Functional consequence of plasmid DNA modified site-specifically with 7-deaza-deoxyadenosine at a single, programmable site

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Abstract

Replacement of a single dA nucleotide positioned at a programmed site in a DNA plasmid with its 7-deaza-analog is described together with its complete resistance to restriction enzymatic cleavage.

Protein-DNA recognition is a fundamental biomolecular interaction that drives critical cellular processes responsible for life. The ability to recognize specific DNA sequences within the vast genome, as the first step to deciphering the encoded genetic information, is critical for the timely expression of genes as proteins and cellular survival.1 In order to gain insights into the nature of these interactions, well-defined and representative DNA substrates containing site-specific modifications are required as probes and they are technically challenging to prepare. We are currently exploring strategies for generating plasmid DNA vectors containing single platinated-DNA adducts that can transfected into live mammalian cells for subsequent functional studies.2 During the course of this work, we carried out a single atom modification on a plasmid by replacing a N7 atom on the heterocyclic ring of a single deoxyadenosine residue with a C–H unit. We report here that this conversion is sufficient to completely abrogate restriction enzyme function.

The methodology described in this communication improves upon a protocol previously described.2 The approach involves formation of a site-specific gap within the plasmid backbone using nicking restriction enzymes and filling in the gap with a synthetic oligodeoxynucleotide containing the platinated DNA adduct (Fig. 1).2, 3 The pGLuc6temG vector was derived from a commercial pCMV-GLuc construct with the intention of preparing a plasmid containing a site-specific monofunctional dG-Pt lesion4 as a probe for live cell experiments. pCMV-GLuc was modified by PCR-mediated deletion to remove the SV40 origin of replication and BspQI restriction sites,5 after which it was directionally ligated with a synthetic 30-bp insertion sequence between HindIII and BamHI sites to yield pGLuc6temG. This insertion sequence contains unique enzyme restriction sites that can be nicked in tandem by Nt.BspQI 16-bases apart on the template strand of the plasmid (Fig. 2). Nt.BspQI is the variant of BspQI that recognizes GCTCTTC(1/4) but is engineered to cleave only one strand of the recognition sequence, specifically at GCTCTTCN↓. Removal of the 16-base nicked strand was achieved by annealing with its complement in high molar excess followed by purification using diafiltration. As part of the vector design, a unique BstAPI restriction site was positioned within the insertion sequence such that it overlaps with the

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location of the intended modified base. In this manner, the modified base can be readily detected by determining the extent to which BstAPI can linearize the plasmid. The gap is filled-in by ligating a 16-base synthetic oligodeoxynucleotide to the plasmid construct with T4 DNA ligase. The covalently closed plasmid product thus formed can be readily purified from the open circular gapped precursor by agarose gel electrophoresis.

We previously reported a strategy for building oligodeoxynucleotides containing purine-rich sequences that are difficult to platinate site-specifically by incorporating 7-deaza-purine bases into their sequences.[6] These bases are isosteric analogs of parent purines in which the N7 atom is replaced by a C–H unit (Fig. 3). They exhibit altered chemical reactivity and stability while maintaining similar biological functionality.[7, 8] For example, 7-deaza-dGTP can substitute for dGTP to clone GC-rich genomic regions to reduce formation of secondary DNA structures that would inhibit conventional PCR. Cisplatin preferentially reacts at the N7 positions of the purine heterocyclic rings, and platination at unwanted purine sites can be averted by substituting them with their corresponding 7-deaza-purine analogs. The 16-base oligodeoxynucleotide strand 5'-CATCCTCTGCTCTTCC required to fill in gapped pGLuc6temG contains dA at the 2-position and dG at the 9-position. We reasoned that, by replacing dA with 7-deaza-dA on the 16-base strand, we could improve the yield and purity of the monofunctional dG-platinated oligodeoxynucleotide preparation by blocking platination at the dA site. We sought to determine whether this minor base modification would adversely affect the subsequent preparation and analysis of the resultant plasmid.

The 16-base oligodeoxynucleotide insertion strands containing either dA or 7-deaza-dA, designated as 16-is and 16-deaza-is respectively, were prepared by conventional phosphoramidite methods and purified by anion-exchange-HPLC.[9] By analyzing the RP-HPLC trace of the enzymatic digests by nuclease S1 and calf intestinal phosphatase (CIP), the two oligodeoxynucleotides were readily distinguished based on the different retention times of dA and 7-deaza-dA (Fig. 3). The 7-deaza-dA nucleotide is more hydrophobic than dA and binds more strongly to the reverse phase C18 column, resulting in a longer retention time.

Insertion of the phosphorylated 16-base oligodeoxynucleotide strands into the gapped plasmid was facile and ligation proceeded in high yield. Gel electrophoretic analysis of isolated gapped pGLuc6temG treated with T4 DNA ligase revealed low levels of covalently closed circular DNA, indicating that the plasmid was effectively gapped. By comparison, the levels of covalently closed plasmid product formation were high when 16-is and 16-deaza-is were added, 73% and 72% respectively (Fig. 5), indicating that the 7-deaza-dA modification did not affect the ligation efficiency of inserts to the gapped plasmid. Ligation of 16-is and 16-deaza-is into gapped pGLuc6temG carried out on a preparative scale yielded covalently closed plasmid products, designated as pGLuc6tem + is and pGLuc6tem+deaza-is respectively, in moderate yields. Analysis of the synthesized plasmids was performed by treating them with BstAPI and the results were compared to those for the parent pGLuc6temG plasmid isolated from bacterial culture. Both 2-dA and 9-dG are situated within the recognition sequence of BstAPI (GCANNNN↓NTGC). Unhindered BstAPI cleaves the pGLuc6temG vector quantitatively at the insertion sequence to yield exclusively linearized plasmid (lin). Both parent pGLuc6temG and pGLuc6temG+is are susceptible to BstAPI cleavage, but pGLuc6temG+deaza-is, which contains a site-specific 7-deaza-dA nucleotide, could not be linearized (Fig. 6).

The ability of 7-deaza-purine bases to impede restriction enzyme digestion has been previously demonstrated. In one study, EcoRI (G↓AATTC) cleaved the octadeoxynucleotide duplex substrate d(GGAATTCC) cleanly but not d(GGAATCC), where G denotes the position of 7-deaza-dG.[10] In another report, PCR was carried out on bacteriophage λ DNA

and pUC19 plasmid using 7-deaza-dGTP in place of dGTP to prepare PCR fragments having exclusively 7-deaza-dG.11 The PCR-amplified products isolated were resistant to a number of Type IIP restriction enzymes containing at least a dG in the recognition sequence, including EcoRI, BamHI, and AccI. Out of the 13 enzymes tested, only 3 were able to cleave the 7-deaza-dG enriched DNA, but no discernible pattern could be established linking the spatial position of dG within the recognition sequence to the susceptibility of enzyme cleavage.

Our system differs from the ones in these earlier reports in that the DNA substrate is a covalently closed plasmid containing a single-atom, site-specific base modification at a unique restriction site. Structurally, pGLuc6temG+deaza-is differs from pGLuc6temG+is only by the replacement of N7 with a C–H unit at one of the dA in the recognition sequence. This substitution is sufficient to prevent linearization by BstAPI, implicating an important role of N7-dA for its activity. Since BstAPI recognizes the interrupted GCANNNN↓NTGC sequence, but cuts between the fourth and fifth unspecified base,12 its inability to cleave the 7-deaza-dA-containing plasmids stems from the failure to recognize the modified base. This result suggests important protein-DNA interactions between the DNA-binding motif and N7-dA in the recognition sequence. Restriction enzymes form a highly cooperative H-bonding network with the bases as well as the sugar-phosphate backbone in their recognition sequence, giving rise to specific protein-DNA interactions.13 BglI, for example, exhibits similar restriction characteristics as BstAPI because it also recognizes a discontinuous 11-bp DNA sequence (GCCNNNN↓NGGC) and cleaves between the fourth and fifth unspecified base to leave 3-bp 3'-overhangs. Crystallographic studies of BglI bound to a 17-base DNA substrate revealed numerous contacts between the enzyme recognition motif with N7-dG on both strands of the DNA substrate at the recognition site via H-bonding with arginine and lysine residues.14 It is plausible that disruption of this intricate network by removal of a N7-dA contact point severely affects the ability of BstAPI to stably bind at its recognition site. Therefore, this technique of controlling and reprogramming a plasmid vector at the level of a single nucleotide level can used to probe highly specific enzyme-DNA interactions.

In summary, site-specific modification of a mammalian expression vector was accomplished by replacing a short single-stranded sequence on the plasmid DNA with a synthetic oligodeoxynucleotide containing the modified base. The N7-dA atom at the BstAPI restriction site was converted to a C–H unit, which rendered the modified plasmid resistant to enzymatic cleavage by BstAPI. The present study extends the investigation of DNA-based structure-activity relationships by a systematic alteration of a nucleotide at known or postulated recognition sites. For example, the effects of the sugar-phosphate backbone on site recognition could be studied by replacing phosphate linkages with phosphothioate ones. In principle, site-specific programming of a plasmid can be easily carried out as long as the modifiednucleotide can be synthesized as part of the oligodeoxynucleotide insert. We anticipate that the method outlined will have broad applicability for mechanistic and biotechnological studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Notes and references

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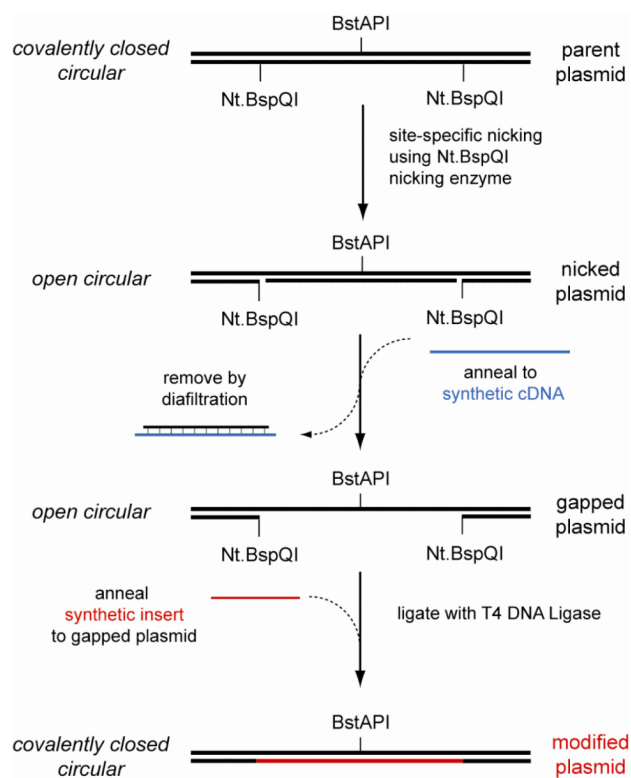14. Newman M, Lunnen K, Wilson G, Greci J, Schildkraut I, Phillips SEV. EMBO J. 1998; 17:5466–5476. [PubMed: 9736624]

Fig. 1.
Overall scheme for preparing site-specifically modified plasmids.

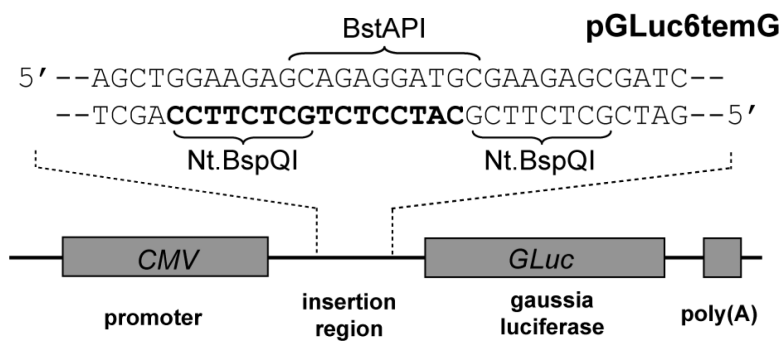**Fig. 2.**
DNA sequence of the insertion region in pGLuc6temG vector; the position of the intended strand for insertion is depicted in boldface font.
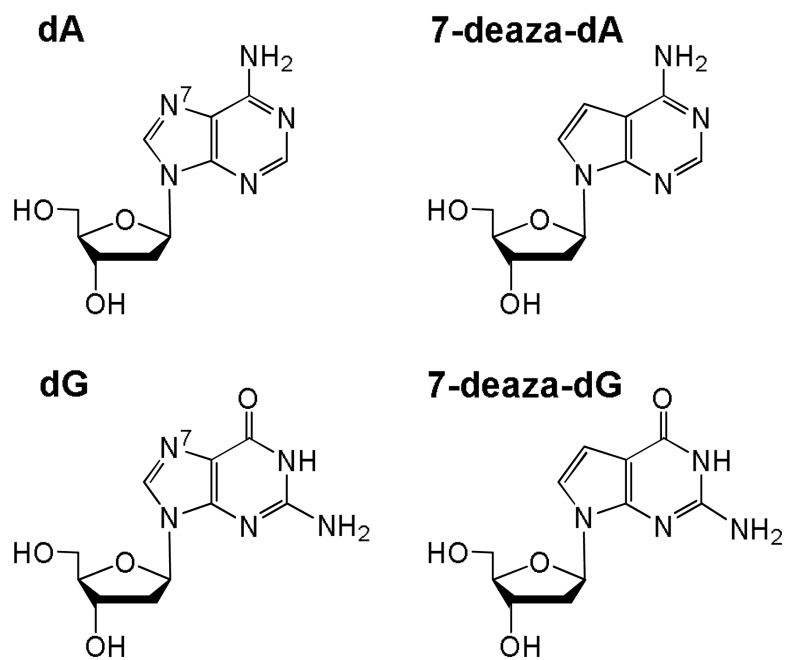
Fig. 3.
Chemical structures of purine deoxynucleosides (left) compared to their 7-deaza-analogs (right).
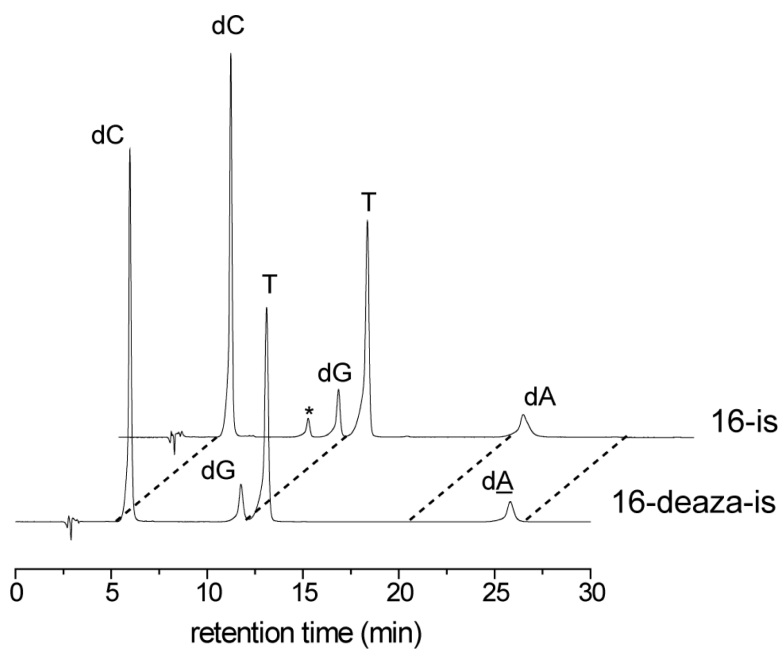
Fig. 4.
HPLC chromatogram of enzymatic digestion of 16-base oligodeoxynucleotide strands by
nuclease S1 and CIP (peak demarcated by an asterisk is an impurity). dA\ is the deaza base.
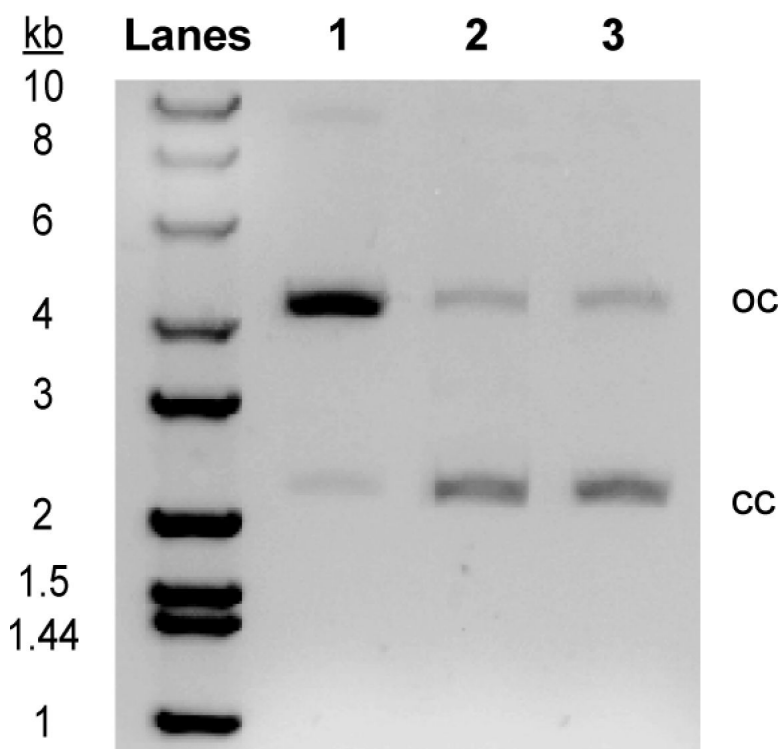
Fig. 5.
Ligation experiment of gapped pGLuc6temG in presence of different inserts with T4 DNA Ligase at 16°C for 12 h; gapped pGLuc6temG alone (lane 1), plasmid + 16-is (lane 2), plasmid + 16-deaza-is (lane 3).
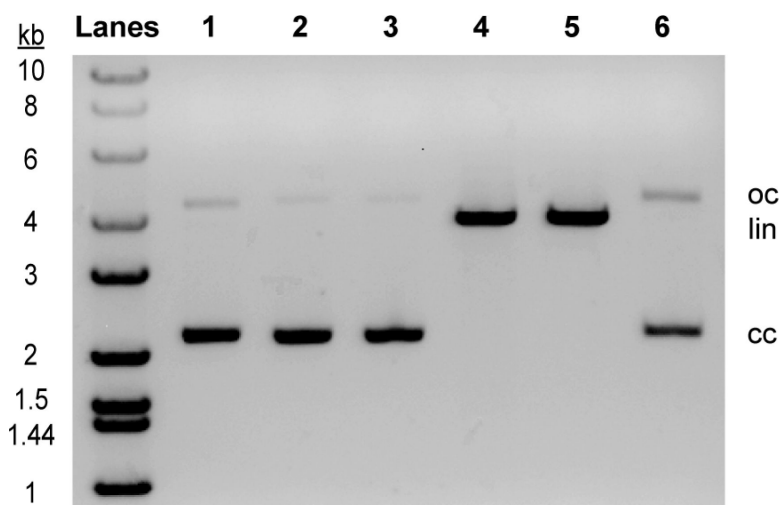
Fig. 6.
Restriction analysis of site-specifically modified plasmids with BstAPI; undigested parent pGLuc6temG (lane 1), pGLuc6temG+is (lane 2) and pGLuc6temG+deaza-is (lane 3), and BstAPI-digest of parent pGLuc6temG (lane 4), pGLuc6temG+is (lane 5) and pGLuc6temG +deaza-is (lane 6).