

## Molecular detection of proteolytic activity of human parechovirus 2A protein by gene expression

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### ABSTRACT

Parechoviruses form one of the nine genera in the *picornaviridae* family, and include two human pathogens: Human parechovirus type1 and 2 (Hpev1 and Hpev2). The genome of picornaviruses encodes a single polyprotein, which undergoes a cleavage cascade performed by virus encoded proteases to give the final virus proteins. The primary cleavage occurs by 2A protein and this step is critical for viral life cycle. Recent sequence analysis suggests that Hpev1 is distinct from other picornaviruses and lacks the motifs believed to be involved in the protease function of 2A. The aim of this study was to analyze proteolytic activity of 2A protein in Hpev1. For this purpose we made several recombinant plasmids contain 2A region of parechovirus type1 genome and expressed in prokaryotic and *in vitro* systems under T7 promoter. Analyzing the expression products by SDS-PAGE revealed just a large single band (90 KDa), the same size as primary translation product. Whereas with plasmids include 3C gene several small bands were observed, indicating that processing had occurred. In conclusion: the results of this work show that Human parechovirus type1 has a processing strategy different from the other members of picornaviruses and in this virus, as in hepatovirus, 2A protein does not have a protease function.

**Keywords:** *Picornaviridae*, Human parechovirus, 2A protein, 3C protein, Gene expression

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### INTRODUCTION

Human parechovirus 1 (Hpev1) is a common pathogen associated with gastrointestinal and respiratory symptoms as well as central nervous system infections (Grist *et al* 1978, Joki *et al* 1998). Hpev1 is a member of the parechovirus genus of the picornaviridae family (King *et al* 2000). It is a small non-enveloped RNA virus with a single-stranded genome of positive polarity, about 7.4 kb in length and a capsid containing 60 copies of each of the

capsid proteins (VP4, VP2, VP3 and VP1, respectively) (Hyypia *et al* 1992). The genome encodes a large polyprotein, which is subsequently processed by virus-specific proteolytic activity to produce polypeptides involved in virus replication and virion assembly (Ghazi *et al* 1998). The capsid proteins are encoded towards the N terminus of the polyprotein and the non structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) are encoded downstream of them (Rueckert *et al* 1996). The 2A polypeptide shows major differences among picornaviruses. In enterovirus and rhinoviruses, the 2A protein is a trypsin-

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like protease that is responsible for the *cis* cleavage at its own N terminus between the VP1 capsid protein and 2A (Palmenberg *et al* 1990). The *trans*-cleavage activity of 2A processes cellular factors, including eukaryotic initiation factor-4G (p220) (Foeger *et al* 2003), TATA-binding protein (Yalamanchili *et al* 1997), and poly (A)-binding protein (Joachims *et al* 1999). The cleavage of eukaryotic initiation factor-4G, and possibly poly(A)-binding protein, contributes to the shut-off of host cell protein synthesis (Joachims *et al* 1999, Barco *et al* 2000). In cardio- and aphthoviruses, 2A is involved in an unusual C-terminal proteolytic processing event between the 2A and 2B proteins (Ryan *et al* 1989 and 1997, Flint *et al* 1997, Hahn *et al* 2001). The 2A protein of hepatovirus has no autocatalytic proteolytic activity (Jia *et al* 1993, probst *et al* 1997). Sequence alignment has revealed that the parechovirus 2A protein differs considerably from the corresponding proteins in other picornaviruses (Stanway *et al* 1994, Ghazi *et al* 1998). In view of the diverse nature of the 2A protein in different picornaviruses it was decided to study proteolytic activity of this protein in Hpev1.

## MATERIALS AND METHODS

**cDNA preparation.** Human Parechovirus type1 (Harris strain) cDNA was prepared by using Maloney Murine Leukemia Virus (M-ML V) reverse transcriptase with a (dt) 17 primer and then amplified by PCR (Polymerase Chain Reaction). The PCR product was ligated into pBS vector by using restriction enzymes which their recognition sites were on the primers (Hyypia *et al* 1992).

**Constructs preparation.** To further analyze the processing of the 2A region in Hpev1, three cDNA constructs (pFG1, pFG1.1 and pFG3) were produced. The first of these was pFG1 (5500 bp length) containing the whole of 2A gene (2700-4200 region), 5'UTR and parts of VP0 and VP1 of

Hpev1 genome in the vector pUBS (Figure 1). The pFG1 construct was made by digestion of a plasmid pBSP2 (including 2A and VP1 genes) with *EcoRI* and *Hind III*. After phenol extraction and ethanol precipitation (Holmes *et al* 1981) this plasmid was ligated to pUBSVP0 (including 5'-UTR and part of VP0) which had been digested with the restriction enzymes *Hind III* and *SmaI*. The ligated DNA was transformed into *E. coli* MC1022 which was competent with calcium chloride (Hanahan *et al* 1991, Inoue *et al* 1990). To identify the correct construct, DNA isolated from the obtained colonies were digested with *PstI* and gel electrophoresed. The correct construct giving three fragments, of sizes 2900, 1400, 1200bp were selected (Figure 2). Then confirmed by PCR (30 cycles in 94 °C one minute, 56 °C one minute and 72 °C one minute) using two primers complementary to 2A gene (Figure 3).

pFG1.1 construct (4828 bp length) was made by digestion of pFG1 with *BamHI* and ligation of the 2000 bp band into pUBS vector, which had been digested with *BamHI* and phosphatase treated. The resulting clone lacks the majority of the 5'UTR (up to position 672). After transformation, DNA was isolated from several white colonies. To identify the correct construct, isolated DNAs were digested separately with *PstI* (bands of 1400 and 2500 bp were expected) and *BamHI* (bands of 2900 and 2000bp were expected) and a clone giving these results (pFG1.1) was identified (Figure 2). pFG3 construct (8638 bp in length) including coding region of 3C protein. This region (4200-7338) of Hpev1 cDNA was excised using *PstI* enzyme and inserted into pUBS vector which was digested with the same enzyme, then this recombinant plasmid and pFG1 were separately digested with *HindIII* and *BpmI* enzymes and were ligated. After transformation into *E. coli* MC1022, DNAs were isolated and digested with *PstI* to identify the correct construct (pFG3) (Figure 2). For final

confirmation, the constructs were sequenced. **Expression in prokaryotic system.** The recombinant plasmids were expressed in Prokaryotic and *In vitro* systems under T7 promoter. To express gene in Prokaryotic system *E. coli* BL21 was used as host. First bacteria was cultured in SOB media (Studier *et al* 1990) containing MgCl<sub>2</sub> and KCl, to entered into logarithmic phase (2-3 hours). Then the recombinant plasmids were transformed into bacteria and induced by 0.1 mmol IPTG (Studier *et al* 1990, Moffat *et al* 1986). From these bacteria incubated with IPTG, after the time periods: zero, one, two and three hours, samples were collected and centrifuged. The precipitation was suspended in lysate buffer containing SDS.

**Expression in *in vitro* system.** These plasmids also were expressed *in vitro* system by using *in vitro* cell free T7/SP6 Transcription/Translation kit, Roche product (Studier *et al* 1990, Moffat *et al* 1986). After expression of plasmids the products were analyzed by SDS-PAGE, followed by Gimsa and silver staining.

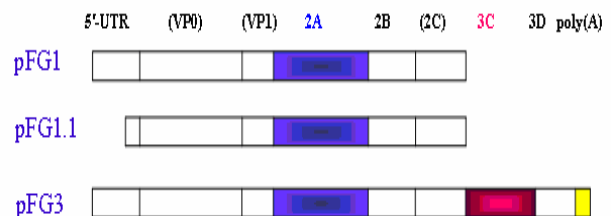
**PCR primers for 2A gene.** Forward primer was 5'-GGTGAATAACGGTACCAGCTGG-3' and reverse primer was 5'-ATTTATAAACTCATGTGGTGATCACAAC-3'.

## RESULTS

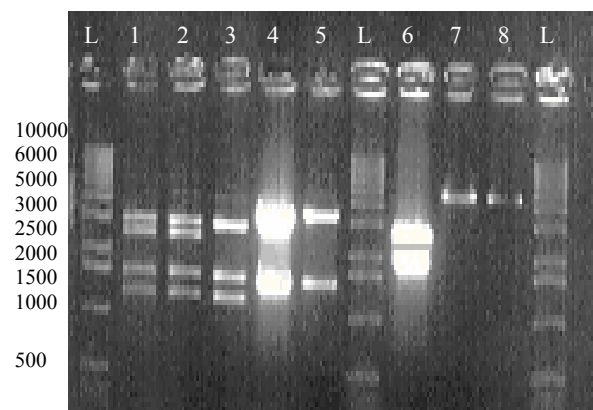
To detect the proteolytic activity of 2A protein, following recombinant plasmids were used: FG1, pFG1.1 (containing 2A gene). pFG1 (5500 nucleotides length) contained all 2A gene, 5'UTR and parts of VP0-VP1-2A-2B-2C in plasmid pUBS (contains the T7 promoter and therefore in the presence of T7 RNA polymerase and ribonucleotide triphosphates, RNA will be synthesized).

Because of previous studies with other viruses showed that the shorter 5'UTR causes an increase in protein synthesis (Alsaadi *et al* 1989) so the construct pFG1.1 was made. Plasmids pFG1 and pFG1.1 were almost identical but pFG1.1 (4828 nucleotides length) lacks the majority of the 5'UTR

up to position 672 nucleotides (Figure 1). The constructs, pFG1 and pFG1.1, were transcribed /translated in the coupled *in vitro* system under T7 promoter.



**Figure 1.** Schematic diagrams of clones produced to analyze polyprotein processing in parechovirus type1. Names in brackets indicate incomplete genes. pFG1 (5500bp) containing 2A gene, pFG1.1 (4828 bp) lacks most of 5'UTR, pFG3 (8638 bp) including 3C gene.

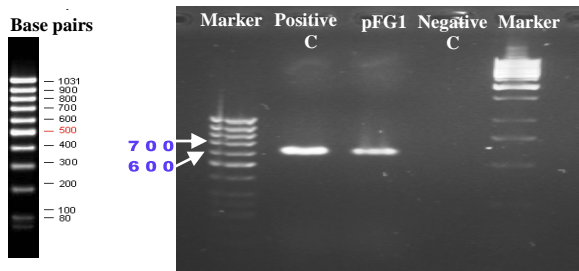


**Figure2.** Restriction enzyme analysis of constructs produced in attempts to investigate whether Hpev1 2A is protease. L (1Kb DNA ladder), tracks 1-5, digested with *PstI* of pFG3 (1), pFG3 (2), pFG1 (3), pFG1.1 (4), pFG1.1 (5), track 6, pFG1.1 digested with *BamHI*, track 7 and 8 pFG1 digested with *HindIII*.

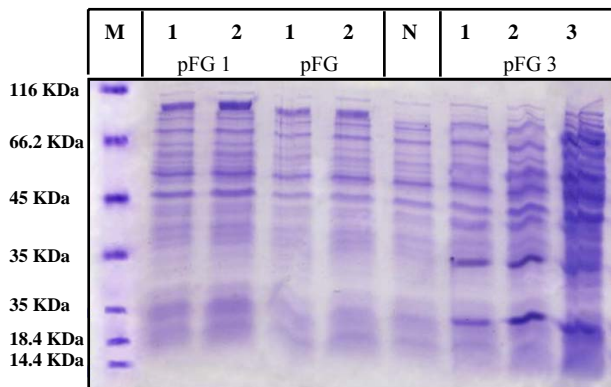
Since no processing of polypeptide containing 2A was observed, the 3C region was added to the cDNA constructed (pFG3) to test whether the system used allows processing to be observed. Plasmid pFG3 (8638 nucleotides length) contained the same parts as pFG1 and also includes 3C region.

After amplification and extraction of DNA, the plasmid was digested separately with restriction enzymes and the length of each band was detected by agarose gel electrophoresis and DNA marker (Figure 2). After transcription / translation in *in vitro*

system, products were analyzed by SDS-PAGE. pFG3 yielded several small bands (20, 30 and 40 KDa) in this system, indicating that after addition of the 3C region processing had occurred (Figure 4 and 5).



**Figure 3.** PCR detection of Hpev1 2A gene. A sample of agarose gel electrophoresis of PCR Product using a specific primer for 2A gene.



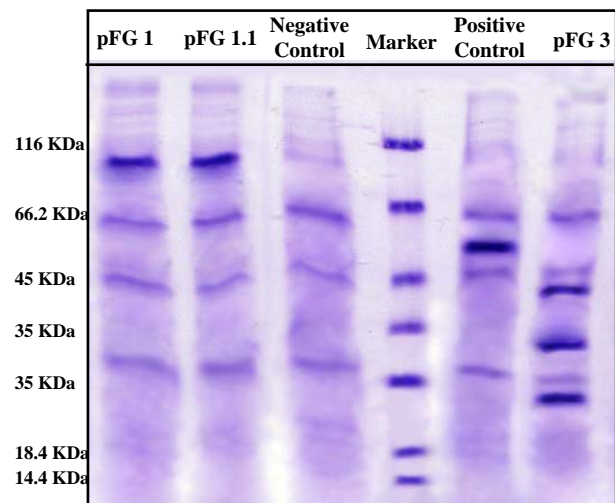
**Figure 4.** PAGE analysis of coupled *in vivo* transcription / translation products. The samples are parechovirus type1 2A containing constructs pFG1 (after 1 and 2 h) pFG1.1 (after 1 and 2 h) pFG3 (after 1, 2 and 3 h), and a negative control containing no DNA (N) . Molecular weight marker (M).

To confirm this result anti protease was added to the reaction (just one band was observed). Prokaryotic system expression of the constructs have also shown that Hpev1 2A has no autocatalytic activity (Figure 4).

## DISCUSSION

The 2A protein coding region in the Picornavirus polyprotein is one of the least conserved loci among

picornavirus genome and the structural and functional properties of the 2A gene products are variable among members of the different genera of the picornaviruses (Palmenberg *et al* 1990, Shirley *et al* 1995, Semler *et al* 2003). *In vitro* translation has been used frequently to study protein processing in picornaviruses and a complete translation / transcription system makes this technique easier to use (Palmenberg 1990, Jia *et al* 1991, Tesar *et al* 1994, Medina *et al* 1993, Ryan *et al* 1997). 2A part of Hpev1 cDNA were placed under the control of the T7 Promoter in the vector pUBS and transcribed /translated in prokaryotic system and a commercially available, coupled *in vitro* cell free system (Roche product). The basic construct used (pFG1) yielded a single protein band in this system.



**Figure 5.** PAGE analysis of coupled *in vitro* transcription / translation products. The samples are parechovirus type 1,2A containing constructs pFG1, pFG1.1, pFG3 and a negative control containing no DNA. Molecular weight marker (M).

This corresponded in size to the complete primary translation product (90 KDa) suggesting that no processing has taken place and therefore that 2A has no protease acting in Hpev1. This is consistent with sequence analysis which shows that the Hpev1 and hepatitis proteins do not contain either protease

consensus sequences, or the peptide-motif (NPGP) seen in cardio and aphtovirus (Palmenberg *et al* 1990, Ghazi *et al* 1998). Construct pFG1.1 was almost identical to pFG1 but removes much of 5' UTR and this improves the efficiency of translation. pFG3 includes the 3C region and this construct should allow an assessment of whether this region is necessary for the cleavage of 2A.

Transcription/translation of pFG3 gave several bands smaller than the full length product (Figure 5) indicating that processing had occurred. Expression of the constructs in prokaryotic system also supports the conclusion that the Hpev1 2A protein does not have a protease activity (Figure 4). It is worth to mention that these results are similar to those previously reported for hepatoviruses (Jia *et al* 1991, Probst *et al* 1997). In hepatoviruses 3C protein seems to be the only virus encoded protease that can catalyze cleavage of all sites in the hepatovirus polyprotein, including the primary cleavage which separates the structural protein precursor (1ABCD2A) from the non-structural proteins.

Since Hpev1 2A is not a protease, recent evidence suggests that 2A has other function in viral replication. 2A appeared to be an essential component in RNA replication as no viral RNA synthesis can be observed by reverse transcription/PCR in cell transfected with RNA lacking this viral polypeptide (Molla *et al* 1993, Yu *et al* 1995). Recent experiments showed that Hpev1 2A binds to viral 3'UTR RNA, a feature that could be important for the function of the protein during Hpev1 replication (Samuliova *et al* 2004). It has been shown recently that the 2A proteins of parechovirus, hepatitis A and Aichi virus three picornavirus groups are related to each other and, additionally, to a recently identified family of cellular H box/NC proteins, which are possibly involved in the control of cell proliferation (Hughes *et al* 2000).

In conclusion, the results of this study show that in human parechovirus, processing of 2A region of the polyprotein is not brought about by 2A protein and is likely to be done by 3C protease. Therefore in this virus, as hepatovirus, 2A protein does not have a proteolytic function and it seems that 3C protease is the only virus encoded protease catalyzing the cleavage of all sites in primary polyprotein.

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