In vivo translational efficiency of different hepatitis C virus 5′-UTRs

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Abstract Initiation of translation in hepatitis C virus (HCV) is dependent on the presence of an internal ribosome entry site (IRES) contained in its 341-nt-long 5′-untranslated region (UTR). This region is very conserved among different isolates and has been used to classify HCV isolates in six different genotypes. These genotypes differ in nucleotide sequence that generally preserve the IRES structure. However, the small differences seen may be biologically and clinically significant as the HCV strains seem to differ from each other in several important ways, such as the behaviour of the viral infection and the response to interferon therapy. Therefore, differences in translational initiation efficiency amongst the various genotypes could provide an explanation for these phenomena. Using a bicistronic expression system we have compared the in vivo translational ability of the three most common European genotypes of HCV (1, 2, and 3). The results show that genotype 3 is less able than 1 and 2 to promote translation initiation. In addition, by site-directed mutagenesis of the sequence of the IRES domain III apical stem loop structure, we have shown that the conservation of the primary nucleotide sequence and not only the structure, is important for the conservation of IRES function.

Key words: Hepatitis C virus: genotype; 5′-Untranslated region; Translation initiation efficiency

1. Introduction

The sequence of the 5′-UTR region of hepatitis C virus [1,2] is highly conserved among different isolates [3] and has been used to classify the existing viral strains in six distinct virus genotypes [4]. Structural studies have shown that it forms a complex secondary structure resembling closely that of the recently isolated GB virus B (GBV-B) [5,6]. Initial studies performed on HCV 5′-UTRs confirmed that it contains an IRES, a structure capable of driving cap-independent translational initiation [7,8]. Several key elements important for its function have been identified as belonging to conserved stem-loop structures [6], a double-stranded region [9] and also a pseudoknot structure [10]. One of the stem-loop elements known as domain III, represents the largest secondary structure with multiple stem loops contained in the HCV IRES [6]. This domain contains several potentially important features for the regulation of translation: a pyrimidine-rich tract (nt 191–199) followed by an AUG triplet located about 20 nucleotides downstream [11], similar to those already observed in Picornavirus IRES elements [12,13], and a region (nt 192–203) complementary to bases 461–471 of human 18S RNA [5]. Deletion of this domain causes the complete loss of IRES functioning [9]. In addition, several deletion analyses were performed to map the minimal IRES sequences [7–10,14–16]. The 5′-end of HCV UTR is generally considered to be positioned at nucleotide 45 but the 3′-boundary has been the subject of some controversy. In fact, unlike what was known for picornavirus IRESs, it was found that HCV IRES also seemed to extend several nucleotides ($\approx$30) in the coding region of the HCV core protein [17]. However, most of the earlier studies performed on HCV IRESs had used monocistronic and bicistronic expression systems which did not contain any HCV core protein sequence and yet displayed a good level of internal translation initiation. The reason for this apparent discrepancy has been addressed in a recent work [6] which provides an explanation for the involvement of the core coding region, and showed that the stability of a conserved stem-loop which is present there (domain IV) is inversely correlated with efficiency of translation initiation. It has been suggested that the usefulness for HCV of possessing such an inhibitory structure would be to contribute in the in vivo regulation of viral translation mediated through a binding with regulatory proteins, either of viral or cellular origin [6]. The existence of such complex regulatory pathways means that any study comparing the translational ability of different 5′-UTRs of HCV should be carried out in in vivo assays. In fact, it has been shown that the HCV IRES function is more efficient in vivo than in vitro [18].

In the present study we have measured the efficiency of translation of different 5′-UTR representative sequences belonging to genotypes 1, 2, and 3. Full-length and truncated 5′-UTR sequences were inserted in a bicistronic expression vector and transfected in COS-1 cells. The results of this analysis suggest that the 5′-UTR of genotype 3 is the IRES least able to promote translation initiation, whilst 1 and 2 had comparable efficiencies. In addition we have also studied three artificial mutants of the domain III of genotype 3. The results of this second study demonstrate that not only the maintenance of domain III secondary structure plays an essential role in IRES functioning but also the primary sequence of the stem is important for translation initiation.

2. Materials and methods

2.1. HCV 5′-UTR sequences employed in this study

HCV 5′-UTR sequences spanning from nucleotide 46 to 341 of the different genotypes were amplified from patients sera [19] and classified accordingly [20]. The amplified products were then cloned in the *Kpn*I/*Hin*dIII pBlueScript SK+ plasmids (Stratagene, La Jolla, CA). The sequence belonging to genotype 1 (UTR-1) had a 100% identity with the already published HCV-1b sequence [21], the sequence for

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Abbreviations: HCV, hepatitis C virus; IRES, internal ribosome entry site; UTR, untranslated region; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction

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genotype 2 (UTR-2) with HCV-2a [22] and the one for genotype 3 with Eb 1 [20].

5′-UTR mutated sequences based on the 5′-UTR of genotype 3 as a template were generated by PCR using pairs of complementary oligonucleotides containing the desired mutation. Three artificial mutants were generated: in the first one (UTR-3/195), the polypyrimidine stretch at the top of domain III was mutated to a stretch of polypurines. In the second one (UTR-3/207), the stem-loop structure underneath domain III was disrupted whilst in the third (UTR-3/188/207), compensatory mutations were introduced to restore base pairing. This double-mutation was obtained using UTR-3/207 as a template. To generate these artificial mutants we used the following pairs of primers: UTR-3/195 (K297S/LOOP-AS; LOOP-S/HAS1), for UTR-3/207 (K297S/STDIS-AS; STDIS-S/HAS1), for UTR-3/188/207 (K297S/STASC-AS; STASC-S/HAS1). The sequence of the above mentioned primers are as follows: 5′-TTGGTACCTGTGAGGAACTACTGTCT-3′ (K297S); 5′-TTGATCCCCCCCCGGACCCG-3′ (LOOP-AS); 5′-CGGGTCCGGGGGGGGATCAA-3′ (LOOP-S); 5′-TTAAGCTTGGTGCACGGTCTACGAGACCT-3′ (HAS1); 5′-GTATTGAGGCCCATGTTCCA-3′ (STDIS-AS); 5′-TGGAACATGGGCCTAATAC-3′ (STDIS-S); 5′-AGAAAGGTGGGCGTCACCC-3′ (STASC-AS); and 5′-GGGTGACGCCCACCTTTCT-3′ (STASC-S).

2.2. pSV GH/CAT expression vectors construction

The different 5′-UTRs were inserted in the pSV GH-CAT bicistronic expression system [23]. The polylinker of this plasmid was modified to insert a *Kpn*I and *Xba*I restriction site and to bring the *Hin*dIII restriction enzyme site adjacent to the ATG nucleotides of the *CAT* reporter gene. This was performed by amplifying the polylinker and part of the *CAT* coding sequence using primers: 5′-CTTAAGGATATCGGTACCTCTAGAAAGCTTATGGAGAAAAAAATCACTGG-3′ and 5′-CGGAATTCCGGATGAGCATTC-3′ and inserting back the amplified product using the *Eco*RV and *Bsp*EI unique restriction sites. Therefore, the six different 5′-UTRs spanning from nucleotide 45 to 341 were inserted in this position: UTR-1 (pSV UTR-1), UTR-2 (pSV UTR-2), UTR-3 (pSV UTR-3), UTR-3/195 (pSV UTR-3/195), UTR-3/207 (pSV UTR-3/207), and UTR-3/188/207 (pSV UTR-3/188/207). A schematic representation of these constructs is shown in Fig. 1A. In a second set of experiments the HCV-BK core protein [24] was amplified by PCR together with genotypes 1, 2, and 3 5′-UTRs in such a way as not to leave any nucleotide between the end of the 5′-UTR and the first codon of the core protein. This approach also gave us the possibility to insert the first 45 nucleotides of each UTR. In brief, domain I was inserted by amplifying from the pSV GH/CAT plasmids described using a 5′-primer (P1) containing the sequence 5′-GCCGCCCCCTGATGGGGGCGACACTCCACCATGAATCACTCCCCTGTGAGGAACT-3′ and a 3′-primer (P2) containing the first four residues of the BK core protein: 5′-ATTCGTGCTCATGGTGCACGGTCTACGA-3′. A second PCR was then performed using as a 5′-primer (P3) its complementary sequence: 5′-TCGTAGACCGTGCACCATGAGCACGAAT-3′ and a 3′-primer (P4) which contained the end of the HSV Tag hinge region in frame with the last three codons of the HCV BK core protein: 5′-TCCGCCAGATGCAGCGGAAGCT-3′. A third PCR was performed to amplify a GSGGG hinge region together with a 13 amino acid HSV-Tag using as 5′-primer (P5): 5′-GGATCTGGCGGAGGTCAGCCTGAACTCGCTCCAG-3′ and as a 3′-primer (P6) containing an *Hin*dIII restriction enzyme site: 5′-TATTAAGCTTTTATCACTAATCTTCCGGATC-3′. The amplified products of P3/P4 and P5/P6 were denatured together and amplified with P3 and P6. The product was an HCV core protein containing a 13 amino acid HSV Tag terminal sequence (QPELAPEDPED) from the herpes simplex virus glycoprotein D joined to the protein with a GSGGG hinge. This product was then denatured together with the P1/P2 amplified product. A last round of amplification was performed using a 5′-primer which contained an *Xba*I cloning site and a T7 promoter (which was inserted as a spacer region from the end of the *hGH* gene): 5′-AAATCTCTAGATAATACGACTCACTAGTGGGTACCGCCAGCCCCCTG-3′ and as a 3′-primer P6. The amplified product was then inserted in the *Xba*I and *Hin*dIII cloning sites in the pSV GH-CAT plasmid and sequenced. Therefore, a second set of plasmids (pSV UTR-1core, pSV UTR-2core, pSV UTR-3core) was then prepared which contained the three different 5′-UTRs fused with the BK-core protein (now becoming the second cistron). A schematic representation of these constructs is shown in Fig. 1B. The HSV-Tag region gave us the possibility to recognize the core protein with a commercially available mouse monoclonal antibody (HSV Tag Antibody, Novagen).

2.3. Transfection of COS-1 cells with the pSV GH/CAT expression systems

The eight 5′-UTRs inserted into the pSV GH/CAT expression vector were used to transfect COS-1 cells using DOTAP (Boehringer Mannheim) according to manufacturer's protocol. Cells (400 000) were seeded in 60 mm dishes and after 24 h they were transfected with 4 μg of each plasmid in a serum-free medium. After 4 h the serum-free medium was replaced with complete medium. Cells were then harvested after 48 h and the human growth hormone (hGH) levels in the cell medium were quantified by a radio-immuno assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). The hGH levels were then used to normalize the amount of cellular lysate used for CAT assays and Western blot procedures. The CAT levels obtained for each construct were performed according to standard protocols [25] and quantified using a phosphoimager (Packard, InstantImager). The relative amounts of core protein produced were recognized by mAb B12.F8 [26] and quantitized using a Bio-Rad GS-670 Densitometer. The Bio-Rad Molecular Analyst computer programme was used to integrate the intensity of the different bands. Cellular extracts used for CAT activity and HCV core protein production were normalized to the level of GH activity. The values in the graphs of Figs. 2 and 3 represent averages from at least three independent experiments with a variation among the different experiments of less than 20%. IRES efficiency of the various genotypes is expressed as a percentage of the total IRES activities in the different experiments.

3. Results

The in vivo translational efficiency of HCV 5′-UTRs belonging to different genotypes of the virus was investigated by transfection assays in COS-1 cells. All UTRs were inserted in the second cistron of the bicistronic expression vector pSV GH-CAT [23] which allowed an accurate standardization of the transfection assays by measuring the amount of hGH released in the culture media. A first set of experiments was performed with the *CAT* enzyme reporter gene under the control of the different UTRs. The results of these transfections show that, whilst genotype 1 and 2 have similar translation efficiencies, genotype 3 is about 40% less effective (Fig. 2A). These experiments suggested a significant difference, but were not definitive as these pSV constructs (Fig. 1A) did not contain the entire HCV 5′-UTR. In fact, they lacked both the first 45 nucleotides (domain I) and the initial core coding region (which contributes to domain IV). Therefore, a new set of constructs (Fig. 1B) was designed to add domain IV by including the HCV-BK core protein [24]. These constructs also included the first 45 nucleotides of the 5′-UTR which, although not considered to be essential for IRES functioning [14], could be involved in the in vivo regulation of translation. The amount of core protein produced by each UTR was measured by Western blot procedure using a monoclonal antibody B12.F8 [26] specific for aa 34–45 of the core protein. The results (Fig. 2B) are even more evident than that observed in Fig. 2A, with genotype 3 being 50% less able to promote translation initiation than genotypes 1 and 2. In addition, as the levels of growth hormone produced by equal amounts of plasmids in the different transfections never varied by more than 20%, a clear indication was provided that the stability of the different transcripts in the transfected cells were comparable.

When the mAb B12.F8 was used to detect the expression of

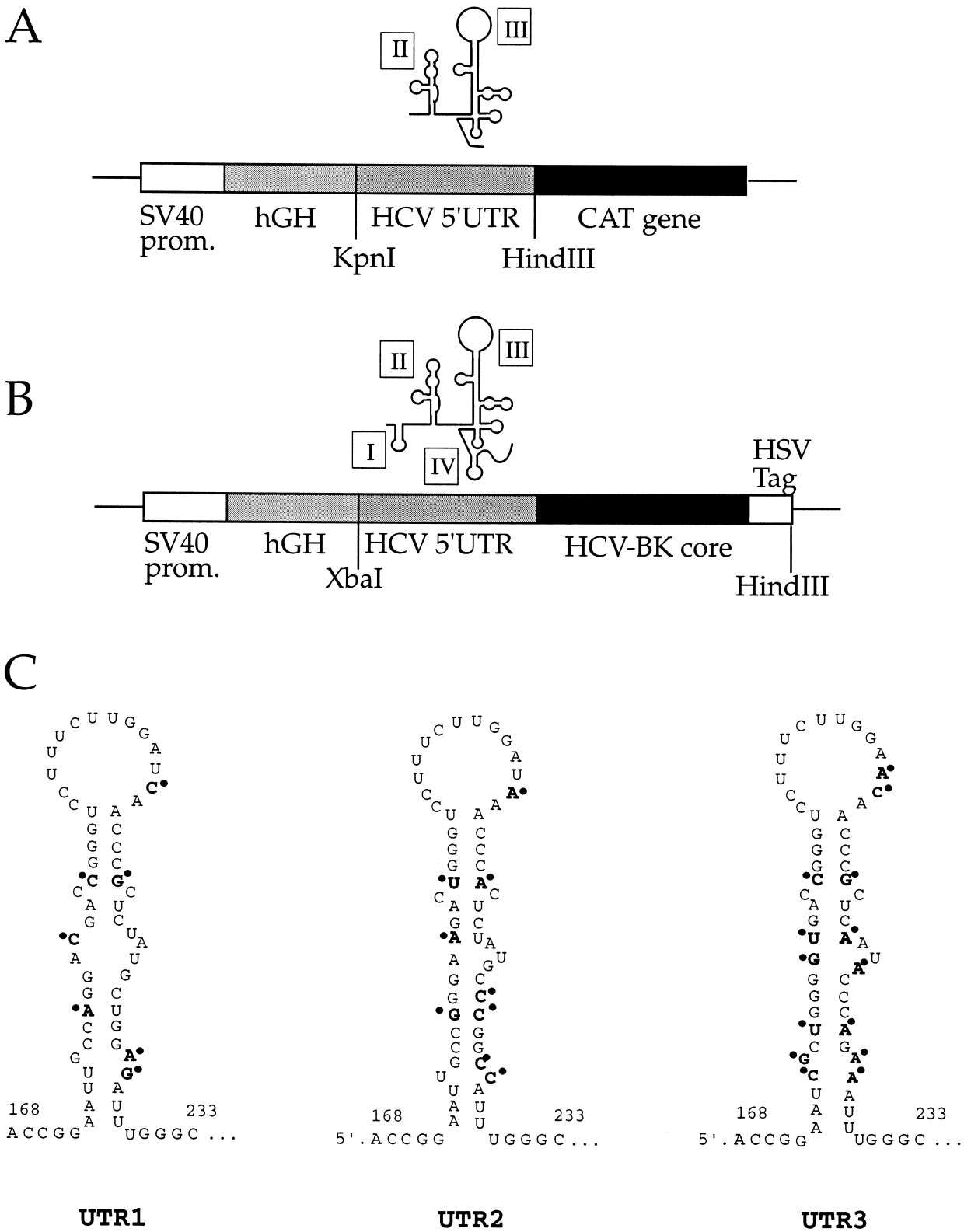

Fig. 1. Schematic representation of the pSV GH-CAT plasmids used for this work. A: The bicistronic portion of the plasmids in which the different 5′-UTRs controlled the expression of the *CAT* reporter gene. B: Diagram of the constructs where the complete 5′-UTRs were fused to the HCV core protein (containing the C-terminal HSV Tag sequence). This allowed the inclusion of domains I and IV, as shown in the drawing above the 5′-UTR box. C: Predicted secondary structures of the different domain III sequences of the HCV 5′-UTRs. The nucleotides differences between the different genotypes are shown in bold letters and marked with an point. UTR1, genotype 1a; UTR2, genotype 2b; UTR3, genotype 3.

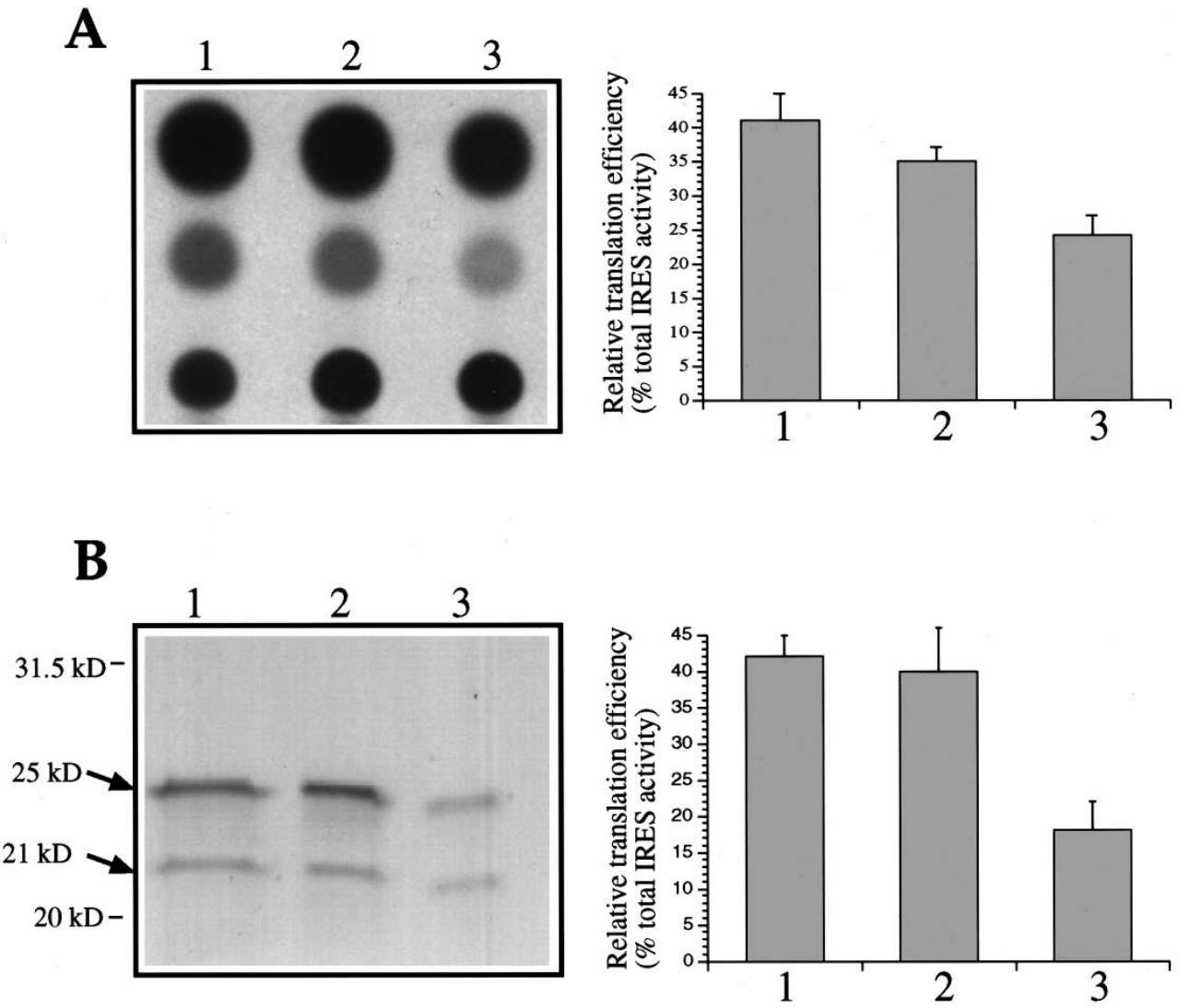

Fig. 2. A: Representative CAT assay using pSV UTR-1 (1), pSV UTR-2 (2), and pSV UTR-3 (3). The amount of cellular extract used in each experiment was normalized using the amount of growth hormone released in the culture media. Relative CAT intensities were measured using a Phosphoimager and the graph on the right represents the mean results of three independent experiments. B: Western blot of COS-1 cells (total cellular extract) transfected with plasmids: pSV UTR-1core (1), pSV UTR-2core (2) and pSV UTR-3core (3). Both the 25 kDa and 21 kDa forms (marked with an arrow) of the core protein were recognized by the core-specific mAb B12.F8. Band intensities were measured using a Bio-Rad Densitometer. The graph on the right shows the results (+SD) of three independent experiments.

the core protein it recognized two distinct bands of 21 and 25 kDa respectively (Fig. 2B). These bands are due to the already described C-terminal proteolytic cleavage of the HCV-BK core protein [27]. Using an anti-HSV Tag specific mAb we have confirmed that this processing occurs at the C-terminal end of the HCV core protein (data not shown). The difference in molecular weight between the processed and unprocessed core protein in our experiments (21 and 25 kDa) is greater than what had been previously described (21 and 23 kDa) due to the addition in our constructs of the HSV Tag sequence (Buratti et al., unpublished results).

As genotype 3 is the least studied of the European most common genotypes we decided to perform, in parallel, some artificial mutations of its domain III. We have mutated the poly-pyrimidine tract (nt 195–200) from UUUCUU to GGGGGG (pSV UTR-3/195). In another mutant, pSV UTR-3/207, the descending arm (ACCCG) of the apical stem (nt 207–211) was mutated to UGGGC, whilst a stem-restoring compensatory mutation was then engineered in pSV UTR-3/188/207. The experiments show that both the pSV UTR-3/195 (Fig. 3A) and pSV UTR-3/207 (Fig. 3B) mutations greatly reduced translation efficiency. Interestingly, the UTR containing the compensatory mutation (UTR-3/188/207) recovers only 60% of the original translational initiation ability (Fig. 3B).

4. Discussion

The aim of this study was to compare the translational initiation ability of representative HCV 5′-UTR sequences belonging to different HCV genotypes (1, 2, and 3). Using a bicistronic expression vector we have shown that the UTR of genotype 3 is approximately 50% less able to promote translation initiation than the UTRs of genotype 1 and 2. In addition, our experiments provide consistent evidence that HCV genotype 1 and 2 show similar efficiencies. The only previous

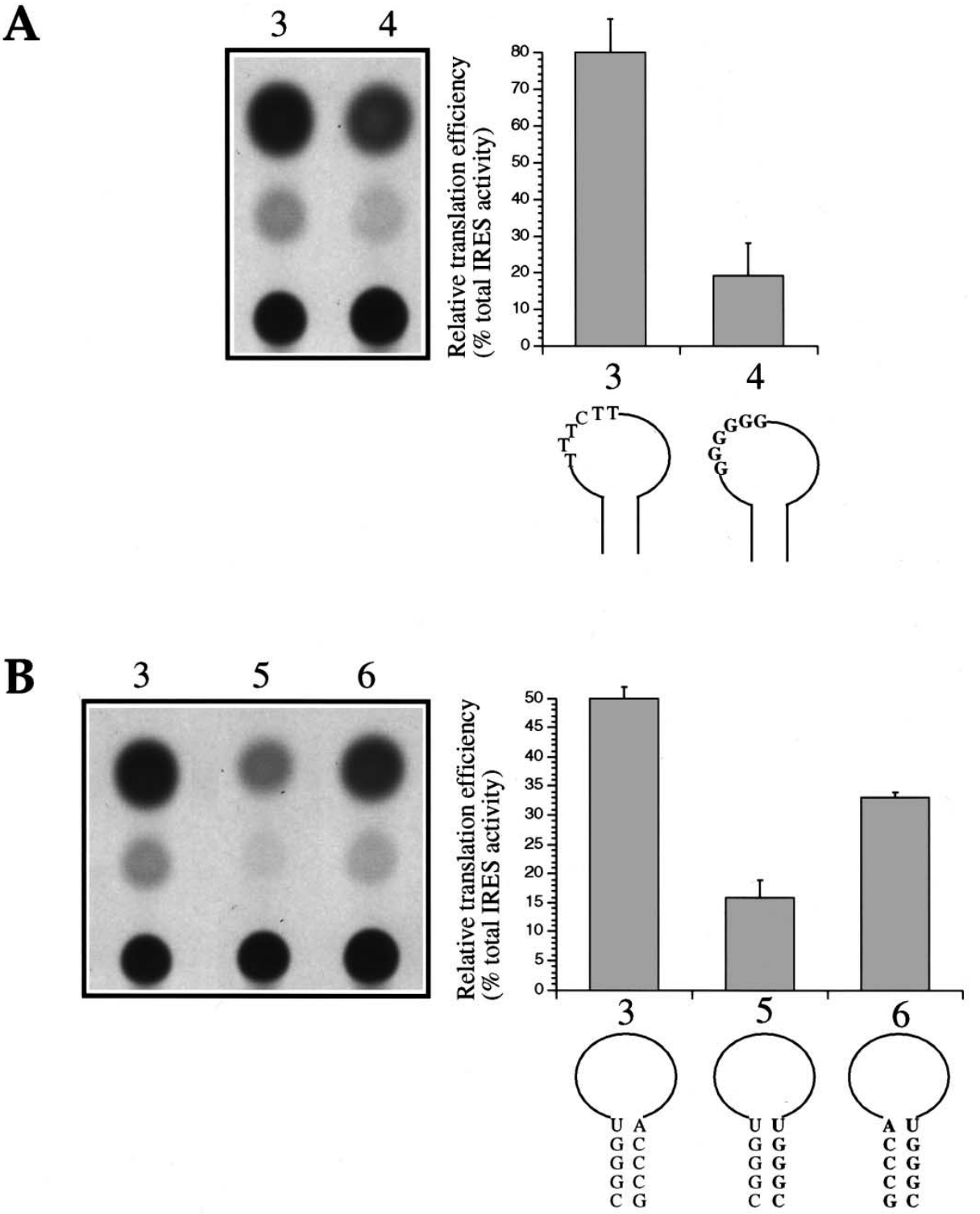

Fig. 3. CAT expression in COS-1 cells after transfection with the different pSV UTR-3 plasmids bearing artificial mutations in the apical stem and apical loop of domain III. A: pSV UTR-3 (3) and pSV UTR-3/195 (4). B: pSV UTR-3 (3), pSV UTR-3/207 (5), and pSV UTR-3/188/207 (6). The inset diagrams show a graphic representation of the different mutations introduced. Relative CAT intensities were measured using a Phosphoimager and the graph on the right represents the mean results of three independent experiments.

comparative study on the efficiency of different genotypes suggested that the IRES of genotype 2 was more efficient than that of genotype 1 [7]. However, it must be considered that these authors used a cell-free protein synthesis system and that, conditions were quite different from those employed by us.

The use of the BK core protein in all the constructs implied that the sequence of position 9–11 in the core region was GAA (typical of genotype 1 and 2) and not ACT (characteristic of genotype 3) [3]. These nucleotide changes are the same (and in the same position) as those observed for the TH33 strain of HCV [28], whose possible domain IV structure had

already been calculated, and which was predicted to minimally alter the structure of the stem-loop [6].

With the use of artificial mutants, we have also focused our attention on the stem-loop structure known as domain III, not only because this is where most nucleotide differences between genotypes occur, but also because this region may be the target for several protein–RNA interactions [29,30]. Previous mutational analysis of this loop [9] showed that the substitution of three nucleotides in the 195–200 polypyrimidine tract had no effect on IRES translational capabilities. Its total deletion completely abolished IRES functioning [9] and this leads to the impression that structural alterations in the apical part of domain III were tolerable. We feel that our mutation analysis in the same region should partially modify this view. In fact, the six-base substitution engineered in mutant UTR-3/195 almost completely abolished translation initiation. Although we cannot rule out that the presence of six Gs could lead to the acquisition of a radically different structure from the proposed models [10] it is possible that, unlike the one previously performed [9], this mutation might be sufficiently severe in order to interfere with the binding of proteins such as PTB [29] or with the suggested complementarity to the human 18S ribosomal sub-unit [5].

Interestingly, whilst base-pair disruption in the stem organization (mutant UTR-3/207) leads to a high reduction of translation initiation, the corresponding compensation in the mutant UTR-3/188/207 only partially recovered the loss. This partial recovery of activity highlights the importance of maintaining not only the secondary structure but also the primary sequence of the stem.

Our results show unequivocally that HCV 5′-UTR belonging to different genotypes can have different translational initiation capabilities. Interestingly, several studies have demonstrated that the HCV genotype of the infecting virus influences the response to IFN-α. In fact, patients infected with genotype 3 usually respond better in comparison with the other infecting genotypes [31]. The mechanism of action of IFN is not clear. A component in translation regulation controls sensitive to IFN and differences between genotypes 1–2 and genotype 3 are an interesting possibility to study. In addition, the changes in translational ability which we observed as a consequence of mutating key regions of the UTR lend more credibility to the emerging view that HCV translation might be dependent on the interaction with either cellular or viral factors. Further work on the nature of these interactions may lead to a better understanding of several characteristics of the different HCV infecting genotypes.

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