

Polypyrimidine tract binding protein is required for feline calicivirus replication in a temperature-dependent manner

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Summary

The interaction of host cell nucleic acid-binding proteins with the genomes of positive stranded RNA viruses is known to play a role in the translation and replication of many viruses. To date however, the characterisation of similar interactions with the
5 genomes of members of the *Caliciviridae* family has been limited to *in vitro* binding analysis. We have now used feline calicivirus (FCV) as a model system to identify and characterise the role of host cell factors that interact with the viral RNA. We demonstrate that polypyrimidine tract-binding protein (PTB) interacts specifically with the 5' sequences of the FCV genomic and subgenomic RNAs. Using RNA
10 interference we demonstrate that PTB is required for efficient FCV replication in a temperature-dependent manner. siRNA-mediated knockdown of PTB resulted in a 15-100 fold reduction in virus titre, as well as a concomitant reduction in viral RNA and protein synthesis at 32°C. In addition, virus-induced cytopathic effect was significantly delayed as a result of a siRNA-mediated reduction in PTB levels. A role
15 for PTB in the calicivirus life cycle was more apparent at temperatures above and below 37°C, fitting with the hypothesis that PTB functions as an RNA chaperone, potentially aiding the folding of RNA into functional structures. This is the first functional demonstration of a host cell protein interacting with a calicivirus RNA.

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25 **Introduction**

The interaction of cellular proteins with RNA sequences and structures plays a critical role in many aspects of the processing, localisation and translation of host cell mRNAs. Often the RNA-binding proteins participating in these interactions are also used by viruses in their own translation and replication mechanisms (Bushell & Sarnow, 2002, Shi & Lai, 2005). Such RNA-protein interactions are often a major determinant of viral tropism, due to cell specific expression of such factors (Gutierrez et al., 1997, Pilipenko et al., 2000). A large number of interactions between host cell proteins and viral RNA structures have now been identified, with some of the best characterised interactions those that regulate translation from viral internal ribosome entry site (IRES) elements (Belsham & Sonenberg, 2000). Of the proteins identified to date, several appear to be involved in the translation and/or replication of more than one family of viruses. For example, polypyrimidine tract binding protein (PTB) has been shown to be required for efficient translation from several picornavirus IRES elements (Belsham & Sonenberg, 2000), but also plays a role in hepatitis C virus IRES-directed translation (Anwar et al., 2000, Gosert et al., 2000) and murine coronavirus replication (Huang & Lai, 1999, Li et al., 1999). In addition to PTB, numerous other RNA-binding proteins, such as unr (Boussadia et al., 2003), poly(rC) binding protein (PCBP) (Parsley et al., 1997), La (Meerovitch et al., 1993), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Yi et al., 2000), hnRNP A1 (Huang & Lai, 2001) and poly (A) binding protein (PABP) (Svitkin et al., 2001) have been identified as essential factors for translation or replication of a number of positive-stranded RNA viruses.

Members of the *Caliciviridae* family of positive-stranded RNA viruses infect a variety of vertebrates. The human caliciviruses (HuCV) are a major cause of acute gastroenteritis in man and are responsible for a large number of outbreaks in hospitals, 50 cruise ships (Widdowson et al., 2004), military settings (Bailey et al., 2005), nursing homes and restaurants (van Duynhoven et al., 2005). Our understanding of the biology of the HuCVs has been somewhat hindered by the lack of a suitable cell culture system (Duizer et al., 2004), although recent advances have demonstrated 55 limited genome replication and packaging (Asanaka et al., 2005). In contrast, feline calicivirus (FCV) and the recently identified murine norovirus 1 (Karst et al., 2003, Wobus et al., 2004) replicate efficiently in cell culture and represent good models with which to study the general replication and translation strategies employed by caliciviruses. To date, the study of RNA-protein interactions that may play critical 60 roles in calicivirus translation and replication has been limited to *in vitro* binding studies. For example, work with the human calicivirus Norwalk virus (NV) has demonstrated that PTB, La, hnRNP L and PCBP-2 interact with 5' sequences of the positive sense genomic RNA (Gutierrez-Escolano et al., 2000). PTB and La also interact with the 3' untranslated region of the NV genome (Gutierrez-Escolano et al., 65 2003). However, the role of these interactions has yet to be determined due to the lack of a permissive cell line or small animal model to support NV replication.

In the present study we have used FCV as a model system with which to identify and dissect the role of RNA-protein interactions involved in the translation and/or replication of members of the *Caliciviridae* family. We show that PTB interacts 70 specifically with the FCV genomic and sub-genomic RNA 5' sequences. Using RNA interference we further demonstrate that PTB is required for the efficient replication of FCV in cell culture, in a temperature-dependent manner. This data is consistent

with a role for PTB as an RNA chaperone to stabilise the folding of the viral RNA into a functional state, and is the first functional characterisation of role for a host RNA-binding protein in calicivirus replication.

Materials and Methods

80 Materials

FCV, strain Urbana, was generated by transfection of RNA transcripts derived from the full length infectious clone pQ14 (Sosnovtsev & Green, 1995) into Crandell-Reese feline kidney (CRFK) cells. Antisera to the FCV RNA polymerase p76 was generated by immunization of New Zealand White rabbits with recombinant p76, purified as described previously (Wei et al., 2001), from a construct kindly provided by Craig Cameron (Penn State). Antiserum to hnRNP I (PTB) was purchased from Santa Cruz Biotechnology. Antiserum to GAPDH was purchased from Ambion. Antiserum to FCV Urbana capsid was kindly supplied by Stanislav Sosnovtsev (NIH, Bethesda).

90 RNA affinity columns

The 5' extremities of the FCV genomic and subgenomic RNAs, corresponding to nucleotides 1-245 and 1-284 of the genomic and subgenomic RNAs respectively, were PCR amplified from the FCV infectious clone pQ14, using the primers detailed in Table 1. Primer pairs consisted of IGRDG 13 and 18 for the genomic 5' end and IGRDG 19 and 28 for the subgenomic 5' end (Table 1). Primers were designed to incorporate a 5' T7 promoter sequence to allow the production of RNA transcripts using T7 RNA polymerase. *E. coli* 5S ribosomal RNA (Roche) was used as non-

specific RNA. RNA transcripts were gel purified as described (Mellits et al., 1990) and 200 µg were coupled to 250 µl (packed bed volume) of cyanogen bromide-activated sepharose 4B (Sigma) as previously described (Kaminski et al., 1995). To
100 isolate proteins that interact specifically with the 5' sequences of the calicivirus genomic and subgenomic RNAs, 50 µl (bed volume) of RNA coupled to sepharose 4B were incubated with 100 µl of nuclease-treated HeLa S10 extract, for 1 h at 4°C. HeLa S10 extracts were prepared as described (Molla et al., 1991). Unbound proteins
105 were removed by extensive washing with S10 buffer (40 mM Hepes pH 8.0, 120 mM KOAc, 5.5 mM MgOAc, 10 mM KCl, 6 mM DTT) and the bound proteins eluted with 100 µl SDS-PAGE sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to PVDF (Millipore) and immunoblotted for the presence of PTB.

110 Purification of GST-PTB

Human PTB1 fused to GST, referred to as GST-PTB herein, was expressed and purified from a construct kindly provided by Richard Jackson (University of Cambridge). The protein was purified from *E.coli* on a glutathione-sepharose column according to the manufacturers' instructions (GE Healthcare). Finally, purified GST-
115 PTB was dialysed against dilution buffer (50 mM Hepes pH 7.6, 1 mM DTT, 1 mM MgCl₂ and 20 % glycerol) and stored at -80°C until required.

Electrophoretic mobility shift assays (EMSAs)

PCR products for the synthesis of radiolabelled FCV 5' positive sense genomic and
120 subgenomic RNA probes, encompassing nucleotides 1-245 and 1-284 of the genomic and subgenomic RNAs respectively, as well as unlabelled competitor RNAs, were generated using the primers detailed in Table 1. Labelled and unlabelled transcripts

were generated by *in vitro* transcription, in the presence of P³² α -GTP where required. All transcripts were purified by electrophoresis on a denaturing urea acrylamide gel, followed by passive elution as described (Mellits et al., 1990) prior to use in EMSA assays. Typically, EMSA reactions contained 85 nM radiolabelled probe, 500 ng GST-PTB, 5 mM Hepes pH 7.6, 25 mM KCl, 2.5 mM MgCl₂, 1mM DTT, 4% glycerol and 10 μ g of yeast tRNA (Sigma). Reactions were incubated at 30°C for 10 minutes prior to separation using a native 4% acrylamide gel (acrylamide:*bis*-acrylamide 19:1) containing 5% glycerol. Gels were dried and exposed to phosphor screens to allow quantification.

RNA interference-mediated knockdown of PTB

For siRNA mediated knock down of PTB expression, CRFK cells were transfected with PTB P1 siRNAs (Domitrovich et al., 2005), target sequence AACUCCAUCAUCCAGAGAA using Lipofectamine 2000 according to the manufacturers' instructions (Invitrogen). Although the sequence of feline PTB is not available, the ability of PTB P1 siRNAs to efficiently lead to a reduction in feline PTB levels would suggest a high degree of sequence conservation. CRFK cells were treated in the same way with siRNAs directed towards GFP as a control (Dharmacon). To monitor effective functional PTB knockdown, siRNA-treated CRFK cells were transfected with a poliovirus type 3 luciferase replicon (Goodfellow et al., 2003) that is defective in replication due to a mutation in the 3' end (mut 4 as described in Meredith et al., 1999) and luciferase levels monitored 16 hours post transfection.

One step growth curve

For the one step growth curve of FCV at 32°C, CRFK cells previously treated with PTB or GFP siRNAs were infected at a density of 10⁶ cells per 35mm dish, with FCV at a multiplicity of infection of 4 TCID₅₀ per cell. At various times post infection, virus yield was measured by TCID₅₀ and the levels of the viral polymerase (p76), capsid, PTB and GAPDH were determined by western blot for each time point.

For the temperature gradient growth of FCV in cells treated with PTB or GFP siRNAs, 2x10⁵ cells were infected in suspension, at a multiplicity of infection of 4 TCID₅₀ per cell. Infections were carried out in an Eppendorf Mastercycler gradient thermal cycler at temperatures between 32 and 39°C with increments of 1°C (±0.1 °C). To maintain the correct pH, culture media was supplemented with 25mM HEPES, pH 7.6. Cells were lysed at 6 hours post infection and analysed by western blot with antisera to FCV p76 to monitor viral protein synthesis, PTB and GAPDH.

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Northern blot analysis

RNA was extracted from cells at various times post infection using the Genelute purification system (Sigma). RNA (1 µg) was treated with glyoxal for 30 minutes at 65°C (Ambion) prior to separation on a 0.8% agarose gel. RNA was transferred to nitrocellulose (Amersham Biosciences) under mild alkaline conditions by capillary transfer (Sambrook & Russel, 2001). RNA was detected using an anti-sense RNA probe consisting of nucleotides 5297-7683, encompassing the entire sub-genomic RNA.

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Results

PTB binds to the 5' sequences of the FCV genomic and subgenomic RNAs.

To identify cellular RNA-binding proteins which play a role in the calicivirus life cycle, affinity columns containing the 5' sequences of the FCV genomic and subgenomic RNAs were generated by coupling *in vitro* transcribed RNAs to cyanogen bromide-activated sepharose 4B. In the absence of any functional data with regards to the position of RNA sequences required for genome translation and/or replication, we chose to use the 5' extremities of both the genomic and subgenomic RNAs (nt 1-245 and 1-284, respectively) as these regions are predicted to contain significant levels of RNA structure (data not shown). Cytoplasmic extracts from HeLa cells were used for affinity purification of host cell factors that specifically interact with the RNA elements from the two regions. HeLa cells were chosen as a source of protein as although not permissive for FCV infection, high levels of virus can be recovered after transfection of VPg-linked FCV RNA (data not shown). Hence, all the necessary factors for efficient genome translation and replication are present and the inability to support FCV infection is likely to reflect a lack of a suitable cellular receptor or a defect in virus entry.

We initially chose to examine the presence of PTB in the eluates from the RNA affinity columns as PTB has previously been shown to bind the 5' and 3' extremities of the NV genome (Gutierrez-Escolano et al., 2000, Gutierrez-Escolano et al., 2003). In addition, the presence of PTB binding site consensus sequences (UCUU) (Singh et al., 1995) within the 5' extremities of both genomic and sub-genomic RNAs (data not shown) further suggested this protein may bind to this region. In agreement with this, PTB was detected in the eluates from the 5' genomic and 5' sub-genomic RNA affinity columns (Fig 1A), whereas only background levels of PTB were recovered

from columns containing the *E.coli* 5S ribosomal RNA or columns lacking RNA (Fig 1A).

200 PTB interacts directly with the 5' extremities of the FCV genomic and sub-genomic RNAs

To confirm that PTB interacts directly with the FCV RNA, rather than an indirect interaction via an additional cellular factor, eletrophoretic mobility shift assays (EMSAs) were performed using recombinant GST-PTB. GST-PTB was found to
205 interact with RNA probes encompassing nucleotides 1-245 and 1-284 of the FCV genomic and sub-genomic RNAs, respectively (Fig 1B and C). GST alone was found not to interact with either of the target RNAs (data not shown). A complex with apparent reduced mobility, present in the absence of recombinant PTB (highlighted with an asterisk in Fig 1B), is likely to represent dimeric probe as it could be resolved
210 by annealing of the RNA probe prior to use (data not shown).

To determine the specificity of the interaction, the effect of adding an excess of unlabelled RNA competitors to EMSA reactions was examined. A non-specific RNA consisting of the poliovirus 2C *cis*-acting replication element (Goodfellow et al., 2000) did not inhibit the interaction of PTB with the genomic or sub-genomic RNA
215 probes (Fig 1B and C). In addition, the 3' sequences of the FCV genome (nucleotides 7574-7683) had no effect on the PTB-5' end complex (data not shown). Homologous unlabelled FCV genomic and sub-genomic RNA 5' extremities were found to inhibit complex formation efficiently, confirming a specific interaction between PTB and the FCV genomic and sub-genomic RNA extremities (Fig 1B and C).

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PTB is required for the efficient replication of caliciviruses in a temperature dependent manner

To analyse a role for PTB in the FCV life cycle, CRFK cells were transfected with siRNAs directed towards PTB and the levels of PTB monitored by western blot analysis. Transfection of PTB-specific siRNAs resulted in an 80% decrease in PTB levels compared to cells transfected with siRNAs directed towards GFP (Fig 2A). To confirm a 'functional effect' of the reduced levels of PTB in CRFK cells, the effect of PTB siRNAs on poliovirus IRES-mediated translation was examined. A poliovirus replicon, defective in replication due to a mutation in the 3' un-translated region, (mut 4 as described in Meredith et al., 1999) was transfected into CRFK cells previously treated with PTB or GFP specific siRNAs (Fig 2B). PTB knockdown resulted in a greater than 200 fold decrease in poliovirus IRES-directed expression of luciferase , in agreement with previous work (Florez et al., 2005).

To examine the functional role of PTB in the FCV life cycle, the effect of PTB knockdown on FCV polymerase (p76) production was examined over a range of temperatures by western blot analysis and quantified by densitometry (Fig 3). PTB siRNAs were found to have a greater effect on p76 levels at temperatures above and below 37°C. Whereas at 37°C p76 levels were 65% of that observed in GFP siRNA transfected cells, at 32°C and 39°C the levels were reduced to 20% and 18% respectively (Fig 3).

The effect of PTB knockdown on FCV replication at 32°C was examined in more detail by analysing virus yield, RNA synthesis and the levels of viral protein (Fig 4). 32°C was chosen for more detailed analysis as at temperatures above 37°C, the replication and yield of FCV (strain Urbana) was significantly reduced (data not shown). At 32°C however, although replication occurred at a reduced rate, similar

levels of virus and viral proteins were produced. PTB knockdown was found to have a dramatic effect on FCV yield at 32°C, resulting in a 100 fold reduction in titre at 6 hours post infection, with a 21 and 15 fold reduction seen at 9 and 18 hours respectively (Fig 4A). Similarly, the appearance of cytopathic effect was inhibited, with a proportion of cells remaining intact after 18 hours of infection in PTB siRNA-treated cells, whereas infection of GFP siRNA-transfected cells resulted in a typical virus induced cytopathic effect (Fig 4B).

The viral polymerase (p76) and major capsid protein were quantified as a measure of the levels of translation from the genomic and sub-genomic RNAs, respectively (Fig 4C). Levels of p76 and capsid were reduced by similar levels in PTB siRNA-transfected cells, resulting in a 6-fold reduction in protein synthesis at 6 hours post infection.

The levels of the FCV genomic and sub-genomic RNAs were examined by northern blot and, as observed for viral protein production, levels were reduced in PTB siRNA-treated cells (Fig 4D and E). Genomic and sub-genomic RNA production was inhibited to the same degree (data not shown), indicating that PTB knockdown did not have a differential effect on sub-genomic RNA synthesis. Genomic RNA levels were 42%, 56% and 75% of the levels observed in GFP siRNA treated cells at 6, 9 and 18 hours post infection, respectively (Fig 4D and E).

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Discussion

In general, the 5' and 3' extremities of viral RNA genomes must fold into defined three dimensional structures in order to adopt a functional state. As a result of structural promiscuity and an abundance of intramolecular interactions, alternative non-functional conformations are also adopted, significantly slowing the appearance of a functional conformation (Treiber & Williamson, 2001). As a consequence, it has been suggested that RNA folding requires the aid of proteins with chaperone activity that possibly trap or resolve misfolded structures (Herschlag, 1995).

The interaction of host cell nucleic acid-binding proteins with the genomes of positive stranded RNA viruses is known to play a role in many aspects of the virus life cycle. The majority of these proteins are predicted to function as RNA-chaperones, allowing the viral RNA to adopt a functional conformation. In the case of cellular or viral IRES elements, the binding of host cell proteins to RNA is thought to lead to structural rearrangements, usually in close proximity to the protein binding site, resulting in the formation of a conformation suitable for translation initiation (Martinez-Salas et al., 2001). These proteins, known as IRES trans-acting factors (ITAFs), include PTB isoforms, poly(rC) binding protein, La, hnRNP K, unr (upstream of *N-ras*), nucleolin, and many others (reviewed in Stoneley & Willis, 2004).

Here we report the identification of PTB as a host cell protein required for efficient calicivirus replication in a temperature-dependent manner. PTB is a regulator of alternative splicing pathways (Lin & Patton, 1995) and is predominantly found in the nucleus, although it can shuttle between the nucleus and cytoplasm (Ghetti et al., 1992). Recent work has demonstrated that phosphorylation of PTB by protein kinase A results in the accumulation of PTB in the cytoplasm (Xie et al., 2003). PTB was found to be evenly distributed between the nucleus and the cytoplasm during

poliovirus infection, probably as a result of the inhibition of host cell transcription and the effects of virus infection on nuclear import (Back et al., 2002). Cleavage of PTB has also been observed during poliovirus infection with the resultant cleavage products inhibiting viral translation (Back et al., 2002). This inhibition is thought to contribute to the switch from translation to replication (Back et al., 2002). Whether a similar cleavage and redistribution of PTB occurs during FCV infection is currently being determined.

A more important role for PTB at temperatures above and below 37°C would agree with the hypothesis that PTB functions as an RNA chaperone, aiding in the correct folding of viral RNA. The lack of a significant effect of PTB siRNAs on FCV replication at 37°C, the temperature at which the virus has been repeatedly passaged, is intriguing. Although viral RNA polymerase levels were reduced to 65% of the levels observed in control siRNA treated cells (Fig 3), no effect on virus titre was observed (data not shown). It is possible that the RNA chaperone activity of PTB is only required in conditions where non-functional RNA structures are stabilised (e.g. temperatures <37°C) or functional structures are destabilised (e.g. temperatures >37°C). It is important to note that although RNA interference-mediated knockdown of PTB significantly reduced PTB levels, detectable PTB remained (Fig 2A). Hence, the PTB remaining after siRNA-mediated knockdown may be sufficient for correct RNA folding at 37°C, but increased levels are required to maintain a functional conformation at non-favourable temperatures or in circumstances where other RNA chaperoning factors are absent. Previous studies on EMCV IRES-mediated translation have demonstrated that although a wild type IRES directing EMCV polyprotein synthesis does not require PTB for efficient translation, an IRES with an enlarged A-rich bulge is highly dependent on PTB (Kaminski & Jackson, 1998). As a result, it

was presumed that PTB plays a significant role in maintaining an appropriate higher order structure for translation initiation only when non-functional conformations are apparent (Kaminski & Jackson, 1998). This observation would fit with our hypothesis
320 that the function of PTB in the calicivirus life cycle is only required under “unfavourable” conditions.

An additional factor that may affect the relative requirement of RNA for a particular RNA chaperone is the expression levels of other interacting host cell factors. The relative expression levels of such factors may be significantly different in primary
325 tissues compared to the levels observed in immortalised cell lines. The temperature of the environment in which the virus replicates is also likely to be a determining factor in the relative contribution of PTB to the virus life cycle. Given that feline body core temperature ranges from 38 to 39°C and can rise to 41.5°C during FCV infection (Poulet et al., 2005), we would predict PTB plays a functional role in FCV replication
330 *in vivo*.

Similar temperature sensitivity of the RNA chaperoning activity of PTB has also been highlighted during *trans*-splicing of the thymidylate synthase group 1 intron (Belisova et al., 2005). Whereas *trans*-splicing occurs in a protein-independent manner at 55°C, the reaction is significantly reduced at 37°C due to an inability of the RNA to fold
335 into a splicing competent conformation. PTB was found to stimulate the rate of *trans* splicing at 37°C by three fold (Belisova et al., 2005). This increased requirement for PTB at reduced temperature was due to stabilisation of RNA structures that were non-functional for splicing.

Previous data on the interaction of PTB with the NV genome demonstrated that PTB
340 interacts with both the 5' and 3' extremities of the viral genomic RNA (Gutierrez-Escolano et al., 2000, Gutierrez-Escolano et al., 2003). However, in the current study

we failed to detect a PTB-3' end interaction by both UV cross-linking and EMSA (data not shown). This may suggest that the caliciviruses differ in their requirements for host cell RNA binding proteins.

345 We have recently reported that caliciviruses use a novel translation initiation mechanism not seen in any other animal RNA viruses, whereby a protein covalently linked to the 5' end of viral RNA (VPg) functions as a proteinaceous cap substitute, recruiting components of the eIF4F complex (Goodfellow et al., 2005). Previous work has also demonstrated an interaction of norovirus VPg with eIF3 (Daughenbaugh et
350 al., 2003) although a role for this interaction in calicivirus translation has yet to be determined. Given the previous reports of a role for PTB in viral translation (reviewed in Stoneley & Willis, 2004), it is possible that PTB also plays a role in calicivirus translation. From the current study we are unable to determine the specific role of
355 PTB in the calicivirus life cycle. This is primarily due to a lack of a specific inhibitor that prevents RNA replication but allows translation to occur, analogous to the effect of guanidine hydrochloride on poliovirus (Rightsel et al., 1961). Preliminary results with rabbit reticulocyte lysates depleted of PTB would suggest that PTB does not play a significant role in calicivirus VPg-dependent translation (data not shown). However, confirmation of this awaits the development of a reproducible method of specifically
360 depleting PTB from translation competent extracts prepared from permissive cells, as rabbit reticulocyte lysates may not faithfully reproduce the effect of PTB depletion on translation alone, due to the increased levels of translation initiation factors and the lack of compartmentalisation. Although previous work has highlighted that the primary role for PTB in the life cycle of many positive stranded RNA viruses is at the
365 level of viral translation (Belsham & Sonenberg, 2000), a role in coronavirus replication has also been observed (Huang & Lai, 1999, Li et al., 1999). Previous

work has also shown that PTB binds to 3' sequences (the "X" sequence) in the HCV genome and may play a role in HCV replication (Domitrovich et al., 2005, Gontarek et al., 1999). Hence it is possible that PTB plays no role in calicivirus translation but
370 instead is required for some aspect of viral RNA replication.

FCV infection generally results in an oral or upper respiratory tract infection (Gaskell et al., 2004), however recent isolates can result in a highly contagious febrile haemorrhagic syndrome (Hurley & Sykes, 2003). FCV vaccines, based on live attenuated or inactivated preparations are available, but whilst they are effective at
375 preventing disease, they do not prevent infection. Antigenic variation has resulted in new strains for which vaccines do not offer protection and there are also concerns that the currently available vaccines may contribute to FCV prevalence (Radford et al., 2001). Hence the current study, in addition to yielding insights into the general biology of the calicivirus life cycle, may allow the rational design of attenuated FCV
380 vaccines, as mutations in PTB binding sites have previously been shown to contribute to poliovirus attenuation (Gutierrez et al., 1997).

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Figure Legends

Figure 1. PTB interacts with the 5' sequences of the feline calicivirus genomic and subgenomic RNAs. Western blot analysis of proteins isolated by RNA affinity columns for the presence of PTB (A). RNA affinity columns were generated by coupling the FCV genomic (5'G), subgenomic (5'SG) or *E.coli* 5S ribosomal RNA (5S rRNA) to sepharose. Proteins bound to RNA affinity columns or sepharose with RNA (-) were separated by SDS-PAGE and immunoblotted with antiserum to PTB. Electrophoretic mobility shift assay (EMSA) demonstrating the interaction of recombinant GST-PTB with the FCV genomic (B) and sub-genomic (C) RNAs. The specificity of complex formation was examined by incubation with non-specific RNA (NS) at 50 fold molar excess or FCV genomic and sub-genomic RNAs at 50, 5 and 0.5 fold molar excess. An asterisk denotes an alternative conformation of the FCV genomic RNA that can be formed in the absence of protein. The interaction of probe (P) with recombinant PTB resulted in the formation of a complex (C) with reduced mobility.

Figure 2. siRNA mediated inhibition of PTB in feline kidney cells. (A) Western blot analysis of cells transfected with siRNA directed towards either GFP or PTB. (B) The effect of either GFP or PTB specific siRNAs on poliovirus IRES-mediated translation was examined using a poliovirus luciferase containing replicon deficient in replication. Results show relative luciferase expression from the replicon expressed as relative light units (RLU).

Figure 3. PTB is required for feline calicivirus replication in a temperature dependent manner. Western blot analysis of cells transfected with either GFP (G) or PTB (P) specific siRNAs at a variety of temperatures using antisera directed towards the viral RNA dependent RNA polymerase p76, PTB or GAPDH. Densitometry was used to quantify the p76 levels. To control for any non-specific effects of transfection of siRNAs or temperature on calicivirus replication, the levels of p76 are expressed as a percentage of the levels observed in the GFP siRNA treated control cells.

Figure 4. Feline calicivirus replication at 32°C is inhibited by PTB siRNAs. (A) One step growth curve analysis of FCV in cells treated with either GFP or PTB specific siRNAs at 32°C. Virus yield was determined by TCID₅₀. (B) Phase contrast microscopy highlighting the delayed cytopathic effect observed in PTB siRNA treated cells. (C) Western blot analysis of the viral capsid, viral RNA polymerase p76, PTB and GAPDH during the course of infection. Note that a precursor of p76, composed of p76 linked to VPg (p89) is also observed. (D) Northern blot detection of viral genomic and sub-genomic RNAs during the course of virus infection. The levels of viral genomic RNA were quantified and expressed relative to the levels observed in the GFP siRNA treated cells at 18 hours post infection (E).

Table 1. Oligonucleotides used during this study

Name	Sequence
IGRDG 13	<u>TAATACGACTCACTATAGGGG</u> TAAAAGAAATTTGAGACAATGTC
IGRDG 18	GGATCTGTCCTTTGGAAG
IGRDG 19	<u>TAATACGACTCACTATAGGGG</u> TGTTTCGAAGTTTGAGCATG
IGRDG 28	ATTCCAGTGCATTGGAGGG

Footnotes: Sense primers were designed to include a T7 promoter sequence (underlined).

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Figure 1 Karakasiliotis *et. al.*

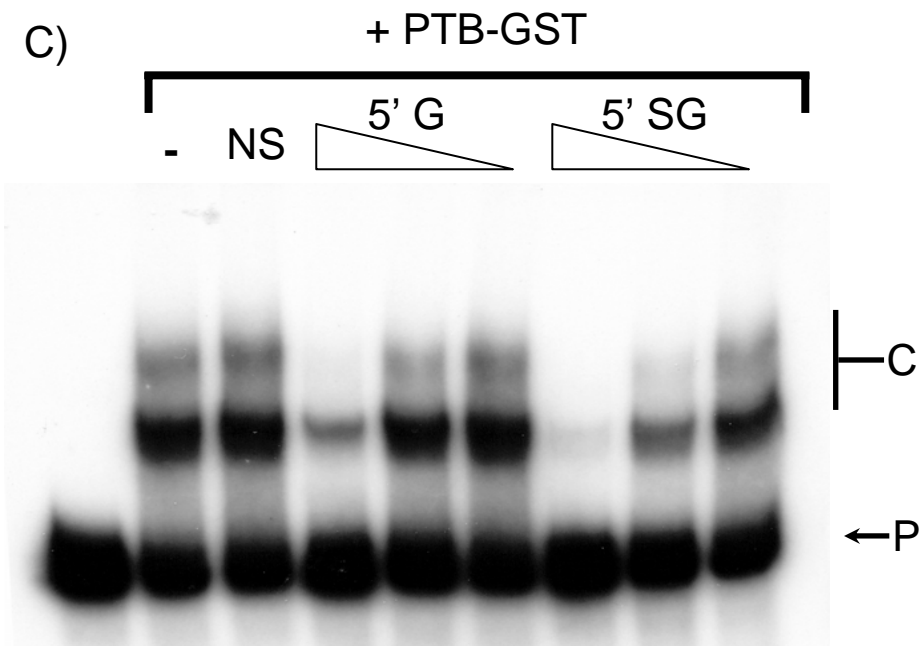
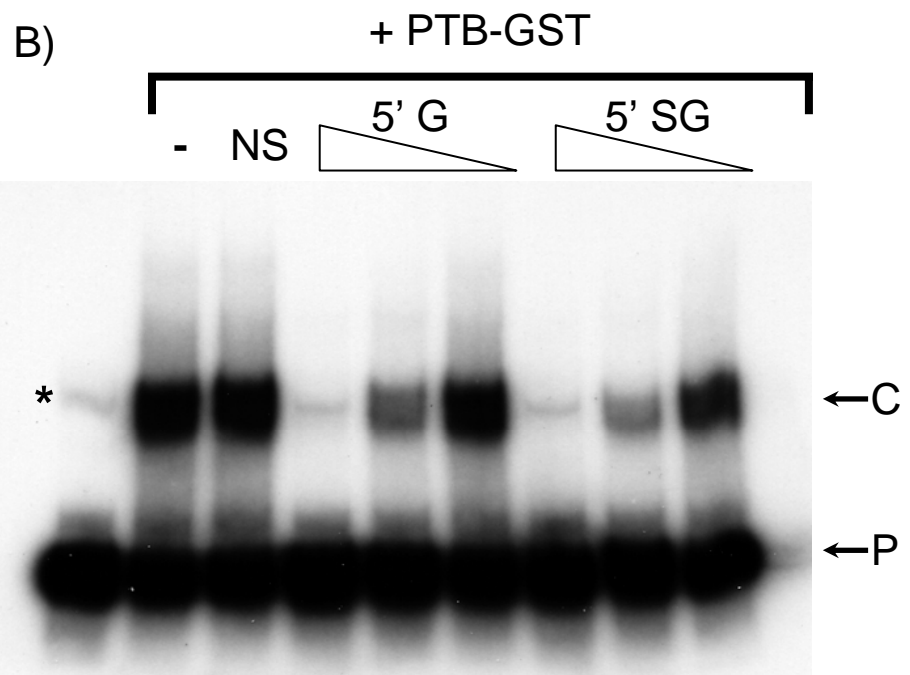
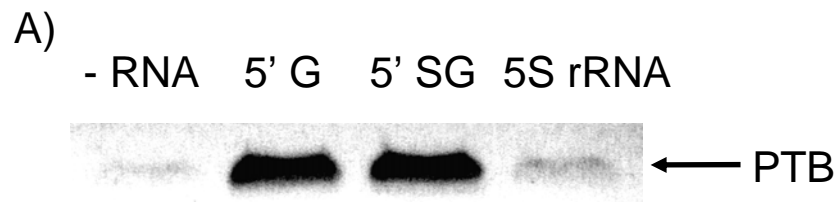


Figure 2 Karakasiliotis *et. al.*

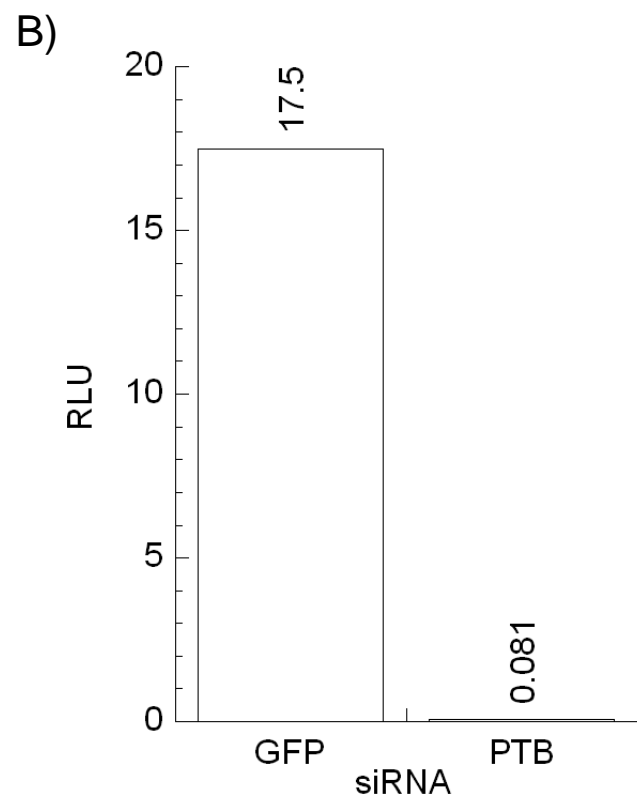
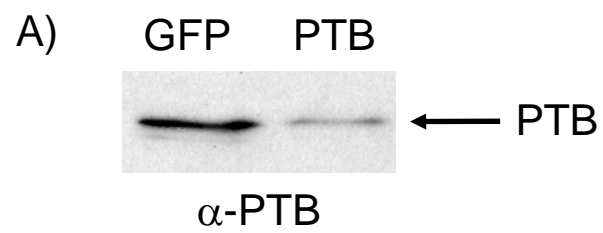


Figure 3 Karakasiliotis *et. al.*

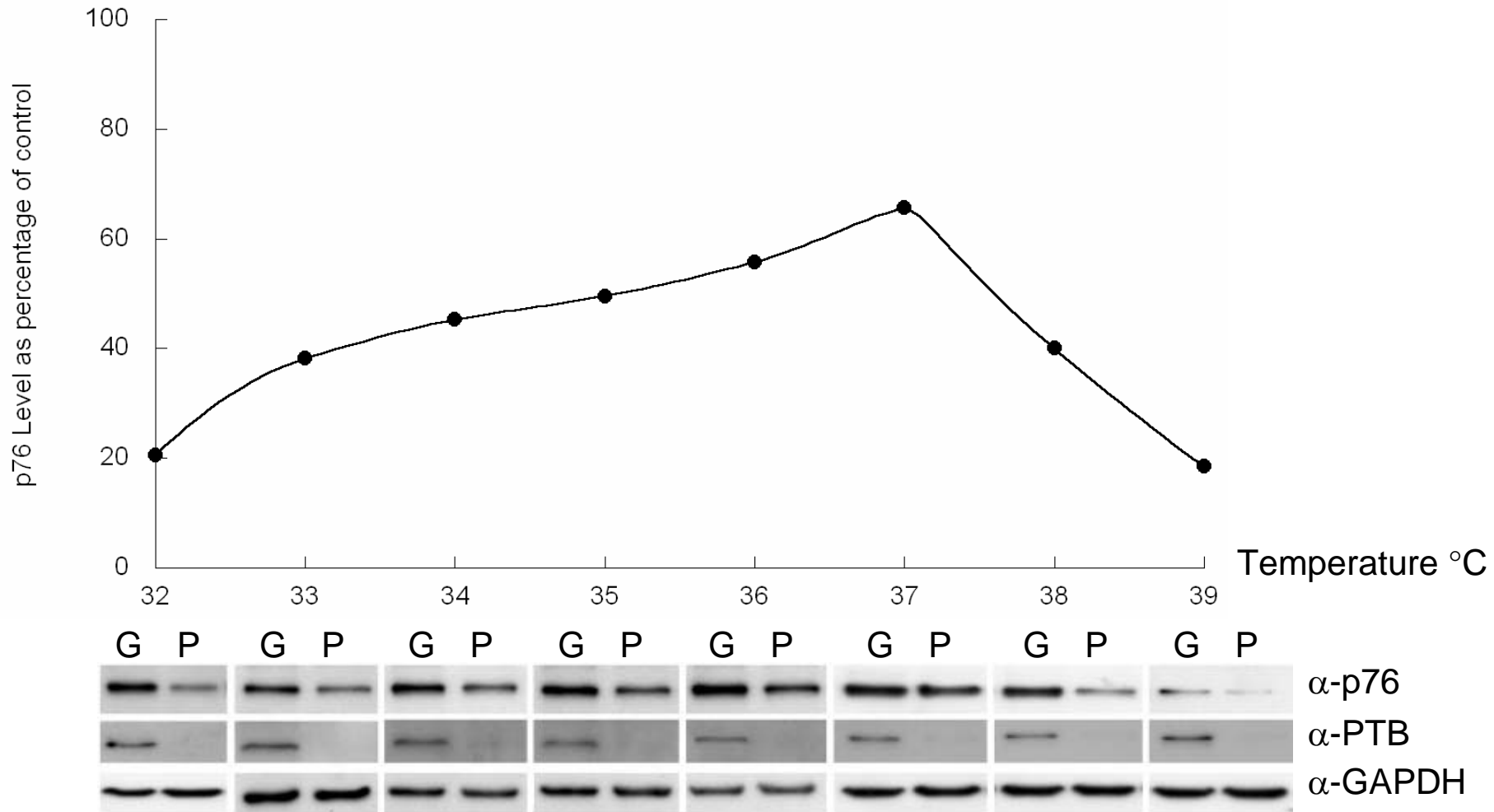


Figure 4 A&B Karakasiliotis *et. al.*

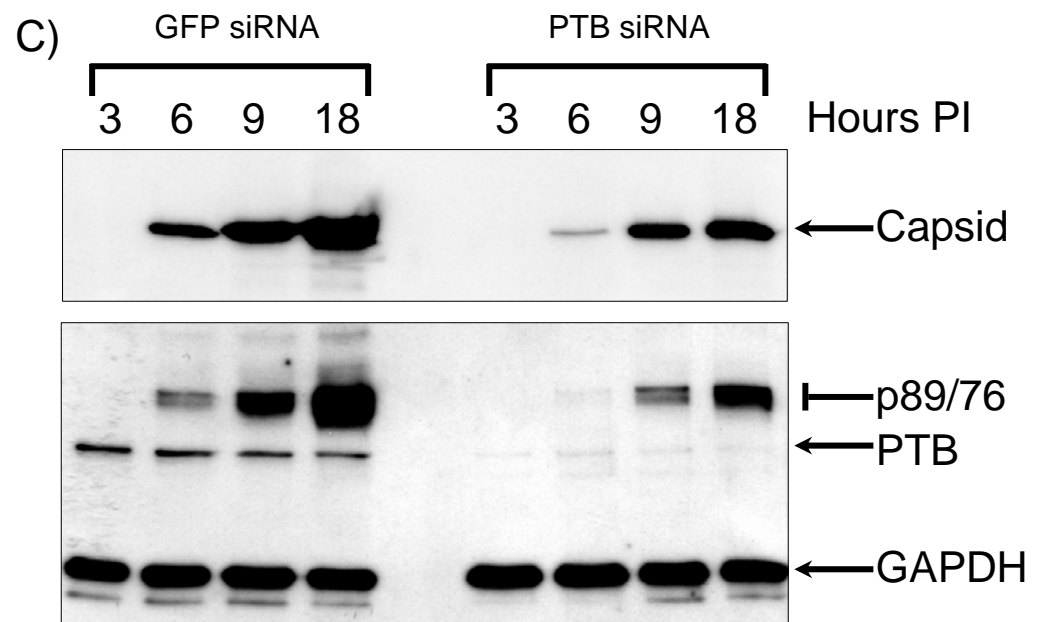
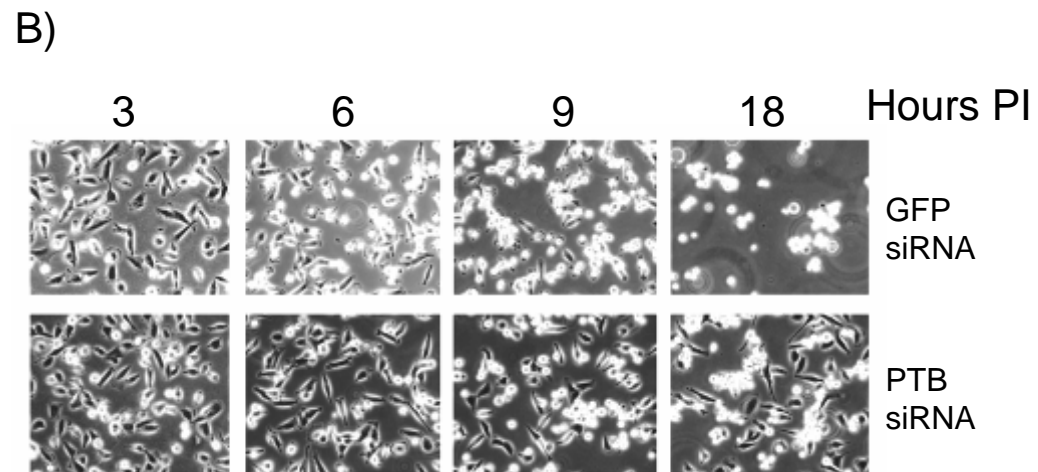
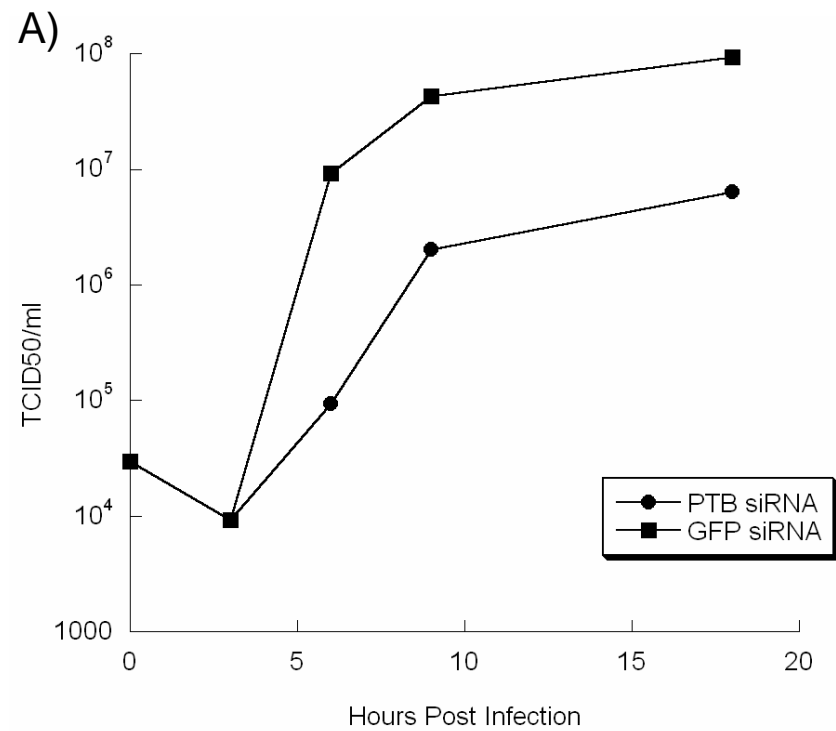
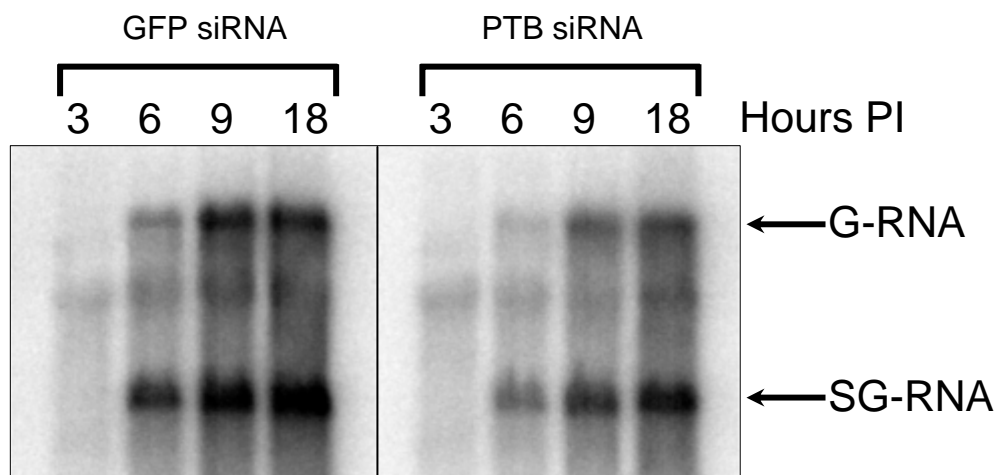


Figure 4 C&D Karakasiliotis *et. al.*

D)



E)

