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Human immunodeficiency virus-1 Rev protein activates hepatitis C virus gene expression by directly targeting the HCV 5′-untranslated region

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ABSTRACT

Coinfection with human immunodeficiency virus-1 (HIV-1) and hepatitis C virus (HCV) accelerates hepatitis C disease progression; however, the mechanism underlying this effect is unknown. Here, we investigated the role of HIV-1 in HCV gene expression and the mechanism involved in this regulation. We discovered that HIV-1 Rev protein activates HCV gene expression. We further revealed that Rev binds to the internal loop of the HCV 5′-untranslated region (5′-UTR) to stimulate HCV IRES-mediated translation.

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1. Introduction

Due to the shared routes of transmission, infection with hepatitis C virus (HCV) is common among human immunodeficiency virus-1 (HIV-1)-infected patients. Infection with HIV-1 may enhance HCV replication since HCV RNA levels are significantly elevated in coinfected patients [1]. Also, HIV-1 seroconversion is associated with sustained increases in HCV viral load [2]. A convenient experimental system for studying the direct interactions between HIV-1 and HCV in a coinfection system is not yet available. However, HIV-1 may adapt to and efficiently replicate in hepatocytes and hepatic stellate cells [3,4]. HCV replication also occurs in extra-hepatic reservoirs, including peripheral blood mononuclear cells (PBMCs) and native human macrophages [5,6]. These reports demonstrate that the two viruses may reside, replicate, and interact in the same cells.

Rev is an essential regulatory protein that is crucial in the life cycle of HIV-1. Rev binds to a specific RNA secondary structure known as the Rev-responsive element (RRE), which is present in unspliced or partially spliced viral RNA. The Rev-RRE complex associates with CRM1 to shuttle from the nucleus into the cytoplasm [7]. Several other cellular proteins (e.g., DDX3, PIMT, and Matrin 3) are directly involved in this process [8–10]. Besides its nuclear export function, Rev has additional, independent stimulatory effects, including the promotion of translation [11].

The HCV 5′-UTR encompasses four structural domains (domains I–IV) (Fig. 1A). Domains II–IV comprise an internal ribosome entry site (IRES) that mediates translation of the HCV open reading frame (ORF). Translation of the HCV ORF is initiated by a 5′ cap-independent mechanism at the HCV IRES [12]. First, the ribosomal 40S subunit binds specifically to the HCV IRES. Next, eukaryotic translation initiation factor 3 (eIF3) is recruited to form the 43S pre-initiation complex. Finally, the ribosomal 60S subunit associates with the 43S pre-initiation complex to form the 80S ribosome [13]. In this study, we show for the first time that Rev binds to the HCV 5′-UTR, enhancing HCV IRES-mediated translation and up-regulating HCV gene expression.

2. Materials and methods

2.1. Cell culture and transfection

Huh7.5.1 [14] cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin, and 100 μg/

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Fig. 1. RNA secondary structure. (A) Schematic diagram of the secondary structure of the RNA 5′-end (nt 1–388) of HCV strain JFH1 genotype 2a, which refers to "full-length HCV IRES RNA." Nucleotides 1–340 correspond to the "HCV 5′-UTR." Internal loop IIIb is boxed. The mutations in the HCV 5′-UTR used in this study are indicated. (B) Schematic diagram of the secondary structure of loop IIIb in the HCV 5′-UTR, internal loop in the HIV-1 RRE, and loop A in HIV-1 5′-UTR. *Abbreviation:* del, deletion.

ml streptomycin sulfate. For transfection, cells were plated in 24-well plates one day prior to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.2. Plasmid construction

Plasmid FL-J6/JFH-5′C19Rluc2Aubi is a monocistronic full-length HCV genome derived from the previously reported infectious genotype 2a HCV genome J6/JFH1 that expresses Renilla luciferase [15]. This plasmid was kindly provided by Dr. Charles M. Rice [16]. The HIV-1 Rev gene was amplified by PCR from PNL4-3, an infectious molecular clone of HIV-1 [17], then inserted into the vector pCMV-Tag 2B. Two mutated Rev genes, Rev-M6 and Rev-M10 [18], were constructed by PCR-based site-directed mutagenesis using the plasmid pCMV-Tag2B-Rev as template, then

inserted into pCMV-Tag 2B. In Rev-M6, four amino acid residues (R41, R42, R43, and R44) were substituted with aspartic acid (Asp) and leucine (Leu), resulting in a two amino acid deletion. In Rev-M10, two amino acid residues (L78 and E79) were substituted with Asp and Leu. The coding sequences of Rev, Rev-M6, and Rev-M10 were subcloned into the bacterial expression vector pET-28a. The monocistronic reporter plasmid pHCV5′ + 3′ was then constructed. It contained the following elements in a 5′ to 3′ order in the vector PMD18-T Simple (Takara, Shiga, Japan): T7 promoter, the complete 5′-UTR (nucleotides [nt] 1–340) plus a 25 amino acid core protein-encoding sequence from strain HCV-2a, ubiquitin, the firefly luciferase (FLuc) gene, and the entire HCV 3′-UTR.

All DNA used for the in vitro transcription of HIV RNA was derived from the plasmid PNL4-3 by PCR. The DNA used for the in vitro transcription of green fluorescent protein (GFP) mRNA

was generated from the plasmid peGFP-C1 by PCR. All DNA used for the in vitro transcription of HCV RNA was derived from the plasmid pHCV5′ + 3′ by PCR. The DNA used for the in vitro transcription of HIV REE RNA was generated from the HIV-1 Env gene (nt 7300–7550) by PCR. The DNAs generated in these reactions were downstream of the T7 promoter sequence.

2.3. In vitro transcription

RNA was transcribed with a MEGAscript™ T7 kit (Ambion, Austin, TX, USA) and purified with a MEGA clear kit (Ambion) according to the manufacturer's protocol. FL-J6/JFH-5′C19Rluc2AUbi RNA was generated essentially as described previously [16]. The RNA probe was labeled at the 3′-end using Biotin-16-UTP (Roche, Indianapolis, IN, USA) and T4 RNA ligase (Promega, Madison, WI, USA) and again purified by gel electrophoresis.

2.4. Luciferase reporter gene assay

To harvest samples for the luciferase assays, cells were washed once with PBS, followed by the addition of 100 µl of lysis buffer (Promega) to each well of the 24-well plate. Each sample (50 µl) was then mixed with luciferase assay substrate (Promega). Luciferase activity was typically measured for 10 s using a luminometer. All assays were performed in triplicate; the results are expressed as the mean ± s.d. of luciferase activity.

2.5. In vivo immunoprecipitation assay

Huh7.5.1 cells were infected with JFH1 for four days and then transfected with pCMV-Tag 2B-Rev. Post-transfection (48 h), the cells were collected. In vivo immunoprecipitation assays were conducted as described previously [19]. The RNA was extracted and analyzed by RT-PCR using HCV RNA-specific primers (forward: 5′-TCGTATGATACCCGATGCT-3′ and reverse: 5′-GTTTGACCCTTG CTGTTGA-3′).

2.6. Protein expression and purification

The plasmids pET-28a-Rev, pET-28a-Rev-M6, and pET-28a-Rev-M10 were transformed into Escherichia coli strain BL21. Protein expression and purification were conducted as described previously [20].

2.7. Native gel mobility shift assay

HCV 5′-UTR RNA was renatured by incubation at 95 °C for 3 min and cooling to 25 °C for 10 min. The RNA and Rev protein were then incubated together in binding buffer (20 mM Tris–Cl [pH 7.5], 5 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 2 U of RNasin) at 37°C for 20 min. The samples were then loaded onto a native 8% polyacrylamide gel and transferred to nitrocellulose membranes (Minipore, Billerica, MA, USA). The membranes were then blocked with 10% fat-free powdered milk in PBST for 30 min, incubated with HRP-Streptavidin (GE) for 1 h, washed, incubated with HRP substrate luminol reagent (Millipore), and analyzed using a Luminescent Image Analyzer (Fujifilm LAS-4000, Tokyo, Japan).

3. Results

3.1. Rev activates HCV gene expression and interacts with HCV RNA

Huh7.5.1 cells were co-transfected with FL-J6/JFH-5′C19Rluc2AUbi RNA and the mRNA for each HIV-1 gene, respec-

tively. The Renilla luciferase activity of FL-J6/JFH-5′C19Rluc2Aubi was measured 12 h post-transfection. Rev stimulated HCV gene expression by 3.8-fold while Tat enhanced HCV gene expression by 2.0-fold. The other HIV-1 proteins had no effect on HCV gene expression (Fig. 2A). To confirm the effect of Rev on HCV gene expression, we constructed Rev-M6 and Rev-M10, two well-characterized dominant-negative Rev mutants [18]. Huh7.5.1 cells were co-transfected with FL-J6/JFH-5′C19Rluc2AUbi RNA and mRNA encoding Rev, Rev-M6, Rev-M10, or GFP, respectively. Wild-type Rev activated HCV gene expression, whereas the two mutants (Rev-M6 and Rev-M10) did not (Fig. 2B). Huh7.5.1 cells were co-transfected with FL-J6/JFH-5′C19Rluc2AUbi RNA and the plasmid PNL4-3 or the control plasmid pUC18. The cells were harvested and analyzed for Renilla luciferase activity at 24 h post-transfection. We found that PNL4-3 stimulated HCV gene expression (Fig. 2C).

We further verified the effect of Rev on JFH1 viral gene expression, a genotype 2a HCV isolate [21]. Huh7.5.1 cells were infected with JFH1 virus and then transfected with Rev, Rev-M6, Rev-M10, or GFP mRNA. The cells were collected and analyzed by Western blotting. The HCV core protein level was increased in the presence of Rev, but not in the presence of Rev-M6, Rev-M10, or GFP (Fig. 2D).

To explore the mechanism of the activation of HCV gene expression by Rev, we investigated whether Rev interacts with HCV RNA using an in vivo immunoprecipitation assay. An HCV-specific product was obtained in the input and supernatant fractions of the samples (Fig. 2E, lanes 1 and 2). This product was also observed in the pellet fraction in the presence of anti-Rev antibodies (Fig. 2E, lane 3), but not in the pellet fraction in the presence of anti-GFP antibodies (Fig. 2E, lane 4) or in the absence of antibodies (Fig. 2E, lane 5).

3.2. The arginine-rich region of Rev binds specifically to HCV 5′-UTR RNA

Labeled 5′-UTR RNA (nt 1–340) was incubated with purified Rev protein at different concentrations (0.05–0.25 µM). An electrophoretic mobility shift assay (EMSA) showed that a protein-RNA complex formed in the presence of Rev protein (Fig. 3A, lane 2), and that the intensity of the complex increased as the concentration of Rev increased (Fig. 3A, lanes 2–4).

We next assessed the binding specificity of Rev to HCV 5′-UTR RNA. First, we analyzed and compared the binding abilities of Rev, Rev-M6, Rev-M10, and bovine serum albumin (BSA) to HCV 5′-UTR RNA. A protein-RNA complex did not form in the presence of BSA (Fig. 3B, lane 5). Wild-type Rev and Rev-M10 bound the HCV 5′-UTR RNA (Fig. 3B, lanes 2 and 4), but the binding activity of the Rev-M6 mutant to HCV 5′-UTR RNA was significantly reduced (Fig. 3B, lane 3).

Second, the binding ability of Rev to the HCV 5′-UTR was evaluated using a competitive EMSA. Unlabeled HCV 5′-UTR RNA competed for the interaction of Rev with the HCV 5′-UTR (Fig. 3C, lanes 3 and 4). Unlabeled HIV-1 RRE RNA also competed with Rev (Fig. 3C, lanes 5 and 6), but GFP mRNA, which served as a negative control, did not affect the interaction (Fig. 3C, lanes 7 and 8).

3.3. Rev binds specifically to the first internal loop (IIIb) of HCV 5′-UTR RNA

To identify the Rev binding region in HCV 5′-UTR RNA, we generated two shorter HCV 5′-UTR RNA fragments A1–120 (nt 1–120) and A1–290 (nt 1–290) (Figs. 1 and 4A, right panels). Our EMSA results showed that a protein-RNA complex formed in the presence
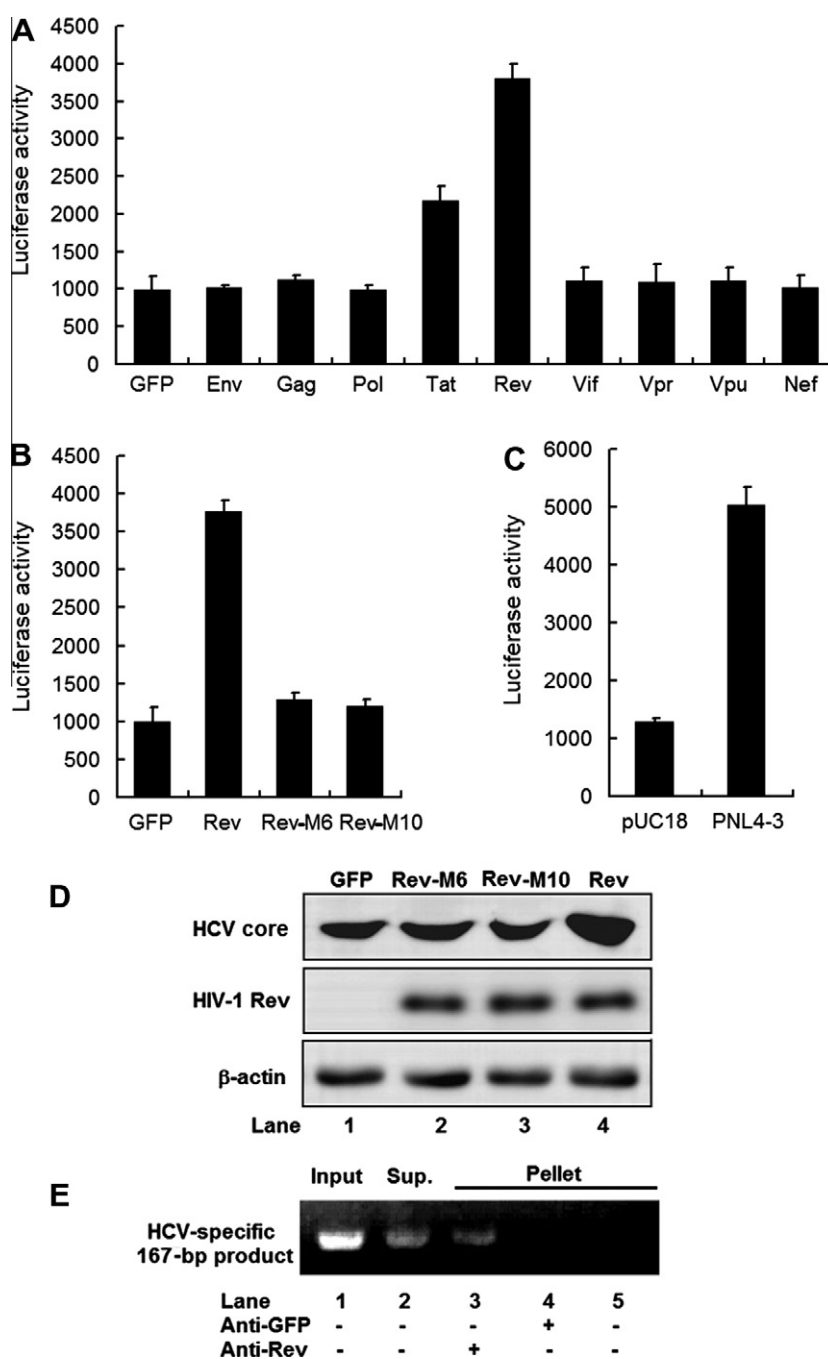
Fig. 2. Rev enhances HCV gene expression. (A) Huh7.5.1 cells were cotransfected with FL-J6/JFH-5′C19Rluc2AUbi RNA (0.1 μg) and HIV gene mRNAs (0.3 μg each) or GFP mRNA (0.3 μg). Renilla luciferase activity was then measured in the transfected cells. (B) Huh7.5.1 cells were cotransfected with FL-J6/JFH-5′C19Rluc2AUbi RNA and mRNA encoding Rev, Rev-M6, Rev-M10, or GFP. (C) Huh7.5.1 cells were cotransfected with FL-J6/JFH-5′C19Rluc2AUbi RNA (0.1 μg) and the plasmid PNL4-3 (0.3 μg) or pUC18 (0.3 μg). (D) Huh7.5.1 cells were infected with JFH1 (MOI = 0.01) for 4 days, and then transfected with mRNA encoding Rev, Rev-M6, Rev-M10, or GFP (1.5 μg each) for 48 h. HCV core, HIV-1 Rev, and β-actin were detected by Western blotting with specific antibodies. (E) The binding ability of Rev to HCV RNA was determined by *in vivo* immunoprecipitation. Coimmunoprecipitated HCV RNA was detected by RT-PCR. *Abbreviation:* Sup., supernatant.

of Rev and labeled full-length HCV 5′-UTR RNA (A*) or labeled mutant HCV 5′-UTR RNA (A1–290*) (Fig. 4A, lanes 2 and 4). However, a protein-RNA complex did not form in the presence of Rev and labeled mutant HCV 5′-UTR RNA (A1–120*) (Fig. 4A, lane 6).

To confirm the Rev binding region in HCV 5′-UTR RNA, we generated a series of HCV 5′-UTR RNA deletion mutants. Mutant A1 (nt 189–206 were deleted) was generated by deleting the terminal loop (IIIb). Mutant A2 (nt 176–221 were deleted) was created by further truncating the first internal loop in domain III. Mutant A3 (nt 144–245 were deleted) was constructed by removing loops

IIIa–c (Figs. 1 and 4B, right panels). All deletion mutants introduce a GAAA sequence at the tip of the truncation as described previously [22]. Our EMSA results showed that a protein-RNA complex formed in the presence of Rev and wild-type HCV 5′-UTR RNA (A*) or mutant A1* (Fig. 4B, lanes 2 and 4), but not with mutant A2* or A3* (Fig. 4B, lanes 6 and 8).

To confirm that the binding site for Rev is located in the first internal loop (IIIb) of the HCV 5′-UTR, two additional mutants were constructed (Figs. 1 and 4C, right panels). We constructed mutant A4, in which two amino acids (A214 and G216) were deleted, and
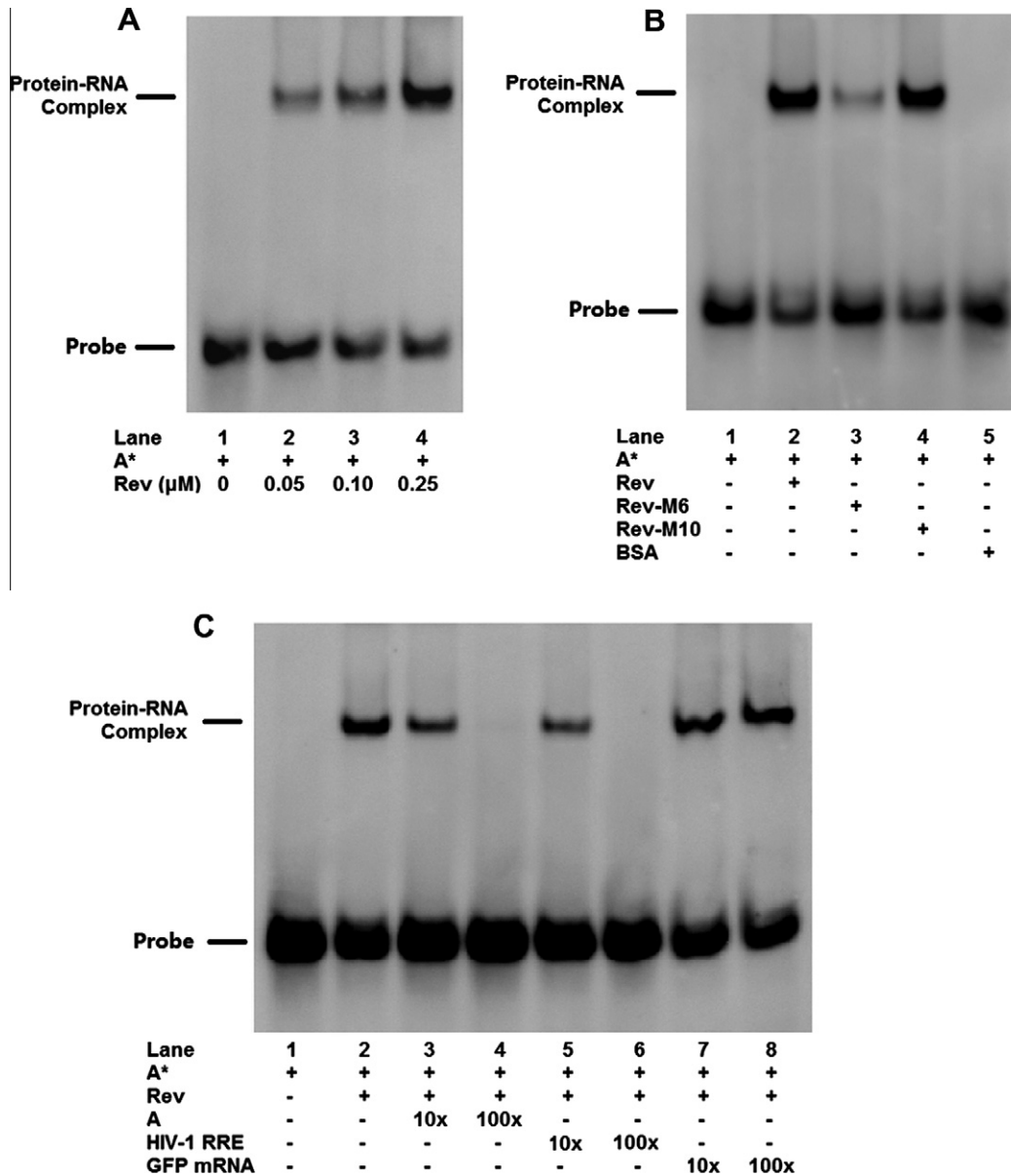
Fig. 3. Binding of Rev to HCV 5′-UTR RNA by EMSA. (A) Labeled HCV 5′-UTR RNA (nt 1–340) (0.1 μg) was incubated with purified Rev (0–0.25 μM). The protein-RNA complex was separated from free RNA and examined by an EMSA. (B) Labeled HCV 5′-UTR RNA (lanes 1–6) was incubated with DMSO (lane 1), Rev (lane 2), Rev-M6 (lane 3), Rev-M10 (lane 4), or BSA (lane 5). (C) Labeled HCV 5′-UTR RNA (lanes 1–8) was incubated with DMSO (lane 1) or Rev (lanes 2–8) in the presence of a 10- or 100-fold molar excess of unlabeled competitor RNA: A (lanes 3 and 4), HIV-1 RRE RNA (lanes 5 and 6), and GFP mRNA (lanes 7 and 8). A* indicates labeled HCV 5′-UTR RNA; A indicates unlabeled competitor HCV 5′-UTR RNA.

mutant A5, in which five amino acids (A180, A181, U213, A214, and U215) were substituted by five purines (G). EMSA revealed that a protein-RNA complex formed in the presence of Rev and the wild-type (A*) or mutant HCV 5′-UTR (A5*) (Fig. 4C, lanes 2 and 6), but not with mutant A4* (Fig. 4C, lane 4). In addition, in the presence of mutant A5*, the intensity of the complex was reduced (Fig. 4C, lane 6 vs. lane 2).

3.4. Rev binds within the HCV 5′-UTR and activates HCV IRES RNA translation

To analyze the role of Rev in the regulation of HCV IRES-mediated translation, we generated three monocistronic reporters, HCV 5′-Luc-3′, HCV 5′-Luc, and HCV Luc-3′ (Fig. 5A). Huh7.5.1 cells were co-transfected with RNA encoding HCV 5′-Luc-3′, HCV 5′-Luc, or HCV Luc-3′ and mRNAs encoding Rev, Rev-M6, Rev-M10, or GFP,

respectively. Cells were collected and the lysates were used for luciferase activity assays. To determine the effect of Rev on HCV IRES-mediated translation, the translation of the three RNAs was set to 1 in the GFP mRNA-transfected cells. The HCV 5′-Luc-3′, HCV 5′-Luc, and HCV Luc-3′ RNAs were not translated in the presence of GFP, Rev-M6, and Rev-M10 (Fig. 5B). In the presence of Rev, translation of the HCV 5′-Luc-3′ and HCV 5′-Luc RNAs was significantly stimulated; however, there was no translational activity of HCV Luc-3′ RNA (Fig. 5B).

To investigate the role of loop IIIb in the activation of HCV IRES-mediated translation regulated by Rev, two HCV 5′-Luc-3′ mutants were generated: HCV 5′-Luc-3′ A4, which contained two adenine deletions within internal loop IIIb of the HCV 5′-UTR, and HCV 5′-Luc-3′ A5, which carried five guanine-to-adenine nucleotide substitutions within internal loop IIIb of the HCV 5′-UTR. Huh7.5.1 cells were co-transfected with HCV 5′-Luc-3′ A4 or HCV 5′-Luc-3′ A5
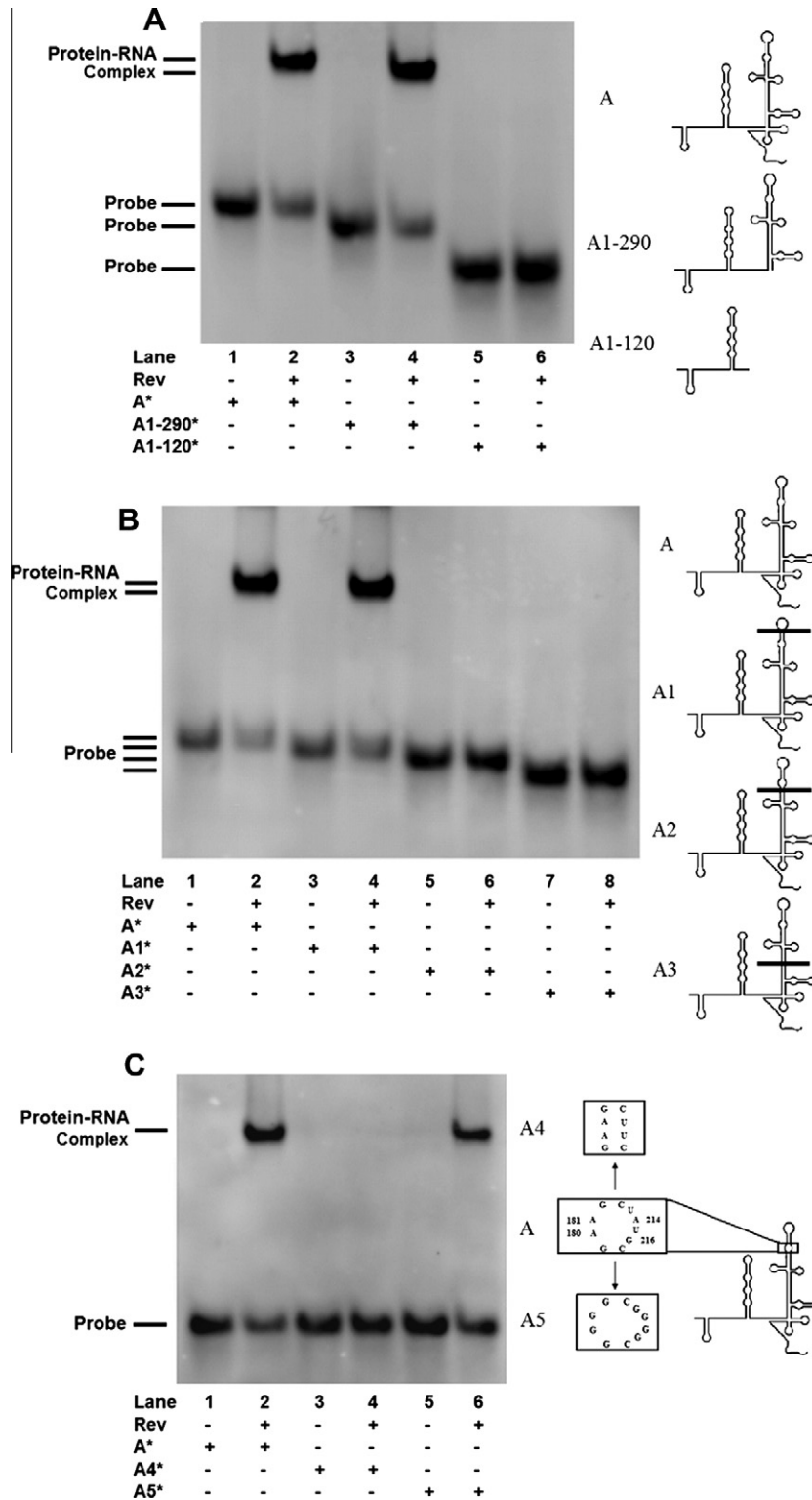
Fig. 4. Effects of mutations in the HCV 5′-UTR on the binding of Rev. (A) Effect of truncations in the HCV 5′-UTR on Rev binding. (B) Effect of sequential deletions in domain III of the HCV 5′-UTR on Rev binding. (C) Effect of mutations in internal loop IIIb of the HCV 5′-UTR on Rev binding.

RNA and Rev or GFP mRNA, respectively. Translation of the three RNAs was set to 1 in the GFP mRNA-transfected cells. In the presence of Rev, the translation of HCV 5′-Luc-3′ RNA was significantly enhanced, while there was no translation of HCV 5′-Luc-3′ A4 RNA (Fig. 5C). In addition, Rev activated the translation of HCV 5′-Luc-3′ A5 RNA, although the level of translation was reduced (Fig. 5C).

4. Discussion

In this study, we demonstrated that HIV-1 Rev protein stimulated HCV gene expression. Rev interacts with both HIV-1 RNA and with the RNAs of heterogenetic viruses. For example, Rev mediates nuclear export through the Rec/cORF-responsive element, an

RRE functional homolog in the 3′-LTR of the human endogenous retrovirus HERV-K [23]. In addition, Rev heterologously promotes expression of the simple beta retrovirus mouse mammary tumor virus (MMTV) and binds directly to RNA in the U3 region [24,25]. Our immunoprecipitation results demonstrate that Rev can bind to HCV RNAs.

An EMSA showed that Rev binds directly to HCV 5′-UTR RNA and that the arginine-rich 41-44 amino acid region of Rev contributes to this binding activity. We generated a series HCV 5′-UTR truncation mutants and analyzed their interactions with Rev. We found that domains IIIa–d of the 5′-UTR were crucial for Rev binding. The three-dimensional structure of loop A in the HIV-1 5′-UTR can be directly superimposed onto that of the HIV-1 RRE internal loop (Rev-binding site) [26], and loop A is also a Rev-binding site (Fig. 1B) [27]. The three-dimensional structure of loop IIIb in HCV is very similar to the RRE internal loop in HIV-1 [28] and loop A in the HIV-1 5′-UTR [26]. Based on this information, we examined whether Rev binds to loop IIIb in the HCV 5′-UTR. Using HCV 5′-UTR deletion mutants, we showed that Rev binds specifically to this region. This suggests that the nucleotide-amino acid interactions involved in the binding of Rev and internal loop IIIb are

analogous to those identified previously in the Rev-RRE or Rev-loop A complex.

In our study, we evaluated the function of Rev in translation mediated by HCV IRES. Consistent with an enhanced polysomal association of RRE-containing RNAs, Rev may also regulate the translation of HIV-1 RNA [11,29]. It is believed that Rev plays a direct role in HIV-1 RNA translation [30]. Employing monocistronic HCV reporter RNAs, we found that Rev specifically stimulated translation mediated by the HCV IRES. Loop A in the HIV-1 5′-UTR is known to specifically bind Rev [27], and it is believed to play a role in the Rev-mediated stimulation of translation [11]. We demonstrated that Rev binds directly to loop IIIb in the HCV 5′-UTR. We next investigated the role of this binding site in the activation of HCV IRES-mediated translation regulated by HIV-1 Rev. We found that Rev stimulated the translation of HCV RNAs. Also, this stimulatory activity was dependent on an intact internal loop (IIIb) in the HCV 5′-UTR. Thus, we conclude that Rev, by binding to the HCV 5′-UTR, enhances HCV IRES-mediated translation.

The mechanism of Rev binding to the HCV 5′-UTR and the activation of translation mediated by HCV IRES is not well characterized. However, Rev is known to enhance the association of
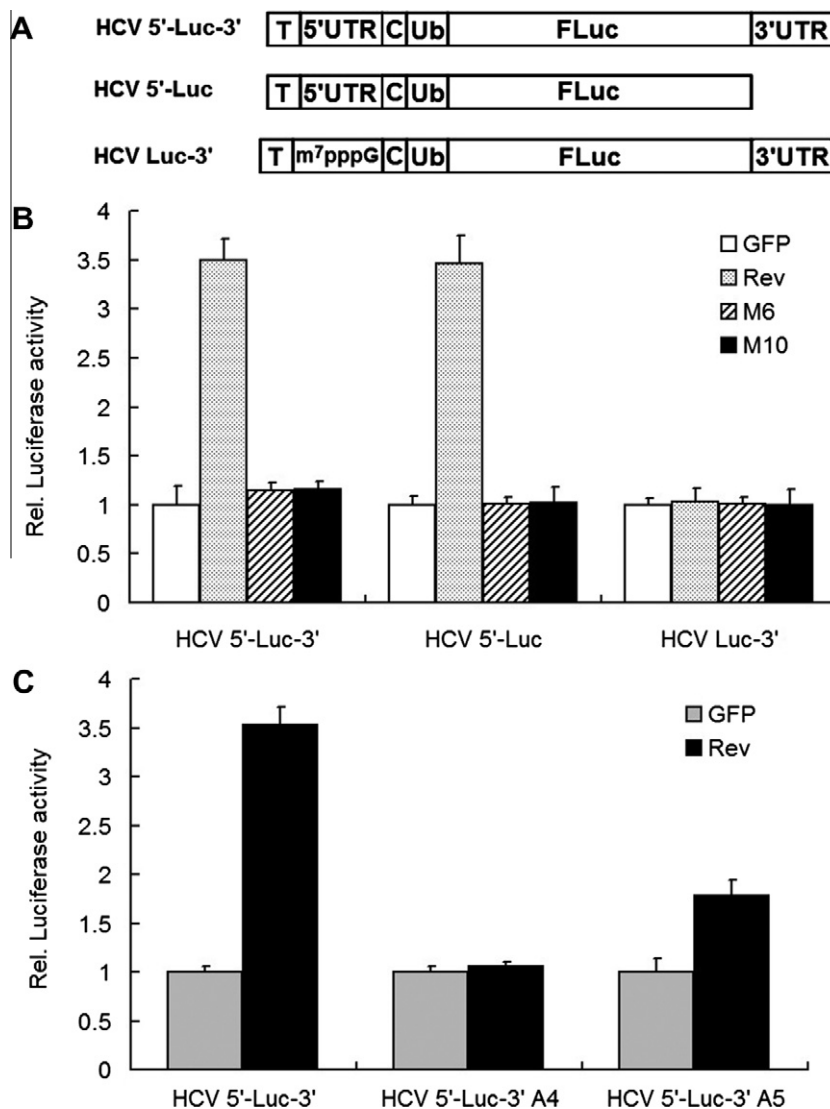

Fig. 5. Rev regulates translation mediated by the HCV 5′-UTR. (A) Schematic diagram of the HCV 5′-Luc-3′, HCV 5′-Luc, and HCV Luc-3′ RNAs. T7: T7 promoter; 5′UTR: the complete HCV 5′-UTR (nt 1–340); C: 25 amino acids of the core protein-encoding sequence from strain HCV-2a; Ub: ubiquitin; FLuc: firefly luciferase gene; 3′ UTR: the entire HCV 3′-UTR. (B) Rev increases translation mediated by the HCV 5′-UTR. (C) Internal loop IIIb is involved in the activation of HCV 5′-UTR-mediated translation regulated by Rev.

Rev-dependent viral RNA with polysomes [31]. It is possible that the binding of Rev to internal loop IIIb acts as a signal that initiates HCV RNA recognition by the translational machinery. The IRES of HCV drives translation by directly recruiting 40S ribosomal subunit and binds to eIF3. Rev may enhance HCV IRES-mediated translation by recruiting components involved in the initiation of translation, including the 40S ribosomal subunit and eIF3. In addition, Rev dramatically stabilizes RRE-containing HIV-1 RNA transcripts in the nucleus [32]. The interaction between Rev and the HCV 5′-UTR may stabilize the IRES secondary structure of HCV, which facilitates the translation initiation complex assembly.

In summary, this study is the first to demonstrate that HIV-1 Rev binds directly to the HCV 5′-UTR and stimulates HCV gene expression. In addition, we found that the binding site for Rev is located in internal loop IIIb of the HCV 5′-UTR. These results provide insight into the coinfection of HIV-1 and HCV.

Acknowledgements

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