Synergism of the 3'-Untranslated Region and an Internal Ribosome Entry Site Differentially Enhances the Translation of a Plant Virus Coat Protein*

Dora Chin-Yen Koh‡‡, Sek-Man Wong¶¶, and Ding Xiang Liu**††

From the ‡Department of Biological Sciences, The National University of Singapore, 14 Science Dr. 4, Singapore 117543, the Institute of Molecular Agrobiology, 1 Research Link, Singapore 117604, and the **School of Biological Sciences, Nanyang Technological University, 1 Nanyang Walk, Block 5, Level 3, Singapore 637616, Republic of Singapore

The use of internal ribosome entry sites (IRESs) is one of the unorthodox mechanisms exploited by viruses to initiate the translation of internal genes. Herein, we report a plant virus exploiting an IRES and its 3'-untranslated region (UTR) to express its internal genes, notably the 3'-proximal viral coat protein gene. Hibiscus chlorotic ringspot virus (HCRSV), a positive-strand non-polyadenylated RNA virus, was demonstrated to harbor a unique 100-nucleotide (nt) IRES, located 124 nt upstream of the coat protein gene, that could function in wheat germ extract, rabbit reticulocyte lysate, and mammalian cells. In comparison with other known IRESs of picornaviruses and eukaryotic mRNAs, this 100-nt IRES is distinctively short and simple. The IRES activity was tested in homologous and heterologous bicistronic constructs, and the expression of the 3'-proximal gene was enhanced when the 3'-UTR was present. When the IRES element was bisected, each half still possessed IRES activity and could initiate internal translation on its own. Site-directed mutagenesis and deletion analyses revealed that the primary sequence within the 5' half was crucial for IRES activity, whereas the primary sequence of the second half and a GNRA motif were non-essential. To our knowledge, this is the first report describing a mechanism whereby an IRES, located in the 3' portion of the virus genome, co-operates with the 3'-UTR to enhance gene expression differentially.

Viruses, being obligate parasites, depend heavily on their host for replication. To compete successfully with cellular mRNAs for translation and to fully utilize their compact genomes, viruses have evolved various mechanisms either to redirect the translational machinery to favor viral transcripts or to regulate the expression of internal genes. Genome partitioning and the use of subgenomic RNAs (sgRNAs) are common mechanisms used by many plant viruses to make their internal genes accessible for ribosomes (1). In addition, several non-orthodox strategies, such as the use of internal ribosome entry sites (IRES) have been exploited by viruses to express multiple genes from a single RNA species.

Hibiscus chlorotic ringspot virus (HCRSV), a member of the genus Carmovirus, is an isometric monopartite plant virus. HCRSV possesses a single-stranded, positive-sense RNA that is non-polyadenylated at the 3' terminus and may not contain a 5' cap. The genomic RNA (gRNA) is 3911 nt long with the potential to encode seven open reading frames (ORFs), including two novel ORFs, ORF(p23) and ORF(p25) (2). Two 3'-cotermminated sgRNA species have been identified. The 3'-UTR of HCRSV was previously reported to differentially enhance the translation of various ORFs on the gRNA and sgRNA, such as ORF(p25) and ORF(p26) (3).

In this study, we report the identification and characterization of a 100-nt IRES that could co-operate with the 3'-UTR of HCRSV to differentially enhance the translation of ORFs on gRNA and sgRNA and a heterologous gene in a bicistronic construct. Located in the 3' portion of the gRNA and 124 nt upstream of the coat protein gene, this IRES element could function in wheat germ extract, rabbit reticulocyte lysate, and mammalian cells. Based on RNA secondary structure predictions, the HCRSV IRES is distinct in comparison with IRESs of picornaviruses and other eukaryotic mRNAs, because it is relatively short and simple. Deletion analyses showed that, when it was bisected, each half of the 100-nt IRES is able to exert the full IRES function independently. Site-directed mutagenesis and deletion analyses revealed a six-nucleotide sequence within a predicted stem structure located in the first half of the IRES element to be crucial for the IRES activity. However, the primary sequence of the second half and a GNRA motif were not essential for the IRES activity. When the IRES element was inserted into homologous and heterologous bicistronic constructs, expression of the second cistron was evident, demonstrating its ability to function as an IRES element. Addition of the 3'-UTR to the heterologous bicistronic construct resulted in the enhancement of the second cistron expression, verifying the co-operation between the IRES element and the 3'-UTR. These results demonstrate, for the first time, that a unique IRES element found in the 3' portion of a virus genome could synergistically co-operate with the 3'-UTR to enhance translation differentially.

GFP, green fluorescent protein; EGFP, enhanced GFP; IBV, infectious bronchitis virus; CP, coat protein; PTB, pyrimidine tract-binding protein; UTR, untranslated region.

* This research was supported by the National Science and Technology Board, Singapore and the National University of Singapore (NUS) through Research Grant R-154-000-111-112. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of the Singapore Millennium Foundation Scholarship and the President's Graduate Fellowship (NUS).

¶ To whom correspondence may be addressed. Tel.: 65-68-74-2976; Fax: 65-77-95-671; E-mail: dbswsm@nus.edu.sg.

†† To whom correspondence may be addressed. Tel.: 65-67-90-3738; Fax: 65-68-96-8032; E-mail: dxliu@ntu.edu.sg.

§ Recipient of the Singapore Millennium Foundation Scholarship and Ding Xiang Liu ** School of Biological Sciences, Nanyang Technological University, 1 Nanyang Walk, Block 5, Level 3, Singapore 637616, Republic of Singapore.
MATERIALS AND METHODS

PCR and Site-directed Mutagenesis—Appropriate primers and template DNA were used in amplification reactions with Pfu DNA polymerase under standard PCR conditions. The PCR reactions were 35 cycles of 94°C for 45 s, 50–55°C for 45 s, and 72°C for 1–3 min. The annealing temperature and extension time were subjected to adjustments accordingly to the melting temperature of the primers used and the length of the PCR fragments synthesized. Site-directed mutagenesis was carried out with two rounds of PCR and two pairs of primer as previously described (4).

SDS-PAGE—SDS-PAGE of in vitro translated products was performed with SDS-(15–17.5%) polyacrylamide gels. The labeled polypeptides were detected by autoradiography of dried gels. 17.5% polyacrylamide gels. The labeled polypeptides were detected by autoradiography of dried gels. 17.5% polyacrylamide gels. The labeled polypeptides were detected by autoradiography of dried gels.

Western Blotting—Western Blotting was carried out according to the manufacturer’s instructions (Promega). Briefly, 1 μg of uncut plasmid DNA was added to a 50-μl reaction mixture that was incubated at 30°C for 60 min in the presence of 50 μCi of [35S]methionine. Equal amounts of templates were used. Reaction products were separated by SDS-PAGE and detected by autoradiography.

Sucrose Gradient Analysis and Polysome Profiling—The integrity of mRNA derived from pHCRSV223inv5’-UTR was analyzed by sucrose gradient analysis as described by Pelletier and Sonenberg (5). In brief, the RNA was linearized and used for in vitro transcription in the presence of α-[32P]UTP. The in vitro synthesized RNA was extracted with phenol-chloroform, precipitated with ethanol, and then incubated in a 50-μl translation reaction containing 35 μl of wheat germ extract at 30°C. The translation mixture was cooled on ice, layered onto a 10–30% (w/v) sucrose gradient (2 ml) in buffer containing 25 mM Tris-HCl (pH 7.6), 100 mM KCl, and 5 mM MgCl2. The gradient was then subjected to centrifugation at 55,000 rpm in a TLS55 rotor (Beckman Instruments) at 4°C for 60 min. Fractions of 200 μl were collected from the top of the gradient and measured at absorbance 260 nm to obtain the polysome profile. Based on the polysome profile, fractions were pooled and RNA was extracted by phenol-chloroform and ethanol precipitation. The transcripts were then resolved on a 1% formaldehyde agarose gel and analyzed by autoradiography.

Results

Evidence for the Synergistic Effect between the 3'-UTR and an Internal Region on Translational Enhancement—The 3'-UTR of HCRSV was previously reported to differentially enhance the translation of gRNA and sgRNAs, increasing the expression of the 3'-located ORFs, such as ORF(p25) and ORF(p38) (3). To understand the mechanisms that control this differential enhancement effect, we first tested if the translation initiation of the 3'-proximal ORFs was dependent on the 5' end. Expression of construct pHCRSV223a5’-UTR, where the 5’-UTR was deleted from the full-length cDNA clone (Fig. 1a), resulted in the production of four major polypeptides of 23, 28, 38, and 25 kDa, similar to the wild type construct pHCRSV223 (Fig. 1b, lanes 1 and 2). These four products were encoded by ORF(p23), ORF(p28), ORF(p38), and ORF(p25), respectively (Fig. 1a). However, the expression of the 5'-proximal ORFs, ORF(p23) and ORF(p28), was significantly reduced, whereas the expression of the 3'-proximal ORF(p25) and ORF(p38) was enhanced (Fig. 1b, lanes 1 and 2). This result indicated that the expression of p23 and p28 was dependent on the 5' end, whereas the expression of p25 and p38 was initiated by an alternative mechanism that was independent of the 5' end. The removal of the 5'-UTR might have partially contributed to the enhanced expression of p25 and p38, because it could have reduced the competition between the initial initiation site and the 5' end. Deletion of the 3'-UTR in construct pHCRSV223a5’-UTR reduced the expression of the p25 and p38 polypeptides (Fig. 1b, lane 3), indicating that the enhanced expression of p25 and p38 observed in pHCRSV223a5’-UTR may be attributed mainly to the presence of the 3’-UTR. When both the 5’- and 3’-UTRs were deleted in construct pHCRSV223a5’UTRA3’UTR, the expression of all the four polypeptides were significantly reduced (Fig. 1b, lane 4).

The presence of an internal region that may co-operate with the 3'-UTR to enhance the expression of p25 and p38 was further analyzed by expression of the construct pHCRSV223inv5’UTR, which was generated by deleting the 5’-UTR and inverting the region containing nt 28–1036 (Fig. 1a). This deletion and inversion abolished the expression of p23
and p28 (Fig. 1b, lane 5). On the contrary, the expression of p25 and p38 was increased (Fig. 1b, lane 5). In addition, two polypeptides p24 and p22.5 were produced (Fig. 1b, lane 5). These two products were observed when pHCRSV80, which contains cDNA clone of sgRNA2, was expressed (2) and may be initiated from the leaky start codon for p25.

To rule out the possibility that the observed expression profile might be due to cleavage of the in vitro transcripts by endonucleases during in vitro translation, the integrity of pHCRSV223inv5'UTR mRNA associated with polysomes was examined by incubation of the α-32P-labeled pHCRSV223inv5'UTR mRNA in a translation reaction containing wheat germ extract at 30 °C for 10 min. After incubation, the RNA was resolved on a 1% formaldehyde agarose gel, and analyzed by autoradiography. The expression of p28 and p25 from pHCRSV223inv5'UTR mRNA was stimulated by incubation with wheat germ extract at 30 °C for 10 min and sucrose density gradient analysis. Based on the polysome profiles, fractions were pooled and RNA was extracted and resolved on a formaldehyde agarose gel. Trace amounts of RNA were detected from the top of the gradient (Fig. 1c, region I and Fig. 1d, lane 2), and small amounts of mRNA could be extracted from fractions containing the 80 S monosomes (Fig. 1c, region II and Fig. 1d, lane 3). As expected, the pHCRSV223inv5'UTR mRNA was found predominantly in the actively translating polysomes (Fig. 1c, region III and Fig. 1d, lane 4). The major RNA species detected in these fractions corresponds to the full-length pHCRSV223inv5'UTR mRNA (Fig. 1d, lanes 1 and 4). Furthermore, a very similar profile between RNA detected in the polysome fraction and the initial α-32P-labeled RNA preparation was observed (Fig. 1d, lanes 1 and 4). These results rule out the possibility that synthesis of polypeptides p38 and p25 from pHCRSV223inv5'UTR mRNA resulted from an endonucleolytic event that occurred between the inverted region at the 5' end and the 3' portion of the messenger. It is likely that an internal initiation event may be responsible for the synthesis of these two products.

Identification of the IRES Element—Deletion analysis of the full-length construct pHCRSV223 was carried out to delineate the region responsible for internal initiation (Fig. 2a). The expression of p28, being 5'-end dependent, was used as an internal control for the internal initiation event and the stability of RNA templates during translation. Deletion of the region from nt 1208–2394 in construct pHCRSV223 stimulated the expression of p38 and p25 (Fig. 2b, lane 2). The expression of p38 was increased by at least 5-fold compared with the control pHCRSV223 (Fig. 2b, lanes 1 and 2). When a further 172-nt region was deleted in construct pHCRSV223Δ2, the expression of p38 was reduced by at least 5-fold that of the control (Fig. 2b, lane 3), indicating that the IRES element was to be located within the boundaries of nt 2394 and nt 2566. When this 172-nt region was specifically deleted in construct pHCRSV223Δ3, a 5-fold reduction in p38 expression was observed (Fig. 2b, lane 4).
Synergy between HCRSV 3'-UTR and an IRES

4), confirming that the IRES element is located within this region.

The region containing the IRES element (nt 2394–2566) was then inserted into a bicistronic construct containing the GFP gene as the 5' cistron and the envelope (E) protein gene of coronavirus IBV as the 3' cistron, giving rise to construct pGFP-IRES-E (Fig. 2a). Construct pGFP-E, containing the GFP gene and the E protein gene in different reading frames, was used as a control (Fig. 2a). Expression of pGFP-E resulted in the detection of GFP (Fig. 2c, lane 1). With the insertion of nt 2394–2566 between the 5' and 3' cistrons, the GFP encoded by the 5' cistron and the E protein encoded by the 3' cistron, were expressed (Fig. 2c, lane 2). These results demonstrated that translation of the E protein was initiated by the inserted sequence.

Co-operation of IRES with the 3'-UTR to Enhance the Expression of the 3' Cistron in the Bicistronic Construct—To test if the identified IRES element was involved in the differential enhancement effect by the 3'-UTR, the 3'-UTR of HCRSV was added into the bicistronic construct pGFP-IRES-E to generate construct pGFP-IRES-E-3'UTR (Fig. 3a). It was observed that addition of the 3'-UTR significantly enhanced the expression of E protein by 4-fold (Fig. 3b, lanes 1 and 2), suggesting the synergistic effect between the 3'-UTR and the IRES element. It was also interesting to note that, although IBV E protein expression was increased, the expression of GFP became significantly reduced by 1.5-fold. The presence of the 3'-UTR and the synergy between the 3'-UTR and IRES could create a competition for translational apparatus, such that the 5'-end-dependent expression of GFP was limited. The expression of both proteins was normalized to that of a background band when quantitative data were presented.

In a parallel experiment, the 3'-UTR was introduced in an antisense orientation to generate pGFP-IRES-E-rev3'UTR (Fig. 3a). The IBV E protein expression was comparable to pGFP-IRES-E (Fig. 3b, lanes 1 and 3). When two copies of the 3'-UTR were tandemly inserted in a head-to-head fashion in construct pGFP-IRES-E-2x3'UTR (Fig. 3a), the translational enhancement effect of the 3'-UTR was not observed (Fig. 3b, lane 4). The expression profiles of both the IBV E and GFP proteins were similar to pGFP-IRES-E (Fig. 3b, lanes 1 and 4). It is possible that the tandemly arranged 3'-UTR segments may form stable double-strand RNA structures by base pairing, thus eliminating essential domains on the 3'-UTR responsible for the synergistic effect.

Previously, we reported that a 6-nt segment within the 3'-UTR was responsible for the translational enhancement effect of the 3'-UTR (3). To test if this 6-nt segment was involved in this synergistic effect, the 6-nt mutation (3659GGGCAG to 3659GCCAG) was introduced, giving rise to the mutant.
construct pGFP-IRES-E-3'UTR(M1) (Fig. 3a). Expression of this construct showed that the 3'-located IBV E protein was comparable to pGFP-IRES-E (Fig. 3b, lanes 1 and 5).

Deletion Analysis of the IRES Activity between nt 2466 and 2566—Detailed dissection of the region from nt 2394 to 2566 was performed by deletion analysis to map the sequence responsible for the IRES effect (Fig. 4a). In construct pHCRSV223Δ4, which had a deletion of the first half of the region consisting of nt 2394–2466, expression of p38 was enhanced by at least 2-fold as compared with pHCRSV223 (Fig. 4b, lanes 1 and 3). Construct pHCRSV223Δ5 contains a deletion of the second half of the region (nt 2466–2566) (Fig. 4a), resulting in a 2-fold reduction in p38 expression when compared with pHCRSV223 (Fig. 4b, lanes 1 and 5). These results indicated that the active IRES element was located in the region from nt 2466 to 2566. This 100-nt region was further bisected in constructs pHCRSV223Δ6 and pHCRSV223Δ7 with deletions of 50 and 45 nt, respectively (Fig. 4a). Expression of the two constructs showed similar levels of the p38 expression to that of pHCRSV223 (Fig. 4b, lanes 1, 4, and 6). This observation implies that the smaller segments, both 50 nt and 45 nt, of the IRES were able to exert full IRES activity independently. Constructs pHCRSV223Δ8 and pHCRSV223Δ9 containing only regions from nt 2466 to 2515 and from nt 2516 to 2566, respectively, were constructed to test the IRES activity of each segment (Fig. 4a). Expression of pHCRSV223Δ8 showed enhanced expression of p38 by 2.5-fold compared with pHCRSV223 (Fig. 4b, lanes 7 and 9). Expression of pHCRSV223Δ9 showed that the p38 expression was enhanced by 1.2-fold compared with pHCRSV223 (Fig. 4b, lanes 8 and 9). It suggests that the region containing nt 2466–2515 possesses stronger IRES activity than the region nt 2516–2566.

Mutational Analysis of the First Half of IRES (nt 2466–2515)—Secondary structure prediction of RNA located within nt 2494 to 2512 (Fig. 5a) was performed by mutating a putative stem loop structure to identify significant motif that may contribute to the IRES activity between nt 2516 and 2566. Nine mutants were constructed based on pHCRSV223ΔM2496 to pHCRSV223ΔM2498 was made in construct pHCRSV223ΔM2 (Fig. 5a), a similar level of p38 expression was observed (Fig. 5b, lanes 1 and 3). Compensatory mutation of 2506ATC2510 to 2506TAG2510 was made based on pHCRSV223ΔM2 to restore the putative stem loop structure, yielding the construct pHCRSV223ΔM3 (Fig. 5a). Expression of this construct resulted in a 5-fold reduction in p38 expression compared with pHCRSV223Δ8 (Fig. 5b, lanes 1 and 4). These results demonstrated that the putative secondary structure of RNA was not responsible for the IRES effect, but rather the sequence located in the region nt 2494–2512 seems to play an important role.

Mutation of the 3 nt from 2496GAT2498 to 2496CTA2498 was introduced into the biologically active cDNA clone of HCRSV, pHCRSV223, yielding the construct pHCRSV223M2496. In vitro transcribed transcripts derived from this mutant were inoculated on kenaf plants, and the plants were regularly observed up to 60 days post-inoculation for the development of viral symptoms. Plants inoculated with the RNA from pHCRSV223 showed severe chlorotic ringspots and vein banding at 14–17 days. Reverse transcription-PCR and sequencing results detected viral replication in the infected leaves. However, plants inoculated with transcripts derived from pHCRSV223M2496 remained uninfected during the whole observation period, and viral transcripts were not detected in reverse transcription-PCR. This result suggests that this mutation affects the infectivity of HCRSV.

Mutational Analysis of the Second Half of IRES (nt 2516–2566) and a Putative GNRA Motif—Systematic site-directed mutagenesis was carried out to identify significant motif that may contribute to the IRES activity between nt 2516 and 2566. Nine mutants were constructed based on pHCRSV223ΔM2496 (Fig. 6a). Expression of these constructs showed that the nucleotide substitutions did not significantly affect the p38 expression (Fig. 6b). Mutations in constructs pHCRSV223ΔM6,
pHCRSV223/H90049M8, and pHCRSV223/H90049M9, where 26–56% nucleotides were substituted resulted in a 1.5-fold reduction in p38 expression compared with the wild type construct pHCRSV223/H9004 (Fig. 6b).

Analysis of the sequence downstream of the IRES element between nt 2566 and the start codon of p38 at nt 2603 revealed the presence of a GAAA motif between nt 2573 and 2576 located in the loop region of a predicted downstream stem loop structure. The GNRA tetraloops, found in all picornavirus IRES secondary structures, are directly involved in IRES activity (10). Mutation of 2573GAAA2576 to 2573CTGC2576 was made in pHCRSV223/H90049M10 (Fig. 6a). Expression of the construct showed that the mutation did not disrupt the level of p38 expression (Fig. 6b, lanes 11 and 12). These results demonstrated that the identified GNRA motif and the primary sequence within the region nt 2516–2566 were not essential for the IRES activity.

**Functionality of the HCRSV IRES Element in Mammalian Cells**—To test if this plant virus IRES element is functional in mammalian cells, the GFP gene was cloned upstream of the IRES region in construct pHCRSV223GFP (Fig. 7a). In a transient expression system (11), COS-7 cells were infected with vaccinia virus, which possesses a T7 polymerase gene, and transfected with T7-promoter driven pHCRSV223GFP plasmid DNA. The expression of GFP was detected in transfected cells when viewed under UV (data not shown). The cells were harvested, and Western blot detection was performed using the expression of β-tubulin gene as a control to normalize the densitometry readings. As shown in Fig. 7b, the expression of both GFP and p38 was detected from pHCRSV223GFP, which
indicates that the IRES element from HCRSV was active in mammalian cells.

The HCRSV IRES activity in COS-7 cells was further analyzed by expression of four deletion constructs (pHCRSV223\textsuperscript{9}H9004\textsubscript{1}, pHCRSV223\textsuperscript{9}H9004\textsubscript{2}, pHCRSV223\textsuperscript{9}H9004\textsubscript{3}, and pHCRSV223\textsuperscript{9}H9004\textsubscript{4}). The results obtained substantiated the observations made in the in vitro studies. As shown in Fig 7c, when the IRES element was deleted in pHCRSV223\textsuperscript{9}H9004\textsubscript{2} and pHCRSV223\textsuperscript{9}H9004\textsubscript{3}, the expression of p38 was abolished (Fig. 7c, lanes 3 and 4). Much enhanced expression of p38 was observed in constructs pHCRSV223\textsuperscript{9}H9004\textsubscript{1} and pHCRSV223\textsuperscript{9}H9004\textsubscript{4} compared with pHCRSV223 (Fig. 7c, lanes 1, 2, and 5).

**DISCUSSION**

RNA viruses, particularly plant RNA viruses, usually contain compact genomes with limited coding capacity. To fully utilize their genetic storage, viruses have evolved strategies to regulate the expression of different ORFs. Previously, we reported that the 3′-UTR of HCRSV could differentially enhance the translation of ORFs located at the 3′ portion of gRNA and sgRNA (3). In this study, we demonstrated that the synergy between the 3′-UTR and an IRES element, located 124 nt upstream of the coat protein (CP) gene, was responsible for the differential translation enhancement effect.

The synergistic effect between the 3′-UTR and the IRES could be likened to that of the interaction between the 5′ and 3′ termini of viral and cellular mRNAs that results in the formation of a circularized mRNA. Evidence has shown that the poly(A) tail binding protein and the 3′-poly(A) tail interact synergistically with the 5′ cap to promote translation initiation and increase translation efficiency by forming a “closed-loop” initiation complex (12, 13). Circularization of mRNA is thought to enhance translation by facilitating the recycling of the initiation factors (12, 13) and allowing new rounds of initiation to occur rapidly (14). This activity is crucial when there is competition between virus and host for translation machinery (15) or when the availability of ribosomes or initiation factors becomes limited (16). Synergy between the poly(A) tail/3′-UTR and IRES elements has also been documented for several viral systems. Bergamini et al. (17) demonstrated that translation driven by the picornavirus IRESs, such as encephalomyocarditis virus, poliovirus, and hepatitis A virus, has been synergistically enhanced by a poly(A) tail. Non-polyadenylated hepatitis C virus with the involvement of the pyrimidine tract-binding protein (PTB) has also adopted this relationship between its 3′-UTR and IRES to enhance translation by 3- to 5-fold (18). The PTB
protein can also bind to IRES regions of several other RNA viruses to regulate their translation (19, 20).

The results presented in this study suggest that the differentially enhanced expression of the HCRSV CP was mediated by the co-operation between the 3′-UTR and IRES. Is this enhancement effect exerted by circularization of the genomic RNA? Mutagenesis studies have shown that an 8-nucleotide region within the first half of the HCRSV IRES may play an important role in IRES activity and determine viral infectivity. One possibility could be that this region might contain sequences complementary to the 18S rRNA. Computer analysis revealed that the region within the IRES from nt 2493 to 2500 (5′-CCTGATTA-3′) has 75% complementarity to the 3′ region of the 18S rRNA of Nicotiana tabacum from nt 1667 to 1674 (5′-TGATC-CGG-3′). The 6-nucleotide segment within the 3′-UTR, which was shown to be responsible for translational enhancement by its predicted base pairing with the 3′ end of the 18S rRNA of N. tabacum (3), was also demonstrated to significantly influence the synergistic effect between the IRES and the 3′-UTR. The regions in which the IRES and 3′-UTR were predicted to base pair with the 18S rRNA are found in the same vicinity, and there is a possibility that their base pairing with the 18S rRNA might circularize the RNA to enhance the translation of the 3′-located ORFs. It has been reported that some mRNA sequences that bind ribosomes through base pairing not only affect translation efficiency but are also able to function as IRESs (21), and mRNA fragments complementary to the 18S rRNA increase their chances of being translated by attaching to the 18S rRNA (22). It is interesting that so far all the IRESs reported to have synergy with either the poly(A) tail or the 3′-UTR are found in the 5′-UTR, suggesting that the HCRSV IRES being found in the 3′ portion of the genome, is unique and distinct. To our knowledge, this is the first report of an IRES element, located at the 3′ portion of the viral genome, which could differentially enhance the expression of downstream ORFs by co-operation with the 3′-UTR.

In comparison to all known viral IRESs that are highly structured, the 100-nucleotide HCRSV IRES is distinctly short and simple. It is interesting to note that the 100-nucleotide IRES can be equally divided into two halves, each containing an IRES activity that could initiate translation internally on its own. Secondary structural analysis revealed a potential stem-loop structure in the 5′ segment. Compensation mutations showed that this structure was not responsible for the IRES activity, but mutations to 3 nt of the stem structure reduced the p38 expression by 2-fold. This indicated that the primary sequence was a contributing element for the IRES function. Substitutions of single nucleotides in other IRES elements have been shown to severely reduce IRES activity (23). Comparison of the second half of IRES with a similar region in turnip crinkle virus revealed 66% sequence similarity particularly in CA-rich regions, which have been reported to enhance the CP expression from sgRNA (24). The presence of CA-rich motifs in the 5′-UTR in the proximity of the initiation codons is a common occurrence in many plant viral RNAs, including turnip crinkle virus (24) and tobacco mosaic virus (25). The CA-rich region within the HCRSV IRES may contribute to the additional length and reduced secondary structure (26) and, therefore, provides a suitable landing pad for ribosomes to initiate at the downstream initiation codon. Dorokhov et al. (27) reported that polypurine (A)-rich sequences, existing as multiple copies of (A)G(A)2(G)12 or G(A)2,5 and found in the IRES element of crucifer infecting tobamovirus, were responsible for promoting internal ribosome entry in plant, yeast, and HeLa cells.

Besides functioning in the wheat germ expression system, the HCRSV IRES was able to function in the rabbit reticulocyte lysate system and mammalian cells. IRES elements identified in crucifer infecting tobamovirus have been reported to be active in the rabbit reticulocyte lysate system (28) and have recently been demonstrated to function in yeast and HeLa cells (27). Although it may be possible for plant viral IRES to function in the rabbit reticulocyte lysate expression system and the
mammalian cell system, RNAs of poliovirus and encephalomyocarditis virus do not function efficiently in wheat germ extract (29, 30). Deletion of sequences upstream of the HCRSV IRES resulted in a much more obvious stimulatory effect on p38 expression in mammalian cells, compared with that observed in the wheat germ system. This could be due to the removal of a certain upstream negative regulatory element that mediates the IRES function more effectively in mammalian cells. Translation of hepatitis C virus RNA was attenuated when a PTB protein binds to an internal pyrimidine-rich tract, which was relieved in the presence of an X region in the 3'-UTR (31). The PTB protein, known to bind an IRES site in the 5'-UTR and the X region in the 3'-UTR to enhance translation (18), is proposed to mediate translation regulation by another mechanism through its interaction with the internally located PTB protein-binding site. It may be possible that such sites that contain negative regulatory elements are located in the immediate upstream region of the HCRSV IRES, such that the removal of these elements resulted in a stimulatory effect of IRES on its 3' proximal gene expression. Alternatively, the presence of secondary structures located upstream of IRES that could determine ribosome accessibility, may also affect IRES activity. Stimulatory effects of IRES on the removal of upstream sequences have been reported in human parechovirus (32).

Evidently, the possibility that the HCRSV CP could be internally translated does not exclude the conventional mechanism controlling the expression of CP via sgRNA. These two mechanisms may operate in concert at different times of the viral life cycle such that internal initiation could direct small amounts of CP to be expressed from gRNA in the early stages of infection for production of virus to effect sufficient systemic infection.

REFERENCES
Synergism of the 3’-Untranslated Region and an Internal Ribosome Entry Site Differentially Enhances the Translation of a Plant Virus Coat Protein
Dora Chin-Yen Koh, Sek-Man Wong and Ding Xiang Liu

doi: 10.1074/jbc.M210212200 originally published online March 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210212200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 30 references, 20 of which can be accessed free at http://www.jbc.org/content/278/23/20565.full.html#ref-list-1