Long-range RNA–RNA interaction between the 5′ nontranslated region and the core-coding sequences of hepatitis C virus modulates the IRES-dependent translation

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ABSTRACT

Hepatitis C virus (HCV) is a positive-sense RNA virus ~9600 bases long. An internal ribosomal entry site (IRES) spans the 5′ nontranslated region, which is the most conserved and highly structured region of the HCV genome. In this study, we demonstrate that nucleotides 428–442 of the HCV core-coding sequence anneal to nucleotides 24–38 of the 5′NTR, and that this RNA–RNA interaction modulates IRES-dependent translation in rabbit reticulocyte lysate and in HepG2 cells. The inclusion of the core-coding sequence (nucleotides 428–442) significantly suppressed the translational efficiency directed by HCV IRES in dicistronic reporter systems, and this suppression was relieved by site-directed mutations that blocked the long-range interaction between nucleotides 24–38 and 428–442. These findings suggest that the long-range interaction between the HCV 5′NTR and the core-coding nucleotide sequence down-regulate cap-independent translation via HCV IRES. The modulation of protein synthesis by long-range RNA–RNA interaction may play a role in the regulation of viral gene expression.

Keywords: HCV; translation; core; IRES

INTRODUCTION

The genome of hepatitis C virus (HCV) is composed of a single-stranded RNA molecule with a positive-stranded polarity, and is ~9600 nucleotides long. By sequence analysis and biochemical studies, HCV has been classified as a member of the *Flaviviridae* family (Rice 1996). The HCV genome contains a unique ORF, which is translated into a polyprotein that is cleaved into functional products by cellular and viral proteases (Bartenschlager and Lohmann 2000; Reed and Rice 2000).

Translation of the polyprotein is directed by an internal ribosomal entry site (IRES) spanning most of the 5′ nontranslated region (5′NTR) in combination with a portion of the core-coding region that augments the IRES-dependent translation (Tsukiyama-Kohara et al. 1992; Wang et al. 1994, 2000; Rijnbrand et al. 1995; Honda et al. 1996a; Lu and

Wimmer 1996; Hwang et al. 1998; Zhao et al. 2000). With the exception of small hairpin structures located within the most 5′ segment of the 5′NTR (domain I), all of the predicted stem-loop structures are essential for the internal initiation directed by HCV IRES (Wang et al. 1994, 1995; Rijnbrand et al. 1995, 2001; Honda et al. 1996a,b; Kamoshita et al. 1997; Rijnbrand and Lemon 2000). Although the 3′ boundary of the IRES is less certain, several studies indicate that the activity of the HCV IRES is dependent on the sequence located immediately downstream of the initiator AUG (Reynolds et al. 1995; Honda et al. 1996a,b; Lu and Wimmer 1996). The initiator AUG is located within the single-stranded segment of a stem-loop (stem-loop IV), which is formed in part by the core protein-coding sequence. Mutations enhancing the stability of this putative stem-loop significantly reduced the translational efficiency, which suggests that the folded and unfolded conformations of stem-loop IV could modulate the efficiency of internal initiation directed by HCV IRES.

A certain amount of controversy exists concerning the role of the core-coding sequence in IRES-dependent translation. The importance of the first 21 nucleotides of the core-coding sequence in IRES-dependent translation was supported by the observation that chimeric polioviruses

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Article and publication are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2185603>.

containing the HCV IRES required at least 21 nucleotides of core-coding sequence for viral replication (Rijnbrand et al. 1995; Lu and Wimmer 1996), and up to 32 nucleotides of the core-coding sequence is known to be absolutely required for efficient IRES-dependent translation (Reynolds et al. 1995; Honda et al. 1996a). On the contrary, efficient IRES activity has been observed in dicistronic constructs containing between three (initiator AUG codon) and eight nucleotides of the core-coding sequence (Tsukiyama-Kohara et al. 1992; Rijnbrand et al. 1995). In addition, it was reported that the inclusion of the nearly full-length core protein-coding sequence significantly suppressed IRES activities in vitro and in vivo assay systems (Wang et al. 1994). The authors showed that core protein-coding sequence, but not core protein, modulates the efficiency of cap-dependent translation directed by the IRES of HCV through a long-range interaction (Wang et al. 1994).

Here, we report that nucleotides 24–38 within the 5'NTR of HCV interact with nucleotides 428–442 of the core-coding sequence, and that this RNA–RNA interaction modulates the HCV IRES-dependent translation. Mutations, which disrupt the stability of this putative interaction, significantly enhanced IRES-dependent translation. Moreover, compensatory mutations, which restore stability, showed translational efficiencies comparable with that of the wild type. This long-range RNA–RNA interaction may play a role in the specific regulation of viral protein synthesis.

RESULTS

Inhibitory effect of the core-coding sequence on IRES-dependent translation

Multiple regions in the core-coding sequence have been reported to affect the HCV IRES-dependent translation. The 5'-end of the core sequence, which constitutes part of domain IV, modulates IRES-dependent translation (Reynolds et al. 1995; Hahm et al. 1998). In addition, a polypyrimidine tract-binding protein is known to interact with a section near the 3'-end of the core-coding sequence, and that this results in the strong inhibition of translation directed by HCV IRES (Ito and Lai 1999). Moreover, this inhibition is relieved by the presence of the X region at the 3'-end.

To further examine the influence of the core-coding sequence on HCV IRES-dependent translation, we constructed the dicistronic reporter vectors [CATΔ/H(39–427)/CAT, CATΔ/H(39–443)/CAT, CATΔ/H(39–480)/CAT, CATΔ/H(1–427)/CAT, and CATΔ/H(1–443)/CAT], which

FIGURE 1. Effect of the core-coding sequence on HCV IRES-dependent translation in dicistronic mRNAs. A schematic diagram of capped dicistronic mRNA (pDC; CATΔ-HCV IRES-CAT) is depicted at top. In vitro translations were performed using 10 nM (lanes 1,3,5) or 40 nM (lanes 2,4,6,7,8) of dicistronic mRNAs containing different portions of the HCV core-coding sequence.

contain the CAT gene with a truncated carboxyl terminus (CATΔ) as the first cistron, IRES and a different portion of the HCV core-coding sequence, followed by a full-length CAT sequence as the second cistron. The CAT-coding sequence was fused in frame with the truncated core sequence to serve as a reporter gene. The translational efficiencies of the dicistronic mRNA transcripts were examined in RRL, in which newly synthesized proteins had been labeled with [³⁵S]methionine. There are equal numbers of methionine residues in each polypeptide. In the cases of the dicistronic mRNAs containing from nucleotide 39 to the core-coding sequence, the translational efficiencies of the hybrid genes were not affected by the expansion of the core sequence (Fig. 1, cf. CAT products in lanes 1, 3, and 5 with those in lanes 2, 4, and 6). As for the dicistronic mRNAs containing from nucleotide 1 to the core-coding sequence, on the other hand, IRES-dependent translation decreased upon expanding the core sequence (Fig. 1, cf. lanes 7 and 8). These results indicate that nucleotides 1–38 of the 5'NTR inhibit IRES-dependent translation of HCV mRNA containing the nucleotides 428–443 of HCV core-coding sequence.

Long-range RNA–RNA interaction between the 5'NTR and the core-coding sequence

To understand the mechanism of translational inhibition by sequences residing ~400 nucleotides apart, we investigated the possibility of a long-range interaction between these two sequences (i.e., nucleotides 1–38 and 428–443). A potential interaction between nucleotides 24–38 and 428–442 of HCV was predicted by the M-fold program (version 3.0) as shown in Figures 2 and 3A. The importance of the long-range RNA–RNA interaction on the translation of HCV

mRNA was further analyzed in vitro by using mutations, which would affect any potential long-range interaction (Fig. 3B). The potential secondary structures of the mutant RNAs are shown in Figure 3A. The mutant and wild-type HCV IRESs were inserted into the intercistronic region of a dicistronic vector expressing *renilla* luciferase (RLuc) translated by a cap-dependent mechanism and firefly luciferase (FLuc) translated by HCV IRES or its derivatives (Fig. 3B, top).

The function of HCV IRES and its derivatives was monitored by measuring RLuc and FLuc activities after in vitro translation reactions. The M1 and M2 mutations, which destabilized the long-range interaction, increased HCV IRES activity compared with the wild-type HCV IRES (Fig. 3B, cf. bars of pR/H[24–442]/F with pR/M1[24–442]/F and pR/M2[24–442]/F). The compensatory mutation pR/M3(24–442)/F, which restores the long-range interaction, reduced HCV IRES-dependent translation (Fig. 3B, pR/M3[24–442]/F). Moreover, the mutation pR/M4(24–442)/F, which stabilizes the long-range interaction, further reduced HCV IRES activity (Fig. 3B, pR/M4[24–442]/F). These relationships strongly suggest that the long-range RNA–RNA interaction between nucleotides 24–38 and 428–442 modulates HCV IRES-dependent translation in vitro.

Changes in the sequence from 24 to 32 seem to influence

HCV IRES activity, which explains the difference in the IRES-dependent translational efficiencies of pR/M1(24–442)/F and pR/M2(24–442)/F, in which M2 mutant shows much higher efficiency than the M1 mutant (Fig. 3B). Note that both pR/M1(24–442)/F and pR/M2(24–442)/F exert weaker long-range interactions than the wild-type construct pR/H(24–442)/F. Moreover, pR/M3(24–442)/F, which exerts a long-range interaction that is similar to that of pR/H(24–442)/F by compensatory mutation, showed weaker translational efficiency than the wild type. These results also indicate the positive effect of the nucleotide sequence between nucleotides 24 and 32 of HCV on HCV IRES function. Alternatively, the mutated region of pR/M1(24–442)/F may have an adverse effect on the IRES function through an unidentified interaction with the core element of HCV IRES.

Finally, the long-range RNA–RNA interaction, and thereby, altered IRES activity were examined in the human hepatoma cell line HepG2 using the same constructs as used in Figure 3C. Forty-eight hours after treating the lipofectamin–DNA complex, cells were lysed by freeze thawing, and RLuc and FLuc activities were measured to monitor translation of the reporter genes (see Materials and Methods). As can be seen in Figure 3C, the effects of each mutation on relative IRES activity in HepG2 cells correlated well with the

FIGURE 2. Proposed secondary and tertiary RNA structures of the HCV 5' NTR and the core-coding sequences. Major structural domains are labeled I, II, III, and IV. The initiator AUG codon in domain IV is depicted by AUG. The hatched and gray boxes indicate the A-rich sequence and the possible long-range RNA–RNA interaction region, respectively.

FIGURE 3. (Legend on facing page)

in vitro results described above. The results of the mutations and the compensatory mutation strongly suggest that nucleotides 24–38 of the 5'NTR anneal to nucleotides 428–442 of the HCV core-coding sequence, and thus modulates HCV IRES-dependent translation in vivo and in vitro.

Phylogenetic conservation of long-range RNA–RNA interaction

The putative long-range RNA–RNA interaction confirmed in our study is also supported by phylogenetic analysis of the published HCV sequences. Of the 184 sequences analyzed (<http://s2as02.genes.nig.ac.jp>), 148 sequences were folded as shown in Figure 4A. Sixteen of these included a U to C change at nucleotide 434, and 35 sequences folded as shown in Figure 4B, in which nucleotides 33–37 annealed to nucleotides 428–432 as shown in Figure 4B. Of these 35 sequences, a G to U change at nucleotide 34 was observed in four cases, and all but one sequence (183 of 184) maintained the overall stem-bulge-stem structure. Interestingly, the A to G change at nucleotide 29 was accompanied by the U to C change at nucleotide 437 in 25 cases. These double mutations allow GC base pairing instead of AU and maintain the same overall secondary structure (Fig. 4A). In addition, exchanges of A29 for G29, C25 for U25, and A440 for G440, were observed in several sequences, and all of these changes maintained the same secondary structure by GU base pairing. This phylogenetic conservation indicates that this long-range RNA–RNA interaction may play a pivotal role in viral proliferation. However, not all of the HCV strains maintain the exactly same secondary structure of RNAs shown in Figure 4, A and B. For instance, some strains contain U to C change at nucleotide 437, resulting in a partial distortion of the bottom stem structure. The effects of the altered long-range interactions derived by the nucleotide changes in the RNA–RNA interacting regions remain to be investigated.

DISCUSSION

In this study, we demonstrate that nucleotides 428–442 of the HCV core-coding sequence anneal to nucleotides 24–38 of the 5'NTR, and that such an interaction modulates IRES-

dependent translation in RRL and HepG2 cells. Mutations destabilizing the RNA–RNA interaction significantly enhanced internal initiation directed by HCV IRES. Compensatory mutations that restored the RNA–RNA interaction lowered the translational efficiency of the dicistronic mRNAs below the level of the wild-type sequence. Curiously, the changes in the sequence nucleotides 24–32 seem to hamper HCV IRES activity partially. The molecular basis of inhibitory effect of the mutations at this region remains to be elucidated.

Several previous reports support our results. Honda et al. (1999) showed that a six-nucleotide deletion spanning nucleotides 32–37 and the substitution of the AG dinucleotide sequence at nucleotides 34 and 35 of HCV-N with GA enhanced its translational activity. Curiously, this inhibitory effect was observed only when the downstream RNA sequence (nucleotides 408–929 of HCV) was included in the experiment. These workers also showed, by using frame shift mutations, that the nucleotide sequence of the transcript, and not the amino acid sequence of the core protein, determined translation efficiency. The inhibition of HCV IRES-dependent translation by the core-coding sequence (from nucleotide 407 to 860) was also shown by Wang et al. using an in vitro translation system and in HepG2 cells (Wang et al. 2000). Because the authors used dicistronic mRNAs containing the full-length HCV 5'NTR, we conclude that the inhibitory effect is likely to be associated with long-range RNA–RNA interaction. Moreover, Zhao and Wimmer (2001) investigated the effect of serial deletions in HCV 5'NTR on HCV IRES function in the context of the replicating poliovirus/HCV chimera, whose translation is directed by the HCV IRES. Deletions up to nucleotide 40 of HCV 5'NTR in the chimeric virus resulted in a virus yield and plaque size comparable with the wild-type poliovirus. On the other hand, deletions up to nucleotides 27, 17, and 9 resulted in medium, small, and minute plaque sizes, respectively. The authors explained this phenomenon on the basis of a putative interaction between the HCV sequence up to nucleotide 31 and the poliovirus cloverleaf, which plays a key role in polioviral replication (Zhao et al. 2000). However, on the basis of the present study, we suggest that this phenomenon may be, at least in part, attributed to long-range RNA–RNA interaction, which inhibits HCV

FIGURE 3. Effect of stabilizing/destabilizing the long-range interaction on HCV IRES-dependent translation. (A) Nucleotide sequences and secondary structures of wild-type and mutant RNAs. Interaction between nucleotides 24–38 and 428–442 of the wild type is shown in H(24–442). The positions of mutated nucleotides are depicted by arrows. Mutations on one side of the annealing region are indicated as M1(24–442), M2(24–442), and M4(24–442). Compensatory mutations on both sides of the annealing region are indicated as M3(24–442). (B) Translational efficiencies of the capped dicistronic mRNAs (RLuc-HCV IRES-FLuc) containing mutations in RRL. A schematic diagram of the dicistronic vector (RLuc-HCV IRES-FLuc) is depicted at top. Translational products synthesized by the dicistronic mRNAs (pR/H[24–442]/F, pR/M1[24–442]/F, pR/M2[24–442]/F, pR/M3[24–442]/F, and pR/M4[24–442]/F) were determined by measuring the activities of FLuc relative to RLuc. The relative ratio of FLuc/RLuc in the in vitro translation products were compared with that of the control (pR/H[24–442]/F). The columns and bars represent the means and standard deviations of triplicate experiments. (C) Translational efficiencies of dicistronic constructs (RLuc-HCV IRES-FLuc) containing mutations in HepG2 cells. Five micrograms of each plasmid DNA was used for transfection by lipofectamin method. About 48 h after transfection, the RLuc and FLuc activities of the cell extracts were measured. The relative ratio of FLuc/RLuc of the sample was compared with that of the control (pR/H[24–442]/F). The columns and bars represent the means and standard deviations of triplicate experiments.

IRES-dependent translation, as the HCV sequence nucleotides 428–442 was present in the chimeric viruses. Further investigation using the chimeric viruses is required to confirm this possibility.

What might be the role of the long-range RNA–RNA interaction in terms of viral replication? First, the long-range interaction may contribute to maintaining viral protein levels at a low concentration. The level of viral protein in HCV patients remains rather low during most of the virus infection period compared with other positive-sense RNA viruses. Moreover, this low level of viral protein synthesis may contribute to the persistence of HCV infection. Second, the long-range interaction may play an important role in the temporal regulation of viral mRNA translation. A switch from translation mode to replication mode of the poliovirus genome RNA (also a member of the positive sense RNA viruses) was shown to be important for viral RNA replication, as continuous translation blocks replication complex movement (Gamarnik and Andino 1998; Barton et al. 1999). The molecular switch was proposed to be achieved by an interaction between polioviral protein 3CD and a cellular protein poly(rC)-binding protein 2 (PCBP2) that augments polioviral IRES-dependent translation (Blyn et al. 1996, 1997; Gamarnik and Andino 1997, 1998, 2000; Parsley et al. 1997; Graff et al. 1998; Silvera et al. 1999; Walter et al. 1999; Herold and Andino 2001), and by the cleavage of a polypyrimidine tract-binding protein (PTB), which is another cellular protein that enhances polioviral IRES activity, by polioviral protein 3C^{pro} (Back et al. 2002). Interestingly,

potential stem-loop structures (nucleotides 388–508) exist in the core-coding sequence, and this region includes the site (nucleotides 428–442) participating in the long-range interaction (Smith and Simmonds 1997; Wang et al. 2000). The first stem-loop structure in this region (nucleotides 388–425) is highly conserved throughout HCV strains (Tuplin et al. 2002). The long-range interaction site (nucleotides 428–442) is preceded by this conserved stem-loop structure and is localized within the second stem-loop structure (nucleotides 427–508) (Wang et al. 2000). The molecular switch, from translation to replication, may be attributed to the alternative secondary structure of the core-coding region. The molecular basis of the translational modulation of HCV mRNA by the long-range interaction remains to be elucidated.

MATERIALS AND METHODS

Plasmid construction

Enzymes used for cloning and modifying DNA were purchased from New England Biolabs Inc. and Boehringer Mannheim.

To construct dicistronic plasmids, pDC/H(1–427), pDC/H(1–443), pDC/H(39–427), pDC/H(39–443), and pDC/H(39–480), a *Bam*HI/*Pst*I fragment of pSK(+)/CATΔ-ICS-CAT (Kim et al. 2000) was ligated to a *Bam*HI/*Pst*I fragment of PCR-amplified 5'NTR containing different portions of HCV IRES, and the core-coding sequence. PCR reactions were carried out using H77C as a template and the following oligonucleotides: H1 and H2 for pDC/H(1–427), H1 and H28 for pDC/H(1–443), H27 and H2 for pDC/H(39–427), H27 and H28 for pDC/H(39–443), and H27 and H3 for pDC/H(39–480) (Table 1).

To construct dicistronic plasmids, pR/H(24–442)/F, pR/M1(24–442)/F, pR/M2(24–442)/F, pR/M3(24–442)/F, and pR/M4(24–442)/F, a *Sall*/*Kpn*I fragment of pRF (Kim et al. 2001) were ligated to *Sall*/*Kpn*I fragments of PCR-amplified 5'NTR containing different portions of HCV IRES and the core-coding sequence. PCR reactions were carried out using H77C as a template and the following oligonucleotides: H58 and H59 for pR/H(24–442)/F,

TABLE 1. Oligonucleotides used in this study

Name	Sequence
H1	5'-AAA CTG CAG CCA GCC CCC TGA TGG GGG-3'
H2	5'-CGC GGA TCC CTG ACC GCC ACC CGG GAA C-3'
H3	5'-GCG GGA TCC GCG CAC ACC CAA TCT AGG-3'
H27	5'-AAA CTG CAG ACT CCC CTG TGA GGA ACT AC-3'
H28	5'-CGC GGA TCC AAC TCC ACC AAC GAT CTG AC-3'
H34	5'-AAA CTG CAG ACT CCA CCA TGA ATC ACT CC-3'
H35	5'-AAA CTG CAG ACA CCT CCT TGA ATC ACT CCC CTG TGA GG-3'
H36	5'-CGC GGA TCC AAC ACC TCC TAC GAT CTG ACC GCC ACC CGG-3'
H58	5'-CGC GTC GAC TCC ACC ATG AAT CAC TCC-3'
H59	5'-CGG GGT ACC ACT CCA CCA ACG ATC TGA C-3'
H60	5'-CGC GTC GAC ACC TCC TTG AAT CAC TCC CCT GTG AGG-3'
H61	5'-CGG GGT ACC ACA CCT CCT ACG ATC TGA CCG CCA CCC GG-3'
H63	5'-CGC GTC GAC TCC ACC AAC GAT CAC TCC CCT GTG AGG AAC-3'

FIGURE 4. Phylogenetic conservation of the predicted secondary structure of nucleotides 24–38 with nucleotides 428–442 of HCV. The nucleotide substitutions relative to the HCV 1a sequence occurring in these regions are indicated by arrows, and the virus genotype associated with this substitution in parenthesis is shown in A. Alternative predicted structures are shown in B.

H60 and H59 for pR/M1(24–442)/F, H58 and H61 for pR/M2(24–442)/F, H60 and H61 for pR/M3(24–442)/F, and H63 and H59 for pR/M4(24–442)/F (Table 1).

In vitro transcription and translation

Plasmid DNAs were purified by polyethylene glycol precipitation and linearized with the appropriate restriction enzymes. The linearized DNAs were then treated with phenol-chloroform and ethanol precipitated. Transcription reactions were performed with T7 RNA polymerase (Boehringer Mannheim) at 37°C for 90 min, as described by the manufacturer. To produce capped mRNAs, 1 mM of m⁷GpppG (Pharmacia Biotech Inc.) was included in the transcription reaction mixture. The concentrations of the RNA transcripts were determined using an UV-spectrophotometer.

Plasmids pDC/H(1–427), pDC/H(1–443), pDC/H(39–427), pDC/H(39–443), and pDC/H(39–480) digested with *Xba*I were used to generate the dicistronic mRNAs containing CATΔ as the first cistron and CAT as the second cistron. Plasmids pR/H(24–442)/F, pR/M1(24–442)/F, pR/M2(24–442)/F, pR/M3(24–442)/F, and pR/M4(24–442)/F digested with *Not*I were used to generate the dicistronic mRNAs containing RLuc as the first cistron and FLuc as the second cistron.

In vitro translations in micrococcal nuclease-treated RRL (Amersham) were performed in 20-μL reaction mixtures containing 10–40 nM (for transcripts from the plasmid pDC series) or 10 nM (transcripts from the plasmid pRF series) of the mRNAs. Translation reactions were carried out at 30°C for 1 h in the presence of [³⁵S]methionine (NEN), and translation products were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. Gels were dried and exposed to Kodak XAR-5 or Agfa Curix RP1 for 12–18 h. In the case of transcripts from the plasmid pRF series, the translation products were analyzed for RLuc and FLuc activities by following the supplier's instructions (Promega).

Cell culture and transfection

Cell monolayers of human hepatoma cell line HepG2 were grown in Earle's modified Eagle's medium (EMEM; GIBCO-BRL), 10% FBS (Hyclone), penicillin, and streptomycin. HepG2 cells were seeded onto 60-mm-diameter tissue culture dishes 24 h before transfection. Five micrograms of each plasmid DNA were used for the transfection, which was conducted by the lipofectamin method according to the manufacturer's instructions (GIBCO-BRL). After treating the mixture of lipofectamin reagent and dicistronic plasmids, the cells were incubated at 37°C for 24 h, and the culture medium was changed for FBS-supplemented EMEM. About 48 h after transfection, the cells were harvested and lysed by freezing-and-thawing. The RLuc and FLuc activities of the cell extracts were measured by using the dual-luciferase reporter assay system (Promega).

ACKNOWLEDGMENTS

The present study was supported in part by grants from the NRL (M10204000018-02J0000-01610) and the MMRG (M10106000056-

02B1700-01210) of MOST, the KRF (KRF-2002-005-C00011), and the KOSEF through PNRC.

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Received November 21, 2002; accepted February 4, 2003.

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