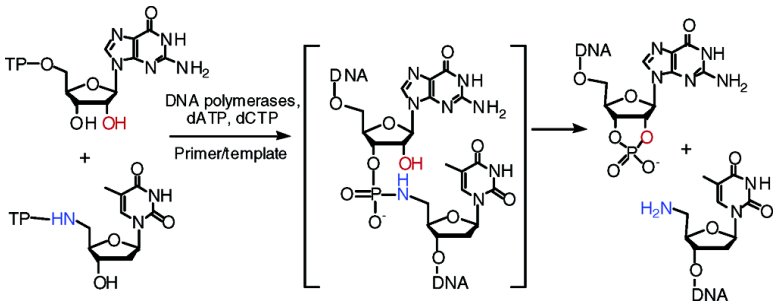
Communication

Sequence-Specific Dinucleotide Cleavage Promoted by Synergistic Interactions between Neighboring Modified Nucleotides in DNA

Jia Liu Wolfe, Bing H. Wang, Tomohiko Kawate, and Vincent P. Stanton

J. Am. Chem. Soc., **2003**, 125 (35), 10500-10501• DOI: 10.1021/ja035646g • Publication Date (Web): 07 August 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 08/07/2003

Sequence-Specific Dinucleotide Cleavage Promoted by Synergistic Interactions between Neighboring Modified Nucleotides in DNA

Jia Liu Wolfe,*,† Bing H. Wang, Tomohiko Kawate, and Vincent P. Stanton, Jr.

Nuvelo, Inc., 60 Hampshire Street, Cambridge, Massachusetts 02139

Received April 15, 2003; E-mail: wolfe@molbio.mgh.harvard.edu

The current repertoire of >3000 restriction endonucleases, which encompasses more than 200 unique sequence specificities, has in many respects revolutionized modern molecular biology.[1] The broad utility of restriction enzymes has inspired much research effort to generate additional sequence specificities that complement the naturally occurring specificities for 4−8 base pair DNA sequences. While attempts of protein engineering proved to be extremely challenging,[2] several alternative strategies for creating specificities for less frequent cleavages (e.g., at 18 base pair sequences) have been moderately successful.[3,4] However, to our knowledge, there has been almost no progress on methods for cleavage of DNA at very simple sequences such as dinucleotides.

In genomic DNA, dinucleotide sequences occur much more frequently than any known restriction site. Consequently, a dinucleotide cleavage method would offer a means to generate small fragments from genomic DNA. For example, while restriction digestion with a four-base-cutter enzyme would on average produce DNA fragments of 4^4 (256) nucleotides, sequence-specific cleavage at dinucleotide sites would produce fragments with an average length of 4^2 (16) nucleotides. The shorter fragments could facilitate downstream analysis such as detection of single-nucleotide polymorphisms (SNPs) or short tandem repeat length polymorphisms (STRs), which have become increasingly important for population and disease genetics studies.[5,6] In particular, analytical platforms using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)[7] would greatly benefit from the use of shorter DNA fragments, given the current detection limit of 50−60 nucleotides in length.[8]

In this study, we designed and examined an approach using chemically modified nucleotides to elicit sequence-specific dinucleotide cleavage. Specifically, a dinucleotide scaffold that is composed of a phosphoramidate group and a nearby 2′-hydroxyl was generated by polymerase incorporation of a ribonucleotide (rNTP) and a 5′-amino-2′, 5′-dideoxyribonucleotide (nNTP) in a 5′ to 3′ orientation. As illustrated in Figure 1, upon protonation, the phosphoramidate may be attacked by the adjacent 2′-hydroxyl functional group to generate a 2′,3′-cyclic phosphate and may result in the cleavage of the phosphoramidate bond. It is this synergistic effect between the two functional groups that confers the sequence specificity and can be manipulated through the choice of modified nucleotides. It is worth noting that a synthetic diuridine phosphoramidate was previously shown to be more prone to hydrolysis than the unmodified diuridine phosphate and the dithymidine phosphoramidate.[9]

Recently, we have shown that all four nNTPs are good substrates of Klenow (exo-) DNA polymerase and each nNTP analogue may separately replace its naturally occurring counterpart during DNA replication.[10] The E710A mutant of Klenow (exo-) has been shown to accept ribonucleotides as substrates.[11] Thus, a mixture of Klenow (exo-) and its E710A mutant was used to incorporate rGTP and nTTP to create the proposed modified dinucleotide framework.

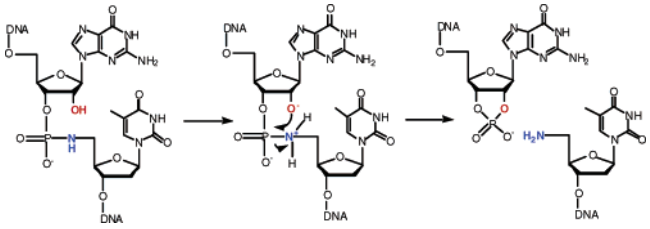


Figure 1. Proposed dinucleotide chemistry that leads to cleavage at a two base sequence such as GT.

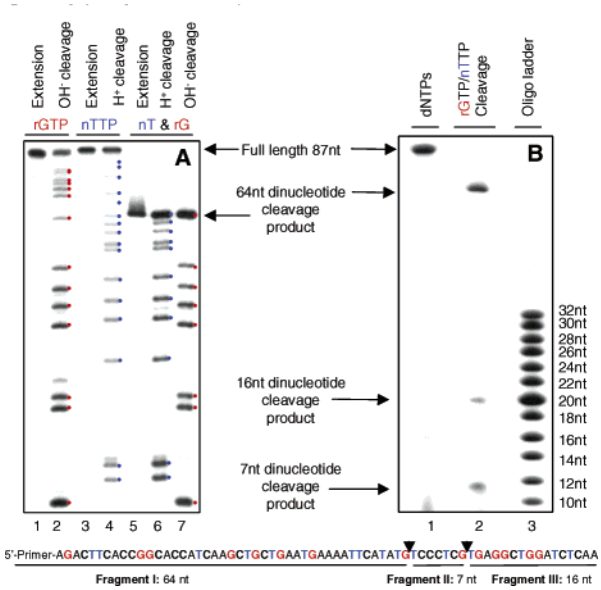


Figure 2. (A) Denaturing PAGE analysis (12%) of extension products using a ^{32}P labeled primer with the modified nucleotide analogues indicated. Cleavage products generated under alkaline (OH^-) or acidic (H^+) conditions are highlighted with red (G) and blue (T), respectively. (B) Denaturing PAGE analysis (12%) of primer extension and cleavage products that were labeled with α-^{32}P-dCTP during polymerization.

A 5′-^{32}P-labeled 20nt primer was annealed to an 87nt template, and primer extension reactions were performed in the presence of rGTP/nTTP, rGTP, or nTTP, and the supplementary dNTPs. The extension products were analyzed by electrophoresis, and the resulting autoradiogram is shown in Figure 2A. Full-length extension products were obtained from either rGTP/d(ACT)TPs or nTTP/d(ACG)TPs, indicating the successful incorporation of rGTP or nTTP, respectively (lanes 1 and 3). Because rNTPs and nNTPs are known to be significantly more labile than corresponding deoxyribonucleotides (dNTPs) under alkaline and acidic conditions, respectively, NH_4OH or acetic acid treatment (lanes 2 and 4) was performed to confirm the replication fidelity. Site-specific cleavage at rG's and nT's generated sequencing ladders for G and T,

† Present address: Department of Molecular Biology, Massachusetts General Hospital, 50 Blossom Street, Boston, MA 02114.

10.1021/ja035646g CCC: $25.00

respectively. It is worth noting that, because rG cleavage occurs at its 3′ side and nT cleavage occurs at the 5′ side, both sequencing ladders contained a 64nt product, as a result of cleavage at the 64th rG and the 65th nT, respectively.

The extension reaction in the presence of both rGTP and nTTP, however, did not show the full-length product. Instead, a very clean 64nt product was observed (lane 5, Figure 2A). The presence of both rGTP and nTTP in this 64nt product was separately confirmed by acidic and alkaline cleavage reactions (lanes 6 and 7 of Figure 2A). Two plausible scenarios for which the product might be generated were considered. One possibility is that the polymerization was terminated after the incorporation of rG at the 64th position; that is, nTTP was not accepted as a viable substrate as a consequence of a rG being immediately upstream. Alternatively, the 64nt fragment may have been created by specific cleavage in situ at the $rG^{64}nT^{65}$ dinucleotide linker after the 65th residue nT was incorporated. To elucidate which process led to the product, α-^{32}P-dCTP was added to the polymerization reaction as an internal label to visualize all of the cleavage products containing C residues and not just the 5′ fragment (Figure 2B). The resulting extension reaction mixture was analyzed (lane 2 of Figure 2B), and three products were observed, each corresponding to an expected GT dinucleotide cleavage product of 64nt, 7nt, and 16nt, respectively. Incomplete cleavage products of 71nt and 23nt were not present, suggesting cleavage was complete. In addition, the relative intensity of the three observed fragments correlated well with the number of C's (i.e., ^{32}P labels) in each fragment. Thus, although the full-length extension product was not directly observed, the data from the internal labeling experiment are consistent with its transient existence. These gel electrophoretic analyses demonstrate that the cleavage was sequence specific as designed and proceeded with surprisingly high efficiency.

To investigate whether this sequence-specific dinucleotide cleavage produced the expected 2′,3′-cyclic phosphate at its 3′-terminus, and to explore the potential applicability of this cleavage strategy to different specificity, we studied rAnT dinucleotide cleavage and analyzed the products using MALDI-TOF MS. A large-scale polymerization reaction was performed using the same Klenow (exo-) mixture, 87nt DNA template, rATP, nTTP, d(CG)TPs, and a 20nt primer. The expected 87nt extension product sequence has six rAnT sites that correspond to seven independent cleavage products ranging from 2nt to 37nt in length (Figure 3). After being purified and desalted, the products were analyzed by MALDI-TOF MS as previously described.[12] Two mass spectra were collected, corresponding to 1000−8000 Da and 5500−15 000 Da mass regions. All of the expected AT dinucleotide cleavage fragments were observed except for the 2nt fragment that was likely lost during the purification process. The observed *m*/*z* data are in excellent agreement with the value expected for each fragment containing a 2′,3′-cyclic phosphate.

In summary, we have developed a conceptually novel dinucleotide cleavage strategy that in principle could be applicable to all nonrepeating dinucleotide sequences. By utilizing sequence-specific, synergistic interactions between 5′-amino-5′-deoxythymidine and ribonucleotides, we demonstrated that specific dinucleotide cleavage may be achieved (e.g., AT or GT) through the use of appropriately modified nucleotides. Although the exact mechanism of this remarkably efficient cleavage is yet to be elucidated, the potential utility of this system is very attractive. For example, using the available human genome sequence information,[13] we could use

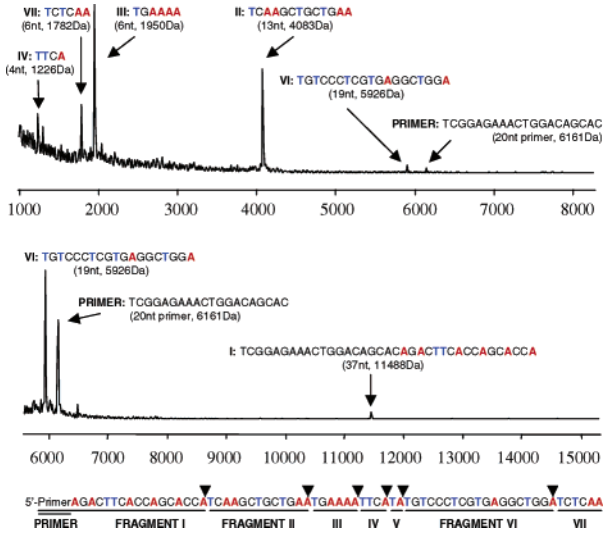


Figure 3. MALDI-TOF MS spectra of AT dinucleotide cleavage products from a 87nt template of human Transferrin Receptor gene: upper panel, 1000−8000 Da; lower panel, 5500−15 000 Da. The sequence of the expected **AT** dinucleotide cleavage products and the corresponding mass data are listed. **AT** dinucleotide cleavage sites are highlighted.

MALDI-TOF MS to analyze dinucleotide cleavage fragments from different individuals, and the mass variations among these fragments could reveal the presence of SNPs without requiring extensive electrophoretic sequencing. Future studies of this system may produce information on polymerase functions, such as substrate competition between two polymerases present in one replication reaction, and the molecular basis for the highly reproducible, specific, and efficient dinucleotide cleavage.

Acknowledgment. E710A Klenow (exo-) polymerase was kindly provided by Dr. C. M. Joyce of Yale University. Drs. C. DeMaria and A. Belenky are acknowledged for their contributions to the project. We thank Drs. A. Ferentz, M. Rook, and T. Zimmermann for critical reading of the manuscript.

Supporting Information Available: Experimental methods used to obtain data shown in Figures 2 and 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Roberts, R. J.; Macelis, D. *Nucleic Acids Res.* **2001**, *29*, 268−269.
(2) Lukacs, C. M.; Aggarwal, A. K. *Curr. Opin. Struct. Biol.* **2001**, *11*, 14−18.
(3) Smith, J.; Bibikova, M.; Whitby, F. G.; Reddy, A. R.; Chandrasegaran, S.; Carroll, D. *Nucleic Acids Res.* **2000**, *28*, 3361−3369.
(4) Ferrin, L. J.; Camerini-Otero, R. D. *Science* **1991**, *254*, 1494−1497.
(5) Chakravarti, A. *Nat. Genet.* **1999**, *21*, 56−60.
(6) Miller, R. D.; Kwok, P. Y. *Hum. Mol. Genet.* **2001**, *10*, 2195−2198.
(7) Hillenkamp, F.; Karas, M. *Int. J. Mass Spectrom.* **2000**, *200*, 71−77.
(8) Laken, S. J.; Jackson, P. E.; Kinzler, K. W.; Vogelstein, B.; Strickland, P. T.; Groopman, J. D.; Friesen, M. D. *Nat. Biotechnol.* **1998**, *16*, 1352−1356.
(9) Thomson, J. B.; Patel, B. K.; Jimenez, V.; Eckart, K.; Eckstein, F. *J. Org. Chem.* **1996**, *61*, 6273−6281.
(10) Wolfe, J. L.; Kawate, T.; Belenky, A.; Stanton, V., Jr. *Nucleic Acids Res.* **2002**, *30*, 3739−3747.
(11) Astatke, M.; Ng, K.; Grindley, N. D.; Joyce, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3402−3407.
(12) Wolfe, J. L.; Kawate, T.; Sarracino, D. A.; Zillmann, M.; Olson, J.; Stanton, V. P., Jr.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11073−11078.
(13) Lander, E. S.; Linton, L. M.; Birren, B.; Nusbaum, C.; Zody, M. C.; Baldwin, J.; Devon, K.; Dewar, K.; Doyle, M.; FitzHugh, W.; et al. *Nature* **2001**, *409*, 860−921.

JA035646G