The two crucial early steps in RNA synthesis by Hepatitis C virus polymerase involve a dual role of residue 405

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Running title: Dual role of residue 405 in initiation by HCV-NS5B
Abstract

The hepatitis C virus (HCV) NS5B protein is an RNA-dependent RNA polymerase (RdRp) essential for replication of the viral RNA genome. In vitro and presumably in vivo, NS5B initiates RNA synthesis by a de novo mechanism then processively copies the whole RNA template. Dissections of de novo RNA synthesis by genotype 1 NS5Bs previously established there are two successive crucial steps in de novo initiation: first dinucleotide formation, which requires a closed conformation; then transition to elongation, which requires an opening of NS5B. We also recently published a combined structural and functional analysis of genotype 2 HCV-NS5Bs (of strains JFH1 and J6) that established residue 405 as a key element in de novo RNA synthesis (Simister et al. 2009, J. Virol 83:11926–11939; Schmitt et al. 2011, J. Virol 85:2565–2581). We hypothesized that this residue stabilizes a particularly closed conformation conducive to dinucleotide formation. Here we report similar in vitro dissections of de novo synthesis for J6 and JFH1 NS5Bs, as well as for mutants at position 405 of several genotype 1 and 2 strains. Our results show that an isoleucine at position 405 can promote both dinucleotide formation and transition to elongation. New structural results highlight a molecular switch of position 405 with long-range effects, resolving the implied paradox of how the same residue can successively favor both the closed conformation of the dinucleotide formation step and the opening necessary to the transition step.
Introduction

The hepatitis C virus (HCV) is an enveloped positive-strand RNA virus belonging to the genus Hepacivirus in the family Flaviviridae (28). The genome of HCV is a 9,600-nucleotide (nt)-long RNA molecule encompassing a single open reading frame (ORF) that is translated primarily into one polyprotein and flanked by nontranslated regions (NTRs). The NTRs are the most conserved parts of the viral genome and play important roles in viral translation and replication. The polyprotein precursor is cleaved by cellular and viral proteases into at least 10 different mature proteins. The nonstructural proteins NS3 to NS5B are associated within a membrane replication complex, in which NS5B is the RNA-dependent RNA polymerase (RdRp). NS5B copies the RNA genome into a complementary negative strand and subsequently uses this negative strand as template for the synthesis of new HCV genomes (for a review, see (21)).

In vitro, recombinant NS5B is a highly processive RdRp that copies RNA templates up to the size of the HCV genome (4). C-terminal deletions of the 21-residue transmembrane helix (NS5BΔC21) are fully active and much easier to produce and purify than the full-length enzyme (37), so that these deletions are used almost exclusively for structural and functional characterization of NS5B. These studies have shown that NS5B can initiate RNA synthesis by a de novo mechanism (19) allowing full copy of the template in a single polymerization round (29)(14). De novo initiation from the 3’ end of the viral positive and negative-strand RNA is likely to be the physiological mode of initiation of RNA synthesis in infected cells (8). Recombinant NS5B is very poorly active overall despite its high processivity and a turnover number comparable to that of other polymerases (7). Dissections of the early steps of RNA synthesis in vitro (29)(14)(12) have shown that this is due to two inefficient steps in de novo
RNA synthesis by NS5B: the first step is *de novo* initiation proper, *i.e.* formation of the first dinucleotide complementary to the last two 3’ bases of the template; the second step is use of this dinucleotide as a primer for further RNA synthesis.

Structural analysis of NS5B led us to propose that these two steps are closely linked to two conformational states of NS5B (14). This RdRp is consistently crystallized in closed conformations that would not allow egress of a double-stranded, template-primer RNA during processive RNA synthesis (6). Key features of the closed conformations are closure of NS5B’s so-called ‘thumb’ domain and occlusion of the egress from the catalytic site by the segment (termed ‘linker’) just upstream of the transmembrane helix. Hence, a conformational transition to a displaced linker and more open thumb must be postulated before NS5B can elongate a dinucleotide primer. On the other hand, dinucleotide formation itself seems to occur in a closed conformation, where NS5B likely provides a protein platform to stabilize the *de novo* initiation complex, similarly to homologous RdRps from double-stranded RNA viruses (5)(35)(14). In accordance with this model, we established a link between a very closed conformation and an unusual *de novo* RNA synthesis efficiency in the JFH1 strain NS5B (34). Introducing the central JFH1 V405I polymorphism into the closely related J6_NS5B recently allowed us to boost J6_NS5B’s *de novo* RNA synthesis *in vitro* and J6_NS5B-based chimeras’ replication in cells.

An infectious J6_NS5B-based chimeric virus was thereby generated with just two nucleotide substitutions (30). We hypothesized that by stabilizing a very closed NS5B conformation, the V405I mutation favors the very first step in *de novo* synthesis (formation of the first phosphodiester bond), with limiting impairment of the subsequent steps (elongation of this dinucleotide primer).
In order to buttress or refute this model, we performed here in vitro dissections of de novo RNA synthesis for J6 and JFH1 NS5Bs, as well as for mutants at position 405 for several genotype 1 and 2 strains. Our functional results show that I405 can improve not only dinucleotide formation, as expected from our model, but also transition to elongation. New structural results, particularly the crystal structure of J6-V405I_NS5B, shed light as to how position 405 is also involved in the transition from initiation to elongation.

**Materials and methods**

**Site directed mutagenesis.**

Obtaining of JFH1_, J6_ and Con1_ NS5B mutants was described in (30). J4_ NS5B-I405V mutant was obtained by using the QuikChange Site-Directed Mutagenesis Kit (Stratagen) and oligonucleotides: AGACACACTCCAGTCAACTCTTGGCTAGGC and TAGCCAAGAGTTGACTGGAGTGTGTCTAGC as forward and reverse primers, respectively.

**Protein expression and purification.**

Expression of NS5B proteins.

NS5B with C-terminal deletions of 21 residues (NS5BΔC21) from HCV strains Con1, JFH1, J4 and J6 were expressed as already reported (30).

Mutant V405I of strain J6 NS5BΔC21 (J6-V405I_NS5B) used in structural work and NS5B full-length of HCV JFH1 (JFH1_NS5B-FL), C-terminally fused to a hexahistidine tag, were expressed in *Escherichia coli* BL21(DE3) or Rosetta(DE3) cells. Glucose (1%) was added to repress NS5B expression in all media except the induction medium. Carbenicillin was used as the antibiotic instead of ampicillin for BL21(DE3) and carbenicillin and chloramphenicol were used.

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for Rosetta(DE3). The bacterial cells were grown to an optical density at 600 nm of 0.6, expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and cells were further incubated 5h for JFH1_NS5B-FL and overnight for J6-V405I_NS5B at 23°C with shaking and sedimented for 10 to 15 min at 6,000 x g. For J6-V405I_NS5B, absolute ethanol was added to a final concentration of 3% at OD 600 = 0.3 to 0.4 and the culture was then placed at the induction temperature.

**Purification of NS5BΔC21.**

NS5BΔC21 used in biochemical analyses were purified as previously described (30)(3). The enzymatic properties studied here were identical whether the purification protocol included a cation exchange chromatography step (3) or not (30). The proteins used in structural work (J6-V405I_NS5B and JFH1_NS5B) were purified as reported in (30).

**Purification of JFH1_NS5B-FL.**

Pellets from 1-liter cultures after induction of the full-length JFH1_NS5B were resuspended in 40 ml lysis buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 20 mM imidazole [Sigma-Aldrich], 1% n-dodecyl-β-D-maltopyranoside [DDM], 20% glycerol, 1 mM DTT, 1 mg/ml lysozyme [Sigma-Aldrich], 25 U/ml benzonase [Merck, Darmstadt, Germany], and protease inhibitors [Complete EDTA free; Roche]). Incubation for 2 h with mild shaking at 4°C was followed by five freeze-thaw steps and a centrifugation for 20 min at 10,000 x g at 4°C. The supernatant was incubated with 1 ml of Ni-Sepharose (GE Healthcare) for 2 h with mild shaking. The 1-ml of Ni-Sepharose was then washed 2 times (2 x 20 ml) with buffer A (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 20 mM imidazole, 0.03% DDM, 20% glycerol, 1 mM DTT). The bound proteins were eluted with 4 ml of buffer B (buffer A with 750 mM imidazole) at room temperature. The fractions containing NS5B-FL were identified by SDS-PAGE and pooled. The pool was loaded
on a FPLC Superose 6 10/300 GL chromatography at a flow rate of 0.4 ml/min. Resin was previously equilibrated with buffer C (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.03% DDM, 20% glycerol, 1 mM DTT). The purified fractions were pooled and diluted to a 150 mM NaCl final concentration and loaded on a FPLC MonoS 5/50 GL chromatography at a flow rate of 0.5 ml/min. Resin was previously equilibrated with buffer C containing 150 mM NaCl. The bound proteins were eluted with 20 ml of a linear gradient of buffer C with 150 mM NaCl and buffer C containing 1 M NaCl at a flow rate of 0.5 ml/min at room temperature. The bound NS5B was eluted at about 700 mM NaCl at room temperature. The fractions containing JFH1_NS5B-FL identified by SDS-PAGE were pooled and concentrated on a 100-kDa-cutoff ultrafiltration unit. Purified JFH1_NS5B-FL was flash frozen by dripping into liquid nitrogen and kept at -80°C until use.

**Protein concentrations.**

Protein concentrations were determined by OD280 with extinction coefficients calculated from the constructs’ sequences.

**Gel-based initiation and elongation assays**

Two templates were used in these assays. They correspond to the 341nt of the 3’end of the minus strand RNA from HCV genotype 1 (G1-C) or 3 (G3-U). They were obtained by *in vitro* transcription as previously described (20). The assay was carried out at 30°C in 20 µl of 20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM DTT, 0.4 U/µl RNasin, 10% DMSO, 0.7 or 1 µM RNA template, 0.7 or 1 µM NS5B. As recombinant proteins were stored in high salt concentration, in all assays NaCl was adjusted to a final concentration of 80 mM. When the initiation phase alone was analyzed, G3-U RNA was used as template and 0.5 mM ATP and 10 µM CTP with 4 µCi [α-
32P] CTP (3000 Ci mmol^{-1}) were the only added nucleotides. At different time points, 4 microliters were collected, the reaction was quenched by adding 1 µl of 100 mM SDS and diluted in 8 µl of 90% formamide, 10 mM EDTA, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol loading buffer. After denaturation at 70°C for 5 min, samples were loaded onto a 22% polyacrylamide gel in TBE buffer containing 7 M urea. The migration was performed at 60 W for 4 h and the gel was submitted to electronic autoradiography using a Pharos apparatus and the Quantity One software (Biorad). For analysis of the elongation phase, the template used was the G1-C RNA. The reaction was performed at 30°C in 20 µl containing 20 mM Tris pH 7.5, 80 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.4 U/µl RNasin, 10% DMSO, 0.5 mM ATP, GTP, 3'dUTP and 100 µM CTP with 4 µCi [α-32P] CTP (3000 Ci mMol^{-1}, Perkin Elmer), 0.7 or 1 µM RNA template, 0.7 or 1 µM NS5B. At different time points, 4 microliters were collected, the reaction was quenched by adding 1 µl of 100 mM SDS and diluted in 8 µl of 90% formamide, 10 mM EDTA, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol loading buffer. Reaction products were fractionated and analyzed as above. The molecular weight markers were synthesized as described in (14).

**Crystallizations**

**Crystals of J6-V405I_NS5B.**

The crystals were obtained by the hanging-drop vapor diffusion method and flash cooled by plunging into liquid nitrogen. Initial crystallization screens, set up using robotics (Cartesian MicroSys) with the vapor diffusion method from 200-µl sitting drops, produced small rods. After optimization of the conditions, the crystals with the best diffraction quality were grown from a 1:1 mixture of protein solution (3.5 mg/ml), and reservoir solution (2.17% PEG 4000, 0.05 M...
Tri-sodium citrate, pH 6.5) in 4-µl hanging drops. Before being flash cooled in liquid nitrogen, the crystals were briefly transferred into a solution containing 15% PEG 4000, 0.05 M Tri-sodium citrate and 30% glycerol.

**Crystals of JFH1_NS5B.**

Crystals were grown as previously reported in 8-12% PEG 3350, 0.2 M sodium phosphate, pH 6.5 (30). Before being flash cooled in liquid nitrogen, the crystals were soaked 30 min into a solution at pH 6.5 containing 12% PEG 3350, 0.05 M ammonium acetate and 35% glycerol. This yielded a different crystal form than previously reported, with two molecules in the asymmetric unit (form pO).

Alternatively, the crystals were soaked 10 min into a solution at pH 6.5 containing 12% PEG 3350, 0.05 M ammonium acetate and 35% glycerol and then transferred for 17 min into the same solution with 1.3 mM of Phosphatidylinositol 4,5-phosphate (C4)(Tebu-bio). This treatment yielded the same crystal form as previously published (form cO with one molecule in the asymmetric unit).

**Structure determinations and refinements**

X-ray diffraction data were collected at beamlines Proxima 1 of the SOLEIL Synchrotron (St. Aubin, Gif-sur-Yvette, France) or ID14 of the ESRF (Grenoble, France). Diffraction data were processed with the XDS package (15). Structures were rebuilt with COOT (11) and refined with phenix.refine (2) with TLS refinement (one TLS group per NS5B molecule) and restrained individual B-factor and positional refinement. X-ray weights were optimized for the final rounds of restrained refinement.

**J6_NS5B-V405I / JFH1_NS5B cO.**
J6_NS5B-V405I crystallized in the same crystal form as J6_NS5B despite the point mutation and different crystallization conditions (30). The J6_NS5B-V405I structure was refined starting from the wild-type J6_NS5B (PDB 2XWH) with initial rigid body refinement. The same test set was retained as for the J6_NS5B dataset.

Similarly, our previous JFH1_NS5B structure in crystal form cO (PDB 2XXD) was used as a starting point for refinement against the higher resolution dataset. The test set for 2XXD was kept and extended to the new high resolution limit.

Molecular replacement was carried out with program Phaser of the PHENIX suite (2) using 2XXD as a search model. Soft (torsion) NCS restraints were enforced in refinement between the two molecules in the asymmetric unit.

**Objective comparisons of structures.**

Differences in main chain conformation between pairs of molecules were objectively assessed using program ESCET (31). As in our previous analysis (30), we used significance values (ESCET “lolim” parameter) of $2 \sigma$ to take into account all significant conformational differences. Differences in side-chain rotamers were collated using the PHENIX structure comparison utility. For each residue with its side chain reported in different rotamers in different structures, we checked that all rotamers were well defined in electron density.
Results

*JFH1_NS5B with a naturally occurring I405 has much higher dinucleotide formation activity than other NS5Bs*

Our previous structure-function comparison of JFH1_ and J6_NS5B indicated that JFH1_NS5B is highly efficient for *de novo* RNA synthesis and that amino acid 405 is a key residue in this reaction (30). Indeed, change of the wt V405 of J6 or Con1 NS5B to isoleucine, as in JFH1, enhanced NS5B-directed *de novo* RNA synthesis. We did not however determine which of dinucleotide formation or transition to elongation step was affected by this change. To analyze more precisely these two initial stages of RNA synthesis, we performed gel-based initiation-elongation assays that allowed quantification of initiation dinucleotide alone or initiation dinucleotide and elongation product. As in our work with genotype 1 enzymes (14), we used templates corresponding to the 3’ end of the HCV negative strand RNA (*i.e.* the complementary sequence to the 5’ NTR). We previously established that these RNAs are suitable for the *in vitro* study of replication of HCV RNA by NS5B, allowing initiation from the 3’-terminal nucleotide with Mg++ as the only divalent cation (14) as expected for synthesis of the positive strand *in vivo* (8). Synthesis of dinucleotide alone was determined by using a 341nt RNA template derived from the 3’end of a genotype 3 HCV (G3-U RNA, Fig 1A). RNA synthesis was run for two hours in the presence of the first two nucleotides incorporated, the lack of GTP preventing the transition to elongation phase (14). Data from such an experiment, illustrated in figure 1B, clearly showed that, consistent with our expectations, JFH1_NS5B is ten times more efficient in this first step of RNA synthesis than J6_ or Con1_NS5B (Table 1).
Mutation V405I boosts dinucleotide formation by J6_NS5B, but not by Con1_NS5B.

Introducing mutation V405I into NS5B of genotype 1 strain Con1 also improved Con1_NS5B de novo RNA synthesis and Con1 HCV replication in cells, although not to the point of infectious particle production (30). Therefore we next checked for both J6_ and Con1_NS5B whether, as predicted by our model, a change of residue 405 from valine to isoleucine would specifically improve dinucleotide formation efficiency. To exclude any spurious effect of V405I on NS5B stability in our assays, we quantified products after 1h and 2h incubation (Fig. 1B). The amount of dinucleotide increased at the same rate for wt and V405I RdRp (2.1 fold between 1h and 2h for wt and V405I Con1_NS5B and 2.3 fold for wt and V405I J6_NS5B) demonstrating that at least in this time period, V405I mutation did not affect enzyme stability.

As predicted, J6-V405I-NS5B indeed synthesized 2 to 2.8 times more dinucleotide than J6_NS5B (Fig 1B and 1C and table 1). However, mutation V405I had no effect on Con1_NS5B’s dinucleotide formation efficiency (Table 1).

Mutation V405I improves de novo RNA synthesis efficiency at the transition to elongation step for both Con1_ and J6_NS5B.

We next determined the effect of V405I mutation on the transition to elongation phase. For that, RNA synthesis was performed with G1-C RNA and the 4 NTP, 3’dUTP being used instead of UTP to arrest RNA elongation at the eleventh position on the template (Fig. 1A). Results obtained in such an experiment clearly showed that the V405I mutation boosted synthesis of elongation product by Con1_NS5B, as indicated by the appearance of the expected 11 nt elongation product for Con1-V405I-NS5B in Fig. 1D. As this mutation had no effect on
dinucleotide synthesis, we may attribute this effect to an improved transition to elongation in Con1_NS5B. Synthesis of the elongation product was also boosted in J6_NS5B by mutation V405I (Fig. 1D). The effect on the transition level was evaluated by determining the ratio between elongation and dinucleotide products after correcting for the number of labeled nucleotides in the products (Fig. 2A, Table 2). With the various enzymes used in this report, we find that there is usually less than 1% and at most a few percent of elongation product per dinucleotide synthesized, consistent with the transition to elongation being the rate-limiting step in de novo RNA synthesis by HCV-NS5B (Table 2). We also find a slight but significant increase of transition by J6-V405I_NS5B compared to the wild type (1.6 fold, p=0.02, Student test). Most striking though is the effect of the V405I mutation on transition by the genotype 1 Con1_NS5B (Fig 1D). In this case, the V405I mutation multiplied by 5 the transition from initiation to elongation (Fig 2A, table 2). Altogether, these data indicated that mutation V405I improves both early steps of de novo synthesis of J6_NS5B whereas Con1_NS5B is markedly improved but only at the transition to elongation step.

**J4_NS5B with a naturally occurring I405 displays enhanced transition compared to J4-I405V_NS5B**

Among 207 NS5B sequences of different genotypes recovered from the European hepatitis C virus database (10), 191 sequences harbor a V405 whereas I405 was only found in 16 sequences, all of them from genotype 1 strains except one (JFH1). The NS5B of HCV J4, a strain known to be infectious in chimpanzees (38), is part of this small genotype 1 NS5B group with a wt isoleucine at position 405. We decided to analyze the early steps of de novo initiation with this polymerase. As illustrated in figure 1D and quantified in Fig. 2A, the wt J4_NS5B displays as
high a transition efficiency as Con1-V405I_NS5B (Table 2). This puts it on a par in this respect with JFH1_NS5B (see below). To confirm the involvement of residue 405 in the transition step, we introduced an I405V mutation in J4_NS5B and analyzed its initiation and elongation products as above. The I405V change did not modify dinucleotide synthesis by J4_NS5B (Fig 1C and 2B) but it decreased elongation product synthesis (Fig 2C). Thus, we found a slight but significant decrease of transition with J4-I405V_NS5B compared to wild-type enzyme (1.5 fold p=0.005 Student test, Fig 2A).

Crystal structure of J6-V405I_NS5B reveals a significantly more open thumb than J6_NS5B

In order to clarify the involvement of residue 405 in de novo RNA synthesis, we solved the crystal structure of J6-V405I_NS5B to a resolution of 1.9 Å (Table 3). This point mutant crystallized in the same crystal form as the wild-type J6_NS5B we previously refined to a resolution of 1.8 Å (30). Nevertheless, there are a number of significant differences in main chain conformation as detected by the ESCET program (31). ESCET is the tool of choice here as it explicitly takes experimental uncertainty into account by first computing estimates of positional errors for all alpha carbons (C\(\alpha\)) based on the structures’ precisions and the C\(\alpha\)’s individual temperature factors, allowing objective assessment of small but significant displacements between two crystal structures. Analyses such as these previously allowed us to pinpoint small but functionally important conformational differences in JFH1_NS5B (34).

At these resolutions, the coordinate errors for C\(\alpha\) are reported by ESCET as 0.075 +/- 0.037 Å for J6_NS5B and 0.101 +/- 0.038 Å for J6-V405I_NS5B, respectively. Only 83% of 561 C\(\alpha\) present in both structures are then in identical positions within experimental error with a cutoff of 2\(\sigma\). We
can thus map rigid blocks in NS5B that have moved with the V405I mutation (32). Apart from a local displacement of some 1.8 Å for the main chain of residues 405-406 (in red on Fig. 3), there is a general opening of the thumb domain (mostly in green on Fig. 3) relative to the palm and fingers domain (in blue on Fig. 3). Thus, the main chain at the top of the thumb moves by 0.8 – 1.0 Å, values similar to the differences in thumb orientation (0.7 – 1.1 Å) between JFH1_NS5B and J6_NS5B (30). Residues 95-98 at the top of the fingers (in yellow on Fig. 3) opposite residue 405 in the thumb also open by a small but significant amount. There is thus a general loosening of the thumb-fingers interaction in this region.

**Mutation V405I also induces a long-range loosening of interactions of the thumb with linker and fingertips**

V405 contributed to buttressing the interaction with residues 95-98 in J6_NS5B, albeit less solidly than I405 in JFH1_NS5B (34). However in J6-V405I_NS5B, I405 retracts against the beta-flap, where it inserts between the apolar parts of the side chains of Asn444 and Glu446 (Fig. 3B). This displacement induces long-range changes in key interactions of the thumb with the other parts of NS5B. The side chains of Asn406 and Ser403 both flip and contribute to rearranging two networks of interaction. The first, main network (Fig. 4A) goes across the beta-flap to the linker: An alternate network of hydrogen bonds (Fig. 4A, top) starts from Asn406 and goes through Asn444, Ser453, Asn442 and to Val564 (while electron density ended at Ser563 for J6_NS5B). At the base of the beta-flap, the main chain shifts at residues 457-458. Asp458 makes a salt bridge to Arg517 in both J6_NS5B and J6-V405I_NS5B. In the latter however, Arg517 is displaced and no longer makes a second salt bridge to Glu541 in helix 540-544 of the linker. A further weakening of the interaction of this helix with the thumb is seen in the loss of part of the
hydrogen bonding with Arg465. Altogether, these rearrangements explain the concerted
displacement of the upper beta-flap and helix 540-544 (in magenta on Fig. 3). Simultaneously
with this remodeling of hydrogen bonding, the new hydrophobic interaction between I405 and
Glu446 induces (Fig. 4A, bottom) a displacement of 0.8 Å of the lower segment 445-448 of the
beta-flap (in dark brown on Fig. 3) and a switch of rotamers for the side chain of Met447, a side
chain that packs against helix 550-554 of the linker. The two-part hydrophobic packing of the
linker against the beta-flap on one side and the fingers on the other is thus altered, with shifts of
the side chains of Ile560 and Phe193.

The second network of interactions that undergoes a marked rearrangement goes from residue
405 towards the fingertips (Fig. 4B). Flipping of the side chain of Ser403 is accompanied by a
shift of segment 399-401. On one side, interaction of Arg401 with Glu18 of the fingertips is
weakened, with the loss of a strong salt bridge. This change is transmitted to the base of the
fingertips through Glu17 and Thr42. On the other side, the side chain of Thr399 flips, inverting
the polar γ1-oxygen for the apolar γ2-methyl. As a consequence, the hydrogen bond to His428 is
lost and the close hydrophobic packing with Leu26 (at the very tip of the fingertips) and Ile432 is
loosened. The shift in position of Ile432 is transmitted (Fig. 4C) to the other side of the same
helix through Met434 and induces a change in rotamers for the side chains of Arg514 and
Leu439. The latter switch contributes to the displacement of the main chain at residues Leu457-
Asp458. Thus the two networks to the fingertips and to the linker are connected at this point and
both contribute to the release of helix 540-544.

In summary, mutation V405I has many subtle but important consequences on the overall
conformation of J6_NS5B. The net effect as seen by the comparison of the two crystal structures
is a slight opening of the thumb with concomitant loosening of both polar and hydrophobic
interactions to the fingers (residue 405 with residues 95-98), outer fingertips (residues 401 and 399/428/432 with residues 18 and 26) and linker (residues 465/517 and 447 with helices 540-544 and 550-554).

*JFH1_NS5B is more efficient than other NS5Bs at both the dinucleotide formation and transition to elongation steps.*

We previously reported that JFH1_NS5B exhibits a 5 to 10 fold higher overall *de novo* synthesis activity *in vitro* compared to the J6 enzyme (34). We show here that this high polymerase activity relies on more efficient dinucleotide synthesis as predicted (Fig 1B), but data presented in figures 5C and 5D show that JFH1_NS5B is also unusually efficient at the transition step. Indeed, JFH1_NS5B is much more efficient at these steps than the other wt enzymes except J4_NS5B (Table 2). As the transition implies removal of the linker from the catalytic cleft and the linker itself connects to the C-terminal transmembrane helix *in vivo*, the transition step in particular will be likely affected by the C-terminus of NS5B. Thus, we tested whether the dinucleotide formation and transition properties of the Δ21 construct also apply to the full-length JFH1_NS5B.

As shown in Fig. 5 and tables 1 and 2, in the same assay conditions JFH1_NS5B-FL still displays about half the transition and about one third the dinucleotide formation efficiencies of JFH1_NS5BΔ21.

*JFH1_NS5B displays conformational variability with similar molecular determinants as those induced by mutation V405I in J6_NS5B.*

In the light of our observations on J6-V405I_NS5B, we next asked whether we could find structural determinants for improved transition in the conformational variability of wt JFH1_NS5B. Two structures are newly available to us for this purpose, with identical sequences
but in different crystal forms (Table 3). The first is a higher resolution structure in the same
crystal form as previously reported (cO). The second new structure is in a new crystal form (pO)
and harbors two molecules in the asymmetric unit. These two molecules are in the same
conformation as reported by ESCET (although differences may be blurred by the soft
noncrystallographic symmetry restraints we used in refinement). However, one of the molecules
in pO has different crystal environments than the one in cO near helix 540-544 and displays
alternate conformations in both helix 550-554 and the immediate surroundings of residue 405. As
a result, near residue 405 JFH1-cO_NS5B closely matches J6-V405I_NS5B while JFH1-
pO_NS5B comes closer in an alternate Asn 406 conformation to J6_NS5B (Fig. 6A). However,
the main chain of JFH1-pO_NS5B comes closer to that of both J6_NS5B and J6-V405I_NS5B
near helix 550-554, while for JFH1-pO_NS5B only the alternate path around the side chain of
Trp550 is present (Fig. 6B). In all structures, the side chain of Trp550 remains in the same
position and contributes to keeping the linker tethered to the thumb (1), but its environment in
the hydrophobic cleft between beta-flap and thumb changes (Fig. 3A, bottom). Differences are
propagated upstream the linker up to Glu541 (Fig. 6C). There, J6_NS5B is the outlier, with the
three other molecules becoming again superimposable.

**Discussion**

HCV is a human pathogen of major importance (13). Numerous studies have therefore been
devoted to the development of anti-HCV drugs and these efforts are now beginning to bear fruit
(24)(25). For years though, research on the HCV life cycle was hindered by the lack of robust cell
culture systems. Con1 was the first strain for which replication of HCV RNA in cells was
achieved and a replicon system developed (18). The first systems allowing a complete HCV cycle
in cells were finally obtained thanks to the discovery (16) and characterization (36)(17)(39) of the HCV JFH (Japanese Fulminant hepatitis)-1 strain. It soon became apparent that the special properties of JFH1 are due to its abnormally high RNA replication capabilities and especially to its polymerase (22). Subsequent studies sought to characterize JFH1_NS5B by systematic comparisons with the closely related J6 strain (34)(23)(30). These studies combined replication in cell culture, activity assays of recombinant proteins and in the case of our own work X-ray crystallography. They established that the key difference between JFH1_NS5B and other NS5Bs from normal strains is a much higher de novo RNA synthesis activity on single-stranded RNA templates by JFH1_NS5B. Our structural analysis allowed us to pinpoint the JFH1 polymorphism I405 as a key element of this JFH1_NS5B property (34) and thus to engineer a chimera based on J6_NS5B but infectious in cell culture with the single amino-acid mutation V405I (30).

In the present report, we compare the de novo RNA synthesis activity by NS5B with either V405 or I405. Precise dissection of the early steps of synthesis of the positive strand from a negative strand template establishes that for strains JFH1, J6 and Con1 also, the most inefficient step in vitro is not synthesis of the initial dinucleotide but its subsequent use as a primer, i.e. transition to elongation. Unexpectedly, mutation V405I in J6_NS5B improves not only dinucleotide synthesis but also transition to elongation. The crystal structure of J6-V405I_NS5B reveals the molecular basis of the effect on transition. It shows that residue 405 may switch from its buttressing position of the thumb-fingers interaction, where it stabilizes a closed conformation of NS5B and hence dinucleotide formation. The new I405 position is associated with a cascade of rearrangements that lead to an opening of the thumb of the same magnitude as the initial difference between JFH1_ and J6_NS5B. A general weakening of the interactions of thumb with linker and fingertips is thus observed. The regions involved were previously reported as important for linker release...
(1) and for transition to elongation (9). Indeed, in the latter study the authors specifically identified the outer fingertips as a locking mechanism for the initiation conformation and residue 428 as involved in initiation but not primer extension (9). The changes we see in J6-V405I_NS5B are thus those that are postulated as the first steps to opening of NS5B upon transition to elongation (14)(6). The fact that they occur in the same crystal form as that of J6_NS5B indicates that they are not the effect of different crystal environments but that V405I stabilizes this more open, pre-elongation conformation. Yet V405I also improves dinucleotide synthesis by J6_NS5B, and therefore also stabilizes its more closed conformation, as predicted. We conclude that the recombinant NS5B exists in solution as a spectrum of very similar conformations in dynamic equilibrium. At any time, most of the population is neither closed enough for dinucleotide formation, nor open enough to reach the conformation conducive to transition. V405I thus manages to improve dinucleotide formation or transition or both by stabilizing one or both of the relevant conformations at the expense of the population in intermediate conformations. In accordance with this view, there is a similarly narrow range of crystallized conformations for NS5B_A21 of genotype 1b, and non-nucleoside inhibitors are seen to bind to such intermediate conformations as those postulated here (6). Further support for this model is brought by our present results with genotype 1b NS5B of strains Con1 and J4. They show that relative to V405, I405 improves the transition of both enzymes without changing the dinucleotide formation efficiency, showing again that one step may be improved without deterioration of the other. Transition efficiency is improved in both strains (J6 and Con1) for which mutation V405I single-handedly boosts both de novo initiation assays and HCV RNA replication in cells (30). Furthermore, JFH1_NS5B is more efficient in transition than these enzymes. The comparisons of JFH1_NS5B structures in different crystal packing environments show that in the JFH1 enzyme,
the changes around residue 405 also correlate with changes in the linker region. Important differences with J6_NS5B however are seen in that the region of helix 550-554 is the most variable in JFH1_NS5B, while it is more subtly involved for J6_NS5B. In contrast, a key regulatory event in linker release in J6_NS5B is shown here to be the loss of a salt bridge to helix 540-544. This salt bridge is never formed at all in JFH1_NS5B because of the functionally important (30) JFH1 K517 polymorphism at a position that is an arginine in most genotype 1 and 2 NS5Bs, including those of J6, Con1 and J4. Thus the coordination of linker movement and thumb opening (14)(30) may be differently regulated in different HCV strains.

Two important questions addressed by studies of the JFH1 strain are the molecular determinants of JFH1_NS5B’s replication properties and, by contrast, the low level of RNA synthesis (or rather the low numbers at any time of replication-competent NS5Bs (7)) in other HCV strains such as J6. The former question is of paramount importance for the development of HCV cell culture systems not dependent on the JFH1 replicase. The latter question is a basic science puzzle that goes far beyond the field of HCV research. Indeed, all viruses that synthesize their replicase together with structural proteins as part of a polyprotein, including all Flaviviridae, are faced with the problem of subsequently preventing unregulated RNA synthesis by the vastly overexpressed replicase. In HCV’s case, this is achieved by keeping nearly all NS5B molecules inactive in the cell. In the replicon system for instance, a huge excess of nonstructural proteins are required to build up functional viral replication complexes but only a few are actually involved in RNA synthesis at a given time point (26). The present work confirms that the molecular basis of this regulation is a double block on de novo RNA synthesis, at the dinucleotide formation and transition to elongation steps. It establishes that both of these steps can be greatly improved in isolated, recombinant NS5B, even with a single point mutation. Transition is clearly
the limiting step in our *in vitro* assays, a result consistent with previous work on our part with genotype 1 enzymes and the same templates used here (14). In contrast, numerous studies using nonviral, more simple templates and dinucleotide primers consistently found that NS5B can extend exogenously added dinucleotide primers more efficiently than single nucleotides in *de novo* initiation (40)(27)(12). This discrepancy may arise from a conserved stem-loop in the 3’ end of the negative strand that leaves the last 4 bases unpaired (33) and likely favors dinucleotide synthesis, but needs to be melted for transition. It is likely that in the context of HCV infection, both dinucleotide formation and subsequent extension actually rely on signals from specific partners for efficient performance at the right time and place.

**Acknowledgements**

We thank Dr. Volker Lohmann for the gift of plasmids encoding JFH1_, J6_, J6-V405I_ and Con1 NS5B. We acknowledge the Structural biology and Proteomics pole of the IMAGIF integrated platform (https://www.imagif.cnrs.fr/?nlang=en) for access to Crystallization and Mass Spectrometry services and Synchrotrons SOLEIL (beamline PROXIMA 1) and ESRF (European Synchrotron Radiation Facility) for generous allocation of beamtime.

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Coordinates and structure factors for the crystal structures described in table 3 are available from the Protein Data Bank under accession codes 4ADP, 4AEP and 4AEX.
References


**Figure legends**

**Figure 1:** Gel-based analysis of initiation dinucleotide and elongation products synthesized by JFH1_NS5B and Con1_ and J6_NS5B wt or V405I. (A) Schematic representation of template RNAs G1-C and G3-U (the 341-nt 3’ ends of genotype 1 and 3 minus strands, respectively). The conserved 3’-proximal stem-loops are indicated. (B) Gel-based initiation assay was performed by incubating 1 µM NS5B and 1 µM G3-U RNA in a reaction buffer containing 500 µM ATP and 10µM $^32$P-CTP at 30°C. Aliquots were removed at different time points and RNA products analyzed on 22% polyacrylamide gel. (C) Quantification of initiation dinucleotide. Following electronic autoradiography, RNA products were quantified with Quantity One software and the mutant/wt ratio determined. The data are the mean of three independent experiments ± standard deviation. (D) Gel-based initiation-elongation assay was performed by incubating 1 µM NS5B with 1 µM G1-C RNA in a reaction buffer containing 500 µM ATP, GTP and 3’dUTP and 100 µM $^32$P-CTP at 30°C. Aliquots were removed at different time points and RNA products analyzed on 22% polyacrylamide gel.

**Figure 2:** Gel-based analysis of initiation and elongation products synthesized by J4_, Con1_ and J6_NS5B wt or mutated at position 405. (A) Quantification of transition from initiation to elongation. Following electronic autoradiography, the initiation dinucleotide and the 11nt elongation products were quantified with Quantity One software and the
elongation/initiation ratio determined. The data are the mean of four independent experiments ±
standard deviation. (B) Gel-based initiation assay with J4_NS5B-wt and I405V was performed as
described in legend of figure 1B excepted that 0.7 µM NS5B and 0.7 µM template were used
instead of 1 µM. (C) Gel-based initiation-elongation assay with J4_NS5B-wt and I405V was
performed as described in legend of figure 1D excepted that 0.7 µM NS5B and 0.7 µM template
were used instead of 1 µM.

Figure 3: Overall displacements between the structures of J6_NS5B and J6-V405I_NS5B.
NS5B is colored by rigid blocks defining the moving parts of the main chain when comparing
J6_NS5B and J6-V405I_NS5B (32). Seven displaced residues (including 405-408 and 457-458)
are not assignable to rigid blocks and are colored red. The seven main rigid blocks are colored in
order of decreasing size blue, green, cyan, magenta, pale yellow, orange and dark gray. Two
small, 4-residue stretches are also detected as rigid blocks and are colored yellow (residues 95-
98) and brown (residues 445-448). (A) Overall views in ribbons representation of J6-
V405I_NS5B in two orthogonal orientations. The linker, fingertips and β-flap are labeled on the
left panel and the fingers, palm and thumb on the right panel. I405 is displayed as spheres. The
regions corresponding to close ups in Fig. 4A, 4B and 4C are indicated on the left panel by
rectangles colored magenta, cyan and green respectively. Helices 540-544 and 550-554 are
labeled on the right panel. (B) Zoom on J6_ (left panel) and J6-V405I_NS5B (right panel) in Cα
trace representation in the orientation of (A), left panel. Side chains are displayed and colored
according to atom type if they are in different rotamers in the two NS5Bs, with carbons colored
by the rigid group to which the residue belongs. Side chains that change environment without
changing rotamer are also displayed, with carbons colored light gray. Hydrogen bonds and salt
bridges that are different in the two structures are displayed as dark gray dotted lines. Residues that shift around residue 405 are labeled.

**Figure 4: Remodeled networks of interaction between J6_NS5B and J6-V405I_NS5B.**

Representation as in Fig. 3B. Relevant residues in each network are labeled and colored by atom type with carbons colored brown (for residues that are in different rotamers in the two NS5Bs) or yellow (for residues that change environment without changing rotamer). (A) Remodeling of interactions going from residue 405 to the linker. Top panels, rearrangement of polar contacts leading to loosening of interactions with helix 540-544. Hydrogen bonds and salt bridges are shown, as well as residues participating in alternate polar interactions. Bottom panels, rearrangement of apolar contacts leading to loosening of interactions with helix 550-554. (B) Remodeling of interactions going from residue 405 to the fingertips. (C) Remodeling of interactions going from the fingertips to the linker.

**Figure 5: Gel-based analysis of initiation dinucleotide and elongation products synthesized by JFH1_NS5B-Δ21wt and JFH1_NS5B-FL.** (A) Gel-based initiation assay with JFH1_NS5B-Δ21wt and JFH1_NS5B-FL was performed as described in legend of figure 1B except that 0.03% dodecyl-maltoside was added in reaction mixture. Δ21wt (-)ATP indicates a mixture incubated without ATP, the initiating nucleotide for G3-U template. (B) Quantification of initiation dinucleotide. Following electronic autoradiography, dinucleotide products were quantified with Quantity One software. The data are expressed as a function of the initiation product synthesized by the JFH1_NS5B-Δ21wt defined as 1. They corresponded to the mean of three independent experiments ± standard deviation. (C) Gel-based initiation-elongation assay with JFH1_NS5B-
Δ21wt and JFH1_NS5B-FL was performed as described in legend of figure 1D except that 0.03% dodecyl-maltoside was added in reaction mixture. Δ21wt (-)GTP indicates a mixture incubated without GTP, the initiating nucleotide for G3-C template. (D) Quantification of transition from initiation to elongation was quantified as described in figure 2A. The data are the mean of three independent experiments ± standard deviation.

Figure 6: Conformations of the residue 405 region and of the linker in two crystal structures of wild-type JFH1_NS5B (cO and pO) and comparison with J6_NS5B and J6-V405I_NS5B. In JFH1-pO_NS5B, Asn406 and the linker downstream of Trp550 display alternate conformations, clearly visible despite the modest resolution of the data and refining to equal occupancies. For clarity, only one of the two alternate conformations in JFH1-pO_NS5B is shown.
Table 1: Dinucleotide formation by various NS5B constructs used in this study.

<table>
<thead>
<tr>
<th>NS5B (HCV strain)</th>
<th>Initiation product</th>
</tr>
</thead>
<tbody>
<tr>
<td>JFH1Δ21-wt</td>
<td>1</td>
</tr>
<tr>
<td>J6Δ21-wt</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>J6Δ21−V405I</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Con1Δ21-wt</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Con1Δ21-V405I</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>J4Δ21-wt</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>J4Δ21-I405V</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>JFH1-FL</td>
<td>0.31 ± 0.04</td>
</tr>
</tbody>
</table>

Gel-based initiation assay was performed by incubating NS5B and G3-U RNA in a reaction buffer containing 500 µM ATP and 10µM $^{32}$P$\alpha$-CTP at 30°C. Aliquots were removed at different time points and RNA products analyzed on 22% polyacrylamide gel. Following electronic autoradiography, RNA products were quantified with Quantity One software. The data are expressed as a function of the initiation product synthesized by the JFH1_NS5B-Δ21wt defined as 1. They corresponded to the mean of three independent experiments ± standard deviation.
Table 2: Elongation/initiation ratios by the various NS5B constructs used in this study.

<table>
<thead>
<tr>
<th>NS5B (HCV strain)</th>
<th>Elongation/initiation product</th>
</tr>
</thead>
<tbody>
<tr>
<td>J6Δ21-wt</td>
<td>0.003 ± 0.000</td>
</tr>
<tr>
<td>J6Δ21−V405I</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Con1Δ21-wt</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>Con1Δ21-V405I</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>J4Δ21-wt</td>
<td>0.021 ± 0.005</td>
</tr>
<tr>
<td>J4Δ21-I405V</td>
<td>0.014 ± 0.004</td>
</tr>
<tr>
<td>JFH1Δ21-wt</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td>JFH1-FL</td>
<td>0.011 ± 0.004</td>
</tr>
</tbody>
</table>

Gel-based initiation-elongation assay was performed by incubating 0.7 or 1 µM NS5B with 0.7 or 1 µM G1-C RNA in a reaction buffer containing 500 µM ATP, GTP and 3’dUTP and 32P-α-CTP at 30°C. Aliquots were removed at different time points and RNA products analyzed on 22% polyacrylamide gel. Following electronic autoradiography, the initiation dinucleotide and the 11 nt elongation products were quantified with Quantity One software and the elongation/initiation ratio determined after correction for the number of labeled nucleotides in the product. The data are the mean of four independent experiments ± standard deviation.
Table 3. Statistics of data collection and refinement

Values for the outer shell are given in parentheses.

<table>
<thead>
<tr>
<th>NS5B/Crystal form/PDB code</th>
<th>J6-V405I/same as J6 wt/4ADP</th>
<th>JFH1/cO/4AEP</th>
<th>JFH1/pO/4AEX</th>
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<tr>
<td>Diffraction source</td>
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<td>ESRF - ID14</td>
<td>SOLEIL - PX1</td>
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<td>Resolution range (Å)</td>
<td>44.19 (1.90-1.95)</td>
<td>46.24-1.80 (1.83-1.80)</td>
<td>40.75-2.41 (2.48-2.41)</td>
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<td>Spacegroup and Unit cell parameters (Å; degrees)</td>
<td>P2₁ 2₁ 2₁ ; a = 64.5, b = 95.6, c = 114.4, α = 90, β = 90, γ = 90</td>
<td>C222₁ ; a = 101.0, b = 110.5, c = 115.3, α = 90, β = 90, γ = 90</td>
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<td>59,618</td>
<td>51,101</td>
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<td>Completeness (%)</td>
<td>99.7 (98.3)</td>
<td>99.5 (99.0)</td>
<td>99.9 (100.0)</td>
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<tr>
<td>Redundancy</td>
<td>6.0 (5.9)</td>
<td>5.3 (5.3)</td>
<td>7.3 (7.2)</td>
</tr>
<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>13.2 (2.0)</td>
<td>11.5 (1.8)</td>
<td>8.9 (1.9)</td>
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<tr>
<td>R_{sym} (%)</td>
<td>11.0 (110.0)</td>
<td>12.3 (120.3)</td>
<td>20.4 (111.7)</td>
</tr>
<tr>
<td>No. of reflections used in refinement</td>
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<td>59,601</td>
<td>51,080</td>
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<tr>
<td>Overall average B factor (Å²)</td>
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<td>32.7</td>
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<tr>
<td>Final R_{work}</td>
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<td>18.7 (32.4)</td>
<td>18.7 (24.7)</td>
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<tr>
<td>No. of reflections for R_{free}</td>
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<td>1717</td>
</tr>
<tr>
<td>Final R_{free}</td>
<td>20.1 (28.15)</td>
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</tr>
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<td>0.006</td>
<td>0.003</td>
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<td>Ramachandran plot analysis</td>
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<td>Most favoured regions (%)</td>
<td>98.0</td>
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<td>Additionally allowed regions (%)</td>
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<td>Disallowed regions (%)</td>
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<td>0.0</td>
</tr>
</tbody>
</table>

* In parentheses, statistics for the highest resolution shell.

In the equation for R_{sym}, only those reflections measured more than once were considered. In the equation for R and R_{free}, the same set of reflections was used for all structures. F_{obs}
are the structure factors deduced from the measured intensities and $F_{\text{calc}}$ the structure factors calculated from the model. $k$ is a scale factor to put the $F_{\text{calc}}$ on the same scale as the $F_{\text{obs}}$. 