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A synthetic molecular system capable of mirror-image genetic replication and transcription

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The overwhelmingly homochiral nature of life has left a puzzle as to whether mirror-image biological systems based on a chirally inverted version of molecular machinery could also have existed. Here we report that two key steps in the central dogma of molecular biology, the template-directed polymerization of DNA and transcription into RNA, can be catalysed by a chemically synthesized D-amino acid polymerase on an L-DNA template. We also show that two chirally mirrored versions of the 174-residue African swine fever virus polymerase X could operate in a racemic mixture without significant enantiomeric cross-inhibition to the activity of each other. Furthermore, we demonstrate that a functionally active L-DNAzyme could be enzymatically produced using the D-amino acid polymerase. The establishment of such molecular systems with an opposite handedness highlights the potential to exploit enzymatically produced mirror-image biomolecules as research and therapeutic tools.

Despite biology's seemingly limitless diversity and the vastness of its territories that permeate into virtually every corner of the Earth, at the fundamental level of biochemistry, all known forms of life are narrowly defined by a single version of molecular machinery based on L-amino acids and D-ribose nucleic acids. Although rare examples of the use of D-amino acids, such as D-aspartic acid in animal brains, and L-sugars, such as L-arabinose in plants, do exist[1,2], the central dogma and most of the biological macromolecules have followed the homochirality established by life's earliest ancestors. Processes that led biology onto this particular chiral path have remained largely elusive, even though experimental evidence for breaking the mirror symmetry has been reported and many theoretical models have been proposed[3–6]. Recently, an *in vitro* selected catalytic RNA capable of incorporating nucleotides in a cross-chiral fashion without enantiomeric cross-inhibition was reported[7]. The fact that no known laws of physics and chemistry preclude biology's use of either of the two chiral systems, mirror-image twins of one another, has led to an intriguing question as to whether a parallel mirror-image world of biology running on a chirally inverted version of molecular machinery could be found in the universe or be created in the laboratory[8].

We reasoned that towards synthesizing a mirror-image biological system, an imperative step would be to reconstitute a chirally inverted version of the central dogma of molecular biology with D-amino acid enzymes and L-ribose nucleic acids—although reconstituting a mirror-image, ribosome-based translation system through the total synthesis of all the ribosomal RNA (rRNA) and protein building blocks is still beyond the current technology, the total chemical synthesis of (small enough) mirror-image polymerases might be feasible. Here we set out to synthesize such a mirror-image polymerase and to test if two steps in the central dogma, the template-directed polymerization of DNA and the transcription into RNA, can be carried out in a synthetic mirror-image molecular system (Fig. 1a).

Recent advances in synthetic organic chemistry have made it possible to chemically synthesize biological macromolecules, such as peptides and nucleic acids of near-arbitrary chirality, revealing the essential properties of mirror-image enzymes and nucleic acids, such as their chiral substrate specificity and biochemical properties that mirror those of their natural counterparts[9–12]. In solid-phase peptide synthesis, owing to the accumulation of resin-bound by-products, peptides longer than 50 residues are often difficult to synthesize directly. Thus, the native chemical ligation (NCL) method was developed to ligate short peptide segments into longer ones through native peptide bonds[13]. Using peptide hydrazides as thioester surrogates[14], the synthesis of D-amino acid peptides as long as 312 residues has been demonstrated[15]. Nonetheless, the complete chemical synthesis of the 832-residue Taq polymerase or the ~604-residue Klenow Fragment is still beyond reach with the current methods. Therefore, we turned to the smallest known DNA polymerase, the 174-residue African swine fever virus polymerase X (ASFV pol X)[16], as a model system for the synthesis of a mirror-image polymerase.

Results and discussion

Complete chemical synthesis and folding of a functional mirror-image polymerase. The first step in our effort to synthesize a mirror-image polymerase was to design a synthetic route for producing the D-amino acid ASFV pol X by assembling three peptide segments in the C- to N-terminus direction using hydrazides as thioester surrogates[15,17–19] (Supplementary Figs 1–5). One of the hurdles that we encountered during the synthesis of the D-ASFV pol X was that the high hydrophobicity of the peptide segment H_2N-[D-Cys^{86}(Acm)-D-Leu^{105}]-CON_2H_3 (Acm, acetamidomethyl (see Supplementary Methods)) significantly affected the synthesis and purification efficiency. We overcame this problem by incorporating an isoacyl dipeptide (isoacyl D-Lys^{95}-D-Thr^{96}) into the segment, which reduced the peptide's hydrophobicity, but in the meantime worked as a traceless modification to the peptide because of the rapid O-to-N acyl shift under NCL conditions at pH ~7 (refs 20, 21), leading to the complete chemical synthesis of D-ASFV pol X.

Having achieved the complete chemical synthesis of D-ASFV pol X, we purified the polypeptide by reversed-phase high-performance liquid chromatography (RP-HPLC). We analysed the polypeptide by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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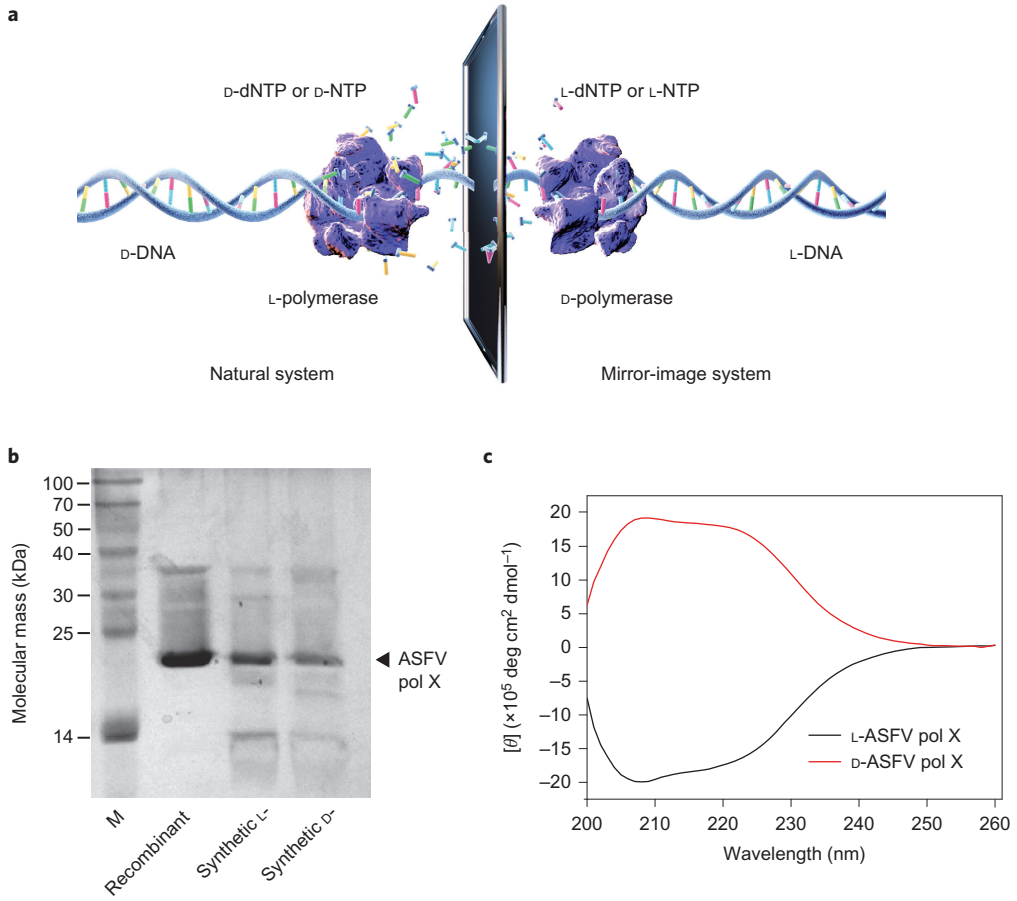

Figure 1 | Two chirally mirrored polymerase systems with synthetic L- and D-ASFV pol X. a, Template-directed primer extension by an L-polymerase on a D-DNA template, and its mirror-image system, the template-directed primer extension by a D-polymerase on an L-DNA template. **b**, Chemically synthesized and folded L- and D-ASFV pol X, as well as purified recombinant L-ASFV pol X from the *E. coli* strain BL21(DE3)pLySs, were analysed by 15% SDS-PAGE (silver stained) with a fraction of unligated peptide segments observed in the synthetic L- and D-ASFV pol X. M, marker. **c**, CD spectra of the synthetic L- and D-ASFV pol X. The CD curves were averaged from three independent measurements and background subtracted.

(SDS–PAGE) (Fig. 1b; with a fraction of unligated peptide segments observed in the synthetic L- and D-ASFV pol X) and electrospray ionization mass spectrometry (ESI-MS) to validate the molecular mass (observed 20,315.9 Da, calculated 20,316.5 Da) (Supplementary Fig. 5). The synthetic polypeptide was folded by successive dialysis against several renaturation buffers that contained different concentrations of guanidine hydrochloride (Gn·HCl) (see Methods). The disulfide bond between D-Cys[81] and D-Cys[86] was formed by air oxidation and validated by ESI-MS (observed molecular mass 20,313.8 Da, calculated 20,314.5 Da) (Supplementary Fig. 6). In parallel, we also synthesized the natural L-ASFV pol X using the same synthetic route, but with L-amino acids, purified and folded by the same procedures. We analysed the peptide sequence of the synthetic L-ASFV pol X by LC-MS/MS to validate our synthetic route (Supplementary Table 1), although the synthetic D-ASFV pol X was not sequenced because it could not be digested effectively by trypsin. In addition, analysis of the L- and D-polymerases by circular dichroism (CD) revealed chirally symmetric spectra, as expected (Fig. 1c), which suggests that the chemically synthesized L- and D-ASFV pol X both folded properly and were mirror images of each other.

Mirror-image DNA polymerization. We next examined and compared the polymerization activities of the two chirally mirrored polymerase systems. The activity of synthetic D-ASFV pol X was tested on an L-DNA template with an L-DNA primer and L-deoxyribonucleotide triphosphates (L-dNTPs) in an achiral buffer (Tris-HCl, pH 7.5) with 20 mM Mg^{2+}, and the same buffer condition was used to test the activity of the L-ASFV pol X system[16]. The L-DNA oligonucleotides and L-dNTPs were obtained commercially, the purity and chirality of which were assessed by HPLC and CD (Supplementary Fig. 7). A technical issue that we encountered when handling the L-DNA primers was that they could not be radiolabelled efficiently through phosphorylation by T4 Polynucleotide Kinase (Supplementary Fig. 8), which has also been reported in previous studies on L-DNA aptamers[22]. Thus, as an alternative approach, we used fluorophores, such as fluorescein amidite (FAM), to label the 5′ end of the L-primers (as well as the D-primers for a fair comparison).

We showed that both the natural and the mirror-image systems extended the corresponding 12 nucleotide (12 nt) primers to the full length of 18 nt in about four hours (Fig. 2a). However, the quantified polymerization efficiency of the mirror-image system, with a measured k_{obs} (observed rate of reaction, defined from the rate of disappearance of primers) of $3.3 \pm 0.3\ h^{-1}$, appeared to be lower than that of the natural one ($k_{obs} = 6.0 \pm 0.8\ h^{-1}$) (Supplementary Fig. 9), and partial stalling at the +1 nt position was observed with both the natural and the mirror-image systems (Fig. 2a). We reasoned that many differences in the purity of the L- and D-amino acids, the D- and L-DNA templates, primers and dNTPs may contribute to the difference in the observed rate of reaction. Indeed, purity differences of the commercially supplied D- and L-DNAs were observed in the HPLC and CD spectra

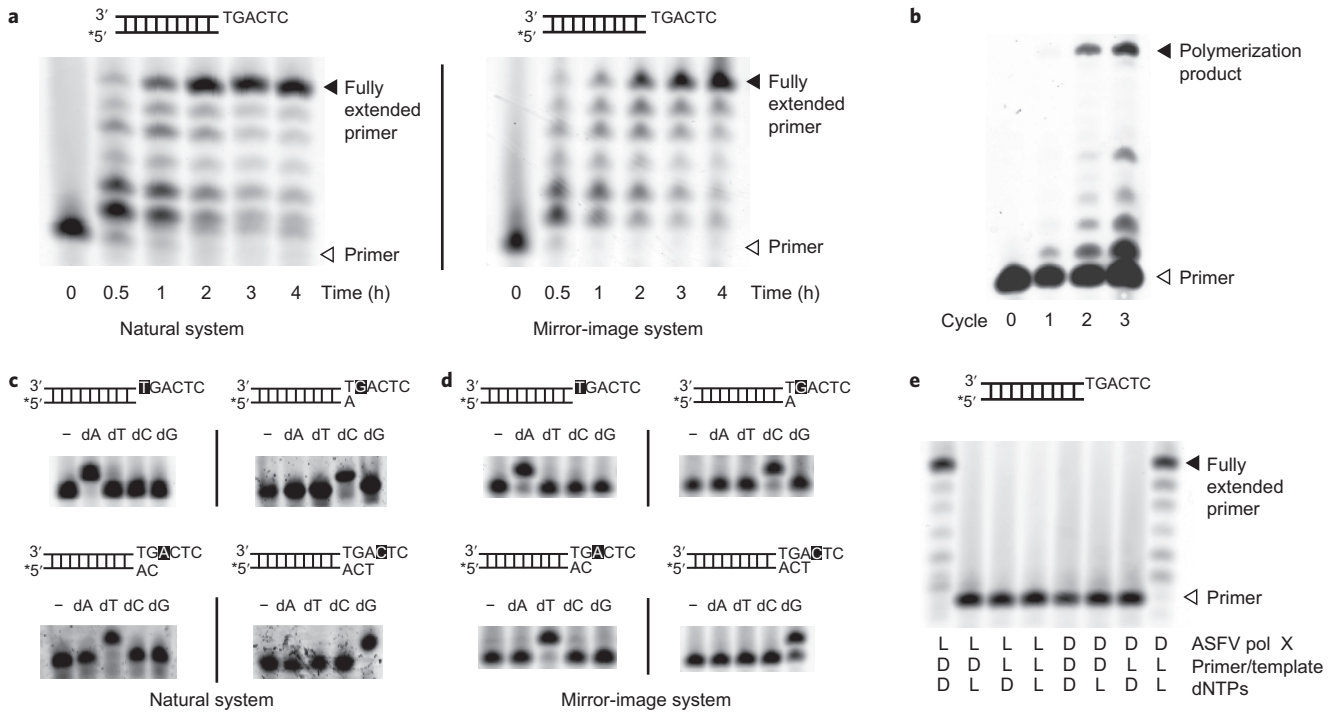

Figure 2 | Template-directed DNA polymerization by synthetic L- and D-ASFV pol X. a, Template-directed primer extension by synthetic L-ASFV pol X (natural system) and D-ASFV pol X (mirror-image system) in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, with the corresponding D- and L-DNA primers, templates and dNTPs, incubated for up to 4 h at 37 °C, and analysed by 20% PAGE in 8 M urea. **b**, Repeated cycles of polymerization by D-ASFV pol X: the amount of FAM-labelled full-length L-DNA products increased by ~2.3-fold (measured by fluorescence). Cycle 0, control lane before the first cycle of the primer extension. **c**,**d**, The nucleotide substrate specificities of synthetic L- and D-ASFV pol X were assayed by individually adding the four D-dNTPs or L-dNTPs (0.2 mM each), respectively, on four different primer/template pairs varying only the next template site for extension (highlighted), incubated for 30 min at 37 °C and analysed by 20% PAGE in 8 M urea. –, control experiment without D-dNTP or L-dNTPs added. **e**, Chiral specificity assay with different chiral combinations of polymerases, primer/template pairs and dNTPs. Reaction conditions were the same as those described in **a** except that the incubation of all the samples was for 12 h at 37 °C. The samples were analysed by 20% PAGE in 8 M urea. *, 5′-FAM label.

(Supplementary Fig. 7). We next validated the molecular mass of the fully extended, purified L-DNA by ESI-MS (observed 5,516.9 Da, calculated 5,517.6 Da (Supplementary Fig. 8)), because most of the current DNA sequencing techniques rely on the use of natural DNA polymerases or ligases and thus are not compatible for sequencing L-DNA directly. Furthermore, we showed that the mirror-image polymerization could be carried out with other primer and template pairs of different lengths and sequences (Fig. 4b and Supplementary Fig. 8), which suggests the robustness and generality of the mirror-image polymerase system despite the lower polymerization efficiency.

Next, we tested if the mirror-image polymerase system was capable of synthesizing more copies of L-DNA molecules through repeated cycles of polymerization. We reasoned that, although ASFV pol X is not thermal stable, a proof-of-concept multicycle polymerization reaction with freshly added enzymes could be achieved. With a small number of polymerization cycles (limited by the necessity to remove and add fresh enzymes, and by the amount of available synthetic enzymes (see Methods)), it is difficult to detect a noticeable increase of the polymerization products, and hence we chose to FAM label one of the L-DNA primers that could only be extended to the full length efficiently when annealed to the enzymatically polymerized L-DNA from the first polymerization cycle (for the experimental design, see Supplementary Fig. 8). We showed that the amount of FAM-labelled full-length L-DNA products increased by ~2.3-fold as measured by fluorescence (Fig. 2b)—in an ideal polymerase chain reaction (PCR)-like reaction this ratio should be approaching three, as shown in Supplementary Fig. 8. These results suggest that more copies of the newly synthesized L-DNA molecules can, indeed, be produced by the mirror-image polymerase system through repeated cycles of polymerization.

Nucleotide substrate specificity and chiral specificity of mirror-image polymerization. It is crucial to confirm that the mirror-image DNA polymerization does occur in a template-directed manner. We tested the nucleotide substrate specificity of the mirror-image polymerization reaction by supplying the four L-dNTPs individually on four different primer/template pairs varying only the next template site for extension (a total of 16 possible combinations (Fig. 2c,d)). We showed that the L-dNTPs could only be incorporated efficiently with the four complementary nucleotides on the template (A:T, T:A, C:G and G:C), with no significant mispairing observed. This result suggests that, even though ASFV pol X has been reported to be more error-prone compared with its peers in the modern polymerase arsenal[23], both the natural and the mirror-image polymerases possess a reasonable nucleotide substrate specificity that obeys Watson–Crick base pairing, and the polymerization reactions were template-directed without an apparent sequence bias (Fig. 2c,d).

To investigate if the natural and the mirror-image polymerization reactions occurred with sufficient chiral specificity, we tested different chiral combinations of L- or D-polymerases, D- or L-DNA primer/template pairs and D- or L-dNTPs (a total of eight combinations (Fig. 2e)). We showed that the primer extension only occurred with the L-polymerase on a D-DNA primer/template pair supplied with D-dNTPs (the natural system) and with the D-polymerase on an L-DNA primer/template pair supplied with

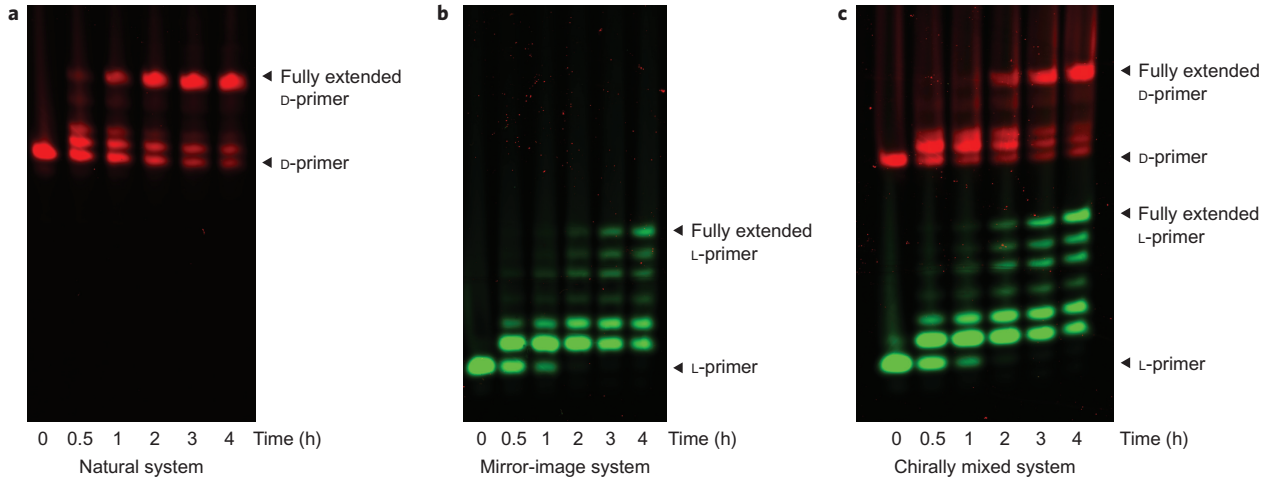

Figure 3 | Polymerization of the two chirally mirrored systems in a racemic mixture. a,b, Primer extension by synthetic L- and D-ASFV pol X with the corresponding D-DNA primer (5′-Cy5 labelled) and L-DNA primer (5′-FAM labelled), templates and dNTPs. The independent reactions were carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl, for up to 4 h at 37 °C, and analysed by 20% PAGE in 8 M urea. **c**, The above two polymerization reactions were carried out in a racemic mixture under the same conditions as described above, with the L- and D-ASFV pol X, D- and L-primers, D- and L-templates and D- and L-dNTPs added, incubated for up to 4 h at 37 °C. The mixed reaction products were analysed by 20% PAGE in 8 M urea and scanned by a Typhoon Trio+ system operated under both Cy5 and FAM scanning modes.

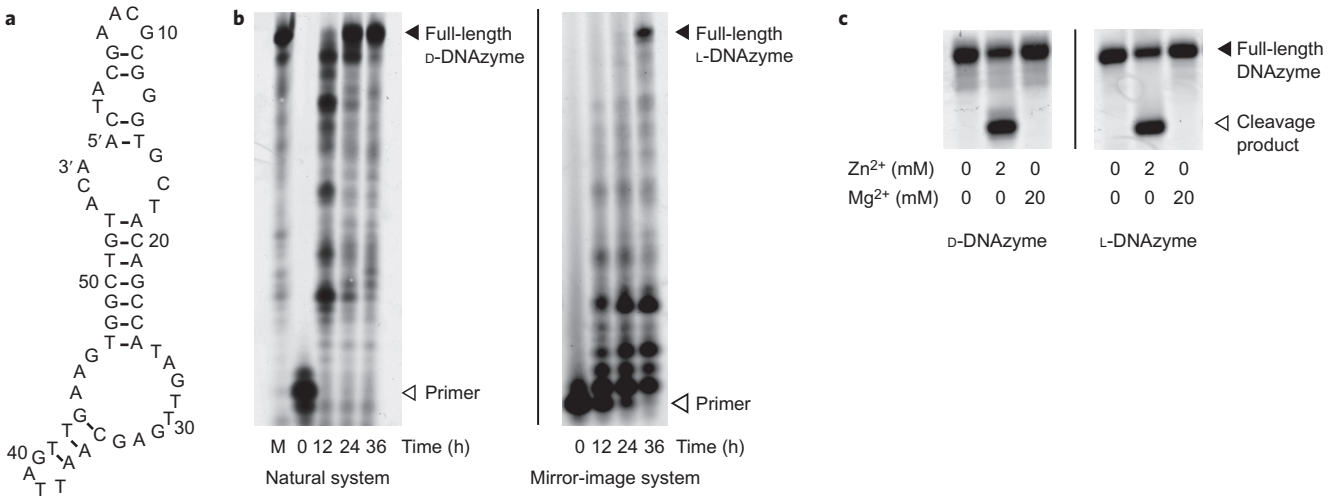

Figure 4 | Enzymatic polymerization of a mirror-image DNAzyme of active functions. a, Sequence and predicted secondary structure of the previously reported Zn^{2+}-dependent self-cleaving DNAzyme[29]. **b**, Primer extension on a 66 nt template to produce the Zn^{2+}-dependent self-cleaving DNAzyme. Both the natural and the mirror-image polymerization reactions were carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl, and analysed by 12% PAGE in 8 M urea. Both the D- and L-primers were 5′-FAM labelled. M, marker: a chemically synthesized 56 nt full-length 5′-FAM-labelled DNAzyme with the same sequence and length as the expected fully extended primer. **c**, Self-cleavage of the enzymatically polymerized Zn^{2+}-dependent D- and L-DNAzymes. The DNAzyme template was designed so that it was 10 nt longer (at the 3′ end) than the fully extended primer for the separation and purification by denaturing urea PAGE. The PAGE-purified, 5′-FAM-labelled D- and L-DNAzymes were incubated in 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM $ZnCl_2$ or 20 mM $MgCl_2$ at 37 °C for 36 h and analysed by 12% PAGE in 8 M urea.

L-dNTPs (the mirror-image system), but not any of the other combinations, even after 12 hours of incubation (Fig. 2e). This result suggests a strict chiral specificity of the natural and the mirror-image polymerases on their substrates for polymerization, a property crucial for the polymerase systems to avoid enantiomeric cross-inhibition, as discussed below.

Polymerization of the two chirally mirrored systems in a racemic mixture. It has been shown previously that different polymerase enzymes possess a range of chiral specificities with enantiomeric nucleotide substrates: some are affected by enantiomeric cross-inhibition whereas others are not[24–27]. The above-demonstrated chiral specificity of ASFV pol X (Fig. 2e) suggests the absence of enantiomeric cross-inhibition in this polymerase system[28]. Indeed, we showed that the two chirally mirrored polymerase systems could operate in a racemic mixture with only small differences of k_{obs} observed (Fig. 3 and Supplementary Fig. 9). Thus, the L- and D-ASFV pol X-catalysed DNA polymerization appeared unaffected by significant enantiomeric cross-inhibition in a racemic mixture.

Enzymatic polymerization of a mirror-image DNAzyme of active functions. To demonstrate further the ability of the mirror-image polymerase system to synthesize long, functional L-DNA molecules, we tested the polymerization on an L-DNA template

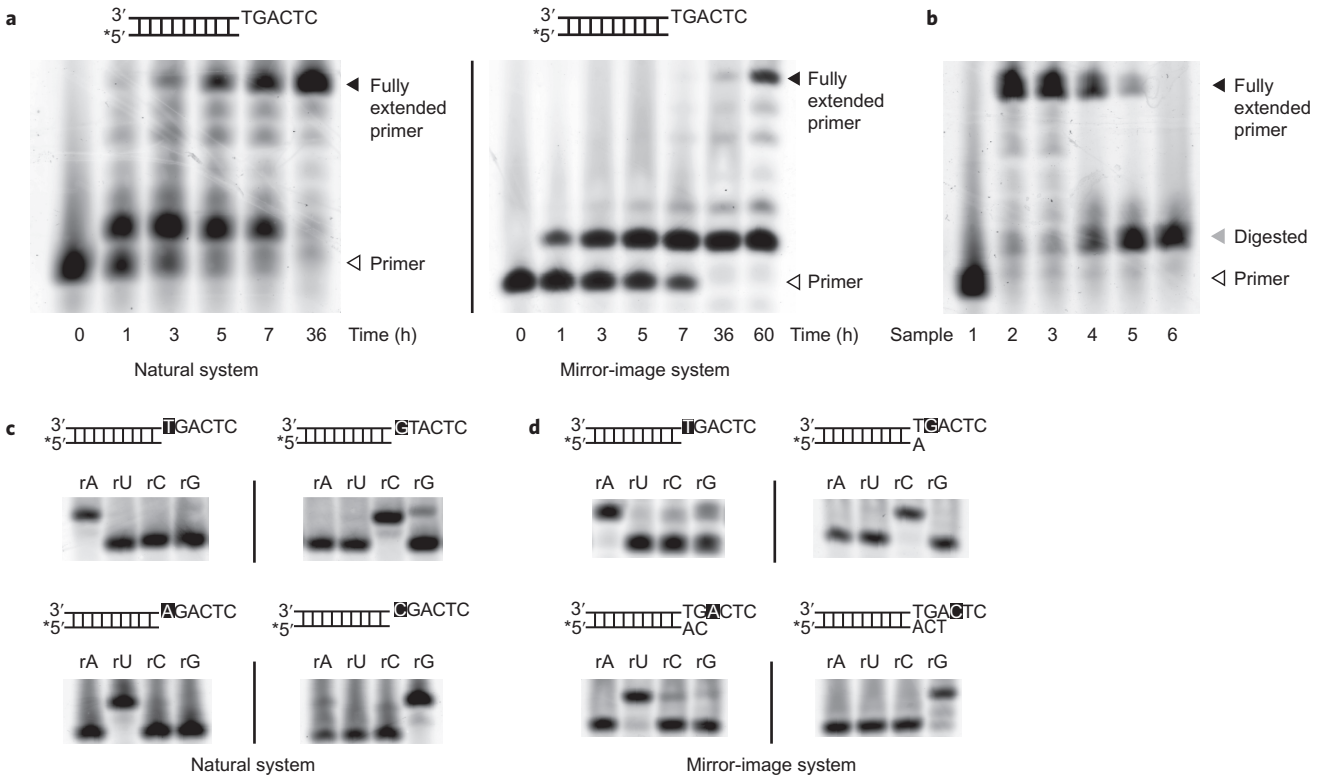

Figure 5 | DNA-templated polymerization of mirror-image RNA. a, DNA-templated RNA polymerization by synthetic L- and D-ASFV pol X with 5′-FAM-labelled D- and L-primers, D- and L-templates and D- and L-NTPs, incubated for up to 60 h at 37 °C and analysed by 20% PAGE in 8 M urea. **b**, RNase A digestion of the full-length extension product. Sample 1, 5′-FAM-labelled 12 nt D-DNA primer; Sample 2, fully extended primer after 36 h of D-primer extension with D-NTP on a D-DNA template; Sample 3, fully extended primer after being heated to 75 °C for 10 min to inactivate the polymerase and RNase inhibitor; Samples 4–6, fully extended primers treated by RNase A at concentrations of 0.01, 0.1 and 1 μg μl^{-1}, respectively, and incubated for 10 min at 23 °C. The digestion reactions were terminated by the addition of 20 units of RNase inhibitor and the products were analysed by 20% PAGE in 8 M urea. **c,d**, The nucleotide substrate specificity of the DNA-templated polymerization of RNA by synthetic L- and D-ASFV pol X was assayed by individually adding the four D-NTPs or L-NTPs (0.2 mM each), respectively, on four different primer/template pairs varying only the next template site for extension (highlighted), incubated at 37 °C for 12 h and analysed by 20% PAGE in 8 M urea. *, 5′-FAM label.

that coded for a previously reported 44 nt Zn^{2+}-dependent self-cleaving DNAzyme (Fig. 4a)[29]. The extension of the L-DNAzyme (to a total length of 56 nt, including a 12 nt primer sequence) was completed in 36 hours (Fig. 4b), which demonstrated the ability of the mirror-image polymerase system to polymerize full-length products on templates of such lengths. A technical problem was encountered when we attempted to separate the synthesized single-stranded (ss) L-DNA from the L-DNA template: efforts to biotin label the 5′ end of the L-DNA template and to separate the ssDNA on a streptavidin column suffered from a low recovery efficiency. To circumvent this problem, we developed an alternative method by using a longer L-DNA template so that the template would be 10 nt longer (at the 3′ end) than the fully extended L-DNAzyme, a sufficient length difference for the separation and purification by denaturing urea PAGE (see Methods). Using the same approach, a D-DNAzyme of the same length and sequence was polymerized by synthetic L-ASFV pol X, purified by PAGE and sequence validated by Sanger sequencing (Supplementary Fig. 8).

We showed that in the presence of 2 mM Zn^{2+}, the L-DNAzyme self-cleaved and the cleavage efficiency was comparable to that of the D-DNAzyme (Fig. 4c). Under control conditions (in a buffer that contained Mg^{2+} without Zn^{2+}), neither the natural nor the mirror-image DNAzymes exhibited self-cleavage activity (Fig. 4c). Such enzymatically produced mirror-image DNAzymes and other nucleic acid polymers may develop into excellent nuclease-resistant research and therapeutic tools for their ability to evade nuclease-mediated degradation, as shown in previous studies on nuclease-insensitive mirror-image aptamers that bind natural biological targets[22,30]. Although the current D-ASFV pol X system is still inefficient and the chemical synthesis of mirror-image polymerases is expensive and time-consuming, if thermal stable, more efficient mirror-image polymerase systems[31] could be developed in the future through improved chemical synthesis schemes, one may harvest mirror-image nucleic acid polymers through enzymatically catalysed PCR or *in vitro* transcription, as an alternative to the current production approach of chemically synthesizing therapeutic L-DNAs/RNAs.

DNA-templated polymerization of mirror-image RNA. Having demonstrated the enzymatic, mirror-image polymerization of L-DNA on an L-DNA template, we asked if L-DNA-templated transcription into L-RNA could also be performed by the mirror-image polymerase. It has been known that a number of DNA polymerases in the X family are capable of incorporating ribonucleotides in a template-directed manner[32], although this has not been demonstrated with ASFV pol X. We first tested if ASFV pol X has RNA polymerase activity by examining the natural L-polymerase on a 5′-FAM-labelled D-DNA primer and a D-DNA template, but supplied by D-nucleoside triphosphates (D-NTPs) instead of D-dNTPs. To our surprise, although less efficient than DNA polymerization, a fully extended primer was obtained in 36 hours (Fig. 5a). The RNA part of the product was digested successfully by RNase A, a ribonuclease that specifically cleaves

3′,5′- but not 2′,5′-phosphodiester linkages, which suggests that the ASFV pol X-polymerized RNA molecules had 3′,5′-phosphodiester linkages (Fig. 5b).

Next, we showed that the polymerization of L-RNA could also be performed by D-AFSV pol X with an L-DNA primer on an L-DNA template supplied with L-NTPs, although this appeared to be less efficient than the mirror-image DNA polymerization reaction. Partial stalling at the +1 nt position was observed with both the natural and the mirror-image systems (and was more pronounced with the mirror-image system) (Fig. 5a). We next tested the nucleotide substrate specificity of the reaction by supplying the four L-NTPs individually, and we showed that the L-RNA polymerization also appeared to be template-directed, although the substrate specificity appeared to be lower than that of DNA polymerization, with a number of mispairing events observed (T:rG, A:rC and so on (Fig. 5d)). The above experiments suggest that the L-DNA-templated transcription into L-RNA with a decent substrate specificity could be achieved by the D-ASFV pol X system.

Conclusions

The mirror-image polymerase system capable of template-directed polymerization of DNA and transcription into RNA described here, along with other reported mirror-image enzyme and nucleic acid systems, have revealed the essential properties and functions of mirror-image biological molecules, many of which are comparable to their chiral-twin counterparts[7,9–12,15,17,22]. Undoubtedly, the efficiency of the current mirror-image polymerase system remains to be improved, particularly for the DNA-templated polymerization of RNA. Although optimizing procedures for the chemical synthesis and folding of the mirror-image polymerase and refining the substrate purity may help to improve the performance of the system, we attribute the efficiency problem mainly to the intrinsic property of ASFV pol X, which has been shown previously[16]. Moreover, the thermal stability of ASFV pol X does not allow PCR to be carried out without the addition of fresh enzymes in each cycle (we tested at 50 °C for one minute (in 3.5 M proline), being the highest temperature condition ASFV pol X can withstand without losing its activity). Thus, optimization of the model mirror-image polymerase should be carried out either through the directed evolution and optimization of the polymerase itself[33], or by choosing other more efficient and thermal stable (yet still small enough) polymerase systems, such as the 352-residue *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) for the complete chemical synthesis[31].

The next step in the effort to reconstitute a chirally inverted version of the central dogma of molecular biology would be to build a mirror-image ribosome-based translation system, possibly through the synthesis of ribosomal RNA and protein building blocks, followed by the *in vitro* self-assembly of the components[34]. Bacterial ribosomes typically consist of a large number (50–80) of small ribosomal proteins and, with the exception of the 557-residue rpsA protein, most of the ribosomal proteins are no longer than 240 residues[35], which makes them suitable candidates for the complete chemical synthesis. If the PCR of longer L-DNA templates and the transcription of longer L-RNAs become feasible, assembled with chemically synthesized ribosomal proteins, a reconstituted mirror-image central dogma would be able to produce all of the components required for building itself, as well as other essential molecular machinery (for example, helicase, DNA primase, DNA ligase and RNA polymerase, and so on). If more efficient versions of these mirror-image enzymes could be developed, a simplest version of mirror-image machinery could become capable of self-replication and evolve into more complex forms as in Darwinian evolution[36]. Even though the synthesis of a completely self-sustained mirror-image cell would still require an enormous number of problems to be solved, if self-sustained mirror-image cells could be created eventually, they may become powerful tools that enable many applications, such as enzymatically producing chiral-specific pharmaceuticals and creating virus-resistant mirror-image bacteria as biofactories.

Methods

Folding of synthetic D-ASFV pol X. Lyophilized D-ASFV pol X (5 mg) was dissolved in 10 ml of 6 M Gn·HCl and dialysed against a series of renaturation buffers that contained 4, 2, 1 and 0 M Gn·HCl. The renaturation buffer also contained 50 mM Tris-HCl, pH 7.4, 40 mM KCl, 6 mM $(AcO)_2Mg$, 0.01 M EDTA and 16% glycerol. Each step of the dialysis took place at 4 °C for ten hours with gentle stirring. The disulfide bond between D-Cys^{81} and D-Cys^{86} was formed during the folding process by air oxidation.

DNA-polymerization assay. The HPLC-purified L-DNA oligonucleotides, L-dNTPs and L-NTPs were purchased from ChemGenes. The polymerization reactions were carried out in a reaction buffer that contained 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 mM KCl, with 0.7 μg of L- or D-ASFV pol X, 2.5 μM FAM-primer12, 2.5 μM template18 and 0.2 mM dNTPs (each), incubated at 37 °C. The reaction was terminated by the addition of EDTA, and immediately mixed with loading buffer that contained deionized formamide, Xylene Cyanol FF and bromophenol blue for PAGE analysis. The reaction products were separated by 20% PAGE in 8 M urea, and visualized by the Typhoon Trio+ system. For testing the nucleotide substrate specificity, all the conditions were as above except that 0.2 mM D- or L-dNTPs (each) were added, and the reactions took place at 37 °C for 30 minutes. To reduce the cost of synthesizing the L-DNA primers, the primers of different lengths used for testing the nucleotide substrate specificity of the mirror-image reaction system were obtained by primer extension of 5′-FAM-L-primer12 by D-ASFV pol X with L-dNTPs and purified by PAGE. The chirally mixed experiment was carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl that contained 0.2 mM dNTPs (each). The natural system contained 0.7 μg of L-ASFV pol X, 2.5 μM D-template26 and D-Cy5-primer20. The mirror-image system contained an equal quantity of D-ASFV pol X, 2.5 μM L-FAM-primer12 and L-template18 for kinetic quantitation, although for gel imaging twice as much the polymerase was used for an optimized covisualization of both reactions in the same gel. The extended FAM- or Cy5-labelled primers were analysed by 20% PAGE in 8 M urea, and scanned by the Typhoon Trio+ system under the 670 BP 30 Cy5 and 520 BP 40 CY2, ECL+ and Blue FAM modes. The repeated cycles of polymerization were carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl that contained 2.672 μg of D-ASFV pol X, a total of 7.5 μM L-FAM-primer11 and 7.5 μM L-reverse11, 1.25 μM L-template27 and 0.4 mM dNTPs (each), incubated at 37 °C for four hours. As ASFV pol X tends to stay bound to the DNA after polymerization (even after being heated to 95 °C, observed by electrophoresis), we had to remove the enzyme by phenol/chloroform extraction followed by isopropanol precipitation after each polymerization cycle (which places a limit on the number of cycles that can be carried out as a result of the limited extraction efficiency). In the second round of polymerization, the reaction system was heated to 95 °C for 30 seconds and then cooled to room temperature for annealing. The polymerization was again initiated by the addition of 0.334 μg of D-ASFV pol X and the sample was incubated at 37 °C for 20 hours. The third cycle of polymerization followed the same procedures. The reaction products were analysed on 20% PAGE (with the sample loading adjusted by the efficiency of phenol/chloroform extraction determined by a control experiment) with Typhoon Trio+. Gel quantitation was performed by the ImageQuant software.

Polymerization of DNAzyme and cleavage assay. The DNAzyme synthesis was carried out in a 100 μl reaction system in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl that contained 14.5 μg of L-ASFV pol X or 28.9 μg of D-ASFV pol X, 5 μM FAM-primer12, 5 μM DNAzymeTemplate and 0.4 mM dNTPs (each), incubated at 37 °C for up to 36 hours. The secondary structure of the Zn^{2+}-dependent self-cleaving DNAzyme is drawn based on Mfold predictions[37]. The marker used was a chemically synthesized 56 nt full-length 5′-FAM-labelled DNAzyme with the same sequence and length as the expected fully extended primer. The fully extended primers were purified by 12% PAGE in 8 M urea. The DNAzyme template was designed so that it was 10 nt longer (at the 3′ end) than the fully extended primer for separation and purification by denaturing urea PAGE. The precipitated PAGE-purified product was dissolved in a buffer that contained 50 mM HEPES, pH 7.0, and 100 mM NaCl, and incubated at 90 °C for two minutes before being cooled on ice for five minutes. The cleavage reactions were initiated by adding an equal volume of buffer that contained 50 mM HEPES, pH 7.0, 100 mM NaCl and 4 mM $ZnCl_2$ or 40 mM $MgCl_2$ (to a final concentration of 2 mM $ZnCl_2$ or 20 mM $MgCl_2$), as indicated. The reaction system was incubated at 37 °C for 36 hours, before being quenched by the addition of EDTA and analysed on 12% PAGE with Typhoon Trio+.

DNA-templated RNA polymerization and RNase A digestion. RNA polymerization by the natural system was carried out in a 10 μl reaction system in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl that contained 0.7 μg of L-ASFV pol X, 2.5 μM D-FAM-primer12, 2.5 μM D-template18, 0.2 mM D-NTPs

(each) and 20 units of recombinant RNase inhibitor, incubated at 37 °C. The mirror-image RNA polymerization was carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 50 mM KCl that contained 1.4 μg of D-ASFV pol X, 5 μM L-FAM-primer12, 5 μM L-template18 and 0.4 mM L-NTPs (each), incubated at 37 °C. The polymerized full-length D-DNA(primer)–RNA chimera, after being heated to 75 °C for ten minutes to inactivate the ASFV pol X and RNase inhibitor, was subjected to RNase A digestion. Various concentrations of RNase A (0.01, 0.1 and 1 μg μl^{-1}) were used, and incubated at 23 °C for ten minutes. The digestion reactions were terminated by adding 20 units of RNase inhibitor and loading buffer that contained deionized formamide, Xylene Cyanol FF and bromophenol blue, and analysed by 20% PAGE in 8 M urea. To examine the nucleotide substrate specificity of the RNA polymerization by D-ASFV pol X, all the conditions were the same as above except that different templates (template18, template18A, template18C and template18G) and individual NTPs were added as indicated, and incubated at 37 °C for 12 hours. Control experiments without D- or L-NTPs added were not performed because the same conditions have already been tested (Fig. 2c,d). To reduce the cost of synthesizing L-DNA primers, the primers of different lengths used for testing the nucleotide substrate specificity of the mirror-image reaction system were obtained by primer extension of 5′-FAM-L-primer12 by D-ASFV pol X with L-dNTPs and purified by PAGE.

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Author contributions

L.L. and W.X. designed the synthetic route and implemented the chemical synthesis of ASFV pol X. Z.W. and W.X. performed the biochemistry and analytical experiments, and contributed equally to this work. T.F.Z. conceived the idea and wrote the paper. T.F.Z. and L.L. designed and supervised the study.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to L.L. and T.F.Z.

Competing financial interests

The authors have filed a provisional patent application related to this work.