PCR amplification of GC-rich DNA regions using the nucleotide analog N⁴-methyl-2'-deoxycytidine 5'-triphosphate

Cyntia R. Flores-Juárez¹, Eva González-Jasso¹, Anaïd Antaramian², and Reynaldo C. Pless¹

¹CICATA, Instituto Politécnico Nacional, Querétaro, Querétaro, 76090, Mexico and ²Instituto de Neurobiología, Universidad Nacional Autónoma de México, Juriquilla, Querétaro, 76230, Mexico

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GC-rich DNA regions were PCR-amplified with *Taq* DNA polymerase using either the canonical set of deoxynucleoside triphosphates or mixtures in which the dCTP had been partially or completely replaced by its N⁴-methylated analog, N⁴-methyl-2'-deoxycytidine 5'-triphosphate (N⁴me-dCTP). In the case of a particularly GC-rich region (78.9% GC), the PCR mixtures containing N⁴me-dCTP produced the expected amplicon in high yield, while mixtures containing the canonical set of nucleotides produced numerous alternative amplicons. For another GC-rich DNA region (80.6% GC), the target amplicon was only generated by re-amplifying a gel-purified sample of the original amplicon with N⁴me-dCTP-containing PCR mixtures. In a direct PCR comparison on a highly GC-rich template, mixtures containing N⁴me-dCTP clearly performed better than did solutions containing the canonical set of nucleotides mixed with various organic additives (DMSO, betaine, or ethylene glycol) that have been reported to resolve or alleviate problems caused by secondary structures in the DNA. This nucleotide analog was also tested in PCR amplification of DNA regions with intermediate GC content, producing the expected amplicon in each case with a melting temperature (T_m) clearly below the T_m of the same amplicon synthesized exclusively with the canonical bases.

GC-rich regions are found in >60% of gene promoters in higher eukaryotes (1). Most housekeeping and tumor suppressor genes, as well as ~40% of tissue-specific genes, contain high-GC sequences in their promoter regions (2). Therefore, the study of GC-rich regions is of great interest in biological and clinical investigations. The amplification of genes of interest by PCR is an essential step in the study of their function and regulation, but DNA regions that are >60% GC require special PCR protocols due to their complex and strong secondary structures that resist denaturation and interfere with

primer annealing. Secondary structures can also block the advance of DNA polymerase during the extension step, resulting in poor amplification of the desired products or in the amplification of shortened PCR products lacking the sequence containing the secondary structure (3–8).

There are a large number of protocols for amplification of GC-rich DNA. Some methodologies involve protocols such as stepdown PCR, slowdown PCR, or PCR with multiple heat pulses during the extension phase (8,9). Certain organic co-solvents such as DMSO, formamide, and ethylene glycol have been shown

to facilitate DNA strand separation by disrupting base pairing, and other agents such as tetraalkylammonium (TAA) salts and betaine have also been used (10–13); however, high concentrations of these additives inhibit DNA polymerase activity (14,15). In a different approach, nucleotide analogs such as 7-deaza-2'-deoxyguanosine (c⁷dGTP), 2'-deoxyinosine 5'-triphosphate (dITP), and N⁴-methyl-2'-deoxycytidine 5'-triphosphate (N⁴me-dCTP) have been reported as being useful by destabilizing G:C base pairs (9,16).

Compared with cytosine, the analog N⁴-methylcytosine (N⁴meC) forms a

METHOD SUMMARY

Here, we report use of the nucleotide analog N⁴-methyl-2'-deoxycytidine 5'-triphosphate to partially or totally replace dCTP in PCR amplification of high-GC content DNA regions resistant to amplification using the canonical set of nucleotides.

less stable base pair with guanine, with a stability similar to that of the A:T base pair (17). The methyl group in N⁴meC can be positioned either *cis* or *trans* to N³; when the methyl group is in the *cis* position it will not allow the formation of the Watson-Crick base pair, resulting in a destabilization of the duplex, whereas in the *trans* position the N⁴-methyl group protrudes into the mayor groove, interfering with the local hydration structure, again destabilizing the DNA duplex (18).

The use of N⁴me-dCTP, the N⁴-methylated analog of dCTP, in the amplification of DNA regions with moderate GC-content has previously been reported (19). Subsequently, PCR amplicons containing N⁴meC were compared with those containing unmodified cytosine for hybridization on microarrays, with the N⁴meC amplicons generally performing better (20).

Here, we examined PCR amplification of GC-rich DNA regions, either using the normal complement of nucleotides in the PCR mixture or replacing dCTP with N⁴me-dCTP, to determine if there is any advantage gained by using this analog. In addition, the effects of some of the above-mentioned additives on PCR were also compared. It was found that the use of N⁴me-dCTP affords substantial benefits in the amplification of DNA sequences with high GC content and that these benefits exceed those attainable with commonly used co-solvents and additives.

Materials and methods

Primer design

Primer pairs were designed to amplify GC-rich segments located in various

human genes (Table 1) using the Primer-Quest program from Integrated DNA Technologies (IDT, Coralville, IA). Primers were selected that contained the fewest possible guanines in their sequence in order to minimize the reduction of the primer hybridization temperature for the DNA template containing N⁴meC (19). Primers were also evaluated for their tendency to form homodimers, heterodimers, and hairpin structures using the OligoAnalyzer program (IDT). Finally, primers were evaluated with primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure their uniqueness within the human genome.

PCR experiments

All PCR experiments were performed in a LightCycler 1.5 thermocycler (Roche, Basel, Switzerland) in borosilicate glass capillaries (Roche). PCR was carried out in a total volume of 10 μ L. The amplification mixtures contained 30 ng of human genomic DNA (Roche); 0.5 μ M of each primer (IDT); PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) (Sigma-Aldrich, St. Louis, MO); 200 μ M of each deoxyribonucleotide (dATP, dGTP, dTTP, and dCTP) (Sigma-Aldrich) or 200 μ M N⁴me-dCTP (TriLink Biotechnologies, San Diego, CA) instead of dCTP in the case of modified amplicons; 1.5 U HotStart Taq DNA polymerase (Sigma-Aldrich); 2.5 mM MgCl₂ (Sigma-Aldrich); and SYBR Green I (Invitrogen, Carlsbad, CA) at a final concentration of 0.4 $\times$.

To evaluate the usefulness of N⁴me-dCTP in the amplification of highly GC-rich target sequences, we selected regions in the human *ARX* and *GNAS1* genes (78.9% GC and 80.6% GC, respectively).

For the comparison between additives, we chose to amplify the *ARX* gene. Additives were used in the following concentrations: 2.5%–10% DMSO (Sigma-Aldrich), 0.5–2 M betaine (Sigma-Aldrich), and 0.5–1.5 M ethylene glycol (Fermont, Mexico City, Mexico).

Other less GC-rich segments were also amplified with N⁴me-dCTP in order to determine the T_m reduction of the amplicons when N⁴meC was incorporated.

Cycling protocols

The standard thermal program used was: 95°C for 1 min; followed by 45 cycles of 95°C for 30 s; 62°C, 64°C, or 66°C for 30 s; and 72°C for 60 s; with a final extension at 72°C for 4 min. The slowdown program (9) used a heating rate of 2.5°C s⁻¹ and a cooling rate of 1.5°C s⁻¹, with temperatures of 95°C for 1 min, then 37 cycles of 94°C for 30 s, annealing for 30 s at the temperatures detailed below, and 72°C for 1 min, followed by a final extension at 72°C for 4 min. The annealing temperature was set at 70°C for the first 2 cycles and then reduced by 1°C every second cycle until 60°C was reached (22 total cycles for 11 steps of decreasing annealing temperature); this was followed by 15 additional cycles with annealing at 60°C.

PCR product characterization

PCR products were evaluated by amplicon melting temperature (T_m), which was obtained from the melting curves (Supplementary Material) provided by the LightCycler 1.5 computer automatically after thermocycling. Furthermore, all PCR products were analyzed by agarose gel electrophoresis: 7- μ L aliquots of the PCR mixtures were applied in loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol) to 2% agarose gels (Invitrogen) in 1 $\times$ TBE buffer (Invitrogen) and electrophoresed in a subcell GT electrophoresis chamber (Bio-Rad, Hercules, CA) at 85 V for 60 min. The gels were stained in aqueous ethidium bromide (0.2 μ g/mL), rinsed with water, and photographed on a transilluminator (320 nm) (Model LMS-20E; Ultra-Violet Products Ltd., Cambridge, UK). For size standards, a 100-bp or 50-bp DNA marker ladder

Table 1. Main characteristics of the primers designed for this study.

Gene	GenBank	Primer sequence 5'-3'	T_m (°C)	Product length (bp)	GC (%)
<i>GNAS1</i>	AH002748	TCCTCCTCCTCTCCACC CGCCGCTCTTATTGCCT	61.6 61.8	124	80.6
<i>ARX</i>	NG_008281	CCCGCAGGCTCCCCTAA CTCATCTTCTTCGTCTCCAG	65.5 61.2	522	78.9
<i>BRAF</i>	NG_007873.2	CTCGGTATAAGATGGCGCGCTGA AGTCGGGAGGGCGGCAGGGT	72.1 71.6	185	74.6
<i>NSMCE2</i>	NT_008046	CCGCCTCTGGTGACTCACGCT GCCGCTCCTGCCAGCCCC	70.3 76.4	308	70.8
<i>TFPI-2</i>	AY044097	CGTCCCCGTCTGGACTACAGGAGAAAG CCCGTGGGCAAGGCGTCCGAGAAAGC	63.3 70.5	267	67.2
<i>ACTB</i>	NG_007992.1	GCTAAGTCCTGCCCTCATT GACACCCACCTTGATCTTCAT	62.0 62.0	220	55.5
<i>GAPDH</i>	NG_007073.2	CTCTCTCCATCCCTTCTCC CCCACCCCTCTCTAAGTCC	60.3 59.9	195	54.4

Figure 1. PCR amplification of the *ARX* gene (522 bp). PCR products were analyzed by agarose gel electrophoresis. (A) Amplification with the normal nucleotides and different hybridization temperatures. Lane M: 100-bp ladder; Lane 1: hybridization at 62°C; Lane 2: hybridization at 64°C; Lane 3: slowdown protocol. (B) Amplification with the slowdown protocol and different ratios of N⁴me-dCTP:dCTP or complete replacement of dCTP with N⁴me-dCTP. Lane M: 100-bp ladder; Lane M₂: 50-bp ladder; Lane 4: N⁴me-dCTP:dCTP (10:1); Lane 5: N⁴me-dCTP:dCTP (5:1); Lane 6: N⁴me-dCTP:dCTP (3:1); Lane 7: N⁴me-dCTP.

from Invitrogen was used. Sequencing was performed at LANGE BIO, Irapuato, Mexico by the Sanger method using capillary sequencing technology.

Results and discussion

Previously, we demonstrated the capacity of N⁴me-dCTP in the reaction mixture to produce specific PCR products with reduced T_m values from DNA regions with moderate GC content (19). Here, GC-rich segments of different human genes were tested by PCR amplification using a reaction mixture containing the canonical set of nucleoside triphosphates at 200 μ M each or one in which dCTP was replaced with N⁴me-dCTP, either completely (200 μ M) or with various proportions of N⁴me-dCTP:dCTP [i.e., 10:1 (182 μ M:18 μ M), 5:1 (167 μ M:33 μ M), or 3:1 (150 μ M: 50 μ M)]. Figure 1

shows the amplification reactions for the *ARX* gene using either a canonical nucleotide mix or mixes containing N⁴me-dCTP in different proportions. PCR amplifications conducted with the canonical complement of nucleotides and different thermal profiles produced spurious electrophoretic bands in all cases (Figure 1A). In contrast, amplifications performed with N⁴me-dCTP in different proportions produced a single specific band of the expected molecular size (Figure 1B). Similarly, when the dCTP was completely replaced by N⁴me-dCTP, the specific band was again obtained (Figure 1B, Lane 7).

Analysis of possible hairpin structures in the *ARX* gene by the OligoAnalyzer program shows the potential for two extensive hairpin structures formed mainly by G:C base pairs in the central region of the desired amplicon (Supplementary Material), with ΔG values of -6.49 and -0.84 kcal/mol at 72°C and 50 mM NaCl. These hairpins might block the advance of DNA polymerase during the extension step, resulting in a shorter amplicon due to the enzyme skipping past the hairpin or other amplicons, including longer ones, due to the opportunistic amplification of less difficult to amplify regions of the genome. In contrast, when N⁴meC is incorporated by the DNA polymerase during PCR, the resulting DNA product acts as a better template for subsequent PCR amplifications, likely as a result of weaker G:N⁴meC base pairs leading to less stable hairpin structures. Upon replacing the G:C pairs in the hairpin-prone regions by A:T pairs (for a rough approximation of the energetics of G:N⁴meC pairs, see Reference 17) the OligoAnalyzer program predicts only a very weak hairpin in the sequence of interest, with $\Delta G = +0.98$ kcal/mol at 72°C and 50 mM NaCl.

The 522-bp PCR product of the *ARX* gene, obtained using N⁴me-dCTP instead of dCTP or with 10:1 N⁴me-dCTP:dCTP, was isolated from the gel and sequenced using the reverse primer. This gave the correct sequence over the length of the amplicon, and it aligned with the sequence in the NCBI Nucleotide database when searched with BLAST, confirming that the desired

Figure 2. PCR amplification of the *GNAS1* gene (124 bp). PCR products were analyzed by agarose gel electrophoresis. (A) Amplification with the normal nucleotides and different hybridization temperatures. Lane M: 100-bp DNA ladder; Lane 1: hybridization at 66°C; Lane 2: hybridization at 64°C; Lane 3: hybridization at 62°C; Lane 4: slowdown protocol. (B) Reamplification of the purified faint band obtained from an initial PCR (with dATP/dGTP/dTTP/dCTP and 10% DMSO) using the slowdown program and different ratios of N⁴me-dCTP:dCTP or replacing dCTP with N⁴me-dCTP. Lane 5: N⁴me-dCTP:dCTP (10:1); Lane 6: N⁴me-dCTP:dCTP (5:1); Lane 7: N⁴me-dCTP:dCTP (3:1); Lane M₂: 50-bp DNA ladder; Lane 8: N⁴me-dCTP.

amplicon had indeed been obtained. It should also be noted that the potential hairpin structures did not interfere with sequencing, probably because these structures were relatively destabilized in the template DNA due to the partial or total replacement of the cytosines with N⁴meCs.

We also compared amplification of the *GNAS1* gene using the different nucleotide mixes. Using the canonical set of nucleotides and testing different PCR programs, the desired 124-bp amplicon was not obtained; instead, the product mixtures contained a number of unwanted larger amplicons, whose identity and distribution varied with the different PCR

thermal programs used (Figure 2A). It was also not possible to produce the desired 124-bp amplicon even when the N⁴me-dCTP nucleotide was used, either with different N⁴me-dCTP:dCTP input ratios or complete replacement of the canonical nucleotide with the N⁴-methylated analog. However, the desired product was obtained by first conducting a PCR with the canonical set of nucleotides in 10% DMSO, isolating the desired faint band from the agarose gel using the QIAquick gel extraction kit from QIAGEN (Hilden, Germany), and reamplifying this purified material by PCR with the same pair of primers without DMSO and either with mixtures of N⁴me-dCTP:dCTP or with N⁴me-dCTP only. This provided satisfactory yields in all cases (Figure 2B). Here, secondary-structure analysis by the OligoAnalyzer program showed one of the two primer hybridization sites to be involved formation of a strong hairpin structure with a neighboring stretch of DNA, which should preclude efficient priming.

Regions with lower GC-content (54.4%–74.6%) from different human

Table 2. Melting temperature (T_m) values for different gene fragments amplified with the normal set of nucleotides or with replacement of dCTP by N⁴me-dCTP.

Gene	Normal amplicon T_m (°C)	Modified amplicon T_m (°C)	ΔT_m (°C)
<i>GNAS1</i>	88.1	78.6	-9.5
<i>ARX</i>	87.0	76.4	-10.6
<i>BRAF</i>	95.1	86.0	-9.1
<i>NSMCE2</i>	95.7	84.4	-11.3
<i>TFPI-2</i>	94.7	85.0	-9.7
<i>ACTB</i>	89.6	79.1	-10.5
<i>GAPDH</i>	87.4	78.7	-8.7

genes (*BRAF*, *NSMCE2*, *TFPI-2*, *ACTB*, *GAPDH*) were amplified using N⁴me-dCTP instead of dCTP. All PCR amplifications yielded the target amplicons. Table 2 shows the T_m values for all fragments amplified in both versions of the PCR mixture, namely, with the canonical set of nucleotides or with complete replacement of dCTP with N⁴me-dCTP. For the amplified fragments, the ΔT_m values obtained for the normal and the modified version of the same amplicon ranged from -8.7°C to -11.3°C; contrary to what might have been expected, these T_m differences

did not correlate with the GC content of the amplicons. Thus, *GNAS1*, the most GC-rich amplicon (80.6% GC), showed a reduction in T_m of 9.5°C when cytosine was replaced with N⁴meC, while the much less GC-rich fragment *ACTB* (55.5% GC), showed a T_m reduction of 10.5°C, suggesting that there are other factors besides the fractional GC-content involved in determining the specific melting-temperature differences of the amplicons.

Previous efforts to amplify GC-rich regions have focused on the use of organic compounds such as dimethyl sulfoxide (DMSO), betaine, ethylene glycol, or formamide, among others. These approaches have generally required optimization of the additive concentration and sometimes even the combination of two or more additives to achieve satisfactory amplification. To evaluate N⁴me-dCTP in comparison with PCR additives, the 522-bp region from the *ARX* gene was amplified using N⁴me-dCTP or using the canonical complement of nucleotides with added DMSO, betaine, or ethylene glycol. Gel electrophoresis (Figure 3, Lane 2)

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Figure 3. Gel electrophoresis of the products of PCR amplification of the 522-bp *ARX* gene region with N⁴me-dCTP or with different additives. Lane 1: PCR amplification with N⁴me-dCTP replacing dCTP, without additives; Lane 2: amplification with dATP/dGTP/dTTP/dCTP. Lanes 3–6: amplification with dATP/dGTP/dTTP/dCTP, with 2.5%, 5%, 7.5%, or 10% DMSO. Lanes 7–10: amplification with dATP/dGTP/dTTP/dCTP, with 0.5 M, 1.5 M, or 2 M betaine; Lanes 11–13: amplification with dATP/dGTP/dTTP/dCTP, with 0.5 M, 1 M, or 1.5 M ethylene glycol; Lanes M: 100-bp DNA marker.

illustrates the difficulty of PCR amplification for this highly GC-rich region (78.9% GC) when neither the nucleotide analog nor any additive is used; at least 3 nonspecific products are evident. The use of N⁴me-dCTP results in amplification of the correct target, and reduces non-specific amplification (Figure 3, Lane 1). Using the canonical set of nucleotides with 2.5%–10% DMSO, as commonly reported (5,21,22), there is a reduction in the number of nonspecific

products, but in no case is the 522-bp fragment obtained as the sole amplification product (Figure 3, Lanes 3–6). DMSO concentrations >10% were not tested, as they are reported to inhibit the DNA polymerase (15). A similar result was observed with betaine (Figure 3, Lanes 7–10), which was evaluated at concentrations of 0.5–2 M (i.e., in the concentration range recommended for this additive) (15,23). Only with a betaine concentration of 2 M was it possible

to significantly reduce the second band at ~650 bp, but in no case was it possible to obtain only the specific 522-bp fragment. Similar results were obtained with ethylene glycol, tested at concentrations of 0.5–1.5 M (Figure 3, Lanes 11–13).

Analyzing T_m values for the specific *ARX* amplicon, we found the lowest T_m value, 76.4°C, occurred with the use of the analog, whereas the values for 10% DMSO, 2 M betaine, and 1.5 M

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ethylene glycol were 82.7°C, 81.5°C, and 83.9°C, respectively. This shows a greater ability of the N⁴meC nucleotide to reduce the stability of the DNA duplex, compared with the additives used at the highest concentrations recommended. Thus, N⁴me-dCTP is more effective than the various additives tested, both in a functional sense, allowing PCR amplification of difficult DNA sequences, and in a physical sense, giving a larger reduction in the T_m values.

The amplicons containing N⁴meC can be used for sequencing or for hybridization experiments (20). As to their use in cloning, this will depend on the particular restriction enzymes used in the cloning scheme, as the majority of restriction enzymes are inhibited by N⁴-methylation of cytosines in their recognition sequence (24,25). In any event, performing PCR with N⁴me-dCTP will be beneficial for hybridizations involving highly GC-rich regions. Regarding the amplification fidelity of N⁴meC-containing DNA, sequencing data showed the expected sequence over hundreds of nucleotides. It should be noted that this does not address potential analog-mediated mutations occurring at lower but significant rates (1:100 or 1:1000 bp), which would have to be examined using cloning experiments. However, the fact that N⁴meC occurs routinely in the DNA of certain microorganisms (26–30) argues for relatively low mutation rates associated with incorporation of this nucleotide analog, at least for the polymerases used by these organisms.

Author contributions

C.R.F.J. performed the experiments. C.R.F.J. and R.C.P. designed the experiments and wrote the initial manuscript. E.G.J. and A.A. analyzed the data and contributed to the discussion and the final text. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Reynaldo C. Pless, CICATA, Instituto Politécnico Nacional, Querétaro, Querétaro, Mexico. E-mail: rcpless@yahoo.com

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