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RNA Synthesized in Calicivirus-Infected Cells Is Atypical of Picornaviruses

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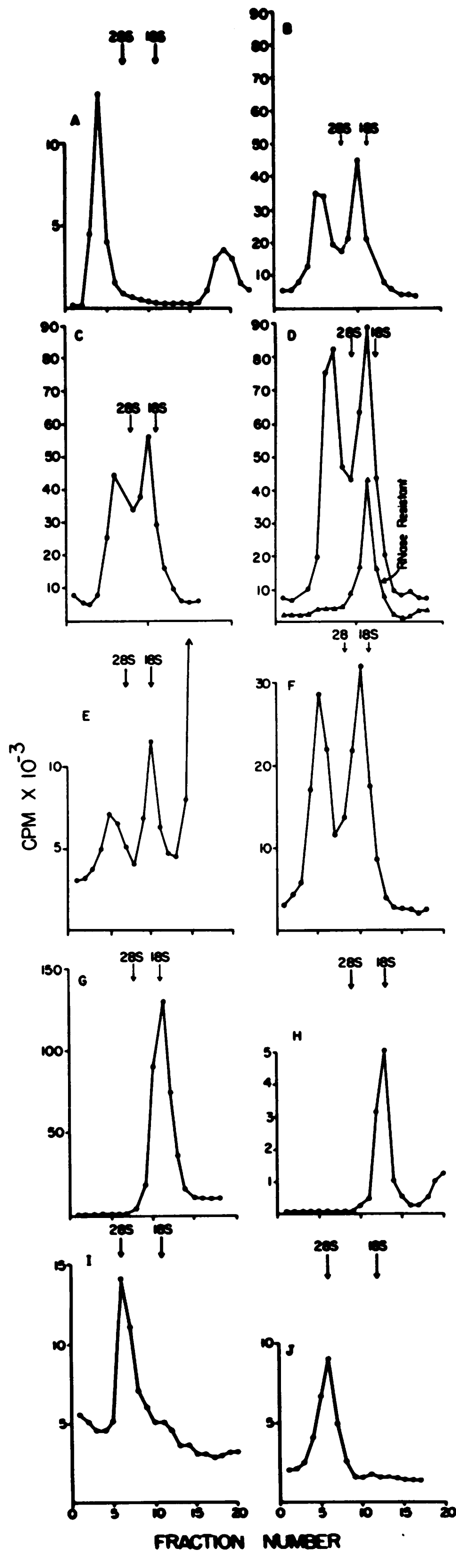
RNA labeled with [^{3}H]uridine from Vero cells infected with San Miguel sea lion virus in the presence of actinomycin D was analyzed by glycerol density gradient sedimentation and polyacrylamide gel electrophoresis. The predominant single-stranded RNA (36*S*, 2.6×10^6 molecular weight) was genome size. There was also a prominent 22*S*, 1.1×10^6-molecular weight, single-stranded component and one or more double-stranded or partially double-stranded classes. Replicative forms, sedimenting at 18*S*, contained single-stranded RNA corresponding to the larger-molecular-weight class. All classes of intracellular RNA and virion RNA were polyadenylated. These findings and results with pig kidney cells infected with vesicular exanthema of swine virus and feline cells infected with feline calicivirus indicate that caliciviruses exhibit a strategy of replication different from typical picornaviruses and supports removal of the caliciviruses from the family *Picornaviridae*.

The caliciviruses, San Miguel sea lion virus (SMSV), vesicular exanthema of swine virus (VESV), and feline calicivirus (FCV), have been considered a provisional genus of *Picornaviridae* (5, 10) despite incomplete knowledge of their molecular biology. The virions, small spherical particles (35 to 40 nm) with cup-shaped surface structures, contain one major capsid polypeptide (3, 14) and a 35-38*S* single-stranded RNA genome (11, 13). Recently, Brown and Black (2) reported an inability to detect precursor proteins to the capsid polypeptide in VESV-infected cells, supporting an earlier suggestion that these viruses differ from the picornaviruses and represent a new family of animal viruses (3). Our finding that multiple classes of RNA are synthesized in cells infected with caliciviruses provides additional evidence for a mode of replication atypical of picornaviruses.

Type 2 SMSV, type A_{48} VESV, and strain F-9 of FCV were propagated in Vero, porcine kidney (PK15), and Crandall feline kidney (FK) cells, respectively. General methodology was as previously described (11, 13-15). For intracellular viral RNA, cells in 3-ounce (ca. 90-ml) prescription bottles were infected at input multiplicities (multiplicity of infection [MOI]) of approximately 1 or 100 and incubated at 37°C (SMSV and VESV) or at 32°C (FCV) in minimal essential medium containing 20 μCi of [^{3}H]uridine and 5 μg of actinomycin D per ml. At various times the cultures were terminated for analysis of RNA as indicated in the figure legends. In preliminary experiments, traditional approaches using cytoplasmic extracts and phenol treatment failed consistently to demonstrate high-molecular-weight RNA (>22*S*), presumably due to RNase in these extracts. We found that a simple procedure of in situ solubilization of cell monolayers with sodium dodecyl sulfate (SDS) yielded consistent patterns of high-molecular-weight RNA. The monolayer was drained of medium and 0.5 ml of 1% SDS in TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl; pH 7.4) or in electrophoresis preparation buffer (4 mM Tris, 2 mM sodium acetate, 1 mM EDTA, 20% glycerol; pH 7.2) was added. This resulted in a viscous extract, which was analyzed immediately unless otherwise noted. More recently, alternative SDS techniques using proteinase K (Beckman Instruments, Inc.), either with (15) or without phenol treatment, gave essentially identical patterns of major RNA components, indicating validity of the simpler in situ approach used in most of our experiments.

A single major peak of labeled RNA with an *S* value of 36 was observed upon sedimentation of SDS-disrupted virions of SMSV (Fig. 1A), confirming earlier estimates (11, 13). The material at the top of the gradient was not characterized but may have represented degraded viral RNA or contaminating 4*S* cell RNA. RNA from infected cells showed two peaks with *S* values of 36 and 22. The amount of label in the two



peaks was approximately equal in extracts of Vero cells infected with SMSV, both peaks increasing as continuous labeling was extended from 3 h (Fig. 1B) to 5 h (Fig. 1C) and 7 h (Fig. 1D). Resedimentation of 36*S* and 22*S* peaks yielded single peaks of corresponding *S* value with only a trace of cross-contamination (not shown). Essentially identical RNA patterns were obtained with FCV in FK cells (Fig. 1E) and VESV in PK15 cells (Fig. 1F). The 36*S* RNA from cells infected with SMSV was degraded by RNase, whereas approximately one-third of the 22*S* RNA was resistant to RNase (Fig. 1D). Treatment with RNase prior to sedimentation also abolished the 36*S* peak and shifted the peak of RNase-resistant material to 18*S* (Fig. 1G). RNA from a 22*S* peak was treated with 2 M LiCl (16) to precipitate single-

FIG. 1. *Sedimentation patterns of calicivirus-specific RNA. (A) SMSV virion RNA. (B, C) RNA from SMSV-infected Vero cells at 3 and 5 h postinfection, respectively. (D) Total and RNase-resistant RNA from SMSV-infected Vero cells 7 h postinfection. (E) RNA from FCV-infected FK cells 12 h postinfection. (F) RNA from VESV-infected PK cells 5 h postinfection. (G) RNA, from 7-h SMSV-infected cells, pretreated with RNase. (H) LiCl supernatant of SMSV 22S RNA. (I) Denatured LiCl supernatant of SMSV 22S RNA in Me$_2$SO gradient. (J) Denatured SMSV 36S RNA in Me$_2$SO gradient. Linear 10 to 30% (wt/wt) glycerol gradients in TEN buffer in a Spinco SW 50L rotor at 45,000 rpm, 2.5 h, 4°C, were used for patterns A through H. Gradients of 10 to 30% (wt/wt) glycerol in 99% Me$_2$SO containing 0.1 M LiCl and 0.001 M EDTA in a Spinco SW 50.1 rotor at 40,000 rpm, 10 h, 22°C, were used for patterns I and J. Curves represent acid-insoluble radioactivity, and arrows indicate positions of Vero cell ^{32}P-labeled rRNA markers. Virion RNA labeled with ^{3}H (A) was from CsCl gradient-purified (14) virus treated with 1% SDS and 200 μg of proteinase K per ml at 21°C for 5 min. For infected cell extracts (B–J), MOI ≈ 100, in situ solubilization was used except as noted. RNase treatment of individual fractions (D) was with 10 μg of pancreatic RNase per ml (Worthington) for 30 min at 37°C. RNase pretreatment (G) was with 10 μg of RNase per ml for 30 min at 37°C, followed by proteinase K, 50 μg/ml, 30 min, at 37°C, of a cytoplasmic extract (2% Nonidet P-40, 15 min at 4°C; clarified at 600 × g, 5 min). The 22S peak fractions of a preparative gradient of RNA from 7-h SMSV-infected cells were adjusted to 2 M LiCl, held for 12 h at 4°C, clarified for 40 min at 10,000 × g, and precipitated with ethanol; a portion of the alcohol precipitate was suspended in TEN buffer and sedimented in an aqueous glycerol gradient (H), and another portion was suspended in 0.1 M LiCl plus 0.001 M EDTA, heated in 99% Me$_2$SO containing 0.1 M LiCl plus 0.001 M EDTA at 100°C for 1 min, and sedimented in a Me$_2$SO-glycerol gradient (I). A portion of the 36S peak fraction of the preparative gradient was similarly denatured and sedimented in Me$_2$SO (J).*

stranded RNA and, if present, partially double-stranded replicative intermediate (RI). RNA remaining in the supernatant, presumably double-stranded replicative form (RF), was completely resistant to RNase and sedimented at 18S (Fig. 1H). Denaturation of the LiCl-soluble RNA by heating in dimethyl sulfoxide (Me_2SO) yielded RNA with sedimentation characteristics in an Me_2SO gradient (Fig. 1I) identical to similarly treated 36S RNA (Fig. 1J). The shift in relative sedimentation rates resulting in cosedimentation of 36S viral and 28S rRNA under denaturing conditions cannot be considered unusual, since similar shifts occur in other systems using a 28S rRNA marker (7).

Polyacrylamide gel electrophoresis of labeled RNA from SMSV (Fig. 2A and B)- and VESV (Fig. 2C)-infected cells revealed two major peaks near the middle of the gel, one or more slowly migrating components, and material barely entering the gel. The MOI and time of infection with SMSV had little effect on the patterns except for differences in relative quantities of the major fractions (Fig. 2A and B and gels not shown). RNase pretreatment eliminated all but the slowly migrating material (Fig. 2D), which presumably represented RF and the double-stranded portions of RI. (Untreated RI should barely enter the gels.) The heterogeneity in double-stranded RNA revealed by electrophoresis (Fig. 2D), in contrast to apparent homogeneity indicated by sedimentation (Fig. 1G), has not been further investigated. The two more rapidly migrating electrophoretic components, which were completely abolished by RNase pretreatment, appeared to be single-stranded RNA with molecular weights of 2.6×10^6 for the major peak and 1.1×10^6 for the smaller peak (assuming Vero 28S rRNA = 1.67×10^6 and 18S rRNA = 0.7×10^6; reference 11). A 36S fraction and a 22S fraction obtained by density gradient sedimentation were subjected to electrophoresis separately. As expected, the 36S peak corresponded to the 2.6×10^6-dalton component (Fig. 2E), and the 22S peak contained the 1.1×10^6-dalton component and slowly migrating RNA (Fig. 2F). In Fig. 2F there appeared to be only one major double-stranded component; however, other 20–22S fractions exhibited greater heterogeneity of double-stranded RNA after electrophoresis (not shown). In addition to the major classes of RNA resolved by gel electrophoresis, minor components also appeared to be present. For example, a shoulder on the leading side of the 1.1×10^6 peak was observed regularly, suggesting RNA of molecular weight 0.6×10^6 to 0.8×10^6 (Fig. 2A, B, C, and F).

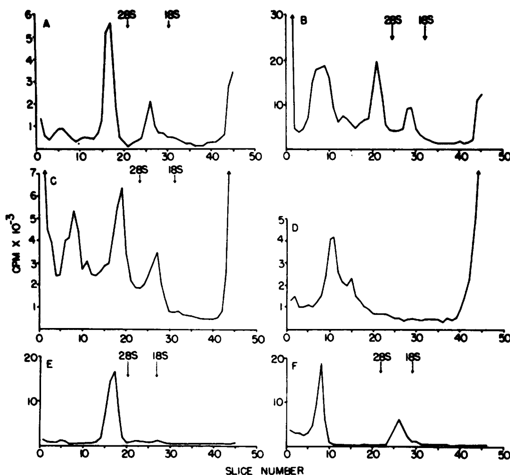


FIG. 2. *Polyacrylamide gel electrophoresis patterns of [^{3}H]uridine-labeled RNA from calicivirus-infected cells. (A) SMSV at an MOI ≈ 100 for 3 h. (B) SMSV at an MOI ≈ 1 for 14 h. (C) VESV at an MOI ≈ 100 for 5 h. (D) SMSV RNA (MOI ≈ 100, 7 h) pretreated with RNase. (E) 36S RNA. (F) 22S RNA. All extracts were prepared by in situ solubilization. For RNase pretreatment (D), RNA was further treated with phenol and precipitated with ethanol, suspended in electrophoresis preparation buffer, and incubated with 10 μg of RNase per ml at 37°C for 30 min. Preparative glycerol density gradient sedimentation of SMSV RNA (MOI ≈ 100, 7 h) provided 36S (E) and 22S (F) RNA. Arrows indicate peak positions of Vero cell ^{32}P-labeled rRNA. Electrophoresis was in 2.4% polyacrylamide gels (13) at 5 V/cm for 145 min; slices were 1.6 mm thick.*

Various SMSV RNA fractions were tested for the presence of polyadenylic acid [poly(A)] sequences, indicative of mRNA (9, 12, 16), by chromatography on oligodeoxythymidylic acid [oligo(dT)]-cellulose (1). Most (70 to 80%) of the virion RNA (Fig. 3A) and intracellular 36S (Fig. 3B) and 22S (Fig. 3C) RNA bound to oligo(dT)-cellulose. Sedimentation analysis of unbound RNA (fractions 2 and 3) from all three chromatograms revealed heterogeneous patterns indicative of degraded molecules. In contrast, bound 36S RNA, both virion and intracellular, showed homogeneous RNA sedimenting at 36S (not shown). Electrophoresis of bound 22S RNA (Fig. 3D) showed poly(A) on both 1.1×10^6-dalton single-stranded RNA and slowly migrating RNA. These results suggest an mRNA function for both 36S and 22S single-stranded RNA. Poly(A) on the slowly migrating fractions, the putative RF and RI, is in accord with poliovirus results (16).

Our finding that two major polyadenylated single-stranded RNAs are synthesized in cells infected with caliciviruses suggests that the

strategy of replication of caliciviruses is more like that of alphaviruses (group A togaviruses) than that of picornaviruses, such as poliovirus and encephalomyocarditis virus. In alphavirus-infected cells, single-stranded forms are 42S, characteristic of virion RNA, and 26S "interjacent" RNA, serving as mRNA for virion polypeptides; double-stranded RF appears to be heterogeneous (8, 12, 17). In contrast, the typical picornaviruses show only virion-size single-stranded RNA (9). In calicivirus-infected cells the 2.6×10^6-dalton 36S RNA appears to be analogous to virion RNA. The 1.1×10^6-dalton 22S single-stranded RNA is probably a viral mRNA similar to the 26S alphavirus RNA. There appear to be some differences in relative quantities and rates of synthesis of the mRNA's in the two systems, but these might be affected by factors such as cell system, MOI, and actinomycin D. The calicivirus 22S RNA appears too large to serve as mRNA for the 60,000- to 70,000-molecular-weight (2, 14) virion polypeptide unless post-translational cleavage occurs. Black and Brown (2) found no high-molecular-weight polypeptides serving as precursors of virion protein but did observe minor polypeptides, which they attributed to incomplete cell protein shutoff. Experiments in our laboratory (M. K. Fretz and F. L. Schaffer, unpublished data) indicate that there are viral-specific polypeptides with molecular weights both greater than and less than 70,000. However, these experiments have not yielded evidence for post-translational cleavage. Again by analogy to alphaviruses, difficulty in demonstration of post-translational cleavage may be due to technical problems (12).

The presence of defective interfering (DI) virus might have marked effects on RNA patterns in calicivirus-infected cells. DI RNA (and the corresponding RF and RI) in alphavirus-infected cells is much smaller than standard virion RNA (6), whereas in a well-studied picornavirus system DI RNA represents about 85% of the genome (4). Possible effects of DI virus were not rigorously excluded in our experiments. However, the high titers of our stocks (10^8 to 10^9 infectious units per ml) and experiments at low input multiplicity of a plaque-purified stock (Fig. 2B) indicate DI virus was not responsible for multiple RNA classes. Where calicivirus DI virus was observed, the size of DI particle RNA was much smaller than virion RNA (M. Jensen and S. R. Coates, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, S41, p. 211; personal communication), again pointing to a similarity to alphaviruses. Analogies to alphaviruses should not be carried to

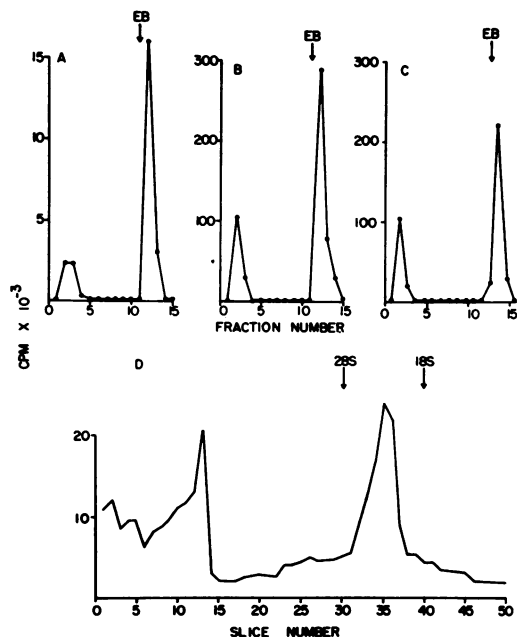


FIG. 3. *Characterization of poly(A)-containing SMSV RNA. Oligo(dT)-cellulose chromatography of (A) virion RNA, (B) 36S cellular RNA, and (C) 22S cellular RNA. (D) Polyacrylamide gel electrophoresis of the eluate of (C). Virion RNA was prepared as in Fig. 1; a preparative glycerol gradient provided 36S and 22S fractions of cellular RNA (in situ solubilization, MOI ≈ 100, 7 h). For chromatography, 0.7- by 2.5-cm columns of oligo(dT)-cellulose (T-3, Collaborative Research) in binding buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4) were washed (0.1 M NaOH, 0.5 M NaCl) and equilibrated with binding buffer; 1 ml of RNA in binding buffer was applied, followed by, consecutively, 10 ml of binding buffer and 5 ml of elution buffer (EB indicated by arrow); fractions of 1 ml each were assayed for radioactivity, and selected fractions were precipitated with ethanol for further analysis. Fractions 12, 13, and 14 (pooled) were subjected to electrophoresis as in Fig. 2.*

extreme in view of the difference in genome size and the enveloped nature and multiple polypeptides of alphavirus virions. Nevertheless, the aspects of RNA and protein synthesis discussed here support the suggestion (2, 3) of classifying caliciviruses apart from picornaviruses.

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