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Further characterization of the virus-specific RNAs in feline calicivirus infected cells

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Summary

The virus-specific RNAs in feline calicivirus (FCV) infected cells were examined to determine the number and forms of RNAs that are synthesized during the infection process. Northern blots of poly(A)$^+$ RNA from 5-h infected cells probed with a cDNA clone derived from the 3′ end of the FCV genome (pCV3) revealed four FCV-specific RNAs that were approximately 8.2 (genomic RNA), 4.8, 4.2 and 2.4 kb in length. Northern blots of poly(A)$^+$ RNA purified from infected cells hourly after infection and probed with pCV3 demonstrated that transcription of all FCV-specific RNAs are detectable at 2 to 3 h post-infection (PI) and that these RNAs reached steady state levels at approximately 4 h PI. The levels of the FCV RNAs then remained relatively constant through 7 h PI, the last time tested, with the exception of the 4.8 and 4.2 kb transcripts which showed a marked increase between 6 and 7 hours PI. Northern blots of dsRNA which had been LiCl-fractionated from pooled total cellular RNA isolated from 5-h and 7-h FCV infected cells, showed two double-stranded RNAs corresponding to the 8.2 kb genomic RNA and the 2.4 kb subgenomic RNA. Preliminary mapping by Northern blotting using cDNA probes derived from varying locations within the FCV genome was done to determine the approximate regions from which the subgenomic RNAs are derived. This analysis indicates that the viral RNAs are nested, co-terminal transcripts with common 3′ ends.

Feline calicivirus; Northern blot; Subgenomic RNA; Double-stranded RNA

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Introduction

The caliciviruses are a family of small viruses which possess a plus-strand, non-segmented, polyadenylated RNA genome. The buoyant densities of these non-enveloped viruses range from 1.36 to 1.39 g/ml (Oglesby et al., 1971; Burroughs and Brown, 1974; Soergel et al., 1975). Members of this family include feline calicivirus (FCV), San Miguel sea lion virus (SMSV), vesicular exanthema virus of swine (VEV) and human calicivirus (Schaffer, 1979).

In characterizations of viral-specific RNAs in calicivirus-infected cells, Ehresmann and Schaffer (1977) demonstrated that there are two calicivirus-specific transcripts in infected cells. Their work involved labeling experiments with FCV, SMSV and VEV-infected cells using [^{3}H]uridine in the presence of actinomycin D and subsequent analysis by glycerol gradient centrifugation and polyacrylamide gel electrophoresis. The largest RNA corresponded to the viral genomic RNA (36 S or approximately 2.6×10^6 daltons) and the other to a subgenomic RNA of approximately 1.1×10^6 daltons (22 S). They also demonstrated that approximately one-third of the viral RNA which sedimented at 18–22 S, was RNase resistant. This was shown to be the same size as the genomic RNA by analysis of the RNA on denaturing gradients and comparison to similarly treated 36 S RNA. Further work in characterization of the RNase-resistant population of RNAs which sedimented at 18–22 S showed that there were two double-stranded RNA species present, one of which corresponded to the genomic RNA and one to the 22 S subgenomic RNA (Ehresmann and Schaffer, 1979). Black et al. (1978) in similar experiments with VEV demonstrated the presence of the 36 S, 22 S and an 18 S RNA. In addition, the 18 S RNA was shown to be RNase-sensitive, suggesting that it could be functioning as message in the infected cell. They also noted the presence of a small amount of RNase-resistant RNA which also sedimented at 18 S. This, in concurrence with Ehresmann and Schaffer (1977), was considered to be double-stranded replicative form RNA.

This communication describes work to further elucidate the scheme of RNA transcription in calicivirus-infected cells through the use of the more sensitive technique of Northern blotting. This work has resulted in the identification of a fourth single-stranded RNA, confirmation of the identity and size of the two double-stranded RNAs and the approximation of the length of each RNA transcript. Preliminary localization of each subgenomic RNA within the FCV genome was done through the hybridization of blotted RNA with cDNA clones originating from different regions of the genome and has demonstrated that the FCV RNAs are nested, co-terminal transcripts.

Materials and Methods

Materials

All restriction endonucleases, *E. coli* DNA polymerase I, *E. coli* DNA polymerase Klenow fragment, T4 DNA ligase, T4 DNA polymerase, *Eco*RI methylase

and *E. coli* DNA ligase were purchased from New England Biolabs (Beverly, MA). AMV reverse transcriptase was purchased from Bio-Rad (Richmond, CA), RNase H was from Bethesda Research Laboratories (Gaithersburg, MD), and RNasin RNase inhibitor was from Promega Biotech (Madison, WI). Lambda *gt*10 and gigapack in vitro packaging system were purchased from Stratagene Cloning Systems (San Diego, CA), [α-^{35}S]dATP was from New England Nuclear (Boston, MA) and [α-^{32}P]dATP was purchased from ICN Radiochemicals (Irvine, CA).

Strains and medium

Feline calicivirus strain CFI/68 FIV (FCV), which was obtained from American Type Culture Collection, was used in all phases of this study. FCV was propagated in Crandall-Reese Feline Kidney cells (CRFK) which were maintained in F-15 Eagle's MEM supplemented with 0.25% lactalbumin hydrolysate and 10% fetal calf serum. FCV was plaque-purified to eliminate defective interfering particles.

E. coli strain DH1 (Hanahan, 1983) was used for plasmid maintenance and propagation and was maintained on Luria broth (LB) agar plates which contained 50 μg/ml ampicillin when plating transformed bacteria. Strain C600 (Appleyard, 1954) was used for plating lambda *gt*10 and was maintained and plated on NZY-amine agar plates. Strain JM107 (Yanisch-Perron et al., 1985) was used for transformation by pUC plasmids (Yanisch-Perron et al., 1985) and was plated on LB plates containing 80 μg IPTG and 0.01% X-gal per plate. JM107 was also used to propagate M13mp18 containing FCV cDNA clones for dideoxy chain termination sequence analysis. JM107 was maintained on minimal medium as previously described (Yanisch-Perron et al., 1985).

Calicivirus propagation and purification

FCV was propagated by infection of CRFK cells, which had been grown to confluence in 490 cm^2 roller bottles, with FCV at an m.o.i. of approximately 0.01 in a total volume of 10 ml. The virus was allowed to adsorb for 2 h at 37 °C. The inoculum was removed and 50 ml of serum-free medium was added and the incubation was continued at 37 °C until CPE was complete (generally within 24 h). The medium was removed and was frozen at −20 °C, thawed at room temperature and the cell debris was removed by centrifugation at 5000 rpm for 10 min at 4 °C in a Sorvall GS-3 rotor. The virus was precipitated from the supernatant by the addition of solid polyethylene glycol (PEG, 8000 M_r) to a final concentration of 10% (w/v) and stirring at room temperature until the PEG was in solution. The precipitate was pelleted by centrifugation at 8000 rpm for 20 min in the GS-3 rotor and the supernatant was discarded. The pellet was resuspended in 10 ml of phosphate buffered saline (PBS, pH 7.2), the insoluble matter was removed by centrifugation at 10000 rpm for 10 min in a Sorvall SA-600 rotor and the supernatant was layered over a CsCl step gradient. The step gradient was formed by layering 5 ml of 1.28 g/ml CsCl over 5 ml of 1.60 g/ml CsCl, both in PBS, and immediately layering the FCV supernatant on the top of the gradient. The virions

($\rho = 1.36$) were banded at the 1.60 : 1.28 interface by centrifugation at 22000 rpm for 3 h at 4 °C in a SW28 rotor. The white viral band was removed and was rebanded in an isopyknic CsCl gradient (1.38 g/ml CsCl) by centrifugation at 48000 rpm for 18 h in a 70.1 TI rotor. The viral band was removed from the gradient and was dialyzed against 1000 volumes of PBS overnight. The purified virions were stored at −70 °C.

Preparation of FCV RNA

Full-length genomic RNA was prepared from isolated virions as previously described (Love, 1976). Total cellular RNA was prepared from FCV-infected and non-infected cells by the guanidine-HCl : CsCl centrifugation procedure as described by Krawetz and Anwar (1984). CRFK cells were grown and inoculated as described above with the exception that the cells were inoculated at an m.o.i. of approximately 10. Total poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography according to Maniatis et al. (1982). FCV double-stranded RNA was LiCl fractionated from total cellular RNA isolated from FCV infected cells as described by Garger and Turpen (1986).

Cloning and screening of FCV cDNA

First-strand cDNA was synthesized from FCV genomic RNA using oligo(dT) as primer and with AMV reverse transcriptase as specified by the supplier. Second-

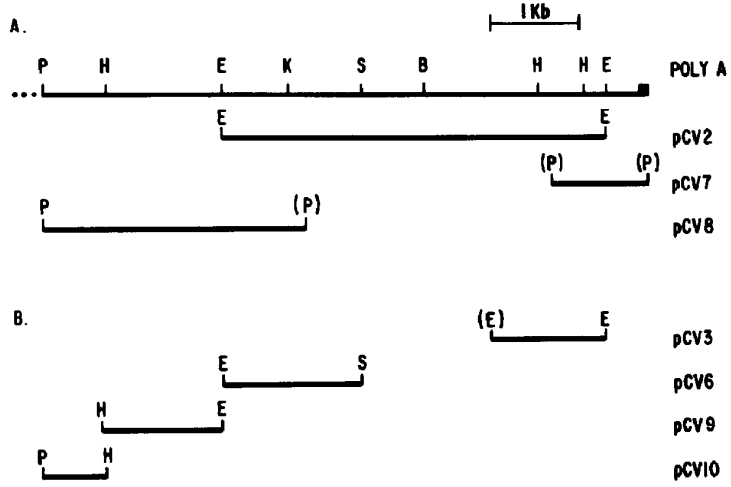

Fig. 1. (A) Physical map of feline calicivirus (FCV) cDNA. Restriction endonuclease cleavage sites are indicated. Dots at 5′ end of genome represent uncloned sequences. Lines beneath the map indicate cDNA clones that were obtained following cloning of reverse transcribed genomic RNA as described in Materials and Methods. (B) Genomic location of cDNA clones that were used in Northern blot analysis of FCV-infected cells. Restriction sites in parenthesis at the ends of the cDNA clones indicate linker sequences. Restriction endonuclease sites are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; and S, *Sph*I.

strand cDNA was synthesized according to Gubler and Hoffman (1983). The ends of the double-stranded cDNA were made blunt with T4 DNA polymerase, the cDNA was treated with *Eco*RI methylase and *Eco*RI synthetic linkers were blunt-end ligated onto the cDNA. The cDNA-linker mixture was digested with *Eco*RI and was precipitated twice with an equal volume of 4 M ammonium acetate and 2 volumes of ethanol. The cDNA was then ligated into lambda *gt*10, in vitro packaged and plated on appropriate *E. coli* strains. The resultant plaques were lifted on nitrocellulose filters and probed with ^{32}P-labeled random-primed cDNA which had been synthesized using the FCV genomic RNA as template as described by Maniatis et al. (1982). A second library was also constructed in the same manner using *Pst*I linkers and ligation into *Pst*I-digested pUC18. This was done because only the large internal *Eco*RI fragment was recovered from the lambda library (Fig. 1).

A synthetic oligonucleotide 20mer, GGAATTTTGCCCCGGGCCCT, synthesized on an Applied Biosystems Oligonucleotide Synthesizer (model 8750), was used to prime first strand cDNA synthesis in order to clone FCV sequences 5′ to those contained within pCV2. This sequence was derived from DNA sequence analysis of the end of the *Eco*RI fragment which corresponded to the 5′ end of the cloned FCV genome. cDNA was synthesized and cloned as described above with *Pst*I synthetic linkers. The cDNA was cloned into pUC18, used to transform JM107 and screened by plating on medium containing IPTG and X-gal as previously described.

Northern blot analysis of viral RNAs

Total cellular poly(A)$^+$ RNA from FCV infected cells (approximately 3 μg) was separated on formaldehyde denaturing agarose gels according to the procedure of Lehrach et al. (1977). The RNA was blotted to nitrocellulose, prehybridized and hybridized with ^{32}P-labeled FCV cDNA clones according to the procedure of Thomas (1980). Autoradiography was done without intensifying screens using Kodak XAR-5 film.

DNA sequencing

The ends of FCV cDNA clones were sequenced using the dideoxy chain termination procedure as described by Williams et al. (1986) to allow synthesis of the synthetic oligonucleotide primer described above for further cloning of the FCV genome and to determine which strand encoded the viral sense strand by location of the cloned poly(A) tail.

Results

Construction of an FCV genomic cDNA library

A cDNA library of FCV sequences was constructed in the lambda cloning vector *gt*10 and was screened with ^{32}P-labeled random-primed cDNA which was synthe-

sized using FCV genomic RNA as the template. A plaque with a strong hybridization signal was isolated and found to contain an *Eco*RI fragment of approximately 4200 bp. This fragment was subcloned into pUC18 for restriction enzyme analysis (Fig. 1A). The resulting plasmid was designated pCV2.

A second cDNA library was constructed using *Pst*I linkers in the plasmid pUC18. White transformants were analyzed for the presence of cDNA inserts by gel electrophoresis and a plasmid containing an insert of approximately 1000 bases was chosen for analysis. This plasmid, pCV7, was subjected to restriction analysis and was found to contain restriction sites in common with pCV2 and also contained a region of approximately 500 bp which contained novel restriction sites (Fig. 1A). The *Pst*I fragment was subcloned into M13mp18 and the ends, in both orientations, were sequenced. One of these sequences showed a poly(A) tract of 46 adenine residues, demonstrating that this clone contained the 3′ end of the FCV genome (data not shown). From this analysis, it was possible to determine which strand encoded the viral sense strand and to place the clones in the proper orientation.

Once it was known which strand encoded the FCV sense strand, the sequence at the *Eco*RI site which corresponded to the 5′ portion of the cloned FCV RNA contained in PCV2 was determined (data not shown). From this information, a synthetic oligonucleotide was synthesized which hybridized to sequences approximately 150 bases 3′ of the 5′-most *Eco*RI sequence on the genomic RNA. This oligonucleotide was used to prime reverse transcription in order to clone sequences 5′ of the *Eco*RI restriction site. The double-stranded cDNA was blunt-ended, cloned with *Pst*I linkers, and analyzed as described above. A plasmid containing an insert of approximately 2800 bases was chosen for further analysis. Restriction endonuclease analysis showed that this clone contained restriction sites in common with the 5′ end of pCV2 as well as a region of approximately 2000 bases of novel sequences. This plasmid was designated pCV8. The results of the restriction endonuclease mapping are illustrated in Fig. 1A. The dots at the 5′ end of the map represent uncloned sequences.

Northern blot analysis of RNA isolated from FCV infected cells

Poly(A)$^+$ RNA from non-infected and FCV infected cells was fractionated on 1.2% agarose gels containing 2 M formaldehyde, blotted to nitrocellulose and probed with nick-translated pCV3 to characterize the FCV-specific RNA species found within infected cells (Fig. 1B). When poly(A)$^+$ RNA from 5-h infected cells was analyzed, four FCV-specific RNAs were observed (Fig. 2, lane a). These bands represent the full-length 8.2 kb genomic RNA and three subgenomic RNAs of 4.8, 4.2 and 2.4 kb. The sizes of the RNAs were approximated by comparison to denatured lambda-*Hin*dIII size markers which were electrophoresed along with the RNA. The RNA used in the Northern blots was prepared by oligo(dT) cellulose chromatography demonstrating that these virus-specific transcripts are polyadenylated. No hybridization was detected in the RNA from non-infected cells (Fig. 2, lane b). Total cellular RNA from both non-infected and FCV infected cells was

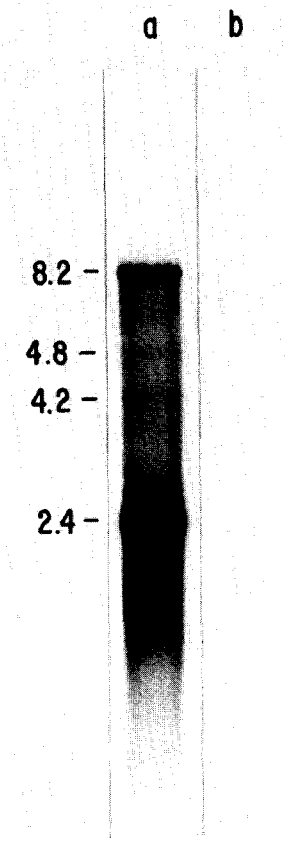

Fig. 2. Northern blot analysis of non-infected and 5 h PI FCV-infected CRFK cells. Poly(A)$^+$ RNA was prepared from FCV-infected (lane a) and non-infected CRFK cells (lane b), fractionated by electrophoresis on a formaldehyde denaturing agarose gel and blotted to nitrocellulose. The probe was radio-labeled pCV3. Denatured lambda DNA cut with *Hin*dIII was co-electrophoresed as size markers. The sizes indicated are in kb.

analyzed in the same manner. This analysis yielded the same result as the poly(A)$^+$ RNA.

In order to examine the transcription of FCV-specific RNAs during the infection process, poly(A)$^+$ RNA was prepared from FCV-infected cells which had been harvested at hourly intervals PI until the time at which the cells were no longer attached to the culture bottle (7 h PI). This RNA was analyzed as described above for the presence of FCV-specific transcripts (Fig. 3). No FCV-specific transcripts were observed in the RNA from mock-infected cells or in the 1 h PI RNA (lanes a and b, respectively). FCV-specific transcripts were detectable at 3 h PI (lane c) and reached steady state levels at 4 h PI (lane d). The RNA levels were consistent for the remainder of the times tested with the exception that there was a large increase in the hybridization signals of the 4.2 and 4.8 kb transcripts at 7 h PI (lane g),

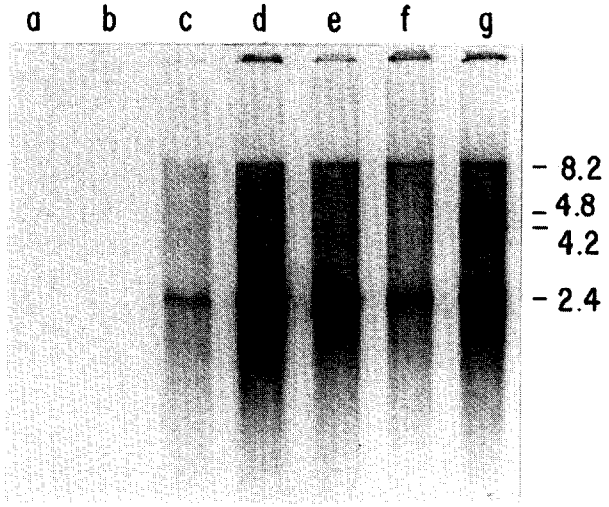

Fig. 3. Northern blot analysis of a time course of FCV infection. CRFK cells were infected at time 0 at an m.o.i. of 10 and cells were harvested hourly for poly(A)$^+$ RNA purification. The RNA was denatured, fractionated by electrophoresis on a denaturing agarose gel and blotted to nitrocellulose. The probe was radio-labeled pCV3. The lanes are 0, 1, 3, 4, 5, 6 and 7 h PI (a through g, respectively). The sizes indicated are in kb.

indicating an increase in copy number of these RNA species. The 2-h PI RNA was not included in this analysis because not enough RNA was obtained from the purification process. However, in other experiments, specific hybridization was observed in 2-h PI RNA indicating that viral replication was taking place. All RNAs observed at later time points were also present at 2 h PI (data not shown).

Double-stranded RNA of FCV

Total cellular RNA from 5 h and 7 h PI FCV infected cells was pooled and the double-stranded RNA was isolated by LiCl fractionation in order to determine the number and size of the FCV double-stranded RNAs. Following fractionation, the double-stranded RNA was denatured and fractionated on a denaturing agarose gel along with poly(A)$^+$ RNA for a size comparison and to illustrate the characteristic four band pattern (Fig. 4, lane a). Two bands were observed in the lane containing the LiCl-fractionated double-stranded RNA (Fig. 4, lane b), one corresponding to the 8.2 kb genomic RNA, and one corresponding to the 2.4 kb RNA. A double-stranded RNA for either the 4.8 kb or the 4.2 kb subgenomic RNA was not detected.

Genomic mapping of FCV transcripts

Northern blots of FCV-infected total cellular poly(A)$^+$ RNA were probed with FCV cDNA clones corresponding to different regions of the FCV genome. This was

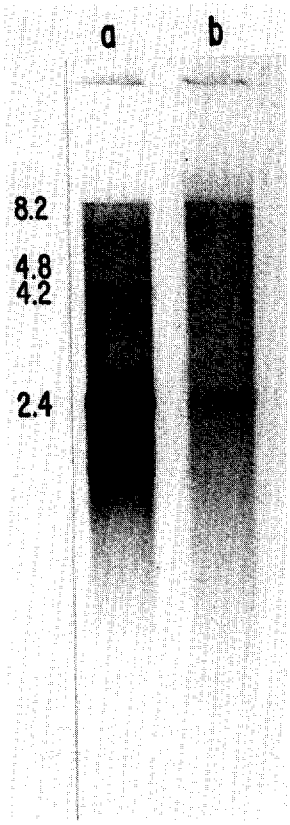

Fig. 4. Northern blot analysis of double-stranded RNAs of FCV. Double-stranded RNAs were LiCl fractionated from pooled 5-h and 7-h PI total cellular RNA as described in the text, denatured and fractionated by electrophoresis on a formaldehyde denaturing agarose gel. Poly(A)$^+$ RNA was included in the adjacent lane for size comparison. The RNA was blotted to nitrocellulose and was probed with radio-labeled pCV3. Lane a contains poly(A)$^+$ RNA and lane b contains LiCl-fractionated dsRNA. The sizes indicated are in kb.

done to approximate the location from which the different subgenomic RNAs are derived. The location within the genome of these cDNA clones is illustrated in Fig. 1B. Probing with pCV3 produced the characteristic 4 bands corresponding to the four FCV RNAs (Fig. 2), while probing with pCV6 resulted in the loss of signal from the 2.4 kb transcript (Fig. 5a). This result demonstrates that the 2.4 kb transcript is derived from within the 3′ 3000 bases of the FCV genome. Figure 5b illustrates the results obtained by probing with pCV9. The genomic RNA is present as is the 4.8 kb transcript. The 4.8 kb transcript is not clearly visible in Fig. 5b but is visible on the original autoradiograph. This indicates that there is only a small region of homology between this transcript and the FCV sequences contained in pCV9. Probing with pCV10 yielded only the full-length genomic RNA (Fig. 5c) indicating that the 4.2 and 4.8 kb transcripts are encoded 3′ of the position of this

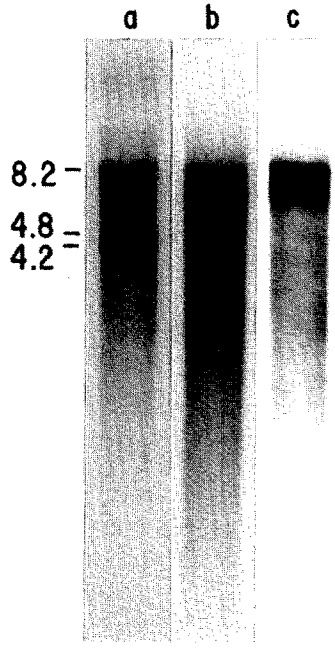

Fig. 5. Preliminary mapping analysis to determine from which portion of the genome the subgenomic RNAs are derived. Poly(A)$^+$ RNA from 5-h PI FCV-infected cells was fractionated by electrophoresis on a formaldehyde denaturing gel, blotted to nitrocellulose and probed with radiolabeled cDNA clones illustrated in Fig. 1B. (a) Probed with pCV6. (b) Probed with pCV9. (c) Probed with pCV10. The characteristic four-band pattern obtained when probed with pCV3 is illustrated in Fig. 2. The sizes indicated are in kb.

cDNA probe and that the 4.8 kb transcript originates within the sequences contained in pCV9. A diagram illustrating the results of this mapping is shown in Fig. 6. The dots at the 5′ end of the transcripts indicate the area where transcription

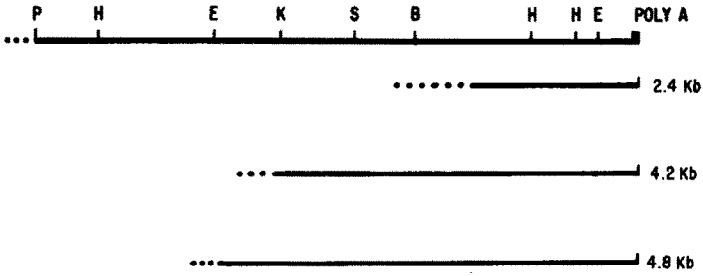

Fig. 6. Physical map of the tentative genomic areas from which the FCV subgenomic RNAs are derived. The solid lines indicate regions that are present in the subgenomic RNA. The dotted areas of the lines indicate regions in which transcription initiation may originate based on the length of the transcripts (Fig. 2) and the mapping hybridization data (Fig. 5).

initiation might originate based on the data obtained from the mapping experiments (Fig. 5) and the length of the RNAs (Fig. 2).

Discussion

Work in the area of the molecular biology of the caliciviruses has lagged behind that of other RNA viruses in recent years. This is especially true of the picornaviruses with which the caliciviruses were at one time classified. There is increasing evidence that the caliciviruses are more widespread and may cause disease more often than was previously believed (Barlough, 1986; Cubbitt et al., 1979; Schaffer et al., 1985; Smith, 1981). Feline calicivirus is ubiquitous in the cat population and has been demonstrated to cause disease (Gillespie and Scott, 1973; Schaffer, 1979). Very little is known concerning these viruses, and studies concerning the molecular biology and the relatedness of these viruses are warranted. This work was done to bring the molecular biology of the caliciviruses up to date and to expand on the work that has been done.

Northern blot analysis of poly(A)$^+$ RNA from FCV infected cells detected four viral RNAs, the full-length genomic and three subgenomic RNAs of 4.8, 4.2 and 2.4 kb in length (Fig. 2). This result is similar to that obtained by Black et al. (1978) in which they observed three bands by sucrose centrifugation. Their radioactivity profile showed highly labeled genomic and small subgenomic RNAs (2.8×10^6 and 0.7×10^6 Da, respectively) and a broad, less intensely labeled middle band (2.2×10^6 Da). This broad middle band probably corresponds to the 4.2 and 4.8 kb transcripts observed by Northern blot hybridization, which yield a low hybridization signal. The low hybridization signal is probably due to lower copy number of these transcripts whether it be from lower stability, lower rate of transcription or both. The strength of the hybridization signal is probably not a good indicator of transcript copy number as it is probable that the larger transcripts, especially the genomic RNA, will not blot as efficiently as the 2.4 kb transcript.

The determine whether the subgenomic RNAs are transcribed uniformly throughout the infection process, poly(A)$^+$ RNA was isolated at hourly intervals post-infection, Northern blotted and probed with pCV3. The four FCV transcripts were observed at 3 h PI, reached steady state levels at 4 h and remained constant until the last time period assayed, 7 h PI (Fig. 3). The exception to this is the 4.2 and 4.8 kb transcripts which showed a large increase in hybridization signal between 6 and 7 h PI. The reason for this is unclear. Two lines of evidence suggest that this is not a late function RNA; (1) these RNAs appear with, and increase in proportion to, the other viral transcripts until 7 h PI at which time the cells are no longer attached to the culture bottle and (2) it has been demonstrated that intracellular virus could be detected by 3 h PI (Studdert et al., 1970), suggesting that all functions necessary for viral replication and assembly are present early in infection. The 8.2 kb genomic RNA appears to be present at roughly the same copy number from 4 h until 7 h PI. This would seem to indicate that the viral RNA is being synthesized at approximately the same rate that virus particles are maturing and being released

from the cell. More detailed kinetic studies are needed to determine this.

Ehresmann and Schaffer (1977) and Black et al. (1978) demonstrated that a population of viral RNA sedimenting at 18–22 S was resistant to degradation by RNase, indicating the presence of a double-stranded replicative form RNA. This population of RNA was presumed to be the replicative form RNA. Ehresmann and Schaffer also mentioned the detection of a second, smaller species of double-stranded RNA in addition to the genomic double-stranded RNA. In investigations to characterize this second double-stranded RNA, Ehresmann and Schaffer (1979) found that this 2 M LiCl-soluble RNA sedimented at 18 S and when denatured, exhibited properties identical to the 1.1×10^6 dalton (22 S) subgenomic RNA. Investigations were done in this study to confirm the size and identities of these double-stranded RNAs and to determine if there are any other double-stranded RNAs present. The results of Northern blot analysis of LiCl fractionated RNA showed that there are two double-stranded RNAs present in FCV infected cells which correspond to the 8.2 kb genomic RNA and 2.4 kb subgenomic RNAs (Fig. 4). This result suggests that the sequences contained in the 2.4 kb subgenomic RNA may be replicated as well as transcribed. This would allow for amplification of these sequences, indicating that they may be needed in larger amounts than can be produced by transcription alone. Fretz and Schaffer (1978) proposed that the capsid protein may be the translational product of the 22 S RNA. Thus, replication of this RNA as well as transcriptional production would allow this RNA to be present in higher copy number and allow a larger amount of the capsid protein to be synthesized. The 2.4 kb RNA is present in high copy number (Figs. 2 and 3) and the capsid protein is the most abundant viral protein present in calicivirus infected cells (Black and Brown, 1977; Fretz and Schaffer, 1978). However, it is yet to be shown that the 2.4 kb RNA is being replicated. In addition, there was no band detected which corresponded to the 4.2 or 4.8 kb transcripts. It is possible that these transcripts are actually specific degradation products of the genomic RNA, or it is also possible that these transcripts lack sequences necessary to be replicated, which seems unlikely as preliminary evidence indicates that they possess the same sequences as those present in the 2.4 kb RNA (Fig. 5).

A potentially unique aspect of calicivirus replication would be the replication of the 2.4 kb subgenomic RNA as indicated by the presence of the subgenomic double-stranded RNA. Subgenomic RNAs produced by other plus-stranded RNA viruses such as the coronaviruses and the togaviruses are produced only by transcription (Ou et al., 1983; Bruton and Kennedy, 1975). Work is currently underway to further characterize the double-stranded RNAs and to understand their role in replication of FCV.

Mapping studies using cDNA probes from different regions of the FCV genome were done to begin to understand the transcriptional strategy of the caliciviruses. It was found that the cDNA clone from the 3′ end of the genome hybridized with all of the FCV transcripts. As the probes used were progressively more 5′ within the genome, there was a progressive loss of hybridization signal from the 2.4 kb to the 4.8 kb transcripts (Fig. 5). This demonstrates that the RNAs are co-terminal, nested sets of transcripts in which the transcript begins at specific points and then

continues to the 3′ end of the genome. This scheme of transcription is similar to that employed by the coronaviruses and the togaviruses (Baric et al., 1987; Brayton et al., 1984; Ou et al., 1983). As in these viruses, the subgenomic RNAs of FCV are thought to act as mRNA for specific polypeptides not translated from the other transcripts (Strauss and Strauss, 1982; Sturman and Holmes, 1983).

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