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Genetic and biochemical diversity in the HCV NS5B RNA polymerase in the context of interferon α plus ribavirin therapy

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Abstract

The hepatitis C virus (HCV) RNA polymerase (RdRp) may be a target of the drug ribavirin and it is an object of drug development. Independent isolates of any HCV subtype differ genetically by ~10%, but the effects of this variation on enzymatic activity and drug sensitivity are poorly understood. We proposed that nucleotide use profiles (G/U ratio) among subtype 1b RdRps may reflect their use of ribavirin. Here, we characterized how subtype 1b genetic variation affects RNA polymerase activity and evaluated the G/U ratio as a surrogate for ribavirin use during pegylated interferon α and ribavirin therapy. Genetic and biochemical variation in the RdRp were compared between responders who would be largely sensitive to ribavirin and relapsers who would be mostly resistant. There were no consistent genetic differences between responder and relapser RdRps. RNA polymerization, RNA binding, and primer usage varied widely among the RdRps, but these parameters did not differ significantly between the response groups. The G/U ratio among a set of subtype 1a RdRps increased rather than decreased following failed therapy, as would be expected if it reflected ribavirin use. Finally, RdRp activity was significantly associated with ALT levels. These data indicate that (1) current genetic approaches cannot predict RNA polymerase behavior, (2) the G/U ratio is not a surrogate for ribavirin use, (3) RdRp activity may contribute to liver disease by modulating viral mRNA and antigen levels, and (4) drug candidates should be tested against multiple patient-derived enzymes to ensure widespread efficacy even within a viral subtype.

Keywords

Hepatitis C virus; RNA binding; RNA polymerase activity; Viral genetics

Hepatitis C virus (HCV) infects over 130 million people world-wide and is a major cause of liver failure and hepatocellular carcinoma (1;2). HCV has an enveloped virion containing a ~9,600 nucleotide long positive polarity RNA genome (2). The virus is highly genetically variable, with six genotypes that have less than 72% identity and multiple subtypes per genotype with ~80–85% identity (3;4). Independent isolates within a subtype are typically ~88–92% identical, and individual genomes in a patient share only 97–99% identity due to

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Disclosures

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HCV's replication as a quasispecies. The impact of this genetic variation on HCV's pathogenesis and response to therapy is poorly understood.

RNA replication is catalyzed by the RNA-dependent RNA polymerase (RdRp) encoded by the NS5B region of the genome. The RdRp has the typical nucleic acid polymerase structure that can be likened to a right hand, but there is an extension that links the fingers to the thumb domains (5;6). Most studies of the RdRp have employed a few common subtype 1b RdRp isolates, and hence the impact of HCV's high genetic variability on its structure and function is poorly understood.

HCV infections are treated with pegylated interferon α plus ribavirin. This year-long and physically demanding therapy clears the virus (sustained viral response, SVR) in only about half of patients infected with genotype 1, the most common genotype in the U.S.A. (7–9). The reasons for the poor success are poorly understood, but they are due to both viral and host factors. Viral factors are known to affect success of therapy because genotype 1 responds to therapy about 50% of the time, whereas response rates for genotypes 2 and 3 are ~80% (8;10;11). We have presented genetic evidence strongly implying that sensitivity to interferon-based therapy differs among independent HCV isolates even within a given subtype (12–14). Host factors include variable drug intake, different antiviral immune responses, and variable interferon responsiveness (15;16). Recently, human genetic differences in the IL28 gene cluster have been closely associated with response to therapy (17–19).

The precise mechanisms by which interferon α controls HCV are not known, but it clearly acts through multiple effectors. Blunting these effectors is essential for HCV to establish a chronic infection, and consequently HCV has evolved numerous mechanisms to limit their induction and/or action (20). The role of ribavirin during HCV therapy is less clear. Ribavirin approximately doubles the SVR rate when given with interferon α, primarily by reducing relapse after drug withdrawal (9;21). At least three mechanisms have been proposed for how ribavirin works (15;22;23). First, it inhibits inosine monophosphate dehydrogenase (IMPDH), which lowers GTP pools in cells. Second, it skews the immune system towards a Th1-like response. Finally, ribavirin is a guanosine analog which can be phosphorylated in cells to ribavirin triphosphate (RTP), which is a substrate for the HCV RdRp (24;25). Although RTP is a very poor inhibitor of the RdRp, incorporation of RMP into the viral RNA can induce mutations and can impair extension of RNA synthesis when it is in the template strand (22;24). The relative importance of these potential mechanisms is the subject of much debate.

We seek to determine whether ribavirin may work against HCV at least in part by being a substrate for the RdRp. We previously asked whether we could detect signatures of differential ribavirin use in patient-derived HCV NS5B sequences or in the biochemical behaviors of their encoded RdRps (26). We examined the NS5B gene from patients who failed interferon α monotherapy and were then re-treated with interferon α plus ribavirin. This approach maximized chances of detecting variations in the NS5B that may modulate ribavirin effectiveness because response to the second round of therapy would be primarily due to ribavirin. We found that amino acid substitutions in the RdRp associated with outcome of therapy clustered asymmetrically on the enzyme, with many variations localized in the fingers domain that can affect nucleotide selectivity in nucleic acid polymerases (27–30). We also observed higher use of GTP compared to UTP (G/U ratio) by some responder RdRps compared to non-responders. Both observations are consistent with the hypothesis that patients who clear HCV during interferon α plus ribavirin therapy are infected with variants whose RdRps use the GTP analog RTP better than enzymes from non-responders.

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Here, we examined treatment-naive patients to determine if these genetic and biochemical patterns were present in typical patients undergoing anti-HCV therapy for the first time.

Materials and Methods

Patient selection and sequence acquisition

Forty HCV 1b-infected participants of the Virahep-C study (12;31) were analyzed. The patients were treated for up to 48 weeks with 180 μg week^{-1} peginterferon α2a (Pegasys; Roche Pharmaceuticals, Nutley, NJ USA) and ribavirin (Copegus; Roche Pharmaceuticals) at 1000 or 1200 mg day^{-1} based on body weight. All patients provided informed, written consent, and this study was conducted in accordance with the Helsinki declaration and was approved by the Saint Louis University Institutional Review Board. Twenty-six patients achieved SVR and 14 were relapsers. SVR was defined as undetectable HCV titres six months following therapy. Relapsers had ≥2.8 $\log_{10}$ declines in viral titers and their titers transiently became undetectable before resurging.

The NS5B sequences were extracted from the full open reading frame sequences we previously reported (12). The patient identification numbers and Genbank identifiers are in Supplementary Table 1.

Genetic and statistical analyses

Phylogenetic trees were created using the Jukes-Cantor genetic distance model. Sequence alignments were performed with ClustalW. Mean pairwise genetic distances were calculated using the p-distance algorithm. Amino acid covariances were identified employing a custom program implementing the observed-minus-expected algorithm, and the covariance networks were analyzed using Cytoscape (13). Structural analyses were performed in Pymol using a 1b HCV RdRp structure (1GX5). Statistical significance was calculated using an independent samples t-test except where indicated. Statistical analyses were done using SPSS v. 15 (SPSS, Inc., Chicago, IL USA) and Prism v. 5.0 (GraphPad, Inc., La Jolla, CA USA).

RdRp expression and purification

Viral RNA was isolated and amplified as described (32). NS5B sequences lacking the C-terminal 21 codons with a C-terminal hexa-histidine tag were cloned into pTrcHis2B (Invitrogen, Carlsbad, CA USA). RdRps were expressed in *E. coli* Bl21 Codon+ Cells (Invitrogen) and purified by nickel-agarose affinity as described (26).

Homopolymeric templated RNA synthesis

RNA synthesis assays were performed as described (26). All biochemical data are summarized in Supplementary Tables 2 and 3.

RNA binding

RdRp (0.001–3 ug) was incubated with 5 mM $MgCl_2$, 25 mM Tris pH 7.5 and 2 pMol radiolabeled poly C on ice for 30 min. The mixture was passed through nitrocellulose (Schleicher& Schuell, Keene, NH USA) and Immobilon-Ny+ membranes (Millipore, Billerica, MA USA), and retained radioactivity was quantified with a phosphorimager. Binding data were fit to the equation $\theta = \theta_{max} [P]/([P] + K_{0.5})$, where θ is the percentage of bound RNA, θ_{max} is the percentage of maximal bound RNA, [P] is the RdRp concentration, and $K_{0.5}$ is the apparent dissociation constant (33) using Prism.

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Results

Patients

This study employed samples and data from genotype 1-infected patients who were treated with pegylated interferon α plus ribavirin as part of the Virahep-C study (31). We compared the NS5B genes and RdRp activities between HCV isolates from SVR patients who cleared the virus and relapsers who initially responded to therapy but in whom HCV resurged during or after treatment. We focused on SVR and relapser patients because our previous study of HCV RdRp activity during therapy (26) implied that differences in use of the guanosine analog ribavirin by the RdRp may influence outcome of therapy. Ribavirin's primary effect is to reduce relapse, approximately doubling the number of patients who achieve SVR (9;21). Therefore, HCV from the relapsers should be relatively insensitive to ribavirin because relapse occurred despite the presence of the drug. In contrast, HCV from most SVR patients should be relatively sensitive to ribavirin. This is because ribavirin was instrumental in driving the virus to extinction in ~50% of these patients, and many of the remaining SVR samples should also be sensitive to ribavirin, although this would have been clinically inapparent because interferon would have cleared the virus by itself.

Few genetic differences between NS5B genes from SVR and relapser patients

We examined the pretherapy consensus HCV NS5B sequences from all subtype 1b Virahep-C SVR and relapse patients (12) for genetic differences that may be correlated with outcome of therapy. Five sequences lacked the C-terminal 35 codons, and hence the sequences were truncated after codon 556 in the genetic analyses to ensure equal representation of all samples.

The genetic variation within the NS5B gene was less than average in the HCV genome, but it was still substantial, with a mean pairwise identity of 97% among these isolates. The amino acid distribution was significantly different between the SVR and relapser groups at only codon 98, where 22 of the SVR sequences had R and four had K, whereas in the 14 relapsers seven were R and seven were K ($p = 0.03$ by χ^2). No clustering of the SVR and relapser sequences could be detected in phylogenetic analyses, no significant differences were found between the SVR and relapser sequences in the number of variations relative to a reference sequence, and the number of variations found uniquely in each class were similar. We recently identified networks of coordinated amino acid substitutions (covariances) in the HCV genome (13). Networks of covarying positions were found in both the SVR and relapser NS5B sequences, but there were few differences between the networks from these two response groups. Therefore, despite substantial genetic variability among the NS5B sequences, there were no significant phylogenetic, diversity, or covariance network differences that could reliably distinguish NS5B genes from the SVR and relapser groups.

Cloning and expression of variant RdRps

We previously found elevated G/U ratios in RdRps from patients who had failed interferon monotherapy but then cleared HCV during re-treatment with interferon α plus ribavirin (26). In the previous study, response to the second round of therapy was primarily due to differential sensitivity to ribavirin, and as obvious genetic differences between the NS5B SVR and non-responder sequences could not be found in the previous cohort either, we extended our examination of the Virahep-C sequences to a biochemical analysis of the RdRps.

Hexahistidine-tagged expression constructs for NS5B genes lacking the C-terminal 21 codons were cloned; this truncation increases RdRp solubility without affecting biochemical activity (34). All clones were sequenced to ensure that the quasispecies variants chosen were

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close to the consensus sequence employed in the genetic analyses. These near-consensus sequence clones were defined as (1) having no more than four amino acid variations relative to their respective patient consensus sequence, and (2) having no differences relative to their patient consensus sequence at positions where variations unique to the SVR or relapser classes were present. The variations relative to the patient sequences are in Supplementary Table 1. The RdRps were expressed in *E. coli* and purified by nickel-affinity chromatography as described (26). Clones expressing adequate RdRp levels were obtained for 18 SVR and 13 relapser patients; all biochemical data for these enzymes are in Supplementary Table 2.

These 31 expression clones were compared to their respective patient consensus sequences to determine if they adequately represented the patient sequences. As expected, there were more variations in the expression clones than in the consensus sequences (an average of 3.2 additional variations for both groups), but these additional patient-derived variations did not cause statistically significant differences between the SVR and relapser clones in the total number of variations or in the mean genetic distances. Therefore, the expression clones are good representations of the SVR and relapser NS5B genes in these patients.

RNA synthesis

The enzymatic activities of the 31 RdRps were assessed by measuring poly-G or poly-U synthesis on primed poly-C or poly-A templates. The RdRps were pre-incubated on ice with the primer/templates for 30 min to form elongation-competent complexes, radiolabeled nucleotides were added, and the reactions were incubated at 30 °C for 90 min. RNAs were collected on nitrocellulose filters, the filters were washed, and retained radioactivity was measured. RNA synthesis was normalized to a reference RdRp from the genotype 1b strain BK (Genbank AF333324). The negative control was an enzymatically-inactive RdRp carrying GDD-GAG mutations in the active site.

We observed over a 300-fold variation in enzymatic activity among the enzymes (Fig. 1A), but the average amounts of poly-G synthesized by the SVR and relapser enzymes were equivalent (35.3% vs. 39.3%, $p = 0.81$). The average poly-U synthesis was almost twice as high in the relapser than SVR enzymes (53.6% vs. 29.3%), but this was not statistically significant ($p = 0.12$) due to the large inter-sample variation. These differences in poly-G and poly-U synthesis imply that there may be differences among the RdRps in their use of the various NTPs. When poly-G synthesis was normalized to poly-U synthesis (G/U ratio), we found that the average G/U ratio was 3-fold higher in the SVR RdRps compared to the relapser enzymes (3.0 vs. 1.0), but this difference again did not achieve statistical significance ($p = 0.14$) due to inter-sample variation (Fig. 1B).

RNA binding

The differences in poly-G synthesis among the RdRps could be due to variations in use of GTP (catalytic rate or GTP affinity) and/or differences in binding to the poly-C template. Therefore, we asked if there were differences in binding of the RdRps to RNA. The proteins were incubated with radiolabeled poly-C, the mixtures were passed through two filters in a slot-blot apparatus, the filters were washed, and retained RNAs were detected by phosphorimager analysis. The top filter was nitrocellulose to collect RNA:protein complexes, and the lower filter was charged nylon to collect unbound RNA. The binding conditions were identical to those used for the polymerization reactions, except that the RNA concentration had to be reduced to 2 pM. The apparent binding constant for the RNA was calculated as the half-maximal RNA binding level ($K_{0.5}$) as described (33). A 66-fold range was observed in RNA binding by the variant RdRps (Fig. 2A and 2B), but there was no significant difference in binding by the SVR and relapser enzymes ($K_{0.5}$ values of 51.6 ±

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11.7 nM and 43.8 ± 34.2 nM, respectively, $p = 0.56$). RdRps with lower $K_{0.5}$ values tended to have higher rates of poly-G synthesis, as would be expected from their better ability to bind the template (Fig. 2C). However, this correlation was weak ($R^2 = 0.11$, $p = 0.06$). Therefore, the large differences in poly-G synthesis among the variant enzymes were not primarily due to differences in template binding.

Primer length preference

RNA synthesis depends on formation of a complex between the RdRp and the primer-template, and this could also have contributed to the variability in RNA synthesis despite pre-incubating the RdRps with the primer-template to minimize this effect. To evaluate whether complex assembly may have been variable among the enzymes, we compared their ability to synthesize poly-G on templates primed with our standard 10 nt-long primer (G10) or a 3 nt primer (G3) (Fig. 3A). The ability of the enzymes to synthesize RNA using the G3 relative to G10 (G3/G10 ratio) varied by 55-fold (Fig. 3B). However, the average G3/G10 ratio for both the SVR and relapse groups was 1.3 ($p = 0.96$), and there was no significant correlation between poly-G synthesis primed by G10 and the G3/G10 ratio ($R^2 = 0.07$, $p = 0.17$). Therefore, there were large differences among the RdRps in how they interact with primers of different lengths, but these differences did not correlate with synthesis of poly-G primed by G10 or outcome of therapy.

Changes in RdRp activity during failed therapy

The preceding analyses were performed with pre-therapy HCV sequences, but the availability of post-therapy sequences for some subtype 1a-infected non-responders in the Virahep-C study (14) allowed us to ask whether evolution in NS5B gene during therapy selected for RdRps with altered biochemical parameters (14). Therefore, we selected seven patients whose NS5B genes accumulated mutations during failed antiviral therapy for biochemical analysis.

Near-consensus pre-therapy sequences were cloned for all seven patients, and then the mutations found in the post-therapy sequence from the same patient were introduced into the pre-therapy clones. The variations in the pre-therapy samples relative to the consensus sequences, mutations that accumulated during therapy and biochemical data for the RdRps are in Supplementary Table 3. The 14 RdRps were expressed and purified as before. Poly-G and poly-U synthesis activities were measured and normalized to the reference RdRp using our standard approaches, except that the amount of the enzyme was increased from 2 to 3.5 μg to partially compensate for the lower activity of the 1a enzymes (34). Poly-G synthesis increased in the post-therapy samples for four of the seven patients and remained relatively stable in the remaining three (Fig. 4A), whereas poly-U synthesis decreased in one and stayed roughly stable in six (Fig. 4B). Overall, the average poly-G synthesis increased significantly in the post-therapy samples (2.64 ± 1.7% to 15.8 ± 14.87%, $p = 0.04$), whereas the average poly-U synthesis did not change significantly ($p= 0.35$). These changes led to large increases in the G/U ratio for four patients (Fig. 4C) and a significant increase in the average G/U ratio (1.12 to 7.78, $p = 0.02$). Therefore, most RdRps accumulated mutations during therapy that elevated their G/U ratios, usually by increasing their ability to synthesize poly-G.

RdRp activity correlates with ALT levels

Finally, we asked if the pre-therapy biochemical activity of the RdRps was correlated with baseline clinical characteristics in the 31 patients for whom biochemical data were available. No correlations were found for poly-G synthesis, poly-U synthesis, the G/U ratio, or poly-C binding with pre-therapy viral loads or Ishak fibrosis scores. A positive correlation was found between the poly-U synthesis and ALT ($R^2 = 0.21$, $p = 0.01$ by Pearson correlation)

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(Fig. 5B), and the correlation between poly-G synthesis and ALT approached significance (R^2 = 0.09, p = 0.10) (Fig. 5A). However, mean pre-therapy ALT levels were not different between the SVR and relapser patients (p = 0.72). Therefore, higher RdRp activity was weakly associated with greater liver damage in these patients, but this effect was not related to treatment outcome.

Discussion

These studies were intended to characterize the natural variation in the RdRp activity among typical HCV patients undergoing pegylated interferon α plus ribavirin therapy, and to evaluate the possibility that ribavirin may act at least in part by being a substrate for the NS5B RdRp following its conversion to RTP. We previously proposed that differences in poly-G synthesis normalized to poly-U synthesis (G/U ratios) among variant RdRps may reflect differences in NTP usage, including RTP usage (26). Unfortunately, the very low usage rate of RTP by the RdRp typically prevents direct measurement of this parameter in the HCV RdRp unless its internal β-loop is deleted (24). Therefore, a specific goal of these studies was to evaluate the biochemical activity of the RdRps for patterns that would either strengthen or refute the possibility that the G/U ratio may be a surrogate for RTP use. We compared NS5B genes from patients who achieved SVR and hence would be largely sensitive to ribavirin to genes from patients who partially responded to therapy but then relapsed and hence would be largely resistant to ribavirin.

No obvious phylogenetic, genetic diversity, or covariance network differences were found between the SVR and relapse groups. Furthermore, the only position within the NS5B gene where a significant difference in the distribution of amino acids between the SVR and relapser sequences was found was at residue 98, but this was just a skewing in the relative frequency of R vs. K. Therefore, although substantial genetic variability was found in these NS5B genes, there were no differences that could reliably distinguish sequences from the SVR and relapser patients.

Consistent with the large genetic variation among the NS5B genes, we observed large differences among the enzymes in every biochemical parameter we assessed. RNA polymerization varied by >300-fold (Fig. 1A), RNA binding by 66-fold (Fig. 2A), and the ability to use short vs. long primers by 55-fold (Fig. 3A). The differences in RNA polymerization could not be fully explained by differential template binding or apparent differences in formation of elongation-competent complexes, and hence they are probably due at least in part to differences in NTP affinity or in the catalytic rates for GTP or UTP. However, none of the variations among these parameters were significantly different between the SVR and relapser groups.

We have speculated that the G/U ratio may be a surrogate for the relative use of RTP as a substrate (26). The average G/U ratio among the Virahep-C SVR enzymes was higher than among the relapsers (Fig. 1B), and this difference approached statistical significance (p = 0.14). This is the second cohort in which we detected a trend toward higher G/U ratios in SVR patients, indicating that a modest elevation in G/U ratios is probably present among enzymes from patients who will clear the virus during antiviral therapy. The biochemical changes leading to elevated G/U ratios can develop in independent viral lineages because there was no phylogenetic clustering of the RdRps with high G/U ratios. The higher G/U ratio among the SVR patients is consistent with our speculation that the G/U ratio may reflect the relative ability to use RTP. However, when we characterized RNA synthesis by paired pre- and post-therapy enzymes from seven subtype 1a-infected Virahep-C patients, we found a large and significant increase in the average G/U ratios in the post-therapy samples (Fig. 4C). This strongly implies that the G/U ratio does not reflect RTP use, because

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therapy should select for RdRps which use RTP less efficiently rather than more efficiently. Therefore, there appears to be a modest biochemical difference between SVR and non-responder enzymes in how they use various NTPs, but this does not reflect their relative ability to discriminate against RTP. One effect of ribavirin is to inhibit IMPDH and hence lower cellular GTP levels (35). Consequently, the increased ability of most of the post-therapy RdRps to use GTP may represent an adaptation to the limited substrate availability induced by therapy. Consistent with this speculation, 76% (13/17) of the mutations that accumulated in the RdRps in which poly-G synthesis increased after therapy mapped to the fingers domain of the enzyme, a domain which assists in nucleotide selectivity and is often the site of nucleoside drug resistant mutations in nucleic acid polymerases (27–30).

Higher poly-U synthesis by the variant RdRps correlated significantly with higher pre-therapy ALT levels ($p = 0.01$), and the correlation between poly-G synthesis and ALT levels approached significance ($p = 0.10$). In contrast, there was no correlation between RNA synthesis and pretherapy viral loads or the fibrosis score. The failure to find correlations with viral load or the Ishak score is not surprising because genome synthesis is only one of many variables determining viral titres, and development of fibrosis is also a very complex process. However, the correlation between RNA polymerase activity and ALT was unexpected. If this correlation is confirmed in other cohorts, it would indicate that RNA synthesis is a substantial contributor to liver damage. The simplest explanation for this correlation would be that the positive-stranded viral RNA is both the viral genome and the mRNA for all viral proteins. Increasing the mRNA concentration would increase the amount of viral proteins in cells, increasing any toxic effects they may have and also increasing the antigen burden on the cells to make them better targets for immune attack.

Finally, the very wide variation in the biochemical parameters we observed in these RdRps from subtype 1b-infected patients indicates that enzymes from the standard HCV isolates employed for drug screening are not always representative of enzymes from viral strains found in the human population, even for the same subtype. Therefore, antiviral drug candidates should be tested against a wide panel of patient-derived enzymes as early as possible during development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Alter MJ. Epidemiology of hepatitis C virus infection. World J Gastroenterol. 2007; 13:2436–2441. [PubMed: 17552026]
2. Lemon, SM.; Walker, C.; Alter, MJ.; Yi, M. Hepatitis C Virus. In: Knipe, DM.; Howley, P.; Griffin, DE.; Lamb, RA.; Martin, MA.; Roizman, B., et al., editors. Fields Virology. 5 ed.. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1253-1304.
3. Simmonds P. Genetic diversity and evolution of hepatitis C virus--15 years on. J Gen Virol. 2004; 85:3173–3188. [PubMed: 15483230]
4. Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol. 1993; 74:2391–2399. [PubMed: 8245854]

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5. Bressanelli S, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, et al. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc Natl Acad Sci U S A. 1999; 96:13034–13039. [PubMed: 10557268]
6. Wang M, Ng KK, Cherney MM, Chan L, Yannopoulos CG, Bedard J, et al. Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. J Biol Chem. 2003; 278:9489–9495. [PubMed: 12509436]
7. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002; 347:975–982. [PubMed: 12324553]
8. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001; 358:958–965. [PubMed: 11583749]
9. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med. 1998; 339:1485–1492. [PubMed: 9819446]
10. Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. Hepatology. 2004; 39:1147–1171. [PubMed: 15057920]
11. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004; 140:346–355. [PubMed: 14996676]
12. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol. 2007; 81:8211–8224. [PubMed: 17522222]
13. Aurora R, Donlin MJ, Cannon NA, Tavis JE. Genome-wide hepatitis C virus amino acid covariance networks can predict response to antiviral therapy in humans. J Clin Invest. 2009; 119(1):225–236. [PubMed: 19104147]
14. Cannon NA, Donlin MJ, Fan X, Aurora R, Tavis JE. Hepatitis C virus diversity and evolution in the full open-reading frame during antiviral therapy. PLoS ONE. 2008; 3:e2123. [PubMed: 18463735]
15. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature. 2005; 436:967–972. [PubMed: 16107837]
16. Mihm U, Herrmann E, Sarrazin C, Zeuzem S. Review article: predicting response in hepatitis C virus therapy. Aliment Pharmacol Ther. 2006; 23:1043–1054. [PubMed: 16611264]
17. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009; 461:399–401. [PubMed: 19684573]
18. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009; 41:1105–1109. [PubMed: 19749757]
19. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009; 41:1100–1104. [PubMed: 19749758]
20. Gale M Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. Nature. 2005; 436:939–945. [PubMed: 16107833]
21. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. 1998; 352:1426–1432. [PubMed: 9807989]
22. Graci JD, Cameron CE. Quasispecies, error catastrophe, and the antiviral activity of ribavirin. Virology. 2002; 298:175–180. [PubMed: 12127780]
23. Pawlotsky JM, Dahari H, Neumann AU, Hezode C, Germanidis G, Lonjon I, et al. Antiviral action of ribavirin in chronic hepatitis C. Gastroenterology. 2004; 126:703–714. [PubMed: 14988824]

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24. Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. J Biol Chem. 2001; 276:46094–46098. [PubMed: 11602568]
25. Miller JP, Kigwana LJ, Streeter DG, Robins RK, Simon LN, Roboz J. The relationship between the metabolism of ribavirin and its proposed mechanism of action. Ann N Y Acad Sci. 1977; 284:211–229. [PubMed: 280136]
26. Cannon NA, Donlin MJ, Mayes LM, Lyra AC, Di Bisceglie AM, Tavis JE. Evidence for action of ribavirin through the hepatitis C virus RNA polymerase. J Viral Hepat. 2009; 16:595–604. [PubMed: 19243495]
27. Jager J, Pata JD. Getting a grip: polymerases and their substrate complexes. Curr Opin Struct Biol. 1999; 9:21–28. [PubMed: 10047577]
28. Steitz TA. DNA polymerases: structural diversity and common mechanisms. J Biol Chem. 1999; 274(25):17395–17398. [PubMed: 10364165]
29. Boyer PL, Sarafianos SG, Arnold E, Hughes SH. Nucleoside analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. J Virol. 2002; 76:9143–9151. [PubMed: 12186898]
30. Sarafianos SG, Das K, Ding J, Boyer PL, Hughes SH, Arnold E. Touching the heart of HIV-1 drug resistance: the fingers close down on the dNTP at the polymerase active site. Chem Biol. 1999:R137–R146. [PubMed: 10322129]
31. Conjeevaram HS, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology. 2006; 131:470–477. [PubMed: 16890601]
32. Yao E, Tavis JE, Virahep C. A general method for nested RT-PCR amplification and sequencing the complete HCV genotype 1 open reading frame. Virol J. 2005; 2:88. [PubMed: 16321149]
33. Huang L, Hwang J, Sharma SD, Hargittai MR, Chen Y, Arnold JJ, et al. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. J Biol Chem. 2005; 280:36417–36428. [PubMed: 16126720]
34. Ferrari E, Wright-Minogue J, Fang JWS, Baroudy BM, Lau JYN, Hong Z. Characterization of soluble hepatitis C virus RNA dependent RNA polymerase expressed in Escherichia coli. Journal of Virology. 1999; 73:1649–1654. [PubMed: 9882374]
35. Lau JY, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology. 2002; 35:1002–1009. [PubMed: 11981750]

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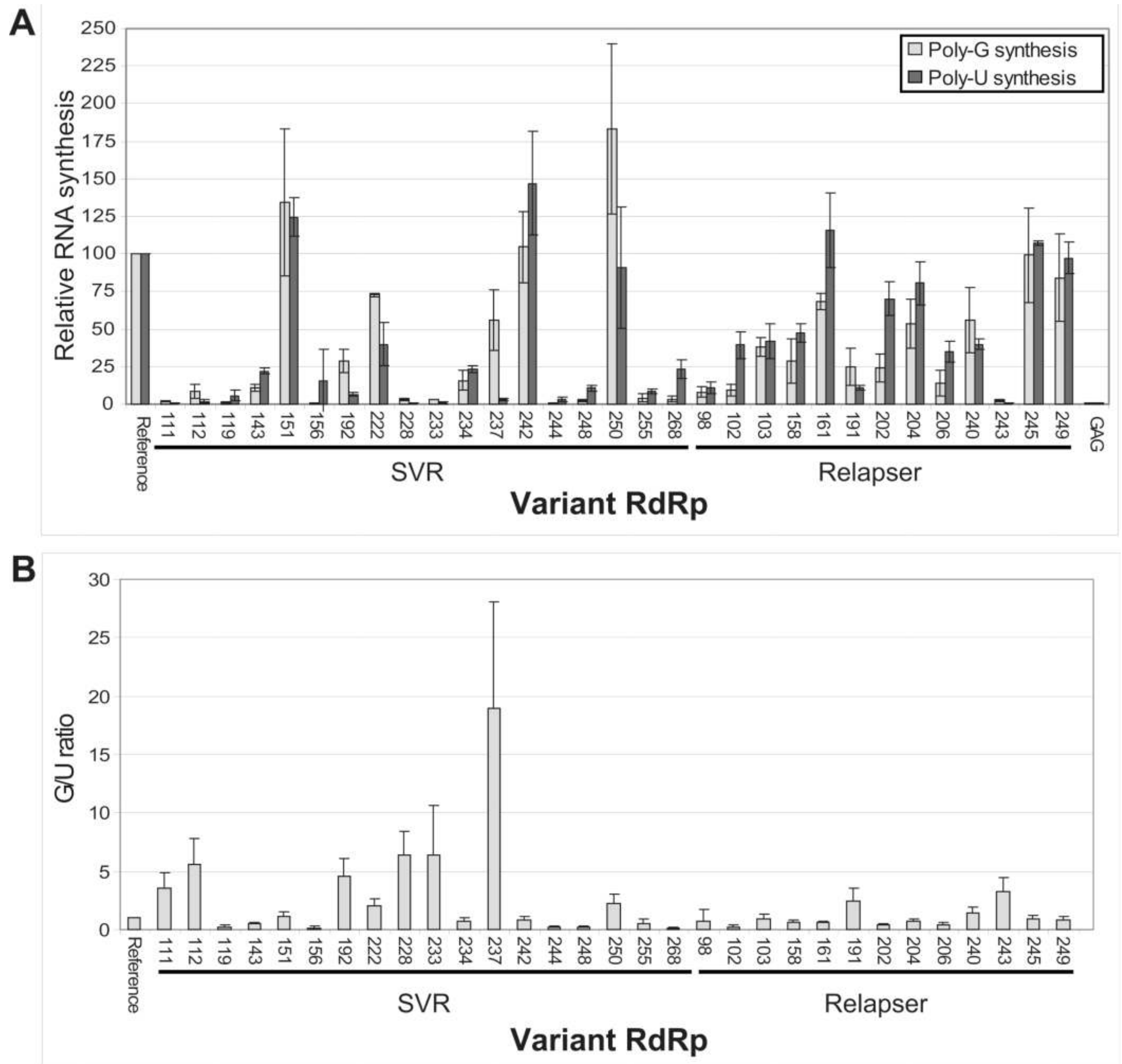

Fig. 1. RNA synthesis activity and G/U ratios of the variant RdRps

A. Activity of the RdRps on homopolymeric templates. The activities of the variant enzymes on primed poly-C or poly-A templates were measured and normalized to the reference RdRp (strain BK). The negative control was an enzymatically-inactive RNA polymerase carrying GDD-GAG mutations in the active site. Error bars are the standard deviation from 3–5 assays. **B.** The G/U ratios for the variant RdRps. All ratios are normalized to the reference RdRp.

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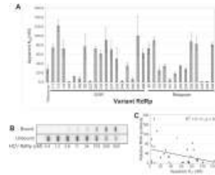

Fig. 2. Poly-C RNA binding by the variant RdRps

Radiolabeled poly-C was incubated with varying concentrations of the HCV RdRp, loaded onto a slot-blot apparatus, and filtered through nitrocellulose (top) and nylon (bottom) membranes. **A.** Poly-C binding by the variant RdRps. Error bars are the standard deviation from 3–5 assays. **B.** Representative RNA binding experiment **C.** Correlation of poly-C RNA binding by the variant enzymes with poly-G synthesis activity.

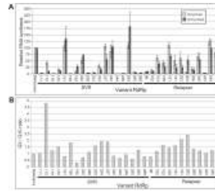

Fig. 3. Effect of primer length on RNA synthesis by the variant RdRps

A. Poly-G RNA synthesis by the variant RdRps on a homopolymeric poly-C template primed with long (10 nt) or short (3 nt) primers, normalized to the reference RdRp. Error bars are the standard deviation from 3–5 assays. **B.** RNA synthesis primed by the G3 primer normalized to RNA synthesis primed by the G10 primer (G3/G10 ratio), normalized to the reference RdRp.

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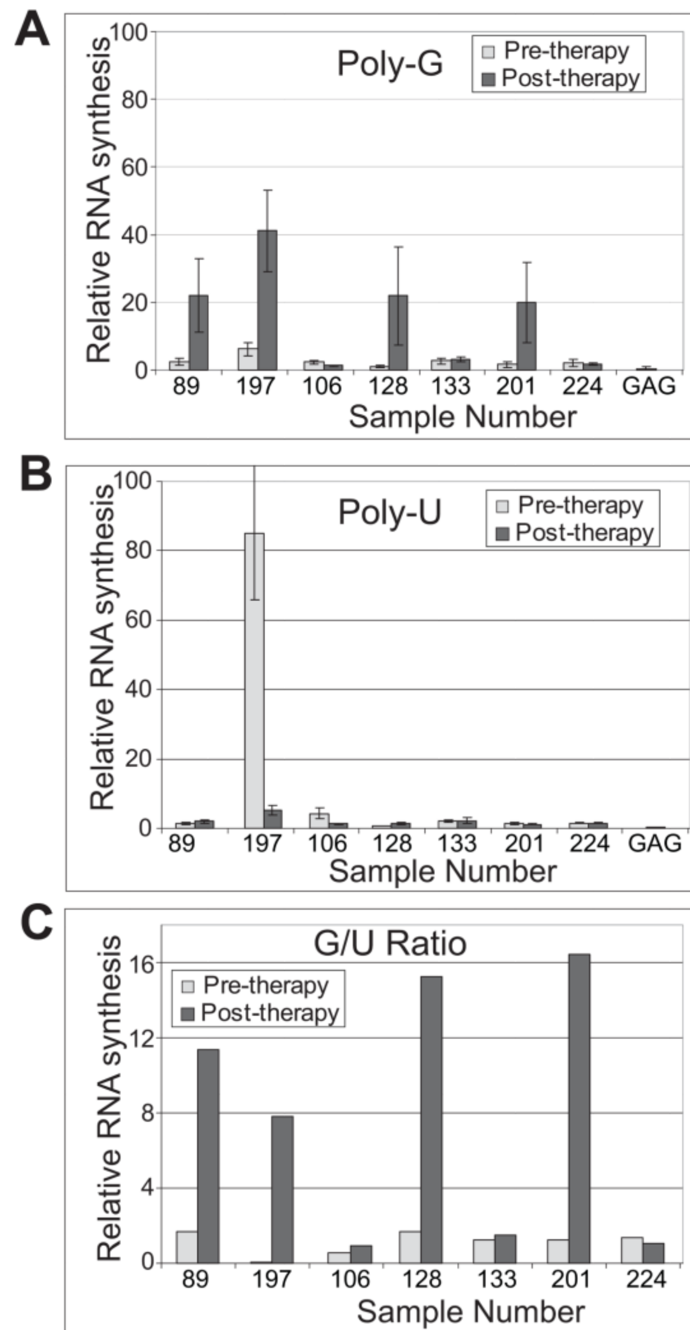

Fig. 4. Changes in RNA polymerase activities among variant HCV RdRps variant after failed antiviral therapy

A. Poly-G synthesis by the paired pre- and post-therapy RdRps, normalized to the reference RdRp. **B.** Poly-U synthesis by the paired pre- and post-therapy RdRps, normalized to the reference RdRp. Error bars in panels A and B are the standard deviation from 3–5 assays. **C.** The G/U ratios for the paired pre- and post-therapy RdRps. All ratios are normalized to the reference polymerase.

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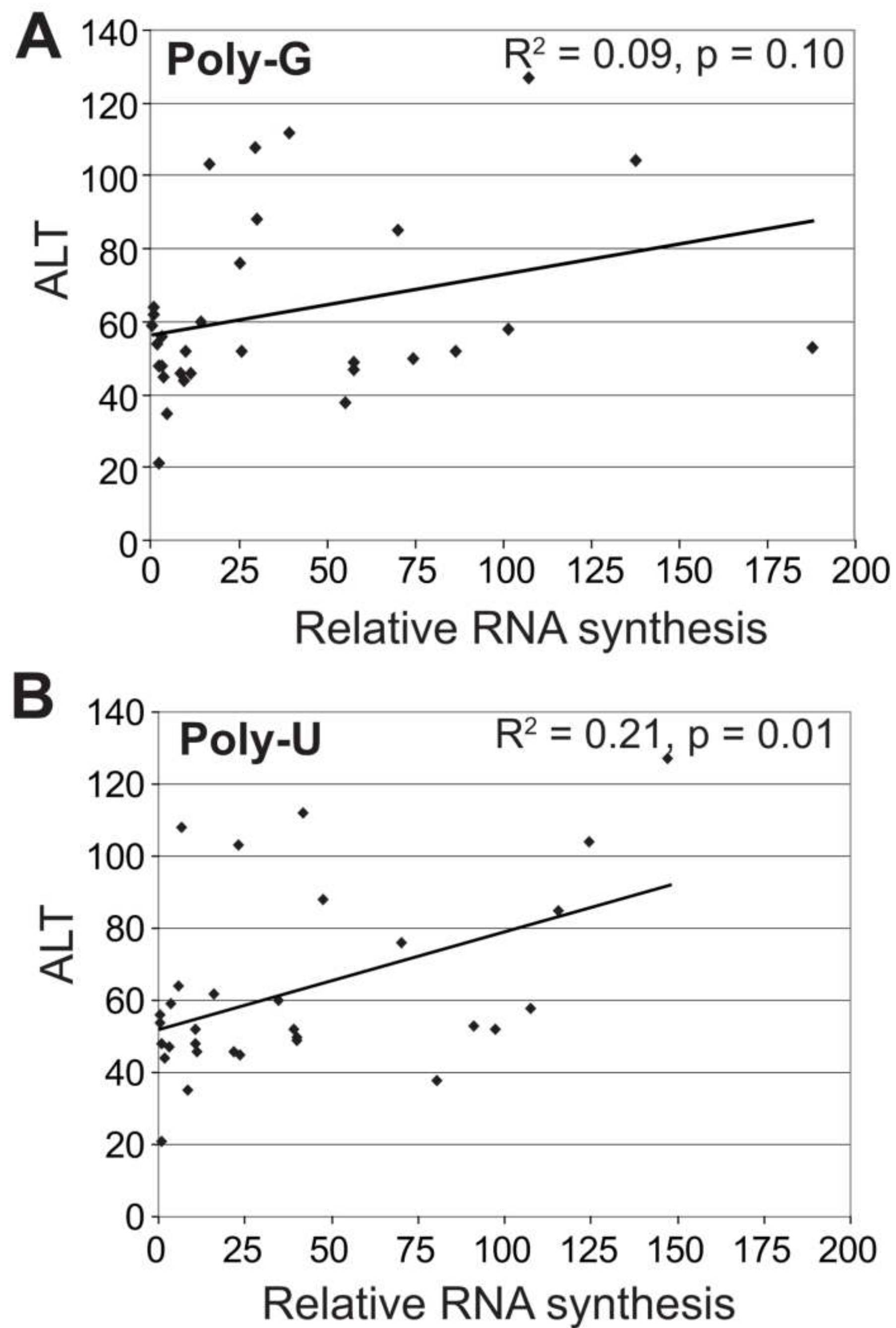

Fig. 5. Correlation of pre-therapy RNA polymerase activities of the variant RdRps with pre-therapy ALT levels from the respective patients

A. Correlation with poly-G synthesis. **B.** Correlation with poly-U synthesis.