Initiation Factor eIF2-independent Mode of c-Src mRNA Translation Occurs via an Internal Ribosome Entry Site*

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Overexpression and activation of the c-Src protein have been linked to the development of a wide variety of cancers. The molecular mechanism(s) of c-Src overexpression in cancer cells is not clear. We report here an internal ribosome entry site (IRES) in the c-Src mRNA that is constituted by both 5’-noncoding and -coding regions. The inhibition of cap-dependent translation by m7GDP in the cell-free translation system or induction of endoplasmic reticulum stress in hepatoma-derived cells resulted in stimulation of the c-Src IRES activities. Sucrose density gradient analyses revealed formation of a stable binary complex between the c-Src IRES and purified HeLa 40 S ribosomal subunit in the absence of initiation factors. We further demonstrate eIF2-independent assembly of 80 S initiation complex on the c-Src IRES. These features of the c-Src IRES appear to be reminiscent of that of hepatitis C virus-like IRESs and translation initiation in prokaryotes. Transfection studies and genetic analysis revealed that the c-Src IRES permitted initiation at the authentic AUG351, which is also used for conventional translation initiation of the c-Src mRNA. Our studies unveiled a novel regulatory mechanism of c-Src synthesis mediated by an IRES element, which exhibits enhanced activity during cellular stress and is likely to cause c-Src overexpression during oncogenesis and metastasis.

Most eukaryotic mRNAs are translated by a cap-dependent mechanism where eIF4F complex binds to the 5’ cap structure through its eIF4E subunit (1, 2). This binding event results in activation of mRNA and assembly of the 48 S preinitiation complex. The 48 S complex scans mRNA in a 5’ to 3’ direction until an appropriate AUG initiation codon is encountered, which is followed by joining of the 60 S subunit (1). Many cellular conditions such as apoptosis, stress, mitosis, heat shock, hypoxia, infections, and nutrient deficiency alter the function of translation initiation machinery. This is largely affected by post-translational modifications (e.g. phosphorylation) and/or cleavage of canonical initiation factors (e.g. eIF4B, eIF3, eIF2a, and eIF4G family members) (1, 3, 4). A considerable number of cellular and viral mRNAs have been shown to be translated by a cap-independent mechanism due to the presence of an IRES3 element in the mRNAs (5, 6). Nearly 125 IRES elements have been described in a variety of species ranging from viruses to humans (see Ref. 3 for lists of IRESs). The IRES elements have been detected in a number of eukaryotic mRNAs that encode proteins involved in signal transduction pathways, gene expression and development, differentiation, apoptosis, and cell cycle or stress response (1, 2, 7). For example, cellular stress causes dephosphorylation of eIF4E and hypophosphorylation of 4E-BPs, both of which are unfavorable for the assembly of translation preinitiation complex by the cap-dependent mechanism (1, 8). However, under these conditions, Bcl-2, X-linked inhibitor of apoptosis, eIF4G, vascular endothelial growth factor, ornithine decarboxylase, platelet-derived growth factor, P73, c-Myc family members, and a whole host of proteins maintain their presence because of their IRES-controlled translation (3, 6, 9–11, 15).

All of the viral and cellular IRESs initiate translation of a downstream open reading frame (ORF) by a cap-independent mechanism despite their rich structural diversities (12). The distinct structural features allow the IRESs to attract a different set of canonical and noncanonical translation factors for their efficient activities and/or regulation. For example, some of the viral and cellular IRESs require initiation factors such as eIF4G and poly(A)-binding protein, whereas others show enhanced activities when these factors are cleaved or their function is inactivated (2). A few of the IRESs seek support from IRES-specific trans-acting factors such as heterogeneous nuclear ribonucleoprotein family members, polypyrimidine tract-binding protein, lupus-associated antigen, and poly(rC)-binding protein for their efficient function (11, 13–15). The IRESs also exhibit variations in the mode of assembly of preinitiation complex. Poliovirus-like IRESs recruit the 48 S preinitiation complex upstream of the initiation site and require scanning of the complex for the initiator AUG codon, whereas an extensively studied encephalomyocarditis virus IRES recruits the preinitiation complex at the initiation site that includes AUG (3, 12). The IRESs of hepatitis C virus (HCV, a hepacivirus), classical swine fever virus (a pestivirus), cricket paralysis virus (a dicistrovirus), and simian picornavirus type 9 constitute a distinct class because of their ability to directly bind and make multiple contacts with the 40 S ribosomal subunit (12, 16–18). The

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assembly of productive initiation complexes on these IRESs is energy-efficient and can ignore the need of several critical translation initiation factors (eIFs 4F/4A/4B/1/1A) that are controlled by a variety of external and internal cellular regulators (15, 19). This “40 S-binding signature” has not been reported for the known cellular IRESs.

Translational dysregulation of a whole host of mRNAs has been observed in many diseases, including cancer. This is caused by a breakdown of the translational control mechanism, aberrant levels of translation factors, and/or undesirable mutations in these factors (2, 9, 20). The level of c-Src protein, a prominent member of the nonreceptor tyrosine kinase family, is known to increase in a variety of tumors (21–25). However, it is not known whether the enhanced expression is regulated by transcriptional and/or post-transcriptional mechanisms. The c-Src protein promotes cell differentiation, tumor growth, metastasis, and angiogenesis (24–27). It activates STAT3, which transcriptionally regulates expression of Bcl-XL, c-Myc, and cyclin D1 leading to activation of anti-apoptotic and cell cycle progression pathways (28, 29). The c-Src activities are also important for promoting vascular endothelial growth factor-associated tumor angiogenesis and protease-associated metastasis (30).

Post-translational modifications such as phosphorylation and myristoylation are key regulators of the c-Src activities. Although nonmyristoylated c-Src readily moves to the nucleus at G0, and at the G1/S phase, myristoylation at the N terminus is required for its membrane attachment and transforming activities (31, 32). The intramolecular interaction between its Src homology 2 domain and phosphorylated Tyr-530 residue (numbered according to GenBankTM accession number NM_198291) at the C terminus induces closed or inactive conformation in the c-Src molecule. Under basal conditions in vivo, 90–95% of Src is found in this state (33). The dephosphorylation of Tyr-530 by protein-tyrosine phosphatase and autophosphorylation of Tyr-419 by its kinase domain causes induction of an enzymatically active, open conformation (25, 27).

The Src gene is composed of 14 exons (34, 35). Transcription of this gene in hepatoma cells from two different promoters and alternative splicing results in mature transcripts that differ only of this gene in hepatoma cells from two different promoters and an enzymatically active, open conformation (25, 27). The dephosphorylation of Tyr-530 by protein-tyrosine phosphatase and autophosphorylation in the c-Src molecule. Under basal conditions in vivo, 90–95% of Src is found in this state (33). The dephosphorylation of Tyr-530 by protein-tyrosine phosphatase and autophosphorylation of Tyr-419 by its kinase domain causes induction of an enzymatically active, open conformation (25, 27).

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In Vitro Transcription—The plasmids p5′Src-FLuc, p5′SrcΔ1-FLuc, p5′SrcΔ2-FLuc, p5′SrcΔ3-FLuc, and p5′SrcΔC-FLuc were linearized with HpaI and transcribed with T7 RNA polymerase to produce luciferase reporter RNAs. The uncapped RNAs were prepared with RiboMax large scale RNA production kit (Promega). The capped RNAs were synthesized in the presence of the ARCA cap analogue using mMessage mMachine ultra kit (Ambion) in accordance with the manufacturer’s instructions. The transcribed RNAs were passed through G-25 column and purified by extraction with phenol: chloroform:isoamyl alcohol followed by water-saturated cold ether. Following precipitation and washing with 70% ethanol, the final preparations were dissolved in RNase-free water and checked for integrity of RNAs by formaldehyde-agarose gel electrophoresis. Concentrations of RNA were determined spectrophotometrically. For preparation of the c-Src NCR probe, 5′-capped RNAs were synthesized in the presence of cap analogue using T7 RNA polymerase.

Cell Culture and Preparation of Cell Lysates—Huh7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1X penicillin/streptomycin and 10% fetal bovine serum (Invitrogen) and maintained at 37 °C and 5% CO₂. HeLa S3 cultures were carried out in spinner flask containing Joklik-modified minimum essential medium Eagle’s (Sigma) supplemented with 5% bovine calf serum, 2% fetal clone II (Hyclone), 1X penicillin/streptomycin, and incubated at 37 °C and 5% CO₂.

HeLa translation lysates (S10) and lysates containing initiation factors (IFs) were prepared according to the protocol described by Barton et al. (38). The rabbit reticulocyte lysate (RRl) nuclease-treated was purchased from Promega. The total lysates from cultured Huh7 cells were prepared using M-PER kit (Pierce) as instructed.

In Vitro Translation of RNAs—The in vitro transcribed wild type 5′Src-FLuc and its mutant derivatives were translated in HeLa cell-free system. The standard HeLa cell-free translation mixtures contain 20 μl of S10, 10 μl of initiation factors, 5 μl of 10× buffer (155 mM HEPES-KOH, pH 7.4, 600 mM potassium acetate, 10 mM ATP, and 2.5 mM GTP, 300 mM phosphocreatine, 4 mg/ml creatine phosphokinase), 20 units of RNasin, 5–10 μg of RNA template in a 40-μl final volume. One microliter of [35S]methionine was added for radiolabeling of the newly synthesized proteins. The translation mixtures were incubated for 1–2 h at 30 °C, and the FLuc activity was assayed using 2-μl aliquots. For detection of protein bands, the samples were subjected to SDS-PAGE followed by autoradiography. For detection of the 5′Src-RLuc RNA expression, a Dual-Luciferase assay protocol (Promega) was employed, and Renilla and firefly luciferase activities were simultaneously assayed. Varying amounts of m7GDP or m7GTP were added in the standard HeLa translation mixtures for inhibition of cap-dependent translation. Unmethylated GDP or GTP served as negative control. Translation of the RNA in RRL was carried out as described in the supplier’s protocol (Promega).

RNA Stability Assay—Equal amounts of 32P-labeled wild type or mutant reporter RNAs were incubated in standard HeLa translation reactions, and total RNAs were extracted from each sample using the RNeasy kit (Qiagen). The recovered RNAs were subjected to formaldehyde-agarose gel electrophoresis followed by autoradiography of the dried gel. The bands of 18 S or 28 S rRNA in each lane were measured by ethidium bromide staining before drying the gels. During transfection experiments, 32P-labeled reporter RNAs (1–2 X 106 dpm) were transfected into Huh7 cells using the standard transfection method, and total RNAs were isolated. The radioactive full-length RNAs were detected by autoradiography.

Transfection of RNA into Cells—Huh7 cells were transfected with in vitro transcribed RNAs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 3, 5, 8, 24, and 48 h post-transfection, the cells were harvested and resuspended with lysis buffer (100 mM potassium phosphate pH 6.8, 1 mM dithiothreitol, 0.5% Igepal). The samples were then subjected to two freeze-thaw cycles, and supernatants were assayed for Luc activities. For fluorescence microscopy, the cells were grown on coverslips (Fisher) followed by RNA transfection. The cells were fixed with 4% formaldehyde 48 h post-transfection, permeabilized, and stained with anti-firefly luciferase monoclonal antibody (Bionovus). A fluorescein isothiocyanate-labeled secondary conjugate was used to visualize the FLuc distribution in the transfected cells.

Isolation of 40 S Ribosomal Subunit—HeLa S10 lysate was prepared from HeLa S3 cells grown in a spinner flask as described by Barton et al. (38). The ribosomes were pelleted from S10 lysate by centrifugation in Ti70.1 rotor (Beckman, 45,000 rpm) for 3 h at 4 °C, and the pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 50 mM KCl, and 4 mM MgCl₂) at a concentration of 150 units/ml measured as A260 as described by Pisuere et al. (39). Puromycin (1 mM) and KCl (0.5 mM) were added, stirred in an ice bath for 10 min, followed by incubation for 10 min at 37 °C. The mixture was then loaded onto a 10–30% sucrose density gradient and centrifuged for 16 h at 4 °C in a Beckman SW28 rotor (22,000 rpm). The peak fractions containing 40 S ribosomes (as determined by the presence of only 18 S rRNA) were pooled and concentrated in Ultracent-100 K (Millipore). The final preparation was dialyzed in buffer C (20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 100 mM KCl, 2 mM MgCl₂, 0.25 M sucrose), aliquoted, and stored at −80 °C (39).

Sucrose Density Gradient Analysis—Capped or uncapped 32P-labeled RNAs were incubated in standard HeLa translation lysates that were treated with 1 mM GMP-PNP for 5 min in an ice bath. The mixtures were then incubated for 15 min at 30 °C, layered onto a 10–30% sucrose gradient in buffer K (20 mM Tris-HCl, pH 8.0, 100 mM potassium acetate, 5 mM magnesium acetate, and 2 mM dithiothreitol), and centrifuged for 3 h at 45,000 rpm and at 4 °C in an SW-51 rotor. Fractions (250 μl) were collected from the bottom of the gradient and analyzed by scintillation counter. Total RNAs from peak fractions were iso-
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A.

![Diagram of c-Src gene organization](image)

**Characteristics of the Computer-generated c-Src RNA Structures**—Our preliminary investigations on c-Src translation (not shown) and several published reports (21, 22, 26, 28) indicate that c-Src level is enhanced in many cell types during stress conditions that impair cap-dependent translation. This observation prompted us to examine cap-independent translation of the c-Src mRNAs. The 5′NCRs in the c-Src transcripts have been shown to be relatively longer than those in most cellular mRNAs (35). Sequence analysis revealed multiple pyrimidine-rich motifs and two cryptic AUGs with short ORFs at positions 147 and 179 in the 350-nt-long type 1A 5′NCR (Fig. 1). Only the AUG located at position 351 is known to serve as the initiator codon in this mRNA. Our nucleotide blast search using the BLASTN position 351 and 353 for nt 1–383, which was found to be similar to the structure obtained for nt 1–410 segment, is shown in Fig. 2. This Y-shaped secondary structure appears to contain three domains designated as domains I–III. We further observed that a large portion of domains I and II were conserved in the structures predicted for all three c-Src segments. In addition, a high degree of conservation in the apical loops contributed by AACAGA (nt 360–366), GUGCCA (SL II, nt 289–294), and UAUUCU (SL III, nt 255–260) motifs was also noticed in the predicted structures. The structures in domain III, however, showed less conservation among various structures generated for the three c-Src segments. A 14-nt pyrimidine (Py)-rich motif (nt 330–344) was located 6 nt upstream of the initiator AUG, which conforms with Py tracts found in many viral IRESs. These characteristics and the Y-shaped architectural features are considered as important elements of many viral and cellular IRESs (42). The predicted structure for the c-Src nt 1–353 that represents the entire 5′NCR and AUG codon lacked a significant portion of domain II structure (structure not shown).

**c-Src mRNA Motif Supports Cap-independent Translation of Reporter RNAs**—FLuc-based reporter mRNAs were engineered to test if the c-Src 5′NCR supports cap-independent translation (Fig. 3A). Because a 33-nt sequence motif downstream of the initiator AUG in the c-Src mRNA forms a conserved stem-loop structure at the translation initiation site (Fig. 2), we included the region with the 5′NCR for engineering a parent reporter 5′Src-FLuc RNA. The RNA contains c-Src nt 1–383 (full-length 5′NCR plus 33 nt of the coding region) that is fused in-frame with luciferase ORF and ends with the poly(A) tail (Fig. 3A). In vitro transcribed capped and uncapped RNAs were translated in rabbit reticulocyte cell-free lysate (45, 46) in the presence of [35S]methionine, and the synthesized products were visualized by autoradiography. As expected, the capped 5′Src-FLuc RNA was translated to produce active luciferase protein (Fig. 3B, lane 3). Interestingly, the uncapped 5′Src-FLuc RNA was also translated but with higher efficiency (Fig. 3B, lane 2) than its capped counterpart (lane 3). During the assay, we used a reporter RNA [5′PV(Δ286–605)-FLuc] that contains PV 5′NCR but lacks a major portion of its IRES element (from nt 286 to 605). Thus, the noncoding region (420 nt) in the mutant PV construct represents nt 1–285 followed by nt 606–
was successfully achieved when the same RNA had 5’ m7G cap structure (Fig. 3B, lane 5). These results clearly demonstrate that the c-Src nt 1–383 allow cap-independent translation of downstream ORF, and its activity is enhanced when cap function is absent.

The translatability of the mRNA constructs was further tested in HeLa cell-free translation lysates that have been widely used for investigating IRES-mediated translation initiation (38). An uncapped reporter FLuc RNA that contains wild type, full-length PV 5’NCR (5’PV-FLuc, Fig. 3C, lane 4) and 5’Src-FLuc (lane 2) were efficiently translated, whereas the mutant 5’PV(Δ286–605)-FLuc again failed to support synthesis of luciferase (lane 3). Next, we examined the c-Src 5’NCR-promoted translation in the context of a dicistronic mRNA (5’Src-RFLuc). The RNA is similar to the monocistronic 5’Src-FLuc RNA except that it contains RLuc ORF and stop codon upstream of the c-Src sequence (Fig. 4A). The in vitro transcribed capped RNA was transfected into hepatoma HuH7 cells for 3 h, and the lysates were subjected to Dual-Luciferase assay. As shown in Fig. 4B, both ORFs were translated in these cells. The capped dicistronic RL-HCV1b and RL-vector RNAs were used as positive and negative controls, respectively, during the transfection. The RL-HCV-1b is similar to the 5’Src-RFLuc (Fig. 4A) except that the translation of downstream FLuc ORF is controlled by the HCV IRES instead of the c-Src IRES. In RL-vector, the c-Src IRES between upstream RLuc and downstream FLuc ORF is deleted. Both of the RNAs produced results as expected (supplemental Fig. S2). In a parallel experiment, total RNAs isolated from the dicistronic 5’Src-RFLuc RNA-transfected cells were subjected to Northern blot analysis using a 32P-labeled oligonucleotide probe that detects the 3’ end of FLuc ORF. The result showed that the dicistronic RNA was intact in the transfected cells (Fig. 4C, lane 3). The migration of isolated RNA was similar to that of in vitro transcribed dicistronic RNA
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![Diagram](image)

**FIGURE 3.** c-Src 5’NCR-mediated translation in cell-free lysates. A, organization of **in vitro** transcribed uncapped reporter RNAs. The 33-nt coding sequence (dotted box, C) and Kozak sequence are shown at translation initiation site. The solid line represents 5’NCR. An, poly(A) tail. B, 5 μg of uncapped (lanes 2 and 4) and capped (lanes 3 and 5) 5’Src-FLuc (lanes 2 and 3) or 5’PV(Δ286–605)-FLuc (lanes 4 and 5) RNAs were translated in RRL for 1.5 h in the presence of [35S]methionine. The FLuc protein bands were visualized by autoradiography after SDS-PAGE. Two μl of the translation lysates were assayed for enzymatic activity (shown as relative light units (RLU)) of FLuc using D-luciferin substrate. Lane 1, translation without exogenous RNA (control). C, translation of uncapped 5’Src-FLuc (lane 2), 5’PV(Δ286–605)-FLuc (lane 3), and 5’PV-FLuc (lane 4) RNAs in HeLa cell-free lysate as described above. The FLuc activity and the protein bands are shown.

![Diagram](image)

**FIGURE 4.** c-Src 5’NCR-mediated translation in Huh7 cells. A, schematic of an **in vitro** transcribed dicistronic reporter mRNA (5’Src-FLuc). B, Huh7 cultured cells (80% confluence, 50-mm dish) were transfected with 10 μg of capped 5’Src-RFLuc for 3 h, and Renilla and firefly luciferase activities were assayed in the cytoplasmic fractions. Each transfection was carried out in triplicate, and the experiment was repeated three times to confirm the results. The cytoplasmic fractions of untransfected cells were used as negative control. C, Northern blot analysis of total RNA isolated from 5’Src-RFLuc-transfected (lane 3) and untransfected (lane 4) Huh7 cells. Lanes 1 and 2 show position of monocistronic 5’Src-FLuc and dicistronic 5’Src-RFLuc RNAs, respectively, as RNA markers. The 32P-labeled oligonucleotide probe was derived from 3’ end of the FLuc ORF. Overexposure of the film during autoradiography (for more than a week) did not show any fragment of the dicistronic mRNA in lane 3.

(4C, lane 2) and was not cleaved into the monocistronic form (lane 1).

The 5’Src-FLuc RNA encodes a chimeric firefly luciferase with amino acids 1–11 (MGSNKSKPDKA) of the c-Src protein at its N terminus (supplemental Fig. S1A). This c-Src motif has been shown to play an important role in membrane localization and translocation of the protein into the nucleus (31, 47). Transfection of an uncapped 5’Src-FLuc RNA into Huh7 cells resulted in the synthesis of luciferase protein that was primarily localized in the nucleus and perinuclear membranes (supplementary Fig. S1B). This observation was in sharp contrast to the diffused cytoplasmic localization of luciferase that was encoded by 5’HCV-FLuc RNA in which translation of FLuc occurs under the control of HCV IRES. The resulting FLuc lacks the c-Src amino acid 1–11 motif. These results suggest that the luciferase synthesized from 5’Src-FLuc RNA contains the c-Src protein motif, which is possible when translation is initiated at the authentic AUG351 (also see Fig. 5 for translation of 5’SrcA1-FLuc).

Identification of an IRES Element in the c-Src mRNA—We introduced several deletion mutations in the c-Src motif of the 5’Src-FLuc RNA to determine its putative IRES function. The mutant 5’SrcΔ1-FLuc is similar to the wild type 5’Src-FLuc RNA except that it lacks the c-Src coding sequence (nt 354–383; Fig. 5A). The mutant 5’SrcΔ1-FLuc contains a 19-nt deletion (nt 344–362). This deletion resulted in the loss of the Kozak sequence and a major portion of SL1 structure at the translation initiation site (Fig. 2 and Fig. 5A). As shown in Fig. 5B, both of the deletions caused dramatic reduction in the synthesis of FLuc (lanes 4 and 7) as compared with the wild type RNA (lane 3). Similarly, the 5’SrcΔ1-FLuc mutant RNA that contains a large deletion (nt 95–348) upstream of initiator AUG also failed to support efficient synthesis of FLuc (Fig. 5B, lane 5). Unlike these mutants, a 5’SrcA1-FLuc RNA that maintains nt 1–47 and 216–383 of the c-Src mRNA showed cap-independent translation of FLuc (Fig. 5B, lane 6) and was comparable with that of wild type 5’Src-FLuc RNA. The predicted structure of this mutant c-Src motif (data not shown) by the M-Fold program showed significant similarities in the domains I and II of the wild type structure (Fig. 2).

To determine the stability of the reporter constructs, we translated 32P-labeled uncapped mutants and wild type RNAs in HeLa cell-free lysates as described above. Total RNAs from each reaction were isolated by the RNeasy column method, and the input probes were visualized by autoradiography. As shown in Fig. 5C (upper panel), the amounts of full-length mutant RNAs recovered (lanes 2–5) were similar or better than that of
the wild type 5’Src-FLuc (lane 1). The quantity of 18 S rRNA (internal control) in each lane had minor variations (Fig. 5C, lower panel). This observation suggests that the mutant RNAs were present in the lysates, yet these RNAs were unable to support translation of FLuc due to absence of essential elements in the c-Src sequence motif. The different band intensities observed for the RNA probes may likely be due to minor differences in stability and/or loss during the purification process. A similar observation was also made during transfection of three mutant RNAs (Δ2, Δ3, and ΔC) into Huh7 cells. Although full-length mutant RNA probes were purified from the transfected cells (Fig. 5F), only Δ3 mutant showed efficient synthesis of reporter FLuc (Fig. 5E). These results further suggest that a functional IRES that is represented by the c-Src motif in Δ3 mutant (Fig. 2, Domains I and I) was capable of directing translation by a cap-independent mechanism in cells as well as in the cell-free lysates. Unlike known cellular IRESs, this IRES requires a coding region for its optimal function.

We carried out kinetic analysis of translation promoted by the wild type and mutant c-Src motifs in HeLa translation lysates. The time course experiment presented in Fig. 5D shows that translation of the 5’Src-FLuc RNA exponentially increased...
Study the assembly of 80 S translation initiation complex on the c-Src IRES in HeLa cell-free translation lysates. Incubation of the lysates with 1 mM GMP-PNP in ice for 5 min resulted in the formation of 43S/48S complexes (Peak I) or 28S rRNA (Peak II) in the absence of exogenous ATP and GTP. Inhibition of translation initiation by 1 mM GMP-PNP did not affect the assembly of 80 S complexes. The complexes were separated by sucrose density gradient centrifugation, and the fractions containing 40 S complexes (Peak I) and 60 S complexes (Peak II) were determined by incorporation of the input RNA probe into the 18 S rRNA. The result clearly established the assembly of the 80 S complex on the c-Src 5' NCR motif, which was not inhibited by 1 mM GMP-PNP. In a similar translation reaction, further reduced GTP and ATP concentrations by omitting the 10× reaction buffer from the translation mixture. This omission caused 10× increase in the GMP-PNP to GTP ratio during translation. Interestingly, the 80 S assembly on the 5'Src-FLuc mRNA probe occurred when the cap structure was absent in the FLuc RNA probe (Fig. 6A, Peak I, broken line with squares; Fig. 6B, lane 5) similar to the standard reaction conditions described above. On the contrary, when a capped FLuc mRNA probe that lacks the c-Src motif was used in a standard translation reaction supplemented with 1 mM GMP-PNP, only 48 S complex was obtained as expected (Fig. 6A, Peak II, solid line with triangles). This conclusion was based on the observation that peak II lacks 28 S RNA, showed lower sedimentation than peak I, and contains only input RNA probe and 18 S rRNA (Fig. 6B, lane 7). In addition, the 48 S complex assembly was considerably reduced when the cap structure was absent in the FLuc RNA probe (Fig. 6A, Peak II, broken line with triangles, and B, lane 6). The GMP-PNP is known to inhibit GTPase function that is required for the assembly of the 80 S complex on a capped mRNA. Thus, the observed cap-independent 80 S assembly on the c-Src IRES is most likely to be independent of eIF2 function. In a similar
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experiment, the assembly of ribosomal complex on a mutant poliovirus 5'NCR containing FLuc (PVΔ286–605-FLuc) was compared with the 5'Src-FLuc RNA in the presence of 1 mM GMP-PNP (Fig. 6, C and D). The uncapped 5'Src-FLuc RNA showed assembly of 80 S in a reproducible manner (Fig. 6, C, Peak I, squares with solid line, and D, lane 3). However, the uncapped PVΔ286–605-FLuc RNA showed a peak at lower sucrose density (Fig. 6, C, Peak II, triangles with broken line; D, lane 4), and the complexes were spread over a wide range of sucrose density, most likely due to varying compositions of ribonucleoprotein complexes formed with the mutant PV RNA.

The assembly of 80 S on the c-Src IRES was further strengthened by our finding that purified 40 S ribosomal subunit directly interacts with the c-Src IRES (Fig. 7A). The purified 40 S subunit was mixed with uncapped 32P-labeled IRES fragment of the 5'Src-FLuc, and bound complex was separated from the free probe by sucrose density gradient centrifugation. Characterization of the peak fractions revealed the presence of 18 S rRNA and the probe in the same peak (Fig. 7A, inset, lane 1), although the free IRES probe showed a peak at lower sucrose density. A nonspecific RNA probe of similar length and nucleotide contents (PVΔ286–605 5'NCR) failed to form the 40 S-RNA binary complex in this assay (Fig. 7B inset, lane 2). These results together provide evidence that the c-Src mRNA contains an IRES element that directly interacts with the 40 S ribosomal subunit and is capable of assembling the 80 S complex during conditions when eIF-2 function is significantly compromised.

**c-Src IRES-mediated Translation Is Enhanced when Cap-dependent Translation Is Inhibited**—The eIF4E protein is a key translation initiation factor that binds the 5' cap structure of an mRNA and initiates assembly of the 48 S preinitiation complex (1). It has been shown that m7GDP inhibits eIF4E function by occupying its cap-binding site. Therefore, cap-dependent translation is efficiently inhibited by the m7GDP cap analogue (44). The cap-dependent translation of a FLuc RNA, which contains the 5’cap and 3’ poly(A) tail at the respective ends of the luciferase ORF but lacks an IRES (5’Cap-FLuc), was inhibited by m7GDP in a dose-dependent manner in RRL (Fig. 8A). In contrast, the HCV IRES-controlled translation of a reporter FLuc ORF (5’HCV-FLuc) was stimulated until a threshold concentration (10 μg) of m7GDP was reached. Above this concentration, both cap-dependent as well as HCV IRES-dependent translations were inhibited. Interestingly, translation of the 5’SrcA3-FLuc RNA (genetic organization shown in Fig. 5A) was considerably enhanced in the presence of m7GDP as observed for the HCV IRES-mediated translation initiation. Similar observations were also made for the uncapped wild type 5’Src-FLuc RNA (not shown).

Next, we examined translation of a capped dicistronic RNA (5’Src-RFLuc; Fig. 4A) in RRL in the presence of increasing concentrations of m7GDP. The wild type c-Src IRES-controlled translation of FLuc was initially enhanced in the presence of m7GDP (5–10 μg) as observed for its monocistronic counterpart, whereas the cap-dependent translation of the upstream RLuc ORF continued to decline with increasing concentrations of m7GDP (Fig. 8B).
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A. % FLuc Activity

B. % Luc Activities

Although the requirement of inhibitor concentration to inhibit overall translation was a little higher than that of the monocistronic RNAs, the stimulation pattern of the c-Src IRES in the presence of m7GDP was similar for both monocistronic and dicistronic templates. During several control experiments (data not shown), we observed that the m7GTP cap analogue (inhibitor) also causes stimulation of the c-Src IRES in HeLa and RRL cell-free translation systems, whereas the unmethylated nucleotides (GTP or GDP) had no effects within the concentration range used in our studies. These results together with those described above (Figs. 3, 5, and 6) established the presence of a functional IRES in the c-Src mRNA that can be activated when cap function is absent or significantly inhibited and/or eIF-2 activity is inadequate in the translation system.

**DISCUSSION**

An overwhelming majority of reports, including polysome-profiling data, strongly advocate for IRES-dependent translation initiation of a subset of cellular mRNAs during cell division, apoptosis, cellular stress, and viral infections where cap-dependent translation initiation is compromised (reviewed in Ref. 15). Unlike known cellular IRESs, the c-Src IRES demonstrated here exhibits many unique attributes that are analogous to the characteristics of HCV-like IRESs. To identify an...
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IRES function in viral and cellular mRNAs, mono- and dicistronic RNA-expressing plasmids have been extensively used during transfection studies. This approach, however, has been a subject of criticism due to expression via cryptic promoters and faulty transcription and splicing of the reporter constructs (51). To avoid spurious results generated by this method, we have used only in vitro transcribed capped and uncapped reporter RNAs for cell-free translation assays, transfection studies, and sucrose density gradient analyses. The transcription reactions were digested with DNase I prior to purification and checked by agarose gel electrophoresis for the absence of DNA contamination in the final RNA preparations. Furthermore, the reporter RNA transcription is under the control of the T7 promoter, and transcription of the RNA from plasmid DNA contamination is not possible in any of the systems used here. We also demonstrated that the wild type and mutant c-Src motif containing reporter RNAs were intact during various translation assays. These measures permitted us to present reliable data for the identification of c-Src IRES.

We established here that the c-Src IRES-controlled translation can be stimulated similar to the HCV IRES when eIF4E function is blocked (Fig. 8). The initiation factor eIF4E has been shown to be a negative modulator of the IRES-mediated translation, and translation of IRES-containing RNAs is accelerated when eIF4E availability is reduced (63). This is likely attributed to a decrease in eIF4F complex formation that may be accompanied by an increased availability of eIF4GI/4G3 or eIF4A RNA helicase or other initiation factors. Based on these observations, we believe that a direct binding of m7GDP to eIF4E in our assay may lead to increased availability of translation factors that are required for efficient activities of the HCV or c-Src IRESs.

Our in vitro studies that defined the presence of an IRES in the c-Src mRNA were further corroborated by the results of transfection of mono- and dicistronic reporter RNAs into hepatoma-derived cells and induction of cellular stress in the transfected cells. Uncapped reporter RNAs containing nt 1–383 of c-Src mRNA at their 5’ ends were efficiently translated in two cell-free translation systems (RRL and HeLa lysates) and in Huh7 cells. Our genetic analysis shows that nt 200–383 of the c-Src mRNA, which harbors initiator AUG (at nt 351), plays a pivotal role in promoting cap-independent translation. An extensive analysis of the secondary and/or possible higher order structures within this region is, however, needed for accurate understanding of its role in loading productive initiation complex. We found that the c-Src IRES promotes assembly of stable 80 S complexes in the absence of cap structure and in the presence of 1 mM GMP-PNP. Under similar conditions, however, only 48 S complex can be trapped on a capped reporter RNA lacking a 5’ NCR or contains a scrambled IRES. Furthermore, similar to the HCV-like IRESs, a direct binding of purified HeLa 40 S with the c-Src nt 1–383 was detected in the absence of initiation factors. These evidence together strongly support the existence of a physiologically relevant IRES element at the 5’ end of c-Src mRNA. The c-Src IRES appears to be functionally similar to the HCV IRES as both IRES elements directly interact with the purified 40 S subunit, require coding region for their functions, promote eIF2-independent assembly of 80 S complex (Figs. 3, 5 and 6) (see Refs. 19, 43, 52, 53 for the HCV IRES function), and are stimulated when eIF4E or eIF2α function is impaired (Figs. 8 and 9). Therefore, our studies reported here present several unique attributes of a cellular IRES that have been demonstrated only for HCV-like IRESs.
The sucrose gradient analyses further provided insights into the mechanism of ribosome assembly on the c-Src IRES. The nonhydrolyzable GTP analogue, GMP-PNP, blocks eIF2-dependent initiation pathway at the 48 S complex stage (18, 39). This effect was clearly evident for the cap-dependent translation initiation of the FLuc mRNA in HeLa cell-free lysates in which the 48 S complex was trapped by 1 mM GMP-PNP treatment (Fig. 6). Thus, the GMP-PNP concentration used here during translation initiation assembly was sufficient to block 80 S assembly by cap-dependent initiation mechanism in the translation mixture. In sharp contrast, assembly of 80 S complex took place on the c-Src IRES in the presence of GMP-PNP or in a reaction mixture containing the analogue but was also deficient in exogenously added ATP and GTP. Generally, 60 S subunits join the 48 S complex to form 80 S only after eIF5-induced GTP hydrolysis and dissociation of the eIF2-GDP complex (53). This step is preceded by ATP-dependent scanning by the 48 S complex to locate the AUG codon (1). From the data presented here, c-Src IRES appears to evade both of the critical energy-dependent steps that are needed for the 80 S assembly by the cap-dependent mechanism. Because the c-Src IRES directly binds the 40 S subunit (Fig. 7) and the structural motifs from flanking regions of the initiator AUG are required for efficient function of the c-Src IRES (Fig. 5), it is highly likely that the 48 S complex formed at this element may not require energy-dependent scanning for the initiator AUG. This notion is supported by the genetic analysis of the c-Src IRES. A 19-nt deletion at translation site in 5’/H9251c-Src-Δ1-FLuc RNA resulted in complete impairment of the IRES function despite the presence of upstream AUG147 and AUG179. Recently, the HCV IRES was shown to switch from classical eIF2-dependent initiation to the eIF2-independent pathway under cellular stress that favors inactivation of eIF2 due to phosphorylation of its α subunit. This alternative pathway was further shown to require only eIF3 and eIF5B (an analogue of bacterial IF2) for Met-tRNAiMet delivery at the P site. Based on these observations, it was proposed that the 80 S assembly on the HCV IRES is analogous to bacteria-like mode of translation initiation (53). In this context, the c-Src IRES appears to follow HCV IRES-like mode of translation initiation when the GTase function of the ternary complex is blocked. This conclusion is further supported by RNA transfection studies in which thapsigargin-led induction of cellular stress in Huh7 cells failed to inhibit the c-Src IRES despite increased Ser-51 phosphorylation of eIF2α as compared with the normal (unstressed) cells (Fig. 9).

The studies presented here demonstrate significant resistance of the c-Src IRES activities to the reduced level of ternary complex and eIF2-α phosphorylation. In contrast to the eIF2-dependent initiation pathway in which the eIF2 complex delivers Met-tRNAi to 40 S subunits in a GTP-dependent manner, the eIF2A has been shown to deliver the Met-tRNAi to 40 S subunits by AUG-dependent and GTP-independent mechanisms (19, 64). In addition, a number of RNA-binding proteins have been shown to stabilize IRES structure and/or promote ribosomal complex assembly (13, 14). A comprehensive analysis is needed to ascertain whether these factors contribute to the reduced ternary complex dependence of the c-Src IRES.

In the cells, stress and serum deprivation causes inhibition of phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway-dependent phosphorylation of eIF4E-BP. The unphosphorylated protein forms a tight complex with eIF4E and prevents its binding to eIF4G and the cap structure (8). Similarly, hypophosphorylation of eIF4E that is controlled by Ras-mitogen-activated protein kinase (MAPK) pathway also reduces its cap-binding ability. Both of these events culminate into suppression of global cap-dependent translation. In addition, phosphorylation of eIF2α by cellular kinases (e.g. PKR, PERK, HRI, and GCN2) in response to various cellular stress and viral infections leads to reduction in the level of ternary complex (eIF2-GTP-Met-tRNAiMet) due to inhibition of guanine nucleotide exchange factor activity (54). Our investigations revealed that 80 S assembly on the c-Src IRES occurs when the functions of eIF2 and eIF4E are inhibited. Therefore, it is possible that the c-Src mRNA can easily escape from tight regulation of both of these translation initiation factors, which may ultimately lead to continued c-Src protein synthesis during adverse conditions (e.g. endoplasmic reticulum stress and starvation). Enhanced c-Src level has been shown to correlate with its activated state in hepatocellular carcinoma (55). Activated c-Src is known to induce phosphorylation of eIF4E-BP1 via phosphatidylinositol 3-kinase/mammalian target of rapamycin and eIF4E via Ras/Raf/extracellular signal-regulated kinase (ERK) pathway, both of which favor cell survival and proliferation (56, 57). Thus, the c-Src IRES controlled translation provides an important recovery mechanism from translational blockade during cellular stress.

It has been shown that the cap-dependent translation of c-Src mRNA is regulated by elements located in its long 3’NCR through interaction with heterogeneous nuclear ribonucleoprotein K (59). It would be interesting to investigate if heterogeneous nuclear ribonucleoprotein K or microRNAs can affect the c-Src IRES-controlled translation through the 3’NCR interactions. Both transcripts of the c-src gene (type 1A and type 1α, Fig. 1A) contain conserved sequences that constitute most parts of the IRES element. However, the extreme 5’ ends in these mRNAs are dissimilar in length and nucleotide composition. It is not known if these sequences play any role in regulating the c-Src translation.

c-Src is an important player in signal transduction pathways that control onco genesis, cell proliferation, and metastasis (24–27). The emerging strategies for treatment of breast, lung, prostate, skin, and other cancers are focused on the inhibition of c-Src activities (23, 58, 60) but not the enhanced supply of c-Src in the tumor cells. Many of the small molecules that target c-Src activities also inhibit other protein kinases and/or show high degrees of cytotoxicity (60). Adaptation for growth during cellular stress is a hallmark feature of many cancer cells, and c-Src has been shown to play a very important role during this process (61). Our study presents c-Src IRES as a new therapeutic target for treatment of cancer. Because the c-Src IRES is located downstream of the cap structure in the mRNA, interference with the IRES structure and/or function will likely result in the inhibition of cap-dependent as well as IRES-de-
dependent c-Src synthesis. This strategy will prevent unabated c-Src supply in the cancer cells and hence is likely to reduce the chances of cancer cell survival.

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