INITIATION FACTOR eIF2-INDEPENDENT MODE OF c-Src mRNA TRANSLATION OCCURS VIA AN INTERNAL RIBOSOME ENTRY SITE (IRES)

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Overexpression and activation of 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 cell-free translation system or induction of ER 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 40S ribosomal subunit in the absence of initiation factors. We further demonstrate eIF2-independent assembly of 80S initiation complex on the c-Src IRES. These features of the c-Src IRES appear to be reminiscent to 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 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 48S pre-initiation complex. The 48S complex scans mRNA in 5’ to 3’ direction until an appropriate AUG initiation codon is encountered, which is followed by joining of the 60S subunit (1). Many cellular conditions such as apoptosis, stress, mitosis, heat-shock, hypoxia, infections and nutrient deficiency alter the function of normal 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, eIF2α and eIF4G family members) (1, 3, 4). A considerable number of cellular and viral mRNAs have been shown to be translated by cap-independent mechanism due to the presence of an IRES element in the mRNAs (5, 6). Nearly 125 IRES elements have been described in a variety of species ranging from viruses to humans (see review 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, 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 pre-initiation complex by the cap-dependent mechanism (1, 8). However, under these conditions, Bcl-2, XIAP, eIF4G, VEGF, ODC, PDGF, PTTSLRE, c-Myc family members, and a whole host of proteins maintain their presence due to their IRES-controlled translation (3, 6, 9, 10, 11, 15).

All of the viral and cellular IRESs initiate translation of a downstream open reading frame (ORF) by cap-independent mechanism in spite of
their rich structural diversities (12). The distinct structural features allow the IRESs to attract different set of canonical and non-canonical translation factors for their efficient activities and/or regulation. For examples, some of the viral and cellular IRESs require initiation factors such as eIF4G and PABP while others show enhanced activities when these factors are cleaved or their function is inactivated (2). A few of the IRESs seek support from ITAFs (IRES-specific trans-acting factors) such as hnRNP family members, PTB, La antigen and PCPB for their efficient function (11, 13-15). The IRESs also exhibit variations in the mode of assembly of pre-initiation complex. Poliovirus-like IRESs recruit the 48S pre-initiation complex upstream of the initiation site and require scanning of the complex for the initiator AUG codon, whereas an extensively studied encaphalomyocarditis virus (EMCV) IRES recruits the pre-initiation complex at the initiation site that includes AUG (3, 12). The IRESs of hepatitis C virus (HCV, a hepacivirus), classical swine fever virus (CSFV, a pestivirus), cricket paralysis virus (CrPV, a dicistrovirus) and simian picornavirus type 9 (SPV9) constitute a distinct class because of their ability to directly bind and make multiple contacts with the 40S ribosomal subunit (12, 16-18). The 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 ‘40S-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 breakdown of translational control mechanism, aberrant levels of translation factors and/or undesirable mutations in these factors (2, 9, 20). The level of cellular Src (c-Src) protein, a prominent member of the non-receptor 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). It has been shown that activated c-Src-focal adhesion kinase (FAK) complex promotes cell mobility, cell-cycle progression and cell survival. The c-Src activities are also important for promoting VEGF-associated tumor angiogenesis and protease-associated metastasis. (30).

Post-translational modifications such as phosphorylation and myristoylation are key regulators of the c-Src activities. While non-myristoylated c-Src readily moves to the nucleus in 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 SH2 domain and phosphorylated Tyr530 (numbered according to NM_198291) residue 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 Tyr530 by protein tyrosine phosphatase (PTP) and autophosphorylation of Tyr419 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 in the extreme 5' ends but encode the same 60 kDa c-Src protein (Fig. 1A). The c-Src Type-1A mRNA contains a 350 nt long 5' noncoding or untranslated region (5'NCR or 5'UTR)) with multiple AUGs located at nt positions 147, 179, and 351 (Fig 1B). However, only AUG351 is used to initiate translation of the c-Src open reading frame (ORF). The Type-1α c-Src transcript contains a 451 nt long 5’NCR and differ with Type-1A only in the first exon (1A or 1α) (35). The second and third exons (1B and 1C) are shared in both transcripts. Hepatoma cells have been shown to express both transcripts (35). The regulatory role(s) of these noncoding/untranslated elements during translation of c-Src mRNAs and their role(s) in c-Src overexpression are not known. Here, we report the presence of an IRES element in the c-Src mRNAs that is constituted by both noncoding and coding regions. This IRES possesses many unique attributes not found in the known cellular IRESs. The data presented here
show that the c-Src IRES features are reminiscent to that of HCV-like IRESs and/or translation initiation in prokaryotes. Thus, our findings have opened up new avenues for investigation on the translation control of c-Src synthesis and its effects on tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs.** Total RNA was isolated from hepatoma-derived Huh7 cell line using Qiagen RNeasy kit. Nucleotides 1-383 of the Type-1A c-Src mRNA were amplified from the total RNA using high fidelity RT-PCR Kit (Promega) and a pair of primers (Sense: 5’CATAGCAAGCTTGGAGGAGCGCCAGGC CGCGTCTGC3’; Antisense: 5’GCACGCTGTCATGAGGACCCTTGGGC TTGCTTTTGTCTACC3’, restriction enzymes sites are underlined). The amplified DNA contains wild type full-length 5’NCR and 33 nt coding region that encodes first 11 aa of the c-Src protein. The PCR products were digested with HindIII and BspHI for ligation into a vector backbone. The backbone was created by digestion of a previously described plasmid pT7C1-DC29-332 (36) with HindIII and NcoI and the PCR products were ligated at this site after restriction digestion. The resulting plasmid p5’Src-FLuc contains T7 promoter at the 5’ end of the c-Src sequence which is followed by ORF of firefly luciferase (FLuc) and ends with oligoA tail. The in vitro transcription of the HpaI digested plasmid by T7 RNA polymerase produces an RNA containing the entire 5’NCR followed by coding sequences representing N-terminus 11 aa (MGSNKSKPKDA) of c-Src fused with the FLuc ORF ending with poly(A) tail. The capped RNAs were prepared with Promega’s Ribonuclease-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’Src-FLuc was linearized with XbaI and transcribed with T7 RNA polymerase in the presence of [α-32P]CTP. The 5’PV(Δ286-605)-FLuc was similarly digested with XbaI and transcribed for preparation of an inactive IRES control probe. The probes were purified using Qiagen RNeasy purification method. The plasmid pRL-HCV1b encodes upstream Renilla luciferase followed by the HCV IRES (nt 1-357 of the HCV genotype 1b) linked to the second reporter FLuc.
(37). The plasmid was linearized with HindIII and transcribed 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 (DMEM) supplemented with 1x Pen/Strep 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 (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 and colleagues (38). The rabbit reticulocyte lysate nuclease-treated (RRL) 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 ul S10, 10 ul IFs, 5 ul 10X 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 RNasin, 5 to 10 ug RNA template in a 40 ul final volume. One microliter [35S]Methionine was added for radio-labeling of the newly synthesized proteins. The translation mixtures were incubated for 1 to 2 hr at 30°C, and the FLuc activity was assayed using 2 ul aliquots. For detection of protein bands, the samples were subjected to SDS-PAGE followed by autoradiography. For detection of the 5’Src-RFLuc 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 RNeasy Kit (Qiagen). The recovered RNAs were subjected to formaldehyde-agarose gel electrophoresis followed by autoradiography of the dried gel. The bands of 18S or 28S rRNA in each lane were measured by ethidium bromide staining before drying the gels. During transfection experiments, 32P-labeled reporter RNAs (1-2x10⁶ dpm) were transfected into Huh7 cells using 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 hours post-transfection, the cells were harvested and resuspended with lysis buffer (100 mM potassium phosphate pH 6.8, 1 mM DTT, 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 Scientific) followed by RNA transfection. The cells were fixed with 4% formaldehyde 48 hours post-transfection, permeabilized and stained with anti-firefly luciferase monoclonal antibody (Bionovus). A FITC-labeled secondary conjugate was used to visualize the FLuc distribution in the transfected cells.

Isolation of 40S ribosomal subunit. HeLa S10 lysate was prepared from HeLa S3 cells grown in a spinner flask as described by Barton and colleagues (38). The ribosomes were pelleted from S10 lysate by centrifugation in Ti70.1 rotor (Beckman, 45,000 rpm) for 3hr at 4 °C and the pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM DTT, 50 mM KCl, and 4 mM MgCl₂) at a concentration of 150 U/ml measured at A260 as described by Pisarev et al. (39). Puromycin (1 mM) and KCl (0.5 M) were added, stirred at 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’s SW28 rotor (22,000 rpm). The peak fractions containing 40S ribosomes (as determined by the presence of only 18S rRNA) were pooled and concentrated in Ultracell-100 K (Millipore).
The final preparation was dialyzed in buffer C (20 mM Tris-HCl pH 7.5, 2 mM DTT, 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 mRNAs were incubated in standard HeLa translation lysates that were treated with 1mM GMP-PNP for 5 min on 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 DTT), and centrifuged for 3 hours at 45,000 rpm and 4 °C in a SW-51 rotor. Fractions (250 ul) were collected from the bottom of gradient and analyzed by scintillation counter. Total RNA s from peak fractions were isolated using Qiagen RNeasy Mini column for analysis of the RNA contents in the fractions.

For 40S-IRES binary interaction assay, the ³²P-labeled c-Src IRES or a nonspecific RNA probe derived from 5'PV(Δ286-605) as scrambled IRES was mixed with purified HeLa 40S subunit in buffer K containing 20 U RNasin in a final volume of 40 ul and incubated for 15 minutes at 30°C. The reaction mixtures were analyzed by sucrose density gradient method as described above. A sample of RNA probe without 40S was used to specify position of the free probes during centrifugation.

RESULTS

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 BLASTN 2.2.20+ program (40) revealed that the exon 1C and 11 amino acid N-terminus coding sequences are highly conserved in humans, chimpanzees and rhesus monkeys (94%-100% identity) whereas mouse sequences in this region are 76-80% identical to the reference human c-Src mRNA sequences (NM_005417.3 and NM_198291).

Using M-Fold program (41), we examined a number of predicted secondary structures representing three segments (nt 1-353, 1-383, 1-410) of the Type-1A c-Src mRNA. A representative structure (dG = -135) 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 domain I, II and III. We further observed that a large portion of the 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 AACAGAGA (nt 360-366), GUGCCA (SL II, nt 289-294) and UAUUC (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) is located 6 nt upstream of the initiator AUG, which conforms 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).

A c-Src mRNA motif supports cap-independent translation of reporter RNAs. Firefly luciferase (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 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 poly(A) tail (Fig. 3A). In vitro transcribed capped and uncapped RNAs were translated in rabbit reticulocyte cell-
In the context of a dicistronic mRNA, we examined the c-Src 5’NCR synthesis of luciferase (lane 3). Next, we were efficiently translated while the mutant 5’Src RNA (5’Src-RFLuc). The RNA is similar to the monocistronic FLuc, Fig. 3C, lane 4) and 5’Src-FLuc (lane 2) were efficiently translated while 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 renilla luciferase (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 hr 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 in stead 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 (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 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 (lane 2), and was not cleaved into monocistronic form (lane 1).

The 5’Src-FLuc RNA encodes a chimeric firefly luciferase with aa 1 to 11 (MGSNKSKPKDA) of the c-Src protein at its N-terminus (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 (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 c-Src aa 1 to 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’SrcΔ1-FLuc).

Identification of an IRES element in the c-Src mRNA. We introduced several deletion mutations in c-Src motif of the 5’Src-FLuc RNA to determine its putative IRES function. The mutant 5’SrcΔC-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’SrcΔΔ1-FLuc contains a 19 nt deletion (nt 344-362). This deletion resulted into the loss of Kozak sequence and a major portion of SLI structure at the translation initiation site (Figs. 2 and 5A). As shown in Fig. 5B, both of the deletions caused dramatic reduction in the synthesis of FLuc (lanes
4, 7) as compared to the wild type RNA (lane 3). Similarly, the 5'SrcΔ2-FLuc mutant RNA that contains a large deletion (nt 95-348) upstream of initiator AUG also failed to support efficient synthesis of FLuc (lane 5). Unlike these mutants, a 5'SrcΔ3-FLuc RNA that maintains nt 1-47 and 216-383 of the c-Src mRNA showed cap-independent translation of FLuc (lane 6) and was comparable to that of wild type 5'Src-FLuc RNA. The predicted structure of this mutant c-Src motif (not shown) by M-Fold program showed significant similarities in the domains I and II of the wild type structure (Fig. 2).

To determine stability of the reporter constructs, we translated 32P-labeled uncapped mutant and wild type RNAs in HeLa cell-free lysates as described above. Total RNAs from each reaction were isolated by 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 18S rRNA (internal control) in each lane had minor variations (lower panel). This observation suggests that the mutant RNAs were although 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 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 (Domains I and II, Fig. 2) was capable of directing translation by cap-independent mechanism in cells as well as in the cell-free lysates. Unlike known cellular IRESs, this IRES requires 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 with time whereas the mutant 5'SrcΔ2-FLuc was translated inefficiently at all time-points. During our investigations (Fig. 5D and 5E), a minor translation was consistently observed for Δ2 mutant RNA. It is possible that sequence motifs that form domain I and translation site in this RNA might have played a role in the residual translation. These motifs are, however, absent in Δ1 and ΔC mutants that are completely incompetent for translation initiation.

Assembly of 80S initiation complex on the c-Src IRES in HeLa cell-free translation lysates is not affected by inhibition of eIF2. Sucrose density gradient analysis was carried out to study assembly of 80S translation initiation complex on the c-Src IRES. The 32P-labeled uncapped 5'Src-FLuc RNA or capped FLuc was incubated for 15 min in HeLa cell-free translation lysates containing 1 mM GMP-PNP, a nonhydrolyzable GTP analogue, which causes accumulation of 48S complex and inhibition of 80S formation on a capped mRNA (18, 43). The reaction mixtures were analyzed for ribosome assembly on the mRNAs by 10%-30% sucrose density gradient centrifugation method. The initiation complexes in the gradient fractions were determined by incorporation of the input RNA probe into the ribosomal complexes (Fig. 6A). A single peak (Peak I, solid line with squares) of ribosomal complex containing the input RNA probe, 18S and 28S rRNA was obtained for the 5'Src-FLuc RNA (Fig. 6B, lane 4). The result clearly established assembly of 80S complex on the c-Src 5'NCR motif, which was not inhibited by 1 mM GMP-PNP (Fig. 6A and 6B). In a similar translation reaction, we further reduced GTP and ATP concentrations by omitting the 10X reaction buffer from the translation mixture. This omission caused 10-times increase in the GMP-PNP to GTP ratio during translation. Interestingly, 80S assembly on the 5'Src-FLuc mRNA probe occurred (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 48S complex was obtained as expected (Peak II, solid line with triangles). This conclusion was
eIF4E protein is a key translation initiation factor when cap-dependent translation is inhibited. Function is significantly compromised. The 80S complex on a capped mRNA. Thus, the GMP-PNP is known to inhibit GTPase function that is required for the assembly of 80S complex during conditions when eIF-2 activity is compromised. 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 5'cap and 3'polyA 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 ug) of m7GDP was reached. Above this concentration, both cap- as well as HCV IRES-dependent translations were inhibited. Interestingly, translation of the 5'Src3-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 ug) as observed for its monocistronic counterpart, whereas cap-dependent translation of the upstream renilla luciferase (RLuc) ORF continued to decline with increasing concentrations of m7GDP (Fig. 8B). 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 mono- and dicistronic templates. During several control experiments (data not shown), we observed that m7GTP cap analogue 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.
c-Src IRES-controlled translation is modestly enhanced during cellular stress that blocks cap-dependent translation. Thapsigargin (TG) causes eIF-2 phosphorylation resulting in global translation inhibition and ER stress response due to inhibition of Ca²⁺-ATPase activities (48, 49). We treated Huh7 cells with 1 µM TG for different time-points (0.5 to 6 hrs) and monitored the status of eIF2α by Western blot. We observed a considerable increase in the phosphorylation level of eIF2α within 30 min of TG-treatment, which remained elevated for 6 hr (Fig. 9A, lanes 2-6, upper panel). The total eIF2α level, on the other hand, was not affected by this treatment (Fig. 9A, lower panel). The enhanced eIF2α phosphorylation may be considered as an indicator for TG-induced cellular stress and reduction in global cap-dependent translation in the treated Huh7 cells. In the subsequent experiments, Huh7 cells were pre-treated with 1 µM TG for 3 hr before transfection with the capped 5'Src-RFLuc or RL-HCV1b RNAs while maintaining 1 µM TG. The RL-HCV1b is similar to the 5'Src-RFLuc except that the translation of FLuc ORF in the RNA is driven by an HCV IRES. The FLuc and RLuc activities were assayed in the cytoplasmic fractions 3 hrs post-transfection. The translation of reporter luciferases in untreated transfected cells (control) were considered as 100% and compared to that of DMSO- or DMSO-TG-treated cells (Fig. 9B). Both the cap-dependent and IRES-dependent (HCV or c-Src) translation was not affected by DMSO treatment of the cells. In contrast, the cap-dependent translation of RLuc was dramatically reduced for both the RNAs due to DMSO-TG treatment of the cells. In these cells, however, the c-Src or HCV IRES-controlled translation of FLuc was moderately enhanced. We also determined expression level of natural c-Src protein in these lysates. A modest increase in the total c-Src protein level was observed in DMSO-TG-treated cell lysates as compared to the untreated (control) or DMSO alone-treated lysates (Fig. 9C). The c-Src mRNA level remained unchanged in these cells (Fig. 9D).

Serum-deprivation of cells also causes suppression of cap-dependent translation due to competitive inhibition of eIF4E binding to the 5' cap structure by poly(A)-specific ribonuclease (PARN) (50), and phosphorylation of eIF2α (4). When Huh7 cells were subjected to serum starvation, the total c-Src protein level was moderately enhanced within 72 hours (Fig. 9E, minus) as compared to the control (plus) although the c-Src mRNA level was not affected (Fig. 9F). These results together complement our findings that the c-Src level in Huh7 cells is modestly enhanced or maintained to steady level under varying cellular stress conditions that are unfavorable for cap-dependent translation. This effect is likely to occur due to increase in the c-Src IRES activities.

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 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 IRES function in viral and cellular mRNAs, mono- and di-cistronic 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 T7 promoter and transcription of the RNA from plasmid DNA contamination is not possible in any of the system used here. We also demonstrated that 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.
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 eIF4G/eIF4A or eIF4A RNA helicase or other initiation factors. Based on these observations, we believe that a direct binding of m7GDP to eIF-4E 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 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 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 80S complexes in the absence of cap structure and in the presence of 1mM GMP-PNP. Under similar conditions, however, only 48S 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 40S with the c-Src nt 1-383 was detected in the absence of initiation factors. These evidence together strongly support 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 40S subunit, require coding region for their functions, promote eIF2-independent assembly of 80S complex (Figs. 3, 5 and 6; see ref. 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 48S 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 48S complex was trapped by 1mM GMP-PNP treatment (Fig. 6). Thus, the GMP-PNP concentration used here during translation initiation assembly was sufficient to block 80S assembly by cap-dependent initiation mechanism in the translation mixture. In sharp contrast, assembly of 80S 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, 60S subunit joins the 48S complex to form 80S only after eIF5-induced GTP hydrolysis and dissociation of eIF2.GDP complex (53). This step is preceded by ATP-dependent scanning by the 48S complex to locate 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 80S assembly by cap-dependent mechanism. Because the c-Src IRES directly binds 40S 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 48S 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’Src-Δ1-FLuc RNA resulted in complete impairment of the IRES function in spite of the presence of upstream AUG147 and AUG179. Recently, the HCV IRES was shown to switch from classical eIF2-dependent initiation to 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-tRNA<sup>Met</sup> delivery at the P site. Based on these observations, it was proposed that the 80S assembly on the HCV IRES is analogous to
bacterial-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 GTPase 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 in spite of increased Ser51 phosphorylation of eIF2α as compared to the normal (unstressed) cells (Fig. 9).

The studies presented here demonstrate significant resistance of the c-Src IRES activities to reduced level of ternary complex (TC) and eIF-2α phosphorylation. In contrast to the eIF2-dependent initiation pathway in which the eIF-2 complex delivers Met-tRNAi to 40S subunits in a GTP-dependent manner, the eIF2A has been shown to deliver the Met-tRNAi to 40S subunits by AUG-dependent and GTP-independent mechanism (ref. 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 TC-dependence of the c-Src IRES.

In the cells, stress and serum-deprivation causes inhibition of PI3K/AKT/mTOR 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-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, HR1 and GCN2) in response to various cellular stress and viral infections leads to reduction in the level of ternary complex (eIF2-GTP-Met-tRNA₅₅₆) due to inhibition of guanine nucleotide exchange factor (GEF) activity (54). Our investigations revealed that 80S assembly on the c-Src IRES occurs when function 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. ER 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 4E-BP1 via PI3K/mTOR and eIF4E via Ras/Raf/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 hnRNP K (59). It would be interesting to investigate if hnRNP K or miRNAs 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 part 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 oncogenesis, 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 degree 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 very important role during this process (61). Our report 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-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.
REFERENCES

FOOTNOTES
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Abbreviations: c-Src, cellular Src; HCV, hepatitis C virus; ER, endoplasmic reticulum, IRES, internal ribosome entry site; Luc, luciferase; NCR, noncoding or untranslated region; ORF, open reading frame; PV, poliovirus. TC, ternary complex.

FIGURE LEGENDS

FIGURE 1. A, Organization of the c-Src gene. Transcription from two promoters (indicated as P) and alternative splicing result in Type-1α (NM_005417) and Type-1A (NM_198291) mRNAs in liver cells. Both transcripts differ only in the 5’ distal region of the 5’NCR. The sequence of the exons 1B and 1C and open reading frame (ORF) are shared in both transcripts (35). AUGi, initiator AUG codon. B, Schematic of the Type-1A 5’NCR. Two cryptic AUGs at nt 147 and 179, the initiator AUG at nt 351, and a pyrimidine-tract (nt 330-344) located 6 nt upstream of the initiator AUG are shown. The gray lines with arrows on both sides show sequence locations in different putative domains whereas similar arrows with black solid lines indicate position of the conserved stem-loop structures as shown in Fig. 2.

FIGURE 2. Computer-assisted folding of the Type-1A c-Src mRNA sequence. The Zucker’s M-Fold program (version 3.2, 41) was used for prediction of the secondary structures representing various segments of the c-Src mRNA. Only a representative structure of the c-Src nt 1-383 that includes the entire 5’NCR followed by 33 nt coding sequence, is shown here. The initiator AUG at position 351 (arrow), and putative stem-loop (SL) structures, domains and nt positions are indicated.

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, Five ug of uncapped (lanes 2, 4) and capped (lanes 3, 5) of 5’Src-FLuc (lanes 2, 3) or 5’PV(Δ286-605)-FLuc (lanes 4, 5) RNAs were translated in RRL for 1.5 hr in the presence of [35S]Methionine. The FLuc protein bands were visualized by autoradiography after SDS-PAGE. Two ul of the translation lysates were assayed for enzymatic activity (shown as RLU) of FLuc using D-luciferine 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.

FIGURE 4. c-Src 5’NCR-mediated translation in Huh7 cells. A, Schematic of an in vitro transcribed dicistronic reporter mRNA (5’Src-RFLuc). B, The Huh7 cultured cells (80% confluency, 50 mm dish) were transfected with 10 ug capped 5’Src-RFluc for 3 hr, 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.
FIGURE 5. Effect of deletion mutations on the c-Src sequence-controlled translation of reporter RNAs.  
A, The organization of in vitro transcribed uncapped wild type reporter RNA (5’Src-RFluc) and the mutants containing various lengths of deletions (Δ) in the c-Src sequences are shown; dashed line, extent of deletion.  AUG, initiator codon is underlined.  
B, The RNAs were translated for 1.5 hr in HeLa lysates in the presence of [35S]Methionine and the FLuc protein bands were visualized by autoradiography after SDS-PAGE.  
C, Stability of the reporter RNAs in HeLa translation lysates.  The 32P-labeled reporter RNAs (~1x10⁶ dpm) were added to a standard HeLa translation mixture for 1.5 hr and total RNAs were isolated by Qiagen RNeasy column method.  Half of the eluted RNA samples (20 µl) were subjected to formaldehyde-agarose gel electrophoresis.  The gel was photographed after ethidium bromide staining for detection of ribosomal RNAs (lower panel), dried and autoradiographed (upper panel).  Reporter RNAs are as indicated for each lane.  
D, Comparison of the kinetics of translation between wild type 5’Src-FLuc and a deletion mutant (5’SrcΔ2-FLuc).  The RNAs were translated in a standard HeLa lysates mixture and FLuc activities were assayed with an aliquot (2 µl) of the reaction at various time-points.  Control, translation lysates without exogenous RNA.  
E, Transfection of uncapped monocistronic c-Src mutant RNAs.  The Huh7 cells (80% confluent in 60 mm culture plates) were transfected in triplicates with uncapped RNAs as indicated and FLuc activities were assayed 3 hrs post transfection in the total lysates.  
F, Relative stability of mutant reporter RNAs in the transfected cells.  The 32P-labeled mutant RNAs (as indicated) were transfected as above.  The total RNAs were isolated and the labeled RNAs were detected by agarose gel electrophoresis followed by autoradiography of the dried gel (upper panel).  Lower panel, the same gel showing 18S rRNA in each lane.

FIGURE 6. Assembly of translation initiation complexes on the c-Src IRES in HeLa cell-free lysates and analysis by sucrose density gradient centrifugation.  
A, The translation mixtures were incubated with 1 mM GMP-PNP on ice-bath for 5 min and in vitro transcribed 32P-labeled RNAs: 5’Src-FLuc (squares in solid line) or capped FLuc (lacking IRES, triangles in solid line) or uncapped FLuc (triangles in broken line) were added to the translation reaction and incubated for 15 min at 30°C.  The complexes formed in the absence of exogenous ATP and GTP on the 5’Src-Fluc probe is shown as squares in dashed line.  The lysates were separated by 10%-30% sucrose density gradient centrifugation and fractions (250 µl) were collected from the bottom of the tube to determine RNA contents.  
B, Total RNAs from the peak fractions (Peak I or Peak II) were isolated and analyzed by agarose gel electrophoresis.  Marker lanes: 1, input 5’Src-FLuc RNA probe; 2, total RNA extracted from HeLa S10 translation lysate showing 18S and 28S rRNAs; 3, 18S rRNA extracted from purified 40S subunit.  The total RNAs extracted from Peak I (fractions 5 and 6) for 5’Src-Fluc probe are shown in lanes: 4, standard translation reaction shown as squares in solid line (section A), and 5, translation reaction deficient in exogenous ATP/GTP (squares in broken line shown in A).  
C, Comparison of ribosomal complex formed at wild type c-Src IRES and a mutant PV 5’NCR used as scrambled IRES in the presence of 1 mM GMP-PNP.  The 32P-labeled uncapped 5’Src-Fluc (squares in solid line) or 5’PVΔ286-605-FLuc (scrambled IRES, triangles in dashed line) RNAs were used in sucrose gradient centrifugation analysis as described above.  
D, The total RNA isolated and resolved by agarose gel electrophoresis is shown.  Lanes 1 and 2, rRNA (as markers) isolated from lysates and purified 40S subunit respectively; lanes 3 and 4, RNA isolated from Peak I and Peak II respectively.

FIGURE 7. Direct binding of the c-Src IRES with purified HeLa 40S ribosomal subunit.  
A, Purified HeLa 40S subunit was mixed with 32P-labeled RNA representing c-Src nt 1-383 (solid line) and subjected to sucrose density gradient centrifugation.  A separate sample without 40S was run to locate position of the free probe (broken line) during sedimentation.  Peak fractions were analyzed for RNA contents (inset).  Inset, lanes: M, 18S rRNA as marker; 1, RNA extracted from 40S plus probe peak; 2, RNA from free probe peak.  
B, An experiment similar to that described in section A but repeated with a scrambled
IRES [5'PV(Δ286-605)] as indicated. Inset: lane M, 18S rRNA; RNA isolated from c-Src IRES probe (lane 1) and scrambled IRES probe (lane 2).

FIGURE 8. Stimulation of the c-Src IRES-controlled mRNA translation when eIF4E function is inhibited. A, Capped RNAs: 5'Src-FLuc (triangle), and FLuc (5'Cap-FLuc, circle) or uncapped 5'HCV-FLuc (square) RNAs were translated in triplicate in the presence of increasing amounts of m7GDP in RRL for 1 hr, and one-tenth of each reaction mixture was assayed for FLuc activity. Average FLuc activity of three reactions is shown for each m7GDP concentration. The FLuc activity in samples without m7GDP was considered as 100% translation and compared with those containing the cap analogue (inhibitor). Similar translation reactions were carried out twice to confirm the results. B, Translation of a capped dual luciferase RNA construct (5'Src-RFLuc) in RRL in the presence of increasing amounts of m7GDP as described above. Relative cap-dependent translation of renilla luciferase (RLuc) and c-Src IRES-dependent FLuc synthesis are shown. Each translation mixture was carried out in triplicate. The results were confirmed by three independent experiments.

FIGURE 9. c-Src IRES-controlled translation is not inhibited during cellular stress. A, Phosphorylation of eIF2α by thapsigargin (TG) treatment. The cytoplasmic lysates (40 μg) from Huh7 cells that were treated with 1 μM TG for 0.5, 1, 2, 4 and 6 hrs (lanes 2-6) were subjected to Western blot analysis using anti-[Phospho-eIF2α(Ser51)] antibody (Cell Signaling, upper panel) and anti-eIF2α antibodies (Sigma, lower panel). Lane 1, 0 min treatment. B, Huh7 cells in triplicate were treated with DMSO alone or 1 μM thapsigargin dissolved in DMSO (DMSO-TG) for 3 hrs followed by transfection with in vitro transcribed capped 5'Src-RFLuc RNA (solid black bar) or RL-HCV1b (solid gray bar). The upstream RLuc in RL-HCV1b RNA is translated by cap-dependent mechanism whereas HCV IRES mediates downstream FLuc translation. The cytoplasmic lysates were assayed for FLuc (IRES-dependent translation) and RLuc (cap-dependent translation) activities 3 hours post-transfection. The activities of FLuc and RLuc in untreated (control) samples were considered as 100% and compared with the solvent alone (DMSO) or TG-treated cells. C, The cytoplasmic lysates from experiments described in section B (above) were subjected to Western blot for the total c-Src protein with monoclonal anti-Src antibody (clone 327, Santa Cruz, upper panel) or anti-actin antibodies (lower panel). D, Northern blot analysis of total RNA extracted from Huh 7 cells described in section B, probed with 32P-labeled oligonucleotide corresponding to nt 320-350 of the c-Src 5’NCR (upper panel). Lower panel shows 18S rRNA in the same samples. E, Total c-Src level in Huh7 cell lysates cultured for 72 hrs in serum-deprived (indicated as minus) or 10% serum containing (plus) media. Western blot was carried out with anti-Src antibody as described above. F, Northern blot for probing c-Src mRNA (as described above) in total RNA extracted from Huh 7 cells cultured in serum-starved (minus) and serum supplemented (plus) regular media. The 18S rRNAs in each lane is shown in the lower panel.
A.

**c-Src gene:**

- Exon 1α
- Exon 1A
- Exon 1B
- Exon 1C

**Regulated by:**

- HNF1α
- Sp1/Spy family

Alternative transcription/splicing

**mRNAs:**

- 5'NCR, 451 nt
- Cap
- ORF
- AUGi

Type-1α

B.

Type 1A 5'NCR:

- AUG147
- AUG179
- AUG351

**Py-tract**

- 330CCCCTGCCTTCTACC344

**Domain III**

(nt 60-225)

**Domain II**

(nt 200-340)

**Domain I**

(nt 346-386)

Figure 1
Figure 3
Figure 4

A. Diagram showing the expression of FLuc and RLuc ORFs. The 5' NCR region is labeled as 5'Src and AUG indicates the initiation codon. The Cap site is labeled as Cap.

B. Bar graph showing luciferase activity with FLuc and RLuc compared to negative controls. The y-axis represents luciferase activity ranging from 0 to 100,000. Bars indicate the activity levels for each condition.

C. Western blot analysis showing the expression of 5'Src-RFLuc and 5'Src-FLuc in transfected and untransfected samples. Lanes 1 to 4 represent different samples.
Figure 5
Figure 5
Figure 5
Figure 6

A.

Fraction number

Percent RNA Probe Bound

Peak I
(80S complex)

Peak II
(43S/48S complexes)

B.

RNA Markers | Peak I | Peak II
---|---|---
- 28S rRNA | - 18S rRNA | - Input Probe

Figure 6
Fraction number

D.

RNA Markers  Peak I  Peak II

- 28S rRNA
- Input Probe
- 18S rRNA

Figure 6
Figure 7
Figure 7
Figure 8
Figure 9

A. TG, hrs 0 0.5 1 2 4 6
eIF2α-P

B. % Luc Activities

5’Src-RFLuc RL-HCV1b

Control DMSO DMSO TG Control DMSO DMSO-TG

IRES-dependent Cap-dependent
Figure 9

C. c-Src -

Actin -

Src/Actin Ratio 1.0 0.8 1.8

D. c-Src RNA -

18S rRNA -

Src mRNA/18S Ratio 1.00 0.96 1.08

E. Serum

+ -

c-Src -

Actin -

Src/Actin Ratio 1 1.7

F. Serum

+ -

c-Src mRNA -

18S rRNA -

Src mRNA/18S Ratio 1.00 0.98
Initiation factor eIF2-independent mode of c-Src mRNA translation occurs via an internal ribosome entry site (IRES)

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