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Evidence for Translation of the Borna Disease Virus G Protein by Leaky Ribosomal Scanning and Ribosomal Reinitiation

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The Borna disease virus antigenome includes five major open reading frames (ORFs) which encode, from 5′ to 3′, the putative nucleoprotein (N), the phosphoprotein (P), the putative matrix protein (M), the major glycoprotein (G), and the RNA-dependent RNA polymerase (*pol*). Whereas the N and P ORFs are translated from monocistronic transcripts, the M, G, and *pol* ORFs are translated from polycistronic transcripts. Expression of the M, G, and *pol* ORFs is dependent upon differential splicing of two introns (intron 1, 94 nucleotides [nt]; intron 2, 1,294 nt). In vitro transcription-translation assays of wild-type and mutant sequences indicated that the G ORF is translated from an unspliced 2.8-kb RNA by leaky ribosomal scanning. Splicing of intron 1 enhances the translation of the G ORF by converting the M ORF into a 13-amino-acid minicistron, a structure that facilitates ribosomal reinitiation.

Borna disease virus (BDV) is an enveloped, nonsegmented, negative-strand RNA virus of the order *Mononegavirales* (1, 4, 6, 14, 20). Its antigenome contains five major open reading frames (ORFs), N, P, M, G, and *pol* (2, 4). The position of each ORF on the antigenome and the biochemical features of the polypeptides encoded by each ORF suggest that ORF N encodes the viral nucleocapsid protein, P encodes the phosphoprotein, M encodes the matrix protein, and G encodes the major glycoprotein involved in viral attachment to and/or penetration of host cells (11, 17, 18, 22, 24). The gene product encoded by the most 5′ ORF on the viral antigenome has not been reported. However, this ORF predicts a protein that contains motifs considered critical to viral RNA-dependent RNA polymerase activity and is therefore expected to encode the viral polymerase (*pol*) (2, 4).

The third BDV transcription unit directs the expression of two primary transcripts, the 2.8- and 7.1-kb RNAs (19). These RNA transcripts are coterminal at the 5′ end yet differ at the 3′ end due to the use of alternative transcriptional termination sites. Whereas the 2.8-kb transcript contains only the M and G ORFs, the 7.1-kb transcript contains the M, G, and *pol* ORFs (2, 19). These primary multicistronic transcripts are posttranscriptionally modified by differential splicing of two introns to generate six additional RNAs (5, 23). Although the unspliced bicistronic primary transcripts most likely serve as M protein messages, they also contain the downstream G ORF and could serve as messages for the G protein (Fig. 1). To determine the extent to which the downstream G ORF is translated from the bicistronic unspliced transcripts and the influence of the upstream M ORF on G ORF translation, a series of in vitro translation experiments was performed by using cRNAs transcribed from intron 1-spliced and unspliced wild-type and mutant cDNA constructs.

The G ORF is present in both the bicistronic unspliced 2.8-kb RNA, which contains the overlapping M and G ORFs, and the monocistronic intron 1-spliced 2.7-kb RNA, which contains only the G ORF. Therefore, to determine the potential for each of these RNAs to serve as a G protein message, in vitro transcription-translation assays were performed with reporter cRNAs representing spliced and unspliced transcripts. These cRNAs were transcribed in vitro by using reporter plasmids pT72.8 and pT72.7 as templates. Plasmids pT72.8 and pT72.7 were constructed by placing the T7 RNA polymerase promoter at the 5′ end of each cDNA insert and an epitope tag (T7Tag) at the 3′ end of the insert in frame with the G ORF (21). The epitope tag was inserted into the G ORF such that the reporter protein product, which represents G ORF initiation, could be detected with anti-T7Tag antibodies. The epitope tag was placed 693 nucleotides (nt) downstream of the M stop codon to allow detection of M-G fusion proteins. The reporter constructs were generated by using total RNA isolated from C6 cells infected with BDV (He80-1) (3) as the template for cDNA synthesis with the oligonucleotide primer oligo(dT)-NBE (21). Thereafter, PCR mutagenesis was performed with oligonucleotide primers T7-9.18 and p57-3010.AS (Table 1) to incorporate the T7 promoter into the 5′ end of cDNAs, representing the unspliced and intron 1-spliced transcripts. The amplicons were separated by agarose gel electrophoresis, purified from the gel, cloned into pBluescript SKII+ prepared with 3′ T overhangs at the *Eco*RV site, and screened for orientation relative to the T3 primer site (16, 21). The fragments from the newly constructed plasmids were released with restriction enzymes *Not*I and *Hin*dIII and subcloned into a reporter vector (pTAG) such that the G protein ORF was in frame with T7-TAG. Plasmid pTAG was generated by PCR amplifying the pET21b (Novagen) polylinker with primers HindTag.sen and Tag.AS, digesting the PCR products with *Hin*dIII and *Xho*I, and ligating the fragments into pBluescript SKII(+) prepared with *Hin*dIII and *Xho*I. Plasmids pT72.8 and pT72.7 were linearized by digestion with *Sal*I prior to in vitro cRNA transcription.

Capped cRNAs were transcribed by using 500 ng of each linearized plasmid as the template in a 50-μl reaction with T7 RNA polymerase in accordance with standard protocols (Promega). To ensure that the cRNAs transcribed with T7 RNA polymerase contained authentic 5′ ends, transcripts were circularized and sequenced across their junctions as previously described (19). This analysis demonstrated that all of the transcripts were authentic and devoid of vector sequence at the 5′ end (data not shown). The capped cRNAs were purified by

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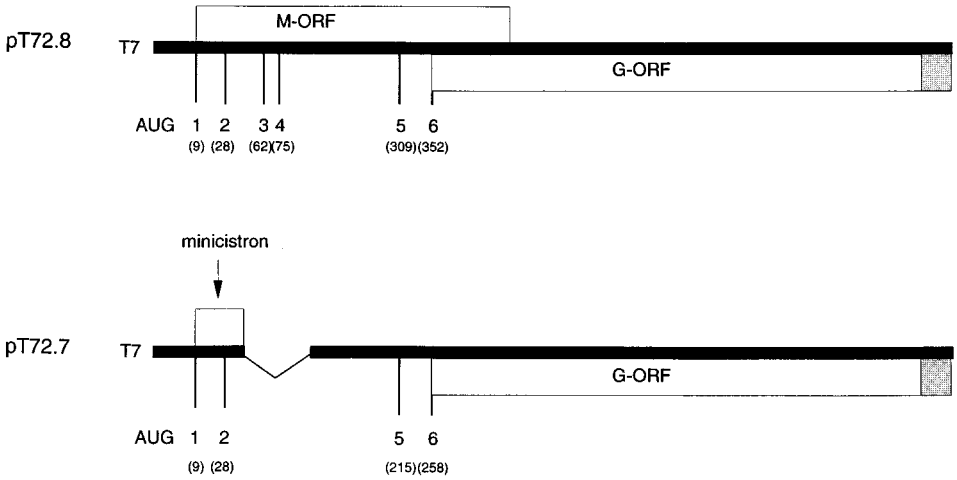

FIG. 1. Schematic diagrams of reporter plasmids that contain cDNA inserts representing the BDV 2.8- and 2.7-kb transcripts. ORFs are represented as open boxes. Each AUG upstream of the G ORF is indicated by a vertical line. Shaded boxes represent the T7Tag epitopes that were cloned in frame with the first 777 nt of the G ORF to facilitate identification of recombinant protein. The distances between the 5′ ends of the mRNAs and the AUGs are in parentheses.

using G-50 desalting columns (5 Prime→3 Prime Inc.), and 500 μg of each cRNA was translated with a rabbit reticulocyte lysate (Promega) in a 50-μl reaction. Each translation reaction was split into four aliquots, and the respective efficiency of translation initiation at the M and G ORFs was determined following immunoprecipitation assays (IPA) with a mouse anti-TAG epitope monoclonal antibody (for G protein initiation; Novagen) and rat anti-M sera (M protein) in accordance with a protocol (9) modified to include the use of protein A-Sepharose (Sigma) for IPA with mouse antibodies and protein G-Sepharose (Sigma) for IPA with rat antisera. The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, autoradiography, and densitometry (Molecular Dynamics). The experiments were repeated twice, and the results of the densitometric analyses for a total of three determinations were normalized to the values obtained by using the pT72.8 reporter construct.

Whereas unspliced cRNA served as a message for both the M and G proteins, spliced cRNA served as a message only for the G protein. Translation initiation at the G protein start codon was three- to fourfold less efficient for the unspliced cRNA relative to the spliced cRNA (Fig. 2 and Table 2). An M-G fusion protein was not detected, suggesting that although the M ORF overlaps the 5′ end of the G ORF by 84 nt, ribosomal frameshifting did not contribute substantively to G protein translation. Furthermore, given the extent of the M ORF and G ORF overlap, it is unlikely that G ORF expression was due to ribosomal reinitiation by back scanning (8). Instead, the data appeared to be most compatible with independent translation of the G ORF from a downstream ORF (dORF).

Examples of dORF expression have been described for several eukaryotic cell and virus transcripts (7–10, 13). The mechanisms by which upstream ORFs (uORFs) are skipped to allow dORF initiation include internal ribosomal entry and leaky ribosomal scanning. In some instances, the uORFs mediate expression of the dORF by facilitating ribosomal reinitiation (8, 9, 13). To determine the mechanisms by which the downstream G ORF is translated in the bicistronic unspliced RNA and how the removal of intron 1 facilitates G protein initiation, a series of mutations was introduced into the spliced (pT72.7) and unspliced (pT72.8) reporter constructs.

The possibility that internal ribosomal entry contributed to translation of the downstream G ORF was addressed by introducing a 56-nt stem-loop 3 nt downstream of the 5′ end of the spliced and unspliced reporter plasmid inserts to prevent the scanning of ribosome initiation complexes that load at the 5′ end of the cRNA (7). Plasmids pT72.8 and pT72.7 were used as templates to introduce stem-loop structures into the 8-nt 5′

TABLE 1. Oligonucleotide primers used for plasmid construction by PCR mutagenesis

Primer	Sequence[a]	Modification(s)	Plasmid(s)
HindTag.sen	5′-CACAAGCTTAGAGATATACATATGGCTAGCATGACTG	*Hin*dIII site	pTAG
Tag.AS	5′-CCTCGAGGTCGACGGTATATCGAGTGCGGCCGCAAGCAAGTCG	*Xho*I site	pTAG
T7-9.18	5′-TAATACGACTCACTATAGAATCACCATGAAT	T7 promoter	pT72.8/pT72.7
p57-3010.AS	5′-CCAAGCTTGTGAACTAGGTCA	*Hin*dIII site	pT72.8/pT72.7
SL38.AS	5′-TGGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTCTTCCAATCACCATG	*Not*I site	pT72.8/pT72.
9.18-1.MC	5′-TAATACGACTCACTATAGAATCACCATGΔATTCA	T7 promoter	pT713aaMC
T7UUG	5′-TAATACGACTCACTATAGAATCACCTTGAA	T7 promoter/A → T	pT7UUG
LUZIab.AS	5′-CAACAGTGTGGGCCAACCAGGGACGATTACCTTGTCCTT	T → A/T → A	pT72.8ΔIab
LUZIab.Sen	5′-GACAAGGTAATCGTCCCTGGTTGGCCCACA	A → T	pT72.8ΔIab
TMC11.AS	5′-CCCATCTGCTGACCCCCTGTCATTGAAGCCATTGGG	AUG insertions	pT7UUUG3AUG/ pT72.8.3AUG
TMC11.Sen	5′-ATGGCTTCAATGACAGGGGGTCAGCAGATGGGACGCG	AUG insertions	pT7UUUG3AUG/ pT72.8.3AUG

[a] Locations of modifications are underlined.

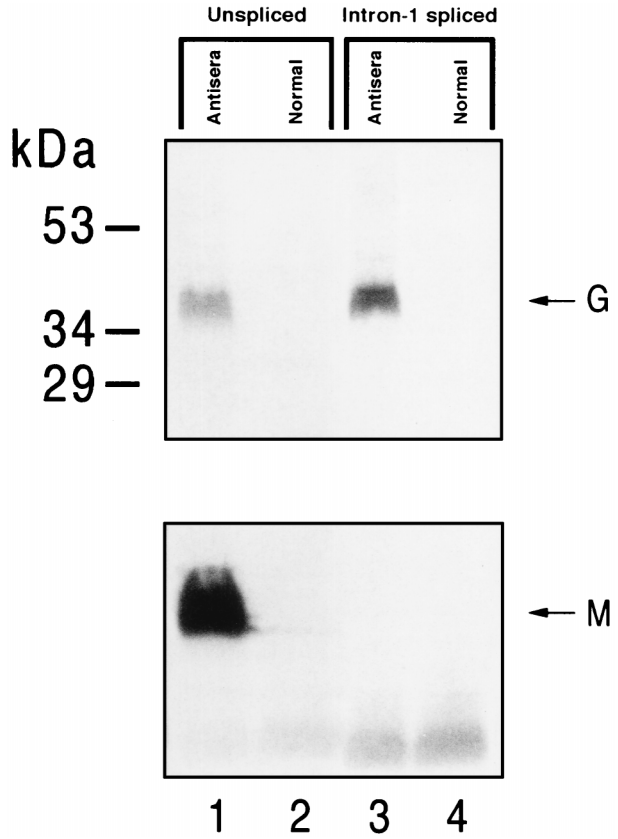

FIG. 2. Both the spliced, monocistronic 2.7-kb and the unspliced, bicistronic 2.8-kb transcripts can serve as mRNA for the G protein. Complementary RNAs were transcribed in vitro by using reporter plasmid templates that represent the authentic, unspliced 2.8-kb transcript (pT72.8; lanes 1 and 2) or the spliced 2.7-kb transcript (pT72.7; lanes 3 and 4) and then translated in vitro in a rabbit reticulocyte lysate system. Translation of the pT72.8 RNA resulted in two protein products: one initiated translation at the G AUG and was detected by immunoprecipitation with a monoclonal antibody against the T7Tag epitope (lane 1, top); the other initiated translation at the M AUG and was detected by immunoprecipitation with rat anti-M sera (lane 1, bottom). Whereas the protein that initiated translation at the G AUG was detected after translation of pT72.7 cRNA (lane 3, top), the protein that initiated translation at the M AUG was not detected by immunoprecipitation (lane 3, bottom). Protein products were not detected after immunoprecipitation with either normal mouse serum (lanes 2 and 4, top) or normal rat serum (lanes 2 and 4, bottom).

untranslated region (UTR) by PCR mutagenesis with the primer pairs p57-3010.AS and SL39.AS (Table 1). The inserts from the newly constructed plasmids were released with the restriction enzymes *Not*I and *Hin*dIII and subcloned into plasmid pTAG. The 5′ end of the insert, which is complementary to the region in pBluescript SKII+ between the T3 RNA polymerase promoter and the *Not*I restriction site, was designed to serve as the template for transcription of a cRNA containing a 56-nt stem-loop at the 5′ terminus. The resulting plasmids (pT72.7SL and pT72.8SL) were linearized and used as templates for cRNA transcription with T3 RNA polymerase. The transcribed cRNAs were then analyzed for the efficiency of G protein translation from spliced and unspliced cRNAs with 5′ UTR stem-loops. Levels of G protein translated from the spliced and unspliced cRNAs with 5′ UTR stem-loops were barely detectable (Fig. 3 and Table 2), indicating that internal ribosomal entry is unlikely to play a significant role in G ORF expression.

In many instances, upstream AUG (uAUG) codons are skipped by ribosomal initiation complexes via leaky scanning. Ribosomal leaky scanning of uAUGs is facilitated by a suboptimal initiation codon context (12). There are four AUGs upstream of the G ORF in the unspliced RNA. With the exception of AUG-1, all are in a poor context relative to the Kozak consensus sequence (Table 3) (12). Although the context of AUG-1 appears to be optimal, its initiation efficiency is compromised by its short distance (8 nt) from the 5′ end of the message. To investigate whether leaky ribosomal scanning could serve as a mechanism for G protein expression in the unspliced RNA, upstream sequences were modified to enhance (delete uAUGs) or inhibit (insert uAUGs) leaky scanning. First, the M protein initiation codon (AUG-1) was converted to UUG in plasmid pT7UUG by PCR mutagenesis of pT72.8 with primers p57-3010 and T7-UUG (Table 1). Second, three uAUGs were placed in frame with the M ORF, between AUG-1 and the G ORF at nt positions 132, 141, and 159 relative to the first nucleotide in the message. These uAUGs were inserted into plasmids pT72.8 (pT72.8.3AUG) and pT7UUG (pT7UUG3AUG) by overlapping PCR mutagenesis with pT72.8 and pT7UUG as the template and primer pairs T3 and TMC11.AS or p57-3010 and TMC11.Sen (Table 1). The efficiency of G protein translation from each cRNA was compared to translation from spliced RNAs containing authentic 5′ UTRs. Whereas in the unspliced cRNA without AUG-1 (pT7UUG), the G ORF expression increased fourfold relative to that in the unspliced cRNA with an authentic 5′ UTR (Fig. 4 and Table 2, pT72.8), the G protein initiation efficiency decreased 2.5-fold when three uAUGs were placed upstream of the G ORF (Fig. 4 and Table 2, pT72.8.3AUG). In addition, when AUG-1 was removed and the three AUGs were present upstream of the G ORF (pT7UUG3AUG), G ORF expression decreased 1.5-fold relative to that of the unspliced transcripts with an authentic 5′ UTR (Fig. 4 and Table 2, pT72.8). The observations that G ORF expression is enhanced by removing

TABLE 2. Densitometric analysis of G protein translational efficiency

Plasmid	5′ UTR	M ORF	Sequence upstream of G ORF	Avg relative level of G ORF initiation ± SEM[a]
pT72.8	Authentic	Intact	Authentic	1.00
pT72.7	Authentic	Spliced	Authentic	3.60 ± 0.65
pT72.8SL	Stem-loop	Intact	Stem-loop	0.03 ± 0.01
pT72.7SL	Stem-loop	Spliced	Stem-loop	0.04 ± 0.02
pT7UUG	Authentic	AUG → UUG	Authentic	4.13 ± 0.61
pT72.8.3AUG	Authentic	Intact	3 AUGs inserted	0.37 ± 0.09
pT7UUG3AUG	Authentic	AUG → UUG	3 AUGs inserted	0.68 ± 0.16
pT713aaMC	Authentic	Minicistron	Authentic	5.80 ± 0.70
pT72.8ΔIab	Authentic	Intact	AUG-3, AUG-4 removed	1.10 ± 0.12

[a] Levels are averages of three experiments and are relative to levels obtained with pT72.8.

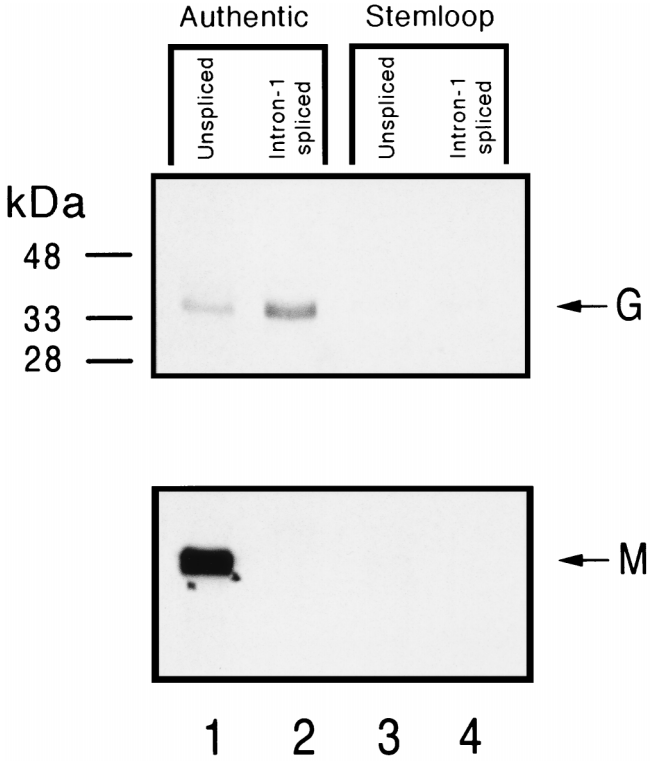

FIG. 3. The G protein is not efficiently translated in vitro by internal ribosomal entry. Proteins were translated in vitro in rabbit reticulocyte lysates from RNAs transcribed by using reporter plasmid templates that represent the authentic, unspliced 2.8-kb transcript (pT72.8; lane 1), the authentic, spliced 2.7-kb transcript (pT72.7; lane 2), the unspliced 2.8-kb transcript (pT72.8SL; lane 3), or the spliced 2.7-kb transcript (pT72.7SL; lane 4) with stem-loop structures located in the 5′ UTR. Proteins that initiated translation at the G AUG (lanes 1 and 2, top) or the M AUG (lane 1, bottom) were detected by immunoprecipitation and served as positive controls. Proteins were not readily detected by immunoprecipitation after translation of cRNA that contained 5′ UTR stem-loop structures (pT72.8SL [lane 3] and pT72.7SL [lane 4]).

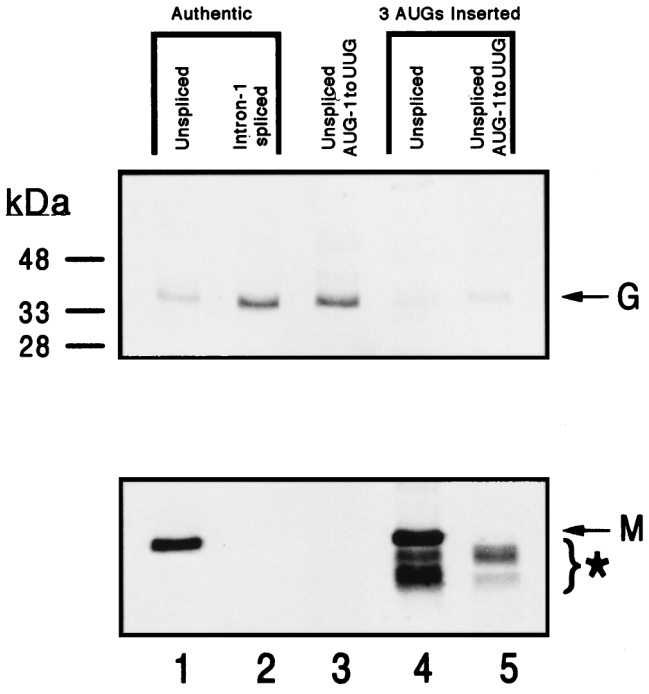

FIG. 4. The G protein is translated in vitro from unspliced bicistronic transcripts by leaky scanning. Proteins were translated in vitro in rabbit reticulocyte lysates from RNAs transcribed by using reporter plasmid templates that represent the authentic, unspliced 2.8-kb transcript (pT72.8; lane 1), the authentic, spliced 2.7-kb transcript (pT72.7; lane 2), the unspliced 2.8-kb transcript with the M AUG converted to UUG (pT7UUG; lane 3), the unspliced 2.8-kb transcript with three AUGs in the optimal context inserted into the M ORF (pT72.8.3AUG; lane 4), or the unspliced 2.8-kb transcript with the M AUG converted to UUG and AUGs in the optimal context inserted into the M ORF (pT7UUG3AUG; lane 5). Proteins that initiated translation at the G AUG (lanes 1 and 2, top) or the M AUG (lane 1, bottom) were detected by immunoprecipitation and served as positive controls. The levels of protein that initiated translation at the G AUG increased after translation of cRNA from pT7UUG (lane 3, top) and decreased after translation of cRNA from pT72.8.3AUG (lane 4, top) relative to authentic cRNA from pT72.8 (lane 1, top). The levels of protein that initiated translation at the G AUG decreased after translation of cRNA from pT7UUG3AUG (lane 5, top) relative to cRNA transcribed from pT7UUG (lane 3, top). The asterisk indicates truncated M protein initiated at the three AUGs inserted into plasmids pT72.8.3AUG (lane 4, bottom) and pT7UUG3AUG (lane 5, bottom).

AUG-1 and reduced after the addition of three uAUGs suggest that leaky scanning is a mechanism by which ribosomal initiation complexes could arrive at the downstream G ORF in the bicistronic unspliced RNAs.

The finding that the absence of intron 1 allows enhanced translation of the G ORF is intriguing, since AUG-1 (the M protein initiation codon) is present in the intron 1-spliced transcripts. Splicing of intron 1 is not predicted to result in a substantive change in RNA secondary structure (data not shown); thus, it is unlikely that removal of intron 1 enhances leaky scanning of the upstream initiation codon (AUG-1). However, two alternative mechanisms could account for the more-than-threefold increase in the translational efficiency of the G protein following the removal of intron 1 (Table 2): (i) splicing of intron 1 places the 13th amino acid residue of the M ORF in frame with a stop codon, resulting in the formation of a minicistron and reinitiation of ribosomes that normally translate the M protein; (ii) splicing of intron 1 removes two uORFs (initiating at AUG-3 and AUG-4) that could repress G protein translation in unspliced RNAs (8).

To test whether the presence of the minicistron formed after splicing of intron 1 could play a role in facilitating G protein translation, a frameshift was introduced into the unspliced reporter construct after the 12th amino acid residue in the M ORF. This mutation (pT713aaMC) created the same minicistron formed following intron 1 splicing without deleting the intervening sequences. Plasmid pT713aaMC was generated by using pT72.8 as the template to introduce the frameshift in the M ORF. As described above, a DNA fragment was PCR amplified with primer pairs p57-3010.AS and 9.18-1.MC (Table 1)

TABLE 3. Context of AUG codons located upstream of G protein ORF

Codon	Context	Predicted ORF length (aa)[b]	
		Unspliced	Intron 1 spliced
Consensus[a]	C RCC AUG G		
AUG-1	C ACC AUG AAU	M ORF	13
AUG-2	U CCU AUG UGG	7	25
AUG-3	C UGG AUG GCC	7	NP[c]
AUG-4	A CUG AUG CUU	120	NP
AUG-5	A UAC AUG UUC	42	42

[a] Kozak consensus sequence; R represents purine.
[b] aa, amino acids.
[c] NP, not present due to splicing of intron 1.

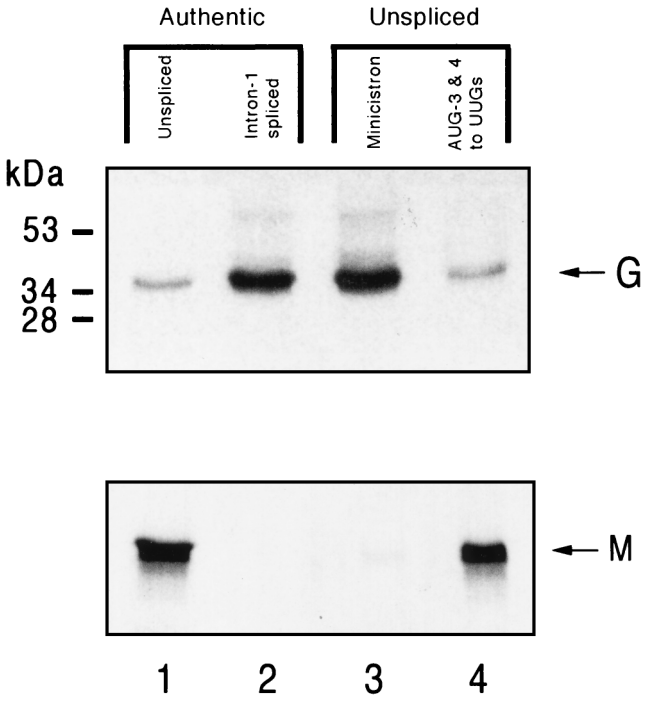

FIG. 5. Splicing facilitates translation of the G protein through formation of a minicistron. Proteins were translated in vitro in rabbit reticulocyte lysates from RNAs transcribed by using reporter plasmid templates that represent the authentic, unspliced 2.8-kb transcript (pT72.8; lane 1), the authentic, spliced 2.7-kb transcript (pT72.7; lane 2), the unspliced 2.8-kb transcript with the M ORF converted to a 13-amino-acid minicistron (pT713aaMC; lane 3), and the unspliced 2.8-kb transcript with the intronic AUGs converted to UUG (pT72.8ΔIab; lane 4). Proteins that initiated translation at the G AUG (lanes 1 and 2; top panel) or the M AUG (lane 1, bottom) were detected by immunoprecipitation and served as positive controls. The levels of protein that initiated translation at the G AUG after translation of cRNA from pT7T713aaMC (lane 3, top) were similar to those observed after translation of cRNA from pT72.7, a construct designed to represent authentic, spliced 2.7-kb transcripts (lane 2, top). The levels of protein that initiated translation at the G AUG after translation of cRNA from pT72.8ΔIab (lane 4, top) were similar to the levels found after translation of cRNA from pT72.8, which represents authentic, unspliced 2.8-kb transcripts (lane 1, top).

and cloned into pTAG. Plasmid pT72.8ΔIab was constructed to test if the two uAUGs in intron 1 interfere with scanning ribosomal initiation complexes. The uAUGs were converted to UUG in the unspliced reporter construct by overlapping PCR mutagenesis using as the plasmid pT72.8 template. An upstream fragment of pT72.8 was amplified by using primer pairs T3 and LUZIab.AS and mixed with an overlapping downstream fragment generated with primer pairs p57-3010 and LUZIab.Sen (Table 1). The mixed overlapping fragments were then used to amplify a full-length fragment with primers T3 and LUZIab.Sen. RNAs transcribed from the pT713aaMC and pT72.8ΔIab constructs were assessed for efficiency of G protein translation. The levels of G ORF expression from pT713aaMC cRNA transcripts were approximately sixfold greater than those obtained with pT72.8 cRNA transcripts (Table 2). In contrast, cRNA from pT72.8ΔIab showed no enhancement of G ORF expression relative to the unspliced reporter construct (Fig. 5). These results demonstrate that the formation of a minicistron enhances G protein translation independently of the presence of intron 1.

Mechanisms of gene expression in negative-strand RNA viruses are extensive and play pivotal roles in regulating virus production, virulence, and tropism. BDV is remarkable for establishing a persistent, nonlytic infection with low levels of production of the M and G proteins (2, 15). The in vitro results presented here suggest that ribosomal reinitiation may also be part of the repertoire of mechanisms for regulating the levels of the M and G proteins in vivo. To explore this possibility, we are pursuing reverse genetic systems suitable for studies of BDV gene expression in infected cells. Whereas abrogation of M protein expression is a direct consequence of intron 1 splicing, up-regulation of G protein expression after intron 1 splicing is dependent upon the formation of the upstream minicistron and not splicing per se. Modulation of the translational efficiency of these proteins by differential splicing of intron 1 may be important for maintenance of tight control of their expression and, in turn, facilitate viral persistence.

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