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Transcription of feline calicivirus RNA

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Summary. We report here the cloning and 3' sequence determination of feline calicivirus strain F9. Subcloning the 3' terminus enabled the production of strand specific probes for RNA synthesis. We extend the number of virus specific RNAs detected intracellularly to 8, and show that numbers 1–5 are represented as negative strands which may serve as templates in the synthesis of these RNAs.

Introduction

The family *Caliciviridae* are non-enveloped viruses of distinctive morphology previously classified with the family *Picornaviridae* [17]. Like the picornaviruses, caliciviruses are positive-stranded and their genomes bear a covalently attached protein (VPg) at the 5' end [18]. However, caliciviruses differ from members of the picornavirus group in that RNA molecules smaller than the genome are synthesized intracellularly [7]. In this respect the caliciviruses may have more in common with the corona- or togaviruses. Ehresmann and Schaffer have studied cells infected with San Miguel sealion virus (SMSV) and reported 4 such species in addition to genomic RNA; a 22S subgenomic single stranded molecule, two double-stranded molecules each consisting of two strands of genomic or subgenomic RNA respectively and a heterogenous partially double-stranded form [8]. Black et al. [1] obtained broadly similar results working with Vesicular exanthema of swine virus (VESV) and indicated that a third single-stranded subgenomic RNA molecule (18S) was present. All the single-stranded molecules were polyadenylated and lacked cap structures at their 5' termini [8]. More recently, Neill and Mengeling have cloned FCV RNA and used these cDNA molecules to identify intracellular virus-specific RNA [13]. In this way these workers have identified three intracellular RNAs (4.8, 4.2, and 2.4 kb) as well as the genome (8.2 kb) and shown that these form a nested set of 3' co-terminal molecules similar to that observed in coronavirus infected cells. We have been working in a similar manner and have also cloned RNA

extracted from purified feline calicivirus (FCV) particles. We have used this to probe FCV-infected cells for the synthesis of virus specific RNA and confirm and extend the observations of Neill and Mengeling. Subcloning of the virus 3' end into a single stranded vector has allowed us to examine the occurrence of positive and negative sense RNA separately. Sequence analysis has shown a small open reading frame (ORF) at the 3' end, which suggested that the nested set could include small, as yet undetected mRNAs.

Materials and methods

Virus and cell culture

Feline calicivirus strain F9 was obtained from Prof. O. Jarrett, University of Glasgow Veterinary School and grown in the feline kidney cell line CRFK (Flow Laboratories, Rickmansworth), in which it produces widespread cpe within 6 h. FCV was plaque-purified by three rounds of single plaque selection in these cells and a working stock of virus (2.8×10^8 pfu/ml) was prepared.

Virus purification

Virus particles were purified for cloning experiments as follows: six confluent roller bottles of CRFK cells were each infected with 2 ml of virus stock. Growth was allowed to proceed for 16 h when the bulk of the cell sheet had been destroyed. Cell debris was removed by sedimentation at 1,500 **g** for 10 min and then at 10,000 **g** for 15 min before virus particles were pelleted at 17,000 **g** for 2.5 h. The virus pellets were then thoroughly resuspended in NT buffer (100 mM NaCl, 10 mM tris-HCl pH 7.5) and the volume adjusted to 10 ml. The virus suspension was then overlayered onto four discontinuous CsCl gradients each consisting of 0.8 ml of CsCl (1.5 g/cm^3) and 1.5 ml of CsCl solution of density 1.3 g/cm^3. Gradients were formed in Sw55 tubes (Ultraclear, Beckman Ltd., High Wycombe) and spun at 50,000 rpm for 90 min at 4 °C. Under these conditions virus sedimented to the interface between the two CsCl solutions although a distinct band was often not visible. The interface regions (in about 1 ml per tube) were then pooled and adjusted to a density of 1.34 g/cm^3 with CsCl. This virus suspension was then centrifuged overnight at 50,000 rpm in the Sw55 rotor and allowed to decelerate without the brake. Under these conditions a tight band was normally visible by scattered light in the central region of the tube and could be removed with a syringe. The band was then diluted with NT buffer and repelleted at 50,000 rpm for 1 h before being resuspended in 0.5 ml of NT buffer. This material contained one protein band corresponding to the virus capsid on Coomassie blue-stained SDS-polyacrylamide gels. RNA was extracted from this purified virus for cloning procedures.

Cloning

Double-stranded cDNA synthesis for cloning was performed using the procedure of Gubler and Hoffman [10] employing oligo-dT as primer for the reverse transcription of 2 µg of virus RNA. cDNA molecules were cloned by the homopolymer tailing method. Approximately 20 C residues were added per end using terminal deoxynucleotidyl transferase (Gibco BRL). Radioactive dCTP was used for this step in order to increase the specific activity of the product. Prepared cDNA molecules were hybridized with oligo dG-tailed. PstI cut plasmid vector pBR322 and introduced into *Escherichia coli* DH5alpha F' (Gibco BRL) rendered competent by the method of Hanahan [11]. Transformed bacteria were selected on media containing tetracycline and screened by colony hybridization [9]. RNA was

purified from CRFK cell cytoplasmic extracts for the preparation of probes with which to select recombinant colonies. Polyadenylated species were selected using poly-U sepharose chromatography. cDNA probes were produced by reverse transcription of 2 μg of cellular A + RNA and omission of the unlabeled dCTP to increase specific activity. Actinomycin D was included at 500 μg/ml to prevent labelling of any sequences derived from contaminating host cell DNA. This protocol led to the production of short transcripts and so to ensure complete sequence representation the oligo dT primer was replaced with random hexanucleotide primers (Pharmacia, Milton Keynes U.K.). A probe specific for the 3' end of the virus, i.e., enriched for sequences immediately adjacent to the poly A tail, was prepared using this procedure with oligo dT as primer.

Sequencing

Any clones to be sequenced were re-cloned into the bacteriophage vectors M13mp18 and mp19 after digestion with appropriate restriction enzymes. Single-stranded template was prepared and sequenced using a Sequenase V2.0 sequencing kit (Cambridge Bioscience, U.K.) exactly as recommended by the manufacturers. All sequence data presented here were confirmed by sequencing both strands. Data was analysed using the PC Gene software package supplied by Genofit SA (Geneva, Switzerland).

Results

RNA extracted from virus particles showed some degradation but a sharp band of 7–8 kb was visible. cDNA synthesis from this RNA led to a broad size range of products. Cloning from this material resulted in the production of many clones which were predominantly small in size (approx. 1 kb). Accordingly the larger molecules of ds cDNA were eluted from the gel and cloned separately. This resulted in the production of 50 clones all greater than 1.5 kb in size, the largest of which pFC 4.2 was found to be 4.5 kb in size. This clone was excised from the vector as two PstI fragments 2.14 and 2.34 kb in length. Restriction mapping of this clone led to the map shown in Fig. 1. Similar analysis showed that all the other clones tested could be aligned on this map and most formed a nested set extending from the right hand end. Those larger than 300 bases possessed the EcoRI site and those larger than 2.14 kb contained the central

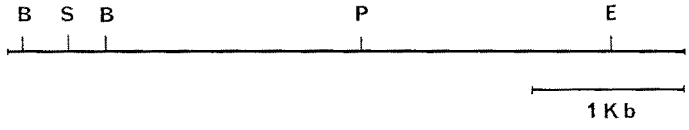

Fig. 1. Restriction map derived for clone pFC 4.2. Clone pFC 4.2 was digested with the enzymes shown individually and in combination. The right hand end of this map corresponds to the 3' end of the genome (see text): *B* BamHI; *E* EcoRI; *P* PstI; *S* SmaI. The EcoRI site appears to be common to this clone and also that derived previously [13], but the central PstI site is found only in pFC 4.2. The SmaI site is present in both clones but is differently located. Both HindIII sites found by Neill and Mengeling are absent in pFC 4.2 which possesses two BamHI sites, neither of which are in the same position as the single site found previously [13]

PstI site as well. This suggested that all clones were derived from a common priming site, most likely the 3' end of the virus.

Accordingly a probe was prepared which was enriched in 3' end sequences as described. This was than hybridized to a PstI restriction digest of clone pFC 4.2 separated on an agarose gel and blotted to nitrocellulose. The result (Fig. 2) indicated that the right hand end of the map presented (i.e., the 2.13 kb fragment) hybridized most strongly to this probe, also indicating that this constituted the 3' end of the virus. Finally this inference was confirmed by subcloning the terminal EcoRI/PstI fragment into M13 and determining its sequence (Fig. 3a). This showed a poly A stretch preceded by a short run of poly C which is exactly that structure expected for cDNA clones derived by this method from an mRNA 3' end. Furthermore, an open reading frame extended towards the 3' end terminating some 45 residues from the poly A tail. A search of known nucleotide sequences as contained in the EMBL database (release no. 19) failed to reveal any significant homology between this sequence and any already determined. Sequence determination from this EcoRI site toward the 5' end in the virus RNA sense encountered a group of closely-spaced termination codons in all frames. This was confirmed by sequencing additional clones covering this region and overlapping the EcoRI site. However, an ORF was observed beyond these termination signals which extended towards the 5' end of the clone (Fig. 3b).

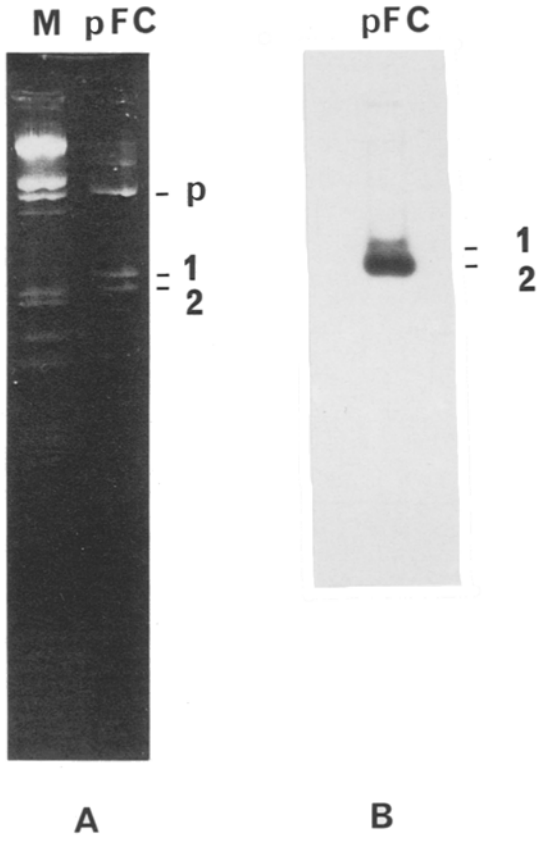

Fig. 2. PstI digest of clone pFC4.2. **A** Ethidium Bromide-stained agarose gel analysis of clone pFC 4.2: *M* EcoRI/HindIII digested lambda DNA molecular weight markers; *pFC* PstI digested plasmid pFC 4.2. The inserted cDNA is excised as 2 fragments 2.34 kb *(1)* and 2.14 kb *(2)* in size. *p* Linearized vector pBR322. **B** DNA from **A** transferred to nitrocellulose and hybridized to 3' end enriched cDNA probe prepared as described

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          10         20         30         40         50         60
           ¦          ¦          ¦          ¦          ¦          ¦
  1 GGAATTGATA GTGATGGGTT CTCTTTTGTT GGTGTTTCTG GCTTTGGTAA ATTAGAATTT

 61 CCCCTTTCTG CCTCCTACAT GGGAATACAA TTGGCAAAGA TCCGGCTTGC CTCTAACATT

121 AGGAGTCCCA TGACTAAGTT ATGAATTCAA TATTAGGCCT GATTGATACT GTTACTAACA
                           Eco R1

181 CTATTGGTAA AGCTCAGCAG ATTGAATTGG ACAAAGCTGC ACTTGGTCAA CAGCGTGAAT

241 TGGCTCTCCA GCGCATTGGC TTGGACCGCC AAGCCTTAAA CAACCAAGTT GAGCAGTTTA

301 ACAAAATTCT TGAGCAAAGG GTACAAGGCC CAATCCAATC CGTTCGCCTT GCGCGCGCGG

361 CTGGATTTAG GGTTGACCCT TACTCATACA CAAACCAAAA CTTTTATGAC GATCAATTAA

421 ATGCAATTAG ACTATCATAT AAAAATTTGT TTAAAATTTG ATCATATATC CCTTTGGGCT

481 GCCGCACCTG CGCCTAACCC CAGGG (A)30 (C)25

Total number of bases is: 505.
DNA sequence composition:   141 A;  112 C;  104 G;  148 T;
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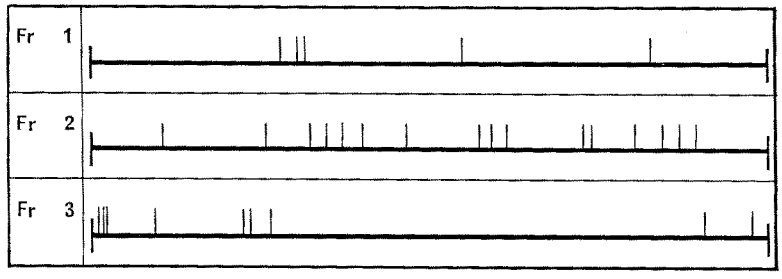
A



B

Fig. 3. A Terminal sequence from clone pFC4.2 presented in the DNA form. The position of the EcoRI restriction site is marked and possible start (ATG) and stop (TGA) codons for the hypothetical 3' open reading frame are indicated. **B** Position of stop codons in the three mRNA-sense frames *(Fr 1–3)* derived from the sequence given above. The short open reading frame is evident in frame 3 and that preceding it in frame 1

This was a puzzling observation since termination at this point would lead to a relatively large non-translated region of 365 bases before the poly A tail.

RNA synthesis in the infected cell was examined by blotting. RNA was extracted from infected CRFK cells at 30 min intervals p.i., separated on an agarose gel, transferred to nitrocellulose and detected using FCV cDNA probes. The probes used in this case were prepared from the EcoRI/PstI restriction

fragment corresponding to the 3' end of the FCV genome and subcloned into M13. Single-stranded sense-specific probes were made by Klenow enzyme transcription of the purified ssDNA templates. Both virion (assumed positive) and complementary (assumed negative) senses were prepared and used separately. Incorporation of radioactivity was similar in both cases and acid precipitable radioactivity was equalized in each hybridization. The results of this analysis are given in Fig. 4. RNA sizes were determined using prokaryote and eukaryote rRNA markers whose length is known from the sequence of their genes [2, 3, 12, 15]. A different pattern of intracellular RNA was observed using probes of each sense.

The virus-sense probe detected the genome and a 2.7 kb virion-sense RNA at early times. Both of these were found in the first sample taken at 30 min p.i. The amounts of these species remained fairly steady until 3 h p.i. when a marked accumulation was observed. Other RNAs appeared at this time and increased

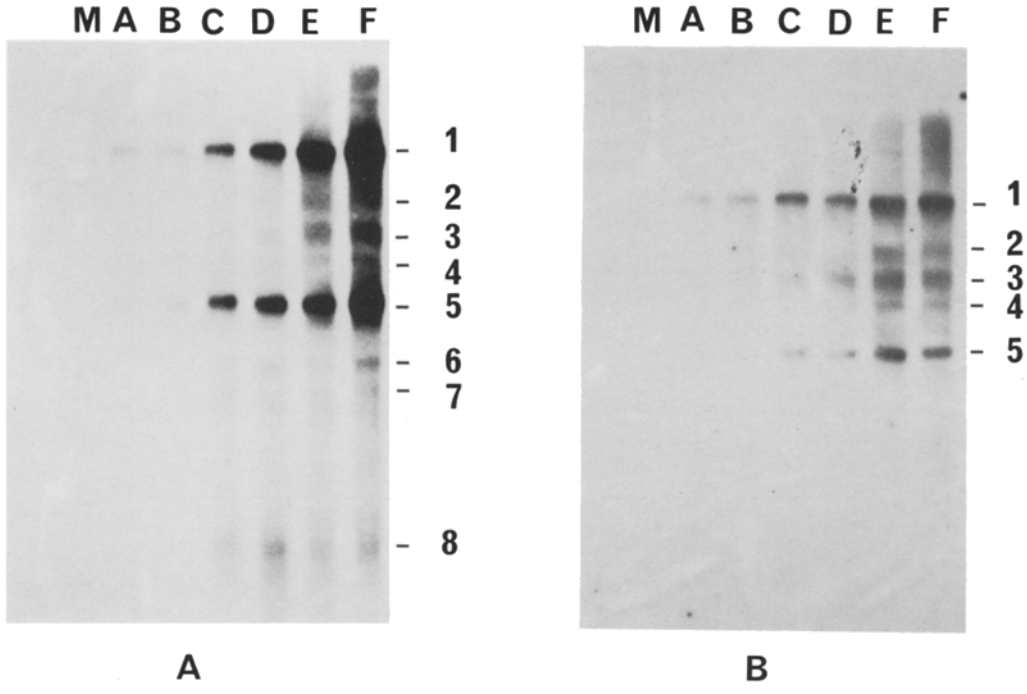


Fig 4. Northern blot analysis of infected cell cytoplasmic RNA. CRFK cells were infected as described in the text and harvested at 30 min intervals. *A–F* RNA from infected cells harvested 30–180 min p.i.; *M* RNA from mock-infected cells. RNA species are numbered 1–8 (genome as no. 1). Cytoplasmic extracts were prepared by disruption in 10 mM Tris (pH 7.5), 10 mM NaCl, 1.5 mM $MgCl_2$ containing 1% NP40, 0.2% sodium deoxycholate, and RNAsein. Nuclei were removed by sedimentation and RNAs were extracted by proteinase K and phenol, denatured with formamide, and analysed in duplicate on a 0.75% MOPS-buffered agarose gel containing 2.2 M formaldehyde. Separated species were transferred to nitrocellulose filters by capillary blotting. Filters were then cut in half and each half hybridized with one of the sense-specific probes described above. **A** Positive stranded RNA; **B** negative sense RNA

Table 1. RNA sizes

RNA no.	Size	Remarks
1	7,100	genome
2	5,300	
3	4,300	
4	3,600	
5	2,700	major species
6	1,900	
7	1,500	
8	550	

in amount as the infection progressed. Eight separate RNAs were detected in this analysis but many were faint. At all times the genome and the 2.7 kb RNAs were the most abundant species as reported [13]. However, previous analysis has revealed only two mRNAs in addition to these dense bands giving a total of 4 RNA species. The experiments described here have identified 4 novel bands. All bands are numbered in order of decreasing size in Fig. 4 (genome is assigned as no. 1). Bands reported by Neill and Mengeling correspond to no. 1, 3, 4, and 5 which are the most readily discernible. Novel bands reported in this study are no. 2 and 6–8. Sizes of these RNAs, derived as the average of multiple determinations, are given in Table 1. The antisense probe detected the genome-sized RNA after 30 min and this preceded the large increase in the virion-sense form of this band. Negative sense 2.7 kb RNA was not clearly detected until 90 min p.i. and thus lagged behind the appearance of the positive form. However, although the positive form of this RNA was present earlier, it did not increase dramatically in abundance until after the appearance of the negative sense. Exposure times required for the negative sense blots were longer than those for the positive sense indicating that positive RNA predominated in the cells.

The genome and the 2.7 kb mRNA (no. 5) are known to be synthesized in double-stranded forms and therefore their detection by both probes was expected. However, the negative strand-specific probe also showed hybridization to subgenomic messages 2–4, but this was not observed until later in infection than the detection of the positive forms of these molecules. Negative sense forms of RNAs 6–8 were not detected at any time. The possibility that probes of each sense recognize both positive and negative sense RNA is discounted since only one probe reacted with RNA extracted from purified virus (not shown). Furthermore, whilst both positive and negative genome-sized RNA can be detected at early times, negative sense 2.7 kb RNA was not observed before 3 h p.i. even though large amounts of its positive form were present during this period.

Discussion

Recently FCV mRNAs have been found to form a 3' co-terminal nested set in common with other virus families [20, 22]. In coronaviruses, successive ORFs

from the 5' end are exposed by an unknown mechanism and translated from the next smallest member of the series. Such a mechanism could explain our finding of a small ORF at the 3' end of the FCV genome and would predict that a small mRNA should be detectable inside FCV-infected cells formed by activation of this ORF. A candidate band was observed in our blotting experiments (band 8, 550 bp). The sequence of FCV determined in the region of this ORF was compared with the known junction sites for members of the family *Coronaviridae* [22]. The short sequence AAACUUU which is found on the 3' side of coronavirus-junction sites and precedes coding information [22] was found in FCV, flanked by an extra A and U. However, unlike coronavirus-junctions, it is not followed by a suitable initiation codon and it occurs well downstream from the termination codons separating the ORFs. It therefore seems unlikely that a mechanism identical to that of the coronaviruses is involved and sequence determination from the intracellular molecules is required to characterize any processing which may occur.

This investigation of intracellular RNA synthesis has shown that the first intracellular virus RNAs detected are the positive and negative sense genome-sized molecules and the positive sense 2.7 kb RNA. Both positive forms were present in approximately the same amount. The negative forms of these two species clearly differ in amount, and that of the 2.7 kb molecule is barely detectable until 90 min p.i. We have recently shown that the sensitivity of the blotting method in our hands approaches that of a single copy per cell [6]. Much of the positive sense genome detected in these experiments at early times could result from infecting virus whose multiplicity (200 pfu per cell) greatly exceeds this limit. However, this explanation cannot apply to the negative form of this molecule, nor to the positive 2.7 kb RNA. Neither of these are part of the virion [4]. It is therefore possible that the first molecule transcribed from the incoming genome may be a full length complementary RNA. The 2.7 kb positive sense molecule could be transcribed from this and subsequently synthesized in its negative form. This model would explain the apparent differences in ratios observed. We consider it likely that all the subgenomic mRNAs (with the exception of 6–8) may be made in a similar manner and are synthesized first as positive strands. These may then be replicated independently via negative stranded forms. This would provide a powerful amplification system and account for the rapid accumulation of all positive sense RNAs which commenced in the second hour post infection onward. This is at the same time as the amplification of protein synthesis we have recently reported [5]. A similar mechanism for RNA transcription has been observed in coronaviruses, toroviruses, and arteriviruses during the preparation of this report [16, 19, 21]. This suggests that the replication strategies of these virus groups may be very similar in principle despite the apparent lack of similarity in sequence at a presumed junction site in the genome of FCV.

Neill and Mengeling [13] observed only three subgenomic RNA bands; however, these workers used a larger probe which did not correspond to the

exact 3' end of the virus and lacked the terminal EcoRI fragment. We have found that the use of larger probes increases the background in the central region of the blots which could have masked minor RNA species. The small RNA (no. 8) was clearly absent in the previous analysis [13] but this RNA is only marginally larger than the 3' EcoRI fragment we have used as a probe and which was absent from the probes used previously. The overlap between this RNA and the probe used by Neill and Mengeling [13] would thus have been small and consequently it may not have been recognized.

There is considerable antigenic variation within the one serotype of FCV and it is likely that a spectrum of antigenic types may exist [14]. The restriction maps generated for the 3' end of FCV strain CF1/68 FIV [13] and strain F9 (this report) also differ and this suggests that these differences will be represented at sequence level. The sizes of the RNAs reported here also differ from those reported by Neill and Mengeling. To some extent this may be accounted for by the different nucleic acid markers employed (ssRNA or ssDNA) in the two studies. However, strain variation in polypeptide size is known and RNA sizes may therefore also differ. Comparative sequence analysis and investigation of any strain-specific pathogenesis is urgently required.

Acknowledgements

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References

1. Black DN, Burroughs JN, Harris TJR, Brown F (1978) The structure and replication of calicivirus RNA. Nature 274: 614–615
2. Brosius J, Palmer ML, Kennedy PJ, Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli.* Proc Natl Acad Sci USA 75: 4801–4805
3. Brosius J, Dull TJ, Noller HF (1980) Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli.* Proc Natl Acad Sci USA 77: 201–204
4. Burroughs JN, Doel TR, Smale CJ, Brown F (1978) A model for vesicular exanthema virus, the prototype of the calicivirus group. J Gen Virol 40: 161–174
5. Carter MJ (1989) Feline calicivirus protein synthesis investigated by Western blotting. Arch Virol 108: 69–79
6. Carter MJ, Willcocks MM, Mitchison HC et al (1989) Is a persistent adenovirus infection involved in coeliac disease. Gut 30: 1563–1567
7. Ehresmann DW, Schaffer FL (1977) RNA synthesized in calicivirus-infected cells is atypical of picornaviruses. J Virol 22: 572–576
8. Ehresmann DW, Schaffer FL (1979) Calicivirus intracellular RNA: fractionation of 18–22S RNA and lack of typical 5'-methylated caps on 36S and 22S San Miguel sea lion virus RNAs. Virology 95: 251–255
9. Grunstein M, Hoegness DS (1975) Colony hybridization: a method for the identification of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 72: 3961–3965
10. Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25: 263–269

11. Hanahan D (1985) Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA cloning, vol 1. IRL Press, Eynsham, Oxford, UK, pp 109–135
12. Hassouna N, Michot B, Bachellerie JP (1984) The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. Nucleic Acids Res 12: 3563–3583
13. Neill JD, Mengeling WL (1988) Further characterization of the virus-specific RNAs in feline calicivirus infected cells. Virus Res 11: 59–72
14. Povey RC (1974) Serological relationship among feline caliciviruses. Infect Immun 10: 1307–1314
15. Raynal F, Michot B, Bachellerie JP (1984) Complete nucleotide sequence of mouse 18S rRNA gene: comparison with other homologs. FEBS Lett 167: 263–268
16. Sawicki SG, Sawicki DL (1990) Coronavirus transcription: subgenomic mouse hepatitis virus replicative intermediates function in RNA synthesis. J Virol 64: 1050–1056
17. Schaffer FL, Bachrach HL, Brown F et al (1980) Caliciviridae. Intervirology 14: 1–6
18. Schaffer FL, Ehresmann DW, Fretz MK, Soergel ME (1980) A protein, VPg, covalently linked to 36S calicivirus RNA. J Gen Virol 47: 215–220
19. Sethna PB, Hung S-L, Brian DA (1989) Coronavirus subgenomic minus strand RNAs and the potential for mRNA replicons. Proc Natl Acad Sci USA 86: 5626–5630
20. Siddell S, Wege H, terMeulen V (1983) The biology of coronaviruses. J Gen Virol 64: 761–776
21. Snijder EJ, Horzinek MC, Spaan WJM (1990) A 3' co-terminal nested set of independently transcribed mRNAs is generated during Berne virus replication. J Virol 64: 331–338
22. Spaan W, Delius H, Skinner M et al (1983) Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. EMBO J 2: 1839–1983

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