

A translation-attenuating intraleader open reading frame is selected on coronavirus mRNAs during persistent infection

(hypervariable 5' terminus/persistence mechanism)

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ABSTRACT Short open reading frames within the 5' leader of some eukaryotic mRNAs are known to regulate the rate of translation initiation on the downstream open reading frame. By employing the polymerase chain reaction, we learned that the 5'-terminal 5 nt on the common leader sequence of bovine coronavirus subgenomic mRNAs were heterogeneous and hypervariable throughout early infection in cell culture and that as a persistent infection became established, termini giving rise to a common 33-nt intraleader open reading frame were selected. Since the common leader is derived from the genomic 5' end during transcription, a common focus of origin for the heterogeneity is expected. The intraleader open reading frame was shown by *in vitro* translation studies to attenuate translation of downstream open reading frames in a cloned bovine coronavirus mRNA molecule. Selection of an intraleader open reading frame resulting in a general attenuation of mRNA translation and a consequent attenuation of virus replication may, therefore, be a mechanism by which coronaviruses and possibly other RNA viruses with a similar transcriptional strategy maintain a persistent infection.

The mechanisms leading to persistent infection by cytoplasmically replicating RNA viruses are not all understood (1). In some cases, persistence is known to result from genetic changes in the virus, but even here the mechanisms giving rise to viral persistence are not all known (1). Coronaviruses are a large family of medically important viruses that by unknown mechanisms are capable of readily establishing persistent infection in cell culture (2). By studying the structure of the common 5'-terminal sequence of coronavirus subgenomic mRNAs throughout establishment of persistent infection, we have observed a genetic change in the leader sequence that we postulate is causally related to the viral persistence.

Coronaviruses are cytoplasmically replicating positive-sense, single-stranded RNA viruses with a 30-kb genome and a strategy of transcription that yields a common leader on genomic and subgenomic mRNAs (reviewed in ref. 3). The function(s) of the coronavirus leader, a sequence of 60–90 nt (depending on the species of coronavirus), is unknown, but several hypotheses proposing a role in subgenomic RNA synthesis have been put forward. (i) From initial studies describing the leader (4, 5), free leader was hypothesized to serve as primer for transcription of subgenomic mRNAs from a minus-strand antigenome molecule (3). (ii) Recently the leader was shown to bind nucleocapsid protein (N), and it was suggested from these studies that N binding may regulate the rate of transcription (6). (iii) We have hypothesized from recent studies (7–10) that antileader, positioned at the 3' end of the minus-strand anti-mRNA or antigenome, carries promoter activity for synthesis of mRNAs as well as genome.

The leader may be multifunctional, however, and it may also play a role in the regulation of translation.

In studies designed to establish the 5'-terminal sequence of bovine coronavirus (BCV) mRNAs, we surprisingly learned that the 5'-terminal 5 nt were both heterogeneous and hypervariable throughout early infection in cell culture and that as persistent infection became established, termini giving rise to a common 33-nt intraleader open reading frame (ORF) were selected. In the virion and during acute infection, the type I terminus (5'-GAUUGUG) predominated, but by 120 days postinfection types III (5'-GAAUAUG) and IV (5'-GAUAUG) predominated, and by 296 days postinfection and beyond, type II (5'-GAUUAUG) predominated and appeared to be stably selected. Termini types II, III, and IV all possess a start codon (underlined) for the intraleader ORF. Although we have not been able to demonstrate existence of the peptide product, the intraleader ORF was shown by *in vitro* translation studies to attenuate the translation of downstream ORFs in a cloned BCV mRNA molecule, perhaps by directly affecting ribosomal scanning. These data lead us to hypothesize that the intraleader ORF may be a selectable element that serves to attenuate mRNA translation and thereby maintain the persistent infection by secondarily attenuating virus replication.

MATERIALS AND METHODS

Preparation of RNA from Virions and Infected Cells. The Mebus strain of BCV was plaque purified three times and then serially passaged four times on human rectal tumor cells to prepare a virus stock (11). Preparations of RNA from pelleted virus and from infected cells were made as described (7, 10). Briefly, cytoplasmic RNA was extracted by the Nonidet P-40 lysis/proteinase K method. Persistently infected cells were established and maintained (7). Briefly, cells that survived the acute infection—i.e., those that did not show cytoplasmic vacuolization, round up, and become detached from the monolayer (an estimated 80%)—were treated with trypsin and passaged at 4 days postinfection and at every fourth day thereafter. After approximately four passages, cells appeared essentially normal except for short periods (e.g., for a period of 8 days at around 120 days postinfection) when cytoplasmic vacuolization, floating cells, and abundant mRNA species reappeared (7).

Sequencing the 5' End of N, M, and S mRNAs. To sequence the 5' end of specific mRNAs, a PCR-enhanced method to amplify the termini before cloning and sequencing was developed (12). Briefly, a separate primer (primer 1) was prepared for each of the N, M (multispanning membrane protein), and S (spike protein) mRNAs such that it would

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Abbreviations: BCV, bovine coronavirus; ORF, open reading frame; PCR, polymerase chain reaction; N, nucleocapsid protein; M, multispanning membrane protein; S, spike protein; I, second protein encoded by the bicistronic N mRNA.

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anneal to a site within the coding region of the mRNA, but near the 5' untranslated region, and used in a primer extension reaction with reverse transcriptase to copy the 5' end of the mRNA. Extension, amplification, and cloning reactions were carried out separately for each mRNA species. Primer 1 for N mRNA bound to a 26-nt region beginning 34 nt downstream from the N initiation codon; for M mRNA, to a 20-nt region beginning 19 nt downstream from the M initiation codon; and for S mRNA, to a 21-nt region beginning 23 nt downstream from the S initiation codon. Extended primers were purified by electrophoresis in a 6% polyacrylamide sequencing gel containing 50% urea and were eluted. Eluted extension products were ligated head-to-tail with T4 RNA ligase and amplified by PCR. For this, a second primer (primer 2), complementary in sequence to primer 1, and a third primer (primer 3), complementary to nt 30–48 of the leader (13), were used in the thermocycling reaction. Amplified products therefore represented the ligated head-to-tail junctions of molecules that contained the first 29 nt of the leader (assuming a leader of consensus length—e.g., one having a type I terminus). The PCR products were ligated into *Sma*I-linearized, nondephosphorylated pGEM-3Zf(–) vector (Promega) DNA and used to transform *Escherichia coli* JM109 cells. An oligodeoxynucleotide homologous to nt 7–33 of the leader (13) was used to screen by colony hybridization for clones containing a leader-specific insert. DNA from the identified colonies was asymmetrically amplified by PCR and sequenced by the dideoxynucleotide chain-termination method using the forward and reverse primers for the pGEM-3Zf(–) vector (14, 15).

Testing the Effect of the Intraleader ORF on Translation of Downstream ORFs. cDNA of BCV N mRNA was cloned into the pGEM-3Zf(–) vector, to give pLN(I) (13). T7 polymerase-generated transcripts of *Mlu*I-linearized pLN(I) began with 5'-GAUUGUG (type I terminus) and ended with a 3' poly(A) of 21 nt. pLN(II), generated from pLN(I) by an oligonucleotide-directed G → A mutation at the fifth nucleotide, yielded transcripts with a 5'-terminal sequence of GAUUAUG (a type II terminus). For quantitative analysis of translation efficiencies, transcripts of pLN(I) and pLN(II) were separately prepared and quantitated before translation. Transcripts were digested with RNase-free DNase (Promega), chromatographed on a Bio-Spin 6 column (Bio-Rad), and quantitated by spectrophotometry. A portion was resolved by formaldehyde/agarose gel electrophoresis and quantitated by blotting and RNA hybridization to ³²P-end-labeled primer 1 of known specific activity (1.9×10^7 cpm/ μ g) in order to normalize the amount of full-length transcript to be used in translation. Blots were quantitatively analyzed with the Ambis radioanalytic imager (7). Proteins were synthesized *in vitro* in rabbit reticulocyte lysate in the presence of [³⁵S]methionine and analyzed by SDS/PAGE as described (13). Quantitative protein measurements were made from duplicate analyses on two independent preparations of RNA transcripts.

Table 1. Distribution of 5' termini found on the N, M, and S mRNAs at various times postinfection

Time post-infection	No. of clones														
	N mRNA					M mRNA					S mRNA				
	I	II	III	IV	Aber.	I	II	III	IV	Aber.	I	II	III	IV	Aber.
Virion*	7 (3)	(2)	—	—	4	6	—	—	(1)	7	3 (1)	5 (8)	—	—	2
18 hr	11 (2)	—	—	—	7	4	2 (1)	—	—	8	6 (11)	—	—	—	10
4 days	9 (11)	(1)	—	—	5	5 (1)	1	—	1	1	8 (7)	(2)	—	—	4
120 days	—	—	13 (4)	5	5	—	—	2 (1)	5	1	1	—	2 (5)	7	7
296 days	—	7 (14)	5 (1)	1	5	—	7 (14)	—	—	—	—	14	1 (1)	—	1
432 days	—	(21)	—	1	—	—	12 (6)	—	—	—	—	(16)	—	—	—

The 5' termini were identified as types I (GAUUGUG), II (GAUUAUG), III (GAAUAUG), and IV (GAUAUG) or as "aberrant" (Aber.), as described in Table 2. Numbers in parentheses indicate clones with a truncated first base.

*Packaged mRNAs were isolated from purified virus harvested at 18 hr postinfection.

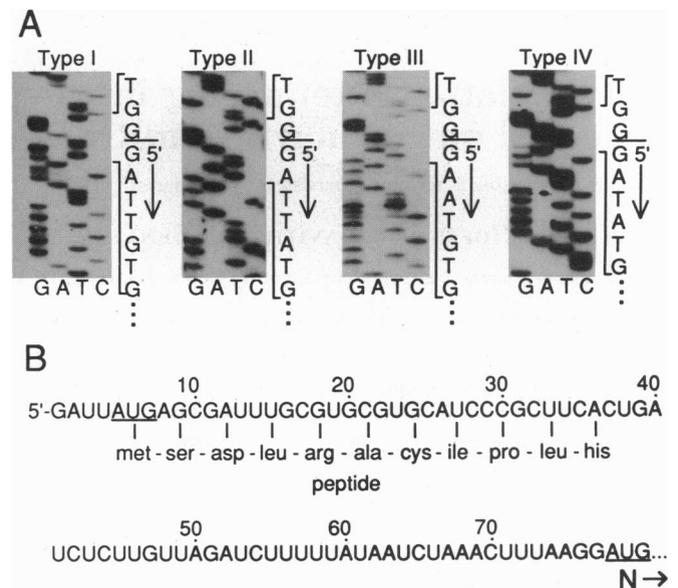


FIG. 1. (A) Four basic types of 5' termini found on N mRNA species. Asymmetrically amplified single-stranded DNAs from cloned 5' termini (shown as head-to-tail ligated products) were sequenced by the chain-termination method. (B) ORF in the leader with a type II terminus on the N mRNA. The peptide potentially translated from the intraleader open reading frame is shown below.

RESULTS

An Intraleader ORF Is Selected During Persistent Infection in Cell Culture. In initial efforts to establish the leader sequence on BCV subgenomic mRNAs, we were unable to resolve an apparent heterogeneous 5'-terminal sequence by chemical sequencing of end-labeled extended primers (unpublished data). To resolve the heterogeneity we developed a method to sequence extended primers that had been first ligated head-to-tail with RNA ligase, amplified by PCR, and cloned (refs. 12 and 14; Fig. 1). The analysis was applied to the N, M, and S mRNA species isolated from virions and from cells in culture during an acute infection (the first 18 hr postinfection) and throughout a period of persistent infection (through 432 days). The results are summarized in Tables 1 and 2 and Fig. 2.

Three major points emerge from these data. (i) The 5'-terminal sequences of mRNA were heterogeneous and hypervariable for over a 120-day period of infection. The heterogeneous termini, furthermore, could be classified into two broad categories. In the first (285 out of a total of 352 clones) were termini that made up one of the four basic types: I (5'-GAUUGUG), II (5'-GAUUAUG), III (5'-GAAUAUG), or IV (5'-GAUAUG) (Fig. 1 and Table 1). Among these were termini with a truncated first base but still identifiable as one

Table 2. Aberrant 5' terminal sequences on N, M, and S mRNAs at various times postinfection

Time post-infection	N mRNA	M mRNA	S mRNA
Virion*	1 5 10 15 -----guguuGAUUU . . . -----uGAGAGAUUU . . . -gccGgccCuGAUUU . . . <i>ggAggGaaugGcGAUUU . . .</i>	1 5 10 15 gGAUUGUGAGCGAUUU . . . aGAUUGUGAGCGAUUU . . . uGAUUGUGAGCGAUUU . . . <i>uaacccUGcauuGAUUU . . .</i> (×3) ggacuuuagcGAUUGuuuuGAUUU . . . GAccAUGcugGuUUU . . . guAccAcGaaauUUU . . . gGAUUGUGAGCGAUUU . . . aGAUUGUGAGCGAUUU . . . (×2) aGAUUUGAGCGAUUU . . . aGAUUUGAGCGAUUU . . . gaccauGcUgGUGAGCGAUUU . . . gaccaugcuGgUGaGcGcuUUU . . . GAccAUGcugGuUUU . . .	1 5 10 15 aaGAUAUGAGCGAUUU . . . ugGAUUGUGAGCGAUUU . . . gGAUAUGAGCGAUUU . . . (×2) aGAUAUGAGCGAUUU . . . aaGAUAUGAGCGAUUU . . . uGAUUGUGAGCGAUUU . . . ucAUUGUGAGCGAUUU . . . (×2) uggcaGAUUGUGAGCGAUUU . . . uggugGAUUGUGAGCGAUUU . . . aGAUAUGAGCGAUUU . . . ugaGAUAUGAGCGAUUU . . . ugagGAUAUGAGCGAUUU . . . -----uCcuUaaa . . . -----AcuuAgaca . . . (×2) cGAUAUGAGCGAUUU . . . aGAUAUGAGCGAUUU . . . gaaUAUAUGAGCGAUUU . . . ccaAaUAUGAGCGAUUU . . . -uUAUAUGAGCGAUUU . . .
18 hr†	-----uaauGgcAUUU . . . -----GgccGuGAUUU . . . -----guUccGguAUUU . . . -----aagcAGCGAUUU . . . -----gGccGgGAUUU . . . -GAUAcuAGCGAUUU . . . <i>guucaaaGUcGguAauGAUUU . . .</i>	gaccuuuagcGAUUGuuuuGAUUU . . . GAccAUGcugGuUUU . . . guAccAcGaaauUUU . . . gGAUUGUGAGCGAUUU . . . aGAUUGUGAGCGAUUU . . . (×2) aGAUUUGAGCGAUUU . . . gaccauGcUgGUGAGCGAUUU . . . gaccaugcuGgUGaGcGcuUUU . . . GAccAUGcugGuUUU . . .	gGAUAUGAGCGAUUU . . . (×2) aGAUAUGAGCGAUUU . . . aaGAUAUGAGCGAUUU . . . uGAUUGUGAGCGAUUU . . . ucAUUGUGAGCGAUUU . . . (×2) uggcaGAUUGUGAGCGAUUU . . . uggugGAUUGUGAGCGAUUU . . . aGAUAUGAGCGAUUU . . . ugaGAUAUGAGCGAUUU . . . ugagGAUAUGAGCGAUUU . . .
4 days†	-AUcGcGAGCGAUUU . . . GAUcGcGAGCGAUUU . . . GAUUGUaAGCGAUUU . . . auuauuAUGAGCGACAU . . . ggagcaGAcUAUaAagGuUUU . . .	GAccAUGcugGuUUU . . .	aGAUAUGAGCGAUUU . . . ugaGAUAUGAGCGAUUU . . . ugagGAUAUGAGCGAUUU . . .
120 days	gcAUUAUGAGCGAUUU . . . guAUUAUGAGCGAUUU . . . gauAUUAUGAGCGAUUU . . . (×3)	gGAUAUGAGCGAUUU . . .	-----uCcuUaaa . . . -----AcuuAgaca . . . (×2) cGAUAUGAGCGAUUU . . . aGAUAUGAGCGAUUU . . . gaaUAUAUGAGCGAUUU . . . ccaAaUAUGAGCGAUUU . . . -uUAUAUGAGCGAUUU . . .
296 days	-GuUAUGAGCGAUUU . . . (×2) auuAUUAUGAGCGAUUU . . . (×3)	-----	-----
432 days	-----	-----	-----

Lowercase sequences are those that do not conform to one of the four basic terminus types. Italicized sequences (except for the underlined bases) are identical to stretches of sequence found within the genome.

*Packaged mRNAs were isolated from purified virus harvested at 18 hr postinfection.

†The 5' sequence uucuauuagcagucaguagcuguuacuaauguuuaggacuuGAUUU . . . was found on one clone of S mRNA at 18 hr, and the sequence uucuauuagcagucaguagcuguuacuaauguuuagguacuuaAUUU . . . was found on an S mRNA clone at 4 days.

of the four basic types. The single-base truncation could have resulted from a premature termination of the reverse transcriptase during synthesis of first-strand cDNA in the cloning procedure or, alternatively, could represent the actual

mRNA terminus, as suggested by the large number of such termini at 432 days postinfection (Table 1). Regardless of origin, for this study, clones truncated by one base were scored as one of the four major types in Table 1.

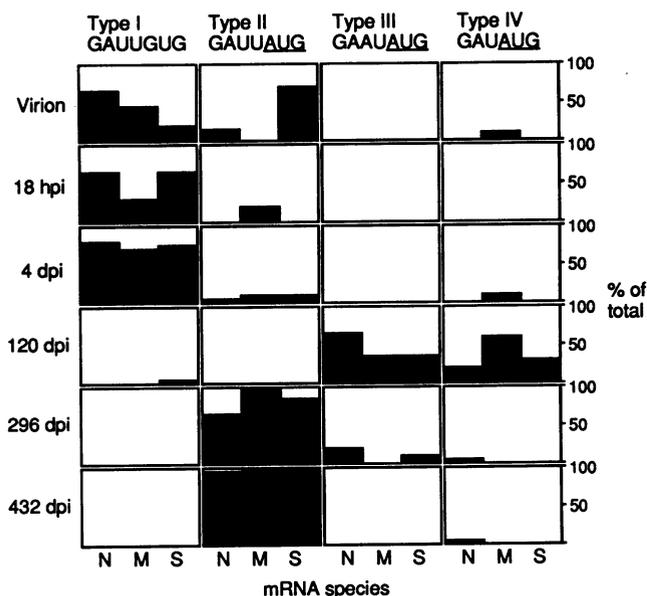


FIG. 2. Occurrence of the four basic types of 5' termini at various times postinfection (hr, hpi; days, dpi). Results for the N, M, and S mRNA species are depicted separately. Aberrant termini listed in Table 1 were included in the total number when percentages were calculated.

In the second category were clones (the remaining 67 out of 352 clones) containing aberrant sequences, some replacing up to 16 nt of the 5'-terminal sequence and some extending 5'-ward from base 1 for a distance of 1-37 nt (Table 2). Of the 39 5'-ward extensions, 30 represented extensions of 1-5 nt on one of the four basic terminus types described in Table 1. The origin of the extended sequences is not apparent, but they appear not to have resulted from an enzymatic copy-back mechanism. Some of the aberrant sequences included stretches of ≥6 nt (identified by italics in Table 2) that could have been derived from within of one of the eight open reading frames in the sequenced 3'-terminal 8955 nt of the BCV genome. The mechanism by which these sequences were incorporated is likewise not understood.

Two pieces of evidence indicate that the sequence heterogeneity was not a result of the method used to make the analyses. First, when the leader sequence with the type I 5' terminus (5'-GAUUGUG) was cloned and the resulting T7 RNA polymerase-generated capped transcripts from a single clone were further cloned and sequenced by the protocol described above, only the type I terminus was recovered in the cloned products (6 of 6 clones) (12). When transcripts of this construct were transfected into uninfected cells, reisolated, and used as templates for cloning, again only the original sequence (18 of 18 clones) was recovered (12). Second, because cloned termini obtained after a long period of persistent infection (prepared both at 296 and at 432 days) had a clearly different makeup (predominantly 5'-GAUU AUG, type

II) than those obtained during acute infection (predominantly 5'-GAUUGUG, type I) (Table 1 and Fig. 2), more than a mere random generation of sequences occurring during our analytical procedure would have had to play a role.

(ii) The 5' termini on the separate N, M, and S messages coevolved coordinately to yield a predominant terminus type at various times postinfection (Table 1 and Fig. 2). In the virion, and early in infection continuing through 4 days postinfection, the predominant terminus was type I (5'-GAUUGUG), and we infer that this may have been the progenitor on the basis of its predominance. Terminus types II (5'-GAUUAUG) and IV (5'-GAUAUG) but not type III (5'-GAAUAUG) were found in low numbers during this time. By 120 days postinfection, the predominant termini were types III (5'-GAAUAUG) and IV (5'-GAUAUG) on all three mRNAs, and by 296 days and remaining through 432 days postinfection the predominant terminus was type II (5'-GAUUAUG) on all three mRNAs.

(iii) Throughout establishment of the persistent infection, leader sequences with a 33-nt internal ORF were selected (Fig. 1). That is, by 120 days postinfection, the vast majority of 5' termini were types II, III, and IV, or the aberrant variants, all with a methionine codon initiating a 33-nt ORF. For types II and III the methionine codon begins at nt 5, and for type IV it begins at nt 4. At 296 days postinfection and beyond, all termini possessed the methionine codon. According to the ribosomal scanning model for initiation of protein synthesis on eukaryotic mRNAs (16), the sequence surrounding the methionine codon fits a consensus that is favorable for initiation of translation, since there is a purine in the -3 position (A in the case of types II and III, and G in the case of type IV) and an A in the +4 position (Fig. 1). The potential peptide Met-Ser-Asp-Leu-Arg-Ala-Cys-Ile-Pro-Leu-His encoded by the ORF is neutral and hydrophilic, and its synthesis would be terminated by a UGA codon.

The Intraleader ORF Attenuates Translation of Downstream ORFs *in Vitro*. To test whether the intraleader ORF is translated into a product and whether it attenuates translation of downstream ORFs, equimolar amounts of the two forms of N mRNA, differing only by the absence or presence of the intraleader ORF (i.e., a type I or a type II terminus), were translated *in vitro* and the products were quantitated. To date, we have found no radiolabeled polypeptide of the size predicted by the intraleader ORF (1.1 kDa) when either [³⁵S]methionine or [³⁵S]cysteine was included in the translation mixture and when products were analyzed in a denaturing gel of 20% polyacrylamide (17). We observed, however, a $47 \pm 5.3\%$ (mean \pm SD) reduction in the amount of N and a $17 \pm 4.5\%$ reduction in the amount of I produced when the intraleader ORF was present (Fig. 3).

DISCUSSION

By exploiting an experimental approach that has enabled us to sequence the 5' ends of individual BCV mRNA species, we have learned that the 5'-terminal sequence of the leader is heterogeneous and hypervariable throughout early infection and during establishment of a persistent infection and that it becomes essentially homogeneous and stable by 432 days of persistent infection. Although the mechanism by which the heterogeneity was generated remains unknown, we have experimentally ruled out the possibility that it was artifactually derived from the procedures of amplification and cloning used in our analysis, since transcripts of cloned constructs demonstrated no such heterogeneity when carried through the procedure (12). We think it is also unlikely that the heterogeneity resulted from a cap-snatching mechanism similar to that described for the myxoviruses (18, 19), bunyaviruses (20, 21), or arenaviruses (22, 23), in which the 5'-terminal sequences are obtained from cellular mRNAs during the transcription process, since this mechanism would have

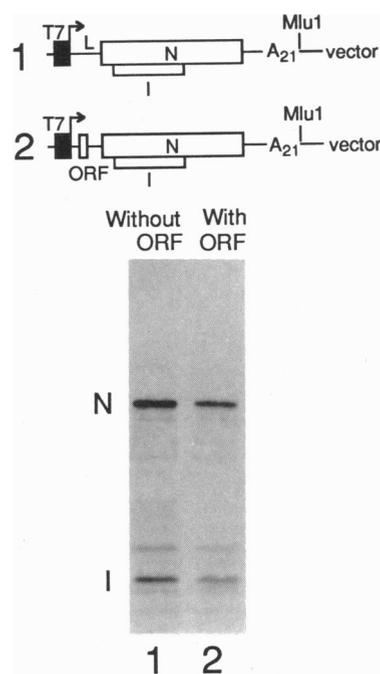


FIG. 3. Effect of the intraleader ORF on the translation of N and I proteins *in vitro* from the bicistronic N mRNA (13). Equal amounts of each full-length transcript (1 μ g) made *in vitro* by T7 RNA polymerase were translated in rabbit reticulocyte lysate in the presence of [³⁵S]methionine, and the radioactive products were analyzed by gel electrophoresis and quantitated by an Ambis radioanalytic imager. Lanes: 1, pLN(I); 2, pLN(II).

yielded a heterogeneous pattern throughout long-term infection and not the observed periodic predominance of one kind of terminus over another.

What is the origin of the 5'-terminal hypervariability? It has been clearly established that the common 5' leader, encoded only at the 5' end of the coronavirus genome, can be the only source of the 5' leader on subgenomic mRNAs when the genome alone is the infectious unit and when mRNAs are transcribed from the antigenome (3-5). Once subgenomic mRNAs are present, however, it becomes less clear what molecules can serve as leader donors, since anti-mRNAs have been shown to possess a minus-strand copy of the leader (9), a potential leader template, and the leader fusion process may function in trans (a process called leader switching) (24). A subgenomic defective interfering RNA, when present (7), could also theoretically serve as a leader donor. Thus, the presence of a wide variety of 5' termini on packaged (virion) mRNAs and on mRNAs extracted during the acute phase of the infection (first 4 days) lead us to conclude that despite our use of stock inoculum that had been prepared from sequentially plaque-purified virus, a variety of preexisting termini from multiply packaged genomes or subgenomic molecules may have been introduced by the infecting virus. Thus, at least two possible mechanisms could explain the apparent hypervariability. In the first, predominant terminus types could have been periodically selected from a preexisting pool. In the second, base changes may have occurred as the result of the high error frequency (10^{-3} to 10^{-4}), inherent among RNA-dependent RNA polymerases (25), giving rise to variant termini that were subsequently selected. The type II terminus (5'-GAUUAUG), for example, could have arisen from a type I terminus (5'-GAUUGUG) by a G \rightarrow A transition at base position 5 and subsequently given rise to a type III terminus (5'-GAAUAUG) by a U \rightarrow A transversion at position 3. A more rigorous analysis of the 5'-end hypervariability must

await the preparation of a virus inoculum that has been derived from a single infectious genomic RNA molecule.

We postulate that selection of leaders with an intraleader ORF during persistent infection is causally related to coronaviral persistence. This idea is based on a large body of experimental evidence showing that an intraleader ORF on eukaryotic mRNAs attenuates translation of the mRNA ORF (16), and secondly on the notion that persistent infection by a virus is promoted when the cytolitic potential of a virus is diminished (1). Conceivably, attenuating coronavirus mRNA translation in this manner would impair virus replication and bring it into equilibrium with cell growth and division. This would be an especially potent mechanism if, as found in this study, the same intraleader ORF arises simultaneously on the genome and all subgenomic mRNAs. Alternatively, attenuating expression of a specific cytopathogenic gene could inhibit the cytolitic (virulent) phenotype.

Persistent infections by cytoplasmically replicating RNA viruses have been correlated with such genetic changes as deletions and rearrangements resulting in subgenomic defective interfering RNAs (reviewed in ref. 26), point mutations in structural proteins affecting viral entry (27) or assembly (28), and point mutations in the 5' untranslated region affecting translation rates (through a non-ORF mechanism) (29). Here we identify a selectable upstream ORF that potentially functions as an attenuator of virulence, leading to persistent infection. Precedent for an intraleader ORF operating in a strain-specific manner to attenuate virulence has been reported for the barley stripe mosaic hordeivirus (30), but in this instance, persistence was not a reported consequence of attenuation. Inspection of known coronavirus leader sequences, all of which were studied during acute lytic infection and show no intraleader ORF (4, 9, 31–35), verify the potential of an intraleader ORF as a selectable element.

Translation-attenuating intraleader ORFs occurring as stable sequence elements in mRNAs of cells and viruses attenuate translation by one of two known mechanisms (16), either or both of which might function in BCV. (i) The peptide product of the intraleader ORF, as in the case of the yeast gene *CPA1* (36), may be responsible for attenuating expression. (ii) The intraleader ORF may directly retard ribosomal scanning or, alternatively, make it necessary for ribosomes to reinitiate translation on the downstream mRNA ORF. The second mechanism has precedent in the regulation of eukaryotic papovavirus (37), cytomegalovirus (38), retrovirus (39), and hordeivirus (30) mRNA expression.

Earlier, it was proposed that attenuation of coronavirus replication and development of persistent infection might result from mRNA replicons behaving as defective interfering RNA and competing with genome for replication machinery (10). This mechanism is still under investigation. We now propose a second (but not mutually exclusive) mechanism by which persistent infections by coronaviruses might be maintained. The question is medically pertinent, since persistence of coronavirus replication in the brain appears to be a component of mouse hepatitis coronavirus-induced demyelinating encephalitis (40) and possibly multiple sclerosis (41, 42). A selectable intraleader ORF might be a general attenuating mechanism used by other viruses, such as arteriviruses, that readily cause persistent infection and share a leader-fusing transcriptional strategy with coronaviruses (43).

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