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Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites

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ABSTRACT

Sequence elements that can function as internal ribosome entry sites (IRES) have been identified in 5' noncoding regions of certain uncapped viral and capped cellular mRNA molecules. However, it has remained largely unknown whether IRES elements are functional when located in their natural capped mRNAs. Therefore, the polysomal association and translation of several IRES-containing cellular mRNAs was tested under conditions that severely inhibited cap-dependent translation, that is, after infection with poliovirus. It was found that several known IRES-containing mRNAs, such as BiP and c-myc, were both associated with the translation apparatus and translated in infected cells when cap-dependent translation of most host-cell mRNAs was blocked, indicating that the IRES elements were functional in their natural mRNAs. Curiously, the mRNAs that encode eukaryotic initiation factor 4G1 (eIF4G1) and 4GII (eIF4GII), two proteins with high identity and similar functions in the initiation of cap-dependent translation, were both associated with polysomes in infected cells. The 5'-end sequences of eIF4G1 mRNA were isolated from a cDNA expression library and shown to function as an internal ribosome entry site when placed into a dicistronic mRNA. These findings suggest that eIF4G proteins can be synthesized at times when 5' cap-dependent mRNA translation is blocked, supporting the notion that eIF4G proteins are needed in both 5' cap-independent and 5' cap-dependent translational initiation mechanisms.

Keywords: cycloheximide; dicistronic mRNA; poliovirus; RNA stability; translation

INTRODUCTION

Translation initiation of mRNAs in eukaryotic cells is a complex process that involves the concerted interaction of numerous factors (Pain, 1996). For most mRNAs, the first step is the recruitment of ribosomal 40S subunits onto the mRNA at or near the capped 5' end. Association of 40S to mRNA is greatly facilitated by the cap-binding protein complex eIF4F. Factor eIF4F is composed of three subunits: the RNA helicase eIF4A (Rozen et al., 1990; Lorsch & Herschlag, 1998a,b), the cap-binding protein eIF4E (Sonenberg, 1988), and the multi-adaptor protein eIF4G (Hentze, 1997; Morley et al., 1997). Because eIF4G can interact with both eIF4E (Mader et al., 1995) and 40S-associated factor eIF3 (Lamphear et al., 1995), 40S subunits are tethered to the 5' end of capped mRNAs via protein–protein interactions. Alternatively, 40S subunits can enter

mRNA molecules at so-called internal ribosome entry sites (IRES) which are normally located hundreds of nucleotides downstream of the capped 5' ends of mRNAs. Such IRES elements have been found in picornaviral (Jang et al., 1988; Pelletier & Sonenberg, 1988; Jackson & Kaminski, 1995), flaviviral (Tsukiyama-Kohara et al., 1992; Rijnbrand et al., 1997), and cellular mRNAs (Macejak & Sarnow, 1991; Oh et al., 1992; Teerink et al., 1995; Vagner et al., 1995; Gan & Rhoads, 1996; Bernstein et al., 1997; Nanbru et al., 1997). An RNA sequence element has been functionally designated as an IRES if it can mediate the translation of a second cistron in a heterologous dicistronic mRNA (Jackson & Kaminski, 1995). However, it is not clear whether all IRES elements identified in such dicistronic assays also function as IRESs in their normal locations in cellular mRNAs. Therefore, a system was needed where the selective translation of normal cellular IRES-containing mRNAs could be monitored.

It has long been known that infection of cells with poliovirus results in the rapid inhibition of host cell translation, yet allows efficient translation of the viral mRNA

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(reviewed in Ehrenfeld, 1996). Several lines of evidence have shown that this translational inhibition is mostly due to an alteration of the cap-binding protein complex eIF4F. For example, the eIF4G component of eIF4F is proteolyzed by viral (Liebig et al., 1993; Haghighat et al., 1996) and, perhaps, cellular proteases (Lloyd et al., 1986; Wyckoff et al., 1990) in infected cells. Furthermore, the affinities of specific eIF4E-binding proteins for eIF4E are enhanced in poliovirus infected cells, resulting in a decreased affinity of eIF4E for eIF4G (Gingras et al., 1996). Both the proteolysis of eIF4G and the sequestration of eIF4E by eIF4E-BPs lead to diminished levels of functional eIF4F complexes in infected cells. As a consequence, translation of cap-dependent translation is severely inhibited (Etchison et al., 1982). However, any cellular mRNA with a reduced requirement for eIF4F for translation initiation or which contains an IRES element, like polioviral mRNA, is predicted to be associated with host cell ribosomes and translated under such a condition. In this study, we show that in addition to the immunoglobulin heavy-chain binding protein BiP mRNA (Sarnow, 1989), the natural IRES-containing c-myc and eIF4GI mRNAs were selectively associated with the translation apparatus during poliovirus infection, demonstrating that c-myc- and eIF4GI-IRES elements function in their normal mRNAs and can confer selective translation to their respective mRNAs under stress conditions when overall cap-dependent translation is inhibited.

RESULTS

Inhibition of host cell translation and proteolysis of eIF4G in cells infected with poliovirus mutant 3NC202

Host cell mRNAs that can be translated cap-independently by internal initiation are predicted to be translated in poliovirus-infected cells at a time when cap-dependent translation of most host mRNAs is inhibited. However, the IRES-containing BiP mRNA was the only mRNA that was observed to be translated in infected cells by inspection of labeled proteins (Sarnow, 1989). The failure to detect translation of other host cell mRNAs in infected cells suggested that steady-state levels of other putative IRES-containing host mRNAs might be low, poorly translated, or less stable in infected cells than in uninfected cells.

To detect cellular IRES-containing mRNAs by a more sensitive means, a system was developed in which inhibition of cap-dependent translation occurred slowly. Figure 1A shows that infection of cultured HeLa cells with mutant poliovirus 3NC202 (Sarnow et al., 1986; Jacobson et al., 1993) at a semi-permissive temperature for this temperature-sensitive virus resulted in a slow gradual inhibition of cap-dependent translation.

FIGURE 1. Inhibition of cap-dependent translation in HeLa cells during poliovirus infection. **A:** Radiolabeled extracts prepared from mock-infected (M) or 3NC202-infected cells (from 1 to 6 h after infection at 37 °C; lanes 1–6) were separated by SDS-PAGE. An autoradiograph of the gel is shown. The migration of marker proteins with known molecular weights are shown to the left of the figure (in kiloDaltons). **B:** Cleavage of eIF4G during poliovirus infection. Aliquots from samples shown in **A** were separated by SDS-PAGE and immunoblotted with antibodies directed against eIF4G. Long arrow indicates the migration of full-length eIF4G, and the arrow head indicates the migration of the cleaved eIF4G products.

For example, synthesis of the prominent 45-kDa actin protein was reduced to less than 10% by 3 h (Fig. 1A, lane 3) after infection. In contrast, the synthesis of viral proteins was apparent by 3 h, and peaked between 4 h (Fig. 1A, lane 4) and 5 h (Fig. 1A, lane 5) after infection. The time of inhibition of host cell translation paralleled the time of proteolysis of eIF4G (Fig. 1B) which could be observed as early as 1 h (lane 1) after infection and was complete by 3 h (lane 3) after infection. In wild-type poliovirus-infected cells, host translation was inhibited by 2 h after infection (G. Johannes & P. Sarnow, unpubl. observations). The slow kinetics of inhibition of host cell translation in 3NC202-infected cells encouraged us to analyze the translation of natural mRNAs that have been shown in the “dicistronic assay” to contain IRES elements in their 5' noncoding regions.

Steady state levels of IRES-containing cellular mRNAs in 3NC202-infected cells

First, the steady-state levels of certain mRNAs which lack or contain IRES elements, as determined by dicistronic assays, were determined at different times in 3NC202-infected cells. Figure 2A shows that the steady state levels of non-IRES-containing actin (Nakajima-Iijima et al., 1985) and junB (Nomura et al., 1990) mRNAs remained constant throughout the infection. Similarly, the levels of IRES-containing BiP (Sarnow et al., 1986; Ting & Lee, 1988) and eIF4G (Yan et al., 1992; Gan & Rhoads, 1996) mRNAs did not change significantly during the infectious cycle. In contrast, IRES-containing c-myc (Arrushdi et al., 1983; Bentley & Groudine, 1986; Nanbru et al., 1997; Stoneley et al., 1998) mRNA levels increased substantially at 3 h after infection, a time when viral 3NC202 mRNA molecules started to accumulate. To determine whether the increase in c-myc mRNA level was due to the virus-induced inhibition of host cell translation, we monitored the steady-state levels of c-myc mRNA in uninfected cells in which translation was inhibited by addition of cycloheximide (Rose & Lodish, 1976). Specifically, cycloheximide was added to mock-infected cells at 1 h after infection and the steady-state levels of various mRNAs were examined at 0.5, 1, 2, 3, and 4 h after cycloheximide addition. Figure 2B shows that treat-

ment of cells with cycloheximide resulted in increased intracellular levels of c-myc at approximately 2 h after drug addition. Therefore, the intracellular accumulation of c-myc mRNA is likely a consequence of virally induced inhibition of host cell mRNA translation. This is in agreement with the observation that c-myc mRNA stability is controlled by a labile destabilizing factor (Dani et al., 1984; Brewer & Ross, 1989).

Association of natural IRES-containing mRNAs with the translation apparatus in infected cells

IRES-containing mRNAs should be translated at 3 h after 3NC202 virus infection, because (1) cap-dependent translation is largely inhibited (Fig. 1), (2) the intracellular levels of IRES-containing mRNAs are not diminished (Fig. 2A), and (3) very little viral mRNA has accumulated at this time in the infected cells (Fig. 2A), to compete with the cellular IRES-containing mRNA for the translational machinery. To test the translation of cellular IRES-containing mRNAs, the polysomal distributions of IRES-containing and control mRNAs were examined in uninfected cells and in cells infected with 3NC202.

Figure 3 shows that all cellular mRNA species could be detected in polysomes which were isolated from uninfected cells (Mock). BiP (2,700 nt) and eIF4G

FIGURE 2. Steady-state levels of mRNAs in poliovirus infected cells. **A:** Northern analysis of mRNAs during 3NC202 poliovirus infection. Total RNA was isolated from mock-infected (M) or 3NC202-infected cells at various times after infection (1–6 h) and hybridized to radiolabeled DNA complementary to actin, Jun B, BiP, eIF4G, c-myc, or polioviral mRNAs. An autoradiograph of the blot is shown. The upper bands in the actin and eIF4G blots likely represent pre-mRNA species that disappear upon poliovirus infection because of the inhibition of polymerase II transcription (Crawford et al., 1981). **B:** Effect of cycloheximide treatment on steady-state levels of mRNAs. Total RNA was isolated from mock-infected HeLa cells at 0 h (lane 0) and 1 h (lane 1) prior to the addition of cycloheximide and at various times after cycloheximide additions (lanes 1.5–5). Addition of cycloheximide after 1 h is indicated by the arrow. RNA isolated from control-treated cells (i.e. no cycloheximide was added) taken in parallel with the 4-h time point is displayed in lane C.

FIGURE 3. Association of mRNAs with polysomes after inhibition of cap-dependent translation. Polysomal distributions of mRNAs lacking (Actin, JunB) or containing (BiP, eIF4G, c-Myc, polio) IRES elements from mock- or 3NC202 poliovirus-infected cells at 2 h, 3 h and 4 h after infection are shown. Absorbance profiles at 254 nm are shown at the top of each panel. Fractions from the top to the bottom of the sucrose gradient are displayed from left to right. Arrows denotes the migration of 60S ribosomal subunits. Gradients were collected in 10 fractions. Equal volumes of each fraction were loaded in each lane of the agarose gels. The RNAs separated in the gels were transferred to membranes and hybridized to individually radiolabeled probes. The autoradiograph was scanned using a Microtek ScanMaker E6 and the resulting image was labeled using Adobe Photoshop version 3.0.

(5,100 nt) mRNAs were mostly associated with polysomal fraction 10, containing the most rapidly sedimenting polysomes. Actin (1,900 nt) and c-myc (2,100 nt) mRNAs predominantly accumulated in polysomal fraction 9. Both of these fractions are considered "heavy polysome" fractions, which contain mRNAs associated with more than seven ribosomes (Fig. 3). In contrast, junB mRNA (1,800 nt) sedimented with polysomal fraction 8, indicating that junB mRNA was associated with fewer ribosomes on average, than was actin mRNA.

To test whether the sedimentation properties of these mRNAs were determined by the number of associated ribosomes, the sedimentation profiles of the mRNAs were examined after sucrose-gradient centrifugation in the presence of 15 mM EDTA, which causes the dissociation of ribosomes from mRNAs without disrupting the majority of nonribosomal RNA-protein complexes (Calzone et al., 1982). Figure 4 shows that treatment with EDTA resulted in the disappearance of mRNAs from polysomal fractions 8–10, with concomitant accumulation of 40S and 60S ribosomal subunits. These findings indicate that the presence of mRNAs in fractions 8–10 in gradients lacking EDTA was because of their association with ribosomes, and not with nonribosomal ribonucleoprotein complexes.

FIGURE 4. Polysomal association of mRNAs in the presence of EDTA. Polysomal analysis was carried out as described in Figure 3 (panel 3 Hr PI), except that 15 mM EDTA was included in the extraction and gradient buffer. The absorbance profile at 254 nm is shown at the top of the panel. The distribution of the various mRNAs within the gradient was monitored by Northern analysis as described in Figure 3. The arrow denotes the migration of 60S ribosomal subunits.

Inspection of Figure 3 shows that polysomes gradually disappear during the infectious cycle of poliovirus. For example, approximately 50% of the polysomes redistributed into 40S and 60S ribosomal subunits by 2 h after infection. Nearly 90% of polysomes were lost by 3 h after infection, and only small amounts of polysomes could be detected by 4 h after infection. The disappearance of polysomes correlated well with the overall inhibition of host-cell mRNA translation shown in Figure 1. Concomitant with the loss of polysomes, actin and junB mRNAs redistributed in the gradient from polysomal (Fig. 3, Mock) to nonpolysomal fractions. For example, the majority of both of these mRNAs redistributed from fractions 9 (actin) or 8 (junB) to fractions 1–6 by 3 h after infection. Thus, the translation of actin and junB mRNAs is predicted to be severely inhibited (see below) as has been observed with most host-cell mRNAs at this time after infection (Fig. 1). In contrast, the redistribution of BiP, eIF4G, and c-myc mRNAs was less dramatic, with much less than half of these three mRNAs sedimenting with nonpolysomal fractions 1–6 at 3 h after infection. Table 1 displays quantitatively the distribution of the mRNAs. Most of the BiP- (74%), eIF4G- (87%), and c-myc- (89%) IRES-containing mRNAs could be found in polysomal fractions 7–10 at 3 h after infection. Although all mRNA species redistributed to the nonpolysomal fractions of the gradient by 4 h after infection, the IRES-containing mRNAs were still more quantitatively associated with polysomal fractions than the non-IRES-containing mRNAs (Table 1). Whether the preferential association of IRES-containing mRNAs with polysomes in infected cells resulted in their preferential translation during inhibition of cap-dependent translation was addressed in the next set of experiments.

Translation of non-IRES- and IRES-containing mRNAs in poliovirus-infected cells

Extracts from cells that were radiolabeled with S^{35} -methionine for 10 min at different times after infection (Fig. 1) were immunoprecipitated with antisera directed

TABLE 1. Association of cellular mRNAs with polysomes in uninfected and poliovirus-infected cells.^a

mRNA	Mock	2Hr PI	3Hr PI	4Hr PI
Actin	100	68	35	25
Jun B	100	62	28	12
BiP	100	91	74	50
c-Myc	100	89	89	63
eIF4G	100	100	87	68

^aData are obtained after quantitation of the phosphorimage shown in Figure 3 using Imagequant software. The relative amount of polysomal RNA found in polysomal fractions 7–10 compared to polysomal fractions in the "Mock" gradient (set as 100) is shown.

against actin, BiP, or c-myc proteins and analyzed in SDS-polyacrylamide gels followed by autoradiography. Figure 5 shows that actin mRNA was translated in mock-treated cells (M) and at 1 h after infection (lane 1). Only a very small amount of actin was synthesized at 2 h after infection, and the synthesis of actin ceased completely by 3 h after infection. This finding is in agreement with the observation that actin mRNA redistributed to nonpolysomal fractions by 3 h after infection (Fig. 3), a time when overall cap-dependent translation was inhibited (Fig. 1). In contrast, both newly synthesized BiP and c-myc proteins could be detected at 3 h after infection. Therefore, the association of natural BiP and

c-myc mRNAs with the translation apparatus in infected cells has functional consequences, that is, the continued synthesis of BiP (Sarnow, 1989) and c-myc proteins when overall cap-dependent translation is inhibited. Unfortunately, several antibodies directed against eIF4G that have proven useful in the detection of eIF4G in Western blot analyses (Fig. 1), were not able to immunoprecipitate radiolabeled eIF4G from uninfected cells (G. Johannes & P. Sarnow, unpubl. observations); thus, the fate of newly synthesized eIF4G could not be examined by this approach. Because eIF4G mRNA was clearly associated with polysomes in infected cells (Fig. 3), we wanted to examine in more detail by which mechanism eIF4G mRNA could be translated in the absence of functional cap binding protein complex.

FIGURE 5. Immunoprecipitation of newly synthesized cellular proteins in 3NC202 poliovirus-infected cells. Cells were pulse-labeled at each hour (lanes 1–6) after infection with ^{35}S -methionine. Extracts were prepared and proteins immunoprecipitated with antibodies directed against actin (Anti-Actin), BiP (Anti-BiP), or Myc (Anti-myc) and separated in SDS-polyacrylamide gels. In addition, proteins were immunoprecipitated from mock-infected cells (M). Labeled proteins in extracts, harvested at 5 h after infection, are displayed in lane I. Arrow denotes the migration of actin (Anti-Actin panel), BiP (Anti-BiP panel), or Myc (Anti-Myc panel) proteins. The migration of proteins with known molecular weights is indicated on the right. The autoradiograph was scanned using a Microtek ScanMaker E6 and the resulting image was labeled using Adobe Photoshop version 3.0.

Isolation of a novel eIF4GI cDNA variant

The eIF4G mRNA was detected in the above experiments with radiolabeled sequences that were complementary to the coding region of eIF4G mRNA, whose cDNA was isolated from a human brain cDNA library (Yan et al., 1992). This eIF4G cDNA displayed an unusually long 5'NCR, 368 nt in length (Fig. 6A), which was subsequently shown to function as an IRES in dicistronic assays in cultured cells (Gan & Rhoads, 1996; Gan et al., 1998). Curiously, radiolabeled sequences complementary to the 5'NCR of eIF4G failed to hybridize to any mRNA species isolated from HeLa cells (G. Johannes & P. Sarnow, unpubl. observations).

Recently, Gradi et al. (1998) published a novel cDNA variant of eIF4G, termed eIF4GI, which was identical to the originally isolated eIF4G cDNA except that it contained an extended N-terminus of 115 amino acids (Fig. 6A). Because the identity between the previously isolated eIF4G and the eIF4GI cDNAs stopped abruptly at a putative canonical splice acceptor site (SA, Fig. 6A), it was suggested by Gradi et al. that the previously isolated eIF4G cDNA contained an unspliced intron at its 5' end that included the PPT and the IRES. Thus, either the eIF4G and eIF4GI cDNAs represent spliced variants of the same RNA transcript, or they are derived from different genes or alleles.

To determine which variant eIF4G mRNAs were associated with polysomes in poliovirus-infected HeLa cells, we isolated cDNAs containing the 5'NCRs of eIF4G genes from a human HeLa cDNA library (see Materials and Methods). Using PCR technology, several cDNAs were isolated that all contained at least the extra nucleotide extensions seen in eIF4GI (Fig. 6A; Gradi et al., 1998). While most of the isolated cDNAs contained the identical 5' terminal sequences found in the eIF4GI cDNA (Gradi et al., 1998), several cDNAs contained longer 5' extended sequences. Figure 6B shows the nucleotide sequence of the longest isolated cDNA, which added an additional 180 nt to the 5' end

FIGURE 6. Organization and nucleotide sequence of variant eIF4G cDNAs. **A:** Sequence organization of eIF4G, eIF4GI, and eIF4GI_{ext} cDNAs. The features of three distinct eIF4G cDNAs are depicted; see text for details. The open boxes denote coding regions. The thin solid line (top diagram) indicates noncoding region, and the thick solid lines (bottom two diagrams) denote sequences in which it is unknown whether they are coding or noncoding. Capital letters denote amino acids and the filled-in triangles the locations of AUG triplets. The locations of a putative splice acceptor (SA) site and a polypyrimidine tract (PPT) in the eIF4G cDNA are shown. **B:** Nucleotide sequence of the 5' terminal eIF4GI_{ext} cDNA. The 5' end of the eIF4GI cDNA isolated by Gradi et al. (1998) is shown in italics at the bottom. AUG triplets are underlined. **C:** Predicted amino acids sequence of the eIF4GI_{ext} sequences shown in **B**. **D:** Sequence comparison of eIF4GI_{long} and eIF4GII. The 5' ends of eIF4GI_{long} and eIF4GII (Gradi et al., 1998) are shown at the top and the bottom (in italics), respectively. The unique 5' terminal 60 nt in eIF4GII and the predicted AUG start codons are underlined.

B

1	UUU CCC CCG GGG CAG ACA GCG CCG GUG GUG UUC AGU ACG CCA CAA GCG ACA
52	CAA <u>AUG</u> AAC ACG CCU UCU CAG CCC CGC CAG CAC UUC UAC CCU AGC CGG GCC
103	CAG CCC CCG AGC AGU GCA GCC UCC CGA GUG CAG AGU GCA GCC CCU GCC CGC
154	CCU GGC CCA GCU GCC <u>CAU</u> <u>GUC</u> UAC CCU GCU GGA UCC CAA GUA <u>AUG</u> <u>AUG</u>
	1 GCU GGA UCC CAA GUA <u>AUG</u> <u>AUG</u>

C

1	Phe Pro Pro Gly Gln Thr Ala Pro Val Val Phe Ser Thr Pro Gln Ala Thr
18	Gln <u>Met</u> Asn Thr Pro Ser Gln Pro Arg Gln His Phe Tyr Pro Ser Arg Ala
35	Gln Pro Pro Ser Ser Ala Ala Ser Arg Val Gln Ser Ala Ala Pro Ala Arg
52	Pro Gly Pro Ala Ala His Val Tyr Pro Ala Gly Ser Gln Val <u>Met</u> <u>Met</u>
	1 Ala Gly Ser Gln Val <u>Met</u> <u>Met</u>

D

1	GUC CCC CCU CCU CAC CCG AAC AUC AGG GCC CUC CAG ACU CAG GCG CCC CAA
52	CAA AUU CCU AGA GGA CCU GUG CAA CAA CCU CUU GAG GAU CGA AUC UUC ACU
1	<u>C AAU CCC ACA GAG UAU UGA UGA GGA AAC UGA AGU</u>
103	CCC GCU GUC UCA GCA GUC UAC AGC ACG GUA ACA CAA GUG GCA AGA CAG CCG
35	<u>UUG GAG CGA UCA CAU CAU UUU CCC AAG</u> GUA ACA CAA GUG GCA AGA CAG CCG
154	GGA ACC CCU ACC CCA UCC CCU UAU UCA GCA CAU GAA AUA AAC AAG GGG CAU
86	<u>GGA ACC CCU ACC CCA UCC CCU UAU UCA GCA CAU GAA AUA AAC AAG GGG CAU</u>
205	CCA AAU CUU GCG GCA ACG CCC CCG GGA CAU GCA UCG UCC CCU GGA CUC UCU
137	<u>CCA AAU CUU GCG GCA ACG CCC CCG GGA CAU GCA UCG UCC CCU GGA CUC UCU</u>
256	CAA ACC CCU UAU CCC UCU GGA CAG AAU GCA GGU CCA ACC ACG CUG GUA UAC
188	<u>CAA ACC CCU UAU CCC UCU GGA CAG AAU GCA GGU CCA ACC ACG CUG GUA UAC</u>
307	CCU CAA ACC CCU CAG ACA <u>AUG</u>
239	CCU CAA ACC CCU CAG ACA <u>AUG</u>

of the eIF4GI cDNA described by Gradi et al. (1998) (Fig. 6A). This new eIF4GI variant was termed eIF4GI_{ext} and the sequence of its 180-nt extension is shown in Figure 6B. The eIF4GI_{ext} leader contains two putative

methionine start codons at positions 196 and 199 (underlined in Fig. 6B) each of which is embedded in optimal sequence context for translational initiation (Kozak, 1989). Two other AUG codons at positions 55 and 170

(underlined in Figs. 6B and 6C) are not in optimal context. However, because the entire sequence upstream of the AUGs at positions 196 and 199 encodes in-frame protein sequence (Fig. 6C), it is not known whether the authentic 5' end of the eIF4G_{ext} gene was isolated.

Polysome association of eIF4GI mRNAs in poliovirus-infected cells

To determine whether the eIF4GI and eIF4G_{ext} cDNA sequences were present in natural mRNAs, we analyzed the polysomal distributions of the eIF4GI and eIF4G_{ext} mRNAs in uninfected and infected cells. Using a radiolabeled probe (eIF4GI-5') that hybridized specifically to eIF4GI cDNA, the polysomal distribution of eIF4GI mRNA was examined in both mock- and virus-infected cells (Fig. 7). The bulk of eIF4GI mRNA was associated with polysomal fraction 10 in uninfected cells (Fig. 7, Mock), indicating that eIF4GI mRNA was efficiently translated in cells. At 3 h after infection with 3NC202 poliovirus, the bulk of eIF4GI mRNA relocated to polysomal fraction 9, suggesting that eIF4GI mRNA was still translated at a time when the bulk of actin

mRNA relocated to nonpolysomal fractions 2–6 (Fig. 7). Similarly, radiolabeled probes that specifically hybridized to eIF4G_{ext} mRNA displayed a polysomal distribution pattern of eIF4G_{ext} mRNA that was identical to that of eIF4GI (G. Johannes & P. Sarnow, unpubl. observations). Because the 5' ends of polysomal eIF4GI mRNAs have not yet been determined, it is not known whether eIF4GI mRNAs with different 5' ends are present in the cell. In any case, these data suggest that eIF4GI mRNAs can be translated cap independently.

Polysome association eIF4GII mRNAs in poliovirus-infected cells

Recently, Gradi et al. (1998) have identified a novel gene, eIF4GII, whose coding region shares an overall similarity of 56% with eIF4GI. Importantly, eIF4GII seems to be a functional homolog of eIF4GI, because eIF4GII can restore cap-dependent translation to extracts in which eIF4GI has been proteolyzed (Gradi et al., 1998).

To monitor the polysomal association of the eIF4GII mRNA in uninfected and infected cells, cDNAs encoding the 5' NCR of eIF4GII were isolated from a HeLa

FIGURE 7. Polysomal distribution of eIF4GI, eIF4GII, and actin mRNAs in poliovirus-infected cells. Polysome analysis was carried out with total cytoplasmic RNA isolated from mock-infected cells (Mock) and from cells 3 h after infection (3 Hr PI) as described in Figure 3. The eIF4GI-5' probe hybridizes specifically to a region in the 5' leader of eIF4GI; the eIF4GII-3' and eIF4GII-5' probes hybridize specifically to regions in the 3'- and 5'-NCR regions of eIF4G-II, respectively. Note that results were obtained after rehybridization of the blot shown in Figure 3. The absorbance profile at 254 nm is shown at the top of each panel. The distribution of the various mRNAs within the gradient was monitored by Northern analysis as described in Figure 3. The arrow denotes the migration of 60S ribosomal subunits.

cDNA library by PCR technology. Figure 6D shows that a 325-nt 5'NCR of eIF4GII was isolated that was different from the originally isolated 5'NCR of eIF4GII, which is predicted to be 257 nt in length (Gradi et al., 1998). In particular, the predicted 5' terminal 60 nt in the originally isolated eIF4GII gene (underlined in Fig. 6D) were replaced with a new 128-nt sequence element (Fig. 6D). This new leader, which is only a putative 5'NCR because no stop codon was found that was in-frame with the predicted start codon at position 325, was termed eIF4GII_{long}.

To determine whether the eIF4GII_{long} sequences were present in natural mRNAs, we analyzed the polysomal distributions of eIF4GII_{long}-containing mRNAs in uninfected and infected cells (Fig. 7). Using radiolabeled probes that hybridized specifically to the eIF4GII_{long} cDNA (Fig. 7: eIF4GII-5'), a polysomal distribution of eIF4GII mRNA was detected in both mock- and virus-infected cells (Fig. 7). Furthermore, probes specific for the 5' (eIF4GII-5') or 3' (eIF4GII-3') NCRs of eIF4GII detected an mRNA species that was of similar size and abundance on polysomes in mock- and in virus-infected cells (Fig. 7). Thus, the data in Figure 7 suggest that both eIF4GI and eIF4GII mRNAs can be translated cap independently.

eIF4GI mRNA contains an internal ribosome entry site

To test whether eIF4GI and eIF4GII mRNAs contain IRES elements that mediate their cap-independent translation in poliovirus-infected cells, the ability of their 5' terminal sequences to direct the translation of the second cistron in dicistronic mRNAs (Macejak & Sarnow, 1991) was examined. First, the 5' terminal 196 nt of eIF4GI_{ext} (Fig. 6B) and the 325 nt of eIF4GII_{long} (Fig. 6D) were placed between the chloramphenicol acetyltransferase (CAT) and luciferase (LUC) cistrons in a dicistronic cDNA which was then transfected into human HeLa cells. Figure 8A shows that the eIF4GI_{ext} sequence element functioned as an IRES with an efficiency that was slightly less than that of the poliovirus IRES (Yang & Sarnow, 1997). On the other hand, the eIF4GII_{long} sequence element promoted the translation of the second luciferase cistron with an efficiency that was slightly less than control ED and ICS sequences. Thus, in contrast to eIF4GI_{ext}, eIF4GII_{long} did not function as an IRES in the dicistronic assay.

Lastly, the integrities of the dicistronic mRNA CAT–LUC mRNAs in transfected cells were examined by Northern analysis using radiolabeled probes that hy-

FIGURE 8. Test for internal ribosome entry using dicistronic mRNA expression. **A:** Expression of dicistronic mRNAs containing various sequences in the intracistronic spacer (ICS) region. In each transfection, observed luciferase activity was normalized to CAT activity. In a typical experiment, CAT activity ranged from 5–20% of conversion of unmodified chloramphenicol to modified chloramphenicol. Luciferase activity ranged from 5,000–10,000 light units from ED-containing constructs to 70,000–100,000 light units for poliovirus IRES-containing constructs. The results are displayed as luciferase light units/CAT units (normalized Luciferase/CAT) for two different experiments. The dicistronic vectors contained ED- or ICS-control sequences (Macejak & Sarnow, 1991), the 5' terminal 325 sequences of the eIF4G-II_{long} cDNA (G. Johannes & P. Sarnow, unpubl. observations), the 5' terminal 196 sequences of the eIF4G-I_{ext} cDNA, or the 5' terminal 747 sequences of the poliovirus cDNA in the intracistronic region. Black and white boxes show results of two independent experiments each performed in triplicate transfections. **B:** Integrity of dicistronic mRNA in transfected cells. Poly(A⁺) mRNA was isolated from cells that were transfected with dicistronic plasmids, and analyzed by Northern blot hybridization using radiolabeled RNA that hybridized to chloramphenicol acetyltransferase (CAT) or luciferase (LUC) sequences. The sequences located in the intercistronic spacer regions are indicated at the top of the figure. The migration of 28S and 18S rRNA is indicated. The autoradiograph was scanned using a Microtek ScanMaker E6 and the resulting image was labeled using Adobe Photoshop version 3.0.

bridized to the first CAT cistron or the second LUC cistron. Figure 8B shows that the dicistronic mRNAs were largely intact, indicating that the translation of the second cistron was not mediated by a subgenomic mRNA. Due to various transfection efficiencies, the intracellular steady-state levels of the different mRNAs changed from experiment to experiment (Yang & Sarnow, 1997). However, the expression of the first CAT cistron was always proportionate to the level of the mRNA. For example, cell extracts prepared from dicistronic CAT-polio-LUC- or CAT-4GII_{long}-LUC-transfected cells displayed similar CAT activity (G. Johannes & P. Sarnow, unpubl. observations).

In summary, the extended 5'NCRs in eIF4GI mRNA permitted internal initiation in the dicistronic assay and, thus, are likely to mediate the cap-independent translation of the natural eIF4GI mRNAs. On the other hand, the extended 5' terminal sequences of eIF4GII did not function as an IRES in the dicistronic assay (Fig. 8A). The failure to detect IRES activity in eIF4GII mRNA may indicate that the extreme 5' end of the mRNA has not yet been identified. Alternatively, the observed cap-independent translation of eIF4GII mRNA could be caused by a low requirement for the cap binding protein complex eIF4F.

DISCUSSION

Although the list of IRES-containing cellular mRNAs is growing (Sachs et al., 1997), it is often not clear whether such IRES elements function in their natural mRNAs. This has been due to the lack of an experimental *in vivo* system in which cap-independent translation of IRES-containing mRNAs can be studied in the absence of ongoing cap-dependent translation of the bulk of the cellular mRNAs. A logical system for studying cap-independent translation is poliovirus-infected cells in which cap-dependent translation is inhibited due to inactivation of eIF4F (Ehrenfeld, 1996). Using the mutant 3NC202 poliovirus that grows much more slowly than wild-type virus (Waggoner & Sarnow, 1998), it was possible to identify a time in the infectious cycle at which cap-dependent host-cell mRNA translation was inhibited, yet relatively low concentrations of viral mRNAs had accumulated. The prediction that the cap-independent translation of natural IRES-containing mRNAs should be allowed under such circumstances was tested and it was found that certain IRES-containing mRNAs such as c-myc were not only associated with polysomes but also translated at 3 h in mutant virus-infected cells. This finding shows that both the BiP IRES (Sarnow, 1989) and c-myc IRES function in their natural mRNAs.

It was found that eIF4G mRNA was translated cap independently in poliovirus-infected cells. Surprisingly, the 5' end of the eIF4G cDNA, which we had isolated, had a different sequence than the 5' end of the eIF4G

cDNA isolated by Yan et al. (1992). In agreement with Gradi et al. (1998), eIF4GI_{ext} mRNA, identified in human HeLa cells, contained the same coding sequences as eIF4G (Yan et al., 1992) but a different 5' NCR. These 5' end sequences in eIF4GI_{ext} functioned as an IRES in the dicistronic assay. Thus, the cap-independent translation of eIF4GI_{ext} mRNA is likely to be conferred by the newly discovered IRES element.

The 5' NCR present in the originally isolated eIF4G cDNA (Yan et al., 1992) clearly encodes a IRES (Gan & Rhoads, 1996; Gan et al., 1998). However, the eIF4G-IRES sequence is different from the eIF4GI_{ext}-IRES detected in this study. The eIF4G-IRES contains a hallmark sequence that can be found in intron RNAs, that is, a polypyrimidine tract located upstream of the splice-acceptor sequence (Gan & Rhoads, 1996; Gan et al., 1998). Whether the eIF4G-IRES encoding RNA species is a pre-mRNA or a functional mRNA that can be translated by ribosomes is not yet known. If functional IRES elements are found in introns, it will be interesting to determine how such unspliced RNAs are translated, because introns are usually not exported from the nucleus into the cytoplasm (Chapon & Legrain, 1992; Hammarikjold, 1997). In this context, it is noteworthy that several IRES-binding proteins, such as La and PTB, are predominantly nuclear and are involved in RNA-processing events. Whether there is any interaction of these proteins with the nuclear export pathway is not known.

To date, we have no evidence for the mechanism by which eIF4GII can be translated cap-independently. The main challenge will be to identify the mechanism that leads to cap-independent translation of mRNAs that are associated with polysomes in infected cells. In addition to IRES-containing mRNAs, mRNAs with unstructured 5' NCRs that require only small amounts of functional eIF4F for their cap-dependent translation, such as heat shock mRNAs (Joshi-Barve et al., 1992), may be identified this way. Studies from *Saccharomyces cerevisiae* have indicated that the poly(A) binding protein (PABP), by interacting with either of the two eIF4G proteins, can greatly facilitate the recruitment of ribosomes to the 5' end of the mRNAs (Tarun & Sachs, 1995, 1996), indicating that the 5' and 3' ends in an mRNA can both regulate the efficiency of translation initiation. Although an interaction of mammalian PABP and eIF4G proteins has not been observed so far, a human PABP-interacting protein (PAIP) that interacts with eIF4A has recently been identified (Craig et al., 1998). Because eIF4A is known to be involved in several steps in translational initiation (Pause et al., 1994), the PABP-PAIP complex was suggested to provide a bridge between the 5' and 3' ends in mammalian mRNAs and perhaps promote reinitiation of ribosomes that had terminated at the 3' end (Craig et al., 1998). If this model is correct, it is conceivable that mRNAs, which are translated by this presumably cap-inde-

pendent reinitiation mechanism, are preferentially enriched in polysomes from infected cells.

The polysomal RNA pool from mutant-infected cells should be useful for several studies. This pool should harbor several known and likely many unknown cellular mRNAs that can be translated cap independently. Indeed, preliminary microarray hybridization experiments designed to detect mRNAs species that are preferentially associated with polysomes from infected cells have indicated that many hitherto unsuspected mRNAs can be translated cap independently (G. Johannes, M. Eisen, P. Brown, & P. Sarnow, unpubl. observations). Several RNA viruses inhibit cap-dependent translation in infected cells. This raises the question whether the translation products of cellular mRNAs that can be translated cap-independently have important functions in the viral infectious cycle and in viral pathogenesis.

MATERIALS AND METHODS

Cells and virus

HeLa cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% calf serum containing $1 \times$ antimycotic and antibiotic solution (Gibco BRL). Mutant poliovirus, 3NC202, was used in all experiments (Sarnow et al., 1986). Cells were infected with poliovirus at a multiplicity of infection of 30 as described (Sarnow et al., 1986).

Cloning technologies

Restriction enzymes (Gibco BRL) were used according to manufacturer's instructions. The eIF4GI 5' terminal sequences were isolated from the Marathon Hu HeLa cell cDNA library provided by Clontech (Palo Alto, California), using a 4GI gene-specific primer complementary to nt 408–442 (Yan et al., 1992) and the AP1 primer (Clontech) which anneals to fixed end sequences of the cDNA. The eIF4GII 5' terminal sequences were isolated using a 4GII-specific primer complementary to nt 239–259 (Gradi et al., 1998) and the AP1 primer. The isolated products were cloned into pCR2.1 vector (Invitrogen) and sequenced. Radiolabeled probes were randomly primed using RadPrime DNA labeling reagents (Gibco BRL). Probes were of the following sequences: BiP: nt 1–220 (Ting & Lee, 1988); Actin: nt 685–1171 (Erba et al., 1986); c-myc: nt 1683–2121 (Watt et al., 1983); Jun B: nt 1051–1130 (Nomura et al., 1990); eIF4G: nt 368–1353 (Yan et al., 1992).

Construction of the dicistronic vectors

The eIF4GI and eIF4GII 5' regions were cloned into the dicistronic CAT/LUC vector as described (Macejak & Sarnow, 1991). Briefly, the 5' terminal sequences of eIF4GI or eIF4GII (Figs. 6 and 8) were amplified in a PCR reaction using 5' primers which contained a terminal *Sal* I site and 3' primers which contained a *Nco* I site encompassing the AUG start codons (AUG₁₉₆ for eIF4GI (Fig. 6) and AUG₂₅₇ for eIF4GII (Gradi et al., 1998)). The fragments were then cloned into the

Sal I and *Nco* I sites in the dicistronic vector (Macejak & Sarnow, 1991) to yield pSVCAT/4G-I_{ext}/LUC and pSVCAT/4G-II_{long}/LUC.

Transient plasmid transfections of cultured cells

HeLa cells were grown to 60–70% confluency in 60 mm plates and transfected with 4 μ g of plasmid DNA using 20 μ g Lipofectin Reagent (Gibco BRL) per plate, following the manufacturer's instructions. Cells were harvested after incubation for 48 h at 37 °C and CAT and LUC activities were determined in the extracts as described (Ausubel et al., 1989).

Radiolabeling of cells and preparation of extracts

At various times after infection, 100-mm plates containing $2\text{--}3 \times 10^6$ cells were washed once with DMEM medium lacking methionine and cysteine. Next, DMEM (–meth, –cys) containing 100 μ Ci/ml of ³⁵S methionine (3,000 Ci/mmol) was added and the plates incubated for 10 min at 37 °C. The medium was removed and the cells washed twice with ice-cold PBS on ice. The cells were scraped into 1 ml of ice-cold PBS, transferred to a 1.5-ml Eppendorf tube, and centrifuged at 1,000 rpm for 5 min at 4 °C. The pellet was resuspended in ice-cold extraction buffer (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP40), vortexed briefly, and incubated on ice for 10 min. Nuclei and debris were sedimented (10,000 rpm, 10 min) and the supernatant was saved. Protein concentration was determined using the Biorad protein reagent (Biorad) with IgG as a standard.

RNA isolation and Northern blot analysis

Total RNA was isolated from $2\text{--}3 \times 10^6$ mock- or poliovirus-infected cells using the Trizol Reagent (Gibco BRL) following the manufacturer's procedure. Briefly, one 100-mm plate was washed twice with PBS and lysed on the plate with 1 ml of Trizol Reagent. After centrifugation in an Eppendorf centrifuge ($12,000 \times g$, 10 min), the RNA was isolated from the aqueous phase and precipitated with isopropanol. The pellet was washed with 75% ethanol and resuspended in 100 μ l of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 ($1 \times$ TE). The RNA was precipitated again in 0.3 M NaOAc and 2.5 volumes of ethanol, washed with 70% ethanol, and resuspended in 100 μ l of $0.1 \times$ TE. The concentration of RNA was determined by spectrophotometry.

Five micrograms of total RNA was separated through a 1.2% formaldehyde-containing agarose gel and transferred to a Zeta probe membrane (Biorad) overnight (Ausubel et al., 1989). The RNA was crosslinked to the membrane by ultraviolet irradiation using a stratalinker (Stratagene) at an energy setting of 125 mJ at 4 °C. The integrity of the RNA and the efficiency of transfer were evaluated by staining the membrane with methylene blue. The blots were hybridized with ³²P-labeled, randomly primed probes in 1 mM EDTA, 5 M Na₂PO₄, 7% SDS overnight at 65 °C, subsequently washed twice with 1 mM EDTA, 40 mM Na₂PO₄, 5% SDS at 65 °C, and twice with 1 mM EDTA, 40 mM Na₂PO₄, 1% SDS at

65°C according to manufacturer's directions (Biorad). Blots were visualized by autoradiography. For the cycloheximide experiment shown in Figure 3, mock-infected cells were incubated with medium for 1 h before the addition of 0.1 mg/ml cycloheximide to the medium. No cycloheximide was added to a control sample which was harvested at the same time as the 4-h cycloheximide sample.

Poly (A⁺) RNA was isolated from nontransfected and transfected HeLa cells using the Dynabeads mRNA Direct kit (DYNAL) as described recently (Yang & Sarnow, 1997).

Polysome analysis

Approximately 2×10^7 cells were mock infected or infected with 3NC202 poliovirus and incubated at 37°C. Prior to harvest, cycloheximide was added to the medium at a concentration of 0.1 mg/ml cycloheximide for 3 min at 37°C. The medium was then removed and replaced with 10 ml of ice-cold PBS containing 0.1 mg/ml cycloheximide, and the plate was placed on ice. All subsequent procedures were carried out on ice. First, the cells were washed twice with ice-cold PBS containing 0.1 mg/ml cycloheximide and lysed directly on the plate after addition of 400 μ l of polysome extraction buffer (15 mM Tris-Cl, pH 7.4, 15 mM MgCl₂, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide and 1 mg/ml heparin). Extracts were transferred to 1.5 ml Eppendorf tubes and incubated on ice for 10 min with occasional mixing. The nuclei and debris were removed by centrifugation at $12,000 \times g$ for 10 min in a microcentrifuge. Supernatants were recovered and 500 μ l aliquots were layered onto 10 ml, 10–50% sucrose gradients composed of extraction buffer lacking Triton X-100. The gradients were sedimented at 35,000 rpm for 190 min in a SW41 rotor at 4°C. The gradients were collected as 1.2 ml fractions by pumping 60% sucrose into the bottom of the gradient and collecting from the top using an ISCO fraction collection system, with concomitant measurement of the absorbance at 254 nm. The fractions were adjusted to 2 ml by the addition of H₂O, followed by the addition of 3 ml of 8 M guanidine HCL and vortexing extensively for 2 min. Next, 5 ml of 100% ethanol was added and the fractions stored at –20°C overnight. The fractions were centrifuged at 10,000 rpm for 25 min to collect the RNA. The pellets were washed with 75% ethanol, carefully resuspended in 400 μ l of TE, pH 7.5, and precipitated again in 0.3 M NaOAC and 2.5 volumes of ethanol. The pellets were washed with 75% ethanol and resuspended in 100 μ l of TE, pH 7.5. Equal volumes of each sample were analyzed by Northern analysis as described above. All quantitations were performed using a Storm phosphorimager and Imagequant software (Molecular Dynamics). All analyses were performed three times and representative experiments are shown. In the EDTA release experiment, MgCl₂ was substituted with 15 mM EDTA in the extraction buffer and the gradient.

Western blot analyses and immunoprecipitations

Approximately 20 μ g of total cell extracts (Waggoner & Sarnow, 1998) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Biorad) at 25 V

for 12 h at 4°C as described (Ausubel et al., 1989). The membranes were incubated with 5% milk in Tris-buffered saline (TBS) overnight at 4°C. The blots were washed three times in TBS, incubated with polyclonal antibodies directed against eIF4G (Gradi et al., 1998) (a gift from N. Sonenberg, McGill University) for 1 h at room temperature, washed, incubated with horseradish peroxidase-conjugated anti-IgG, and visualized using the ECL kit following manufacturer's directions (Amersham Life Sciences).

Immunoprecipitations were performed with 20 μ g of ³⁵S-methionine-labeled extracts using excess amount of antibodies directed against GRP78, myc, or actin (Santa Cruz Biotechnology) following the manufacturer's procedure, except that the final wash solution included 500 mM NaCl. The pellet was then resuspended in 30 μ l of SDS-PAGE loading buffer and boiled for 10 min. The beads were removed by short centrifugation and the supernatants loaded into the wells of a 10% SDS-PAGE gel. The gel was dried and visualized after autoradiography.

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