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While DNA circuits are becoming increasingly useful as signal transducers, their utility is inhibited by their slow catalytic rate. Here, we demonstrate how RecA, a recombination enzyme that catalyzes sequence specific strand exchange, can be used to increase circuit rates up to 9-fold. We also show how the introduction of RNA into DNA circuits further controls the specificity of RecA strand exchange, improving signal-to-noise.

There has been increasing interest in the development of non-enzymatic nucleic acid circuits for computation and for diagnostic applications[1-11]. However, in general strand exchange circuits are slower than corresponding cellular pathways, in part because they lack acceleration by enzyme catalysis. For example, we have previously shown that a circuit known as catalytic hairpin assembly (CHA) is a robust signal amplifier and transducer, able to detect specific nucleic acid sequences and output a variety of signals[8]. CHA circuits can also be combined with isothermal amplification reactions for real-time detection and signal transduction[12]. Nonetheless, CHA is greatly limited in rate, with reactions running to completion in a few hours[4, 5, 8]. For more complex computational and diagnostic circuits, execution times as long as 60 hours may prevail[3, 9-12]. If the rates of DNA circuits could be accelerated, particularly by enzymatic catalysis, many applications in diagnostics and therapeutics could be further developed. To this end, we have pursued the use of Recombinase A protein (RecA) as a generalized rate enhancer for DNA circuits.

E. coli RecA is well-known for facilitating homologous recombination in the cell. RecA monomers form a filament along single-stranded DNA in an ATP-dependent fashion, and can catalyze strand exchange between the ssDNA and homologous regions of dsDNA[13]. The RecA filament-ssDNA complex has been shown to recognize as few as 6 base pairs of homology[14, 15]. While RecA has ATP-ase activity, it does not hydrolyze ATP in the process of catalyzing strand exchange;[16] instead, RecA hydrolyzes ATP during the release of DNA[13]. Its interactions with short oligonucleotides, such as those that might be involved in DNA circuits, have been investigated extensively, and it has been observed that nucleation can occur on as few as 17 nt of ssDNA, with stable filaments forming at or above lengths of 30 nt when ATP is used as a cofactor[17, 18].

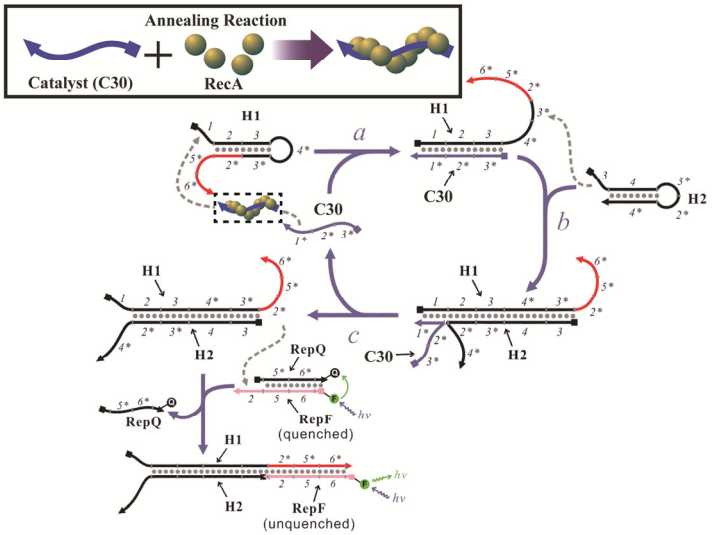


Figure 1 Catalytic Hairpin Assembly (CHA) supplemented with RecA protein. RecA monomers form a filament along the single stranded catalyst (C30, solid box) in a pre-annealing step or during the CHA reaction (dashed box). (a) The RecA:DNA catalyst complex enhances the rate of H1 unfolding by increasing the rate of strand exchange between catalyst and H1, ultimately leading to the opening of the H1 hairpin. (b) H2 catalyzes the release of C30 for subsequent RecA annealing and CHA reactions via toehold-mediated strand exchange with newly exposed regions in H1. The exposed 3' tail of H1 displaces the quenching strand RepQ from the double-stranded reporter complex, generating a fluorescent signal.

Due to its efficacy at catalyzing strand exchange with short DNA, we hypothesized that RecA could form filaments on single-stranded components of our CHA reactions, especially the ssDNA catalyst, potentially increasing the rate at which reactions progressed. The CHA circuit components were identical to previous efforts[8], except that 3 nucleotides of the trinucleotide sequence GTG were added to each end of a 24 nt catalyst (C1) to create C30 (Figure 1). It was thought that this catalyst might better fulfill the 30 nt minimum length required for stable RecA filament formation. Note that dATP was used in place of ATP throughout the entirety of this study, as it has been shown to enhance RecA function[19].

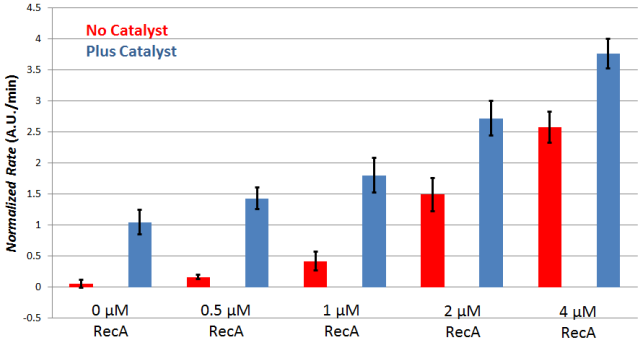
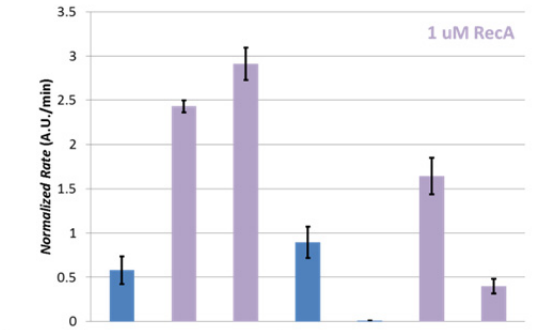


Figure 2. Normalized kinetic rates of CHA reactions with and without catalyst in the presence of increasing concentrations of RecA. Positive signal (+C30) is in blue, while background (no C30 catalyst) is in red.

To test whether RecA might accelerate CHA, we set up reactions with the protein and initiated these reactions by the addition of H1 (Figure 2, blue). However, background increased with RecA concentration at an even faster rate, ultimately reducing the signal-to-noise ratio (Figure 2, red). We hypothesize that at higher concentrations, RecA promotes the interactions of circuit components, either non-specifically or via exposed single-stranded regions.

RecA is known to assemble as a stable filament on single-stranded DNA to initiate recombination[17]. Therefore, we hypothesized that annealing RecA to the single-stranded catalyst in a pre-annealing reaction before addition to CHA would further enhance RecA catalysis of the strand exchange circuit (Figure 1, Annealing Reaction). A protocol was established in which reactions were performed in 2 steps. First, a 10 nM concentration of C30 was annealed with 1 uM RecA (Figure 1, solid box) in the presence 2 mM dATP and RecA buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.6), a buffer from New England Biolabs designed for RecA-DNA annealing reactions. This reaction created a RecA-C30 complex (Figure 1, dotted box) that was predicted to increase the rate of the CHA reaction as compared to C30 alone. After annealing, a final concentration of either 2.5 nM C30-RecA complex or C30 alone was used to initiate CHA reactions. These latter reactions were assembled as previously described in TNaK buffer (20 mM Tris pH 7.5, 140 mM NaCl, 5 mM KCl) with 50 nM H1, 400 nM H2, and 50 nM RepF:RepQ, and supplemented with 2 mM ATP and 4 mM $MgCl_2$ to accommodate RecA[17]. CHA reactions were attempted in the RecA buffer used for annealing, but this led to high levels of background (data not shown). Some 1 µM of a dA-dT dsDNA blocker of length 21 bp was also included to prevent non-specific adsorption of reaction components. Readings (1/minute for 45 minutes) were taken on a Tecan Infinite M200 Pro plate reader. Kinetic data was normalized



	Catalyst Annealing 37°C, 10min			No Catalyst Annealing			
Catalyst (2.5 nM)	+	+	+	+	--	+	--
RecA (1 uM)	--	+	+	--	--	+	+
dATP (2 mM)	--	--	+				

Figure 3. Normalized kinetic rates for pre-annealed RecA with CHA. RecA protein was incubated with 10 nM Catalyst (C30) at 37oC for 10 min, then diluted down to a final concentration of 2.5 nM in the CHA reaction. The row marked 'dATP' indicates the addition of dATP to the annealing reaction. Purple indicates the presence of RecA in the reaction.

to a scale from 0 to 100. Kinetic rates represent the slope between 10 and 20 minutes (see also Methods).

Annealing to the minimal, single-stranded RecA substrate C30 at 37°C for 10 min (Figure 3, 3rd Column) increased the rate of the CHA reaction 5-fold over a control annealing reaction with no RecA (Figure 3, 1st column), and 3.25-fold over regular CHA without RecA or annealing (Figure 3, 4th column), values greater than the rate increase seen from simply including RecA in the CHA reaction (~1.8-fold, Figure 3, 6th column/4th column). The addition of denatured RecA protein did not increase rates (Figure S1). Annealing even in the absence of dATP, a necessary cofactor for RecA filament formation, still increased rates compared to no annealing (6th column). RecA filaments form by four to five RecA monomers first nucleating a filament by binding ssDNA (the rate-limiting step), followed by elongation of the filament with the addition of ATP-bound RecA monomers[20]. Given this, we hypothesize that the rate-limiting nucleation step can occur in the pre-annealing reaction even without dATP, allowing the filaments to quickly form in the subsequent CHA reaction where dATP is present; this would explain the rate difference between column 2 and column 6 in Figure 3.

We attempted to further optimize the reaction by using various concentrations of RecA during annealing and allowing longer annealing times. However, neither modification yielded significant improvements in rate (Figure S2). The temperature of the annealing reaction was lowered to 17°C to improve filament stability and potentially increase rates, but this was also ineffective (data not shown).

Longer ssDNA sequences have been shown to improve filament stability[17]. Since catalyst sequences may be flanked by other DNA sequences in many applications[12], we designed a 90 nt long catalyst where the same 24 nt catalyst sequence found in C30 was flanked at each end by eleven 3 nt repeat units (GTG). This trinucleotide sequence has previously been demonstrated to improve the stability of RecA filaments[21]. The longer catalyst was tested in CHA reactions

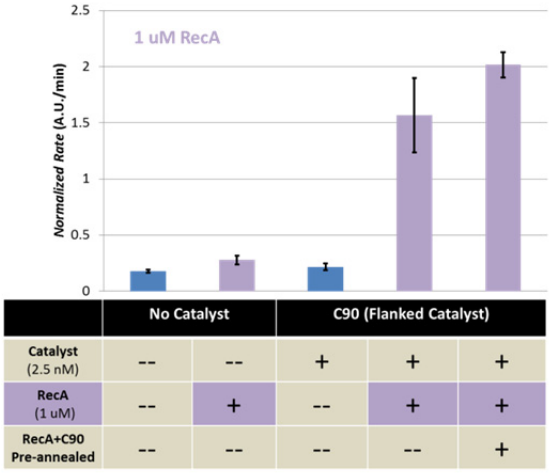


Figure 4. Normalized kinetic rates for an augmented catalyst with CHA. The smaller catalyst (C30) was flanked by 30bp DNA repeats on either side, creating C90. Annealing reactions were performed for 10 min at 37oC (+) with 10 nM C90 (+). Purple indicates the presence of RecA in the reaction.

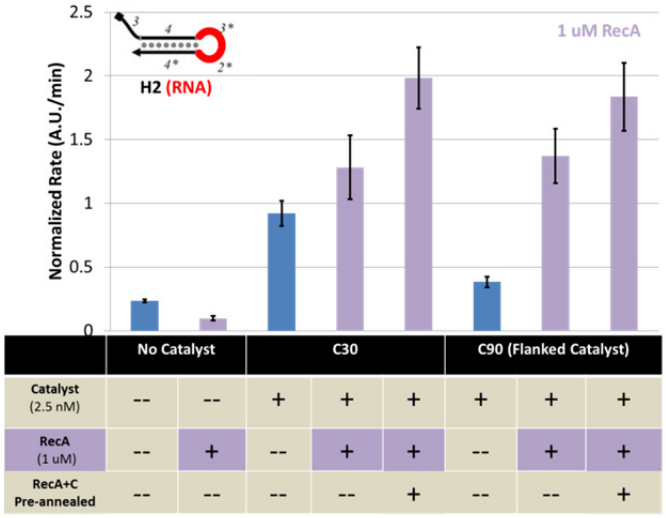


Figure 5. Normalized Kinetic rates of RecA-enhanced CHA reactions with a chimeric H2 with an RNA loop (pictured). All other circuit components and reaction conditions were the same as in Figure 4.

both with and without annealing. In the absence of catalyst annealing, the C90 catalyst showed a 7.2-fold rate increase in the presence of RecA (Figure 4, 4th column/3rd column), and 9.1-fold in the presence of annealing (Figure 4, 5th column). This improvement in response was largely due to the fact that the longer catalyst showed very little activity over background without RecA (Figure 4, 3rd column and 1st column).

While RecA shows obvious utility in speeding up reaction rates, it also often increases background, leading to decreased signal/noise ratios (Figures 2-4). We hypothesized that single-stranded components of the CHA hairpins (toeholds, loops) might be interacting with RecA, and that these complexes led to the CHA cascade being non-specifically triggered. In an effort to investigate this, we designed and synthesized H1 and H2 hairpins that did not possess toeholds (Figure S3). These were then introduced in place of regular hairpins in CHA reactions without RecA. As expected, both hairpins blocked the CHA cascade. When RecA was then added to these reactions, toehold-less H1 with RecA showed background levels similar to its toehold-bearing counterpart, while toehold-less H2 showed a much higher background level, even in the absence of the catalyst sequence (Figure S4).). This would seem to indicate that the single-stranded loop in H2 is serving as an unanticipated site for RecA complex formation, allowing the loop of H2 to interact with H1 via RecA-mediated strand exchange.

In order to fabricate a circuit in which RecA would not interact with the loop of H2, we introduced RNA in place of DNA in the loop (Figure 5). RecA is known to bind weakly to and hydrolyze ATP poorly with RNA substrates[22, 23]. In the RNA-modified circuit, the background reaction rate in the presence of RecA was 3-fold lower than that of the corresponding DNA circuit (Figure 5 vs. Figure 4, 2nd columns). Surprisingly, this background level was even lower than in negative reactions without RecA (Figure 5, 1st column). However, RecA-mediated increases in rate were not as great with RNA loops as with DNA loops; pre-annnealing C30 with RecA increased rates 2.2-fold relative to no RecA, while pre-annealing with C90 led to a 4.8-fold rate improvement (Figure 5, 5th column/3rd column and 8th column/6th column). Even with reduced rates, the signal-to-noise ratio increased to 20-fold for RecA-Annealed C30 and C90 with RNA-modified substrates (3 to 6-fold above the comparable values with DNA hairpins) (Figure 5).

Conclusions

Nucleic acid circuits are gaining traction as useful analytical and diagnostic tools. For example, CHA reactions are proving useful as real-time transducers for isothermal amplification reactions, with detection limits as low as 20 pM of a nucleic acid analyte[8] and greatly improved surety due to reduced detection of non-specific amplicons[24]. However, the relatively slow speed of DNA circuits limits their application, especially for point-of-care diagnostics. Rolling Circle Amplification (RCA) with CHA serves as a detector for a variety of diagnostic targets,[12, 25, 26] but with reactions that typically take four or more hours. By adding RecA to real-time CHA transducers for isothermal amplification reactions, it should now prove possible to shorten analysis times to less than an hour. The addition of RecA should also further improve the specificity of isothermal amplification reactions[27].

Various combinations of polymerases, restriction enzymes, ligases, and nucleases have been incorporated into DNA circuits[7, 28-30]. For example, Kim and Winfree created various types of transcriptional oscillators using T7 RNA Polymerase to generate RNA and RNAseH to destroy it[29]. Similarly, DNA nanomotors that move along a DNA track often require restriction enzymes and ligases to provide catalysis and motive force[31, 32].

While RecA has been previously used for its ability to coat single-stranded DNA and thereby aid in materials formation[33, 34], the addition of RecA to nucleic acid circuits should now generally accelerate nucleic acid computations. The introduction of chimeric RNA substrates—such as the 16 bp-long loop of H2 in our circuit—controls background RecA activity in a novel way and should thereby allow enzymatic catalysis to be introduced into almost any circuit. However, given previous RecA kinetic studies it was surprising that the loop regions described in this study could serve as substrates for RecA.

Notes and references

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