Toward building a chirally inverted version of the central dogma of molecular biology, Zhu and colleagues realized the transcription of a mirror-image gene into L-RNA and reverse transcription into L-DNA by synthetic D-polymerases. They enzymatically transcribed full-length mirror-image 5S rRNA and demonstrated a comprehensive mirror-image transcription, reverse transcription, PCR, and sequencing toolbox.
SUMMARY
The flow of genetic information between DNA, RNA, and protein lays the molecular foundation of life. Here, we show the transcription of a mirror-image gene into L-RNA and reverse transcription into L-DNA by synthetic D-polymerases based on designed mutants of the DNA polymerase Dpo4. Furthermore, the reverse-transcribed L-DNA was amplified by mirror-image PCR and sequenced by nucleobase-specific chemical cleavage. As a step toward the synthesis of a mirror-image ribosome, we enzymatically transcribed full-length mirror-image 5S rRNA. The realization of a comprehensive mirror-image transcription, reverse transcription, PCR, and sequencing toolbox will enable the directed evolution of nuclease-resistant L-RNA aptamers and ribozymes and spur the development of other functional mirror-image molecular systems.

INTRODUCTION
Biology’s remarkably skewed choice of chirality has led to the exciting prospect of building a mirror-image form of life in the laboratory.1–3 Toward this goal, an imperative step is to establish a chirally inverted version of the central dogma of molecular biology.4 We initially realized mirror-image genetic replication and transcription with a chemically synthesized D-amino acid African swine fever virus polymerase X (ASFV pol X).5 However, owing to the intrinsically low processivity of this polymerase, only the template-directed copying of a 44-nt L-DNA and transcription of a short, 6-nt L-RNA were achieved.4 More recently, the D-amino acid version of the smallest known thermostable polymerase capable of performing polymerase chain reaction (PCR), Sulfolobus solfataricus P2 DNA polymerase IV (Dpo4), has been chemically synthesized by us and others,5–7 enabling more efficient L-DNA polymerization, including mirror-image PCR (MI-PCR) despite that the synthesis of larger polymerases is still beyond current technology.

We reasoned that to establish a complete mirror-image central dogma of molecular biology, a more efficient system capable of enzymatically transcribing mirror-image genes is required since even the shortest ribosomal RNA (rRNA) in Escherichia coli (5S rRNA, 120 nt) is too long to be chemically synthesized.8 It is known that most DNA polymerases discriminate against nucleoside triphosphates (NTPs) through bulky side-chain residues in their active sites by steric clash, and mutating the steric gate amino acid into a less bulky one may result in loss of NTP discrimination, thus greatly enhancing its DNA-dependent RNA polymerase activity.9,10 Therefore, we set out to realize an efficient mirror-image transcription (MI-transcription) system by testing different steric gate mutant versions of Dpo4.

While studying the mirror-image transcription system, we discovered that Dpo4 also possesses RNA-dependent DNA polymerase activity, which led to the development of mirror-image reverse transcription (MI-reverse transcription) and mirror-image PCR (MI-PCR). This allowed us to establish a comprehensive mirror-image biochemical toolbox that will be invaluable in the development of other functional mirror-image molecular systems.

The Bigger Picture
The realization of mirror-image biology systems may open the next frontier for biological discovery and technical innovation, and a crucial challenge in this new venture is to establish a chirally inverted version of the central dogma of molecular biology. Here, we realized the transcription and reverse transcription of a mirror-image gene, a stepping stone toward building the mirror-image central dogma. The transcription of full-length mirror-image 5S ribosomal RNA also marks an initial step toward building a mirror-image ribosome. The demonstrated enzymatic transcription of long, nuclease-resistant L-RNA, which currently cannot be obtained using other methods including chemical synthesis, may enable various applications of nuclease-resistant L-aptamer biosensors and drugs, and facilitate future studies on ribozyme and aptamer function and structure using their mirror-image version as an orthogonal model system that does not require RNase decontamination.
of a mirror-image reverse transcription (MI-RT) system. Reverse transcription is an important modification to the initial concept of the central dogma and is also widely used in various important biotechniques such as reverse transcription PCR (RT-PCR). Thus, the development of efficient mirror-image transcription and reverse transcription systems has both conceptual and practical significance to the effort of building mirror-image biology systems.

RESULTS

Designed Mutants of Dpo4 Enable Efficient Transcription

It has been previously reported that mutating the 12th residue tyrosine of Dpo4 (Figure S1) into alanine (Y12A) results in an increase of >200 fold in matched ribonucleotide incorporation efficiency, and a more recent study suggested that mutation into serine (Y12S) results in more efficient transcription. Another study compared different mutations of the steric gate tyrosine of Tgo DNA polymerase and found that mutation into glycine (Y409G) results in the highest transcription efficiency. Thus, we experimentally compared the efficiency and fidelity of mutant Dpo4 transcription systems by constructing three different mutations, alanine (Y12A), glycine (Y12G), and serine (Y12S), in the Dpo4-5m polymerase (a mutant version of Dpo4 modified to facilitate its chemical synthesis). We expressed and purified the three mutant versions of Dpo4-5m from E. coli and examined their transcription efficiency in the natural chirality system by extending a 21-nt 5'-fluorescein (FAM)-labeled RNA primer on a 41-nt single-stranded DNA (ssDNA) template, as well as a 19-nt 5'-FAM-labeled RNA primer on a 120-nt ssDNA template coding for the E. coli 5S rRNA. The results of primer extension experiments suggested that all three mutants increased the DNA-dependent RNA polymerase activity of this enzyme significantly (Figures 1A and 1B) and Y12S possesses the highest RNA polymerase activity (Figure S2).

Next, we examined the transcription fidelity of these three mutants by reverse transcribing the DNase I-digested transcription product by Superscript III high-fidelity reverse transcriptase, amplifying by Q5 high-fidelity DNA polymerase, and sequencing the amplicons by Sanger sequencing. To eliminate contamination from any residual DNA template that evaded the DNase I digestion, a wobble base pair was designed between the RNA primer and ssDNA template so that the reverse-transcribed and PCR-amplified DNA carries an A at this site, as opposed to a G on the template DNA (Figure S3A). The sequencing results suggested that the Y12S mutant possesses a lower transcription error rate (0.15%), compared with that of the Y12A (0.74%) and Y12G (0.32%) mutants (Table S1). Overall, the transcription error rates are an order of magnitude higher than that of DNA replication by wild-type (WT) Dpo4 and Dpo4-5m (on the order of 10^-6). Moreover, it has been known that Dpo4 catalyzes non-templated nucleotide addition to the 3'-terminus of blunt-end DNA. To test whether the transcription product of Y12S mutant contains 3'-protruding bases (which is important for the preparation of functional RNAs, such as transfer RNAs (tRNAs), whose 3'-termini are important for their functions), we ligated a DNA adaptor (in the natural chirality system) to the 3'-terminus of the transcription product for reverse transcription and Sanger sequencing (Figure S3B). The sequencing results suggested the absence of additional bases in the majority of the transcription product, as analyzed by denaturing PAGE (Figure 1B).

Transcription of Mirror-Image 5S rRNA by Synthetic Mutant D-Amino Acid Dpo4

Based on the above results, we chose the Y12S mutation along with the previously determined five point mutations (C31S, S86C, N123A, S207A, and S313A) for
efficient mirror-image transcription. The chemical synthesis of this mutant D-amino acid Dpo4 (D-Dpo4-5m-Y12S) was carried out using the previously reported synthesis strategy and characterized by reversed-phase high-performance liquid chromatography (RP-HPLC) (Figure S4A) and electrospray ionization mass spectrometry (ESI-MS) (Figure S4B). After folding by successive dialysis against a series of renaturation buffers that contained 4, 2, 1, 0.5, 0.25, and 0 M GlnHCl and purification by heat-precipitation and Ni-NTA column chromatography, the polymerase was further characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1E) and circular dichroism (CD) (Figure 1F). We next tested the
transcription activity of the D-polymerase with a 19-nt 5'-FAM-labeled L-RNA primer on a 120-nt L-ssDNA template coding for the 5S rRNA. Full-length 120-nt L-5S rRNA was observed after 3 h of reaction, and the additional full-length product was obtained after incubation for 18 h (Figures 2 B and S6A). The full-length L-RNA was subsequently purified by denaturing PAGE to remove the L-ssDNA template and partially extended products. As expected, the transcription product was indeed resistant to natural RNase H digestion (Figure 2 B), suggesting that enzymatically transcribed L-RNAs are promising for various practical applications, such as nuclease-resistant aptamer biosensors and drugs. \(^{16}\)

**Reverse Transcription of L-RNA by Synthetic Mutant D-Amino Acid Dpo4**

A number of DNA polymerases are known to incorporate deoxynucleotide triphosphates (dNTPs) in an RNA-templated manner, \(^{17–19}\) although this activity has not been demonstrated with Dpo4. While testing the transcription activity of Dpo4 mutants in the natural chirality system, we used different combinations of primer and template pairs (DNA/DNA, DNA/RNA, and RNA/RNA) as controls (Figures 1 A, 1C, and 1D). To our surprise, Dpo4-5m was able to catalyze the extension of a DNA primer on an RNA template with dNTPs, while the three Y12 mutants (Y12A/G/S) possess much lower reverse transcription activity (Figure 1 C) and none of the polymerases possess RNA-dependent RNA polymerase activity (Figure 1 D). Because
reverse transcriptases often have lower fidelity than DNA-dependent DNA polymerases, we examined the reverse transcription fidelity of Dpo4-5m in the natural chirality system by amplifying the reverse-transcribed DNA using Q5 high-fidelity DNA polymerase and sequencing the amplicons by Sanger sequencing (Figure S3C). We measured a reverse transcription error rate of ~2.6% (Table S1), approximately two orders of magnitude higher than that of DNA replication by WT Dpo4 and Dpo4-5m.5,13,14

Encouraged by the reverse transcription results in the natural chirality system, we tested reverse transcription in the mirror-image system. We used the previously transcribed and purified 5'-FAM-labeled L-5S rRNA as template, and 5'-Cy5-labeled L-DNA as primer, which was extended to full length by D-Dpo4-5m after incubation for 36 h (Figures 2C and S6B). As expected, the L-DNA products were not digestible by natural DNase I (Figure 2C). Notably, the L-RNA template was also extended (Figure 2C), likely due to non-templated nucleotide addition to the 3'-terminus by Dpo4-5m (Figure S5B).

Developing a Comprehensive Mirror-Image Transcription, Reverse Transcription, PCR, and Sequencing Toolbox

The broad application of reverse transcriptases in molecular biology has been propelled by the introduction of RT-PCR. Here, we set out to test the ability of the Dpo4-based mirror-image PCR system6 to amplify the reverse-transcribed 120-nt L-DNA. We show that PCR amplification of the reverse-transcribed L-DNA resulted in a target band in sieving agarose gel electrophoresis with the expected length of 120 bp, which increased in intensity with cycle numbers of up to 35, while the negative controls without polymerase or reverse transcription product resulted in no amplification product (Figure 2D). Notably, the same D-Dpo4-5m was used for both mirror-image reverse transcription and PCR, thus simplifying the system in that it can be achieved using one D-polymerase, minimizing experimental cost and effort required to meet the future needs of mirror-image molecular applications.

Mirror-Image RNA Sequencing

Next, we tested whether the reverse-transcribed and amplified L-DNAs could be used for sequencing, in an attempt to realize L-RNA sequencing using the L-DNA sequencing approach we have recently developed.22 We carried out the mirror-image reverse transcription experiment with a synthetic 76-nt L-tRNA template and a 5'-FAM-labeled L-DNA primer supplied with L-dNTPs, which was extended to full length after incubation for up to 24 h (Figure 3A). We were able to amplify the reverse-transcribed L-DNA by mirror-image PCR, and the amplification product was indeed resistant to natural DNase I digestion (Figure 3B). Next, we carried out the same mirror-image PCR experiment except that one of the primers was FAM-labeled at the 5'-terminus, followed by mirror-image sequencing (MI-seq) using a set of nucleobase-specific chemical cleavage reactions. We show that the expected sequence of the mirror-image reverse-transcribed and PCR-amplified L-DNA was determined by denaturing PAGE analysis (Figure 3C). Therefore, the sequence of synthetic L-RNAs can be determined using this complete mirror-image reverse transcription, PCR, and sequencing toolbox.

DISCUSSION

We have demonstrated the transcription of a mirror-image gene into L-RNA, as well as reverse transcription of L-RNA into L-DNA by synthetic D-polymerases, based on designed mutants of Dpo4. The efficient mirror-image transcription system may
enable enzymatic preparation of L-RNA molecules to further enable clinical applications of nuclease-resistant aptamer biosensors and drugs or studies on mirror-image or cross-chiral ribozymes and aptamers. The enzymatically transcribed L-5S rRNA shown in this study could be used as one component in a future effort to assemble a mirror-image ribosome, a step toward the realization of mirror-image life. The development of a mirror-image reverse transcription system serves as an important amendment to the mirror-image central dogma of molecular biology, with both conceptual and practical significance to the mirror-image biology field. For example, mirror-image reverse transcription may be applied to the quantification of L-RNAs when used in conjunction with mirror-image PCR to enable mirror-image quantitative reverse transcription PCR (MI-RT-qPCR). Furthermore, integrating a suite of mirror-image transcription, reverse transcription, PCR, and sequencing tools, as we have demonstrated in this study, may lead to many exciting applications. For example, if the efficiency and fidelity of the polymerases can be further improved, a mirror-image systematic evolution of ligands by exponential enrichment (MI-SELEX) scheme will enable direct in vitro selection of L-DNA or L-RNA aptamers against biological targets as potential research and therapeutic tools.

Figure 3. Mirror-Image Reverse Transcription, PCR Amplification, and Sequencing of L-tRNA
(A) Extension of a 14-nt 5’-FAM-labeled DNA primer on a synthetic 76-nt L-tRNA catalyzed by synthetic D-Dpo4-5m, carried out at 65°C for up to 24 h. The reverse transcription product was further treated by natural DNase I and analyzed by 12% denaturing PAGE in 8 M urea.
(B) Mirror-image PCR amplification of the reverse transcription product of L-tRNA by D-Dpo4-5m, sampled from multiple cycles. The mirror-image PCR product was further treated by natural DNase I and analyzed by 3% sieving agarose gel electrophoresis and stained by GoldView, with cycle numbers from which they were sampled indicated above the lanes. NC1, negative control without reverse transcription product. NC2, negative control without polymerase. M, DNA marker.
(C) Mirror-image sequencing by the Maxam-Gilbert approach. Four different sequence-specific cleavage reactions (C+T, C, A+G, A>G) were carried out and the cleaved products were analyzed in two sections (indicated by two colors) by 12% and 20% denaturing PAGE in 8 M urea, respectively.
While the relatively low fidelity and processivity of the Dpo4-based transcription and reverse transcription systems are sufficient for the preparation of short sequences of up to a couple of hundred nucleotides, they are apparently insufficient for the preparation and sequence validation of longer L-RNAs such as the 1.5-keb 16S and 2.9-keb 23S rRNAs. Polymerase engineering approaches, such as directed evolution, may be applied to discover polymerase mutants with improved fidelity and processivity. Moreover, it is technically difficult to prepare ssDNA templates longer than a couple of hundred nt and to purify transcribed RNA from the DNA template without the help of DNase. Through pushing the technology of protein chemical synthesis to the limit, efforts to chemically synthesize other polymerases, such as the T7 RNA polymerase for transcription and murine leukemia virus (MLV) reverse transcriptase for reverse transcription, may help address these issues.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-DNA and L-RNA oligos (Table S2) were synthesized on a MerMade-192e DNA synthesizer with L-deoxynucleoside or ribonucleoside phosphoramidites (ChemGenes, MA, USA). L-dNTPs and L-NTPs were purchased from ChemGenes. D-DNA and D-RNA oligos were ordered from IDOBIO (Beijing, China). Superscript III high-fidelity reverse transcriptase was purchased from Thermo Fisher Scientific (MA, USA) and Q5 high-fidelity DNA polymerase was purchased from New England Biolabs (MA, USA). All the DNA and RNA oligos were purified by HPLC and denaturing PAGE. The PAGE DNA Purification Kit was purchased from Tiandz (Beijing, China). Natural Dpo4-5m and Dpo4-5m-Y12A/G/S were expressed in *E. coli* and purified as described before. D-Dpo4-5m and D-Dpo4-5m-Y12S were chemically synthesized and folded as described before.

**Primer Extension by Dpo4 Mutants**

In the primer extension experiments with 41-nt templates in the natural chirality system, all the reactions were performed in a buffer containing 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 0.1 mg/ml BSA, 800 μM (each) dNTPs or NTPs, 0.5 μM 5’-FAM-labeled DNA or RNA primer, 1 μM DNA or RNA template, and ~500 nM polymerase. Prior to the addition of polymerase, the reaction system was heated to 95°C for 2 min and slowly cooled to 4°C for annealing. Primer extension reactions took place at 65°C for 1 h. The reactions were stopped by the addition of a loading buffer containing 98% formamide, 0.25 mM EDTA, and 0.0125% SDS, and the products were analyzed by 12% denaturing PAGE in 8 M urea and scanned by a Typhoon Trio⁺ system operated under Cy2 mode.

**Mirror-Image Transcription**

The transcription of L-5S rRNA was performed in a buffer containing 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 0.1 mg/ml BSA, 100 μM (each) L-NTPs, 0.5 μM 5’-FAM-labeled L-RNA primer, 0.5 μM L-ssDNA template, and ~500 nM Dpo4-5m-Y12S. Prior to the addition of polymerase, the reaction system was heated to 95°C for 2 min and slowly cooled to 4°C for annealing. Primer extension reactions took place at 65°C for up to 18 h. The transcription product was digested by 1 U RNase H (Thermo Fisher Scientific, USA) at 37°C for 5 min. The reactions were stopped by the addition of a loading buffer containing 98% formamide, 0.25 mM EDTA, and 0.0125% SDS, and the products were analyzed by 12% denaturing PAGE in 8 M urea and scanned by a Typhoon Trio⁺ system operated under Cy2 mode. The transcription product was purified by denaturing PAGE at 55°C for up to 24 h to fully remove the L-ssDNA template and partially extended products.
Mirror-Image Reverse Transcription

The reverse transcription of L-5S rRNA and L-tRNA was performed in a buffer containing 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 0.1 mg/ml BSA, 800 µM (each) L-dNTPs, 0.5 µM 5'-FAM-labeled or 5'-Cy5-labeled L-DNA primer, 1 µM L-RNA template, and ~500 nM D-Dpo4-5m. Prior to the addition of polymerase, the reaction system was heated to 95°C for 2 min and slowly cooled to 4°C for annealing. Primer extension reactions took place at 65°C for up to 36 h. The products were digested by 1 U DNase I (New England Biolabs, USA) or RNase H (Thermo Fisher Scientific, USA) at 37°C for 5 min. The reactions were stopped by the addition of a loading buffer containing 98% formamide, 0.25 mM EDTA, and 0.0125% SDS, and the products were analyzed by 12% denaturing PAGE in 8 M urea and scanned by a Typhoon Trio+ system operated under Cy2 and Cy5 mode.

Mirror-Image PCR

The mirror-image PCR was performed in a buffer containing 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 3% DMSO, 0.1 mg/ml BSA, 200 µM (each) L-dNTPs, 1 µM (each) L-DNA primers, 1 µL mirror-image reverse transcription product, and ~500 nM D-Dpo4-5m. Prior to the addition of polymerase, the reaction system was heated to 95°C for 2 min and slowly cooled to 86°C. The PCR program settings of L-5S rRNA were 86°C for 3 min (initial denaturation), 86°C for 30 s (denaturation), 58°C for 3 min (annealing), and 65°C for 14 min (extension) for up to 35 cycles. The PCR program settings of L-tRNA were 86°C for 3 min (initial denaturation), 86°C for 30 s (denaturation), 50°C for 3 min (annealing), and 65°C for 8 min (extension) for up to 40 cycles. The PCR products were digested by 1 U DNase I (New England Biolabs, USA) at 37°C for 5 min. All the products were analyzed by 3% sieving agarose gel electrophoresis and stained by GoldView (Solarbio, China). The negative controls were performed without polymerase or reverse transcription product.

Mirror-Image Sequencing

The mirror-image reverse transcription products were amplified by mirror-image PCR and one of the primers was 5'-FAM labeled. The PCR products were purified by denaturing PAGE. An aliquot of 1 µL FAM-labeled L-DNA (10 µM) was mixed with 1.5 µg carrier E. coli genomic DNA and kept on ice. For the C+T reaction, the mixture was denatured by heating to 95°C for 2 min followed by quick chilling on ice, an aliquot of 20 µL 80% hydrazine hydrate was added and the mixture was incubated at 45°C for 3.5 min. For the C-specific reaction, the mixture was denatured by heating to 95°C for 2 min followed by quick chilling on ice, an aliquot of 10 µl 4 M NH₂OH·HCl (pH adjusted to 6.0 by trimethylamine) was added and the mixture was incubated at 90°C for 35 s. For the A+G reaction, an aliquot of 20 µL 66% formic acid was added and the mixture was incubated at room temperature for 3 min. For the A>C reaction, an aliquot of 10 µL 1.5 M NaOH/1 mM EDTA was added and the mixture was incubated at 90°C for 8 min. The C+T and C reactions were quenched by adding 200 µL 0.3 M sodium acetate, 2 µL glycogen (10 mg/ml), 2 µL EDTA (10 mM, pH 8.0), 5 µL yeast tRNA (10 mg/ml), and 1 mL absolute ethanol. The A+G reaction was quenched by adding 200 µL 0.3 M sodium acetate, 2 µL glycogen (10 mg/ml), 5 µL yeast tRNA (10 mg/ml), and 1 mL absolute ethanol. The A>C reaction was quenched by adding 100 µL 1 M sodium acetate, 2 µL glycogen (10 mg/ml), 5 µL yeast tRNA (10 mg/ml), and 1 mL absolute ethanol. The mixture was chilled in liquid nitrogen for 10 min and the DNA was precipitated by centrifugation at 12,000 rpm for 10 min and washed by 1 mL absolute ethanol. The residual ethanol was removed by evaporation, and the pellet was dissolved into 100 µL 1 M piperidine and...
incubated at 90°C for 30 min (C, A+G, A>C reactions) or into 120 μL 1 M piperidine and incubated at 90°C for 50 min (C+T reaction). After lyophilization, the remaining pellet was dissolved in a denaturation buffer containing 98% formamide, 0.25 mM EDTA, and 0.0125% SDS. The products were analyzed by 12% or 20% PAGE in 8 M urea and scanned by a Typhoon Trio+ system operated under Cy2 mode.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and two tables and can be found with this article online at https://doi.org/10.1016/j.chempr.2019.01.001.

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AUTHOR CONTRIBUTIONS
M.W., W.J., and X.L. performed the experiments. W.J. discovered the reverse transcription activity of Dpo4. J.W., B.Z., C.F., and L.L. synthesized the D-Dpo4 mutants. All authors analyzed and discussed the results. T.F.Z. designed and supervised the study and wrote the paper.

DECLARATION OF INTERESTS
T.F.Z., X.L., W.J., and M.W. have filed a provisional patent application related to this work.

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REFERENCES AND NOTES


