Translation of Eukaryotic Translation Initiation Factor 4GI (eIF4GI) Proceeds from Multiple mRNAs Containing a Novel Cap-dependent Internal Ribosome Entry Site (IRES) That Is Active during Poliovirus Infection*

Received for publication, December 14, 2004, and in revised form, March 4, 2005
Published, JBC Papers in Press, March 8, 2005, DOI 10.1074/jbc.M414014200

Marshall P. Byrd‡, Miguel Zamora, and Richard E. Lloyd§
From the Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030

Eukaryotic translation initiation factor 4GI (eIF4GI) is an essential scaffolding protein required to recruit the 43 S complex to the 5’-end of mRNA during translation initiation. We have previously demonstrated that eIF4GI protein expression is translationally regulated. This regulation is mediated by cis-acting RNA elements, including an upstream open reading frame and an IRES that directs synthesis of five eIF4GI protein isoforms via alternative AUG initiation codon selection. Here, we further characterize eIF4GI IRES function and show that eIF4GI is expressed from several distinct mRNAs that vary via alternate promoter use and alternate splicing. Several mRNA variants contain the IRES element. We found that IRES activity mapped to multiple regions within the eIF4GI RNA sequence, but not within the 5’-UTR per se. However, the 5’-UTR enhanced IRES activity in vivo and played a role in initiation codon selection. The eIF4GI IRES was active when transfected into cells in an RNA form, and thus, does not require nuclear processing events for its function. However, IRES activity was found to be dependent upon the presence, in cis, of a 5’ m7guanosine-cap. Despite this requirement, the eIF4GI IRES was activated by 2A protease cleavage of eIF4GI, in vitro, and retained the ability to promote translation during poliovirus-mediated inhibition of cap-dependent translation. These data indicate that intact eIF4GI protein is not required for the de novo synthesis of eIF4GI, suggesting its expression can continue under stress or infection conditions where eIF4GI is cleaved.

The initiation of eukaryotic protein synthesis is a highly regulated process utilized to control global protein synthesis and to differentially control expression from specific mRNAs. The majority of cellular mRNAs translate cap-dependently by virtue of a 5’ m7G-cap, which is specifically recognized and bound by eukaryotic translation initiation factor (eIF)4E (eIF4E). During translation initiation eIF4E is part of a protein complex, termed eIF4F, which also contains the RNA helicase eIF4A, and the large scaffolding protein eIF4G (for a review see Ref. 1). eIF4G, in particular, is crucial for recruitment of the 40 S ribosomal subunit, 5’-3’ interactions on mRNA and AUG initiation codon selection (2).

Two genes encode eIF4G, and their protein products are termed eIF4GI and eIF4GII. eIF4GI comprises the majority of total eIF4G in cells as revealed by examination of protease cleavage products of purified eIF4F (3). eIF4GII can be functionally divided into three domains, separated by caspase-3 cleavage sites (3, 4). The N-terminal cleavage product binds to polyadenosine-binding protein (PABP), whereas the central portion encodes the eIF4E interaction sequences and a HEAT repeat domain responsible for eIF3, eIF4A, and RNA interactions (6, 7). The C-terminal cleavage fragment encodes a second eIF4A binding region and can recruit the mitogen-activated protein (MAP); MAP kinase-interacting kinase (MNK) (8). It has been previously demonstrated that the central domain encompassing the eIF4E, eIF3, and eIF4A binding domains is sufficient to support cap-dependent translation initiation (9).

eIF4G plays a central role in translation initiation, thus eIF4G function is frequently targeted to regulate protein synthesis. Many regulatory processes modulate eIF4G function by displacement of proteins normally bound to eIF4G. For example, during serum starvation and encephalomyocarditis virus (EMCV) infection, dephosphorylation of eIF4E-binding protein (4E-BP) results in the removal of eIF4E from eIF4G. This, in turn, leads to a down-regulation of global cap dependent translation (10, 11). Alternatively, the function of eIF4G can be modified by proteolytic cleavage, which separates specific eIF4G binding domains from each other. For instance, rhinovirus, poliovirus (PV), Coxsackievirus B3 (CVB3), and foot and mouth disease virus (FMDV) encode proteases that cleave eIF4G and separate the eIF4E and PABP binding domain from the central domain, resulting in a down-regulation of cellular, cap-dependent translation initiation (12–16). Similarly, the protease expressed by human immunodeficiency virus (HIV) cleaves eIF4G, separating the N-terminal (including the eIF4E interaction site) and C-terminal regions from the eIF3-binding central fragment, also resulting in a dramatic inhibition of host translation (17). Other cellular proteases activated during PV infection have also been shown to cleave eIF4GI, separating the eIF4E and PABP interaction domains from the remainder of the protein in a cleavage similar to that mediated by PV 2A protease (19).

* This research was supported by National Institutes of Health Grants AI 50237 and GM 59803. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a NIAID, National Institutes of Health Training Grant T32 AI07471 in molecular virology.
§ To whom correspondence should be addressed: Dept. of Molecular Virology and Microbiology, Rm. 860E, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-8993; Fax: 713-798-5075; E-mail: rlloyd@bcm.tmc.edu.

The abbreviations used are: eIF, eukaryotic translation initiation factor; UTR, untranslated region; ORF, open reading frame; MOI, multiplicity of infection; IRES, internal ribosome entry site; PABP, polyadenosine-binding protein; ITAF, IRES transactivating factors; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; GFP, green fluorescent protein; RL, Renilla luciferase; FL, firefly luciferase; uORF, upstream open reading frame.
Control of translation initiation can also be mediated via proteolytic cleavage of eIF4GI in infected cells. During apoptosis, caspase cleavage of several translation initiation factors, including eIF4G, results in a down-regulation of cap-dependent translation. In this case, eIF4GI cleavage by caspase-3 separates putative regulatory regions in the N and C termini from the core domain, which binds to eIF4E, eIF3, and RNA (3, 18).

During virus-induced inhibition of cap-dependent translation most cellular mRNAs cannot be effectively translated; however, viral RNAs continue to translate via specialized RNA sequences that mediate translation initiation. During PV infection, cellular protein synthesis is down-regulated by the viral 2A and 3C proteases, which cleave eIF4G and PABP, respectively (13, 15, 20, 21). Translation of the viral polyprotein is maintained by an internal ribosome entry site (IRES) present in the 5′-untranslated region (UTR) of PV RNA (22). The PV IRES can mediate translation initiation independent of a mG cap and eIF4E by recruitment of the translation apparatus directly to the IRES. RNA-binding proteins (e.g. polypyrimidine tract-binding protein (PTB) and poly(rC)-binding protein 2 (PCBP2)), known as IRES transactivating factors (ITAFs), have been shown to stimulate PV IRES function (23–25). A variety of other viral RNAs have been shown to translate from IRES elements (e.g. hepatitis C virus (HCV), EMCV, rhinovirus, hepatitis A virus (HAV) and HIV), and ITAFs are also implicated in these processes (for a review see Ref. 26).

More recently, it has been demonstrated that the translation of certain cellular mRNAs is also maintained during cap-dependent translation inhibition, (e.g. during viral infection) (27). Two known mechanisms of translation initiation operate under conditions when cap-dependent scanning has been down-regulated. First, like their viral counterparts, some cellular mRNAs continue to translate via discontinuous scanning of translation initiation from the second cistron of dicistronic RNAs (22). As has been demonstrated for viral IRESes, these cellular IRESes do not require cap-dependent scanning and recruit ITAFs to enhance their function (31). However, other mRNAs continue to translate via a discontinuous scanning mechanism, known as shunting (e.g. Hsp70), which requires the presence of a mG-cap (32). As currently defined, translation shunting elements do not function in dicistronic RNAs, and consist of two sequences, the cap proximal shunt donor and AUG proximal shunt acceptor which enable scanning ribosomes to bypass or jump over large regions of RNA leader sequences (32). Both IRES and shunting mechanisms can mediate translation initiation when cap-dependent scanning is repressed, however, the fundamental mechanism by which this is accomplished differs (33, 34).

Several reports have described IRES activity in eIF4GI RNA; however, confusion has been generated because some investigations tested mutually exclusive and distinct regions of the eIF4GI gene. The original sequence reported for human eIF4GI mRNA contained a 5′ leader that was reported to encode an IRES (35, 36). This cDNA sequence did not contain the complete eIF4GI ORF, encoded only the smallest of the eIF4GI isoforms (isoform e), and was subsequently suggested to contain intron sequences appended to the ORF (37, 38). These intron sequences were later shown to have promoter activity that could have been misinterpreted as IRES activity in dicistronic assays (39). However another group demonstrated that native eIF4GI mRNA co-sedimented with polysomes during poliovirus infection, suggesting that eIF4GI protein expression does not require cap-dependent scanning (33). Further experiments in our laboratory also supported the hypothesis that eIF4GI mRNA contained IRES activity in a separate region of the mRNA that mapped to the ORF (38).

In order to clarify the eIF4GI expression scheme we characterized the eIF4GI mRNA species expressed in HeLa cells. Here we demonstrate that eIF4GI is expressed from multiple mRNA species that originate from three separate promoters and are further altered by alternate splicing that modifies the leader sequences on mRNAs. We further characterized the IRES element embedded within the ORF on most of the eIF4GI mRNAs. We show that these eIF4GI RNA sequences directed translation initiation internally when placed in dicistronic constructs and this translation was resistant to inhibition of cap-dependent translation in vivo. These data indicate that this eIF4GI translation control element is by convention an IRES. However, we found that the eIF4GI IRES-like element also shares characteristics with shunting sequences, in that it only functions within mG-capped RNA. Taken together our finding support a complex model of eIF4GI translation regulation via both cap-dependent scanning and IRES-mediated mechanisms. Interplay between these mechanisms may dynamically control eIF4GI expression in response to changing cellular translation conditions. Further, an IRES allows the cell to regulate global translation via eIF4GI cleavage and still continue translation of eIF4GI to restore those levels.

**Experimental Procedures**

**RT-PCR**—HeLa cell S10 lysates were extracted with TRIzol reagent (Invitrogen) and total cytoplasmic RNA precipitated according to the protocol provided by the manufacturer. RNA was subjected to reverse transcription (RT) with either avian myeloblastosis virus or moloney murine leukemia virus reverse transcriptase (Promega); in each case the reactions were primed with random decamers (Ambion). RT reactions were then subjected to polymerase chain reaction (PCR) using the Master Mix Taq polymerase (Promega) with a common 3′ primer (5′-gccgaagtctacgcgtctg3′) and 5′ primers selected to amplify endogenous transcribed from each of the three eIF4GI promoters, α-promoter (5′-cgaaattgtttcgactgtctg3′), β-promoter (5′-ctgttgtcgcctggc caggccc3′), or γ-promoter (5′-ttctgactgggctggctggc3′). DNA products were separated by 2% agarose gel electrophoresis, excised, and purified using a gel extraction kit (Qiagen). Purified DNA was ligated into the pNL Easy vectors (Promega) with a common 5′ primer (5′-CCTGTGCCCCGCTGG3′). DNA sequencing of the recovered, amplified DNA was performed using standard oligonucleotides which annealed to the T7 and SP6 sequences that flank each insert (Lon Star Labs).

**DNA Constructs**—pRL-HL is a kind gift from S. M. Lenox (University of Texas Medical Branch, Galveston, TX) and has been previously described (40). pBS/US6Apal is from Y. Shi (Harvard Medical School, Boston, MA), and has also been described (41). pBS/US6-Rli was constructed via sequential insertion of two sets of annealed oligonucleotides. Briefly, oligonucleotides 5′-tgatgagcttgctgata3′ and 5′-agctaatagccgctcctc3′ are annealed and ligated into pBS/US6Apal, which was prepared by digesting with Apal, blunting with T4 polynu-erase, and then digesting with HindIII. This new construct was isolated, digested with HindIII and EcoRI, and then annealed oligonucleotides 5′-agctaatagccgctcctccttg3′ and 5′-aatctaaagtgaattcgtagattga3′ were ligated into the plasmid to generate pBS/US6-Rli. This plasmid mediates DNA polymerase III expression of a ~50-nucleotide RNA hairpin, which is processed by Dicer and subsequently directs degradation of RL ORF containing transcripts via RNA interference (RNAi) (41, 42).

Construction of pRL-XIAp-FL has been detailed previously (43). pSV-βGal, pGL3 Control, and pGL-CMV were obtained from Promega. The construction of pRL-HL and pRL-revHL, which each have a 35-nucleotide polyadenosine tract following the FL ORF were detailed previously (28). pRL-FL was constructed by removing the Neol fragment from pRL-GFP (38) (encoding the CMV promoter and RL) and insertion into the Neol site of pGL3-Enhancer (Promega). pRL-4Go-FL was constructed by obtaining the ~780-bp NolI fragment from pGEM-Teasy-4Go (obtained by RT-PCR) that was inserted into the Bsp1201 site of pRL-GFP to generate pRL-4Go-GFP. 5′ PCR primer was designed based on RACE analysis of HeLa mRNA. Subsequently the ~2.0-kb PsI-Agel fragment from pRL-4Go-GFP was ligated into the PsI-Agel sites of pRL-FL, to produce pRL-4Go-FL.
Nucleic Acid Transfection—DNA transfections into cells were performed using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. RNA was transfected into cells as previously described (46) using the NEBRI-C reagent (Sigma). Poliovirus Infection and Time Course—Preparations of Poliovirus type 1 Mahoney were prepared as previously described (19), and titers were determined by plaque assay on HeLa S3 cell monolayers. Infections at the indicated multiplicity of infection (MOI) were performed in serum-free Dulbecco’s modified Eagle’s medium for 0.5 h followed by replacement of the infection medium with serum containing media. Metabolic labeling of cells was carried out in Dulbecco’s modified Eagle’s medium lacking methionine (Sigma) supplemented with dialyzed 10% fetal bovine serum and 100 μM Tran-label [35S]methionine/ cysteine (Met/Cys) (ICN).

Immunoprecipitation—Cells were lysed in passive lysis buffer (PLB) (Promega) for 10 min at 22 °C, and nuclei removed by centrifugation at 10,000 × g for 10 min. Cell lysate supernatants were precleared by incubation in PLB plus protease inhibitors and 25 μl of 50% protein A-agarose (Sigma) and rotated overnight at 4 °C. After removal of the protein A-agarose beads by sedimentation, 5 μl of monoclonal anti-AFP antibody (Calbiochem) were added to each reaction in addition to 25 μl of fresh protein A-agarose beads (50% suspension), and samples were incubated for 2 h at 4 °C with rotation. After being allowed to settle, beads were washed once with 1 ml of PLB, and twice with 1 ml of IP buffer. Nuclei were washed with 250 μl of NacI, 0.5% Nonidet P-40, and 0.05% deoxycholic acid. Protein was eluted in SDS-PAGE loading buffer.

Luciferase Assay—Luciferase assay were preformed as previously described (28) using a dual luciferase assay kit (Promega).

In Vitro Translation—Translation of transcript RNAs in rabbit reticulocyte lysate (RRL) (Promega) was carried out at 30 °C for 1 h as previously described (38). Preparation of HeLa cell translation lysates was accomplished as described in (21). In vitro translation of transcript RNAs in HeLa cell lysate was performed at 35 °C as described (21). HeLa lysates were supplemented with mGpppG, ApppG, BSA, or Cox-sackie virus B3 (CBX3) 2A protease (2Aaa”) where indicated in the figure legend. The production and purification of CBX3 2Aaa” from bacteria had been previously detailed (45).

Antibodies and Immunoblots—Rabbit anti-eIF4GI antibodies were raised to peptides (RPDDRSQGAIADRPGPLPGEHC) that coupled to keyhole limpet hemocyanin (ProSci, San Diego) using a Imject maleimide activated mcKLH kit (Fierce). This antisera was used in experiments detailed in Figs. 1, 9, and 10. Rabbit anti-eIF4GI antibodies were also obtained from L. Carrasco, University of Cordoba, Cordoba, Spain (Fig. 11). Immunoblots were performed as described previously (38).

RESULTS
eIF4GI Protein Is Expressed from Multiple mRNAs—eIF4GI RNA has been reported to contain IRES activity by several groups, however, there has been confusion generated by reports that discuss two distinct eIF4GI sequences that may lie within unique mRNAs. In order to clarify the eIF4GI expression scheme and determine which sequences may modulate eIF4GI expression and contain authentic IRES elements, we mapped cDNA sequences onto the now complete eIF4GI locus on human chromosome 3. We found that the eIF4GI gene is composed of 33 exons and that all splice sites were in agreement with known splice consensus sequences (46). Furthermore, comparison between human and mouse eIF4GI gene sequences reported in GenBank™ indicated that all exon-intron boundaries are completely conserved in the corresponding murine locus on chromosome 16. Fig. 1A shows a graphical representation of the human locus, with the region corresponding to the 5' leader expanded to show further detail. Analysis of EST sequences suggested the presence of at least three active eIF4GI promoters, which we have labeled α, β, and γ. The existence of the α promoter and the novel exon 1 sequences have not been reported previously, but can be deduced from analysis of EST sequences. We previously reported a complete eIF4GI mRNA sequence that is denoted here as originating from the β promoter. Not that identification of the new α promoter caused us to re-number the eIF4GI exons to reflect
the additional 5’ exon (compared with exon numbering reported in Ref. 38). All nucleotide numbering herein is based on the eIF4GI β transcript, which is the longest, and is consistent with the numbering scheme as presented in (38). The original eIF4GI IRES sequence studied by Gan and Rhoads (36) that we later identified to contain a cryptic promoter corresponds to the region denoted γ in Fig. 1A (39) and was confirmed to lie within an intron between exons 7 and 8 of the eIF4GI gene. We cloned the putative intronic β and γ promoter sequences from human Bac clone RP 11-125E8 into a FL reporter vector (pGL-3) to assay promoter activity in transfected cells. We found that both β and γ intronic sequences did contain significant promoter activity. Furthermore, neither sequence required an enhancer element for significant activity. This indicated that multiple eIF4GI RNAs may be expressed in cells, and that the leader sequences of these RNAs vary.

Our analysis of GenBank™ EST and cDNA sequences also indicated that eIF4GI mRNAs are alternatively spliced within the 5’ leader region (Fig. 1B). Thus, we sought to identify the potential splice variants of eIF4GI mRNA expressed in HeLa cells. HeLa cytoplasmic RNA was subjected to RT-PCR using primers that would anneal to transcripts produced from each of the eIF4GI promoters. Interestingly, multiple RT-PCR products were recovered that were subcloned and sequenced.

Graphical representations of six recovered eIF4GI mRNAs are shown in Fig. 1B. Four of the sequences we isolated from HeLa cells were predicted from existing GenBank™ data. One transcript originating from the β promoter was recovered from HeLa cells. This was not alternatively spliced and corresponded to the full-length eIF4GI 5’ sequence we previously reported (38). In contrast, four unique transcripts were produced from the α promoter and differed by alternate splicing. The longest α promoter transcript encoded an ORF that was identical to the complete eIF4GI ORF previously reported, though its 5’-UTR was unique (38). The remaining three α promoter transcripts encoded N-terminally truncated eIF4GI ORFs. Interestingly, we also recovered a γ promoter transcript from HeLa cells that contained the full 5’-UTR reported by Gan et al. appended to exon 8. Thus, the proposed “intronic IRES” sequence can be found on one mRNA that is expressed in HeLa cells, but is spliced out of all other forms of eIF4GI mRNA. It is still unclear if this sequence contains bona fide IRES activity because it has not been tested in RNA form in cells. Taken together these data indicate that eIF4GI protein can be translated from a variety of mRNAs, and that multiple mRNAs may be simultaneously expressed in cells.

In addition to the regulation of eIF4GI mRNA expression by alternative promoter usage and splicing, we have previously shown that eIF4GI protein expression is subject to translational regulation. We demonstrated that each of the five in-
frame AUGs in the 5’-end of eIF4GI mRNA can be used to initiate translation, thus giving rise to five eIF4GI protein isoforms with progressively shorter N termini (38). Typically, all five eIF4GI isoforms are not discernible by immunoblot. However, cleavage of eIF4GI by viral 2Apro allows sufficient separation of the N-terminal cleavage fragments in gels for each of the isoforms to be seen (Fig. 1D, lane 2). Thus, each individual eIF4GI mRNA described above may allow translation of a set of eIF4GI isoforms. This scheme has been graphically depicted in Fig. 1B, along with notation indicating which eIF4GI protein isoforms may be derived from each mRNA.

cis-Acting elements within the eIF4GI leader also modulate translation of eIF4GI isoforms. We previously found that the eIF4GI 5’-UTR derived from the β promoter down-regulates eIF4GI expression (38). This leader region is predicted to contain a large stable stem loop that would likely inhibit scanning of ribosomes (Fig. 1C). Interestingly, the 5’-UTR in transcripts from the α promoter also has a predicted strong secondary structure which may down-regulate cap-dependent scanning (Fig. 1C) (48), though this structure is smaller and less stable than the β leader structure (Fig. 1C). Translational regulation of eIF4GI is also partially mediated by a uORF that we demonstrated is actively translated from an AUG in exon 2 (Fig. 1, A and B, indicated by an asterisk). Previous data indicated that in β promoter-derived transcripts, ribosomes can initiate at the uORF and down-regulate overall eIF4GI translation, with the greatest effect on translation of the “a” isoform. As shown in Fig. 1B this uORF is maintained in most of the eIF4GI mRNA splice variants though the length of the uORF varies. These alterations in the size and overlap of the uORF and the primary eIF4GI ORF may alter protein isoform expression from these mRNA splice variants. The α leader also encodes an additional uORF close to the 5’-end, however, the AUG is in poor context, and may be too close to the 5’-cap on some transcripts for efficient initiation to occur (49). Finally, the IRES activity identified within the 5’-eIF4GI mRNA sequence may further alter the eIF4GI expression pattern from each mRNA (33, 38). Previous data indicated that IRES activity is encoded in sequences expressed within exons 3–8, thus, many of the alternatively spliced eIF4GI mRNAs may encode a functional IRES (38). Taken together, these findings illustrate that eIF4GI translation is regulated by a complex set of cis-acting elements originating from several mRNA species.

Mapping of eIF4GI IRES Activity in Vitro—We cloned the eIF4GI 5’ leader sequences (including exons 1–8) into a RL-FL dicistronic reporter in order to better define the translational regulation of eIF4GI isoform expression and investigate IRES activity. To confirm that fusion of extensive eIF4GI amino acid sequences to FL did not adversely effect FL enzymatic activity, we translated monocistronic FL or 4GΔ275-FL RNA (in which the first 199 amino acids of eIF4GI are fused to FL) in RRL (Fig. 2B, lanes 1 and 2, respectively). Comparison of FLuc

---

**Fig. 2. Mapping eIF4GI cis-regulatory elements in vitro.** A, schematic representation of dicistronic RNAs used for mapping studies. The RL ORF is labeled and indicated in gray, and the FL ORF is in black. In-frame eIF4GI initiation codons are indicated by black arrowheads above each diagram. uORFs are represented below each diagram, arrowheads indicate initiation codons, the ORF length is indicated by the line, and the stop codon by the bar terminating each line. The RNA hairpin in hpFL-4GΔ275-FL is graphically depicted. C, capped monocistronic RNAs T7-FL (left) or 4GΔ275-FL (right) were translated in RRL, in the presence of [35S]Met/Cys. Translation reactions were either assayed for FL activity or separated by SDS-PAGE, and gels exposed to film. Translation products were quantitated by luciferase activity or by scanning densitometry of autoradiograms (S.D.). In each case FL translation was normalized to one, and the relative translation activity of p4GΔ275-FL calculated. C, capped RNAs transcribed from linearized plasmids were used to program RRL in vitro translation reactions, then analyzed by 10% SDS-PAGE, and autoradiography. The upper panel depicts an autoradiogram of translated FL and FL fusion proteins, while the lower panel depicts RL translation products taken from the same gel. The construct tested in each reaction is indicated below the panels, and in the case of pRL-4Gα-FL the eIF4GI isoforms corresponding to each FL fusion protein are indicated to the right. The numbers on the left indicate the migration of molecular mass markers in kDa. D, densitometric analysis of eIF4GI-FL fusion proteins in B were quantitated using Image J. 3’ deletion constructs are depicted with gray bars and 5’ deletions with white bars. Total values for FL-fusion bands were divided by values for RL bands to control for equal gel loading and then graphed.
enzymatic activity to protein expression levels indicated there was no significant difference between the activity of FL alone or when fused with the eIF4GI N terminus (Fig. 2B). In three separate experiments when activity was calculated by densitometry or by luciferase activity results never varied by more than 10%. These data are consistent with previous observations that N-terminal FL fusions do not significantly diminish FL enzymatic activity (50).

In order to define sequences that mediate eIF4GI IRES activity, we analyzed a series of dicistronic pRL-FL RNAs containing sequences corresponding to RNAs produced from the eIF4GI α and β promoters (Fig. 2A). When dicistronic RNAs containing all 8 exons were translated in RRL, the α and β promoter-derived leader sequences were found to produce identical fusion translation products corresponding to all five eIF4GI isoforms (plus native FLuc originating from its own AUG) (Fig. 2C, compare RL-4Gβ-FL to RL-4Ga-FL, lanes 9 and 10). Thus, inclusion of the α or β leader region did not detectably alter overall protein translation, vary alternate AUG codon usage or apparent IRES activity in a dicistronic context in vitro.

Deletion of eIF4GI 5′ and 3′ sequences in dicistronic RNAs altered the mobility of product eIF4GI-FL fusion proteins based on the removal of initiation codons (5′ deletion), or the deletion of intervening amino acids (3′ deletion) respectively. Interestingly, several of these deletion constructs (RL-4G610A-FL, RL-4G497-FL, RL-4G697-FL, Fig. 2C, lanes 3, 5, and 6) still contained IRES activity in this assay. Further, deletion of eIF4GI nucleotides 1–696 (pRL-4G697-FL, lane 6) abrogated alternate AUG usage, despite the presence of the AUG codons corresponding to the “d” and “e” isoforms. On the other hand, all other deletion constructs maintained the ability to initiate at each of the eIF4GI AUGs that were present. These data indicate that sequences between nucleotides 497 and 697 may be necessary for alternative AUG usage by the eIF4GI IRES sequence.

As previously observed, removal of either α or β eIF4GI 5′-UTRs (pRL-4GΔ275-FL) enhanced translation from the “a” isoform initiation codon since the uORF inhibits efficient initiation on the “a” AUG (38) (Fig. 2C, lane 8). Similar results were observed when eIF4GI nucleotides 1–497 were deleted (pRL-Δ497-FL, lane 5), because initiation on the most 5′- AUG (“c”) was enhanced (compare with pRL-4Ga-FL and pRL-4Gβ-FL, lanes 9 and 10).

To distinguish re-initiation, which occurs in RRL (see pRL-FL in Fig. 2C, lane 2), from IRES activity per se, we placed a thermodynamically stable ($\Delta G = -60$ kcal/mol) hairpin upstream of the RL ORF to block 5′ scanning (51). Fig. 2C shows that inclusion of the RNA hairpin was able to reduce upstream RL translation by greater than 95% (lane 7), however translation of downstream FL fusion proteins was reduced by less than 50% (values quantitated by scanning densitometry) (38). These data are similar to observations with other IRES elements and indicate ribosomes can initiate on FL and RL AUGs independently (28, 44). Thus, although a fraction of FL translation in this assay may be caused by re-initiation, IRES activity is present.

We quantitated overall IRES activity (adding contributions from each eIF4GI-FL fusion protein) in translation by scanning densitometry (Fig. 2D). HCV IRES activity (pRL-HL) was relatively weak in RRL compared with a negative control (pRL-FL). This may be caused by a lack of specific ITAFs in RRL compared with other lysate systems, as has been previously observed (52, 53). In contrast, all IRES activity from eIF4GI sequences was more robust and stronger than the HCV IRES in this assay (38). IRES activity in constructs containing the two different (α or β) eIF4GI leader regions was similar (compare pRL-4Gα-FL and pRL-4Gβ-FL), though removal of the leader stimulated IRES activity in the dicistronic context in RRL. This latter result was in contrast to in vivo experiments reported below. Large 5′ or 3′ deletions in RNAs reduced overall IRES activity (pRL-Δ697-FL and pRL-339A-FL). Together, these data indicate that the eIF4GI IRES is active (relative to HCV IRES) in vitro, suggesting that it may not require ITAFs that are limiting for other IRESs in this system. Further, eIF4GI sequences between nucleotides 497 and 697 may be crucial for directing alternative AUG usage. However, the sequences responsible for eIF4GI IRES activity could not be specifically identified by these experiments.

**Expression of Dicistronic eIF4GI Deletion Constructs in Vivo**—To further examine which regions of eIF4GI RNA are required to direct internal ribosome entry, dicistronic DNA constructs were transfected into 293T cell monolayers. Cells were harvested 48 h post-transfection, and lysates assayed for luciferase activities (RL and FL). The HCV IRES (pRL-HL) was used as a positive control for IRES activity, whereas pRL-FL controlled for the level of re-initiation that may occur on the dicistronic RNAs in vivo. The data show the HCV IRES had significantly higher activity than all eIF4GI dicistronic reporters in vivo, contrasting to in vitro results (Fig. 3). The eIF4GI α and β leader sequences were found to have similar IRES activity in vivo (compare pRL-4Gα-FL to pRL-4Gβ-FL) which agreed with in vitro data. Furthermore, deletion of the nucleotides from 611–873 (pRL-4G610A-FL) had little effect on IRES activity. However, deletion of nucleotides 340–873 (pRL-4G339A-FL), which leaves only the α 5′-UTR and part of exon 3 intact, completely abrogated IRES activity. These data suggest that the minimal IRES sequences may be between nucleotides 339 and 610 (e.g. within exons 4, 5, or a portion of exon 6).

However, the effect of 5′ deletions was quite different than 3′ deletions. All 5′ deletions tested displayed some IRES activity, but none totally abrogated activity (Fig. 3). Each deletion construct, Δ275 (deleting the 5′-UTR), Δ497 (deleting exons 1–4 and part of 5), and Δ697 (in which only exons 7 and 8 remain) displayed activity that was significantly above that of pRL-FL control vector ($p > 0.001$). A single internal deletion construct was also tested (RL-4G65Δ-FL) in which sequences encoding exon 5 were removed. Surprisingly, this construct displayed higher IRES activity than full-length sequences, possibly indicating the presence of negative regulatory elements within exon 5. Taken together, these data suggest that at least two
The eIF4GI sequences encode a weak promoter or splice site. Indicated test DNA reporter constructs were co-transfected into 293T cells along with pBS/κGal and either pBS/U6ApaI (+) or pBS/U6-RLi (+). Control cells were co-transfected with two test plasmids, (pRL-CMV and pG3L) to express RL and FL from independent RNAs. Forty-eight hours post-transfection, cells were harvested and assayed for FL, RL, and β-galactosidase enzymatic activity. Values for FL and RL were divided by β-galactosidase values to normalize for transfection efficiency. The amount of remaining FL and RL activity from constructs, when co-transfected with pBS/U6-RLi, is noted as a percent above the appropriate bar graph.

IREs elements may lie in the eIF4GI 5’-UTR, one on exon 4 that is stronger, and one in exons 7–8 that is weaker. Further, though the eIF4GI 5’-UTR is not necessary or sufficient for IRES activity, it does appear to enhance IRES activity when exons 3–8 are all present.

The eIF5GI 5’ Leader Encodes a Weak Cryptic Promoter or Splice Site—We wanted to rule out confounding effects contributed by possible cryptic promoters or splice sites in eIF4GI sequences expressed from dicistronic vectors. eIF4GI-FL fusion constructs lacking the CMV promoter were tested in transfection experiments, and were found to have very low activity as compared with pGL3-control plasmid. This low activity likely resulted from the presence of the SV40 enhancer region in these plasmids because it was comparable to activities from transfections of pGL3-enhancer plasmid with no inserted sequences (data not shown). We further analyzed RNA integrity in vivo using an siRNA method (28, 43). Previous Northern analyses indicated that dicistronic DNA constructs containing eIF4GI sequences (nucleotides 275–873) are expressed in transfected cells as a single RNA (38). However, Northern blots may not be sensitive enough to detect minor transcription or splice products (48). Therefore, dicistronic DNA expression vectors were co-transfected with an RNAi vector targeting Renilla luciferase. This resulted in silencing of RL expression by 70–80% (Fig. 4). This RL-specific RNAi response lowered expression of RL and FL by equivalent amounts in cells co-transfected with pRL-XIAP-FL and eIF4G-FL, further supporting the hypothesis that the eIF4GI leader requires a m7G cap for translation in vitro. In contrast, FL expression increased when pRL-XIAP-FL was co-transfected with the RNAi vector, indicating that significant levels of FL expression originated on monocistronic or aberrantly spliced RNAs not containing the RL target sequence. Aberrant splicing from this construct has been previously demonstrated (43). When pRL-4Gα-FL was examined, expression of RL RNAi reduced RL activity to 19% of control values. FL activity also was reduced, but only to 49% of control values. This incomplete reduction of FL activity indicated that most of the FL protein was produced from intact dicistronic RNA, however a subset was also translated from RNA resistant to RL RNAi silencing. Similar results were observed previously when the Bcl-2 5’-UTR was tested by this siRNA assay, and this sequence was subsequently shown to encode an active splice acceptor site (44). These data indicate that in cells transfected with pRL-4Gα-FL at least some of the mRNA produced is not dicistronic, therefore a weak cryptic promoter or splice site is encoded within the eIF4GI IRES sequence.

The eIF4GI IRES Is Active in Dicistronic RNAs Directly Transfected into Cells—Based on the results above, we further examined eIF4GI sequences for IRES activity using capped and polyadenylated dicistronic RNAs directly transfected into 293T cell monolayers, as previously described (43). RNAs were labeled with [32P]ATP so that their condition could be examined after transfection. Denaturing gel analysis of test RNA recovered from transfected cells showed the RNA was still full-length and was not processed (e.g. spliced) within the cell (Fig. 5B), in agreement with previously published data (43). The RNA doublet observed in the RL-HCV-FL and RL-revHL lanes is the result of early termination by T7 polymerase in vitro, and is also observed in non-transfected RNAs (data not shown). In Fig. 5A, FL values were normalized to RL to control for transfection efficiency. We found that by this assay eIF4GI IRES activity was about 35–40% as active as the HCV IRES, and well above the background seen in a negative control construct (RL-revHL). As in previous experiments the two different eIF4GI leaders (RL-4Gα-FL and eIF4Gβ-FL) again displayed similar IRES activity. Examination of several of the eIF4GI deletion constructs by this method yielded results that were analogous to those seen with DNA transfection experiments, shown in Fig. 3 above. Specifically, the removal of the entire 5’-UTR (RL-4GΔ275-FL and RL-4GΔ697) reduced IRES activity, but did not totally abrogate it, and deletion of nucleotides 610–873 (RL-4GΔ610-FL) had little effect on IRES activity. Additionally, the deletion of exon 5 sequences enhanced IRES activity as seen previously in DNA transfections. Taken together, the RNA transfection data support mapping studies performed by DNA transfection experiments and are also consistent with the presence of IRES sequences within exons 3–8. Additionally these studies suggest that nuclear modification, which is hypothesized to be important in the function of some IRES elements, is not essential for the function of the eIF4GI IRES.

The eIF4GI IRES Displays a Novel Cap Dependence—In the course of RNA transfection experiments we compared expression of 32P-labeled RNAs that were capped with either m7GpppG, or AppppG, as a stabilized control for uncapped RNA. Surprisingly, we found that when A-capped RNAs containing eIF4GI IRES sequences were transfected into cells, the IRES activity was specifically inhibited 10-fold compared with m7G-capped RNA (Fig. 6A). We have previously shown that capped and A-capped RNAs transfected into cells are stable (28). We found that less AppppG capped RNA was recovered from cells following our protocol, however it is not clear if this is reflected a reduction in either transfection or stability in these RNAs (Fig. 6B). This minor difference, however, did not correlate with FL expression from the HCV IRES, which was higher with AppppG-capped RNA (Fig. 6A) and was unlikely to account for greater than 10-fold reduction in synthesis of FL from the eIF4GI IRES constructs (pRL-4Gα-FL and p4Go-FL). Taken together, these data suggest that the eIF4GI leader requires a m7G cap for its activity, both in monocistronic and dicistronic constructs.

The eIF4GI Leader Requires a Cap for Translation in Vitro—The apparent cap-dependent phenotype of the eIF4GI IRES does not fit current models for IRES-dependent translation initiation, therefore we further explored its cap requirements in vitro. It has been previously observed that translation in RRL does not proceed via the stringent cap-dependent mechanism that predominates in cells (54), thus we assayed translation directed by the eIF4GI leader in the cap-dependent HeLa
In agreement with previous observations we found that monocistronic FL transcripts translated 6-fold more robustly when capped with m7GpppG than with ApppG (Fig. 7B). On the other hand monocistronic RNA encoding the HCV IRES upstream of FL translated with similar efficiency regardless of the presence of the m7G cap structure (pHL in Fig. 7B). Translation directed by the eIF4GI leader was highly cap-dependent in the HeLa lysate system since m7GpppG- capped RNAs translated 8-fold more efficiently than their ApppG-capped counterparts. These differences could not be explained by a difference in RNA stability because, as previously observed, 32P-labeled RNAs extracted after translation reactions were found to be intact in each case (Fig. 7C). These data mirror the results of RNA transfection experiments, and suggest that translation directed by the eIF4GI leader is dependent upon a cis m7G cap.

**Exogenous m^7^GpppG Does Not Inhibit Translation Directed by the eIF4GI Leader**—To further characterize the cap dependence of eIF4GI translation control elements we conducted translation experiments in HeLa cell lysates in which cap-dependent translation was competitively inhibited by the addition of excess cap analog. As expected, addition of 0.1 mM and 0.25 mM m^7^GpppG inhibited translation of FL and RL from monocistronic mRNAs (pT7-FL and pRL in Fig. 8, A and B, respectively). Translation inhibition for each of these RNAs was reached 6–7-fold at 0.25 mM m^7^GpppG (Fig. 8C). The

**FIG. 5.** The eIF4GI IRES is active in dicistronic RNAs transfected into cells. 32P-labeled, capped, T7 run-off transcripts from indicated DNAs (which each contained templated poly(A) tails) were transfected into 293T cells. Eight hours post-transfection, cells were harvested either into passive lysis buffer for enzymatic assay or TRIzol for RNA analysis. A, FL activity/RL activity has been graphed for each indicated RNA construct as a measure of relative IRES activity. Error bars represent S.D. from the mean for three experiments. The deletion constructs are depicted with gray (3') or white (5' deletion) bars. B, 2 μg of total RNA extracted from transfected cells were analyzed on a 1% agarose-glyoxal-Me2SO gel, transferred to nylon membrane, and exposed to film (upper panel). As a control for equal loading, the membrane was also stained with methylene blue to visualize 18 S rRNA (lower panel).

**FIG. 6.** The eIF4GI IRES is cap-dependent in vivo. m^7^GpppG- or ApppG-capped, 32P-labeled T7 transcripts from the indicated constructs were transfected into 293T cells (in each case RNA included templated poly(A) tails). Eight hours post-transfection, cells were harvested and assayed for luciferase activity or extracted with TRIzol for RNA Analysis. A, values for FL activity from each transfection were calculated and normalized based on the amount of RNA (cpm) that was isolated from cells following transfection (calculated by scintillation counting). Results for monocistronic and dicistronic RNAs are each from three separate experiments. B, 32P-labeled RNA was isolated from cells and separated by 1% agarose-glyoxal-Me2SO gel electrophoresis, as described in Fig. 5. The migration of 28 S and 18 S rRNA is indicated on the left.

**FIG. 7.** Translation from the eIF4GI 5' leader is cap-dependent in vitro. A, schematic of monocistronic polyadenylated RNAs. B, m^7^GpppG or ApppG capped RNAs transcribed from the indicated plasmids were translated in HeLa cell lysates. The fold stimulation in translation activity mediated by m^7^G-cap (versus A-Cap) was calculated for three separate experiments, averaged and graphed. Error bars indicate S.D. C, the indicated 32P-labeled RNAs were extracted from HeLa cell lysates after 30 min of translation and analyzed by denaturing gel electrophoresis/autoradiography. Membranes were stained with methylene blue to visualize 18 S rRNA (bottom panel) as a loading control, and the migration of 18 S and 28 S rRNA is indicated to the left.
addition of 0.25 mM AppG however did not inhibit translation from the monocistronic RNAs (Fig. 8, A and B). Conversely, translation directed by the HCV IRES (pRL-HL) was enhanced by the addition m7GpppG, while RL translation from the first cistron was simultaneously reduced. This reduction in RL translation by m7GpppG was also observed in experiments with dicistronic pRL-4G-FL RNA, where RL translation was inhibited by ~4-fold (Fig. 8C). However, downstream FL translation directed by the eIF4GI sequences was slightly enhanced by competitive inhibition with cap analog. This demonstrates resistance of eIF4GI sequences to cap-analog translation restriction and independence from the upstream cistron, two hallmarks of known IRES elements. Interestingly, translation from monocistronic p4G-FL was also insensitive to cap-analog inhibition (Fig. 8, A and C). These data suggest that while the translation mediated by the eIF4GI leader requires a m7G-cap in cis, it is not inhibited by exogenous m7G-cap in trans. This is in contrast to translation of control monocistronic RNAs (pT7-FL and pRL) in which translation was reduced in dose-dependent fashion by m7G-cap in trans. However, the behavior of the eIF4GI IRES was also distinct from the HCV IRES that was stimulated by exogenous m7G-cap.

Translation from the eIF4GI IRES Is Enhanced by 2A Protease Activity—We next sought to determine if the activity of the eIF4GI IRES was also dependent upon intact eIF4GI protein in the translation lysate. Test RNAs were translated in HeLa cell lysate that had been pretreated with increasing amounts of recombinant Coxackievirus 2A protease (2Apro), which rapidly cleaves eIF4GI, causing inhibition of cap-dependent translation. To monitor the activity of the 2Apro in these experiments, eIF4GI integrity was assessed prior to translation and immediately after translation was stopped (Fig. 9C). These data show eIF4GI cleavage was complete at high 2Apro concentrations after 5 min of incubation, but not at the lower concentration. In both cases eIF4GI cleavage was complete following translation (35 min) indicating that physiologically relevant concentrations of active 2Apro were used.

We found that addition of 2Apro moderately inhibited translation of capped mRNAs, reducing FL activity from pT7-FL by about 2-fold (Fig. 9, A and D). Similarly, upstream RL translation from dicistronic RNAs, pRL-HL and pRL-4G-FL (Fig. 9, B and D) was also inhibited 2-fold. We have previously shown that more significant inhibition of translation in HeLa lysates requires both 2Apro and viral 3Cpro (which cleaves poly(A)-binding protein) (21).

Conversely, addition of 2Apro enhanced translation directed by the HCV IRES in monocistronic RNA (pHL in Fig. 9, A and D) or had no effect in dicistronic RNA (pRL-HL in Fig. 9, A and D). These results are consistent with known requirements of the HCV IRES, which include eIF3, but not eIF4G. Similarly, translation of FL from monocistronic p4G-FL was enhanced ~2-fold by the addition of 2Apro. Interestingly, however, FL synthesis from dicistronic pRL-4G-FL was strongly enhanced by 2Apro, reaching a maximal enhancement of ~6-fold. Also, the eIF4GI IRES activity from dicistronic RNAs, which was weak in the HeLa lysate system, was stimulated to levels similar to those obtained for monocistronic p4G-FL following 2Apro treatment. These data are consistent with the report that some native eIF4GI mRNA remains polysome-associated during PV infection, at times when eIF4GI is cleaved (33). Furthermore, despite requiring an m7G-cap in cis, the eIF4GI IRES activity does not require intact eIF4GI, and is activated by 2A protease when cap-dependent translation is inhibited.

The eIF4GI IRES Retains Activity after Shutoff of Cap-dependent Translation in Vivo—In order to directly assess eIF4GI IRES translation activity during times when eIF4GI is cleaved we determined its activity during PV infection. For these experiments, monocistronic expression vectors producing eIF4GI fusion proteins were transfected into 293T cells and cells were subsequently infected with poliovirus. At regular intervals post-transfection cells were metabolically labeled with Tran label [35S]Met/Cys and fusion proteins were immunoprecipitated from lysates. This protocol allowed us to determine which of the five eIF4GI AUG initiation codons were utilized in vivo under these conditions. Unfortunately, repeated transfections of RNA encoding eIF4GI-FL fusion proteins did not yield enough translation product to be visualized clearly by immunoprecipitation. Therefore, we transfected cells with monocistronic DNA constructs encoding two eIF4GI-EGFP fusion proteins, diagramed in Fig. 10.

We found that by 5-h postinfection endogenous mRNA translation and translation of EGFP in transfected cells had shutoff by over 75% (Fig. 10, A and D). However in cells transfected with p4Go-EGFP or p4GΔ275-EGFP translation of eIF4GI-EGFP fusion proteins was maintained at control levels. Furthermore, all five isoforms of eIF4GI were synthesized from
both RNA constructs during PV infection. Early in infection before eIF4GI was cleaved (2 h) or in mock-infected cells there was a difference in translation of p4G/H9251-EGFP and p4G/H9004275-EGFP construct mRNAs. The full-length eIF4GI leader RNA mediated initiation at all five eIF4GI initiation codons (indicated a-e), and alternate initiation codon selection among codons was relatively equivalent (Fig. 10D). In contrast, p4G/H9004275-EGFP RNA translated predominantly from the first AUG corresponding to the eIF4GI isoform "a" and alternate AUG selection was partly suppressed. These data are consistent with our previous results, which indicated that the eIF4GI 5'-UTR and uORF down-regulates translation initiation at the AUG corresponding to the "a" isoform in favor of the other initiation codons (Fig. 2) (38). Following PV infection and cleavage of endogenous eIF4G (Fig. 10C), both RNAs translated similarly to mock-infected cells (Fig. 10D, time points 3–5 h). Thus, translation of eIF4GI-fusion proteins isoforms is not affected by poliovirus infection under conditions where eIF4GI protein is cleaved and cap-dependent scanning is inhibited.

**Translation of Endogenous eIF4GI Is Maintained During Poliovirus Infection**—It has been reported that some eIF4GI mRNA remains polysome-associated during PV infection, however, the nature of eIF4GI isoforms expressed in infected cells are unknown. We directly assayed eIF4GI IRES mediated translation in PV-infected cells via pulse-label/immunoprecipitation experiments. First, at times when nascent eIF4GI was cleaved, translation of eIF4GI was maintained (Fig. 11A). Translation of isoforms a, b, and c was observed, and the
identities of these bands were confirmed by Western blot of immunoprecipitated proteins that were separated by SDS-PAGE. However, a contaminating background protein migrates just below the cleaved eIF4GI c isoform, so the d and e isoforms could not be resolved (data not shown). However, translation of the d and e isoforms is likely maintained, as evidenced by the intense band for the full-length eIF4GI d/e observed in Fig. 11, A and B.

Surprisingly, we found that after eIF4GI cleavage is complete (by Western blot, Fig. 11, C and D) nascent full-length eIF4GI can still be detected by IP (see Fig. 11D, 3.5 and 4.7 h time points). This apparent delay in the cleavage of newly synthesized eIF4GI was observed in all experiments and may indicate that newly translated eIF4GI does not assume a conformation susceptible to protease cleavage, or may not rapidly enter a protein complex that enhances its susceptibility (e.g., binding to eIF4E) (55). We also noted that full-length isoforms d and e were more strongly labeled during infection than isoforms a-c. Since the 2Apro cleavage products of eIF4GI isoforms d and e could not be resolved, we cannot determine if translation of these isoforms is enhanced during PV infection, or if their cleavage following synthesis is less efficient than the larger (a-c) eIF4GI isoforms. Finally, we found that eIF4GI translation was completely shut off late in infection (by 6 h) in high MOI infections (Fig. 11B). This is consistent with observations that late in PV infection all translation, even that of viral polyproteins, is shutdown. Taken together, these data indicate that native eIF4GI mRNA is efficiently translated at times when eIF4GI protein is cleaved and cap-dependent translation is suppressed. All native eIF4GI isoforms were expressed during PV infection, suggesting that alternative AUG selection on eIF4GI mRNA is maintained during infection.

**DISCUSSION**

We report here that eIF4GI expression can be controlled via multiple mechanisms. eIF4GI mRNA is produced from three alternative promoters in HeLa cells and sequence data from GenBank indicates that the length of the 3′-UTR can be altered based on two alternative polyadenylation cleavage sites. Further, we demonstrate that eIF4GI mRNAs are alternatively spliced in HeLa cells to generate an array of mRNAs from which eIF4GI may translate. These RNAs are subject to further regulation at the level of translation initiation. Full-length eIF4GI mRNAs can translate up to five eIF4GI protein isoforms via alternative AUG selection. Furthermore, translation is modulated by the presence of an IRES that is embedded in the ORF and a regulatory 5′-uORF (38). By deletion studies we were able to demonstrate that eIF4GI IRES activity may be encoded in at least two regions within the mRNA and the stronger activity may map to exon 4. However, alternative AUG selection required sequences encompassed by a 200-nucleotide region of the eIF4GI mRNA between nucleotides 497 and 697. Despite the ability of eIF4GI RNA sequences to direct translation initiation from dicistronic RNAs, the IRES(s) only functions efficiently within capped RNAs. We also demonstrated that eIF4GI IRES activity is enhanced when eIF4G is cleaved by viral 2Apro. Accordingly, eIF4GI translation was maintained during PV infection, despite complete cleavage of endogenous eIF4G.

Recently, the validity of some reported IRES elements have come under heavy scrutiny in the literature. In particular it has been pointed out that the dicistronic DNA test used in defining and mapping IRES sequences could, in fact, indicate the presence of promoter or splice acceptor sequences (48, 56). We and other groups have shown that when dicistronic DNA expression vectors are used to assess the activity of IRES sequences, the presence of cryptic promoters and splice acceptor sites can yield apparent IRES activity. Furthermore, in some cases analysis of dicistronic RNA integrity by Northern blot was insufficient to rule out the presence of weak promoters or inefficient splice acceptor sites (43, 57). Thus, we used an siRNA method to determine that the eIF4GI sequences in question did contain a weak promoter or splice site that was active in vivo (Fig. 4). As a result, we confirmed the presence of an IRES using transfected dicistronic RNA. The results obtained from transfection of dicistronic DNAs and RNAs were in close agreement, indicating that aberrant production of monocistronic FL RNA from the eIF4G-dicistronic DNA vectors constituted only a small fraction of the observed FL activity. Additionally, since dicistronic RNA and DNA transfections yielded similar results it is unlikely that nuclear RNA processing events are required for the translation regulatory activity of eIF4G sequences (25).

Our results suggest that the translation control regions in the eIF4G1 leader and ORF share characteristics of both an IRES and a shunt element. As expected for a canonical IRES, the eIF4G1 sequences direct internal translation initiation in dicistronic constructs. These results have been recapitulated via two methods of in vitro analysis (RRL and HeLa lysate translations) and two in vivo analyses (DNA and RNA transfection of dicistronic constructs). These analyses yielded similar results; the eIF4G1 sequences could direct internal initiation at low basal levels (compared with the HCV IRES) (Figs. 3, 5, 8, and 9). However, basal translation could be enhanced by the addition of 2Apro in in vitro translation experiments (Fig. 9).
in direct opposition to RNAs that are cap-dependent, where translation was blocked by 2Apro. Translation directed by the eIF4GI sequences in dicistronic RNAs was not dependent on translation of the upstream cistron, as demonstrated by inhibition of RL translation via a stable RNA hairpin (Fig. 2 and Ref. 38) or via cleavage of eIF4G with 2Apro (Fig. 9). As expected for an IRES, addition of exogenous cap (m7GpppG) to HeLa in vitro translation reactions inhibited first cistron translation, but had little effect on translation of the second cistron directed by eIF4GI sequences. Based on these data the eIF4GI RNA sequences examined here could be categorized as a functional IRES.

However, the eIF4GI translation control element also shares certain characteristics with cap-dependent translation shunts. Translation mediated by eIF4GI RNA sequences was found to be dependent upon a cis-acting m7G cap structure, both in vivo (Fig. 6) and in vitro (Fig. 7). These results cannot be explained by a model requiring simple cap-dependent scanning, since the eIF4GI sequences mediated internal initiation from dicistronic RNAs. However the phenotype of this control element is reminiscent of shunt-mediated translation initiation, in that shunt elements promote cap-dependent translation initiation and allow ribosomes to “jump-over” large regions of 5’ RNA sequence (58). Despite their typical cap-dependence, some ribosomal shunts can also facilitate translation during PV infection or when eIF4F function is restricted, a characteristic shared with both eIF4GI sequences and many other IRES sequences (34, 59). However, invoking a shunt model for eIF4GI translation initiation is problematic since shunt elements typically require a cap-proximal donor sequence upon which ribosomes initially scan prior to shunting (47). In the dicistronic constructs analyzed in this study, no eIF4GI sequences are included in the short RL 5’-UTR.

Based upon these observations we propose that the eIF4GI translation control element is most accurately defined as a cap-dependent IRES. There are several ways in which a m7G-cap structure might participate in the function of the eIF4GI IRES, independent of its role in cap-dependent scanning. It is possible, for instance, that the eIF4GI IRES requires eIF4F in a m7G-bound conformation for IRES activity. This hypothesis would explain why m7G is required by the IRES in cis, however excess m7G did not inhibit IRES function in trans (Fig. 8). In either case eIF4F would be present in a “cap-bound conformation”. However, this hypothesis is too simplistic to explain the continued activity of the IRES when eIF4GI is cleaved and thus separated from eIF4E and the cap (e.g. during PV infection or in vitro 2Apro treatment). It is possible that 2Apro-cleaved eIF4GI assumes a conformation similar to when it is bound to eIF4E and the m7G cap. It has been shown previously that the HAV IRES, which requires intact eIF4F in a cap-unbound state, is inactive both when eIF4G is cleaved (by FMDV 1Apro) or when eIF4F is bound to m7G-cap (5). The data here may support a model in which 2Apro cleaved eIF4GI is in a conformation similar to that of eIF4F-m7G bound eIF4G. Alternatively it is possible that the m7G-cap plays a novel structural role in the eIF4GI IRES itself. However, this is less likely because in dicistronic RNAs the cap structure would be required to simultaneously initiate translation of the first cistron, and participate in interactions with the IRES downstream.

We have attempted to elucidate the elements within the eIF4GI 5’ leader that control eIF4GI protein expression. In vitro deletion mapping of eIF4GI IRES activity (Fig. 2) demonstrated that multiple regions within the eIF4GI RNA may mediate internal initiation. However, these experiments did demonstrate that the region between nucleotides 497 and 697 is important for alternative initiation codon selection on AUGs corresponding to the d and e isoforms. In a construct lacking these sequences (RL-4GΔ697-FL) IRES activity was exhibited, however, translation initiation occurred only on the FL AUG (Fig. 2B). This 200-nucleotide region may constitute a placement domain for an IRES because ribosomes could then scan to the downstream d and e initiators.

In accordance with our previously published data, we found that the eIF4GI 5’-UTR was not absolutely required for IRES activity (Figs. 2, 3, and 5), and indeed when tested in isolation the 5’-UTR had no IRES activity (Fig. 3). Thus, the IRES sequences are embedded within the eIF4GI ORF, implying that ribosomes must be placed upstream of the IRES(s) location to translate the longer isoforms (e.g. a or b). Our data does indicate that the eIF4GI 5’-UTR modulates the isoform expression profile from eIF4GI RNA, specifically down-regulating expression of the a isoform (38). This phenomenon is observed in both monocistronic and dicistronic expression vectors, thus, the 5’-UTR is not solely involved in regulation of cap-dependent scanning (Figs. 2 and 10). Based upon the IRES-enhancing effect demonstrated for the 5’-UTR sequence, we propose that these sequences play a role in placement of ribosomes by the IRES(s).

Clearly, the regulatory strategy controlling expression of eIF4GI is very complex, involving transcription, splicing and translation regulation. Each level of regulation can potentially control total eIF4GI expression and modulate expression of individual protein isoforms. For instance, splicing can modulate the isoform coding potential of mRNAs, in addition to determining which translation regulatory elements are present to regulate protein synthesis. It is important to note that translation initiation on native eIF4GI mRNAs likely occurs by both canonical cap-dependent scanning and via the eIF4GI cap-dependent IRES. The interplay between these two mechanisms would provide control over eIF4GI expression, which would be responsive to cellular translation conditions. When cellular cap-dependent translation is ongoing, translation of eIF4GI could occur partly via cap-dependent scanning, but translation would be limited by the secondary structure of the 5’-UTR and presence of the regulatory uORF. On the other hand when cap-dependent scanning is down-regulated and nascent eIF4GI cleaved, as during stress or PV infection, the eIF4GI IRES would allow for continued de novo synthesis of eIF4GI protein. This scheme would allow for eIF4GI cleavage and/or degradation, as a means of translation control, while the cell still retains the ability to synthesize and replenish intact eIF4GI de novo.

It is clear from our results that regulation of eIF4GI gene expression extends to controlling the synthesis of specific protein isoforms. It follows that eIF4GI isoforms may have distinct functions within the cell (e.g. the e isoform lacks the PABP binding domain and may promote translation initiation of non-polycadenylated mRNAs or during PABP-independent conditions). Further investigation is required to elucidate the functional differences between the five eIF4GI polypeptides and to determine the contribution of each to cellular translation control.

Acknowledgment—We thank P. Younan for technical assistance.

REFERENCES
1. Prevot, D., Darlix, J.-L., and Ohlmann, T. (2003) Biol. Cell 95, 141–156
2. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pliipenko, E. V., Shtatsky, I. N., Agol, V. I., and Helen, C. U. T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7029–7036
3. Mariissen, W. E., and Lloyd, R. E. (1998) Mol. Cell. Biol. 18, 7565–7574
4. Clemens, M. J., Bushell, M., and Morley, S. J. (1998) Oncogene 17, 2921–2931
5. Ali, I. K., McKendrick, L., Morley, S. J., and Jackson, R. J. (2001) J. Virol. 75, 7854–7863
6. Imataka, H., Grafilo, A., and Sonenberg, N. (1999) EMBO J. 17, 7480–7489
7. Marconigrino, J., Lomakin, I. B., Sonenberg, N., Pestova, T. V., Helen, C. U., and Burley, S. K. (2001) Mol. Cell 7, 193–203
8. Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999) EMBO J. 18, 270–279
18. Bushell, M., Poncet, D., Marissen, W. E., Flotow, H., Lloyd, R. E., Clemens, J., Zamora, M., Marissen, W. E., and Lloyd, R. E. (2002) J. Virol. 62, 4495–4499
20. Kuyumcu-Martinez, N. M., Joachims, M., and Lloyd, R. E. (2002) Mol. Cell. Biol. 27, 21975–21983
21. Kuyumcu-Martinez, N. M., Van Eden, M. E., Younan, P., and Lloyd, R., E. (2002) J. Virol. 76, 165–177
22. Pelletier, J., and Sonenberg, N. (1988) Biochemistry 27, 9768–9773
23. Hellen, C. U. T., Witherell, G. W., Schmid, M., Shin, S. H., Pestova, T. V., Gil, A., and Wimmer, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7642–7646
24. Blen, L. B., Towner, J. S., Semler, B. L., and Ehrenfeld, E. (1997) J. Virol. 71, 6243–6246
25. Stoneley, M., Suhkhankulova, T., Le Queuxe, J. P., Coldwell, M. J., Jopling, C. L., Belsham, G. J., and Willis, A. E. (2000) Nucleic Acids Res. 28, 687–694
26. Kean, K. M. (2003) Biol. Cell 95, 129–139
27. Jhaveri, B. G., Carter, M., Ebers, M., Brown, P., and Sarnow, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13118–13123
28. Van Eden, M. E., Byrd, M. P., Sherrill, K. W., and Lloyd, R. E. (2004) RNA 10, 469–481
29. Yang, Q., and Sarnow, P. (1997) Nucleic Acids Res. 25, 2800–2807
30. Nanburo, C., Lafon, I., Audigier, S., Gensac, M. C., Vagner, S., Huez, G., and Prats, A. C. (1997) J. Biol. Chem. 272, 32061–32066
31. Stoneley, M., and Will, A. E. (2004) Oncogene 23, 3200–3207
32. Yueh, A., and Schneider, R. J. (2000) Genes Dev. 14, 414–421
33. Johannes, G., and Sarnow, P. (1998) RNA 4, 1590–1513
34. Castrillo, J. L., and Carrasco, L. (1987) J. Biol. Chem. 262, 7328–7334
35. Yan, R., Rychlik, W., Etchison, D., and Rhodes, R. E. (1992) J. Biol. Chem. 267, 23226–23231
36. Gan, W., and Rhodes, R. E. (1996) J. Biol. Chem. 271, 623–626
37. Gradi, A., Imataka, H., Svitkin, Y. V., Rom, E., Raught, B., Merino, S., and Sonenberg, N. (1998) Mol. Cell. Biol. 18, 334–342
38. Byrd, M. P., Zamora, M., and Lloyd, R. E. (2002) Mol. Cell. Biol. 22, 4499–4511
39. Han, B. and Zhang, J. T. (2002) Mol. Cell. Biol. 21, 7372–7384
40. Honda, S., Kanzaki, S., Matsushita, E., Kobayashi, K., Abell, G. A., and Lemon, S. M. (2000) Gastroenterology 118, 152–162
41. Sui, G., Sodhoo, C., Affar, E. B., Gay, F., Shi, Y., Forrester, W. C., and Shi, Y. (2002) Proc. Natl. Acad. Sci. 99, 5515–5520
42. Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P. D. (2004) Science 306, 1377–1380
43. Van Eden, M. E., Byrd, M. P., Sherrill, K. W., and Lloyd, R. E. (2004) RNA 10, 720–730
44. Sherrill, K. W., Byrd, M. P., Van Eden, M. E., and Lloyd, R. E. (2004) J. Biol. Chem. 279, 29066–29074
45. Joachims, M., van Breugel, P. C., and Lloyd, R. E. (1999) J. Virol. 73, 718–727
46. Faustino, N. A., and Cooper, T. A. (2003) Genes Dev. 17, 439–437
47. Deminguez, D. I., Ryahova, L. A., Poggini, M. M., Schmidt-Puhta, W., Futterer, J., and Hohn, T. (1998) J. Biol. Chem. 273, 3669–3678
48. Kazm, M. (2001) Mol. Cell. Biol. 21, 1899–1907
49. Kazm, M. (1991) J. Biol. Chem. 266, 19867–19870
50. Eu, J. and Andrade, J. (2001) Luminescence 15, 57–63
51. Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1995) J. Biol. Chem. 270, 3200–3207
52. Ali, N., and Siddiqui, A. (1995) J. Biol. Chem. 270, 720–730
53. Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1995) Mol. Cell. Biol. 15, 5777–5788
54. Ali, N., and Siddiqui, A. (1995) J. Biol. Chem. 270, 6367–6375
55. Pudi, R., Abhiman, S., Srivivasan, N., and Das, S. (2003) J. Biol. Chem. 278, 12321–12340
56. Michel, Y. M., Poncet, D., Pirun, M., Kean, K. M., and Borman, A. M. (2000) J. Biol. Chem. 275, 32268–32276
57. Haghhighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., and Sonenberg, N. (1998) J. Virol. 70, 8444–8450
58. Kazm, M. (2003) Gene (Amst.) 318, 1–23
59. Han, B., and Zhang, J. T. (2002) Mol. Cell. Biol. 22, 7372–7384
60. Schneider, R. J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 901–914, Cold Spring Harbor Laboratory Press, New York
61. Yueh, A., and Schneider, R. J., (1996) Genes Dev. 10, 1557–1567