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Hijacking hepatitis C viral replication with a non-coding replicative RNA

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ABSTRACT

The current treatments used against RNA viruses have a limited efficacy and are often hampered by the induction of side-effects. The specific delivery of antiviral proteins in infected cells should increase their efficiency and reduce their impact on healthy cells. Here, we describe the development of a new approach which takes advantage of the viral replication machinery to specifically target the antiviral protein expression to the infected cells. The strategy is based on the delivery of a non-coding (−)RNA carrying the structures required for the binding of the viral replication complex and the complementary sequence of an antiviral gene. The viral replication complex replicates the (−)RNA similarly to the viral genome to give a coding (+)RNA from which the antiviral protein will be expressed. As non-infected cells do not express the replication complex, this specific machinery can be used to target virus-infected cells without affecting healthy cells. We show that this approach can be successfully applied to the hepatitis C virus. In both replicon-harboring cells (genotype 1b) and JFH-1 infected cells (genotype 2a), nrRNAs induced a strong decrease in genomic RNA and viral protein NS5A. These effects were correlated with a strong activation of several interferon-stimulating genes.

1. Introduction

Numerous (+)RNA viruses use an RNA-dependent RNA polymerase (RdRp) included in a replication complex (RC) to replicate their genome. Replication consists in two steps: first, the (+)RNA is the template for the synthesis of a non-coding (−)RNA and second, the latter serves as a replication intermediate used by the RC to synthesize new (+)RNAs. This RNA synthesis mechanism requires the recognition by the RC of specific binding sites often localized at the 3′ ends of the (+) and (−)RNAs (Ortin and Parra, 2006). The couple formed between the RC and these binding sites is specific to the virus and is not exist in non-infected cells. This feature could be leveraged to deliver antiviral molecules in infected cells using a non-coding (−)RNA harboring the RC binding sites and carrying the complementary sequence of an antiviral gene. From this (−)RNA, the RC will synthesize a coding (+)RNA which will be translated to produce the antiviral protein. Because only infected cells express the RC, healthy cells should not produce coding RNA and will not be altered. In this work, we explore the feasibility of using nrRNA on hepatitis C virus (HCV)-infected cells.

The HCV genome consists in a single-stranded (+)RNA that contains a unique long ORF that is translated as a polyprotein of about 3010 amino acids (Clarke, 1997) and is flanked by two untranslated regions (UTR). The polyprotein is processed by viral and cellular proteases into the structural and non-structural proteins. The non-structural proteins from NS3 to NS5B (NS5B being the viral RdRp) associate with cellular proteins to form the replication complex (RC). Sequences required for replication have been identified in the 5′ UTR (Friebe et al., 2001; Luo et al., 2003) and in the 3′ UTR (Cai et al., 2004; Friebe and Bartenschlager, 2002; Yi and Lemon, 2003) and we have already described a minimal sequence required for efficient replication and expression of a reporter gene (Dumas et al., 2007). We thus designed RNA molecules able to allow the expression of antiviral proteins only in HCV-infected cells. This restricted expression is ensured by the use of non-coding replicative RNAs named “nrRNAs” harboring the binding sites for the RC. The replication of these (−)RNAs allows the synthesis of coding (+)RNAs that could then be translated to produce antiviral proteins. Since the RC is specific to infected cells, the effects of these antiviral molecules should be restricted to these cells, thereby increasing their efficiency and decreasing their side-effects.

Abbreviations: HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; RC, replication complex; UTR, untranslated region.

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The success of this strategy depends on the ability of the viral RC to replicate an exogenous RNA in place of the viral genome. This capacity has long been thought to be compromised in HCV-infected cells and in the commonly used replicon cellular model, where replication takes place in lipid raft structures inaccessible to external proteins (Aizaki et al., 2004; El-Hage and Luo, 2003). Nevertheless, at least in the replicon cellular context, the replication machinery is accessible to other RNA templates, since we previously showed that a minigenome RNA constituted by a reporter gene flanked by HCV 5′ and 3′ UTRs was efficiently replicated in these cells (Dumas et al., 2007). Thus, the primary prerequisite for testing the strategy is fulfilled and opens the way to selectively expressing a protein in infected cells.

Anti-HCV treatment, which associates interferon-α (IFN-α) and ribavirin, is known to be more efficient than interferon monotherapy, but it induces a sustained response in only 42–82% of infected people, depending on HCV genotypes (Shepard et al., 2005). Moreover, adverse effects hamper the use of these molecules. In this report, we show that expressing IFN-α and one of its regulatory factors through these non-coding RNAs is efficient against HCV replication in two current cellular models of HCV replication.

2. Materials and methods

2.1. Plasmids and vectors

The pGEM-T/5UTR-EGFP-3UTR and pGEM-T/5UTR-H2AE-3UTR vectors were constructed as previously described (Dumas et al., 2007). The ricin chain A gene was obtained by PCR amplification using Ric_Start and Ric_Stop primer (Table 1). It was introduced into the pGEM-T/5UTR-EGFP-3UTR plasmid by exchanging the EGFP gene with ricin chain A gene between BamHI and XbaI sites to obtain the pGEM-T/5UTR-Ric-3UTR plasmid. The IFN-α gene and the IRF-1 gene coding for the interferon regulatory factor 1 were obtained by PCR amplification from pDONR201-IFN-A17 and pDNR-Dual-IRF-1 plasmids (DF/HCC DNA Resource Core, Harvard Medical School) using IFN-S plus IFN-AS and IRF-1-S plus IRF-1-AS primers respectively (Table 1).

2.2. In vitro transcription

PCR primers 5UTR_Start and T7_3UTR were designed to introduce a T7 RNA polymerase promoter in the correct orientation (Table 1). PCR was performed with the Taq Phusion DNA polymerase (Ozyme). RNAs were synthesized using the MEGAscript kit (Ambion). DNA templates were digested with DNase I for 15 min. After acidic phenol/chloroform extraction, the RNAs were precipitated with LiCl. The purity and the integrity of RNAs were determined by analysis on a 5% polyacrylamide gel containing 7 M urea in TBE buffer (90 mM Tris–borate pH 8.0, 1 mM EDTA). The RNA used as a control in transfection experiments consists in an RNA molecule of the same length as the nrRNA (1500 nts) and constituted by part of the β-galactosidase RNA without the HCV UTRs. The RNA used to quantify the transfection efficiency consists in the already described (+)5UTR-EGFP-3UTR minigenome (Dumas et al., 2007).

2.3. Cell culture and transient RNA transfection

All cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin (50 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Transient transfections were done by seeding 24-well or 96-well plates respectively with 10^5 or 2.5×10^4 of the appropriate cells, 24 h before transfection. Each RNA transfection point was performed using 900 ng of RNA combined with 2.1 μl of DMRIE-C (Invitrogen) for 24-well plates and 50 ng of RNA combined with 0.5 μl of DMRIE-C for 96-well plates, according to the manufacturer's instructions. To determine the efficiency of transfection, cells were transiently transfected with (+)5UTR-EGFP-3UTR minigenome. Fluorescence intensity was directly measured 24 h post-transfection by flow cytometry (Beckton-Dickinson, FACS Calibur).

Table 1
Oligonucleotides used in cloning and PCR experiments. The sequence corresponding to the T7 RNA polymerase promoter is underlined.

Oligonucleotides	Sequence 5′–3′
Ric_Start	CACACAGGATCCATATTTCCCAAACAATACCCAATC
Ric_Stop	ATATATTCTAGATTACTAAAACTGTGAGCTCGG
IFN_S	ATATATATCTAGACTAGGCGGGCTGCTCCAGCTCC
IFN_AS	ATATATTCTAGATCAATCCTTCCTCCTTAATATTTTTTGC
IRF-1_S	ATATATGGATCCCCCATCACTCGGATGCGCATG
IRF-1_AS	ATATATTCTAGACTACGGTGCACAGGGAATGGC
5UTR_Start	GCCAGCCCCCGATTGGGGGCG
T73UTR	TAATACGACTCACTATAGGACTTGATCTGCAGAGAGGCCAG
NS5B_S	CCATAGTTACTCTCCAGGTGAGATC
NS5B_AS	GTGTTTAGCTCCCCGTTCA
NS3_S	AAGTCTTTGGAGCGGTGCAA
NS3_AS	TGTCTCAACGGGGATGAAAT
SFRS4_S	AAAAGTCGGAGCAGGAGTCA
SFRS4_AS	CTCTTCCTGCTCTTCCTCTT
MxA_S	ATC GAC CTC ATT GAC TCC C
MxA_AS	TAA CTG ACC TTG CCT CTC C
OAS2_S	AGA GCA ATG GGA AAT GGG G
OAS2_AS	GTC ACT GAA GAA GAG GAC AAG
ISG15_S	TGG ACA AAT GCG ACG AAC
ISG15_AS	CAT GAA CAC GGT GCT CAG

2.4. Establishment of cell lines

The Huh7/Rep5.1 cell line was established after electroporation with the Rep5.1 RNA replicon (kindly provided by R. Bartenschlager) and selection with 500 μg/ml G418. The cells were aliquoted and frozen after 1 month of culture. The Huh7/Rep5.1 cell line was cured from the Rep5.1 replicon by a 3-week treatment with 150 U/ml interferon-α. The resulting cell line was name Huh7/QR. Cured Huh7 cells (Huh7/QR) were transfected with 1 μg of the JFH-1 RNA (kindly provided by Wakita et al., 2005) and 3 μl of DMRIE-C as described by the supplier. After 4 days of culture, the viral production was quantified by quantitative RT-PCR then, after expansion, the virus-producing cells were aliquoted and frozen.

2.5. Coomassie blue staining of cell colonies

Hygromycin-resistant cells were washed twice in PBS and fixed in 3% formaldehyde for 10 min at room temperature. After two more PBS washes, cells were stained for 3 min with Coomassie blue. Petri dishes were then unstained with 10% methanol/10% acetic acid, dried, and colonies were counted.

2.6. RNA isolation

Total RNA was extracted from cells using the total RNA isolation reagent (Ademtech) according to the manufacturer's instructions and then resuspended in 35 μl of H_2O treated with diethylpyrocarbonate.

2.7. Quantitative RT-PCR

The NS5B-S and NS5B_AS primers (Table 1) were designed to amplify part of the NS5B region of the HCV replicon RNA. The NS3_S and NS3_AS (Table 1) were designed to amplify part of the NS3 region of the HCV JFH-1 RNA. Because several housekeep-

ing genes including GAPDH and β-actin are deregulated by HCV replication, we chose to normalize the number of HCV RNA on the SFRS4 RNA whose level has been shown to be relatively stable in HCV-infected cells (Waxman and Wurmbach, 2007). SFRS4_S and SFRS4_AS primers (Table 1) were used. Reactions were performed in 25 μl using SYBRGreen (iScript one-step RT-PCR kit, Bio-Rad), with primers at 300 nM, under the following conditions: Step 1, RT 10 min at 50 °C; Step 2, 5 min at 95 °C; Step 3, 40 cycles of 10 s at 95 °C, 30 s at 61.5 °C, and 30 s at 72 °C. SYBRGreen fluorescence was measured after 30 s at 84 °C except for NS5B replicon RNA amplification, where SYBRGreen fluorescence was measured at the end of the elongation step at 72 °C. NS5B replicon, NS3 JFH-1, and SFRS4 copy number were determined by comparison with specific serially diluted transcripts included in the RT-PCR analysis. To ensure that no inhibitor was present in extracted RNA, qRT-PCR reactions were performed on 5 μl of cellular RNA diluted 1:10, 1:30 and 1:90. The copy number was calculated as the mean of this triplicate after applying the dilution factor.

Part of the human ISG15 ubiquitin-like modifier gene (Genebank accession no. NM_005101), the common part of the 2′-5′-oligoadenylate synthetase 2 transcript variants 1, 2 and 3 (Genebank accession nos. respectively NM_016817, NM_002535, and NM_001032731) and part of the MxA human p78 protein (Genebank accession no. M33882.1) were amplified using ISG15_S/ISG15_AS, OAS2_S/OAS2_AS, and MxA_S/MxA_AS (Table 1). Reactions were performed in 25 μl using SYBRGreen (iScript one-step RT-PCR kit, Bio-Rad), with primers at 300 nM, under the following conditions: Step 1, RT 10 min at 50 °C; Step 2, 5 min at 95 °C; Step 3, 42 cycles of 10 s at 95 °C, 30 s at 61 °C, and 30 s at 72 °C. SYBRGreen fluorescence was measured after 30 s at 68 °C. To ensure that no inhibitor was present in extracted RNA, RT-qPCR reactions were performed on 5 μl of cellular RNA diluted 1:5, 1:15, and 2:45. The fold induction of ISG mRNA was calculated with the ΔΔCt formula against SFRS4 housekeeping gene and a control condition where cells were transfected with part of the β-galactosidase RNA as control.

2.8. Cytotoxicity analysis

After 48 h of culture, the medium of transfected cells was replaced by 80 μl of fresh medium and 20 μl of CellTiter (Promega) were added to each well. The optical density was measured 1 h later using a 492-nm filter. The number of viable cells was calculated from a standard curve established by the same method, 4 h after seeding 5×10^3–10^5 living cells.

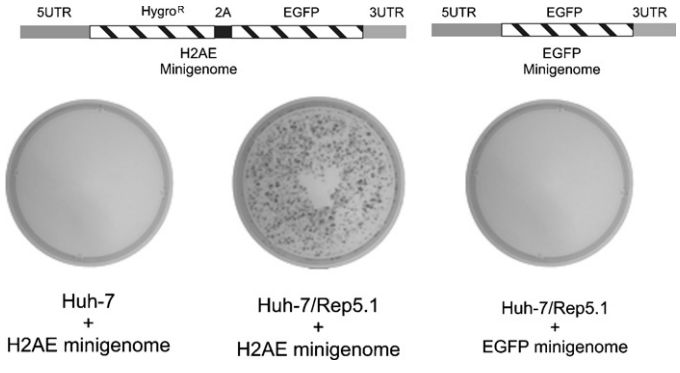

Fig. 1. H2AE minigenomes are not replicated in cells devoid of RC. Upper panel: schematic representation of the H2AE and EGFP minigenomes. Lower panel: 1 μg of the H2AE (+)minigenome was transfected into 10^5 Huh7 or Huh7/Rep5.1 cells with 3 μl DMRIE-C. As a control, 10^5 Huh7/Rep5.1 cells were transfected with the EGFP (+)minigenome in the same conditions. After 21 days of culture in the presence of 50 μg/ml hygromycin, colonies were stained with Coomassie blue. Results are representative of at least 3 independent experiments.

2.9. Immunolabeling

Huh7/Rep5.1 cells were plated on coverslips at 75,000 cells/well in 24-well plates, transfected as described above, and then cultured for 72 h. After two washes with PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and pH 7.4), cells were fixed with 200 μl of 3% paraformaldehyde for 15 min. Cells were washed twice, then incubated with 200 μl of PBS 1× Saponin 0.1% for 10 min. Cells were immediately incubated for 1 h with 200 μl of anti-NS5A antibody in PBS plus 10% fetal calf serum for 1H (Brass et al., 2002) and washed three times with PBS plus 5% fetal calf serum. Cells were incubated for 1 h with anti-mouse IgG antibody labeled with Alexa 488 and after 4 washes in PBS, coverslips were sealed onto glass slides using 5 μl of Fluorescence Mounting Medium (FluoroMount, Interchim) with 2 pg ml^{-1} 4′-6-diaminido-2-phenylindole (DAPI, Sigma). Cells were observed with a Zeiss Axiovert 40 using the 40× lens.

3. Results

3.1. Non-coding replicative RNAs are harmless for healthy cells

It should be possible to restrict the expression of an antiviral protein in the infected cells using a non-coding (−)RNA harboring the HCV sequences required for efficient replication by the HCV RC. After a replication step by the RC, this non-coding (−)RNA should generate a coding (+)RNA which, due to the IRES activity contained in the HCV 5′ UTR, would be translated and give rise to the antiviral protein. For these reasons it was named “non-coding replicative RNA” or nrRNA. For this strategy to be efficient, the healthy cells should not be affected.

Previous results using RNA minigenome, a nrRNA complementary strand, already indicated that no replication occurs in healthy cells (Dumas et al., 2007). This reporter RNA minigenome (H2AE), where the HCV UTRs flanked the sequences encoding hygromycin phosphotransferase and EGFP both linked by the FMDV 2A sequence, was used to reinforce this finding with a more sensitive approach. We transfected Huh7 cells and Huh7 cells constitutively expressing the RC from replicon (Huh7/Rep5.1) with this H2AE minigenome. Then the cells were cultured in the presence of hygromycin. After 21 days of selection, no hygromycin-resistant cell colonies were visible upon transfection of Huh7 cells with the (+)H2AE minigenome, while Huh7/Rep5.1cell colonies resistant to hygromycin and G418 were always observed (Fig. 1). In repeated experiments, the number of cell colonies replicating the H2AE minigenome varied from 2898 to 9753 for 10^5 transfected cells and 1 μg RNA, while no colony was obtained with the EGFP minigenome. We concluded that the minigenome could only be replicated in RC-expressing cells.

To rule out the existence of a weak replication in cells devoid of RC, a gene with a strong cellular toxicity was used. A suicide nrRNA was designed to express ricin A chain (RicA), which carries the cytotoxic activity of this plant toxin. One RicA molecule inactivates 1500 ribosomes in 1 min, driving the cell where it is expressed to death (Olsnes et al., 1975). Because RicA is not able to pass through the plasma membrane without ricin B chain, its cytotoxic effect is restricted to the infected cells without impacting the surrounding ones. These features ensured high toxicity even after a low level of replicative activity. Transfection of naïve Huh7 cells devoid of RC by this nrRNA showed that only 4 ± 5% cells died (Fig. 2, white histogram). The same non-specific cytotoxicity was observed with the control RNA ($n = 9$, $p \leq 0.01$, Mann–Whitney), demonstrating that the RicA nrRNA did not affect cells devoid of HCV RC, since a very weak expression of RicA should lead to cell death. Conversely, transfection of cells expressing the RC like Huh7/Rep5.1 cells (Fig. 2, grey histogram) or JFH-1 infected cells (Fig. 2, black

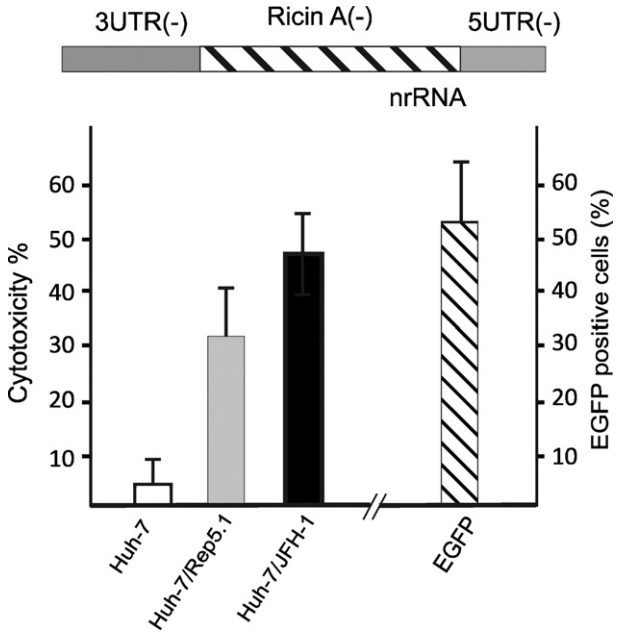

Fig. 2. nrRNAs have no effect on healthy cells. Upper panel: schematic representation of Ricin A nrRNA. Lower panel: 100 ng of RicA nrRNA were transfected into 25,000 Huh7 or Huh7/rep5.1 cells with 0.5 μl DMRIE-C in a 96-well plate. The percentage of cytotoxicity was measured after 48 h of culture as described in Section 2 with EGFP minigenome (+)RNA as zero reference. For Huh7 and Rep5.1 harboring cells, results are the mean (±standard deviation) of 9 independent experiments and 3 independent experiments for JFH-1 infected cells. To measure the level of transfected cells, 900 ng of (+)EGFP minigenome were transfected into 100,000 Huh7/Rep5.1 cells in the same conditions as above. After 24 h, percentage of positive cells was measured by flow cytometry (dashed histogram) ($n = 9$).

histogram) with RicA nrRNAs led to a high toxicity, since 31 ± 10% and 45 ± 8% of the cells died, respectively. Since the control (+)EGFP minigenome showed only 53% ±12 ($n = 9$) of fluorescent cells, it could be concluded that RicA nrRNAs were efficient against almost all transfected cells. This result demonstrates that the nrRNAs are replicated and then lead to the expression of the ricin A chain in HCV-infected cells, while no replication occurs in healthy cells. Of note, RicA nrRNAs seemed particularly active on JFH-1 infected cells, although the nrRNA UTRs used (genotype 1b) were of different genotypes from the viral RC (genotype 2a).

3.2. nrRNAs allow efficient delivery of antiviral proteins to infected cells

IFN-α is highly active against HCV replication in cell culture and can easily cure not only genotype 2a JFH-1 infected cells but also genotype 1b replicon-harboring cells (Blight et al., 2000; Kato et al., 2007). As HCV is known to inhibit the cellular pathways that induce the IFN antiviral response, the addition of IFN-α is efficient to reduce viral replication. This makes this molecule a good candidate for evaluating the nrRNA strategy. It was recently shown that IFN-α17 induces better activation of the interferon-stimulating genes (ISGs) than the commonly used IFN-α2a (Dubois et al., 2009). We thus constructed an IFN-α17 nrRNA to study whether IFN can be delivered in this way. Moreover, we tested nrRNAs designed to express interferon regulatory factor-1 (IRF-1) which has already been shown to be able to decrease HCV replication (Kanazawa et al., 2004). IRF-1 belongs to a family of transcription factors that stimulate not only the expression of IFN but also numerous factors involved in the cellular antiviral response. Since HCV is known to inhibit the activation of ISGs through the viral cleavage of TRIF and CARDIF (Ferreon et al., 2005; Meylan et al., 2005), directly delivering of IRFs to infected cells thanks to nrRNAs will restore the cellular antiviral response.

We thus constructed antiviral nrRNAs consisting in the antisense sequences of either IFN-α17 or IRF-1 flanked by the complementary sequences of the 5′ and 3′ UTRs from genotype 1b HCV (Fig. 3). The antiviral activity of IFN-α17 and IRF-1 nrRNAs was first evaluated by transfecting them in Huh7/Rep5.1 cells expressing the genotype 1b RC. After 48 h of culture, cell viability did not differ from cells transfected with control RNA (data not shown). Results of quantitative RT-PCR (qRT-PCR) experiments indicated that transfection of the IFN-α17 or IRF-1 nrRNAs decreased replicon RNA by 68 ± 18% and 58 ± 16%, respectively (Fig. 3). Because the transfection of the control (+)EGFP minigenome led to only 53 ± 12% ($n = 13$) of EGFP-expressing cells (Fig. 3, dashed histogram), we concluded that the observed inhibition affected almost all transfected cells.

To confirm these results at the viral protein level, the nonstructural viral protein 5A (NS5A) expression was analyzed in nrRNA transfected cells by immunofluorescence. Seventy-two hours post-transfection of the IFN-α17 or IRF-1 nrRNAs, the expression of NS5A significantly decreased in transfected cells (Fig. 4). Not all the Huh7/Rep5.1 cells were revealed by the fluorescent antibody, thus showing the heterogeneity of expression of the viral protein in this cellular model, as already described for NS5B (Pietschmann et al., 2001). Because only half of the cells were transfected, not all cells were subject to inhibition by IFN or IRF-1 nrRNA. However, the fluorescence intensity was strongly reduced in many of the cells. Thus, the decrease in replicon RNA was accompanied by a decrease in the viral protein expression in nrRNA transfected cells.

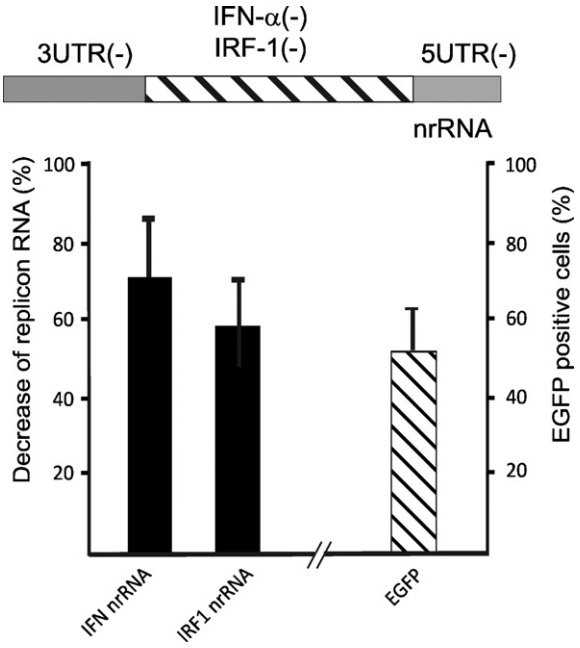

Fig. 3. Activity of IFN and IRF-1 nrRNAs on replicon-harboring cells. Upper panel: schematic representation of IFN-α17 and IRF-1 nrRNAs which consist in the complementary sequence of the 5′ and 3′ UTRs from HCV genotype 1b (Con1) flanking the antisense sequence of interferon-α or interferon regulatory factor-1. Lower panel: 900 ng of the IFN-α and IRF-1 nrRNAs and a control RNA were transfected into 10^5 Huh7/Rep5.1 cells with 2.1 μl DMRIE-C in a 24-well plate. Forty-eight hours post-transfection, the copy number of replicon subgenomic RNA was quantified by qRT-PCR performed on the NS5B coding sequence and normalized with the SFRS4 housekeeping gene. Results are presented as the percentage of inhibition calculated from a control RNA (mean ± SEM, $n = 8$ independent experiments). To measure the level of transfected cells, 900 ng of (+)EGFP minigenome were transfected in Huh7/Rep5.1 cells in the same conditions as above. After 24 h, percentage of positive cells was measured by flow cytometry (dashed histogram) ($n = 9$).

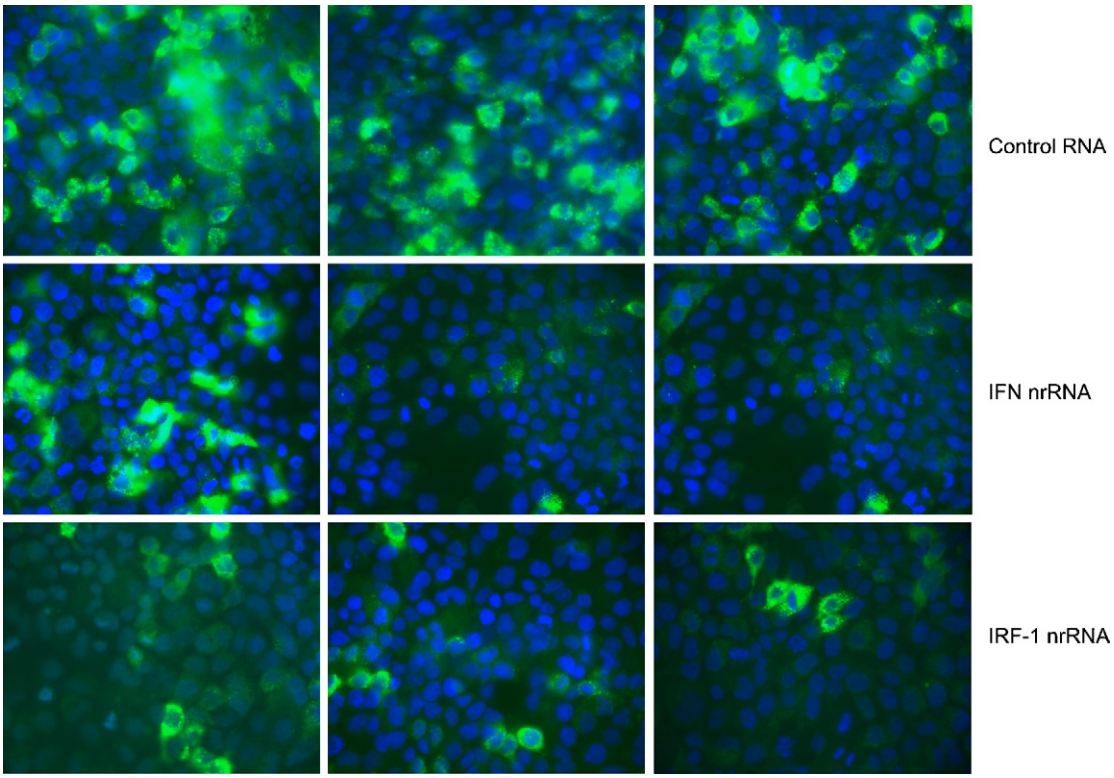

Fig. 4. Effect of IFN-α and IRF-1 nrRNAs on NS5A protein expression. Huh7/Rep5.1 cells were transfected with in vitro synthesized RNA transcripts of the nrRNAs. Cells were immunostained for NS5A expression (green) 72 h post-transfection of the nrRNAs or with a control RNA. Nuclei are counterstained with DAPI (blue). Three fields from one representative experiment out of three independent fluorescence microscopy experiments are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We concluded that the nrRNAs could exert their antiviral activity in genotype 1b replicon-harboring cells.

Because the nrRNA UTRs originates from a genotype 1b strain, the question was whether they could be recognized by the RC from a different genotype. To test this hypothesis and to validate the nrRNA strategy in the context of a complete replication cycle, we performed similar experiments on genotype 2a JFH-1 infected Huh7 cells. Transfections of the IFN-α and IRF-1 nrRNAs decreased the JFH-1 viral RNA level by 51 ± 15% and 38 ± 14% respectively (Fig. 5). Again the level of inhibition was close to the percentage of transfected Huh7 cells, indicating that all the transfected cells were affected by the nrRNA antiviral activity.

3.3. *nrRNAs activity is mediated by Type I interferon pathway activation*

As IFN-α17 or IRF-1 nrRNAs exert an antiviral activity after transfection in Huh7/Rep5.1 or Huh7/JFH-1 cells, we analyzed the induction of three different ISGs that are involved in the Type I IFN signaling pathway in these transfected cells. Quantification of ISG15, OAS2 and MxA performed 48 h post-transfection of nrRNAs showed a very weak activation with the control EGFP nrRNA. The level of induction did not exceed 2 to 3-fold for these three genes in Huh7/Rep5.1 cells and 4-fold in JFH-1 cells, while IFN-β gene was not affected. Introduction of IFN-α17 or IRF-1 nrRNAs in Huh7/Rep5.1 led respectively to 41- and 6-fold induction of OAS2, 51- and 11-fold induction of MxA and 21- and 6-fold induction of ISG15 mRNA, compared to cells transfected with control RNA. In JFH-1 infected cells, IFN-α17 or IRF-1 increased ISG mRNA levels respectively by 90- and 34-fold for OAS2, 20- and 12-fold for MxA and 24- and 18-fold for ISG15. Despite large fluctuations observed between the different experiments as shown by the error bars, IFN-α17 nrRNAs showed a significantly higher stimulation than the

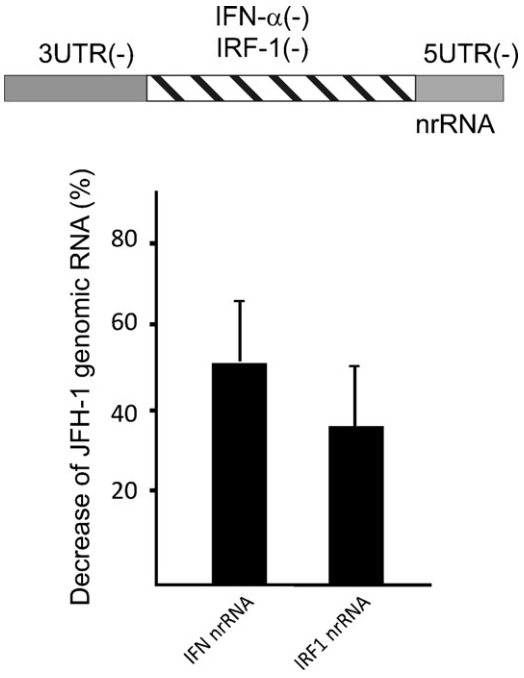

Fig. 5. Effect of IFN-α and IRF-1 nrRNAs on viral RNA synthesis in JFH-1 infected cells. Upper panel: schematic representation of IFN-α and IRF-1 nrRNAs. Lower panel: 900 ng of IFN-α or IRF-1 nrRNAs and a control RNA were transfected into 10^5 JFH-1 infected Huh7 cells with 2.1 μl DMRIE-C in a 24-well plate. Forty-eight hours post-transfection, the copy number of genomic RNA was quantified by qRT-PCR (mean ± SEM, $n = 5$ independent experiments) performed on the NS3 coding sequence and normalized with the SFRS4 housekeeping gene.

control EGFP nrRNA. This clearly indicates a specific stimulation of the Type I IFN signaling pathway through IFN-α17 nrRNAs. In the case of IRF-1 nrRNA, the statistical analysis was less convincing for JFH-1 but at least for OAS2 and ISG15 genes with the replicon, the results are close to the threshold of significance and point to the stimulation of these ISGs. Taken together, these results indicate that IFN and IRF-1 nrRNAs induce activation of the Type I IFN signaling pathway in HCV-infected cells, leading to the disappearance of the viral RNA genome.

4. Discussion

This report shows that nrRNAs can be replicated by the HCV RC and used to deliver antiviral proteins to HCV-infected cells. This approach based on the use of a minus-strand RNA involves the specific RdRp activity of the HCV replication complex. Consequently the synthesis of these exogenous proteins is strictly dependent on the HCV replication machinery, since only cells supporting viral RNA replication are able to produce a (+) coding strand. On the other hand, the use of an extremely sensitive approach using nrRNAs containing the ricin A gene showed that healthy cells were not affected by these nrRNAs, since the same non-specific cytotoxicity was observed with a control RNA ($p < 0.01$, Mann–Whitney). If few uninfected cells were to be able to replicate these (−)RNAs, for example by a hypothetical cellular RdRp activity, this replication would be so weak that it is unlikely that IFN or IRF-1 nrRNAs would perturb normal cells. This also raises the question of the stability of nrRNAs in infected cells when using the IFN or IRF-1 nrRNAs. Indeed, because the nrRNAs reduced the number of subgenomic or genomic HCV RNAs, the amount of RC decreased. The stronger the nrRNA antiviral activity, the weaker the RC activity becomes, until the disappearance of the nrRNA and/or the virus. The nrRNA should win out in this battle so the half-life of the RC and the stability of the cellular antiviral response induced by the nrRNA should be in keeping with this outcome. The level of viral inhibition observed in the first days of the cell culture is thus essential to the therapeutic activity of the nrRNA. In this regard, IFN-α17 nrRNAs seemed to be more efficient than IRF-1 nrRNAs since in the case of both replicon-harboring cells (Fig. 1) and JFH-1 infected cells (Fig. 3), the decrease in the viral RNA and the activation of ISGs was slightly lower with IRF-1 nrRNA than with IFN nrRNA. Nevertheless, this difference could be due to the fact that IFN-α17 expressed its activity in an autocrine and paracrine manner on the IFN receptors of surrounding cells, even though it is secreted at a very low level by IFN nrRNA transfected cells. Indeed, IFN could not be detected in the culture supernatant (Human IFN ELISA Ready-SET-Go, eBioSource, results not shown).

Although the expression of the IRF-1 gene in Huh7 cells harboring HCV replicon has shown its efficacy (Kanazawa et al., 2004), other activators of the innate immune response could be used. The activation pattern of the Type I IFN signaling pathway could be different in JFH-1 and Rep5.1 cells, with a greater response for MxA in Rep5.1 cells and for OAS2 in JFH-1 cells. Nevertheless, the great variability of the innate immune response shown by the error bars in Fig. 6, which likely depends on the state of the cell culture and the variation in the replication level of the virus and the replicon (Pietschmann et al., 2001), suggests that these results should be interpreted with caution. Work is in progress to search for better antiviral activity with other IRF genes, especially with IRF-3 and IRF-7, which activate the Type I IFN genes more widely and the cellular antiviral response to viral pathogens more generally.

nrRNAs could be efficient against the Rep5.1 replicon and the JFH-1 virus by reducing either the number of genomic RNA copies with IFN or IRF nrRNAs, or by killing the cells expressing the RC when using RicA nrRNA. Thus, the genotype 1b origin of the nrRNA UTRs shows that it could be efficient outside of its genotype context. Irrespective of whether IFN or IRF-1 nrRNAs were used, its activity did not seem to differ significantly when studied on the genotype 1b replicon (Fig. 1) or on the genotype 2a virus (Fig. 3). In the case of RicA nrRNA, the activity seemed even better in the heterologous genotype 2a context than when the genotype of the nrRNA and that of the RC was the same (Fig. 5). Nevertheless, in the latter case, the high level of RNA replication in JFH-1 infected cells could probably explain this difference. We have previously shown that a HCV minus-strand RNA from genotype 1 is more efficiently replicated in vitro by the genotype 3a NS5B polymerase than the homologous genotype 3a RNA (Masante et al., 2008). Thus, although genotype-specific signals have been described in the 5′ and 3′ UTRs

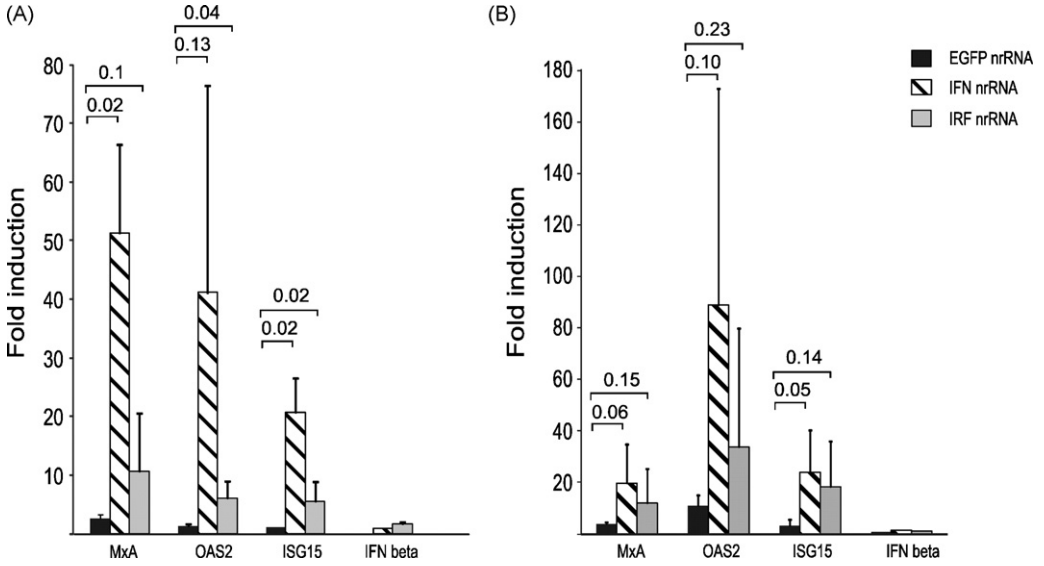

Fig. 6. Effect of IFN-α17 and IRF-1 nrRNAs on ISGs induction. Induction of ISGs was measured by qRT-PCR by the ΔΔCt method as described in Section 2. Nine hundred nanograms of the IFN-α and IRF-1 nrRNAs or a control RNA were transfected into 10^5 Huh7/Rep5.1 cells with 2.1 μl DMRIE-C in a 24-well plate. Forty-eight hours post-transfection, MxA, OAS2, ISG15 and IFN-β mRNAs were quantified by qRT-PCR and normalized with the SFRS4 housekeeping gene. Results obtained with control EGFP (black histogram), IFN (hatching histogram) and IRF-1 (gray histogram) nrRNAs are presented as the fold induction calculated from the control RNA (mean ± STDEV) for (A) replicon-harboring cells (left panel, $n = 3$–5 independent experiments), and (B) JFH-1 infected cells (right panel, $n = 4$ independent experiments). Results of statistical analysis (Student's test) are shown in each column.

of HCV (Binder et al., 2007), our results indicate that the nrRNAs are sufficiently replicated in a heterologous genotype context to fulfill their mission. Since genotype 1b is relatively distant from genotype 2a (Simmonds et al., 2005), it is likely that nrRNAs can act on viruses from other genotypes. In addition, given that this approach does not directly target the viral proteins, the appearance of resistant mutations is rather unlikely. This approach should thus be relatively independent of viral variability.

In this study, we used RNA molecules to express antiviral proteins. In spite of the nuclease sensitivity of RNA molecules, this approach has a dual advantage compared with a DNA strategy (Zhang et al., 2005). Firstly, the presence of a eukaryotic promoter in the DNA sequence corresponding to the HCV 5′ UTR (Dumas et al., 2003) would allow transgene expression in healthy cells in the absence of RC. Secondly, the high RNase content of the cell cytoplasm should lead to the disappearance of the remaining nrRNAs when the production of viral RC is stopped. Thus, nrRNAs should cure the infected cells and then disappear, leaving them intact.

Since viral pathogens use the host cell machinery to synthesize viral proteins and because the viral and eukaryotic enzymes are structurally and functionally close, specific inhibitors are difficult to develop. Active molecules against these pathogens are therefore still relatively lacking. The targeting of different steps of the viral cycle such as entry, translation of viral proteins, genome replication or encapsidation requires the development of specific approaches for each virus. The complex formed between RC and the viral genome is specific to the virus and is not present in the host cell before infection. The new approach described here capitalizes on this peculiarity. The efficiency of this approach to HCV may be demonstrated using antiviral proteins. It is likely that antiviral RNAs such as ribozymes or RNA aptamers could thus be delivered to infected cells. Replicon cellular models have been developed for other RNA viruses such as West Nile virus (Shi et al., 2002), Dengue virus (Pang et al., 2001), Sindbis virus (Bredenbeek et al., 1993) or Kunjin virus (Anraku et al., 2002), so this approach should be applicable to other RNA viruses by creating nrRNAs containing the RC binding sites specific to the targeted virus.

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