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Cell-Free Synthesis of Polypeptides Lacking an Amino-Terminal Methionine by Using a Dicistroviral Intergenic Internal Ribosome Entry Site

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An amino-terminal methionine corresponding to a recombinant AUG initiation codon sometimes affects the functions of proteins. To test the performance of translation mediated by a dicistroviral internal ribosome entry site (IRES), which initiates protein synthesis with elongator tRNAs, we optimized the conditions for cell-free translation. Although the IRES is 188 nucleotides long, a further 50 nucleotides of the upstream sequence stabilized translation efficiency. Optimal ion concentrations were affected by the sequences of the constructs. In a wheat-germ system, IRES-mediated translation produced 78 μg/ml of firefly luciferase from the AUG-deleted sequence, suggesting that dicistroviral IRESs will be able to yield polypeptides with a specific N-terminal amino acid other than methionine.

Key words: cell-free protein synthesis, *Dicistroviridae*, internal ribosome entry site, translation initiation, wheat germ extract.

Abbreviations: Fluc, firefly luciferase; IGR, intergenic region; IRES, internal ribosome entry site; PSIV, Plautia stali intestine virus; Rluc, *Renilla* luciferase; TSV, Taura syndrome virus; WGE, wheat germ extract.

Protein synthesis from cloned genes is a popular technique in biochemical research. In normal mRNAs, the initiation of translation absolutely requires an initiator tRNA (*1*, *2*). The N-terminal methionine corresponding to a recombinant AUG initiation codon, however, sometimes affects the functions of proteins that do not carry an N-terminal methionine in their authentic form, such as secreted proteins and cleaved proteins. For example, methionylation of interleukin-2 affects the interaction between its N- and C-termini (*3*), methionylation of a lipolytic enzyme reduces its maximum velocities towards substrates (*4*), and methionylation of a lysozyme reduces its solubility (*5*). To obtain proteins with an authentic N-terminal amino acid, various methods have been developed (*6–11*), although some of these methods have limitations in terms of the type of N-terminal amino acid or require several experimental steps to produce the desired N-terminal amino acid in addition to the normal protein synthesis procedure.

Plautia stali intestine virus (PSIV) is a member of the family *Dicistroviridae* (*12*). The intergenic region (IGR) of dicistroviral RNA genomes contains an internal ribosome entry site (IRES) (*13–16*). It has been generally believed that most of IRES elements form tertiary structures and recruit ribosomes at an internal part of the mRNA independently of the 5′ cap structure (*17*). The IGR-IRES elements of dicistroviruses consist of about 200 nucleotides containing four stem-loops and three pseudoknots (*18*). Unlike normal mRNAs, protein synthesis mediated by the IGR-IRES elements of dicistroviruses starts at AUG-unrelated codons by virtue of their own tertiary structure formation (*19–22*). A pseudoknot comprising a 5-base-pair interaction is formed at the –1 position of the viral coding region (*19*). This pseudoknot is located at the 3′ half of the IGR-IRES and determines the reading frame of the mRNA (*19*) by positioning at the peptidyl tRNA site on eukaryotic ribosomes (*20*).

The whole structure of the IGR-IRES (*21*, *22*) has been believed to mimic the functions of eukaryotic initiation factors and initiator tRNA, because the IGR-IRES of cricket paralysis virus, a member of the family *Dicistroviridae,* can start elongation cycles in the absence of any initiation factors or initiator tRNA (*23*, *24*). In addition, an *in vitro* experiment has shown that there is no restriction to the first decoded triplet in the IGR-IRES–mediated translation initiation, in other words, IGR-IRES–mediated translation can synthesize polypeptides that have any desired N-terminal amino acid (*25*). These previous observations suggest that IGR-IRES elements would be useful for obtaining authentic polypeptides *in vitro* from cloned genes.

Previous analysis of the IGR-IRES of PSIV showed that, although the viral coding sequence could be replaced with AUG-deleted exogenous sequences, the efficiency of IGR-IRES-mediated translation was decreased by such replacements, suggesting that translation is sensitive to the flanking sequences (*25*). To adapt IGR-IRES–mediated translation to a eukaryotic cell-free translation system, we have tested the effects of various 5′- and 3′-flanking sequences, ion concentrations, and amounts of template RNA. Under our optimized conditions, IGR-IRES–mediated translation produced a protein product as efficiently as a cap-dependent translation mechanism, suggesting that it will be possible to synthesize polypeptides with a specific N-terminal amino acid

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other than methionine by using the IGR-IRES of dicistroviruses.

MATERIALS AND METHODS

Plasmid Construction—Coding sequences of chloramphenicol acetyltransferase (CAT), enhanced green fluorescent protein (EGFP), and *Renilla* luciferase (Rluc) were used to test the effect of exogenous sequences. A pCAT3-Control Vector (Promega), pEGFP (Novagen), and a modified pRL-null vector (Promega), which had been altered from adenine to guanine at nucleotide 312 by site-directed mutagenesis to create a *Nco*I recognition sequence, were digested with *Nco*I and *Xba*I. Fragments containing the coding sequences were ligated into a large fragment of pT7LUC (*13*), which had been digested with *Nco*I and *Xba*I, to generate pT7CAT, pT7EGFP, and pT7Rluc, respectively. The PSIV sequence, nucleotides 5950 to 6240, was amplified by PCR from pT7CAT-5375 (*13*) using a forward primer with a *Hin*dIII sequence in the 5′ part and a reverse primer with a *Nco*I sequence in the 5′ part. The amplified fragments were digested with *Hin*dIII and *Nco*I, and ligated into the corresponding sites of pT7LUC to generate pT7-5950IRES6240-Fluc. This plasmid, containing the coding sequence for firefly luciferase (Fluc), was digested with *Hin*dIII, blunted with Klenow enzyme, and digested with *Eco*RI. The resultant 1.8-kb fragment, 5950IRES6240-Fluc, was ligated into pT7CAT, pT7EGFP, and pT7Rluc, which had been digested with *Xba*I, blunted and digested with *Eco*RI, to generate pT7CAT-5950IRES6240-Fluc, pT7EGFP-5950-IRES6240-Fluc, and pT7Rluc-5950IRES6240-Fluc, respectively. The deletion of nucleotides 5950 to 5959, 5968, 5978 and 5988 was generated by ligation of the phosphorylated PCR products.

For the performance test in the improved WGE, the CAT coding sequences were deleted from pT7CAT-IRES-ΔaugFluc and pT7CAT-IRES-ΔaugRluc (*25*). Large fragments obtained by the *Hin*dIII and *Eco*RV digestion of these plasmids were blunted and then ligated to generate pT7IRES-ΔaugRluc and pT7IRES-ΔaugFluc, respectively. Silent mutations were introduced by PCR-based mutagenesis, yielding pT7IRES-ΔaugFluc(mut) and pT7IRES-ΔaugRluc(mut), respectively.

In Vitro Transcription and Translation—Uncapped and capped RNAs were prepared as described (*16*). In vitro translation was done with WGEs obtained from Promega and Toyobo (PROTEIOS™) according to the manufacturers' recommendations. Fluc and Rluc activities were measured by a Steady-Glo Luciferase Assay System and a *Renilla* luciferase assay system (Promega).

To evaluate upstream sequences and salt concentrations, uncapped RNAs (40 pmol/ml) and amino acids (20 μM each) were incubated for 60 min at 26°C, and Fluc activities in 5 μl of the reaction mixtures were measured. To examine the effect of the amount of RNAs, Fluc activities in 1 μl of reaction mixtures containing various concentrations of RNAs were measured. To perform protein synthesis with a dialysis system, 100 μl of a reaction mixture containing 64 pmol of uncapped RNA was loaded into a cup of a Micro DispoDialyzer system (Spectrum, MWCO; 15 kDa). The cup was immersed in 3 ml of PROTEIOS™ Buffer (Toyobo) and incubated at 25°C. After 24 h, 9 μl of a solution containing 64 pmol of template RNA and 30 μg of creatine kinase was added, and the mixture was incubated for a further 24 h. The final amounts of luciferases were enzymatically determined as described

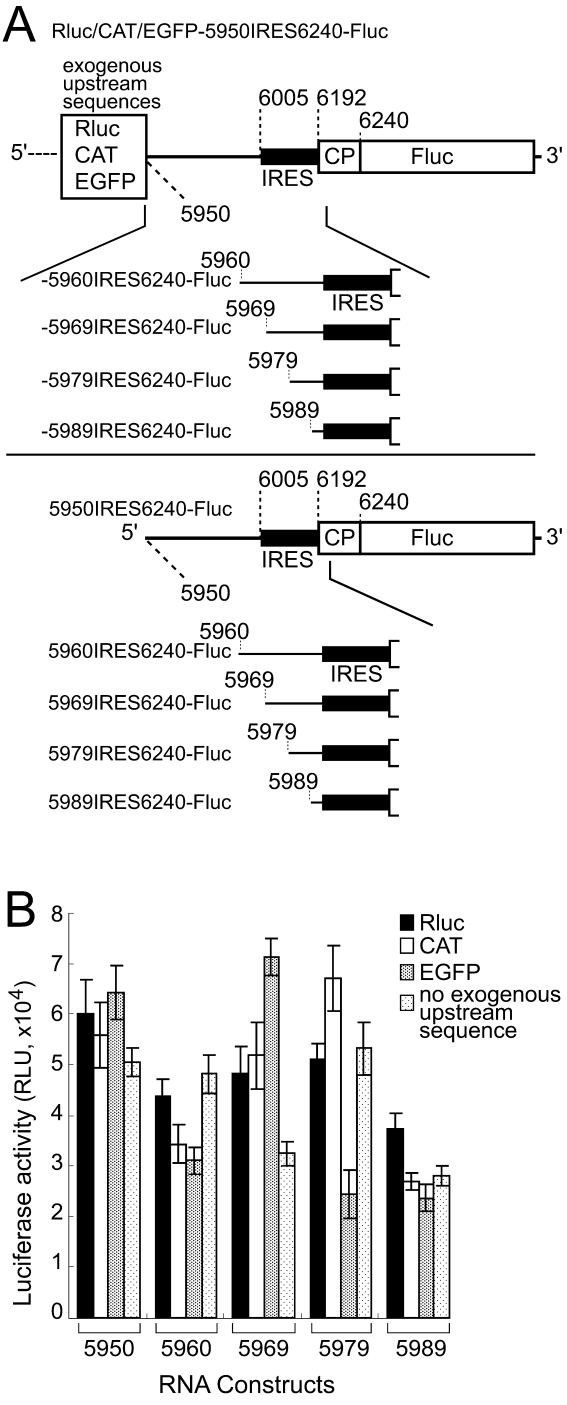

Fig. 1. **Effect of different 5′ upstream sequences on IGR-IRES–mediated translation.** A: Diagrams of RNA constructs. The numbers indicate nucleotide positions corresponding to the PSIV genome. The open reading frame of the capsid protein (CP)–Fluc fusion protein was used to evaluate translation efficiency. B: Comparison of translation efficiency of the constructs shown in A. Fluc activities in each reaction mixture are indicated as the mean ± SD (n = 3).

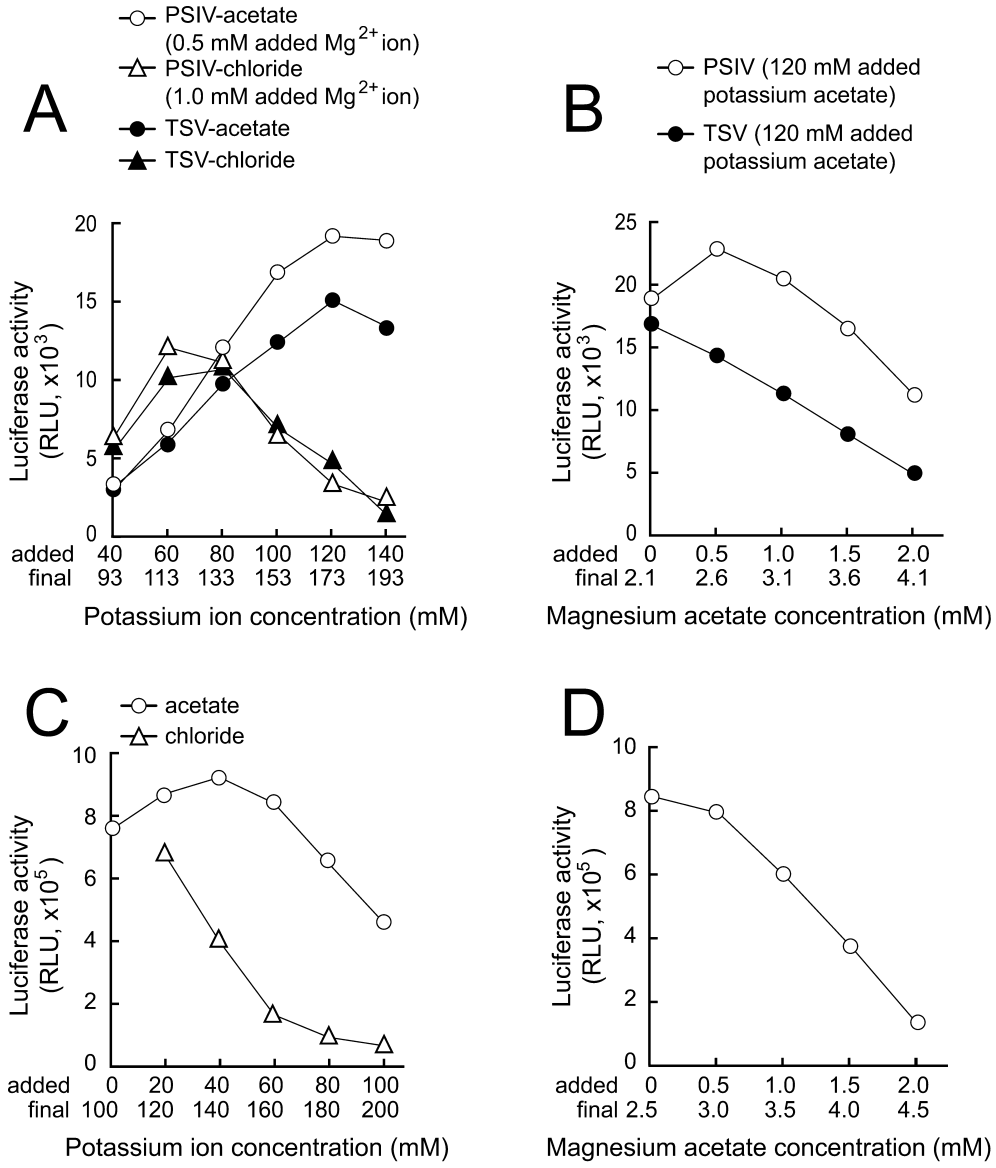

Fig. 2. **Efficiency of IGR-IRES-mediated translation at different ion concentrations.** A: Effect of potassium chloride and potassium acetate concentrations on translation in the Promega WGE. B: Effect of magnesium acetate concentrations on translation in the Promega WGE. C: Effect of potassium ion concentrations on translation in PROTEIOS™. D: Effect of magnesium acetate concentrations on translation in PROTEIOS™.

(*25*) using standards for Fluc (Toyo B-net) and Rluc (Chemicon).

RESULTS

Stabilization of Translation Efficiency by Longer PSIV Upstream Sequences—In a previous deletion analysis that estimated the length of the IGR-IRES, about 250 nucleotides were suggested to be necessary for internal initiation (*13*). However, the predicted secondary structures of the IGR-IRES elements in dicistroviruses suggested that conserved structures were formed in the central 200 nucleotides (*18*) and no structural conservation was detected in the downstream or upstream sequences. A recent study has shown that the 3′ downstream sequence, the viral capsid coding sequence, is not absolutely required for IGR-IRES–mediated translation, although replacing exogenous downstream coding sequence did reduce translation efficiency (*25*). In contrast, the effect of the 5′ upstream sequence on IGR-IRES–mediated translation has not been elucidated. To analyze the effect of various upstream sequences on IGR-IRES–mediated translation, the 3′ ends of the Rluc, CAT, and EGFP coding sequences were inserted at nucleotides 5950, 5960, 5969, 5979, and 5989, that is, at positions located 55-, 45-, 36-, 26-, and 16-bases, respectively, upstream of the 5′ end of the PSIV-IRES (Fig. 1A, upper). To determine the effect of these exogenous sequences on IGR-IRES-mediated translation efficiency, constructs without exogenous sequences were also examined (Fig. 1A, lower).

We found that the average Fluc activities of the four 5989 constructs were lower than those of the 5950 constructs (Fig. 1B). When the 3′ end of the EGFP sequence was located at nucleotide 5979, the Fluc activity was about 2.5×10^4, less than half of that of the CAT construct. A more than twofold difference was also observed among the 5969 constructs. For the 5960 and 5950 constructs, the difference in Fluc activities was normalized, but the 5950 constructs showed a higher average Fluc

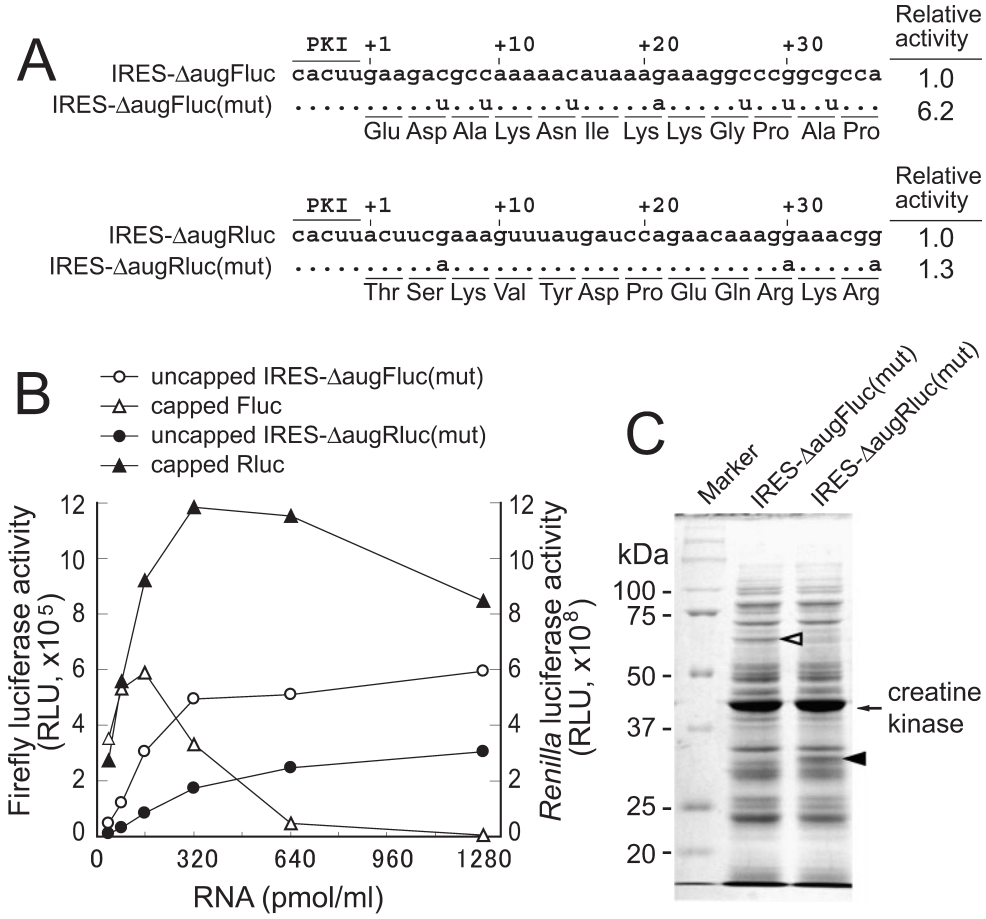

Fig. 3. **Direct synthesis of proteins lacking an N-terminal methionine by the IGR-IRES.** A: Nucleotide sequences at the 5′ regions of coding sequences translated by the IGR-IRES. The translation initiation site immediately downstream of the pseudoknot I (PK I) is marked as +1. Silent mutations that decreased the GC content and the relative translation efficiency of these mutants, measured by enzymatic activities, are shown. B: Effect of RNA concentration on the canonical ribosome scanning and IGR-IRES–mediated initiation mechanisms. C: Detection of IGR-IRES–mediated translation products. After 48 h of incubation, 4-μl aliquots of the reaction mixtures were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. Open and closed triangles indicate Fluc and Rluc, respectively.

activity than the 5960 constructs. In the previous preliminary deletion assays, there was no significant difference in translation activities when a longer 5′ part of the PSIV sequence, nucleotides 5950–5375, was retained (*13*). Taken together, these observations suggest that about 50 nucleotides of the viral upstream sequence can stabilize the translation activity of the IGR-IRES of PSIV.

Effect of Potassium and Magnesium Concentration—The concentration of potassium and magnesium salts affects translation efficiency (*26*, *27*). To optimize the efficiency of the IGR-IRES–mediated translation, we changed the salt concentrations in commercially available WGE systems and monitored the translation efficiency of 5950-IRES6240-Fluc RNA by measuring Fluc activity. Because the IGR-IRES of Taura syndrome virus (TSV) is structurally distinct from that of PSIV (*16*), the translation efficiency of transcripts from pIGR-CP-Fluc (*16*) containing the IGR-IRES of TSV was also monitored.

We first examined ion conditions using the Promega WGE. This extract endogenously contains 53 mM potassium acetate and 2.1 mM magnesium acetate in a standard reaction mixture. The highest Fluc activities were observed at 173 mM of potassium acetate (Fig. 2A) and 2.6 mM magnesium acetate for translation by the PSIV-IRES (Fig. 2B). In preliminary assays, the amount of template RNA, 10, 40, or 160 pmol/ml, hardly affected optimal ion concentrations. When the potassium ion concentration was adjusted with potassium chloride, the optimum concentration was 113 mM (Fig. 2A). Although the requirement for potassium ions was similar for both PSIV and TSV IGR-IRES–mediated translation (Fig. 2A), the magnesium ion requirement differed. For TSV, the optimum magnesium ion concentration was found to be less than 2.1 mM (Fig. 2B). In comparison with PSIV, the IGR-IRES of TSV contains an additional GC-rich side-stem in domain 3 (*16*). Because correct folding of the IRES region is required for effective translation, the difference in the GC content in domain 3 may account for the different magnesium ion requirements.

To examine whether the ion concentrations optimized for PSIV in the Promega WGE are universally applicable, we tested another cell-free system (PROTEIOS™, Toyobo). This system endogenously contains 100 mM potassium acetate and 2.5 mM magnesium acetate in a standard reaction mixture. Because the WGE in PROTEIOS™ has been prepared by an improved method (*28*), the translation efficiency was about 40-fold higher than in the Promega WGE for our constructs (Fig. 2, CD and AB). The optimal final concentration of potassium acetate for translation in PROTEIOS™ was 140 mM (Fig. 2C), 30 mM lower than that for translation in the Promega WGE (Fig. 2A). We could not determine the optimal final concentration of magnesium acetate because it was lower than the endogenous concentration (Fig. 2D). Extrapolation of the curve in Fig. 2D, however, suggests that it

might be close to 2.5 mM, a value that is almost identical to that determined with the Promega WGE (Fig. 2B).

We also carried out similar experiments using the constructs IRES-ΔaugFluc and IRES-ΔaugRluc (Fig. 3A). These constructs contain AUG-deleted Fluc and Rluc coding sequences immediately downstream of the pseudoknot I (PK I) of the PSIV-IRES. Optimal ion concentrations for these two constructs were within ± 20% of those of the 5950IRES6240-Fluc RNA construct (data not shown), suggesting that exogenous sequences affect the optimal salt concentrations for IGR-IRES–mediated translation. Taken together, these results indicate that optimal salt concentrations are distinct in each cell-free system and are also affected by the nucleotide sequences of constructs. Recently, a higher potassium chloride requirement (130 mM) was reported for a system using rabbit reticulocyte lysate and the IGR-IRES of cricket paralysis virus (CrPV) (*29*). In reticulocyte lysate, we also found that the IGR-IRES of PSIV showed a potassium chloride requirement similar to that of CrPV (data not shown).

Production of Non-Methionine Protein Using the Improved WGE System—Because the efficiency of IGR-IRES–mediated translation has been shown to be improved by a lower GC content in the 5′ part of the AUG-deleted EGFP coding sequence (*25*), we introduced silent mutations that reduced the GC content in the 5′ region of the Fluc and Rluc coding sequences. These mutations resulted in a 6.2- and 1.3-fold increase in luciferase activities for Fluc and Rluc, respectively (Fig. 3A). These results show that the introduction of silent mutations that decrease the GC content in the 5′ part of an exogenous coding sequence is a potential method by which to improve the efficiency of translation mediated by the IGR-IRES.

We next tested optimal RNA concentrations in the improved WGE system. Fluc and Rluc activities increased in the reaction mixtures with increasing amounts of uncapped IRES-ΔaugFluc(mut) and IRES-ΔaugRluc(mut) RNAs, respectively (Fig. 3B). In contrast, in reaction mixtures containing capped RNAs, Fluc and Rluc activities showed a maximum at 160 and 320 pmol/ml, respectively. For the Fluc coding sequence, the IGR-IRES was able to produce translation products as efficiently as a cap-dependent canonical ribosome scanning mechanism at higher RNA concentrations. For the Rluc coding sequence, however, the efficiency of IGR-IRES–mediated translation was about one-fifth of that of the scanning mechanism.

When IRES-ΔaugRluc(mut) and IRES-ΔaugFluc(mut) RNAs were translated by a dialysis membrane–based system, proteins with the expected molecular masses were observed by Coomassie Brilliant Blue staining on a polyacrylamide gel (Fig. 3C). Quantification of the translation products from the RNAs showed that 78 μg/ml of Fluc and 88 μg/ml of Rluc, respectively, were produced in the reaction mixture.

DISCUSSION

Insertion of a dicistroviral IGR-IRES sequence immediately upstream of the coding sequence achieves cell-free synthesis of proteins without an N-terminal methionine. Several methods have been reported to obtain proteins that do not have an N-terminal methionine, including (i) cleavage of N-terminal methionine by a chemical transamination reaction and phenylene-1,2-diamine treatment (*6*); (ii) generation of mutated initiator tRNAs that have anticodon triplets corresponding to codons other than AUG and that accept amino acids corresponding to the mutated anticodon triplets (*7*); (iii) modification of methionine aminopeptidases to permit larger penultimate amino acids for cleavage (*8*); and cleavage of the leader peptide sequence in a fusion protein by proteases such as (iv) a picornaviral 2A protease (*9*), (v) inteins (*10*), and (vi) deubiquitylating enzymes (*11*). Methods (i) and (ii) are complex because they require skilled techniques for organic chemistry and for producing a mutated initiator tRNA. Furthermore, in methods (iii), (iv), and (v), there are restrictions on the available N-terminal amino acid that can be used because proteolytic cleavage does not occur when unfavorable residues are located in cleavage sites. Although method (vi) would be suitable for high-throughput analysis, most deubiquitylating enzymes do not cleave proline at the N-terminus. In contrast to these methods, IGR-IRES–mediated production does not limit the initiating amino acid (*25*) and does not require additional experimental procedures.

Previous replacement of the viral capsid coding sequence with exogenous coding sequences showed that the translation efficiencies of constructs with these exogenous sequences were less than 10% of the construct containing the viral coding sequence (*25*). We consider that this deficit may be accounted for by the requirement for RNA folding in IGR-IRES–mediated translation. The exogenous sequences interact with the IRES sequence, and thus the amount of translation product decreases because part of the tertiary structure of the IRES is distorted by these interactions (*25*). The normal function of the IGR-IRES of dicistroviruses is to translate viral capsid proteins. Because capsid proteins of dicistroviruses are one of the most abundantly produced viral components in infected cells (*30*), we assume that the nucleotide sequence comprising the IRES has evolved to produce capsid proteins, in other words, the nucleotide sequences of native IRES elements are optimized for their own viral coding sequences. A few nucleotides in the IGR-IRES that form loop sequences in domains 2a and 2b are highly conserved among dicistroviruses (*22*), indicating that such nucleotides are essential for IRES function. Most of the other nucleotides in helical regions, however, are not conserved. This implies that it may be possible to create an IGR-IRES sequence for the effective translation of a specific coding sequence. For example, where the efficiency of IGR-IRES–mediated translation was very low in comparison with that of cap-dependent translation, as observed in for ΔaugRluc(mut) RNA (Fig. 3B), in vitro evolution methods (*31*, *32*) or sequence replacement of helical regions in the IGR-IRES may be used to improve translation efficiency.

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REFERENCES

1. Kozak, M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187–208
2. Hershey, J.W.B. and Merrick, W.C. (2000) Translational Control of Gene Expression (Sonenberg, N., Hershey, J.W.B. and Mathews, M.B., eds.) pp. 33–88, Cold Spring Harbor Laboratory Press, New York
3. Endo, S., Yamamoto, Y., Sugawara, T., Nishimura, O., and Fujino, M. (2001) The additional methionine residue at the N-terminus of bacterially expressed human interleukin-2 affects the interaction between the N- and C- termini. *Biochemistry* **40**, 914–919
4. Märki, F. and Hanulak, V. (1993) Recombinant human synobial fluid phospholipase A_2 and N-terminal variant: kinetic parameters and response to inhibitors. *J. Biochem.* **113**, 734–737
5. Mine, S., Ueda, T., Hashimoto, Y., and Imoto, T. (1997) Improvement of the refolding yield and solubility of hen egg-white lysozyme by altering the Met residue attached to its N-terminus to Ser. *Protein Eng.* **10**, 1333–1338
6. Nishimura, O., Suenaga, M., Ohmae, H., Tsuji, S., and Fujino, M. (1998) An efficient chemical method for removing N-terminal extra methionine from recombinant methionylated human growth hormone. *Chem. Commun.* 1135–1136
7. Mayer, C., Köhrer, C., Kenny, E., Prusko, C., and RajBhandary, U.L. (2003) Anticodon sequence mutants of *Escherichia coli* initiator tRNA: effects of overproduction of aminoacyl-tRNA synthetases, methionyl-tRNA formyltransferase, and initiation factor 2 on activity in initiation. *Biochemistry* **42**, 4787–4799
8. Liao Y., Jeng, J., Wang, C., Wang, S., and Chang, S. (2004) Removal of N-terminal methionine from recombinant proteins by engineered *E. coli* methionine aminopeptidase. *Protein Sci.* **13**, 1802–1810
9. de Felipe, P., Hughes, L.E., Ryan, M.D., and Brown, J.D. (2003) Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. *J. Biol. Chem.* **278**, 11441–11448
10. Wood, D.W., Wu, W., Belfort, G., Derbyshire, V., and Belfort, M (1999) A genetic system yields self-cleaving inteins for bioseparations. *Nat. Biotech.* **17**, 889–892
11. Catanzariti, A.M., Soboleva, T.A., Jans, D.A., Board, P.G., and Baker, R.T. (2004) An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Protein Sci.* **13**, 1331–1339
12. Mayo, M.A. (2002) A summary of taxonomic changes recently approved by ICTV. *Arch. Virol.* **147**, 1655–1656
13. Sasaki, J. and Nakashima, N. (1999) Translation initiation at the CUU codon is mediated by the internal ribosome entry site of an insect picorna-like virus in vitro. *J. Virol.* **73**, 1219–1226
14. Domier, L.L., McCoppin, N.K., and D′Arcy, C.J. (2000) Sequence requirements for translation initiation of *Rhopalosiphum padi* virus ORF2. *Virology* **268**, 264–271
15. Wilson, J.E., Powell, M.J., Hoover, S.E., and Sarnow, P. (2000) Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. *Mol. Cell. Biol.* **20**, 4990–4999
16. Hatakeyama, Y., Shibuya, N., Nishiyama, T., and Nakashima, N. (2004) Structural variant of the intergenic internal ribosome entry site elements in dicistroviruses and computational search for their counterparts. *RNA* **10**, 779–786
17. Hellen, C.U.T. and Sarnow, P. (2001) Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev.* **15**, 1593–1612
18. Kanamori, Y. and Nakashima, N. (2001) A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation. *RNA* **7**, 266–274
19. Sasaki, J. and Nakashima, N. (2000) Methionine-independent initiation of translation in the capsid protein of an insect RNA virus. *Proc. Natl Acad. Sci. USA* **97**, 1512–1515
20. Wilson, J.E., Pestova, T.V., Hellen, C.U.T., and Sarnow, P. (2000) Initiation of protein synthesis from the A site of the ribosome. *Cell* **102**, 511–520
21. Jan, E. and Sarnow, P. (2002) Factorless ribosome assembly on the internal ribosome entry site of cricket paralysis virus. *J. Mol. Biol.* **324**, 889–902
22. Nishiyama, T., Yamamoto, H., Shibuya, N., Hatakeyama, Y., Hachimori, A., Uchiumi, T., and Nakashima, N. (2003) Structural elements in the internal ribosome entry site of *Plautia stali intestine virus* responsible for binding with ribosomes. *Nucleic Acids Res.* **31**, 2434–2442
23. Pestova, T.V. and Hellen, C.U.T. (2003) Translation elongation after assembly of ribosomes on the cricket paralysis virus internal ribosome entry site without initiation factors or initiator tRNA. *Genes Dev.* **17**, 181–186
24. Jan, E., Kinzy, T.G., and Sarnow, P. (2003) Divergent tRNA-like element supports initiation, elongation, and termination of protein biosynthesis. *Proc. Natl Acad. Sci. USA* **100**, 15410–15415
25. Shibuya, N., Nishiyama, T., Kanamori, Y., Saito, H., and Nakashima, N. (2003) Conditional rather than absolute requirements of the capsid coding sequence for initiation of methionine-independent translation in *Plautia stali* intestine virus. *J. Virol.* **77**, 12002–12010
26. Borman, A.M., Bailly, J.L., Girard, M., and Kean, K.M. (1995) Picornavirus internal ribosome entry segments: comparison of translation efficiency and the requirements for optimal internal initiation of translation *in vitro*. *Nucleic Acids Res.* **23**, 3656–3663
27. Jackson, R.J. (1991) Potassium salts influence the fidelity of mRNA translation initiation in rabbit reticulocyte lysates: unique features of encephalomyocarditis virus RNA translation. *Biochim. Biophys. Acta* **26**, 345–358
28. Madin, K., Sawasaki, T., Ogasawara, T., and Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl Acad. Sci. USA* **97**, 559–564
29. Pöyry, T.A.A., Kaminski, A., and Jackson, R.J. (2004) What determines whether mammalian ribosomes resume scanning after translation of a short upstream open reading frame? *Genes Dev.* **18**, 62–75
30. Moore, N.F., Reavy, B., Pullin, J.S.K., and Plus, N. (1981) The proteins expressed by different isolates of Drosophila C virus. *Virology* **112**, 411–416
31. Nemoto, N., Miyamoto-Sato, E., Husimi, Y., and Yanagawa, H. (1997) In vitro virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. *FEBS Lett.* **414**, 405–408
32. Xu, H., Petersen, E.I., Petersen, S.B., and El-Gewely, M.R. (1999) Random mutagenesis libraries: optimization and simplification by PCR. *Biotechniques* **27**, 1102–1108