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Detection of negative-stranded subgenomic RNAs but not of free leader in LDV-infected macrophages

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Abstract

The mechanism of synthesis of the seven subgenomic mRNAs of lactate dehydrogenase-elevating virus (LDV) was explored. One proposed mechanism, leader-primed transcription, predicts the formation of free 5'-leader in infected cells which then primes reinitiation of transcription at specific complementary sites on the antigenomic template. No free LDV 5'-leader of 156 nucleotides was detected in LDV-infected macrophages. Another mechanism, independent replication of the subgenomic mRNAs, predicts the presence of negative complements to all subgenomic mRNAs in infected cells which might be generated from subgenomic mRNAs in virions. Full-length antigenomic RNA was detected in LDV-infected macrophages by Northern hybridization at a level of < 1% of that of genomic RNA, but no negative polarity subgenomic RNAs. Negative complements to all subgenomic mRNAs, however, were detected by reverse transcription of total RNA from infected macrophages using as primer an oligonucleotide complementary to the antileader followed by polymerase chain reaction amplification using this sense primer in combination with various oligonucleotide primers complementary to a segment downstream of the junction between the 5' leader and the body of each subgenomic RNA. It is unclear whether these minute amounts of negative subgenomic RNAs function in the replication of the subgenomic mRNAs. They could also be by-products of the RNA replication process. Finally, no subgenomic mRNAs were detected in LDV virions.

Keywords: Negative-strand RNA; Lactate dehydrogenase-elevating virus; Subgenomic mRNA

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Lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (FAV), simian hemorrhagic fever virus (SHFV) and porcine reproductive and respiratory syndrome virus (PRRSV) belong to an as yet unnamed new family of enveloped, positive-stranded RNA viruses (Plagemann and Moennig, 1992; Plagemann, 1994). Morphologically these viruses resemble togaviruses, whereas their genome organization and expression are similar to those of coronavirus. Virus replication involves the formation of a 3'-coterminal nested set of 6 or 7 subgenomic mRNAs, mRNAs 2 to 7 and one additional mRNA (l-1) for LDV (see Fig. 1A). Each subgenomic mRNA possesses a 5'-leader derived from the 5'-end of the genome and a poly(A)-tail at the 3'-end. Only the ORF at the 5'-end of each mRNA is thought to be translated (Plagemann and Moennig, 1992), but experimental evidence supporting this view is lacking. ORF 7 encodes the nucleocapsid protein, ORF 6 a non-glycosylated envelope protein, ORF 5 the major envelope glycoprotein, ORF 2 a minor envelope glycoprotein and ORFs 3 and 4, glycoproteins of unknown function (Plagemann and Moennig, 1992; de Vries et al., 1992; Plagemann, 1994). mRNA1-1 is unique for LDV. It carries the 3'-end of ORF 1b (Fig. 1).
and can potentially be translated to yield a protein consisting of the C-terminal end of the replicase protein (Chen et al., 1993). mRNAs 2-7 contain non-coding segments of 9 to 135 nucleotides between the 5' leader and the translation initiation codon (Plagemann, 1994). The arrangement of ORFs 2-7 in the genome differs from those of the coronavirus ORFs. Each overlaps with its neighbors (overlaps range from 1 to 200 nucleotides; see Fig. 1A), whereas in coronaviruses the ORFs are separated by non-coding intergenic sequences (Lai, 1990).

The mechanism by which the subgenomic mRNAs of LDV and related viruses are produced has not been resolved, as is also the case for coronaviruses (Joo and Makino, 1992; Jeong and Makino, 1992; Hofmann et al., 1993; Makino and Joo, 1993; Yokomori et al., 1992). One mechanism, leader-primed transcription (Lai, 1990), proposes that the 5'-leader is synthesized from a full-length antisense genome. This 5'-leader then "falls off" the template and rebinds to complementary segments in the minus-strand template towards its 5'-end, acting as primer for resumption of transcription (see Fig. 1A). The model predicts that the 5'-leader possesses a specific sequence at its 3'-end which is repeated further downstream in the genome, preceding each of ORFs 2-7 where the leader becomes joined to the body of each of the mRNAs and that each mRNA contains this junction segment. Our previous results for LDV were not consistent with these predictions. We found that there is only a single heptanucleotide junction for each mRNA where the 5'-leader becomes joined to its body (Chen et al., 1993). However, the heptanucleotides preceding ORFs 1-1, 2, 3, 4, 6 and 7 differed by 1 or 2 nucleotides from each other and from the heptanucleotide at the 3'-end of the 5'-leader, 5'-UAUAACC-3' (V in Fig. 1A), and the junctions of the individual mRNAs were identical to the former rather than the latter (Chen et al., 1993). Most of the nucleotide differences were present in the first 4 nucleotides of the heptanucleotide junctions, indicating that the joining of the 5'-leader to the bodies of the mRNAs occurs within the 5'-end of the heptanucleotide junction segment (Chen et al., 1993). Similar observations have been reported for PRRSV (Meulenberg et al., 1993) and bovine coronavirus (BCV; Hofmann et al., 1993). These results raise two main difficulties with respect to the leader-primed transcription model. First, how does the leader specifically recognize the single, often incompletely complementary junction sites for each subgenomic mRNA on the minus-strand template (D in Fig. 1A); and how does it distinguish them from many similar, but apparently non-functional, sites at other places in the template? Second, assuming that such specific primer binding occurs, resumption of transcription beginning in the first four nucleotides of the junction segment would require back-trimming of the primer and factors other than base pairing to hold the primer to the template (Lai, 1990).

Another prediction of the leader-primed transcription model is the formation of free leader in infected cells and indeed free leaders of 70-82 and 130-250 nucleotides have been found in cells infected with mouse hepatitis virus (MHV), another coronavirus (Baric et al., 1985). In the present study, we have searched for free leaders in LDV-infected macrophages. The 5'-leader of the strain of LDV used in the present study, LDV-P (Brinton-Darnell and Plagemann, 1975), is 156
nucleotides long, inclusive of the 3'-heptanucleotide involved in junction formation (Chen et al., 1994). LDV-P RNA was extracted from 6-h LDV-P-infected macrophage cultures and analyzed by Northern hybridization using an oligonucleotide complementary to a segment in the 5'-leader (A1260) as described previously (Chen et al., 1993). The probe hybridized to genomic RNA as well as to all seven subgenomic mRNAs but no free 5'-leader of 156 nucleotides or longer was detected (Fig. 2A), even upon longer exposure of the blots (data not shown). Results similar to those in Fig. 2A, second lane were obtained in several experiments. In one experiment (Fig. 2A, first lane) the RNA from infected macrophages was electrophoresed for only a short time to facilitate detection of a 5'-leader containing RNA smaller than the smallest subgenomic mRNA, but none was detected.

Fig. 2. Failure to detect free 5'-leader in LDV-infected macrophage cultures (A) and of subgenomic mRNAs in LDV virions (B and C). A: Total RNA extracted from 6-h LDV-P-infected macrophage cultures was electrophoresed for 0.75 or 2.5 h and then analyzed by Northern hybridization using as probe 32P-labeled oligonucleotide A1260 which is complementary to a segment in the 5'-leader (Chen et al., 1994). B: Plasma pooled from about 30 1-day LDV-P-infected mice was clarified by centrifugation at 10,000 × g for 10 min. LDV was collected from the supernatant by ultracentrifugation through 0.5 M sucrose in TNE and its RNA was phenol-chloroform-extracted (Chen et al., 1994). The virion RNA (lane 2) along with RNA extracted from 6-h LDV-infected macrophages (lane 1) was electrophoresed for 2.5 h and analyzed by Northern hybridization using the 32P-labeled A1260 probe as in A. C: RNA extracted from two other plasma pools of LDV-P was electrophoresed and ethidium bromide stained and the gel viewed under ultraviolet light.
Combined, our results indicate that if the subgenomic mRNAs are formed via a leader-primed transcription mechanism, the process is more complex than initially envisioned (Lai, 1990). This conclusion is also indicated by recent studies with MHV (Joo and Makino, 1992; Jeong and Makino, 1992; Makino and Joo, 1992; Yokomori et al., 1992) and BCV (Hofmann et al., 1993). For example, our failure to detect significant amounts of free 5'-leader in LDV-infected macrophages might indicate that the formation of the 5'-leader, its binding to the transcription reinitiation sites and reinitiation of transcription is a coupled process which might be directed by secondary structure of the antigenome template and perhaps a combination of viral and cellular proteins. The same combination of factors may be involved in the transcription reinitiation process per se, which would require back-trimming of the heptanucleotide on the 5'-leader before transcription is reinitiated. Base pairing of complementary junction segments is clearly insufficient to explain a leader-primed synthesis of the subgenomic mRNAs.

Another postulated mechanism proposed for the generation of subgenomic mRNAs of some coronaviruses, which might be equally applicable to LDV and related viruses, is self-replication (Hofmann et al., 1990; Sethna et al., 1991; Sawicki and Sawicki, 1990). Since the 3'-ends of genomic and antigenomic RNAs probably contain replicase recognition sites required for RNA replication initiation, and the genome and the subgenomic mRNAs possess identical 5' and 3' ends, one might expect that both become replicated by the replicase. This hypothesis was supported by the finding that up to 10% of the total viral RNA in cells infected with BCV (Hofmann et al., 1990), transmissible gastroenteritis virus (TGEV; Sethna et al., 1991) and MHV (Sawicki and Sawicki, 1990) represented minus strands identical in size to genomic and subgenomic mRNAs. Furthermore, the antisense subgenomic RNAs contained an antileader (Sethna et al., 1991) and were present in replicative intermediates (Sawicki and Sawicki, 1990). Also, all subgenomic mRNAs, but not their antisense complements, were found in purified RNase-treated virions of BCV (Hofmann et al., 1990) and TGEV (Sethna et al., 1991). These could function as subgenomic replicons. Conversely, in other studies, only insignificant amounts of subgenomic mRNAs were detected in purified MHV and only small amounts of full-length antigenomic RNA in MHV-infected cells (Lai, 1990; Yokomori et al., 1992). The discrepancies between these findings have not been resolved.

Whether subgenomic mRNAs are packaged into virions may depend on whether they possess a nucleotide sequence that functions as packaging signal. The packaging signal of MHV is located in the 3'-end of ORF 1b (Van der Most et al., 1991; Fosmire et al., 1992), and is thus not present in the subgenomic mRNAs. Where it is located in the BCV and TGEV genomes is not known. It could be located in a segment also present in the subgenomic mRNAs and thus may allow packaging of the positive-stranded but not negative-stranded subgenomic RNAs. A similar situation appears to pertain to togaviruses, which, like LDV, possess a cubical nucleocapsid rather than a helical nucleocapsid typical for coronaviruses. The Sindbis virus 26S subgenomic mRNA is not packaged into virions because the packaging signal is located in a genome segment that is not present in the
subgenomic mRNA (Weiss et al., 1989). In contrast, the Aura alphavirus subgenomic mRNA is efficiently packaged presumably because it contains a packaging signal (Rümenapf et al., 1993).

In the case of LDV, subgenomic mRNAs are not packaged into virions. Earlier studies had already indicated that primarily or only 48-S genomic RNA is recovered from purified [3H]uridine-labeled virions (Brinton-Darnell and Plagemann, 1975). In the present study, no subgenomic mRNAs were detected in purified LDV whether the RNA extracted from virions was directly analyzed for size by gel electrophoresis (Fig. 2C) or by Northern hybridization analysis using the 5′-leader-specific oligonucleotide A1260 as probe (Fig. 2B, lane 2), which hybridizes to all eight LDV mRNAs (Fig. 2A, B, lane 1). Only 14-kb genomic RNA was recovered from purified virions. The subgenomic mRNAs are not packaged probably because they lack a packaging signal.

On the other hand, we have detected subgenomic minus strands to all subgenomic mRNAs in LDV-infected macrophages but only by means of highly sensitive reverse transcription (RT)/polymerase chain reaction (PCR) technology. Thus, these minus strands are present only in minute amounts in infected cells. We first searched for negative-stranded RNAs in LDV-infected macrophages by Northern blot analyses using a negative-strand-specific riboprobe. Riboprobes specific for sense and antisense RNAs were prepared by cloning cDNA 4–55, which represents a 3′-terminal end fragment of the LDV genome of 437 nucleotides (Kuo et al., 1992), in opposite directions into the BamHI site of pBluescript II KS(+) and then transcribing the HindIII-digested plasmids with T7 RNA polymerase in the presence of [α-32P]ATP (Kuo et al., 1992). The riboprobes were hybridized to total RNA extracted from 6-h LDV-infected and uninfected macrophage cultures (Chen et al., 1993). Whereas the positive-strand-specific riboprobe hybridized specifically to all eight LDV mRNAs, the minus-strand-specific riboprobe hybridized mainly non-specifically to 28- and 18-S rRNA, even though the hybridization and washing conditions and time of exposure of the blots were the same (Fig. 3A). Only a minute degree of specific hybridization to a full-length negative strand (antigenome) could be detected and clearly only after longer exposure (Fig. 3B). Weak hybridization of the negative-strand-specific probe to additional RNAs was apparent, but it was non-specific since similar hybridization was observed with total RNA from both infected and uninfected macrophages. There was nothing inherently wrong with the negative-strand-specific riboprobe since it hybridized to cDNA 4–55 with the same intensity as the positive-strand-specific riboprobe (Fig. 3C). The reason for the strong non-specific hybridization of the negative-strand-specific riboprobe to rRNA and other cellular RNAs is unclear. Increases in stringency of hybridization reduced about equally its hybridization to the cellular RNAs and the antigenome and no homologies were detected between the negative-strand-specific probe and murine rRNAs or other sequences in the GenBank data base. We also used negative-strand-specific oligonucleotides in Northern blot hybridization. However, their sensitivity was too low to even detect the antigenome (data not shown).

Since the Northern analyses indicated that, if subgenomic negative RNAs are
generated during LDV infection, they can be present only in minute amounts, we turned to RT/PCR technology to search for them (for outline of strategy see Fig. 1B). Total RNA extracted from 6-h LDV-infected macrophages was reverse transcribed using as primer a sense oligonucleotide, representing a segment in the 5'-leader (5'-CGAACTCCTACTATACCTCCCTCT-3'; E1003), which therefore is complementary to the antileader. The first-strand products were amplified by PCR with oligonucleotide E1003 in combination with antisense oligonucleotides that are complementary to segments within the ORF or the segment between the ORF and the 5'-leader junction of each subgenomic mRNA (Table 1). Each primer set should yield a PCR product of a certain size from the antisense strand of a specific LDV mRNA (Table 1). The strategy is illustrated for the amplification of the antisense strand to mRNA 2 in Fig. 1B using nucleotide A1360 as antisense primer in combination with oligonucleotide E1003. The predicted PCR product of this combination is 114 nucleotides long and specific for antisense RNA 2. Similarly, the C1001 and E1003 primer pair should yield a PCR product of 96 nucleotides from antisense RNA 2 (Fig. 1B) and similar larger PCR products could be generated with the various primer pairs, in each case from the antisense strand of the next larger mRNA stated in Table 1.

The PCR products were directly analyzed by gel electrophoresis (Fig. 4A and B) and by Southern hybridization analysis using a leader-specific internal oligonucleotide as probe (Fig. 4C) to ascertain the size of the PCR products as well as
Table 1
Antisense oligonucleotides used in combination with antileader-specific sense oligonucleotide E1003 for the detection of negative-stranded genomic and subgenomic RNAs and sizes of predicted PCR products

| Detection of negative-strand RNAs | Antisense oligonucleotide b | Size of expected product (bp) |
|----------------------------------|-----------------------------|------------------------------|
| 1 (Genomic)                      | E1002: 5'-end of ORF-1a     | 240                          |
| 1-1                              | PI-2: 3'-end of ORF-1b      | 503                          |
| 2                                | A1360: Segment just upstream of ORF-2 | 114 |
| 3                                | C1001: Segment just upstream of ORF-3 | 96  |
| 4                                | E1837: 5'-end of ORF-4      | 218                          |
| 5                                | C1008: Middle of ORF-5      | 329                          |
| 6                                | E1005: Middle of ORF-6      | 304                          |
| 7                                | A1238: 5'-end of ORF-7      | 251                          |

a For details of experiment see text.

b Except for E1002, all oligonucleotides are complementary to a segment downstream of the junction segment of the indicated mRNA. For exact location see Chen et al. (1993). E1005 is an antisense oligonucleotide corresponding to nucleotides 2968–2990 in Chen et al. (1993).

Fig. 4A, lanes 1, 3 and 4 show that PCR products of the size (about 240, 250 and 300 bp) predicted for the appropriate primer pairs were generated indicating the presence of the antigenome and the antisense complements of mRNAs 7 and 6, respectively. Two control PCR reactions were conducted to rule out accidental priming of oligonucleotide E1003 on the positive-stranded mRNAs in the RT reaction, even though this seemed unlikely since E1003 does not exhibit significant complementarity to any segment in the LDV genome. In one control reaction, oligonucleotide E1003 was paired with sense oligonucleotide E1004, which represents a segment close to the 5'-end of the LDV genome, that is upstream of the segment represented by E1003 (Chen et al., 1993). No PCR product was generated (Fig. 4A, lane 2). In the other control reaction, oligonucleotide E1004 was paired with antisense oligonucleotide E1002 which is complementary to a segment in the 5'-end of ORF1a (Chen et al., 1994). This combination should yield a 317-bp PCR product from the antisense cDNA transcript of genomic LDV mRNA (Chen et al., 1994) if such had been aberrantly primed by E1003. It should not yield a 317-bp PCR product from cDNA transcripts of the antigenome and none was generated in the present reaction (data not shown).

Fig. 4B, lanes 1–5 show that PCR products of the sizes (about 500, 115, 100, 220 and 330 bp, respectively) predicted for the appropriate primer pairs were also generated, indicating the presence of antisense complements to mRNAs 1–1, 2, 3, 4 and 5, respectively. The authenticity of the PCR products was confirmed by Southern hybridization analyses of the PCR products with an internal oligonucleotide (A1260), which is complementary to the 3'-end of the 5'-leader, as probe (Fig. 4C). Two smaller than expected PCR products were produced with oligonucleotides E1003 and C1008 (specific for antisense RNA 5) and several smaller than
Fig. 4. Detection of antigenome and of negative complements to all subgenomic mRNAs using RT/PCR technology. The details of the approach are described in the text and Fig. 1B. In brief, total RNA extracted from 6-h LDV-P-infected macrophage cultures was reverse transcribed using as primer oligonucleotide E1003 which is complementary to the antileader. The first-strand product was amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and as primers E1003 in combination with antisense oligonucleotides specific for each of the 8 LDV mRNAs (see Table 1) under conditions described previously (Chen et al., 1993). They typically consisted of 30–40 cycles with 1 min at 50–52°C for primer annealing, 2–3 min at 72°C for primer extension and 1 min at 94°C for denaturation of double-stranded products. In A and B, the PCR products were electrophoresed, ethidium bromide stained and the gel viewed under ultraviolet light. In C, they were analyzed by Southern hybridization using ³²P-labeled leader-specific oligonucleotide A1260 as probe which recognizes an internal segment in each PCR product. The Southern blot was for the same PCR products shown in B but was prepared from a different, though comparable, gel. This might have caused some differences in the signal intensity of the corresponding bands in B and C. The primer pairs used for PCR and the size of their predicted products were: in A, lane 1: E1003/E1002 (240 bp); 2: E1002/E1004 (none); 3: E1003/A1238 (251 bp); 4: E1003/E1005 (304 bp); and in B and C, lane 1: E1003/PI-2 (530 bp); lane 2: E1003/A1360 (114 bp); lane 3: E1003/C1001 (96 bp); lane 4: E1003/E1837 (218 bp); lane 5: E1003/C1008 (329 bp); lane 6: E1003/E1002 (240 bp; as lane 1 in A).

expected PCR products with oligonucleotides E1003 and PI-2 (specific for antisense RNA 1–1) as primers (Fig. 4B, lanes 5 and 7, respectively). The origins of these additional PCR products have not been elucidated. They were LDV-specific (Fig. 4C) and thus could have been generated from aberrant antisense RNAs or from aberrant first-strand products. Southern analysis also identified two minor LDV-specific PCR products of unknown origin in addition to the PCR product of the antisense strand of mRNA 4 (Fig. 4C, lane 4). The fastest moving bands in Fig. 4A and B represent residual primers which did not hybridize to the A1260 probe (Fig. 4C).

As illustrated in Fig. 1B and discussed already, PCR amplification with the primer sets shown in Table 1 could yield products that are larger than those indicated, in each case, from the next larger antisense RNA. Significant amounts of these larger PCR products were not detected in the experiment just discussed (Fig. 4) but were obtained in another similar experiment. In this experiment, PCR amplification with oligonucleotide primers E1003 and C1001 yielded the expected
product of about 630 bp specific for antisense RNA 2 (see Fig. 1B) in addition to
the about 100-bp product specific for antisense RNA 3, and PCR amplification
with oligonucleotides E1003 and B1837 as primers yielded an about 720-bp
product specific for antisense RNA 3 in addition to the about 220-bp product
specific for antisense RNA 2. Overall, these results prove that antisense strands to
all subgenomic LDV mRNAs, including mRNA 1–1, which carries the 3′-end of
ORF-1b, are produced in LDV-infected macrophages. However, they are pro-
duced only in minute amounts (< < 1% of the level of subgenomic mRNAs).
Therefore, it is unclear whether they could play a significant role in the replication
of the subgenomic mRNAs. They could also be non-functional by-products of the
replicative process accumulating in double-stranded replicative forms, which is a
common occurrence in the replication of positive-stranded RNA viruses. Further-
more, recent studies with MHV defective interfering RNAs have indicated that
MHV subgenomic mRNAs cannot be replicated because they are lacking the
complete 850-nucleotide-long 5′-end and a discontinuous segment in ORF1a which
are required for the replication of genomic RNA (Lin and Lai, 1993). Whether a
similar situation holds for LDV is not known.

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