Cleavage of adenine-modified functionalized DNA by type II restriction endonucleases

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ABSTRACT

A set of 6 base-modified 2-deoxyadenosine derivatives was incorporated to diverse DNA sequences by primer extension using Vent (exo-) polymerase and the influence of the modification on cleavage by diverse restriction endonucleases was studied. While 8-substituted (Br or methyl) adenine derivatives were well tolerated by the restriction enzymes and the corresponding sequences were cleaved, the presence of 7-substituted 7-deazaadenine in the recognition sequence resulted in blocking of cleavage by some enzymes depending on the nature and size of the 7-substituent. All sequences with modifications outside of the recognition sequence were perfectly cleaved by all the restriction enzymes. The results are useful both for protection of some sequences from cleavage and for manipulation of functionalized DNA by restriction cleavage.

INTRODUCTION

Nucleic acids containing modified nucleobases are of great current interest and find applications in chemical biology, bioanalysis or nanotechnology and material science (1–5). Apart from chemical synthesis, base-modified DNA can be prepared enzymatically (6,7) by polymerase incorporations of base-modified nucleoside triphosphates (dNTPs). Number of functional groups, i.e. aminoalkyls (8–10), guanidine (11), imidazole (12), acridone (13), biotin (14), carbohydrates (15), amino- and nitrophenyl groups (19) fluorescent labels (20,21), spin labels (22) and Ru/Os(bpy)3 complexes (23,24), alkynes (25,26) and azide (27,28) functionalities for subsequent Click-Chemistry and Staudinger ligations, etc., have been attached at position 5 of pyrimidine or at position 7 of 7-deazapurine dNTPs via diverse linkers. These 5-modified derivatives of pyrimidines and 7-deazapurines were successfully incorporated to DNA by primer extension (PEX) or PCR. On the other hand, 8-substituted purine dNTPs were repeatedly shown (16,29,30) to be poor substrates and only relatively small substituents (Br or Me) were generally tolerated by the polymerase (29) and were incorporated to DNA which, despite the presence of 8-substituents, still preserved B-conformation.

Type II restriction endonucleases are highly specific enzymes that recognize short, usually palindromic, sequences of 4–8 bp and cleave the DNA phosphodiester bond within or close to the recognition sequence (31–35). DNA methylation (at C-5 of C and at N6 of A) causes resistance and protection against some restriction enzymes, while others are not sensitive to it and cleave even methylated DNA. There is only very little knowledge about cleavage of other types of base-modified DNA. Komatsu et al. (36) published a study on resistance of 8-hydroxy-adenosine, 8-methoxy-adenosine and 8-methoxy-guanosine containing octadeoxyribonucleotides to cleavage by EcoRI. DNA containing 7-deazaadenine (37–43) or 7-deazaguanine (38) in recognition sequence was repeatedly reported to be resistant to some endonucleases apparently due to the lack of N7 atom capable of formation of H-bonds in major groove. Some other analogs of adenine, e.g. 3-deazaadenine (44,45) or 2- and 2,6-diaminopurine (37), were also incorporated into oligonucleotides for studying interactions with restriction endonucleases. Presence of 5-halogenated pyrimidines in DNA was well tolerated by restriction enzymes without significant inhibition of the cleavage (46,47). On the other hand, presence of glucosylated hydroxymethylcytosine and hydroxymethyluracil in palindromic sequence resulted in reduction of cleavage of DNA by EcoRI (1). Moreover, some DNA adducts (e.g. the malondialdehyde-deoxyguanosine adduct) were reported to partially reduce cleavage by EcoRI (49). DNA duplexes containing photoactive derivatives of deoxyuridine were prepared (50–52) and successfully applied as photo-crosslinking probes for restriction endonucleases.

In order to be able to prepare any desired DNA sequence containing one or several modifications at specific positions in the duplex by enzymatic incorporation of
modified dNTPs and to introduce base-modifications to plasmids or genomic DNA, standard DNA manipulations (cleavage by restriction endonucleases, ligation etc.) must be performed. Since only very scarce data on the ability of different restriction enzymes to cleave base-modified DNA are available in the literature, we have decided to prepare DNA duplexes containing base-modified adenine derivatives and analogues bearing different functional groups in major groove (position 8 of modified adenine derivatives and analogues bearing different functional groups in major groove (position 8 of modified DNA are available in the literature, we have must be performed. Since only very scarce data on the
tions (cleavage by restriction endonucleases, ligation etc.)
stored at 0°C in D2O (referred to dioxane as internal standard, δH = 3.75 p.p.m., 6C = 69.3 p.p.m., standard for 31P NMR was external H2PO4). Mass spectra were measured on LCQ classic (Thermo-Finnigan) spectrometer using ESI or Q-Tof Micro (Waters, ESI source, internal calibration with lockspary).

**MATERIALS AND METHODS**

**General**

NMR spectra were measured on a Bruker Avance 600 (600 MHz for 1H and 151 MHz for 13C nuclei) and a Bruker 500 (500 MHz for 1H, 125.7 MHz for 13C and 202.3 for 31P) in D2O (referred to dioxane as internal standard, δH = 3.75 p.p.m., 6C = 69.3 p.p.m., standard for 31P NMR was external H2PO4). Mass spectra were measured on LCQ classic (Thermo-Finnigan) spectrometer using ESI or Q-Tof Micro (Waters, ESI source, internal calibration with lockspary).

**Synthesis and characterization of modified dNTPs**

*Synthesis of 2'-Deoxy-7-ethyl-7-deazaadenosine 5'-O-triphosphate (dA7EtTP).* 2'-Deoxy-7-ethyl-7-deazaadenosine (146 mg, 0.53 mmol) was suspended in trimethyl phosphate (1 ml) in argon purged vial at 0°C and POCl3 (52 μl, 0.54 mmol) was added. The mixture was then stirred at 0°C for 60 min, an ice-cooled solution of (NH4)2HPO4 (1100 mg, 2 mmol) and Bu4N (0.4 ml, 1.7 mmol) in dry DMF (4 ml) was added and the mixture was stirred at 0°C for another 60 min. Then the mixture was quenched by addition of 2 M aqueous TEAB (2 ml) and the solvents were evaporated in vacuum and the residue was co-distilled with water three times. The product was isolated on DEAE Sephadex column (150 ml) eluting with a gradient 0–1 M TEAB, several times co-distilled with water and converted to sodium salt form (Dowex 50WX8 in Na+ cycle) followed by freeze-drying from water gave white solid product 2'-deoxy-7-ethyl-7-deazaadenosine 5'-O-triphosphate (109 mg, 34%). MS(ESI⁺): 603 (100, M + K⁺), 581 (109, M + Na⁺) and 561 (100, M + H⁺) as major peaks. 1H NMR (499.8 MHz, D2O, δH = 7.1, phosphate buffer, ref_dioxane = 3.75 p.p.m.): 1.45 (t, 3H, H-2); 2.76 (m, 1H, J = 7.9, J2,a,3 = 6.1, H-2,b); 2.72 (m, 1H, J = 14.0, J2,b,1 = 6.1, H-2,b); 2.66 (m, 1H, J = 14.0, J2,a,1 = 7.9, J2,a,3 = 6.1, H-2,a); 3.69 (s, 1H, H-2); 3.61 (d, 1H, J = 14.0, J2,a,3 = 6.1, H-2,a); 4.11 (dt, 1H, J = 11.4, J1p,H-5p = 5.1, H-5,b); 4.18 (dd, 1H, J = 11.4, J1p,H-5p = 6.3, J5,a,4 = 4.2, H-5,a); 4.25 (m, 1H, H-4); 4.77 (m, 1H, H-3); 6.72 (dd, 1H, J1,2 = 7.9, 6.1, H-1'); 7.46 (m, 1H, H-3p); 7.52–7.59 (m, 5H, H-8 and H-o,m-P); 8.20 (s, 1H, H-2). 13C NMR (125.7 MHz, D2O, δC = 7.1, phosphate buffer, ref_dioxane = 69.3 p.p.m): 41.32 (CH-2); 83.30 (d, J,C,p = 6.0, CH-5'); 84.60 (CH-4'); 114.10 (CH-1'); 129.00 (C-5'); 131.10 (CH-2); 134.50 (C-3'); 152.90 (C-6').

**PES experiment**

The reaction mixture (40 μl) contained Vent(exo-) DNA polymerase (New England Biolabs, 0.1 U), natural dNTPs (dGTP, dCTP and TTP, Fermentas, 0.2 mM), modified surrogates of dATP (derivatives of 7-deaza-dATP (7-deaza dATP, Jena Bioscience), 0.2 mM, 8-modified dATP 1 mM), primer (Sigma-Aldrich oligo), sequence see Table 1 and 2, 0.15 μM), template (Sigma-Aldrich oligo, sequence see Tables 1 and 2, 0.225 μM) in 1 × Thermopol reaction buffer. Primer was labeled by

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Table 1. Templates and primer used to construct DNA with modification in recognition sequence

| Template primer | Restriction endonuclease | Sequence          |
|-----------------|--------------------------|-------------------|
| temp unlawful   | Acll                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | EcoRI                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | KpnI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PspGI                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PstI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PvuII                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | Rsal                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | SacI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | Scal                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | SphI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | KpnI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |

*Bold: target palindromic sequence for restriction endonuclease, underlined - position of the modification in product.

Table 2. Templates and primers used to construct DNA with modification outside the recognition sequence

| Template primer | Restriction endonuclease | Sequence          |
|-----------------|--------------------------|-------------------|
| temp unlawful   | HaeIII                   | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | EcoRI                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | EcoRI                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PvuII                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PvuII                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | PvuII                    | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | Rsal                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | SacI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |
| temp unlawful   | SacI                     | 5′-ACGACGACACCGCC-3′ |
| prim unlawful   |                         | 3′-GCTTACGACCGGT-5′ |

*Bold: target palindromic sequence for restriction endonuclease, underlined - position of modification in product.

use of [γ-32P]-ATP according to standard techniques. Reaction mixtures were incubated for 30 min at 60°C in a thermal cycler. The reaction mixtures were then divided into two portions (each 20 μl). The stop solution [40 μl, 80% (v/v) formamide, 20 mM EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol] was added to the first portion which was then analyzed using 12.5% denaturing PAGE. The second portion was used in following cleavage reaction.

Cleavage by restriction endonucleases (general procedure)

The second portion of products of PEX experiment was mixed with 1 × reaction buffer supplied by manufacturer relevant to restriction endonucleases [for cleavage by Sacl, PstI and KpnI the BSA (100 μg/ml, 0.2 μl) was added] and one of 11 types of restriction endonucleases (New England Biolabs, 2U). Reaction mixture was incubated at 37°C for 60 min and then the stop solution was added (40 μl, 80% (v/v) formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol). Products of cleavage by restriction endonucleases were analyzed using 12.5% denaturing PAGE. Visualization was performed by phosphoimaging.

Cleavage by EcoRI and PspGI

Second portion of products of PEX experiment with temp E c.E1.E2.PG was purified on spin columns (MicroSpin™ G-25 Columns, GE Healthcare) and then
mixed with 1 × reaction buffer supplied by manufacturer relevant to restriction endonucleases and restriction endonucleases (2 U). Reaction mixture was incubated at 37°C for 60 min (in case of PspGI at 85°C for 60 min) and then the stop solution was added (40 μl, 80%[v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol). Products of cleavage by restriction endonucleases were analyzed using 12.5% denaturing PAGE. Visualization was performed by phosphoimaging.

Kinetics of cleavage of unmodified and modified DNA by KpnI and SacI

The reaction mixture (70 μl) contained Vent (exo-) DNA polymerase (New England Biolabs, 0.1 U), natural dNTPs (dGTP, dCTP and TTP, Fermentas, 0.2 mM), modified surrogates of dATP (derivatives of 7-deaza-dATP, Jena Bioscience), 0.2 mM, 8-modified dATPs were previously found to be poor substrates for DNA polymerases, only derivatives bearing small bromine (dA8BrTP) or methyl (dA8MeTP) groups (29) were selected.

RESULTS

Two types of adenine-modifications of dATP were selected for the study: 8-substituted dATP and 7-substituted 7-deaza-dATP derivatives. As 8-substituted dATPs were previously found to be poor substrates for DNA polymerases, only derivatives bearing small bromine (dA8BrTP) or methyl (dA8MeTP) groups (29) were selected. On the other hand, a variety of alkyne or aryl groups are known to be tolerated by polymerases at position 7 of 7-deazaadenine dNTPs and, therefore, we have tested four examples bearing different groups varying in size and electronic effects: unsubstituted 7-deaza-dATP (dA7TP) and its 7-ethyl (dA7EtTP), 7-phenyl (dA7PhTP) and 7-(3-nitrophenyl) (dA7NO2TP, 19) derivatives (Chart 1).

Preparation of the two unknown dNTPs is shown in Scheme 1. dA7EtTP was prepared by the Sonogashira cross-coupling reaction (54) followed by standard triphosphorylation. dA7PhTP was prepared by the Suzuki-Miyaura cross-coupling reactions of 7-iodo-7-deaza-dATP with phenylboronic acid in analogy to the previously reported synthesis of dA7NO2TP (19).

In each experiment, the whole set of six modified dATP derivatives was used in PEX using Vent (exo-) polymerase that was previously reported to be the most versatile enzyme for efficient incorporation of these types of base-modified dNTPs. In all experiments, the unmodified dATP was used as positive control (A). The sequences (Tables 1 and 2) were designed in order to contain the modified adenine either within or outside of the palindromic recognition sequence. In all cases, the PEX proceeded very well to give fully extended products which were analyzed on denaturating PAGE (Figures 1–3 and Supplementary Data).

Eleven type-II restriction endonucleases, AfeI (Abdurashitov et al., unpublished observations; Stickel and Roberts, unpublished observations), EcoRI (55–58), HaeIII (59,60), KpnI (61–63), PspGI (64,65), PstI (66), PvuII (67), Rsal (37), SacI (68), Scal (69) and SphI (70), were selected and tested for the ability to cleave the sequences containing modified A in the recognition sequence. Products of PEX were without further purification treated with restriction enzymes and the products were analyzed on PAGE. Table 3 and Figure 1 (Supplementary Figure S1) summarize the results of these restriction cleavage experiments. Virtually all the

Chart 1. dNTPs used in construction of studied DNA.
Scheme 1. Synthesis of 7-deza-7-modified dATP.

restriction enzymes were able to cleave all sequences containing 8-modified adenine derivatives (Br or Me) showing that these small functional groups in position 8 do not affect the ability of the enzyme to recognize and cleave the corresponding sequence in contrast to previous results of Komatsu et al. (36) who reported that 8-substituents blocked the cleavage by EcoRI. The ability of enzymes to cleave those sequences was not surprising since these compounds still possess the N7 suitable for H-acceptor interaction with the enzyme in the same way as in unmodified A. On the other hand, 7-deazaadenine derivatives lack this crucial N-atom and it was previously reported by Seela (38) that the presence of an unsubstituted 7-deazaadenine (H) in recognition sequence blocks the cleavage by some restriction enzymes. In our experiments, DNA sequences containing 7-deazaadenine (H) were efficiently cleaved by most of the enzymes (PvuII, RsaI, PspGI, KpnI, PsiI, SacI and SphI) and partly cleaved by AfeI. Only two of the studied enzymes (SacI and EcoRI) were found to be unable to cleave the modified sequences containing this base. This clearly shows that the N7 nitrogen is not crucial for the recognition of the sequence by most of these enzymes.

Replacement of the hydrogen at position 7 of 7-deazaA by acetylene (E) resulted in blocking the cleavage of the sequences not only by SacI and EcoRI, but also by AfeI, PvuII and PsiI. Introduction of even more bulky phenyl (Ph) or nitrophenyl (NO₂) groups completely restrained any cleavage by most restriction enzymes. The only exception was PspGI enzyme which was found to be able to cleave sequences containing all the tested modified adenines.

In most cases, the cleavage was perfectly specific and occurred in the expected positions in the sequence even when the crude PEX products were not purified prior to treatment with a restriction enzyme. Only in case of EcoRI and PspGI, we have observed a star activity (71), resulting in cleavage at different positions when crude unpurified PEX products were used (even in case of unmodified DNA sequences used as positive control). Therefore, in these cases, the crude PEX products were first purified on MicroSpin™ G-25 Columns and the purified DNA was then treated with the restriction enzyme to give correct specific cleavage at expected positions (Supplementary Figure S2).

Table 3. Summary of results of cleavage of DNA containing modification within the recognition sequence by restriction enzymes

| Enzyme target sequence | A | H | E | Ph | NO₂ | Br | Me |
|------------------------|---|---|---|----|-----|----|----|
| AfeI                   | + | + | - | -  | -   | +  | -  |
| 3'-TCG/CAG-5'         | + | + | - | -  | -   | +  | +  |
| PvuII                  | + | + | - | -  | -   | +  | +  |
| 3'-GTC/GAC-5'         | + | + | + | -  | -   | +  | +  |
| RsaI                   | + | - | - | -  | -   | +  | +  |
| 3'-CA/TG-5'           | + | - | - | -  | -   | +  | +  |
| SacI                   | + | - | - | -  | +   | -  | -  |
| 3'-TCA/TAG-5'         | + | + | + | +  | +   | -  | -  |
| PspGI                  | + | - | - | -  | -   | -  | -  |
| 3'-GGAC/CA G-5'       | + | - | - | -  | -   | -  | -  |
| EcoRI                  | + | - | - | -  | +   | -  | -  |
| 3'-CTTAA/G-5'         | + | + | + | -  | +   | -  | -  |
| KpnI                   | + | - | - | -  | -   | -  | -  |
| 3'-C/TATGG-5'         | + | - | - | -  | -   | -  | -  |
| PsiI                   | + | - | - | -  | -   | -  | -  |
| 3'-G/ACGTC-5'         | + | - | - | -  | -   | +  | +  |
| SacI                   | + | - | - | -  | +   | -  | +  |
| 3'-C/TCGAG-5'         | + | + | + | -  | +   | -  | +  |
| SphI                   | + | + | + | -  | +   | -  | +  |
Figure 1. Denaturing PAGE analysis of PEX products (lanes 2, 4, 6, 8, 10, 12 and 14) and products of PEX followed by cleavage with 6 restriction endonucleases (lanes 3, 5, 7, 9, 11, 13 and 15).
In order to compare the kinetics of cleavage of natural and modified DNA, we have designed simple experiments determining conversions of cleavage in timescale 1, 2, 5, 10 and 30 minutes. Two enzymes (KpnI and SacI) were selected for cleavage of unmodified DNA and DNA containing 7-ethynyl-7-deazaadenine base (E) in the recognition sequence as the most bulky modification tolerated by these two enzymes (Supplementary Figure S3). The reactions were stopped at the particular times by adding of urea and EDTA (72) and the conversions were semi-quantitatively calculated from the PAGE analysis. In general, the reaction rates were not significantly different neither for unmodified and modified sequences with the same enzyme nor for the same modification with two different enzymes. In all cases ~50% conversion was observed in 1–2 min and virtually full conversion in 30 min. The kinetic experiment with SacI endonuclease showed expected slightly faster cleavage of natural DNA compared to the modified sequence. On the other hand, KpnI unexpectedly cleaved modified DNA somewhat faster than the unmodified one.

The same set of modifications was also tested in restriction cleavage of DNA containing the modification outside of the recognition sequence. We have selected five enzymes (HaeIII, PvuII, RsaI, SacI and EcoRI) and for each of them sequences containing a modified A next to the recognition sequence were designed and prepared by PEX (Table 2). For the latter four enzymes, sequences containing two unmodified bases between the modification and recognition sequence were also prepared for comparison (Table 2). All these DNA sequences were subjected to cleavage by restriction enzymes to show that virtually all of them were perfectly and specifically cleaved by all these enzymes (Table 4, Figure 3). The bands of cleaved products using EcoRI are rather weak due to some loss of material during the additional purification of the PEX product (needed to prevent the star activity) but the absence of the band of PEX product clearly indicates full conversion of the cleavage. Only using SacI enzyme in combination with DNA modified by bulky Ph or NO2 modifications in adjacent position to the recognition sequence, the cleavage was only partial (Supplementary Figure S4).

Table 4. Summary of results of cleavage of DNA containing modification outside the recognition sequence by restriction enzymes

| Enzyme target sequence | A | H | E | Ph | NO2 | Br | Me |
|------------------------|---|---|---|----|-----|----|----|
| HaeIII 3’-ACC/GG-5’   | + | + | + | +  | +   | +  | +  |
| PvuII RePv1 3’-AGTC/GAC-5’ | + | + | + | +  | +   | +  | +  |
| RsaI ReRs1 3’-AC/TG-5’ | + | + | + | +  | +   | +  | +  |
| RsaI ReRs2 3’-ATGC/A/G-5’ | + | + | + | +  | +   | +  | +  |
| SacI ReSa1 3’-AC/TGGAG-5’ | + | + | + | +  | +   | +  | +  |
| SacI ReSa2 3’-ATGCTGA/G-5’ | + | + | + | +  | +   | +  | +  |
| EcoRI ReEc1 3’-ATTTAA/G-5’ | + | + | + | +  | +   | +  | +  |
| EcoRI ReEc2 3’-ATGCTTAA/G-5’ | + | + | + | +  | +   | +  | +  |
In order to test the possibility of protection of a certain sequence from cleavage by introduction of modification in presence of another copy of the same sequence, we have performed an experiment using radiolabeled template temp$^{EcoI}$ and primer prim$^{B}$. PEX experiments using modified dATPs built up DNA duplexes containing two copies of palindromic sequence GGTACC where one of them was containing a modified A in one strand, while the other one was unmodified. All these DNA sequences were then treated with endonuclease KpnI (Figure 4). The DNA containing unmodified A was cleaved at both sites and only the radiolabeled short 14-mer oligonucleotide was observed on PAGE (lane 3). Similar situation was observed for sequences containing 8-substituted adenines (Br or Me) and 7-deazaadenines (H or E) but in these cases, a weak band of longer 35-mer ON was also observed. In case of the bulkiest modifications (Ph or NO$_2$), the cleavage of the modified sequence was almost fully prevented and the longer 35-mer ON was the only product of restriction cleavage (lanes 9 and 11). This clearly shows that the modified sequence was protected from the cleavage by the phenyl-modifications of 7-deazaadenine in presence of another unmodified copy of the same sequence that was still cleaved under these conditions.

**DISCUSSION**

The motivation for this study was the future incorporation of the base-modified nucleotides into large DNA (plasmids, genomic DNA) by combination of PEX incorporation and cleavage by restriction enzymes with enzymatic ligation. The results clearly show that there is surprisingly high tolerance of several restriction endonucleases to the presence of 8- or 7-modified purine analogs even in the recognition sequence. The DNA containing 8-modified adenine is usually cleaved as well as unmodified sequences. On the other hand, 7-substituted
7-deazaadenines are tolerated only by some enzymes depending on the nature and size of the substituent. While sequences containing unsubstituted 7-deazaadenine are cleaved by most enzymes (except for EcoRI and SacI), introduction of more bulky acetylene group results in blocking of the cleavage also by AfeI, PvuII and PstI. Large phenyl or nitrophenyl group at position 7 completely block cleavage by all tested enzymes except for PspGI. Kinetic experiments with KpnI and SacI revealed only rather minor differences in the rate of cleavage of unmodified and modified (E) sequences showing that the reactions are completed within 30 min.

The explanation of the differences in tolerance of different enzymes to different modification and classification of the enzymes is very complicated. All the enzymes used are type-II restriction endonucleases from the same class of enzymes. However, each of the enzymes specifically recognizes a different sequence containing a different number of adenines and cleaves the sequences in a different position. In other words, this means that each enzyme has a totally different substrate and thus the data for two different enzymes are not easily and directly comparable. Nevertheless, some conclusions and at least presumptive explanations can be made. The high tolerance of PspGI to the presence of all types of modified adenines (even those containing bulky phenyl groups) can be rationalized by the fact that this is the only enzyme from our set which recognizes a 5-bp duplex composed of CCAGG sequence in one strand and CCTGG in the opposite strand (all the other enzymes recognize palindromic sequences of 4 or 6 bp). On the other hand, the two least tolerant enzymes (EcoRI and SacI) which are blocked even by the presence of 7-deazaadenine (and certainly also by its all substituted derivatives) are both characterized by recognition sequences containing two adenines (in our cases two modifications). The lack of cleavage of sequences containing 7-deazaadenines can be either explained by the presence of two modifications or by the need of specific H-bond(s) to N7 of adenine(s) for the recognition by the enzyme. The largest group of enzymes tolerates the presence of 7-deazaadenine but is blocked either by all 7-modifications (AfeI, PvuII and PstI) or by aromatic 7-modifications (RsaI, KpnI, SacI and SphI). They all recognize palindromic sequences containing one adenine and apparently do not require an H-bond interaction with N7 (since they all tolerate the presence of 7-deazaadenine). The minor differences in tolerance to the presence of bulky C7 modifications may reflect a different size and shape of the active site of each enzyme.

On the other hand, the ability of some 7-deazaadenines to block the cleavage is also potentially useful and may be utilized in protection of certain sequences from cleavage even in the presence of identical (but unmodified) sequences required to be cleaved. This was proved by one example of a specific protection of one copy of a sequence in presence of another copy that is still cleaved by KpnI enzyme. During the revision of this manuscript, a communication by Ang and Lippard (73) appeared reporting on protection of a plasmid containing a sequence site-specifically modified by 7-deazaadenine from cleavage by BstAPI restriction enzyme. These and our present results confirm that the site-specific modification of DNA by deazaadenines is a very useful tool in manipulation of diverse DNA sequences. By the proper choice of the sequence, modification and restriction enzyme we can either achieve cleavage or protection of the modified sequences.

Also of interest and importance was the finding of star activity of EcoRI and PspGI enzymes, that was observed when crude PEX products were used as substrates. It can be only speculated whether the cleavage of such crude products at unexpected positions was caused just by the presence of metal cations or other additives used for PEX or whether the presence of unreacted dNTPs and primers or DNA polymerase may have also some effect. In any case, the purification of the PEX products solved this problem and the cleavage proceeded with the correct sequence specificity.

While the presence of modification within the recognition sequence leads to blocking of the cleavage in some cases, introduction of the modification next to the recognition sequence does not restrain the cleavage in any case. This means that there is a big potential of the functionalized DNA to be manipulated by restriction enzymes if one can select a suitable enzyme specific to sequence close to the modification. The ligation of the modified DNA fragments by DNA ligases is the subject of our ongoing study.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.
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