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Short Communication

Bovine coronavirus I protein synthesis follows ribosomal scanning on the bicistronic N mRNA

Savithra D. Senanayake, David A. Brian *

Department of Microbiology, University of Tennessee, Knoxville, TN 37996-0845, USA

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Abstract

The mRNA encoding the 49-kDa nucleocapsid protein (N) of the bovine coronavirus is bicistronic. A 23-kDa protein, termed the I protein for the 'internal' open reading frame (ORF), is also synthesized but in the +1 reading frame beginning 61 nt downstream of the N start codon. Sequences flanking the N and I start codons suggest that the I ORF might be accessed by scanning ribosomes passing over the N start codon. Here we test this idea and demonstrate with translation studies both *in vitro* and *in vivo* that the I protein is synthesized according to the leaky scanning model for initiation of translation on the subgenomic N mRNA molecule. © 1997 Elsevier Science B.V.

Keywords: Coronavirus bicistronic N mRNA; Nucleocapsid protein; Ribosomal scanning

It has been demonstrated that the bovine coronavirus (BCV) nucleocapsid (N) gene, transcribed as mRNA 7, encodes both the 49-kDa N and 23-kDa 'internal' (I) proteins from overlapping reading frames (Senanayake et al., 1992). It has also been shown with I-specific antipeptide serum that the I protein is synthesized in virus-infected cells and with immune calf serum that infection in calves induces an antibody response to I protein

(Senanayake et al., 1992). This along with the fact that the I open reading frame (ORF) is conserved in the genome of several strains of the closely related murine coronavirus (Homberger, 1995; Kunita et al., 1993; Parker and Masters, 1990; Skinner and Siddell, 1983) suggests to us that the I protein probably plays a biological role. Its role cannot be a universal one required for coronavirus replication, however, since the I ORF is interrupted by a stop codon at base position 49 in MHV-JHM (Parker and Masters, 1990; Skinner and Siddell, 1983) and at base position 181 in

* Corresponding author. Tel.: +1 423 9744030; fax: +1 423 9744007; e-mail: dbrian@utk.edu

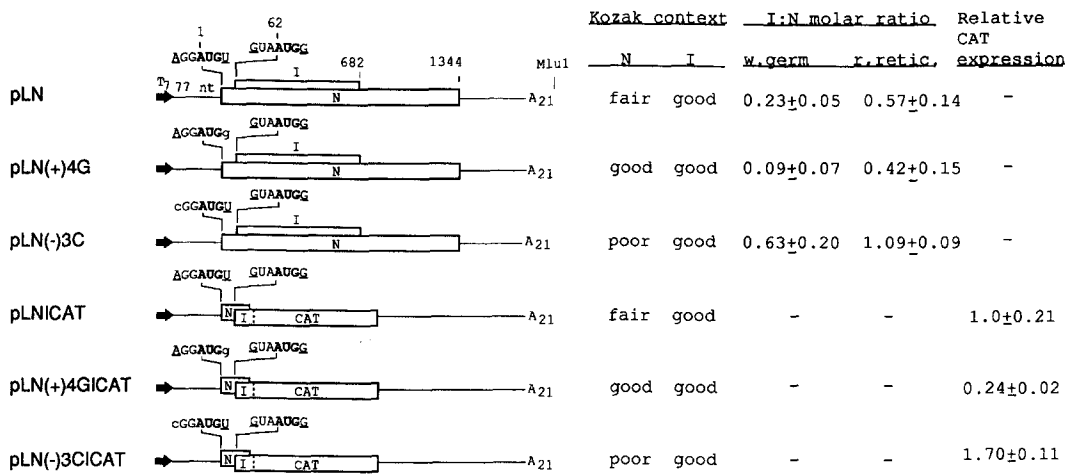


Fig. 1. Summary of mutant constructs used to measure the rate of translation from the I start codon. The wild-type bases at positions -4 and $+3$ relative to N and I start codons, shown in bold, are underlined. Mutated bases are shown in lower case letters. The sequences and the predicted quality of the sequence contexts for the initiation of translation from the N and I start codons according to the observations of Kozak (1989) are shown in the first column to the right. The molar ratios of I:N synthesized in vitro and the relative amounts of CAT protein synthesized in vivo from the different constructs are shown in the second and third columns. The standard deviation from the mean from four separate experiments is shown. W. germ, wheat germ lysate; r. retic., rabbit reticulocyte lysate.

HCV-OC43 (Kamahora et al., 1989), it does not appear to be present, at least in a similar form, in the TGEV-related or IBV-related coronaviruses (Bridgen et al., 1993). Additionally, in HCV-OC43 the I start codon has undergone mutation to AUC. Nevertheless, bases at the -3 and $+4$ positions flanking the N and I start codons, putatively important in regulating translation initiation by a ribosomal scanning mechanism (Fig. 1; Kozak, 1989), are fully conserved in BCV, HCV-OC43, MHV-A59, and in all sequenced strains of murine coronavirus (Homerger, 1995; Kunita et al., 1993; Parker and Masters, 1990; Skinner and Siddell, 1983), and in the closely related turkey coronavirus (Verbeek and Tijssen, 1991). The sub-optimal context for the initiation of N synthesis (A at -3 , U at $+4$) and the optimal context for the initiation of I synthesis (G at -3 , G at $+4$) would suggest a scanning mechanism for the entrance of ribosomes onto the I ORF (Kozak, 1989) and a possible means of regulating the abundance of I relative to N. Alternatively, the synthesis of I could result from an internal ribosomal entry mechanism, possibly similar to that documented for mRNA 3 of IBV (Le et al., 1994;

Liu and Inglis, 1992) and mRNA 5 of MHV (Leibowitz et al., 1988; Thiel and Siddell, 1994), which would have different implications for the control of I abundance. With internal entry, all plus-strand molecules could, in theory, serve as templates for synthesis of I.

To determine whether the I ORF is approached by ribosomes entering the N mRNA molecule from its 5' terminus, translation was examined by two in vitro and one in vivo experimental method. In the first in vitro method, the degree of inhibition of N and I synthesis in the presence of cap analog was examined. If BCV mRNAs are capped as described for MHV (Lai and Stohlman, 1981), and if both N and I are synthesized by ribosomes entering the mRNA by a cap-dependent mechanism, then translation of both proteins should be competitively inhibited by a methylated cap analog in the in vitro translation mix (Jackson et al., 1995). To test for inhibition, $0.4 \mu\text{g}$ of capped synthetic transcripts of N message were translated in vitro in $25 \mu\text{l}$ of wheat germ extract (Promega) in the presence of $0.2 \text{ mM m}^7\text{G}(5')\text{ppp}(5')\text{G}$ cap analog (New England Biolabs). For this, construct pLN was used in which the sequence identi-

cal to N mRNA, complete with a 65 nt leader beginning as 5'GAUUGUG and a 3' poly(A) tail of 21 nt, was cloned under the T7 RNA polymerase promoter into the pGEM3Zf(–) vector (Promega) (Hofmann et al., 1993; Senanayake et al., 1992). Proteins labeled in the presence of [³⁵S]methionine (> 800 Ci/mmol; ICN) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels of 10% polyacrylamide and autoradiograms were quantitated with the BioRad GS-670 Densitometer, Molecular Analyst System (BioRad Laboratories), using the transillumination mode. Since N and I each contain eight methionine residues (Senanayake et al., 1992), signal intensities directly reflect relative molar amounts. In the presence of cap analog, synthesis of N was inhibited by 89% and I by 65% (Fig. 2) suggesting that ribosomes had entered in a cap-dependent manner and scanned to initiate translation of both N and I.

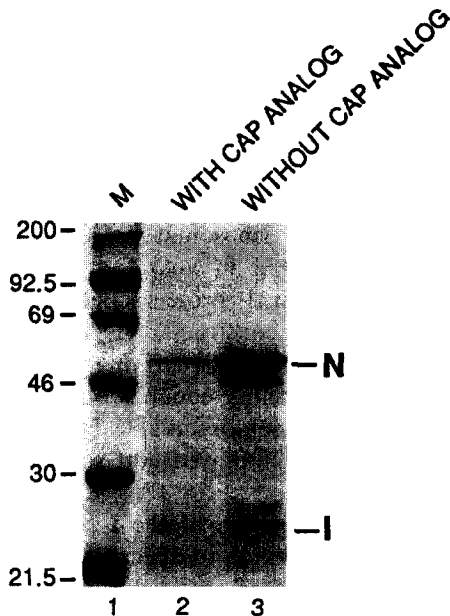


Fig. 2. Inhibition of N and I protein synthesis by a competitive methylated cap analog during translation in vitro. Equal amounts of capped synthetic transcripts representing the wild type N mRNA were translated with [³⁵S]methionine in the absence or presence of the methylated cap analog m⁷(5')ppp(5')G. The products were analyzed by electrophoresis and autoradiography. M, protein molecular weight markers.

In the second in vitro approach, the sequence context surrounding the N start codon was changed by site-directed mutagenesis to create in one case a more favorable context (U→G at +4), and in the second case a less favorable context (A→C at –3) for the synthesis of N (Fig. 1; Kozak, 1989), and the effects of these changes were noted on the ratio of I to N synthesized in vitro from the same transcript. If ribosomes were to enter and scan from the 5' terminus, the ratio of I to N should be profoundly influenced by the context of the N start codon (Kozak, 1986). For preparation of mutants, mutant primer A, 5'GTTAGATCTTTTATAATCTAACTTA(A/C)GGAT(G/C)(G/T)CTTTTACTCC3', which anneals to bases 47–91 in the minus strand of pLN, and primer B, 5'CCTTCTGGGGCTCGTCAAGATTCCCA3', which anneals to bases 1109–1135 in the genome sense (plus) strand of pLN, were used in a mutagenesis polymerase chain reaction (PCR). For this, 10 ng of pLN plasmid DNA was used in a 20 μl, 25 cycle reaction with 20 pmol each of primers A and B. The amplified DNA was digested with *Bgl*II and the resulting 622 nt fragment was recovered by electroelution and ligated into *Bgl*II-cut pLN vector. Capped transcripts prepared from transformants with G in the +4 position, named pLN(+4)G, and C in the –3 position, named pLN(–)3C, were used for translation analysis in wheat germ and rabbit reticulocyte lysate systems (Promega). Translation products were quantitated as described above.

In both the wheat germ and rabbit reticulocyte lysate systems it was observed that as the Kozak context of the N start codon improved, the amount of I synthesized diminished, and vice versa. As shown in Fig. 3, lane 2, and summarized in Fig. 1, for every molecule of N synthesized in wheat germ lysate from the pLN(+4)G construct, 0.09 molecules of I was made. This is compared with 0.23 molecules for the wild type construct pLN and 0.63 molecules for construct pLN(–)3C in which the N start context was made less favorable (Fig. 3, lanes 3 and 4). In rabbit reticulocyte lysate, these values were 0.42, 0.57 and 1.09, respectively (Fig. 3, lanes 5–7). The

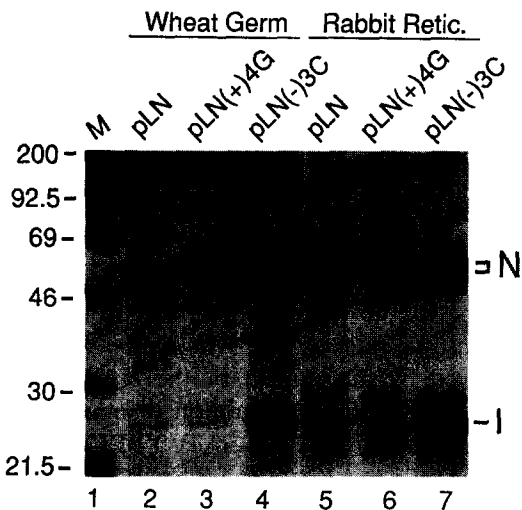


Fig. 3. Effect of mutated N ORF flanking sequences on the translation rate of I protein accumulation in vitro. Capped synthetic transcripts representing wild type N mRNA (pLN) or mutant mRNAs were translated with [³⁵S]methionine in wheat germ lysate or rabbit reticulocyte lysate as indicated, and products were analyzed by electrophoresis and autoradiography. M, protein molecular weight markers.

slower rate of migration noted for N made in the rabbit reticulocyte lysate is the result of in vitro phosphorylation (Senanayake, unpublished data). These results demonstrate the strong influence of the N start codon context on the amount of I made and suggest that ribosomes have scanned through the N initiator codon to reach the I ORF.

To determine whether the same effects of the N start codon sequence context could be observed in vivo, the chloramphenicol acetyl transferase (CAT) gene was placed in-frame with the I ORF in constructs pLN, pLN(+)-4G, and pLN(-)-3C, to form pLNICAT, pLN(+)-4GICAT, and pLN(-)-3CICAT, respectively, and synthesis of CAT in vivo was measured using a vaccinia virus T7 polymerase-mediated transient expression system (Elroy-Stein and Moss, 1990). To generate pLNICAT, the 654 nt CAT gene was inserted in-frame within the I ORF in pLN at the *Xba*I site located 94 nt downstream from the start of N. For this, the CAT gene was removed from pCM4 (Pharmacia), blunt-ended with mung

bean nuclease, and ligated into *Xba*I-cut and blunt-ended pLN. The *Xmn*I-cut, N start codon-containing fragment of pLNICAT was replaced either with *Xmn*I-cut pLN(+)-4G to form pLN(+)-4GICAT or with pLN(-)-3C to form pLN(-)-3CICAT. Lipofectin (GIBCO-BRL)-mediated transfection was carried out as recommended by the manufacturer. Briefly, 5 μ g of supercoiled plasmid DNA quantitated spectrophotometrically was transfected into vaccinia virus (strain vWR)-infected T7 polymerase-expressing OST7-1 cells (Elroy-Stein and Moss, 1990) in 35 mm dishes of cells at 80% confluency. Vaccinia virus was used at a multiplicity of 5 PFU/cell 1 h prior to transfection and Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Atlanta Biologicals) was added at 5 h posttransfection. Cell extracts were prepared in lysis buffer at 24 h posttransfection and CAT activity was measured with the Promega cell lysis and enzyme assay kit (Promega). C14-labeled products were quantified by spotting onto nitrocellulose membrane and scanning with the AMBIS instrument (AMBIS Systems, CA). The relative amounts of CAT activity observed were 0.24, 1, and 1.7 for constructs pLN(+)-4GICAT, pLNICAT (which carries the wild-type N flanking sequences), and pLN(-)-3CICAT, respectively (Figs. 1 and 4), suggesting that a ribosomal scanning mechanism was being used in vivo for the synthesis of the downstream I protein. Because of the nature of these constructs, a truncated N protein of 39 amino acids carrying only the single initiator methionine codon would be made. This product was not observable by the experimental procedures employed and was, therefore, not quantified.

Although the function of the I protein is unknown, it is reasonable to speculate that its biological role may be associated with that of N. The expression of I by a scanning mechanism on the N mRNA rather than by internal ribosomal entry would seem to assure that N and I are maintained in constant relative amounts. This mechanism, therefore, might be one selected by evolution if the two proteins are required stoichiometrically.

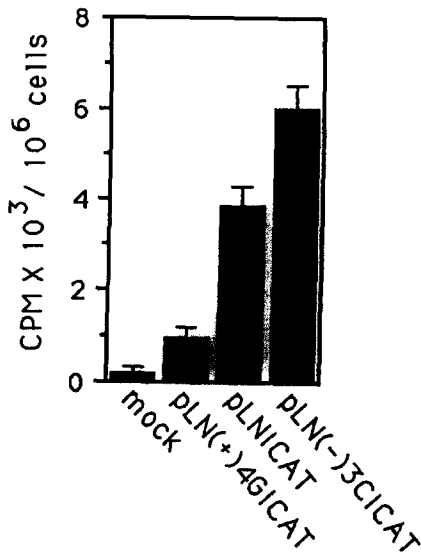


Fig. 4. Effect of mutated N ORF flanking sequences on the rate of CAT accumulation in transfected cells. Equal amounts of plasmid DNA were transfected into vaccinia virus-infected T7 polymerase-expressing OST7-1 cells and cell lysates prepared at 24 h posttransfection were measured for CAT activity. Each assay was performed in triplicate and bars indicate the standard deviation from the mean.

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