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Mechanism of Forced-Copy-Choice RNA Recombination by Enteroviral RNA-Dependent RNA Polymerases

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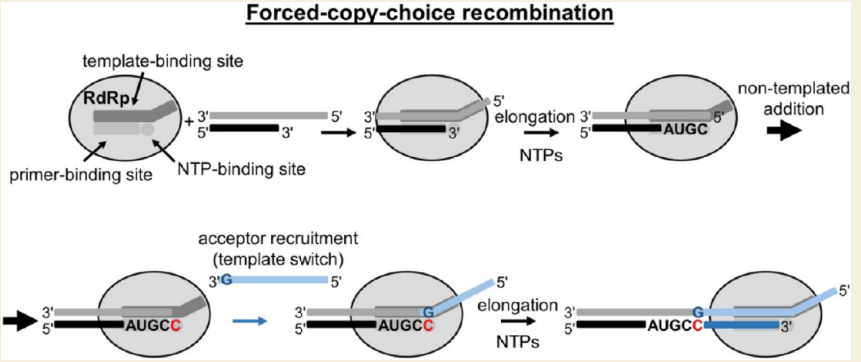

ABSTRACT: Forced-copy-choice recombination occurs at the end of a template, differing from copy-choice recombination, which happens at internal positions. This mechanism may produce full-length genomes from fragments created by host antiviral responses. Previous studies from our laboratory demonstrated that poliovirus (PV) RNA-dependent RNA polymerase (RdRp) switches to an "acceptor" template *in vitro* when initiated on a heteropolymeric RNA-primed "donor" template. Surprisingly, recombinants showed template switching from the 3′-end of the donor template. We have developed a primed-template system to study PV RdRp-catalyzed forced-copy-choice RNA recombination. PV RdRp adds a single, nontemplated nucleotide to the 3′-end of a blunt-ended, double-stranded RNA product, forming a "plus-one" intermediate essential for template switching. Nontemplated addition of CMP was favored over AMP and GMP (80:20:1); UMP addition was negligible. A single basepair between the plus-one intermediate and the 3′-end of the acceptor template was necessary and sufficient for template switching, which could occur without RdRp dissociation. Formation of the plus-one intermediate was rate limiting for template switching. PV RdRp also utilized synthetic, preformed intermediates, including those with UMP 3′-overhangs. Reactions showed up to five consecutive template-switching events, consistent with a repair function for this form of recombination. PV RdRp may exclude UMP during forced-copy-choice RNA recombination to preclude creation of nonsense mutations during RNA fragment assembly. Several other picornaviral RdRps were evaluated, and all were capable of RNA fragment assembly to some extent. Lastly, we propose a structure-based hypothesis for the PV RdRp-plus-one intermediate complex based on an elongating PV RdRp structure.

KEYWORDS: *RNA virus, viral evolution, RNA recombination, template switching, RNA-dependent RNA polymerase, RNA repair, forced-copy-choice recombination*

INTRODUCTION

A common property of most, if not all, positive-strand RNA viruses of bacteria, plants, and animals is the ability to recombine.[1−4] Based primarily on mechanistic studies performed using poliovirus (PV), RNA recombination is thought to use a template-switching mechanism in which the RNA-dependent RNA polymerase (RdRp) initiates RNA synthesis on a *donor* template then switches to an acceptor template to complete RNA synthesis.[5] This type of recombination is referred to as copy-choice recombination because the RdRp has a choice in the template that it copies (Figure 1A). This template switch requires sequence complementarity between the 3′-end of nascent RNA and

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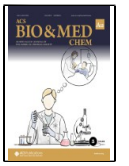

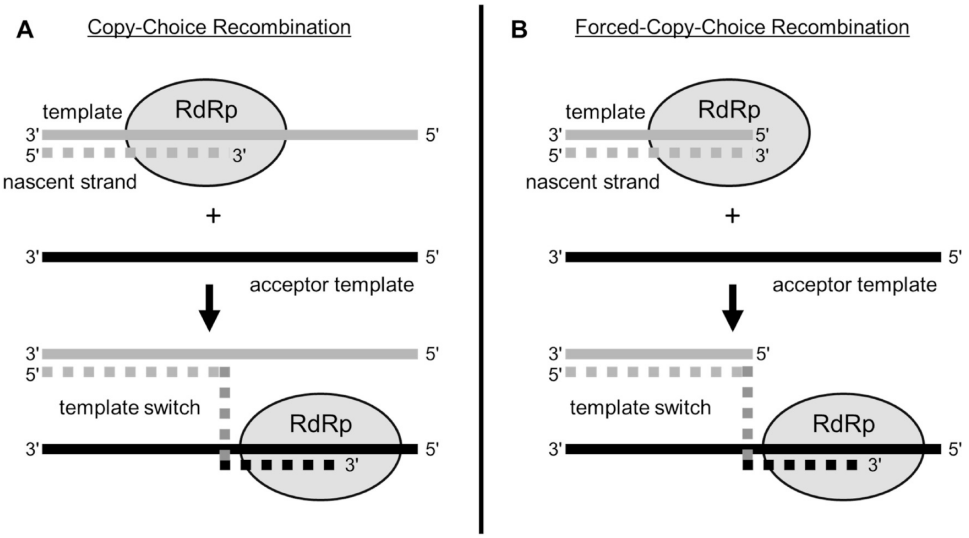

Figure 1. Viral RNA recombination can occur by either a copy-choice or a forced-copy choice mechanism. During viral RNA synthesis, the RdRp elongates the nascent RNA strand while copying a template strand and can subsequently switch templates by either a copy-choice or a forced-copy-choice mechanism. (A) Copy-choice recombination occurs with the RdRp switching templates at an internal position while continuing to extend the nascent RNA strand by copying the acceptor template. (B) Forced-copy-choice recombination occurs when the RdRp reaches the end of the initial template, for example at a premature end caused by a ribonuclease. The RdRp continues to extend the nascent RNA by switching templates and copying the acceptor template. Both models depict switching templates to an internal position on the acceptor template, however this can also occur at the 3′-end of the acceptor template. In the case of forced-copy-choice recombination this would be an end-to-end template switching event.

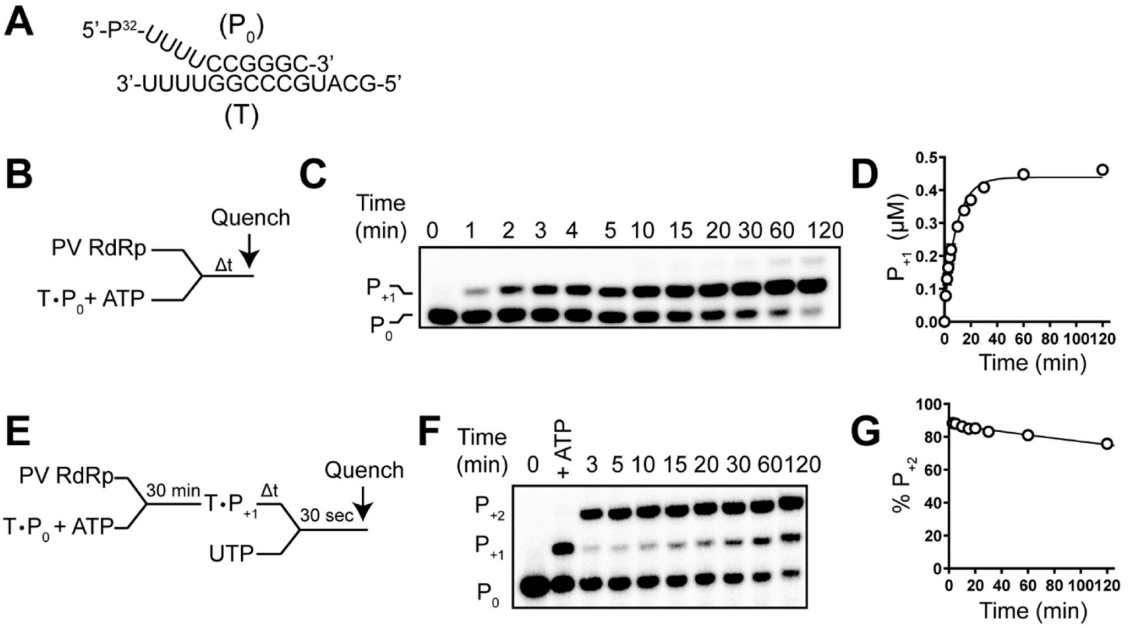

Figure 2. Establishment of a primed-template to study template switching by PV RdRp. (A) Primed-template used in this study. The primer (P_0) is a 10-nt RNA and template (T) is a 14-nt RNA; sequences are shown. The annealed primed-template forms a 6-bp duplex with a 4-nt 5′-template overhang. The RNA primer was labeled on the 5′-end with ^{32}P. (B) Schematic of assay to measure assembly of the elongation complex. Primer extension was initiated by adding PV RdRp with primed-template in the presence of ATP for various amounts of time and then quenched. (C) Analysis of reaction products by denaturing PAGE. The positions of the unextended primer P_0 and extended primer P_{+1} are indicated. (D) Quantitative analysis of the kinetics of assembly by monitoring product RNA (P_{+1}) formation. The data were fit to a single exponential yielding an observed rate constant of 0.10 ± 0.01 min^{-1}. The half-life for assembly is ln 2/kobs = 0.693/0.1 min^{-1} = 7 min. Mean of four replicates are shown. (E) Schematic of assay to measure stability of the elongation complex. Elongation complexes were assembled by adding PV RdRp with primed-template in the presence of ATP for 30 min. After formation of P_{+1}, UTP was added after various amounts of time and reactions quenched after 30 s. (F) Analysis of reaction products by denaturing PAGE. The positions of the unextended primer P_0 and extended primers P_{+1} and P_{+2} are indicated. (G) Quantitative analysis of elongation complex stability over time by monitoring the percentage of product RNA (P_{+2}) formed relative to total product RNA (P_{+1} + P_{+2}). The data were fit to a single exponential yielding an observed rate constant of 0.00128 ± 0.00010 min^{-1}. The half-life for complex dissociation is ln 2/kobs = 0.693/0.00128 min^{-1} = 540 min. Mean of three replicates are shown.

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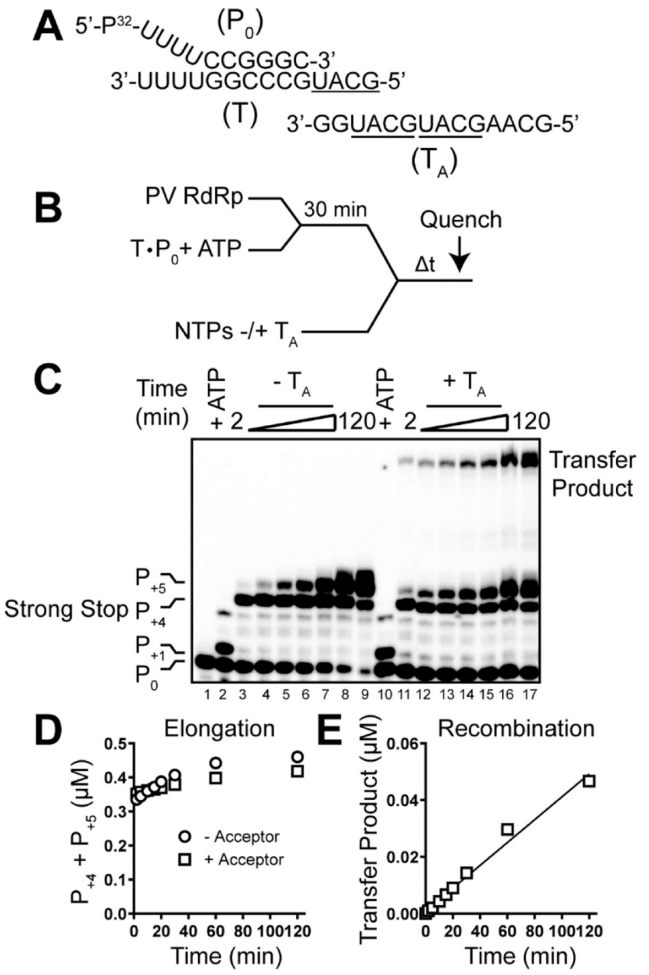

Figure 3. PV RdRp template-switching assay. (A) Primed-template and acceptor template (T_A) RNAs used to assess template switching by PV RdRp. The first four templating bases of the primed-template and two, sequential sites (UACG) in the acceptor template are underlined. (B) Schematic of assay used to measure template switching. Elongation complexes were assembled by adding PV RdRp with primed-template in the presence of ATP for 30 min at which point acceptor template and all four NTPs were added. Reactions were allowed to proceed for various amounts of time and then quenched. (C) Analysis of reaction products from reactions performed in the absence or presence of acceptor template by denaturing PAGE. Time points shown are 2, 5, 10, 20, 30, 60, and 120 min. The positions of the unextended primer (P_0), extended primer (P_{+1}), strong-stop product (P_{+4}), strong-stop product with the addition of a nontemplated nucleotide (P_{+5}), and transfer product are indicated. The transfer product is only observed in reactions performed in the presence of acceptor template (T_A). (D, E) Quantitative analysis of the formation of strong-stop (P_{+4} + P_{+5}) products (Elongation) or transfer product (Recombination) as a function of time. The rate of formation of transfer product was fit to a line with a slope of $4 \pm 1 \times 10^{-4}$ min^{-1}. Mean of three replicates are shown.

the acceptor template (Figure 1A). When the acceptor template is a different RNA molecule than the donor template, an intermolecular recombinant is produced, carrying some genetic traits from both the donor and acceptor (Figure 1A). In contrast, when the acceptor template is the same RNA molecule, an intramolecular recombinant is produced that harbors a deletion relative to the original donor template. This mechanism is at least one source of defective enteroviral genomes.[1,6−8] Both intermolecular and intramolecular recombination appear to be triggered by the same molecular mechanisms, including nucleotide misincorporation and incorporation of certain nucleotide analogs by the RdRp.[9−13]

The selective pressure of forcing viruses to maintain the capacity to recombine, particularly in cell culture, is unclear. PV mutants with defects to copy-choice RNA recombination can replicate as well or slightly better than wild-type virus in cell culture.[13−18] However, these viruses are highly attenuated in vivo.[14,17−19] The function for copy-choice RNA recombination in the viral lifecycle and/or pathogenesis is also unclear.[4] One possibility is that recombination purges accumulated mutations caused by RNA synthesis by the error-prone RdRp.[14,20−22] One recent study from our group showed that copy-choice RNA recombination could be selectively impaired by mutations in RdRp-coding sequence, but this PV mutant exhibited impaired infection dynamics in cell culture relative to wild-type.[23] The reason that other mutants failed to reveal such a phenotype may reflect the magnitude of the recombination defect, how recombination was measured, and/or impact on some as yet-to-be-defined property of the RdRp required for RNA recombination.[23]

A second type of recombination is forced-copy-choice RNA recombination. This mechanism occurs when an elongating RdRp reaches the end of the donor template. This end is often not the authentic end of the genome; rather, it may have been created by nucleolytic cleavage of the donor template (Figure 1B).[24−26] An obligatory step of reverse transcription used by retroviruses requires a forced-copy-choice mechanism.[25−28] Reverse transcriptase (RT) initiates DNA synthesis using a tRNA primer annealed to the primer-binding site of the RNA genome at a site located a few hundred nucleotides or so from the 5′-end.[26,27] The product of this RT-catalyzed elongation reaction is a strong-stop DNA that uses complementarity between its 3′-end and that of the 3′-end of the genome in a forced template switch.[26−28] This reaction has been mimicked *in vitro* using synthetic nucleic acids and purified RT from human immunodeficiency virus (HIV).[9,24,29,30] A hallmark of this reaction *in vitro* was the addition of a nontemplated nucleotide to the strong-stop DNA product that was present in the product of template switching.[10,24,30] However, no function of the nontemplated nucleotide was proposed, as the forced template switch was thought to require substantial complementarity between strong-stop DNA and acceptor template.[10,24,30]

Since the description of the mechanism of HIV RT-catalyzed forced-copy-choice recombination, evidence has accumulated for the existence of a terminal transferase-like activity of RTs broadly that permits addition of nontemplated nucleotides to the 3′-end of strong-stop DNA.[24,28,31−33] Interestingly, the preferred nucleobase and number of nucleotides added vary.[24,28,31−33] Most recently, studies of a group II intron-encoded RT have shown that basepairing between a single, nontemplated nucleotide on the donor template and the 3′-end of an acceptor template is sufficient for template switching.[34]

Enteroviruses initiate RNA synthesis using a VPg peptide[35,36] or VPg-containing precursor[37] and a structured template located in protein 2C-coding sequence of the genome.[38−41] The resulting diuridylylated VPg must then switch to the 3′-end of the viral genome to produce full-length, negative-strand RNA. This process is akin to the first steps of retroviral reverse transcription described above. Efforts to

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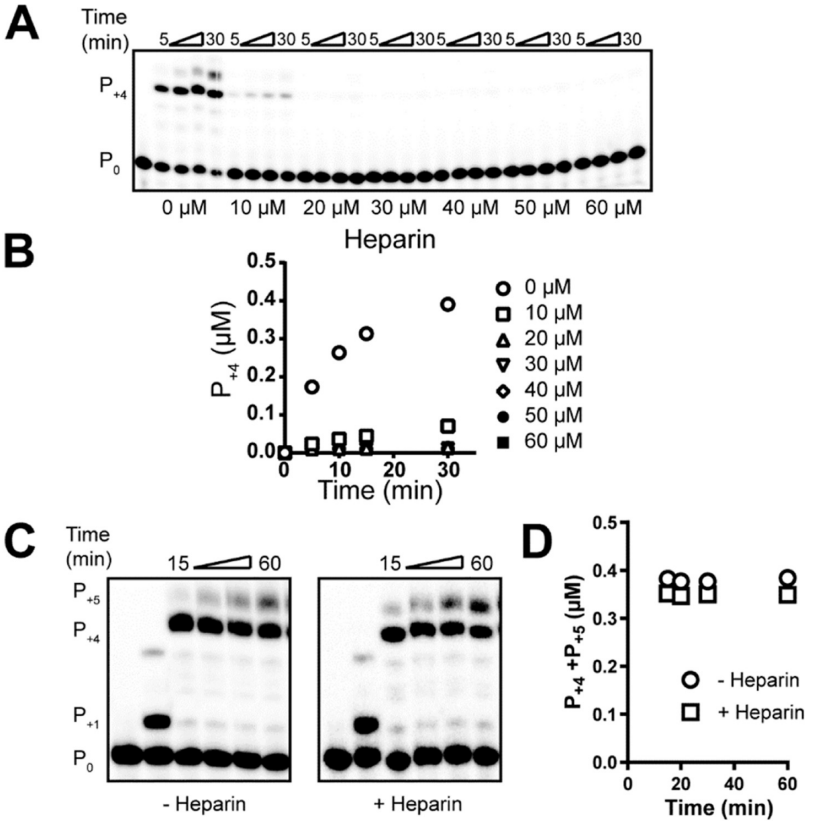

Figure 4. Heparin inhibits complex assembly but not elongation. (A) Complex assembly in the presence of increasing concentrations of heparin. Primer extension was initiated by adding PV RdRp with primed-template in the presence of all four NTPs and the indicated amounts heparin for 5, 10, 15, and 30 min and then quenched. Shown is the analysis of reaction products resolved by denaturing PAGE. (B) Quantitative analysis of the kinetics of assembly by monitoring product RNA (P_{+4}) formation in the presence of increasing concentrations of heparin. Mean of three replicates are shown. (C) Elongation in the presence of heparin. Elongation complexes were assembled by adding PV RdRp with primed-template in the presence of ATP for 30 min. After formation of P_{+1}, all four NTPs with or without heparin were added and reactions quenched at 15, 20, 30, and 60 min. Shown is the analysis of reaction products resolved by denaturing PAGE. (D) Quantitative analysis of the kinetics of elongation by monitoring product RNA (P_{+4}) formation in the presence of heparin. Mean of three replicates are shown.

mimic copy-choice RNA recombination using synthetic RNA templates and purified PV RdRp demonstrated template switching *in vitro*.[13,42] However, subsequent studies suggested forced-copy-choice RNA recombination as the likely mechanism observed.[13]

Here, we report the development of a primed-template system that permits a more rigorous evaluation of the mechanism of forced-copy-choice RNA recombination catalyzed by PV RdRp. To our surprise, the mechanism used by PV RdRp and other picornaviral RdRps is strikingly similar to that described for the group II intron-encoded RT.[34] We present a structure-based hypothesis for the mechanism of enteroviral RdRp-catalyzed, forced-copy-choice RNA recombination. Using this mechanism, enteroviral RdRps can assemble RNA fragments together randomly. We discuss the possibility of using this reaction as a last-ditch mechanism to maintain a full-length viral genome when faced with nucleolytic damage.

MATERIALS AND METHODS

Materials

RNA oligonucleotides were from Horizon Discovery Ltd. (Dharmacon). T4 polynucleotide kinase was from ThermoFisher. [γ-^{32}P]ATP (6000 Ci/mmol) was from PerkinElmer. Nucleoside 5′-triphosphates (ultrapure solutions) were from Cytiva. Heparin was from MilliporeSigma. All other reagents were of the highest grade available from MilliporeSigma, VWR, or Fisher Scientific.

RNA Purification

RNA substrates were suspended in 90 mM Tris, 90 mM Boric Acid, 2 mM EDTA, 90% formamide and resolved by denaturing polyacrylamide gel electrophoresis on an 18.5% acrylamide/1.5% bis(acrylamide) gel containing 7 M Urea and 1X TBE. RNA bands were visualized by UV shadowing (260 nm) and excised from the gel. Excised gel samples were crushed and RNA was eluted by using an Elutrap Electroelution System (GE Healthcare). The RNA was concentrated by Sep-Pak C18 Classic Cartridge (Waters) and solvent was evaporated in a Speed Vac Concentrator (Savant). RNA samples were deprotected by adding 500 mM Acetic Acid and heating to 65 °C for 15 min, then adding 660 mM Tris base and heating to 65 °C again for 15 min. Final preparation of RNA samples involved desalting by running through G25 Sephadex equilibrated with 10 mM Tris pH 8.0, 1 mM EDTA (Sigma-Aldrich) column.

5′-^{32}P-Labeling of RNA Substrates

RNA oligonucleotides were end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Reaction mixtures, with a typical volume of 50 μL, contained 0.5 μM [γ-^{32}P]ATP, 10 μM RNA oligonucleotide, 1X kinase buffer, and 0.4 unit/μL T4 polynucleotide kinase. Reaction mixtures were incubated at 37 °C for 60 min and then held at 65 °C for 5 min to heat inactivate T4 PNK.

Annealing of dsRNA Substrates

dsRNA substrates were produced by annealing 10 μM RNA oligonucleotides in $T_{10}E_1$ [10 mM Tris pH 8.0 1 mM EDTA] with 50 mM NaCl in a Progene Thermocycler (Techne). Annealing reaction mixtures were heated to 90 °C for 1 min and slowly cooled

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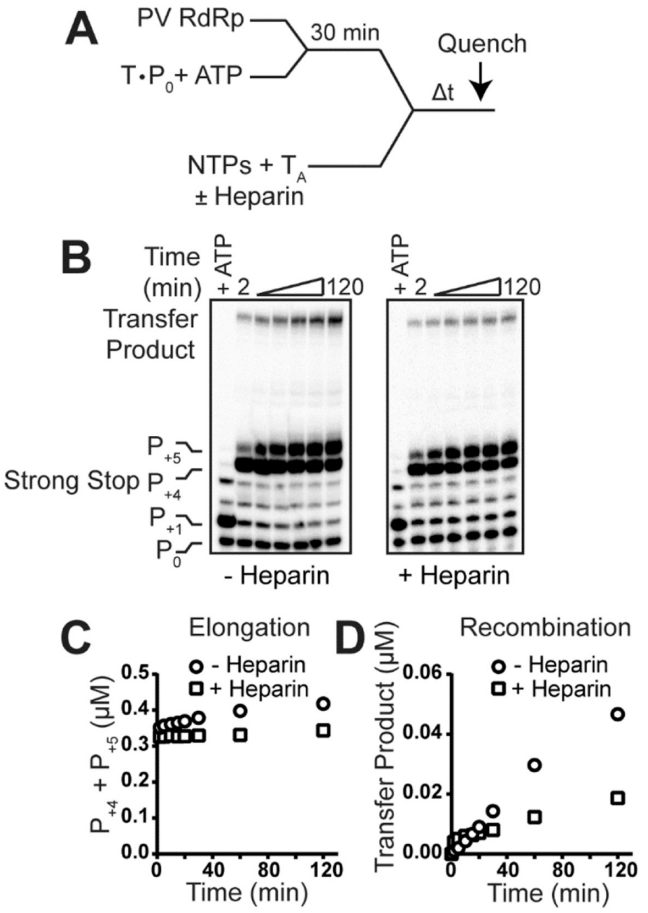

Figure 5. PV RdRp-catalyzed template switching can occur by a heparin-resistant mechanism. (A) Schematic of template-switching assay performed in the absence or presence of heparin. Elongation complexes were assembled by adding PV RdRp with primed-template in the presence of ATP for 30 min at which point acceptor template and all four NTPs were added in the presence or absence of heparin. Reactions were allowed to proceed for various amounts of time and then quenched. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed in the absence or presence of heparin. Time points shown are 2, 5, 10, 30, 60, and 120 min. The positions of the unextended primer (P_0), extended primer (P_{+1}), strong-stop product (P_{+4}), strong-stop, "plus-one" product (P_{+5}), and transfer product are indicated. The transfer product is observed in reactions performed in the presence of heparin. (C, D) Quantitative analysis of the formation of strong-stop products or transfer-product as a function of time in the absence or presence of heparin. The total amount of transfer product in the absence and presence of heparin at 120 min was 0.047 and 0.019 μM, respectively. Mean of three replicates are shown.

(5 °C/min) to 10 °C. Specific scaffolds are described in the figure legends.

Expression and Purification of PV RdRp

Expression and purification of WT and K359R PV RdRps were performed essentially as described previously.[43,44]

Expression and Purification of Picornaviral RdRps

Expression and purification of Picornaviral RdRps (CVB3, EV-D68, RV-C15, RV-A16, and FMDV) were performed essentially as described previously.[45−47] The following accession numbers are provided as reference sequences for the indicated Picornaviral RdRps: CVB3: JX312064.1; EV-D68: KT347249.1; RV-C15: GU219984; RV-A16: L24917.1; and FMDV: AJ133357.1.

In Vitro RdRp-Primed-Template Complex Assembly Assays

Elongation complexes were assembled by incubating 5 μM WT or mutant poliovirus polymerase with 0.5 μM RNA primed-template duplex and 500 μM ATP for various amounts of time before being quenched by addition of 50 mM EDTA. Reactions included heparin (0 to 60 μM) during assembly or after assembly of enzyme onto RNA duplex. All and subsequent reactions described were performed at 30 °C in 50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 60 μM $ZnCl_2$.

Complex Stability Assay

Elongation complexes were assembled by incubating 5 μM WT or mutant poliovirus polymerase with 0.5 μM RNA primed-template duplex and 500 μM ATP. At various time points, 500 μM UTP was added and the reaction was quenched 30 s later by addition of 50 mM EDTA.

In Vitro Template-Switching Assay

Elongation complexes were assembled by incubating 5 μM WT or mutant poliovirus polymerase with 0.5 μM RNA primed-template duplex and 500 μM ATP for 30 min (Mix 1). Template-switching reactions were initiated by adding 60 μM of RNA acceptor template and 500 μM CTP, GTP, UTP and 25 μM heparin (Mix 2) and quenched at the listed times by addition of 50 mM EDTA.

Denaturing PAGE Analysis of Reaction Products

An equal volume of loading buffer (85% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol) was added to quenched reaction mixtures and heated to 90 °C for 5 min prior to loading 5 μL on a denaturing 23% polyacrylamide gel containing 1X TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) and 7 M urea. Electrophoresis was performed in 1X TBE at 90 W. Gels were visualized by using a PhosphorImager (GE) and quantified by using ImageQuant TL software (GE).

Sanger Sequencing and Small RNA-Seq of RNA Products

In vitro template switching assays were performed with 5′-phosphate-terminated primers and 3′-deoxy-terminated acceptor templates. Reactions were quenched with EDTA after 60 min, reaction products were resolved by denaturing PAGE and both strong-stop and transfer products were gel purified. RNA products were either cloned and sequenced following the small RNA cloning kit miRCat-33 protocol (Integrated DNA Technologies) or total purified RNA was submitted for small RNA-Seq next generation sequencing (Illumina, 2 × 150 bp, ~350 M PE reads, single index) performed by Genewiz. Sanger sequencing results (>100 sequences) were aligned to the following reference primer and transfer product sequence (5′-UUUUCCGGGC-3′; 5′-UUUUCCGGGCAUGCCCAUGCAUG-CUUGC-3′). Results from NGS used the following pipeline for analysis: data was evaluated with FastQC, trimmed with bbduk.sh (BBmap suite) to remove adapter sequences (both Illumina universal and small RNA adapter), sequences were clustered using clumpify.sh (BBMap suite), each sequence was treated differently which did not allow any substitutions, and the top 500 sequences identified (sequence, count, and length).

Data Analysis

All gels shown are representative, single experiments that have been performed at least three to four individual times to define the concentration or time range shown with similar results. In all cases, values for parameters measured during individual trials were within the limits of the error reported for the final experiments. Data were fit by either linear or nonlinear regression using the program GraphPad Prism v7.03 (GraphPad Software Inc.).

RESULTS

New Primed-Templates to Study Template Switching by the Enterovirus RdRp

When our laboratory began studying PV RdRp, we pursued the use of conventional primed-templates: a stable duplex with a

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Table 1. Sequence Analysis of Strong-Stop and Transfer Products[a]

A. Strong-stop product using primer (5′-UUUUCCGGGC-3′) without acceptor RNA				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGC**			67
2.	UUUUCCGGGC**AUGC**<u>**C**</u>		+C	15
3.	UUUUCCGGGC**AUGC**<u>**G**</u>		+G	12
4.	UUUUCCGGGC**AUGC**<u>**A**</u>		+A	5
5.	UUUUCCGGGC**AUGC**<u>**U**</u>		+U	1
B. Strong-stop product using primer (5′-UUUUCCGGGC-3′) with 3′-GGUACGUACGAACG-5′ acceptor RNA				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGC**			42
2.	UUUUCCGGGC**AUGC**<u>**C**</u>		+C	7
3.	UUUUCCGGGC**AUGC**<u>**G**</u>		+G	7
4.	UUUUCCGGGC**AUGC**<u>**A**</u>		+A	3
5.	UUUUCCGGGC**AUGC**<u>**U**</u>		+U	nd
C. Transfer product using primer (5′-UUUUCCGGGC-3′) with 3′-GGUACGUACGAACG-5′ acceptor RNA				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGC**<u>**C**</u>**CAUGCAUGCUUGC**		+C	63
2.	UUUUCCGGGC**AU**c**CAUGCAUGCUUGC**	G to C		14
3.	UUUUCCGGGC**AUGCCAUGCAUGCUUGC**			6
D. Transfer product using primer (5′-UUUUCCGGGC-3′) with 3′-GGUACGUACGAACG-5′ acceptor RNA + heparin				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGC**<u>**C**</u>**CAUGCAUGCUUGC**		+C	17
2.	UUUUCCGGGC**AU**c**CAUGCAUGCUUGC**	G to C		8
3.	UUUUCCGGGC**AUGCCAUGCAUGCUUGC**			4

[a]The last column indicates the total number of each sequence obtained. Bold nucleotides indicate extension of primer. Underlined nucleotides indicate non-templated nucleotide addition and lower case indicates misincorporation (identified explicitly in the indicated columns). nd: not detected.

10-nt or longer 5′ overhang to serve as template.[48] To our surprise, the RdRp partitioned in an orientation such that it was bound to the duplex portion of the substrate instead of at the primed-template junction.[49] When bound to the duplex, the enzyme was able to add one or more nontemplated nucleotides depending on the divalent cation used in the reaction.[49] These observations led to development of a symmetrical, primed-template (sym/sub) that had a duplex formed by a self-complementary sequence with 5′-overhangs on both strands.[48] This substrate proved to be the first that could be used for pre-steady-state kinetic analysis of PV RdRp-catalyzed nucleotide incorporation.[50−52] Unfortunately, using this substrate to study template switching is complicated by the fact that one cannot distinguish primer from template because of the self-complementary nature of the RNA used.

To address this problem, we have created primed-templates that are no longer self-complementary (Figure 2A). To the 5′-end of the primer (P_0) and 3′-end of template (T), we added four uridine nucleotides to prevent the enzyme from binding in an unproductive conformation, although the addition to the primer turned out not to be essential (Figure S1). Assembly of PV RdRp on this primed-template to form a stable elongation complex was slow ($t_{1/2}$ = 7 min) (Figure 2B−D), but the resulting elongation complex was very stable ($t_{1/2}$ = 540 min) (Figure 2E−G).

To this primed-template we added an acceptor template (T_A) that was used previously with sym/sub.[13] The acceptor template was designed to study copy-choice RNA recombination, with two sequential sites that would be complementary to the product produced by elongation of the primer (Figure 3A). Later, it became clear that, while this system supported template switching, it was not mimicking copy-choice RNA recombination.[13] Rather, it was mimicking forced-copy-choice RNA recombination.[13]

We mixed RdRp, primed-template, and ATP (first templated nucleotide) for a time sufficient to produce a one-nucleotide-extended product (P_{+1}) (Figure 3B) whose fate could be monitored (lane 2 of Figure 3C). In the presence of all four nucleotides but the absence of acceptor template, two major products formed (lanes 2−9 of Figure 3C). The first product was consistent with the RdRp elongating to the end of template; we refer to this product as strong-stop or P_{+4} (Figure 3C). Over time, the strong-stop product was extended by one nucleotide in a nontemplated fashion; we refer to this product as the product of nontemplated addition, the "plus-one" product, or P_{+5} (Figure 3C). In the presence of the acceptor template, we observed a product of template switching, referred to as the transfer product (Figure 3C). Elongation was equally efficient in the absence and presence of acceptor template (Figure 3D). Template switching occurred at a rate of 4 x10^{-4} μM/min using 5 μM of PV RdRP (Figure 3E).

To determine if template switching occurred processively—that is, without dissociation of the enzyme used to produce the strong-stop and/or plus-one products, we required a trap for free or dissociating enzyme. Using sym/sub, heparin worked well.[13,42] With the new substrate, heparin showed complete inhibition at 20 μM (Figure 4A,B). When we formed the elongation complex and then added heparin along with the remaining three nucleotides, heparin exhibited no effect on the production of strong-stop or plus-one products (Figure 4C,D). We repeated this experiment in the presence of the acceptor template (Figure 5). Again, neither formation nor utilization of the elongation complex was impaired in the presence of heparin (Figure 5B,C). While production of the transfer

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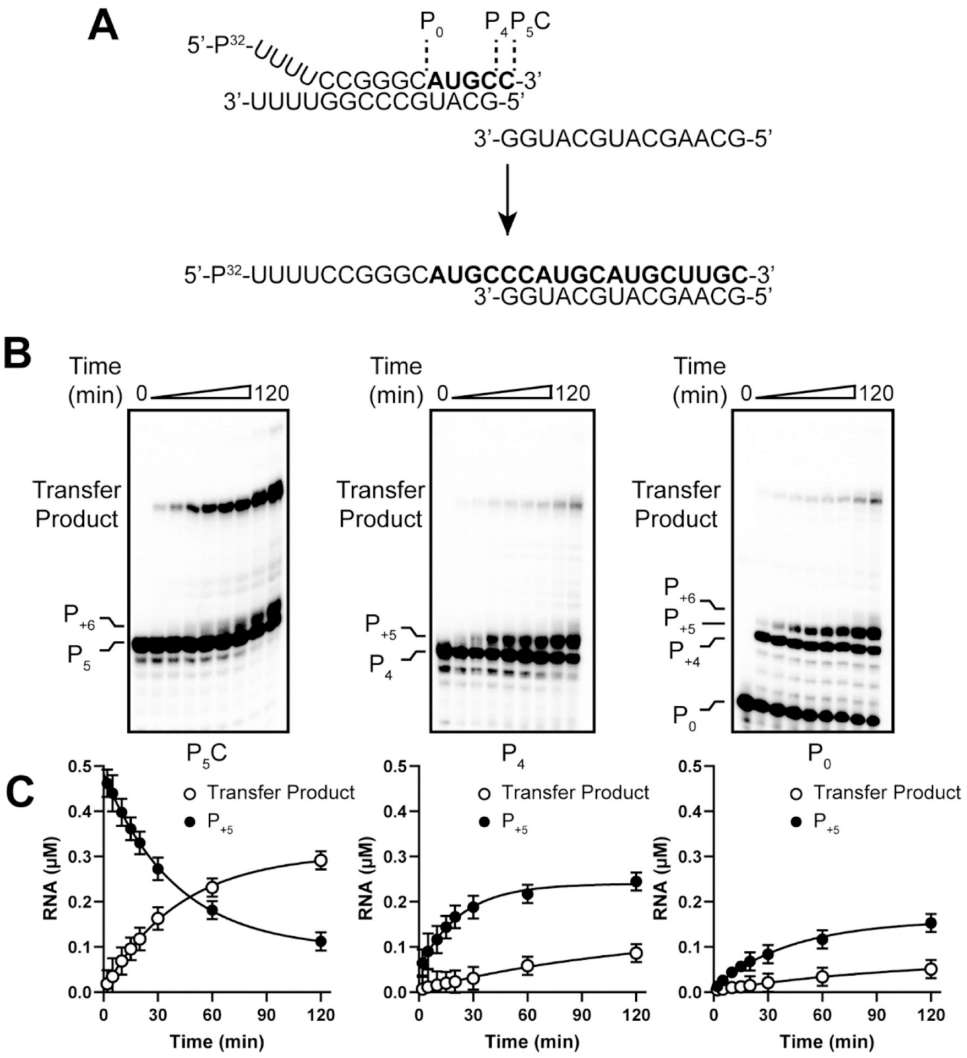

Figure 6. Plus-one product (P_{+5}) as an intermediate in forced-copy-choice recombination. (A) Primers of varying length (P_0, P_4, and P_5C) used to assess template switching by PV RdRp. P_4 and P_5C are consistent with strong-stop products produced during elongation: blunt-ended (P_4) or 3′-overhang formed by nontemplated addition ("plus-one" product, P_5C). (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5C, P_4, and P_0 primed-template duplexes. Time points shown are 2, 5,10, 15, 20, 30, 60, and 120 min. The positions of the unextended primer, strong-stop product (P_{+4}), strong-stop, "plus-one" and "plus-two" products (P_{+5} and P_{+6}), and transfer product are indicated. (C) Quantitative analysis of the formation of transfer product as a function of time from template-switching reactions performed with P_5C, P_4, and P_0 primed-template duplexes. Reactions using P_5C primed-template duplexes resulted in transfer products 6-fold greater than reactions with P_0 or P_4. The data were fit to a single exponential yielding observed rate constants: P_5C (Transfer Product) 0.025 ± 0.005 min^{-1}; P_4 (Transfer Product) 0.008 ± 0.001 min^{-1} and P_{+5} 0.04 ± 0.01 min^{-1}; P_0 (Transfer Product) 0.009 ± 0.001 min^{-1} and P_{+5} 0.02 ± 0.01 min^{-1}. Mean of three replicates are shown. Error bars represent standard deviation.

product was not completely resistant to heparin, at least 10% of the complexes were (Figure 5D).

Plus-One Product as an Intermediate Required for Forced-Copy-Choice RNA Recombination

In order to gain insight into how the transfer product was formed, we cloned and sequenced the strong-stop, plus-one, and transfer products from reactions performed under a variety of conditions, including in the absence and presence of heparin, as described under Materials and Methods (Table 1). As expected from the gel-based assays, the sequence(s) observed for strong-stop and plus-one products were as expected, and the presence of acceptor template did not alter the sequence(s) observed (compare section A to section B in Table 1). In the presence of all four ribonucleoside triphosphates, each could be used for nontemplated addition. Addition of CMP and GMP were most efficient. Addition of AMP was easily detected. We only observed one example of UMP addition (sections A and B of Table 1). The sequence of the transfer product could only be explained as follows: (1) strong-stop RNA is produced; (2) nontemplated additions occur, producing plus-one products; (3) the plus-one product capable of basepairing to the 3′-end of acceptor template is selected; and (4) continued synthesis leads to production of a 28-nt RNA transfer product (sections C and D of Table 1). Therefore, we suggest that the plus-one product is an obligatory intermediate for forced-copy-choice RNA recombination (Figure 6A). Use of the plus-CMP product was preferred in the absence and presence of heparin (compare section C to section D in Table 1). We observed a few other transfer-product sequences that may have arisen from misincorporation or utilization of a truncated template during

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Table 2. Sequence Analysis of RNA Products from Small RNA-Seq[a]

products using primer (5′-UUUUCCGGGC-3′) with 3′-GGUACGUACGAACG-5′ acceptor RNA					
	sequence	length	counts	frequency	type
1.	UUUUCCGGGC**AUGCCCAUGCAUGCUUGC**	28			expected transfer product with nontemplated C
2.	UUUUCCGGGC	10	4799326 (0.352)	0.519	primer
3.	UUUUCCGGGC**A**	11	52306 (0.00385)	0.000394	extended primer n + 1 A
4.	UUUUCCGGGC**g**	11	6900 (0.000506)	0.000233	extended primer n + 1 G misincorporation
5.	UUUUCCGGGC**c**	11	3522 (0.000258)	0.000132	extended primer n + 1 C misincorporation
6.	UUUUCCGGGC**u**	11	916 (0.000177)	0	extended primer n + 1 U misincorporation
7.	UUUUCCGGGC**AUGC**	14	1521234 (0.111)	0	strong-stop n + 4 AUGC
8.	UUUUCCGGGC**AaGC**	14	256414 (0.0188)	0	strong-stop n + 4 with A misincorporation
9.	UUUUCCGGGC**AgGC**	14	40643 (0.00298)	0	strong-stop n + 4 with G misincorporation
10.	UUUUCCGGGC**AUGCC**	15	107854 (0.00791)	0	strong-stop + nontemplated C “plus-one”
11.	UUUUCCGGGC**AUGCA**	15	27648 (0.002028)	0	strong-stop + nontemplated A “plus-one”
12.	UUUUCCGGGC**AUGCG**	15	1564 (0.000114)	0	strong-stop + nontemplated G “plus-one”
13.	UUUUCCGGGC**AUGCCCAUGC**	20	219758 (0.0161)	0	nontemplated C + transfer product (truncated)
14.	UUUUCCGGGC**AUGCCCAUGCAUGCUUGC**	28	108704 (0.00791)	0	nontemplated C + transfer product (full-length)

[a]Representative sequences that correspond to primer, extended primer, strong-stop, “plus-one”, and transfer products, both truncated and full-length, are listed. The list is sorted from shortest to longest sequence. The sequence, length, counts, frequency, and type are indicated. Bold nucleotides indicate extension of primer. Underlined nucleotides indicate non-templated nucleotide addition and lower case indicates misincorporation. The top 500 sequences are reported in Supporting Data Tables: NGS_3A and NGS_3A_Control. All T’s in the sequence in supplemental tables have been converted to U’s as reported in the Table below

Table 3. Sequence Analysis of Transfer Products Using Different Acceptors[a]

A. Transfer product using primer (5′-UUUUCCGGGC-3′) with 3′-GCUACGUACGAACG-5′ acceptor RNA				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGCCGAUGCAUGCUUGC**		+C	11
A. Transfer product using primer (5′-UUUUCCGGC-3′) with 3′-GAUACGUACGAACG-5′ acceptor RNA				
		misincorporations	nontemplated	# of reads
1.	UUUUCCGGGC**AUGCCUAUGCAUGCUUGC**		+C	12

[a]The last column indicates the total number of each sequence obtained. Bold nucleotides indicate extension of primer. Underlined nucleotides indicate non-templated nucleotide addition and lower case indicates misincorporation (identified explicitly in the indicated columns). nd: not detected

strong-stop RNA synthesis (see lines 2 and 3 of sections C and D of Table 1).

Isolation of RNA products from gels, cloning, and then sequencing clearly can introduce bias into the results. Therefore, we pursued direct analysis of RNA substrates, intermediates, and products from reactions performed in vitro using small RNA-Seq as described under Materials and Methods. The first observation was that even analysis of just the primer alone revealed a lot of background (see Table S1). We observed 500 different sequences in the preparation of purified primer defined from 10^7 reads; only five million or 50% of those represented the sequence ordered (see Table S1). Given this complexity, we only present data for sequences whose origin can be explained. The incorporation of the first, correct nucleotide (AMP) was quite faithful, representing 82% of primers extended by one nucleotide (line 3 of Table 2). GMP, CMP, and UMP misincorporation occurred at a frequency of 11, 6, and 1%, respectively (lines 4−6 of Table 2). We observed strong-stop RNA product of the correct sequence, which represented 84% of recognizable 14-nt sequences (line 7 of Table 2). Others had a misincorporation (lines 8−9 of Table 2). This analysis revealed a strong, unexpected bias for the addition of nontemplated nucleotides. Nontemplated addition of CMP was preferred (79% of events), and nontemplated addition of UMP was below the limit of detection (less than 1% of events) (line 10 in Table 2). Nontemplated addition of AMP (20%) and GMP (1%) was observed (lines 11 and 12 of Table 2). Finally, in addition to the expected 28-nt transfer product (33% of total), we also observed a 20-nt transfer product that accumulated (67% of total).

The structure of the intermediate formed and capable of using a 3′-GMP-terminated acceptor template is shown in Figure 6A. We asked if this intermediate could be used directly by PV RdRp in the presence of acceptor to form a transfer product. PV RdRp used the plus-one intermediate (P_5C in Figure 6B) without delay. A P_{+6} product was also observed; however, it is unclear whether this product derived from a templated or nontemplated event (Figure 6B). In contrast, the formation of transfer product by utilization of a P_{+4} blunt-ended product that was preformed (P_4 in Figure 6C) or formed by extension of the P_0 primer was limited by the rate of formation of the P_{+5} product. The sequencing of the transfer product produced using different acceptors also supported the addition of a plus-one product as an intermediate required for forced-copy-choice RNA recombination (Table 3). These data are consistent with formation of the plus-one intermediate as the rate-limiting step for PV RdRp-catalyzed forced-copy-choice RNA recombination.

Connection between RdRp Nucleotide-Incorporation Fidelity and Forced-Copy-Choice RNA Recombination

The requirement for a plus-one intermediate for forced-copy-choice RNA recombination to occur provided a potential

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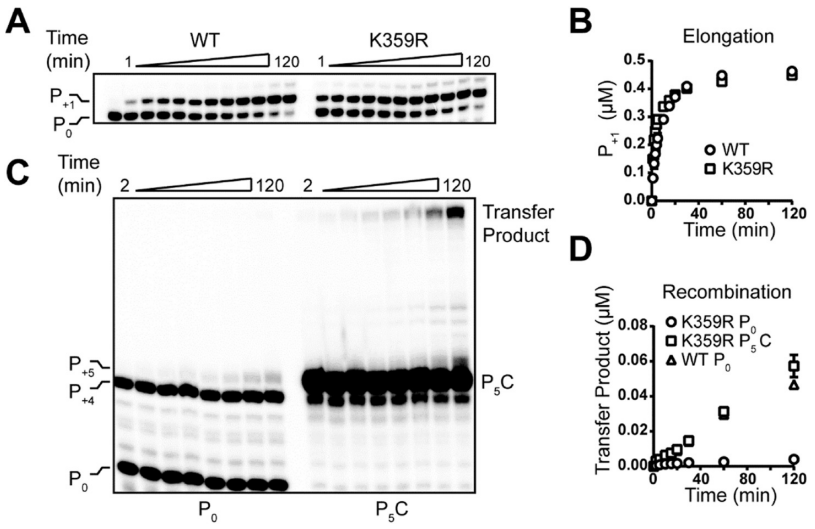

Figure 7. High-fidelity polymerase derivative fails to catalyze forced-copy-choice recombination because of its inability to produce the plus-one intermediate. (A) Comparison of complex assembly between WT and K359R PV RdRp by monitoring primer-extension activity, formation of P_{+1} RNA product. Shown are the reaction products resolved by denaturing PAGE. Time points shown are 1, 2, 3, 4, 5, 10, 15, 20, 30, 60, and 120 min. (B) Quantitative analysis of the kinetics of assembly by monitoring product RNA (P_{+1}) formation using either WT or K359R PV RdRp. There are no differences in the kinetics of assembly. (C) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_0 or P_5C primed-template duplexes using K359R PV RdRp. Time points shown are 2, 5, 10, 15, 20, 30, 60, and 120 min. The positions of the unextended primers (P_0 or P_5C), strong-stop product (P_{+4}), "plus-one" product (P_{+5}), and transfer product are indicated. The amount of "plus-one" product (P_{+4}) for K359R was 0.026 μM. Transfer product is only observed in reactions that used P_5C-primed-template duplexes. (D) Quantitative analysis of the formation of transfer product as a function of time from template-switching reactions performed using P_0 and P_5C primers with K359R PV RdRp and P_0 primers with WT PV RdRp. Mean of three replicates are shown. Error bars represent standard deviation.

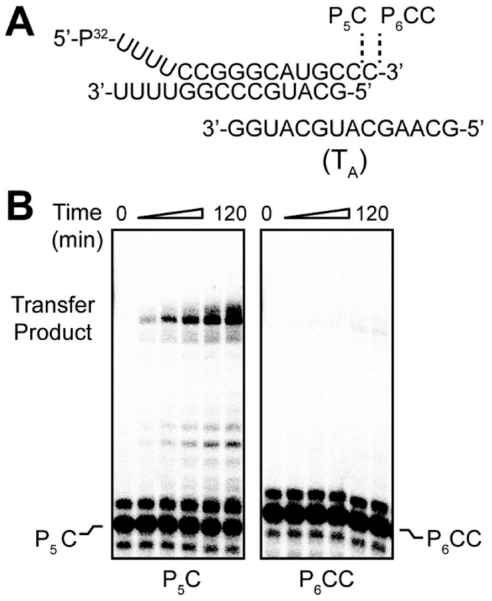

Figure 8. Only a single, nontemplated nucleotide supports forced-copy-choice RNA recombination. (A) Duplexes containing 3′-overhangs of one (P_5C) or two (P_6CC) nucleotides were used to assess template switching by PV RdRp. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5C and P_6CC primed-template duplexes. Time points shown are 5, 15, 30, 60, and 120 min. The positions of the unextended primers (P_5C and P_6CC) and transfer product are indicated.

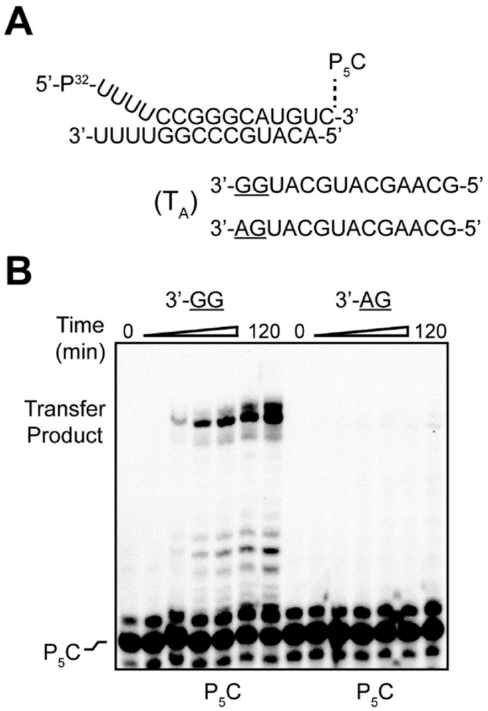

Figure 9. Basepairing to the 3′-end of the acceptor RNA is required for template switching. (A) P_5C intermediate and acceptor template RNAs with unique 3′-terminal ends (3′-GG or 3′-AG end) used to assess template switching by PV RdRp. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5C primed-template duplex and 3′-GG or 3′-AG acceptor template RNA. Time points shown are 2, 5, 10, 30, 60, and 120 min. The positions of the unextended primer and transfer product are indicated.

explanation for the inability of high-fidelity RdRp variants to support this type of RNA recombination.[13] To test this possibility, we used a well-characterized, high-fidelity PV RdRp derivative, K359R.[43] This derivative assembled and elongated

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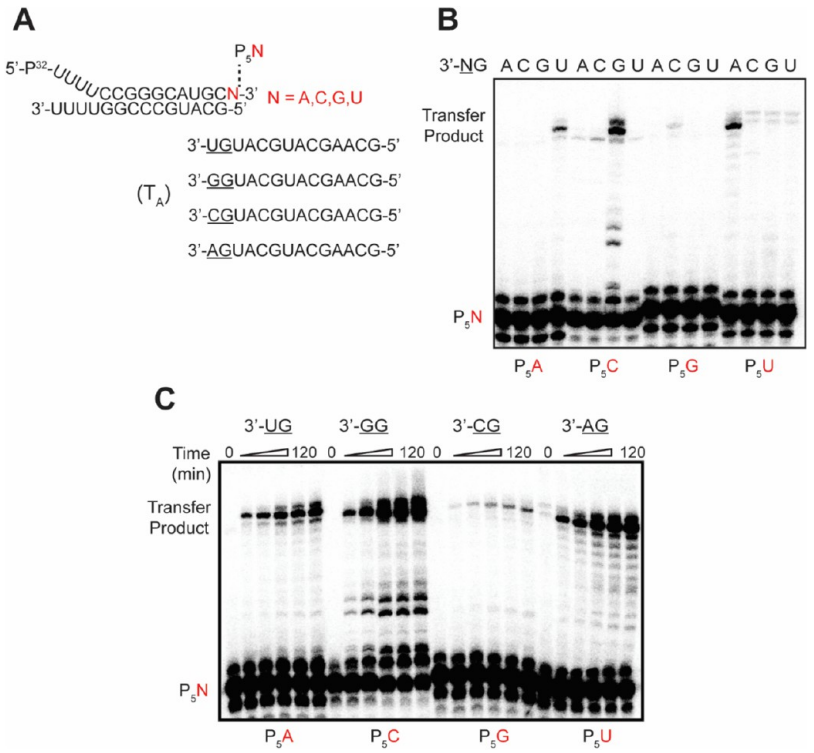

Figure 10. Single basepair between the intermediate and acceptor template is sufficient for template switching. P_5N intermediates with unique 3′-terminal overhangs (*N* = A, C, G, and U in red) and acceptor template RNAs with unique 3′- termini (3′-UG, 3′-GG, 3′-CG, and 3′-AG) used to assess template switching by PV RdRp. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5N intermediates (P_5A, P_5C, P_5G, and P_5U) and the indicated acceptor template RNAs. The positions of the unextended primer and transfer product are indicated. Transfer product is only observed when a basepair can form between the primer and acceptor template RNA. (C) Comparison of the amount of transfer product RNA produced over time from template-switching reactions performed with P_5N intermediates and the indicated acceptor template RNAs. Time points shown are 5, 15, 30, 60, and 120 min.

primed templates as well as WT (Figure 7A,B).[43] However, unlike WT RdRp (0.13 μM), K359R RdRp produced very little P_{+5} product (0.03 μM) after reaching the end of template (panel P_0 in Figure 7C and Figure S2). If production of the plus-one intermediate is the only defect for the high-fidelity RdRp, then the K359R RdRp should use the P_5C intermediate. K359R RdRp used this intermediate to produce transfer product (panel P_5C in Figure 7C). Importantly, the rate of transfer product accumulation was equivalent to WT RdRp (Figure 7D), consistent with the inability of K359R RdRp to produce the plus-one intermediate being the sole defect associated with the failure of this derivative to support forced-copy-choice RNA recombination.

Determinants of the Intermediate Required for Efficient Forced-Copy-Choice RNA Recombination

We performed a series of experiments with modified intermediates and acceptor templates to identify determinants of each promoting template switching by PV RdRp.

Only a Single, Nontemplated Addition Supports Template Switching. We synthesized an intermediate containing a two-nucleotide 3′-overhang (P_6CC in Figure 8A). Addition of this extra nucleotide completely inhibited formation of transfer product (Figure 8B). Please note that the exposure time used was increased to maximize sensitivity. The band that appears above the primary, labeled primer in these and subsequent experiments represent no more than 10% of the total and likely derives from incomplete deprotection of the synthetic RNA. This band was observed in all images below derived from long exposures.

Basepairing between the Intermediate and Acceptor Template Is Required for Template Switching. To determine the importance of basepairing for acceptor template utilization, we created a terminal C:A mispair (Figure 9A). This acceptor template failed to produce transfer product (Figure 9B). We followed up this experiment with a more systematic analysis of the sufficiency of a single basepair for template switching. We created intermediates with each 3′-overhangs containing a different nucleotide (Figure 10A). We then combined each intermediate individually with acceptor templates containing a different, terminal nucleotide (Figure 10A). Only intermediates and acceptor templates with complementary nucleotides yielded transfer product (Figure 10B). In all cases, transfer product accumulated over the 2 h period monitored; however, the efficiency of each reaction varied (Figure 10C). While a C:G (intermediate/acceptor) basepair was most efficient (compare panel P_5C in Figure 10C to other panels), the G:C basepair was least efficient (compare panel P_5G in Figure 10C to other panels). The molecular basis for the observed differences in efficiency remains unclear and requires further investigation. By changing the context in which the terminal nucleotides of the intermediate and acceptor template were presented, we observed context-dependent changes in the efficiency of production of transfer product (Figure 11). However, these changes were subtle (Figure 11). Finally, we asked if the ribose hydroxyls of the terminal nucleotide of the acceptor template contributed to the efficiency of acceptor template utilization (Figure 12A). We found that neither the 3′−OH nor the 2′−OH were required

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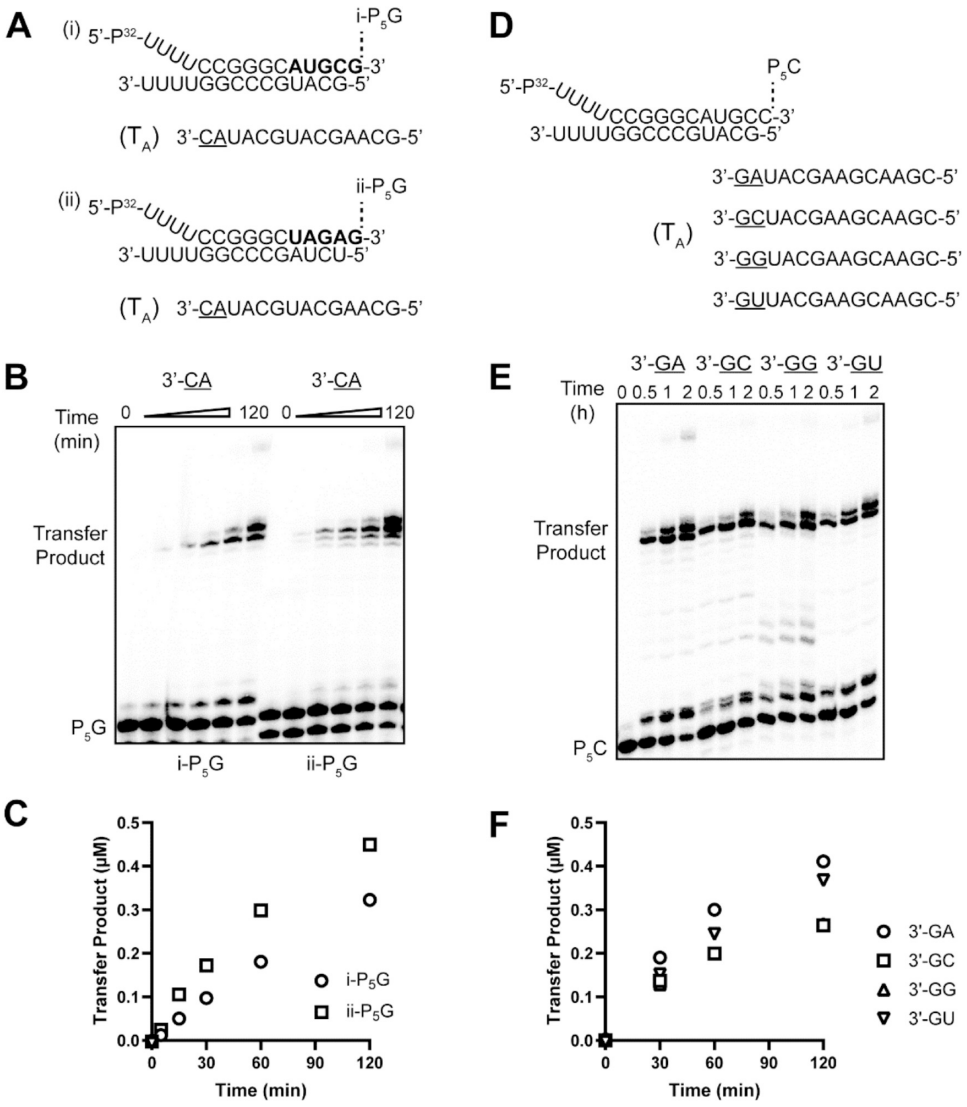

Figure 11. Efficiency of template switching varies depending on the sequence of the adjacent nucleotide. (A) P_5G intermediates with unique 3′-terminal ends (sequence in bold is different) and acceptor template RNA used to assess template switching by PV RdRp. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5G intermediates and 3′-CA acceptor template RNA. Time points shown are 5, 15, 30, 60, and 120 min. The positions of the unextended primer and transfer product are indicated. (C) Quantitative analysis of the formation of transfer product as a function of time from template-switching reactions performed with i-P_5G and ii-P_5G primed-template duplexes. (D) P_5C intermediate and acceptor template RNAs with unique 3′-terminal ends (3′-GA, 3′-GC, 3′-GG, and 3′-GU ends) used to assess template switching by PV RdRp. (E) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5C intermediate and acceptor template RNAs. The positions of the unextended primer and transfer product are indicated. (F) Quantitative analysis of the formation of transfer product as a function of time from template-switching reactions performed with P5C intermediate and indicated 3′GN acceptor RNAs.

(Figure 12B). Both the 3′-dG-terminated and the 2′-dG-terminated acceptor templates supported production of transfer product with the same efficiency as the 3′-G-terminated acceptor template when the intermediate was produced during the reaction (panels P_0 in Figure 12B) or the intermediate was produced synthetically (panels P_5C in Figure 12B).

Observation of Multiple, Template-Switching Events

The experiments performed to this point suggested that formation of the transfer product is limited by the rate of formation and accumulation of an intermediate with a 3′-overhang complementary to the 3′-terminal nucleotide of the acceptor template. These observations suggest that the efficiency of template switching might be increased by skewing nucleotide pools to favor formation of the intermediate complementary to the acceptor template. When CTP was present at 2 mM and the other NTPs present at 20 μM, transfer product formed efficiently with the two different acceptor templates used (Figure 13A and lanes labeled CTP in Figure 13B). When ATP was present at 2 mM instead of CTP, template switching did not occur (lanes labeled ATP in Figure 13B). We observed two template-switching events with both acceptor templates only when CTP was present at 2 mM (#1 and #2 transfer products in lanes labeled CTP in Figure 13B). Interestingly, as many as five, consecutive, template-switching events occurred with the T_{A2} acceptor template (#1−#5 transfer products in lanes labeled T_{A2} and CTP in Figure 13B). The difference between T_{A1} and T_{A2} is that the former

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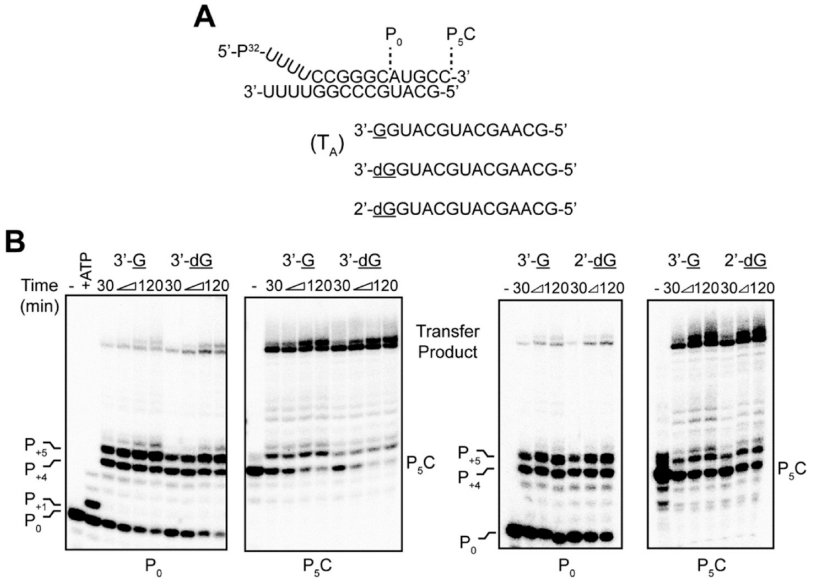

Figure 12. 3′-deoxy and 2′-deoxy-terminated acceptor templates can serve as substrates for template switching. P_0 primed-templates, P_5C intermediate, and acceptor template RNAs with either a 3′−OH, 3′-deoxy, or 2′-deoxy terminated 3′-end used to assess template switching by PV RdRp. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with either P_0 primed-templates or P_5C intermediate and acceptor template RNAs. Time points shown are 30, 60, 90, and 120 min (3′-G to 3′-dG) and 30, 60, and 120 min (3′-G to 2′-dG). The positions of the unextended primer, strong-stop product (P_{+4}), strong-stop, "plus-one" product (P_{+5}), and transfer product are indicated.

terminates as follows: 3′···CG-5′, and the latter terminates as follows: 3′···GC-5′. We synthesized a third, subtly longer acceptor template terminating with 3′···GC-5′ (T_{A3} in Figure 13A). This acceptor template also supported robust template switching only in the presence of 2 mM CTP, yielding again as many as five, template-switching events (#1-#5 transfer products in lanes labeled T_{A3} and CTP in Figure 13B).

Template Switching from the Ends of Templates Resembling Viral Plus-and Minus-Strand RNAs

While contemplating the function of forced-copy-choice RNA recombination in infected cells, we considered the possibility that this process served as a last-ditch response to rescuing viable genomes in the face of extraordinary RNA damage. For example, cell-based, innate-immune mechanisms will introduce oxidants into the infected tissue, causing base modifications and perhaps even scission of the phosphodiester backbone. If this is the case, then the ability of the polymerase to distinguish between a natural 5′-end and a damaged end might be advantageous. The 5′-end of PV plus-strand RNA is a VPg-pUpU dinucleotide that is formed by using the hairpin present in 2C-coding sequence as template (Figure 14A). The 5′-end of minus-strand RNA is a poly(U) stretch templated by the poly(A) tail of plus-strand RNA (Figure 14A). We constructed primed-templates with 5′-ends that mimic these natural sequences and their complementary sequences (Figure 14B). The design of the experiment is indicated in Figure 14C. Running PV RdRp into a uridine dinucleotide or an adenine dinucleotide led to robust template switching (T-U_2 and T-A_2 in Figure 14D). While a stretch of uridine residues presented no impediment to template switching (T-U_{10} in Figure 14E), a stretch of adenine residues diminished the efficiency of formation of an elongation-competent polymerase complex as well as template switching (T-A_{10} in Figure 14E). Whether or not there is something special about the presence of a poly(A) tail remains unclear and future studies will need to be designed to address this question more deeply than we can do here.

Beyond the PV RdRp

It is very clear that all picornaviruses interrogated for their ability to perform copy-choice RNA recombination in cells do so.[53,54] To the best of our knowledge, this study reports the first observation of forced-copy-choice RNA recombination by a viral RdRp. While far beyond the scope of this first study to investigate the details of this mechanism of recombination for multiple picornaviruses, to maximize impact of this study, we evaluated the ability of other picornavirus polymerases to catalyze template switching by initiating from the standard primed-template (Figure 15) or from the P_5C intermediate (Figure 16). The additional picornavirus RdRps evaluated were: Coxsackievirus B3 (CV−B3), enterovirus-D68 (EV-D68), human rhinovirus-C15 (RV-C15), RV-A16, and foot-and-mouth-disease virus (FMDV). There was clear evidence for template switching for all RdRps tested (Figures 15 and 16). RV-A16 RdRp was by far the best at template switching (see RV-A16 in Figures 15 and 16). The RdRps from CVB3, EV-D68 and RV-C15 were all efficient at primer extension (see P_{+4} in Figure 15). However, production of the transfer product by these enzymes, even when starting from the P_5C intermediate, was weak. Sorting out the basis for these differences between the various RdRps will require a study devoted specifically to this question.

DISCUSSION

Why do positive-strand RNA viruses and retroviruses recombine? Is recombination an evolved trait to maintain viral fitness of the viral population or a "mechanistic by-product" of the biochemical and biophysical properties of the viral polymerase required for genome replication?[4] The missing piece to this puzzle is the absence of a clear mechanistic description of viral RNA recombination. This circumstance is now changing.[12] Recent studies of copy-choice

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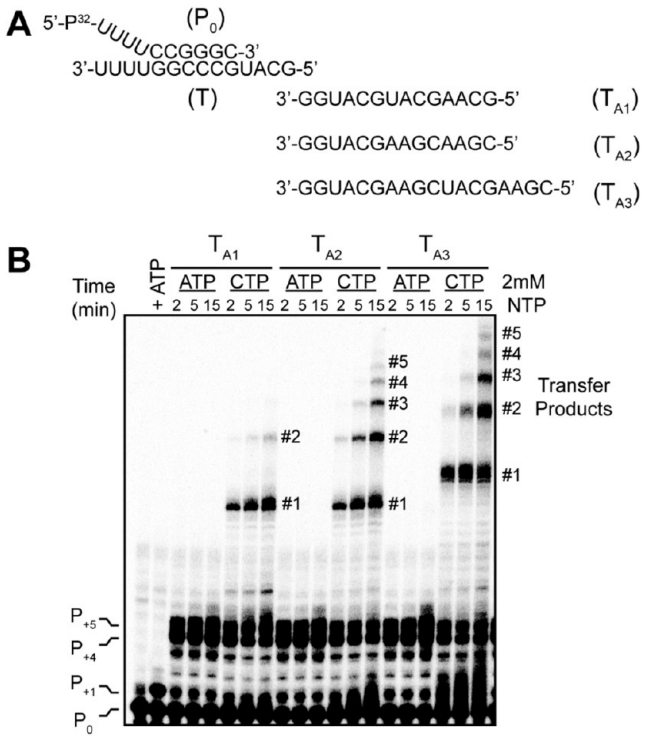

Figure 13. Altering the concentration of NTPs increases the frequency of template switching. (A) Primed-template and acceptor template RNAs (T_{A1},T_{A2}, and T_{A3}) used to assess template switching by PV RdRp. The sequence of the acceptor templates each have a 3′-terminal GMP residue but differ slightly in the remaining sequence. (B) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_0 primed-templates and acceptor template RNAs. The concentration of NTPs were 20 μM each nucleotide with an additional 2 mM ATP or CTP. Reactions containing 2 mM CTP promoted nontemplated nucleotide addition and the ability of PV RdRp to multiple template switching events using the 3′GMP terminated acceptor template. The positions of the unextended primer, strong-stop product (P_{+4}), strong-stop, "plus-one" product (P_{+5}), and transfer products (#1-#5) are indicated.

RNA recombination revealed an unexpected mechanism of template switching.[12] In response to nucleotide misincorporation, some fraction of elongation complexes "backtrack," releasing the 3′-end of nascent RNA from the template. The 3′-end is then extruded through the nucleotide-entry channel into the solution, where hybridization of a complementary template can occur to complete the template-switching process.[12] The mechanism of forced-copy-choice RNA recombination reported here is equally unexpected. The use of a blunt-ended duplex with a nontemplated nucleotide added to the 3′-end of one strand to form the intermediate required for template switching (Figure 17). A single basepair is sufficient for this template-switching event to occur (Figure 17). In both types of RNA recombination, the properties of the polymerase required for recombination are in many ways independent of the requirements for elongation and are triggered by nucleotide misincorporation. We would like to suggest here that viral RNA recombination may be an evolved trait.

The very first paper published from our laboratory showed that PV RdRp was able to use template switching to make products greater than unit length when homopolymeric primed templates were used.[42] Inspired by the mechanistic studies of Peliska and Benkovic on template switching by HIV RT,[24] we added an acceptor template complementary to the 3′-end of nascent RNA produced from a self-complementary (symmetrical) primed template referred to as sym/sub.[13,48] We demonstrated an acceptor template-dependent RNA product, formation of which was compromised by randomizing the acceptor template sequence.[13] However, efforts to sequence the recombinant products were complicated by the symmetrical nature of the starting primed template.[13] The sequencing data that we were able to obtain suggested a forced-copy-choice mechanism instead of a copy-choice mechanism.[13]

Forced-copy-choice recombination is a concept that originates from a description of retroviral reverse transcription (Figure 1).[25,26,28] Initiation of genome replication is primed by a tRNA bound to the retroviral genome a few hundred nucleotides or so from the 5′-end. Initiation from the tRNA is forced to terminate when the 5′-end is reached. To continue synthesis the strong-stop product must be transferred to the 3′-end of the retroviral genome. To our knowledge, this type of recombination has never been invoked as a mechanism used by positive-strand RNA viruses. However, initiation of genome replication by enteroviruses requires a priming event that occurs at an internal position of the genome instead of the 3′-end.[36]

We established and validated a primed-template system that could be used to determine the type of RNA recombination that was occurring and the ability to elucidate its mechanism (Figures 1−5). Nontemplated addition of a single nucleotide to the 3′-end of nascent RNA once the polymerase reached the end of template was quite robust (e.g., P_{+5} in Figure 3C and Tables 1 and 2). A PV RdRp derivative (K359R) defective for nontemplated addition was also defective for template switching in this assay (Figure 7). This was the first indication that the P_{+5} product with the nontemplated addition might be an obligatory intermediate for template switching by a forced-copy-choice mechanism. In fact, neither the blunt end nor a two nucleotides-extended product supported template switching, but the P_{+5} intermediate worked quite well for both WT RdRp (Figures 6 and 8) and K359R RdRp (Figure 7). A single Watson−Crick basepair was sufficient for the RdRp to match the intermediate with an appropriate acceptor template and extend the intermediate to the end of the acceptor template (Figures 9 and 10) and beyond (Figure 13). As is common when nucleic acid is a component of a reaction, the context in which the 3′-terminal nucleotide is presented, its nearest neighbors, modulate the efficiency of template switching (Figures 11 and 12). Formation of the basepair mattered most; the nature of the sugar configuration mattered least (Figure 12). Finally, all picornavirus RdRps tested were able to catalyze template switching by a forced-copy-choice mechanism whether the reaction was launched from a primed template (Figure 15) or the P_{+5} intermediate (Figure 16).

The mechanism of forced-copy-choice RNA recombination catalyzed by PV and other enterovirus RdRps reported here (Figure 17) is essentially identical to the forced-copy-choice DNA recombination catalyzed by the reverse transcriptase encoded by a group II intron.[34,55] Nontemplated addition to a blunt-ended elongation product was essential for efficient template switching, and formation of a single basepair was sufficient for efficient template switching.[34] Different RTs are known to do this reaction, but the nature of the nucleotide incorporated and the number of nucleotides added

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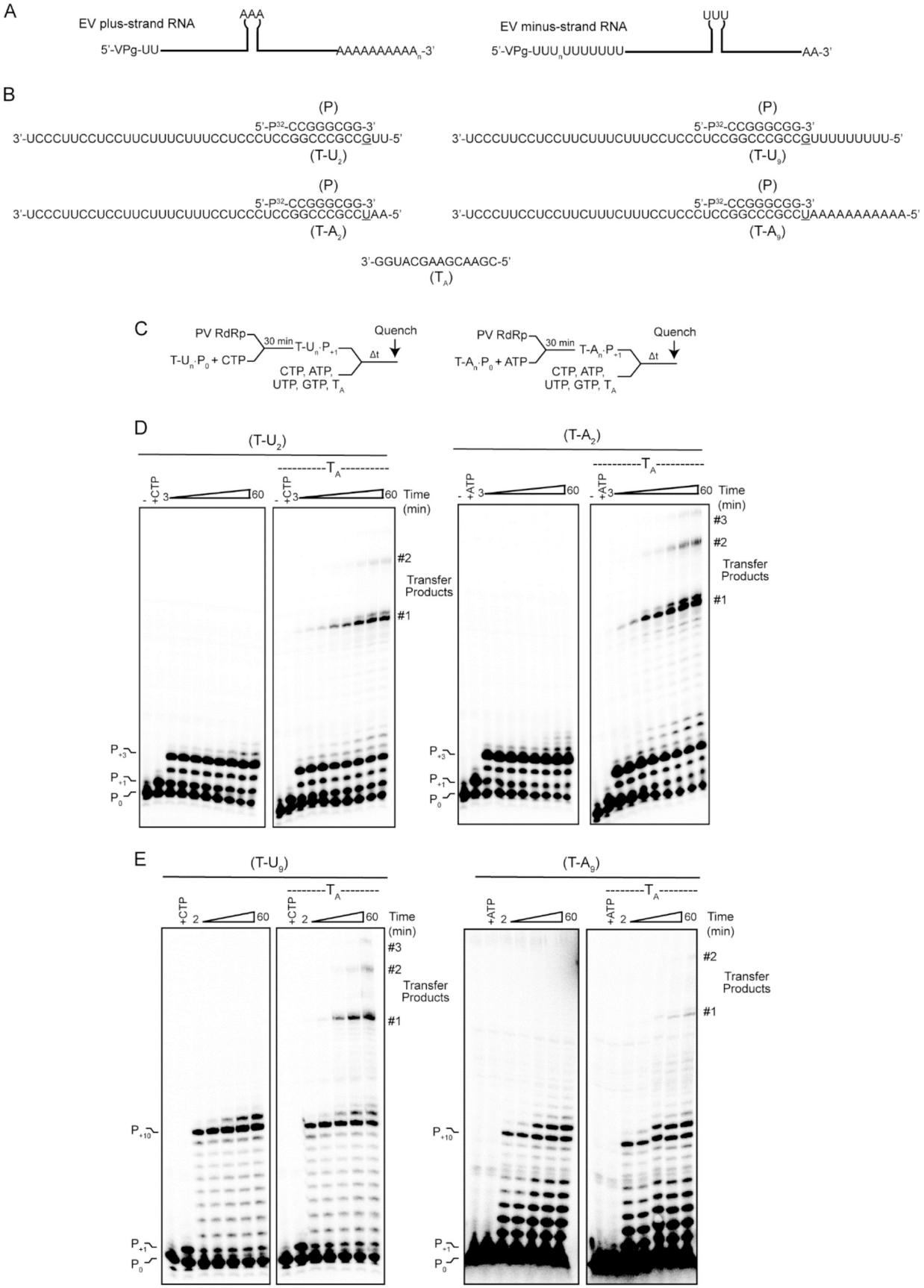

Figure 14. Impact of repeated nucleotides on template switching by PV RdRp. (A) Schematic of the enterovirus plus-strand and minus-strand RNAs. There are two uridylate residues covalently linked to VPg on the 5′-end of plus-strand and a 3′-poly-A tail of ill-defined length. There is an internal cis-replication element (CRE, oriI), that contains three adenylate residues on the apical stem-loop that serves as template to prime production of VPg-pUpU that is then transferred to the 3′-end of plus-strand RNA to produce minus-strand RNA. The 5′-end of minus-strand

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Figure 14. continued

RNA contains VPg-pUpU and a poly-U stretch of ill-defined length; the 3′-end has two adenylates. (B) Primer and templates used in this study. The primer (P) is an 8-nt RNA and templates (T-U_2, T-A_2, T-U_9, and T-A_9) are 31-nt and 38-nt RNAs; sequences are shown. The annealed primed-template forms an 8-bp duplex with a 3-nt or 10-nt 5′-template overhang. The first templating base is underlined. The RNA primer was labeled on the 5′-end with ^{32}P. (C) Schematic of assay. Primer extension was initiated by adding PV RdRp with primed-template in the presence of the first nucleotide to be incorporated, CTP or ATP for 30 min. After formation of P_{+1}, the remaining NTPs (20 μM) and CTP (2 mM) were added in both the absence and presence of acceptor template for various amounts of time and then reactions were quenched. (D,E) Analysis of reaction products by denaturing PAGE using P-T-U_2, P-T-A_2 (panel C) and P-T-U_9, P-T-A (panel D). Time points shown are 3, 6, 9, 12, 15, 30, 45, and 60 min (panel C) and 2, 5, 15, 30, and 60 min (panel D). The positions of the unextended primer P_0, extended primers P_{+1}, strong-stop products (P_{+3} and P_{+10}), and transfer product are indicated.

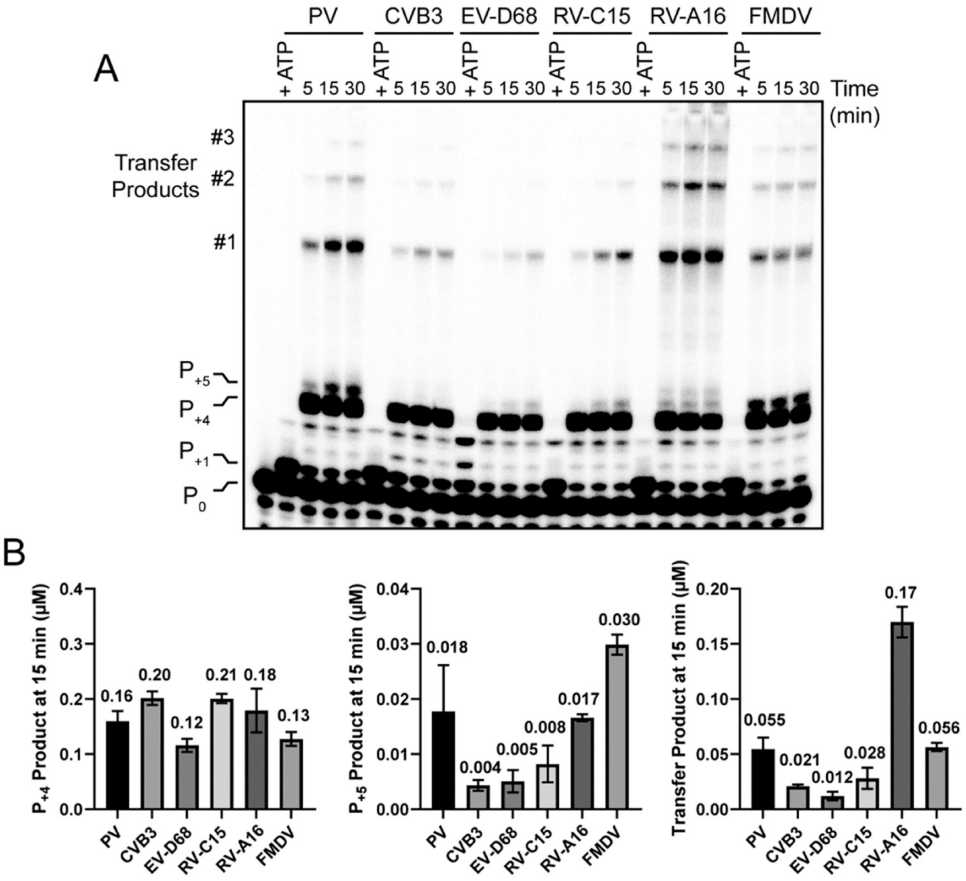

Figure 15. Template switching by picornaviral RdRps. (A) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_0 primed-template, acceptor template RNA, and the indicated RdRp. The concentration of NTPs were 20 μM each with an additional 2 mM CTP. Reactions promoted nontemplated nucleotide addition and the ability of the RdRp to perform multiple template switching events using the 3′GMP terminated acceptor template. The positions of the unextended primer, plus-one product (P_{+1}), strong-stop product (P_{+4}), strong-stop, "plus-one" product (P_{+5}), and transfer products (#1-#3) are indicated. (B) Quantitative analysis of the formation of P_{+4}, P_{+5} and transfer product at 15 min from template-switching reactions performed with the indicated RdRps.

vary.[34,55−57] Therefore, it was quite surprising to see that both the enterovirus RdRp and group II intron RT used the exact same mechanism.

A few years ago, Lambowitz and colleagues reported the structure of the group II intron-encoded RT poised for template switching.[34] Because RT and the RdRp share substantial conservation in overall topology and conserved structural motifs and corresponding functions, we compared the RT model to a model of the PV RdRp elongation complex.[58] The PV RdRp elongation complex enabled formulation of a structure-based hypothesis for RdRp-catalyzed template switching during forced-copy-choice RNA recombination (Figure 18A). The RdRp binds to the strong-stop RNA product (step 1), then adds a nontemplated nucleotide (step 2), and finally recruits an acceptor template capable of basepairing with the nontemplated nucleotide added (step 3). The acceptor template and nascent basepair should have the same extensive interactions experienced by the template and nascent basepair of the elongation complex (Figure 18B). Importantly, this analysis revealed several residues in conserved sequence/structure motifs F (red) and G (green) that are conserved across the enterovirus RdRps that should be critical to the positioning of the acceptor template and nascent basepair for catalysis (Figure 18C).

One interesting difference between RdRp and RT-catalyzed template switching is that the RdRp appears to rarely incorporate UMP as the nontemplated nucleotide but RT uses all four apparently equally.[9,24,34,55−57] We were unable to

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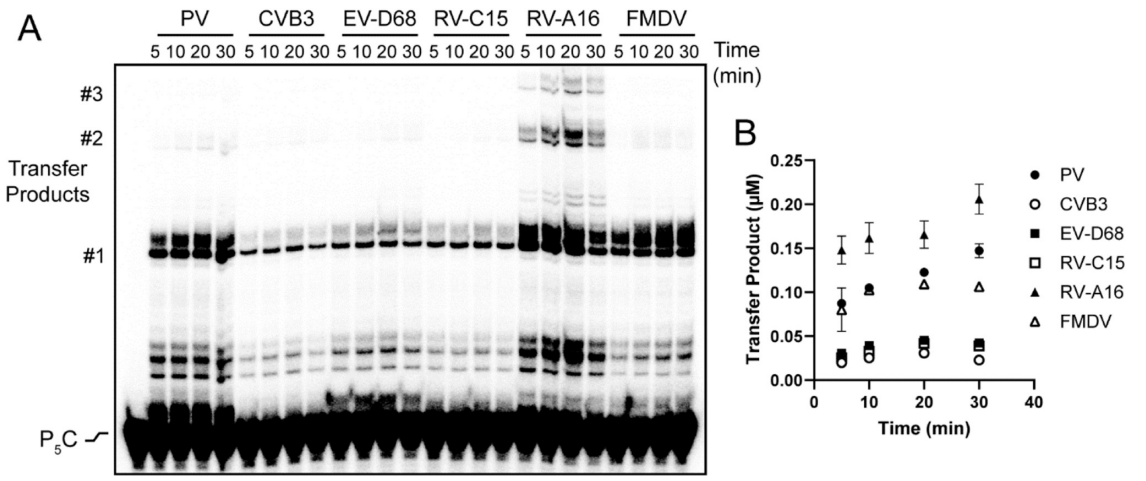

Figure 16. Utilization of the P_5C intermediate by picornaviral RdRps. (A) Analysis of reaction products by denaturing PAGE from template-switching reactions performed with P_5C intermediate, acceptor template RNA, and the indicated picornavirus RdRp. The concentration of NTPs were 500 μM each. Reactions promoted the RdRps to template switch. The positions of the P_5C intermediate and transfer products (#1-#3) are indicated. (B) Quantitative analysis of the formation transfer products from template-switching reactions performed with the indicated RdRps.

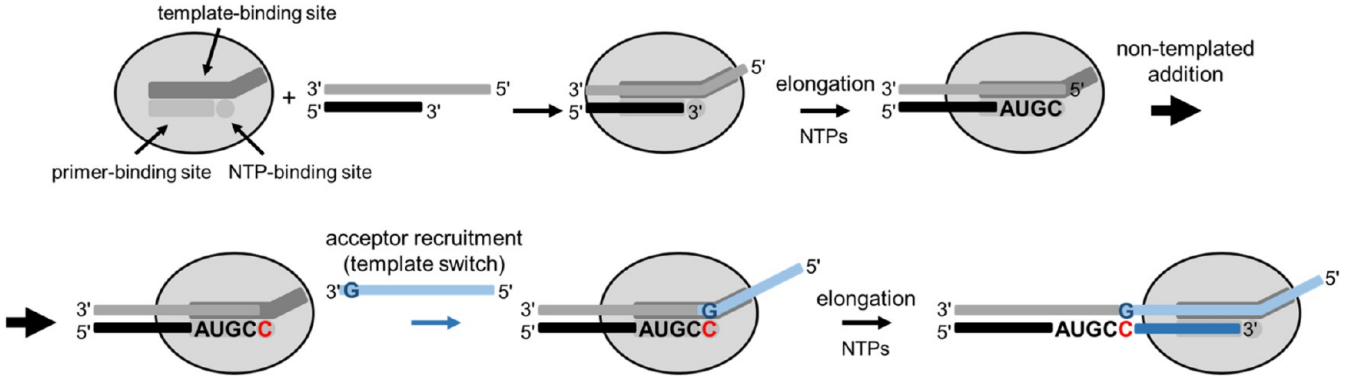

Figure 17. Model for enteroviral polymerase-catalyzed forced-copy-choice recombination. Mechanism for recombination as a method of rescuing viral RNA genomes following partial degradation or from abortive synthesis. Using poliovirus RdRp as a model, we have shown that the polymerase extends the nascent RNA strand to the end of its template, and then proceeds to add nontemplated nucleotides. From this process a minimal 1 nt-long recombination junction is formed between the 3′-end of the nascent RNA and the 3′-end of the secondary acceptor template where elongation continues.

observe a P_{+5} (15-nt) intermediate ending with UMP (see rows 10−12 in Table 2). Enteroviruses and other picornaviruses produce their proteins as a single polyprotein that is cleaved post-translationally. Inadvertent incorporation of a stop codon during recombination would be lethal to the virus. One possibility is that the inability to introduce UMP by nontemplated addition may have evolved to preclude introduction of a stop codon, all of which begin with a uridine. RdRps and RTs are thought to have evolved from a common ancestor.[59−61] For RdRps to be incapable of adding UMP and for RTs to be capable of adding UMP suggests this activity must have happened after the split into the two lineages. We know that the problem is UMP addition and not utilization because a P_5U intermediate is used by PV RdRp (Figure 10). Moreover, evidence for UMP misincorporation from an internal position of template was detectable even at a frequency of 0.00009 (see row 6 in Table 2). This observation supports the notion that RdRp-catalyzed forced-copy-choice RNA recombination has evolved and perhaps continues to evolve independent of other known functions of the polymerase.

Another distinguishing feature of the specificity of PV RdRp for nontemplated addition of nucleotides is the preference for CMP incorporation over other nucleotides (compare lines 10−12 in Table 2). Many RdRps have been reported to exhibit terminal ATP adenylyltransferase activity. We have assumed that this reflected the polymerase "A-rule," which states that when in doubt, for example in the absence of a template or in the presence of a lesion in the template, polymerases incorporate AMP.[62,63] This rule also extends to blunt-ended duplex substrates.[56] Indeed, a commercial strategy for cloning PCR products relies on nontemplated addition of AMP by Taq polymerase.[64] While the RT encoded by a group II intron only adds a single, nontemplated nucleotide of any type,[34] the RT from Moloney murine leukemia virus adds three cytidine residues.[57] This observation further supports the notion that mechanisms of recombination evolved independently of the mechanism of elongation. Our data with the picornavirus RdRps support a similar conclusion. Although all RdRps tested produced equivalent amounts of strong-stop product (P_{+4} in Figure 15), the efficiency of nontemplated addition of nucleotides and template switching varied widely (P_{+5} and Transfer Products in Figure 15). Explanation of the differences

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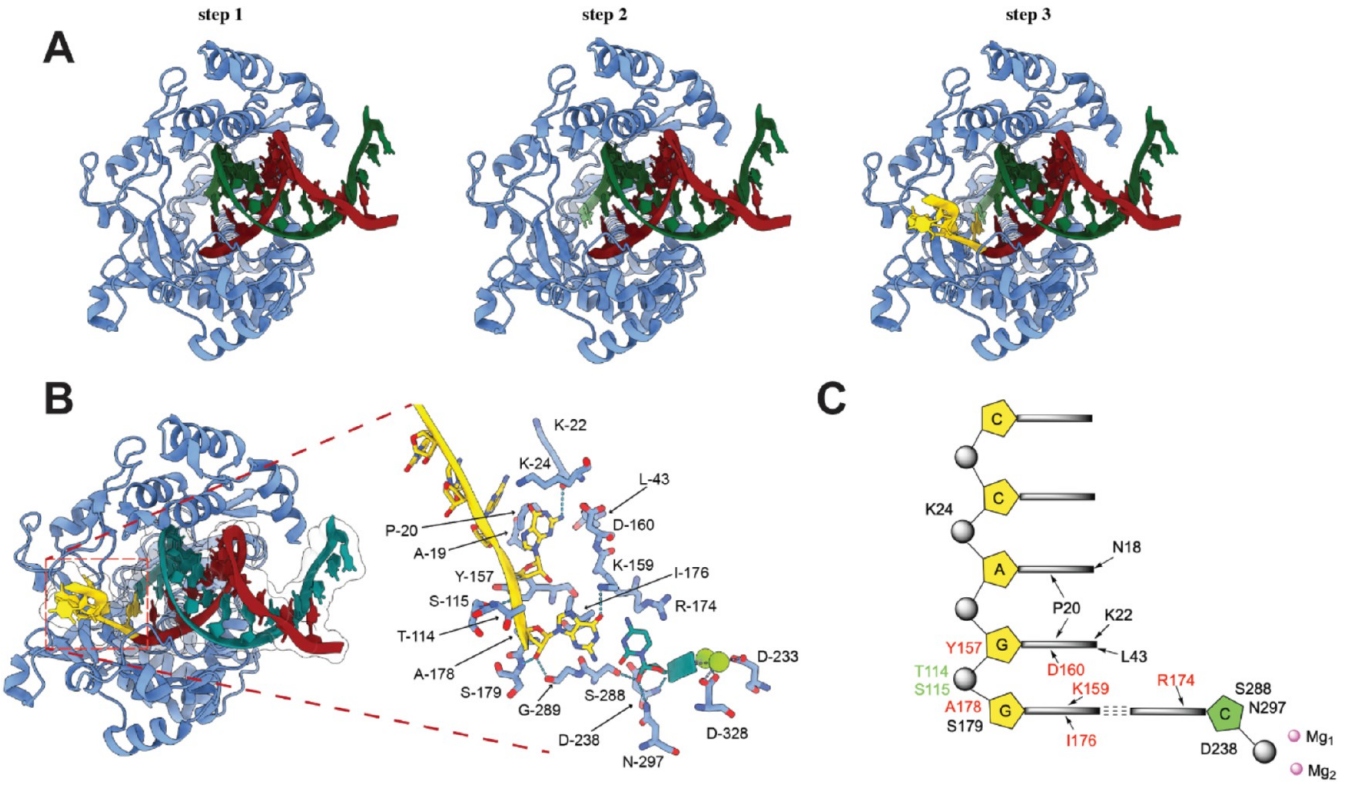

Figure 18. Structural model for forced-copy-choice recombination. (A) Using an existing structure of PV RdRp with primed template (30L7), we have produced snapshots of the proposed steps of forced-copy-choice recombination. PV RdRp extends the nascent RNA strand (green) to the end of its template (red) [step 1], and then adds a nontemplated nucleotide [step 2]. Binding of the template strand (yellow) to the PV-RdRp-recombination intermediate complex leads to formation of 1-bp duplex, extension from which produces the recombinant RNA [step 3]. (B) Structural model for the recombination intermediate (step 3) in which PV RdRp extends the nascent RNA strand (green) 1 nt beyond the template (red) and the recombination junction is formed between the 3′-end of the nascent RNA (green) and the 3′-end of the secondary acceptor template (yellow). Boxed region showing the interactions of PV RdRp amino acid side chains with acceptor template. (C) Schematic of the basepairing between the recombination intermediate (green) and template (yellow). Residues stabilizing this interaction originate from conserved sequence/structural motifs F (red) and G (green).

observed between RdRps will require future studies. Do all of these RdRps follow the C-rule? When the P_{+5} intermediate does not accumulate, does this mean that the addition of the nontemplated nucleotide is inefficient or does this mean that recruitment of the acceptor template is more efficient?

Forced-copy-choice recombination is thought to have evolved as an RNA repair mechanism that will assemble full-length genomes from fragments.[65] The evidence for this with HIV is substantial.[26,27] For such a mechanism to work, more than one copy of the genome is required, minimally a donor template and an acceptor template. Of course, each HIV virion contains two copies of the genome. Our studies of the RdRp show quite convincingly that the forced-copy-choice mechanism permits assembly of RNA fragments (Figures 13 and 15). Our work shows that a single nontemplated nucleotide is sufficient to serve as a repair mechanism, but how this disruption might impact the reading frame is not known. Other mechanisms may exist that "resolve" these additions.[8] Now, we also know that enteroviruses spread by a nonlytic mechanism with many virions contained in vesicular carriers of different origins. Multiplicities of infection much greater than one as afforded by nonlytic spread makes an RNA repair mechanism feasible. More work in this area will be needed. For PV, we have mutants capable of only copy-choice or forced-copy-choice recombination that may be useful for exploring the role of RNA recombination in virus biology.[23]

If forced-copy-choice recombination contributes to RNA repair, then it might be advantageous to distinguish unnatural genome ends created by nucleolytic cleavage from natural genome ends (Figure 14A). With the exception of an oligo(rA)-terminated RNA, all natural genome ends appeared competent for recombination (Figure 14). It is unclear if the result with the oligo(rA)-terminated RNA reflects the inability to extend the corresponding duplex with this sequence. Multiple preparations yielded the same result (data not shown). More work will be required to clarify this matter.

The capacity for the enterovirus RdRp to use a one-nucleotide-extended, double-stranded nucleic acid as a primer for extension of a template and assembly of multiple fragments suggests a few applications for this reaction. First, introduction of adapters, fluorophores, peptides, anything that can be attached to an oligo can be covalently linked to the 5′-end of product RNA. We are particularly intrigued by the ability to use the forced-copy-choice mechanism of the RdRp to prepare samples for analysis by nanopore technologies. Typically, evaluation of a ssRNA by nanopore begins with the addition of adapters and use of RT to convert the ssRNA to an RNA-DNA hybrid, ending with ligase treatment to remove any nicks. The need to use RT to make a double-stranded product relates to removal of any secondary structure in the RNA that would create unique signatures when detected by the nanopore instrument. Of course, RT is not the most processive

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polymerase. Replacing RT with an RdRp may permit even longer templates to be evaluated and would also produce a second strand of RNA that could be analyzed, thus bolstering the reliability of interpretations made by analyzing data derived from the first strand of RNA.

■ ASSOCIATED CONTENT

Data Availability Statement

All data are incorporated into the article and its online Supporting Information. Constructs and data sets presented in this study are available upon request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.5c00049.

Comparison of assembly using primed templates with and without additional uridine residues (Figure S1); comparison of the formation of plus-one product (P_{+5}) and RNA recombination by WT and K359R RdRps (Figure S2) (PDF)
NGS3A_Control (Table S1)(XLSX)
NGS_3A The top 500 sequences from small RNA-Seq (Table S2) (XLSX)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PV	Poliovirus
RdRp	RNA-dependent RNA polymerase
RT	reverse transcriptase
VPg	viral protein genome-linked
CV−B3	Coxsackievirus B3
EV-D68	Enterovirus-D68
RV-C15	Human rhinovirus-C15
RV-A16	Human rhinovirus-A16
FMDV	Foot-and-mouth-disease virus
PAGE	polyacrylamide gel electrophoresis
AMP	adenosine monophosphate
CMP	cytidine monophosphate
GMP	guanosine monophosphate
UMP	uridine monophosphate
EDTA	ethylenediaminetetraacetic acid

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