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Functional analysis of the interaction between HCV 5′UTR and putative subunits of eukaryotic translation initiation factor eIF3

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ABSTRACT

Translation initiation in Hepatitis C Virus is controlled by the presence of an internal ribosome entry site element (IRES) principally located in its 5′ untranslated region (UTR). Mutation/deletion analyses have shown that the integrity of this structure is essential for initiation of cap-independent protein synthesis. We have developed a strategy to swap the position of the two major domains (II and III) on the 5′UTR sequence. The aim was to further characterize this mechanism by preserving domain-specific interactions but possibly losing contacts that require any interdomain geometry. The expression of dicistronic mRNAs containing these different UTRs showed that the positioning of the different domains on the 5′UTR is essential for efficient IRES functioning. We then used these mutants to identify cellular factors implicated in IRES activity. Using UV crosslinking assays we found that domain III makes direct contact with two proteins (p170/p120) which can be associated with efficient IRES activity. In particular, we have mapped the binding sites of these proteins and shown that p120 binds to the apical loop segment of domain III, whilst p170 binds in the stem portion, independently of domain III position or context. Finally, we provide evidence showing that p170 and p120 represent two subunits of eukaryotic initiation factor eIF3: p170 and p116/p110.

INTRODUCTION

Initiation of protein synthesis in the HCV virus (1,2) is controlled by a 341 nucleotide (nt) long 5′UTR sequence, a very highly conserved region among the different isolates (3). This region forms an internal ribosome entry site (IRES) structure capable of binding the 40S ribosomal subunit directly to the mRNA, allowing a mechanism of cap-independent translation initiation (4). Although the precise boundaries of the HCV IRES have not yet been clearly identified (5–11), general consensus considers its 5′ boundary to begin at nt 25–46 of the RNA molecule, whilst the 3′ boundary extends ~30 nt in the core protein coding region (12).

Structural studies have shown that the IRES forms a complex secondary structure resembling closely that of the recently isolated GB virus B (GBV-B) (13,14), with the 5′UTR RNA folding upon itself to form several well defined stem–loop domains (I, II, III and IV), a double-stranded region (7) and a pseudoknot (8). As expected, the integrity of some of these features has been observed to be critical for the ability of HCV RNA to promote internal initiation and/or its control (7,8,10,15). One possibility for explaining the loss in IRES activity following mutation/deletions of these regions is that they implicate a loss of interaction between specific cellular factors and the HCV RNA.

To date, the search for such factors has been mainly focused on the major domains of HCV RNA and the principal target has been domain III, being the largest secondary structure with multiple stem–loop elements, and because its deletion was already known to cause complete loss in IRES activity (7). Significantly, this domain contains nucleotide sequences potentially important for RNA–protein interactions: a polypyrimidine-rich tract sequence (nt 191–199) followed by an AUG triplet located ~20 nt downstream (16), resembling those already observed in Picornavirus IRES elements (17,18), and a region (nt 192–203) complementary to bases 461–471 of human 18S RNA (13). Two proteins of 87 and 120 kDa (named p87 and p120 respectively) have been observed to bind specifically domain III, but their identity is still unknown (19). The binding site of p87 has been determined to be the polypyrimidine sequence in the apical loop of domain III but its binding did not seem to affect translation initiation of the HCV genome, suggesting that it may be implicated in other processes such as viral replication. On the other hand, translation competition assays performed in the probable binding region of p120 (nt 131–278) suggested that this protein might be involved in translation initiation (19).

An association between protein binding and IRES activity has also been recently described to exist also for domain II, the other main stem–loop domain of HCV 5′UTR, with the identification of a 25 kDa protein binding specifically to one of its stem–loop elements. Mutational analysis has shown that this interaction is essential for correct IRES functioning (20).

An alternative approach for the search of potentially important cellular factors for HCV IRES activity has also been that of testing cellular factors already known to be involved in the regulation of translation of other viruses. Presently, there is now experimental evidence of an interaction of p52 (La antigen) with domain IV of the HCV RNA (21), or PTB (22) with polypyrimidine sequences. However, it must be noted that the exact significance of the interaction between PTB and HCV 5′UTR is still unclear (23).

The aim of the present study was to investigate whether the positioning but not the integrity of the different domains on HCV 5′UTR was also critical for IRES activity. In order to accomplish this aim we have developed a strategy that allows to swap the

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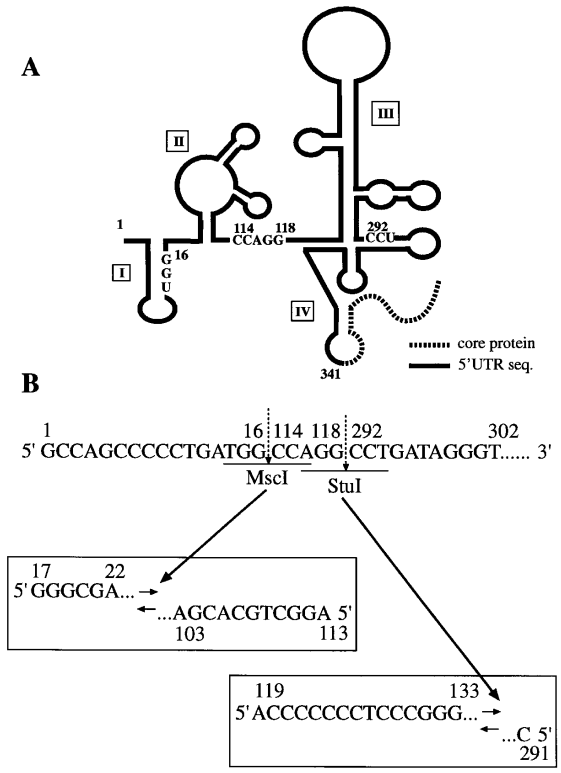

Figure 1. (A) Secondary structure model of HCV 5′UTR with the nucleotide positions that were joined together to create the *Msc*I and *Stu*I restriction enzyme sites (allowing the insertion of foreign sequences in the exact position of domain II and III respectively). Major structural domains are indicated by Roman numerals I–IV. (**B**) Linear representation of the template in which the numbering of nucleotides has been maintained as in the original wild type UTR. Both enzyme sequences have been underlined and the dashed arrows indicate the exact cutting site. The two lower boxes show the leader sequences that have to be added to the 5′ end of the primers used to amplify the sequences to be inserted in each position. These leader sequences are necessary in order to restore the deleted regions of the 5′UTR sequence.

position of domain II with domain III without altering any of the key elements that are important for IRES functioning. Our findings indicate clearly that the order of the different domains on HCV 5′UTR is essential for correct translational initiation even though some protein–RNA interactions are not affected. In addition, these constructs have proved useful in identifying two proteins that specifically bind the apical region of domain III (p170 and p120). Through UV crosslinking studies performed on mutant UTRs we show that this binding only requires the nucleotide sequence of the domain III itself independent of its position and context, and that this interaction contributes significantly to the efficiency of HCV IRES functioning. Finally, we provide evidence that these two proteins correspond to subunits p170 (24) and p116/p110 (25,26) of eukaryotic initiation factor eIF3 (24–28).

MATERIALS AND METHODS

Plasmid construction of HCV 5′UTR mutants

The strategy used to swap the positions of domain II and III on the HCV 5′UTR was to create a 'template' sequence (5′MscStu) lacking both these domains and replacing them with *Msc*I and *Stu*I restriction sites (Fig. 1A). This template was amplified from plasmid pSV UTR-1core (15) using as a 5′ oligo the sequence: 5′-GCCAGCCCCCTGATGGCCAGGCCTGATAGGGT-3′ and as a 3′ primer an oligo corresponding to the end of the core protein sequence: 5′-TATTAAGCTTTTATCACTAATCTTCCGGATC-3′. This amplified product was then cloned in the *Xba*I/*Hin*dIII sites of plasmid pUC18 and sequenced. The domain II sequence (nt 23–102) to be inserted in the *Stu*I restriction site was then amplified from the pSV UTR-1core plasmid using two sense and antisense primers (5′oligo: 5′-**ACCCCCCCTCCCGGG**CACTC-CACCAT-3′ and 3′oligo: 5′-**C**CACTCATACTAACGCCA-3′) which contained 5′ leader sequences (marked in bold) aimed at restoring the nucleotides deleted during the formation of the *Stu*I restriction site (Fig. 1B). This amplified sequence was then inserted in the *Stu*I site of 5′MscStu template, creating plasmid 5′MscStu domII, and sequenced for correct orientation. Plasmid 5′MscStu domIII/domII was then created by inserting in the *Msc*I site of 5′MscStu domII the amplified sequence of domain III (nt 134–290) from pSV UTR-1core using the following 5′ and 3′ primers: 5′-**GGGCGA**AGAGCCATAGT-3′, 5′-**AGGCTGCACGA**AGT-ACCACAAGG-3′ (respectively) (Fig. 1B). Similarly to the primers used for inserting domain II, these primers also contained 5′ leader sequences (in bold lettering) aimed at restoring the nucleotides deleted during the formation of the *Msc*I site. Finally, plasmid 5′MscStu domIII was obtained by cloning in the *Msc*I site of the template 5′MscStu the amplified domain III sequence. Each of these different 5′UTR sequences were then inserted in the *Xba*I/*Hin*dIII sites of the pSV GH bicistronic expression system (29) for transfection experiments. A basic diagram of all these mutants is shown in Figure 2A.

Mutant 5′S/L was obtained by inverting the apical stem–loop structure of 5′wt domain III. In this mutant the nucleotides from position 188 to 199 were inverted with respect to those in positions 200–211. This was accomplished by using two primers (5′ oligo: 5′-CTAGGTTCTTTCCTGGGCCTCTATGCCT-3′, 3′oligo: 5′-GAAAGAACCTAGTTGGGCGTCGTCCTGG-3′) which overlapped each other in the central region. Each primer was then used in a separate PCR reaction to amplify the 5′ and 3′ regions of HCV UTR. The two amplified products were then annealed and used in a final PCR reaction to obtain a full length HCV UTR. This product was then inserted in the pSV UTR-1core plasmid (15) and entirely sequenced to avoid unwanted mutations. The other stem–loop mutants used in this study UTR-3/207 and UTR-3 188/207 and the UTR-3 sequence have already been described (15). In order to standardize the UV cross-linking patterns and IRES activity with those of the other plasmids used in this study these two mutations were also inserted in the pSV UTR-3core plasmid (15). A basic diagram of all these domain III mutants is shown in Figure 2B.

All the UTRs described above were also cloned in the *Xba*I/*Hin*dIII restriction enzyme sites of the polylinker of pBluescript II KS+ (Pharmacia). As negative control for the UV crosslinking assays we used a *Sma*I (nt 609)–*Hin*dIII fragment obtained from the original pSV UTR-1core construct also cloned in the corresponding sites of pBluescript II KS+ (this construct was named Blscore). Finally, the amplified domain III sequence (nt 134–290) was cloned in both orientations in the *Sma*I restriction site of pBls II SK+ plasmid. The two resulting plasmids were named 5′domIIIS and 5′domIIIAS respectively for the sense and antisense orientation with respect to the T7 promoter.

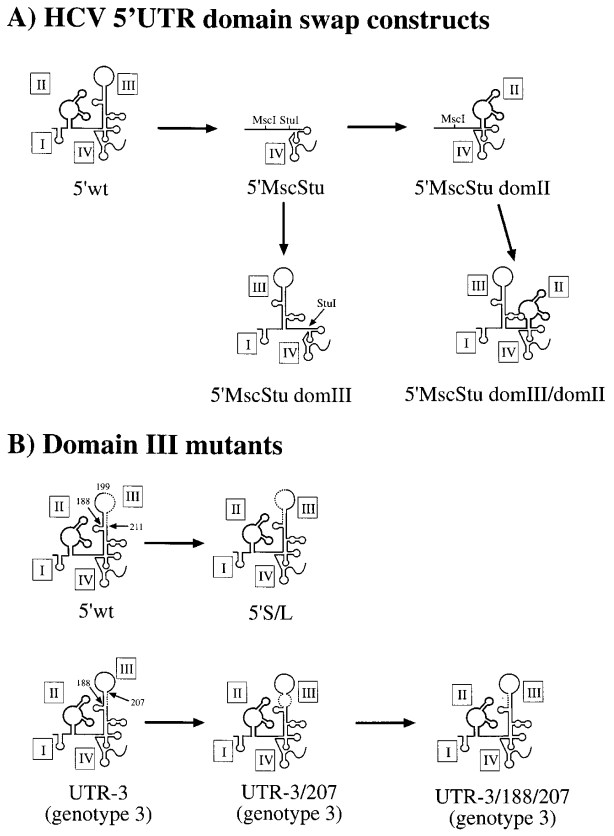

Figure 2. Schematic representation of the 5′UTR wild type and mutant structures used in this study. (**A**) UTRs used in the part of the study concerning the effect of swapping the positions of domain II and III. (**B**) Domain III apical stem–loop mutants used to study the interaction between p170/p120 and HCV 5′UTR. The arrows indicate how the different mutants were obtained from the original sequence. The dotted line indicates the sequence changes introduced in the stem–loop (upper panel) or in the stem alone (lower panel). Major structural domains are indicated by Roman numerals I–IV.

Transfection of COS-1 cells with the pSV GH mutants

The different 5′UTR sequences inserted into the pSV GH expression vectors were used to transfect COS-1 cells using DOTAP (Boehringer Mannheim) as previously described (15). The hGH levels were quantified by an ELISA assay kit (Boehringer Mannheim) and used to normalize the amount of cellular lysate in the western blot procedures. The core protein produced was recognized by MAb B12.F8 (30) kindly provided by Prof. M.U.Mondelli (University of Pavia, Italy) and evidentiated on Kodak autoradiographic film by ECL chemioluminescence analysis (Amersham Life Science) according to the manufacturer's instructions. The film was subsequetly scanned using an Imaging Densitometre GS-670 (Bio-Rad) and each band was quantified using the Molecular Analyst program for Macintosh computer. Each transfection was repeated three times and standard deviation values, where indicated, were calculated using the Statview SE program for Macintosh computers.

Transcription of the pBluescript II KS+ plasmids

Plasmids 5′wt, 5′MscStu, 5′MscStu domII, 5′MscStu domIII, 5′S/L, 5′MscStu domIII/domII, UTR-3, UTR-3/207 and UTR-3/188/207, and Blscore were linearized by digestion with *Hin*dIII. Plasmid Bls domIIIS and Bls domIIIAS were linearized with *Bam*HI. Transcription with T7 RNA polymerase (Stratagene) was performed in the presence of [^{32}P]UTP and purified on a Nick column (Pharmacia) according to manufacturer's instructions. The labelled RNAs were then precipitated and resuspended in RNAse-free water. The specific activities of labelled RNA preparations were in the range of 4×10^6 c.p.m./μg of RNA.

Ribosomal salt wash preparation and UV crosslinking assay

Ribosomal salt wash extracts were prepared from 6 g of COS-1 cells grown in suspension with 10% calf serum as described in (31) and the total protein concentration of the extract was then measured using a Bio-Rad Protein Assay (Bio-Rad).

The UV crosslinking assay was performed by adding [^{32}P]UTP-labelled RNA probes (1×10^6 c.p.m. per incubation) in a water bath for 15 min at 30°C with 20 μg of the different protein extracts in a 30 μl final volume. Final binding conditions were 20 mM HEPES pH 7.9, 72 mM KCl, 1.5 mM $MgCl_2$, 0.78 mM magnesium acetate, 0.52 mM DTT, 3.8% glycerol, 0.75 mM ATP and 1 mM GTP and 2 μg of *Escherichia coli* tRNA as an aspecific competitor. In the competition experiments cold RNA was also added as a competitor 5 min before addition of the labelled RNAs (the amount used for the three data points was 1, 2.5 and 5 μg, unless specifically stated). Samples were then transferred in the wells of an HLA plate (Nunc, InterMed) and irradiated with UV light on ice (800 000 kJ, ~5 min) using a UV Stratalinker 1800 (Stratagene). Unbound RNA was then digested with 30 μg of RNAse A (Sigma) and 6 U of RNAse T1 (Sigma) by incubating at 37°C for 30 min in a water bath. Samples were then analyzed by 8% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography. Purified eIF3 was a kind gift from Dr Tatyana Pestova (New York University Health Center, USA).

RESULTS

Effects of domain II and III positioning on HCV IRES activity

Most studies performed on HCV IRES activity have principally involved the use of mutation/deletions on the structure of the different 5′UTR domains. However, the *trans*-domain interactions, if any, have not been explored and there is to date no experimental data on the effect of moving the position of the major domains inside the 5′UTR. To better characterize the cap-independent translation of HCV virus, we designed a series of 5′UTR constructs in which the positions of the two principal domains (II and III) was swapped with respect to that found in the original 5′UTR. This strategy (Fig. 1) was intentionally designed to make sure that in the final construct none of the secondary structures critically important for IRES functioning, such as pseudoknot or helical regions, would be altered. Specific domain interactions should then be preserved while contacts that require a determined interdomain geometry will be lost. Therefore, we prepared a 5′UTR template sequence (5′MscStu) which lacked both domain II and III but contained two different insertion sites (*Msc*I and *Stu*I). Starting from this 5′MscStu template we sequentially inserted back the nucleotide sequences of domain II and III to obtain a series of plasmids in which the positions of these domains was changed with respect to the original wild type UTR (a schematic representation of these UTRs is shown in Fig. 2A). All

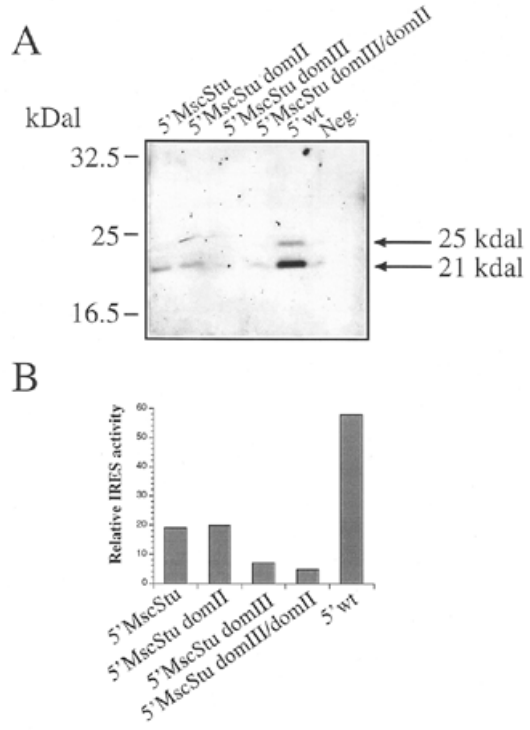

Figure 3. (**A**) Transfection in COS-1 cells of the mutant 5′UTRs in which the position of domain II and III has been swapped with respect to the wild-type 5′UTR. The two arrows indicate the position of the mature (21 kDa) and precursor (25 kDa) HCV core protein. The proteins were recognized using MAb B12.F8 and evidentiated through autoradiography using ECL staining. (**B**) Relative quantification of the amount of protein produced using a Bio-Rad Imaging Densitometre GS-670.

these mutant UTRs included as reporter gene a core protein of the HCV BK isolate to measure the IRES activity, and whose presence also allowed the formation of domain IV. The IRES activity of each 5′UTR was then assayed by measuring the amount of HCV core protein produced in transfection experiments in COS-1 cells. As already discussed in a previous work (32) the expression of this core protein produces a mature HCV core protein of 21kDa and a precursor form of 25 kDa due to the C-terminal inclusion of an HSV Tag sequence. These UTRs were all inserted in the bicistronic expression plasmid pSV GH (29) that allowed the standardization of the different transfection efficiencies by measuring the amount of growth hormone produced in the culture media.

Figure 3 shows that all the domain swap and deleted (Fig. 2A) UTRs produce very small amounts of core protein when compared to the wild type 5′UTR sequence (5′wt). The plasmids containing the template sequence (5′MscStu) and domain II (5′MscStu domII) could be observed to produce lower but significant amounts of core protein when compared to wild type. However, the amount of protein produced was observed to decrease uniformly as the length of the mutated 5′UTR increased, falling to almost undetectable levels in the 5′MscStu domIII/domII construct. Considering that this mutant is of the same length and base composition of the wild type HCV 5′UTR sequence it is this construct that should be considered as the genuine negative control for HCV IRES function. Therefore, rather than reduced IRES activity, the expression observed with 5′MscStu and 5′MscStu dom II can be ascribed to the small length of these intracistronic sequences, which allows the translation of the second cistron by a carry-through mechanism. This possibility has already been described to happen in mammalian cells (33).

Identification of cellular factors that bind specific domains of HCV 5′UTR

The presence in these mutated 5′UTRs of selected stem–loop domains made them particularly suitable for the identification of domain specific cellular factors possibly involved in the regulation of HCV IRES activity. Therefore, each RNA was produced by transcription with T7 RNA polymerase and then used in a UV crosslinking assay with ribosomal salt wash extracts from COS-1 cells. Figure 4A shows that, among the numerous factors that could be crosslinked to all the different RNAs, a protein of molecular weight of ~170 kDa (p170) could be observed to crosslink with only three UTRs: 5′wt, 5′MscStu domIII and 5′MscStu domIII/domII. Interestingly, these RNAs were the only ones which contained the domain III sequence. In fact, no binding could be detected with an RNA containing nt 609–974 of the HCV core coding region (Blscore) but also with the 5′MscStu and 5′MscStu domII RNAs (Fig. 4A).

To examine the specificity of this interaction we performed competition experiments which show that the p170 protein could be readily competed away by the addition of increasing amounts of cold 5′wt RNA, but not by equal amounts of 5′MscStu template RNA (Fig. 4B). Competition was observed to occur also when 5′MscStu domIII was added but not with 5′MscStu domII (data not shown). Most importantly, these competition analyses evidentiated that another protein of molecular weight of ~120 kDa (p120) could be seen to bind to the 5′wt RNA in an identical manner to p170. This band was not evident in shorter gel runs (i.e. Fig. 4A) due often to the presence of another labelled band migrating lower but very closely to the p120 factor.

Minimal domain binding requirements of p170 and p120

The minimal nucleotide sequence sufficient to allow the binding of these two proteins was tested by cloning the domain III nucleotide sequence (nt 134–290) in the sense and antisense orientation of the *Sma*I site of Bls KS+ plasmid (5′domIIIS and 5′domIIIAS respectively). The RNAs transcribed from these plasmids were then subjected to UV crosslinking assays with the ribosomal salt wash preparation from COS-1 cells. Figure 5A shows that 5′domIIIS (lane 3) was capable of binding p170/p120 in the same way as 5′wt RNA (lane 2). On the other hand, the antisense RNA transcribed from 5′domIIIAS (lane 4) did not show any binding activity, just like the 5′MscStu template (lane 1). The specificity of these interactions is demonstrated in Figure 5B, which shows that only cold 5′domIIIS was capable of competing p170/120 from 5′wt, and that no competion could be observed with cold 5′domIIIAS.

Taken together, these findings indicate that the binding of the two cellular proteins p170/p120 to the HCV 5′UTR requires only the presence of the domain III nucleotide sequence, and that it does not need any additional structural requirement from the other structures/domains of HCV 5′UTR.

Two identical proteins could also be observed when using a HeLa ribosomal salt wash extract (data not shown), indicating that p170/p120 were not COS-specific proteins.

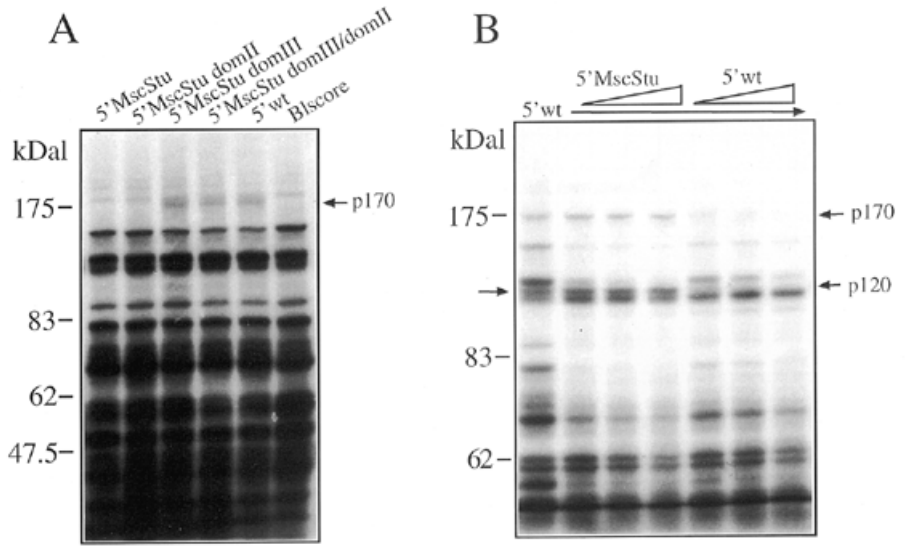

Figure 4. (**A**) Crosslinking assay using a COS-1 ribosomal salt wash extract with all the mutant UTRs carrying different combinations of domain II or III in swapped positions. The arrow indicates the p170 protein observed to bind only to 5′MscStu domIII (lane 3), 5′MscStu domIII/domII (lane 4) and 5′wt (lane 5). No binding could be observed with the 5′MscStu RNA (lane 1), 5′MscStu dom II (lane 2) or Blscore, the negative control RNA (lane 6). (**B**) Binding and competition experiment using cold 5′wt and 5′MscStu RNA. This figure shows the UV crosslinking pattern of ribosomal salt wash extract with 5′wt (lane 1) in the presence of cold 5′MscStu RNA (lanes 2–4) and cold 5′wt RNA (lanes 5–7). The positions of both p170 and p120 are indicated by arrows.

Delineating the nucleotide and structural requirements for the binding of p170/p120 to the HCV domain III sequence

In order to better define the binding sites of p170/p120 on the HCV 5′UTR we used several mutants in which the apical stem–loop structure of domain III was artificially modified (a schematic diagram is shown in Fig. 2B). In a previous study (15) we have already reported that mutants in the apical stem–loop sequence of genotype 3 5′UTR displayed different translational efficiencies (as measured by CAT assay). In particular, a mutation in the stem structure (UTR-3/207) severely inhibited IRES activity, which could be only partially recovered by restoring the stem structure through a compensatory mutation (UTR-3/188/207). These mutants, together with the wild type genotype 3 UTR sequence (UTR-3), were then inserted in a pSV plasmid containing the HCV core protein as reporter gene and assayed both in transfection and in UV-crosslinking assays. In addition to the genotype 3 mutants we also prepared a previously undescribed mutant (5′S/L) from genotype 1 UTR, where the 5′ stem–loop structure starting from nt 188 to 199 was exchanged with the corresponding 3′ sequence (nt 200–211).

Figure 6A shows that the binding pattern observed for mutant UTR-3/188/207 was the one most similar to that detected for the wild type 5′UTR (5′wt), being capable of binding both the p170 and the p120 protein. On the other hand, the 5′S/L mutant was observed to bind only p170, but never p120 and mutant UTR-3/207 could not bind any of the two proteins.

We then investigated the ability of each of these mutants to compete the p170/p120 proteins when bound to their respective UTR wild type sequences.

Figure 7A shows that cold UTR-3/188/207 RNA was capable of competing (although with reduced efficiency compared to cold UTR-3 RNA) the p170/p120 proteins. On the other hand, no competition could be observed when cold UTR-3/207 RNA was used. The ability of each mutant to direct IRES translation of the HCV core protein is shown in Figure 7B and, as already previously observed in the case of the CAT assays (15) the UTR-3/207 mutant showed a very reduced efficiency cap-independent translation, an

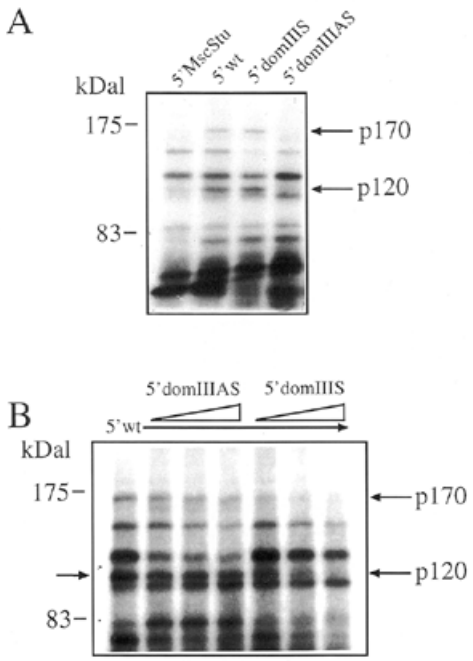

Figure 5. Reactivity of an isolated (nt 134–290) domain III region with a COS-1 ribosomal salt wash extract. (**A**) UV crosslinking pattern of a sense domain III RNA (5′domIIIS) and an antisense domain III RNA (5′domIIIAS) compared with the pattern obtained from the wild type UTR sequence (5′wt) and template sequence without domain II and III (5′MscStu). The arrows indicate the positions of p170/p120. (**B**) UV crosslinking competition analysis on HCV wild type 5′UTR (5′wt) (lane 1) using cold 5′domIIIAS RNA (lanes 2–4) and cold 5′domIIIS RNA (lanes 5–7). The arrows indicate the positions of p170/p120.

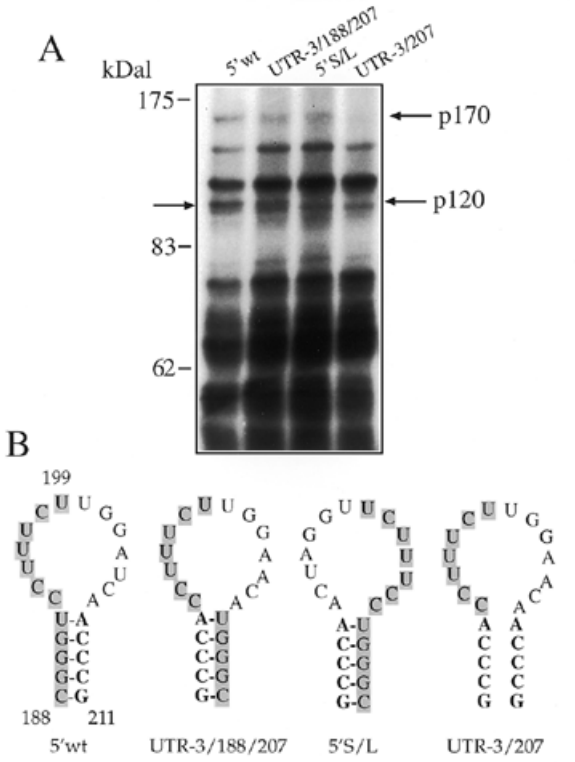

Figure 6. (**A**) UV crosslinking assay with the COS-1 ribosomal salt wash extract and the different domain III apical stem–loop mutants: 5′wt (lane 1), UTR-3/188/207 (lane 2), 5′S/L (lane 3) and UTR-3/207 (lane 4). The arrows indicate the position of p170/p120. (**B**) Nucleotide sequence (nt 188–211) of the domain III apical stem–loop region of each mutant; nt 188–199 are shadowed in order to better follow the changes in their position in the different mutants.

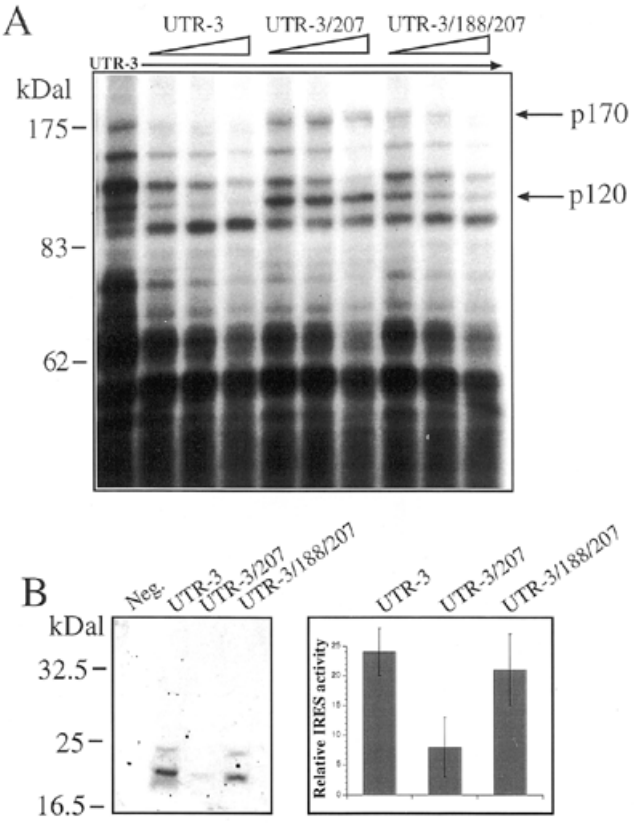

Figure 7. (**A**) shows a competition UV crosslinking assay using genotype 3 HCV 5′UTR (UTR-3, lane 1) in the presence of cold UTR-3 RNA (lanes 2–4), cold UTR-3/207 RNA (lanes 5–7) and cold UTR-3/188/207 (lanes 8–10) with a COS-1 ribosomal salt wash extract. The position of p170/p120 is marked by an arrow. (**B**) Relative IRES activity of these three UTRs when inserted in the pSV GH plasmid and transfected in COS-1 cells. The core proteins expressed were recognized using MAb B12.F8 and evidentiated through autoradiography using ECL staining. The right panel shows the relative quantification of the amount of protein produced using a Bio-Rad Imaging Densitometre GS-670.

ability that could only be partially recovered by the stem-restoring mutation in plasmid UTR-3/188/207.

Finally, it must also be noted that no differences in UV cross-linking patterns could be detected between UTR-3, UTR-3/207 and UTR-3/188/207 when we used an S100 extract from COS-1 cells (data not shown), indicating clearly that the only differences in binding patterns between these UTRs are represented by the p170/p120 proteins.

Figure 8A shows the results of an analogous UV crosslinking competition experiment using cold 5′S/L RNA showing that this RNA is unable to compete either p170 or p120 from 5′wt RNA, although it is quite capable of binding to p170 though with lower efficiency (as shown in Figs 6A and 8B). Interestingly, this inability to bind p120 or compete p170/p120 from 5′wt is not associated with a significant loss of IRES activity with respect to the 5′ wild type sequence (Fig. 8C). However, this observation reflects the fact (discussed in later experiments) that p170/p120 are part of the same protein complex and therefore the ability to bind p170 alone is not sufficient to displace the p170/p120 complex from the wild type UTR. In fact, Figure 8B shows that cold 5′S/L RNA is quite capable to compete p170 from labelled 5′S/L RNA and the specificity of this competition is indicated by the inability of 5′MscStu RNA to compete p170 also from 5′S/L (Fig. 8B).

p170/p120 correspond to subunits p170 and p116/p110 of eukaryotic translation factor eIF3

The fact that these two proteins could only be clearly observed in a UV crosslink assay by using ribosomal salt wash preparation suggested that they could belong to the family of canonical eukaryotic translation initiation factors. A comparison with the known molecular weights for the subunits of all these factors (28) showed that the most likely candidates were subunits p170 and p116/p110 of factor eIF3. In keeping with this possibility, when our ribosomal salt wash preparation was fractionated with ammonium sulphate the p170 protein could be localized only in the 0–40% fraction, the same fraction where the eIF3 factor is known to precipitate (Fig. 9A). Finally, a UV crosslinking analysis of a purified fraction of eIF3 (34) showed that subunits p170 and p116/p110 migrated in this assay in the same way as p170/p120 in the ribosomal salt wash preparation (Fig. 9B).

Interestingly, HCV 5′wt RNA UV cross-linked with purified eIF3 could be seen to crosslink also with two other subunits of eIF3, p66 (Fig. 9B) and p47 (data not shown). The other labelled

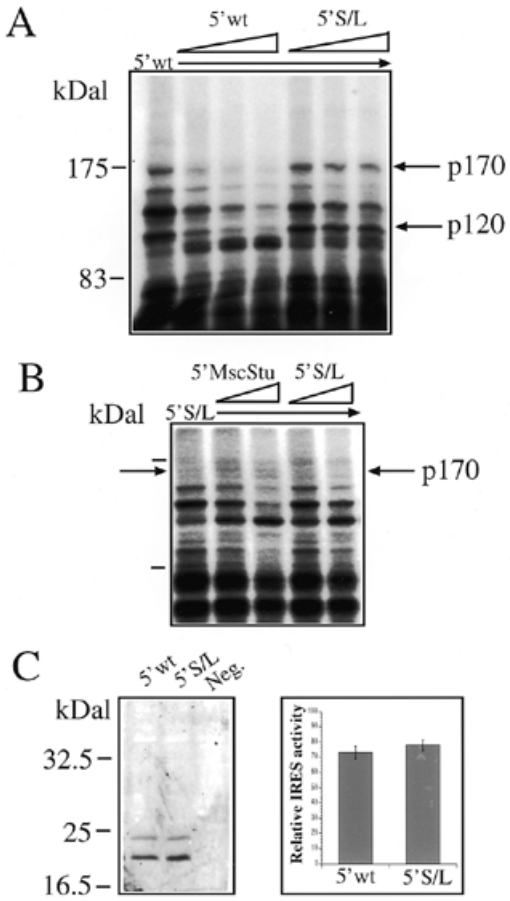

Figure 8. (**A**) Competition UV crosslinking assay using wild type HCV 5′UTR sequence (5′wt, lane 1) in the presence of cold 5′wt RNA (lanes 2–4), and cold 5′S/L RNA (lanes 5–7) with a COS-1 ribosomal salt wash extract. The position of p170/p120 is marked by an arrow. (**B**) Competition UV crosslinking assay using labelled 5′S/L RNA (5′S/L, lane 1) in the presence of cold 5′MscStu RNA (lanes 2–3, respectively 2.5 and 5 µg of RNA), and cold 5′S/L RNA (lanes 5–6, respectively 2.5 and 5 µg of RNA) with a COS-1 ribosomal salt wash extract. The position of p170 is marked by an arrow. The two horizontal lines on the right represent the molecular weight markers (175 and 83 kDa respectively in descending order). (**C**) Relative IRES activity of these two UTRs when inserted in the pSV GH plasmids and transfected in COS-1 cells. The core proteins expressed were recognized using MAb B12.F8 and evidentiated through autoradiography using ECL staining. The right panel shows the relative quantification of the amount of protein produced using a Bio-Rad Imaging Densitometre GS-670.

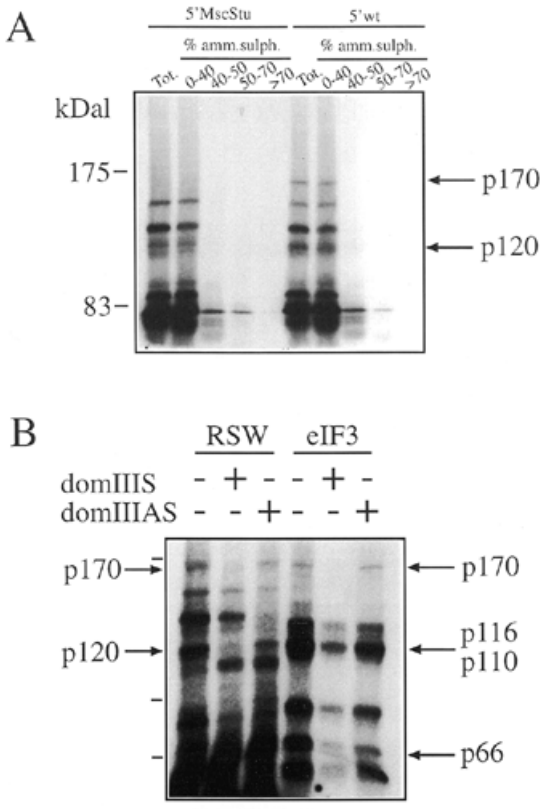

Figure 9. (**A**) Crosslinking assay of 5′MscStu and 5′wt RNA with a COS-1 ribosomal salt wash extract (Tot.) and the different fractions using ammonium sulphate (0–40, 40–50, 50–70 and >70%). The arrow indicates the position of p170/p120. (**B**) Competition UV crosslinking assay using wild type HCV RNA (5′wt) ribosomal salt wash (RSW) or a purified fraction of eIF3 (eIF3) in the presence of cold domIIIS RNA (5 µg) and cold domIIIAS RNA (5 µg) as competitors. The arrows on the left show the positions of p170 and p120 in the ribosomal salt wash extract, whilst the arrows on the right show the positions of p170, p116/p110 and p66 in the purified eIF3 fraction. The horizontal lines on the right represent the molecular weight markers (175, 83 and 62 kDa respectively in descending order).

bands in Figure 9 are probably degradation products of p170, which is known to be very sensitive to proteolysis and indeed is underrepresented if compared with p116/p110.

DISCUSSION

Initiation of translation of Hepatitis C Virus RNA is a cap-independent process which involves the presence of an IRES element to mediate internal entry of ribosomes. The boundaries of this IRES element but also the structural requirements for its efficient functioning have been the subjects of several studies (5–11,15,20). However, very little is known on the importance of the localization of the different stem–loop domains on the structure of the 5′UTR, with recent studies focusing only on the translation window positioning of the AUG codon with respect to the rest of the IRES (35,36).

Effect of swapping the position of domain II and III on IRES activity

We have performed a study on the effect of swapping the positions of the two principal domains (II and III) on IRES activity. The results show clearly that correct positioning of these two domains on the 5′UTR structure is essential for translation to occur efficiently and showed that some interactions are not abolished by the relative position of the domains and hence the RNA–protein interaction requirements are fullfilled by the limited domain structure. UV crosslinking studies were then performed to attempt a characterization of cellular factors that might contribute to explain this loss of activity. In addition, this study showed that some of the mutants and the wild type sequence were able to bind two proteins of molecular weight of ~70 and 120 kDa (p170/p120). It must also be pointed out that two identical proteins were also observed in crosslinking assays using

a ribosomal salt wash extract from HeLa cells (data not shown), ruling out the possibility that either p170 or p120 could be considered COS-1 specific proteins. Interestingly, the only binding requirements for these proteins was the presence of the domain III structure. A possible reservation of such an experimental approach is whether the secondary structure of the swapped domains is conserved with respect to the original 5′UTR structure. However, the observation that these two proteins could bind the active wild type sequence but also the inactive 5′MscStu domIII and 5′MscStu domIII/domII mutant makes it likely that the structure of this domain in the mutants is conserved. Furthermore, computer modelling of the modified IRES showed significantly the same main domains. This was expected, since base composition and sequence were conserved. Taken together, these results suggest that correct positioning of the different proteins on the HCV IRES structure plays a role in the correct formation of the translation complex.

Structural specificities for p170/p120 binding to HCV 5′UTR

A first interesting finding of investigating the binding specifitity of p170/p120 has been that these two proteins require only the domain III nucleotide sequence for binding to occur, as observed by cloning only the domain III sequence in pBluescript. This fact suggests that domain III is able to fold upon itself in an autonomous process, one that does not require the presence of other particular HCV domain/nucleotide sequences.

We then investigated the ability of domain III to bind or compete for these proteins using artificial mutants of its apical stem–loop structure. The results of this analysis are summarized in Table 1

By comparing the p120 binding activities of wild type 5′wt and mutants UTR-3/188/207 and 5′S/L it is clear that the binding site of this subunit is localized exclusively in the apical loop structure of domain III (Fig. 6). In addition, although our mutants do not allow us to determine unambigously the specific binding sequence, the observed loss of binding following the inversion in mutant 5′S/L clearly indicates that it is positioned either at nt 200–206 or 193–200. However, it is important to note that the nucleotide sequence is not the only determinant for p120 binding. In fact, although the UTR-3/207 mutant was not altered in the apical loop portion with respect to 5′wt it was clearly unable to bind the p120 protein, showing that maintenance of correct secondary structure is just as important as the primary sequence.

On the other hand, the binding of p170 does not seem to involve the loop structure, but rather the stem portion extending below the apical loop. The exact amount of stem necessary for this binding is currently under study but it probably extends below nt 195 since changes in the sequence of the stem structure (mutant UTR-3/188/207 and 5′S/L) do not seem to affect the interaction. However, considering the fact that the ability of cold UTR-3/188/207 RNA to compete p170/p120 from 5′wt RNA is reduced with respect to the wild type HCV RNA, it is at least likely that the nucleotide sequence of the stem at position 195 may be partially involved in the binding of p170.

These observations are in keeping with recent crystallography findings concerning the characteristics of RNA–protein interaction, which indicate that for specific recognition to occur several constraints must be observed beside nucleotide sequence, such as orientation and spatial separation of the bases (37).

Table 1. Binding of p170/p120 to the different UTRs and ability of each UTR to compete p170/p120 from their respective wild type sequences

5′UTRs	Reactivity			
	Binding		Competition	
	p170	p120	p170	p120
5′wt/UTR-3	+	+	+	+
UTR-3/188/207	+	+	+/–	+/-
5′S/L	+	–	–	–
UTR-3/207	–	–	–	–

p170/p120 correspond to the p170 and p116/p110 subunits of eukaryotic translation factor eIF3

Eukaryotic translation factor eIF3 is the largest protein synthesis initiation factor consisting of at least 10 polypeptide chains (p170, p116, p110, p66, p48, p47, p44, p40, p36 and p35) that give a final molecular weight of ~650 kDa (24–28). The fact that a functional eIF3 preparation lacking the p170 subunit has been recently been described (38) raises the possibility that this protein is not a true subunit of eIF3 but a factor associated with a particular eIF3 functional form. Originally, the function of eIF3 was thought to be confined to the maintenance of a 60S-free pool of 40S subunits, making them available for participation in the initiation process and stabilizes the binding of the ternary complex. However, it also binds other initiation factors being implicated in the correct positioning of these factors on the mRNA (39).

We have provided evidence that the p170/p120 proteins correspond to two subunits of eukaryotic translation factor eIF3: p170 and p116/p110.

This is the first time that p170 has been described to possess an independent ability to bind RNA, since only two proteins of eIF3 have been identified to possess an RNA binding ability, p116 and p66 (26,27). It will be interesting to further investigate this RNA binding ability since it may represent a unique difference between IRES elements and cap dependent mRNAs.

It should be noted that the resolution obtained in our autoradiographic gels does not allow to state clearly whether our p120 observed protein corresponds to the p116 or p110 subunit of eIF3. Although work is currently in progress to clarify this issue it is probable that our p120 protein corresponds to p116, considering that this factor contains a well known RNA recognition motif (RRF) (26), something that has not been identified in p110 (25).

In addition, it is possible that our p120 protein is the same reported in a previous work (19) and which was shown to bind in the domain III region, although we failed to detect the p87 protein described by these authors. It may be possible that p87 is a degradation product of p170 which is well known to be very sensitive to proteolysis.

Finally, using a purified fraction of eIF3 we have observed binding also for the p66 and p47/p44 subunits. However, in our UV crosslinking experiments using the ribosomal salt wash extract we were never able to obtain any direct evidence for the binding of the p66/p47/p44 subunits (Figs 4–8), in fact no proteins of these approximate molecular weights could be observed to behave similarly to p170/p120 in any of our competition experiments. Although we cannot rule out the possibility that also the p66 and p47/p44 subunits bind specifically to HCV 5′UTR we must also consider the possibility that binding of these subunits to the wild type HCV 5′UTR could represent an artefact caused by the absence of aspecific protein competitors. These competitors could well be

present in abundance in the ribosomal salt wash preparation but not in the purified protein fraction. Further work will be aimed at clarifying this issue.

While this work was in progress the interaction of complete eIF3 with domain III of the Hepatitis C Virus 5′UTR was reported (40). Toe-print analysis showed that the 40S ribosomal subunit alone could bind the HCV IRES to form an initiation complex at the authentic initiation codon. The binding of eIF3, although not essential for this process, was determined to be needed for assembly of an active 80S complex on the HCV IRES. Considering that eIF3 is stoichiometrically associated with free 40S ribosomal subunits in the cytoplasm the binding of these two components to the HCV IRES may enhance the entire entry process of the initiation codon in the ribosomal P site (40). This consideration is in keeping with our observation that mutant UTR-3/207, though unable to bind both p170 and p116/p110, shows a severely reduced but does not totally abrogate IRES activity. In addition, our study extends this finding by identifying the eIF3 subunits principally involved, providing a first functional analysis of this binding indicating its importance for IRES activity.

Relationship between binding of p170/p120 and IRES activity

We have already described (15) that disruption of the stem structure in mutant UTR-3/207 leads to severe loss in IRES activity. In the present work we show the inability of both p170 and p120 to bind this RNA giving a probable functional reason. Interestingly, the 5′S/L mutant binds specifically but weakly p170 and p120 binding is not detected by UV-crosslinking. However, there is no significant effect on IRES activity, suggesting that both binding sites have to be lost before severe inhibition of IRES activity can occur. This result can be explained with the fact that p170 and p116 are not only part of the same eukaryotic initiation factor but are also known to interact specifically with each other (26). Therefore, our results suggest that successful binding of at least one of the two subunits would be sufficient for mantaining both factors in a position favourable for the IRES mechanism to function effectively. In fact, considering the already known protein–protein interactions between these subunits it is likely that binding of p170 probably implies the presence in position of p120, though perhaps not at a distance sufficient to make direct contact with the RNA (and therefore not being detected by a UV crosslinking assay). This interpretation would also be consistent with the inability of this mutant to compete p170 from the wild type RNA. In fact, both results agree to indicate that in the wild type RNA the p170 protein is presumably kept in place also by direct binding to p116/p110 and therefore cannot be displaced by 5′S/L RNA. Thus, only UTRs similar to UTR-3/188/207 which are capable of binding with both factors would be expected to displace p170/p120 from the RNA (as shown in Fig. 7).

Currently, we are defining the minimal interaction requirement of these factors with domain III. The understanding of the specific HCV IRES initiation strategy may show peculiarities that may help to devise IRES specific inhibitors.

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