Effect of the reversion of NS5A S2204I mutation on the replication of hepatitis C virus

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Effect of the reversion of NS5A S2204I mutation on the replication of hepatitis C virus

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Aeri Chung

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This certifies that the Master's Thesis of
Aeri Chung is approved.

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The Graduate School
Yonsei University

June 2014

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하루의 대부분을 실험실에서 보내며 함께 울고 웃었던 우리 실험실 멤버들!! 고민이 있을 때 마다 귀 기울여 주고 조언을 아끼지 않은 숙인 언니, 짓궂은 장난도 다 받아주고 이것저것 신경 써준 성훈 오빠, 덜렁대던 나에게 짜증 한 번 내지 않고 가르쳐준 나의 첫 사수 혜림 언니, 먼저 졸업했지만 소주 한 잔 하며 누구보다 허심탄회하게 얘기할 수 있었던 동갑내기 친구 신화, 언제나 찡찡거리는 나를 달래준 애교 많은 보라, 알면 알수록 허당끼 가득한 문혁 오빠, 조용하면 너무 허전한 실험실의 활력소 막내 경주, 많은 시간을 함께 보내지 못해 아쉬운 혜정 언니와 다영 언니. 지구상의 수많은 사람들 중 깊은

인연으로 만나 2년 반이라는 시간을 그대들과 함께 해서 정말 즐거웠고 좋은 추억 많이 만들어 줘서 너무 고맙습니다. 각자의 위치에서 남은 과정 잘 마무리 하시고 언젠가 다시 만나게 된다면 지금보다 더 행복하고 더 나은 모습으로 볼 수 있기를 바랍니다.

나에게 없어서는 안 될 정신적 지주이자 가족과도 같은 최강우정카라파 멤버들 초희, 희영, 진영, 수진, 선영, 시재. 너희들이 있어 언제나 든든하고 행복하다. 앞으로도 우정 변치 말고 멋지게 살자 사랑한다!! 같은 대학교부터 대학원까지 들어와 진심으로 걱정해주고 기도해준 꼬꼬 가민. 서로 바빠 자주 만나지 못해도 만날 때마다 폭풍수다로 큰 즐거움을 준 여민이와 찬우에게도 고맙다고 말하고 싶어.

마지막으로 제가 여기까지 올 수 있도록 늘 곁에서 응원하고 믿어주신 세상에서 가장 든든한 지원군인 아빠, 지혜로움으로 항상 딸의 앞길을 밝혀주시는 엄마, 무뚝뚝하고 못난 동생을 항상 걱정해주는 하나뿐인 우리 언니. 사랑하는 우리가족에게 감사와 사랑의 마음을 전합니다.

지면이 부족하여 언급하지 못한 분들을 포함하여 제게 힘이 되어주신 모든 분들의 앞날에 늘 하느님의 축복이 함께하길 기도하겠습니다.

2014년 6월

정애리 드림

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<ABSTRACT>

Effect of the reversion of NS5A S2204I mutation on the replication of hepatitis C virus

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Background & Aims: The nonstructural protein 5A (NS5A) of hepatitis C virus is a phosphoprotein that is required for both RNA replication and virion assembly. Differential phosphorylation has been proposed as a molecular switch that modulates the two functions of NS5A protein. Efficient replication of H77 (genotype 1a) in Huh7.5 cells requires five cell culture-adaptive mutations, including K2040R andS2204I in NS5A protein. However, replication of JFH1 (genotype 2a) in cell culture system does not require any cell culture-adaptive mutations. Therefore in this study, we tested whether the reversion of K2040R and S2204I cell culture-adaptive mutations of H77S NS5A to wild-type sequence have any effect on viral RNA replication in diverse genotypic backgrounds.

Method: We generated various JFH1-based H77 NS5A chimeras, including both K2040R and S2204I cell culture-adaptive mutations (JFH1/H5A), single amino acid substitution mutations (JFH1/H5A/RK andJFH1/H5A/IS), and a wild-type sequence (JFH1/H5A/RKIS). Huh-7.5 cells were transfected with

in-vitro transcribed viral RNA from the respective recombinant plasmid DNA and viral replication was evaluated by *Gaussia* luciferase reporter assay.

Results: The efficiency of viral replication was severely impaired in cells transfected with JFH1/H5A. The reversion of S2204I to a wild-type sequence (JFH/H5A/IS) restored viral RNA replication capacity. Similar levels of replication efficiency was observed for the constructs bearing both R2040K and I2204S substitution mutations (JFH1/H5A/RKIS). However, R2040K substitution alone (JFH1/H5A/RK) did not affect the viral replication rate. Focus-forming assay showed that no infectious virus particles were released from cells transfected with the JFH1/H5A and JFH1/H5A/RK. In contrast, infectious virus particles were efficiently released by cells transfected with the JFH1/H5A/IS and JFH1/H5A/RKIS, the titer of which was comparable to that of wild-type JFH1. Consistent with these results, immunoblot analysis demonstrated no significant differences in the expression levels of NS5A protein in cells transfected with JFH1/H5A/IS, JFH1/H5A/RKIS.

Conclusion: S2204I cell culture-adaptive mutation, conferring high viral replication in H77 strains, had negative effect in the context of JFH1. This result suggests that phosphorylation of serine residue at position 2204 in NS5A protein is important for efficient replication of JFH1 RNA.

Key words: hepatitis C virus, HCV, NS5A, cell culture-adaptive mutation, viral replication

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I. INTRODUCTION

Approximately 3% of the world's population (130-170 million people) is infected with hepatitis C virus (HCV). In most infected individuals, this RNA virus establishes persistent infection that can lead to chronic liver disease, including advanced fibrosis, cirrhosis, hepatocellular carcinoma[1,2]. Current standard therapy for HCV, consisting of pegylated alpha interferon typically used in combination with ribavirin, is poorly tolerated and has low efficacy against the most prevalent HCV variants[3]. A triple combination including direct–acting antiviral (DAA) might increase cure rates but also bear risks of side effects, viral resistance and different efficacy depending on HCV genotypes[4-6].

HCV is an enveloped positive-sense, single-stranded RNA virus and classified within the genus *Hepacivirus* of the *Flaviviridae* family, which includes *Flavivirus* and *Pestivirus.* HCV isolates are classified into seven major genotypes (1-7, ~30% sequence divergence) and numerous subtypes (a, b, etc, ~20% sequence divergence)[7]. Genotype 1 is the most prevalent in the world.

Many HCV patients in the United States (70%), Japan (75%), southern Europe (70%), and northern Europe (50%) are infected with genotype 1 virus.

The 9.6-kb viral genome contains a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). The ORF is translated via an internal ribosome entry site (IRES) and the resulting polyprotein is cleaved co- and post-translationally by both viral and cellular proteases to yield three structural (core, E1 and E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins[1,8]. Although NS5A is known as a multi-functional protein that interacts with a variety of host cell proteins involved in apoptosis, cell signaling, and lipid trafficking[9,10] during the viral life cycle, its enzymatic activity and the mechanism of regulation are still poorly understood. NS5A consists of three structural domains, separated by two low-complexity sequences (LCSI and LCS II). Domain I is a highly structured domain that contains a zinc-binding motif, and is indispensable for RNA replication. The crystal structure shows that domain I is present as two distinct dimer configurations with a putative RNA binding groove located at the dimer interface[11]. Unlike Domain I, Domains II and III are poorly characterized and less conserved. While domain II is essential for RNA replication, domain III is dispensable for RNA replication, but it is important for virion assembly[12,13].

NS5A is a phosphoprotein that exists as two distinct forms according to their apparent molecular weights as 56 kDa and 58 kDa by SDS-PAGE gels, which correspond to the basally and hyperphosphorylated forms, respectively. Basally phosphorylated NS5A (p56) is phosphorylated at one or more residues, around the center and nearby C-terminus (amino acid 2200 to 2250 and 2350 to 2419) while hyperphosphorylated NS5A (p58) is believed to be phosphorylated at conserved serine cluster within LCS I (S2197, S2201, S2204). Differential phosphorylation has been proposed as a regulator modulating different functions of NS5A in RNA replication and virion assembly[14-16].

The first selectable subgenomic replicon system allowing persistent

RNA replication in a human hepatoma cell line, Huh-7, was derived from the Con 1 isolate (genotype 1b)[17]. In this replicon system, cell culture-adaptive mutations clustering in the NS5A were identified to confer robust RNA replication through the selection and sequencing of G418-resistant cell clones. When these mutations were introduced into the parental construct, replication capacity was enhanced to 0.2-10% in transfected cells, as compared with 0.0005% for the original Con 1 replicon.

Among these mutations, S2204I mutation dramatically increased HCV RNA levels to ~20, ~300, and ~400 fold relative to the negative control at 24, 48, and 96 hours post-transfection[18]. This mutation has been demonstrated as essential for efficient replication of H77 isolate (genotype 1a), as well as genotype 3a and 4a in cell culture[19,20], and serine residue at position 2204 within NS5A, a major site of hyperphosphorylation, is conserved throughout most isolates of HCV. Taken together, these studies suggest S2204I mutation has an important role in HCV RNA replication.

JFH1 isolate (genotype 2a) came from a Japanese patient with fulminant hepatitis and is capable of very efficient RNA replication in Huh-7-derived cell culture without adaptive mutation[21,22]. In contrast, H77 requires five cell culture-adaptive mutations, two within NS3 (Q1067R, V1651I), one in NS4A (K1691R), and two in NS5A (K2040R, S2204I)[19].

Recently, two research groups have reported conflicting results for the apparently similar NS5A chimeras. They carried out the same experiment by replacing NS5A of JFH1 (genotype 2a) with NS5A of H77 (genotype 1a). When the RNA transcribed from this chimeric DNA were transfected into Huh-7-derived cell line, Okamoto et al[23]. showed a slightly reduced RNA replication level, whereas Kim et al[24]. showed a significant impairment in replication capacity. A careful examination of these two studies found that the former used a wild-type H77 sequence but the latter used H77 containing K2040R and S2204I cell culture-adaptive mutations within NS5A.

Thus, we hypothesized that two adaptive mutations (K2040R, S2204I) are the main cause of the opposite results described above. To demonstrate this possibility, we developed various JFH1-based recombinants, harboring NS5A of H77, including R2040K and S2204I or wild-type sequence. Using these recombinant viruses, we assessed the effects of the reversion of adaptive mutations on HCV RNA replication and virus production.

II. MATERIALS AND METHODS

1. Plasmids

pH77S, a cell culture-adapted infectious molecular clone of genotype 1a HCV was described previously[19]. pH77S.3 is a modified version of this plasmid that has an enhanced capacity for the production of infectious virus in cell culture and that contains an additional N476D mutation in E2 and lacks the Q1067R adaptive mutation in NS3[25]. pJFH1[22] and pH77S were used to generate JFH1/H5A, which contains two cell-culture adaptive mutations, K2040R, S2204I within NS5A[19,26]. Arginine-to-lysine substitution mutation at position 2040 and isoleucine-to-serine (or threonine) substitution mutation at position 2204 were introduced into the JFH1/H5A, yielding JFH1/H5A/RK, JFH1/H5A/RKIS, and JFH1/H5A/RKIT, respectively. The substitution mutations were generated by QuickChange Site-Directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) using the following mutagenesis primers (Table 1). Proline-to-alanine substitution mutation at position 2004 glycine-to-alanine substitution mutation at position 2309 were introduced into the JFH1/H5A/RKIS, yielding JFH1/H5A/P2004A and JFH1/H5A/G2309A. The substitution mutations were generated by QuickChange Site-Directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) using the following mutagenesis primers (Table 1). For monitoring of RNA replication

efficacy, *Gaussia* luciferase (GLuc) reporter gene was inserted into the JFH1/H5A recombinant DNA by NsiI and XbaI restriction enzyme. DNA sequencing verified the integrity of the manipulated sequence and the presence of the intended substitution mutations.

2. Cells

Huh-7.5, a subline of Huh7, was grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin and streptomycin at 37°C in a 5% CO_2 environment.

3. RNA transcription and transfection

Plasmid DNAs were linearized by XbaI restriction digestion. Synthetic RNA was transcribed with T7 MEGA-Script kit (Promega, Madison, WI, USA) after linearizing plasmid with XbaI (New England Biolabs, Ipswich, MA, USA). Following treatment with RNase-free DNase to remove template DNA and precipitation of the RNA within lithium chloride, the RNA was transfected into Huh-7.5 cells Twenty-four hours before transfection, 3×10^5 cells per well were seeded in 6-well culture dishes. Briefly, 1.25μg of *in vitro*-transcribed RNAs were mixed with TransIT-mRNA transfection reagent and boost reagent (Mirus Bio LLC, Madison, WI, USA) in 250μL Opti-MEM (Gibco, Rockville, MD, USA). The mixture were incubated for 3 min at room, and added dropwise into the Huh-7.5 cells. Six hours after transfection, the culture medium was replaced with fresh medium and the supernatant was collected at different time points (24, 48, 72 h).

4. Virus titration

Culture supernatants were collected 3 days after transfection, and the titer of infectious virus was determined by the inoculation of onto naïve

Huh-7.5 cells seeded 1 day previously in 48-well culture dishes (1 x 10^5cells/well). Three days after inoculation, infected cells were stained for the presence of intracellular HCV core protein by immunofluorescence microscopy, as described below. The titer of infectious virus was determined from the number of infected foci observed at each dilution and is expressed as focus-forming unit (FFU) per ml.

5. Immunofluorescence detection of intracellular HCV antigen

For immunofluorescence, transfected cells were washed twice in PBS and fixed in methanol : acetone (1:1) at room temperature for 8min. Cells were then washed twice in PBS and incubated with anti-core antibody; 1:300 (Thermo scientific, Pittsburgh, PA, USA) diluted in 3% BSA for 1h 30min at 37℃. Cells were washed with PBS three times and incubated with goat anti-mouse IgG conjugated to Alexa Flour-488 diluted in 3% BSA; 1:200 (Invitrogen, Grand Island, NY, USA) for 1h at 37℃. Cells were washed 3 times in PBS and then nucleus was counterstained with DAPI (4',6-diamidino-2-phenylindole), and the stained cells were examined under the fluorescence microscope (Olympus America, Melville, NY, USA).

6. Immunoblots

To extract total proteins from transfected cells, 200 μL of 1X RIPA buffer (Cell Signaling, Danver, MA, USA) with 0.1mM PMSF was added to each well of the 6-well culture dishes. Cells were incubated on ice for 10 min and the lysates were transferred to 1.5 ml tubes and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube and stored at -20°C. The extracted proteins were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Pittsburgh, PA, USA). The membrane was incubated with 5% skim milk in TBST at room

temperature for 1 h for blocking and then probed with the 9E10 mouse monoclonal anti- NS5Aantibody (kindly provided by Charles Rice). Actin was also probed with anti-actin antibody (Cell signaling, Danver, MA, USA) as a loading control.

7. *Gaussia* luciferase reporter assay

Following RNA transfection, cell culture supernatant fluids were collected and fresh media added at 24h, 48h and 72h. Secreted *Gaussia* luciferase (GLuc) activity in the supernatant was measured by a BioLux Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA, USA). GLuc assay solution was prepared by adding BioLux GLuc Substrate to BioLux GLuc Assay Buffer (1:100) and mix well by inverting. Luminescence was measured after shaking (1 sec), delay (5 sec) and integration (10 sec). Twenty microliters of collected supernatant were transferred into 96-well white plates. Fifty microliters of GLuc assay solution were added to the supernatant and the luminescence signal was promptly measured by using microplate reader.

Table 1. Sequence of mutagenesis PCR primers for amino acid substitution

Mutations	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')
R2040K	GAT CAC TGG ACA TGT CAA AAA CGG GAC GAT GAG G	CCT CAT CGT CCC GTT TTT GAC ATG TCC AGT GAT C
I2204S	CAG CTC CTC GGC TAG CCA GCT GTC CGC TCC ATC TCT C	GAG AGA TGG AGC GGA CAG CTG GCT AGC CGA GGA GCT G
I2204T	CTA TGG CCA GCT CCT CGG CTA CCC AGC TGT CCG CTC CAT C	GAT GGA GCG GAC AGC TGG GTA GCC GAG GAG CTG GCC ATA G
P2004A	CAA GCT CAT GCC ACA ACT GGC TGG GAT TCC CCT TGT GTC	GAC ACA AAG GGA ATC CCA GCC AGT TGT GGC ATG AGC TTG
G2309A	CAC CTG TGG TCC ATG CCT GCC CGC TAC CAC C	GGT GGT AGC GGG CAG GCA TGG ACC ACA GGT G

III. RESULTS

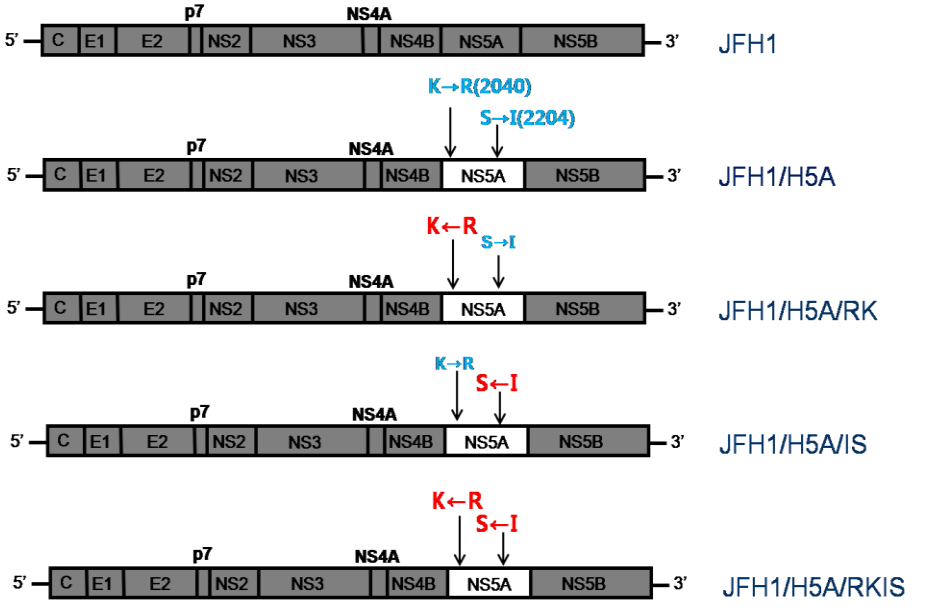
1. Effect of the reversion of NS5A cell culture-adaptive mutations on RNA replication & virus production

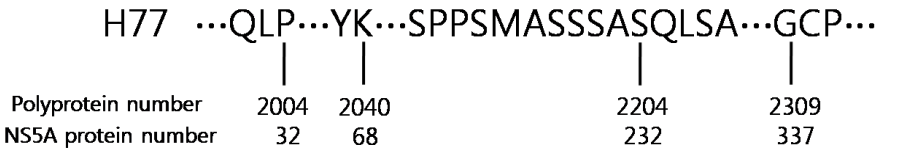
We prepared a chimeric construct harboring the NS5A region of the H77S (genotype 1a) in the JFH1 background (genotype 2a). This chimeric RNA (JFH1/H5A) did not replicate and produce infectious virus in Huh-7.5 cells[24]. This RNA also contained both K2040R and S2204I (K68R and S232I, respectively based on NS5A protein sequence) cell culture-adaptive mutations, which were shown previously to enhance replication of genotype 1a replicon[19,26]. To test whether the substitution of K2040R and S2204I mutations in NS5A of H77S with wild-type sequence recovers RNA replication capacity within the JFH1 background, we generated JFH1/H5A/RK, JFH1/H5A/IS and JFH1/H5A/RKIS constructs by introducing R2040K and I2204S substitution mutations, singly or in combination, into JFH1/H5A chimera (Fig. 1A).

To determine the impact of NS5A swapping and the reversion mutation (K2040R, S2204I) on RNA replication, synthetic RNA transcribed from these recombinant plasmid DNAs were transfected into Huh-7.5 cells, a Huh-7-derived cell line[27,28]**.** The RNA replication efficiency of each recombinant DNA was evaluated by *Gaussia* luciferase (GLuc) reporter assay. The RNA replication of wild-type JFH1 RNA-transfected cells increased efficiently up to 48h, but decreased after 48 h post-transfection. As expected, the chimeric JFH1/H5A RNA, which contains the NS5A sequence of H77S within the JFH1 background showed impaired RNA replication[24]. In contrast, introduction of I2204S substitution mutation into JFH1/H5A (JFH/H5A/IS) restored HCV RNA replication and a very similar replication level was observed for construct bearing both R2040K and I2204S substitution mutations (JFH1/H5A/RKIS) over 72 h post-transfection. However, R2040K substitution alone (JFH1/H5A/RK) did not alter RNA replication (Fig. 1B, upper).

To assess the effect of NS5A swapping and substitution mutations on the production of infectious viral particles, naïve Huh-7.5 cells were inoculated with the conditioned medium collected at 72 h post-transfection. The infected cells were identified 3 days later by immunofluorescence of HCV core protein and the titer of released virus was measured by focus-forming assay[26]. No infectious virus particle was released from cells transfected with the JFH1/H5A or JFH1/H5A/RK, suggesting impairment in RNA replication. In contrast, the titer of infectious virus released into the conditioned medium of JFH1/H5A/IS or JFH1/H5A/RKIS-transfected cells was comparable to that of JFH1 (Fig. 1B, lower). Interestingly, the replication kinetics of JFH1/H5A/IS, JFH1/H5A/RKIS was similar to that of H77S.3 rather than that of JFH1 (Fig. 1C).

A


p7
NS4A
5'
C
E1
E2
NS2
NS3
NS4B
NS5A
NS5B
3'
JFH1
K→R(2040)
S→I(2204)
JFH1/H5A
K←R
S→I
JFH1/H5A/RK
K→R
S←I
JFH1/H5A/IS
K←R
S←I
JFH1/H5A/RKIS


H77 ···QLP···YK···SPPSMASSSASQLSA···GCP···
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2040
2204
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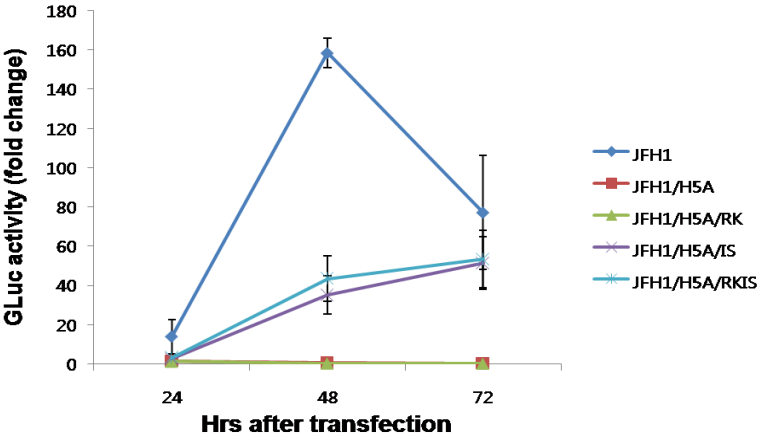
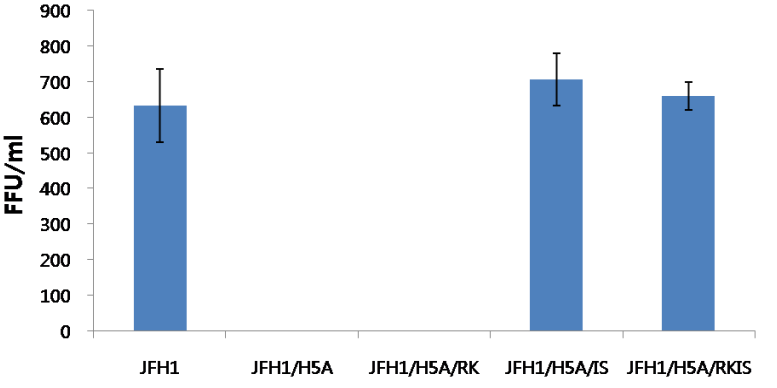
NS5A protein number
32
68
232
337

B


GLuc activity (fold change)
180
160
140
120
100
80
60
40
20
0
24
48
72
Hrs after transfection
JFH1
JFH1/H5A
JFH1/H5A/RK
JFH1/H5A/IS
JFH1/H5A/RKIS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700
600
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C

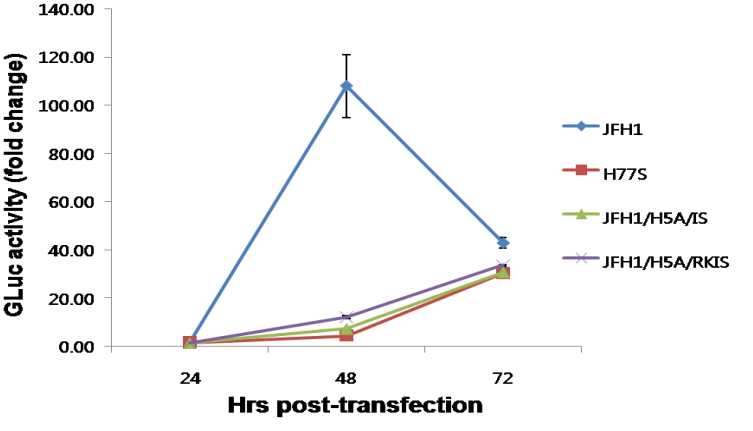
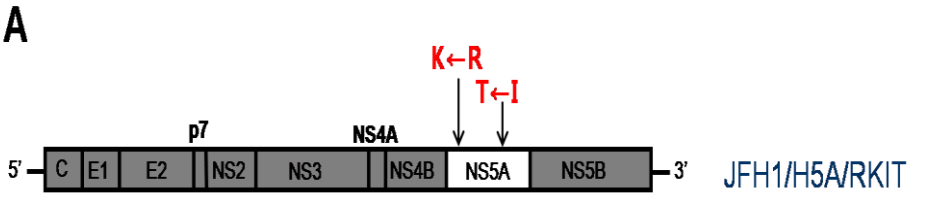

GLuc activity (fold change)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
24
48
72
Hrs post-transfection
JFH1
H77S
JFH1/H5A/IS
JFH1/H5A/RKIS

Figure 1. Evaluation of replication and virus production of recombinant constructs. (A) A schematic diagram of various recombinant constructs is shown. The entire NS5A was exchanged between H77S (genotype 1a) and JFH1 (genotype 2a) and the substitution mutations in NS5A are indicated with arrows. “RK” refers to the R2040K; “IS” refers to the I2204S; “RKIS” refers to the R2040K/I2204S double mutations. The number of amino acid was counted based on either H77 polyprotein sequence (K2040R, S2204I) or NS5A protein sequence (K68R, S232I). (B) Huh-7.5 cells were transfected with *in-vitro* transcribed RNA from the recombinant plasmid DNAs and RNA replication was evaluated by GLuc reporter assay (upper). Infectivity of virus released into supernatant of transfected Huh-7.5 cells (lower panel). (C) Comparison of RNA replication kinetics of recombinant constructs.

2. In vitro replication fitness of IS & IT reversion mutants

Recently, a chimpanzee infected with cell culture-derived genotype 1a virus (H77S.2) containing 6 cell culture-adaptive mutations showed that S2204I mutation in NS5A was replaced by threonine during 8 weeks of infection. After 62 week, the original S2204I adaptive mutation had completely reverted to the wild-type serine[29]. Since isoleucine (ATC) was mutated to threonine (ACC) first, not directly to wild-type serine (TCC), we hypothesized that threonine may have some positive effect on RNA replication. To investigate this possibility, isoleucine at position 2204 was substituted with threonine in JFH1/H5A/RK (named as JFH1/H5A/RKIT) (Fig. 2A). The GLuc activity of this recombinant construct was comparable to that of JFH1/H5A/IS, JFH1/H5A/RKIS, suggesting a similar level of replication efficiency (Fig. 2B, upper). We also examined the virus production of JFH1/H5A/RKIT. Consistent with the RNA replication data, virus production was restored by introduction of threonine at 2204 (Fig. 2B, lower).


A
K←R
T←I
p7
NS4A
5'
C
E1
E2
NS2
NS3
NS4B
NS5A
NS5B
3'
JFH1/H5A/RKIT

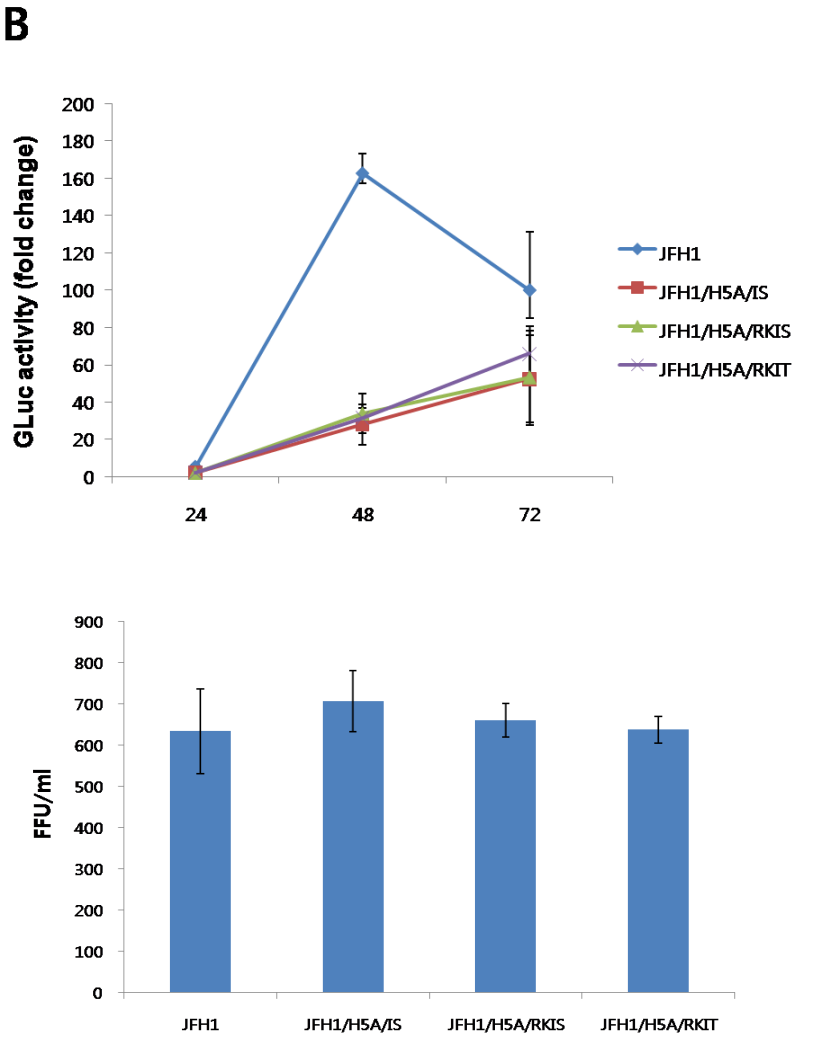

B
GLuc activity (fold change)
200
180
160
140
120
100
80
60
40
20
0
24
48
72
JFH1
JFH1/H5A/IS
JFH1/H5A/RKIS
JFH1/H5A/RKIT
FFU/ml
900
800
700
600
500
400
300
200
100
0
JFH1
JFH1/H5A/IS
JFH1/H5A/RKIS
JFH1/H5A/RKIT

Figure 2. The effect of threonine at 2204 on RNA replication and virus production. (A) A schematic presentation of JFH1/H5A/RKIT construct is shown. “RKIT” refers to the R2040K/I2204T double mutation. (B) Huh-7.5 cells were transfected with in-vitro transcribed RNA from JFH1/H5A/RKIT and RNA replication was assessed by GLuc reporter assay (upper). Infectivity of virus released into supernatant fluids of transfected Huh-7.5 cells by focus-forming assay (lower).

3. Abundance of NS5A and ratio of basally& hyper-phosphorylated form

We next analyzed whether the reversion of NS5A cell culture-adaptive mutations and threonine substitution at 2204 affected expression and ratio of basally phosphorylated (56 kDa) and hyperphosphorylated NS5A (58 kDa) species by immunoblot. Consistent with RNA replication shown in Fig.1B and 2B, similar abundance of NS5A was observed for cells transfected with JFH1/H5A/IS, JFH1/H5A/RKIS, and JFH1/H5A/RKIT. A much stronger NS5A signal, appearing as two distinct bands (presumably basally and hyperphosphorylated isoforms), was detected in the lysate of genotype 2a JFH1-transfected cells than in lysates from the other recombinant constructs (Fig. 3).

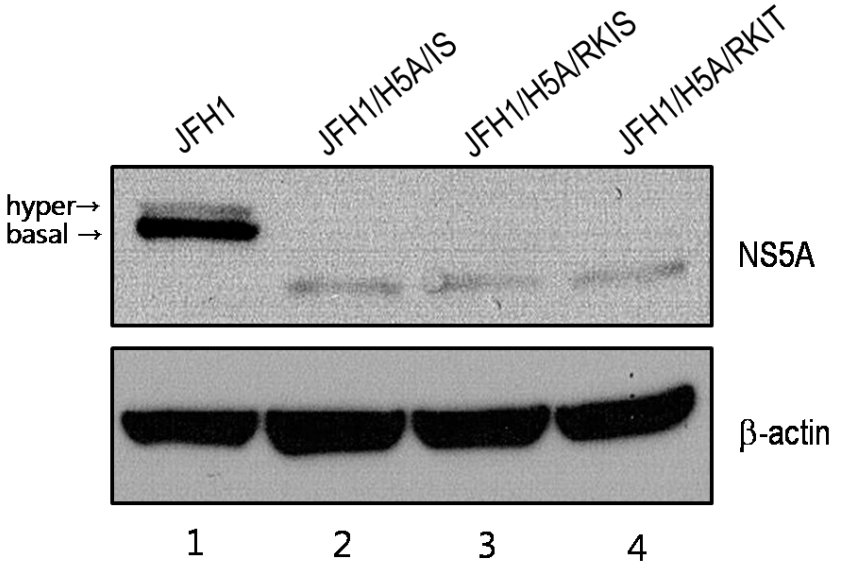


Figure 3. NS5A expression in Huh-7.5 cells transfected with JFH1, JFH1/H5A/IS, JFH1/H5A/RKIS, and JFH1/H5A/RKIT RNAs. Immunoblot analysis of cell lysates that were obtained at 72 h post-transfection was performed with anti-NS5A (9E10). β–actin was used as a loading control. Using 7.5% SDS-PAGE gel allowed separation of basally (lower band) and hyperphosphorylated (upper band) form of NS5A.

4. Trans-complementation of NS5A

A replication-defective JFH1 replicon with S2204I substitution mutation can be *trans*-complemented by co-transfection of a helper replicon encoding a functional wild-type NS5A[30]. A recent study demonstrated that JFH1 replicon containing S2204I was efficiently intragenically *trans*-complemented by replication-defective NS5A alleles, P32A and G337A (P2004A andG2309A,respectively based on HCV polyprotein sequence) (Fig. 4, adapted from Fridell et al)[31]. These results prompted us to examine whether JFH1/H5A and JFH1/H5A/RK, which showed impairment in RNA replication and infectious virus production in Fig. 1B, could also be intragenically *trans*-complemented by the P2004A or G2309A allele. To test this hypothesis, we introduced two substitution mutations, proline-to-alanine at 2004 and glycine-to-alanine at 2309 within the JFH1/H5A/RKIS background (JFH1/H5A/RKIS/P2004A and JFH1/H5A/RKIS/G2309A) (Fig. 5A). Synthetic RNA transcribed from these two recombinant constructs and JFH1/H5A, JFH1/H5A/RK were co-transfected into Huh-7.5 cells, and RNA replication was evaluated by GLuc activity. In agreement with the previous study, JFH1/H5A/RKIS/P2004A or JFH1/H5A/RKIS/G2309A was severely impaired in RNA replication (Fig. 5B). However, co-transfecting JFH1/H5A or JFH1/H5A/RK with JFH1/H5A/P2004A or JFH1/H5A/G2309A did not make any detectable change in replication, which is opposite to the results obtained by Fridell et al[31] (Fig. 5B).

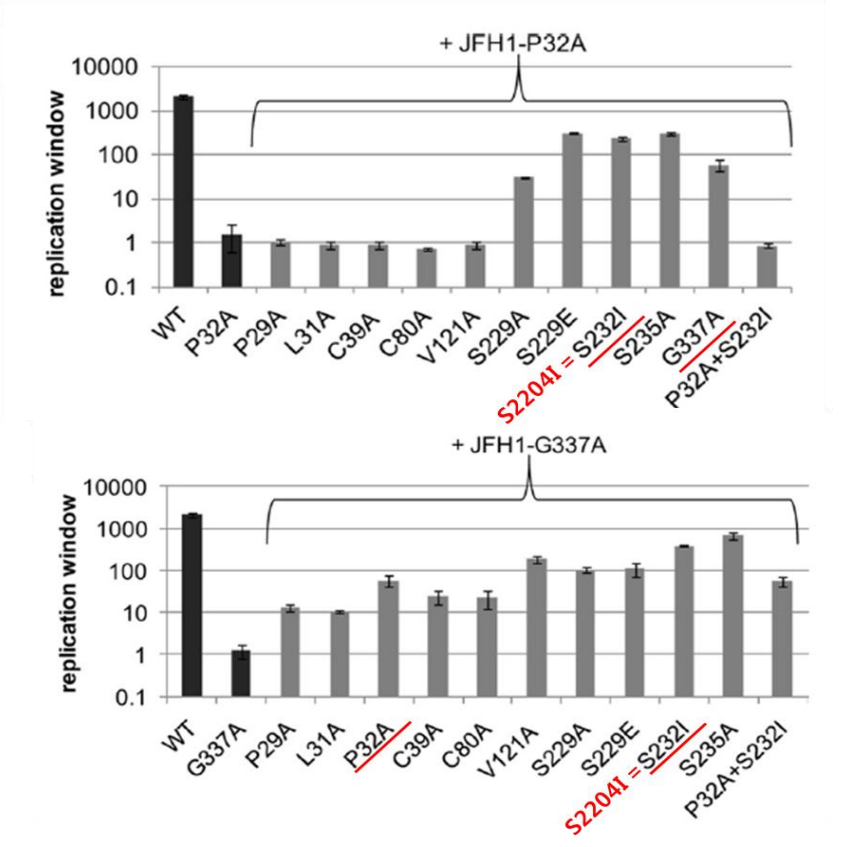
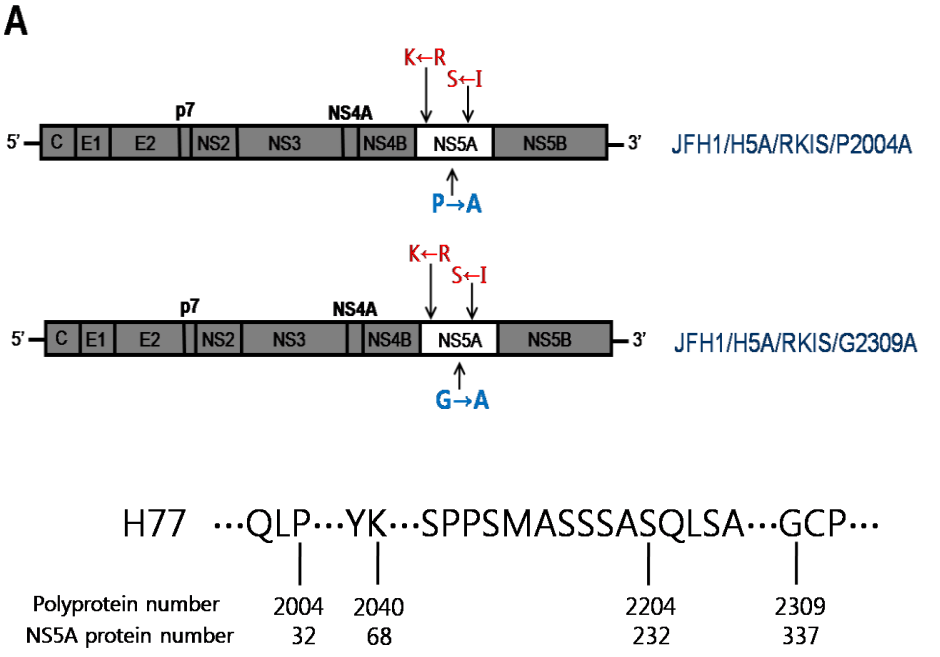
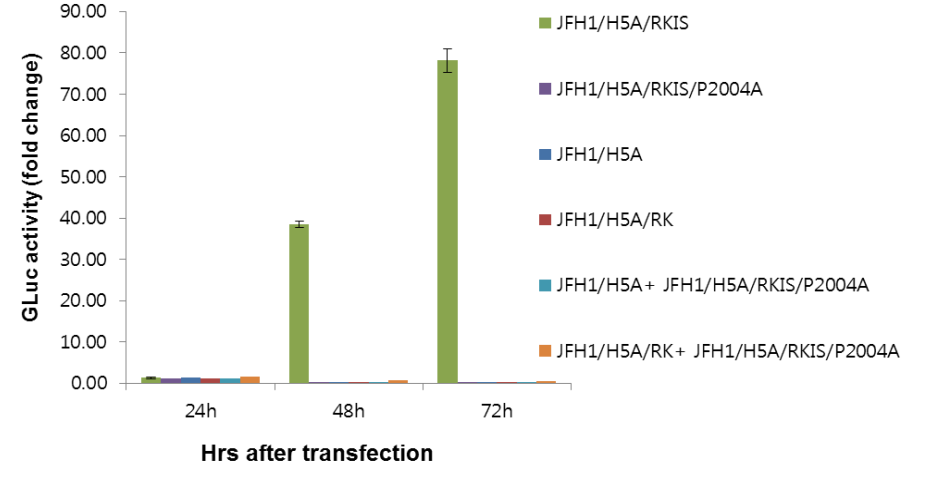


Figure 4. Intragenic complementation of replication-null NS5A alleles. Combinations of replication-null Rluc replicons with the indicated amino acid substitutions in NS5A were transiently expressed in Huh-7.5 cells (adapted from Fridell et al).


A
K←R
S←I
p7
NS4A
5'
C
E1
E2
NS2
NS3
NS4B
NS5A
NS5B
3'
JFH1/H5A/RKIS/P2004A
P→A
K←R
S←I
p7
NS4A
5'
C
E1
E2
NS2
NS3
NS4B
NS5A
NS5B
3'
JFH1/H5A/RKIS/G2309A
G→A
H77 ···QLP···YK···SPPSMASSSASQLSA···GCP···
Polyprotein number
NS5A protein number
2004
32
2040
68
2204
232
2309
337


B
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
GLuc activity (fold change)
24h
48h
72h
Hrs after transfection
JFH1/H5A/RKIS
JFH1/H5A/RKIS/P2004A
JFH1/H5A
JFH1/H5A/RK
JFH1/H5A+ JFH1/H5A/RKIS/P2004A
JFH1/H5A/RK+ JFH1/H5A/RKIS/P2004A

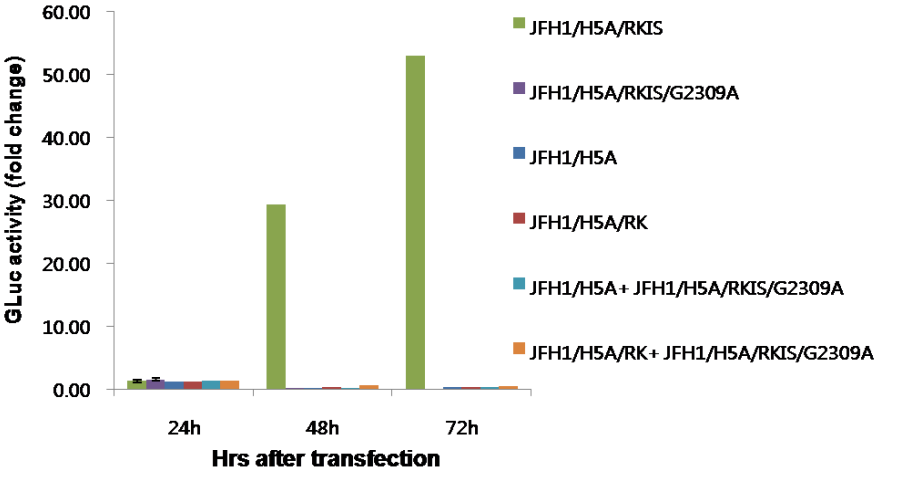


Figure 5. Trans-complementation of replication-defective JFH1/H5A and JFH1/H5A/RK. (A) A schematic presentation of JFH1/H5A/RKIS/P2004A and JFH1/H5A/RKIS/G2309A constructs used for this study. The number of amino acid was counted based on either H77 polyprotein sequence (P2004A, G2309A) or NS5A protein sequence (P32A, G337A). (B) Combinations of *in-vitro* transcribed RNA from replication-defective constructs were transfected into Huh-7.5 cells.

IV. DISCUSSION

In this study, we evaluated whether the reversion of K2040R and S2204I cell culture-adaptive mutations of H77S NS5A to wild-type sequence have any effect on HCV RNA replication in diverse genotype backgrounds.

First, we generated various JFH1-based recombinant plasmid in which the RNA region encoding the NS5A harboring K2040R and S2204I mutations was from the genotype 1a, H77S and the remainder was from genotype 2a, JFH1 (JFH1/H5A). The combination of these two adaptive mutations enabled robust RNA replication in H77[19,26], while they completely abolished RNA replication and infectious virus production in JFH1/H5A chimera (Fig. 1A). As shown in Fig. 1B, the reversion of K2040R in JFH1/H5A to wild-type sequence did not have any impact on RNA replication and virus production in cells transfected with JFH1/H5A/RK. Although K2040R mutation, identified from genotype 1a H77, permitted RNA replication in combination with other cell culture-adaptive mutations, its single effect on different isolates of HCV genotypes has not been reported. In contrast to JFH1/H5A/RK, the reversion of S2204I to wild-type sequence (JFH1/H5A/IS) significantly enhanced both RNA replication and virus production. When we introduced both reversion mutations (JFH1/H5A/RKIS), there was no additional effect on RNA replication, compared to JFH1/H5A/IS. Therefore, this result indicates that the reversion of S2204I is a key determinant for RNA replication of NS5A recombinant chimera. Although S2204I mutation is known to promote efficient viral RNA replication in genotype 1a, 1b, 3a, and 4a viruses[19,20,26], it has a negative effect on the replication of genotype 2a, JFH1. This finding is consistent with recent study that JFH1 with S2204I amino acid substitution was severely impaired in both hyperphosphorylation and RNA replication[31]. Therefore, phosphorylation of serine-2204 and NS5A hyperphosphorylation are important for efficient JFH1 RNA replication. In support of this notion, other studies suggest that some hyperphosphorylation may be required for replication, albeit reduction in

hyperphosphorylation has generally been associated with robust replication of Con1[32]. We also found that replication of JFH1/H5A/IS and JFH1/H5A/RKIS steadily increase up to 72 h post-transfection with a linear pattern, but JFH1 replication increased substantially up to 48 h then decreased after 48 h post-transfection. Since the RNA replication kinetics of these recombinant constructs were similar to that of H77S rather than that of JFH1, these data suggest that NS5A may be responsible for RNA replication kinetics (Fig. 1C).

Since phosphorylation of NS5A is a conserved feature among different HCV isolates and also among other members of the *Flaviviridae* (for NS5), it has been proposed that phosphorylation of NS5A has an important role, modulating multiple NS5Afunctions in viral life cycle[16,33]. During preparation of this paper, another study was published describing a chimpanzee infected with cell culture-derived genotype 1a virus (H77S.2) containing 6 cell culture-adaptive mutations including S2204I. This study showed that S2204I cell culture-adaptive mutation in NS5A was partially substituted by threonine (I2204T) within 8 weeks of infection and then the original S2204I adaptive mutation was completely reverted to the wild-type serine by 62 weeks[29]. Intriguingly, NS5A is mainly phosphorylated on serine and to a much lesser extent, on threonine residue[34]. Therefore, we investigated whether threonine residue is able to support RNA replication and infectious virus production. Cells transfected with synthetic JFH1/H5A/RKIT RNA showed similar level of both RNA replication and infectious virus production, compared to those of JFH1/H5A/RKIS (Fig. 2). These results suggest that phosphorylation at amino acid 2204 of NS5A is important and also led us to conclude that there is no difference in *in-vitro* viral replication fitness between threonine and serine residue. A study by Ross-Thriepland et al. showed that a serine at residue 2204 is a phosphorylated amino acid by mass spectrometry analysis in JFH1 virus[15]. Although there is no biochemical evidence of phosphorylation at threonine-2204, the replacement of isoleucine with threonine before its final

reversion to serine in the H77S.2-infected chimpanzee suggests that phosphorylation at this residue is important. Nonetheless, additional biochemical experiments are required to answer whether phosphorylation occurs at threonine 2204. Despite no distinguishable difference of in-vitro viral fitness between serine and threonine at amino acid 2204 in JFH1/H5A chimeras, serine seems to be more fit *in-vivo* models[29]. Consistent with this notion, a previous study showed that cell culture-adaptive mutations including S2204I promote replication of Con1 in Huh7 cells while impairing its replication fitness *in-vivo*[35].

We also assessed the effects of the reversion mutations on NS5A expression and the relative abundance of basally and hyperphosphorylated NS5A. Immunoblot analysis showed that there was no significant difference in expression of NS5A by introducing these mutations (Fig. 3). Some cell culture-adaptive mutations in NS5A confer higher RNA replication efficiency by altering NS5A hyperphosphorylation status[18,36]. However, other studies indicate that HCV RNA replication was inhibited if hyperphosphorylation is further reduced by introduction of additional adaptive mutation[37] or by treatment with NS5A hyperphosphorylation inhibitors[32]. Collectively, these data suggest that a critical ratio between basally and hyperphosphorylated NS5A is important for productive HCV RNA replication. Interestingly, NS5A of H77S virus, containing S2204I mutation, is expressed as a single band[24]. Given that the hyperphosphorylation of NS5A is a highly regulated process, which requires the expression of an intact NS3-5A polyprotein[38,39], and interaction between NS5A and the upstream structural proteins affect the NS5A phosphorylation status[24], NS5A in other genotypic backgrounds may have different phosphorylation patterns. However, when NS5A of H77S was placed in the JFH1 background (JFH1/H5A/IS, JFH1/H5A/RKIS, and JFH1/H5A/RKIT), NS5A was still expressed as a single band.

A recent study demonstrated that replication defect of the

S2204I-JFH1 replicon can be intragenically *trans*-complemented, by co-transfecting of replication-defective replicon bearing P2004A or G2309A substitution[31]. We decided to investigate the effect of co-transfecting JFH1/H5A or JFH1/H5A/RK, which showed severe RNA replication defect (Fig. 1), with different replication-defective constructs containing P2004A or G2309A. However, no RNA replication was observed in any combination of these recombinant constructs (Fig. 5). The reason why *trans*-complementation did not occur by P2004A and G2309A substitution mutations in our study is not known.

V. CONCLUSION

S2204I cell culture-adaptive mutation, which enables robust RNA replication in genotype 1 (Con1, H77) HCV strains, completely abolished RNA replication in the chimeric context in the background of genotype 2a, JFH1. However, the reversion of S2204I to wild-type sequence restored RNA replication. These results suggest that phosphorylation of serine-2204 is important for efficient JFH1 RNA replication. The effect of the reversion of S2204I on HCV RNA replication in JFH1 background should be taken into account when designing new chimeric constructs using JFH1 and different genotype isolates of HCV that contains a NS5A S2204I mutation.

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< ABSTRACT(IN KOREAN)>

NS5A S2204I 변이의 치환이 C형 간염 바이러스의 복제에 미치는 영향

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정 애 리

배경 및 목적: C형 간염 바이러스의 비구조 단백질인 NS5A는 인산화된 단백질로서 RNA 복제와 바이러스 입자 조립의 두 과정에 모두 필요하다. 그리고 인산화 정도의 차이는 이러한 NS5A의 두 가지 기능을 조절하는 일종의 스위치 역할을 할 것이라 생각되고 있다. 유전자형 1a에 해당하는 H77은 Huh7.5 세포에서의 효율적 복제를 위해 NS5A 내의 K2040R, S2204I를 포함한 5개의 cell culture-adaptive 변이를 필요로 한다. 반면에, 유전자형 2a에 해당하는 JFH1은 어떠한 cell culture-adaptive 변이 없이도 세포배양 내에서 복제가 가능하다. 본 연구에서는 H77S의 NS5A에 위치한 K2040R과 S2204I변이를 야생종 서열로 치환시킬 경우 다양한 유전자형 배경에서 C형 간염 바이러스의 복제능력에 어떠한 영향을 미치는지 알아보고자 하였다.

방법: JFH1의 NS5A를 K2040R과 S2204I cell culture-adaptive 변이를 갖고 있는 H77의 NS5A로 바꾼 JFH1/H5A, 아미노산을 하나씩 치환시킨 JFH1/H5A/RK, JFH1/H5A/IS와 야생종 서열을 갖는

JFH1/H5A/RKIS를 제작하였다. 제작한 재조합 플라스미드 DNA로부터 만들어진*in-vitro* transcribed RNA를 Huh7.5 세포에 transfection 시킨 후 *Gaussia* lucifease reporter assay를 통해 RNA 복제능력을 측정하였다.

결과: JFH1/H5A를 transfection 시킨 세포에서는 RNA 복제가 전혀 일어나지 않았다. S2204I를 야생종 서열로 치환시킨 JFH1/H5A/IS에서는 RNA 복제능력이 복구되었고, K2040R과 S2204I를 함께 야생종 서열로 치환시킨 JFH1/H5A/RKIS 에서도 비슷한 수준으로 복제가 일어남을 확인하였다. 하지만 K2040R만 단독으로 치환시켰을 때에는 어떠한 변화도 일어나지 않았다. Focus-forming assay를 수행한 결과, JFH1/H5A 와 JFH1/H5A/RK를 transfection 시킨 세포에서는 전염성을 갖는 바이러스 입자가 배출되지 않았다. 반면에 JFH1/H5A/IS와 JFH1/H5A/RKIS를 transfection 시킨 세포에서는 야생종 JFH1과 비슷한 수준으로 전염성을 갖는 바이러스 입자가 배출되었다. 이러한 결과들과 상응하게 immunoblot을 통해 JFH1/H5A/IS와 JFH1/H5A/RKIS를 transfection 시킨 세포에서 NS5A 발현에 큰 차이가 없음을 확인하였다.

결론: H77에서 효율적인 RNA 복제가 가능하게 하는 S2204I cell culture-adaptive 변이가 JFH1에서는 부정적인 영향을 미쳤다. 이 결과는 JFH1의 효율적인 RNA복제에 있어 NS5A내 2204번에 위치한 세린기의 인산화가 중요하다는 점을 제시한다.

핵심 되는 말: C형 간염 바이러스, NS5A, cell culture-adaptive 변이, RNA 복제

PUBLICATION LIST

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