was dispensable. Δ 415-901 and Δ 657-901 of N^{pro} region caused a reduction of 50% and 35%, respectively.

Materials and Methods

Construction of plasmid for the expression of bicistronic mRNAs : The structure of plasmid pBi5'BVDV has been described previously[11]. Briefly, it expresses from a SP6 promoter a bicistronic mRNA transcript containing the chloramphenicol acetyltransferase (CAT) and luciferase (LUC) coding sequences seperated by the 5' 901 nucleotides (nt) of BVDV genome, which correspond to the 5' UTR and N^{pro} region and 4 amino acids of capsid protein. All DNA manipulations necessary to construct plasmid was performed according to standard protocols[13]. Plasmid was transformed into *E coli* strain JM109[14].

Construction of deletion mutants of N^{pro} region : pBi Δ 386-901 (Nomenclature used previously, pBiORF Δ 386-901), pBi Δ 415-901 and pBi Δ 657-901 were obtained by site- directed mutagenesis on the plasmid pBi5'BVDV as described using *E coli* strain mutS[15]. The sequence of the mutant strand selection oligo nt, which eliminates the unique NdeI site in pGEM-Luc plasmid derivatives, was as follow: 5'GTACTGAGAGTGCACCAACTGCGGTGTGAAATAC3'. The sequence of the mutagenic oligo nt is as follows (the dash interrupting each of the nt sequences separates the 3' nt preceding the deletion from the 5' nt following the deletion; Δ 386-901, 5'GTTTTTGGCGTCTTCCAT-TGTCCATGTA-CAGCAGAG3'; Δ 415-901, 5' GTTTTTGGCGTCTTCCAT-GTATAAAAGTTCATTTGTGATC3'; and Δ 657-901, GTT-TTTGGCGTCTTCCAT-CTGGTAAAATAGTGGCCCTGG 3'. The resulting mutants were confirmed by nt sequencing as described previously[16].

Cell culture, transfections and transient expression assays : CV-1 cells (African green monkey kidney) and BT cells (Bovine turbinate) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Plasmid DNA (0.050μg) was introduced by using Lipofectamine (GIBCO-BRL) into the infected cells with vaccinia virus recombinant vSP6[17,18] expressing SP6 RNA polymerase. After 16h postinfection, cell extracts were prepared by three cycles of freezing and thawing and assayed for CAT[19] and LUC activity as described by manufacturer (Promega). Quantitation of CAT activity was performed by thin layer chromatography and liquid scintilation counting. Relative IRES activity ratios were calculated as: R = (LUC activity from mutant IRES/LUC activity from wild-type IRES)/(CAT activity from mutant IRES/CAT activity from wild-type IRES).

Western blotting analysis for detection of luciferase : To detect the LUC, we performed with same manner as described previously (Chon, 1998). Cell lysates from 10^6 cells

prepared as described for transient expression assay were mixed with double-concentration Laemmli's sample buffer; aliquots (10μl) were separated on 8% slab SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane using a semidry system (BioRad). The membrane was processed with antibodies against firefly luciferase (Promega) and developed with a chemiluminescent reagent as described previously[20]. Protein concentration in lysates was determined by amido black staining exactly as described previously[21].

Results

Mutagenesis of N^{pro} region : To investigate whether the N^{pro} region plays a role in translation into BVDV, we performed with site-directed mutagenesis to delete the N^{pro} region. Based on our previously reported plasmid pBi5'BVDV[11], we constructed plasmids in which the two reporter genes within the bicistronic mRNA sequences were seperated by 5' UTR containing deletions of each ORF as indicated in Fig 1. To identify the expression of LUC from wild-type and mutant plasmids, each of the plasmids was transfected into vSP 6 infected CV-1 cells. As shown in Fig 2A, LUC was expressed from pBi5'BVDV and pBiΔ386-901, respectively. Also, N^{pro}-LUC fusion protein was detected in the immunoblot probed with antibodies against firefly luciferase from pBiΔ415-901 and pBiΔ657-901. By 8% slab SDS-PAGE, however, these translational products did not detect the different migration by molecular sizes.

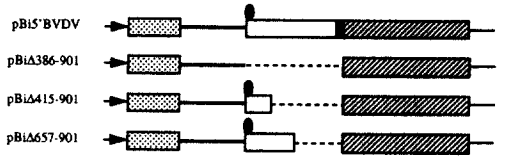

Fig 1. The diagram depicts the deletion of N^{pro} region. The structure of plasmid pBi5'BVDV has been described previously[11]; it express from a SP6 promoter(→) a bicistronic mRNA transcript containing the CAT( ) and LUC( ) coding sequences separated by the BVDV 5' UTR( ), N^{pro}( ) and 4 amino acids capsid coding sequences( ). ATG codon is indicated by closed circle( ). Mutated forms of the N^{pro} region were deleted by site-directed mutagenesis with oligo nt(△386-901, △415-901 and △657-901) as described in Materials and Methods.

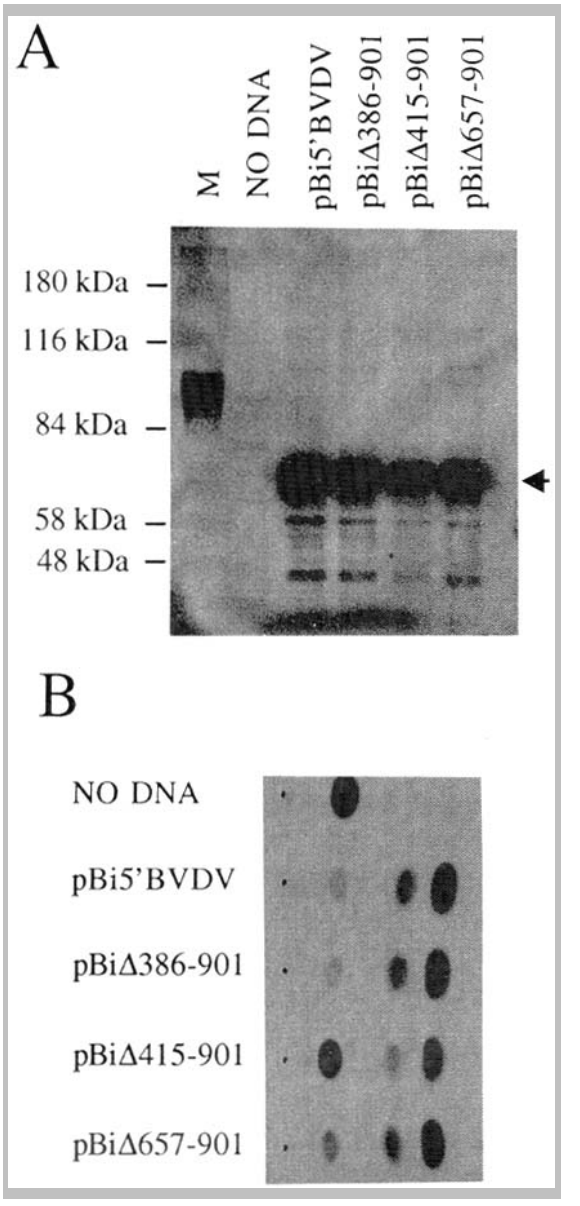

Fig 2. Expression of LUC and transfection efficiency of N^{pro} region mutants *in vivo*. CV-1 cells were transfected with wild-type or mutants shown in Fig 1 and lysated after 16h post-tinfection. A. Cell lysates were separated by 8% slab SDS-PAGE, analyzed by immunoblotting using rabbit antibodies against firefly luciferase, and developed with a chemiluminescent reagent. B. As a control for transfection efficiency, CAT assay was performed with 2μl lysates by thin layer chromatography.

To determine the transfection efficiency of plasmids, CAT assays were performed with thin layer chromatography and liquid scintillation counting with same cell lysates used for western blotting. By a 5' end dependent manner, 5' cap dependent translation, the RNA transcribed from these constructs is translated into the first cistron, which is CAT gene. CAT activity should exactly consider the RNA level because these mutant plasmids are identical at the 5' end. Fig 2B showed CAT activity for wild-type and mutant plasmids as indicated in panel by thin layer chromatography. Wild-type and three mutant plasmids showed only 2% difference of CAT activities. This was confirmed by liquid scintillation counting (Data not shown).

IRES activity of N^{pro} deletion mutants : To analyse the different mutants of N^{pro} region, CAT activity was used to normalize for variations in transfection efficiencies, serving to normalize to LUC activity. A relative IRES activity ratio was calculated as follows : R = (LUC activity from mutant IRES/LUC activity from wild-type IRES)/(CAT activity from mutant IRES/CAT activity from wild-type IRES). All values are based on at least three different experiments (Fig 3).

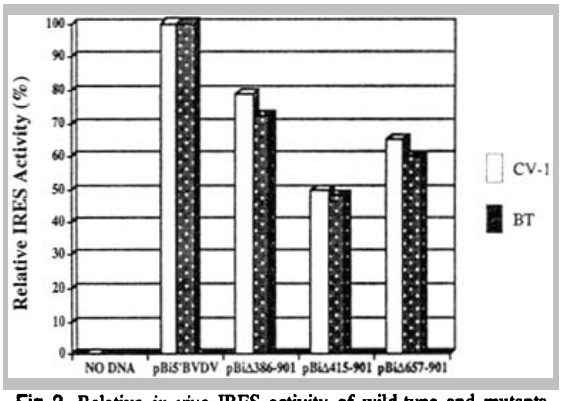

Fig 3. Relative *in vivo* IRES activity of wild-type and mutants of N^{pro}. Transient expression assays in lysates of vSP6 infected CV-1 cells or BT cells transfected with wild type or mutant plasmid DNA, as indicated, were determined. These data were used to calculate the IRES activity ratio of each mutant relative to the activity of the wild-type IRES. The IRES activity of pBi5'BVDV is artificially set at 100%.

Plasmid pBi Δ 386-901, deletion of N^{pro} and 4 amino acids of capsid coding sequences, led to only 21% loss of IRES activity. Also, Plasmid pBi Δ 415-901 reduced IRES activity by only 35%. The results showed that deletion of Δ 386-901 and Δ 415-901 had no detrimental effect on the internal translation initiation activity of the BVDV IRES *in vivo*. On the other hand, plasmid pBi Δ 657-901 reduced IRES activity by 50% only but did not completely abrogate. The results showed that the IRES activity of mutational N^{pro} region was not significantly different from those mutant plasmids. These results obtained indicate that the IRES activity does not completely affect by N^{pro} region.

Discussion

Deletion of the sequence 3' to the initiator AUG (Δ 386-901) resulted in a modulate loss of BVDV IRES function, above 20%. Interestingly, a more stringent requirement for sequences 3' to the AUG ORF was reported for efficient hepatitis C virus (HCV) IRES activity[22]. However, other reports show almost wild-type levels of translation in the absence of this region[23,24].

Pestova *et al*[25] proposed a model in which proper positioning of the initiator AUG, locking it in place for initiation of translation. Ribosomal protein S3, probably the protein referred to as 25 kDa protein, is thought to participate in the interactions with RNA sequences 3' of the AUG[25]. The initiator AUG codon is thought to be required for the proper binding of the 43S ribosome subunit, and its topology relative to the other structual elements of the IRES seems crucial for efficient IRES function.

Translation reduction of up to 50% was measured for mutations of N^{pro} region. This indicates that some N^{pro} coding sequences are required for optimal IRES function. This result is consistent with the 3' boundary of the HCV IRES reported by Reynolds *et al*[22]. These findings may indicate that the N^{pro} region up to nt 415 or more sequences contributes to the translation of BVDV RNA. In this report, we present data showing that the N^{pro} region in BVDV is not essential for BVDV RNA translation and the 3' boundary of the BVDV IRES is expanded into N^{pro} region. Here, we suggest that N^{pro} may not play a major role in BVDV replication.

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