All five cold-shock domains of unr (upstream of N-ras) are required for stimulation of human rhinovirus RNA translation

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Efficient translation of human rhinovirus-2 (HRV-2) RNA from its internal ribosome entry site (IRES) depends on the presence of cellular trans-acting factors upstream of N-ras (unr) and polypyrimidine-tract-binding protein. unr contains five cold-shock domains (CSDs) and is predicted to act as an RNA chaperone, allowing the HRV-2 IRES to attain the correct conformation for ribosome binding. To investigate the role of each of the CSDs in IRES-dependent translation, five unr mutants, each harbouring a point mutation in a different CSD, were generated. All five mutants were severely impaired in their ability to bind to the IRES and to stimulate translation from it. This showed that the ability of unr to function as an activator of HRV-2 RNA translation requires the RNA-binding activity of all five CSDs.

INTRODUCTION

Translation of picornavirus RNAs occurs by internal initiation, in which ribosomes are able to bind directly to an internal ribosome entry site (IRES) on the RNA independently of the 5′ end (Jang et al., 1988; Pelletier & Sonenberg, 1988). All picornaviruses to date have been shown to contain an IRES of approximately 450 nt in their 5′-UTR. Picornavirus IRESs can be divided into two major groups, the entero-/rhinovirus IRESs and the cardio-/aphthovirus IRESs, plus a minor group consisting of the hepatitis A virus IRES. There is considerable primary sequence identity and strong conservation of secondary structure amongst the 5′-UTRs of each group, but very little homology between the groups (Jackson & Kaminski, 1995). Internal initiation of translation can be demonstrated in vitro using a dicistronic mRNA assay in a cell-free system such as rabbit reticulocyte lysate (RRL). No viral proteins are required, but most of the canonical translation initiation factors, with the exception of the cap-binding protein eIF4E, are required for picornavirus IRES-dependent translation (Pestova et al., 1996; Ohlmann et al., 1996). The hepatitis A virus IRES is also different in this respect, demonstrating some dependence on eIF4E for translation (Ali et al., 2001). In addition to the canonical initiation factors, trans-acting factors from the host cell may be required for efficient picornavirus translation initiation. The requirement for trans-acting factors varies greatly between the different groups of IRESs, and even within groups of closely related viruses the factors that stimulate IRES-dependent translation can differ (Hunt et al., 1999; Pilipenko et al., 2000).

For example, cardio- and aphthovirus RNAs are translated efficiently in RRL (Kaminski et al., 1990), whereas entero- and rhinovirus RNA translation is inaccurate and inefficient in this system (Dorner et al., 1984; Borman & Jackson, 1992), unless the RRL is supplemented with HeLa cell cytoplasmic extract.

The dicistronic mRNA assay was used as a functional assay in the purification of two activities from HeLa cell extract that bound to the human rhinovirus-2 (HRV-2) 5′-UTR and stimulated translation from the HRV-2 IRES (Hunt & Jackson, 1999). These were identified as polypyrimidine-tract-binding protein (PTB; Hunt & Jackson, 1999) and upstream of N-ras (unr; Hunt et al., 1999).

unr is encoded by a gene so closely linked to N-ras that it was thought that there might be co-ordinated transcription of the two (Jeffers et al., 1990), although transcriptional interference of N-ras by unr has proved to have only a weak effect (Boussadia et al., 1997). It is an essential gene: the homozygous knockout is embryonic lethal in mice, but only at around 10 days, which implies that unr is not essential for general cell viability and cell division, but must be essential for certain stages in differentiation (Boussadia et al., 1997). It has a broad tissue and cell-type distribution (Lopez-Fernandez et al., 1995), and is largely cytoplasmic (Jaccurmin-Sablon et al., 1994). There are two known isoforms differing by the inclusion or exclusion of the optional exon 5 (Boussadia et al., 1993) with a 10:1 ratio in abundance of the smaller to larger isoforms. unr is a member of the cold-shock family of single-stranded nucleic acid-binding proteins (Doniger et al., 1992). However, it is exceptional amongst this family in that it has five copies of the cold-shock domain (CSD), each of which has an unusual amino acid sequence signature motif, FFH, in part of the
RNA-binding surface, whereas all other members of the family have FHV in this position (Doniger et al., 1992). The occurrence of this unusual motif in all five CSDs implies that unr arose by repeated duplication of a protein that originally had just one domain.

To investigate the function of unr in translation of HRV-2 RNA, the role of each individual CSD was studied by the generation of five mutants, each with a point mutation in a different CSD. The effect of mutation of each of the CSDs on the ability of unr to stimulate translation from the HRV-2 5′-UTR and its sub-domains, was studied.

**METHODS**

**Plasmid constructs.** pHJV10-611 encodes a monocistronic mRNA consisting of nt 10–611 of the HRV-2 5′-UTR followed by a slightly truncated form of the coding region for influenza virus non-structural protein 1 (NS1). pHJV10-611 was linearized with BamHI prior to transcription to generate transcripts consisting of the HRV-2 5′-UTR (from nt 10) terminating in -AU/GGAUC, where AUG is the translation initiation codon, pHJV-d2, -d5 and -d5+6 encode monocistronic mRNAs consisting of subdomains 2, 5 and 5+6 of the HRV-2 5′-UTR upstream of NS1 (E. C. Brown, S. L. Hunt & R. J. Jackson, unpublished results). These were generated by PCR of nt 102–176, 439–547 and 439–611 of the HRV-2 5′-UTR, respectively, and subcloning into a pGEM-based vector upstream of the NS1 coding region. Linearization of these constructs with BamHI prior to transcription was carried out to generate transcripts consisting of the individual subdomains for use in gel-shift and UV cross-linking assays.

**Site-directed mutagenesis.** This was done with a QuikChange site-directed mutagenesis kit (Stratagene). Complementary oligonucleotides were designed that encompassed the proposed site of mutation: CSD 1*: 5′-CCTACTTCATAG-3′ and 5′-GATGTGTTGCTGCATGTTGATGAC-3′; CSD 2*: 5′-GAGGCGATTGGCGGGCTTAAAGGAAGGTGATG-3′ and 5′-CATCACCTTTTCAATAGCGCAGGATCCGCT-3′; CSD 3*: 5′-GATGTGTTGCTGCATGTTGATGAC-3′ and 5′-GAGGCGATTGGCGGGCTTAAAGGAAGGTGATG-3′; CSD 4*: 5′-GATGTGTTGCTGCATGTTGATGAC-3′; CSD 5*: 5′-GATGTGTTGCTGCATGTTGATGAC-3′. Reactions were set up according to the supplier’s recommendations. The reaction underwent 16 cycles of amplification at 95°C for 30 s, 55°C for 1 min and 68°C for 2 min per kb plasmid length, in a thermal cycler. The reaction mixture was incubated with DpnI at 37°C for 1 h, and 1/10 of the reaction volume was used to transform *Escherichia coli* TG1 cells.

**In vitro transcription and translation.** Uncapped mRNAs for use in translation assays were generated by *in vitro* transcription of the linearized plasmid template with T7 RNA polymerase, as described previously (Dasso & Jackson, 1989). Translation assays were carried out in RKL that had been treated with micrococcal nuclease according to the protocol of Jackson & Hunt (1983), and were set up as described previously (Hunt & Jackson, 1999).

**UV cross-linking assays.** High specific activity 32P-labelled RNA probes were synthesized as described previously (Hunt & Jackson, 1999) and the yield of RNA was quantified as described by Dasso & Jackson (1989). All RNA probes were adjusted to a concentration of 10 nM. UV cross-linking reactions were carried out according to Hunt & Jackson (1999), with a final concentration of 1 nM RNA probe.

**Gel-shift assays.** RNA probes were synthesized as described above for UV cross-linking, except that after quantification the probes were adjusted to a concentration of 2 nM. Gel-shift reactions were set up containing 10 mM HEPES/KOH, pH 7.5, 3 mM MgCl2, 5% glycerol, 1 mM DTT, 90 mM KCl, 1 μg *E. coli* 23S rRNA, 16 units RNA-guard (Amersham Biosciences), the protein(s) to be assayed and 2 fmol 32P-labelled probe, in a final volume of 10 μl (final concentration of RNA probe 0.2 nM). The reactions were incubated at room temperature for 15 min, separated by native TBE-PAGE at 4°C and analysed by autoradiography.

**Overexpression of recombinant proteins.** His-tagged PTB (pET28aPTB) was overexpressed in *E. coli* BL21(DE3) cells and purified on Ni2+-NTA agarose (Qagen) according to the supplier’s recommendations.

**RESULTS**

**Construction of CSD mutants of unr**

Amino acid sequence alignment of the five CSDs of unr with the archetypal CSD, CspB from *Bacillus subtilis* (Fig. 1), showed that 7 aa were conserved in the CSDs of unr. These conserved residues were found in the ribonucleoprotein (RNP)-1 and RNP-2 motifs of each CSD, and are Y/FGI (RNP-1) and FHH (RNP-2), separated by a 9 or 10 aa spacer. These were identical to the corresponding RNP motifs of CspB (Schindelin et al., 1993; Schnuchel et al., 1993) with

**Fig. 1.** Alignment of the five CSDs of unr and CspB. The first five lines show the five CSDs of unr, aa 37–90, 166–215, 329–379, 499–548 and 654–704, respectively. The lower line shows aa 15–63 of *B. subtilis* CspB. The conserved RNP-1 and RNP-2 motifs are shown in grey; other conserved amino acids are in bold type. The underlined phenylalanine residue is absolutely conserved in all CSDs and is critical for RNA-binding activity of the domain. This residue was mutated to alanine in the unr CSD mutants, as described in the text.
the exception of FFH replacing FVH in RNP-2 in all five domains.

The design of the mutants was based on work by Schröder et al. (1995) carried out on B. subtilis CspB. These authors used site-directed mutagenesis and gel-shift assays to show that an F17A mutation (within the RNP-1 motif) affects the RNA-binding activity of CspB most severely, and even a conservative F17Y mutation reduces RNA binding considerably. Two-dimensional NMR showed that the overall structure of the CSD was not altered by the mutation except in the close vicinity of the mutated amino acid (Schröder et al., 1995). Therefore, in our study, the residue corresponding to F-17 was mutated, by site-directed mutagenesis, to alanine in each of the five CSDs of unr, namely F-39, F-168, F-331, F-501, and F-656. The mutants were referred to as 1*, 2*, 3*, 4*, and 5*, indicating proteins with mutations in CSDs 1, 2, 3, 4, and 5, respectively. The mutant unr ORFs (with a C-terminal His tag) were subcloned into baculovirus transfer vectors and used to co-transfect S9 insect cells with linearized baculovirus DNA, so that recombinant baculoviruses were generated that were capable of expressing the mutant unr proteins.

**Translation activity of unr CSD mutants**

The wild-type and mutant unr proteins were used to supplement RRL programmed with a dicistronic mRNA transcribed in vitro from linearized pXLIHRV10-611 such that cyclin B2 was the upstream cistron, translated by a 5'-end-dependent scanning mechanism, and NS' was the downstream cistron, whose translation was dependent on the efficient use of the HRV-2 IRES. Wild-type unr is known to stimulate translation of the NS' cistron in this system by 1-3- to 1-8-fold, but this stimulation is greatly increased (6-8-fold) if the system is also supplemented with PTB (Hunt et al., 1999). Therefore, the unr CSD mutants could be tested for translation stimulation activity on their own, and in combination with PTB, by comparison of the yields of NS' relative to cyclin B2. Fig. 2(a) shows the results of this dicistronic mRNA assay. Fig. 2(b) represents the results of three independent experiments with quantification of the yield of radio-labelled NS' and cyclin B2 by densitometric analysis.

With unsupplemented lysate, cyclin B2 was synthesized efficiently but the yield of NS' was very low (Fig. 2, lane 1). Addition of 200 nM recombinant PTB alone (lane 2) increased the yield of NS' to approximately twofold that of the unsupplemented reaction, whereas supplementation with 20% (v/v) HeLa cytoplasmic S100 extract, estimated to provide 40 nM PTB and 10 nM unr (lane 3), raised the yield of NS' to 7-5-fold that of the control reaction. Addition of recombinant wild-type unr alone (200, 100, 50 nM; lanes 4-6) also increased the yield of NS' to 1-5- to 1-8-fold that of the unsupplemented reaction, whereas addition of wild-type unr and 200 nM PTB together (lanes 7-9) gave synergistic stimulation of translation of the NS' cistron: the yield of NS' was sixfold that obtained with unsupplemented lysate. Addition of 10 nM unr and 40 nM PTB, such as was estimated to be present in the lysate supplemented with HeLa extract, resulted in detectable but low-level stimulation of translation (data not shown), most probably because a proportion of the protein in the recombinant preparation was inactive. Higher concentrations of recombinant protein were used in these assays to see the maximal effect of the CSD mutants. It has also been shown that poly(C)-binding protein 2 can enhance the stimulation caused by unr and PTB in this assay (Hunt et al., 1999), which perhaps accounts for the difference between supplementation with HeLa extract and the unr/PTB combination.

All the CSD mutants were severely impaired in their ability to stimulate translation of the NS' cistron when added in combination with PTB: the yield of NS' was 2–3-fold that observed with unsupplemented lysate (Fig. 2b, lanes 13–15, 19–21, 25–27, 31–33 and 37–39). Indeed, CSD mutants 4* and 5* could not stimulate translation beyond approximately twofold even in the presence of 200 nM protein, indicating that they had achieved their maximum activity. When added on their own to the lysate, the CSD mutants stimulated IRES-dependent translation by less than 1-5-fold. Although this was less than the stimulation caused by wild-type unr alone, the difference between the effects of wild-type unr and the mutants was less clear than in the presence of PTB, because of the low levels of NS' product. The results shown here were reproducible with several different protein preparations. These results clearly showed that the point mutation of a single CSD of unr is enough to restrict the translation stimulation activity of the entire protein: all five CSDs are required for unr to be active in stimulating HRV-2 RNA translation.

**Effect of unr mutants on wild-type unr activity**

As each of the five mutant proteins retained four intact CSDs with the potential to bind to RNA, it was possible that they might bind to the HRV-2 5'-UTR and inhibit the activity of wild-type unr in driving IRES-dependent translation. This was tested by carrying out translation assays in which a basal level of translation stimulation was achieved by addition of 25 nM wild-type unr and 200 nM PTB, and the mutant proteins were added at concentrations of 50, 100 or 200 nM (Fig. 3). As a control, 50, 100 and 200 nM wild-type unr was added instead of mutant proteins, and a dose-dependent increase in the stimulation of translation was seen (lanes 21–24). Addition of unr mutants 1* and 2* did not have any inhibitory effect on translation at any concentration tested (lanes 6–8 and 9–11). Mutants 3*, 4* and 5* inhibited translation by approximately 25% (lanes 12–14 and 15–17), an effect seen with a twofold molar excess of mutant to wild-type unr protein, whereas mutant 5* inhibited, again by about 25%, but only when added at eightfold molar excess over wild-type unr (lanes 18–20). Thus, although some inhibition of translation from the HRV-2 IRES was seen with some of the mutants, in no case was the inhibition as strong as would be expected if they were acting as dominant negative mutants but, rather, it
would seem that some of them can act as weak competitors against wild-type unr.

**Binding of unr mutants to the full-length HRV-2 5′-UTR and to its subdomains**

Since the mutant proteins appeared to have at best a weak competitive effect rather than a strong dominant negative action, we next compared the physical interaction of the wild-type and mutant unr proteins with the full-length HRV-2 5′-UTR and with individual 5′-UTR subdomains. The HRV-2 5′-UTR has a complex RNA secondary structure that can be divided into six stem–loop subdomains (Belsham & Jackson, 2000) and we have recently shown that wild-type unr binds to subdomains 2, 5 and 5+6 of the 5′-UTR (E. C. Brown, S. L. Hunt & R. J. Jackson, unpublished results).

In gel-shift assays with 400 nM wild-type unr and with the full-length HRV 5′-UTR as probe, all of the probe was shifted into two slower-migrating complexes, suggesting the formation of complexes with either one or two molecules of unr bound to the RNA (Fig. 4, lane 2). Each of the five unr mutants was able to shift approximately half of the probe into a complex corresponding to the faster migrating of the two seen when wild-type unr was used (the complex presumed to represent one molecule of unr bound to the RNA), but no secondary shifts were observed (Fig. 4, lanes...
Cold-shock domain mutants of HRV-2 unr

3–7). Therefore, all the mutant proteins showed reduced activity in binding the HRV-2 5'-UTR, although little difference was detected between the five mutants. The relatively high concentrations of wild-type and mutant unr were used to see binding of the mutant proteins to the RNA probe. At lower concentrations, wild-type unr was able to give the shifts shown, but the mutants were unable to shift the RNA. These results were reproducible with a number of different protein preparations. The gel-shift assays were also carried out in the presence of PTB. This resulted in a primary PTB shift and a supershift with both wild-type unr and the mutants (data not shown). PTB thus did not affect the trend of RNA binding by wild-type unr and the mutants.

The five CSD mutants were then used in gel-shift assays with subdomains 2, 5 and 5+6 of the HRV-2 5'-UTR. We hypothesized that if we could detect differences between the mutants binding to certain subdomains, then we might be able to identify which CSD of unr binds to which region of the 5'-UTR. Each unr CSD mutant was titrated through concentrations of 70–350 nM on each of the subdomain RNA probes to detect any subtle differences in binding of the mutants to the individual subdomains. Table 1 summarizes the results of the unr CSD mutants binding to the HRV-2 5'-UTR and its subdomains. Binding of wild-type unr to individual subdomains was approximately 10-fold weaker than to the full-length 5'-UTR (E. C. Brown, S. L. Hunt &

![Fig. 3. Inhibition of wild-type unr activity by CSD mutant unr proteins. (a) Lanes: 1, unsupplemented RRL programmed with a cyclin B2–HRV-2 IRES–NS' dicistronic mRNA; 2, RRL supplemented with 20% (v/v) HeLa cell extract; 3 and 4, 25 nM recombinant wild-type unr and 200 nM recombinant PTB respectively. The reactions in lanes 5–20 contain both 25 nM unr and 200 nM PTB, and reactions 6–20 also contain 50, 100 or 200 nM unr CSD mutants 1*–5*. Lanes 21–24, control experiment with 25 nM unr and 200 nM PTB supplemented with 50, 100 or 200 nM wild-type unr. The positions of cyclin B2 and NS' are shown to the right of the autoradiograph. Lane M, markers (kDa). (b) Densitometric analysis of the relative yield of NS' in three independent experiments. Lane numbers correspond to those of the autoradiograph in (a).
The results were represented only semi-quantitatively, since the low signal of the shifted bands in comparison to the large excess of free probe made accurate quantification impossible. Wild-type unr gave a relatively strong primary shift and a weak secondary shift on subdomain 2 at all concentrations tested. In contrast, none of the CSD mutants gave a secondary shift but varied in the strength of their primary shift. The CSD 3* and 5* mutants gave a primary shift in which the signal was relatively strong at the higher concentrations of protein. The CSD 1*, 2* and 4* mutants gave weaker primary shifts that were barely detectable at 70 nM protein, but increased with protein concentration. The results with the subdomain 5 and 5 + 6 probes were similar to those using the subdomain 2 probe, i.e. the hierarchy of binding by the mutants was the same regardless of the RNA probe used: wild-type > CSD 3* and CSD 5* > CSD 1*, 2* and 4*.

The results of UV cross-linking assays, in which the ability of wild-type unr and the CSD mutants to be cross-linked to the full-length HRV-2 5’-UTR was tested, are shown in Fig. 5. All unr proteins were present at 100 nM. The strength of the cross-linking signal with the wild-type protein was much greater than that of any of the five mutant proteins. Of the five mutant proteins, the CSD 5* mutant gave a slightly stronger signal than the other mutants, and the CSD 4* mutant a slightly weaker signal, with the others giving a band of approximately the same intensity, intermediate between the CSD 5* and CSD 4* mutants; 250 nM unrip protein, which has no RNA-binding activity, could not be cross-linked to the probe (lane 7).

The titration of mutant proteins on subdomains 2, 5 and 5 + 6 was also carried out in UV cross-linking assays, and Fig. 6 summarizes the results of cross-linking to the HRV-2 5’-UTR subdomains. Wild-type and mutant unr proteins were added to UV cross-linking reactions at 70 to 280 nM and the intensity of the radiolabelled unr bands was measured by densitometric analysis. These measurements are shown as the relative intensity of the mutant unr bands compared with the wild-type unr band for each RNA probe, averaged over the range of unr concentrations. The mutant unr proteins were labelled less strongly than wild-type unr by each of the subdomain probes. CSDs 1*, 2*, 3* and 5*

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†Two shifted bands.

R. J. Jackson, unpublished results). The results were thus represented only semi-quantitatively, since the low signal of the shifted bands in comparison to the large excess of free probe made accurate quantification impossible. Wild-type unr gave a relatively strong primary shift and a weak secondary shift on subdomain 2 at all concentrations tested. In contrast, none of the CSD mutants gave a secondary shift but varied in the strength of their primary shift. The CSD 3* and 5* mutants gave a primary shift in which the signal was relatively strong at the higher concentrations of protein. The CSD 1*, 2* and 4* mutants gave weaker primary shifts that were barely detectable at 70 nM protein, but increased with protein concentration. The results with the subdomain 5 and 5 + 6 probes were similar to those using the subdomain 2 probe, i.e. the hierarchy of binding by the mutants was the same regardless of the RNA probe used: wild-type > CSD 3* and CSD 5* > CSD 1*, 2* and 4*.

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were labelled very similarly by all the subdomains tested. However, CSD 4* showed much weaker cross-linking to subdomain 2 and especially to subdomains 5 and 5+6, consistent with the results obtained using the full-length 5'-UTR as a probe (Fig. 5).

While the majority of the in vitro binding assays indicated that the mutant unr proteins were binding the RNA probes in a weak and non-specific manner, the fact that the CSD 4* mutant cross-linked so poorly to the subdomain 5 and 5+6 probes suggested that CSD 4 in the wild-type protein may be important in binding subdomain 5 of the HRV-2 5'-UTR. To test whether the specific binding sites of CSD 4* and the other mutants could be mapped on the 5'-UTR, modification interference analysis was carried out. Subdomains 2, 5 or 5+6 were chemically modified using dimethyl sulphate, diethyl pyrocarbonate or hydrazine to determine whether modification of specific nucleotides would prevent binding of the mutant unr proteins to the RNA probe in gel-shift assays. This technique was used successfully to identify the nucleotides of subdomains 2 and 5 that were involved in binding wild-type unr to the HRV-2 5'-UTR (E. C. Brown, S. L. Hunt & R. J. Jackson, unpublished results). When applied to the mutant unr proteins, no differences in nucleotide modification were observed between the RNA bound by any of the mutant proteins and the input RNA (data not shown). These results suggested that the CSD mutants of unr were indeed binding the RNA in a non-specific manner.

**DISCUSSION**

Five mutants in a single CSD of unr were constructed and each was predicted to be deficient in RNA-binding activity.

The results of in vitro dicistronic mRNA translation assays in RRL comparing the ability of wild-type unr and the CSD mutants to stimulate translation from the HRV-2 IRES showed that mutation of a single CSD severely reduced the ability of the protein to stimulate translation; this was particularly clear when the mutants were added in combination with PTB. It might have been expected that there would be some redundancy between the five CSDs of unr, such that only mutation of key CSDs, or multiple CSD knockouts, would be severely detrimental to the activity of the protein. However, this was not the case, indicating that each of the five CSDs of unr has an important role in its translation stimulation activity.

Despite the fact that each of the mutants still contained four active RNA-binding domains, the mutant proteins were not able to significantly prevent wild-type unr binding to the IRES and performing its role in translation. Thus, the RNA-binding activity of the mutant proteins is greatly affected by the knockout of any single CSD. There were differences in that CSD mutant proteins 1 and 2 did not inhibit translation at all, even at high concentrations, whereas mutants 3, 4 and 5 inhibited translation a little, up to 25%, when the mutant protein was eightfold in excess of the wild-type protein. This may indicate that mutation of CSDs 1 and 2 of unr results in...
a lower binding affinity for the full-length IRES than mutation of CSDs 3, 4 and 5.

These results were corroborated by gel-shift assays, in which all the mutants were impaired in their ability to bind to the full-length IRES. Wild-type unr was able to shift all the probe into two complexes, probably corresponding to one and two molecules of unr binding to the IRES, respectively, at the concentration tested, but none of the mutants was able to shift more than half of the probe, and they were able to form the primary complex only, which probably corresponds to a single molecule of unr binding to the IRES. Thus, it seems that mutation of a single CSD of unr prevents the formation of a physiological (unr)2–IRES complex, and this may account for the low translation stimulatory activity of the mutants. The results of complementation assays testing the ability of combinations of unr CSD mutants to stimulate HRV-2 IRES-dependent translation suggested that both molecules of unr in a putative (unr)2–IRES complex require all five CSDs for activity.

Since binding of all the mutants to the full-length IRES was similar, binding to individual subdomains of the IRES over a range of concentrations was investigated to judge any differences between the binding activity of the five mutant proteins and hence determine whether individual CSDs of unr could be mapped to individual subdomains of the HRV-2 5′-UTR. The gel-shift assays indicated that there were differences in the ability to bind RNA, with CSD mutants 3, 4 and 5 able to shift the subdomain probes better than mutants 1 and 2. This is in agreement with the results of the translation assay, in which CSD mutants 1 and 2 were unable to inhibit the activity of wild-type unr. These results are also validated by the work of Triqueneaux et al. (1999), in which CSD 1 of unr was shown to have higher RNA-binding activity than CSD 5. However, differences between binding to each of the subdomains were less evident, and the same hierarchy of binding activity was seen in each case. This suggests that the mutant unr proteins may be binding to these subdomains non-specifically, reflecting the way they interact with the full-length IRES. Although the UV cross-linking assays did not show exactly the same trends as the gel-shift assays, again the trend was consistent among the different IRES subdomains used as a probe. In these experiments, the CSD 4* mutant was labelled less strongly by the RNA probes than were the other mutant proteins, perhaps suggesting that there are fewer U residues where CSD 4 is binding the RNA. The lack of discrimination between the subdomain RNAs indicated that the unr mutants are binding in a non-specific manner. This was also confirmed by modification interference assays, in which no specific nucleotides were detected that affected binding of the unr mutant proteins to the HRV-2 5′-UTR.

To conclude, mutation of any of the five CSDs of unr has a severe detrimental effect on the activity of unr in stimulating translation from the HRV-2 IRES. In vitro binding assays show that the affinity of the mutants for the IRES or any of its subdomains is greatly reduced in comparison with the wild-type protein, and that the reason for the poor translation activity may be the inability of the protein to form a physiological protein–IRES complex.

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REFERENCES


