

## INHIBITION OF INTERNAL ENTRY SITE (IRES)-MEDIATED TRANSLATION BY A SMALL YEAST RNA: A NOVEL STRATEGY TO BLOCK HEPATITIS C VIRUS PROTEIN SYNTHESIS

Saumitra Das<sup>1</sup>, Michael Ott<sup>2</sup>, Akemi Yamane<sup>1</sup>, Arun Venkatesan<sup>1</sup>, Sanjeev Gupta<sup>2</sup> and Asim Dasgupta<sup>1</sup>

Department of Microbiology, Molecular Genetics and Immunology, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1747<sup>1</sup>, <sup>2</sup>Department of Medicine, Albert Einstein, College of Medicine of Yeshiva University, Bronx, New York 10461-1602

Received 9/18/98 Accepted 9/23/98

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### 1. ABSTRACT

The observation that poliovirus mRNA is not translated in the yeast *Saccharomyces cerevisiae* has led to the discovery of a small RNA (60 nt, called IRNA, inhibitor RNA) which was later shown to specifically inhibit internal ribosome entry site (IRES)-mediated translation of naturally uncapped mRNAs. Translation of cellular capped mRNAs was not significantly inhibited by IRNA. IRNA also specifically inhibited hepatitis C virus (HCV) IRES-mediated translation *in vitro* and *in vivo*. A hepatoma cell line constitutively expressing IRNA was refractory to infection by a chimeric poliovirus (PV/HCV) in which PV IRES is replaced by HCV-IRES. In contrast, a PV/EMCV chimeric virus containing the EMCV IRES was not significantly inhibited in the IRNA-hepatoma cell line compared to the control hepatoma cells. UV-crosslinking studies showed that the IRNA binds a number of cellular proteins that appear to be important for IRES-mediated translation. Interaction of these proteins with the viral IRES elements is believed to be important in recruiting ribosomes to the 5' UTR of viral RNAs. The binding of the purified La autoantigen to the HCV IRES element was efficiently and specifically

competed by IRNA. These results provide a basis for development of novel drugs effective against HCV infection.

### 2. INTRODUCTION

Mammalian plus strand RNA viruses cause a multitude of infectious diseases in humans and animals, and currently, no effective vaccination is available against the majority of these viruses. The RNA genomes of some of these viruses (picorna and flaviviruses) are translated by a common mechanism known as internal initiation of translation where the ribosomes bind to an internal sequence within the 5'-untranslated region (UTR) near the initiator AUG of the viral RNA. This sequence is termed internal ribosome binding site (IRES) (48). This method of eukaryotic protein synthesis is different from the cap-dependent translation of cellular mRNAs. This fundamental difference could serve as a target for antiviral agents that would preferentially stop viral proliferation without affecting the host. Studies from several laboratories suggest the importance of specific interaction of cellular proteins with the IRES elements of the viral RNAs. Apparently there is

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no resemblance in the RNA sequence amongst different IRES elements, yet they exhibit some common features both at the level of cis-acting RNA elements and transacting factors. Identification and characterization of specific protein factors that are involved in this process are necessary for a better understanding of this relatively new phenomenon of eukaryotic protein synthesis. We believe one or more common factors may be required exclusively for IRES-mediated translation and once identified, these factors may be targeted to inhibit viral translation.

Recently, we have isolated a small RNA (I-RNA) from the yeast *Saccharomyces cerevisiae*, which specifically inhibits translation programmed by the IRES sequences of different viral RNAs, including those inducing common cold, poliomyelitis and infectious and chronic hepatitis (HAV and HCV). The inhibitor RNA does not act as an antisense RNA. Rather, it specifically binds certain cellular proteins required for internal initiation of translation thereby preventing them from interacting with the 5'-untranslated region (5'-UTR) of viral RNA.

Since the naturally occurring small yeast RNA (I-RNA) appears to block translation of the viral RNA but does not significantly affect cellular capped mRNA translation, it is clearly important to determine whether the I-RNA or its derivatives can be used as antivirals against viruses that use IRES mediated translation. We have recently provided evidence that the I-RNA can fold into a stable secondary structure, which mimics part of the viral 5'-UTR, and thus competes for protein binding (Venkatesan, Das and Dasgupta, unpublished). Thus, determination of its three dimensional structure might lead to the design of small molecules which would be biologically more effective in inhibition of viral RNA translation. The normal function of IRNA in yeast is not known. Some of the yeast genes are reported to be internally initiated (27, 28), and thus it is possible that IRNA may regulate the expression of these genes by inhibiting internal initiation of translation.

### 2.1. Internal initiation of translation in eukaryotes

To date, three different modes of initiation of eukaryotic mRNA translation have been discovered. The majority of the eukaryotic mRNAs are translated by the scanning mechanism whereby 40S ribosomal subunits bind to the 5'-cap structure and then scan the mRNA in a 5' to 3' direction until an appropriate initiator AUG is encountered (34). The second method is the reinitiation mechanism where after translating an open reading frame, the 40S subunits resume scanning and start initiation at a further downstream AUG codon; the best example is the yeast GCN4 gene (21, 26). The third mechanism is known as the internal initiation. Certain viral and cellular mRNAs contain sequences in their 5'-NCR that can direct cap-independent translation. The sequences mediating the internal recruitment of ribosomes have been termed the internal ribosome entry site (IRES) and can be located hundreds of nucleotides downstream from the 5'-end of mRNA. The first example of initiation of translation by

internal binding of ribosomes was found in picornaviral mRNAs (5,6,9,19,20,32,36,37,49,56). In sharp contrast to cellular mRNA translation, the naturally uncapped picornaviral RNAs translate inside infected cells in an environment where cap dependent translation of the majority of cellular capped mRNAs is impaired due to cleavage of the cap binding protein complex (39). It is through IRES mediated translation that picornaviral proteins are synthesized in infected cells, while cellular protein synthesis is inactivated.

Since internal initiation of translation is carried out by the cellular translational apparatus in uninfected cells, the question arises whether cellular mRNAs can be translated by a similar cap-independent manner. It is possible that some capped mRNAs can be translated by both cap dependent scanning and internal ribosome binding mechanisms. Such RNAs may contain IRES sequences in their 5'-non-coding regions that allow them to be translated at times when eIF-4F is nonfunctional and cannot bind to the 5'-cap structure. Alternatively, an IRES located within the coding region of mRNA could render the mRNA functionally polycistronic and translation could result in the production of several protein products. In fact, there are reports that the cellular mRNAs encoding immunoglobulin heavy chain binding protein (38), *Drosophila* antennapedia gene (45), the mouse androgen receptor (24), and the mammalian fibroblast growth factor 2 gene (59) utilize IRES mediated initiation of protein synthesis. Recently, Chen and Sarnow have demonstrated that the mammalian ribosomes can translate a covalently closed circular mRNA if it includes a functional IRES element (11).

### 2.2. The structure of IRES elements in different groups of viruses

The 5'-UTR plays a major role in translation of picornaviral RNAs. Usually the 5'-UTR is relatively long (varies from 600 - 800) and can fold to generate structures consisting of a number of stems and loops. Interestingly though, the primary sequences are not conserved but the RNA secondary structures are very similar, suggesting a possible role of RNA folding in virus translation. On the basis of the sequence and structural similarities of the IRES elements, the picornaviruses can be classified into three groups, 1) enterovirus and rhinovirus, 2) cardiovirus and aphthovirus, and 3) hepatitis A virus (30). A pyrimidine rich tract (25 nt) upstream of the initiator AUG is found to be conserved in the 5'-UTR of almost all picornaviral RNAs. Whether these conserved residues in loops and bulges are essential for the internal ribosome entry is not clear (1,29,33). A number of cellular protein factors have been shown to interact with the IRES elements but how they influence internal initiation is yet unclear. It is possible that the interaction helps in proper RNA folding and/or maintaining the higher order secondary structures of the 5'-UTR RNA during translation. There are reports (but no direct evidence) that the IRES/protein interaction might help in assuming correct RNA folding to facilitate IRES/rRNA interaction. In fact, results from different laboratories have shown the existence of significant pseudoknot structures common among EMCV, TMEV,

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FVDV and HAV which might assume an efficient binding structure for the ribosomes during translation initiation. The base pairing between human 18S rRNA and the IRES elements of picornaviruses is another plausible model (30,31,53).

Hepatitis C virus (HCV), a member of the flavivirus group, is the causative agent of hepatitis and is transmitted through blood. HCV infection often leads to chronic hepatitis, cirrhosis of liver and hepatocellular carcinoma. HCV 5'-UTR is 341 nt long and is highly structured amongst different strains and isolates (10). The exact boundaries of the IRES is somewhat controversial; requirement of a short length of 5'-proximal HCV polyprotein coding sequences has been reported for efficient IRES function (35, 58).

### 2.3. Cellular proteins that interact with the 5'-UTR of the viral RNAs

Studies from several laboratories suggest the importance of specific interactions of cellular proteins with various elements containing secondary structures within the 5'-UTR of viral RNAs. Two nuclear proteins have been identified that are apparently required for the efficient usage of the IRES by the host cell translation apparatus. One of them is a 52 Kd polypeptide (p52) which has been identified as the La autoantigen (41). The other cellular protein p57 appears to be identical to the polypyrimidine tract binding protein (PTB) (25). La is involved in RNA polymerase III transcription termination and PTB is a part of the nuclear spliceosome complex involved in RNA polymerase II transcript splicing. It is believed both La and PTB have dual roles in cytoplasmic mRNA translation and nuclear RNA biogenesis (11). Both La and PTB could be immunodepleted from in vitro translation extracts resulting in loss of picornaviral mRNA translation. Interestingly, translation in these depleted extracts could not be restored with the addition of purified La or PTB (25,57). These findings suggest that La and PTB may stimulate the IRES element in concert with additional factors to which they are tightly bound. The stimulatory effect of La and PTB on picornavirus IRES could be due to the recruitment of La and PTB associated factors to the IRES. Such factors could then directly mediate the binding of ribosomal subunits to the IRES. In contrast, recent studies using mutants having deletions of different stem loop domains of poliovirus 5'-UTR demonstrated that interactions of neither p57 nor p52 is absolutely required for internal ribosome binding (22,23,50). Thus, functional significance of La and PTB in IRES-mediated translation is not clearly established and possible involvement of other RNA-protein and/or protein-protein interactions are being explored. In fact, several additional RNA binding proteins has been shown to be crosslinked to type 1 and type 2 IRES elements of poliovirus including p37, p39, p48, p70, p80, p100 and p110 (7,16,18,44). The 37 kD HeLa cell protein that binds to the IRES of HAV has been identified as cellular Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) (55).

Another protein of molecular mass 39 kD (binds to stem-loop IV of polio virus 5'-UTR) has been identified as

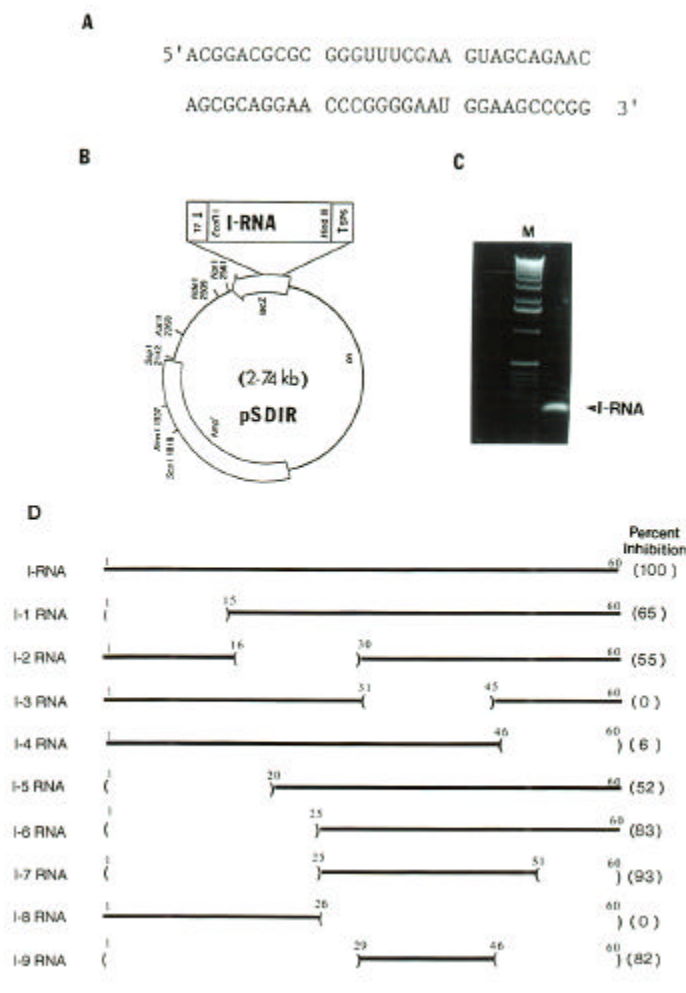
poly(rC) binding protein (PCBP2) (8, 17, 51). One or more of these cellular protein factors also bind to Hepatitis C 5'-UTR and to the regulatory elements of human T-cell leukemia virus type 2 RNA (45,52,60,61,62). Thus, it will be interesting to identify the common cellular protein factors that specifically bind to all IRES elements with some sort of functional significance. There are few reports on the requirement of tissue specific transacting factors in IRES-mediated translation. It has been observed that liver-specific factors can stimulate the IRES function of the hepatitis A virus (20).

### 2.4. Requirement of canonical eIFs in IRES-mediated translation

Initiation of eukaryotic protein synthesis involves the assembly of 80S initiation complex containing initiator tRNA<sub>i</sub><sup>MET</sup>, 40S and 60S ribosomal subunits at the initiation codon. In cap-dependent translation a 43S complex is formed that consists of eIF3 and a ternary complex of Met-tRNA<sub>i</sub><sup>MET</sup>: eIF2: GTP bound to the 40S subunit. In the next step the 43S complex binds to the 5'-end of the mRNA at the 5' m7GpppX cap structure. The cap-dependent binding of ribosomes requires at least eukaryotic initiation factor eIF4F and ATP. eIF4F consists of three subunits, eIF4A, eIF4E, and eIF4G. eIF4A exhibits RNA-dependent ATPase activity, and together with eIF4B, RNA helicase activity. The eIF4G subunit coordinates the binding of eIF4F to the m7GpppCap by interacting with eIF4A, eIF4E, eIF3 and the RNA. The eIF4B-induced helicase activity of eIF4F melts the RNA secondary structure to generate a single stranded(ss) region near the cap. eIF4B then interacts with 18S rRNA to guide the 40S ribosomal subunit to the ss region of the mRNA (3,47,51,54).

In case of cap-independent translation, the IRES elements promote binding of the 43S complex to a position far from the 5' cap, and closer to the initiator AUG. Stimulation of EMCV- IRES mediated translation by one or more eIFs has been shown. eIF2/2B and eIF4B binding to the EMCV and FMDV IRES have been reported from different laboratories (4,43,54). In a recent report on in vitro reconstituted IRES-mediated translation, it was demonstrated that eIF2, eIF3 and eIF4F were required for initiation whereas eIF4B and to a lesser extent PTBP stimulated the process (51). Interestingly, translation of capped mRNA by the scanning mechanism absolutely requires the intact eIF4F holoenzyme complex. Infection with enteroviruses, rhinoviruses, or FMDV results in cleavage of the eIF4G component of the eIF4F rendering it inactive in promoting initiation on capped mRNA. However, the cleaved factor (incomplete eIF4F complex) stimulates IRES-dependent translation more efficiently than the intact eIF4F holoenzyme. It is believed that internal initiation is driven by the C-terminal cleavage product of eIF4G with its associated eIF4A. Despite the subtle differences, the overall similarity of the initiation factor requirements suggests that the two mechanisms can not be fundamentally very different (30). It

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**Figure 1.** Sequence, cloning and deletion analysis of IRNA. The nucleotide sequence (A), cloning (B), and *in vitro* transcription of IRNA from the clone pSDIR (C) are shown. Panel D shows IRNA deletion mutant constructs. The nucleotide positions are indicated for each mutant. The numbers in parentheses indicate percentage of translation inhibition by mutant IRNAs compared with wild type IRNA (100%), calculated by averaging results from three independent experiments. Reprinted with permission from *J. Virology*, Vol. 68, p.7200-7211, 1994, and Vol. 10, p. 1624-1632, 1996, ASM.

is probably the first step i.e. the binding of the 43S complex to the internal sequence, that constitutes the primary difference between cap-dependent and IRES-mediated translation.

### 3. TRANSLATION OF VIRAL mRNA IN YEAST

#### 3.1. Poliovirus RNA is not translated in yeast

The discovery that the yeast *Saccharomyces cerevisiae* contains a potent inhibitor of IRES translation occurred through scientific serendipity. While trying to express the infectious poliovirus cDNA in the yeast, it was observed that the yeast *Saccharomyces cerevisiae* was incapable of translating poliovirus RNA (12). A poliovirus cDNA construct, pBM POLIO (cloned into pBM258 vector, which contained the sequences for replication in both *E. coli* and yeast) under the control of the GAL10

promoter, and a control plasmid (pBM258) were transformed into *S. cerevisiae*. Transformed cells were grown in galactose to induce expression of poliovirus RNA from the cDNA (from GAL10 promoter) by the yeast RNA polymerase. Although full-length viral RNA was synthesized in cells transformed with pBMPOLIO, no viral protein could be detected in the cell.

The inability of yeast cells to translate poliovirus and P2CAT RNA *in vivo* was further recapitulated *in vitro* and appeared to be due to one or more translational inhibitors present in yeast cells. In fact, addition of small amounts of yeast cell lysates to the HeLa cell translation reaction prevented translation of RNAs containing the PV5'-UTR (p2CAT RNA) whereas cap-dependent translation of CAT RNA (devoid of the PV 5'-UTR sequences) remained unaffected. These observations

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suggested that the translational inhibitory effects are mediated through PV 5'-UTR and the inhibitor was capable of acting in trans. Furthermore, addition of excess HeLa lysate was capable of rescuing the inhibition whereas addition of increasing amounts of template RNA to the translation reaction did not. Thus, the inhibitor appeared to interact with a component of the translational machinery rather than directly bind to the input viral RNA as an antisense RNA. In an attempt to purify the transacting factor (the inhibitor), the yeast cell lysates were fractionated on a DEAE sephacel column and the inhibitory activity was eluted with 1M KCl containing buffer. The partially purified inhibitor was further characterized and shown to be heat stable and resistant to phenol extraction, proteinase K digestion and DNase treatment, but sensitive to RNase digestion. These results suggested that the inhibitor is most probably an RNA molecule (12).

A later publication from this laboratory reported further purification of the DEAE sephacel purified fraction of the yeast inhibitor (15). Total RNA obtained from the 1M KCl fraction of the DEAE sephacel was treated with DNase and proteinase K followed by phenol extraction and alcohol precipitation. The purified total RNA was end labeled and resolved by 20% PAGE / 8M urea gel electrophoresis. Each RNA band was eluted from gel slices and tested for the inhibitory activity against PV-IRES driven translation. A single band associated with the inhibitory activity was identified and sequenced. On the basis of the RNA sequence of the small RNA molecule (60nt) a synthetic clone was prepared (figure 1). RNA derived from the synthetic clone (pSDIR) by *in vitro* transcription (I-RNA) was shown to block the translation of poliovirus RNA both *in vitro* and *in vivo* (15). When HeLa monolayer cells were cotransfected with PV RNA and I-RNA, poliovirus specific proteins were not detected by immunoprecipitation in transfected cells. Also, host protein synthesis was restored in cells cotransfected with PVRNA and I-RNA compared to complete shut off seen in cells transfected with PV RNA alone. Furthermore, the *in vivo* effect of I-RNA was reversed by addition of equimolar amounts of antisense I-RNA in the cotransfection experiment (15). These findings strengthened the idea that the purified yeast inhibitor RNA specifically blocked viral RNA translation without adversely affecting host cell protein synthesis.

### 3.2. I-RNA specifically inhibits internal initiation of translation

To examine if the cloned and purified yeast inhibitor RNA (I-RNA) preferentially inhibits internal initiation of translation, its effect on translation from a bicistronic messenger was determined. For this purpose, bicistronic constructs containing CAT and luciferase (Luc) genes flanked by the IRES sequences of different picornaviruses were used. Translation of Luc is initiated internally and that of CAT is initiated in a cap- dependent manner. In the presence of I-RNA, significant inhibition of Luciferase synthesis was observed, whereas synthesis of CAT was almost completely unaffected in each case (13,

15, and Das & Dasgupta, unpublished observation). Monocistronic constructs having IRES sequences upstream of a reporter gene were tested for inhibition by I-RNA. As expected, the 5'-UTR of poliovirus (p2CAT)- and the Bip mRNA(Bip)-mediated translation of the reporter genes was significantly inhibited by the addition of I-RNA. In contrast, addition of equivalent amounts of I-RNA did not inhibit cap dependent translation of CAT, Luciferase or the yeast  $\alpha$ 36 mRNA. Interestingly, EMCV-IRES mediated translation (pCITE) of the reporter gene was not inhibited by I-RNA (12).

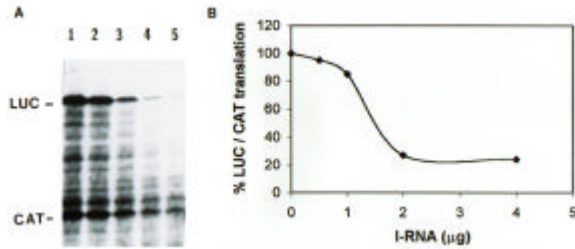
### 3.3. Minimum sequences required for I-RNA activity

To determine I-RNA sequences required for inhibition of poliovirus IRES-mediated translation, a nested set of 15 nt long deletions were generated. The effect of these truncated RNAs on *in vitro* translation programmed by P2 CAT RNA containing poliovirus 5' UTR was determined (13). The deletion analysis suggested that the minimum sequence required to inhibit PV IRES-mediated translation resides between nucleotides 30-45 (figure 1). This notion was supported by two observations. First, a deletion mutant (I-3 RNA) which contained the entire I-RNA sequence except nucleotides 31-45, was totally inactive in inhibiting viral IRES-mediated translation. Secondly, a truncated I-RNA (nt 30-45, I-9 RNA) retained considerable amount of translation-inhibitory activity (figure 1). However, a 25 nt. long truncated RNA (I-7 RNA) containing the I-9 RNA sequence appeared to be more active particularly *in vivo* (see figure 1). The shorter I-9 RNA was only 50% as active as I-RNA *in vivo*. The structure(s) of I-RNA or its truncated derivatives may be important in IRES-mediated translation-inhibition. The fact that addition of an extra ten nucleotides to the 3'-end of I-7 RNA (nt. 26-50) significantly reduces its (I-6 RNA, nt. 26-60) translation-inhibitory activity may be indicative of alteration of the structure of this RNA. Similarly, addition of another 5 nucleotides to the 5'-end of I-6 RNA drastically reduces its (I-5, nt. 20-60) ability to inhibit translation (figure 1).

### 3.4. Inhibition of HCV IRES-mediated translation by IRNA *in vivo* and *in vitro*

To test the possibility that IRNA might interfere with the HCV IRES-mediated translation, human hepatocellular carcinoma cells (Huh-7) were transiently cotransfected with 3 plasmids: a reporter gene expressing luciferase programmed by the HCV-IRES element (pCD HCV-luc), SV40/ $\beta$ -gal plasmid to measure transfection efficiency, and the plasmid expressing IRNA (pCDIR.Ribo.  $\Delta$ T7). All transfections were performed in triplicates and contained equal amounts of the luciferase reporter and  $\beta$ -gal plasmids. Increasing concentrations of the pCDIR.Ribo.  $\Delta$ T7 plasmid were used in various reactions and the total amount of DNA in each reaction was kept constant by addition of an appropriate amount of a non-specific DNA (pCDNA3). Following transfection, luciferase activity was measured in cell-free extracts. At the lowest concentration of the IRNA plasmid, inhibition of luciferase activity from the pCD HCV-

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**Figure 2.** IRNA selectively inhibits HCV IRES-mediated translation *in vitro*. (A) a bicistronic construct containing the HCV IRES flanked by CAT and Luciferase (LUC) genes was transcribed by T7 RNA polymerase, and the bicistronic mRNA was translated *in vitro* in HeLa lysate in the absence (lane 1) and presence of 0.5, 1, 2 and 4 µg of IRNA (lanes 2, 3, 4 and 5, respectively). (B) The results in Figure 2A were quantitated and the ratios of LUC to CAT are presented as percentage of LUC/CAT translation against various concentration of IRNA. Reprinted with permission from J. Virology, Vol. 72, p. 5638-5647, 1998, ASM.

luc plasmid was approximately 50% compared to the control (14). However, at the highest concentration, 90% of luciferase activity was inhibited. Translation of luciferase from a control plasmid (pCDNA3-luc) without having the HCV-IRES was not significantly inhibited by IRNA. Expression of either ribozyme alone or a nonspecific RNA having similar length as IRNA, did not interfere with luciferase expression (14). These results suggested that HCV-IRES-mediated translation was specifically inhibited by IRNA in hepatoma cells, whereas cap-dependent translation of luciferase from the control plasmid lacking the HCV IRES element was not significantly affected by IRNA (14).

To confirm the results obtained *in vivo*, the effect of IRNA on HCV-IRES-mediated translation was determined *in vitro*. A bicistronic construct consisting of the HCV IRES flanked by CAT and luciferase genes was used in this experiment. While synthesis of CAT is mediated by cap-dependent translation, the downstream luciferase synthesis occurs by HCV IRES-mediated translation. Translation was measured by quantitating radioactivity incorporated into luciferase (Luc) and CAT polypeptides at 0, 0.5, 1, 2 and 4 µg of IRNA (figure 2). The specific inhibition of luciferase synthesis was normalized by determining the ratio of luciferase to CAT at each IRNA concentration. At one microgram IRNA, luciferase synthesis was inhibited to 20% of the control, whereas at 2 µg IRNA, specific inhibition of HCV IRES-mediated luciferase synthesis was 76% compared to the control. Although both luciferase and CAT synthesis were inhibited by IRNA *in vitro*, luciferase synthesis was affected much more than CAT at higher concentrations of IRNA. The inhibition of cap-dependent translation by IRNA could be due to its interaction with general RNA-binding proteins which have been implicated in facilitating cap-dependent translation. In addition, IRNA's interaction with La may affect AUG start site selection during translation initiation, as

suggested by recent studies from different laboratories (40, 61).

### 3.5. Constitutive expression of I-RNA in eukaryotic cells

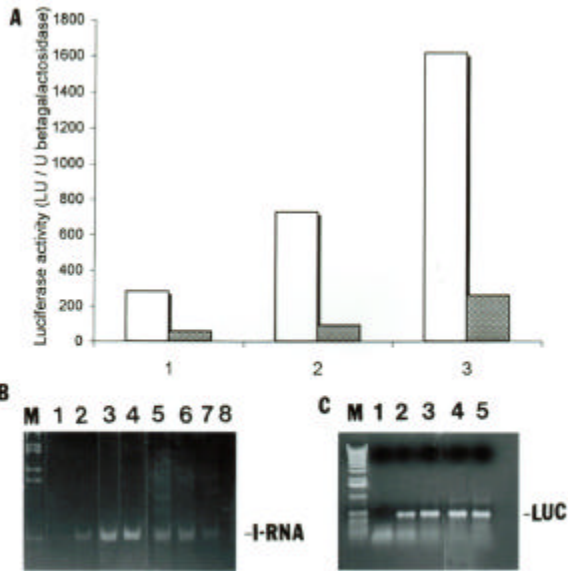
To determine the long term effect of expression of IRNA in hepatoma cells, cell lines constitutively expressing IRNA were generated using a pCDNA based vector (14). The cell line (pCDIR) made initially contained the T7 sequences at the 5'-end of the IRNA gene. Another cell line was made in which the hepatitis delta ribozyme sequence was added at the 3'-end of IRNA sequence for generation of the exact 3'-end (pCDIR-Ribo). A third cell line was prepared using the pCDIR-Ribo construct lacking the T7 promoter sequences (pCDIR-Ribo-ΔT7) (14). The control cells and cell lines expressing IRNA were cotransfected with HCV-IRES-Luc and β-gal DNA. Cell-free extracts were used to measure both luciferase and β-gal activities. Luciferase expression was plotted after normalizing with respect to β-gal activity. Approximately 60% inhibition of HCV-IRES-mediated translation was observed in the pCDIR cells compared to the control. The cells expressing IRNA with hepatitis delta antigen showed 65% inhibition of HCV-IRES-mediated translation. In cell lines expressing IRNA-Ribo without the T7 promoter sequence, almost 81% inhibition of luciferase expression was observed. These results clearly show that IRNA interferes with expression of luciferase programmed by HCV IRES. A titration of the reporter construct (HCV-IRES-Luc) in the cell line pCDIR-Ribo- T7 consistently showed 80-85% inhibition of HCV IRES-mediated translation of luciferase (figure 3). No significant inhibition of cap-dependent translation from the pCDNA-Luc construct was observed with cell lines expressing IRNA (14). That constitutive expression of IRNA in hepatoma cells is not detrimental to these cells is supported by continued viability of these cell lines for the last 6-8 months. Moreover, overall cellular transcription and translation were not significantly altered in the cell lines compared to the control cells (14).

### 3.6. Cells expressing I-RNA are refractory to PV and PV/HCV chimeric virus infection

To determine the effect of IRNA on HCV IRES-mediated translation during virus infection, the pCDIR.Ribo.ΔT7 cells were infected with a chimeric poliovirus (PV/HCV 701) in which PV IRES is replaced by the HCV IRES. PV/HCV 701 contained the 5'-cloverleaf structure of PV, followed by HCV IRES (nt. 9-332) plus 123 amino acids of HCV core protein followed by the entire poliovirus ORF plus the 3'-UTR and poly (A) (35). Translation of viral proteins in cells infected with PV/HCV chimeric virus is mediated by the HCV IRES element. Huh-7 control cells and the hepatoma-IRNA cells (pCDIR.Ribo.ΔT7) were infected with polio and PV/HCV chimeric viruses. Following infection, cell free extracts were prepared from infected and mock-infected cells which were then used to further infect HeLa monolayer cells. Plaques characteristic of wt PV (panel A) and PV/HCV 701 (panel B) were apparent in HeLa cells infected with cell-free extract from control hepatoma cells (figure 4). Evidently



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**Figure 3.** HCV-luciferase reporter dose response and quantitation of IRNA and Luciferase mRNA in the hepatoma cell line expressing IRNA constitutively. (A) one, 2 and 3 µg of the pCD HCV-luc reporter plasmid were transfected into Huh-7 hepatoma cells (dotted bar) or the IRNA-expressing hepatoma cell line (pCDIR.Ribo.ΔT7) (white bars). A control β-gal plasmid was also cotransfected to normalize transfection efficiency. The ratio of luciferase ( $\times 10^3$  light units [LU]) to β-gal activities were plotted against various concentrations of the reporter plasmid. (B) IRNA expression level in the cell line was detected by RT-PCR; (lane 1) no IRNA; (lanes 2-4), 1, 2.5 and 5 ng of purified IRNA. (Lanes 5 to 7), 2, 1.5 and 1 µg of total RNA from the cell line expressing IRNA. (Lane 8) contained 2 µg of total RNA from control hepatoma cells. (C) Luciferase mRNA in control (lanes 4) versus IRNA-expressing cells (lane 5) was determined by RT-PCR. Lanes 1-3 show no RNA, and 1 and 10 ng of luciferase mRNA control, respectively. Reprinted with permission from J. Virology, Vol. 72, p. 5638-5647, 1998, ASM.

**Table 1.** Inhibition of PV/HCV plaque formation by IRNA

Chimeric virus	Virus Titer (pfu/ml)		
	Huh-7	Hepatoma-IRNA	Hepatoma-ribozyme
PV/HCV	$5.9 \times 10^8$	$1.0 \times 10^6$	$5.2 \times 10^8$
PV/EMCV	$1.8 \times 10^8$	$0.8 \times 10^8$	$1.6 \times 10^8$

$3 \times 10^5$  cells in 30 mm plates were infected with 150 microliter of  $2.5 \times 10^4$  pfu/ml PV/HCV or PV/EMCV chimeric viruses for 24 hours. Cell free extracts were prepared from infected cells and virus titers were determined by infecting  $2 \times 10^6$  HeLa cells and counting plaques after 48 hours of infection. Each titer is an average of 3 experiments.

viral replication was drastically affected in the cell line expressing IRNA (Hepatoma-IRNA) with either virus (figure

4). In a parallel experiment, the virus titers were measured using the serial dilution method and the results demonstrated more than 100 fold decrease in virus yield in IRNA-expressing hepatoma cells compared to the control cells. While the control hepatoma cells showed extensive damage after infection, the cells expressing IRNA were significantly protected from the cytopathic effect of the chimeric virus (figure 4). Thus, hepatoma cells constitutively expressing IRNA were significantly resistant to both PV and PV/HCV chimeric virus under the conditions used for infection (14).

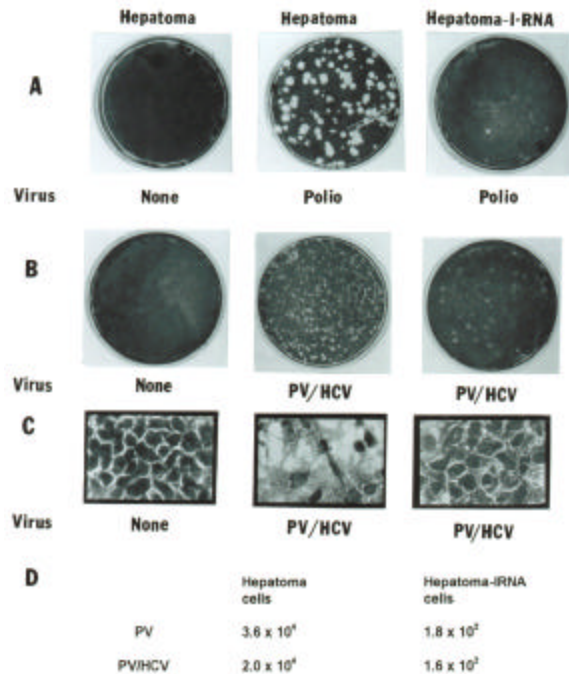
To rule out the possibility that the cloned hepatoma cell line is simply less permissive to support viral infectious life cycle, its ability to support replication of another chimeric poliovirus was examined. For this purpose a chimeric PV [PV1 (ENPO)] containing the EMCV IRES was used (14). We had previously shown that EMCV IRES-mediated *in vitro* translation was not inhibited by IRNA (12). Both the PV/HCV and PV/EMCV chimeric viruses were used to infect the Huh-7 control, hepatoma-IRNA and hepatoma cells expressing only the ribozyme. As can be seen in table 1, the PV/HCV chimeric virus titer was reduced approximately 100 fold in hepatoma-IRNA cells compared to Huh-7 control cells. In contrast, the PV/EMCV virus titer was not significantly reduced in hepatoma-IRNA cells compared to Huh-7 cells. This is consistent with our previous finding that EMCV IRES-mediated translation is not inhibited by IRNA *in vitro* (12). Also, the hepatoma-ribozyme cell line was as active in supporting PV/HCV (or PV/EMCV) replication as the control Huh-7 cells (14). These results suggest that the cloned hepatoma cell line expressing IRNA is not simply less permissive to support virus replication in general. These results also suggest that viral RNA synthesis or protein processing is not inhibited by IRNA.

To confirm the results obtained with the plaque assay, viral proteins were labeled with [ $^{35}$ S] methionine during infection of Huh-7 and hepatoma-IRNA cells with the PV/HCV and PV/EMCV chimeric viruses. Labeled capsid proteins were then immunoprecipitated using anti (PV) capsid antiserum and analyzed by SDS-PAGE (14). Quantitation of the results showed that the inhibition of individual capsid protein synthesis in hepatoma-IRNA cells infected with PV/HCV varied from 60% (VP0), 82% (VP1), 78% (VP2) and 77% (VP3) compared to that in Huh-7 control cells. Consistent with our plaque assay results, only marginal inhibition of capsid protein synthesis was observed with the PV/EMCV chimeric virus (lanes 3 and 4).

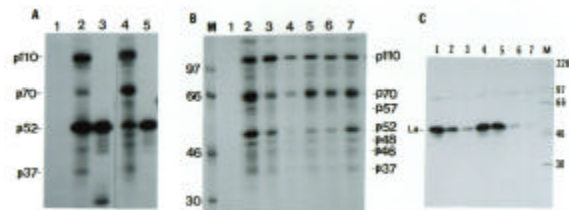
### 3.7. I-RNA binds proteins that are critical for IRES mediated translation

Since the I-RNA sequence is not complementary to the 5'-UTR sequences of the viral RNAs and is therefore, not likely to act as an antisense RNA, we determined whether I-RNA was capable of binding cellular proteins believed to be required for IRES-mediated translation. To determine whether I-RNA binds similar polypeptides as observed with the IRES elements, UV crosslinking studies were performed using HeLa ribosomal salt wash and labeled 5'-UTR or I-

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**Figure 4.** Hepatoma cells constitutively expressing IRNA prevent PV and HCV-PV chimera infection. Huh-7 control cells or the IRNA-expressing hepatoma cell line (pCDIR-Ribo.  $\Delta$ T7) were infected with either poliovirus (PV) or HCV-PV chimera (B and C). After 72 h, cells were stained for the observation of cytopathic effect (C) or cell extracts were made to further infect HeLa monolayer cells for plaque assay (A and B). (D) Average virus titers obtained from 3 independent plaque assays are shown. Reprinted with permission from *J. Virology*, Vol. 72, p.5638-5647, 1998, ASM.



**Figure 5.** IRNA binds cellular proteins that interact with HCV 5' UTR. (A)  $^{32}$ P-labeled IRNA (lanes 1 to 3) and HCV 5' UTR (lanes 4 and 5) were UV-crosslinked to HeLa S10 proteins (lanes 2 and 4) and purified La protein (lanes 3 and 5). (B) Competition UV-crosslinking studies were performed with  $^{32}$ P-labeled HCV UTR RNA and HeLa S10 proteins in the absence (lane 2), and presence of 250 and 500 fold molar excess of unlabeled HCV UTR (lanes 3 and 4), and 250 and 500 fold molar excess of unlabeled IRNA (lanes 5 and 6) or 500 fold molar excess of a non-specific RNA. Lane 1 contains no proteins. (C)  $^{32}$ P HCV-UTR UV-crosslinked to purified La proteins (lane 1) were competed with 100 (lanes 2, 4, 6) and 200 (lanes 3, 5 and 7) fold molar excess of unlabeled IRNA (lanes 2 and 3), non-specific RNA (lanes 4 and 5) and HCV 5' UTR RNA (lanes 6 and 7). Reprinted from *J. Virol.* Vol. 72, p. 5638-5647, 1998, ASM.

RNA. It was observed previously that both PV-5' UTR and I-RNA bound polypeptides of apparent molecular masses of 80, 70, 52, 43 and 37 kDa (15). That the polypeptides bound by PV-5' UTR and I-RNA were similar was confirmed by competition experiments using labeled I-RNA and unlabeled 5' UTR. Almost all polypeptides bound to labeled I-RNA could be competed out by inclusion of unlabeled UTR during the assay. However, similar amounts of a nonspecific RNA were totally ineffective in inhibiting crosslinking of I-RNA to these polypeptides. These results suggest that similar polypeptides (80, 70, 52 and 37 kDa) bind to both I-RNA and viral 5' UTR.

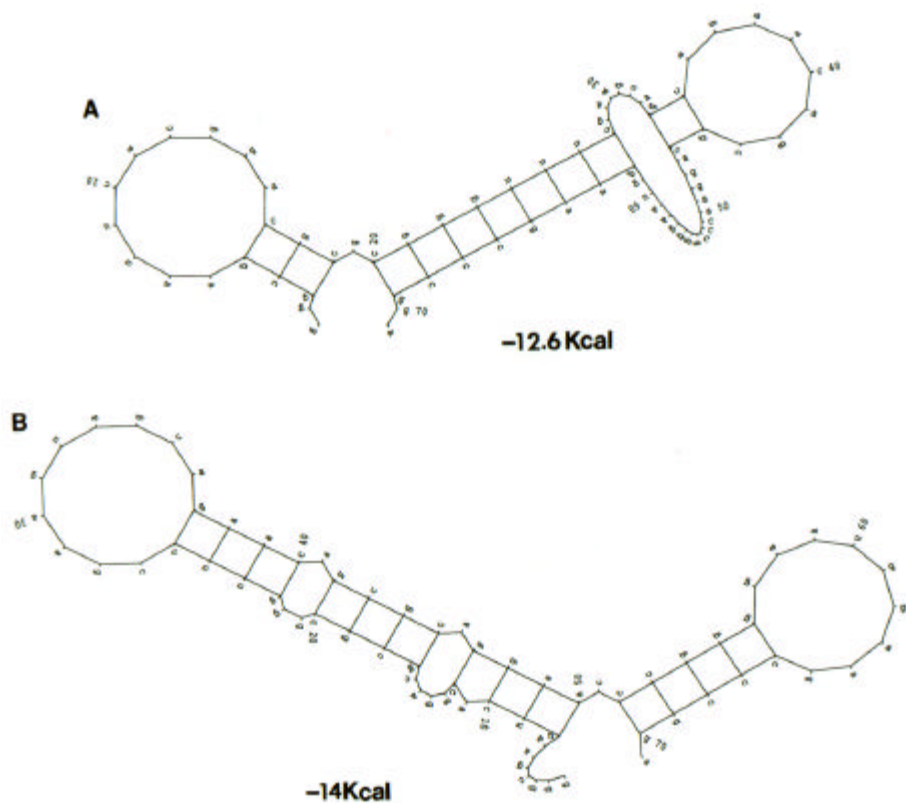
Comparison of protein binding by active and inactive truncated I-RNA mutants suggested that in addition to the La protein (52 kDa), three other polypeptides 80, 70 and 37 kDa might influence the translation inhibitory activity of IRNA (13). Both active (I-7 and I-9) and inactive (I-4 and I-8) mutants bind to two common polypeptides having molecular masses of 52 and 37 kDa. However, I-7 and I-9 bound a 80 kDa protein whereas the 70 kDa protein is only bound by I-4 and I-8 mutants. It is possible that the binding of 80 kDa protein to PV5' UTR is important for IRES mediated translation. This binding might require La and 37 kDa and/or other polypeptides. I-4 and I-8 RNA may not efficiently inhibit because of the inability to interact with the 80 kDa polypeptide. Identification and characterization of this 80 kDa protein might reveal the actual mechanism of I-RNA mediated translation inhibition. Furthermore, when  $^{32}$ P I-RNA was crosslinked to HeLa RSW protein, it was observed that I-RNA also binds (albeit weakly) to two other cellular proteins, PTB and poly(rC) binding protein which have been shown to be critical for picornavirus IRES mediated translation (unpublished observation).

When [ $^{32}$ P] labeled HCV-IRES was used in the UV-crosslinking experiment, major protein-nucleotidyl complexes were observed at 110, 70, and 52 kDa and minor bands were detected at 100, 57, 55, 48, 46 and 37 kDa (figure 5A, lane 4). Similar complexes were also observed when [ $^{32}$ P] labeled IRNA was used as the probe (figure 5A, lane 2). When purified La was used in the UV-crosslinking experiment, both  $^{32}$ P IRNA and  $^{32}$ P HCV-IRES bound the La protein which comigrated with the p52 detected in the S10 fraction (figure 5A, Lanes 3 and 5). Unlabeled HCV-IRES competed with the labeled probe ( $^{32}$ P HCV-IRES) for binding to p110, p70, p57 (a doublet), p52, p48, p46 and p37 (figure 5B). Unlabeled IRNA strongly competed with [ $^{32}$ P] HCV-IRES for the binding of p52, whereas weak competition was observed with p70, p57, p48, p46 and p37 (figure 5B). A non-specific RNA was not as effective as HCV-IRES or IRNA in the competition assay. Approximately 80% of La (p52) bound to [ $^{32}$ P] HCV-IRES was competed with unlabeled IRNA, whereas only 22% competition was observed with a nonspecific RNA. For other proteins (p48, p46, p37, p70, p110), however, specific competition with IRNA was marginal compared to the control. Competition experiments with purified La protein showed that IRNA was a very effective competitor for



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La



**Figure 6.** Predicted secondary structures of IRNA. Two computer predicted secondary structures of IRNA are shown. Recent results show that the form depicted in (A) is the actual secondary structure of IRNA.

binding to HCV IRES (figure 5C). These results suggest that IRNA specifically competes with HCV-IRES for La binding, an observation consistent with a recent result that La specifically stimulates HCV IRES-mediated translation *in vitro* (2).

UV crosslinking studies with the 5'-UTR of different picornaviruses and I-RNA in HeLa cell-free extracts (S10 or RSW) demonstrated a very similar binding profile in each case. The only difference was that the La protein bound more strongly to the enterovirus and rhinovirus 5'-UTR whereas PTB bound more strongly to the 5'-UTR of cardioviruses (Das & Dasgupta, unpublished observation). Since poliovirus infection shuts off cap-dependent translation of the cellular mRNAs, HeLa extracts derived from virus-infected cells are only active in IRES-mediated cap-independent translation. It was observed that I-RNA inhibited translation of P2CAT RNA to 20% of the control. This inhibition was reversed by addition of purified La protein to almost 90% of the control (13). These results suggest that I-RNA-La protein interaction plays a major role in inhibition of IRES-mediated translation. However, further experimentation is necessary to investigate whether other factors that interact with I-RNA are required for picornavirus IRES-mediated translation. We are currently trying to deplete HeLa cell-free translation extract of IRNA binding

proteins by passing it over an I-RNA affinity column. It would be interesting to see whether the readdition of purified proteins or different fractions back to the translation reaction would result in restoration of IRES-mediated translation.

## 4. STRUCTURE OF IRNA

### 4.1. Structure of I-RNA is important for the inhibitory activity

Since both the picornaviral 5'-UTR and I-RNA bind similar proteins involved in internal initiation of translation, the obvious question is whether there is any sequence homology between these RNAs. We found no significant homology of I-RNA with any picornaviral RNA. Furthermore, the antisense I-RNA (complementary sequence of I-RNA) was found to be almost equally active in blocking IRES-mediated translation (Venkatesan, Das and Dasgupta, unpublished). Taken together, it is plausible that I-RNA might fold into a secondary structure that could be very similar to a portion of the IRES element of the picornaviral RNAs. In fact, the secondary structure predicted by the Zuker MFOLD modeling program shows that a stem loop formed by intramolecular folding of I-RNA resembles the conserved core structure of the picornavirus IRES element (Ref. 54 and unpublished results) (figure 6). Thus it appears that the structure rather than the primary

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sequence of I-RNA molecule is the major determinant for selective inhibition of IRES mediated translation.

### 4.2. I-RNA encoding gene in *Saccharomyces Cerevisiae*

Sequence analysis of the region spanning the active site of I-RNA (at 16-60) have been found to be highly homologous to an yeast chromosome 3 fragment while the leader sequence (nt 1-15) may be encoded by a chromosome 12 fragment. It is possible that the I-RNA encoding gene originates from two different chromosomes, however since IRNA was originally isolated from the ABYS1 strain of *S. cerevisiae*, further experimentation is necessary to understand whether this discontinuity in sequence is due to trans splicing or simply reflects a strain variation between ABYS1 and the data base strain.

## 5. PRESPECTIVE

Since the naturally occurring small yeast RNA (I-RNA) appears to block translation of the viral RNA but does not significantly affect cellular capped mRNA translation, it would be interesting to determine whether I-RNA or it's derivatives can be used as an antiviral agent against viruses that exclusively use IRES-mediated translation for synthesis of viral proteins. The inhibitor RNA does not act as an antisense RNA, rather it interacts with cellular protein factors that are necessary for IRES-mediated translation. We strongly believe that the I-RNA can fold into a stable secondary structure which mimics part of the viral 5'-UTR RNA and thus compete for protein binding. Thus, determination of its 3-dimensional structure might lead to design of a more stable derivative of I-RNA or small molecules which would be biologically more effective in inhibition of viral RNA translation. The knowledge gained by studying I-RNA/cellular protein interaction would help in understanding the mechanism of IRES mediated translation.

What is the normal function of I-RNA in yeast? Some of the yeast genes are reported to be internally initiated. It is possible I-RNA regulates the expression of these genes by inhibition of internal initiation of translation. The normal function of I-RNA in yeast is not known. However, sequence spanning the active site of I-RNA have been found to be highly homologous with an yeast chromosome 3 fragment (in the strain used in the database). I-RNA was originally isolated from the *S. cerevisiae* ABYS1 strain. The yeast genome database search suggests that a chromosome 12 fragment could code for the leader sequence (1-15 nt.), whereas the activity domain of I-RNA (15-60 nt.) has been identified in a chromosome 3 fragment. Further experimentation is necessary to understand whether this discontinuity in sequence is due to trans splicing or simply strain variation.

Future studies would be directed towards identification of the common factors involved in IRES mediated translation in different systems, evaluate the potential of the I-RNA as a general inhibitor of IRES mediated translation and eventually design an effective antiviral agent targeting this unique mechanism of viral RNA translation.

## 6. ACKNOWLEDGMENTS

This work was supported by the grant AI-18272 and AI-38056 from the National Institutes of Health to AD. S.G. was supported by grant DK 46952 and the Irma T. Hirschal Charitable Trust. AY was supported by the Cota Robles Fellowship and AV was a Howard Hughes Medical Institute Medical Student Training fellow and was also supported by the NIH Medical Scientist Training Program grant NRSA/GM08042-15. We thank W. Tsai and other members of the Dasgupta laboratory for their support and E. Berlin for excellent secretarial work. We also thank E. Wimmer, M. Gromeier, F. Lasher and H-Lu for PV/HCV, PV/EMCV viruses. We thank Aleem Siddiqui for the PT7-DC 1-341 HCV bicistronic construct.

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**Key words:** IRES, HCV, Translation-inhibitor, Hepatitis, Hepatitis C, Hepatitis C virus, Virus, RNA, Protein synthesis

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Send correspondence to: Dr Asim Dasgupta, Department of Microbiology Immunology and Molecular Genetics, UCLA School of Medicine, Los Angeles, CA 90095-1747, Tel: (310)-206-8649, Fax: (310)-206-3865, E-mail: [dasgupta@ucla.edu](mailto:dasgupta@ucla.edu)