J. Biochem. **120**, 22-25 (1996)

Identification of a Ribosomal Frameshift in *Leishmania* RNA Virus 1-4[1]

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Received for publication, May 7, 1996

Double-stranded *Leishmania* RNA virus 1-4 (LRV 1-4) has at least four open reading frames (ORFs). The two small ORFs located near its 5′ terminus, ORF1 and ORFx, could encode 34- and 60-amino acid polypeptides, respectively. ORF2 encodes an 82-kDa major capsid protein, and ORF3 encodes a 98-kDa polypeptide which contains the consensus sequence for RNA-dependent RNA polymerases of plus-strand and double-stranded RNA viruses. The complete sequence of LRV 1-4 shows that ORF2 and ORF3 overlap by 71 nucleotides, and that ORF3 lacks a potential translation initiation site, suggesting that the viral polymerase may be synthesized as a 180-kDa fusion protein with the virus capsid. In this report, we present evidence for the synthesis of a fusion protein through a ribosomal frameshift. *In vitro*-translation experiments and immunostudies involving antiserum against the viral capsid protein demonstrated that the overlapping 71 nucleotides of ORF2 and ORF3 are contained in a region which promotes translational frameshifting. Computer analysis of the putative frameshift region revealed a potential pseudoknot structure located within the overlapping 71 nucleotide sequence.

Key words: capsid protein, *Leishmania* RNA virus, pseudoknot structure, ribosomal frameshift, RNA-dependent RNA polymerases.

Double-stranded RNA viruses have been discovered in some strains of the parasitic protozoa, *Leishmania guyanensis* and *L. braziliensis* (*1-3*). These viruses were designated as *Leishmania* RNA virus 1 (LRV 1), and have recently been classified as members of the Totiviridae family of viruses (*4*). Virions consist of an apparent icosahedral capsid of *ca.* 30 nm diameter containing a 5.3 kb double-stranded RNA molecule, substantially less abundant plus-strand RNA and unique RNA-dependent RNA polymerase (RDRP) activity (*5, 6*). The RNA molecule and polymerase activity were co-purified on sucrose and CsCl gradients with viral particles (*7*). As in other double-stranded RNA viruses, the viral polymerase is involved in two functions: transcription, which generates single-stranded transcripts of positive polarity, and replication, *i.e.* the synthesis of transcripts of the opposite orientation yielding double-stranded RNA (*8*). Electroporation of virus particles into uninfected and heterologously infected strains of *Leishmania* produces only a transient infection (*9*). This virus appears to be regulated and maintained at a low copy number within the infected cells (*10*).

Recently, the entire nucleotide sequences were determined for two different *Leishmania* viruses, LRV 1-1 (*11*) and LRV 1-4 (*12*). The molecular organization of LRV 1-4 indicates that the double-stranded RNA genome contains at least four open reading frames (ORFs) on the plus-strand mRNA (Fig. 1A) (*12*). ORF1 and ORFx, located near the 5′ terminus, could encode 34- and 60-amino acid polypeptides, respectively (*12*). No function has yet been identified for the small 5′ ORFs. Western blot analysis revealed a major viral capsid protein of 82 kDa, the size predicted from sequence information to be encoded by ORF2 (*13*). Recently, the capsid protein alone was expressed and shown to spontaneously assemble into virus-like particles (*14, 15*). ORF3 of the LRV 1-4 RNA genome may encode the RNA-dependent RNA polymerase (RDRP) associated with purified LRV 1 particles. The amino acid sequence of the putative LRV 1-4 RDRP (ORF3) has several motifs that are conserved among numerous viral RNA-dependent RNA polymerases (*16*). Interestingly, ORF3 does not contain a consensus translation initiation sequence (*17, 18*), and overlaps the end of ORF2 by 71 nucleotides (Fig. 1A). Sequence analysis of the ORF2-ORF3 overlap indicated that this region contains a putative slippery site and a pseudoknot structure (Fig. 1B) (*12*). This suggests that a ribosomal frameshift produces a *gag-pol* type fusion protein in LRV 1-4 RNA, as seen in yeast viruses (*19, 20*) and *Giardiavirus* (*21*).

Sequencing of LRV 1-1 has confirmed a similar molecular organization to that of LRV 1-4 except for the presence of

[1] This study was supported by grants from Yonsei University, and for the Basic Science Research Institute Program (BSRI-95-4419) from the Ministry of Education, Korea.

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a single ORF at the 5′ end (*11*). ORF1, which encodes a 72 amino-acid polypeptide, may initiate translation by ribosome binding at internal sites. ORF2 encodes the 82 kDa major viral coat protein. The predicted amino acid sequence of ORF3 exhibits the greatest similarity to that of the yeast ds RNA virus (*11*). In this report, we present evidence that ORF2-ORF3 fusion proteins are produced through a ribosomal frameshift during *in vitro* translation of *Leishmania* transcripts.

Plasmid pBS-RF1, which contains the overlapping 71 nucleotide frameshift region of LRV 1-4, was constructed as follows: a 259 nucleotide sequence containing the proposed frameshift region corresponding to nt. 2565–2823 of the LRV 1-4 genome (*12*) was reverse-transcribed and then amplified by PCR using two primers (5′-CCGGAATTCGTTGAGTTACCACTAGCCCCA-3′ and 5′-CCGGAATTCAACTATCGCTGGCATCTGGGA-3′) with *Eco*RI sites added at the 5′ ends for cloning purposes. The cDNA fragment was digested with *Eco*RI and then inserted in frame into the *Eco*RI site of the pMAL vector (New England Biolabs), resulting in plasmid pMAL-RF1. From this plasmid, a *Bgl*II-*Sal*I fragment containing a part of the maltose binding protein coding region (Mal E gene of the pMAL vector) and a frameshift signal was ligated into *BamH*I-*Sal*I-digested pBS to create plasmid pBS-RF1 (Fig. 2). In this way, the capsid reading frame is in frame with the first AUG of the coding region under the control of the T7 promoter of pBS. This plasmid codes for a 30.8 kDa protein when the capsid (ORF2) reading frame is maintained in the absence of a frameshift. However, a +1 translational frameshift within the LRV 1-4 sequence would yield a 42.7 kDa fusion protein (Fig. 2). From pBS-RF1, a 259 nucleotide *Eco*RI fragment was deleted to create plasmid pBS-RF0, which lacks the frameshift region as a negative control. A 33 kDa protein is expected to be

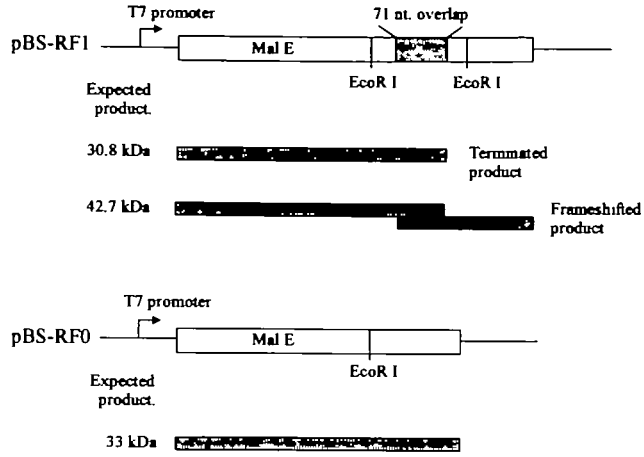

Fig. 2. **Features of plasmids used for frameshift analysis.** In plasmid pBS-RF1, a 259 nucleotide sequence containing the putative ribosomal frameshift region was reverse-transcribed, PCR-amplified and then inserted at the 3′-end of the Mal E gene (see text for details). A part of the Mal E gene and the LRV 1-4 frameshift region are under the control of a T7 RNA polymerase promoter. The T7 RNA polymerase promoter is depicted as an arrow. In the absence of a frameshift, pBS-RF1 codes for a 30.8 kDa terminated protein translated by the ORF2 reading frame. In the case of a translational frameshift to the +1 reading frame, pBS-RF1 codes for a 42.7 kDa fusion protein. Plasmid pBS-RF0, which does not contain the frameshift region, codes for a 33 kDa protein.

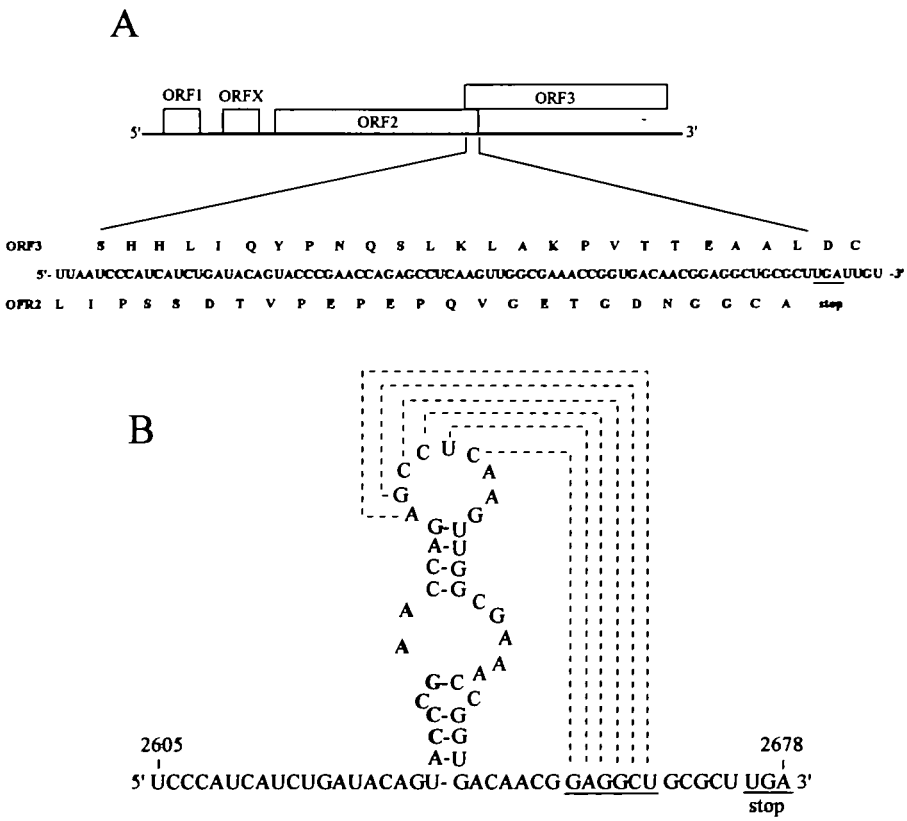

Fig. 1. **Schematic representation of the open reading frames of the LRV 1-4 genome.** (A) The 5283 nucleotide double-stranded RNA genome of LRV 1-4 contains four open reading frames (ORFs) on the plus mRNA strand. ORF2 (encoding the major capsid protein) overlaps ORF3 (encoding viral RNA-dependent RNA polymerase) by 71 nucleotides. The nucleotide sequence of the overlapping region, and the amino acid sequences encoded by the ORF2 and ORF3 frames are shown below. The stop codon (UGA), which is in frame with the capsid reading frame, is underlined. (B) The predicted pseudoknot structure of the LRV 1-4 plus strand responsible for ribosomal frameshifting is shown. Dashed lines indicate base pairs formed during pseudoknot formation and the putative slippery sites (CCCGAA) are indicated by bold letters.

produced by this plasmid (Fig. 2).

Two plasmids, pBS-RF1 and pBS-RF0, were examined as to their ability to direct frameshifting in an *in vitro*-translation system. *In vitro*-translation products were synthesized using the TNT-coupled reticulocyte lysate system (Promega) in a mixture containing 1 μg of circular DNA template, 100 units of T7 RNA polymerase and [^{35}S]methionine (Amersham) in a total volume of 50 μl, according to the manufacturer's recommendations. After incubation for 2 h at 30°C, the samples were resolved on a 15% SDS-PAGE gel and autoradiographed. In Fig. 2, the predicted sizes of the translation products of pBS-RF1 with or without ribosomal frameshfting were 42.7 and 30.8 kDa, respectively. As shown in Fig. 3, two polypeptides were detected after *in vitro*-translation of T7 transcripts derived from pBS-RF1 (indicated by arrowheads in lane 3). The sizes of the products suggest that the faster migrating band (30.8 kDa) is the terminated product translated by the ORF2 reading frame and the slower migrating band (42.7 kDa) is the result of a translational frameshift in the +1 reading frame. Taken together with sequence analysis of LRV 1-4 RNA, these results indicate that frameshifting occurred exclusively in the +1 or − 2 direction as predicted (*12*). When pBS-RF0 DNA was used as a template for the *in vitro*-translation reaction as a negative control, a translation product of around 33 kDa was detected (indicated by an arrowhead in lane 4). A transcription-translation reaction with 1 μg luciferase DNA (lane 1) as a positive control or without added DNA template as a negative control (lane 2) was also performed. The frameshift efficiency of the pBS-RF1 template in reticulocyte lysates appears to be higher than that of any other known viral frameshift similarly tested *in vitro*. However, we do not know whether or not the extent of frameshifting of LRV 1-4 observed in reticulocyte lysates reflects the actual frameshifting efficiency in *Leishmania* cells.

To further confirm the ribosomal frameshifting within the overlapping 71 nucleotide sequence of LRV 1-4, *in vitro*-translated products were immunoprecipitated with various polyclonal antisera. Since plasmid pBS-RF1 contains a part of the maltose binding protein gene (Mal E gene) and the 3′ region of the capsid gene, both the terminated and frameshift products translated in reticulocyte lysates were expected to be precipitated by either anti-Mal E product antiserum or anti-capsid antiserum (*13*). The *in vitro*-translated products were incubated with polyclonal antisera for 1 h at 4°C. The antigen-antibody complexes were then incubated with protein A-Sepharose beads, CL4B (Pharmacia), prepared according to the manufacturer's instructions. After incubation for 2 h at 4°C, unbound antigen was removed by extensive washing and bound antigen was eluted by boiling in the SDS-PAGE sample buffer. The samples were directly resolved on a 15% SDS-PAGE gel and autoradiographed. As shown in Fig. 4, two *in vitro*-translated products derived from pBS-RF1 (approximately 31 and 43 kDa) were immunoprecipitated by both Mal E product antiserum (lane 4) and capsid antiserum (lane 3), but not by preimmune serum (lane 1) or topoisomerase II antiserum (lane 2). In contrast, the *in vitro*-translated product (approximately 33 kDa) derived from pBS-RF0 was precipitated only by the Mal E product antiserum (lane 7), but not other antisera (lanes 8-10). These results further support that the slower migrating band in lanes 3-5 was produced through ribosomal frameshifting.

The results presented here demonstrate that an LRV1 capsid-RDRP protein can be synthesized through frameshifting, at least *in vitro*, and that nucleotide sequences derived from the junction of the LRV capsid and polymerase genes stimulate this event. This finding along with the homology to other viral systems suggests that a fusion protein is likely produced *in vivo*. It was previously shown that two viral proteins were detected in a Western blot assay with an antibody generated against purified virus particles (*13*). The major band was approximately 82 kDa, the size predicted for the putative capsid protein from sequence information for ORF2. The minor band was approximately 180 kDa, the size predicted for a capsid-polymerase fusion protein. However, Western analysis of the baculovirus-expressed capsid protein also revealed a protein of approximately 180 kDa, indicating that this protein might be a dimer of the capsid protein (*14*).

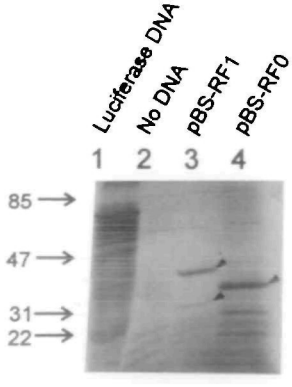

Fig. 3. **Frameshift analysis of *in vitro*-translated products.** Autoradiogram of a 15% SDS-PAGE gel containing ^{35}S-labeled translated products from pBS-RF1 (lane 3) and pBS-RF0 (lane 4). The reactions were performed with luciferase DNA (lane 1) and without DNA as controls (lane 2). Molecular sizes are indicated in kilodaltons.

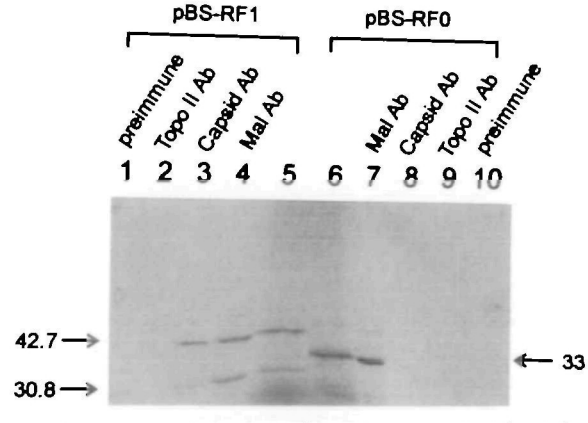

Fig. 4. **Immunoprecipitation of *in vitro*-translated products.** *In vitro* products translated from pBS-RF1 (lanes 1-4) or pBS-RF0 (lanes 7-10) were immunoprecipitated with anti-Mal E product antibodies (lanes 4 and 7), anti-capsid antibodies (lanes 3 and 8), anti-topoisomerase II antibodies (lanes 2 and 9), or preimmune serum (lanes 1 and 10). *In vitro*-translation products from pBS-RF1 (lane 5) and pBS-RF0 (lane 6) were also loaded.

Although these results clouded the initial interpretation of the frameshift evidence, taken together with the frameshift data for related Totiviruses such as yeast viruses and *Giardiavirus*, our results strongly suggested that ORF3 is expressed as a fusion product with the capsid protein through ribosomal frameshifting. Computer analysis of the 71 nucleotide ORF overlap sequence revealed a putative ribosomal slippery region (nt. 2625-2630) and a downstream pseudoknot (Fig. 1B). These regions are also conserved in LRV 1-1 (*12*). Interestingly, the reading frame of the overlap sequence suggests a +1 or −2 frameshift model for the synthesis of the LRV 1 capsid-polymerase fusion protein, which is different from the −1 frameshifting found in GLV (*21*), yeast viruses (*19, 20*), and retroviruses (*22, 23*). Although we present evidence for fusion protein production of LRV 1-4 *via* a ribosomal frameshift in *in vitro*-translation reactions, it remains to be determined whether it is a true reflection of the *in vivo* situation.

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