Rate-limiting pyrophosphate release by hepatitis C virus polymerase NS5B improves fidelity

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The hepatitis C virus RNA-dependent RNA polymerase NS5B is responsible for the replication of the viral genome. Previous studies have uncovered NTP-mediated excision mechanisms that may be responsible for aiding in maintaining fidelity (the frequency of incorrect incorporation events relative to correct), but little is known about the fidelity of NS5B. In this study, we used transient-state kinetics to examine the mechanistic basis for polymerase fidelity. We observe a wide range of efficiency for incorporation of various mismatched base pairs and have uncovered a mechanism in which the rate constant for pyrophosphate release is slowed for certain misincorporation events. This results in an increase in fidelity against these specific misincorporations. Furthermore, we discover that some mismatches are highly unfavorable and cannot be observed under the conditions used here. The calculated fidelity of NS5B ranges between $10^{-4}$–$10^{-9}$ for different mismatches.

Polymerase fidelity plays an important role in maintaining the integrity of the genome during replication. Viral polymerases face a unique challenge in balancing a sufficiently high fidelity to reduce the frequency of lethal mutations while still allowing for sufficient genetic variation for the virus to escape the host immune response and evolve resistance against antiviral therapeutics. Although extensive analysis has revealed the mechanism and kinetic basis for fidelity of DNA-dependent DNA polymerases, few studies have quantified the fidelity of RNA-dependent RNA polymerases including HCV (1). In particular, very little quantitative data are available to define the SARS-coronavirus fidelity (2–4) or the kinetics of nucleotide incorporation in vitro (5, 6).

In vivo measurements of HCV RNA viral replication show a mutation rate in the range of $10^{-6}$–$10^{-4}$ substitutions/nucleotide site/cell infection (7, 8). Average measurements of the mutation rate of the hepatitis C virus (HCV) are $3.5 \times 10^{-5}$ substitutions/nucleotide site/cell infection, indicating an average substitution of 0.36 mutations/genome replication cycle (2). These measurements, however, are biased against lethal mutations and may not reflect the true fidelity of the HCV RNA-dependent RNA polymerase (NS5B). Attempts to measure the fidelity of NS5B using in vitro methods have been previously published (1). In this method, an RNA template was designed so that NS5B will extend a dinucleotide primer using two of the four nucleotides up to 15 nucleotides and then pause because of the absence of the next complementary nucleotide. However, in prior studies the elongation complex was not isolated from unincorporated nucleotide used during the de novo initiation process, and the studies were conducted at a low NaCl concentration in which the active elongation complex is insoluble, which can complicate analysis of single-nucleotide incorporation. Here, we explore the fidelity of NS5B by measuring the kinetics of all 12 possible misincorporations using an isolated NS5B/9-nt primer/20-nt template elongation complex in an optimized buffer. Using these methods, we show that the rates of misincorporation vary widely for different mismatches. In addition, we found that the initial rate measurements underestimate the range of fidelity because of a previously unknown slow pyrophosphate release step seen with four of the mismatches. This slow pyrophosphate release allows for the reversal of chemistry to occur and contributes an increase in fidelity up to two orders of magnitude.

Results

To probe NS5B fidelity, a pre-steady-state kinetic analysis was performed to characterize incorporation of the four complementary nucleotides to serve as a basis for comparison with all 12 misincorporation events. The kinetics of the incorporation of complementary nucleotides allowed for the determination of the kinetic parameters $k_{\text{pol}}$ and $K_{d,\text{app}}$ to afford calculation of $k_{\text{cat}}/K_{m}$. For measurement of misincorporation, the results are separated into two groups: low-fidelity misincorporations and high-fidelity misincorporations. Lower fidelity afforded estimates of $k_{\text{cat}}$, $K_{m}$, and $k_{\text{cat}}/K_{m}$, whereas higher-fidelity misincorporations only afforded estimates of $k_{\text{cat}}/K_{m}$ or in some cases, showed no evidence for misincorporation.

Correct incorporation of NTP

Correct incorporation of CTP:G and UTP:A (G and A are the templating bases) were previously measured and reported (9). Incorporation of ATP:U and GTP:C were measured by rapidly mixing the NS5B/9-nt/20-nt elongation complex with the complementary nucleotide using rapid quench-flow methods. The data were fit globally using Scheme 1 to determine the apparent equilibrium dissociation constant for ground-state binding ($K_{d,\text{app}} \approx k_{-1}/k_{1}$), the maximum rate of incorporation ($k_{\text{pol}} = k_{\text{cat}} = k_{j}$), and the specificity constant for correct incorporation ($k_{\text{cat}}/K_{m} = k_{\text{pol}}/K_{d,\text{app}}$). The results for these
incorporation reactions are summarized on Table 1. The results for incorporation of ATP:U and GTP:C are shown in Fig. 1, A and B, respectively. The apparent affinity for ATP is the weakest of the four nucleotides ($K_{\text{d,app}} = 572 \pm 88 \mu M$). This weak apparent affinity can be overcome by the high concentration of ATP in the cell (≈3 mM) (10). The rate constant of incorporation $k_{\text{pol}}$ was measured to be $8 \pm 1 \text{ s}^{-1}$. These results give a $k_{\text{pol}}/K_{\text{d,app}}$ of $0.014 \pm 0.003 \text{ s}^{-1}$, the lowest of the four correct incorporations, but commensurate with the high cellular ATP concentrations. The kinetic parameters of GTP:C incorporation were similar to CTP:G. The apparent affinity for CTP:G misincorporation ($K_{\text{d,app}} = 2.8 \pm 0.4 \text{ mM}$), whereas the rate constant of misincorporation was $37$-fold slower compared with correct incorporation with a $k_{\text{pol}} = 0.41 \pm 0.04 \text{ s}^{-1}$. This misincorporation is more efficient than CTP:A ($k_{\text{pol}}/K_{\text{d,app}} = 0.359 \pm 0.007 \text{ s}^{-1}$). Both UTP:U and GTP:U mismatches have similar specificity constants, $k_{\text{pol}}/K_{\text{d,app}}$ (UTP:U = $4.5 \pm 0.6 \times 10^{-6} \text{ s}^{-1}$) is the slowest rate of polymerization of the four ($k_{\text{pol}} = 0.017 \pm 0.002 \text{ s}^{-1}$; $470$-fold slower compared with correct incorporation). This results in GTP:U mismatches being less favorable than CTP:A and ATP:C mismatches ($k_{\text{pol}}/K_{\text{d,app}} = 2.2 \pm 0.2 \times 10^{-6} \text{ s}^{-1}$). UTP:U mismatches were shown to be as efficiently incorporated as GTP:U, but UTP:U had a $K_{\text{d,app}} = 20 \pm 2 \mu M$, the weakest of the four in this group, although the rate constant of polymerization was greater ($k_{\text{pol}} = 0.089 \pm 0.01 \text{ s}^{-1}$) than GTP:U. Both UTP:U and GTP:U mismatches have similar specificity constants, $k_{\text{pol}}/K_{\text{d,app}}$ (UTP:U = $4.5 \pm 0.6 \times 10^{-6} \text{ s}^{-1}$). Because correct incorporation of ATP has the lowest specificity constant of the four correct incorporations ($k_{\text{pol}}/K_{\text{d,app}} = 0.014 \text{ s}^{-1}$), misincorporation of GTP:U and UTP:U showed fidelity similar to the rest of the misincorporations in this category ($1.5 \pm 0.3 \times 10^{-4}$ and $3.2 \pm 0.8 \times 10^{-4}$, respectively).

### High-fidelity misincorporations

High-fidelity misincorporations are broken down into two subcategories: 1) no misincorporation observed and 2) slow pyrophosphate release. For those misincorporation reactions in which no extension of the 9-nt primer was observed by the end of the time course, we set a lower limit of detectable incorporation at 1% of the starting material after incubation with 5 mM nucleotide for 900 s. This allowed estimation of an upper limit on the rate of misincorporation to afford maximal estimates of $k_{\text{cat}}/K_{\text{m}}$ values (Table 3). Four misincorporations fall under this subcategory: GTP:G (Fig. 4A), GTP:A (Fig. 4B), CTP:C (Fig. 4C), and CTP:U (Fig. 4D). We set a lower limit of $K_{\text{m}}$ for these misincorporations at $5 \mu M$ nucleotide and an upper limit for the rate constant of polymerization ($k_{\text{pol}}$ at $0.000002 \text{ s}^{-1}$). This gives an estimated $k_{\text{cat}}/K_{\text{m}}$ value of $4 \times 10^{-9} \text{ s}^{-1}$. Using these estimations, we calculate fidelity of $\leq 1 \times 10^{-8}$ for GTP:G ($\leq 1$ in 90,000,000), $3.9 \times 10^{-8}$ for GTP:A ($\leq 1$ in 26,000,000), $4 \times 10^{-8}$ for CTP:C ($\leq 1$ in 240,000,000), and $2.9 \times 10^{-7}$ for CTP:U ($\leq 1$ in 3,000,000). Averaging these misincorporations
by the frequency of their respective bases in the HCV genome, these would occur only once in every 1400–83,000 replication cycles. It is quite possible that these results may change as a function of local RNA sequence.

The second subcategory in this group shows a slow rate constant of pyrophosphate release ($k_3$ in Scheme 1), which leads to slower net rates of misincorporation. The kinetics of incorporation are biphasic (Fig. 5A), and the amplitude of the fast reaction phase increases hyperbolically as a function of increasing concentrations of nucleotide (Fig. 5B). The amplitude dependence on NTP concentration implies that incorporation is reversibly linked to nucleotide binding. Because pyrophosphate release is largely irreversible at the concentrations formed during a single turnover, the amplitude dependence can be attributed to a slow rate constant for pyrophosphate release allowing polymerization ($k_2$) and the reverse of chemistry ($k_{-2}$) to come to equilibrium linked to nucleotide binding. This behavior has been observed in other polymerases such as 8-oxo-dGTP incorporation by human mitochondrial polymerase γ (11) and by HIV RT when reverse transcribing an RNA template (12) but not to the best of our knowledge has not been observed in HCV NS5B.

The concentration dependence of the observed rate and amplitude of the reaction affords resolution of the forward and reverse rate constants for the chemistry step and the rate constant for pyrophosphate release, supported by confidence contour analysis (Fig. 5C) (25). This slower rate constant of

**Figure 1. Incorporation of ATP and GTP by NS5B.** The above plots show the incorporation of (A) ATP (15.6, 31.3, 62.5, 125, 250, and 500 μM, red to magenta) and (B) GTP (6.3, 12.5, 25, 50, and 100 μM, red to cyan). Incorporation was measured by rapid quench-flow methods and fit globally using Scheme 1 by KinTek Explorer. The solid lines show the best-fit results. Kinetic parameters are summarized in Table 1.

**Figure 2. Example of analytical fit of low-fidelity misincorporations.** The above plots show and example of fitting a low-fidelity misincorporation (CTP:A) using analytical functions. A, the product versus concentration fit to a single exponential function demonstrating all concentrations go to the same end point. B, when plotting the amplitude versus concentration, no change in amplitude with increasing concentration is observed. C, the rate increases hyperbolically as a function of concentration, allowing the data to be fit to obtain estimates of $K_{d, app}$ and $k_2$.

**Table 2**

| Template base | Incoming base | $K_{d, app}$ (μM) | $K_{pol}$ (s$^{-1}$) | $K_{pol}/K_{d, app}$ (μM$^{-1}$s$^{-1}$) | Fidelity |
|---------------|---------------|-------------------|---------------------|-------------------------------------|----------|
| A             | U             | 320 ± 60          | 33 ± 5              | 0.10 ± 0.02                         | 1        |
| C             | C             | 7600 ± 1000       | 0.36 ± 0.04         | (4.7 ± 0.8) × 10$^{-5}$              | (4.6 ± 1) × 10$^{-4}$ |
| U             | A             | 15 ± 3            | 15 ± 0.9            | 0.99 ± 0.2                          | 1        |
| U             | G             | 2800 ± 400        | 0.41 ± 0.04         | (1.5 ± 0.3) × 10$^{-4}$              | (1.5 ± 0.4) × 10$^{-4}$ |
| U             | A             | 570 ± 90          | 8 ± 1               | 0.014 ± 0.003                       | 1        |
| U             | G             | 7700 ± 300        | 0.017 ± 0.002       | (2.2 ± 0.2) × 10$^{-6}$              | (1.5 ± 0.3) × 10$^{-4}$ |
| U             | U             | 20,000 ± 2000     | 0.089 ± 0.01        | (4.5 ± 0.6) × 10$^{-6}$              | (3.2 ± 0.8) × 10$^{-4}$ |

*Rate constants for correct incorporation are from Villalab et al., 2019 (9).*
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pyrophosphate release serves to lower $k_{cat}/K_m$ by allowing reversal of chemistry and release of the bound nucleotide, thereby increasing fidelity as described below.

The four misincorporations that are classified in this subcategory are ATP:G (Fig. 6A), UTP:G (Fig. 6B), UTP:C (Fig. 6C), and ATP:A (Fig. 6D). The kinetic parameters are summarized in Table 3. All four misincorporations showed weak apparent nucleotide affinities compared with the $K_{d, app}$ of their respective correct incorporation reactions. The weakest apparent affinity was UTP:G ($K_{d, app} = 6 \pm 1$ mm), followed by ATP:A ($K_{d, app} = 4.7 \pm 0.6$ mm), ATP:G ($K_{d, app} = 4 \pm 0.5$ mm), and finally UTP:C ($K_{d, app} = 3.7 \pm 0.6$ mm). The ATP:G misincorporation showed the highest fidelity of the four in this subcategory. This is attributable to the rate constant for the reverse of chemistry ($k_{-2} = 0.0081 \pm 0.001$ s$^{-1}$) greater than the forward reaction ($k_2 = 0.0045 \pm 0.0006$ s$^{-1}$). The rate constant of pyrophosphate release is also the slowest compared with the other three misincorporations in this category ($k_3 = 0.00057 \pm 0.0001$ s$^{-1}$).

Including pyrophosphate release in the model leads to the following equation for the specificity constant:

$$k_{cat}/K_m = \frac{K_1k_2k_3}{k_{-2}+k_3} \quad (Eq. 2)$$

When $k_{-2} < k_3$, $k_{cat}/K_m = k_1k_2$.

Using Equation 1, $k_{cat}/K_m$ for this misincorporation was calculated to be $(7.4 \pm 2) \times 10^{-8}$ $\mu$m$^{-1}$ s$^{-1}$. This gives a fidelity of

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**Figure 3. Four low-fidelity misincorporations by NS5B.** The plots show the four misincorporation reactions that are classified as low-fidelity: (A) CTP:A, (B) ATP:C, (C) GTP:U, and (D) UTP:U (500, 1000, 2000, 4000, and 5000 $\mu$m NTP). The designation CTP:A represents incorporation of CTP opposite a template A, for example. The data were fit globally using Scheme 1 using KinTek Explorer. The solid lines show the best-fit results from fitting. Kinetic parameters are summarized in Table 2.

**Table 3**

| Template base | Incoming base | $K_{d, app}$ (mM) | $k_2$ (s$^{-1}$) | $k_{-2}$ (s$^{-1}$) | $k_3$ (s$^{-1}$) | $K_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) | Fidelity |
|---------------|---------------|-------------------|-----------------|-------------------|-----------------|---------------------------------|----------|
| G            | C$^a$         | 26 ± 3            | 10 ± 0.8        | –                 | –               | 0.38 ± 0.05                    | 1        |
|              | A             | 4000 ± 500        | 0.0045 ± 0.0006 | 0.0081 ± 0.001   | 0.000857 ± 0.0001 | $(7.4 \pm 2) \times 10^{-8}$ | $(2.0 \pm 0.8) \times 10^{-7}$ |
|              | U             | 6000 ± 1000       | 0.014 ± 0.002   | 0.015 ± 0.002    | 0.0021 ± 0.0006 | $(2.1 \pm 1) \times 10^{-7}$  | $(7.8 \pm 3) \times 10^{-7}$  |
| G$^b$        | ≥ 5000        | ≤ 0.00002         | –               | –                 | –               | $4 \times 10^{-9}$            | $1.1 \times 10^{-8}$          |
| C            | G$^b$         | 15 ± 3            | 15 ± 0.9        | –                 | –               | 0.99 ± 0.019                  | 1        |
|              | U$^b$         | 3700 ± 600        | 0.023 ± 0.004   | 0.017 ± 0.007    | 0.0066 ± 0.003  | $(1.6 \pm 0.7) \times 10^{-6}$ | $(1.7 \pm 0.8) \times 10^{-6}$ |
|              | C$^b$         | ≥ 5000            | ≤ 0.00002       | –                 | –               | $4 \times 10^{-9}$            | $4.1 \times 10^{-9}$          |
| A            | U$^a$         | 320 ± 60          | 33 ± 5          | –                 | –               | 0.1 ± 0.02                    | 1        |
|              | A$^a$         | 4700 ± 600        | 0.0048 ± 0.0006 | 0.0021 ± 0.0004  | ≤ 0.0017        | $(4.6 \pm 1) \times 10^{-7}$  | $(4.4 \pm 2) \times 10^{-6}$  |
|              | C$^b$         | ≥ 5000            | ≤ 0.00002       | –                 | –               | $4 \times 10^{-9}$            | $3.9 \times 10^{-8}$          |
|              | U$^b$         | 570 ± 90          | 8 ± 1           | –                 | –               | 0.014 ± 0.003                | 1        |

$^a$ Rate constants for correct incorporation are from Villalab et al., 2019 (9).

$^b$ Limits are set by estimating 1% substrate turnover by the end of the observed time course.
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Discussion

In this study, we explored the fidelity of NS5B by measuring the kinetics of incorporation of the cognate base pairs and all 12 mismatches. We used a pre-steady-state kinetic analysis to monitor the extension of a primer over time and fit the results globally using Scheme 1 using KinTek Explorer. From this we were able to determine the apparent equilibrium dissociation constant ($K_{d\,\text{app}}$), the maximum rate constant of chemistry ($k_{\text{pol}} = k_3$), and in some cases, the reverse of chemistry ($k_{-2}$) and rate constant of pyrophosphate release ($k_4$). Using these results, and by using Equation 2 for mismatches that show slow pyrophosphate release, we are able to calculate the specificity constant ($k_{\text{cat}}/K_m = k_{\text{pol}}/K_{d\,\text{app}}$) for low-fidelity mismatches. The fidelity for each misincorporation was calculated by dividing the specificity constant for the misincorporation by the specificity constant of the correct incorporation for a given templating base (Equation 1).

Some similarities were observed between this work and previously reported results for incorporation by NS5B. The specificity constant for correct incorporation of CTP, UTP, and ATP were within 2-fold of each other, and both identify ATP as having the lowest specificity constant of the four incorporations. Also, both works not only identify the GTP:U wobble mismatch as one of the most likely mismatch to occur but also suggest that the reverse mismatch (UTP:G) is less likely to occur (1).

We found that under our conditions and using an isolated elongation complex, the fidelity of NS5B ranges from $10^{-4}$–$10^{-5}$ depending on the incoming nucleotide and templating base. This range indicates that NS5B may have a higher fidelity than previously thought ($10^{-3}$–$10^{-4}$) (1). Furthermore, in contrast to these previously reported results, we identified four misincorporations that are more likely to occur than others and a mechanism for increasing fidelity previously unknown to NS5B. Normalizing each misincorporation by the frequency each base occurs in the HCV genome gives an average of 0.19 substitutions/genome replication cycle, ~1.9-fold lower than in vivo measurements (2).

The four lowest fidelity misincorporations (CTP:A, ATP:C, GTP:U, and UTP:U) all had a fidelity in the $10^{-4}$ range. Each of these four misincorporations had a measured apparent nucleotide dissociation constant significantly higher than the physiologically concentrations of ribonucleotides. This weak apparent affinity is especially key in discriminating against accumulation of UTP:U misincorporations. CTP:A and ATP:C showed a much faster rate constant of misincorporation of the four and also had the highest efficiency compared with the other two. These were also the most likely of the four to be extended after the misincorporation has occurred (data not shown). These four lower-fidelity mutations may account for mutations that lead to genetic drift.
Previous work measuring the misincorporation frequencies during replication of an HCV replicon indicate that NS5B preferentially makes transition mutations over transversion mutations (1). Three of the four low-fidelity misincorporations that lead to transition mutations (CTP:A, ATP:C, and GTP:U) are in agreement with this observation. However, we measured UTP:G mismatches to be higher-fidelity misincorporations. This difference may reflect an influence of local sequence context on the fidelity of a specific mismatch.

A U → A mutation is required for the S282T substitution that affords resistance to sofosbuvir, a nucleotide analog critical for treatment of HCV infections (13, 14). This mutation could arise because of a UTP:U mismatch during (−) strand synthesis or by an ATP:A mismatch during (+) strand synthesis. The UTP:U misincorporation occurs readily, but the weak apparent binding affinity cannot be saturated at the low physiological concentration of UTP relative to ATP (570 μM and 3 mM, respectively) (10). Moreover, the ratio of (+) strand RNA to (−) strand RNA in vivo can reach up to 1000:1 depending on the cell type (15–17). Therefore, it would be more likely for the misincorporation necessary to form the S282T resistance variant to occur during (+) strand synthesis with an ATP:A misincorporation. However, this misincorporation has a slow pyrophosphate release rate that increases discrimination against misincorporation. These results may explain why the S282T mutation has not been observed in clinical samples, although there are certainly many reasons for the failure to observe this mutation in the clinic.

Four misincorporation reactions were so largely unfavorable that they were not observed above background during our measurements (GTP:G, CTP:C, GTP:A, and CTP:U). We set limits on the rate constant of incorporation at $k_2$ to make an estimate on the fidelity of these four misincorporations. The fidelity ranged from $10^{-7}$ to $10^{-9}$. After normalizing these misincorporations by the frequency of the templating bases in the HCV genome, it is reasonable to assume that these mutations require a large viral population (such as during an active infection) to accumulate significantly. One possible explanation for these mismatches being so unfavorable is that NS5B may have evolved to heavily discriminate against...
these four misincorporations because of a possible lethal effect they may have. However, the exact reason for this large discrimination is still unclear, and more work needs to be done to address the effect these four misincorporations would have in vivo. We have not examined sequence context effects. It is possible that the mutation frequency may change as a function of sequence context, especially for those sites showing undetectable misincorporation. Nonetheless, our studies in a single-sequence context provide an order of magnitude estimate of fidelity and define the mechanistic basis for selectivity.

Interestingly, a slow rate constant of pyrophosphate release was observed for four misincorporation reactions (ATP:G, UTP:G, UTP:C, and ATP:A). The slow rate constant of pyrophosphate release allows the reverse of chemistry to occur, which increases the fidelity of these misincorporations by decreasing $k_{cat}/K_m$ by up to two orders of magnitude. This phenomenon has not been previously observed in NS5B.

Fidelity of DNA polymerization catalyzed by HIV RT is a function of a nucleotide-induced conformational change preceding chemistry (18). It is not yet known whether a conformational change step may govern fidelity for NS5B. If it does, then our $K_{d, app}$ values must be considered as $K_m$ values, not true $K_d$ values (which was never intended in any case). The values measured would still be valid, but the interpretation for the mechanistic basis for understanding $K_m$ would change, as described for HIV RT (18, 19).

NS5B does not contain an exonuclease domain to correct misincorporations. However, we have previously demonstrated an ATP-mediated excision mechanism for removing nucleotides at the 3′-end of a primer strand (9, 20). It is unknown what contribution, if any, this excision reaction makes toward increasing fidelity. Our studies on measuring this effect have been limited by an inability to efficiently generate an elongation complex containing a mismatch at the 3′-end of a primer strand. Although we have yet to identify the contribution of
this mechanism toward fidelity, our work has at least identified that NS5B is able to increase fidelity on certain base pairs by slowing down the release of pyrophosphate after the chemical step for misincorporation.

This work serves as a basis for understanding and measuring the fidelity of other viral RNA-dependent RNA polymerases, including the SARS-coronavirus-2. In particular, application of the methods outlined in this paper will better define the fidelity and the mechanism and specificity constant for incorporation of remdesivir triphosphate (5, 6) currently used to treat COVID-19 (21).

**Experimental procedures**

**RNA templates, expression, and purification of NS5BΔ21**

RNA templates and GG dimers used were obtained from Dharmaco (Chicago, IL) and were desalted and decapped prior to delivery. The sequences of the RNA oligomers used are shown in Table 4. NS5BΔ21 was expressed, purified, and stored at −80 °C as previously described (9). Throughout the text we refer to NS5BΔ21 as simply NS5B.

**Replicates**

All experiments were performed at least two times to ensure reproducibility of the results.

**Assembly and purification of NS5BΔ21/9-nt primer/20-nt template elongation complex**

The NS5B elongation complex with a 9-nucleotide primer and 20-nucleotide template (NS5B/9-nt/20-nt, Table 4) was assembled using an extension-and-pause set-up and initiated using either a 20 μM pG or 1 mM GTP start method (9). All reactions were mixed in a buffer containing 40 mM Tris-HCl, pH 7.0, 20 mM NaCl, 5 mM DTT, and 2 mM MgCl2 and centrifuged at 16,000 rpm for 5 mins. The purified complexes were resuspended in a buffer containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, and 2 mM MgCl2.

**Incorporation and misincorporation of nucleoside triphosphates by NS5BΔ21**

The elongation complex was assembled, washed, and resuspended in elongation buffer for each of the four RNA templates as previously described (22). To measure the rate constant of NTP incorporation, the elongation complex was rapidly mixed with a solution containing the complementary NTP using KinTek RQF-3 rapid quench-flow elongation buffer. Reactions were carried out at 30 °C and quenched with 50 mM EDTA. The rate constants for misincorporation are slow and therefore were measured by hand-mixing methods. Elongation complex was mixed with an equal volume of a solution containing the NTP in elongation buffer. Reactions were allowed to proceed at 30 °C, and 10-μl aliquots were removed from the reaction and mixed with 40 μl of 50 mM EDTA at given time points. The samples were heat denatured by incubating at 95 °C for 5 min and loaded onto a 16% polyacrylamide gel containing 7 M urea. Electrophoresis, drying, exposing, and quantification were performed as previously described (9).

**Data analysis**

The kinetics of nucleotide incorporation into a primer by NS5B were fit using KinTek Explorer based on a rapid equilibrium substrate binding mode (Scheme 1) (19, 23, 24). To determine the $K_{d, app}$ for NTP, the rate constant of binding ($k_1$) was assumed to be diffusion limited and locked at 100 μM$^{-1}$s$^{-1}$, and the rate constant of dissociation ($k_{-1}$) was allowed to vary during fitting. The $K_{d, app}$ was then determined by dividing the rate constant of dissociation by the rate constant of binding ($K_{d, app} = k_{-1}/k_1$). The maximum rate constant of polymerization at saturating nucleotide concentration was determined by allowing $k_2$ to vary during the fitting process. For incorporation of complementary NTP, pyrophosphate release ($k_3$) was assumed to be fast and not rate-limiting and was locked at 100 s$^{-1}$. The rate constants for the reverse of chemistry ($k_{-3}$) and rebinding of pyrophosphate ($k_{-3}$) were modeled as irreversible steps and held constant at 0 s$^{-1}$. For misincorporations that do not appear to be limited by the release of pyrophosphate, the rate constant of product release ($k_{3}$) was locked at 100 s$^{-1}$, and the reverse of chemistry ($k_{-3}$) and pyrophosphate rebinding were modeled as irreversible steps by locking their rate constants at 0. For these reactions, the specificity constant ($K_{cat}/K_m$) was determined by dividing the rate constant of chemistry by the apparent equilibrium dissociation constant ($k_{pol}/K_m = k_{2}/K_{d, app}$). For misincorporations that have a slow pyrophosphate release, the reverse of chemistry ($k_{-3}$) and the rate constant of pyrophosphate release ($k_{3}$) were allowed to vary during the

**Table 4**

**RNA templates**

| Template          | Underline RNA Template                  |
|-------------------|----------------------------------------|
| 20-nt CG template | 3'-CCUAUAAUUGGGGAAAUGCUAA-3'          |
| 20-nt UA template | 3'-CCUCUCUUUGGGGAAAUGCUAA-3'          |
| 20-nt GC template | 3'-CCUAUAAUUGGGGAAAUGCUAA-3'          |
| 20-nt AU template | 3'-CCACACACACACACACACACACACACACACACAA-3' |

The underlined base indicates where the extension-and-pause reaction stops during NS5B/9-nt/20-nt elongation complex formation. The base in red indicates the templating base for single incorporation reaction.

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20-nt AU template 3'-CCUAUAAUUGGGGAAAUGCUAA-3' 20-nt CG template 3'-CCUAUAAUUGGGGAAAUGCUAA-3' 20-nt UA template 3'-CCUAUAAUUGGGGAAAUGCUAA-3' 20-nt GC template 3'-CCUAUAAUUGGGGAAAUGCUAA-3' 20-nt AU template 3'-CCACACACACACACACACACACACACACACACAA-3'
fitting process. Because of the low pyrophosphate concentration relative to nucleotide concentration, the re-binding of pyrophosphate was determined to be negligible and locked at 0. For these reactions, \( k_{\text{cat}}/K_m \) was determined by Equation 2 (11). Fidelity was determined by dividing the specificity constant of a misincorporation by the specificity constant for correct incorporation (Equation 1).

Data availability

All data generated during this study are included in this article.

Author contributions—B. V. conceptualization; B. V. and K. A. J. data curation; B. V. formal analysis; B. V. and K. A. J. investigation; B. V. methodology; B. V. writing-original draft; B. V. and K. A. J. writing-review and editing; K. A. J. resources; K. A. J. supervision; K. A. J. validation; K. A. J. project administration.

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Conflict of interest—K. A. J. is president of KinTek Corporation, which provided the RQF-3 rapid quench-flow instrument and KinTek Explorer software used in this study.

 Abbreviations—The abbreviations used are: HCV, hepatitis C virus; NS5B, nonstructural protein 5B.

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