Chemical Amplification (CHAMP) by a Continuous, Self-Replicating Oligonucleotide-Based System

David Albagli,* Reuel Van Atta, Peter Cheng, Bingfang Huan, and Michael L. Wood

NAXCOR, 4600 Bohannon Drive, Suite 220 Menlo Park, California 94025

Received February 11, 1999

A self-replicating chemical system requires elements of molecular recognition, reversible self-assembly and an efficient reaction mechanism for forming next-generation templates.[1] Polynucleotide oligomers, capable of reversible, sequence-specific duplex formation, inherently provide two of these elements and are often used in studies of such systems.[2] Moreover, polynucleotide-based systems may be relevant to prebiotic models,[3] and useful for nucleic acid diagnostics.[4]

Regarding the coupling mechanism, many chemical-based methods have been reported, most of which use thermally activated nucleophilic or electrophilic reagents.[5] Typically these reagents show indiscriminate reactivity, leading to side products or inactive components, unless a "feeding" mechanism[6] is employed where the selected reactants are supplied fresh each cycle. Photoinitiated reaction chemistry offers the advantage of selective and repeatable activation of the reactive molecule. Although there are several reports of photochemical systems with modest success in effecting template-driven photoligation, none of these achieve amplification or self-replication.[7]

Herein we report a continuous, closed tube, self-replicating chemical-based (nonenzymatic) amplification method, termed CHAMP, that is initiated by the presence of a specific nucleic acid sequence.

CHAMP employs a (2 + 2) photocycloaddition reaction between a coumarin derivative and thymidine to join two oligonucleotide probes covalently. Coumarins, psoralens, and related compounds undergo efficient photochemical reactions with nucleic acids,[8] and have been used as labeling reagents in many applications.[9] A series of cross-linker compounds have been developed that contain a coumarin moiety and two functional groups which enable incorporation of the compound into an oligonucleotide backbone by automated solid-phase DNA synthesis.[10] The cross-linker compound replaces a regular nucleotide unit (Figure 1a), with the coumarin positioned for reaction with a thymidine base in the opposing strand. The evidence thus far indicates that a coumarin probe must hybridize for the covalent cross-linking reaction to occur. The corollary is that intramolecular reaction of the coumarin is not observed, unless the sequence promotes hairpin formation in a region that includes the coumarin.

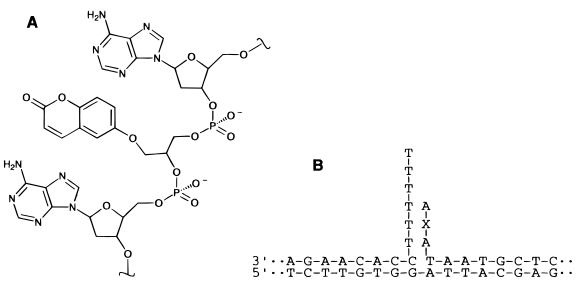

Figure 1. (a) Chemical structure of the coumarin cross-linking compound within an -AXA- sequence. (b) Three-armed junction formed by probe−probe−target hybridization.

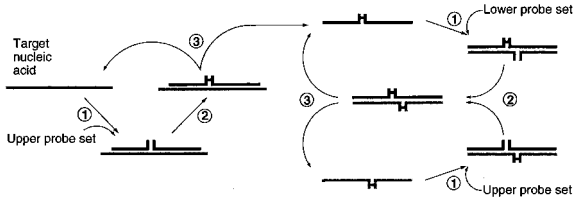

Figure 2. Linear and geometric CHAMP processes. Two probes (1) hybridize to adjacent regions of a nucleic acid target and form a three-arm junction where the third arm is a short duplex formed by the two probes. Next, (2) irradiation covalently cross-links the two probes, after which the sample is heated to (3) denature the probe−probe−target complex and free the target for the next cycle. By providing a second set of probes complementary to the first, one cross-linked probe set serves as a target for the other, and the process becomes a self-replicating cycle.

The CHAMP method directs hybridization of the probes to adjacent positions on the nucleic acid template such that a three-arm junction is created (Figure 1b). The arm consisting of the probe−probe duplex contains the cross-linking reactants, coumarin and thymidine. Thus, template-specific duplex formation organizes the reactants for efficient and selective photochemical cross-linking. Without such a stem, we have found that a cross-linker at a probe terminus will react with bases in the target strand.

A three-arm junction, however, is not as well-structured as a duplex or a four-arm Holliday junction.[11] Base-pairing is disrupted up to five bases removed from the junction, sensitizing the DNA to single-strand cleavage reagents and nucleases in this region. Nevertheless, cross-linking is specific and efficient in the short arm of a three-armed structure comprising as few as three bases.[12]

The first step toward a self-replicating system is to demonstrate that the reaction joining two probes on a single-stranded template can produce amplified amounts of cross-linked probe−probe product. By repeatedly cycling the conditions to sequentially promote hybridization, reaction, and denaturation, amplification is achieved in a closed system.[13] The linear CHAMP mechanism is illustrated in the left side of Figure 2.

* To whom correspondence should be addressed. E-mail: dalbagli@aol.com.

(1) (a) Kuhn, H.; Waser, J. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 500. (b) Szathmary, E.; Smith, J. M. J. *Theor. Biol.* **1997**, *187*, 555.

(2) von Kiedrowski, G.. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 932. (b) Zielinski, W. S.; Orgel, L. E. *Nature* **1987**, *327*, 346.

(3) Orgel, L. E. *Acc. Chem. Res.* **1995**, *28*, 109.

(4) (a) Abramowitz, S. *Trends Biotechnol.* **1996**, *14*, 397. (b) Tang, Y.-W.; Procop, G. W.; Persing, D. H. *Clin. Chem.* **1997**, *43*, 2021.

(5) (a) Gryaznov, S. M.; Schultz, R.; Chaturvedi, S. K.; Letsinger, R. L. *Nucleic Acids Res.* **1994**, *22*, 2366. (b) Achilles, T. A.; von Kiedrowski, G. A. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1198. (c) Dolinnaya, N. G.; Sokolova, N. I.; Ashirbekova, D. T.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3067. (d) Chu, B. C. F.; Orgel, L. E. *Nucleic Acids Res.* **1988**, *16*, 3671.

(6) (a) Luther, A.; Brandsch, R.; von Kiedrowski, G. *Nature* **1998**, *396*, 245. (b) Li, T.; Nicolauo, K. C. *Nature* **1994**, *369*, 218. (c) Ferris, J. P.; Hill, A. R.; Liu, R.; Orgel, L. E. *Nature* **1996**, *381*, 59.

(7) (a) Liu, J.; Taylor, J.-S. *Nucleic Acids Res.* **1998**, *26*, 3300. (b) Letsinger, R. L.; Wu, T.; Elghanian, R. *Nucleosides Nucleotides* **1997**, *16*, 643. (c) Royer, G. P.; Morrison, L. E., Cruickshank, K. A. U.S. Patent 5,449,602, **1995**. (d) Lewis, R. J.; Hanawalt, P. S. *Nature* **1982**, *298*, 393.

(8) Cimino, G. D.; Gamper, H. B.; Isaacs, S. T.; Hearst, J. E. *Annu. Rev. Biochem.* **1985**, *54*, 1151.

(9) (a) Pieles, U.; Sproat, B. S.; Neuner, P.; Cramer, F. *Nucleic Acids Res.* **1989**, *17*, 8967. (b) Cotten, M.; et al., *Virology* **1994**, *205*, 254. (c) Kobertz, W. R.; Essigmann, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 5960.

(10) (a) Saba, D.; Glass, R. S.; Yabusaki, K. K. U.S. Patent 5,082,934, **1992**. (b) Zehnder, J.; Van Atta, R.; Jones, C.; Sussman, H.; Wood, M. *Clin. Chem.* **1997**, *9*, 1703.

(11) (a) Duckett, D. R.; Lilley, D. M. J. *EMBO J.* **1990**, *9*, 1659. (b) Leontis, N. B.; Kwok, W.; Newman, J. S. *Nucleic Acids Res.* **1991**, *19*, 759. (c) Zhong, M.; Kallenbach, N. R. *J. Mol. Biol.* **1993**, *230*, 766.

(12) In an equilibrated solution of two probes and a target, an 80% yield of cross-linked probe−probe product is obtained in ∼4 min irradiation. The amplification experiments however use a container with a more restricted light acceptance, resulting in cross-linking rates half as fast. Data for the extent of reaction vs irradiation time is available in the Supporting Information.

(13) Whereas a hybridization period is not strictly necessary, having one improves the overall amplification efficiency for a fixed total irradiation period (data not shown).

10.1021/ja990434y CCC: $18.00
Published on Web 07/14/1999

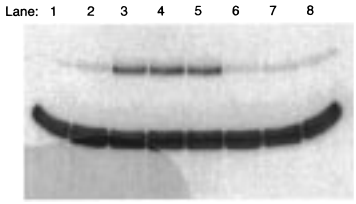

Figure 3. Linear CHAMP as a function of thermal cycling. Eight identically prepared samples were subjected to varied thermal cycling conditions and then analyzed by dPAGE and autoradiography. Lane 1, one cycle: 7 min at 40 °C, 10 min at 40 °C with irradiation, and 3 min at 84 °C; lane 2, two cycles: as above; lane 3−5, 10 cycles: as above; lane 6−8, 10 cycles: 7 min at 40 °C, 10 min at 40 °C with irradiation. Probe 1 (0.5 pmol), 5′-TTTATAAAAAGCTCGTAATATGCAAGAAX-AAAA; probe 2 (0.75 pmol), 5′-TTTTTTTTTCATTGTAAGCAGAA-GACTTA; target (10 fmol), 5′-TAAGTCTTCTGCTTACAATGAACT-TGCATATTACGAGCTTTTTATAAA. Probe 1 was 5′-end radiolabeled with ^{32}P-phosphate.

The results of an experiment testing linear CHAMP are shown in Figure 3.[14] An amplified amount of cross-linked product was observed only in samples denatured between irradiations. In the samples not denatured each cycle, the amount of product plateaued at 1-fold amplification (1:1 ratio of cross-linked product to target), indicating that all of the target strands were occupied with cross-linked probes and that no product was produced independently of a target strand. Through 10 complete cycles, amplification proceeded with >50% efficiency per cycle to yield a ∼5.2-fold increase in detectable product over the amount of target.

By including in the reaction a second probe set complementary to the first, the same three-step cycle yields an exponential increase in the amount of detectable product. This geometric CHAMP process is illustrated in Figure 2.

The efficiency of geometric CHAMP may be different from the individual linear CHAMP reactions, depending on how well the cross-linked product (vs single-stranded DNA) functions as a template for its complementary probe set. We have found that the cross-linking reaction depends on the offset between the two three-arm junctions. No offset affords a four-arm, or Holliday, junction, which surprisingly did not yield detectable reaction products. Introduction of a three to ten base offset between three-arm junctions however, provided a structure that supported the cross-linking reaction in the complementary probe set. The conditions of the experiment must be adjusted (primarily via temperature), however, to prevent the four probes from coming together independently of the target or cross-linked product.

The results of an experiment demonstrating geometric CHAMP are illustrated in Figure 4.[14] The geometric probe set has cross-linkers on complementary probes, with the arms offset by seven bases. Only one target strand was included, and the labeled probe was homologous to the target strand. Thus, the target strand could not directly cause the formation of labeled cross-linked product.

The autoradiogram for the experiment and a graph of the amount of product generated by the self-replicating reaction are shown in Figure 4. The amount of cross-linked product formed increased with each cycle to ultimately yield an appreciable amount of product. In contrast, when no target was present to initiate the process, no amplification was observed. Similarly, even with target present, if the sample was not denatured between irradiations, then no amplification occurred. The nonlinear response at early cycles evident in Figure 4b is due to the prevalence of the linear CHAMP reaction (single-strand templated reaction) prior to the production of significant amounts of the cross-linked product. In later cycles there is an exponential increase in product, indicating the geometric CHAMP reaction (cross-linked-product templated reaction) is the dominant reaction.

(14) Notes regarding the cross-linker compound, and probe synthesis and purification have been published.[10] For all other details see the Supporting Information.

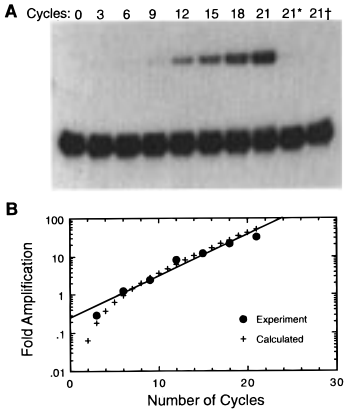

Figure 4. Geometric CHAMP as a function of thermal cycling. Samples containing a geometric probe set were subjected to a varied number of cycles of: 6 min at 51 °C, 12 min at 50 °C with irradiation, and 20 s at 86 °C. The first eight samples (0−21 cycles) contain target; the ninth sample (21* cycles) contains no target; and the tenth sample (21† cycles) contains target but was not denatured during any cycle. (a) Autoradiogram of the dPAGE analysis. (b) Plot of the observed amplification (●) and the theoretical yield (+) for linear and geometric processes initiated by single-stranded target assuming 25% efficiency for all reactions. The solid line is an exponential fit to the experimental data. Probe 1 (1.5 pmol), 5′-TCGCCGATGAGTTCGACATTCCACATACGAGCCCTTTCTCG; probe 2 (1.0 pmol), 5′-CGAGAXATATCACATCGACCTTGGTTTT-TAAATC; probe 3 (1.0 pmol), 5′-GATTTAAAAACCAAGGTCGAT-GTGATAGGGCTCGAXA; probe 4 (1.5 pmol), 5′-TTTTTTTTATGTG-GAATGTCGAACTCATCGGCGA; target (10.0 fmol), 5′-GATTT-AAAAACCAAGGTCGATGTGATAGGGCTCGTATGTGGAATGT-CGAACTCATCGGCGAT. Probe 4 was 5′-end radiolabeled with ^{32}P-phosphate.

Other experiments have demonstrated that geometric CHAMP can be initiated by both target strands and both cross-linked products. When the labeled probe pair is complementary to the target, reaction products appear after the first cycle, but when the labeled probe pair is homologous to the target, reaction products are not observed until the second cycle. The data and these observations indicate that the probes operate according to the self-replicating cycles of Figure 2 to produce exponentially increasing amounts of cross-linked products.

In summary, the coumarin−thymidine photocycloaddition reaction proceeds efficiently near a three-arm junction and is an effective means for covalently joining two DNA probes that form such a branched structure upon binding to adjacent regions of a nucleic acid target. Moreover, incorporating the reactive agent into a stem structure eliminated the occurrence of side reactions between the cross-linker probe and the template. Amplification of cross-linked product occurred upon repeating the three-step cycle of hybridization, irradiation, and denaturation in a continuous fashion, without the need to separate intermediate products or add fresh reagents. Including a second probe set complementary to a first set transformed the linear amplification process into a geometric, self-replicating system. Also, initial tests of CHAMP in hundreds of clinical sample matrices showed no inhibitory effect on the photochemical-based amplification system, suggesting this methodology may be useful for clinical nucleic acid diagnostics.

Further investigations into the sequence dependence in and around the junction, the nature of the stem, and the structure of the cross-linker on the reaction efficiency are in progress.

Acknowledgment. We thank Stephen J. Lippard and Peter M. Southern for critical comments on the manuscript.

Supporting Information Available: All experimental details and graph of the cross-linking reaction time dependence (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA990434Y