Proofreading Activity of *Pfu* Thermostable DNA Polymerase on a 6-*O*-Methylguanine-Containing Template Monitored by ESI-FTICR Mass Spectrometry

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Chromosomal DNA damage, in the form of base alkylation, can be caused by a number of chemical agents.[1,2] Because the damage produced by DNA alkylating agents is not necessarily "bulky", DNA polymerases can often bypass or replicate beyond the site of alkylation.[3] In the case of single-nucleotide substitutions, the induction of sequence alterations occurs during DNA replication when the polymerase encounters a modified form of a base.[1,4] The ability of commonly used polymerase chain reaction (PCR) enzymes to bypass DNA base alkylation has not been widely studied.

One commonly studied DNA adduct is 6-*O*-methyl (6-*O*-me) deoxyguanine (dG), formed in vivo by methylnitrosurea and tobacco smoke.[3,5] This is considered to be an important precarcinogenic lesion.[2] 6-*O*-me dG is well known to induce a base mismatch, with thymine at the complementary position, by mammalian polymerase β, T4 and the Klenow DNA polymerase.[6,7] While 6-*O*-me dG-dC is a stronger hydrogen-bonding pair than 6-O-me dG-dT, structural data indicate that the 6-O-me dG-dT pair is more similar to a Watson–Crick base pair.[8,9] The effect of 3′-5′ exonuclease function, or "proofreading" activity, has been suggested to enhance mutagenesis by favoring dT incorporation, consistent with exonuclease discrimination based on structural distortion.[7]

Electrospray ionization mass spectrometry (ESI-MS) is an excellent tool for precision characterization of oligonucleotides. The ability to analyze PCR products with this technique has been demonstrated by several groups.[10–12] In order for this approach to detect DNA modification-induced sequence alteration, it must be able to identify mass differences corresponding to single-nucleotide deletions and substitutions. This has been demonstrated by using PCR products of over 80 base pairs in length, with Fourier transform ion cyclotron resonance (FTICR) mass spectrometers.[13–15]

The DNA polymerases from several thermophilic bacteria are well studied due to their utility for PCR amplification, an essential tool in molecular biology research.[16] Among them, *Pfu*, from *Pyrococcus furiosis*, is probably the most widely used due to its high fidelity, partly arising from its native proofreading activity.[16] We have examined the effect of 6-*O*-me dG on *Pfu* by using a 50-mer oligonucleotide template for PCR. The amplification products from templates with and without the 6-*O*-me dG base were examined by ESI-FTICR MS to identify the presence of single-nucleotide substitutions. An exonuclease negative (*exo*$^-$) form of *Pfu* was used to determine if this activity was required for the amplification products observed.

Two forms of a 50-mer template were synthesized for PCR amplification, one lacking and one containing a 6-*O*-me dG. It was first determined whether the 6-*O*-me dG template could be resolved from the unmodified version when both are present in equimolar amounts (Figure 1). The presence of the modified base resulted in a 14 Da size difference between the templates and is illustrated in the deconvoluted spectra.

PCR amplification of the 50-mer template produced two strands, measuring 15 095.62 ± 0.04 and 15 668.66 ± 0.03 Da for their most abundant isotope (data not shown), compared with the predicted values of 15 095.58 Da and 15 668.64 Da, respectively. This mass accuracy would allow identification of single-base substitutions for much larger PCR products. The high level of mass accuracy resulted from frequency correction (e.g. calibration) by using the 3$^-$ charge state of an oxidized bovine insulin alpha chain as an internal standard, similarly to in a previous report.[17]

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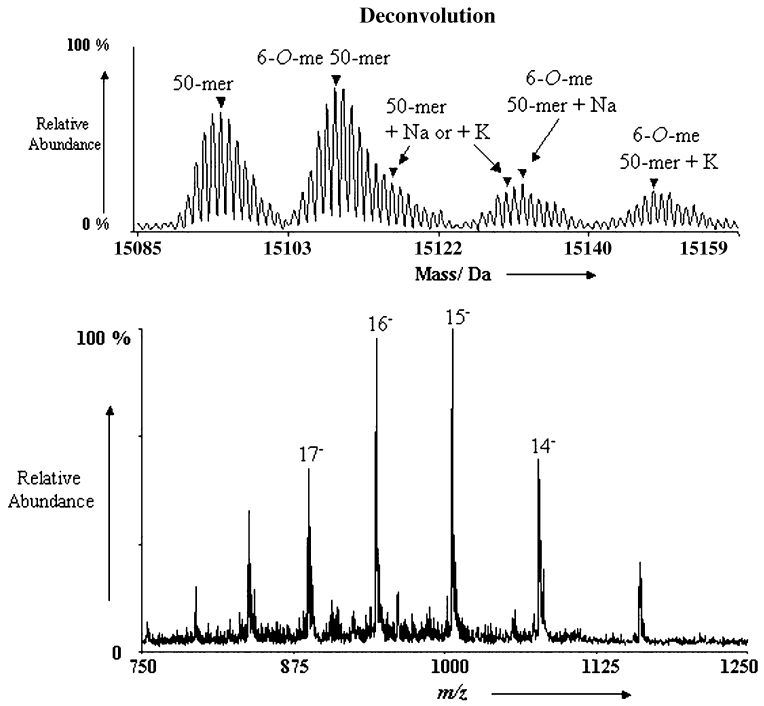

Figure 1. *The mass spectrum of a 1:1 mixture of the dG-containing 50-mer and the 6-O-me dG-containing 50-mer oligonucleotide. The electrospray solution contained approximately 450 ng μL^{-1} of each oligonucleotide. The deconvoluted spectrum is given (top) with the most abundant isotope of each indicated (black triangles) as well as adducts (gray triangles) of each.*

PCR amplification of the 6-*O*-me dG template resulted in two types of products, one identical in mass to the 50-mer template/compliment strands and an additional set of peaks with masses of 15079.56 ± 0.02 and 15683.60 ± 0.01 Da (Figure 2). These additional masses were 15.98 Da smaller than the 50-mer template and 14.96 Da larger than its complementary strand, respectively. This mass shift indicates a transition from a dG-dC base pair (50-mer/50-mer complement) to a dA-dT base pair, which would have a theoretical mass shift of −15.99 Da and +15.01 Da, respectively. These assignments agree with previous reports of a 6-*O*-me dG base inducing a mismatch with a thymine during DNA synthesis with other polymerases.[1,18]

The PCR enzyme used for the initial amplification was native *Pfu,* which contains a 3′ to 5′exonuclease proofreading activity. The contribution of the proofreading activity to the production of both types of PCR products was unknown for this enzyme. Therefore, an amplification of both templates was also performed with recombinant *exo*⁻ *Pfu* enzyme (lacking proofreading activity). Figure 3 shows a comparison of the two deconvoluted spectra for the coding strands of the 6-*O*-

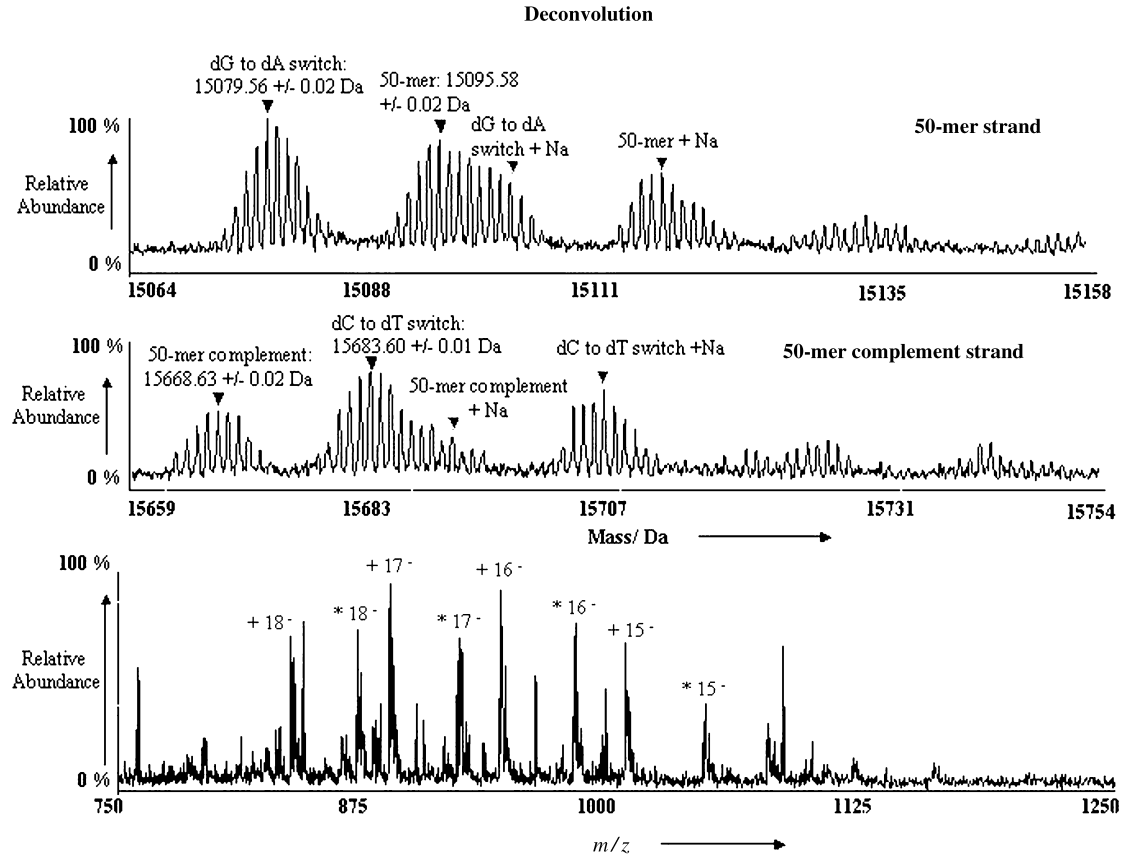

Figure 2. *Mass spectrum of the* Pfu*-amplified PCR product with the 6-O-me dG 50-mer as template. Charge states 15⁻ to 18⁻ are labeled for the 50-mer (*) and 50-mer complement (+) strands (bottom plot). A series of peaks corresponding to two distinct products for each strand can be seen in the deconvoluted mass spectra from 15050 to 15150 Da for the 50-mer and 15650 to 15750 Da for the 50-mer complement strands (top two plots). The most abundant isotopes in each distribution are indicated with black triangles.*

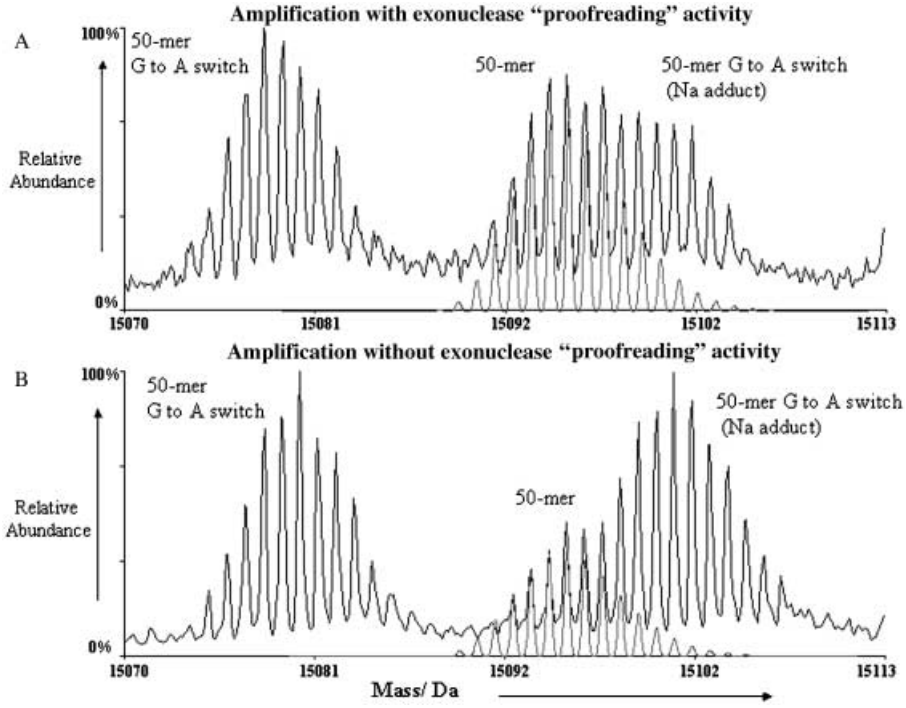

Figure 3. *Comparison of deconvoluted mass spectra for the 50-mer template strand of products amplified from the 6-O-me dG-containing template with native* Pfu *polymerase (A) and the* exo$^-$ Pfu *enzyme (B). The actual mass spectra for each are given as black lines and the theoretical isotope distribution for the 50-mer template strand is given in gray.*

me dG template, amplified with native (Figure 3 A) and *exo*$^-$ (Figure 3 B) *Pfu* polymerase. Amplification with a nonproofreading enzyme resulted in a small amount of nontemplate-encoded 3′ nucleotide addition (data not shown), with adenine being added preferentially. However, amplification of the 6-*O*-me dG-containing template with the *exo*$^-$ *Pfu* enzyme yielded a similar result to that obtained with the native *Pfu*: the dG to dA substitution in the 50-mer strand was clearly present. While partially obscured by the base-substituted strand's sodium adduct, the unsubstituted 50-mer strands were also evident. The theoretical isotope distribution is in good agreement with the experimental data for the 50-mer template (shown in gray). It should be noted that the abundance of the dG-to-dA-substituted 50-mer strand appears greater after amplification with *exo*$^-$ *Pfu*, but is within a factor of 2 of the *exo*$^+$ *Pfu* amplified products when the abundances of the adducted species for each strand are considered. In order for a more precise quantitative measurement of product abundance to be made, further reduction of cation adducts would be required. Methods to reproducibly eliminate adducts for even larger PCR products have been reported.[13,14]

In summary, we have examined the effects of a 6-*O*-me dG base modification on PCR amplification by using *Pfu* DNA polymerase. The previously reported effect of *O*-me dG on DNA polymerization is the induction of a base substitution from dG-dC to dA-dT.[1,7] While this has been well characterized with several DNA polymerases, exonuclease activity on this base modification by PCR enzymes has not been studied. ESI-FTICR was used to investigate whether this phenomenon could be observed by mass spectrometry and whether exonuclease activity was a factor. Amplification of the 6-*O*-me dG 50-mer produced both the dG-dC and dA-dT containing products, as seen by the masses for each strand. Furthermore, this is independent of exonuclease activity for this polymerase. The relative amount of base-pair-substitution product appears to be two-fold greater without the *Pfu* exonuclease activity as compared to the abundance of comparable isotopic peaks. This would seem to contradict earlier findings for the Klenow polymerase, in which exonuclease negative polymerase had four-fold less base substitution than the wild-type polymerase at the highest concentrations of modified-base-containing template studied.[7]

Experimental Section

Polymerase chain reaction: The synthetic oligonucleotide template and primers were synthesized at the oligonucleotide synthesis facility at the University of South Carolina, Department of Biology. The 50-mer oligonucleotide used as a template for PCR amplification had the sequence 5′-ACATCTTACACATCACCACTTAAACTG*GAATCTTCCCATACATTCAATCC-3. This oligonucleotide strand (phosphoramidite template or PCR amplicon) is referred to as the "50-mer" template. Position 27 in the sequence (asterisk) is the location of the 6-*O*-me dG for the "6-*O*-me dG" 50-mer template. The forward and back primers had the sequences 5′-ACATCTTACACATCACC-3′ and 5′-GGATTGAATGTATGGG-3′, respectively. For the PCR reactions, 3 ng of template was used with a reaction buffer containing, at final concentration, $MgSO_4$ (1.5 mM), KCl (10 mM), $(NH_4)_2SO_4$ (10 mM), tris-HCl (20 mM), dNTP (250 μM of each), primer (100 μM of each), and *Pfu* or *exo*$^-$ *Pfu* DNA polymerase (1 U; Stratagene, La Jolla CA). PCR reactions were carried out with a Perkin Elmer series 9000 thermocycler in a total reaction volume of 100 μL .

PCR product purification: Prior to PCR product purification, 250 μL of reaction volume was pooled. Ethanol precipitations were carried out by adding ammonium acetate to a final concentration of 500 mM, followed by addition of a 15-fold excess of 100% ethanol, and the mixture was placed at −80 °C for 4 h. Following precipitation, the DNA was centrifuged to a pellet at 10000 *g* for 20 minutes, washed with 80% ethanol and resuspended in 50 μL of 10 mM ammonium acetate. The precipitation step was repeated once, and then microdialysis against ammonium acetate (10 mM) was carried out with a 13 kDa molecular weight cut-off dialysis fiber (Spectrum Inc., Houston, TX) to reduce salt adducts, as previously described.[19]

Electrospray ionization (ESI) Fourier transform ion cyclotron resonance (FTICR) mass spectrometry: Prior to electrospray ionization, acetonitrile was added to each sample to a final concentration of 50% with triethylamine, piperidine, and imidazole all added to a final concentration of 25 mM. A potential of −1.8 kV was applied to a 25 μL syringe needle with a sheath SF_6 gas flow to reduce corona discharge. Instrument tuning was performed with a solution of oxidized alpha chain of bovine insulin in 50% acetonitrile (10 μM). All FTICR experiments were performed by using a 7 T FTICR mass spectrometer equipped with an Odyssey data system (Finnigan FTMS, Madison, WI) and an elongated cubic cell. The instrument has been described in detail elsewhere.[20] Ions were accumulated by biasing the front and back trapping plates at −2 and −3 V and cooled for 5 s by a pulse of N_2 through a piezoelectric valve (Lasertechniques Inc, Albuquerque, NM). The trapping voltages were set to −0.5 V for broadband frequency-chirp excitation (35 Hz μs^{-1} from 21600 to 216000 Hz) and detection (313.726 kHz acquisition rate, 256 K data points).

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