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RNA Sequence Determinants of a Coupled Termination-Reinitiation Strategy for Downstream Open Reading Frame Translation in *Helminthosporium victoriae* Virus 190S and Other Victoriviruses (Family Totiviridae)\(^\text{V}\)

Hua Li,\(^1\) Wendy M. Havens,\(^1\) Max L. Nibert,\(^2\) and Said A. Ghabrial\(^1\)*

Plant Pathology Department, University of Kentucky, Lexington, Kentucky 40546,\(^1\) and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115\(^2\)

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The genome-length, dicistronic mRNA of the double-stranded RNA fungal virus *Helminthosporium victoriae* virus 190S (genus Victorivirus, family Totiviridae) contains two long open reading frames (ORFs) that overlap in the tetranucleotide AUGA. Translation of the downstream ORF, which encodes the RNA-dependent RNA polymerase (RdRp), has been proposed to depend on ribosomal reinitiation following termination of the upstream ORF, which encodes the capsid protein. In the current study, we examined the RNA sequence determinants for RdRp translation in this virus and demonstrated that a coupled termination-reinitiation (stop-restart) strategy is indeed used. Signals for termination-reinitiation are found within a 32-nucleotide stretch of RNA immediately upstream of the AUGA motif, including a predicted pseudoknot structure. The close proximity in which this predicted structure is followed by the upstream ORF’s stop codon appears to be especially important for promoting translation of the downstream ORF. The normal strong preferences for an AUG start codon and the canonical sequence context to favor translation initiation appear somewhat relaxed for the downstream ORF. Similar sequence motifs and predicted RNA structures in other victoriviruses suggest that they all share a related stop-restart strategy for RdRp translation. Members of the genus Victorivirus thus provide new and unique opportunities for exploring the molecular mechanisms of translational coupling, which remain only partly understood in this and other systems.

The vast majority of eukaryotic mRNAs are monocistronic and, hence, contain only one translated open reading frame (ORF). Some, however, contain two or more ORFs and have an associated mechanism, such as ribosomal frameshifting or coupled termination-reinitiation (stop-restart), to promote regulated translation of the downstream ORF(s). Such multicistronic mRNAs are especially common among eukaryotic RNA viruses.

In coupled termination-reinitiation, ribosomes translate the upstream ORF of an mRNA, but upon termination, a proportion of the 40S subunits remain bound to the mRNA and go on to reinitiate at the start codon of the downstream ORF (see review in reference 39). This strategy has been shown to promote the translation of downstream ORFs in the mRNAs of several different plus- or minus-stranded RNA mammalian viruses, including those that encode protein VP2 or VP10 of certain caliciviruses (30, 33, 34), the BM2 protein of influenza B virus (21, 40), and the M2-2 proteins of respiratory syncytial virus (RSV) and other pneumoviruses (1, 15, 16). One example of a plus-stranded RNA viral gene that employs such translational coupling has also been described (17). A stop-restart strategy has been previously proposed for downstream ORF translation from the dicistronic mRNA of the double-stranded RNA (dsRNA) fungal virus *Helminthosporium victoriae* virus 190S (HvV190S) (45), and identifying RNA sequence determinants for that strategy in HvV190S and related viruses is the focus of this report.

HvV190S is the prototype strain of the genus Victorivirus, family Totiviridae (14). It was previously classified within the genus Totivirus and for several years was the only virus in that genus to have been isolated from a filamentous fungus rather than from a yeast or smut. Recently, however, HvV190S and 10 other, related viruses from filamentous fungi have been recognized and grouped together as members of the genus Victorivirus.

The ~5,200-bp dsRNA genome of HvV190S is nonsegmented and comprises two large overlapping ORFs, both of which are contained within the same genome-length mRNA (Fig. 1A). The upstream ORF (ORF1) encodes the capsid protein (CP), whereas the downstream ORF (ORF2), which is in the -1 frame relative to ORF1, encodes the RNA-dependent RNA polymerase (RdRp) (23). Several lines of evidence indicate that ORF1 translation initiates at the AUG codon at positions 290 to 292 (23, 24), even though this codon (underlined below) resides in an unfavorable context (26) for translation initiation (UCCAUGU). More-
HvV190S ORF1 terminates at the UGA codon at positions 2606 to 2608 (23, 24).

Although a single ORF encodes CP, the particles of HvV190S contain three different forms of CP: p88, p83, and p78 (11). Both in vivo and in vitro studies have indicated that p88 and p83 are phosphorylated, whereas p78 is not (13). The results of in vitro translation studies with either the denatured HvV190S dsRNA or the full-length in vitro transcript have further indicated that p88 is the primary translation product (11, 13). Expression studies in bacterial and eukaryotic systems have confirmed this finding and have also shown that p88 undergoes cleavages near its C terminus to generate p83 and p78 (23, 24). Based on these findings, it has been proposed that the sites for phosphorylation reside within a C-proximal region that is shared by p88 and p83 but is removed from p78 by further proteolysis.

The RdRp-encoding ORF2 of HvV190S has its first in-frame AUG codon overlapping the CP stop codon in the tetranucleotide sequence AUGA. Unframe AUG codon overlapping the CP stop codon in the further proteolysis.

The RdRp-encoding ORF2 of HvV190S has its first in-frame AUG codon overlapping the CP stop codon in the tetranucleotide sequence AUGA at positions 2605 to 2608 (23). Unlike numerous viruses from other genera in the family Tetranaqueae, which express the RdRp as a CP/RdRp fusion protein consequent to -1 or +1 ribosomal frameshifting (25, 29, 35, 48, 49), the HvV190S RdRp is expressed as a separate, nonfused protein (23). When the RdRp ORF is expressed in Escherichia coli from a monocistronic construct employing its predicted start codon (HvV190S nt positions 2605 to 2607), the translation product is indistinguishable in both size and serological reactivity from the HvV190S particle-associated RdRp (23).

Although coupled termination-reinitiation has been proposed as the strategy by which the downstream RdRp ORF is expressed from the dicistronic mRNA of HvV190S (45), the RNA sequence determinants have not been examined to date. The current study identifies several of those determinants and thereby provides the first direct demonstration of coupled termination-reinitiation by a dsRNA virus. Similar RNA sequence motifs and predicted structures in other victoriviruses suggest that they all share a related stop-restart strategy for translation of RdRp from its downstream ORF. Members of the genus Victorivirus thus provide new and unique opportunities for exploring the molecular mechanisms of translational coupling, which remain only partly understood in this and other systems.

**MATERIALS AND METHODS**

**Fungal and bacterial strains.** Two *H. victoriae* strains that differ in virus content were used. Strain A-9 (ATCC 42018), a disease isolate that contains both virusivirus HvV190S and chrysoivirus HvV145S, was used as a source of HvV190S virions and HvV190S proteins CP and RdRp. Strain B-2ss (ATCC 42020), which is virus free, was used in all transformation experiments with recombinant plasmids harboring a full-length cDNA clone of the wild-type or mutant HvV190S genome. All fungal cultures were maintained on potato dextrose agar supplemented with 0.5% (wt/vol) yeast extract. *E. coli* strain DH5α was used to propagate plasmids containing recombinant HvV190S cDNA.

**Construction of transformation vector and plasmid constructs.** A transformation expression vector for *H. victoriae* was briefly described by Ghabrial (10). The vector, designated p190S (Fig. 1B), comprises a full-length cDNA clone of the HvV190S genome inserted under the control of a gpd1 promoter from Cochliobolus heterostrophus and a trpC terminator from Aspergillus nidulans. The vector also contains a hygromycin (HygB) selection marker gene.

To introduce site-directed, insertion, or deletion mutations into the region flanking the AUGA stop-restart motif in the HvV190S cDNA, an adaptation of the overlap-extension protocol using PCR was used (19, 20). Two unique restriction-enzyme sites, for cleavage by FseI and StuI at nt positions 2171 (434 nt upstream of the AUGA) and 2990 (385 nt downstream of the AUGA) in the HvV190S cDNA, respectively, were selected to border the region for mutagenesis (Fig. 2). A three-step PCR amplification procedure was used to generate mutated fragments (Fig. 2, AB, CD, and AD) with two common primers (Fig. 2, indicated by a and c; see primer types b and c in Table SI at the URL given above). These mutant primers have terminal complementarity, allowing overlapping fragments can be fused together in a subsequent extension reaction that includes the outside primers a and d (Fig. 2). The sequences of all mutant products were verified by sequencing as described by Zhao et al. (51).

**Fungal transformation.** Protoplasts were prepared from virus-free *H. victoriae* strain B-2ss as described by Zhao et al. (51). Protoplast transformations with expression vector p190S (containing full-length HvV190S cDNA), one of the p190S-derived constructs, or empty vector were also carried out as described by Zhao et al. (51) using the polyethylene glycol-mediated DNA uptake method of Yoder (50). Ten to 15 transformants per vector construct were transferred from the regeneration plates to new plates containing potato dextrose agar supplemented with 0.5% (wt/vol) yeast extract and also containing 50 µg/ml hygromycin. At least three of these hygromycin-resistant transformants were then selected for further studies at each concentration of each construct.

**Preparation of viral particles.** Purified HvV190S virions were isolated from virus-infected *H. victoriae* strain A-9, and VLPs were isolated from *H. victoriae* strain B-2ss that had been transformed with plasmid p190S (containing full-length HvV190S cDNA) or one of the p190S-derived constructs. Virions and VLPs were purified from 14-day-old stationary cultures grown in potato dextrose broth supplemented with 0.5% (wt/vol) yeast extract as described previously (12).

Particle fractions were collected from the sucrose density gradients and pelleted by overnight centrifugation at 40,000 rpm in a Beckman 50Ti rotor, and the pellets were resuspended in 50 mM Tris-HCl buffer (pH 8.0).

**Western blotting.** Western blotting was performed as described by Huang et al. (24). Briefly, equal amounts of proteins from fungal samples were prepared in...
The free energy value for each predicted structure, as provided by the respective default settings as implemented at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi. RNA virus 2 (SsRV2) was predicted using the program RNAfold with the .csse.uwa.edu.au/. The long stem-loop structure in victorivirus using the program DotKnot (version 1.2) (47) as implemented at http://dotknot/plant-pathology/ghabrial/ and a series of mutagenic primers containing nucleotide mismatches represented by green rectangles (indicated by b and c; see primer types b and c in Table S1 at the URL given above) were designed to introduce a variety of mutations into the region flanking the AUGA overlap. For each mutant construct, fragment AB was synthesized using the common forward primer FseI-a-F and the persistent mutagenic type b primer for reverse priming, fragment CD was synthesized using the persistent mutagenic type c primer for forward priming and the common reverse primer StuI-d-R, and finally, fragment AD was synthesized using the amplified fragments AB and CD as templates and the common primer pair FseI-a-F and StuI-d-R. All AD fragments were doubly digested with FseI and Stul and cloned into the similarly digested vector p190S.

SDS loading buffer (10 times more protein was used for detecting RdRp than for detecting CP), boiled for 3 min, and analyzed by SDS-PAGE using 7.5% polyacrylamide gels. After blotting to a polyvinylidene fluoride membrane, the blots were probed with antiserum to CP or RdRp (23). All Western blotting experiments were repeated at least twice and with two or three independent transformants per vector construct.

RNA isolation and Northern blotting. Total RNA was extracted from virus-infected or vector-transformed fungal mycelia using TRizol reagent (Invitrogen) according to the manufacturer’s protocol. The full-length plus-stranded RNA transcript of HvV190S (~5.2 kb) was synthesized in vitro using 17 RNA polymerase and plasmid pT7HV190S containing full-length HvV190S cDNA as template (23, 46). RNA samples (15 μg) were then fractionated on a denaturing agarose gel. Blotting to Hybond-N+ membranes and hybridization were carried out as described by Zhao et al. (51). PCR fragments corresponding to the CP ORF or the RdRp ORF of HvV190S cDNA were amplified and randomly radiolabeled using [α-32P]dCTP for probing the blots.

RESULTS

Efficient expression of HvV190S RdRp in the native host. Full-length cDNA clones of the HvV190S genome were previously analyzed for protein expression in different cell systems (23, 24, 45). Translation of CP from the upstream ORF of the dicistronic mRNA was detected in E. coli, Schizosaccharomyces pombe, and Spodoptera frugiperda (insect) cells. Translation of RdRp from the downstream ORF, on the other hand, was detected in S. pombe cells but not in bacteria, suggesting that eukaryotic host factors are required. RdRp translation in insect cells was not tested.

To investigate the mechanism of downstream ORF translation in the native host fungus, full-length HvV190S cDNA was first cloned into a fungal transformation/expression vector under the control of a fungal gpdI promoter and a fungal trpC terminator (Fig. 1B). This recombinant plasmid, designated p190S, was then transformed into a virus-free isolate of H. victoriae. Following transformation, the viral genome was integrated into the host DNA as verified by Southern blotting. In addition, transcription of the dicistronic mRNA followed by translation into CP and RdRp were respectively demonstrated by Northern and Western blotting (see below). Viral dsRNA replication was not launched in these transformants, however, and only empty virus-like particles (VLPs) accumulated in the transformed hyphae, albeit to substantially higher levels than in natural infections (10). As in natural infections, the primary translation product of ORF1, p88, was phosphorylated and proteolytically processed to generate phosphorylated p83 and nonphosphorylated p78. Thus, although the transcript generated in the transformants is expected to be capped and polyadenylated, its translation products are indistinguishable from those of the noncapped and nonpolyadenylated wild-type transcript.

Western blotting of empty VLPs from different H. victoriae transformants detected both CP and RdRp in sizes and amounts similar to those of the proteins present in viral particles purified from naturally infected H. victoriae (Fig. 3A). VLPs from three transformants per vector construct were subjected to routine Western blotting, with reproducible results (Fig. 3A, for example). The successful expression of both CP and RdRp from plasmid p190S in H. victoriae suggested that this dicistronic cDNA construct, as well as the corresponding transformation and expression system, could be used to explore the mechanism of RdRp translation from the downstream ORF. Notably, no CP/RdRp fusion products larger than the nonfused RdRp (92 kDa) were detected by Western blotting using either CP- or RdRp-specific antiserum.

The possibility that RdRp is translated from a subgenomic mRNA was previously ruled out in a study of RdRp expression in S. pombe (45). Nonetheless, to verify that RdRp was expressed from genome-length HvV190S mRNA in H. victoriae, Northern blotting was performed. Total RNAs were prepared from virus-infected H. victoriae, p190S transformants, and empty-vector transformants and then hybridized to CP- or RdRp-specific gene probes. A strong band corresponding to genome-length mRNA (~5.2 kb) was detected for the infected strain, as expected, as well as for all of the p190S transformants (Fig. 3B). In contrast, no band consistent with a subgenomic
mRNA that might serve as an alternative or preferred template for RdRp translation was seen.

Initiation codons for HvV190S RdRp translation from dicistronic mRNA. Previous studies have shown that when the RdRp ORF is expressed in E. coli from a monocistronic construct employing its predicted start codon (HvV190S nt positions 2605 to 2607), the translation product is indistinguishable in both size and serological reactivity from the particle-associated RdRp of HvV190S (23). In addition to the start codon at 2605 to 2607 (AUG1), however, ORF2 contains two other in-frame AUG codons that are fairly close to its 5’ end: AUG2 at nt positions 2686 to 2688 (codon 28 relative to AUG1 as codon 1) and AUG3 at nt positions 2803 to 2805 (codon 67). Moreover, no out-of-frame AUG codons are found over this interval. To verify that HvV190S RdRp translation initiates from AUG1 in its native host, mutations of these three AUG codons were introduced into plasmid p190S by site-directed mutagenesis. After transformation of the mutated plasmids into virus-free H. victoriae, viral proteins prepared from wild-type strain and p190S transformants and subjected to SDS-PAGE and Western blotting. Because of the preponderance of CP relative to RdRp in the particle samples, 10 times more sample was loaded into each gel lane for detecting RdRp. The designsations of different proteins are given, and sizes of protein standards are indicated on the right. HvV190S, viral proteins prepared from wild-type strain A-9; p190S-T1, -T2, and -T3, viral proteins prepared from three different p190S transformants. (B) Northern blot analysis with total RNA isolated from the wild-type strain and p190S transformants. Hybridization was carried out using CP- or RdRp-specific DNA probes corresponding to ORF1 and ORF2, respectively. Empty vector lacking the HvV190S cDNA sequence served as a negative control (EmptyV). Full-length HvV190S transcripts synthesized in vitro served as a positive control (HvV190S-transcript).

FIG. 3. Expression of CP and RdRp from plasmid p190S transformed into H. victoriae. (A) Western blot analysis of viral proteins expressed from plasmid p190S in a virus-free H. victoriae isolate using CP- or RdRp-specific antiserum. Virus particles were purified from the wild-type strain and p190S transformants and subjected to SDS-PAGE and Western blotting. Because of the preponderance of CP relative to RdRp in the particle samples, 10 times more sample was loaded into each gel lane for detecting RdRp. The designations of different proteins are given, and sizes of protein standards are indicated on the right. HvV190S, viral proteins prepared from wild-type strain A-9; p190S-T1, -T2, and -T3, viral proteins prepared from three different p190S transformants. (B) Northern blot analysis with total RNA isolated from the wild-type strain and p190S transformants. Hybridization was carried out using CP- or RdRp-specific DNA probes corresponding to ORF1 and ORF2, respectively. Empty vector lacking the HvV190S cDNA sequence served as a negative control (EmptyV). Full-length HvV190S transcripts synthesized in vitro served as a positive control (HvV190S-transcript).

Mutation of AUG1 to GUG in construct pStartM1 resulted in an RdRp smaller in size than that expressed from the wild-type construct (Fig. 4A). Mutation of AUG2 to GUG in construct pStartM2, on the other hand, did not influence the RdRp size (Fig. 4A). The yields of RdRp from the wild-type, pStartM1, and pStartM2 constructs were all similar. Mutation of both AUG1 and AUG2 to GUG in construct pStartM3, however, severely reduced RdRp expression (Fig. 4A). These results suggest that GUG cannot serve as an effective reinitiation codon at either the AUG1 or the AUG2 position of the downstream RdRp ORF and also that AUG2 but not AUG3 can serve as an effective reinitiation codon when AUG1 is mutated, probably due to a requirement for proximity of the stop and restart signals, as further characterized below. Lastly, these results are consistent with previous ones in E. coli (23) in indicating that RdRp translation, in this case by HvV190S in its
native host, initiates from AUG1, which is located in the AUGA stop-restart motif (nt positions 2605 to 2607).

Effects of noncanonical start codons and context on HvV190S RdRp translation from dicistronic mRNA. In light of recent evidence for the effective use of noncanonical start codons in another system utilizing coupled termination-reinitiation for downstream ORF translation (40), we were interested to study this phenomenon in the HvV190S system. When we used site-directed mutagenesis to mutate AUG1 to AU in construct pStartM4, we found that the expressed RdRp was indistinguishable in both size and yield from the wild-type protein (Fig. 4B). This suggested that translation from the downstream ORF could effectively initiate from the noncanonical start codon AUG1 replacing AUGU1. To verify that with pStartM4 RdRp, translation was indeed effectively initiating from AU at the AUG1 position rather than from AUG2, we also generated construct pStartM5, in which AUG2 was mutated to the ineffective start codon GUG (Fig. 4A) shows preceding evidence that GUG is ineffective). The RdRp expressed from pStartM5 was again indistinguishable in both size and yield from the wild-type protein (Fig. 4B). As a final test of the use of AU as an effective start codon in this system, we additionally generated construct pStartM6, in which AUGU1 was mutated to the ineffective start codon GUG and AUG2 was mutated to AU. In this case, a smaller RdRp was expressed, though at a yield similar to that of the wild type (Fig. 4B), consistent with the use of AU as an effective start codon at the AUG2 position.

The finding of efficient reinitiation from the AU codon replacing either AUG1 or AUG2 prompted us to review whether other such potential reinitiation codons are present in the AUG1-AUG2 region of the native HvV190S sequence. In fact, over this interval, there are no in-frame AU codons or other in-frame codons with seemingly strong reinitiation potential, such as ACG. There are, however, two out-of-frame AU codons and three out-of-frame ACG codons between AUG1 and AUG2, though soon followed by stop codons in both other frames, which would have terminated any translation that might have started from these noncanonical start codons. In the AUG2-AUG3 interval, there are again no in-frame AU codons, but there is one in-frame ACG at codon 49.

In the work reported by Powell et al. (40), the authors also determined that the sequence context immediately surrounding the reinitiation codon has little effect on the efficiency of downstream ORF translation. To address this issue in HvV190S, we changed the relatively good context in the wild-type construct, GGGACAAUGA (purine at position 3, pyrimidine at position 2, initiator AUG underlined), to poorer contexts in constructs Kozak-3, GGGCAAAUGA (pyrimidine at 3, mutation in boldface), and Kozak-2, GGGAAAUAUGA (purine at 2, mutation in boldface). The yields of RdRp expression were similar for wild-type and mutant Kozak-3 and were in fact reproducibly increased to some degree with mutant Kozak-2 (Fig. 4C). These findings suggest that changes in the sequence context immediately surrounding the reinitiation codon did not have deleterious effects on the efficiency of downstream ORF translation in HvV190S. The somewhat higher yield of RdRp in mutant Kozak-2 is possibly explained by a preference for A at position 2 in filamentous fungi (3).

Termination of CP translation is essential for reinitiation for RdRp translation. In all of the mutant constructs shown in Fig. 4, the stop codon of ORF1 remains in place, albeit changed from UGA to UAG in constructs pStartM4 and pStartM5 (Fig. 4B). To assess the influence of uncoupling termination of CP translation from reinitiation for RdRp translation, the authentic UGA stop codon of ORF1 (at nt positions 2606 to 2608 in the AUGA stop-restart motif) was mutated to UGG. As a result, ORF1 was expected to terminate at the next in-frame stop codon, which is UAG at nt positions 2606 to 2608 in the AUGA stop-restart motif) was translation, the authentic UGA stop codon of ORF1 (at nt positions 2606 to 2608 in the AUGA stop-restart motif) was mutation (Fig. 4, the stop codon of ORF1 remains in place, albeit changed from UGA to UAG in constructs pStartM4 and pStartM5 (Fig. 4B). To assess the influence of uncoupling termination of CP translation from reinitiation for RdRp translation, the authentic UGA stop codon of ORF1 (at nt positions 2606 to 2608 in the AUGA stop-restart motif) was mutated to UGG. As a result, ORF1 was expected to terminate at the next in-frame stop codon, which is UAG at nt positions 2606 to 2608, 20 codons downstream of the native ORF1 stop (Fig. 5, construct pStop +20). As expected, the resulting full-length CP product (~97 kDa) was larger than the primary translation product of wild-type ORF1, p88, though it was still processed to p83 and p78, consistent with the fact that the proteolysis to generate these smaller forms occurs near the CP C terminus (23, 24). Notably, no RdRp was detected with construct pStop +20 (Fig. 5), suggesting that uncoupling the stop-restart signals in the AUGA motif severely reduces the expression of the downstream ORF.

To further study the effects of the position of the CP stop codon relative to the RdRp reinitiation codon, we generated a series of mutants in which a stop codon in frame with ORF1 was introduced at various positions upstream or downstream of the ORF2 start codon, with the authentic UGA stop codon of ORF1 mutated to UGG in all cases (Fig. 5). In constructs pStop +8 and pStop +2, the inserted stop codon was respectively positioned 8 or 2 codons downstream of the ORF2 start codon, whereas in constructs pStop –2, pStop –8, and pStop –12, the inserted stop codon was respectively inserted 2, 8, or 12 codons upstream of the ORF2 start codon. The results showed that proximity of the CP stop codon to its wild-type
position is required for reinitiation for RdRp translation, since little or no RdRp was seen with constructs pStop+8, pStop−8, and pStop−12. One caveat is that in the case of mutant pStop−8, relocation of the stop codon by 8 codons upstream of the RdRp start codon disrupts a predicted pseudoknot structure in this region, which we have also shown to be important for reinitiation (see below). Notwithstanding this caveat, these results imply that the relative location of the CP stop codon is important for the efficiency of reinitiation for RdRp translation, consistent with a coupled termination-reinitiation mechanism.

A pseudoknot structure is predicted upstream of the AUGA motif and appears important for reinitiation for RdRp translation. RNA structure predictions using the programs HPKNOTTER (22) and DotKnot (47) suggested that an H-type pseudoknot may closely precede the AUGA stop-restart motif in HvV190S (Fig. 6). As for other pseudoknots, this one is predicted to be formed when nucleotides in a hairpin loop form base pairs with nucleotides in a single-stranded region outside the hairpin to form a second stem adjacent to the hairpin stem. To address whether this predicted pseudoknot plays a role in reinitiation for RdRp translation, multiple mutations were introduced in an effort to disrupt the structure (Fig. 7A, PK0). Indeed, no pseudoknot was predicted by the program HPKNOTTER for the mutated sequence in PK0. Upon analysis with three different transformants of construct PK0, little or no RdRp was seen (Fig. 7A), suggesting the importance of the predicted pseudoknot sequence for ORF2 reinitiation.

We were next interested to determine whether RdRp expression could be restored if a wild-type version of the predicted pseudoknot sequence were reintroduced into construct PK0 downstream of the mutated one. Two constructs were made (Restore1 and Restore2), both having a new, wild-type pseudoknot-to-AUGA cassette inserted downstream of the mutated sequence and the native AUGA position. In Restore1, the native AUGA motif was mutated to AUAG, which nonetheless retains the CP stop codon at its authentic position; as a result, the inserted wild-type version of the predicted pseudoknot sequence is now upstream of this stop codon, mimicking the native organization (Fig. 7B). Much stronger restoration of RdRp expression was seen with this mutant, which also showed CP expression of the expected, larger size. From these results, we conclude that the predicted pseudoknot structure, which is conserved among victoriviruses (see below), is an important determinant for promoting translation of downstream ORF2 and that it must be located upstream of the CP stop codon for optimal activity. Our results, however, do not rule out a role for the primary sequence of the region that includes the predicted pseudoknot.

Effect of the spacer length between the predicted pseudoknot and the AUGA motif on reinitiation for RdRp translation. The length of the spacer sequence between the predicted pseudoknot and the AUGA motif in HvV190S is 9 nt. To address whether spacers smaller or larger than 9 nt would affect RdRp expression, a set of deletion and insertion mutants were constructed and analyzed (Fig. 8). The deletion of 3 nt (leaving a 6-nt spacer) had little effect on RdRp expression from constructs Spacer6-1 and Spacer6-2, whereas the deletion of 6 nt (leaving only a 3-nt spacer) essentially abolished RdRp expression from construct Spacer3. Reciprocally, the insertion of one duplicate spacer region (giving an 18-nt spacer) had little effect on RdRp expression from construct Spacer18, whereas the insertion of two duplicate spacer regions (giving a 27-nt spacer) essentially abolished RdRp expression from construct Spacer27. Based on these results, we conclude that the length of spacer sequence between the predicted pseudoknot
and the AUGA motif is important, with spacers smaller than 6 nt or larger than 18 nt not supporting RdRp expression. Whether the sensitivity to spacer length is determined by the distance between predicted pseudoknot and CP stop codon and/or between predicted pseudoknot and RdRp start codon is not addressed by this experiment, but based on preceding findings (e.g., effective initiation of RdRp from AUG2, as shown by the results in Fig. 4), we interpret that when the spacer length is larger than that in wild-type HvV190S, the spacing between the predicted pseudoknot and the CP stop codon is the more relevant determinant.

Other victoriviruses contain similar sequence motifs for coupled termination-reinitiation for RdRp translation. The sequences of 9 of the 10 viruses so far assigned to the genus Victorivirus (14) have an H-type pseudoknot predicted by HPKNOTTER (22) and DotKnot (47) at nearly the same position as in HvV190S (Fig. 9). The only exception is Sphaeropsis sapinea RNA virus 2 (SsRV2), which instead contains a predicted long stem-loop structure at nearly the same position (Fig. 9). In all 10 viruses with a predicted pseudoknot, including HvV190S, the hairpin stem (Fig. 6 and 9, stem 1, shown in cyan) is more conserved in the number of base pairs involved in stem formation (3 or 4) than is stem 2 (Fig. 6 and 9, shown in orange), which comprises from 2 to 7 bp. Both stems, however, show a preponderance of G·C base pairs. The spacer lengths between the predicted pseudoknots (or stem-loop) and the CP stop codons in all of the victoriviruses are very similar, ranging from a minimum of 8 nt in Magnaporthe oryzae virus 1 (MoV1) to a maximum of 12 nt in Chalara elegans RNA virus 1 (CeRV1). The spacer lengths between the predicted pseudoknots (or stem-loop) and the RdRp start codons in all of the victoriviruses are also similar, ranging from a minimum of 6 nt in Sphaeropsis sapinea RNA virus 1 (SsRV1) and SsRV2 to a maximum of 13 nt in MoV1. Although 7 of the 11 victoriviruses contain the AUGA stop-restart motif, the remaining 4—CeRV1, MoV1, SsRV1, and SsRV2—show a small difference: the stop and start codons are close together but do not overlap (2-nt spacers in each). Moreover, in 3 of these viruses, CeRV1, SsRV1, and SsRV2, the start codon comes first, whereas in MoV1, the stop codon comes first. These observations suggest that each victorivirus, similar to the prototype HvV190S, expresses its RdRp from the downstream ORF via a coupled termination-reinitiation mechanism, even though some variation in the relevant signals (predicted pseudoknot versus stem-loop and overlapping versus nonoverlapping stop and restart codons) is tolerated.

**DISCUSSION**

We have previously reported evidence that the dsRNA fungal virus HvV190S expresses its RdRp from the downstream ORF (ORF2) of its dicistronic mRNA via an internal initiation mechanism, proposed to be coupled termination-reinitiation (23, 24, 45). Moreover, as inferred from shared AUGA or similar such sequence motifs involving the ORF1 (CP) stop
codon and the ORF2 start codon, this means of RdRp expression has been thought to be shared by at least 10 other viruses closely related to HvV190S and constituting the genus Victo-

virina in the family Totiviridae (14). Despite the previous evi-
dence, however, the occurrence of translational coupling in
these viruses has not been widely appreciated.

In this study, we identify at least some of the RNA sequence
determinants for the stop-restart strategy of HvV190S. This
process requires that the CP stop and RdRp start codons
remain relatively closely spaced and that a predicted RNA
pseudoknot structure is also present within close proximity
upstream of the CP stop codon. Translation of the upstream
(CP) ORF and termination at its stop codon are absolute
requirements for reinitiation at the RdRp start codon, distin-
guishing this process from internal ribosome entry site (IRES-
mediated initiation (18), as well as from leaky scanning (28)
and ribosomal shunting (43). Leaky scanning can be further
ruled out since there are >20 AUG codons in a reasonably
favorable context upstream of the RdRp start codon. The fact
that RdRp is expressed from its downstream start codon as a
separate, nonfused protein also distinguishes this process from
ribosomal frameshifting (6) and in-frame read-through of
termination codons (8), which generate fusion proteins.

Translation of the downstream RdRp of HvV190S via cou-

pled termination-reinitiation represents the first example of
this expression strategy to be identified in a dsRNA virus.

The stop-restart strategy for expressing RdRp allows
HvV190S to produce two proteins from a single mRNA. Fur-
thermore, this strategy allows HvV190S to regulate the level of
RdRp expression (lower) relative to CP expression (much
higher), consistent with the fact that only 1 or 2 RdRp mole-
cules are present in each HvV190S virion versus 120 CP mol-
ecules (4). We predict that the abundance of RdRp molecules
must be regulated so that the appropriate copy number is
available for proper assembly and competence for replication
(7). Although it is not known how the plus-strand RNA of
HvV190S is packaged into assembling progeny virions, it is
thought by analogy to Saccharomyces cerevisiae virus L-A, the
prototype of genus Totivirinae and family Totiviridae, that
the RdRp would recruit the progeny plus-strand RNA for pack-
aging (9). We have recently demonstrated protein-protein in-
teractions between HvV190S CP and RdRp using the yeast
two-hybrid system (H. Li and S. A. Ghabrial, unpublished
data).

The identification of a predicted RNA structure (pseudoknot
or long stem-loop) as a key determinant of the stop-
restart mechanism in HvV190S and other victorviruses is an
exciting aspect of this study. Results indicate that the predicted
structure element must be located closely upstream of the CP
stop codon, presumably so that it can help to localize the
terminated ribosome for reinitiation at a downstream start
codon (see review in reference 39). Even though the restart
codon is very nearby the predicted structure element (only 6 to
13 nt downstream) and CP stop codon (within 2 nt) in all of
the wild-type victorviruses, the fact that HvV190S RdRp can
initiate at the AUG2 position, which is 90 nt downstream of the
predicted pseudoknot (80 nt downstream of the CP stop
codon) when the AUG1 codon is mutated to GUG suggests
that the very close juxtaposition seen in the wild-type viruses is
not strictly required, though it may be favored for other rea-
sions that we have yet to identify. There does appear to be a
limit to how far downstream the restart codon can be located,
however, in that when both AUG1 and AUG2 are mutated to
GUG, reinitiation does not occur effectively at the AUG3
position, which is 207 nt downstream of the putative pseudo-
knot in HvV190S. Our results additionally indicate that the
normal strong preferences for AUG start codons and the ca-
nonical flanking sequences for translation initiation appear
somewhat relaxed in the case of HvV190S RdRp. Localization
of ribosomal components as aided by the predicted RNA struc-
ture after termination at the CP stop codon presumably con-
tributes to these relaxed preferences by making the ribosomal
components more readily available for reinitiation.

Although translational coupling has also been shown to oc-
cur with certain eukaryotic cellular mRNAs, this process is
only efficient when the upstream ORF is short (27). The pre-

ence of short upstream ORFs in the 5′ UTR of mRNAs is
typically used as a means of downregulating the expression of
the downstream ORF (5). The translational coupling observed
with many viral mRNAs, however, is distinct from that of
eukaryotic cellular mRNAs in that the upstream ORF is typ-
ically longer and encodes a functional protein (1, 21, 30,
33, 40).

Among viral mRNAs, the mechanism of translational cou-
pling has been best studied with certain caliciviruses. In these
viruses, 84 to 87 nt of RNA sequence immediately upstream of
the overlapping stop-restart codons, termed the “termination
upstream ribosome binding site” (TURBS), are required for

efficient reinitiation (30, 31, 33, 34). Two distinct regions within
the TURBS have been shown to play significant roles. Motif 2
is not conserved among different viruses, and its function re-
mains unknown. Motif 1, on the other hand, is conserved and
is complementary to a small single-stranded region at the tip of
helix 26 of 18S rRNA, which is juxtaposed to mRNA in the
translating ribosome (32). This complementary region is
thought to tether the mRNA to the 40S subunit, allowing time
for it to acquire the initiation factors necessary for downstream
ORF translation (33, 34). There is also evidence that the
TURBS is involved in recruiting eukaryotic initiation factor 3
ev3) and eIF3/40S complexes (41). The eIF3 complex plays
multiple roles in translation initiation, including dissociating
the 60S and 40S ribosomal subunits after termination (36, 37),
and it is therefore possible that the TURBS is involved in both
ribosome dissociation/recycling and 40S tethereing (39).

A mechanism for stop-restart similar to that of caliciviruses
has been proposed for influenza B viruses (40). Stop-restart in
pneumoviruses, however, appears to occur by a distinct mech-
anism. At present, the evidence suggests that a large RNA
structure element in a region upstream of stop and restart
codons is required for reinitiation of translation in pneumovi-
ruses (15, 16), but the nature of this structure and how it works
to promote reinitiation remain unknown.

Our findings with regard to the stop-restart strategy of
HvV190S and other victorviruses are well in line with those for
the other RNA viruses described in the preceding paragraphs,
including tighter constraints on the position of the termination
codon versus the position of the reinitiation codon and a re-
 laxation of sequence preferences for the reinitiation codon.
Also of particular note among all of these viruses is the con-
sistent importance of a region of sequences closely upstream of
the stop and restart codons. Interestingly, in the case of victoriviruses, this specific region, 32 nt in length, is predicted to contain a well-defined RNA structure, either a predicted pseudoknot or a predicted long stem-loop structure, and we have provided both experimental evidence (via mutational analysis) and genetic evidence (via sequence comparisons among different victorivirus strains) for the importance of the predicted structure. Our results, however, do not yet rule out a role for other sequences upstream of the predicted RNA structure in the translation of the downstream RdRp ORF. In other viral mRNAs, including ones from other members of the family Totiviridae, pseudoknots are known to be structural features that promote the translation of an alternative ORF by −1 ribosomal frameshifting (6, 49). Ribosomal pausing induced by the pseudoknot is thought to be important in at least some of those cases (38).

In the case of HvV190S and other victoriviruses, we considered the possibility that part of the predicted RNA structure upstream of the stop and restart codons may interact with the host 18S rRNA, given previous findings with caliciviruses and influenza B viruses (30, 33, 34, 39, 40). Interestingly, a portion of loop 2 in the predicted pseudoknot of HvV190S (Fig. 6) has the sequence CUGAUGC, which is complementary to nt positions 909 to 915 of the Cochliobolus (anamorph: Helminthosporium) sativus 18S rRNA (44). However, (i) the HvV190S sequence, CUGAUGC, is not conserved among other victoriviruses (Fig. 9), (ii) the identified region of Cochliobolus 18S rRNA does not align with the region of mammalian 18S rRNA implicated in the stop-restart mechanisms of caliciviruses and influenza B viruses, and (iii) according to RNA folding predictions, the identified region of Cochliobolus 18S rRNA forms part of a stem, unlike the single-stranded region of mammalian 18S rRNA implicated in the stop-restart mechanisms of caliciviruses and influenza B viruses. We therefore consider it unlikely, albeit possible, that the role of the predicted RNA structure is for base pairing with 18S rRNA as a part of the stop-restart mechanism of victoriviruses.

The mechanism by which the predicted pseudoknot or stem-loop can promote coupled translation in victoriviruses thus remains unknown. The nonpolyadenylated victorivirus mRNA with a stop codon in its middle is reminiscent of a nonsense-containing mRNA without poly(A) binding protein (PABP). Amrani et al. (2) have shown that in the absence of PABP, terminated ribosomes tend to linger on mRNA in the vicinity of the stop codon. We therefore envision that the secondary structure in the victorivirus mRNA upstream of the CP stop codon may function to prevent terminated ribosomes from sliding backwards on the mRNA, helping to keep them positioned near the RdRp initiation codon. Although this is an attractive model, the HvV190S mRNA derived from transformants in our experimental system is expected to be polyadenylated. Furthermore, the close proximity of the CP stop codon to the predicted RNA structure would seem likely to cause the ribosome to interfere with refolding of the predicted RNA structure immediately following the termination of CP translation. It is possible that previously proposed, as-yet- unidentified host factors (45) may play a role in refolding and stabilizing the predicted RNA structure, as well as in retaining the terminated ribosome, following CP translation.

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