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# Identification of constrained peptides that bind to and preferentially inhibit the activity of the hepatitis C viral RNA-dependent RNA polymerase

Anthony Amin,[a,*] Joe Zaccardi,[b] Stanley Mullen,[a] Stephane Olland,[b] Mark Orlowski,[a] Boris Feld,[a] Patrick Labonte,[a] and Paul Mak[a]

[a] *Department of Infectious Disease, Wyeth Research, 401 N. Middletown Road, Pearl River, NY 10965, USA*
[b] *Department of Biological Chemistry, Wyeth Research, 401 N. Middletown Road, Pearl River, NY 10965, USA*

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**Abstract**

A class of disulfide constrained peptides containing a core motif FPWG was identified from a screen of phage displayed library using the HCV RNA-dependent RNA polymerase (NS5B) as a bait. Surface plasmon resonance studies showed that three highly purified synthetic constrained peptides bound to immobilized NS5B with estimated $K_d$ values ranging from 30 to 60 $\mu$M. In addition, these peptides inhibited the NS5B activity in vitro with $IC_{50}$ ranging from 6 to 48 $\mu$M, whereas in contrast they had no inhibitory effect on the enzymatic activities of calf thymus polymerase $\alpha$, human polymerase $\beta$, RSV polymerase, and HIV reverse transcriptase in vitro. Two peptides demonstrated conformation-dependent inhibition since their synthetic linear versions were not inhibitory in the NS5B assay. A constrained peptide with the minimum core motif FPWG retained selective inhibition of NS5B activity with an $IC_{50}$ of 50 $\mu$M. Alanine scan analyses of a representative constrained peptide, FPWGNTW, indicated that residues F1 and W7 were critical for the inhibitory effect of this peptide, although residues P2 and N5 had some measurable inhibitory effect as well. Further analyses of the mechanism of inhibition indicated that these peptides inhibited the formation of preelongation complexes required for the elongation reaction. However, once the preelongation complex was formed, its activity was refractory to peptide inhibition. Furthermore, the constrained peptide FPWGNTW inhibited de novo initiated RNA synthesis by NS5B from a poly(rC) template. These data indicate that the peptides confer selective inhibition of NS5B activity by binding to the enzyme and perturbing an early step preceding the processive elongation step of RNA synthesis.


## Introduction

The hepatitis C virus (HCV) is the major etiological agent of transfusion-acquired non-A, non-B hepatitis (Alter and Mast, 1994; WHO, 1996). Approximately 4 million people in the United States and 170 million people worldwide are infected with HCV (WHO, 1996). About 15% of those infected will spontaneously resolve the disease, but 85% with acute asymptomatic infection will progress to chronic hepatitis, of which 20% will develop cirrhosis that can progress to hepatocellular carcinoma (Farci et al., 1992). Currently, the best available therapy for HCV infection is the combination therapy of pegylated interferon and ribavirin, which is effective in only up to half of the patients evaluated (McHutchinson et al., 1998; Poynard et al., 1998). Therefore, there is a clinical need to develop efficacious specific therapies against this virus. To a large degree, studies on the biology of the virus has been limited due to the lack of an efficient cell-culture system for viral propagation, intrinsic low level of viral replication, and the genetic diversity associated with the virus. The development of a subgenomic HCV replicon in cell culture (Blight et al., 2000; Lohmann et al., 1999, 2001), as well as an animal model which consists of immunodeficient scid mice with transplanted chimeric human livers (Mercer et al., 2001), will prove useful to evaluate potential drug candidates that inhibit replication of the virus.

* Corresponding author.
*E-mail address:* labontp@wyeth.com (P. Labonte).

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Our current understanding of the function of viral encoded proteins has been derived from in vitro studies (Bartenschlager and Lohmann, 2000; Reed and Rice, 2000). The HCV genome consists of a positive-strand linear RNA of about 9.4 kb (Choo et al., 1991; Kato et al., 1990). It encodes a single open reading frame that is preceded by an untranslated region (UTR) of 341 nucleotides at the 5′ end, and the 3′ end containing a variable region, a poly (U/C) tract, and a 98× tail (Kolykhalov et al., 2000; Tanaka et al., 1996; Yamada et al., 1996). The polyprotein, comprising 3010–3040 amino acids, is processed co- and posttranslationally by cellular and viral proteinases into at least 10 mature polypeptides (Bartenschlager et al., 1993; Grakoui et al., 1993a; Tanji et al., 1995; Tomei et al., 1993). The polyprotein contains the following order of proteins: $NH_2$-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Grakoui et al., 1993b; Tomei et al., 1993). Among these viral proteins, the HCV RNA-dependent RNA polymerase (NS5B) has been the major focus of investigation since it is an attractive target for antiviral intervention. Our current understanding of the function of viral-encoded proteins has been derived from in vitro studies (Bartenschlager and Lohmann, 2000; Reed and Rice, 2000). However, further studies on adaptive amino acid substitutions of these proteins generated during passage of the HCV subgenomic replicon in hepatic cells could potentially reveal information on genetic interactions among viral and host proteins (Blight et al., 2000; Lohmann et al., 2001).

Although our knowledge of the mechanism of HCV replication in the viral life cycle is limited, studies on other plus-strand RNA viruses suggest that the positive RNA strand genomic RNA serves as a template for synthesis of the minus RNA strand (antisense). The minus RNA strand, in turn, serves as the template for synthesis of multiple copies of the progeny plus strands that are subsequently packaged into virions (Porter, 1993; Strauss and Strauss, 1986; Wimmer et al., 1993). Studies using highly purified preparations of the full-length NS5B polymerase have revealed some biochemical features of the catalytic properties of the enzyme (Behrens et al., 1996; Lohmann et al., 1998). Recent studies have shown that removal of the C-terminal 21 acid hydrophobic tail of the enzyme resulted in a soluble protein that is catalytically more active when compared to the full-length enzyme (Carroll et al., 2000; Tomei et al., 2000). The enzyme can catalyze complementary-strand RNA synthesis from a self-primed single-stranded nonviral RNA template by a “copy-back” mechanism to form a duplex RNA hairpin (Behrens et al., 1996; Carroll et al., 2000; Labonte et al., 2002) and can utilize short oligonucleotide-primed RNA template for complementary-strand RNA synthesis (Ferrari et al., 1999; Zhong et al., 2000). In addition, the enzyme can catalyze de novo synthesis from a nonprimed template, which may have implications for initiation of HCV genome replication (Kao et al., 2000; Luo et al., 2000; Zhong et al., 2000). The crystal structure of the enzyme indicates that the canonical palm, fingers, and thumb domains are conserved among other polymerases (poliovirus 3D-pol and HIV-reverse transcriptase). However, the HCV polymerase contains an additional structure that encircles the active site. This peculiar structure has also recently been observed on the distantly related double-strand RNA polymerase of bacteriophage $\phi$6 and is likely to influence the mechanism of replication (Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999; Butcher et al., 2001).

In this study, we used phage-displayed peptide technology to identify small peptides that bind to NS5B and inhibit its function. The utility of the phage-display technology has been exemplified in applications for the identification of protein:protein contact sites (Hoess et al., 1993), peptide mimics of nonpeptide ligands (Devlin et al., 1990; Hoess et al., 1993; Oldenburg et al., 1992; Scott et al., 1992), and for mapping of epitopes (Cwirla et al., 1990; Felici et al., 1991; Scott and Smith, 1990). In addition, protein variants with altered affinity or specificity have been isolated from libraries of random mutants (Choo and Klug, 1995; Roberts et al., 1992; Soumillion et al., 1994). We report here for the first time a class of constrained peptides that bind to and preferentially inhibit the activity of the HCV polymerase. Our analysis indicates that these peptides inhibit a step in the formation of a preelongation complex by the enzyme and the 3′OH end of the RNA substrate. Peptide ligands that interact with specific sites on polymerase can serve as a valuable tool to study mechanism of enzyme function, as well as for target validation and the development of a high-throughput screen assay.

## Results

### *Identification of peptides that bind to NS5B*

To maximize the chance of identifying peptides that bind to NS5B polymerase, a C-terminal hexahistidine-tagged version lacking the C-terminal 21 amino acid residues was purified to homogeneity in a biologically active nonaggregated form. Fig. 1A shows a Coomassie dye stained gradient SDS–PAGE (4–20%) of the final protein preparation. Lanes 1 and 8 contain molecular weight markers, whereas lanes 2–7 contain increasing amounts of the protein ranging from 2.5 to 40 $\mu$g. At the highest concentration used, only a 66-kDa band corresponding to NS5B (del 21-His) was detected, which illustrates the near homogeneity of the protein preparation. In addition, sucrose gradient density analysis revealed that the NS5B sedimentation profile was consistent with the protein being monomeric in solution (data not shown).

The purified enzyme was assayed for its ability to catalyze RNA synthesis using a 369–nt RNA substrate called pOF1213 (57) by a copy-back mechanism as reported previously by others (Bartenschlager and Lohmann, 2000; Butcher et al., 2001) and as described under Materials and

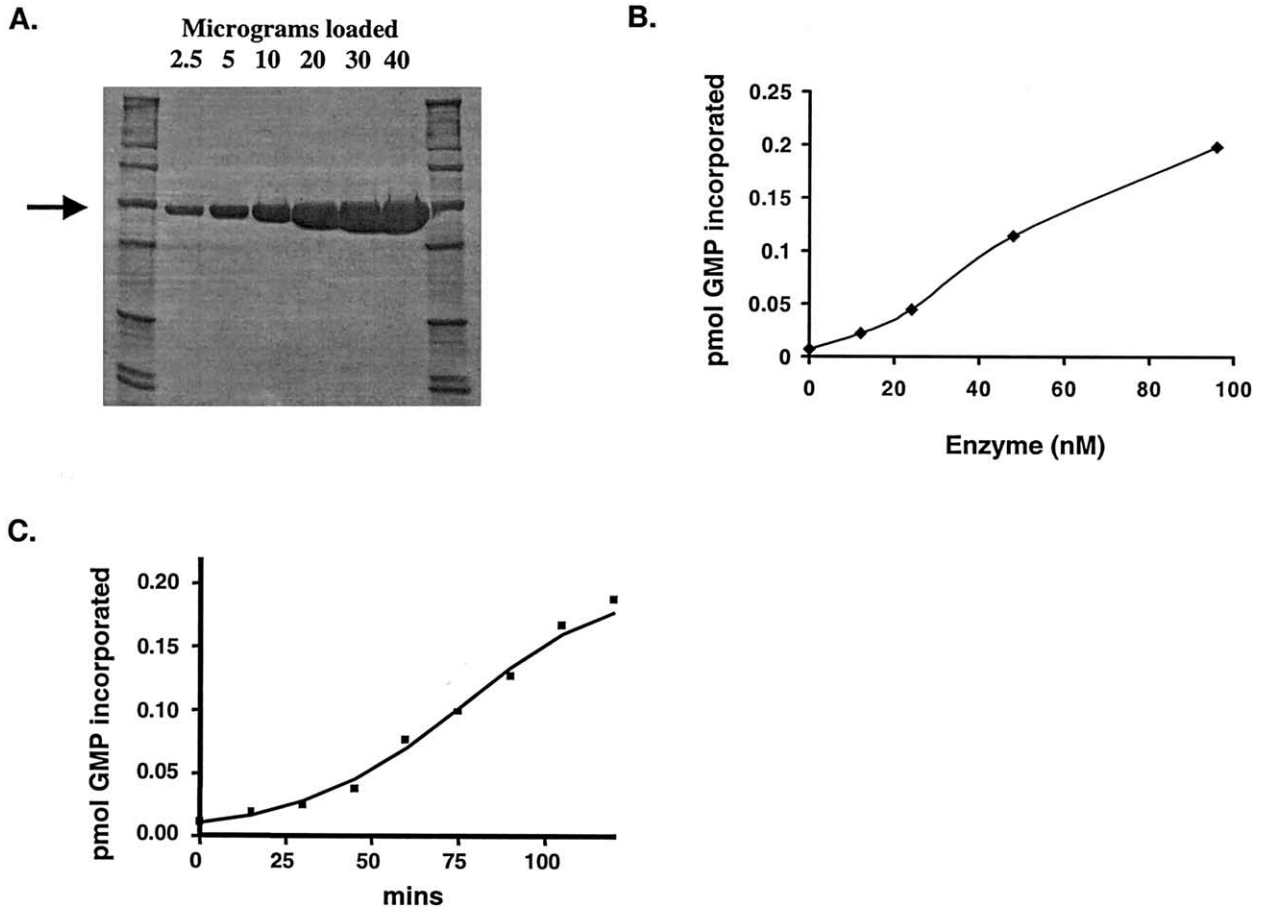


Fig. 1. Purified HCV NS5B is active in catalyzing RNA synthesis using a 396-nucleotide self-primed pOF1213 RNA substrate. (a) Purified HCV NS5B polymerase. Increasing amounts of purified polymerase were subjected to electrophoresis on a SDS–PAGE (4–20%). The gel was stained with Coomassie blue R. The arrow indicates the position of the NS5B protein (66 kDa); molecular weight markers (Bio-Rad) were used in the outer lanes. (b) Graph of pmol of GMP incorporated into product, as a function of NS5B concentration (nM). Increasing concentrations of polymerase were incubated with 10 nM pOF1213 RNA substrate, 100 $\mu$M rATP, rUTP, rCTP, and 5 $\mu$M rGTP and 1.25 $\mu$Ci [$\alpha^{33}$P]rGTP for 2 h at room temperature. The pmol of GMP incorporated into product was determined as described under Materials and methods. (c) Graph of pmol GMP incorporated at a fixed concentration of NS5B as a function of time. 24 nM of the enzyme was incubated with the same amounts of substrates indicated above and the yield of product determined at 20-min intervals over 120 min as described above.

methods. Fig. 1B shows the yield of $^{33}P$-labeled product increased linearly as a function of enzyme concentration over 2 h. A time course of product formation using 24 nM of enzyme over 120 min in Fig. 1C shows that the product yield is biphasic, with a slow increase over the first 40 min, followed by an increase in the rate from 40 to 120 min. As will be shown later, this first slow phase may indicate a slow rate of preelongation complex formation between NS5B and the dinucleotide self-primed hairpin substrate. These results indicate that the highly purified polymerase preparation was active in catalyzing copy-back RNA synthesis of a pOF1213 self-primed template.

This enzyme preparation was used to isolate peptide ligands from a heptamer-constrained phage-displayed library as described under Materials and methods. For the panning experiments, the purified protein was immobilized on polystyrene plates via an interaction of its C-terminal hexahistidine with the immobilized $Ni^{2+}$ so that the polymerase would be oriented to the phage-displayed peptides in solution. After the binding reaction, the wells were blocked with BSA to minimize the recovery of plastic interacting peptides. After the third cycle of panning, phage DNA were isolated from individual plaques and sequenced to deduce the displayed peptides. The results of the sequence of nine constrained heptamer peptides recovered are shown in Fig 2. The sequence alignment of these peptides revealed the presence of a conserved FPWG motif. For subsequent experiments, we have focused on the first three peptides based on their profiles of binding and inhibition of NS5B.

*Analysis of the binding of peptides to NS5B by Biacore*

Since the experiments above suggested that the peptides displayed as part of the pIII coat protein of the phage head can bind to NS5B, we evaluated whether highly purified preparations of these disulfide-constrained heptameric pep-

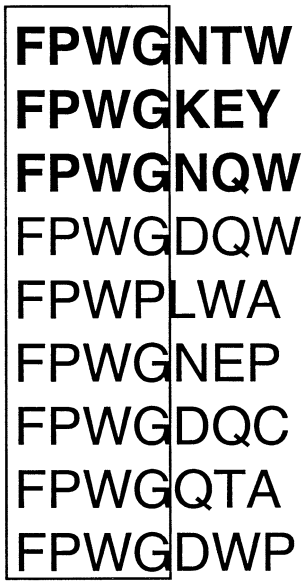


Fig. 2. Alignment of the 7-mer disulfide-constrained peptides deduced from phage clones isolated from a phage-displayed library. After the third panning, the eluted phage particles were plated, and 40 independent plaques were amplified in *E. coli* and the phage DNA was isolated and the displayed peptide sequences were deduced following determination of the N-terminal sequence of the recombinant PIII. Based on the profiles of binding and inhibition, the first three peptides in bold were chosen for further characterization in this study.

tides could bind to NS5B in vitro. The nomenclatures of the purified synthetic peptides as well as their corresponding sequences used in this study are shown in Table 1.

The interactions of a representative subset of these peptides with the NS5B protein were evaluated by the Biacore surface plasmon resonance. Our initial strategy for the Biacore experiments was to immobilize the C-terminal biotinylated peptides to a streptavidin-coated CM5 chip and to measure its interaction with the polymerase in solution. Unfortunately, the NS5B interacted strongly and nonspecifically with the chip, thus precluding the use of this analytical approach. Therefore, we used an alternative approach in which the HCV polymerase and human serum albumin (as a negative control) were immobilized in separate channels and subsequently challenged with increasing concentrations of peptides. For these experiments, the peptides were injected over a range of concentration from 10 to 100 $\mu$M over the channels and the $K_d$s and stoichiometry of binding were estimated by fitting with the steady-state affinity model included in the Biacore Evaluation software. As shown in Figs. 3A, B, and C, respectively, peptides KEY (S-S), NTW (S-S), and NQW (S-S) bound with varying affinities ($K_d$ ranging from 33 to 56 $\mu$M) to the enzyme with a stoichiometry consistent with a 1:1 binding event. Interestingly, the linear version of NQW peptide also bound to HCV polymerase with a $K_d$ value of 64 $\mu$M (Fig. 3C). These peptides did not exhibit any specific interaction with immobilized human serum albumin (data not shown). The fits for these peptides all indicate that the stoichiometry of the significant binding event was 1:1. However, at higher peptide concentrations, there were indications of additional weaker nonspecific interactions with the enzyme. Taken together, the data suggest that these four peptides interacted with the NS5B polymerase in vitro.

### *Constrained peptides preferentially inhibited the HCV RdRp*

Since these peptides bound to NS5B, we assessed whether they can inhibit NS5B activity in vitro. Moreover, if they were highly selective for NS5B, we would expect them to be significantly less potent in inhibiting the enzymatic activities of other polymerases, which are known to contain conserved structural elements. The enzymes used in this study included HIV reverse transcriptase, calf thymus polymerase $\alpha$, human polymerase $\beta$, and RSV polymerase. DTT was omitted from these assays to maintain the integrity of the disulphide-constrained peptides during the reaction. In addition, the assays contained high concentrations of BSA (50 $\mu$g/ml, see Materials and methods) that would mitigate any inhibition due to nonspecific interactions of the peptide with proteins.

The constrained and linear versions of the peptides NTW, KEY, NQW, and the core motif (FPWG) were synthesized and evaluated in the assays described above. These peptides were titrated in the assays over a range from 0.146 to 150 $\mu$M and their $IC_{50}$ values determined. As shown in Table 2, the constrained peptide with the core motif inhibited only the HCV polymerase with an $IC_{50}$ value of 50 $\mu$M. Peptides NTW (S-S), KEY (S-S), and NQW (S-S) inhibited the NS5B assay with $IC_{50}$ values ranging from 6 to 22 $\mu$M, but they had values of $>$139 $\mu$M for DNA polymerases and HIV reverse transcriptase. Interestingly, peptide NQW (S-S) also inhibited the RSV polymerase, another RNA-dependent RNA polymerase. However, replacing Q with T rendered the peptide, NTW (S-S), highly specific for HCV polymerase. Also, the linear version of these peptides, NTW

Table 1
Summary of peptide sequences and their nomenclature used in this study

| Name | Peptide sequence |
|---|---|
| NTW (S-S) | ACFPWGNTWCGGK (constrained) |
| NTW (L) | ACFPWGNTWCGGK (linear) |
| KEY (S-S) | ACFPWGKEYCGGK (constrained) |
| KEY (L) | ACFPWGKEYCGGK (linear) |
| NQW (S-S) | ACFPWGNQWCGGK (constrained) |
| NQW (L) | ACFPWGNQWCGGK (linear) |
| FPWG (S-S) | CFPWGC (constrained) |
| FPWG (L) | CFPWGC (linear) |

*Note.* The indicated disulfide-constrained peptides or their linear counterparts were synthesized with an additional A and an additional GGK at their N- and C-terminal ends, respectively. To simplify the nomenclature, peptides were designated by the three amino acids adjoining the FPWG motif. Constrained or linear peptides were designated as (S-S) or (L), respectively.

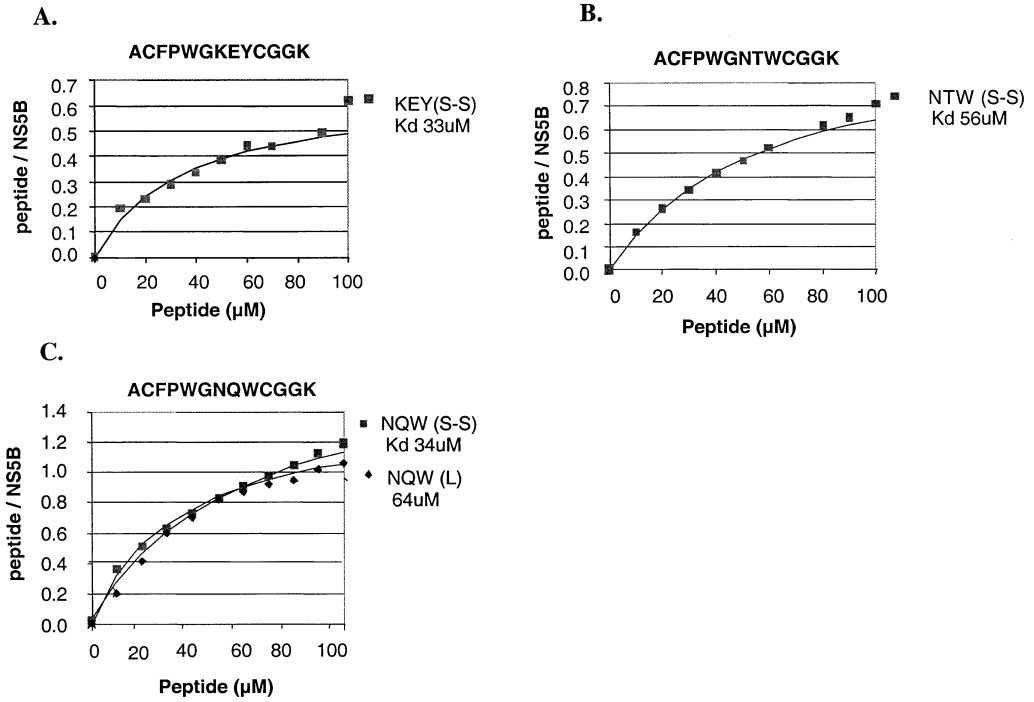


Fig. 3. Analysis of HCV NS5B and peptide interactions using the Biacore surface plasmon resonance technology. The HCV polymerase and human serum albumin were immobilized on separate chambers of the CM5 chips by amine coupling, and then increasing concentrations of the constrained and linear version of peptides were analyzed for their ability to interact with the enzyme as described under Materials and methods. The graphical relationship of moles bound peptide/moles immobilized NS5B ratio as a function of the respective $\mu$M peptide concentrations are shown for peptides KEY (S-S) (A), NTW (S-S) (B), NQW (S-S), and NQW (L) (C). The estimated $K_d$ values for these peptides are also indicated.

(L) and KEY (L), had an $IC_{50} > 150$ $\mu$M in all the polymerase assays, indicating that the constrained structure conferred selective inhibition of these peptides in the NS5B assay. Surprisingly, the NQW (L) was inhibitory in the NS5B assay ($IC_{50} = 37$ $\mu$M), but not in the other assays (with $IC_{50} > 150$ $\mu$M). At this point, we are uncertain whether this peptide is inhibiting the assay by a mechanism similar to its constrained peptide counterpart. Taken together, there is a good correlation between the binding affinity of the peptides and their selective inhibition of the HCV polymerase.

### *Identification of residues critical for the inhibitory effect of the NTW (S-S) peptide*

One of the bioactive peptide NTW (S-S) was chosen as a prototype to identify residues that are essential for its inhibitory activity. Constrained synthetic peptides in which each of the F, P, W, G, N, T, and W residues were substituted for an A (Ala scan) were designated as F1A, P2A, W3A, G4A, N5A, T6A, and W7A, respectively. The $IC_{50}$ for each peptide was evaluated in the HCV polymerase assay as described previously, and the data are shown in

Table 2
Effects of peptides on polymerase assays

| Name | Peptide sequence | HCV pol | HIV-RT | c-pol $\alpha$ | h-pol $\beta$ | RSV pol |
|---|---|---|---|---|---|---|
| NTW (S-S) | AC*FPWG*NTWCGGK (S-S) | 13.0 | >150 | >150 | >150 | >150 |
| NTW (L) | AC*FPWG*NTWCGGK (linear) | >150 | >150 | >150 | >150 | >150 |
| KEY (S-S) | AC*FPWG*KEYCGGK (S-S) | 22.0 | >150 | >150 | >150 | >123 |
| KEY (L) | AC*FPWG*KEYCGGK (linear) | >150 | >150 | >150 | >150 | >150 |
| NQW (S-S) | AC*FPWG*NQWCGGK (S-S) | 6.0 | >150 | >149 | >139 | >53.0 |
| NQW (L) | AC*FPWG*NQWCGGK (linear) | 37.0 | >150 | >150 | >150 | >150 |
| FPWG (S-S) | C*FPWG*C (S-S) | 50.0 | >150 | >150 | >150 | >150 |
| FPWG (L) | C*FPWG*C (linear) | >150 | >150 | >150 | >150 | >150 |

*Note.* Peptides were titrated into the various enzyme assays (HCV polymerase, HIV reverse transcriptase, calf thymus polymerase $\alpha$, human polymerase $\beta$, and RSV polymerase) at final $\mu$M concentrations of 0.29, 0.58, 1.17, 2.34, 4.69, 9.40, 18.75, 37.5, 75, and 150 in duplicates. The yield of RNA synthesis was quantitated. The numbers represent $IC_{50}$ values as described under Materials and methods.

ACFPWGNTWCGGK
1 2 3 4 5 6 7

| Peptide Sequence | Peptide | $IC_{50}$ (μM) |
|---|---|---|
| AC**A**PWGNTWCGGK | F1A | >200 |
| ACF**A**WGNTWCGGK | P2A | 39 |
| ACFP**A**GNTWCGGK | W3A | 43 |
| ACFPW**A**NTWCGGK | G4A | 7.4 |
| ACFPWG**A**TWCGGK | N5A | 40 |
| ACFPWGN**A**WCGGK | T6A | 11 |
| ACAPWGNT**A**CGGK | W7A | 80 |
| ACFPWGNTWCGGK | Control | 10 |

Fig. 4. Profile of Alanine scan analysis of the NTW (S-S) peptide with respect to inhibition of HCV polymerase. Peptides containing the appropriate alanine substitutions in the heptamer residues as indicated were tested for their ability to inhibit the NS5B enzyme as described previously in Table 2. The mean $IC_{50}$ values of the corresponding peptides are indicated along with the control parent peptide NTW (S-S).

Fig. 4. When compared to the unsubstituted control peptide ($IC_{50}$ = 10 μM), the A substitution at F1 within the core motif resulted in a significant loss of inhibitory activity as reflected in an $IC_{50}$ of >200 μM. The A substitution at W7 also resulted in a dramatic reduction in inhibition with an $IC_{50}$ of 80 μM, which is eightfold less than the parent peptide. The A substitution at P2, W3, and N5, respectively, also resulted in approximately fourfold reduction of activity compared to the parent peptide, whereas the substitutions at G4 and T6 had no effect. These data indicate that F1 within the core motif is essential for the inhibitory effect of the NTW (S-S) peptide, although other residues within the heptamer provide synergy to the inhibitory profile of the molecule.

### *Peptides inhibit an early step in the function of the HCV polymerase: primer template ternary complex*

Based on our current understanding of the reaction pathways of a number of polymerases, the HCV NS5B polymerase likely assembles at the 3′OH end of the self-primed RNA substrate to form a preelongation complex. Thereafter, the enzyme can catalyze complementary-strand synthesis using the pool of the four ribonucleotide triphosphates to yield a copy-back product. To define a step of the reaction pathway inhibited by the peptides, we used a "burst kinetic" assay that measures the yield of products and the rate of elongation carried out by the enzyme primer:template complex in one round of RNA synthesis from the pOF1213 substrate. For this experiment, the reaction was staged so that the polymerase was prebound to the dinucleotide self-primed substrate to enrich for the "preelongation complex," followed by the addition of rNTPs ($\gg K_m$) and heparin to restrict the polymerase to one round of RNA replication (Tomei et al., 2000; Butcher et al., 2001) . Heparin prevents loading of the enzyme onto the template, during the course of RNA synthesis process.

Fig. 5A shows a plot of the picomoles of GMP incorporated into product as a function of time of preincubation of polymerase with pOF1213 from 1 to 4 h, in burst reactions sampled in intervals from 1 to 10 min. An analysis of the size of products derived from timed burst reactions after preincubation of the enzyme and pOF1213 for 4 h is shown in Fig. 5B. Together, the data indicate that the yield of product increased linearly with time of preincubation from 1 to 4 h, implying that formation of the preelongation complex is a slow process under these conditions. The burst of RNA synthesis was completed in 5 min, with conversion of the pOF1213 to a copy-back product twice its size, suggesting that the enzyme synthesized RNA at about 68 nts/min at room temperature. We observed a 1-min lag of synthesis, which could be due to incorporation of the 83 U's (nonradioactive) that preceded incorporation of the first labeled [$^{33}$P] GMP into the template.

It is conceivable that the peptides could affect preelongation complex formation, or the elongation reaction catalyzed by the enzyme. To discriminate between these two possibilities, we designed two experiments using a 4-h period of preincubation of template with the enzyme, with different orders of peptide addition (see Table 3). In the "peptide prebinding" experiment, the peptide was preincubated with the polymerase and RNA template for 4 h, and the reaction was initiated with NTPs for 15 min, a time sufficient for a complete burst. The data of $IC_{50}$ indicate that the constrained peptides NTW (S-S), KEY (S-S), and NQW (S-S) were inhibitory in the assay with $IC_{50}$ of 8.4, 39.1, and 8.82 μM, respectively. These $IC_{50}$ values correlated with the $IC_{50}$ obtained in the straight incubation assay that was carried out in excess substrates (see Table 2).

The results above suggest that the peptides may inhibit the formation of the preelongation complex, or an event required for its transition to the elongation complex. To address whether the peptides can inhibit the processive elongation reaction catalyzed by polymerase, a "peptide postbinding" experiment was done. The enzyme was preincubated with the RNA template for 4 h to form the active preelongation complex, followed by the addition of peptides and incubation for 15 min. Subsequently, rNTPs were added to the reactions followed by incubation for 15 min. The results from this experiment showed that these peptides at a concentration of 150 μM had no significant effect on the burst assay. These data led us to conclude that once the preelongation complex was formed, it became refractory to inhibition by the peptides. Therefore these peptides appear to inhibit the formation of the preelongation complex, but not the elongation reaction catalyzed by the enzyme.

### *Peptide NTW (S-S) inhibits de novo synthesis using poly(C) as a template*

In vivo, the replication of the HCV viral genome is likely to be initiated in the absence of primer by a de novo mechanism (Kao et al., 2000; Luo et al., 2000; Zhong et al., 2000). We have recently shown that the NS5B polymerase

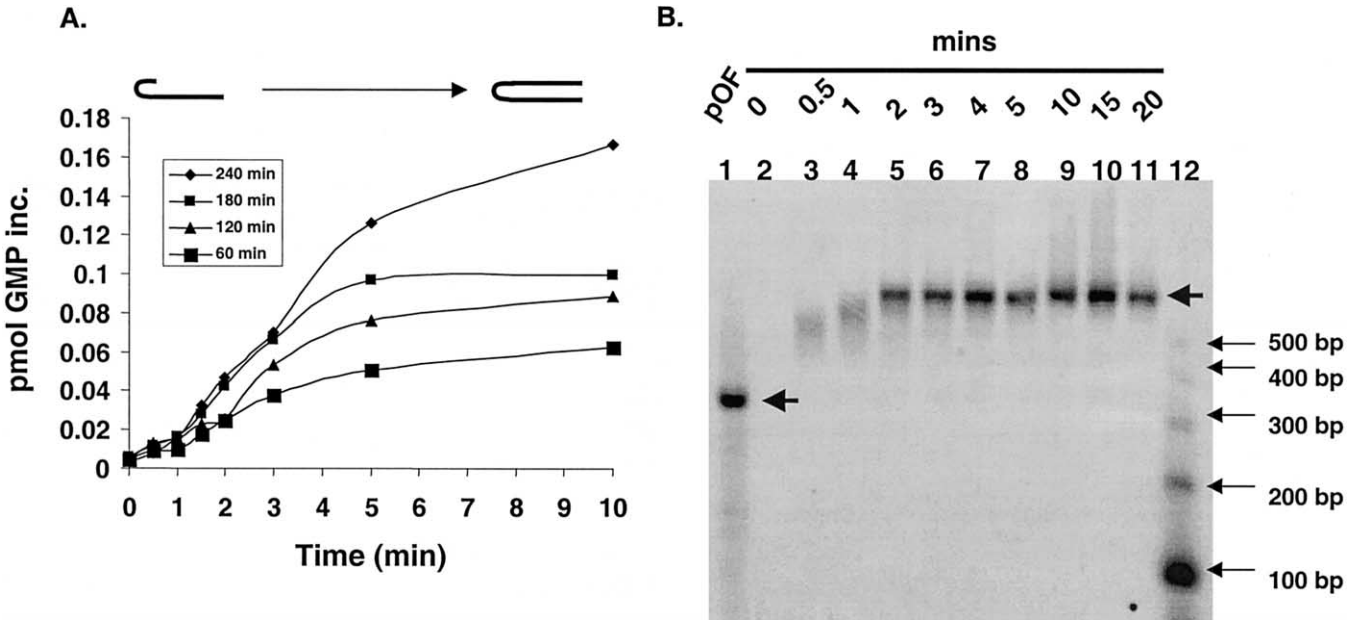


Fig. 5. Burst assay catalyzed by NS5B polymerase. (A) Graph showing pmol GMP incorporated as a function of time of burst RNA synthesis after a time course of preincubation of NS5B with pOF1213 RNA substrate. 48 nM of the HCV polymerase was preinubated with 20 nM of the pOF1213 substrate in 25 $\mu$l of reaction buffer in the absence of nucleotide triphosphates for 0.5, 1, 2, 3, and 4 h at room temperature. Thereafter, each reaction was made up to 50 $\mu$l with the addition of 100 $\mu$M ATP, UTP, CTP, and 5 $\mu$M GTP and 10 $\mu$Ci [$\alpha^{33}$P]rGTP and 0.4 ng/ml heparin. The reactions were terminated at 0.5, 1, 2, 1.5, 2, 3, 5, and 10 min with EDTA to a final concentration of 83 mM and processed as described under Materials and methods. (B) Gel analysis of denatured products derived from reactions sampled in a time course of burst RNA synthesis. The reactions were assembled as described above except that the enzyme was preincubated with pOF1213 for 4 h and the burst reactions were terminated as indicated. The RNA was recovered from samples and subjected to agarose gel electrophoresis as described under Materials and methods. Lane 1 contains the labeled pOF1213 RNA substrate shown by the arrow and lane 12 contains $^{33}$P-labeled RNA standards. The arrow pointing to the band in lane 11 indicates the position of the copy-back product.

can catalyze de novo initiation of RNA synthesis using a poly (C) RNA substrate with rGTP (Labonte et al., 2002). Using this assay, the NTW (S-S) peptide was evaluated for its ability to inhibit this reaction catalyzed by NS5B as described under Materials and methods. The results shown in Fig. 6 show that the peptide inhibited the de novo reaction with an $IC_{50}$ of 10 $\mu$M, which is similar to that obtained in the assays using the self-primed pOF1213 RNA substrate. Since the peptide NTW (S- S) did not inhibit the elongation process as shown previously, it is likely that the peptide interferes with the formation of a preinitiation complex with NS5B and the 3′OH end (nonprimed) of the poly(C) template.

## Discussion

In this study, we have identified for the first time a class of heptamer-constrained peptides from a phage-displayed library screen using HCV NS5B polymerase as bait. These peptides contained a core motif FPWG at the N-terminal region of the sequence. BIACORE SPR analysis showed that NTW (S-S), KEY (S- S), and NQW (S-S) bound to the enzyme, consistent with a 1:1 ratio at low concentrations, with apparent $K_d$ ranging from 33 to 64 $\mu$M. However, at higher peptide concentrations, NQW (S-S) showed evidence of additional weaker binding events, which could easily represent nonspecific interactions. Their contribution

Table 3
Effects of peptides on the burst assays catalyzed by HCV polymerase

| Name | Peptide sequence | Prebinding | Postbinding |
|---|---|---|---|
| NTW (S-S) | ACFPWGNTWCGGK (S-S) | 8.4 | >150 |
| KEY (S-S) | ACFPWGKEYCGGK (S-S) | 39.1 | >150 |
| NQW (S-S) | ACFPWGNQWCGGK (S-S) | 8.82 | >150 |

*Note.* The indicated peptides were tested in the HCV polymerase assays that were staged to assess their effects on the formation of preelongation complex (prebinding) or the elongation reaction carried out by the preelongation complex (postbinding). In the prebinding assay, increasing concentrations of peptides were incubated with pOF1213 RNA substrate for 4 h at room temperature, followed by the addition of heparin and the nucleotide triphosphates. After 15 min, the reactions were stopped and quantitated. In the postbinding assay, the enzyme was incubated with the RNA substrate for 4 h at room temperature, followed by incubation with increasing concentrations of peptides for 15 min. Heparin and nucleotide triphosphates were added followed by incubation for 15 min, after which the yield of products were determined. The numbers represent mean $IC_{50}$ values in $\mu$M.

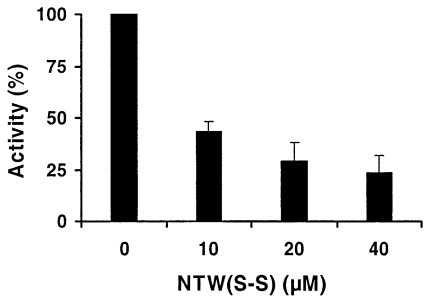


Fig. 6. Inhibition of de novo synthesis of HCV polymerase by NTW(S-S) peptide. In a 50 $\mu$l reaction, 20 nM of the HCV polymerase was incubated with 1.32 $\mu$g/ml of poly (C) and 250 $\mu$M GTP in presence of 0 to 40 $\mu$M of NTW(S-S) peptide. The reaction was incubated at 37°C for 2 h and terminated with EDTA and the pmol GMP incorporated was determined as described previously.

to the binding response complicates the interpretation of the fit for this peptide and they appear to dominate the response in the linear form of the peptide. Hence, it is possible that the estimated $K_d$ for NQW (S-S) could be lower than that reported here. It should also be noted that the random amine coupling procedure used to immobilize the enzyme to the CM5 chip has the potential of modifying or obscuring the suspected binding site of the peptides in an undetermined percentage of the immobilized enzyme. If the $K_d$ values of the modified population of enzyme were weaker than the true $K_d$, what in fact we would observe as a $K_d$ would be an average of the total population of enzyme. However, since the weaker interactions contribute little to the overall response, they probably would not affect the fitted $K_d$, whose determination is not directly related to the absolute concentration of active enzyme on the surface. As mentioned previously, since the HCV NS5B interacted strongly with the CM5 matrix, we were unable to do the preferred reciprocal binding experiment to measure the interaction between the native protein and the immobilized peptides.

More importantly, our results indicated that the constrained peptides NTW (S-S), KEY (S-S). NQW (S-S), and the linear peptide NQW (L), which bound to the NS5B, also inhibited the HCV NS5B enzyme activity in a selective manner. Thus, there is a good correlation between the ability of the peptides to bind NS5B enzyme and their ability to inhibit the enzyme activity in vitro. Given the high degree of similarity in structure and function among other polymerases, the selective inhibition of the HCV polymerase exhibited by these peptides supports the notion of selective binding of the enzyme. It should be noted that the $>50$ $\mu$g/ml BSA were included in the enzyme assays to mitigate any nonspecific binding of peptide to the enzyme during the course of the reaction. Since the linear peptides NTW (L) and KEY (L) did not inhibit the enzyme activity, we suggest that the tertiary peptide structure conferred by the disulfide loop was essential for their inhibitory properties. In support of this, the addition of DTT in the assays with NTW (S-S) and KEY (S-S) led to a dramatic reduction of their inhibitory property, likely due to the reduction of the disulfide bond leading to conversion to the noninhibitory linear counterparts (data not shown). In contrast, both the linear and the constrained versions of the peptide NQW were able to bind HCV polymerase and inhibit the enzyme activity in vitro. At this point, it is not known whether these two peptides inhibited the HCV polymerase activity through the same binding pocket or a different binding site. Future work will focus on cocrystalization of these peptides with the HCV polymerase.

We have also addressed the amino acid requirement of the peptides for NS5B inhibition, by alanine substitution analysis of the heptamer sequence of the NTW (S-S) peptide. The alanine scans of FPWGNTW showed that the F1 within the core motif was the most critical for the inhibitory effect of the peptide. However, contributory effects of P2, W3, N5, and W7 were noted, while G4 and T6 were accommodated by A. The null effect of the G4-to-A change may not be surprising since this can be considered a conservative change. Thus, we infer that the above-mentioned residues could synergistically contribute to the inhibitory property of the peptide. In addition, a measurable selective inhibition with the constrained core motif FPWG (SS) was observed, which indicates that this core sequence is critical for the inhibitory property of the peptides (Table 2).

Our analysis indicated that these constrained peptides blocked the formation of the preelongation complex, consisting of HCV polymerase bound to the dinucleotide self-primed RNA substrate, or a transition step required for the preelongation complex to catalyze processive RNA synthesis. Our data suggest that this is a slow or rate-limiting step of the reaction. However, once the preelongation complex was preformed, the peptide had no effect on RNA synthesis catalyzed by this binary complex. Initial attempts to examine the effects of these peptides on the Huh7 cell-based replicon were not successful due to the lack of an efficient system to deliver the peptides into the cells. However, recent advances on the generation of a cell-free replication system may allow further characterization of the inhibitory peptides described in the present study (Ali et al., 2002, Hardy et al., 2003; Shi et al., 2003). During the last few years, both nucleoside and nonnucleoside inhibitors for HCV NS5B polymerase have been identified (Lesburg et al., 2000). These compounds exhibited an in vitro anti-NS5B activity with $IC_{50}$ values in the micromolar range, which is comparable to the values reported for the inhibitory peptides in the present study. More recently, a class of heterocyclic agent (benzo-1,2,4-thiadiazine) has been shown to be a potent and highly specific inhibitor of NS5B (Dhanak et. al., 2002; Gu, et al., 2003). This agent interacts directly with NS5B apoenzyme as well as the RNA-bound form of NS5B and inhibits RNA synthesis in a noncompetitive manner. The actual mechanism of peptide inhibition needs to be clarified. It is likely that the peptides inhibited enzyme activity when bound to a site that blocked an allosteric step required for enzyme function. We have shown that

the NTW (S-S) peptide inhibited the de novo synthesis catalyzed by the enzyme, which suggests that it likely inhibited the formation of a preinitiation complex between the enzyme and the 3′OH end of the linear poly (C) substrate. Unlike the inhibition profile of the heterocyclic agent, the inability of the peptides to block RNA synthesis by the binary complex (post-binding data) suggests that these peptides might interfere with the NS5B–template interaction to mediate inhibition.

The HCV polymerase is thought to function as a complex with the nonstructural proteins and likely host factors to facilitate viral genome replication in liver cells, or permissive cells, and current strategies are aimed toward developing therapeutics for inhibiting HCV genome replication. In principle, the NS5B polymerase or any of its associated factors could be a target for small molecule inhibitor development. The peptides identified in this study are useful reagents to identify a binding pocket in the HCV polymerase through cocrystallization studies and to further probe the mechanism of enzyme function. By modeling complementary interactions of the cocrystal structures of the respective peptide:polymerase complex, it may be possible to obtain information useful in the rational design of high-affinity binders that could potentially translate into the design of small molecule inhibitors.

## Materials and methods

### *Miscellaneous*

The NS5B protein used in this study was derived from the HCV BK strain, genotype 1b (Takamizawa et al., 1991). The construction of NS5B with a 21 amino acid C-terminal truncation, and a C-terminal hexahistidine tag, will be described elsewhere (Olland, S., unpublished data). The protein was expressed in *Escherichia coli* and purified to homogeneity using $Ni^{2+}$ affinity chromatography, followed by a porous heparin sepharose (HS) column. The final protein preparation was 5 mg/ml in 50 mM Tris pH 7.5, 0.3 M NaCl, 50% glycerol, 1 mM DTT and was >95% homogeneous based on mass spectometry analysis and SDS–PAGE analysis (Olland et al., unpublished data). Protein concentration was determined using the Bio-Rad reagent with known BSA protein concentrations as reference standard as described by the manufacturer. Disulfide constrained and linear peptides of greater than 95% purity were synthesized by Anaspec (CA). The peptides were dissolved in water or DMSO for stocks of 2.5 or 5 mM, aliquoted as 25 μl, and stored at −70°C. Peptides were not used in experiments after three freeze–thaw cycles.

### *Screening for peptide ligands of NS5B*

The Ph.D-7-mer phage display constrained peptide library from New England BioLabs (Cat. No. 8110) was used for the isolation of peptides that bind to NS5B according to an optimized protocol described by the manufacturer. The constrained library consisted of about $1.9 \times 10^9$ peptide sequences with about 20 copies of each peptide in 10 μl of the supplied phage. About 1.5 μg of NS5B (del 21-His) in 200 μl of TBS was immobilized on $Ni^{2+}$-coated 96-well plates (Pierce) for 2 h at 4°C by shaking gently. The wells were washed twice with 200 μl TBS buffer, and the plates blocked with 250 μl of TBS containing 5 mg/ml BSA (Boehringer Mannheim Corp.) for 2 h at 4°C. Thereafter, the wells were washed two times with 250 μl of TBS buffer. About $2 \times 10^{11}$ phage particles in 200 μl of TBS containing 0.01% Tween 20 were added to the wells, which were then incubated at 23°C for 1 h. The wells were washed five times with TBS containing 0.5% Tween to remove unbound phages. The bound phage was eluted with 200 μl of 0.2 M glycine (pH 2.2) at room temperature for 6 min and immediately neutralized with 1 M Tris pH 9.1. The phage was amplified and titered, and the panning procedure repeated three times, after which independent phage clones were isolated. DNA were purified from 40 independent phage isolates and the sequences corresponding to the displayed peptides were determined.

### *Biacore analysis of peptide–NS5B interaction*

Experiments to measure the interaction between the peptides and NS5B polymerase by surface plasmon resonance (SPR) were carried out on a Biacore 2000 instrument (Biacore Inc., Uppsala, Sweden). All sensor chips, coupling reagents, and buffers were purchased from Biacore Inc. Human serum albumin was obtained from Sigma Chemicals (St. Louis, MO). The running and dilution buffer for all operations was 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20, pH 7.4. Coupling reactions were carried out at flow rates of 5 μl/min. All four channels of a CM5 sensor chip were activated by a 7-min injection of a mixture of 0.2 M *N*-ethyl-*N*′-(3-diethyaminopropyl) carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS). The NS5B protein in a solution of 0.01 M sodium acetate, pH 5.0, was injected over a single channel. Human serum albumin in 0.01 M sodium acetate, pH 4.0, was injected in a separate channel. No protein was injected in the remaining two channels to provide two matrix background controls. A typical binding level for both proteins was approximately 8000 resonance units (RU). Remaining esters were deactivated by an injection of 1 M ethanolamine over all four channels.

Peptides were injected over all four channels for 1 min at a flow rate of 50 μl/min. This provided sufficient time for the interaction of peptide with NS5B to reach equilibrium. Each peptide was injected over a range of concentrations between 10 and 100 μM. An equilibrium response was recorded as the average value for the last 10 s of the injection. A similar response from the control channel was subtracted from this value to compensate for minor matrix interactions and buffer mismatch. For samples that were

diluted in DMSO, an additional correction factor generated from a standard curve of various DMSO concentrations was introduced as per Biacore's recommendations.

Since RU is directly proportional to molecular weight, the response of each peptide was converted to a molar equivalent. The moles of peptide bound to the enzyme in a particular injection were divided by the moles of NS5B permanently immobilized on the chip. This value was plotted versus the concentration of the injected peptide, which allowed an evaluation of the stoichiometry of the interaction. This curve was fitted with the steady-state affinity model included in the Biacore Evaluation software to provide a dissociation constant ($K_d$). Off-rates were too rapid to be measured reliably on the Biacore but appeared to be on the order of 1 $s^{-1}$.

*HCV NS5B polymerase assays*

The HCV polymerase assays used in peptide evaluation were carried out in 50 $\mu$l containing 20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 100 $\mu$g/ml BSA, 20 units Rnasein, 3% DMSO, 1 mM rATP, 4 mM rUTP, 0.08 mM rCTP, and 0.05 mM and 2 $\mu$Ci of [$\alpha^{33}$P]rGTP (3000 Ci/mmol, 10 mCi/ml) and 3 nM of the pOF1213 RNA substrate (Baginski et al., 2000). Increasing concentrations of the respective peptides were incubated with the enzyme for 15 min with the rNTPs, followed by the addition of pOF1213 RNA substrate and [$\alpha^{33}$P]rGTP to start the reaction. The reactions were incubated for 2 h at room temperature and subsequently stopped by the addition of EDTA to 83 mM. The $IC_{50}$ values of peptides were determined from triplicate samples using the Data Analysis Toolbox 1.0 from MDL Information System Inc.

Burst assays that measured the elongation reaction catalyzed by the NS5B enzyme were done in the presence or absence of increasing concentrations of peptides under the same buffer conditions as described above. For this assay, the enzyme was preincubated with pOF1213 RNA for 4 h to enrich for the binary preelongation complex, and the elongation reaction was initiated by the addition of the 10 $\mu$M rNTPs containing 25 $\mu$Ci [$\alpha^{33}$P]rGTP (3000 Ci/mmol, 10 mCi/ml). Peptides were added either during the incubation period of NS5B with pOF1213 (peptide prebinding) or after the preincubation period (peptide postbinding). The reactions were stopped at different times by the addition of EDTA to 83 mM.

The de novo synthesis assay with NS5B polymerase was performed in 50 $\mu$l containing 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 20 nM NS5B enzyme, 1.32 $\mu$g/ml Poly(C) (Sigma), 20 U Rnasin, 50 $\mu$g/ml BSA, 0–40 $\mu$M of peptide, 250 $\mu$M rGTP, and 2.5 $\mu$Ci [$\alpha^{33}$P]rGTP (3000 Ci/mmol, 10 mCi/ml). Reactions were incubated at 37°C for 120 min and stopped with 10 $\mu$l of 0.5 M EDTA. Samples were captured on 96-well DEAE filters (Millipore) by filtration and washed five times with 250 $\mu$l of 0.5 M $NaPO_4$ buffer (pH 7.0). The plates were air-dried for 10 min and 50 $\mu$l of Scintisafe Plus 50% (Fisher Scientific) was added for scintillation counting using a beta-counter (Wallac).

*Assays for calf thymus polymerase $\alpha$, human polymerase $\beta$, RSV polymerase, and HIV reverse transcriptase*

All assays for the polymerases to be described were carried out at their respective $K_m$s of NTPs and nucleic acid substrates. The calf thymus polymerase $\alpha$ assay was carried out in a 50 $\mu$l reaction containing 60 mM Tris pH 8, 5 mM Mg acetate, 0.3 mg/ml BSA, 1 mM DTT, 0.1 mM spermidine, 12 $\mu$M dATP, 1.2 $\mu$M dCTP, 1.5 $\mu$M dGTP, 2.7 $\mu$M dTTP, 0.1 u enzyme, 0.5 $\mu$Ci [$\alpha^{33}$P]dCTP (NEN, 3000 Ci/mmol, 10 mCi/ml), and 0.54 $\mu$g calf thymus DNA (Pharmacia) for 2 h at room temperature. The human polymerase $\beta$ assay was carried out in 50 $\mu$l of reaction containing 50 mM Tris pH 8.8, 10 mM $MgCl_2$, 100 mM KCl, 0.4 mg/ml BSA, 1 mM DTT, 1% glycerol, 5 $\mu$M dATP, 10 $\mu$M dCTP, 4 $\mu$M dGTP, and 16 $\mu$M dTTP, 0.04 u (1 $\mu$g) polymerase (Trevigen), 0.75 $\mu$g CT DNA (Sigma), and 0.5 $\mu$Ci [$\alpha^{33}$P]dATP (3000 Ci/mmol, 10 mCi/ml) for 2 h at room temperature. The HIV RT assay was carried out in 20 $\mu$l reaction containing 50 mM Tris pH 8, 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1.2 $\mu$M dATP, 0.7 $\mu$M dGTP, 0.18 $\mu$M dCTP, 0.8 $\mu$M dTTP, 0.36 $\mu$l primer:template stock (Origen), 10 u RNAsein (Promega), 0.25 $\mu$Ci [$\alpha^{33}$P]dCTP (NEN, 3000 Ci/mmol, 10 mCi/ml), 50 $\mu$g/ml BSA, 0.007 u (0.82 $\mu$g) RT (Worthington) at room temperature for 2 h. The RSV polymerase (kindly provided by Dr. Clayton Huntley) reaction was carried out in 60 $\mu$l containing 50 mM HEPES/KOH pH 8, 100 mM KOAc, 5 mM MgOAc, 5% glycerol, 1 mM DTT, 0.75 $\mu$g/ml Actinomycin D, 1 mM ATP, 140 $\mu$M GTP, 100 $\mu$M UTP, 1 $\mu$M CTP, 0.25 $\mu$Ci [$\alpha^{33}$P]CTP, and 0.2 $\mu$l (1 $\mu$g) of RSV polymerase preparation at room temperature for 2 h.

All reactions were quenched with 0.1 M EDTA, captured on 96-well DEAE filters (Millipore) by filtration, and washed five times with 250 $\mu$l of 0.5 M $NaPO_4$ buffer (pH 7.0). The plates were air-dried for 10 min and 50 $\mu$l of Scintisafe Plus 50% (Fisher Scientific) was added for scintillation counting using a beta-counter (Wallac).

*Agarose gel analysis of RNA products from burst reaction*

Burst reactions were stopped with equal volumes of phenol:chloroform (1:1) and precipitated with 10 $\mu$g of glycogen, 1/10 volume of 3 M sodium acetate, and 2.5 volume of ethanol at −70°C for 2 h. Samples were centrifuged at 15,000 rpm for 15 min at 4°C to collect the RNA pellet. The RNA pellet was gently rinsed with 75% ethanol and subsequently recovered by centrifugation. The pellet was air dried and resuspended into 10 $\mu$l of 1× Glyoxal Loading dye (Ambion), heated at 85°C for 10 min, and subjected to electrophoresis on a 2% glyoxal agarose gel using NorthernMax Gly/gel Prep Running Buffer (Ambion). The gel was dried and exposed to X-OMAT film with

a screen overnight. For quantitative analysis the gel was exposed to Kodak K-screen for 4 h and scanned using a Bio-Rad phosphorImager.

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## References

Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., Miyano, Y., 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure 7, 1417–1426.

Ali, N., Tardif, K.D., Siddiqui, A., 2002. Cell-free replication of the hepatitis C virus subgenomic replicon. J. Virol. 76, 12001–12007.

Alter, M.J., Mast, E.E., 1994. The epidemiology of viral hepatitis in the United States. Gastroenterol. Clin. N. Am. 23, 437–455.

Baginski, S.G., Pevear, D.C., Seipel, M., Sun, S.C.C., Benetatos, C.A., Chunduru, S.K., Rice, C.M., Collett, M.S., 2000. Mechanism of action of a pestivirus antiviral compound. Proc. Natl. Acad. Sci. USA 97, 7981–7986.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus (Review). J. Gen. Virol. 81, 1631–1648.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J., Jacobsen, H., 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J. Virol. 67, 3835–3844.

Behrens, S.-E., Tomei, L., De Francesco, R., 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. 15, 12–22.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R.L., Mathieu, M., De Francesco, R., Rey, F.A., 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. USA 96, 13034–13039.

Butcher, S.J., Grimes, J.M., Makeyev, E.V., Bamford, D.H., Stuart, D.I., 2001. A mechanism for initiating RNA-dependent RNA polymerization. Nature 410, 235–240.

Carroll, S.S., Sardana, V., Yang, Z., Jacobs, A.R., Mizenko, C., Hall, D., Hill, L., Zugay-Murphy, J., Kuo, L.C., 2000. Only a small fraction of purified hepatitis C RNA-dependent RNA polymerase is catalytically competent: implication for viral replication and in vitro assays. Biochemistry 39, 8243–8249.

Choo, Q.I., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R.M., Barret, P.J., Weiner, A.J., Bradley, D.W., Kuo, G., Houghton, M., 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88, 2451–2455.

Choo, Y., Klug, A., 1995. Designing DNA-binding protein on the surface of filamentous phage. Curr. Opin. Biotechnol. 6, 431–436.

Cwirla, S.E., Peteres, E.A., Barett, R.W., Dower, W.J., 1990. Peptides on phage: a vast library of peptides for identifying ligands. Proc. Natl. Acad. Sci. USA 87, 6378–6382.

Dhanak, D., Duffy, K.J., Johnson, V.K., Lin-Goerke, J., Darcy, M., Shaw, A.N., Gu, B., Silverman, C., Gates, A.T., Nonnemacher, M.R., Earnshaw, D.L., et al., 2002. Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 41, 38322–38327.

Devlin, J.J., Panganiban, L.C., Devlin, P.E., 1990. Random peptide libraries: a source of specific protein binding molecules. Science 249, 404–406.

Farci, P., Alter, S., Govindarajan, S., Wong, D.C., Engle, R., Lesniewski, R.R., Mushahwar, I.K., Desai, S.M., Miller, R.H., Ogata, N., Purcell, R.H., 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science 258, 135–140.

Felici, F., Castagnoli, L., Musacchio, A., Japelli, R., Cesarini, G., 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. J. Mol. Biol. 222, 301–310.

Ferrari, E., Wright-Minogue, J., Fang, J.W.S., Baroudy, B.M., Lau, J.Y.N., Hong, Z., 1999. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. J. Virol. 73, 1649–1654.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993a. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67, 1385–1395.

Grakoui, A., McCourt, D.W., Wychowski, C., Feinstone, S.M., Rice, C.M., 1993b. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. 67, 2832–2843.

Gu, B., Johnson, V.K., Gutshall, L.L., Nguyen, T.T., Gontarek, R.R., Darcy, M.G., Tedesco, R., Dhanak, D., Duffy, K.J., Kao, C.C., Sarisky, R.T., 2003. Arresting initiation of HCV RNA synthesis using heterocyclic derivatives. J. Biol. Chem. 278, 16602–16607.

Hardy, R.W., Marcotrigiano, J., Blight, K.J., Majors, J.E., Rice, C.M., 2003. Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon- containing hepatoma cells. J. Virol. 77, 2029–2037.

Hoess, R., Brinkmann, V., Hardel, T., Pastan, I., 1993. Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3. Gene 128, 43–49.

Kao, C.C., Yang, X., Kline, A., Wang, Q.M., Barket, D., Heinz, B.A., 2000. Template requirements for RNA synthesis by a recombinant hepatitis C virus RNA- dependent RNA polymerase. J. Virol. 74, 11121–11128.

Kato, M., Hijikata, M., Ootsuyama, Y., Nakagama, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Proc. Natl. Acad. Sci. USA 87, 9524–9528.

Kolykhalov, A.A., Mihalik, K., Feinstone, S.M., Rice, C., 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. 74, 2046–2051.

Labonte, P., Axelrod, V., Agarwal, A., Aulabaugh, A., Amin, A., Mak, P., 2002. Modulation of hepatitis C virus RNA-dependent RNA polymerase activity by structure-based site-directed mutagenesis. J. Biol. Chem. 277, 38838–38846.

Lesburg, C.A., Radfar, R., Weber, P.C., 2000. Curr. Opin. Investig. Drugs. 1, 289–296.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6, 937–943.

Lohmann, V., Roos, A., Körner, F., Koch, J.O., Bartenschlager, R., 1998. Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus. Virology 249, 108–118.

Lohmann, V., Körner, F., Koch, J.O., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Lohmann, V., Körner, F., Dobierzewska, A., Bartenschlager, R., 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J. Virol. 75, 1437–1449.

Luo, G., Hamatake, R.K., Mathis, D.M., Racela, J., Rigat, K.L., Lemm, J., Colonno, R.J., 2000. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J. Virol. 74, 851–863.

McHutchinson, J.G., Gordon, S.C., Schiff, E.R., Schiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S., Albrecht, J.K., 1998. Interferon alpha 2b alone or in combination with ribavirin

as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Eng. J. Med. 339, 1485–1492.

Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human liver. Nat. Med. 7, 927–933.

Oldenburg, K.R., Loganathan, D., Goldstein, I.J., Schultz, P.G., Gallop, M.A., 1992. Peptide ligands for a sugar-binding protein isolated from a random peptide library. Proc. Natl. Acad. Sci. USA 89, 5593–5397.

Porter, A.G., 1993. Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. J. Virol. 6, 6917–6921.

Poynard, T., Marcellin, P., Lee, S.S., Neiderau, C., Minuk, G.S., Ideo, G., Bain, V., Heathcote, J., Zeuzem, S., Trepo, C., Albrecht, J., 1998. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 hours for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 352, 1426–1432.

Reed, K.E., Rice, C.M., 2000. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. 242, 55–84.

Roberts, B.L., Markland, W., Ley, A.C., Kent, R.B., White, D.W., Guterman, S.K., Ladner, R.C., 1992. Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. Proc. Natl. Acad. Sci. USA 89, 2429–2433.

Scott, J.K., Smith, G.P., 1990. Searching for peptide ligands with an epitope library. Science 249, 386–390.

Scott, J.K., Loganathan, D., Easley, R.B., Gong, X., Goldstein, I.J., 1992. A family of concanavalin A-binding peptides from a hexapeptide epitope library. Proc. Natl. Acad. Sci. USA 89, 5398–5402.

Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M.C., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77, 4160–4168.

Soumillion, P., Jespers, L., Bouchet, M., Marchand-Brynaert, J., Winter, G., Fastrez, J., 1994. Selection of beta-lactamase on filamentous bacteriophage by catalytic activity. J. Mol. Biol. 237, 415–422.

Strauss, E.G., Strauss, J.H., 1986. In: Schlesinger, S.S., Schlesinger, M.J., (Eds.). The Togaviridae and Flaviviridae, Plenum Press, New York, pp. 35–90.

Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., Okayama, H., 1991. Structure and organization of the hepatitis C virus genome isloated from human carriers. J. Virol. 65, 1105–1113.

Tanaka, T., Kato, N., Cho, M.-J., Sugiyama, K., Shimotohno, K., 1996. Structure of the 3′ terminus of the hepatitis C virus genome. J. Virol. 70, 3307–3312.

Tanji, Y.M., Hijikata, M., Satok, S., Kaneko, T., Shimotohno, K., 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. 69, 1575–1581.

Tomei, L., Failla, C., Santolini, E., De Francesco, R., La Monica, N., 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J. Virol. 67, 4017–4026.

Tomei, L., Vitale, R.L., Incitti, I., Serafini, S., Altamura, S., Vitelli, A., De Francesco, R., 2000. Biochemical characterization of a hepatitis C virus RNA- dependent RNA polymerase mutant lacking the C-terminal hydrophobic sequence. J. Gen. Virol. 81, 759–767.

Wimmer, E., Hellen, C.V.T., Cao, X., 1993. Genetics of poliovirus. Ann. Rev. Genetic. 27, 453–436.

World Health Organization 1996. Hepatitis C. Seroprevalence of Hepatitis C virus (HCV) in a population sample. Wkly. Epidemiol. Rec. 71, 346–349.

Yamada, N., Tanihara, K., Takada, A., Yorihuzi, T., Tsutsumi, M., Shimorura, H., Tsuji, T., Date, T., 1996. Genetic organization and diversity of the 3′ noncoding region of the hepatitis C virus genome. Virology 223, 255–261.

Zhong, W., Ferrrari, E., Lesburg, C.A., Maag, D., Ghosh, S.K.B., Cameron, C.E., Lau, J.Y.N., Hong, Z., 2000. Template/primer requirements and single nucleotide incorporation by hepatitis C virus nonstructural protein 5B polymerase. J. Virol. 74, 9134–9143.

Zhong, W., Uss, A.S., Ferrari, E., Lau, J.Y.N., Hong, Z., 2000. De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. J. Virol. 74, 2017–2022.