Binding of the La autoantigen to the hepatitis C virus 3’ untranslated region protects the RNA from rapid degradation in vitro

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We have analysed hepatitis C virus (HCV) RNAs in an in vitro RNA degradation assay. We found that the 3’ end of positive polarity HCV RNA is sensitive to cytosolic RNases whereas the 3’ end of negative polarity HCV RNA is relatively stable. Interaction of the HCV 3’ untranslated region with the cellular La protein prevented premature degradation of the HCV RNA. One may speculate that HCV RNAs interact with La protein in infected cells to prevent premature degradation of the viral RNAs.

Introduction

Hepatitis C virus (HCV), a member of the family Flaviviridae, is the principal agent of non-A non-B hepatitis (Houghton, 1996). Infections become chronic in approximately 50% of patients and commonly result in active hepatitis that may develop into liver cirrhosis and hepatocellular carcinoma (Houghton, 1996). The HCV genome is approximately 9.5 kb and contains a unique open reading frame (ORF) that is translated into a polyprotein which is cleaved cotranslationally into functional products by viral and cellular proteases (Bukh et al., 1996; Clark, 1997; Rice, 1996). The ORF is flanked by the 5’ and 3’ untranslated regions (UTRs), which contain sequences that are involved in transcription and translation initiation (Lai, 1998; Lemon & Honda, 1997; Wang & Siddiqui, 1995).

Most eukaryotic cellular mRNAs contain a Cap structure at the 5’ end and a poly(A) tail at the 3’ end. These structures have multiple functions and affect splicing, transport, translation and stability of mRNAs. For example, the Cap and the poly(A) tail act synergistically to promote translation of cellular mRNAs (Sachs, 1997; Wickens et al., 1997). The HCV RNAs lack a poly(A) tail and Cap structure, but the 5’ UTR contains an internal ribosome entry site (IRES) that is required for initiation of translation of HCV mRNAs (Lai, 1998; Lemon & Honda, 1997; Wang & Siddiqui, 1995). In addition, it appears that the HCV 3’ UTR stimulates translation initiation at the HCV IRES (Ito et al., 1998).

The Cap and the poly(A) tail interact with cellular proteins that protect the mRNAs from exonucleolytic degradation (Sachs, 1997; Wickens et al., 1997). Decapping and de-adenylation precede mRNA degradation (Ross, 1995). Thus, the presence of the Cap and the poly(A) tail on the mRNAs results in protection of the mRNAs from RNases and prevents untimely degradation of the cellular mRNAs. For example the poly(A) binding protein binds to the poly(A) tail and inhibits premature mRNA degradation. Interestingly, the poly(A) binding protein stabilizes mRNAs in the absence of a poly(A) tail, if tethered to the mRNA (Coller et al., 1998), demonstrating that it is the poly(A) binding protein and not the poly(A) tail itself that protects the mRNA from degradation. Since HCV mRNAs lack Cap and a poly(A) tail, they may be sensitive to degradation and therefore may interact with cellular proteins that prevent premature degradation. We used a recently described in vitro RNA degradation assay that reproduces regulated mRNA stability (Ford et al., 1999; Ford & Wilusz, 1999) to study the half-life of in vitro-synthesized HCV RNAs representing the 3’ ends of HCV RNAs of positive and negative polarities.

Our results show that the 3’ end of positive polarity HCV RNA is sensitive to cytosolic, cellular RNases and that the cellular La protein binds to the HCV 3’ UTR and inhibits premature degradation of the viral mRNA.

Methods

Plasmids. Plasmids from which HCV 3’ ends of either polarity could be synthesized in vitro were generated as follows: the HCV 3’ UTR was PCR amplified from the infectious clone pCV-H77C (Yanagi et al., 1997) with oligonucleotides HCV3UTRS (5’ GATATCCTACCCG-AGGGGTAGGCATCTA 3’) and HCV3UTRA (5’ CGTCGACATGACTCTGCAGAGG 3’), generating a 256 nt fragment (nt 9343–9599)
µ

510 mM HEPES pH 7 with radiolabelled RNA (10
modifications. Briefly, recombinant protein or cell extract was incubated
RNAs were resuspended in water. UV cross-linking was carried out
was performed with T7-RNA polymerase in the presence of 
[52x268]In vitro 

part of the two proteins.

his tagged in the N-terminal

translational start codons, was inserted in-frame with the His tag in

promoter and the insert. There are no additional sequences at the 3

vector. This is the sequence between the T7 promoter and the cloning

contains 68 nucleotides at the 5
at room temperature followed by UV-irradiation in a Stratagene linker for

of this RNA. The hnRNP C1 or the La open reading frame, from the

of HCV RNA of negative-strand polarity (Fig. 1). The 3
end that are derived from the pCR21

and the pellet was resuspended in buffer A containing 0
90 min at 100000

KCl releases proteins associated with the cell organelles and microsomes.

The supernatant from this centrifugation step was designated P100. S100

KCl releases proteins associated with the cell organelles and microsomes.

The cells were pelleted in a Beckman centrifuge at 6000 g for 2 h at 37 °C.
The pellets were resuspended in ice-cold PBS and lysed by 30 s bursts of sonication
followed by incubation in 1% Triton X-100 on ice. Cell debris was
removed by centrifugation and the supernatants were filtered through a
0.45 µm filter and loaded onto HiTrap chelating columns. Bound proteins
were eluted with EDTA–phosphate buffer pH 7.4 (1 mM Na2HPO4,
1 mM NaH2PO4, 50 mM NaCl) with the following different concentrations
of EDTA: 20, 40, 60 and 100 mM, respectively. The protein concentrations
were determined by comparison with a serial dilution of BSA on Coomassie-stained SDS–polyacrylamide gels.

Preparation of cell extracts. Cytoplasmic extract was prepared by lysis of HeLa cells in a Dounce homogenizer in buffer A (20 mM Tris
pH 7.8, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol and
2 mg/ml Aprotinin) followed by centrifugation at 10000 g for 10 min
(Dignam et al., 1983). The supernatant was collected and centrifuged for
90 min at 100000 g. The supernatant from this step was designated S100 and
the pellet was resuspended in buffer A containing 0.05 M KCl
and centrifuged through 30% sucrose. Resuspension of the pellet, consisting
of the majority of intact cell organelles, in buffer A containing 0.05 M
KCl releases proteins associated with the cell organelles and microsomes.
The supernatant from this centrifugation step was designated P100. S100 contains the soluble portion of the cell, the cytosol. The protein
concentration was determined by the Bradford method.

Western blotting. This was done as described previously (Tan &
Schwartz, 1995) except that an anti-La monoclonal antibody, LA4B0 (ICN
Pharmaceuticals), was used as primary antibody at a dilution of 1:1000. Horseradish peroxidase-conjugated anti-mouse Ig antibody (Amersham)
was used as secondary antibody at a dilution of 1:10000. The samples were
boiled prior to loading on 12% SDS–polyacrylamide gels. Bands
were visualized by using the ECL detection system (Amersham).

RNA degradation assay. An in vitro RNA degradation assay that
had been shown previously to reproduce intracellular RNA degradation
was used (Ford et al., 1999; Ford & Wilusz, 1999). One fmol of
radiolabelled HCV RNA was incubated in a total volume of 20 µl
containing 2% polyvinyl alcohol, 0.8 mM ATP, 17.5 mM creatine
phosphate and 1.4 mg/ml cellular extract at 30 °C. Reactions were
stopped by addition of stop buffer (400 mM NaCl, 25 mM Tris–HCl
pH 7.4 and 0.1% SDS) and the samples were subjected to phenol–
chloroform extraction followed by ethanol precipitation. The BSA that
was added in some experiments was a commercially available product
that had been acetylated to inactivate nucleases (Life Technologies). The
samples were loaded on denaturing 6% urea–polyacrylamide gels and
RNA levels were monitored by autoradiography. All experiments were
performed several times and in triplicate. In all experiments shown here, the standard error was less than 20%.

Results

The 3' end of positive-polarity HCV RNA is degraded rapidly in vitro

To study the stability of HCV RNAs, RNAs representing the 3' ends of positive- and negative-polarity HCV RNA were synthesized as described in Methods and analysed in an RNA degradation assay that has been shown previously to faithfully reproduce degradation of labile cellular mRNAs (Ford et al., 1999; Ford & Wilusz, 1999). Radiolabelled 3'(+) and 3'(-) RNAs (Fig. 1) encoding 395 nt and 257 nt of the 3' ends of positive- and negative-polarity HCV RNA, respectively, were tested. First, the 3'(+) RNA was analysed using cytoplasmic extract from HeLa cells. As can be seen from the results (Fig. 2a), the HCV RNA 3'(+) was rapidly degraded in the S100 extract but not in the P100 extract. The extracts were prepared from HeLa cells according to standard procedures (Dignam et al., 1983). The 3'(+) RNA was stable in buffer A, as expected. Quantification of the RNA levels demonstrated that the 3'(+) RNA has a substantially shorter half-life in the S100 extract (Fig. 2b), under the conditions used here, than the 3'(-) RNA, which was relatively stable in the S100 extract (Fig. 2d). In contrast, both the 3'(+) RNA and the 3'(-) RNA were relatively stable in the P100 extract and in buffer A (Fig. 2b, d). All experiments were performed several times in triplicate. In all experiments shown here, the standard error was less than 20%. Similar experiments were performed with 3'(+) RNA in a commercially available rabbit reticulocyte lysate (Promega) and the RNA was found to be as stable as in buffer A alone (data not shown), indicating that the RNases present in S100 are absent or inactive in rabbit reticulocyte lysate (Promega). We concluded that the 3' end of the positive-RNA strand of HCV is unstable and degraded rapidly in vitro.

The La autoantigen protects the HCV mRNA from degradation in vitro

Cellular mRNAs contain a poly(A) tail at the 3'-end, which is bound by the poly(A) binding protein that protects the mRNA from premature degradation (Coller et al., 1998). The HCV 3' UTR interacts with cellular proteins (Lai, 1998) and it is reasonable to speculate that the HCV 3' UTR interacts with cytoplasmic factors that prevent premature degradation of the newly synthesized, unpolyadenylated HCV mRNAs and/or genomic RNAs. Two nuclear proteins named heterogeneous nuclear ribonucleoprotein C (hnRNP C) (Gontarek et al., 1999) and polypyrimidine tract-binding protein (PTB) (Chung & Kaplan, 1999; Gontarek et al., 1999; Ito & Lai, 1997; Luo, 1999; Tsuchihara et al., 1997) have been shown to interact with the U-rich region and the conserved region, respectively, in the HCV 3' UTR. The role of these two proteins in the HCV life-
Fig. 3. The La protein protects HCV RNA from degradation. (a) Radiolabelled 3’ (+) RNAs were synthesized as described previously (Spångberg et al., 1999) and tested in the RNA degradation assay (Ford et al., 1999; Ford & Wilusz, 1999) as described in the legend to Fig. 2 (a), in the absence or presence of the HCV 3’ UTR-binding proteins hnRNP C and La. Recombinant, His-tagged hnRNP C and La (Spångberg et al., 1999) were used. (b) Densitometric analysis of the results shown in (a). (c) Addition of serially diluted La protein demonstrated that as little as 2 fmol La protein prevented degradation of 1 fmol HCV RNA. (—) represents the amount of RNA that was added to the RNA degradation assay. (d) Addition of 0-5 pmol BSA to the RNA degradation assay demonstrated that BSA had no effect on the degradation of HCV RNA. (e) UV cross-linking of the recombinant His-hnRNP C and His-La to radiolabelled HCV 3’ UTR probe 3’ (+). Competition with 10-fold serially diluted specific competitor 3’ (+) and unspecific competitor C2 is shown. C2 is derived from the human papillomavirus type 1 late 3’ UTR (Sokolowski et al., 1997). That the interaction between La and RNA 3’ (+) is specific has been shown previously (Spångberg et al., 1999). MW, molecular mass marker.

cycle is at present not clear. In addition, we have identified a cellular protein, the La autoantigen, which interacts specifically with the U-rich region in the HCV 3’ UTR (Spångberg et al., 1999). To investigate whether binding of proteins to the HCV 3’ (+) RNA protects the RNA from degradation in the in vitro RNA instability assay, we included 0.5 pmol of recombinant His-tagged La (Spångberg et al., 1999) or His-tagged hnRNP C protein in the assay in the presence of S100 extract and 1 fmol of radiolabelled 3’ (+) RNA. Interestingly, the His-tagged La protein efficiently protected the HCV RNA from degradation, resulting in prolonged half-life of the HCV 3’ RNA (Fig. 3a, b). Addition of serially diluted La protein demonstrated that as little as 2 fmol La protein prevented degradation of the HCV RNA (Fig. 3c). In contrast, the HCV 3’ (+) RNA was unstable in the presence of hnRNP C (Fig. 3a, b). Both hnRNP C and La have been shown to bind to the U-rich region of the HCV 3’
La stabilizes HCV RNA

Fig. 4. Partially purified La protein from HeLa cells protects HCV RNA from degradation. HeLa cell cytoplasmic extracts were prepared as previously described (Spångberg et al., 1999) and fractionated on HiTrap Q columns. Western blotting with monoclonal La antibody (4B6; ICN Pharmaceuticals) was used to identify fractions containing La. F, flow through fraction from the HiTrap Q column; C, total cytoplasmic extract prepared as described in Methods. (a) UV cross-linking of fraction number 13 to the HCV probe 3′(+) shows that La is the only HCV 3′ UTR-binding protein in that fraction. (b) UV cross-linking and Western blotting were performed as previously described (Spångberg et al., 1999). (c) Radiolabelled 3′(+) RNAs were synthesized as described previously (Spångberg et al., 1999) and tested in the RNA degradation assay as described in the legend to Fig. 2(a), in the absence or presence of La protein partially purified from HeLa cells. (d) Densitometric analysis of the results shown in (d).

UTR (Gontarek et al., 1999; Spångberg et al., 1999), but only La protects the RNA from rapid degradation, demonstrating that this effect was specific for the La protein. Fig. 3(e) shows that recombinant His-tagged hnRNP C and His-La bind to the HCV 3′ UTR. The La protein may bind with higher affinity than hnRNP C to the HCV RNA or it may have the ability to form a specific ribonucleoprotein complex that prevents premature degradation of the mRNA. The presence of high concentrations of protein in the reaction mixture does not affect the half-life of the HCV RNA as shown by the results obtained in the presence of 0.5 pmol BSA (Fig. 3 d). Incubation of RNA with this amount of BSA under the conditions used for the RNA degradation assay did not affect the RNA levels (data not shown).

To verify these results with La protein produced in human cells, cytoplasmic extracts of HeLa cells were prepared as previously described (Spångberg et al., 1999) and fractionated on HiTrap Q columns (Pharmacia Biotech). UV cross-linking to HCV probe 3′(+) and Western blotting with monoclonal La antibody (4B6; ICN Pharmaceuticals) were used as described previously to identify fractions containing La (Fig. 4a, b) (Spångberg et al., 1999). The La-containing fractions were concentrated and included in the in vitro degradation assay. Addition of similar levels of the partially purified La protein from fraction 14 to the RNA degradation assay resulted in inhibition of HCV RNA degradation (Fig. 4c, d), confirming the results obtained with recombinant His-tagged La.

In conclusion, by using an in vitro RNA degradation assay we have shown that the 3′ end of positive-polarity HCV RNA is sensitive to cytosolic, cellular RNases and that the cellular La protein binds to the HCV 3′ UTR and inhibits premature degradation of the viral mRNAs.

Discussion

HCV RNAs are uncapped and unpolyadenylated and as such are sensitive to premature degradation in the infected cell. Here we show that the HCV 3′ UTR is rapidly degraded in vitro in an S100 extract whereas the 3′ end of negative-polarity HCV RNA is not. The latter sequence may be more stable as a result of extensive secondary structures whereas the HCV 3′ UTR contains the U-rich region (Yamada et al., 1996) which
may confer sensitivity to RNases. However, the HCV 3′ UTR ends with a conserved sequence that folds into stem–loops (Ito & Lai, 1997) which may aid in the protection of the mRNAs from exonucleases. The roles of the various regions in the HCV 3′ UTR in the degradation of the HCV RNA remain to be elucidated.

In yeast (Saccharomyces cerevisiae) cells, 5′–3′ and 3′–5′ exonuclease pathways appear to be distinct (Jacobs et al., 1998; Mitchell et al., 1997). However, both pathways start with a shortening of the poly(A) tail. This step is followed either by removal of the Cap and 5′–3′ exonuclease degradation or complete removal of the poly(A) tail and 3′–5′ exonuclease degradation. In addition to these two pathways, a poly(A) tail-independent endonucleolytic pathway has been described. Less is known about mRNA degradation in mammalian cells. The premature degradation of mammalian mRNAs containing mRNA instability elements appears to start with deadenylation, followed by degradation of the mRNA body (Ross, 1995). Many mRNAs lacking poly(A) tail also lack Cap, suggesting that the 5′–3′ exonuclease degradation pathway described for yeast cells is also operational in mammalian cells. It has also been shown that RNA instability elements in the 3′ UTR of cellular mRNAs may be attacked by endonucleases (Binder et al., 1994). The HCV 3′ UTR may be the target of both exo- and endonucleases since it lacks a poly(A) tail and contains a U-rich region, a hallmark of many unstable cellular mRNAs (Ross, 1995). Interestingly, a number of mammalian RNases found in the RNaseA super family show a clear preference for poly(U) as a substrate over the other homoribopolymers (Sorrentino & Libonati, 1997). The RNase4 family strongly prefers poly(U) over other RNA substrates (Hofsteenge et al., 1998). For this reason, one may speculate that the poly(U) tract, which is conserved among various HCV sequences, is particularly sensitive to RNases and must be protected from rapid degradation.

The presence of both exo- and endonucleases in cell extracts has been described (Brewer, 1999; Ford et al., 1999). We speculate that the RNases degrading the HCV 3′(+ ) RNA may be similar to those described by Ford et al. (1999) which target deadenylated RNAs with AU-rich RNA instability elements for rapid degradation. Indeed, the HCV 3′(+) RNA contains a U-rich region which is not present in the HCV 3′(−) RNA and which may be the target for the cellular RNases as discussed above. In contrast, RNases that have been described previously to be located in the P100 fraction of mammalian cell cytoplasm extracts (Brewer & Ross, 1988; Wennborg et al., 1995) apparently do not target the HCV RNAs for degradation. Binding of the La protein to the U-rich region may protect the HCV RNA from premature degradation by both exo- and endonucleases in the infected cell.

There are also cellular mRNAs that lack poly(A) tails. Histone mRNAs constitute a rare example of an unpolyadenylated cellular mRNA. They contain a stem–loop structure at the 3′ end that interacts with cellular factors. Production of histone protein is restricted to the S-phase in the cell cycle and ceases at the end of the S-phase, partly as a result of rapid degradation of histone mRNAs. Specific degradation of histone mRNAs in vitro has been reproduced and was shown to be dependent on cellular factors in an S130 extract (McLaren et al., 1997). This effect was augmented by the addition of histone protein. Interestingly, these investigators showed that addition of La protein to their in vitro histone degradation assay significantly stabilized the histone mRNA (McLaren et al., 1997). Similarly to results presented here, these investigators demonstrated that the presence of the La protein stabilized an unpolyadenylated RNA in an in vitro RNA degradation assay.

The La protein binds to RNA polymerase III-synthesized RNAs such as tRNAs, 5S rRNAs, U6 snRNA and the cytoplasmic Y RNAs. The target for the La protein on these RNAs is an oligouridylate sequence at their 3′ ends. Interestingly it has been proposed that the La protein stabilizes the Y RNAs and the U6 snRNA. Small cytoplasmic Y RNAs are normally found in Ro ribonucleoprotein particles (RNP)s together with Ro and La proteins. These RNAs are specifically and rapidly degraded in apoptotic cells (Rutjes et al., 1999). In conjunction with degradation, the La protein is released from the complex, suggesting that the La protein may protect the Y RNAs from premature degradation (Rutjes et al., 1999). In addition, studies on the role of the yeast La homologous protein 1 (Lhp1p) and its effects on RNA polymerase III transcripts in yeast have shown that La stabilizes newly synthesized U6 snRNAs and acts as a chaperone during assembly of the RNA into U6 snRNPs (Pannone et al., 1998).

The La protein has also been shown to interact with unspliced RNAs produced by hepatitis B virus (HBV) (Heise et al., 1999), a DNA virus that, similar to HCV, replicates in the liver of the infected individual. It was shown that La binds to a stem–loop structure located in a sequence on the HBV RNAs that had been shown previously to regulate the levels of HBV RNAs. Treatment of cells expressing HBV RNAs with inflammatory cytokines such as interferon-γ and tumour necrosis factor-α suppresses HBV gene expression by reducing the half-lives of the HBV RNAs. Interestingly, it appeared that cytokine-induced signal transduction pathways regulate the stability of the HBV RNAs by affecting the binding of the La protein to a 91 nucleotide RNA element on the HBV RNAs (Heise et al., 1999). The authors speculated that the La protein affects the HBV RNA half-life, constitutively and in response to cytokines (Heise et al., 1999). Here we have shown that the HCV 3′ UTR is rapidly degraded in vitro and that binding of the La protein to the HCV 3′ UTR stabilizes the HCV RNA. It would be interesting to investigate if inflammatory cytokines affect the HCV RNA half-lives in hepatocytes and if the effects on the HCV RNAs are mediated by the La protein.

In summary, the La protein has been shown to stabilize unpolyadenylated cellular RNAs, i.e. histone mRNAs, Y RNAs and U6 snRNAs (McLaren et al., 1997; Pannone et al., 1998; Rutjes et al., 1999) and here we show that La protects HCV
RNAs from premature degradation by interacting with sequences in the HCV 3′ UTR.


References


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