

# The NS5A Protein of Hepatitis C Virus Transcriptionally Upregulates the AGR3 Gene Expression

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## ABSTRACT

The non-structural protein 5A (NS5A) of hepatitis C virus (HCV) is a multifunctional protein involved in the HCV lifecycle and pathogenesis. The precise molecular mechanisms of NS5A-mediated pathogenesis still remain to be clarified. In this study, we performed cDNA microarray analysis on NS5A-expressing HEK293 cells and the non-expressing control to screen the possible cellular genes dysregulated by NS5A. Subsequent quantitative real time PCR (qRT-PCR) analysis on NS5A-expressing cells and the control confirmed that NS5A upregulated the *anterior gradient homolog 3* (AGR3) mRNA expression. The domain III of NS5A was responsible for the activation of AGR3 gene expression. AGR3 mRNA expression levels were upregulated also in Huh7.5 cells harboring a full-genome HCV-1b RNA replicon (FGR) and in those infected with HCV-2a. Moreover, AGR3 promoter activity was activated in NS5A-expressing cells, FGR-harboring cells and HCV-infected cells. Taken together, our present results suggest that HCV NS5A transcriptionally activates the cancer-associated AGR3 gene. This may be a novel mechanism of HCV-mediated pathogenesis, especially hepatocarcinogenesis.

## INTRODUCTION

Hepatitis C virus (HCV) infection is associated with severe liver disease, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (1). HCV is an enveloped virus with single-stranded, positive-sense RNA that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome is approximately 9.6 kb in length and encodes a single polyprotein with ~3000 amino acids (aa), which is co- and post-translationally processed by cellular and viral proteases into 10 individual viral proteins: the structural proteins, core, E1 and E2, and p7, the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (2).

HCV NS5A is a pleiotropic protein involved in not only viral replication and assembly, but also HCV-induced pathogenesis. NS5A inhibits interferon (IFN) antiviral activity, dysregulates glucose and lipid metabolism, and induces HCC through interactions with numerous host proteins (3). However, the exact mechanism by which NS5A induces HCC remains to be fully clarified.

The anterior gradient 3 (AGR3) gene encodes a protein, AGR3, also known as breast cancer membrane protein 11 (BCMP11) (4). It belongs to the protein disulfide isomerase (PDI) family involved in relieving endoplasmic reticulum (ER) stress by regulating protein disulfide bond formation, protein folding and maturation (5). AGR3 has been implicated in breast, prostate and ovary tumorigenesis (4, 6-8).

In this study, we adopted microarray assay to screen the possible genes dysregulated by NS5A. Subsequent quantitative real time PCR (qRT-PCR) analysis confirmed that NS5A upregulated the AGR3 gene expression. Also, HCV replication and HCV infection induced AGR3 gene expression and promoter activity. Our results thus provide a clue to clarify a novel molecular mechanism of HCV-induced pathogenesis.

## MATERIALS AND METHODS

### Plasmids

Myc-tagged NS5A expression plasmid, HA-tagged full-length NS5A and NS5A deletion mutant expression plasmids were generated as described previously (9). The expression plasmids of the other HCV proteins were as described elsewhere (10).

### Cell lines and virus

The human hepatoma-derived cell lines Huh7.5 (11) (kindly provided by Dr. Charles M. Rice, The Rockefeller University, New York, NY, USA) were cultured in Dulbecco's modified Eagle's medium, and HEK293 cells were in Eagle's minimum essential medium. Both media were supplemented with 10% heat-inactivated fetal bovine serum, 50 IU/ml penicillin, 50 µg/ml streptomycin and 0.1 mM nonessential amino acids. Huh7.5 cells harboring HCV full genomic RNA replicon (FGR), those cured by IFN treatment and HCV-infected cells were prepared as described previously (12). pFK-I389neo/Core-3'/5.1 (a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) and pFL-J6/JFH1 (a kind gift from Dr. Rice) were used to generate FGR and infectious HCV, respectively. Virus infection was performed at a multiplicity of infection (m.o.i.) of 2. For transfection experiments, cells were transfected with plasmid DNA using X-tremeGENE 9 DNA Transfection Reagent (Roche) according to the manufacturer's protocol.

### Immunoblot analysis

Immunoblot analysis was performed as described previously (9). Mouse monoclonal antibodies (MAbs) used in this study were anti-Myc MAb (9E10; Santa Cruz Biotechnology), anti-NS5A MAb (MAB8694; Millipore) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MAb (MAB374; Millipore). A rabbit polyclonal antibody (PAb) against NS5A (12) was also used. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG (Molecular Probes). For chemiluminescence detection, ECL Western blotting detection reagent (GE Healthcare) was used.

### qRT-PCR

qRT-PCR was performed as described previously (13). The primers used were as follows: AGR3 (NM\_176813), 5'-CTGGAGGATTGTCAATACTC-3' (nt 202 to 221) and 5'-GCATAAGGTTTAGCATGAT-3' (nt 286 to 304); PDE7B (NM\_018945), 5'-AAGGCTGCTTCGTGGAATTA-3' (nt 246 to 265) and 5'-TCCATTTGTCAAGCGATCAA-3' (nt 365 to 384); APOB (NM\_000384), 5'-ACCTCCAGAACATGGGATTGC-3' (nt 3785 to 3805) and 5'-GGGCTGGTGCCTAACAGTC-3' (nt 3942 to 3961); NEUROG2 (NM\_024019), 5'-CGCATCAAGAAGACCCGTAG-3' (nt 322 to 341) and 5'-GTGAGTGCCAGATGTAGTTGTG-3' (nt 472 to 494); GAPDH (NM\_002046), 5'-GCCATCAATGACCCCTTCATT-3' (nt 196 to 216) and 5'-TCTCGCTCCTGGAAGATGG-3' (nt 326 to 344).

### Luciferase reporter assay

A human AGR3 promoter-luciferase reporter plasmid pAGR3-GLuc was constructed by cloning a 3.2 kb genomic fragment that encompasses the human AGR3 promoter region from -2991 to +208 into the pEZXP-G04 vector plasmid (GeneCopoeia) containing Gaussia luciferase (GLuc) and secretory alkaline phosphatase (SEAP). Cells were transfected with the pAGR3-GLuc construct and re-fed with fresh medium at 24 h post-transfection. After 48 h, the culture medium was collected and assayed for luciferase activity using a Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia). Luciferase activity was measured by a GloMax™ 96 Microplate Luminometer (Promega). GLuc activity was normalized to SEAP activity.

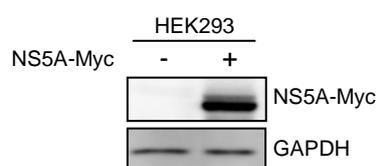
### Statistical analysis

Results were expressed as the means ± standard errors of the means (SEM). Statistical significance was evaluated by analysis of variance (ANOVA) and was defined as a *P* value of <0.05.

## RESULTS

### Microarray analysis for screening possible genes dysregulated by NS5A

To determine the possible effects of HCV NS5A expression on host gene expression, we performed cDNA microarray analysis to screen cellular genes dysregulated by NS5A. A subclone of HEK293 cells was transfected with a Myc-tagged NS5A expression plasmid or the control empty vector, and microarray gene expression profiling analysis was performed. The expression of NS5A-Myc was confirmed by immunoblot analysis (Fig. 1).



**Fig. 1.** Preparation of cells used for microarray analysis to screen the possible genes dysregulated by NS5A. HEK293 cells were transfected with a Myc-tagged NS5A expression plasmid or the control vector. Cell lysates harvested 48 h post-transfection were used for immunoblot analysis with anti-Myc antibody. The cellular RNA was extracted for cDNA synthesis and microarray gene expression profiling analysis was performed.

## NS5A UPREGULATES AGR3 GENE EXPRESSION

Some selected candidate genes either upregulated or downregulated by NS5A are shown in Tables I and II. The AGR3 (BCMP11) gene expression was most upregulated by NS5A in HEK293 cells (Table I).

Table I. Selected candidate genes upregulated by NS5A

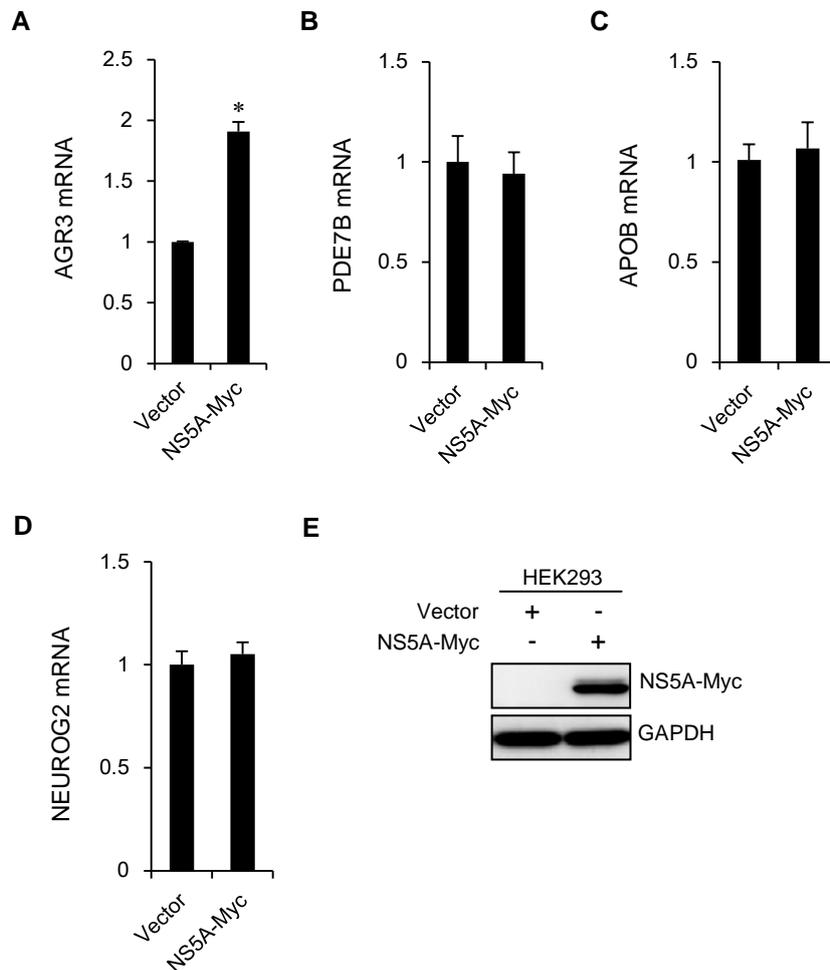
Accession No.	Gene name	Description	NS5A /Vector
NM_176813	AGR3	anterior gradient homolog 3	76.6
NM_006840	LILRB5	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5	66.3
XM_293449	LOC646303	similar to chromosome Y open reading frame 16	59.9
NM_000145	FSHR	follicle stimulating hormone receptor	41.3
NM_016388	TRAT1	T cell receptor associated transmembrane adaptor 1	40
NM_018945	PDE7B	phosphodiesterase 7B	16.5
NM_000125	ESR1	estrogen receptor 1	15.8
NM_001039768	LCA10	lung carcinoma-associated protein	11.4
NM_000384	APOB	apolipoprotein B (including Ag(x) antigen)	4.1
NM_181504	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	1.4

Table II. Selected candidate genes downregulated by NS5A

Accession No.	Gene name	Description	NS5A /Vector
NM_025145	C10orf79	chromosome 10 open reading frame 79	0.2
NM_152754	SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	0.2
XM_927678	LOC653160	Hypothetical protein LOC653160	0.2
NM_001029996	MGC33657	similar to hypothetical protein	0.2
XM_926017	LOC642521	similar to nuclear DNA-binding protein	0.2
XM_496109	LOC440330	hypothetical LOC440330	0.2
NM_024019	NEUROG2	neurogenin 2	0.2
NM_001644	APOBEC1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	0.2
NM_182631	LOC348840	hypothetical protein LOC348840	0.2
NM_173539	ZNF596	zinc finger protein 596	0.2

### NS5A activates AGR3 mRNA expression

To verify the results of the microarray analysis, we transfected HEK293 cells with a Myc-tagged NS5A expression plasmid or the control vector, and performed qRT-PCR to determine the mRNA expression levels of several candidate genes, such as AGR3 (Fig. 2A), PDE7B (Fig. 2B), APOB (Fig. 2C) and NEUROG2 (Fig. 2D). The expression of Myc-tagged NS5A was shown in Fig. 2E. The qRT-PCR results confirmed that NS5A indeed significantly enhanced AGR3 mRNA expression (Fig. 2A). In this connection, it should be stated that the AGR3 protein could hardly be detected by immunoblot analysis due probably to low expression level (data not shown).

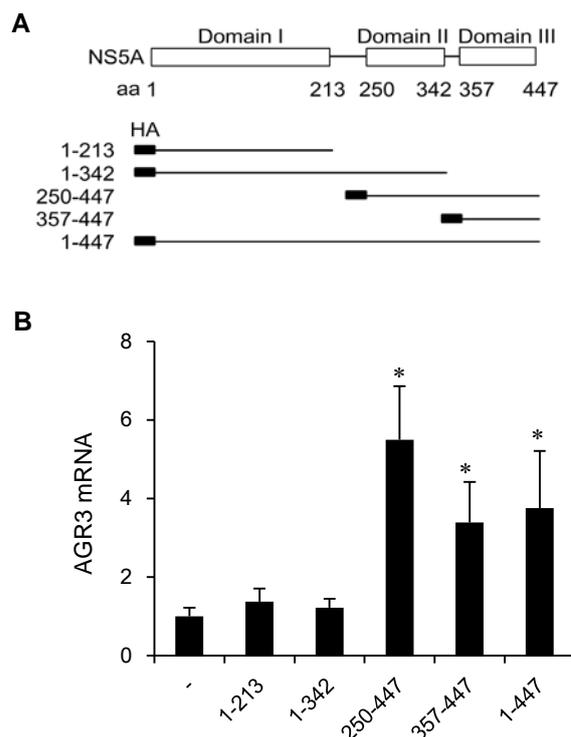


**Fig. 2.** NS5A induces AGR3 mRNA expression. HEK293 cells were transfected with a Myc-tagged NS5A expression plasmid or the control vector. After 48 h, the mRNA expression levels of AGR3 (A), PDE7B (B), APOB (C) and NEUROG2 (D) were measured by qRT-PCR and normalized to GAPDH mRNA. The value for the control cells was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$ . (E) Expression of Myc-tagged NS5A was detected by immunoblotting with anti-Myc antibody.

### The domain III of NS5A is responsible for the upregulation of AGR3 mRNA expression

Structural analysis has revealed that NS5A consists of three domains (14). We constructed various HA-tagged NS5A deletion mutants (Fig. 3A) and sought to determine which domain(s) of NS5A contributed to the upregulation of AGR3 mRNA expression. HEK293 cells were transfected with NS5A deletion mutant expression plasmids and AGR3 mRNA expression levels were analyzed. As shown in Fig. 3B, domain III (aa 357-447), domains II and III (aa 250-447) and full-length of NS5A (aa 1-447) upregulated AGR3 mRNA expression. On the other hand, domain I (aa 1-213) and domains I and II (aa 1-342) of NS5A failed to upregulate AGR3 mRNA expression. This result suggests that domain III of NS5A is responsible for the upregulation of AGR3 mRNA expression.

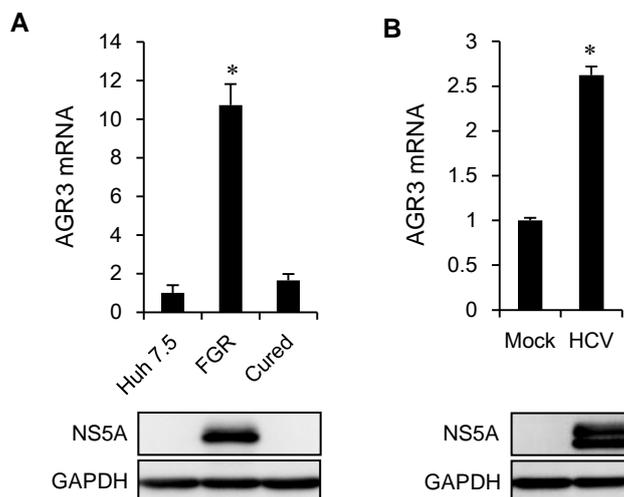
## NS5A UPREGULATES AGR3 GENE EXPRESSION



**Fig. 3.** Domain III of NS5A upregulates AGR3 mRNA expression. (A) Schematic diagram of full-length and deletion mutants of NS5A. (B) HEK293 cells were transfected with expression plasmids for HA-tagged full-length or deletion mutants of NS5A. AGR3 mRNA expression levels were measured by qRT-PCR and normalized to GAPDH mRNA. The value for the control cells was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$ .

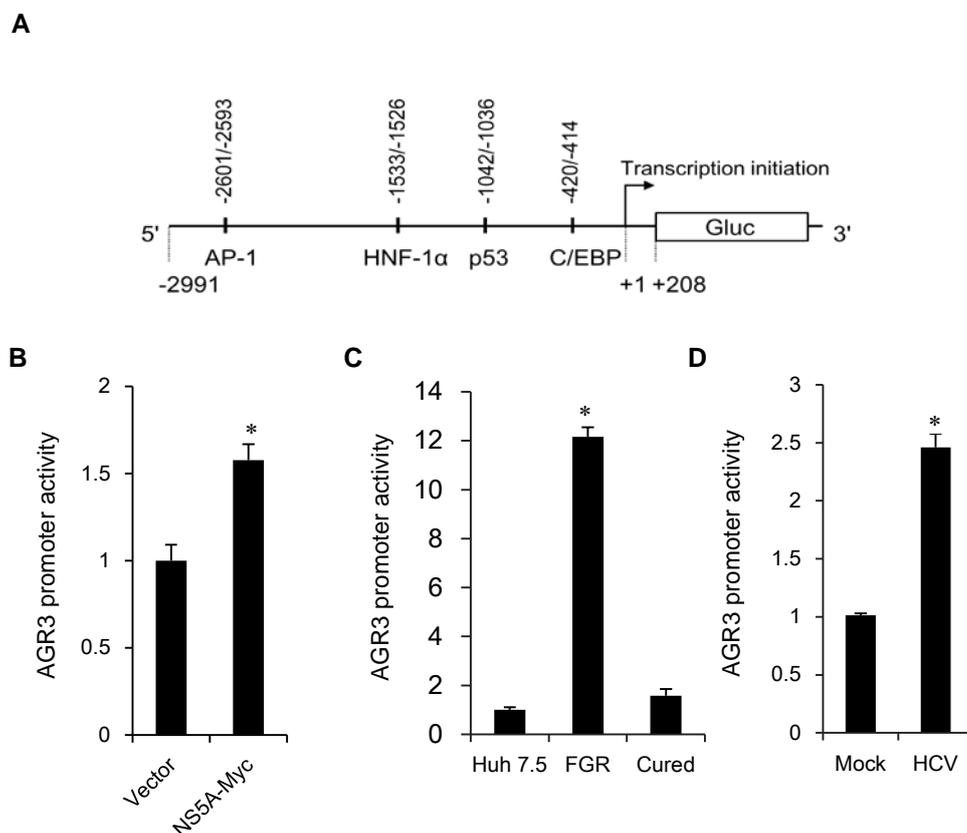
### AGR3 mRNA expression is upregulated in FGR-harboring cells and HCV-infected cells

To evaluate the effect of HCV replication on AGR3 mRNA expression, we performed qRT-PCR analysis on Huh7.5, FGR-harboring and IFN-cured FGR cells. HCV replication remarkably activated AGR3 mRNA expression in FGR-harboring cells, compared with the IFN-cured FGR cells (Fig. 4A). We further examined whether HCV infection also upregulated AGR3 mRNA expression. The AGR3 mRNA expression level was increased by 2.5-fold in HCV-infected cells at 3 days post-infection (dpi), compared with the mock-infected control (Fig. 4B). These results suggest that HCV replication and infection activate the AGR3 gene expression.



**Fig. 4.** HCV replication and infection upregulate AGR3 mRNA expression. (A) qRT-PCR analysis was performed to measure AGR3 mRNA expression levels in Huh7.5, FGR-harboring and IFN-cured cells. AGR3 mRNA expression levels were normalized to GAPDH mRNA and the value for Huh7.5 cells was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$  compared with IFN-cured cells. Elimination of HCV replication in IFN-cured cells was confirmed by immunoblotting with anti-NS5A antibody. (B) Huh7.5 cells were infected with HCV (m.o.i. = 2) and qRT-PCR analysis was performed at 3 dpi. The value for the mock-infected control cells was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$  compared with mock-infected control. NS5A expression in HCV-infected cells was confirmed.

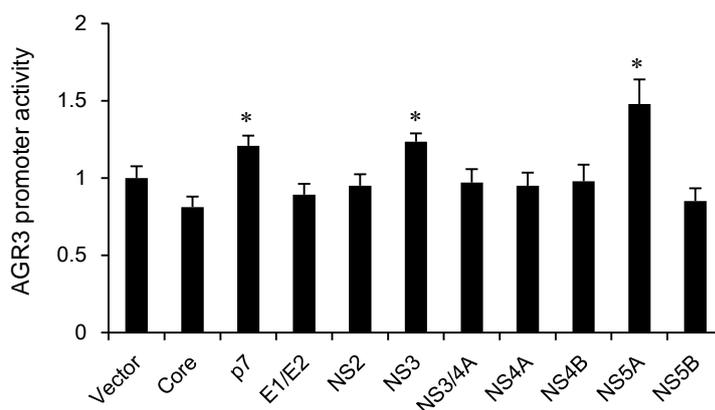
To evaluate the effect of NS5A on AGR3 promoter activity, we constructed a human AGR3 promoter-luciferase reporter plasmid, pAGR3-GLuc, that encompasses the human AGR3 promoter DNA fragment (-2991 to +208) (Fig. 5A). HEK293 cells were transfected with pAGR3-GLuc and pEF1A-NS5A-Myc or the control vector. The luciferase reporter assay indicated that NS5A significantly induced the AGR3 promoter activity (Fig. 5B). In FGR-harboring cells, HCV replication markedly enhanced AGR3 promoter activity, compared with the IFN-cured FGR cells (Fig. 5C). Consistently, HCV infection also significantly induced the AGR3 promoter activity (Fig. 5D).



**Fig. 5.** NS5A, HCV replication and infection upregulate AGR3 promoter activity. (A) Schematic diagram of the AGR3 promoter and the luciferase reporter gene. Potential binding sites for certain transcription factors, such as AP-1, HNF-1 $\alpha$ , p53 and C/EBP, are shown. The AGR3 promoter DNA fragment (-2991 to +208) was fused to Gaussia luciferase gene of the pEZX-PG04 vector plasmid. (B) HEK293 cells were transfected with pAGR3-Gluc reporter plasmid and a Myc-tagged NS5A expression plasmid or the control vector. (C) Huh7.5, FGR-harboring and IFN-cured cells were transfected with pAGR3-Gluc reporter plasmid. The cells were re-fed with fresh medium at 24 h post-transfection and the culture medium was collected for luciferase assay at 72 h post-transfection. Luciferase activities were measured and the value for the control vector-transfected cells (B) or Huh7.5 cells (C) was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$ . (D) Huh7.5 cells were transfected with pAGR3-Gluc reporter plasmid and re-fed with fresh medium at 24 h post-transfection. The cells were then infected with HCV or left uninfected (Mock), and luciferase assay was performed at 3 dpi. The value for the mock-infected control was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from three independent experiments. \*,  $P < 0.01$ .

#### Effects of various HCV proteins on AGR3 promoter activity

To investigate whether the other HCV proteins than NS5A affect the AGR3 promoter activity, HEK293 cells were transfected with pAGR3-GLuc and various HCV proteins expression plasmids. The luciferase assay was performed. The result obtained revealed that, in addition to NS5A, p7 and NS3 weakly but significantly activated AGR3 promoter activity (Fig. 6). The other HCV proteins did not significantly affect AGR3 promoter activity.



**Fig. 6.** Effects of various HCV proteins on AGR3 promoter activity. HEK293 cells were transfected with pAGR3-GLuc and various HCV proteins expression plasmids. The cells were re-fed with fresh medium at 24 h post-transfection and the culture medium was collected for luciferase assay at 72 h post-transfection. Luciferase activities were measured and the value for the vector control cells was arbitrarily expressed as 1. Data represent the means  $\pm$  SEM of the values from two independent experiments. \*,  $P < 0.01$ .

## DISCUSSION

HCV proteins interact with a variety of host cell proteins to modulate a wide range of cellular activities, including cell signaling, transcription, cell proliferation, apoptosis, membrane rearrangements and translational regulation (15). HCV NS5A is a pleiotropic protein involved in HCV lifecycle and modulation of cellular signaling pathways by interacting with various host proteins. In this study, we performed the cDNA microarray analysis to screen cellular genes dysregulated by NS5A. qRT-PCR analysis and luciferase assay confirmed that NS5A transcriptionally activated the AGR3 gene expression. Moreover, we demonstrated that HCV replication and infection also enhanced AGR3 promoter activity and upregulated AGR3 mRNA expression.

NS5A has been reported to modulate all three main MAPK signaling pathways, i.e., ERK, JNK and p38MAPK pathways, mediating potentially opposing cellular functions (3). We previously demonstrated that HCV infection promoted hepatic gluconeogenesis through NS5A-mediated activation of JNK/c-Jun signaling pathway (10). Also, other investigators recently reported that HCV infection, through NS5A involvement, upregulated expression of the C/EBP $\beta$  transcription factor (16, 17). Interestingly, the potential binding sites for the JNK/c-Jun-driven transcription factor AP-1 and the C/EBP transcription factor are located in the promoter region of the AGR3 gene (Fig. 5A). It is reasonable, therefore, to speculate that NS5A-induced upregulation of AGR3 gene expression is mediated through activation of AP-1 and/or upregulation of C/EBP expression. Further studies are needed to elucidate the precise mechanism of NS5A-mediated transactivation of the AGR3 gene in more detail. It should also be noted that, in addition to NS5A, p7 and NS3 significantly activated AGR3 promoter activity, though to lesser extents compared to NS5A (Fig. 6). NS3 has been reported to activate the ERK and JNK signaling pathways and the AP-1 transcription factor (18, 19). It is likely, therefore, that NS3 also upregulates AGR3 mRNA expression at least partly through activation of AP-1.

AGR3 belongs to the protein disulfide isomerase family, which is responsible for facilitating thiol-disulfide exchange. The protein disulfide isomerases are predominantly expressed in the ER and play important roles in protein disulfide bond formation, a highly conserved process involved in protein folding (5). During viral proliferation, large amounts of viral proteins are synthesized and processed through the ER in a relatively short period of time, which often causes ER stress (20). Although HCV-induced ER stress can lead to cell death, NS5A has been reported to utilize multiple mechanisms to inhibit apoptosis (21-23). It is possible, therefore, that NS5A upregulates AGR3 expression to attenuate ER stress through sustaining the proper protein folding process, thereby playing a role in inhibiting HCV-mediated apoptosis. Further studies are needed to clarify the issue.

It was recently reported that AGR3 protein expression was clearly detected in intrahepatic bile duct cholangiocytes, but barely, if any, in hepatocytes of normal liver tissues by immunohistochemical analysis (8). Huh7.5 cells, which we used for HCV infection in this study, were derived from a well differentiated hepatocellular carcinoma (11). AGR3 protein expression was barely detected in Huh7.5 cells by immunoblot analysis due probably to the low expression level (date not shown). However, it should be emphasized that

AGR3 mRNA expression was clearly detected in Huh7.5 cells, which was significantly upregulated by HCV RNA replication and HCV infection in our study (Fig. 4).

In conclusion, our present data suggest that HCV NS5A transcriptionally upregulates expression of the cancer-associated gene AGR3, which is involved in HCV-induced pathogenesis.

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