Triggered amplification by hybridization chain reaction

Robert M. Dirks[†] and Niles A. Pierce[‡§]

Departments of [†]Chemistry and [‡]Applied and Computational Mathematics and Bioengineering, California Institute of Technology, Pasadena, CA 91125

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We introduce the concept of hybridization chain reaction (HCR), in which stable DNA monomers assemble only upon exposure to a target DNA fragment. In the simplest version of this process, two stable species of DNA hairpins coexist in solution until the introduction of initiator strands triggers a cascade of hybridization events that yields nicked double helices analogous to alternating copolymers. The average molecular weight of the HCR products varies inversely with initiator concentration. Amplification of more diverse recognition events can be achieved by coupling HCR to aptamer triggers. This functionality allows DNA to act as an amplifying transducer for biosensing applications.

Biosensors require both a recognition component for detection and a transduction component for readout. In gene chips, recognition is performed by single-stranded DNAs that screen for complementary nucleic acid fragments, and transduction is typically performed by optical or electrochemical means (1, 2). Nucleic acid aptamers obtained by *in vitro* selection methods (3, 4) generalize this recognition capability to a wide range of target analytes (5, 6) and are amenable to optical transduction approaches (7, 8). Here, we demonstrate that DNA can also play the transduction role via an amplification approach termed hybridization chain reaction (HCR). This class of mechanisms suggests the possibility of constructing biosensors solely from unmodified single-stranded DNA.

Single-stranded DNA is a versatile construction material (9) that can be programmed (10–15) to self-assemble into complex structures (10, 16–23) driven by the free energy of base pair formation. Synthetic DNA machines can be powered by strand displacement interactions initiated by the sequential introduction of auxiliary DNA fuel strands (24–31). Typically, various DNA strands begin to associate as soon as they are mixed together. Catalytic fuel delivery provides a conceptual approach to powering autonomous DNA machines by storing potential energy in loops that are difficult to access kinetically except in the presence of a catalyst strand (32). In the system described here, monomer DNA building blocks are mixed together but do not hybridize on an experimental time scale. Exposure of an initiator strand triggers a chain reaction of hybridization events similar to living chain polymerization but without covalent bond formation. This system introduces the concept of triggered self-assembly of DNA nanostructures.

Methods

System Specifications. DNA sequences were designed by using a combination of criteria (15): sequence symmetry minimization (10), the probability of adopting the target secondary structure at equilibrium (12), the average number of incorrect nucleotides at equilibrium relative to the target structure (15), and hybridization kinetics (33). The sequences for the basic HCR system of Fig. 1 and the aptamer HCR system of Fig. 2 are shown in Table 1. The aptamer system required new sequence designs to ensure compatibility with the fixed sequence of the aptamer. For the kinetic studies of Fig. 1*c*, $H1^{2AP}$ is used in place of H1 with the third base (A) replaced with 2-aminopurine (34).

DNA was synthesized and purified by Integrated DNA Technologies (Coralville, IA). For the basic HCR system of Fig. 1, concentrated DNA stock solutions were prepared in buffer that was later diluted to reaction conditions: 50 mM Na_2HPO_4/0.5 M NaCl (pH 6.8). For the ATP aptamer HCR system of Fig. 2, concentrated stock solutions were later diluted to reaction conditions: 5 mM $MgCl_2$/0.3 M NaCl/20 mM Tris (pH 7.6).

Native Gel Electrophoresis. Samples were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use. The 1% agarose gels of Figs. 1*d* and 2*b* contained 0.5 μg of ethidium bromide per ml of gel volume and were prepared by using 1× SB buffer (10 mM NaOH, pH adjusted to 8.5 with boric acid) (35). Agarose gels were run at 150 V for 60 min and visualized under UV light. The native polyacrylamide gel of Fig. 2*c* was a 10% precast gel made with 1× TBE buffer (90 mM Tris/89 mM boric acid/2.0 mM EDTA, pH 8.0). The gel was run at 150 V for 40 min in 1× TBE, stained for 30 min in a solution containing 0.5 μg of ethidium bromide per ml, and viewed under UV light. For the reactions of Fig. 1*d*, stock solutions of I, H2, and H1 were diluted in reaction buffer to three times their final concentrations (see legend), and 9 μl of each species was combined, in that order (27-μl reaction volume). For Fig. 2*b*, DNA species were combined to yield 1 μM concentrations in 27 μl of reaction buffer, with additions made in the following order: buffer and/or (I or I^{ATP}), H1, and then H2 (note that I and I^{ATP} interact with H2 rather than H1). In this case,1 μl of 40 mM ATP, 40 mM GTP, or water was added to each reaction, as appropriate, for a total reaction volume of 28 μl. Reactions were incubated at room temperature for 24 h before running 24 μl of each product on a gel. Reactions for the polyacrylamide gel of Fig. 2*c* were performed at half volume, and the entire reaction volumes were loaded on the gel.

Fluorescence Kinetics. Fluorescence data were obtained by using a fluorometer from Photon Technology International (Lawrenceville, NJ), with the temperature controller set to 22°C. Excitation and emission wavelengths (34) were 303 nm and 365 nm, respectively, with 4-nm bandwidths. Stock solutions of 0.40 μM $H1^{2AP}$ and 0.48 μM H2 were prepared in reaction buffer, heated to 90°C for 90 sec, and allowed to cool to room temperature for 1 h before use. For each experiment, 250 μl of $H1^{2AP}$ was added to either 250 μl of H2 or 250 μl of reaction buffer. These 0.20 μM $H1^{2AP}$ solutions were allowed to sit at room temperature for at least 24 h before taking fluorescence measurements. The initial signal was obtained after rapidly pipetting the sample in the cuvette to obtain a stable fluorescence baseline. After acquiring at least 2,000 sec of this baseline, runs were paused for ≈1 min to add 20 μl of initiator (either 20 μM or 2.5 μM) and allow mixing by rapid pipetting. The final reaction volume was 520 μl for all experiments.

Abbreviation: HCR, hybridization chain reaction.

[§]To whom correspondence should be addressed at: California Institute of Technology, Mail Code 114-96, Pasadena, CA 91125. E-mail: niles@caltech.edu.

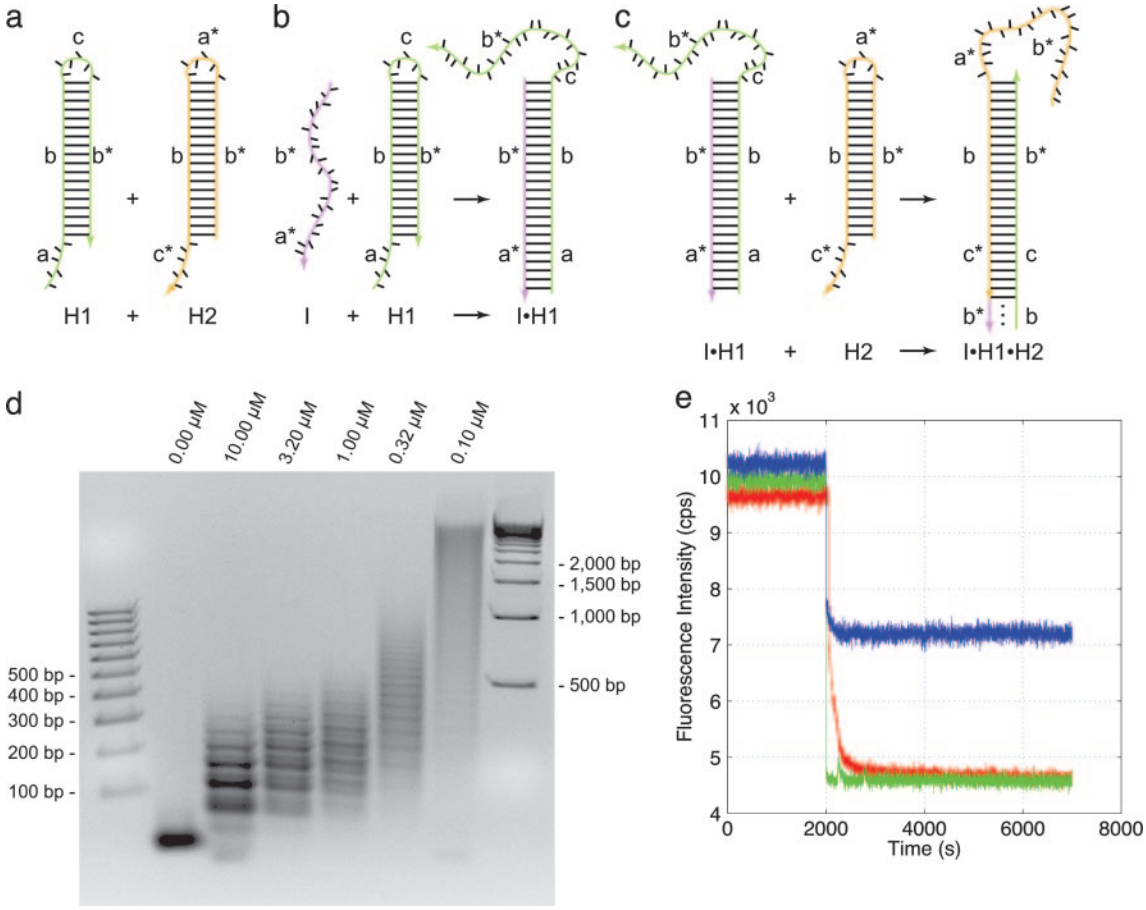

Fig. 1. Basic HCR system. (*a–c*) Secondary structure schematic of HCR function. Letters marked with * are complementary to the corresponding unmarked letter. (*a*) Hairpins H1 and H2 are stable in the absence of initiator I. (*b*) I nucleates at the sticky end of H1 and undergoes an unbiased strand displacement interaction to open the hairpin. (*c*) The newly exposed sticky end of H1 nucleates at the sticky end of H2 and opens the hairpin to expose a sticky end on H2 that is identical in sequence to I. Hence, each copy of I can propagate a chain reaction of hybridization events between alternating H1 and H2 hairpins to form a nicked double-helix, amplifying the signal of initiator binding. (*d*) Effect of initiator concentration on HCR amplification. Lanes 2–7: six different concentrations of initiator (0.00,10.00, 3.20, 1.00, 0.32, and 0.10 μM) in a 1 μM mixture of H1 and H2. Lanes 1 and 8: DNA markers with 100-bp and 500-bp increments, respectively. (*e*) HCR kinetics. The hairpin monomers do not hybridize before triggering by initiator [($H1^{2AP}$ + 1.2× H2) + 0.5× I, red]. The same quenched baseline is achieved without HCR by adding excess initiator to $H1^{2AP}$ in the absence of H2 ($H1^{2AP}$ + 4.0× I, green). Addition of insufficient initiator to $H1^{2AP}$ provides only partial quenching ($H1^{2AP}$ + 0.5× I, blue).

Results

The simplest HCR mechanism employs two hairpin species (H1 and H2 in Fig. 1 *a–c*). The key to this system is the storage of potential energy in short loops protected by long stems. This situation contrasts with that for molecular beacons (36), where short stems protect long loops to allow the target nucleotide to bind in the loop and open the beacon. In the present HCR system, each hairpin is caught in a kinetic trap, preventing the system from rapidly equilibrating. Introduction of an initiator strand (I) triggers a chain reaction of alternating kinetic escapes by the two hairpin species corresponding to "polymerization" into a nicked double helix. Amplification of the initiator recognition event continues until the supply of H1 or H2 is exhausted. The average molecular weight of the resulting polymers is inversely related to the initiator concentration (Fig. 1*d*), sug-

Table 1. HCR systems

System	Strand	Sequence*
Basic	H1	5′-TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGGCGTG-3′
	H2	5′-AGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTACTTTG-3′
	I	5′-AGTCTAGGATTCGGCGTGGGTTAA-3′
Aptamer†	H1	5′-CATCTCGGTTTGGCTTTCTTGTTACCCAGGTAACAAGAAAGCCAAACC-3′
	H2	5′-TAACAAGAAAGCCAAACCGAGATGGGTTTGGCTTTCTTGTTACCTGGG-3′
	I^{ATP}	5′-CCCAGGTAACAAGAAAGCCAAACCTCTTGTT*ACCTGGGGGAGTATTGCGGAGGAAGGT*-3′
	I	5′-CCCAGGTAACAAGAAAGCCAAACC-3′

*In the hairpin sequences, loops are underlined and sticky ends are overlined.
†Aptamer nucleotides (8) are italicized.

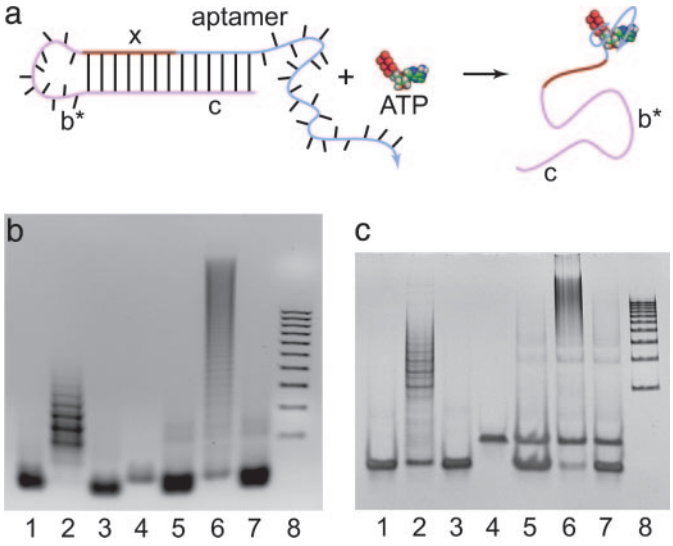

Fig. 2. Aptamer HCR system. (*a*) Aptamer trigger mechanism. Binding of the DNA aptamer (blue) to ATP (37) exposes a sticky end (8) (magenta) that triggers the HCR mechanism of Fig. 1 by opening hairpin H2. The region x (red) is introduced to help stabilize the trigger in the absence of analyte (8). The region b* includes both the hairpin loop and the portion of the stem complementary to x. (*b* and *c*) ATP detection via HCR. Agarose (*b*) and acrylamide (*c*) gels demonstrate amplification of ATP recognition, with the former providing better resolution of HCR products and the latter providing better resolution of unreacted species. Reactions are performed with 1.4 mM ATP and GTP and all DNA species at 1 μM. Lane 1: the hairpins do not polymerize when mixed (H1 + H2). Lane 2: addition of simple initiator triggers HCR (H1 + H2 + I). Lane 3: hairpins with ATP (H1 + H2 + ATP). Lane 4: aptamer initiator with ATP (I^{ATP} + ATP). Lanes 5 and 7: weak spurious HCR in the absence of ATP (H1 + H2 + I^{ATP}) or the presence of GTP (H1 + H2 + I^{ATP} + GTP), respectively. Lane 6: strong HCR amplification of ATP recognition (H1 + H2 + I^{ATP} + ATP). Lane 8: DNA ladder (100–1,000 bp in 100-bp increments).

gesting the potential for quantitative sensing. This inverse relationship follows from the fixed supply of monomer hairpins, but the phenomenon can be observed after 10 min, when the supply has not yet been exhausted.

The kinetics of HCR can be explored by using fluorescence quenching. The adenine analog, 2-aminopurine (2AP), fluoresces when single-stranded but is significantly quenched when in a stacked double-helical conformation (34). Monomer usage can be monitored as polymerization occurs by replacing H1 with the labeled hairpin $H1^{2AP}$ (obtained by substituting 2AP for an A in the sticky end of H1). Monitoring 2AP fluorescence is preferable to using standard end-labeled strands because the local environment of quenched 2AP will be the same regardless of whether I or H2 performs the quenching. In contrast, dyes tethered to the ends of strands may have different fluorescent properties based on their position (terminal or internal) in the HCR polymer.

The hairpin monomers $H1^{2AP}$ and H2 do not hybridize in the absence of initiator (Fig. 1*e*). Addition of I to the hairpin mixture at a lower concentration leads to fluorescence quenching via HCR. The same quenched baseline is achieved without HCR by combining $H1^{2AP}$ with excess I. In this case, each I molecule causes one fluorescent signaling event by binding to $H1^{2AP}$; with H2 present, HCR performs fluorescent amplification, allowing each I molecule to alter the fluorescence of multiple hairpins. Addition of insufficient initiator to $H1^{2AP}$ (at the same concentration as for the first experiment) provides only partial quenching, demonstrating that HCR, and not I alone, is responsible for exhausting the supply of $H1^{2AP}$ monomer. The variation in initial fluorescence intensities is ≈10% across the three experiments. The off/on kinetic behavior of this HCR system suggests that these hairpin constructs may also be useful for delivering fuel to autonomous DNA machines.

More diverse biosensors can be created by triggering HCR with molecular recognition events based on DNA or RNA aptamers (3, 4). Fig. 2*a* depicts a scheme for HCR amplification of ATP detection using an aptamer construct (8, 37) that exposes an initiator strand upon binding ATP. Fig. 2 *b* and *c* demonstrates successful detection of ATP, as well as specificity in differentiating ATP from GTP.

Discussion

These data suggest that HCR can be described by a coarse-grained, structure–function relationship in which secondary structure (as opposed to tertiary atomic coordinates) is sufficient to determine the qualitative interactions of components in the system. This property represents a significant advantage in using nucleic acids as a construction material (9). The basic HCR system of Fig. 1 and the aptamer HCR system of Fig. 2 have identical hairpin secondary structures, with stems of length 18 and loops of length 6 (sticky ends match loop lengths); both sets of hairpins are stable as monomers when mixed but undergo HCR in response to triggering. The sequence identity between analogous hairpins in these systems is only 30%, corresponding to 9 of 30 independent positions (25% identity would be expected for random sequences).

A series of experiments on hairpins with different combinations of loop and stem lengths gave widely different results (data not shown). For example, systems with stems of length 18 and loops of length 8 or with stems of length 10 and loops of length 6 were not stable at room temperature (monomers polymerized overnight in the absence of initiator). If the loops were too small (e.g., of length 4 with stems of length 10), polymerization in the presence of initiator occurred very slowly, if at all. While sequence and tertiary structure are central to aptamer function (37), secondary structure appears to be the appropriate level of description to represent and design the core functionality of HCR. Further experimental studies of this structure–function relationship are warranted.

The concept of HCR is not limited to polymerization of monomer hairpins. The HCR mechanism depicted in Fig. 1 represents linear growth in response to initiator. It is possible to envision more complicated sets of monomers that would undergo triggered self-assembly to create branched structures corresponding to quadratic growth or even dendritic systems exhibiting exponential growth. Nonlinear amplifiers of this type are currently under investigation.

Here, HCR detection was performed by gel electrophoresis or fluorescence quenching. By using inexpensive synthesized DNA components and standard gel electrophoresis equipment, HCR schemes could potentially serve as economical and easily adopted amplifiers for molecular detection. Alternatively, it may be possible to develop a nanogold-based colorimetric assay (38, 39) for HCR that could be used for readout in nonlaboratory settings.

HCR amplification could be applied to a wide range of sensing applications by developing a general approach to aptamer triggering. Furthermore, the trigger need not be based on molecular recognition because any physical process that exposes initiator strand will suffice. It may sometimes be useful to employ HCR for both amplification and capture, using the resulting DNA polymers to remove the analyte from solution. For some applications in which detection is the primary objective of amplification, HCR may have the potential to become an attractive protein-free, room-temperature alternative to PCR (40).

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