The structure and function of a cis-acting element located upstream of the IRES that influences Coxsackievirus B3 RNA translation

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We have investigated the importance of a conserved hexa-nucleotide stretch in the apical loop within stem-loop C (SL C, nt 104–180), upstream of the ribosome landing site, on CVB3 IRES function. The deletion or substitution mutation at this apical loop resulted in significant decrease in IRES activity. Both the mutant IRES RNAs failed to interact with certain trans-acting factors. Furthermore, expression of CVB3 2A protease significantly enhanced IRES activity of the wild type, but the effect was not so pronounced on the mutant IRESs. It is possible that the mutant RNAs were unable to interact with some trans-acting factors critical for enhanced IRES function. Interestingly, the local structure of the IRES RNA was not significantly altered due to substitution mutation. Taken together, it appears that the SL C/c apical loop is critical for CVB3 IRES function.

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Introduction

Internal initiation of translation in Coxsackievirus B3 (CVB3) has been shown to be influenced by various cis-acting elements present in the 5′ untranslated region (5′UTR) of the single-stranded plus-sense genomic RNA. The core element of the 'internal ribosome entry site' (IRES) in CVB3 RNA has been putatively mapped between nt 432–639 of the 5′UTR (Liu et al., 1999). This region was shown to possess several critical cis-acting elements (Yang et al., 1997) which includes a pyrimidine-rich tract and an AUG triplet (AUG591) about 25 nucleotides downstream of the pyrimidine-rich tract. Similar cis-acting elements have been found to be conserved at the 3′ border of IRES elements belonging to other members of the Picornaviridae (Jackson and Kaminski, 1995). Interestingly, the pyrimidine-rich tract in CVB3 has been shown to harbor a stretch of sequences from nucleotide 566–577, which was found to be complimentary to nt 1815–1825 of the 18S ribosomal RNA and therefore termed as Shine–Dalgarno-like sequence (Yang et al., 2003).

Earlier, we have demonstrated the putative 48S ribosomal assembly site around nt 570 on the CVB3 5′UTR, near the SD-like sequence (Bhattacharyya and Das, 2005). Also, as observed for Poliovirus RNA, the IRES activity of CVB3 was found to be weak in Rabbit Reticulocyte lysate (RRL), which is stimulated by exogenous addition of recombinant La protein (Ray and Das, 2002).

The boundary of IRES element in the 5′UTR has been found to differ among the different members of the family Picornaviridae. In Enteroviruses (which includes CVB3) and Rhinoviruses the 5′UTRs are smaller in length (compared to the Aphthoviruses and Cardio-viruses) and the IRES occupies a greater percentage of the 5′UTR. In Poliovirus, the Ribosome-landing-pad was shown to contain a core region spanning nucleotides 134–585 of the 743 nucleotides long 5′ UTR (Nicholson et al., 1991). Similarly, in CVB3 the IRES has been shown to occupy a significant part of the 5′UTR, with the 5′ boundary being within 309–432 and the 3′ boundary between 639–670 of the genomic RNA (Liu et al., 1999). However, a more recent study suggests that CVB3 IRES is actually larger and its 5′ boundary lies between nt 76–125, similar to that of poliovirus IRES (Hunziker et al., 2006). Previously, in vitro studies showed deletion of 1–249 nucleotides of the 5′UTR significantly reduced the efficiency of translation while deletion of 1–529 or 250–529 did not (Yang et al., 1997). However, deletion of 1–249 and 250–529 of the CVB3 viral genome showed down-regulation of the IRES mediated translation to similar extent in vivo (Liu et al., 1999).

The cis-acting elements located upstream of the putative core IRES element has also been shown to interact with some critical trans-acting factors which are believed to modulate the IRES efficiency in different picornaviruses (Meerovitch et al., 1993; Witherell et al., 1995; Belsham and Sonenberg, 1996). Specifically, the cloverleaf structure present at the 5′ terminus of the viral genome is a multifunctional
element that interacts with viral and cellular proteins to form a ribonucleoprotein (RNP) complex, which is required for the initiation of both negative and positive-strand RNA synthesis (Andino et al., 1993, Herold and Andino, 2001). Additionally, it has been speculated that the cloverleaf RNA structure binds to a critical trans-acting factor, PCBP2, which might be involved in mediating the switch from viral translation to replication (Garnamrik and Andino, 1998).

Considering the hyper variability of RNA viruses, it is possible that essential cis-acting elements could be phylogenetically conserved to maintain RNA structure critical for internal initiation (Martinez-Salas et al., 2004). Several studies have shown that majority of the conserved and essential residues are located in numerous loops and bulges within IRES elements (Jang, 2006). In poliovirus (PV) and rhinoviruses the importance of SL C and SL D (SLII and IV) has been well documented. In fact mutation of a conserved loop sequence in SLII was shown to drastically affect the PV-IRES function (Nicholson et al., 1991). Also, the sequences encompassing SL D and SL G were shown to harbor elements critical for the poliovirus IRES function (Haller et al., 1993).

CVB3 5′UTR RNA has been predicted to fold into several stem–loop structures (SL A–K). Earlier we have demonstrated that a GAGA apical loop at the SL H (581–624 nt), maintains the integrity of the IRES structure and facilitates its interaction with critical trans-acting factor (such as La) to help in internal initiation of translation (Bhattacharyya and Das, 2006). Here, we have investigated the contribution of the SL C (104–180 nt, also represented as SLJ) structures upstream of the core IRES element (432–639, SL E–I). It has been reported earlier that sequences within SL C region are important in determining virulence in CVB3 (Dunn et al., 2000, 2003). Also, in another recent report, a long range RNA–RNA interaction has been speculated involving the domain II (encompassing SL C) and domain V (encompassing SL F–G) (Bailey and Tapprich, 2007). Alignment of the SL C–D (104–208 nt) structure showed a hexa-nucleotide stretch conserved with the consensus of RHVCCA. Probing the structure of the RNA with structure specific modifying chemicals showed this stretch to constitute an apical loop in SL C (104–180), termed as SL C/c (147–152 nt). Deletion of this hexa-nucleotide loop (∆SL C/c) showed significant reduction in IRES activity. Also, substitution mutation in this loop showed specific changes in cellular protein binding profile and consequent reduction in IRES activity. Although, expression of CVB3 2A protease significantly enhanced the IRES activity of the wild type, the effect was not so pronounced on the mutant, suggesting the inability of the mutant IRES to bind some trans-acting factors critical for enhancing IRES activity. It appears that the SL C might be involved in some tertiary interaction with the neighboring region which is critical for IRES function.

Results

The 5′ border of the CVB3 IRES lies in between SL C–D of the 5′UTR RNA

The 5′ boundary of the CVB3-IRES has been shown to be within 309–432 and the 3′ boundary within 639–670 of the genomic RNA (Liu et al., 1999). However, conclusions about the IRES boundary using genomic RNA with deletions in the 5′UTR might be complicated, since such elements might have role in the replication of the virus as well.

In order to delineate the 5′ border of the CVB3 IRES, several deletion clones were generated in the bicistronic constructs containing either the WT 5′UTR or mutant ASL A–B 5′UTR (where SL A–B were deleted) or mutant ASL A–D 5′UTR (where SL A–D were deleted). These constructs were cloned in a vector without any eukaryotic promoter (pBluescript). To compare the IRES efficiency of the wild and the mutant 5′UTR in vivo in HeLa cells, the RNAs were expressed from the respective bicistronic constructs in the cytomegalovirus using recombinant vaccinia virus (VTF7/3) encoding T7 RNA polymerase. For this purpose, HeLa monolayer cells were first infected with VTF7.3 (MOI 10) incubated for 1 h and subsequently respective bicistronic DNAs were transfected using Tfx-20 reagent (Promega). After 24 h the cells were harvested, lysed and the activities of Renilla and Firefly luciferase enzymes were assayed using Dual luciferase assay system (Promega). The relative luciferase ratio (F-luc/R-luc) was taken as the index of IRES activity and the efficiency of the mutants was plotted as a percentage of that of the WT. The results suggest that the IRES activity drops by 40% with the deletion of SL A–B and is almost abrogated upon further deletion of SL C and D (in ∆SL A–D) (Fig. 1). The results indicate that in addition to a role in replication of the genomic RNA, the cloverleaf (SLA–B) might have role in influencing IRES function. Further, the SL C–D seems to encompass critical cis-acting RNA elements, which are essential for significant IRES activity in vivo.

SL C contains a conserved apical loop with the sequence consensus of RHVCCA

The stem–loop structures in the IRES RNA are thought to maintain a scaffold, which is recognized by the trans-acting factors that are critical for internal initiation of translation. Since deletion of SL A–B decreased the IRES efficiency to a significant extent and that of SL A–D almost abrogated the activity (Fig. 1), sequences corresponding to SL C–D in the 5′UTR of various strains of Coxsackievirus were aligned in Clustal W (Fig. 2). A particular stretch of trinucleotide ‘CCA’ was found to be highly conserved among the sequences aligned. The three

![Fig. 1](image_url)
upstream nucleotides of CCA showed a consensus of RHV (R representing purine; H representing C or T or A; V representing G or A or C) among the sequences compared. A closer scrutiny revealed the R is represented predominantly by A (in 21 out of 22 sequences), position H is represented predominantly by a pyrimidine (in 19 out of 22 sequences) and the position V is represented predominantly by a purine (in 21 out of 22 sequences). When the position of the conserved CCA element was mapped on the MFOLD predicted structure of CVB3 5′UTR RNA, the CCA was found to be the 3′ segment of a hexa-nucleotide loop in the apical part of SL C (SL C/c) (data not shown).

In order to investigate whether this predicted loop lies in single stranded region or double stranded region of the CVB3-5′UTR, the RNA was partially modified using Dimethyl Sulphate (DMS) as described previously (Bhattacharyya and Das, 2005). Results showed exclusive RT pauses at residues A219, C210, C208, A200, C183, C182, A171, A168, A167, A147–A152, C64 and A26 of the RNA (Fig. 3A compare lanes 2 and 3 with lane1). Increased RT-pause was observed at other places, but since extension of RT in these regions was inhibited without modification (Fig. 3A compare lanes 2 and 3 with lane1). Interestingly, extensive modiﬁcation of the RNA at positions C203, U163, C154, C141, A114 and A32 (Fig. 3B, compare lanes 1 and 2). Interestingly, extensive modiﬁcation was observed at nucleotides 147–152. Consequently, these residues appeared to be in the single stranded region of the RNA (Fig. 3A, compare lanes 1 and 2). When the results of the DMS modiﬁcation of RNA were plotted on the predicted secondary structure of the CVB3 5′UTR RNA (Fig. 3B) the nucleotide 147–152 were found to constitute the apical loop. To conﬁrm that the nucleotides surrounding this hexa-nucleotide stretch are double stranded, partial digestion of the RNA was performed with the double-stranded RNA speciﬁc enzyme RNase V1 using in vitro transcribed CVB3-5′UTR RNA as described previously (Bhattacharyya and Das, 2005). RNase V1 cleaves at double-stranded and stacked single-stranded regions of the RNA. The results showed stops due to the cleavage of the RNA at positions C203, U163, C154, C141, U130, A114 and A32 (Fig. 3B, compare lanes 1 and 2). Interestingly, regions of the RNA around the SL C/c apical loop showed extensive cleavage with RNase V1 (C141 and C154, Fig. 3B, compare lanes 1 and 2) suggesting these nucleotides constitute the double stranded stem structure. Therefore, modiﬁcation of the 5′UTR RNA with a single-strand speciﬁc chemical and a double-stranded RNA speciﬁc enzyme strongly suggested that the nucleotides 147–152 of the CVB3 5′UTR RNA represent an apical loop, closed by a CG base pairing between nucleotides C146 and G153 (Fig. 4).

Mutation at the SL C/c apical loop affects IRES activity

In order to investigate the role of the conserved hexa-nucleotide in the SL C, deletion and substitution mutant were generated (Fig. 5, panel A). To test whether the mutation could result in conformational alteration in the CVB3 5′UTR RNA, computer based PFOLD prediction analysis was performed. Because of limitation on length of the input RNA in PFOLD, sequence corresponding to only SL C was used for folding stimulation. Folding of the RNA corresponding to only SL C from the individual constructs suggested that the ΔSL C C RNA might have structural alteration compared to the wild type at regions both proximal to the loop and around the SL C/c loop (data not shown). Since the PFOLD analysis suggested that the deletion of SL C/c loop might result in structural alteration, we have generated a point mutation mSL C (nt152A-C), which would additionally help us investigating the effect of mutation in the conserved CCA in apical loop within SL C/c. However, the mSL C RNA containing the substitution mutation (nt152A-C) showed high similarity in RNA structure with that of the wt RNA, except at the first and last nucleotide position of the SL C/c loop (data not shown).

To investigate the importance of SL C/c in influencing the CVB3 IRES activity, both the mutant 5′UTRs were cloned as monocistronic construct upstream of firefly luciferase reporter gene in pCDNA3 vector and also as intercistronic region in pBluescript bicistronic plasmid constructs (Fig. 5, panels B and C). In vitro transcribed RNAs from the wild-type (wt) and mutant monocistronic constructs were used in the in vitro translation assay using Rabbit Reti culoctye Lysate (RRL, supplemented with HeLa S10). F-Luc activities were measured and were plotted as an index of IRES activity. Results showed drastic decrease (~90%) in IRES activity of both mutants as compared to wild type (Fig. 5, panel B). To investigate the effect of mutation in vivo, transient transfection assays were performed using the bicistronic plasmid DNAs in HeLa cells using recombinant vaccinia virus system expressing T7 RNA polymerase. 24 h post transfection, the cells were lysed and the levels of R-Luc and F-Luc activities were assayed by Dual-luciferase assay system (Promega). The relative luciferase ratio (F-luc/R-luc) was taken as the index of IRES efﬁciency, and the efﬁciency of the mutants of CVB5′UTR were plotted as a percentage of that of the wild type. Results showed approximately 90% decrease in IRES activity of the 5′UTR upon deletion of the hexa-nucleotide apical loop in SL C (ΔSL C/c, Fig. 5, panel C); while substitution mutant mSL C (nt152A-C)
showed about 50% decrease in IRES activity. However, the R-Luc (the product of first cistron) activity was not affected significantly ruling out the possibility of destabilization of mRNA due to the deletion and point mutation at the intercistronic (IRES) region of the bicistronic RNA. The results suggest that the integrity of hexa-nucleotide apical loop (SL C/c) could be essential for the IRES activity of CVB3-5'UTR.

Fig. 4. Schematic representation of MFOLD prediction of wt SL C. The predicted secondary structure of the SL C part of the CVB3 5'UTR generated by MFOLD (Zuker et al., 1999). The putative cleavage point of the RNase VI (arrows) and the nucleotides modified with DMS (filled circles) are indicated. The position of the apical SL C/c loop is also indicated.
To confirm the above observation, RNA transfection experiment was performed using *in vitro* transcribed monocistronic RNAs generated from the respective DNA constructs. The firefly luciferase activities were measured after 8 h and plotted to compare the relative efficiency of the wt and mutant IRESs (Fig. 6, panel A). The absolute values (obtained from three independent experiments) used in the graphical representation have also been shown in the Table below the graph (Fig. 6, panel B). Interestingly, expression of CVB3 2A proteases significantly enhanced the IRES activity of the wild type, but the effect was not so pronounced on the ΔSL C/c IRES mutant. Apparently, the mSL C RNA could partially rescue the IRES activity in presence of 2A protease but to a lesser extent compared to the wt IRES activity. Additionally, the levels of the cap-dependent translation of host cell mRNAs. Interestingly, the cleaved part of eIF4G has been shown to assist ribosome assembly at the IRES element and thus expression of 2A protease also leads to stimulation of the IRES function of Poliovirus RNA (Borman et al., 1997; Ziegler et al., 1995). In order to investigate the relative IRES activity of the wt and the SL C/c mutants in cells expressing CVB3-2A protease, the respective bicistronic plasmids were transfected into HeLa cells with 500 ng concentration of the 2A protease encoding plasmid pcDNA3-2A. 24 h post-transfection the cells were lysed and the luciferase activities were assayed using DLR assay system (Promega). The results of three independent experiments have been averaged. The fold increase in the relative luciferase activity (ratio of F-Luc to R-Luc) in presence of 2A protease was calculated over the values obtained in control experiment in absence of 2A protease (Fig. 7, panel A). The absolute values (F-luc and R-luc) with standard deviation have been shown in the table below the graph (Fig. 7, panel B).

Effect of CVB3 2A<sup>Ppo</sup> expression on wild-type and mutant IRES function

The picornavirus 2A protease has been shown to cleave the translation initiation factor eIF4G, resulting complete shut-off of the
respective bicistronic transcripts made in the transfected cells in absence and presence of Coxsackievirus 2A protease was monitored by RT-PCR analysis (Fig. 7, panel C). The reporter gene RNA levels were found to be same in presence or absence of 2A protease, suggesting that the effect could be solely at the translation step. Additionally, to confirm the levels of transfected bicistronic RNAs in cells in presence and absence of CVB3-2A protease, Northern blot hybridization was performed. Later the filter was stripped and re-probed with actin specific probe to detect the actin band as loading control. Densitometric analysis of the band intensities (bicistronic RNA/actin) clearly demonstrated that there was no appreciable change in the RNA levels in presence or absence of CVB3 2A protease expression (Fig. 7D).

It is possible that mutation at the hexa-nucleotide loop in SL C/c might impair the ability of binding to some ITAF critical for enhanced IRES activity upon eIF4G cleavage due to 2Apro expression.

Cellular protein binding with the wild-type and mutant IRES elements

In order to get better insight on the effect of mutations on the cellular protein interaction with the IRES elements, UV cross-linking assay was carried out using [32P] labeled wt and mutant IRES RNAs and HeLa S10 extract. ΔSL C/c and mSL C/c RNA showed reduced interaction with several cellular polypeptides of approximate molecular mass of 56, 64 and 90 kDa respectively (Fig. 8, panel A compare...
lanes 2 and 3 with lane 1). However, the binding with other proteins was not affected significantly. Interestingly, both the mutants showed similar changes in protein binding profile compared to control.

To further confirm, competition UV cross-linking experiment was performed. For this purpose, 250 and 500 fold molar excess of unlabeled competitor RNA corresponding to the WT and ΔSL C/c 5′ UTR, was allowed to compete with [32P] labeled WT CVB3-5′UTR RNA for binding to S10 proteins. The result showed, unlabeled WT 5′UTR RNA (250 and 500 fold molar excess) was able to successfully compete out binding with majority of the trans-acting factors as expected (Fig. 8B, compare lanes 2, 3 and 4). However, similar fold molar excess of the unlabeled ΔSL C/c mutant 5′UTR RNA failed to significantly compete binding with p56, p64 and p90 (Fig. 8B, compare lanes 3 and 2, 6 and 7). Interestingly, the binding of p56 with the wt CVB3 IRES appears to be tighter compared to p64 and p90, thus with 250 fold molar excess of cold self RNA only 40% and with 500 fold 52% competition was observed, whereas the p64 and p90 was successfully competed out at this concentration. Taken together, the results strongly suggest that deletion of the apical loop, SL C/c, leads to loss of interaction with the proteins p56, p64 and p90 in HeLa cells.

Additionally, UV cross-linking experiment was repeated with S10 extract prepared from control cells and cells transfected with plasmid encoding 2A protease. Several cellular proteins binding with the wt and mutant IRES RNAs were found to be reduced in presence of 2A protease as indicated (compare lanes 3 and 5 with corresponding controls in Fig. 8, panel C). The interactions of p90 and p64 with the mutant RNAs were further reduced in presence of 2A protease. Interestingly, p56 interaction with the wt IRES RNA, and the substitution mutant was not reduced even when 2A protease treated S10 extract was used. However, under similar condition, p56 interaction with the deletion mutant was significantly reduced. It is tempting to speculate that some of these polypeptide binding might be involved in enhancing IRES function of the wt IRES RNA in presence of 2A protease. Inability of the mutant RNA (especially ΔSL C/c) to bind to these proteins could be the reason behind inefficient IRES function even in presence of 2Aprotease. However, further experiments are necessary to consolidate possible function of the respective cellular proteins in CVB3-IRES function.

**Discussion**

The efficiency of the IRES elements of picornaviruses has been shown to be highly influenced by the integrity of several cis-acting RNA elements. Involvement of the local secondary structure in modulating IRES function has been reported earlier in some viral RNAs. (Malnou et al., 2002; Lopez de Quinto, 1997). It has also been shown that structural organization of viral IRES depends on the integrity of a GNRA motif (Fenández-Miragall and Martínez-Salas, 2003).

The current study demonstrates that deletion of 85nt from 5′ end of the 5′ UTR results in almost 50% decrease in IRES activity. Also, it appears that the stem–loop C (SL C, nt 104–180) structure upstream of the core IRES element (nt 432–639, SL E–I) contains critical cis element for mediating internal initiation of translation. Thus, although earlier the 5′ boundary of CVB3 IRES was delineated between 309–432 (Liu et al., 1999), our observation is more consistent with the recent study which suggests that the 5′ boundary lies between nt 76–125 (Hunziker et al., 2006). It is possible that the apical SL C/c loop might be involved in long range RNA–RNA interaction to facilitate IRES function. The cis-acting elements within SL C/c loop whose tertiary interactions might have been disrupted due to substitution of even a single nucleotide, leading to significant reduction in IRES activity. Interestingly, a recent report has predicted long range RNA–RNA interaction involving domain II (encompassing SL C) and domain V (encompassing SL F–G) in CVB3 RNA (Bailey and Tapprich, 2007). In fact, at similar positions in poliovirus, in the loop sequences of SLIII (AAAACA, nt 145–150) and SLVI (5′-AUAUCC, nt 494–499) were shown to be critical for viral RNA translation (Klinck et al., 1997). Implications of both deletion and point mutation on the structural conformation of the SL C structure was evaluated using prediction program PPfold (data not shown). Although, not much changes in RNA secondary structure was predicted in case of substitution mutant mSL C, it also showed significant reduction in IRES activity along with reduced interaction with some trans-acting factors. This suggests that the mutation might have resulted in changes in tertiary structure of the RNA which in turn could have affected cellular protein binding.

Cap-independent translation is dependent on interaction of various trans-acting factors with the scaffold of the IRES RNA. It can...
be visualized that any given region of the RNA could be important for either having a direct interaction with a particular trans-acting factor or may be responsible for the maintenance of optimal structure which would facilitate such interaction. The first 3 nucleotides of the SL C loop described in this report (5'-ACACA-3') are partially conserved among various CV isolates, while the rest are absolutely conserved. Interestingly, previous studies also showed the presence of such conservation among a wide range of entero and rhinoviruses which could be important for translation initiation (Klinck et al., 1998). Further, the NMR structure of this loop was shown to be unaltered by naturally occurring substitutions, in the 5' proximal half (Klinck et al., 1998).

Several non-canonical cellular protein factors have been shown to specifically interact with picornavirus IRES elements and influence their function, which includes La (p52), PTB (p57), unr (p97), PCBP2 (p39), PABP (reviewed in Vagner et al., 2001). Some of these factors are also involved in switching the function from translation to replication of the viral RNA. Additionally, several canonical initiation factors have been shown to interact with enterovirus IRES elements, such as eIF3, elf4F, cleaved part of elf4F etc. Also, several other cellular proteins have been shown to influence IRES function in general. DAP5, an 86 kDa protein has been shown to be an ITAF for the IRES mediated translation of its own mRNA (Henis-Korenblit et al., 2002). hnRNP C1/ C2 (44 kDa) has also been shown to interact and influence IRES function (Scheepens et al., 2007). However, the identity of the proteins (p90, p64, and p56) interacting differentially with wild-type and mutant CVB3-5' UTR is not clear at this moment.

Among Picornaviruses, the translation from the viral IRES gets stimulated upon co-expression of the entero-rhinovirus 2A protease or the cardio-apthovirus L proteinase. The factor responsible for such stimulation is the cleavage of translation initiation factor elf4F, which leads to a shut-off of the host cell protein synthesis. However, a more direct role of such protease cleavage could also be envisaged, by a mechanism involving increase in the abundance of an ITAFs that enhances IRES activity. Future research on identification and characterization of p90, p64 and p56 could explain the role of such putative host-specific factors; de novo-synthesis or proteolytic activation of which leads to a higher efficiency of viral genome translation.

It is possible that requirement of cis-acting elements and interaction with specific trans-acting factors might vary with the course of viral infection. The piconaviruses which are positive stranded RNA viruses, viral RNA has to translate with the available trans-acting factors. However, if cis-acting elements are modified then the interaction with trans-acting factor may be changed. It is shown in case of poliovirus sabin vaccine that even single point mutation in the IRES may change the requirement of the virus ultimately affecting the viral RNA translation (Guest et al., 2004). In case of poliovirus infection early phase, the viral RNA has to compete with cap dependent translation machinery in host cell. In later phase, the viral translation can compete effectively with cap dependent translation and cleavage of elf4F (reviewed by Hershey and Merrick, 2000) knocks out its interaction with Poly (A) binding protein, PABP (Kuyumcu-Martinez et al., 2004), which then favors IRES mediated translation. Also, in poliovirus infection as viral propagation proceeds, PTB is cleaved by viral proteases and it may be the reason for switching from viral genome translation to viral genome replication (Back et al., 2002). Taken together it appears that the integrity of cis-acting elements and its interaction with trans-acting factor are required for viral RNA translation and replication and the requirements could vary with the course of viral infection.

Methods

Plasmid constructs

The 5'UTR deletions ΔSL C/c and SL C/c mutations were generated by megaprimer-mediated PCR mutagenesis. A megaprimer spanning nucleotide 1–162 with deletion of nucleotides 147–152 was amplified using the primer FL-F (5'-GGCCGACTTATTTAAACAGCTT-3') as forward primer and d151-R (5'-AAAAACGTGGGCCACCGTGACTG-3') as reverse primer. The megaprimer was resolved and eluted from agarose gel and used in a second PCR reaction with the FL-R primer to amplify the 5'UTR with nucleotides 147–152 deleted. The mutant 5' UTR was cloned into pCDNA3 and the pBluescript bicistronic vector in similar manner as described before. A megaprimer was generated using CVBwt forward (5'-GGCCGACTTATTTAAACAGCTT-3') primer and mSL C reverse primer (5'-AAAAACGTGGGCCACCGTGACTG-3'). This megaprimer was used as forward and wt reverse (5'-GGCCGACTTATTTAAACAGCTT-3') primer was used in second PCR to generate the mSL C construct. The mutant 5'UTR was cloned into pCDNA3 monocistronic and the pBluescript bicistronic vector in similar manner as described before. The portion of the CVB3 genome encoding for the amino acids corresponding to 2A protease was amplified from the CVB3 cDNA using the primers CV2A-F (5'-ATTAGATTCCGGCCGCTTGG-3') and CV2A-R (5'-AGCGAATTTCGTTCCATTGGCATC-3'). The amplified fragment was digested with BamHI and EcoRI and cloned in BamHI/EcoRI digested pCDNA3-His-C. The activity of 2A protease was verified by translating in vitro from the above clone followed by specific cleavage activity (data not shown). The monocistronic plasmids were linearized by restriction enzyme XhoI and transcribed in vitro using T7 RNA polymerase.

In vitro transcription and translation

Monocistronic plasmid DNA containing Wt-5'UTR, ΔSL C/c or mSL C upstream of F-Luc DNA were linearized downstream of F-Luc by XhoI enzyme and transcribed using T7 RNA polymerase at 37 °C for 90 min.

In vitro translation of Wt, ΔSL C/c and mSL C CVB3 5'UTR F-Luc RNAs were carried out in micrococcal nuclease treated RRL (Promega corporation W1). Briefly, 12.5 μl reaction containing 1 μg of template RNA, 8.75 μl of RRL supplemented with 10% v/v HeLa S10 extract, 0.5 μl each of minus methionine and minus leucine and 10 U of RNasein. The reaction mixtures were incubated at 30 °C for 90 min. The luciferase values were determined in Luminometer.

For RNA transfection in HeLa cells polyadenylated monocistronic RNAs were synthesized in vitro using T7 polymerase (New England biolabs). For this purpose the template DNA was generated from the respective monocistronic DNA constructs by PCR using T7 forward primer and the reverse primer containing the sequences corresponding to 20 nt of the 3' end of F-luc gene followed by 30 nt of polyA sequences. The PCR amplified products were gel eluted and used as template in the in vitro transcription reaction as described above.

Transient transfection in HeLa cells

The transfection of bicistronic plasmid cloned in the eukaryotic promoter less vector pBluescript (containing T7 promoter), in the HeLa monolayer cells were carried out as described before (Dhar et al., 2007; Fuerst et al., 1986). Essentially, 106 cells were infected with a recombinant vaccinia virus (VTF7.3, with MOI 10), incubated at 37 °C with 5% CO2 for 60 min for the viruses to infect the cells. Subsequently the cells were transfected with 2 μg of the respective bicistronic plasmid DNAs using Tfx-20 reagent (Promega, Madison, USA). 24 h post-transfection cells were lysed and the activity of luciferase enzymes were assayed using dual-luciferase reporter assay system (Promega, Madison, USA).

For co-expression of 2A protease, the plasmid encoding CVB3 2A protease (500 ng) was co-transfected with the pCDNA3.1 bicistronic plasmids. 24 h post transfection the cells were harvested, lysed with passive lysis buffer and the luciferase activities were assayed using Dual Luciferase reporter assay system (Promega, Madison, USA) in a TD20/20 luminometer according to manufacturer's protocol.
For RNA transfection, 2 μg of polyadenylated monocistronic RNAs were transiently transfected into HeLa cells using the trans-messenger kit (Qiagen) according to manufacturer's protocol. 3 h post transfection the cells were washed and DMEM medium containing 10% FBS was added. After 8 h, cells were washed and lysed by 200 μl passive lysis buffer and firefly luciferase activities were measured.

**Hela S10 preparation and UV cross-linking**

HeLa S10 extract was prepared following the protocol described earlier (Ray and Das, 2002). RNA-protein interaction studies were performed as described earlier (Pudi et al., 2005). Briefly, Riboprobes (3 × 10⁵ cpm) were incubated with 20.0 μg of HeLa S10 extract in 1× binding buffer containing 2 μg of Yeast-tRNA as non specific competitor incubated at 30 °C for 10 min. The RNA-protein complexes were then UV cross-linked and analyzed in SDS-10% PAGE followed by phosphorimaging.

**Ribonuclease probing and DMS modification**

Probing the secondary structure of the RNA has been performed as described before (Bhattacharyya and Das, 2005). 10 μg of in vitro transcribed Wt 5′UTR in nucleic digestion buffer, denatured at 65 °C and slowly cooled to room temperature. The RNA was then digested with 0.05 μ of RNase V1 (Ambion) for 10 min at 4 °C. The digested and undigested RNA was precipitated and reverse transcribed with a 32P end labeled reverse primer annealing to 235–250 nucleotides of the 5′UTR. The cDNAs were precipitated and resolved in a 7 M Urea gel. Polyadenylated RNA was added. After 8 h, cells were washed and lysed by 200 μl of DNA polymerase (Bangalore Genei). DNA polymerase (Bangalore Genei).

Reverse transcription was done using M-MLV RT (Promega) followed by PCR with Taq DNA polymerase (Bangalore Genei).

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**References**


