Hepatitis C virus polymerase–polymerase contact interface: Significance for virus replication and antiviral design

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Abstract
The hepatitis C virus (HCV) replicates its genome in replication complexes located in micro-vesicles derived from endoplasmic reticulum. The composition of these replication complexes indicates that proteins, both viral and cellular in origin, are at high concentrations. Under these conditions, protein–protein interactions must occur although their role in the replication pathways is unknown. HCV RNA-dependent RNA-polymerase (NS5B) initiates RNA synthesis in these vesicles by a de novo (DN) mechanism. After initiation, newly synthesized dsRNA could induce conformational changes that direct the transition from an initiating complex into a processive elongation complex. In this report, we analyze the role played by NS5B–NS5B intermolecular interactions controlling these conformational rearrangements. Based on NS5B protein–protein docking and molecular dynamics simulations, we constructed mutants of residues predicted to be involved in protein–protein interactions. Changes at these positions induced severe defects in both the activity of the enzyme and the replication of a subgenomic replicon. Thus, mutations at the interaction surface decreased both DN synthesis initiation and processive elongation activities. Based on this analysis, we define at an atomic level an NS5B homomeric interaction model that connects the T-helix in the thumb subdomain of one monomer, with the F-helix of the fingers subdomain in other monomer. Knowing the molecular determinants involved in viral replication could be helpful to delineate new and powerful antiviral strategies.

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1. Introduction
The phosphoryl transfer reaction of all nucleic acid polymerases is catalyzed by a two-metal ion mechanism (Steitz, 1999). In this model, the correct structural conformation must be maintained during the replicative process to promote the formation of the phosphodiester bond (Steitz, 1999). RNA-dependent RNA-polymerases (RdRPs) catalyze the phosphoryl transfer reaction between ribonucleotides in an RNA template-dependent fashion (Ng et al., 2008). This step is critical during the replicative cycle of positive-strand RNA viruses (RNA(+)), including those infecting humans, and therefore is a key target to develop new antiviral agents (Ferrer-Orta et al., 2006). Viral and cellular proteins that together form still poorly understood replication complexes (RCs) perform RNA virus replication. The viral replication machinery is located on intracellular membranes to both increase the efficiency of replication and to protect the RNA intermediate from cellular defences (Denison, 2008). Like other RNA(+) viruses, hepatitis C...
virus (HCV) RCs have been described to be associated with endoplasmic reticulum (ER)-derived micro-vesicles (Quinkert et al., 2005). One viral component of the HCV RCs, the non-structural protein 5B (NS5B), contains a GDD signature motif and displays enzymatic activity that is typical of an RdRP (Behrens et al., 1996; Lohmann et al., 1997). Thus, NS5B coordinates the phosphoryl transfer reaction during viral genome replication.

HCV NS5B exhibits a so-called right-hand shape, which is common to many nucleic acid polymerases (Ferrer-Orta et al., 2006; Ng et al., 2008). In common with other viral RdRPs, the fingers and thumb domains are connected by the $D_1$ and $D_2$ loops of the fingers, which thus are named fingertips (Fig. 1A) (Ferrer-Orta et al., 2006). Initiation of synthesis of HCV RNA in vivo occurs by a de novo (DN) mechanism (Luo et al., 2000; Zhong et al., 2000), probably in a similar manner as previously described for the phi6 polymerase (Butcher et al., 2001). Two structural motifs in HCV NS5B are essential for DN synthesis, the β-loop and C-terminus (residues E437 to I454 and L545 to R566, respectively, numbered as in strain HC-J4 from genotype 1b (O'Farrell et al., 2003)) (Fig. 1A, color magenta). Both structures are directed to the active site and constitute the platform where the first nucleotide should be positioned (Bressanelli et al., 1999; Lesburg et al., 1999; O'Farrell et al., 2003). Once the first nucleotide is added, these two domains must be relocated to provide sufficient space to house a double-stranded RNA (dsRNA), as has been recently described for the crystal structure of a binary dsRNA–NS5B complex (Mosley et al., 2012) (Fig. 1B). This relocation involves a complex structural rearrangement in NS5B and therefore is thought to be the most critical step during RNA replication (Bressanelli et al., 1999; Lesburg et al., 1999; O'Farrell et al., 2003) (Fig. 1B). Structural and biochemical analyses have shown that GTP and some NS5B residues (mainly I405) may play a critical role in dinucleotide formation and the transition from DN initiation to elongation (Harrus et al., 2010; Scrima et al., 2012; Simister et al., 2009). Recently, in accordance with the proposal that I405 mediates DN initiation without GTP modulation (Simister et al., 2009), a clear correlation of a closed-thumb conformation of HCV NS5B and the efficiency of the enzyme for DN RNA synthesis has been reported (Schmitt et al., 2011).

Conversions from open to closed conformations are important in the catalytic cycle of nucleic acid polymerases (Doublie et al., 1999). Open and closed conformations of viral nucleic acid polymerases have been described for the human immunodeficiency virus reverse transcriptase (HIV RT) and HCV NS5B, among others (Biswal et al., 2005; Huang et al., 1998). The significance of HCV NS5B open and closed conformations is controversial. Some groups have suggested movement of the thumb subdomain to explain the transition among these conformations (Biswal et al., 2005), whereas other groups argue that the open conformation also needs large rearrangements of the $D_1$ loop to allow the accommodation of the newly synthesized dsRNA (Chinnaswamy et al., 2008). The recently solved crystal structure of a β-loop-deleted mutant in complex with dsRNA seems to indicate that a template-primer RNA can be placed into the active site of HCV NS5B without

![Fig. 1. NS5B structure and reaction products. (A) HCV NS5B polymerase. Crystal structure of the NS5B polymerase (HC-J4, 1NB4.pdb, O'Farrell et al., 2003) showing its domain features in different colors: fingers (blue), palm (red), thumb (green), loops $D_1$ and $D_2$ (gray), and β-loop and C-terminal subdomain (magenta). Catalytic aspartic acid residues at positions 220, 318, and 319 in the palm domain are shown as sticks. (B) Structure alignments. Alignment of the apo form of NS5B (red; 1NB4.pdb) with the NS5B–dsRNA form (gray; 4E7A.pdb, Mosley et al., 2012) is shown. Nucleic acid structure was omitted for clarity. (C) HCV polymerase reaction products. M, LE19 RNA labelled at the 5' end with $^{32}$P. −GTP and +GTP, reactions performed in the absence or presence of GTP to obtain primer extension (PE) only (−GTP) or de novo (DN) and PE products (+GTP). Template sequence (in black) and expected newly synthesized product sequences (in red) for DN and PE reactions are shown.](image-url)
alteration of the conformation of the fingertips (Mosley et al., 2012). Additionally, the template channel, the entry site for the incoming nucleotide and exit channel for the newly synthesized dsRNA, can be positioned in this crystal structure without significant changes to the A1 loop. When structures of NS5B in the apo and binary complex are compared, the greatest structural change involves the thumb subdomain (Fig. 1B). Specifically, the HCV NS5B–dsRNA structure shows a dramatic rearrangement of loop residues 397–405, which connects the primer buttress helix with the primer grip helix, as well as movement of several helices in the thumb domain, including the helix T (Mosley et al., 2012). Interestingly, residue His502 has been shown to be involved in intermolecular NS5B–NS5B interactions (Bellon-Echeverria et al., 2010; Qin et al., 2002; Wang et al., 2002). The structural determinants for these interactions remain elusive, although some findings point towards head-to-tail contacts which involve elements from fingers and thumb subdomains (Clemente-Casares et al., 2011; Wang et al., 2002). Polymerase–polymerase interactions have been related to polymerase function in the RNA(+) poliovirus ( Hobson et al., 2001; Lyle et al., 2002; Pata et al., 1995; Spagnolo et al., 2010). Similarly, HCV NS5B–NS5B interactions have been related to its RdRP activity, and have been proposed to be potential targets for some non-nucleoside inhibitors directed against NS5B ( Bellon-Echeverria et al., 2010; Biswal et al., 2006; Clemente-Casares et al., 2011; Gu et al., 2004; Love et al., 2003; Qin et al., 2002; Wang et al., 2002).

With these antecedents, we have proposed a model for NS5B–NS5B interactions, and defined the amino acid residues that play a key role in these contacts. We have studied the contribution of these residues towards NS5B RdRP activity and its RNA synthesis ability, both in vitro and in vivo. We have also tested the effect of these residues on DN initiation and primer extension (PE) activities of HCV NS5B. Based on our data, we define at a molecular level the contact surface between two HCV NS5B monomers. Importantly, the NS5B–NS5B interaction surface helps to understand the molecular mechanisms involved in efficacy and resistance of some non-nucleoside antiviral drugs directed against HCV NS5B as well as defines itself as a target for new antiviral drugs.

2. Material and methods

2.1. NS5B A21 polymerase, mutants and plasmid construction

The plasmid containing the gene-encoding region of HCV NS5B from genotype 1 with a deletion of 21 aa at the C-terminal end (NS5BA21) was kindly provided by Dr. Nyanguile (Pauwels et al., 2007). This polymerase (pJ4-HC strain) was mutagenized to introduce changes at positions R114, E128, D129, E437, E440, and H502. Previously obtained construct (from other studies) with the desired point mutations in NS5B were digested with the restriction enzymes KpnI and StuI, and the DNA fragments were ligated into previously obtained construct (from other studies) with the desired changes at positions R114, E128, D129, E437, E440, and H502. Pre-

2.2. Protein expression and purification

Proteins used in this study were overexpressed and purified as previously reported ( Bellon-Echeverria et al., 2010) in an AKTA FPLC (GE Healthcare). Aliquots of the purest and most concentrated protein samples were adjusted to 50% glycerol and stored at ~80°C until use. All purification processes were followed by SDS–PAGE and Coomassie brilliant blue staining. Protein was quantified by SDS–PAGE gel imaging and protein determination using the Brad- ford assay.

2.3. In vitro RdRP and single-round replication assays

RNA polymerase assays were performed using the symmetric substrate LE-19 (sequence 5’ UGUUAUAUUAUUGUAAAC 3’), which is capable of both primer-extension and DN initiation (Fig. 1C). Except when indicated otherwise, LE-19 RNA was pre-incubated for 15 min in a reaction mixture containing 20 mM MOPS, pH 7.3, 5 mM MnCl2, 35 mM NaCl, 100 mM NS5B and 500 µM GTP. Reactions were started by adding 100 µM ATP and UTP and 1 µCi [γ-32P]CTP (3000 Ci mmol, PerkinElmer Life Sciences). In addition, samples for single-round analysis were supplemented with 200 µg/ml heparin after pre-incubation. Reactions were carried out in a final volume of 10 µl. Reactions were stopped using EDTA/formamide loading buffer at different times as indicated in each experiment. Products were resolved using denaturing polyacrylamide (23% PAA, 7 M urea) gel electrophoresis. Gels were exposed to phosphorimager screens and scanned with Typhoon (Molecular Dynamics). Alternatively, X-ray autoradiography films (Amersham Hyperfilm ECL) were used. Quantification was performed from samples derived from experiments done and resolved by gel electrophoresis in parallel. Quantification of band intensities was carried out on a phosphorimager employing ImageQuant software (GE Healthcare).

2.4. Protein–protein docking and Molecular dynamics

The publicly available X-ray crystal structure of the apo-form of the HC-J4 RNA polymerase (1NB4) ( O’Farrell et al., 2003) was obtained from the Protein Data Bank (Berman et al., 2000). The ClusPro Server (Comeau et al., 2004a,b; Kozakov et al., 2006, 2010) was used for re-docking NS5B without pre-established interaction residues. The output PDB files were used to calculate the binding energy of the generated complexes, removing clashes (using the strongest van der Waals parameters) with FoldX (Schymkowitz et al., 2005). The dimer with the best-predicted interaction energy was used in the subsequent molecular dynamics (MD) analysis. The complex was prepared using the tleap program in AMBER10 (Salomon-Ferrer et al., 2013). The parameter set used was the biomolecular force field ff99bsc0 (Perez et al., 2007). The complex was soaked in a rectangular box of TIP3P water molecules with a margin of 12 Å along each dimension. Cl− ions were added to neutralize the system. This yielded about 170,000 atoms for the system. The solvated complex was equilibrated by carrying out a short minimization, consisting of 50 ps of heating and 50 ps of density equilibration with weak restraints on the complex, followed by 500 ps of constant pressure equilibration at 300 K. All simulations were run with shake on hydrogen atoms, a 2 fs time step, and langevin dynamics for temperature control. Production phase of the simulation was carried out using the same conditions as during the previous equilibration, and lasted a total of 2 ns with coordinates being recorded every 10 ps. In order to calculate interaction energy and solvation free energy for the complex, both MM/GBSA and MM/PBSA were used as described in Gohlke and Case (2004).

2.5. In vitro transcription, electroporation and colony formation

After digestion with Scal, the replicon DNAs (WT and mutated variants) were extracted with phenol–chloroform to remove RNase activity. The in vitro transcription was performed with 1 µg of
digested DNA and the Megascript T7 kit (Ambion), following the manufacturer’s instructions. The reaction was incubated for an additional 2 h at 37 °C after supplementation with 40 U of T7 RNA pol (Ambion). The RNA obtained was purified with the Megaclear kit (Ambion), quantified, and stored at −80 °C until use.

The Huh-7 Lunet cell line employed in this study was kindly provided by R. Bartenschlager (Institute for Virology, Johannes-Gutenberg University, Mainz, Germany). Cells were electroporated with transcribed RNA from WT replicon or replicons with NS5B mutations E128A, D129A, and H502A, using Electrobuffter kit (Cell projects, Iberlabo, Madrid, Spain), in accordance with the manufacturer’s instructions. Briefly, exponentially growing cells were harvested and washed with electroporation medium. Four micrograms replicon RNA plus 6 μg of carrier Huh-7 RNA (Huh-7 total RNA) were mixed with the electroporation medium and added to an aliquot of 4 × 10⁶ cells. The cell suspension was transferred to a cuvette (Bio-Rad Laboratories, Madrid, Spain) and pulsed once with Gene Pulser II (Bio-Rad; settings: 975 μF; 270 V). After electroporation, 2 × 10⁶ (undiluted sample), 2 × 10⁵ (1:10 dilution), and 1 × 10⁵ (1:20 dilution) cell suspensions were seeded in Petri dishes containing Dulbecco’s modified Eagle’s medium (DMEM) (Sigma–Aldrich Química, Madrid, Spain) with 10% fetal bovine serum (Hyclone, Fisher Scientific, Madrid, Spain), supplemented with 1 M Hepes (Sigma), 1% glutamine, non-essential amino acids and antibiotics (Lonza, Madrid, Spain) and cultured at 37 °C in 5% CO₂ incubator. Twenty-four hours later, the DMEM culture medium was replaced with fresh medium containing G418 (Labclinics, Barcelona, Spain), and then refreshed twice per week for three weeks, after which colony quantification was performed by crystal violet staining.

3. Results

3.1. HCV NS5B is a cooperative polymerase

Previously, we and others have shown that ionic strength drives the transition of HCV NS5B from monomeric to oligomeric state (Bellon-Echeverría et al., 2010; Cramer et al., 2006). Furthermore, we have determined that a NaCl concentration range from 25 mM to 100 mM was optimal for HCV NS5B RdRP activity (Clemente-Casares et al., 2011). Based on these premise, we analyzed the DN and PE activities of HCV NS5B as a function of its concentration at low (35 mM) and high (100 mM) NaCl concentration. These experiments were done with template LE19 in the presence of GTP (the first nucleotide, Fig. 3B, lane 2, and Fig. 3C, lane 3) or GTP + UTP (the nucleotides that will be part of the initial dinucleotides during the preincubation period, only the PE product was obtained (Fig. 3B, lanes 2 and 3, and Fig. 3C, lanes 4 and 6). When the reaction was pre-incubated with nucleotides (Fig. 3B, lanes 2 and 3, and Fig. 3C, lanes 4 and 6). When the reaction was performed in the presence of heparin and without nucleotides during the preincubation period, only the PE product was obtained (Fig. 3B, lane 4, and Fig. 3C, lane 8). This result was not surprising, since only this kind of product can be synthesized when (i) GTP is lacking in the preincubation reaction, and (ii) heparin functions as a NS5B trap, thereby preventing any new synthesis when GTP and the other nucleotides are added after the pre-incubation phase (Fig. 3B, lane 4, and Fig. 3C, lane 8).

Fig. 2. HCV NS5B is a cooperative enzyme. (A–C). NS5B titration reactions. De novo (A) and primer extension (B) products obtained by WT NS5B at increasing concentrations of NaCl and at 35 mM NaCl concentration. (C) Effect of ionic strength on NS5B titrations. Relative DN and PE activities at increasing concentrations of WT NS5B protein and at indicated NaCl concentrations are shown. All reactions were carried out for 15 min (Fig. S1). Squares represent DN activity values whereas triangles represent PE products. Solid and dashed lines correspond to 35 mM and 100 mM NaCl concentration, respectively. Values correspond to the mean and SD of at least three independent experiments.

DN cooperativity, and PE to a lesser extent, are dependent on ionic strength.

In the next experiment, we included heparin in the reactions to function as a trap, and allowing only a single cycle of RNA polymerization. A schematic diagram of the experiment is depicted in Fig. 3A and results are presented in Fig. 3B and C. In the absence of heparin, when multiple cycles of RNA polymerization were allowed (Fig. 3B, lanes 1–3), DN activity was almost stable or even increased depending on the pre-incubation conditions, such as, in the absence of nucleotides (Fig. 3B and C, lane 1) or presence of GTP (the first nucleotide, Fig. 3B, lane 2, and Fig. 3C, lane 3) or GTP + UTP (the nucleotides that will be part of the initial dinucleotide, Fig. 3B, lane 3 and Fig. 3C, lane 5). By contrast, the PE product decreased when the reaction was pre-incubated with nucleotides (Fig. 3B, lanes 2 and 3, and Fig. 3C, lanes 4 and 6). When the reaction was performed in the presence of heparin and without nucleotides during the preincubation period, only the PE product was obtained (Fig. 3B, lane 4, and Fig. 3C, lane 8). This result was not surprising, since only this kind of product can be synthesized when (i) GTP is lacking in the preincubation reaction, and (ii) heparin functions as a NS5B trap, thereby preventing any new synthesis when GTP and the other nucleotides are added after the pre-incubation phase (Fig. 3B, lane 4, and Fig. 3C, lane 8). Pre-incubation with GTP in the presence of heparin by contrast, yielded both the DN and PE products (Fig. 3B, lane 5, and Fig. 3C, lanes 9 and 10). Interestingly, when the reaction was pre-incubated with the nucleotides GTP and UTP in the presence of heparin, only DN products were obtained as shown in Fig. 3B (lane 6) and C (lane 11).
The one-cycle conditions described above allowed us to explore the effect of reaction temperature on DN or PE activities separately. Under these one-cycle conditions, we tested the formation of DN and PE products at a range of temperatures in the absence or presence of GTP + UTP during the pre-incubation period (Fig. 3D and E). At the lowest temperature tested (20 °C), low and almost equivalent levels of DN and PE products were observed. At 25 °C, PE products were 6-fold more prevalent than DN products. PE product formation peaked at 30 °C and then gradually decreased at 35 °C and 40 °C. DN products were virtually non-existent at temperatures of 30 °C or higher (Fig. 3E).

3.2. WT RdRP activity can be complemented in trans

If protein–protein contacts play a role in modulation of RNA-polymerase activities, then it should be possible to regulate these activities by adding mutants with a lethal defect in activity (e.g., D220A). Functional polymerase–polymerase contacts of the poliovirus 3D protein have been previously demonstrated using this strategy (Spagnolo et al., 2010). We followed a similar approach to investigate the DN and PE activities of wild type HCV NS5B in the absence or presence of increasing concentrations of the lethal mutant D220A using LE19 as a template. To perform this experiment, we chose a WT NS5B concentration (100 nM) which yielded very low amounts of product, and determined whether supplementation we chose a WT NS5B concentration (100 nM) which yielded very low amounts of product, and determined whether supplementation of wild type HCV NS5B polymerase with catalytically inactive NS5B could create functional polymerase oligomers (Fig. 4). When the lethal mutant was present in the reaction, cooperation between WT and D220A enzymes was readily detected as an increase in the amount of DN and PE products (Fig. 4A and B). To determine the specificity of this trans complementation, we constructed and assayed the double mutant D220A/H502A (DH).
independent experiments. Reactions were carried out for 15 min (Fig. S1). Band values were obtained by using the ImageQuant software as described in Section 2.

The specificity of the reaction was lastly tested by performing the experiment with an unrelated lethal mutant (D338A) of the 3D polymerase from the foot-and-mouth-disease virus (FMDV) (Ferrer-Orta et al., 2009, 2004). In this experiment, the levels of the DN and PE products were unaltered despite the presence of the lethal FMDV polymerase. This result thus demonstrates that NS5B polymerase function can be complemented in trans by a catalytically inactive NS5B mutant, and this effect is lost in the presence of a NS5B mutant lethal for both activity and NS5B–NS5B interactions.

3.3. Molecular dynamics

Fingers and thumb domains in NS5B have been previously related to NS5B–NS5B interactions (Clemente-Casares et al., 2011; Wang et al., 2002). These interactions may be responsible for the cooperative nature of RNA synthesis shown by NS5B. In order to search for a more energetically favorable pose for this interaction, we performed a first round of protein–protein docking analysis. Results were obtained without applying any restrictions to the model. Interestingly, among the best-scoring results, interactions between helices F and T appeared most likely. After this, a short molecular dynamics simulation (2 ns) in explicit solvent was carried out to obtain a relaxed model, and MM/PBSA calculations were performed to determine the NS5B dimerization energy. The energetically most favourable model with a total free energy of binding of −102 kcal mol⁻¹ and a calculated buried surface area of 961.9 Å² is presented in Fig. 5. Energy contributions to the mean binding energy for the 250 snapshots (Table S1) were in accordance with results obtained for other relevant protein–protein interactions such as Ras–Raf (Gohlke and Case, 2004) and the human immunodeficiency virus protease (Wang and Kollman, 2000). Several residues from both monomers are present in the model of a NS5B homodimer comprising of interacting amino acids from helices F (part of the fingers domain) and T (part of the thumb domain), as well as residues from the β-loop and fingertip (Fig. 5A). The majority of these residues are fully conserved among different genotypes. Monomer A contributes the conserved residues R114, H118, D125, E128, and D129 from helix F, as well as residues F101, P265, and Y276. Monomer B contributes the conserved amino acids I23, P25, and R32 from the fingertips, E437, and E440 from the β-loop, and R503, and R505 from helix T. These residues establish interactions mainly by ionic interactions. Only residues F101, P265, and Y276 are involved in hydrophobic interactions (Fig. 5B).

3.4. RNA-polymerase activity of NS5B bearing mutations located at the contact surface

Taking into account the favoured energy model and our previous results (Bellon-Echeverria et al., 2010; Qin et al., 2002) we constructed mutants of residues from helices F and T. Specifically, positions E128, D129, and H502 were mutated to alanine, E437 and E440R mutated to arginine and position R114 was mutated to glutamate. To test the effect of these mutations on the cooperation of DN RdRP activity, we performed polymerase assays at increasing concentrations of the mutant proteins. In Fig. 6A, we depict the increase in DN initiation activity as a function of NS5B protein concentration. For the WT NS5B, its DN activity exhibited a 5-fold increase as its concentration increased from 50 nM to 100 nM, and remained steady up to 150 nM of HCV NS5B protein concentration. This sigmoidal curve allowed us to calculate the Hill coefficient as reported for Fig. 2C. By contrast, R114E mutant exhibited linearity for DN product formation at all NS5B protein concentrations tested. The other mutants exhibited relatively poor DN activities compared to the WT or R114E NS5B. Of these, E128A, D129A, and E440R exhibited lower than 15% DN activities relative to the WT NS5B. Of these, E128A, D129A, and E440R exhibited lower than 15% DN activities relative to the WT NS5B (Fig. 6A), while E440R and H502A displayed ~30% and 40% DN activity, respectively relative to WT NS5B at the highest protein concentration tested (Fig. 6A). This data indicates that mutations at the contact surface of NS5B polymerase results in impairment of DN initiation activity of the protein, albeit to different extents depending on the contact residue mutated.

Since DN initiation activity was diminished in the surface mutants (Fig. 6A), we were curious to know the status of PE activity and the transition from initiation to elongation in these mutants. With this objective, we investigated and analyzed the ratio of DN to PE activities of the different mutants, and compared them to the value obtained for the WT protein. Towards this end, we performed experiments in the absence or presence of GTP to compare the amount of product obtained from DN activity (plus GTP) to that of PE (minus GTP) (Fig. 6B). Mutants R114E, E437R, and E440R displayed very low levels of PE and they were not included in this analysis. Mutants E128A, D129A, and H502A, showed lower DN and PE activities than WT NS5B (Fig. 6B). The diminution was more pronounced for PE than for DN initiation, and this manifested in an increase in their DN/PE ratios (Fig. 6B).
Fig. 5. Molecular modeling and protein docking. (A) Model showing the energetically most favorable dimer. In white is shown one monomer and in black the other. Helix T from monomer in white is colored black, and helix F from black monomer is colored in white. A zoom of the interaction surface is shown in the lower part of the figure, showing the side chains of the most important residues forming part of the interaction surface as well as the β-loop of the monomer in white. (B) Scheme showing the most energetically plausible NS5B–NS5B interactions. The upper part corresponds to domains and amino acids from one monomer and the lower part to an interactive second monomer. The most important NS5B domains such as fingertips, helixes, and the β-loop stem are indicated. Interactions corresponding to ionic side chain-side chain hydrogen bonds, main chain-side chain hydrogen bonds, and hydrophobic interactions are as indicated. Encircled black amino acid position numbers indicate residues that are totally conserved among genotypes, encircled gray amino acid positions indicate residues that are mostly conserved among genotypes, dashed-encircled gray amino acid positions indicate residues that are not conserved. Ionic protein–protein interactions were studied with the Protein Interaction Calculator (PIC) web server (Tina et al., 2007).
3.5. Effect of mutations in NS5B–NS5B contact sites on cell colony formation in Huh7 Lunet cells

To determine the effects of mutations in NS5B in a biologically more relevant context, mutations E128A, D129A, and H502A were engineered into the replication-competent subgenomic HCV RNA. Plasmids were linearized, RNA was obtained by in vitro transcription, and in vitro transcripts were used to transfect Huh7 Lunet cells by electroporation followed by selection for G418 resistance (Lohmann et al., 1999a). Replication of the subgenomic HCV replicon RNA in Huh7 Lunet cells results in expression of neomycin phosphotransferase, which renders Huh7 Lunet cells resistant to the antibiotic G418 (Lohmann et al., 1999a). Three different conditions were analyzed in triplicate (2 μg RNA, and 1/10 and 1/20 dilutions thereof) as described in Materials and Methods. The results showed a moderate effect of the E128A mutation, and a severe or even lethal effect of mutations D129A and H502A (Fig. 7). By comparing the transfections with undiluted and diluted RNA, we could infer that the colony forming ability of H502A subgenomic replicon decreased by 10–20-fold with respect to WT, whereas mutation D129A was lethal. These results indicate that some positions at the surface of the polymerase, which could form part of the putative NS5B–NS5B contact sites, may be important for the replication of the HCV subgenomic replicon in Huh7 Lunet cells.

4. Discussion

Viruses have different strategies to initiate genome replication. HCV initiates its genome synthesis in vitro, and presumably in vivo, by a DN mechanism requiring the replicase, template and triphosphate nucleotides (Luo et al., 2000; Zhong et al., 2000). The structural requirements for DN initiation have been broadly described for the bacteriophage φ6 RNA-dependent RNA polymerase (Butcher et al., 2001). In this model, some domains of the RNA polymerase act as a platform where the first two nucleotides are positioned in the correct orientation to form the phosphodiester bond and generate the dinucleotide. After completion of the first phosphodiester bond, protein domains responsible for initiation must be rearranged to allow the accommodation of the primer-template RNA (Butcher et al., 2001). HCV NS5B domains acting as the initiation platform have also been described by extensive
mutational and biochemical analyses (Ranjith-Kumar and Kao, 2006). The β-loop and C-terminal domain have been pinpointed as the structural elements responsible for DN initiation of HCV RNA (Harrus et al., 2010; Hong et al., 2001; Ranjith-Kumar et al., 2002). Both domains must be accurately positioned to allow NS5B to synthesize the dinucleotide. Moreover, both domains have to be relocated thereafter to allow NS5B to elongate in a template-dependent manner the newly synthesized short primer.

Consistent with previous observations, we have demonstrated cooperativity in RNA synthesis (Figs. 2 and 3) (Bellón-Echeverría et al., 2010; Clemente-Casares et al., 2011; Chinnaswamy et al., 2010; Gu et al., 2004; Qin et al., 2002; Wang et al., 2002). We have also shown that low levels of wild type NS5B activity can be enhanced by trans-complementation with the D220A lethal mutant, but this activity is decreased if the lethal mutant also carried the H502A mutation (mutant DH), which abrogates NS5B–NS5B interaction (Fig. 4). By using molecular dynamics and in silico docking we propose a model in which helix F residues from one NS5B molecule stabilize the β-loop, fingertips, and T helix from the thumb subdomain of a second molecule, to ensure DN initiation (Figs. 5 and 8). Thus, residues R114, E128, D129, E437, E440, and H502 among others would be involved in these protein–protein interactions. These NS5B interactions can be supposed to be pangenomic, although genotype 2a harbors serine at position 502 and 510 of NS5B. Previously, we have demonstrated that NS5B from HCV genotype 2a exhibited the β-loop, fingertips, and T helix from the thumb subdomain of a second molecule, to ensure DN initiation (Figs. 5 and 8). Thus, residues R114, E128, D129, E437, E440, and H502 among others would be involved in these protein–protein interactions. These NS5B interactions can be supposed to be pangenomic, although genotype 2a harbors serine at position 502 and 510 of NS5B. Previously, we have demonstrated that NS5B from HCV genotype 2a exhibited a lower interaction level than that from genotype 1b (Clemente-Casares et al., 2011). Furthermore, the genotype 2a NS5B mutation E125K could compensate at least in part the deleterious effect of H502S and 2011). Furthermore, the genotype 2a NS5B mutation E125K could compensate at least in part the deleterious effect of H502S and R503, which form part of the putative allosteric GTP binding site (Bressanelli et al., 2002; Gai et al., 2005). The existence and exact function of this allosteric GTP binding site is controversial. Activation of HCV NS5B as well as other RNA(+) virus polymerases by GTP has been described previously (Chinnaswamy et al., 2010; Choi et al., 2004; Harrus et al., 2010; Lohmann et al., 1999b; Morin et al., 2012; Nomaguchi et al., 2003). GTP-driven activation of NS5B must occur at the DN initiation step because increasing concentrations of this nucleotide specifically inhibited PE activity on LE19 RNA template (Ranjith-Kumar et al., 2002). We have also shown that GTP inhibits NS5B–NS5B interaction (Bellón-Echeverría, 2011) (Fig. S2). This inhibition is dependent on the number of phosphates of the nucleoside as well as on the concentration of the triphosphate nucleotide. Similar observations have been previously reported when NS5B was resolved by gel filtration chromatography in the absence or presence of GTP (Chinnaswamy et al., 2010). Dr. Kao and co-workers also observed an increase in the thermal stability of NS5B in a GTP-concentration-dependent manner (Chinnaswamy et al., 2010). Moreover, mutations at positions R32, E128, D129, Y276, H502, and R503, which form part of the putative allosteric GTP binding site, showed severe defects in their phenotypes when

Recent findings have indicated that I405 in HCV NS5B might be responsible for the high DN initiation efficiency shown by the JFH1 isolate (Scrima et al., 2012; Schmitt et al., 2011). The side chain of this amino acid possibly stabilizes the β-loop by intra-molecular interactions in a position that favours DN synthesis (Fig. 8). However, in the majority of the isolates described to date, a valine instead of isoleucine is present at this position (Waheed et al., 2012). Moreover, residues E18, I405, W397, and H428, among others, also interact with the β-loop or fingertips to maintain the internal structure that is needed for the correct positioning of initiation factors (mainly nucleotides, template, and divalent cations) (Chinnaswamy et al., 2008; Simister et al., 2009). According to our results, inter-molecular interactions between helix F from the fingers subdomain of one NS5B molecule and helix T from the thumb subdomain of a second molecule could play the same role as I405 in the intra-molecular model. Consistent with this hypothesis, several mutations at positions in the vicinity of the F helix in the fingers subdomain have been correlated with resistance to the site II NS5B inhibitor drug filibuvir. This drug binds to the thumb domain of NS5B and selects resistant mutations at the binding site as well as, and more intriguingly, at positions 110 and 134 in the fingers domain (Shi et al., 2009). The NS5B–NS5B interactions described in this study could be useful to understand these results.

Part of the NS5B–NS5B interaction surface overlaps with the putative allosteric GTP binding site (Bressanelli et al., 2002; Gai et al., 2005). The existence and exact function of this allosteric GTP binding site is controversial. Activation of HCV NS5B as well as other RNA(+) virus polymerases by GTP has been described previously (Chinnaswamy et al., 2010; Choi et al., 2004; Harrus et al., 2010; Lohmann et al., 1999b; Morin et al., 2012; Nomaguchi et al., 2003). GTP-driven activation of NS5B must occur at the DN initiation step because increasing concentrations of this nucleotide specifically inhibited PE activity on LE19 RNA template (Ranjith-Kumar et al., 2002). We have also shown that GTP inhibits NS5B–NS5B interaction (Bellón-Echeverría, 2011) (Fig. S2). This inhibition is dependent on the number of phosphates of the nucleoside as well as on the concentration of the triphosphate nucleotide. Similar observations have been previously reported when NS5B was resolved by gel filtration chromatography in the absence or presence of GTP (Chinnaswamy et al., 2010). Dr. Kao and co-workers also observed an increase in the thermal stability of NS5B in a GTP-concentration-dependent manner (Chinnaswamy et al., 2010). Moreover, mutations at positions R32, E128, D129, Y276, H502, and R503, which form part of the putative allosteric GTP binding site, showed severe defects in their phenotypes when

Fig. 8. Model of NS5B–NS5B interaction. (A) Zoom in at the interaction surface. Interacting parts of two NS5B monomers (gray and khaki) are depicted. Side chains from residues that are involved in the interaction between a monomer and its binding partner are shown in red and blue, respectively. Residues E18 and I405 are shown in green, whereas the β-loop is depicted in orange.
introduced in an HCV replicon (Fig. 7) [Cai et al., 2005; Ma et al., 2004; Qin et al., 2001]. Some of these positions are also related to the NS5B–NS5B contacts described in this study (Figs. 5 and 8). These studies argue in favor of a closed conformation of the polymerase and GTP concentrations as among the prime regulators of DN initiation by NS5B.

NS5B–NS5B interactions described in this study (Figs. 5 and 8) do not hamper RdRP activity. The rNTP entry channel, template channel, and dsRNA exit channel described recently [Deval et al., 2007; Mosley et al., 2012] are accessible in the NS5B homodimer (Fig. S3 A–C). The NS5B residues involved in the putative interaction with NS5A [Qin et al., 2001], and postulated to be critical for NS5A binding, have also been mapped in this model (Fig. S3 D). Therefore, monomers in the complex could carry out the RNA–polymerase reaction in this scenario when NS5B interacts with itself, as the rNTP and template can gain access into the NS5B catalytic active site, and the dsRNA product can exit from it. The observed NS5B–NS5B interaction does not exclude the possibility that only some monomers are performing DN initiation, whereas other molecules could acquire the conformation for PE. Interestingly, dsRNA exit channel has been mapped at two different locations, one through the channel open by the C-terminal end movement (Mosley et al., 2012) and the other through the rNTP entry channel [Vaughan et al., 2012]. Our data suggests the occurrence of both options. This may be visualized as a closed NS5B initiating by a DN mechanism the synthesis of a dsRNA comprising of a template primed with a short oligonucleotide. This dsRNA could then exit through the rNTP exit channel, get transferred to a NS5B in an open conformation where it could then get elongated and exit through the channel open by the C-terminal end movement. This paradigm however needs to be validated experimentally by using structural and biophysical approaches, and might help to clarify the structural determinants and dynamics of the HCV replication complex.

Polymerase–polymerase interactions have been previously reported for other RNA(+) viruses including poliovirus [Hobson et al., 2001; Spagnolo et al., 2010], calicivirus [Kaiser et al., 2006], and foot-and-mouth-disease virus [Bentham et al., 2012]. In this study, we have shown that homomeric NS5B interactions are important both for RdRP activity and in vivo replication of a HCV subgenomic replicon. In addition, we have defined the surfaces involved in this interaction in silico, and demonstrated their importance by biochemical and cellular HCV–replication experiments. This model may explain the allosteric role of GTP in NS5B RdRP catalyzed reactions as well as the reported discrepancies of the dsRNA exit channel. In conclusion, we have described a novel strategy by which NS5B polymerase stabilizes itself in a conformation suitable for DN initiation of HCV RNA replication. Data presented in this study provides mechanistic clues as to how the HCV macromolecular replication complex may rearrange during HCV replication as well as the mechanism of some resistant mutations located far from the binding site of the drug. Furthermore, the NS5B–NS5B contacts proposed herein could be the target for new therapeutic interventions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.04.009.

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