Short communication

Detection of the ORF3 polypeptide of feline calicivirus in infected cells and evidence for its expression from a single, functionally bicistronic, subgenomic mRNA

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Feline calicivirus (FCV) is a small positive-stranded RNA virus within the family Caliciviridae. Its genome is 7690 nucleotides in length and encodes three open reading frames (ORFs). The smallest, ORF3, is located at the extreme 3' end of the genome and can potentially encode a polypeptide of approximately 12 kDa. In this paper, we report the identification of an ORF3-encoded polypeptide in FCV-infected cells using an antiserum raised against a bacterially-expressed bacteriophage T7 gene 10–ORF3 fusion protein. Although a small mRNA of 0.5 kb, which could potentially encode ORF3, has been described, reports on the number and size of FCV subgenomic RNAs have varied considerably. To clarify the situation, RNAs from FCV-infected cells were labelled in vivo using [32P]orthophosphate, an approach which provided definitive data. Only two RNA species were detected, the genomic RNA and a subgenomic mRNA of 2.4 kb. The 5' end of the subgenomic mRNA was mapped to position 5227 on the genomic RNA using RNA sequencing and primer extension methods. RNA isolated from FCV-infected cells in which no subgenomic RNA smaller than 2.4 kb was detectable directed the synthesis in rabbit reticulocyte lysate of the ORF3-encoded polypeptide. Furthermore, a synthetic RNA copy of the 2.4 kb subgenomic mRNA of FCV, containing both ORF2 and 3, directed the synthesis of both ORF2 and ORF3 polypeptides in the in vitro translation system. These data strongly suggest that ORF3 is expressed from the 2.4 kb subgenomic RNA and that this RNA is functionally bicistronic. The possible mechanisms by which ORF3 is expressed are discussed.

The caliciviruses are a family of positive-sense ssRNA viruses whose genomes are approximately 7-8 kb in length. Members include feline calicivirus (FCV), rabbit haemorrhagic disease virus (RHDV), Norwalk virus (NV), San Miguel sealion virus (SMSV) and vesicular exanthema of swine virus (VESV). The complete sequence of FCV has been determined and encodes three potential coding ORFs (Carter et al., 1992a). The largest, ORF1, is expressed as a polyprotein which contains homologues to picornavirus non-structural genes 2C, 3C and 3D (Neill, 1990; Carter et al., 1992a). ORF2 encodes the capsid precursor which is processed proteolytically to generate the mature capsid protein (Carter et al., 1992b). ORF3, located at the 3' end of the genome, is 318 nt long and potentially encodes a basic polypeptide of approximately 12 kDa. The predicted protein shows 90–94% identity at the amino acid sequence level amongst five different FCV strains (Seal et al., 1993). A homologous small ORF is also present at the 3' end of NV, RHDV, VESV and SMSV (Carter, 1990; Meyers et al., 1991; Neill, 1992; Jiang et al., 1993) and is conserved at the amino acid sequence level within members (Milton et al., 1992; Neill, 1992; Seal et al., 1993). The conservation of this hypothetical polypeptide argues that it plays a functional role.

Calicivirus replication involves the synthesis of at least one subgenomic RNA. Analysis of RNA from SMSV-infected cells revealed four classes of RNA molecules; 36S genomic RNA, a 22S subgenomic ssRNA molecule and RNAs corresponding to double-stranded forms of genomic and subgenomic molecules (Ehresmann & Schaffer, 1977, 1979). Similar species were detected in VESV-infected cells (Black et al., 1978). Analysis of RHDV-associated RNAs revealed only the genomic RNA and a 3' co-terminal subgenomic RNA of 2.2 kb.

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The situation with FCV appeared to be more complex. In addition to genomic RNA, Northern blot analysis has identified a number of additional mRNAs in different studies. These include 3' co-terminal RNAs of 4.8, 4.2 and 2.4 kb (Neill & Mengeling, 1988) and 5.3, 4.3, 3.6, 2.7, 1.9, 1.5 and 0.55 kb (Carter, 1990). Double-stranded forms of the genomic RNA and the 2.4 kb message (Neill & Mengeling, 1988; Neill et al., 1991) and negative strands corresponding to the genomic and 5.3, 4.3, 3.6 and 2.7 kb subgenomic RNAs were also detected (Carter, 1990). Thus there are considerable differences between the number of RNA species detected in FCV-infected cells and those found in cells infected by a number of other caliciviruses. These discrepancies may have resulted from differences in the RNA isolation methods used in the analyses and the sources of viral RNA. In view of the differing reports on the number and size of FCV RNAs we decided to clarify the situation using [32P]orthophosphate labelling of RNA synthesized in FCV-infected cells.

Confluent Crandell-Reese feline kidney (CRFK) cell monolayers starved of phosphate were infected with FCV strain F9 at an m.o.i. of 10. The cells were incubated in phosphate-free Glasgow MEM supplemented with 2% fetal calf serum, [32P]orthophosphate (80 µCi/ml) and actinomycin D (2 µg/ml). At 6 h post-infection (p.i.), RNA from infected or mock-infected monolayers was isolated using guanidinium isothiocyanate, oligo(dT)-selected using the Promega PolyATtract mRNA Isolation System, separated on a 1% agarose–formaldehyde gel and the RNAs visualized by autoradiography (Fig. 1 a). Two RNA species of approximately 7.6 kb and 2.4 kb were detected in infected cells; no other RNA species were detected even after prolonged exposure. Primer extension analysis and RNA sequencing mapped the 5' end of the 2.4 kb subgenomic mRNA to position 5227 on the FCV genome (Fig. 1 b) as has been reported previously for FCV strain CFI68 (Neill et al., 1991). The sequence at the 5' end of the 2.4 kb mRNA is closely related to the sequence at the 5' end of the genomic RNA and this relationship extends into the coding regions of ORFs 1 and 2. The related sequences at the 5' end of genomic and subgenomic RNAs may play a role in the transcription of these mRNAs. Sequences similar to those found at the 5' end of both genomic and subgenomic RNAs were not found elsewhere within the FCV genome, supporting the idea that the synthesis of another subgenomic mRNA is unlikely.
Fig. 2. (a) Testing of the rabbit anti-ORF3–gene 10 fusion protein antiserum. pTPH5 encoding ORF3 was transcribed \textit{in vitro} and the RNA translated in RRL. The products were immunoprecipitated with anti-ORF3–gene 10 antisera, its ‘prebleed’ serum or rabbit anti-FCV antisera and electrophoresed alongside total lysate. (b) Identification of ORF3 in FCV-infected cell lysates. Mock- and FCV-infected cell lysates labelled with $[^{35}\text{S}]$methionine in the presence of actinomycin D were analysed directly or immunoprecipitated with rabbit anti-FCV antisera or rabbit anti-ORF3–gene 10 fusion protein antiserum. (c) Expression of ORF3 from \textit{in vitro} translation of RNA isolated from FCV-infected cells. RNA in which no species smaller than 2.4 kb could be detected was translated in RRL. The products were either analysed directly or immunoprecipitated with rabbit anti-ORF3–gene 10 fusion protein antiserum. All products were electrophoresed through a 17.5% SDS–polyacrylamide gel and visualized by autoradiography. Where indicated, low and/or high molecular mass (L/HMM) $^{14}\text{C}$-protein markers (Amersham) were used.

Fig. 1(c) shows the FCV ORFs and RNA transcripts; the 2.4 kb subgenomic mRNA comprises ORFs 2 and 3. To investigate whether ORF3 is expressed in FCV-infected cells, antisera were raised in rabbits against bacterially expressed ORF3–gene 10 fusion protein. A cDNA encoding ORF3 was cloned downstream of and in-frame with the bacteriophage T7 gene 10 coding sequence of the bacterial expression vector pET3xc. The ORF3–gene 10 fusion polypeptide was used to immunize rabbits.

To test the reactivity of the anti-ORF3 serum, a cDNA encoding ORF3 was cloned downstream of the T7 promoter of plasmid pGEM. This vector was linearized downstream of ORF3, transcribed with T7 polymerase and the RNA translated in the rabbit reticulocyte lysate (RRL) cell-free translation system (Promega) at 30 °C for 1 h in the presence of 0.75 μCi/μl $[^{35}\text{S}]$methionine. The translation product was immunoprecipitated with either the anti-ORF3 serum, its ‘prebleed serum’ or a rabbit anti-FCV antiserum. The immunoprecipitates were analysed on a 17.5% SDS–polyacrylamide gel alongside the total lysate and the products visualized by autoradiography (Fig. 2a). A product migrating at approximately 8 kDa was recognized specifically by the anti-ORF3 serum but not by ‘prebleed’ serum or rabbit anti-FCV antiserum. Having established the reactivity of the antiserum, we tested whether ORF3 is expressed in FCV-infected cells. CRFK cells were infected or mock-infected with FCV at an m.o.i. of 10 in the presence of 100 μCi/ml $[^{35}\text{S}]$methionine and 2 μg/ml actinomycin D. At 6 h p.i., the cells were harvested, immunoprecipitated with anti-ORF3 or rabbit anti-FCV antiserum and analysed on a 17.5% SDS–polyacrylamide gel alongside cell lysates (Fig. 2b). A polypeptide of approximately 8 kDa was immunoprecipitated by the anti-ORF3 antiserum in the infected cell lysate but not in the mock-infected lysate, confirming that ORF3 is expressed in FCV-infected cells. Furthermore, the polypeptide had the same molecular mass as the ORF3 product synthesized \textit{in vitro}. As expected, the anti-FCV antiserum recognized a product of the size expected for the FCV capsid protein. Products co-migrating with the capsid and ORF3 proteins were clearly visible in the unprecipitated infected cell lysate. From densitometry, we
Fig. 3. A synthetic 2.4 kb subgenomic mRNA can direct the synthesis of both ORF2 and ORF3 in RRL. (a) Schematic representation of plasmid pTC8, which encodes a cDNA of the 2.4 kb subgenomic mRNA of FCV downstream of a T7 promoter. (b) Position of the transcriptional start site in relation to the T7 promoter. (c) Plasmid pTC8 was linearized with SacI, transcribed in vitro with T7 RNA polymerase, translated in RRL in the presence of [35S]methionine and immunoprecipitated with rabbit anti-FCV or rabbit anti-ORF3 antisera. The immunoprecipitations and total lysate were analysed on a 17.5% SDS-polyacrylamide gel and the products visualized by autoradiography.

estimate that ORF3 is expressed at a level of approximately 10% of ORF2, taking into account that ORF2 contains 14 methionines, but ORF3 only one.

The template RNA for the production of ORF3 is uncertain. It has been suggested that a shorter subgenomic RNA of 0.5 kb may be present in FCV-infected cells (Carter, 1990) which could serve as the template. However, when oligo(dT)-selected FCV RNA was translated in RRL and the products immunoprecipitated with anti-ORF3 antisera (Fig. 2c), the ORF3 product was clearly seen despite the fact that in the mRNA used for the translation, no RNAs smaller than 2.4 kb were present (Fig. 1). It is therefore unlikely that ORF3 is expressed from a smaller subgenomic mRNA. This is supported by the demonstration that both ORF2 and ORF3 can be translated from the 2.4 kb subgenomic RNA alone. In this experiment, a cDNA encoding the full-length 2.4 kb subgenomic mRNA was cloned in pGEM downstream of a T7 promoter to create plasmid pTC8 (Fig. 3a). The cDNA was cloned in such a way that a synthetic transcript is generated which contains the authentic 5' end of the 2.4 kb mRNA (Fig. 3b). pTC8 was linearized with SacI, transcribed in vitro with T7 polymerase and translated in the RRL in the presence of [35S]methionine. The translation reaction was immunoprecipitated with anti-FCV or anti-ORF3 antisera, the products analysed on a 17.5% SDS-polyacrylamide gel and visualized by autoradiography (Fig. 3c). The 2.4 kb synthetic mRNA transcribed from pTC8, a single species stable during in vitro translation, directed the synthesis of both ORF2 and ORF3 polypeptides. Taking into account the methionine content of the two proteins, ORF3 is expressed at approximately 10% of the level of ORF2, a similar ratio to that seen in infected cells.

In this report we confirm that ORF3 is expressed in FCV-infected cells and has an apparent molecular mass of 8 kDa. Expression of ORF3 also occurs following in vitro translation of oligo(dT)-selected RNA isolated from FCV-infected cells in which no small subgenomic RNA (which could encode solely ORF3) was detected. ORF3 expression must therefore occur from either the genomic or subgenomic RNA. A synthetic 2.4 kb RNA encoding ORF2 and 3 could direct the translation of polypeptides from both ORFs in RRL, indicating that this RNA is functionally bicistronic. It is unlikely that ORF3 is expressed from smaller RNAs generated by degradation since it is expressed efficiently in infected cells, from RNA isolated from infected cells and from
RNA translated in vitro. The RNA cleavage would need to occur efficiently just 5' of the initiation codon of ORF3 as other AUG codons are present as close as 11 nt upstream.

How might the expression of ORF2 and 3 be achieved from a single mRNA species? It has been suggested (Neill, 1992) that ORF3 could be expressed by a -1 ribosomal frameshift which would result in an ORF2–ORF3 fusion polypeptide. This seems unlikely, since no such product was detected either in infected cells, in in vitro translations of RNA isolated from FCV-infected cells or from in vitro translation of the synthetic 2.4 kb subgenomic mRNA. Furthermore, signals characteristic of known -1 ribosomal frameshift signals (Brierley, 1995) are absent from the ORF2–ORF3 junction. It also seems unlikely that ORF3 is expressed by leaky scanning (Kozak, 1989). Although the ORF2 initiation codon is in a non-ideal context (GAGCAUGU), the scanning complex would have to fail to initiate at numerous AUGs within the ORF2 coding region, many of which are in good contexts. Two other mechanisms can be proposed to account for ORF3 expression. ORF2 overlaps ORF3 by 4 nt where the initiation codon of ORF3 overlaps the termination codon of ORF2 within the sequence AUGA. This close coupling of termination and initiation codons suggests that the two events may be linked and that expression of ORF3 may occur via a termination–reinitiation mechanism. Reinitiation of translation at a downstream AUG codon has been found to take place in both naturally occurring and artificially constructed bicistronic mRNAs (Peabody & Berg, 1986; Kozak, 1987; Horvath et al., 1990).

A second possibility is that ORF3 is expressed by an internal initiation type mechanism. There have been several reports of internal initiation occurring in eukaryotic RNAs, the best studied examples being of translational initiation on picornavirus RNAs (Oh & Sarnow, 1993). These RNAs are uncapped and have unusually long 5'-untranslated regions (UTR) which direct internal ribosome entry to initiate protein synthesis. RNA secondary structure and sequence elements within the UTR have been shown to be important in this process. It has been demonstrated that the 3c polypeptide of the coronavirus infectious bronchitis virus (IBV) is expressed by internal initiation on a capped mRNA (Liu & Inglis, 1992). In this case, the internal ribosome entry site lies within functional coding sequences (3a and 3b) which are positioned upstream of 3c. Similar elements believed to be important in the internal entry of ribosomes in picornviruses have been found within the 3a and 3b sequences of IBV (Liu & Inglis, 1992). For ORF3 to be expressed by internal initiation, elements within ORF2 must be present which direct internal ribosome entry.

References


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