JOURNAL OF VIROLOGY, July 1995, p. 4399–4406 Vol. 69, No. 7
0022-538X/95/$04.00+0

Mutational Analysis of the J-K Stem-Loop Region of the Encephalomyocarditis Virus IRES

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Received 9 January 1995/Accepted 12 April 1995

Cap-independent translation of encephalomyocarditis virus (EMCV) RNA is controlled by a segment of the 5′ untranslated region termed the internal ribosomal entry site, or IRES. The IRES contains a series of stem-loop structural elements. The J and K stems (EMCV bases 682 to 795), near the center of the IRES, are well conserved among all cardio-, aphtho-, and hepatoviruses. We have examined the biological roles of these elements by constructing mutations within the J-K sequences of EMCV and testing the mutations for activity in translation, translation competition, UV cross-linking, and viral infectivity assays. Mutations near the helical junction of J and K proved severely detrimental to both cellular translation and cell-free translation of downstream cistrons. The same mutations reduced the ability of the IRES to compete for cellular factors in competition assays and reduced the infectivity of viral genomes carrying these lesions. A mutation in the terminal loop of J gave similar results. In contrast, mutations within the terminal loop of K had minimal impact on in vitro translation activity and IRES competitive ability. However, in vivo analysis of the K-loop mutations revealed deficiencies during cellular translation and further showed markedly reduced infectivity in HeLa cells. UV cross-linking experiments identified a 49-kDa protein which interacts strongly with the J-K region, but the identity of this protein and its contribution to IRES activity are unclear.

Encephalomyocarditis virus (EMCV) is a member of the *Cardiovirus* genus of the positive-sense picornaviruses. As with other picornaviruses, efficient translation of EMCV RNA starts through a mechanism different from that used by most eukaryotic mRNAs. Protein synthesis from viral RNA begins after ribosomes bind in a cap-independent manner to an internal ribosomal entry site (IRES) within the 5′ untranslated region of the genome (2, 3, 16, 31). The IRES of EMCV is composed of a series of RNA structural elements, designated H to N (Fig. 1), which can be transferred as a unit to other eukaryotic cistrons (7). As part of an mRNA, the IRES confers to downstream cistrons the high efficiency of translation that is a hallmark of the parental EMCV genome (9, 16, 20).

The 5′ border of the EMCV IRES has been mapped by deletion analysis to a region near base 400 of the genome. This location corresponds to stem-loop H, about 430 bases 5′ to the polyprotein initiation site (7, 16). Some studies suggest that stem-loop H is not essential for IRES function because excision of H only modestly reduces protein synthesis (7). On the other hand, wild-type and mutated H elements which differentially cross-link to a 57-kDa cellular protein have binding efficiencies that correlate roughly to the translational activity of the IRES (4, 18). The interactive protein has been identified as the pyrimidine tract-binding protein (PTB), which was previously characterized because of its involvement in pre-mRNA processing (14). Although PTB is clearly involved in IRES function (41), it also binds to regions of the 5′ untranslated region other than H, and the significance of these interactions has not been clearly defined. Most likely, the H segment helps stabilize the overall structure of the IRES, aids in general protein binding, and thus facilitates translation initiation.

The 3′ borders of all picornavirus IRESs are characterized by a conserved sequence motif, Y*n*-X*m*-AUG, containing a polypyrimidine tract (Y*n*), a spacer element (X*m*, 14 to 22 bases), and an AUG codon (18, 19). This particular AUG initiates polyprotein translation in cardio-, aphtho-, and hepatoviruses, but for entero- and rhinoviruses initiation begins at the next AUG, 30 to 150 bases 3′ of the motif (15, 17). Motif integrity is critical for IRES function. Mutations of the Y*n* tract disrupt EMCV, foot-and-mouth disease virus, and poliovirus translation in vitro, while insertions within the X*m* region of poliovirus also adversely affect translation and reduce the plaque size of virus (18, 21, 24, 32). The Y*n*-X*m*-AUG region interacts with several cellular proteins. With poliovirus, the 52-kDa La autoantigen binds at or near this sequence (22, 25). The La protein has not been proven to be required for cardiovirus translation, however (40), and instead the Y*n*-X*m*-AUG of EMCV binds PTB (41). With foot-and-mouth disease virus RNA, which contains an IRES structure similar to that in EMCV, binding of a 57-kDa protein (believed to be PTB) to mutated Y*n* tracts also correlates with the translational efficiency of the IRES (23), suggesting a direct role for this binding in translation initiation.

Without a doubt, a completely functional IRES is achieved through concerted contributions from multiple structures and sequences within the described boundaries. Unfortunately, little is known about the roles of the stem-loop structures in the interior of an IRES. For EMCV, nucleotide substitutions and linker insertions within the lower helical segments of stem I (bases 451 to 678) have only minimum effects on translation, but mutations within the distal triad of I-terminal loops (bases 520 to 600) are strongly disruptive (7). Stems J and K (EMCV bases 682 to 785) are also important because mutations within J or the terminal loop of K (bases 746 to 750) or near the J-K helical junction (e.g., near base 777) reduce protein synthesis (7, 27, 39). Assignment of more detailed function to these segments has been difficult because of the overall size of the IRES and because of the tacit assumption that multiple IRES

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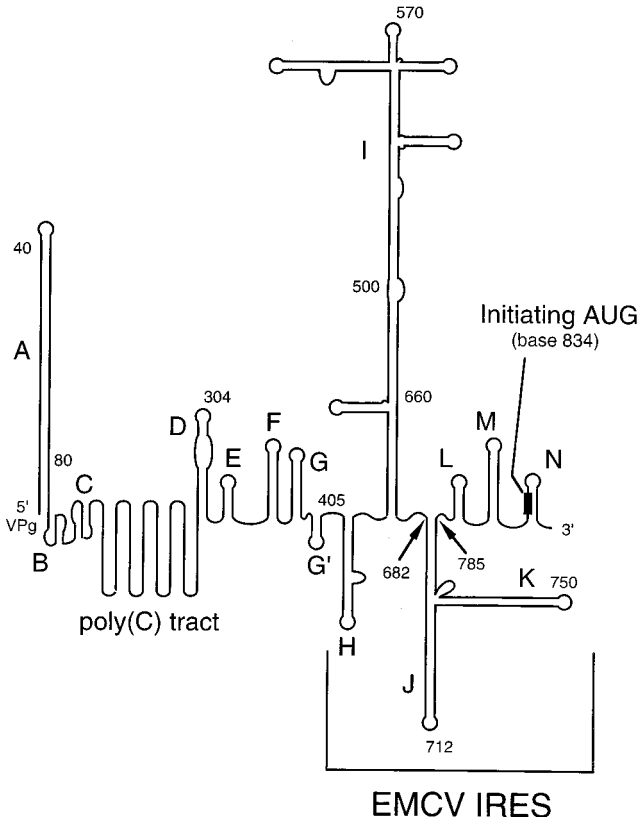


FIG. 1. 5′ UTR of EMCV-like cardioviruses. This schematic is derived from a structural model in reference 7.

elements probably act synergistically to bring about translation initiation.

Still, there are testable behaviors unique to some of these regions. In cell-free extracts, J-K-containing RNAs (bases 682 to 785) possess potent inhibitory effects on exogenous translating mRNAs (capped or uncapped) (7, 10, 39). We have proposed that this inhibition might be caused by J-K-dependent sequestering of cellular factors required for translation. Indeed, it also has been suggested that J-K may provide a binding site for complexes of eukaryotic initiation factors 2 or 2B (eIF-2/2B) (39). The activity hints at possible involvement of J-K sequences in a host-protein shutoff mechanism during natural EMCV infection as well as participation in IRES-directed translation (7). We have pursued these directions more fully and now describe a larger mutational study of the EMCV J-K elements. Using cellular and cell-free translation reactions, translational competition and protein cross-linking assays and infectious cDNA constructions, we provide new evidence that specific RNA regions within the J-K structure are critical to IRES function and viability of EMCV.

MATERIALS AND METHODS

Plasmid construction. Standard recombinant methods were used (1, 38). The sequences of EMCV-R (accession number M81861) and plasmid pEA1, containing EMCV bases 335 to 4229 inserted between the *Eco*RI and *Bam*HI sites of pBS+, have been described previously (7). Digestion of pEA1 with *Eco*RI followed by mung bean nuclease digestion and religation removed the vector's endogenous *Eco*RI site. Site-directed mutagenesis converted viral base 670 from a T (U in the RNA) to an A and created a new *Eco*RI site just upstream of the J-K region sequences (plasmid pEA1.1Eco). The *Eco*RI-*Bam*HI fragment from this plasmid (viral bases 667 to 4229) was then inserted into the multiple cloning site of pBS+ to create vector pEA1.667, convenient for the introduction of substitutions by primer-directed mutagenesis. The G701T mutation (G to T at viral base 701) and G716T mutation destroyed *Kpn*I and *Pfl*MI restriction sites, respectively, while G728C, C733G, T747A, and A772C created *Ava*I, *Bsa*HI, *Nsi*I, and *Hpa*I sites. Deletions were also introduced into pEA1.667 and its derivatives by digestion of appropriate restriction fragments with mung bean nuclease. Deletions 727Δ4 (in which base 727 is the first base of a 4-base deletion), 743Δ9, and 768Δ4 used *Ava*I, *Nsi*I, and *Hpa*I sites created by the G728C, T747A, and A772C mutations, respectively. 709Δ7 was created at a naturally occurring *Pfl*MI site. All constructions were confirmed by sequencing.

Plasmid pMengoEMC containing infectious mengovirus and EMCV chimeric sequences has been described previously (8, 28). Plasmid pM/E was derived from pMengoEMC by the addition of a poly(A) tail and removal (through *Bal* 31 digestion) of nonviral sequences at the 5′ end of the genome. pM/E joins the first 339 bases of mengovirus to the remainder of the EMCV genome (viral bases 414 to 7835 from pMengoEMC) at a common *Avr*II site. The viral fragment is oriented within pBS+ such that reactions with T7 RNA polymerase produce positive-sense RNA transcripts containing just 2 heterologous bases (GG) at the 5′ end and 5 heterologous bases (*Sal*I site) linked 3′ to a poly(A) tail (17 bases). The sequences of all cloning junctions and the entire viral IRES were confirmed for all plasmids. The specific infectivity of pM/E-derived RNA ($\approx 10^4$ PFU/μg) is about 1,000 fold higher than that for pMengoEMC (8).

To produce RNAs for in vitro translation and translation competition assays, *Eco*RI-*Bst*BI restriction fragments containing EMCV bases 670 to 1187 inclusive of the individual pEA1.667-derived J-K mutations were used to replace analogous fragments within pEA1.1Eco. To measure effects on virus viability, the J-K mutations were transferred into pM/E by replacing the *Avr*II-*Bss*HII restriction fragment (EMCV bases 415 to 862) of pM/E with equivalent fragments containing the J-K mutations.

The firefly luciferase gene (pGEM-luc; Promega) was used as a reporter to monitor the effects of the J-K mutations on in vivo translation of downstream cistrons. A/T tailing with terminal transferase at a *Stu*I restriction site just 3′ of the luciferase-encoding region produced a poly(A) of 25 nucleotides. The 1,763-bp *Not*I-*Sal*I fragment, which encodes the luciferase gene and termini, was then inserted into the pEA1.1Eco plasmids containing the J-K mutations, replacing the *Msc*I-*Sal*I fragment. The resulting plasmids (pLuc) contained EMCV bases 335 to 837 followed by a complete luciferase cistron. Protein synthesized from these constructs contained nine heterologous amino acids at the amino terminus of the luciferase protein.

Transcription of plasmids. Plasmid DNAs used for in vitro translation assays (pEA1.1Eco) were linearized with *Bam*HI. Plasmid DNAs used for in vivo translation assays (pLuc) or infectivity assays (pM/E) were linearized with *Sal*I. Plasmids used to generate IRES-only transcripts for translation competition assays (pEA1.1Eco) were digested with *Nco*I. The DNAs were used to program standard T7 RNA polymerase (Bethesda Research Laboratories) runoff transcription reactions containing [α-^{32}P]CTP or [α-^{32}P]UTP. Reactions were terminated by DNase I followed by phenol-chloroform extraction and precipitation with ethanol. Reaction efficiency was monitored by scintillation counting after adsorption to Whatman DE81 filters (38). Transcripts were analyzed for size and purity by standard agarose gel electrophoresis.

Reticulocyte translations. Cell-free translation reactions in reticulocyte extracts were as described previously (7, 30). Briefly, transcript RNA (1.0 to 1.2 μg) programmed reticulocyte extracts (15 μl) containing [^{35}S]methionine (1 μCi/μl). After 60 min at 30°C, the reactions were stopped and the incorporation of radiolabel into acid-insoluble products was quantitated (29). Translation competition assays in reticulocyte extracts were performed as described previously (7) by using EMCV transcripts (viral bases 667 to 4229) or globin mRNA as the template. The competing RNAs were EMCV transcripts containing viral bases 335 to 848, inclusive of the above-described mutations. When eIF-/2B was added (to 0.5 μg each), the competitor RNA was the wild-type EMCV transcript (bases 335 to 848) and incorporation was averaged from duplicate samples (±5% variance).

UV cross-linking. pEA1.667 plasmids were linearized with *Dsa*I and used to program T7 reactions containing [α-^{32}P]UTP (1.2 μCi/μl). The transcripts labeled at a specific activity of 1×10^7 to 2×10^7 dpm/μg. Transcripts, containing EMCV bases 667 to 797, were sized on agarose gels and then purified by using RNAid protocols (Bio 101). Radiolabeled transcript RNAs (32 ng, 0.76 pM) were incubated in 5 μl of a reticulocyte extract for 5 min at room temperature. The extracts were irradiated at 254 nm with 690,000 μJ/cm^2 in a UV Stratalinker 1800 (Stratagene). Extracts were treated with 0.5 μg of RNaseA for 12 min at 37°C. Laemmli sample buffer (30 μl) was added, and samples were treated for 5 min at 100°C before separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% Laemmli gels) and visualization by autoradiography (6). Individual bands were quantitated by densitometry using a Molecular Dynamics Personal Densitometer. Polyclonal antiserum to human La protein was a gift from Edward Chen (Scripps Research Institute, La Jolla, Calif.).

Infectivity assays. Confluent HeLa cell monolayers on 60-mm plates were washed with phosphate-buffered saline (PBS) followed by Hanks' balanced salt solution and then treated with DEAE-dextran (0.5 mg/ml) plus RNA (0.004 to 1.5 μg, derived by transcription of pM/E plasmids) in Hanks' balanced salt solution (250 μl). The plates were incubated for 30 min at room temperature, washed with PBS, overlaid with 2.5 ml of P5 liquid medium containing 0.8% agarose, and then overlaid with an additional 2.5 ml of P5 (37). The plates were incubated at 37°C for 30 h under 5% CO_2 to allow for plaque development.

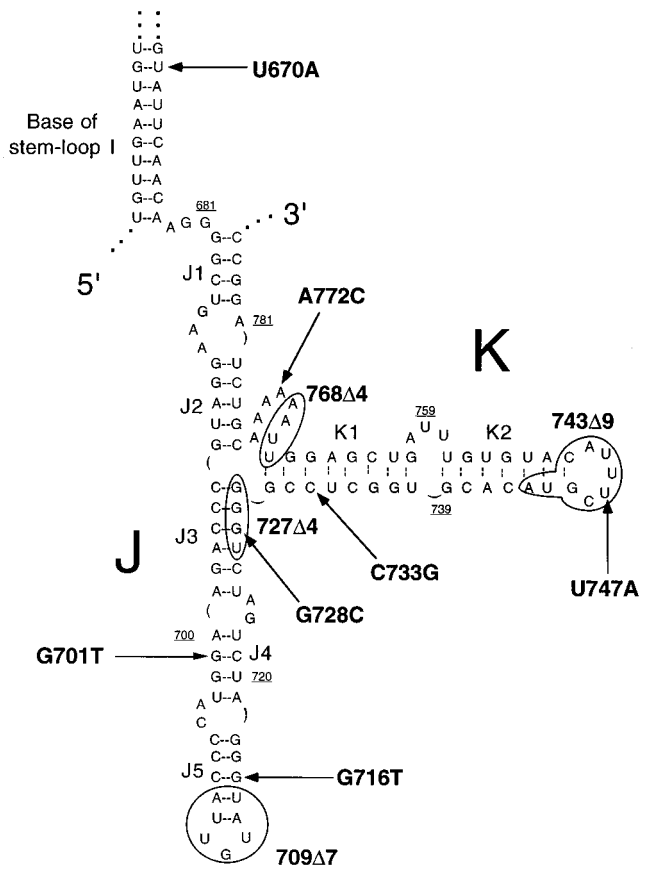


FIG. 2. Close-up of the EMCV J-K domain. Point mutations (arrows) and deletion mutations (circled) were created as described in the text. Helical segments of the model are numbered J1 to J5 and K1 and K2 for reference.

Luciferase assays (in vivo translation). Confluent HeLa cell monolayers (60-mm plates) were transfected by using lipofectACE as the transfection medium. LipofectACE (Bethesda Research Laboratories; formerly called transfectACE) was made as described by Rose et al. (35). Transcript RNAs (0.4 μg of RNA per sample) from pLuc plasmids were mixed with lipofectACE (5 μl) and Hanks' balanced salt solution (245 μl), and the solution incubated with cells for 30 min at room temperature. The monolayers were washed twice with PBS and then overlaid with 2 ml of liquid P5 medium. After 90 min at 37°C, under 5% CO_2, the medium was removed and the monolayers were washed twice with PBS. Lysis buffer (1×, 250 μl per plate; Promega) was added, and incubation continued at room temperature for 10 min. The lysate was clarified by centrifugation and assayed for luciferase activity in an Analytical Luminescence Laboratory Moonlight 2010 luminometer. The instrument automated the injection of luciferin (100 μl, 470 μM; Promega) into the reaction sample (16-μl cell lysate) and recorded the luminescence reaction.

RESULTS

Characterization of J-K domain mutations. To facilitate transfer of engineered J-K mutations into different plasmid contexts, EMCV base 670 (U) was mutated to an A, creating a new *Eco*RI site just 5′ of the J-K region in a viral cDNA (pEA1.1Eco). This mutation altered 1 base near the bottom of stem-loop I in RNA transcripts that contained it (Fig. 2). When added as a template to reticulocyte translation assays or tested in translation competition assays (Table 1), RNA with U670A was as active as the wild-type sequence, indicating that the chosen location was not critical to in vitro IRES activity. Six additional point mutations and four deletion mutations were then engineered into cDNAs containing U670A. The mutations targeted specific segments within the J-K region, including helices J3, J4, J5, K1, and K2, the J and K terminal loops, and the bifurcation loop of adenine residues between the J and K stems (Fig. 2). Optimal and suboptimal computer-aided RNA foldings predicted that these mutations would perturb only local secondary structure (i.e., helices or loops), without gross rearrangement of the larger domain or the IRES (data not shown).

TABLE 1. Characterization of mutations within the J and K stem-loops of the EMCV IRES

Mutation (location)	Relative protein synthesis[a] (%)	Translation of template		Relative luciferase activity[b] (%)
		EMCV RNA	Globin mRNA	
None				
No added RNA		100	100	
Wild type	100	18	20	
A670U (S-L[c] I)	104	22	21	100
G701U (helix J4)	91	91	55	44
G716U (helix J5)	93	50	22	42
G728C (helix J3)	55	92	77	18
C733G (helix K1)	79	42	24	47
U747A (K loop)	85	24	19	19
A772C (A bulge)	67	94	79	37
709Δ7 (helix J5, J loop)	49	97	59	11
727Δ4 (helix J3)	22	113	73	0.6
743Δ9 (helix K2, K loop)	100	37	23	8
768Δ4 (A bulge)	29	116	94	0.4

[a] In vitro translation.
[b] In vivo translation.
[c] S-L, stem-loop.

In vitro translation of RNAs containing J-K mutations. The effects of mutation on IRES function were assessed in reticulocyte reactions programmed with saturating levels (70 μg/ml) of T7 RNA transcripts. The use of saturating RNA levels ensured that minor variance in transcript concentration would not influence translational efficiency. Parallel experiments with subsaturating RNA levels established that mutant versus wild-type relative translational profiles were generally similar to those obtained with saturating RNA concentrations, but with greater variance (less reproducibility) among individual samples (data not shown). As described in Materials and Methods, the complete panel of mutant transcripts was tested in three separate translational experiments by using fresh preparations of RNA (70 μg/ml) each time. The averaged results for synthesis activity, relative to a wild-type EMCV IRES, are shown in Table 1. The standard deviations ranged from 4 to 12%. Thus, any mutation scoring lower than 80% should be considered impaired in translation.

Point mutations in helices J4 (G701U) and J5 (G716U) and the 9-base deletion (743Δ9) in the terminal loop of K produced RNAs that directed protein synthesis to levels that were 91, 93, and 100%, respectively, of pEA1 or U670A. Mutation U747A, in the terminal loop of K and within the region deleted by 743Δ9 was also quite active (85%). For all four of these mutant RNAs, translational efficiencies similar to those for wild-type transcripts were observed even when the transcript concentrations were reduced to subsaturating levels (35 μg/ml) (data not shown). The remaining point mutations, C733G in helix K1, A772C in the adenine loop, and G728C in helix J3, synthesized progressively less protein in our assays. Their levels were 79, 67, and 55%, respectively, of the level for the wild-type transcripts. Deletions overlapping the most sensitive of these regions, 727Δ4 and 768Δ4, had even more deleterious effects,

reducing protein synthesis to 22 and 29%, respectively. The final tested mutation (709Δ7), a 7-base deletion that removed 2 bp from helix J5 and the terminal loop of J, translated with an efficiency equivalent to only 49% of the efficiency of the parental RNA. Together, the data mark the bifurcation region of the J-K domain, including the adenine bulge and helices J3 and K1, as particularly sensitive to mutation as monitored in cell-free assays. The terminal loop of J, but not of K, was also sensitive.

Translation competition assays. Intact, EMCV IRES RNAs are effective inhibitors of cell-free translations programmed with exogenous capped or uncapped mRNAs. The responsible sequences localize within the J-K domain (7) but have not been mapped in detail. Plasmids containing the J-K mutations were linearized with a restriction enzyme (*Nco*I) that cleaved at viral base 848, just after the AUG that initiates the polyprotein. Short IRES-containing transcripts (EMCV bases 335 to 848) inclusive of the mutations were produced. The RNAs were purified, quantitated, and then added to translation extracts in a 5-fold molar excess when EMCV RNA was used as the template (pEA1) or 1.9-fold molar excess when globin mRNA was used as the template. With wild-type IRES sequences, these ratios inhibited translation of the respective templates to 20% of that observed in their absence (Table 1).

On the whole, the competition data are consistent with those from translation experiments. That is, mutations that disrupted translational activity of an IRES also disrupted its competitive activity against exogenously translating templates. With both globin and EMCV templates, for example, mutations G716U, C733G, U747A, and 743Δ9 were efficient competitors and reduced translation of the template RNAs to less than 50%. Mutations G728C, A772C, 709Δ7, 727Δ4, and 768Δ4, which were less effective at directing translation as part of an IRES, were also less inhibitory in the competition assays. Against EMCV templates, this set of mutations reduced the translational activity by less than 8%. Against globin templates, which are more sensitive to competition by a functional EMCV IRES (7), they reduced the activity between 6 to 41%, depending upon the size and location of the mutation. The correlation between losses of translational and inhibitory activities is consistent with suggestions that a single required IRES function, perhaps the sequestering of a cellular factor, is disrupted by the most active J-K mutations. In such a case, translation and inhibition are expected to vary as coordinate functions.

Mutation G701U, however, did not fit this pattern. This change allowed translation of downstream cistrons with high efficiency (91%), yet it was only weakly effective as a competitor against EMCV translation (91%). Globin translation was more strongly affected, but the inhibitory activity (reduced to 55%) was still not as strong as might be expected for a good translational IRES (e.g., G716U). The inhibition results with mutants G728C, A772C, 709Δ7, 727Δ4, and 768Δ4 and the anomalous results with mutant G701U suggested two interpretations. Either the cell-free translation and competition assays were actually measuring somewhat different J-K functions or the in vitro translational assays were surprisingly insensitive to particular changes in the IRES.

In vivo translation of J-K domain mutations. To distinguish these possibilities, an alternative assay was developed to more closely mimic natural in vivo translation during viral infection of cells. A luciferase reporter gene was cloned into cDNA plasmids containing the EMCV IRES and each of the J-K mutations. After transfection of HeLa cells, the RNA transcripts were allowed to synthesize luciferase under the control of various IRESs, and luciferase-mediated oxidation of luciferin within cellular extracts was quantitated with a luminometer. Transfection conditions, transcript concentration, and reaction time for the luciferase assays were optimized to give a strong, reproducible signal in control samples (data not shown). Addition of lipofectACE to the transfection medium was an assay advantage, because high levels of luciferase expression were obtained with as little as 0.4 μg of the wild-type RNA transcript per 6×10^6 cells. Approximately 1% of the input RNA sample could be recovered from within transfected cells by this technique, a level nearly 100 times higher than that reported for DEAE-dextran procedures (11, 35). The transfection efficiency did not vary significantly for any tested IRES RNA sequence (data not shown).

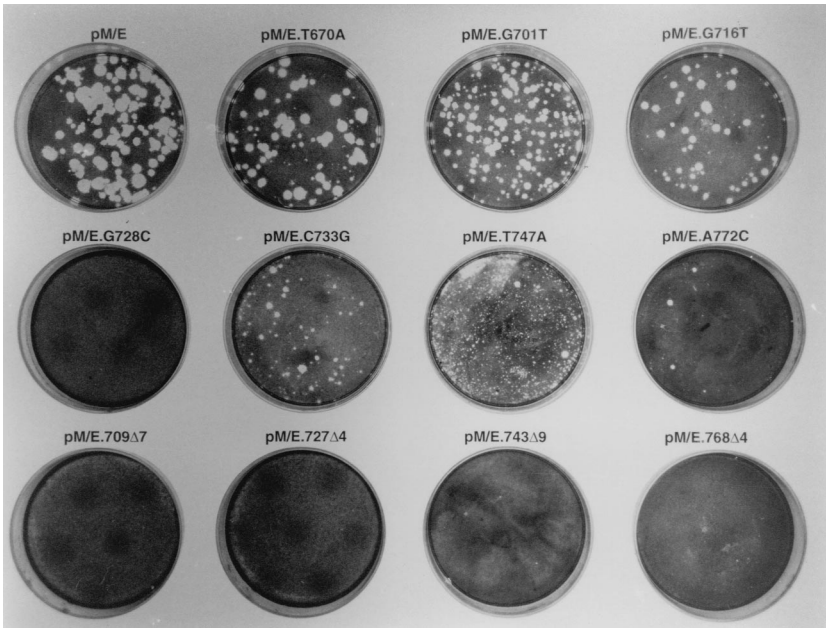
Under our conditions, luciferase transfection assays proved very sensitive to mutational changes within the IRES (Table 1). Compared with the parental IRES containing the U670A mutation (base of stem-loop I), all tested J-K mutations were less than 50% as effective at directing luciferase synthesis. Mutant G701U, which translated efficiently in vitro but gave anomalous results in the competition assays, synthesized only 44% the parental level of luciferase in HeLa cells. G716U, C733G, and A772C, which were also active reticulocyte templates, were reduced to 42, 47, and 37%, respectively, when required to synthesize protein in cells.

The four mutations that were the least efficient translators in vitro were even less efficient in luciferase assays. G728C, 709Δ7, 727Δ4, and 768Δ4 produced 18, 11, 0.6, and 0.4%, respectively, of the enzyme directed by the parental IRES. Consistently, these mutations were among the least efficient translational competitors, too. Mutations U747A and 743Δ9 within the terminal loop of K gave unexpected results. Both sequences were good templates in reticulocyte extracts and strong competitors against mRNA translation. However, in HeLa cells, they produced only 19 and 8% as much luciferase as the parental IRES, respectively, and clearly implicate this loop in the in vivo translational process. The fact that these defects were inapparent in cell-free assays seems to indicate that reticulocyte extracts, optimized with saturating template, tRNA, energy systems, and perhaps other required factors, are far less responsive to changes in the IRES sequence than the translational environment of HeLa cells.

Infectivity assays. It could be argued that artificial reporter genes like luciferase still don't provide an entirely natural test for relative IRES activity, even if assayed in vivo. To confirm whether the translational phenotypes of the luciferase assays showed similar defects in the context of viral genomes, the J-K mutations were shuttled into pM/E plasmids. These cDNAs contain IRES and polyprotein sequences from EMCV and 5′ S fragments from the closely related mengovirus. The use of short 5′ mengovirus segments is an effective cloning trick to bypass difficulties with EMCV's long poly(C) ($C_{115}UCUC_3UC_{10}$) (7). Transcripts from parental pM/E contain a poly(C) of $C_{13}UC_{10}$ and are highly infectious. They produce about 10^4 PFU/μg of RNA and have large plaques characteristic of EMCV (28).

Transfections with full-length RNAs showed that U670A behaved like the wild-type sequence (Fig. 3). It gave large plaques with a high specific infectivity. The G701U and G716U mutants, which were efficient translators in vitro but somewhat less effective in luciferase assays, produced slightly smaller plaques, with good infectivity. C733G translated 79% as efficiently as the wild type in vitro and produced even smaller plaques in the context of a viral genome. RNAs with A772C (65% relative in vitro translation) produced plaques that were barely visible at 30 h posttransfection and typically required 50 to 60 h to attain a medium size. The few plaques observed at 30 h on these plates were sequenced and shown to be faster-growing revertants. The G728C mutation (55% in vitro trans-

A.



B.

RNA	Specific Infectivity (PFU/μg)
M/E	1.0×10^4
U670A	1.4×10^4
G701U	1.4×10^4
G716U	6.8×10^3
G728C	0.7
C733G	2.1×10^3
U747A	1.1×10^3
A772C	168
709Δ7	0.7
727Δ4	0.0
743Δ9	0.0
768Δ4	0.0

FIG. 3. Infectivity of J-K mutations. (A) By using DEAE-dextran techniques, HeLa cell monolayers were transfected with 4 to 40 ng (pM/E, pM/E.T670A, pM/E.G701T, pM/E.G716T, and pM/E.C733G) or 1.5 μg (pM/E.G728C, pM/E.T747A, pM/E.A772C, pM/E.709Δ7, pM/E.727Δ4, pM/E.743Δ9, and pM/E.768Δ4) of genome-length RNA transcripts. Monolayers were stained with crystal violet at 30 h after transfection. Plates from pM/E.G728C, pM/E.709Δ7, pM/E.727Δ4, and pM/E.768Δ4 were stained at 60 to 70 h after transfection. (B) The specific infectivities of the RNAs were averaged from three determinations.

lation) and 709Δ7 deletion (49% in vitro translation) gave very tiny plaques after 60 to 70 h and only at a specific infectivity of about one plaque per 1 to 2 μg of RNA. Some of these plaques were also sequenced as IRES revertants. The most severe in vitro translation mutations, 727Δ4 (22% in vitro translation) and 768Δ4 (29% in vitro translation), failed to yield any plaques even with very high transcript concentrations or with exogenous lysis and serial transfer of the transfected cells. In agreement with the luciferase data, mutations U747A and 743Δ9 in the terminal loop of K gave small plaques or no plaques, respectively.

Protein interactions with the J-K domain. Several IRES-binding proteins have been identified through UV cross-linking techniques. The p57 PTB protein interacts with IRESs from EMCV and poliovirus (13, 18, 40, 41), and the La autoantigen is required for accurate translation of poliovirus in reticulocyte extracts (40). Logically, the mutational sensitivity of the J-K domain in translation and translational competition assays suggested that this region should have special protein binding features. Radiolabeled J-K transcripts (EMCV bases 667 to 797) containing the mutant sequences were synthesized and reacted with translationally competent reticulocyte extracts (Fig. 4). After UV cross-linking and nuclease digestion, each RNA had transferred label to a single protein band, designated p49, according to its migration on calibrated gels. Appearance of this band was sensitive to proteinase K and dependent upon UV irradiation (data not shown). Neither human or rabbit polyclonal antiserum to human La protein was able to precipitate the p49-RNA complex from a cross-linking reaction (data not shown).

Within experimental limitations, densitometric quantitation of band intensity showed that all tested J-K mutant sequences reacted as strongly with p49 as the wild-type sequence (Fig. 4; compare lane 7 with lanes 8 to 13). Surprisingly, control samples also showed that p49 binding to J-K RNA was not highly specific. While a 30-fold molar excess of a nonradiolabeled J-K transcript reduced the band signal to 30% (lane 2), competition with a nonspecific transcript of 273 nucleotides from the pBS+ vector (lanes 3 and 4) was even more effective. Minus-strand EMCV transcripts from the P2 polyprotein region were also competitive at similar concentrations (lanes 5 and 6). If p49 binding correlates with IRES activity, it is not a manner detectable by UV reactions with mutant sequences in the context of an intact J-K fragment. The indiscriminate binding to mutant and wild-type sequences, as well as to nonspecific RNAs, suggests that p49 may be a general RNA-binding protein rather than a contributor to J-K function.

Still, it was of interest to see if a required RNA size or location of the p49 interaction could be more precisely mapped by creating a series of 3′ nested deletions within the J-K sequences. Transcript 667-769 terminated within the adenine bulge. Transcripts 667-747, 667-733, 667-730, and 667-713 terminated within the K loop, helix K1, the 3′ and 5′ boundaries of helix J3, and J loop, respectively. An internal deletion transcript (Δ710-730), which removed the 3′ halves of helices J3, J4, and J5 was also created. Relative to the full-length J-K transcripts (667-797), all deleted sequences bound p49 with lower efficiency (Fig. 5). The shortest transcripts, especially those which deleted stem K and impinged on stem J, were the weakest binders (lanes 5 and 6). The internal deletion sequence, Δ710-730, cross-linked to p49 with an efficiency equivalent to 71% of the efficiency of the wild type. Thus, all dele-

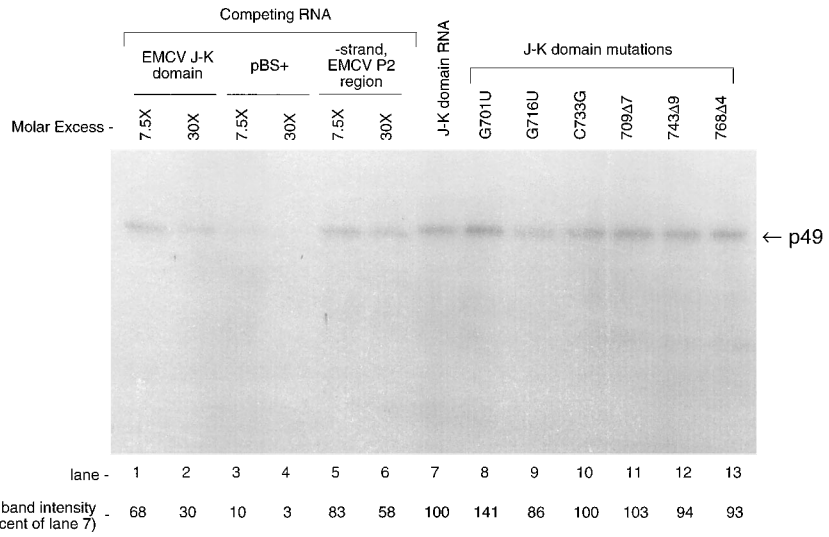


FIG. 4. UV cross-linking to J-K RNA. ^{32}P-labeled RNA transcripts (EMCV bases 667 to 797) were incubated with rabbit reticulocyte lysate (5 μl), irradiated with UV light (254 nm), digested with RNase A (0.5 μg), and analyzed by SDS-PAGE and autoradiography. The sample in lane 7 contained 30 ng (8.1 pM) of the wild-type J-K transcript. Samples in lanes 1 to 6 contained the same labeled transcript and, in addition, the indicated molar excesses of unlabeled, competing RNA. The EMCV J-K domain competitor contained EMCV bases 667 to 797 (identical to those in the probe). The pBS+ RNA was a 273-nucleotide transcript derived from the pBS+ vector. The − strand, EMCV P2 region RNA was a 115-base transcript from the minus-sense P2 region of EMCV. Samples in lanes 8 to 13 contained 30 ng of radiolabeled J-K transcripts with the indicated mutations. All bands were quantitated by densitometry.

tions which left either the J or the K stem intact were roughly equivalent in binding efficiency. The results suggest that p49 may have multiple binding sites within J-K or perhaps, more simply, that p49 requires little more than a short stacked helical region for RNA recognition.

Reactions in vitro with eIF-2 and eIF-2B. Initiation factors 2 and 2B (eIF-2/2B) are also required elements in the translation of eukaryotic mRNAs and have roles in picornavirus IRES function (12, 36). It also has been reported that ribosomal salt wash fractions enriched for these factors can rescue EMCV-directed chloramphenicol acetyltransferase synthesis and globin mRNA synthesis that have been inhibited by EMCV IRES fragments, in assays similar to those described above. Additional data from a multiple substitution mutation in the K loop tentatively mapped the eIF-2/2B activity to the K region of the IRES (39).

We have tested this hypothesis using translation and translational competition assays, augmented by highly purified eIF-2 and eIF-2B components (provided by Albert Wahba, University of Mississippi Medical Center, Jackson, Miss.). The data in Table 2 are presented as average counts per minute for replica samples (variance, ≈5%) and were reproducible in duplicate experiments. Relative to Table 1, a value of 180,000 cpm is analogous to 100% incorporation (wild type, relative

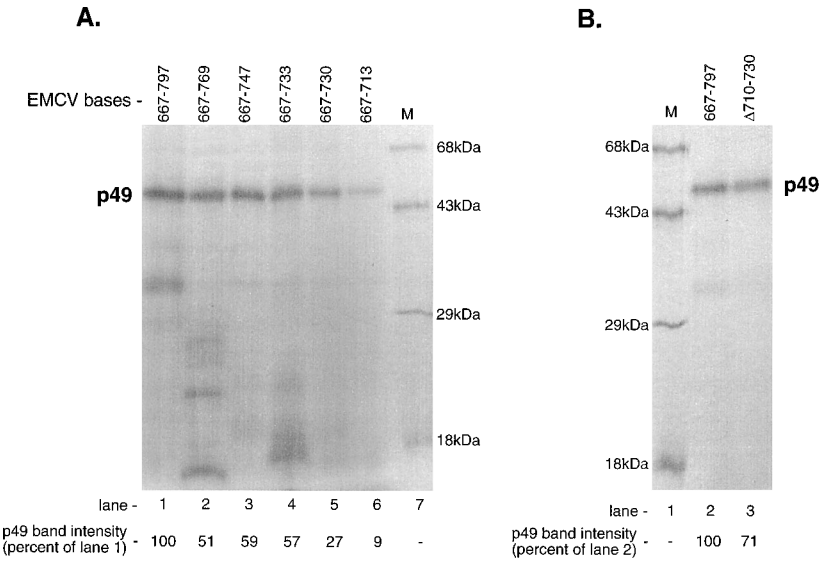


FIG. 5. UV-cross-linking to J-K fragments. ^{32}P-labeled transcripts containing the indicated EMCV bases were incubated with reticulocyte extracts, irradiated with UV light, and prepared as for Fig. 4. (A) Equimolar amounts (8.1 pM) of radiolabeled RNAs with the indicated EMCV bases were used as probes (lanes 1 to 6). Lane 7, radiolabeled protein markers (M). (B) The 667-797 transcript control (lane 2) and marker (M) (lane 1) are similar to lanes 1 and 7 of panel A, respectively. The RNA probe in lane 3 contained EMCV bases 667 to 797 but with a deletion of bases 710 to 730. All bands were quantitated by densitometry.

TABLE 2. Translation and translation competition with added eIF-2 and eIF-2B

Template and added factor(s)[a]	EMCV IRES competitor	[^{35}S]Met incorporation (cpm [10^3]/μl of lysate)
EMCV RNA transcript		
No added factor	−	184
No added factor	+	103
eIF-2	−	325
eIF-2B	−	383
eIF-2 + eIF2B	−	369
eIF-2	+	121
eIF-2B	+	119
eIF-2 + eIF2B	+	102
Globin mRNA		
No added factor	−	56
No added factor	+	6
eIF-2 + eIF2B	−	120
eIF-2 + eIF2B	+	11

[a] 0.5 μg per reaction.

protein synthesis), or about 12% ^{35}S conversion into protein. Either factor, added singly or in combination, boosted in vitro protein synthesis from EMCV and globin mRNA templates about twofold. For EMCV the enhanced synthesis represents about 25% conversion of available methionine into protein.

However, neither factor, even when added at these high concentrations (0.5 μg per reaction), could relieve competition from an exogenous EMCV IRES. The fact that translation was still inhibited and not rescued by the factors, argues that eIF2/2B are not sequestered by J-K and are probably not the limiting cellular factors specifically responsible for J-K-dependent competitive phenomena, as was previously hypothesized (39). Rather, our data suggest that these particular factors probably cycle normally and in a required manner during IRES-directed cell-free translation, aiding in initiation, but without direct or irreversible binding to the J-K portion of the template.

DISCUSSION

Cap-independent translation of EMCV RNA is mediated by a 450-nucleotide segment that lies 5′ to the first AUG of the polyprotein open reading frame. Analyses of the IRES have generally focused on structural motifs near the 5′ and 3′ borders which constitute only 20% of the intact segment. The more central I, J, and K motifs have recognizable homologs in all cardio-, aphtho-, and hepatoviruses (5, 33) but are not common to the rhino- or enteroviruses, which use a different set of 5′ structures for initiation of cap-independent translation (34). Our interest in the cardiovirus J-K segment came from preliminary findings that an insertion mutation at base 777 disrupted EMCV translation in reticulocyte extracts and ablated IRES inhibitory activity in translational competition assays (7). We have now tested a larger panel of mutations in four different in vitro and in vivo assays and confirm a special role for this domain in IRES-directed translation.

In particular, the conserved oligo(A) bifurcation loop between the J-K stems and the adjacent J3 helix proved highly sensitive to mutation. Deletions or point mutations in either location disrupted translation of downstream cistrons in cell-free and cellular systems and rendered viral genomes noninfectious. The J4, J5, and K1 helices were also sensitive to substitution, but viable virus, albeit with reduced plaque phenotypes, was still recovered. A point mutation in loop K gave smaller plaques than those for the J4, J5, and K1 mutations, although genomes with deletions in either terminal loop were dead. To summarize, no tested alterations in the J-K region went unpunished. Some locations were more refractory to change than others, but each engineered sequence clearly manifested a defective translational phenotype, especially when measured as part of a luciferase mRNA in vivo or within the context of a viral genome after transfection.

These results were actually surprising because initial experiments with reticulocyte extracts (Table 1) had suggested that some mutations might identify segments not critical for IRES translation. G701U (J4), G716U (J5), U747A (K loop), and even 743Δ9 (9-base deletion in the K loop) directed protein synthesis efficiently in cell-free extracts, and all except G701U were also effective inhibitors against capped and uncapped messages in translational competition assays. We believe that the data highlight an important caveat for use of in vitro assays to predict in vivo phenotypes. Our reticulocyte lysates have been optimized heuristically for maximum translation of EMCV proteins (7, 30). When combined with saturating RNA levels, the system synthesizes nearly as much protein as the template on a microgram basis. But the robust activity of an in vitro system can easily mask mutational impact unless constructions are also tested in more discriminatory assays. The responsiveness of in vivo reactions to the entire panel of J-K mutations probably reflects real, natural limitations in cellular factor binding, energy generation, or substrates that are not rationed as stringently in the cell-free systems and point to important quantitative or qualitative differences between reticulocytes and transfected HeLa cells.

The dichotomy was especially apparent for mutations in the K loop (U747A and 743Δ9), which did not register at all during in vitro assays but were clearly defective in vivo. On the basis of a substitution of this loop, Scheper et al. proposed a vital IRES function for K as part of a binding site for initiation factor complexes containing eIF-2/2B (39). Our competition data do not support a direct binding role for these factors because addition of up to 0.5 μg of purified eIF-2/2B, singly or in combination, could not relieve the in vitro competitive pressure of an exogenous IRES. Nevertheless, the in vivo translation (luciferase) assays do confirm a primary role for the K loop in IRES translation.

One hypothesis implicit in the J-K studies was that this segment probably binds and sequesters required translational factors, and thus competitively down regulates the template activity of exogenous mRNAs. The competition data using mutated IRESs support this idea, because, for the most part, the J-K mutations were less effective competitors than the wild-type sequences. RNA fragments containing only J-K are almost as competitive as an intact IRES in these assays (7). UV cross-linking was therefore used to tag reticulocyte proteins that might interact with J-K, and a 49-kDa protein-RNA complex was identified. However, this complex formation could be inhibited with nonspecific RNA sequences and, moreover, did not correlate in any obvious way with the translational efficiency or inhibitory activity of any J-K mutations.

The results lead to speculation concerning the role of p49 in EMCV translation. Although interaction with the EMCV IRES could be due to general RNA-binding properties, p49 might actually be important for translation. By analogy, the La autoantigen (p52) is known to bind to a region of the poliovirus IRES which, when mutated, reduces translational efficiency but does not decrease p52 binding (24, 25, 32, 40). Nevertheless, p52 is now thought to be essential for poliovirus translation (26). Perhaps with p49, subtle binding changes to the J-K domain not detectable through UV cross-linking could still

influence the efficiency of EMCV translation. It is also possible that p49 binds J-K in a region not tested by mutation, thus preventing observation of changes in binding which might correlate with translation efficiency. In recent preliminary experiments, we have observed that J-K RNA can be bound by a HeLa cell protein, approximately of the same size as p49, and are currently investigating whether this protein might be synonymous with the human La autoantigen. Antibodies to human La did not react with p49 from reticulocyte lysates; however, this does not preclude its identity as the rabbit homolog of any of these proteins. The amino acid sequences of the human, bovine, mouse, and rat La proteins for which GenBank sequences are available are less than 82% identical, and it is very possible that a rabbit analog with this degree of conservation would go undetected with these antibodies. A positive identification and assessment of the role of p49 await future purification.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grant AI-17331 to A.C.P. and NIH training grant GM-07215 to M.A.H.

We thank Albert Wahba for the generous gift of eIF-2/2B and Edward Chen for providing antisera to human La protein.

REFERENCES

1. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1993. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64:**5389–5395.
3. **Borman, A., and R. J. Jackson.** 1992. Initiation of translation of human rhinovirus RNA: mapping the internal ribosome entry site. Virology **188:** 685–696.
4. **Borovjagin, A. V., A. G. Evstafieva, T. Y. Ugarova, and I. N. Shatsky.** 1990. A factor that specifically binds to the 5′ untranslated region of encephalomyocarditis virus RNA. FEBS Lett. **261:**237–240.
5. **Brown, E. A., S. P. Day, R. W. Jansen, and S. M. Lemon.** 1991. The 5′ non-translated region of hepatitis A virus RNA: secondary structure elements required for translation in vitro. J. Virol. **65:**5828–5838.
6. **Cleveland, D., S. Fischer, M. Firschner, and U. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252:**1102–1106.
7. **Duke, G. M., M. A. Hoffman, and A. C. Palmenberg.** 1992. Sequence and structural elements that contribute to efficient encephalomyocarditis viral RNA translation. J. Virol. **66:**1602–1609.
8. **Duke, G. M., and A. C. Palmenberg.** 1989. Cloning and synthesis of infectious cardiovirus RNAs containing short, discrete poly(C) tracts. J. Virol. **63:**1822–1826.
9. **Elroy-Stein, O., T. R. Fuerst, and B. Moss.** 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. Proc. Natl. Acad. Sci. USA **86:**6126–6130.
10. **Evstafieva, A. G., T. Y. Ugarova, B. K. Chernov, and I. N. Shatsky.** 1991. A complex RNA sequence determines the internal initiation of encephalomyocarditis virus RNA translation. Nucleic Acids Res. **19:**665–671.
11. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofectin: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84:**7413–7417.
12. **Golini, F., S. S. Thach, C. H. Birge, W. C. Merrick, and R. E. Thach.** 1976. Competition between cellular and viral mRNAs in vitro is regulated by a messenger discriminatory initiation factor. Proc. Natl. Acad. Sci. USA **73:** 3040–3044.
13. **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.
14. **Hellen, C. U. T., G. W. Witherell, M. Schmid, S. H. Shin, T. V. Pestova, A. Gil, and E. Wimmer.** 1993. A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. Proc. Natl. Acad. Sci. USA **90:**7642–7646.
15. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15:**477–483.
16. **Jang, S. K., H. G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636–2643.
17. **Jang, S. K., T. Pestova, C. U. T. Hellen, G. W. Witherell, and E. Wimmer.** 1990. Cap-independent translation of picornaviral RNAs: structure and function of the internal ribosomal entry site. Enzyme **44:**292–309.
18. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. **4:**1560–1572.
19. **Kaminski, A., M. T. Howell, and R. J. Jackson.** 1990. Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. EMBO J. **9:**3753–3759.
20. **Kaufman, R. J., M. V. Davies, L. C. Wasley, and D. Michnick.** 1991. Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucleic Acids Res. **19:**4485–4490.
21. **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.
22. **Levy, M., C. Aubert, and M. Brahic.** 1992. Theiler's virus replication in brain macrophages cultured in vitro. J. Virol. **66:**3188–3193.
23. **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.
24. **Meerovitch, K., R. Nicholson, and N. Sonenberg.** 1991. In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J. Virol. **65:**5895–5901.
25. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3:**1026–1034.
26. **Meerovitch, K., Y. V. Svitkin, H. S. Lee, F. Lejbkowicz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. D. Keene, and N. Sonenberg.** 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J. Virol. **67:**3798–3807.
27. **Oudshoorn, P., A. A. Thomas, G. C. Scheper, and H. O. Voorma.** 1990. An initiation signal in the 5′ untranslated leader sequence of encephalomyocarditis virus RNA. Biochim. Biophys. Acta **1050:**124–128.
28. **Palmenberg, A. C., and J. E. Osorio.** 1994. Cardioviral poly(C) tracts and viral pathogenesis. Arch. Virol. **90:**67–77.
29. **Palmenberg, A. C., and R. R. Rueckert.** 1982. Evidence for intramolecular self-cleavage of picornaviral replicase precursors. J. Virol. **41:**244–249.
30. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67:**247–256.
31. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
32. **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.
33. **Pilipenko, E. V., V. M. Blinov, T. M. Dmitrieva, 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.
34. **Pilipenko, E. V., V. M. Blinov, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168:**201–209.
35. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10:**520–525.
36. **Rosen, H., G. Di Segni, and R. Kaempfer.** 1982. Translational control by messenger RNA competition for eukaryotic initiation factor 2. J. Biol. Chem. **257:**946–952.
37. **Rueckert, R. R., and M. A. Pallansch.** 1981. Preparation and characterization of encephalomyocarditis virus. Methods Enzymol. **780:**315–325.
38. **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.
39. **Scheper, G. C., A. A. Thomas, and H. O. Voorma.** 1991. The 5′ untranslated region of encephalomyocarditis virus contains a sequence for very efficient binding of eukaryotic initiation factor eIF-2/2B. Biochim. Biophys. Acta **1089:**220–226.
40. **Svitkin, Y. V., K. Meerovitch, H. S. Lee, J. N. Dholakia, D. J. Kenan, V. I. Agol, and N. Sonenberg.** 1994. Internal translation initiation on poliovirus RNA: further characterization of La function in poliovirus translation initiation. J. Virol. **68:**1544–1550.
41. **Witherell, G. W., A. Gil, and E. Wimmer.** 1993. Interaction of polypyrimidine tract binding protein with the encephalomyocarditis virus mRNA internal ribosomal entry site. Biochemistry **32:**8268–8275.