Identification of an Essential Region for Internal Initiation of Translation in the Aphthovirus Internal Ribosome Entry Site and Implications for Viral Evolution

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Translation of aphthovirus RNA is initiated at an internal ribosome entry site (IRES) element, preceding the first functional AUG initiation codon. The effect of mutations at the base of domain 3 of the aphthovirus IRES on translation activity has been analyzed by site-directed mutagenesis and expression of bicistronic RNAs in transfected cells. The results have shown that the enhanced IRES activity associated with a single pyrimidine transition fixed in a persistent aphthovirus variant (E. Martínez-Salas, J. C. Sáiz, M. Dávila, G. J. Belsham, and E. Domingo, *J. Virol.* 67:3748–3755, 1993) is base specific. Mutations predicted to destabilize the base of domain 3 were detrimental to IRES function, but subsequent restoration of the RNA structure gave rise to fully competent IRES. In contrast, single or multiple mutations that did not affect predicted helical structures modified the relative efficiency of translation by at most 10-fold, suggesting that primary sequence also plays a role in IRES activity. A correlation between the energy of stabilization of the IRES structure and the efficiency of translation has been noted. None of the 15 mutations studied reached a level of initiation of translation comparable to that of the IRES from the persistent variant. The results indicate a critical participation of the base of domain 3 in the activity of the aphthovirus IRES, with a strong effect of secondary or higher-order structures and minor effects of primary structure.

The long 5' untranslated region of picornavirus RNAs contains a sequence of 400 to 500 nucleotides that directs internal initiation of translation (32). This region, termed the internal ribosome entry site (IRES), is predicted to form extensive secondary structures and contains several unused AUG codons (reviewed in references 18, 20, and 43). On the basis of phylogenetic comparisons and biochemical probing of secondary structures, two types of picornavirus IRES have been defined. Type 1 includes IRES of enteroviruses and rhinoviruses, and type 2 includes those of aphthoviruses and cardioviruses. They differ in sequence, structure, and the location of conserved regions across the element (20). However, both IRES types include one pyrimidine tract located upstream of the initiator AUG within the protein synthesis starting window (18, 36). The secondary structure and probably also the tertiary structure (23) play a critical role in IRES activity. RNA-RNA interactions have been suggested on the basis of observed *trans* complementation between poliovirus and foot-and-mouth disease virus (FMDV) RNA IRES deletion mutants (9, 40). In addition, RNA-protein interactions are involved in the mechanism of cap-independent internal initiation of translation (7, 17, 28, 44) and, in FMDV, p57/pTB (polypyrimidine tract-binding protein) and eukaryotic initiation factor 4B (eIF-4B) form a complex involving several IRES stem-loop structures (24, 29).

Most mutations in the IRES, either from natural variants or produced by site-directed mutagenesis, either were neutral or led to an impairment of translation initiation (reviewed in reference 43). For example, of a total of 27 reported mutations

in the polypyrimidine tract of several picornaviruses, 8 were neutral and 19 were detrimental (21, 22, 31, 34, 37). A similar negative effect was observed with most mutations affecting predicted domain structures (15, 31). Only in a few cases did nucleotide substitutions or insertions result in an increase of initiation of translation (13, 14). One such case was observed in an FMDV variant isolated after prolonged persistence in BHK-21 cells (26). Persistence of FMDV (clone C-S8c1) in cell culture is characterized by a coevolution of the host cells and the resident virus whereby the cells become increasingly resistant to the virus and the virus acquires a hypervirulent phenotype for the parental BHK-21 cells (5, 6). Hypervirulent variant

TABLE 1. Primers used in the site-directed mutagenesis

Primer ^a	Sequence (5'–3') ^b	Template	Position ^c
NR4	CACGAGCTCAGCAGGTTTC		–470
ND6	CCGAGCTCAGGGTCATTAATTG		–1
ND9	GAGCCAAATGCAGTACAAAGTG	pBIR	–366
ND10	GAGCCAAATGTAGTACAAAGTG	pBIR	–366
ND11	CCAAAGGCAGTACAAAGTGTACC	pBIR	–363
ND12	CCAAAGGAANTACAAAGTGTACC	pBIR	–363
ND13	GGAGCCAAACGCAGTACAAAG	pBIC	–367
ND14	GTCACCAACATTTGGGTACCAG	pBIR	–156
ND15	GTCACCAACATTTGGGTACCAG	pND12	–156
ND16	GGAGCCAAACTCAGTACAAAG	pBIR	–367
ND17	GGAGCCAAACCCAGTACAAAG	pBIR	–367
ND18	GTCACCAGCGTGTGGGTACCAG	pBIR	–156

^a Oligonucleotides were synthesized by Isogen Bioscience bv, Amsterdam, The Netherlands.

^b Boldface letters indicate nucleotides not present in the FMDV sequence, added to create a *SacI* restriction site (underlined). N indicates any nucleotide at the corresponding position. The sequences of the different mutant IRES obtained are given in Fig. 1A.

^c Nucleotide position at the 5' end of the primer relative to the initiator AUG in the FMDV C-S8c1 IRES sequence (10), where A is given as position +1.

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FIG. 1. (A) Diagram of the plasmids used in this work. The name of the plasmids and their relevant features are indicated. Symbols: $\overrightarrow{\text{---}}$, transcription start sites; T7, Tk, CAT, luciferase, promoters and marker genes used in the constructs (26). The box between CAT and luciferase depicts the IRES element; letters within the box indicate the nucleotides which differ between C-S8c1 and the mutants tested. Numbers indicate relevant positions within the IRES. Only the differences between each mutant (plasmid series pND) and the C-S8c1 IRES (pBIC) are indicated. The steps followed in the construction of each plasmid are detailed in Materials and Methods. (B) Scheme of the predicted secondary structure (M-Fold program) of the IRES of FMDV C-S8c1, with the location (thick tracing) of the mutations which distinguish the IRES from C-S8c1 and from mutants described in this work. Domain numbering is that proposed by Pilipenko et al. (35).

R100 included two point mutations in the IRES element, a U-to-C transition at position -376 and an A-to-G transition at position -15 (counting as +1 the adenosine of the first functional AUG [see Fig. 1]). Expression studies with bicistronic constructs indicated that the pyrimidine transition at position -376, which affected the base of domain 3 (see Fig. 1), was responsible for a 1.5- to 5-fold increase in the IRES activity of FMDV R100 relative to its parental FMDV C-S8c1 element (26). Given the rarity of naturally occurring mutations which result in enhanced initiation of translation and previous evidence that domain 3 is essential for IRES activity of FMDV (22), it was of interest to explore the effect of additional substitutions at the base of domain 3 of the IRES of FMDV C-S8c1 with two main objectives: first, to explore the possible role of the primary sequence and of the stability of this domain in initiation of translation, and second, to assess the tolerance to nucleotide substitutions of a regulatory domain of FMDV RNA. Here we report results of a site-directed mutagenesis analysis of the aphthovirus IRES.

MATERIALS AND METHODS

Plasmid constructions. The IRES region of bicistronic constructs pBIC and pBIR (26) was subjected to site-specific mutagenesis by PCR amplification basically as described by Perrin and Gilliland (33) with minor modifications. Briefly, 1 to 10 pg of linearized template was PCR amplified with the desired mutagenic oligonucleotide and primer NR4 (Table 1) in PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 μ M each deoxynucleotide). Either equal molar amounts of primers or a 1:100 molar ratio was used in the PCR and subjected to 30 cycles of 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min with 2.5 U of AmpliTaq (Cetus). The PCR product was diluted in 2 ml of distilled water and transferred to a Centricon 30 microconcentrator (Amicon) to remove excess primers prior to its use in a second PCR as the 5'-flanking primer, with an equal molar amount of primer ND6 as the partner in the reaction. The annealing temperature in the second PCR was increased to 55°C, and no new addition of template was required. However, when the first PCR was carried out with equal molar amounts of primers, linearized plasmid template was added at a 1:30 molar ratio to the megaprimer molecule. The products of the second PCR were digested with *Sac*I, purified by agarose gel electrophoresis, and ligated to the large *Sac*I fragment of pBIR, upstream of the luciferase gene, to produce the desired constructs (given the same name as the corresponding mutagenic oligonucleotide described in Table 1 with the prefix p). In some cases, the incorporation of the mutagenic nucleotide was screened by differential hybridization with ³²P-labeled oligonucleotide (39) at two temperatures, one permissive for all

inserted molecules and one restrictive for nonmutagenized molecules. The orientation of the mutant IRES elements was determined by *NcoI-HindIII* digestion. Prior to expression analysis, the nucleotide sequence of the entire length of each IRES under study was obtained by primer extension and dideoxy chain termination with T7 DNA polymerase. Chloramphenicol acetyltransferase (CAT) and luciferase expression from bicistronic mRNAs transcribed either from the T7 or the Tk promoter (see Fig. 1A) was quantitated as a relative measure of IRES activity (26).

Transient-expression assays. The cell lines used have been described previously (26). When transcription from the T7 promoter was desired, BHK-21 or BSC40 monolayers were infected with the vaccinia virus recombinant vTF7-3 (11) 1 h before transfection with the relevant plasmids. Lipofectin (Bethesda Research Laboratories)-mediated transfection was carried out as specified by the supplier. Usually, 0.25 μ g of the appropriate plasmid DNA(s) was used to transfect monolayers of about 2×10^5 cells. Fetal calf serum (5%) was added to the monolayer 5 to 6 h later. Cell extracts were prepared 18 and 24 h posttransfection in 200 μ l of 0.5% Nonidet P40–120 mM NaCl–50 mM Tris-HCl (pH 7.8). Experiments were performed on triplicate plates, and each experiment was repeated at least four times.

Luciferase assays were carried out as described by Martínez-Salas et al. (25), with a model 2010 luminometer (Analytical Luminescence). CAT activity was determined as described previously (30), except that the reaction was adjusted to 0.08 M sodium borate (pH 9) and 4 M NaCl at the end of the incubation period to decrease the background. Appropriate dilutions of the extracts were chosen to be in the linear range of the assay. The results are presented as luciferase activity relative to CAT activity, measured in the same extract. In the experiment measuring expression from the Tk promoter, the enzyme activities were further normalized to the amount of protein measured in the cell extracts by the Lowry method.

RESULTS

Specificity of the point mutation which causes an increase in IRES activity. A U-to-C transition at position –376 of the IRES of FMDV C-S8c1 is responsible for the increased internal initiation of translation in the persistent variant R100 (26). To test whether the enhancement of IRES activity was base specific, a series of mutants with substitutions at the base of domain 3 of the aphthovirus IRES were generated by site-directed mutagenesis (Fig. 1). Most of the mutants were derived from pBIR (Table 1), the plasmid with the IRES element of the persistent FMDV R100. Mutant pND13-1 was constructed from pBIC to reproduce by site-directed mutagenesis the pyrimidine transition at position –376, which is also present in pBIR (26). Comparison of CAT and luciferase activities expressed from the bicistronic constructs corresponding to mutants pND13-1, pND16-1, pND17-6, pBIC, and pBIR indicates that only the U-to-C transition, not U-to-A or U-to-G transversions, at position –376 conferred increased IRES activity (Fig. 2A). The presence of additional point substitutions at position –67 (mutant pND16-2) or –268 (mutant pND17-4) had no significant effect (compare Fig. 1 and 2). The specificity of the pyrimidine transition was seen in both BHK-21 and BSC40 cells, although the effect was more pronounced in the latter, as previously noted in the comparison between pBIC and pBIR (26). No difference in relative activity was observed when expression was directed by the Tk promoter or by the T7 promoter in concert with recombinant vaccinia virus infection (Fig. 2B). Expression from the T7 promoter is about 10^3 -fold higher than from the Tk promoter (26). Thus, the change at position –376 leading to enhanced IRES activity in FMDV R100 is base specific.

Destabilization of the base of domain 3 leads to defective IRES. To test whether destabilization of the base of domain 3 could affect IRES activity, the series of mutants pND9-3, pND11-4, pND10-3, and pND12-5 were constructed (Fig. 1A). In these mutants, the base of domain 3 is expected to be progressively open, because of two or three noncanonical or unfavorable C-C, C-A, and C-U pairings (Fig. 3A). The IRES activity of these mutants was severely impaired relative to that of pBIC both in BHK-21 and in BSC40 cells (Fig. 3B). The adverse effect on translation efficiency seems to correlate with

FIG. 2. Relative efficiency of translation of luciferase by FMDV IRES point mutations at position –376. (A) The indicated plasmids (constructs depicted in Fig. 1) were transfected into BHK-21 cells previously infected with vTF7-3. Cell extracts were prepared 18 and 24 h after serum addition, as detailed in Materials and Methods. The luciferase activity produced by 1×10^4 to 2×10^4 transfected cells was normalized to the CAT activity present in the same extract. (B) Luciferase activity produced in BSC40 cells from either the T7 promoter (black rectangles) or the Tk promoter (striped rectangles). Assays were carried out as described in Materials and Methods. Error bars correspond to the standard error of the mean. “Relative efficiency” refers to the activity produced by pBIC.

perturbation of secondary structure rather than with primary sequence. In particular, the presence of a G-U pair between residues –374 and –163 in mutant pND14-7 resulted in a translation efficiency comparable to that of pBIC (Fig. 3B). Furthermore, very low IRES activity, which means at least a 3-log-unit decrease in activity relative to pBIC, was observed with mutant pND12-1. This mutant, in addition to maintaining the substitutions of pND12-5 at the base of domain 3, includes a C-to-G transversion at position –373 (Fig. 3). This transversion is expected to affect the stability of the base of domain 2 (compare Fig. 1B and 3), which also contributes to IRES function (22). Thus, the aphthovirus IRES region at the base of

FIG. 3. (A) Predicted secondary structure at the base of domain 3 of mutant IRES, obtained with the M-Fold program. Outlined symbols depict mutated nucleotides relative to pBIC. (B) Analysis of the effect of point mutations in the FMDV IRES on the efficiency of translation of bicistronic mRNAs in BHK-21 and BSC40 cells. vTF7-3-infected cells transfected with the plasmids indicated (depicted in Fig. 1) were processed as described in the legend to Fig. 2.

domain 3 is critical for function and very intolerant to substitutions which tend to perturb the structure of this domain.

Neutral mutants maintain the predicted structure. A series of mutations designed to restore the predicted structure at the base of domain 3 in defective mutants led to a functional IRES (Fig. 4). Relative to the activity of pBIC, pND14-8, pND14-11, pND15-10, pND15-4, and pND18-5 showed no significant or very modest differences in activity. The comparisons emphasize again the contribution of the secondary structure of this region to IRES activity. In particular, mutant pND12-5 showed a 100-fold-lower activity than its derivatives pND15-4 or pND15-10 (compare Fig. 3 and 4); both mutants had the predicted helical regions at the base of domain 3. Mutants pND15-4 and pND15-10 differed in seven and six mutations, respectively, from pBIC, with no significant consequence with regard to their ability to initiate translation. None of the mutants tested evidenced any increase in IRES activity over the

activity displayed by pBIR, the construct which includes the IRES sequence found in persistent FMDV R100.

A correlation between predicted IRES stability and translation efficiency. The entire IRES RNA (nucleotides -458 to -1) was folded by using the M-fold program of the Genetics Computer Group package (45). The energy of stabilization of the entire IRES of C-S8c1 (in pBIC) was calculated by using the program M-fold. The value obtained was $\Delta G = -1,147.8$ kcal mol⁻¹ ($-4,802.4$ kJ mol⁻¹). Then the same calculation was carried out for each of the mutants studied. Values ranged from $\Delta G = -1,040.4$ kcal mol⁻¹ ($-4,353.0$ kJ mol⁻¹) to $-1,202.7$ kcal mol⁻¹ ($-5,032.1$ kJ mol⁻¹). The difference between the ΔG of each mutant and that of pBIC, divided by the ΔG of pBIC, was then plotted against the relative translation efficiency (Fig. 5A). In all the mutants, the base of domain 3 was the only part of the IRES affected in terms of predicted secondary structure, except for pND12-1, pND17-4, and

FIG. 4. (A) Predicted secondary structure at the base of domain 3 of mutant IRES, according to the M-Fold program. Outlined symbols depict mutated nucleotides relative to pBIC. (B) Analysis of the effect of mutations on the efficiency of translation of bicistronic mRNAs. BHK-21 and BSC40 cells, previously infected with vTF7-3, were transfected with the indicated plasmids (Fig. 1). Cell extracts were processed as described in the legend to Fig. 2.

pND16-2, in which additional changes were observed, as indicated above. The results show three main groups of sequences corresponding to different levels of IRES activity. Decreased stability of the base of domain 3 was clearly associated with low activity. Within each group, however, up to 10-fold differences, which could be partly assigned to the cell type (the values in BSC40 cells are systematically slightly larger than in BHK-21 cells [Fig. 5A]) or to the nucleotide sequence of each particular mutant (Fig. 5), were observed. The comparison emphasizes that translation efficiency forms a plateau at relative energy increments above zero (Fig. 5A). The translation efficiencies of FMDV C-S8c1 or R100 are associated with domain 3 sequences which are close to the maximum translation efficiency, at least among the range of substitutions analyzed.

DISCUSSION

The results of site-directed mutagenesis have shown that the region around nucleotide -376 of the aphthovirus IRES, located at the predicted base of domain 3 (Fig. 1B), plays a critical role in internal initiation of translation. A strong decrease in IRES activity was seen in mutants with nucleotide substitutions predicted to destabilize the helical region at the base of domain 3 (Fig. 3). The need for a helical structure at this domain is supported by a strong correlation between the efficiency of translation and the energy of stabilization predicted for the corresponding mutant IRES (Fig. 5). Restoration of a helical structure resulted in recovery of IRES activity; thus, the decrease in activity of 100-fold seen in mutant pND12-5 relative to pBIC was converted to a one- to twofold increase in pND15-10 (Fig. 4).

Additional, albeit less pronounced, effects were also noted among mutants which maintained a similar predicted structure but differed in nucleotide sequence (Fig. 3, compare mutants pND11-4 and pND14-7). In particular, the increase in IRES activity in the persistent FMDV variant R100 could be achieved only by the U-to-C substitution but not by the U-to-A or U-to-G substitution at residue -376 (Fig. 2), in spite of the very similar effects of the three substitutions on predicted stability (Fig. 5B). Enhanced functional activities associated with noncanonical Watson-Crick base pairs have been documented in other biological systems (1, 3, 12, 38). Within any of the energy levels, differences in translation efficiency have been observed (Fig. 5A); these must presumably arise from effects of the primary structure. These comparisons suggest very subtle effects of position -376 on IRES activity. However, in the absence of knowledge about the tertiary structure of IRES elements, it is difficult to define in molecular terms the structural perturbations triggered by single or multiple mutations and their possible effect on RNA-RNA and RNA-protein interactions (2, 14, 17, 42). Furthermore, synergy between loops for binding of cell factors has been recently documented for the poliovirus IRES (16). Such a synergy could contribute to the very low IRES activity of mutant pND12-1, a variant with a potentially important alteration at the base of domain 2 in addition to domain 3 (Fig. 3).

The comparative results presented here for 17 variant sequences suggest a very limited tolerance of the IRES region analyzed to accept point mutations that disrupt the predicted structure and remain functional. A high proportion of deleterious mutations have recently been reported for the helical J-K

FIG. 5. (A) Relative efficiency of cap-independent translation as a function of the relative increment of free energy of each mutant IRES. Mean values of luciferase activity were corrected for CAT activity and are given relative to pBIC for each experiment. The value of energy of the optimal entire IRES structure ($\Delta G_{37^\circ\text{C}}$ in kilocalories per mole) produced by the M-fold program was used to calculate the relative increment of energy as the difference between the energy of each mutant RNA and that of the control pBIC divided by the energy of pBIC as detailed in the text. (B) Influence of the energy increment associated with each of the four possible nucleotide substitutions at position -376 on the relative translation efficiency of the corresponding IRES.

junction of the encephalomyocarditis virus IRES (19). Previous studies with poliovirus revertants selected *in vivo* suggested that extensive sequence rearrangements can lead to restoration of IRES function (14, 15, 37). Point mutations and block rearrangements may occur at high frequency during RNA virus replication, but they will not be noticeable in progeny RNA unless the parental genome shows an important selective disadvantage relative to the rearranged genome (8). A second important consideration in the mutations observed is viral population size. The substitution at position -376 fixed in this region in the persistent FMDV R100 was probably one of the

very few compatible with high IRES activity. If such a single mutation occurred at the expected frequency of about 10^{-4} , its fixation would be compatible with the viral population size involved in persistence, which was variable but ranged from 10^4 to 10^6 PFU per cell passage (4). In contrast, double or quadruple substitutions, the minimum number needed to restore a functional domain (Fig. 4), are expected to occur at frequencies of 10^{-8} and as low as 10^{-16} , respectively, which are too low for these types of variants to be present and become dominant during persistent infections of FMDV in cell culture. The results of site-directed mutagenesis of the IRES reported here extend to a regulatory region the evidence for strong limitations of variation previously documented for the capsid-coding region of FMDV (27, 41). Such limitations must operate in both coding and noncoding regions of the picornavirus genomes, with many mutations leading to profound losses of fitness (8, 43). However, the results also illustrate the high connectivity of sequence space with respect to fitness gain. A single compensatory mutation is often sufficient to rescue a viral genome from near defectiveness to full functionality.

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REFERENCES

- Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak. 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* **67**:529-536.
- Borovjagin, A. V., M. V. Ezrokhi, V. M. Rostapshov, T. Y. Ugarova, T. F. Bystrova, and I. N. Shatsky. 1990. RNA-protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA. *Nucleic Acids Res.* **19**:4999-5005.
- Costa, M., and F. Michel. 1995. Frequent use of the same tertiary motif by self-folding RNAs. *EMBO J.* **14**:1276-1285.
- de la Torre, J. C., M. Dávila, F. Sobrino, J. Ortín, and E. Domingo. 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. *Virology* **145**:24-35.
- de la Torre, J. C., E. Martínez-Salas, J. Díez, A. Villaverde, F. Sobrino, E. Rocha, M. Dávila, and E. Domingo. 1988. Coevolution of cells and viruses in a persistent infection of foot-and-mouth disease virus in cell culture. *J. Virol.* **62**:2050-2058.
- Díez, J., M. Hofner, E. Domingo, and A. I. Donaldson. 1991. Foot-and-mouth disease virus strains isolated from persistently infected cell cultures are attenuated for mice and cattle. *Virus Res.* **18**:3-8.
- Dildine, S. L., and B. L. Semler. 1992. Conservation of RNA-protein interactions among picornaviruses. *J. Virol.* **66**:4364-4376.
- Domingo, E., and J. J. Holland. 1994. Mutation rates and rapid evolution of RNA viruses, p. 161-184. *In* S. S. Morse (ed.), *Evolutionary biology of viruses*. Raven Press, New York.
- Drew, J., and G. J. Belsham. 1994. *trans* complementation by RNA of defective foot-and-mouth disease virus internal ribosome entry site elements. *J. Virol.* **68**:697-703.
- Escarmís, C., M. Toja, M. Medina, and E. Domingo. 1992. Modifications of the 5' untranslated region of foot-and-mouth disease virus after prolonged persistence in cell culture. *Virus Res.* **26**:113-125.
- Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122-8126.
- Giver, L., D. Bartel, M. Zapp, A. Pawul, M. Green, and A. D. Ellington. 1993. Selective optimization of the Rev-binding element of HIV-1. *Nucleic Acids Res.* **21**:5509-5516.
- Glass, M. J., X. Jia, and D. F. Summers. 1993. Identification of the hepatitis A virus internal ribosome entry site: *in vivo* and *in vitro* analysis of bicistronic RNAs containing the HAV 5' noncoding region. *Virology* **193**:842-852.
- Gmyl, A. P., E. V. Pilipenko, S. V. Maslova, G. A. Belov, and V. I. Agol. 1993. Functional and genetic plasticities of the poliovirus genome: quasi-infectious RNAs modified in the 5'-untranslated region yield a variety of pseudorevertants. *J. Virol.* **67**:6309-6316.
- Haller, A. A., and B. L. Semler. 1992. Linker-scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. *J. Virol.* **66**:5075-5086.
- Haller, A. A., and B. L. Semler. 1995. Stem-loop structure synergy in binding cellular proteins to the noncoding region of poliovirus RNA. *Virology* **206**:923-934.

17. Hellen, C. U. T., T. V. Pestova, M. Litterst, and E. Wimmer. 1994. The cellular polypeptide p57 (pyrimidine tract-binding protein) binds to multiple sites in the poliovirus 5' nontranslated region. *J. Virol.* **68**:941–950.
18. Hellen, C. U. T., and E. Wimmer. 1995. Enterovirus genetics, p. 25–72. *In* H. A. Rotbart (ed.), *Human enterovirus infections*. ASM Press, Washington, D.C.
19. Hoffman, M. A., and A. Palmenberg. 1995. Mutational analysis of the J-K stem-loop region of the encephalomyocarditis virus IRES. *J. Virol.* **69**:4399–4406.
20. Jackson, R., M. T. Howell, and A. Kaminski. 1990. The novel mechanism of initiation of picornavirus RNA translation. *Trends Biochem. Sci.* **15**:477–483.
21. Kaminski, A., G. J. Belsham, and R. J. Jackson. 1994. Translation of encephalomyocarditis virus RNA: parameters influencing the selection of the internal initiation site. *EMBO J.* **13**:1673–1681.
22. Kuhn, R., N. Luz, and E. Beck. 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. *J. Virol.* **64**:4625–4631.
23. Le, S., J. Chen, N. Sonenberg, and J. V. Maizel. 1992. Conserved tertiary structure elements in the 5' untranslated region of human enteroviruses and rhinoviruses. *Virology* **191**:858–866.
24. Luz, N., and E. Beck. 1991. Interaction of a cellular 57-kilodalton protein with the internal translation initiation site of foot-and-mouth disease virus. *J. Virol.* **65**:6486–6494.
25. Martínez-Salas, E., E. Linney, J. Hassell, and M. L. DePamphilis. 1989. The need for enhancers in gene expression first appears during mouse development with formation of the zygotic nucleus. *Genes Dev.* **3**:1493–1506.
26. Martínez-Salas, E., J. C. Sáiz, M. Dávila, G. J. Belsham, and E. Domingo. 1993. A single nucleotide substitution in the internal ribosome entry site of foot-and-mouth disease virus leads to enhanced cap-independent translation in vivo. *J. Virol.* **67**:3748–3755.
27. Mateu, M. G., J. Hernández, M. A. Martínez, D. Feigelstock, S. Lea, J. J. Pérez, E. Giralt, S. Stuart, E. L. Palma, and E. Domingo. 1994. Antigenic heterogeneity of foot-and-mouth disease virus serotype in the field mediated by very limited sequence variation at several antigenic sites. *J. Virol.* **68**:1407–1417.
28. Meerovitch, K., Y. V. Svitkin, H. S. Lee, F. Lejbkowitz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. K. Keene, and N. Sonenberg. 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J. Virol.* **67**:3798–3807.
29. Meyer, K., A. Petersen, M. Niepman, and E. Beck. 1995. Interaction of eukaryotic initiation factor eIF-4B with a picornavirus internal translation initiation site. *J. Virol.* **69**:2819–2824.
30. Neumann, J. R., C. A. Morency, and K. O. Russian. 1987. A novel rapid assay for chloramphenicol acetyl transferase gene expression. *BioTechniques* **5**:444–447.
31. Nicholson, R., J. Pelletier, S. Le, and N. Sonenberg. 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. *J. Virol.* **65**:5886–5894.
32. Pelletier, J., and N. Sonenberg. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature (London)* **334**:320–325.
33. Perrin, S., and G. Gilliland. 1990. Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. *Nucleic Acids Res.* **18**:7433–7438.
34. Pestova, T. V., C. U. T. Hellen, and E. Wimmer. 1991. Translation of poliovirus RNA: role of an essential *cis*-acting oligopyrimidine element within the 5' nontranslated region and involvement of a cellular 57-kilodalton protein. *J. Virol.* **65**:6194–6204.
35. Pilipenko, E. V., V. M. Blinov, B. K. Chernov, T. M. Dimitrieva, and V. I. Agol. 1989. Conservation of the secondary structure elements of the 5'-untranslated region of cardio and aphthovirus RNAs. *Nucleic Acids Res.* **17**:5701–5711.
36. Pilipenko, E. V., A. P. Gmyl, S. V. Maslova, G. A. Belov, A. N. Sinyakov, M. Huang, T. D. K. Brown, and V. I. Agol. 1994. Starting window, a distinct element in the cap-independent internal initiation of translation on Picornaviridae RNA. *J. Mol. Biol.* **241**:398–414.
37. Pilipenko, E. V., A. P. Gmyl, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol. 1992. Prokaryotic-like *cis* elements in the cap-independent internal initiation of translation on picornavirus RNA. *Cell* **68**:119–131.
38. Pley, H. W., K. M. Flaherty, and D. B. McKay. 1994. Three-dimensional structure of a hammerhead ribozyme. *Nature (London)* **372**:68–74.
39. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. Stone, D. M., J. W. Almond, J. K. Brangwyn, and G. J. Belsham. 1993. *trans* complementation of cap-independent translation directed by poliovirus 5' noncoding region deletion mutants: evidence for RNA-RNA interactions. *J. Virol.* **67**:6215–6223.
41. Verdaguer, N., M. G. Mateu, D. Andreu, E. Giralt, E. Domingo, and I. Fita. 1995. Structure of the major antigenic loop of foot-and-mouth disease virus complexed with a neutralizing antibody: direct involvement of the Arg-Gly-Asp motif in the interaction. *EMBO J.* **14**:1690–1696.
42. Weeks, K. M., and D. M. Crothers. 1993. Major groove accessibility of RNA. *Science* **261**:1574–1577.
43. Wimmer, E., C. U. T. Hellen, and X. Cao. 1993. Genetics of poliovirus. *Annu. Rev. Genet.* **27**:353–436.
44. Witherell, G. W., and E. Wimmer. 1994. Encephalomyocarditis virus internal ribosome entry site RNA-protein interactions. *J. Virol.* **68**:3183–3192.
45. Zuker, M. 1989. On finding all suboptimal foldings of an RNA molecule. *Science* **244**:48–52.