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Lactate dehydrogenase-elevating virus (LDV): subgenomic mRNAs, mRNA leader and comparison of 3'-terminal sequences of two LDV isolates

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Summary

The 3'-terminal 1314 nucleotides of the genome of one isolate of lactate dehydrogenase-elevating virus, LDV-P, has been derived by sequence analyses of cDNAs from several genomic libraries and compared to that of another LDV isolate, LDV-C (Godeny et al. (1990) *Virology* 177, 768–771). The 3'-non-coding segment of 80 nucleotides of the two LDV genomes is identical, whereas marked, but varying nucleotide and amino acid divergence is apparent in the three upstream overlapping open reading frames (ORF). The third ORF from the 3'-end exhibits only 82% nucleotide and 90% amino acid identity, whereas the 3'-terminal ORF, which encodes the nucleocapsid protein, exhibits approximately 99% amino acid identity. The second 3'-terminal ORF encodes an 18.8 kDa protein which lacks N-glycosylation sites but possesses 2 or 3 potential transmembrane helices in the N-terminal half of the molecule. A similar membrane organization is observed for the corresponding protein of equine arteritis virus and the M protein of mouse hepatitis virus. The sequence analyses combined with Northern hybridization analyses of RNA from LDV-infected macrophages and spleens of LDV-infected mice indicate that the three ORFs encoded by the 3'-terminal end of the LDV genome are expressed via the three smallest mRNAs (mRNAs 6–8) of the seven subgenomic mRNAs of LDV (mRNAs 2–8), which range in size from about 0.8 to

3.6 kb. All mRNAs have been shown to carry poly(A)-tracts and a common leader sequence. The seven mRNAs were produced in infected macrophage cultures concomitantly with genomic LDV RNA. Maximum LDV RNA synthesis was observed between 6 and 8 h post-infection. The same seven subgenomic mRNAs were detected in macrophages infected with three different isolates of LDV, but different relative amounts of some of the mRNAs were produced. The relative proportions of molecules of mRNAs 1–8 present in 6 h LDV-P-infected macrophages were about 13, 5, 5, 8, 6, 11, 11 and 27% of the total, respectively.

Lactate dehydrogenase-elevating virus; 3'-Terminal sequence; mRNA; Mouse; Open reading frame

Introduction

Lactate dehydrogenase-elevating virus (LDV) is a member of a new group of positive-stranded RNA viruses (Plagemann and Moennig, 1992). Other members of the group are equine arteritis virus (EAV) and most likely simian hemorrhagic fever virus (SHFV). On the basis of virion structure and size (50–60 nm diameter) and the polarity and size (12–13 kb) of their RNA genomes, LDV and EAV have been tentatively placed in the *Togaviridae* and SHFV in the *Flaviviridae* (Westaway et al., 1985a,b). However, recent studies have shown that in genome organization and mode of replication, EAV (den Boon et al., 1991) and LDV (Godeny et al., 1989, 1990; Kuo et al., 1991) resemble coronaviruses and toroviruses. Their genomes are expressed via formation of a 3'-coterminal nested set of 6 or 7 subgenomic mRNAs. The smallest mRNA of EAV and LDV encodes VP-1, the nucleocapsid protein. LDV, EAV and SHFV share other properties (see Plagemann and Moennig, 1992). The 3'-end of the genomes of all three viruses are polyadenylated and their virions are composed of three proteins of similar size: VP-1 (12–14 kDa); VP-2, a non-glycosylated envelope protein of 17–19 kDa; and VP-3, a group of glycosylated envelope proteins that range in size from 25–44 kDa. VP-3 of LDV probably represents a single gene product that varies in size due to different extents of glycosylation, but the presence of more than one structural glycoprotein has not been ruled out (Plagemann and Moennig, 1992). Other shared properties of LDV, EAV and SHFV are that they replicate primarily in macrophages and that all three can cause long term persistent infections without clinical symptoms in their natural hosts. LDV, for example, invariably establishes a viremic, life-long asymptomatic infection in all strains of mice. The persistent infection is maintained by the cytocidal replication of LDV in a renewable subpopulation of macrophages that seems to be the only permissive cell type in the host (Plagemann and Moennig, 1992; Onyekaba et al., 1989).

In the present study, we have further characterized the mRNAs formed in LDV-infected macrophage cultures and mice infected with three isolates of LDV that differ greatly in neurovirulence, and we have identified a 5'-leader that is

associated with all mRNAs. We also present the sequence of the 3'-terminal 1314 nucleotides of the genome of one isolate of LDV (LDV-P) that encodes three overlapping open reading frames (ORFs) and have compared its most 3'-terminal 1064 nucleotide sequence with that reported for another more neurovirulent isolate of LDV (LDV-C; Godeny et al., 1990).

Materials and Methods

Mice

Four- to 6-week-old Swiss mice were purchased from Sasco, Inc. (Omaha, NE) or Harlan Sprague Dawley, Inc. (Madison, WI). Suckling 10- to 14-day-old Swiss mice were raised in the animal facilities of the Department of Microbiology, University of Minnesota.

Virus

LDV-P was originally isolated in this laboratory (Brinton-Darnell and Plagemann, 1975). A highly neurovirulent variant of LDV, LDV-v (Contag and Plagemann, 1989), was isolated from a paralyzed C58/M mouse that had been infected with LDV-M originally obtained from Dr. W. Murphy (Murphy et al., 1983, 1987). An avirulent variant of LDV-M, LDV-a, was also obtained from Dr. W. Murphy (Murphy et al., 1987). LDV concentrations were determined by an end point dilution assay in mice as described previously (Plagemann et al., 1963). LDV titers are expressed as 50% infectious dose (ID_{50}). Each LDV variant was 'cloned' via the end point dilution assay; virus was isolated from a mouse receiving the highest dilution that resulted in infection. Groups of 10–50 Swiss mice were inoculated with each variant cloned in this manner. Their plasma was harvested 20–24 h p.i. and used to infect macrophage cultures.

Macrophage cultures

Resident peritoneal macrophages were harvested and cultured in 10-cm Petri plates with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 10% (v/v) L-cell conditioned medium as source of colony stimulating factor-1 as described previously (Stueckemann et al., 1982). Where indicated, the cultures were infected with LDV after 1 day in culture at a multiplicity of infection of $\geq 100 ID_{50}/macrophage$ as described previously (Stueckemann et al., 1982).

LDV-specific cDNAs

The cDNAs used in the present study came from three independently prepared genomic LDV-P libraries (Kuo et al., 1991). In one case, LDV-P RNA was reverse transcribed using oligo(dT) as primer and the cDNAs were cloned into the plasmid

pIBI24. cDNAs 4-55, 5-14, 4-11, 4-6, 4-14, 4-35 and 4-37 are part of this library. cDNAs L-192 and L-194 are from a genomic library prepared with LDV-P RNA primed with random oligodeoxynucleotides derived from calf thymus DNA and were cloned into pUC19. The third library was prepared from LDV-P RNA primed with hexanucleotide random primers and the cDNAs were cloned into λ ZAPII. cDNAs H63-15, G44, G11, M154, M172, 455-5 and 455-6 are from this library. cDNAs 341-3-4, 300-1-1, 300-2-1 and 344S8-2 were generated by polymerase chain reaction (PCR) amplification. Total RNA extracted from 6-h LDV-P-infected macrophage cultures or semi-purified LDV-P was reverse transcribed with purified avian myeloblastosis virus reverse transcriptase (Pharmacia, Piscataway, NJ) using a number of different antisense primers: 5'-GTGCTGCATCTGACTGACA-3' (B1151), 5'-CGGCATTGCGAAGCAACGCACTAA-3' (B1835), and 5'-ATCACAAAATTCTAGGCCTCCCATT-3' (B1836), which are complementary to sequences at the 3'-terminus of the genome, the 5'-end of ORF-1 and the 5'-end of ORF-2, respectively (see Fig. 2). The products were amplified with appropriate upstream sense oligonucleotides using an Ericomp Twin Block System thermal cycler set for 30 cycles and AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) as recommended by the supplier. The amplified products were purified by gel electrophoresis, cloned into the PCR 1000 vector using a TA cloning kit from Invitrogen (San Diego, CA) and sequenced in both orientations using the Sequenase Version 2.0 (USBC, Cleveland, OH) or TaqTrak (Promega, Madison, WI) sequencing systems as described by the manufacturers. Other cDNAs were subcloned into pBluescriptII or into M13mp19 and sequenced in the same manner (Kuo et al., 1991). cDNA pBR11 was generated by reverse transcription of poly(A)-selected RNA from 6-h LDV-infected macrophage cultures using oligonucleotide B1151 as primer, followed by second strand DNA synthesis and cloning of the cDNA into pBluescriptII.

Northern blot hybridization

Total RNA was extracted from cultures of uninfected and LDV-infected macrophages (generally from two 10-cm culture plates at one time) or from the spleens of LDV-infected mice by the acid-guanidinium thiocyanate method as described previously (Contag et al., 1986; Contag and Plagemann, 1989). Poly(A) containing RNA was isolated from spleens using the 'Fast Track' isolation kit from Invitrogen. Northern blot hybridizations of glyoxylated RNA were conducted as described previously (Contag et al., 1986; Contag and Plagemann, 1989; Kuo et al., 1991) using a number of LDV-specific cDNA probes or an RNA probe complementary to the sense strand of cDNA 4-55. cDNA 4-55 was subcloned in the appropriate orientation into pBluescriptII and transcribed in the presence of [α - 32 P]ATP (Amersham Corp., Arlington Heights, IL) using the T7 promoter of the plasmid. All cDNA probes were labeled by random priming (Feinberg and Vogelstein, 1983) with a kit (Prime-a-Gene) and [α - 32 P]dATP according to the procedure described by the manufacturer (Promega) and hybridized sequentially to Northern blots at 50°C in 50% formamide or in a hybridization solution containing

1% (w/v) bovine serum albumin, 1 mM EDTA, 0.5 M sodium phosphate (pH 7.2) and 7% (w/v) sodium dodecylsulfate (SDS; Church and Gilbert, 1984). The hybridized blots were autoradiographed using Kodak XAR-5 film and, where indicated, the intensity of the bands of the autoradiograms quantitated. The exposed X-ray films were placed over a light source and the image captured with a single capacity high resolution video camera. The images were scanned using the density profile plot analysis of the NIH Image program (version 1.41). The plots of relative density were corrected for film background density and that resulting from non-specific hybridization using the WINGZ spreadsheet program (version 2.10) and the areas under the density peaks integrated.

Results and Discussion

Nucleotide sequence of 1.3-kb 3'-end of LDV-P genome

Fig. 1A illustrates the alignment of various LDV-specific cDNA clones from which the nucleotide sequence of the 3'-terminal 1314 nucleotides of the genome of LDV-P was derived (Fig. 2). Clones representing the 3'-terminus of the LDV genome contained poly(A) tracts consisting of 13–27 AMP (Fig. 1A). Only six nucleotide ambiguities arose in the sequence analysis even though the clones came from several different sources. The sources included three genomic LDV-P cDNA

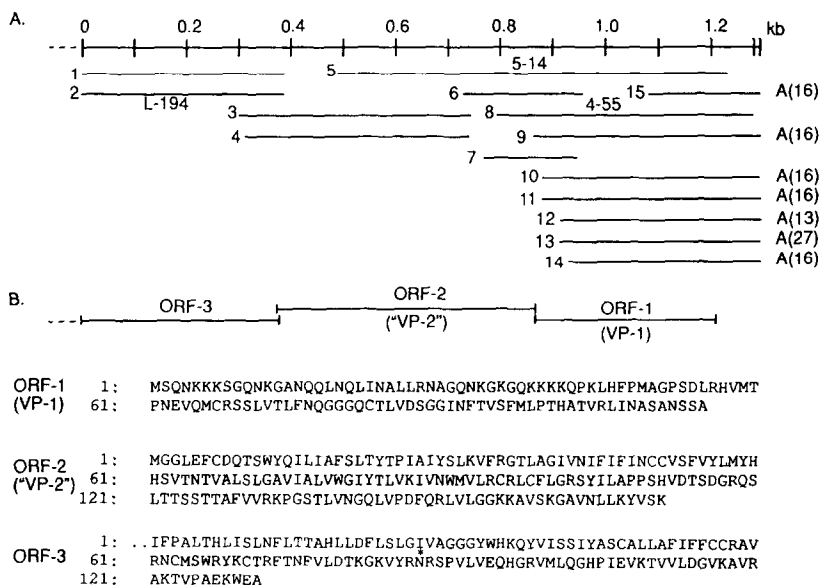


Fig. 1. Alignment of various cDNAs representing the 3'-terminus of the genome of LDV-P and the ORFs predicted by the sequence (A) and the amino acids encoded by the ORFs (B). The cDNAs in (A) are numbered as follows: 1, 344s8; 2, L-194; 3, 300-1-1; 4, 300-2-1; 5, 5-14; 6, 341-3-4; 7, pBR11; 8, 4-55; 9, 455-6; 10, G11; 11, H63-15; 12, M172; 13, 455-5; 14, G44; and 15, M154. 'A' at the 3'-end indicates presence of a poly(A) track with the number of A indicated in parentheses. * Potential N-glycosylation site in ORF-3 (B).

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ldv-p : AATTTTCCAGCTTTGACTCACTTAATTTCCCTTGAATTTCTTACCACCGCCACTTGT 60
ldv-c : .....

ldv-p : AGATTTTCTTTCATTTGGGCATTGTAGCTGGAGGAGCTATTGGCATAAACAGTATGTCAT 120
ldv-c : .....

ldv-p : TAGCTCAATTTATGCAAGTTGTGCCCTTCTCGCCTTCATTTTCTTCTGCTGTAGAGCAGT 180
ldv-c : .....

ldv-p : TAGGAATGTATGAGCTGGAGATATAAGTGCACCAGATTTACAAATTTTGTCCCTGATAC 240
ldv-c : .....

ldv-p : CAAAGGAAAAGTCTACCGGAACCGCAGTCCGGTTCGGTGGAAACAGCATGGCCGAGTCAT 300
ldv-c : .....-T-TT-----T-C--C--T--T--G----C--AAA---AT-

ldv-p : GCTACAAGGCCACCCAATTGAGGTTAAGACCGTTGTGCTCGATGGTGTAAAGCCGTGCG 360
ldv-c : --G-----A-C-----G---A-----C---C---T--C--

ldv-p : GGCTAAAACCGTCCCTGCCGAAAAATGGGAGCCCTAGAATTTTGTGATCAAACCTCCTCG 420
ldv-c : -----T-A-----
                        VP-2 →
ldv-p : TATCAGATACTGATTGCCCTTAGTTTGACTTATACCCCAATTGCCATCTACAGCCCTAAG 480
ldv-c : -----TT-C-----T-----T--T-A--A

ldv-p : GTTTTTCGTTGGCACCITTTGGCIGGTATGTAAATATTTTATTTTATAAAATGTTGTGTT 540
ldv-c : -----A-----T-----A

ldv-p : AGTTTTGTGTATTTGATGTACCATCTCTGTTACCAACACAGTTGGCCTGTCTCTCGGA 600
ldv-c : -----T-----C---TT---AT-G--T

ldv-p : GCTGTTATTGCCCTCGTGGGGCATTACACTCTTGTCAAGATAGTAAACTGGATGGTA 660
ldv-c : ---G---T--T-----C---G-----TG-T--T--G

ldv-p : CTCAGATGCCGGTGTGCTTCCTAGGTAGAAGTTACATCCTAGCCCCACCCAGCCACGTTG 720
ldv-c : A---G-----

ldv-p : GACACCTCTGACGGACGTCAGAGCCTAAACCACATCGTCAACAACCGCCTTTGTGGTTAGG 780
ldv-c : -----G-----T-----

ldv-p : AAGCCAGGTAGTACCCTTGTGAACGGGCAGTTGGTCCCGGACTTTCAAAGACTCGTGCTT 840
ldv-c : -----A-----

ldv-p : GGGGGCAAGAAGGCCGTATCAAAGGAGCGGTTAACCTTTTGAAGTATGCTCAAATAAA 900
ldv-c : -----G-----T-----A-----
                        VP-1 →
ldv-p : GAAGAAAACCGCCCAAGATAAAGGGCAAACCGCAACTTAATCACTTGATTAATGCATT 960
ldv-c : -----GG---T-----C-----G--G--

ldv-p : GCTTCGCAATGCCGGACAGATAAAGGAAAGGGGCAGAAAAAGAAGCAGCCCAAGCT 1020
ldv-c : -----A-----A-G-A-

ldv-p : GCACCTCCCTATGGCCGGACCATCTGATCTCCGCCATGTGATGACCCCTAATGAGGTACA 1080
ldv-c : --T---C---A-T-----C-G-G---T---T--C--C---T--

ldv-p : AATGTGTAGATCCTCATTTGGTAACTTGTTCATCAATCAAGTGGTGGACAGTGTACCTTAGT 1140
ldv-c : G-----C---T-----G--C-T--T---G--C-A--T-----C--A--G--

ldv-p : TGACTCCGGAGGGATCAATTTCACTGTCAGTTTCATGCTTCCAACGCATGCCACTGTTCG 1200
ldv-c : -----T-T-A-A-----C-G-----T-G-----A-C--

ldv-p : GCTAATTAATGCTTCTGCTAATCTTCTGCTTAAGAGTTACAATGTAAGTCATGTGCAGTC 1260
ldv-c : -T-----A-----G-----

ldv-p : AGATGCAGCGACTCAGCCTTTTGTAAATTAATTCGATTGGCTGGGCCGGAATF 1314
ldv-c : .....

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Fig. 2. Nucleotide sequence of 3'-end of LDV-P genome and comparison to that of LDV-C (T = U). The initiation and/or the termination codons for ORFs 1 to 3 are underlined. The lines above the sequence indicate the putative TAACCA and TAAAACC junctions between the 5'-leader and the bodies of mRNAs 7 and 8.

libraries which were generated independently over a 3 year period. Three clones were generated independently by reverse transcription/PCR amplification of total RNA and one clone by reverse transcription of poly(A)-selected RNA from LDV-P-infected macrophages. In each case of nucleotide ambiguity, one clone out of three contained an alternate nucleotide. These were G instead of A, G instead of T, A instead of C, A instead of G, T instead of C, and G instead of A at nucleotide positions 223, 325, 331, 591, 639 and 648, respectively. It is of interest that in the first four cases, one of the LDV-P nucleotides was the same as found in the genome of LDV-C (see below). The nucleotide differences in the LDV-P cDNAs did not affect the coding specificities of the overall consensus sequence. It encodes two complete overlapping ORFs (ORFs 1 and 2) and one incomplete ORF (ORF-3) overlapping ORF-2 (Fig. 1). ORF-1 and ORF-3 are read in the same frame, whereas ORF-2 is read in another frame. ORF-1 and ORF-2 overlap by four amino acids and ORF-2 and ORF-3 by three amino acids. This arrangement is the same as previously reported for another isolate of LDV, LDV-C (Godeny et al., 1990).

The most 3'-terminal ORF encodes a highly basic protein of 115 amino acids (12.4 kDa) which has previously been identified as VP-1 by limited N-terminal sequence analysis of purified VP-1 from LDV-C (Godeny et al., 1990). The second ORF encodes a 171-amino acid protein (18.8 kDa) and the third ORF a protein of more than 131 amino acids (Fig. 1B). The identity of the latter two proteins is uncertain. However, on the basis of its apparent molecular weight, the absence of potential N-glycosylation sites and the presence of two or perhaps three membrane spanning segments (amino acids 12–31, 39–58 and 68–87; Fig. 3A), it could be the non-glycosylated envelope protein, VP-2. The comparative protein of EAV as well as the M protein of mouse hepatitis virus (MHV) A59 possess three similar putative transmembrane helices in the amino terminal half of the molecule (Fig. 3B, C). Other considerable similarities in the hydrophobicity plots of the ORF-2 proteins of LDV and EAV are apparent (Fig. 3A, B), even though there exist no significant amino acid homologies between these two proteins or between either of them and the M protein of MHV.

The third ORF of LDV seems to encode a glycoprotein since it possesses at least one potential N-glycosylation site (Fig. 1B). The third reading frame of the nucleotide sequence contains two additional potential small ORFs, one located within ORF-1 (nucleotide 954–1048) and encoding 35 amino acids, and the other overlapping the 3'-end of ORF-1 and the 3'-non-coding segment (nucleotide 1210–1284) and encoding 25 amino acids. However, examination of the sequences surrounding the AUG initiation codons of these ORFs indicates that they represent poor translation initiation signals (Kozak, 1989).

Comparison of the 3'-terminal 1065-nucleotide sequences of LDV-P and LDV-C and of the amino acids encoded by their ORFs

Comparison of the 3'-nucleotide sequences and of the encoded amino acids of LDV-P and LDV-C (1065 nucleotides; Godeny et al., 1990) revealed some interest-

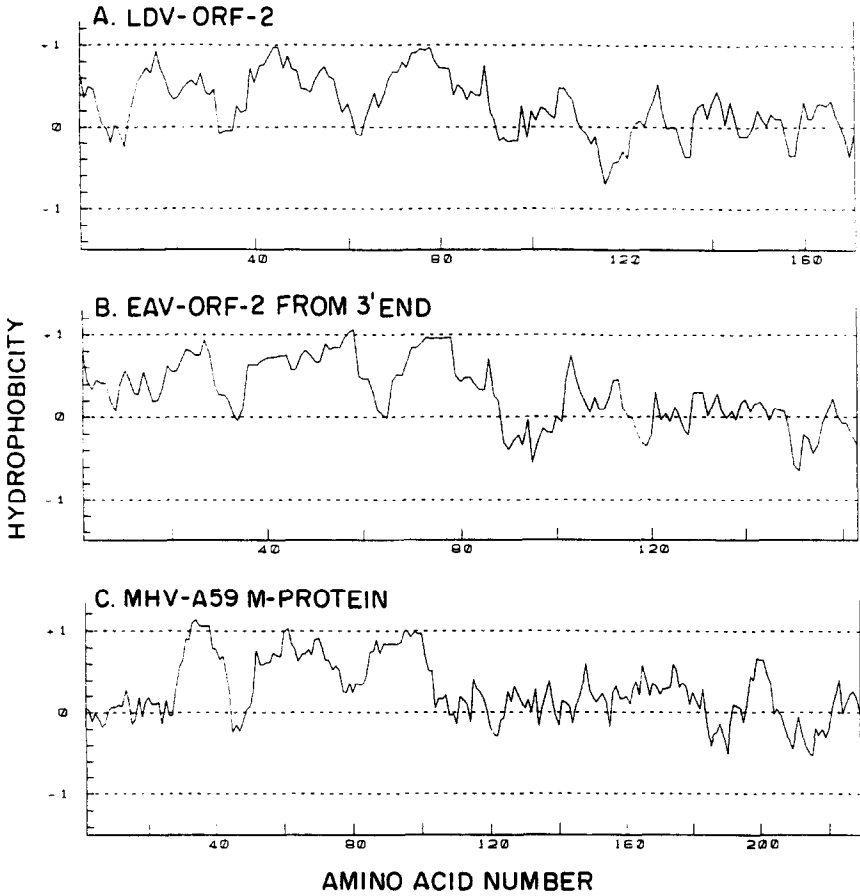


Fig. 3. Hydrophobicity plot according to Eisenberg et al. (1984) of the protein predicted by LDV ORF-2 (A). Two adjacent transmembrane helices of 21 amino acids each (amino acids 38–58 and 68–88) were identified on the basis of a mean hydrophobicities of 65 and 67, respectively. A third segment (amino acids 12–32) with a mean hydrophobicity of 49 may also qualify as a membrane spanning helix (Eisenberg et al., 1984). B and C show similar hydrophobicity plots for the corresponding protein of EAV (den Boon et al., 1991) and the M protein of MHV A59 (GenBank accession number J02252 X00509), respectively.

ing clues as to the potential genetic variability of LDV (Table 1). First, the 3'-non-coding 80 nucleotide segment of the two LDV variants is identical. Thus, this segment is highly conserved probably because of structural restrictions in the replicase attachment site or in other vital functions. In contrast, the ORFs for VP-1 of the LDV variants exhibit only 86% nucleotide identity, but close to 99% amino acid identity. Thus, most of the nucleotide differences are silent. This is not the case for the ORF of 'VP-2'. The differences in amino acids (4.6%) are not much less than the nucleotide differences (7.2%). The results suggest that the amino acid sequence of VP-1 needs to be conserved, perhaps because of specific

TABLE 1

Nucleotide differences between ORFs 1-3 and 3'-non-coding segment of LDV-P and LDV-C and of amino acids encoded by ORFs

The values were derived from the sequences shown in Figs. 1 and 2. D/T = Different/Total.

Segment	Nucleotide differences								Amino acid differences	
	D/T	%	A-G	C-U	A-C	A-U	C-G	U-G	D/T	%
	3'-non-coding	0/81	-							
ORF-1 (VP-1)	48/345	13.8	15	17	3	6	3	4	2/115	1.3
ORF-2 ('VP-2')	37/513	7.2	9	12	1	10	2	3	7/171	4.6
ORF-3 (Partial)	26/144	18.1	3	8	6	3	3	3	5/48	10.4

interactions of VP-1 with the viral RNA in the virion core, whereas VP-2, as an integral envelope protein, can accommodate greater variability. The greatest differences in both nucleotides and amino acids are observed in the partial third ORF which probably encodes a glycoprotein and may allow even greater variability. Further studies of additional LDV variants are required to determine whether these conclusions are valid. It might also be of interest that the nucleotides of ORFs 1 and 3 of the two variants diverged much more than those in the ORFs for VP-2 (Table 1). However, the different nucleotide divergence in these ORFs does not seem to be associated with specific nucleotide differences; A-G, C-U and A-U differences predominated in both ORFs 1 and 2, though the specific nucleotide differences were somewhat differently distributed in ORF-3 (Table 1).

Correlation between organization of 3'-terminal ORFs and subgenomic mRNAs 6-8

Northern hybridization analyses of RNA extracted from 6-8 h LDV-infected macrophage cultures using different cDNA probes was used to probe the relationship between the three ORFs encoded by the 3'-terminal end of the LDV genome and the subgenomic mRNAs. In a previous study (Kuo et al., 1991) it was shown that cDNA 4-55 representing the 3'-terminus of the LDV genome (see Fig. 1A) hybridizes to genomic LDV RNA of 12-13 kb, as well as to the nested set of seven subgenomic RNAs that range in approximate size from 0.8 to 3.6 kb (see Fig. 4). Furthermore, cDNAs L-194 and L-192 were found to hybridize only to the five and four largest subgenomic RNAs, respectively. We have extended these Northern hybridization analyses using additional cDNAs as probes. cDNA 5-14 which overlaps with cDNA 4-55 (see Fig. 1A) also hybridized to all subgenomic RNAs, whereas a 5'-end 345-nucleotide restriction fragment of cDNA 5-14 hybridized only to the six largest subgenomic RNAs (Fig. 4). These hybridization patterns correlate well with the location of these cDNAs in relation to the three 3'-terminal ORFs of the LDV genome (see Fig. 1A). Thus, mRNAs 8, 7 and 6 encode ORF-1 (VP-1), ORF-2 and ORF-3, respectively. cDNA L-192 must represent a sequence upstream of the three ORFs and therefore hybridized only to RNAs 1-5. We have also shown previously that cDNAs 4-11, 4-6 and 4-35, which encode continuous ORFs, hybridize only to genomic RNA (Kuo et al., 1991; see Fig. 4) and thus represent sequences upstream of the ORFs encoded by the mRNAs. They proba-

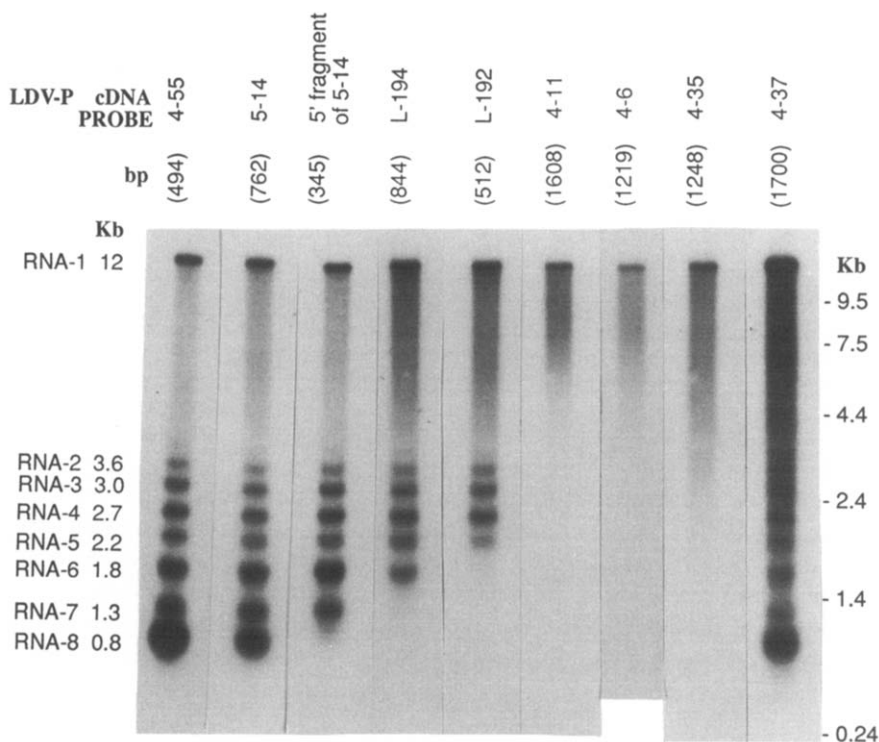


Fig. 4. Northern hybridization analysis of total RNA from 6-h LDV-infected macrophages from 2- to 4-week-old mice. The blot was sequentially hybridized with the indicated cDNA probes (see Fig. 1 and text) as described under Materials and Methods.

bly represent sequences equivalent to ORFs 1a and 1b of the coronaviruses and EAV that encode a putative replicase (Spaan et al., 1988; den Boon et al., 1991). This view is supported by the finding that the predicted polypeptide encoded by the ORF of cDNA 4-11 contains replicase and zinc finger motifs that are characteristic of the replicases of positive strand RNA viruses (Kuo et al., 1991). The LDV replicase motif exhibits 75% amino acid identity with that of the 1b protein of EAV (Kuo et al., 1991). cDNA 4-35 also encodes a polypeptide with a cysteine-rich region (Kuo et al., 1991).

LDV mRNA leader

All subgenomic mRNAs of coronaviruses and of EAV possess a 5'-terminal untranslated leader sequence that is derived from the 5'-end of the genome (Spaan et al., 1988; den Boon et al., 1991). In contrast, the subgenomic mRNAs of toroviruses seem to lack a leader sequence derived from the 5'-end of the genome (Snijder et al., 1991a). The size of the mRNA leaders of various coronaviruses range from about 60 to 75 nucleotides (Spaan et al., 1988), whereas that of EAV is 207 nucleotides long (den Boon et al., 1991).

The following results indicate that the subgenomic mRNAs of LDV also possess a 5'-leader sequence as reported for coronaviruses and EAV. We found that cDNA 4-37 hybridized to all subgenomic RNAs (Fig. 4), in spite of not exhibiting sequence homology with any of the other cDNAs. A terminal restriction fragment of about 1 kb of cDNA 4-37 also hybridized to all subgenomic RNAs, whereas another cDNA of 812 nucleotides (4-18) that represents the opposite end of cDNA 4-37 hybridized only to genomic RNA (data not shown). This result coupled with the knowledge of the coding sequences of RNAs 6-8 indicates that the restriction fragment of cDNA 4-37 encompasses part of an untranslated leader that is joined to the 5'-end of the bodies of the mRNAs. This conclusion has been confirmed by demonstrating that a genomic segment represented by the 5'-terminal sense strand of cDNA 4-37 becomes linked during mRNA synthesis to the bodies of mRNAs 5, 6 and 8 via a common UAACCA sequence and to mRNA 7 probably via a UAAAACC sequence (Chen et al., in preparation), which are located upstream of the AUG initiation codons of their respective ORFs. In the case of mRNAs 8 and 7, the junction sequences precede the initiation codons of their ORFs by 104 and 14 nucleotides, respectively (see Fig. 2). If we assume that the LDV leader is of about the same size as that of EAV (207 nucleotides) LDV mRNAs 8 and 7 should be about 770 and 1150 nucleotides long, respectively. The sizes of mRNAs 8 and 7 estimated from gel electrophoretic profiles (0.8 and 1.3 kb respectively, see Fig. 4) agree approximately with the predicted values.

Subgenomic poly(A)-containing RNAs in spleens of LDV-infected mice

Previous studies indicated that the spleen is one of the primary organs of the mouse containing LDV-permissive macrophages which become rapidly infected and destroyed upon initial exposure of a mouse to LDV (Chan et al., 1989; Plagemann and Moennig, 1992). At 1 day p.i. large amounts of genomic LDV RNA have been detected in spleens by Northern hybridization analyses of total spleen RNA but no subgenomic RNAs were detected since cDNA 4-11 was used as hybridization probe (Contag and Plagemann, 1989; Contag et al., 1989).

In the present study, total RNA as well as poly(A)-selected RNA extracted from spleens of 18-h LDV-infected mice was analyzed by Northern hybridization using cDNA 4-55, the 3'-end-specific cDNA, as hybridization probe. The results indicate that the same seven genomic LDV RNAs are produced in the spleen of infected mice as in macrophage cultures and that all mRNAs, like genomic LDV RNA (Contag et al., 1986; Brinton et al., 1986), possess a poly(A)-tract (Fig. 5A; X-ray films were exposed to the blot of poly(A)-selected RNA for two different lengths of time to document the presence of genomic RNA and all seven mRNAs).

In the hybridization analysis of total spleen RNA, two additional weak bands were observed at about 1.5 and 4.4 kb. These hybridization bands have often been encountered in Northern hybridization analyses to varying extents. They appear to reflect binding of the probes to ribosomal RNA which is present in high concentrations in the total cell RNA preparations. This conclusion is indicated by comparing

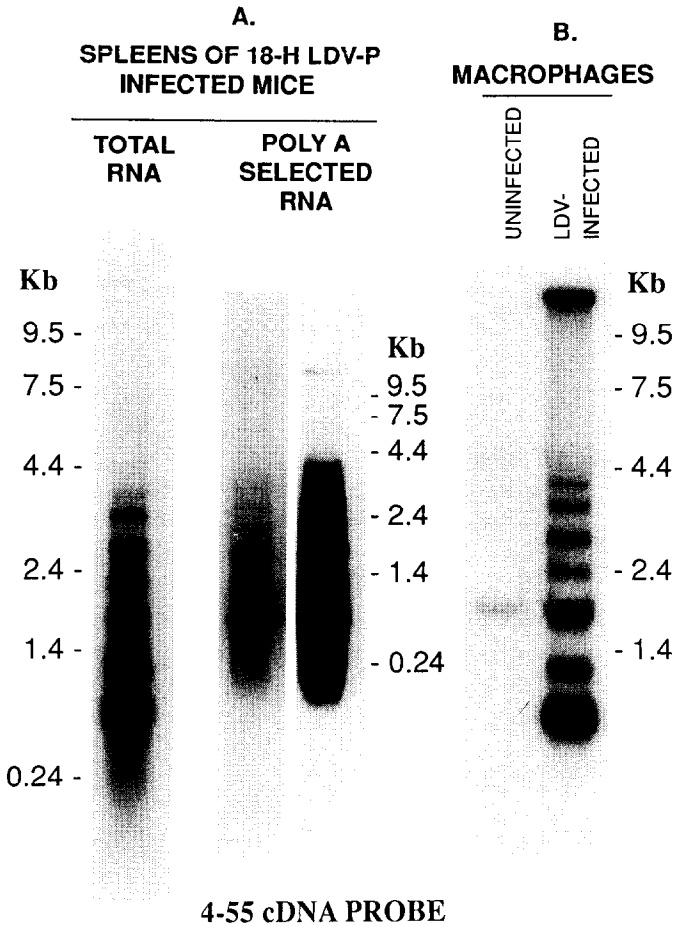


Fig. 5. Northern hybridization analyses of total RNA and poly(A)-selected RNA from spleens of 18-h LDV-infected mice (A) and total RNA extracted from uninfected and 6-h LDV-infected macrophage cultures (B). The blots in A were hybridized with the 3'-end-specific cDNA 4-55 (see Fig. 1) and in B with a (+) strand-specific RNA probe prepared to cDNA 4-55.

the hybridization of the probes to blots of total RNA extracted from uninfected and LDV-infected cells or mouse tissues (Fig. 5B).

Relative proportions of LDV-P RNAs in infected macrophages

The data in Fig. 5B have been used to estimate the relative proportions of the eight LDV-P RNAs in infected macrophages. Video-captured images of the Northern blots of RNA from LDV-infected and uninfected macrophages that were hybridized with the 3'-end-specific RNA probe (Fig. 5B) were scanned for density. The profile for RNA from LDV-infected macrophages was corrected for the profile obtained with RNA from uninfected cells, i.e. for hybridization of the probe to 1.5 and 4.4 kb ribosomal RNAs. Then the area of each peak was

integrated and its proportion of the total calculated. These values represent the relative molecular concentrations of each LDV RNA since each RNA possesses only a single copy of the sequence with which the probe hybridizes. This analysis indicated that at 6 h p.i. RNAs 1–8 represented about 13, 5, 5, 8, 6, 11, 11 and 27% of the total LDV-specific RNA. Thus, as observed for coronaviruses and EAV (Spaan et al., 1988; Hoffman et al., 1990; de Vries et al., 1990), the nucleocapsid mRNA (RNA-8 in the case of LDV) was the predominant RNA molecule synthesized. RNAs 3–5 were produced in similar concentrations and RNAs 6 and 7 in about twice these concentrations. Similar results were obtained in repeated experiments with LDV-P, at least with respect to the relative proportions of RNAs 3–8 (see Figs. 4, 5A and later Fig. 7). Some variations were observed in the relative proportions of RNAs 1 and 2. The variations in RNA-1, the genomic RNA, we attribute to an inefficiency in the blotting of this relatively large RNA molecule. The variation in the apparent presence of RNA-2 is unclear.

Time course of synthesis of LDV genomic and subgenomic RNAs in infected macrophages

LDV RNA synthesis was investigated in two different ways, by radiolabeling and by Northern hybridization analysis. In the first approach, replicate cultures of LDV-infected macrophages were treated with actinomycin D at various times p.i. and then incubated with [5-³H]uridine for 2 h. Total RNA of the macrophages was extracted with SDS and analyzed by zone sedimentation in sucrose density gradients (Fig. 6A–E). The results indicate that little LDV-specific RNA was produced before 4 h p.i., whereas maximum amounts were produced between 6 and 8 h p.i. The results are in agreement with those reported previously using this approach (Stueckemann et al., 1982). The present results, however, also demonstrate that LDV-specific RNA that sedimented slower than the 42S genomic RNA was produced concomitantly with the latter; it represented about 50% of the total LDV-specific RNA whether the macrophages were incubated with [³H]uridine from 4–6, 6–8 or 8–10 h p.i. This broad band of slower sedimentating RNA probably represented the seven subgenomic LDV mRNAs. Though sucrose density gradient centrifugation does not allow the separation of the individual mRNAs, the radioactivity profiles suggest that all LDV-specific RNA were synthesized at a similar rate. This conclusion is supported by Northern hybridization analyses of RNA extracted from infected macrophages using cDNA 4-55 as a 3'-end specific probe (Figs. 4, 5 and 6F). All subgenomic RNAs were produced concomitantly in a similar time course. Maximum amounts of LDV RNAs were present at 8 h p.i. The subsequent disappearance of the LDV mRNAs probably reflected degeneration of the LDV-infected macrophages and their phagocytosis by residual macrophages (Onyekaba et al., 1989).

The total weight of the subgenomic mRNAs as calculated from the Northern hybridization profiles represented about 40% of the amount of total viral RNA that was produced. This value agrees with the estimate (50%) derived from the sucrose density gradient analyses of [³H]uridine-labeled LDV RNA (Fig. 6A–E).

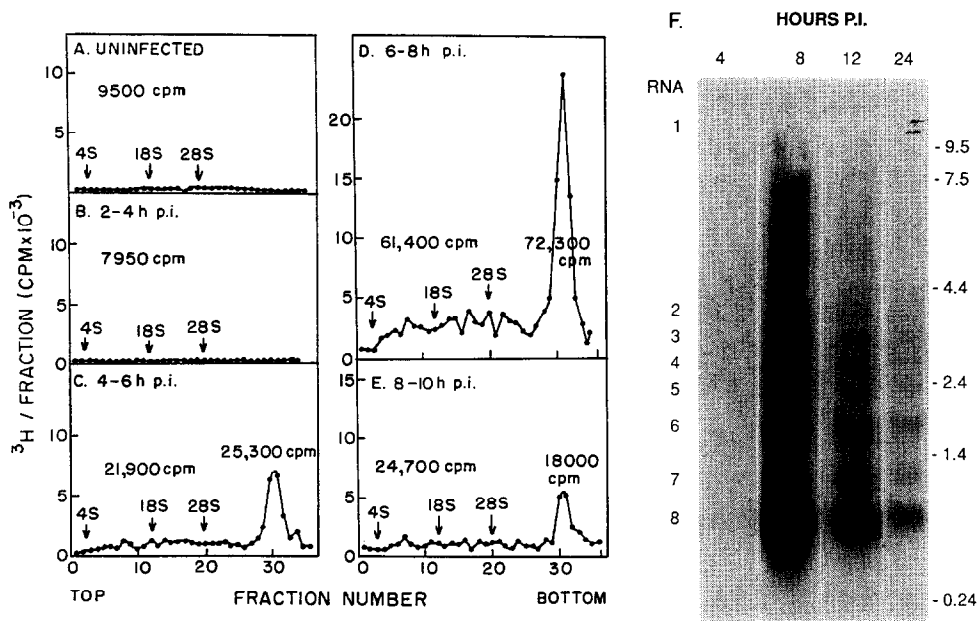


Fig. 6. Analyses of LDV RNA synthesized in macrophage cultures at the indicated times p.i. (A-E). Replicate LDV infected macrophage cultures were supplemented with actinomycin D ($1 \mu\text{g}/\text{ml}$) 0.5 h before incubation for 2 h with [^3H]uridine ($10 \mu\text{Ci}/\text{ml}$). After labeling the medium was discarded, the cells were lysed in a buffered solution containing 1% SDS and the lysates analyzed by zone centrifugation in 0.15–0.9 M sucrose density gradients containing SDS as described previously (Tong et al., 1977). Fractions from the gradients were analyzed for radioactivity in acid-insoluble material. (F) Total RNA extracted from replicate cultures of macrophages at the indicated times p.i. with LDV was analyzed by Northern hybridization using cDNA 4-55 as probe.

Subgenomic RNAs produced in macrophages infected with different variants of LDV

We have compared mRNA synthesis in macrophages infected with three different variants of LDV, LDV-a, LDV-v, and LDV-P in order to explore the possibility that one or more of the subgenomic LDV RNAs we have identified in LDV-P-infected macrophages are defective interfering RNAs. This possibility needed serious consideration since defective interfering RNAs in the molecular weight range of the subgenomic mRNAs arise rapidly in cells during undiluted passage of coronaviruses and toroviruses (van der Most et al., 1991; Makino et al., 1991; Snijder et al., 1991a) and since only six subgenomic RNAs have been detected in cells infected with EAV (den Boon et al., 1991) rather than the seven we find in LDV-P-infected cells. Furthermore, for an efficient infection with LDV, cultured macrophages must be exposed to very high multiplicities of infection (100 – 1000 mouse $\text{ID}_{50}/\text{macrophage}$; Tong et al., 1977; Onyekaba et al., 1989) and stocks of LDV generally consist of plasma harvested from mice 20–24 h p.i. with 10^6 $\text{ID}_{50}/\text{mouse}$.

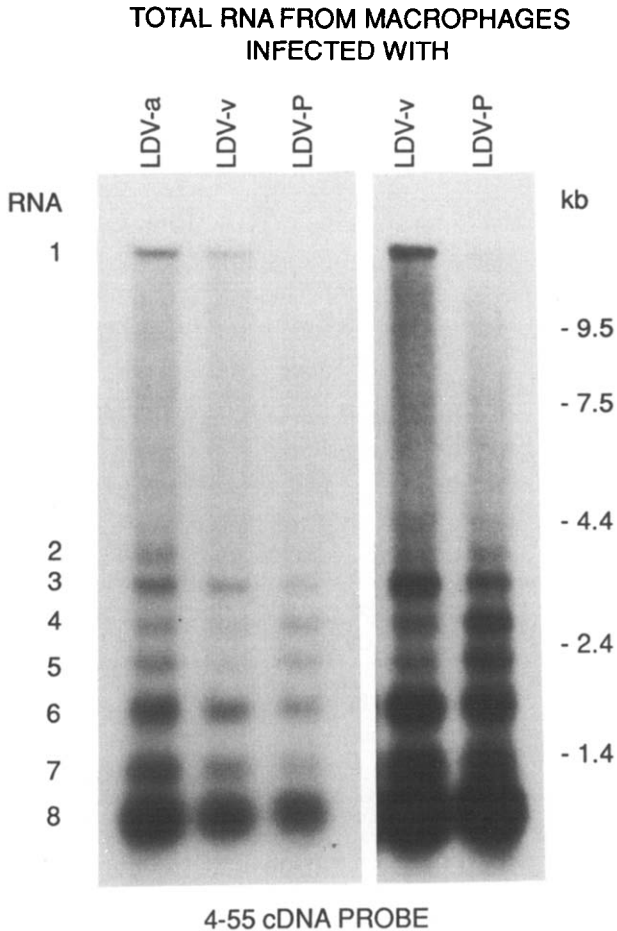


Fig. 7. Northern hybridization analyses of total RNA extracted from macrophage cultures 6-h p.i. with LDV-a, LDV-v or LDV-P using cDNA 4-55 as probe. The right hand lanes represent longer exposures of the left lanes.

However, we detected the same seven subgenomic mRNAs in macrophages infected with LDV-a and LDV-v as in macrophages infected with LDV-P (Fig. 7). Thus, it seems very unlikely that any of the subgenomic RNAs represents a defective interfering RNA. Furthermore, the subgenomic RNAs induced by the three LDV isolates showed approximately identical rates of migration. Thus, they differed little, if at all, in molecular weight. However, we observed that the relative amounts of the various mRNAs induced by the three LDV isolates differed significantly (Fig. 7). This difference has been observed in Northern hybridizations of at least two independently prepared RNA samples for each LDV isolate (data not shown). In both instances, the amounts of RNAs 4 and 5 were lower in macrophages infected with LDV-v than with the other two LDV isolates. Whether this difference is related to any specific properties of these LDV variants is not

known. All three variants replicate about equally in macrophage cultures, but they differ greatly in neurovirulence for C58 mice (Murphy et al., 1987; Plagemann and Moennig, 1992) and in antigenic specificity (Cafruny and Plagemann, 1982). LDV-a is completely avirulent under conditions where the most neurovirulent isolates (LDV-M, LDV-C, LDV-v) cause paralytic disease in close to 100% of infected C58 mice (Plagemann and Moennig, 1992; Anderson, Palmer and Plagemann, unpublished data). Other LDV isolates, such as LDV-P, exhibit an intermediate degree of neurovirulence (see Plagemann and Moennig, 1992). Furthermore, LDV-M (and other LDV isolates) is only poorly neutralized by polyclonal rabbit anti-LDV antibodies prepared to LDV-P which effectively neutralizes the infectivity of the latter (Cafruny and Plagemann, 1982).

Although the genome organization and expression via a 3'-nested set of mRNAs of LDV and EAV resemble those of the coronaviruses and toroviruses, considerable differences exist between these groups of viruses in these properties. The overlapping organization of the ORFs 1 to 3 of EAV and LDV differs from that of the coronaviruses, which are separated by untranslated intergenic sequences (Spaan et al., 1988; Makino et al., 1991; Snijder et al., 1991b). The common junction sequences between leader and the bodies of the mRNAs of the coronaviruses also differ from those of LDV and EAV in that they are generally longer, may contain a number of repeats and seem to be more variable for different mRNAs. Also, the leader sequence of EAV and most likely of LDV is 3-4 times larger than those of coronaviruses. The function(s) of the leaders in virus replication and the mechanism of synthesis of the 3'-nested set of mRNAs of these viruses are not fully understood. One coronavirus model suggests that the mRNAs are generated from a genome length negative strand via a leader primed transcription mechanism (Baric et al., 1983). It proposes that the viral RNA polymerase synthesizes the leader, which 'falls off' the minus strand template but then acts as a site-specific primer for the synthesis by the polymerase of the subgenomic mRNAs at specific initiation points. There is also evidence that the subgenomic mRNAs form their own replicons (Sethna et al., 1989; Sawicki and Sawicki, 1990). The ORFs of the mRNAs of the torovirus, Berne virus, are also preceded by a common sequence. However, this sequence may function as a core promoter for internal transcription initiation rather than as a leader junction, since the mRNAs of Berne virus seem to lack a 5'-leader, even though the viral genome possesses a 5'-untranslated segment of about 700 nucleotides (Snijder et al., 1991a). EAV and LDV also differ from coronaviruses and toroviruses in the size of their genomes, the size and structure of their virions and structural proteins and the symmetry of their nucleocapsids (Kuo et al., 1991; Plagemann and Moennig, 1992).

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