Cryptic AUG is important for 48S ribosomal assembly during internal initiation of translation of coxsackievirus B3 RNA

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We have investigated the possible role of a conserved cis-acting element, the cryptic AUG, present in the 5’ UTR of coxsackievirus B3 (CVB3) RNA. CVB3 5’ UTR contains multiple AUG codons upstream of the initiator AUG, which are not used for the initiation of translation. The 48S ribosomal assembly takes place upstream of the cryptic AUG. We show here that mutation in the cryptic AUG results in reduced efficiency of translation mediated by the CVB3 IRES; mutation also reduces the interaction of mutant IRES with a well characterized IRES trans-acting factor, the human La protein. Furthermore, partial silencing of the La gene showed a decrease in IRES activity in the case of both the wild-type and mutant. We have demonstrated here that the interaction of the 48S ribosomal complex with mutant RNA was weaker compared with wild-type RNA by ribosome assembly analysis. We have also investigated by chemical and enzymic modifications the possible alteration in secondary structure in the mutant RNA. Results suggest that the secondary structure of mutant RNA was only marginally altered. Additionally, we have demonstrated by generating compensatory and non-specific mutations the specific function of the cryptic AUG in internal initiation. Results suggest that the effect of the cryptic AUG is specific and translation could not be rescued. However, a possibility of tertiary interaction of the cryptic AUG with other cis-acting elements cannot be ruled out. Taken together, it appears that the integrity of the cryptic AUG is important for efficient translation initiation by the CVB3 IRES RNA.

INTRODUCTION

IRES-mediated translation was first discovered in poliovirus (Pelletier et al., 1988) and subsequently in other picornaviruses (Jang et al., 1988; Borman & Jackson, 1992; Yang et al., 1997). The 5’ UTR of the genomic RNA of these viruses is naturally uncapped, highly structured and contains multiple AUGs, which are poorly conserved not just between closely related species but even between different strains or different isolates of the same serotype (Pöyry et al., 1992). The variation in nucleotide sequence of the IRES element belonging to different picornaviruses is wide. Although the primary sequence has such moderate conservation, there is a stronger conservation of the secondary structure (Agol, 1991). Depending on the structure, the IRES elements are classified into four groups named type I (enteroviruses and rhinoviruses), type II (apthoviruses and cardioviruses), type III (hepatitis A virus) and type IV (porcine teschovirus serotype 1) (Fitzgerald & Semler, 2009). IRES elements have been shown to possess a three-dimensional structural scaffold of RNA, which recruits ribosomes through the interaction of cis-acting RNA elements and various trans-acting cellular factors.

Supplementary figures are available with the online version of this paper.

The coxsackievirus B3 (CVB3) 5’ UTR harbours the IRES, which mediates internal initiation of translation (Liu et al., 1999; Chen et al., 2008). The ribosomes are recruited upstream of the AUG triplet at 591 (AUG591) of the CVB3 5’ UTR, also called the cryptic AUG, after which they scan downstream for about 150 nt, before initiating translation at the initiator AUG (Bhattacharyya & Das, 2005; M’hadheb-Gharbi et al., 2007). Mfold structure of CVB3 IRES and its secondary structure showed a high degree of similarity to that of poliovirus 5’ UTR (Skinner et al., 1989). However, the region corresponding to stem–loop (SL) G (nt 519–560) and H (nt 581–624) of CVB3 was represented with a single stem–loop (SLG, nt 559–624) in poliovirus (Pilipenko et al., 1992; Liu et al., 1999). Interestingly, the deletion of the first 63 nt from the CVB3 5’ UTR had a negative effect on translation, whereas deletion of nt 250–529 enhanced it marginally (Yang et al., 1997, 2003; Liu et al., 1999). Interestingly, a recent study on the secondary structure of the CVB3 5’ UTR suggests long-range pairing interaction that could link domain II to domain V within the 5’ UTR (Bailey & Tapprich, 2007). In poliovirus it has been observed that mutation of the cryptic AUG codon results in a small plaque phenotype and reduces the translation efficiency by ~70% (Pelletier et al.,...
1988; Meerovitch et al., 1991; Nicholson et al., 1991). This suggests that AUG harbours important information for efficient translation and influences the efficiency of ribosome recruitment by the poliovirus IRES. The length between the pyrimidine-rich tract and the silent or cryptic AUG is very important for ribosome recruitment since insertion of nucleotides into this region compromises the infectivity of the poliovirus. Also, the distance between the cryptic AUG in the oligopyrimidine/AUG tract and the initiator codon is critical for the neurovirulence of poliovirus (Slobodskaya et al., 1996). Earlier, from our laboratory, we have shown the toe-print corresponding to the 48S ribosomal assembly site at nt U570 (Bhattacharyya & Das, 2006). IRES trans-acting factors La and polypyrimidine tract-binding protein (PTB) have been shown to be important for CVB3 IRES activity. Results have also demonstrated that mutation in the PTB-binding pockets in CVB3 3' UTR affects the translation enhancing activity of the 3' UTR (Verma et al., 2010).

In the present study, we have investigated a possible role of this cryptic AUG present in the 5' UTR of CVB3 RNA. Here, we show that mutation in the cryptic AUG reduces the efficiency of translation mediated by the CVB3 IRES. Mutation in the cryptic AUG moiety also reduces the interaction of mutant RNA with the La protein. We have demonstrated here that the 48S ribosomal toe-print is weaker in the cAUG mutant RNA. Our results suggest a marginal alteration in the secondary structure in the mutant RNA due to mutation. It appears that integrity of the cryptic AUG is important for mediating precise La binding and 48S ribosomal complex formation for efficient translation initiation by the CVB3 IRES.

**RESULTS**

**Mutation at the cryptic AUG moiety affects IRES activity**

In order to investigate the role of the conserved cryptic AUG present in the SLH of the CVB3 5' UTR, a substitution mutant was generated in which AUG was mutated to AAG (nt592U→A) (Fig. 1a). The mutant 5' UTR was cloned into the intercistronic region of the bicistronic construct in a eukaryotic expression plasmid pcDNA3.1. The bicistronic mRNA encodes Renilla luciferase (Rluc) translated by a cap-dependent mechanism and Firefly luciferase (Fluc) is translated by the IRES element of the CVB3-5' UTR, which is cloned in between the two cistrons. To investigate the effect of the mutation *ex vivo*, transient transfection assays were performed using the bicistronic plasmid DNAs in HeLa cells. Results showed an approximately 60% decrease in IRES activity upon mutation in the cryptic AUG in SLH (Fig. 1b). However, the Rluc (the product of the first cistron) activity was not affected significantly, ruling out the possibility of destabilization of mRNA due to the point mutation at the intercistronic (IRES) region of the bicistronic RNA (Fig. 1c).

To further confirm the above observation, both the wild-type (wt) and mutant 5' UTRs were cloned as a monocistronic construct upstream of the Fluc reporter gene in the pcDNA3 vector. RNA transfection experiment was performed using *in vitro* transcribed monocistronic RNAs generated from the respective DNA and capped Rluc RNA was taken as control for transfection. The Fluc and Rluc activities were plotted separately. Luciferase activity was measured from the lysate and Fluc and Rluc values were plotted separately. The values in lysate transfected with CVB3-Fluc were taken as 100 and that in others plotted as the number of folds increase or decrease above it.
plotted as fold increase or decrease of the control. The luciferase activities were measured after 8 h and plotted to compare the relative efficiency of the wt and mutant IRESs (Fig. 1d). The results suggest a significant decrease in IRES activity of the cryptic AAG mutant, whereas Rluc, which is translated by cap-dependent mode of translation, did not change, suggesting that the effect of the mutation is specific.

Compensatory mutations in SLH could not rescue IRES activity of the mutant cryptic AUG RNA

In order to investigate the specific function of the cryptic AUG in mediating internal initiation of translation, we have generated some compensatory and non-specific mutations in SLH particularly around the cryptic AUG. We have generated a substitution mutation at nt585C→A in order to generate another AUG to restore IRES function. Similarly A at 617 was mutated to U (nt617A→U) to restore Watson–Crick base pairing that was disrupted because of the mutation in the cryptic AUG moiety. Both mutations were generated in the background of the cryptic AUG mutation (Fig. 2a, b). Additionally, we have generated a non-specific mutation in wt 5′ UTR RNA, where U at 612 was mutated to A (nt612U→A) (Fig. 2a, b). To investigate the effect of these mutations in influencing the CVB3 IRES activity, mutant 5′ UTRs were cloned into the intercistronic region in the bicistronic plasmid constructs. Transient transfection assays were performed using the bicistronic plasmid DNAs in the HeLa cell line. Results showed a decrease in IRES activity of the mutant nt585C→A and nt617A→U that had been generated in the cryptic AAG 5′ UTR backbone, while non-specific mutant nt612U→A did not show any effect on IRES activity (Fig. 2c). The Rluc activity (result of cap-dependent translation) did not show any significant change as expected (Fig. 2c). Additionally, relative luciferase ratio (Fluc/Rluc) was taken as the index of IRES efficiency and plotted separately. Results showed a decrease in IRES activity of the mutant nt585C→A and nt617A→U, while non-specific mutant nt612U→A did not show any effect on IRES activity (Fig. 2d). Since mutants nt585C→A and nt617A→U had been generated in the cryptic AUG 5′ UTR, it is possible that the integrity of the cryptic AUG is required for efficient IRES activity and thus compensatory mutation could not restore IRES activity. This integrity could be required for its interaction with trans-acting factors that might be necessary for the 48S ribosome assembly.

Mutation at the cryptic AUG moiety reduces binding with La

The CVB3 5′ UTR has been shown to interact with various cellular trans-acting factors, some of which have been characterized. These include La autoantigen (Ray & Das, 2002), PTB (Verma et al., 2010) and poly (rC)-binding protein-2 (Zell et al., 2008). In order to investigate the possible effect of the mutation in the cryptic AUG on the interaction of HeLa S10 extract proteins, we performed an UV-cross-linking assay using body-labelled wt and mutant 5′ UTR RNA. The results suggest that polypeptides ranging from 36 to 120 kDa interact with the CVB3 5′ UTR RNA (Fig. 3a). Interestingly, a polypeptide of molecular mass ~52 kDa showed reduced binding with the mutant cryptic AUG RNA, which was characterized earlier as La autoantigen and shown to be important for CVB3 IRES function. In order to investigate whether the 52 kDa interacting
polypeptide was La autoantigen, we immunoprecipitated the UV-cross-linked ribonucleoprotein complex with polyclonal antibody against La, where PTB was used as positive control. The result suggests that the 52 kDa polypeptide can be immunoprecipitated with the La polyclonal antibody (Fig. 3b). Further, the UV-cross-linking experiment with the wt or mutant IRES RNA with recombinant purified La protein showed a considerable decrease in La protein binding due to mutation (Fig. 3c). The result was reconfirmed with the filter-binding assay using the recombinant La protein, which showed a considerable decrease in the affinity of mutant RNA ($K_d \approx 0.7 \mu M$) compared with wt CVB3 IRES RNA ($K_d \approx 0.2 \mu M$) (Fig. 3d). Additionally, the competitive UV-cross-linking experiment using unlabelled wt and mutant RNA further confirms that the cAUG mutation affects La protein binding (Supplementary Fig. S1a, available in JGV Online). However, interaction with PTB does not seem to be affected significantly (Supplementary Fig. S1b, c, available in JGV Online).

Partial silencing of endogenous La RNA affects CVB3 IRES-mediated translation in HeLa cells

Earlier it has been demonstrated in our laboratory that La influences IRES-mediated translation of the CVB3 5’ UTR, by in vitro studies by supplementation of recombinant La (Ray & Das, 2002), and ex vivo studies by partial silencing of La mRNA (Bhattacharyya & Das, 2006). Since the interaction of the La protein is weaker with mutant RNA, we have investigated the IRES activities of the wt and mutant RNA in cells that are partially silenced of La gene expression. Co-transfection of plasmid DNA corresponding to the wt or mutant CVB3 5’ UTR bicistronic construct was performed in HeLa cells, with pre-characterized siRNA targeting endogenous La mRNA (Costa-Mattioli et al., 2004). A non-specific siRNA was used as negative control. The results showed a significant reduction of CVB3 IRES activity (up to 50 %) with partial silencing of La in a dose-dependent manner (Fig. 4b). The reduction in La was assayed by immunoblotting of the lysate using polyclonal antibody against La (Fig. 4c). A $\beta$-actin probe was used as a loading control. Interestingly, the IRES activity of the mutant bicistronic construct was further reduced due to silencing of La (Fig. 4b). However, the cap-dependent translation of Rluc was not affected. These results further demonstrate that the interaction of the La protein has a specific effect on IRES-mediated translation of the CVB3 RNA. Even a single point mutation in the critical cis-acting RNA element cryptic AUG reduces the interaction with La protein and affects IRES activity significantly.

Effect of mutations at the cryptic AUG on the local structure

In order to investigate possible alterations in secondary structure due to mutations in the cryptic AUG moiety,
chemical modification and enzymic digestion of RNA were performed. For this purpose, in vitro transcribed RNA corresponding to the wt 5′ UTR and mutant cryptic AAG or non-specific mutant nt612U→A CVB3 5′ UTR RNAs was incubated with the chemical probing reagent, dimethyl sulphate (DMS). After treatment, the RNAs were incubated with the chemical probing reagent, dimethyl sulphate (DMS). After treatment, the RNAs were precipitated with alcohol and then reverse-transcribed with a 32P-end labelled reverse primer annealed to nt 624–640 using avian myeloblastosis virus (AMV)-RT. DMS is known to modify C residues that are in the single-stranded region, the in vitro transcribed CVB3 5′ UTR RNA was partially digested with RNase T1, which cleaves downstream of single-stranded Gs of the RNA (Fig. 6a). To investigate the paired bases in the double-stranded regions, the CVB3 5′ UTR RNA was partially digested with the enzyme RNase V1 (Fig. 6b), which cleaves double-stranded and stacked single-stranded regions. To map the putative cleavage sites, the partially cleaved RNAs were precipitated with alcohol and sodium acetate and then reverse-transcribed by AMV-RT using an end labelled primer that annealed to the 624–640 nt of the RNA. The cDNAs were run on an 8 M urea–8 % PAGE. RNase T1 was found to cleave downstream of G560, G587, G603, G605 and G607. The band intensity of G560 and G587 in the mutant was marginally higher compared with wt and non-specific 612 mutants, suggesting a possible alteration in the secondary structure (Fig. 6a, compare lanes 2 and 3 with 5 and 6 and 8 and 9). The partial digestions with RNase V1 indicated putative cleavage points corresponding to nt C456 and C493. These bands were specific to RNase V1 as these were present with the same intensity in wt and non-specific 612 mutants (Fig. 6b, compare lanes 2 and 3 with 5 and 6 and 8 and 9). Interestingly, an inherent RT pause was observed at A591 only in the case of the mutant cAAG RNA in all the enzymic and chemical modifications, which could be due to a bulge in the RNA structure formed by a mutation immediately downstream of nt592U→A. Overall, the results suggest that the local secondary structure of the mutant RNA is altered to some extent.

Ribosome loading on the IRES is compromised by a mutation in the cryptic AUG in the SLH domain of the CVB3 5′ UTR

The ribosome recruitment on the CVB3 IRES has been shown to have important determinants in the region immediately downstream of nt 565 within the 5′ UTR (Iizuka et al., 1991; Yang et al., 1997). This region includes the pyrimidine-rich tract and a nucleotide stretch 567–577 that has been shown to act as a Shine–Dalgarno (SD)-like sequence in the internal initiation of translation of CVB3 RNA (Yang et al., 2003). The 48S ribosomal complex in CVB3 IRES RNA has been mapped around U570 and U579 using ribosomal toe-printing assays (Bhattacharyya & Das, 2005). In order to investigate a possible effect of mutation on the cryptic AUG in the 48S ribosomal complex formation, a ribosomal assembly reaction was performed using a construct CVB3 ΔP1 (1–803 nt), which has part of the P1 mRNA.[γ-32P]UTP-labelled CVB3 ΔP1 (1–803 nt) and [γ-32P]UTP-labelled CVB3 cAUG mutant ΔP1 (1–803 nt) were incubated with rabbit
reticulocyte lysate (RRL) supplemented with HeLa S10 and 5′-guanylyl imidodiphosphate (GMP-PNP) and then fractionated by sucrose-gradient centrifugation (Pudi et al., 2004). GMP-PNP is a non-hydrolysable GTP analogue that inhibits translation initiation at the 48S stage by preventing the release of eIF2. Reactions were then fractionated using an ISCO-density-gradient fractionation system. In the presence of the cAUG mutant (Fig. 7c), the 48S ribosomal complex was reduced when compared with wt (Fig. 7a), suggesting that the mutant prevented the 48S ribosomal complex assembly on the CVB3 IRES. However, to further confirm 48S assembly GMP-PNP was added to the reactions. Addition of GMP-PNP abolished the 80S peak in the mutant (Fig. 7d) compared with wt (Fig. 7b), demonstrating that only the 48S complex was being assembled. nt585C

A

A Fluc RNA was used as a control showing that compensatory mutation could not restore the 48S ribosomal complex assembly on the CVB3 IRES. For further characterization, the ribosome toe-printing assay was performed in the presence of RRL as described before (Bhattacharya & Das, 2006). A comparison of the RT pauses specific for the 48S ribosomal complex U570 and A579 in the wt, mutant and control RNA lanes showed that the intensity of pauses with the addition of RRL at U570 and U579 was significantly reduced in the case of the mutant cryptic AAG RNA, while control RNA nt612U

A

A was not reduced (Supplementary Fig. S2a, available in JGV Online; compare lanes 6, 4 and 2). Densitometry analysis of the bands reconfirmed the observation (Supplementary Fig. S2c, available in JGV Online). Further, the formation of the ribosome assembly at the initiator AUG was blocked before the joining of the 60S ribosomal subunit using GMP-PNP as described previously (Dmitriev et al., 2003). In the presence of GMP-PNP, toe-print analysis showed a relative increase in the toe print at position +16 (nt A762) compared with the cryptic AAG mutant (Supplementary Fig. S2b, available in JGV Online), which indicates the presence of stalled 48S complex at this position. Densitometry analysis of the bands at position A762 reconfirms the observation (Supplementary Fig. S3b, available in JGV Online). The result is consistent with the earlier observation that mutating some critical nucleotides at this position affects translation initiation (Yang et al., 2003).

Fig. 5. Chemical modification of the wt, mutant cAAG or non-specific mutant nt612U→A 5′ UTR RNA using DMS. (a) Unmodified wt (lane 1), cAAG (lane 4) and 612 (lane 7) or DMS modified wt (lanes 2 and 3), cAAG (lanes 5 and 6) and 612 (lanes 8 and 9) 5′ UTR RNA were reverse-transcribed with end labelled primer corresponding to nt 624–640 and the cDNAs were resolved in parallel with a sequencing ladder obtained by using the same end labelled primer. The strong stops corresponding to the reverse-transcriptase pauses due to DMS modification appear one base 3′ of the modified base. The numbering in the middle of the gel refers to the modified residues of the 5′ UTR RNA. The numbers to the left of the gel indicate the positions of the C residues of the 5′ UTR sequence.
Enzymic digestion of the wt, mutant cAAG or non-specific mutant nt612 U 5' UTR RNA using RNase T1 or RNase V1.

(a) Undigested wt (lane 1), cAAG (lane 4) and 612 (lane 7) or RNase T1 digested wt (lanes 2 and 3), cAAG (lanes 5 and 6) and 612 (lanes 8 and 9) 5' UTR RNA were reverse-transcribed with end labelled primer corresponding to nt 624–640 and the cDNAs were resolved in parallel with a sequencing ladder obtained by using the same end labelled primer. The strong stops corresponding to the reverse-transcriptase pauses due to RNase T1 appear one base 3' of the cleaved base. The numbering to the right side of the gel refers to the modified residues of the CVB3 5' UTR RNA. The numbers to the left of the gels indicate the position of the C residues of the CVB3 5' UTR sequence.

(b) Undigested wt (lane 1), cAAG (lane 4) and 612 (lane 7) or RNase V1 digested wt (lanes 2 and 3), cAAG (lanes 5 and 6) and 612 (lanes 8 and 9) 5' UTR RNA was reverse-transcribed with end labelled primer corresponding to nt 624–640 and the cDNAs were resolved in parallel with a sequencing ladder obtained by using the same end labelled primer. The strong stops corresponding to the reverse-transcriptase pauses due to RNase V1 appear one base 3' of the cleaved base. The numbering to the right side of the gel refers to the modified residues of the CVB3 5' UTR RNA. The numbers to the left of the gels indicate the position of the C residues of the CVB3 5' UTR sequence.

(c) Schematic representation of CVB3 5' UTR domain V and domain VI, showing the positions of modifications and cleavages by DMS, T1 and V1, respectively.
DISCUSSION

Involvement of local secondary structure in modulating IRES function has been reported earlier in some viral RNAs. It has been demonstrated earlier that the SLH domain (nt 582–625) and the SD-like sequence (nt 566–577) is largely inaccessible to nucleases and base modifying reagents, suggesting the presence of a higher order structure in this region. Here, we have shown that a mutation in the critical cis-acting RNA element, the cryptic AUG within the SLH domain does alter the secondary structure of RNA marginally, but functionally it reduces the IRES activity significantly because of less interaction with La autoantigen and reduction in assembly of the 48S ribosomal complex. Additionally, the mutations affected the interaction of the human La protein and the IRES RNA at this region, leading to a reduction in the efficiency of ribosome entry at the SD-like sequence. The cellular non-canonical factors (such as La) have been speculated to act as RNA chaperones, which consequent to their interaction modulate the folding of the IRES RNA towards a structure with a higher capacity of recruiting ribosomes internally (Belsham & Sonenberg, 1996).

In poliovirus, the La protein has been speculated to have a role in facilitating the assembly of the 48S ribosomal complex on the IRES RNA. In CVB3 it performs a similar function, as the La protein also stimulates translation of the CVB3 IRES-mediated translation. Comparisons of the nuclease mapping data from wt and mutant 5′ UTR RNAs showed a partial change in secondary structure in the cryptic AAG mutant IRES RNA around the SLH. This reinforces the contention that the integrity of the cryptic AUG moiety might be important for the recruitment of ribosomes by IRES.

The mutation at the cryptic AUG reduced the interaction with the La protein; this could have a drastic consequence on ribosome loading at the SD-like sequence. In fact our ribosome toe-printing assay showed a considerable decrease in the band intensity corresponding to the 48S ribosomal complex in the mutant RNA, reinforcing the idea that the binding of the La protein near the SD-like sequence might influence proper folding and exposure of the IRES. Partial silencing of La reduced the availability of the endogenous La protein. Mutation at the cAUG led to low affinity binding with the La protein (does not abrogate binding), thus lower abundance of the La protein (due to partial silencing) showed more effect on the mutant IRES function. Although the mutation at the cryptic AUG did not show appreciable change in binding with PTB, the possible involvement of other trans-acting factors with this cis-acting element is not ruled out. Therefore, the possibility of involvement of this cis-acting RNA element with some other region of the RNA in a long range RNA–RNA interaction cannot be ruled out.

METHODS

Cell lines and transfection. HeLa-S3 was maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) with 10 % FBS.
For RNA transfection in HeLa cells, monocistronic RNAs were synthesized in vitro using T7 polymerase (New England Biolabs). For RNA transfection, 2 μg monocistronic RNA was transiently transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Post-transfection (3 h), the cells were washed and DMEM medium containing 10 % FBS was added. After 8 h, cells were washed with PBS and lysed with 200 μl passive lysis buffer and the luciferase activities were measured (DLEady TD-20/20 Luminometer; Turner Designs).

**Plasmid constructs and in vitro transcription.** The CVB3 cDNA was obtained from Dr Nora Chapman, University of Nebraska, USA. The 5’ UTR (1–741 nt) was cloned into an upstream Luc and downstream Fluc between HindIII and EcoRI sites of the pcDNA3.1 vector, to obtain the CVB3 bicistronic plasmid. The plasmid encoding β-galactosidase, pSV40-beta-Gal (Promega), was co-transfected to normalize the transfection efficiency.

Mutant CVB3 cDNA 5’ UTR having the cryptic AAG mutation was generated by megaprimer-mediated PCR-mutagenesis. To generate the cryptic AAG 5’ UTR, the 5’ UTR primer was used as the forward primer (5’-GCCGGAGAGCTTATAAACACG-3’) and the CVB3 cryptic AAG primer was used as the reverse primer (5’-GTCCCTTAAGGAGGAGATTAGG-3’). Pfx polymerase (Invitrogen) was used for the PCR amplification using the following cycling conditions: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 45 s. The megaprimer thus obtained was used for the second PCR with the CVB3 wt 5’ EcoRI (5’-GCCGGAATTCCTTCGTGATATCA-3’) as the reverse primer to generate the 5’ UTR with the cryptic AAG mutation. The amplified mutant 5’ UTR was gel eluted and digested with HindIII and EcoRI enzymes. Then it was ligated with pcDNA3 vector digested with HindIII and EcoRI enzymes and the intergenic region of the bicistronic plasmid. Mutants nt585C→A, nt612C→A and nt617A→G were generated similarly by megaprimer-mediated PCR.

For the cloning of the mutant cAUG CVB3 ΔP1 (1–803 nt), a site-directed mutagenesis kit (Strategene) was used according to the manufacturer’s protocol. For in vitro transcription, the plasmid was linearized with XhoI.

For monocistronic constructs, the wt and mutant CVB3 5’ UTR was cloned upstream of Fluc, in pcDNA3 to obtain CVB3 5’ UTR-Fluc. For RNA transfection in HeLa cells, monocistronic RNAs were reverse transcribed with AMV-RT and the transcription using T7 RNA polymerase.

**Filter binding.** Filter binding was performed as described previously (Pudi et al., 2004). Briefly, the χ-32P-labelled wt and mutant CVB3 5’ UTR RNA were incubated with increasing concentrations of purified recombinant proteins at 30 °C for 15 min in RNA-binding buffer [containing 5 mM HEPES, pH 7.6, 25 mM KCl, 2 mM MgCl2, 3.8 % (v/v) glycerol, 2 mM DTT and 0.1 mM EDTA] and loaded onto nitrocellulose filters equilibrated with 2 ml RNA-binding buffer. The graph was plotted with protein concentration (nanomolar) on the x-axis and the percentage of counts retained on the y-axis. Each experiment was repeated three times.

**La silencing and Western blotting.** Co-transfection of siLa with bicistronic plasmid was performed as described previously (Bhattacharyya & Das, 2006). For transfection, 50 and 100 nM of siLa (Dharmacon) and 1 μg bicistronic DNA were co-transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Post-transfection (36 h), the cells were lysed and luciferase activity was assayed by using the DLR assay system (Promega). La expression was analysed by using a polyclonal anti-La antibody and β-actin was detected by using a monoclonal anti-actin antibody (Sigma). The signal was detected by using an ECL detection kit (Amersham Pharmacia).

**Ribosome assembly.** χ-32P-labelled CVB3 ΔP1(1–803 nt) IRES RNA and CVB3 ΔP1(1–803 nt) cAUG mutant IRES RNA (3 × 107 c.p.m.) were added to 25 μl translation reaction containing 17.5 μl RRL supplemented with 10 % (v/v) HeLa S10 extract in the presence or absence of 2.5 mM GMP-PNP. The reactions were incubated at 30 °C for 15 min, diluted to 150 μl with gradient buffer (20 mM Tris/HCl pH 7.5, 100 mM KCl, 3 mM MgCl2 and 1 mM DTT) and overlaid on a 5–30 % sucrose gradient. The ribosomal complex was sedimented by ultracentrifugation for 3 h at 30000 r.p.m. (TH-641 Eq.40805718; Sorvall) at 4 °C. Fractions (50 μl) were collected using a fraction collecting system (Iscoc Teledyne) and the radioactivity was measured in a liquid scintillation counter.

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