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Template-directed addition of nucleosides to DNA by the BfiI restriction enzyme

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ABSTRACT

Restriction endonucleases catalyse DNA cleavage at specific sites. The BfiI endonuclease cuts DNA to give staggered ends with 1-nt 3′-extensions. We show here that BfiI can also fill in the staggered ends: while cleaving DNA, it can add a 2′-deoxynucleoside to the reaction product to yield directly a blunt-ended DNA. We propose that nucleoside incorporation proceeds through a two-step reaction, in which BfiI first cleaves the DNA to make a covalent enzyme–DNA intermediate and then resolves it by a nucleophilic attack of the 3′-hydroxyl group of the incoming nucleoside, to yield a transesterification product. We demonstrate that base pairing of the incoming nucleoside with the protruding DNA end serves as a template for the incorporation and governs the yield of the elongated product. The efficiency of the template-directed process has been exploited by using BfiI for the site-specific modification of DNA 5′-termini with an amino group using a 5′-amino-5′-deoxythymidine.

INTRODUCTION

The fundamental reaction catalysed by Type II restriction endonucleases is the hydrolysis of the phosphodiester backbone of DNA at specific sequences, yielding 3′-hydroxyl and 5′-phosphoryl termini (1). The vast majority of restriction enzymes, such as EcoRI or EcoRV, require a metal ion co-factor (2) and stereochemical analyses have revealed that DNA is cleaved with inversion of configuration, implying direct attack of water at the scissile phosphate without an enzyme–DNA covalent intermediate (3,4). BfiI is a type IIS restriction enzyme (5) that cleaves DNA downstream of its recognition sequence as follows,

5′ – A-C-T-G-G-G-n-n-n-n-n↓n – 3′
3′ – T-G-A-C-C-C-n-n-n-n↑n-n – 5′

[where n denotes any nucleotide (nt) and the arrows mark the points of cleavage], to leave products with 1-nt 3′ extensions (6). (However, while BfiI usually cuts the top strand 5 nt away from the recognition site, it will on rare occasions cut it 6 or 7 nt away.) A single active site is used site to cut both strands in a fixed order, first the bottom strand and only later the top strand (7). However, unlike other restriction enzymes, BfiI functions without metal ions (8). BfiI and its isoschizomer BmrI belong to the phospholipase D (PLD) family of enzymes (9–11), whose members catalyse hydrolysis and/or transesterification reactions on a broad range of phosphodiester substrates (12–14). Like other members of the PLD family, BfiI catalyses not only phosphodiester hydrolysis but also transesterifications. The latter proceed with retention of stereoconfiguration at the phosphorus, indicating, uniquely for a restriction enzyme, a two-step mechanism (15). We proposed that BfiI initially uses a conserved active site histidine to attack the scissile phosphate, forming a covalent phosphohistidine enzyme–DNA intermediate with displacement of the 3′-OH leaving group: the intermediate is subsequently resolved by nucleophilic attack with either water or an alcohol, to yield hydrolysis or transesterification products, respectively (15).

During DNA cleavage in the presence of ethanol or glycerol, BfiI generates transesterification products with an alcohol moiety covalently attached to the 5′-terminal DNA phosphate (15). However, to prevail over water as the attacking nucleophile, intermolecular transesterification reactions require high concentrations of the alcohol, in the molar range. BfiI also catalyses an intra-molecular transesterification reaction that joins the 5′-phosphate from the scissile bond in the bottom strand to the 3′-OH of an extended top strand, to produce a DNA hairpin (15). The highest yield of hairpin was achieved on DNA substrates containing self-complementary sequences in the protruding 3′-section of the top strand. These presumably form snap-back structures that hold the 3′-OH terminus of the top strand in position to attack the covalent

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intermediate between the protein and the 5′-phosphate on the bottom strand. This finding suggests that base pairing may play a crucial role in positioning the incoming nucleophilic 3′-OH group.

We ask here whether the 3′-OH group of a 2′-deoxynucleoside can act as an acceptor for inter-molecular transesterification by BfiI and if so, whether transesterification reactions with nucleosides can be enhanced by base pairing between the nucleoside and a DNA template. We find that, after cutting DNA to produce 1-nt 3′-overhangs, BfiI can attach a deoxynucleoside from solution to the 5′-terminal phosphate of the cleaved DNA to generate a blunt-ended product. This transesterification reaction is governed by base pairing of the incoming nucleoside with the protruding 1-nt 3′- extension, which serves as a template. In contrast to inter-molecular transesterifications with ethanol or glycerol, the template-direction incorporation proceeds efficiently at low (millimolar) concentrations of the complementary deoxynucleoside.

MATERIALS AND METHODS

Materials

All nucleosides used in this study were purchased from Sigma–Aldrich (St. Louis, MO): thymidine (dT); 2′-deoxycytidine (dC); 2′-deoxyguanosine (dG); 2′-deoxyadenosine (dA); 2′,3′-dideoxyadenosine (2′,3′-ddA); 2′,5′-dideoxyadenosine (2′,5′-ddA); adenosine (A) and 5′-amino-5′-deoxythymidine.

Oligodeoxynucleotides were purchased from Metabion, (Martinsried, Germany). They were 5′-labelled with [γ-^{33}P]ATP (Hartmann Analytic, Braunschweig, Germany) and polynucleotide kinase, or 3′-labelled with [α-^{32}P]ddATP (GE Healthcare, Chalfont St. Giles, UK) and terminal deoxynucleotidyl transferase (Fermentas UAB, Vilnius, Lithuania): the labelled products were gel-purified (15). Duplexes were obtained by annealing complementary pairs of oligonucleotides.

BfiI was purified as before and its concentrations are given in terms of the dimer (9).

Transesterification reactions

Transesterification reactions were performed by mixing radiolabelled oligoduplexes (1–2 nM) with BfiI enzyme (20 nM) in reaction buffer (20 mM potassium phosphate, pH 7.4, 120 mM KCl, 1 mM EDTA, 0.025 mg/ml BSA). Where appropriate the reactions also contained the relevant nucleoside at $\leq$4.0 mM. After 2 h at 25°C, the samples were quenched with phenol/chloroform or loading dye solution (7). Separation of DNA hydrolysis and transesterification products was performed by denaturing PAGE as described previously (7). Radiolabelled DNA was detected and quantified by using the FLA-5100 phosphorimager (Fujifilm, Tokyo, Japan).

Mass spectrometry

Reactions contained 250 pmol of a 30-bp oligonucleotide duplex (Figure 1) and 250 pmol BfiI in 250 µl reaction buffer that either lacked any nucleoside or were supplemented with one of the following (at 1 mM): dT for the reaction on 30T/30A; dA for 30A/30T; dC for 30C/30G; dG for 30G/30C. After 2 h at 25°C, the reactions were stopped by phenol/chloroform extraction. The samples were desalted by NAP-5 and NAP-10 gel-filtration columns (GE Healthcare) in 0.1 M triethylammonium acetate (pH 7.0) and concentrated by rotary evaporation. Mass spectra of the samples were acquired on a Voyager-DE STR MALDI-TOF mass spectrometer (PE Biosystems, Foster City, CA, USA) using a nitrogen laser operating at 337 nm. The matrix solution was freshly prepared 3-hydroxypicolinic acid (3-HPA, puriss p/a/grade; Sigma–Aldrich, St. Louis, MO,) at 50 mg/ml in a 50:50 mixture of acetonitrile (Rathburn, Walkerburn, Scotland) and deionized water. Diammonium citrate (5 mg/ml in deionized water; Sigma, St. Louis, MO, USA) was added to the matrix in the ratio of nine parts of HPA to one part of diammonium citrate. Sample ($\sim$10 pmol/µl) and matrix, 0.5 µl of each, were spotted onto the sample plate. The spectrum was acquired over the range 3000–6000 Da under negative linear conditions with an accelerating voltage of 20000 V and an extraction delay time of 175 ns.

RESULTS AND DISCUSSION

The DNA alcoholysis reactions catalysed by BfiI were previously studied on minimal oligoduplex substrates in which the top strand was truncated prior to the primary site of cleavage 5 bases downstream of the recognition sequence. These duplexes thus contained a scissile phosphodiester bond only in the bottom strand (15). Significant amounts of DNA-alcohol adducts were observed only at relatively high alcohol concentrations ($\sim$1 M) and their yields decreased with time as the terminal phosphodiester bond between the alcohol and the bottom DNA strand was subsequently hydrolysed by the enzyme (15). The ability of BfiI to catalyse DNA alcoholysis and intramolecular strand transfer reactions prompted us to investigate whether BfiI could catalyse transesterification with a deoxynucleoside as the alcohol and, if so, whether the incorporation of the deoxynucleoside could be enhanced by complementary base pairing with a DNA template. If the latter were realized, the 1-nt 3′-extension left upon DNA cleavage by BfiI would be filled in to yield directly a blunt-ended product.

BfiI cleavage of double-strand DNA in the presence of deoxynucleosides

In the first set of experiments, BfiI was tested against a 30-bp oligoduplex 30T/30A (Figure 1A): after first cutting the bottom strand of this duplex (7), BfiI cleaves the top strand to liberate a product that carries on its bottom strand a 1-nt 3′-extension, a single A residue. The 30T/30A duplex was end-labelled in either bottom or top strands, and the cleavage reactions were carried out in solutions that either lacked a deoxynucleoside or contained one of the following: dA, dT, dC or dG.

The 30T/30A duplex that had been 5′-end labelled in the bottom strand was cleaved by BfiI in both the absence and presence of nucleoside to yield only one radiolabelled

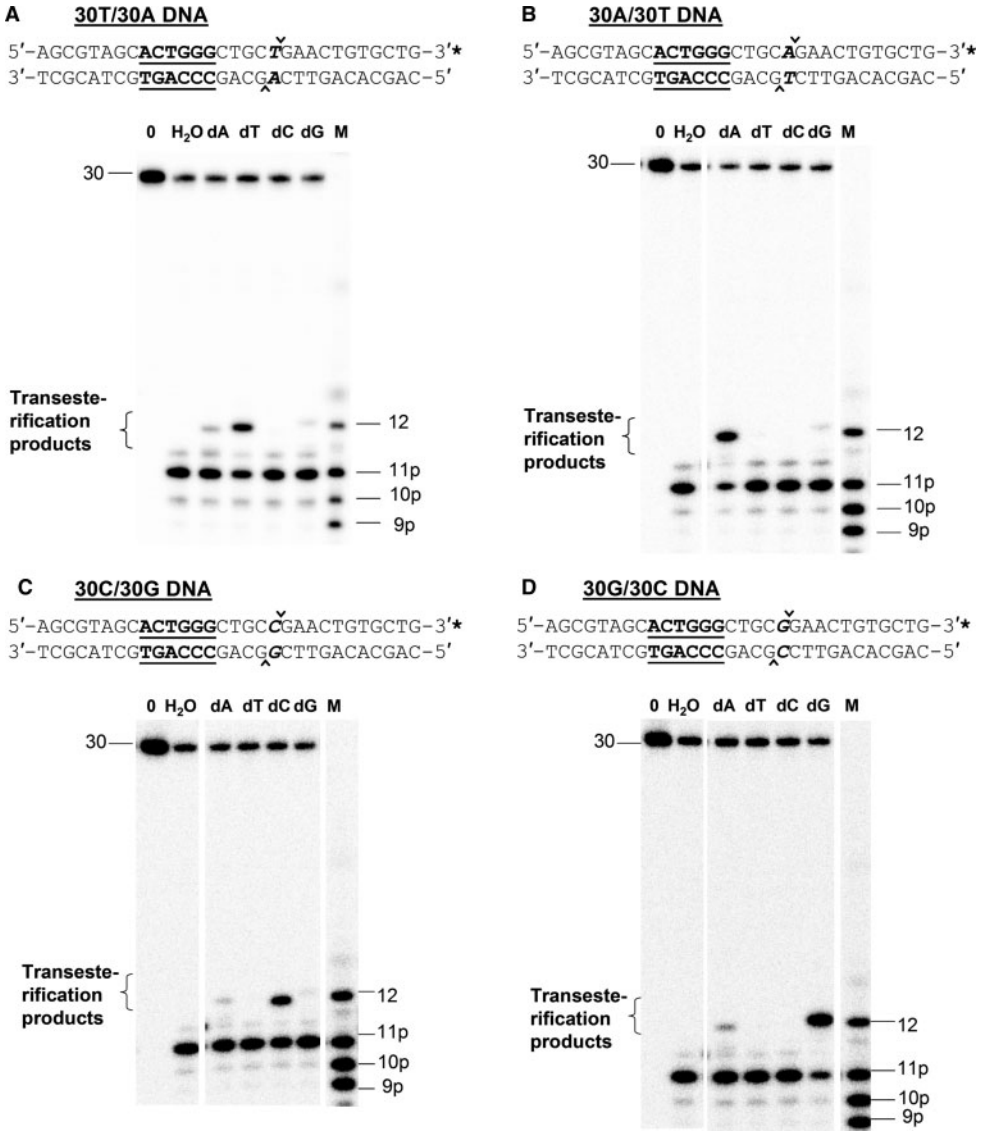

Figure 1. Products from BfiI reactions in the presence of deoxynucleosides. In all four panels (**A–D**), the oligoduplex substrate for BfiI is shown: the recognition sequence for BfiI is in bold and underlined; the points of top and bottom strand cleavage are marked with a v-sign (∨) and a caret-sign (∧), respectively and the base pair that gives rise to the 1-nt 3′-extensions is in bold italic; the 3′-^{32}P label in the top strand is noted by an asterisk (*). The reactions, contained BfiI enzyme (20 nM) and the oligoduplex indicated (1 nM): in (A), 30T/30A; in (B), 30A/30T; in (C), 30C/30G; in (D), 30G/30C. They also contained either no deoxynucleoside (lanes marked H_2O) or 1 mM nucleoside: dA, dT, dC or dG (lanes marked dA, dT, dC and dG, respectively). After 2 h in reaction buffer at 25°C, the reactions were quenched and the samples analysed by denaturing PAGE: the phosphorimager record of each gel is shown. Lane 0 lacks BfiI enzyme. Lane M contains chemically synthesized 5′-phosphorylated oligonucleotides of 11, 10 and 9 nt (bands 11p, 10p and 9p) that correspond to the BfiI products of top DNA strand hydrolysis 5-, 6- and 7-nt downstream of the recognition site. The non-phosphorylated oligonucleotide 12 corresponds to the 11-nt hydrolysis product extended by one nucleoside. The weak bands in the reaction lanes between the 12 and 11p positions correspond to the top DNA strand–glycerol adduct (~3% of total reaction products) (15). Trace amounts of glycerol (~0.1% vol/vol) were introduced into the reactions with the glycerol-containing protein stock solutions.

product, the expected DNA hydrolysis product 12-nt long. For the same duplex labelled at its 3′ terminus, the only radiolabelled product from the latter reaction was the expected 18-nt fragment (data not shown). Similarly, when the top strand of 30T/30A was 5′-labelled, the reactions with and without added nucleoside again yielded only one major radiolabelled product, the expected 19-nt hydrolysis product (data not shown). However, when the top strand in the 30T/30A duplex was radiolabelled at its 3′-terminus, the reactions in the presence of nucleoside yielded additional products with lower electrophoretic mobilities than the expected 11-nt fragment generated in the absence of any nucleoside (Figure 1A). The yield of the novel product depended on the deoxynucleoside present in the reaction mixture: with 30T/30A as the substrate, the highest yield was obtained with dT, the nucleoside complementary to the 1-nt 3′-extension (an A residue), while dA, dG and dC all gave vastly lower amounts.

Equivalent reactions were carried out on 30A/30T, on 30C/30G and on 30G/30C (Figure 1B–D). BfiI cleaves each of these substrates to leave 1-nt 3′-extensions on the bottom strand: a single T in the case of 30A/30T, a G from

30C/30G and a C from 30G/30C. All three of these duplex were 3′-labelled in the top strand and the cleavage reactions carried out in the absence of any nucleoside or in the presence of one of the four canonical 2′-deoxynucleosides; dA, dT, dC or dG. None of these substrates yielded extra products when cleaved in the absence of nucleoside but all three gave rise to additional products in the presence of one or more of the deoxynucleosides tested. With 30A/30T, much more of the additional product was formed with dA in the reaction than with dT, dC or dG (Figure 1B). Conversely, 30C/30G gave the highest level of elongated product with dC (Figure 1C) and 30G/30C with dG (Figure 1D). Thus in all cases, the extra low-mobility product was formed best when the deoxynucleoside added to the reaction mixture was complementary to the nucleotide at the 3′-extension in the bottom strand.

Identification of additional products

Since extra products were detected only when the top strand of 30T/30A was 3′-labelled and not when it was 5′-labelled, the additional low-mobility products generated from the top strand may be transesterification products formed by the transfer of the 11-nt fragment to a nucleoside rather than to water. To examine this possibility, all four of the substrates tested above were again cleaved by BfiI in either the absence or presence of a deoxynucleoside, but this time the products were analysed by mass spectrometry (Figure 2) rather than gel electrophoresis. The deoxynucleoside present with each duplex was the one that generated the highest yield of the additional low-mobility product: dT for the reaction on 30T/30A, dA for 30A/30T, dC for 30C/30G and dG for 30G/30C.

When the products from the reactions in the absence of deoxynucleoside were analysed by mass spectrometry, the spectra revealed species with the masses expected for the 11-nt 3′-terminal product from the top strand and for the 12-nt 5′-terminal product from the bottom strand, in approximately equimolar amounts (Figure 2). However, when the reactions in the presence of deoxynucleoside were analysed by mass spectrometry, an additional species was detected in all cases, whose mass corresponded to sum of the masses of the 11-nt top strand product and the additional deoxynucleoside: in parallel, the unmodified 11-nt top strand product was now present at a lower level than the 12-nt bottom strand product (Figure 2). The mass spectra are thus fully consistent with the view that the additional low-mobility products contain the 11-nt product fragment joined to the cognate nucleoside in a transesterification reaction.

Putative reaction scheme

Previous studies had shown that BfiI contains separate domains for DNA recognition and cleavage (16) and that the DNA cleavage unit acts sequentially on the two strands of a DNA substrate, first cutting the bottom strand 4-nt downstream of the recognition site and then

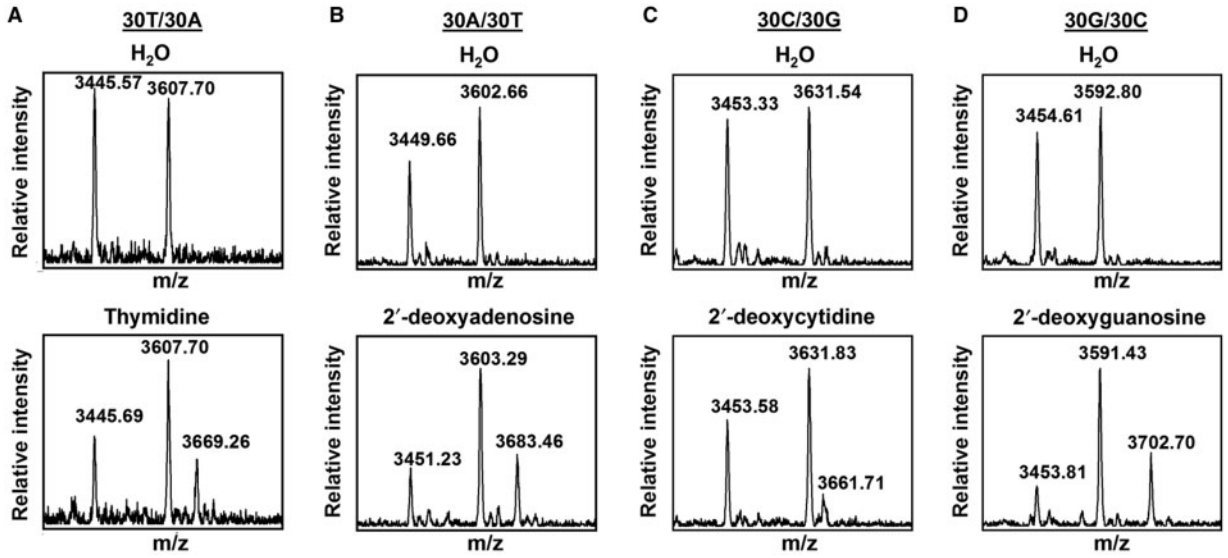

Figure 2. MALDI-TOF analysis of DNA cleavage products. Reactions of BfiI on one of the following duplexes were carried out, and the products analysed by MALDI-TOF, as described in 'Materials and methods' section. The spectra shown display only the 11- and 12-nt products from the top and bottom strands, respectively. The intact 30-nt strands and both 18- and 19-nt products fell outside the range shown. The buffers contained either no nucleoside (the spectra labelled H_2O) or one from dT, dA, dC or dG (as indicated by each spectrum). (**A**) The duplex was 30T/30A and the added nucleoside dT. The theoretical masses of the 5′-phosphorylated 11-nt and 12-nt cleaved products, from top and bottom strands respectively, are 3451 (observed, 3446) and 3613 (observed, 3608). Attachment of dT to the 5′-end of the 11-nt product from the top strand should increase its mass by 224 (observed, by 223). (**B**) The duplex was 30A/30T and the added nucleoside dA. The theoretical masses of the 11-nt top strand and 12-nt bottom strand products are 3451 (observed, 3450) and 3604 (observed, 3603). Attachment of dA to the 5′-end of the top strand product should increase its mass by 233 (observed, by 232). (**C**) The duplex was 30C/30G and the nucleoside dC. The theoretical masses of the 11-nt top strand and 12-nt bottom strand products are 3451 (observed, 3453) and 3629 (observed, 3632). Attachment of dC to the 5′-end of the top strand product should increase its mass by 209 (observed, by 208). (**D**) The duplex was 30G/30C and the nucleoside dG. The theoretical masses of the 11-nt top strand and 12-nt bottom strand products are 3451 (observed, 3455) and 3589 (observed, 3593). Attachment of dG to the 5′-terminus of the top strand product should increase its mass by 249 (observed, by 249).

the top strand 5 nt away to leave 1-nt 3′-overhangs (7). BfiI hydrolyses phosphodiester bonds by means of a covalent intermediate, so its attack on the phosphodiester bond in the top strand yields initially BfiI bound covalently through a phosphohistidine linkage to the 5′-phosphate at the site of cleavage.

We propose that, after cutting the bottom strand, BfiI attacks the phosphorous at the scissile bond in the top strand to form the covalent intermediate (Figure 3). This expels the 3′-OH leaving group to yield the DNA product cleaved in both strands but with BfiI still attached to the 5′-phosphate: the product possesses a 1-nt 3′-extension, a dA in the case of the 30T/30A substrate. The next stage during the reaction on 30T/30A may then involve dT diffusing from solution into the enzyme active site, to form the complementary Watson–Crick base pair with the 3′-protruding nucleoside of the bottom strand, the dA. Finally, the 3′-OH group of dT acts as a nucleophile to attack the covalent phosphohistidine intermediate, so releasing the BfiI protein and forming a thymidine-DNA adduct (Figure 3). The blunt-end transesterification product escapes from the active site and cannot be hydrolysed further by BfiI.

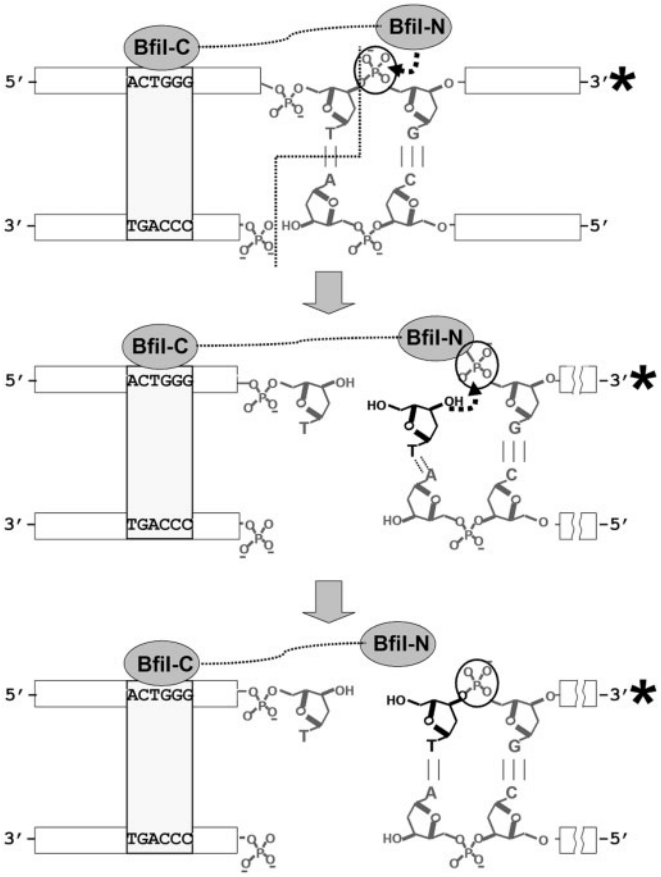

Figure 3. Putative reaction mechanism. The scheme shows the proposed pathway for the reaction of BfiI on 30T/30A DNA in the presence of dT. The two strands of the duplex are marked as horizontal blocks, with the recognition sequence highlighted in grey: the chemical natures of the scissile phosphodiester bonds are given in full. The BfiI endonuclease is shown as a C-terminal DNA-binding domain connected by a linker to the N-terminal catalytic domain: BfiI-C and BfiI-N, respectively, both as grey ovals. After cutting the bottom strand, BfiI-N makes a nucleophilic attack (dashed arrow) on the scissile phosphate (encircled) to form a covalent intermediate, with concomitant release of the 3′-leaving group. The adenine base in the 1-nt 3′-extension base pairs with a dT from free solution (in black), which in turn uses its 3′-OH group to make a nucleophilic attack on the covalent intermediate (dashed arrow): this joins the dT to the 3′-terminus to give a blunt-ended DNA product, whilst displacing the enzyme from the DNA.

The mechanism in Figure 3 supposes that a key factor in the binding of dT to the active site of BfiI during its reaction on 30T/30A is the formation of the correct base pair with the 3′-protruding dA on the bottom strand. If so, then DNA substrates containing other canonical base pairs (A:T, C:G and G:C) at the 5th position downstream of the target site should promote the incorporation of dA, dC and dG nucleosides, respectively. This expectation matched the observations with the 30A/30T, the 30C/30G and the 30G/30C substrates: each of these gave rise to elongated product only with the nucleoside complementary to the 3′-extension (Figure 1B–D). It thus appears that Watson–Crick base pairing acts as a checkpoint for the selection of the incoming nucleoside in these transesterification reactions.

Reactive hydroxyl group

The reaction scheme in Figure 3 implies that the 3′-OH group of the incoming deoxynucleoside acts as the nucleophile in the transesterification reaction. Indeed, if a correct Watson–Crick base pair between the incoming nucleoside and the complementary base in the protruding DNA strand is formed, it has to position the incoming nucleoside in the place previously occupied by the 3′-terminal nucleotide of the top strand (Figure 3). In this case, the 3′-OH moiety would be properly positioned to resolve the phosphohistidine intermediate, but not the 5′-OH. Nevertheless, to determine which OH group on the nucleoside acts as the alcohol, the 30A/30T substrate, which incorporated dA very much better than any other 2′ deoxynucleoside tested (Figure 1B), was used as a substrate for BfiI in reactions containing different adenosine derivatives: dA, 2′,5′-ddA, 2′,3′-ddA and A (Figure 4A).

High yields of nucleoside-DNA adducts were observed only in the reactions containing dA or 2′,5′-ddA, which both contain a 3′-OH group (Figure 4A). In contrast, 2′,3′-ddA, which lacks a 3′-OH group, gave only a minute amount of transesterification product, possibly by means of a 5′-OH attack or due to traces of contaminating 2′-dA. Adenosine, on the other hand, contains both 2′- and 3′-OH groups yet still gave low yields of transesterification product. Maybe the 2′-OH group is too bulky to be accommodated in the active site of BfiI. The dispensability of the 5′-OH group in the nucleoside transfer reaction was further validated by finding that 5′-amino-5′-deoxythymidine, which contains an amino group in place of a 5′-OH, was incorporated efficiently into the cleavage product from the 30T/30A duplex, a substrate that favours thymidine derivatives (Figure 4B). These studies thus show that transesterification by BfiI to a complementary nucleoside occurs primarily to the 3′-OH moiety of the nucleoside.

Incorporation of a 2′-deoxynucleoside into DNA via its 3′-OH group should generate a DNA fragment with a free

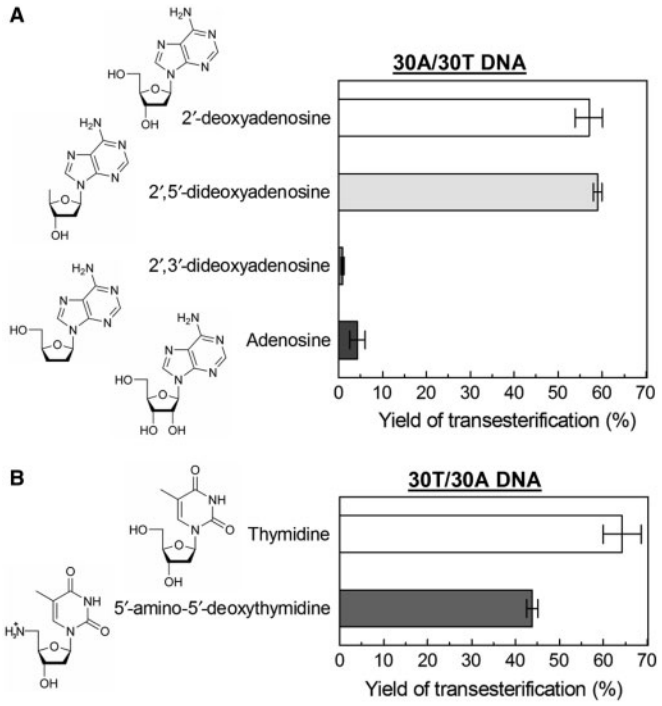

Figure 4. Identification of the reactive hydroxyl group. (**A**) Reactions of BfiI on the 30A/30T DNA duplex, which optimally incorporates adenine nucleosides, were carried out as described in 'Materials and methods' section in the presence of 2′-dA, 2′,5′-ddA, 2′,3′-ddA and A, each at 1 mM. The samples were analysed as in Figure 1 and the percent of the cleaved DNA converted to the additional low mobility product was recorded as 'yield of transesterification'. (**B**) The reactions were conducted as in (A) except that the duplex was 30T/30A DNA, which optimally incorporates thymine nucleosides, and the added nucleosides was either dT or 5′-amino-5′-deoxythymidine, each at 4 mM.

5′-terminal OH, whilst the hydrolysis product generated by BfiI carries a 5′-terminal phosphate. To identify the 5′-terminal moiety, the duplex 30T/30A was cleaved by BfiI in the presence of dT as in Figure 1A and the additional low-mobility product gel-purified. The product then was tested with calf intestine alkaline phosphatase and with polynucleotide kinase. Alkaline phosphatase cleaves phosphomonoesters and can thus remove 5′-phosphates, while polynucleotide kinase uses ATP to phosphorylate 5′-OH groups in DNA. The elongated product was resistant to phosphatase, but was phosphorylated by kinase (Supplementary Figure 1), thus indicating a DNA with a 5′-OH group. This observation confirms that BfiI attaches the 3′-OH group of a deoxynucleoside to the 5′-terminal phosphate of the cleaved DNA, to give a blunt-ended DNA fragment with a 5′-OH group. The phosphorylated oligonucleotide could in turn act as a substrate for T4 DNA ligase when paired with appropriate oligodeoxynucleotides to give a nicked duplex and the ligated product could be cleaved by a restriction enzyme whose recognition sequence spanned the site of ligation (Supplementary Figure 1). Since both T4 ligase and restriction enzymes are highly sensitive to structural and/or chemical irregularities in DNA, the DNA-nucleoside adducts formed by BfiI seems to have retained all of the features of *bona fide* DNA.

Templated versus non-templated incorporation of nucleosides by BfiI

To determine the extent to which transesterification to a 2′-deoxynucleoside can be assisted by a DNA template, reactions were carried out on either duplex DNA substrates that are cleaved by BfiI to give 1-nt 3′-extensions (Figure 1) or on a 3′-tailed substrate,

5′-AGCGTAGCACTGGGCTGCTGAACTGTGCTG-3′
3′-TCGCATCGTGACCCGACG-5′

(30/18), which lacks the target phosphodiester bond 4-nt downstream of the recognition sequence (underlined) in the bottom strand but which can still be cleaved 5 nt away in the top strand. The duplex substrates can allow for template-assisted addition of a nucleoside onto the phosphate at the site of top-strand cleavage, through base pairing with the 1-nt extension in the bottom strand, but the 3′-tailed substrate lacks any template capable of assisting nucleoside incorporation (Figure 5A). Thus, by comparing the yields of transesterification products from the reaction on a duplex in the presence of varied concentrations of the nucleoside complementary to its 1-nt 3′-extension with that from the reaction on 30/18 in the presence of the same nucleoside, it is possible to assess the extent to which the bottom-strand template assists the addition of nucleoside to the top-strand product (Figure 5B–E). In order to reveal the transesterification products, both the duplexes and the tailed DNA were 3′-end labelled in the top strand (as in Figure 1).

The yields of elongated products from the duplex substrates all increased in hyperbolic fashion with the concentration of whichever deoxynucleoside was complementary to the single-strand extension generated by BfiI cutting that duplex: i.e. the addition of dG to the product from 30G/30C (Figure 5B), dC to 30C/30G (Figure 5C), dT to 30T/30A (Figure 5D) and dA to 30A/30T (Figure 5E). The concentration dependencies were fitted to a hyperbolic function,

$$P = \frac{P_{\max} \times [\mathrm{dN}]}{(K_{\mathrm{D}} + [\mathrm{dN}])}$$

where P is the observed yield of transesterification product at deoxynucleoside concentration [dN], P_{max} the yield at saturating [dN], and K_{D} the dissociation constant for the nucleoside binding to the BfiI/DNA complex. The best fits were obtained with the values for K_{D} and $P_{\max}$ given in Table 1. The K_{D} value for the incorporation of dT onto a DNA with an adenine template was similar to that for dC with a guanine template, with both pyrimidines showing lower affinities for the complementary DNA–enzyme complex than either purine. For example, the affinity for dT with an adenine template was lower than that for dA with a thymine template (Table 1). If the affinity for the incoming nucleoside is due solely to the strength of the base pairing interaction with the template nucleotide, then the G:C pairings ought to have given lower K_{D} values than the A:T pairings, and the A:T pair should have equalled the T:A pair. Neither of these expectations was realized so other factors must govern the affinities. The low K_{D} values for the bulky purine bases are perhaps due to stacking interactions with the neighbouring bases

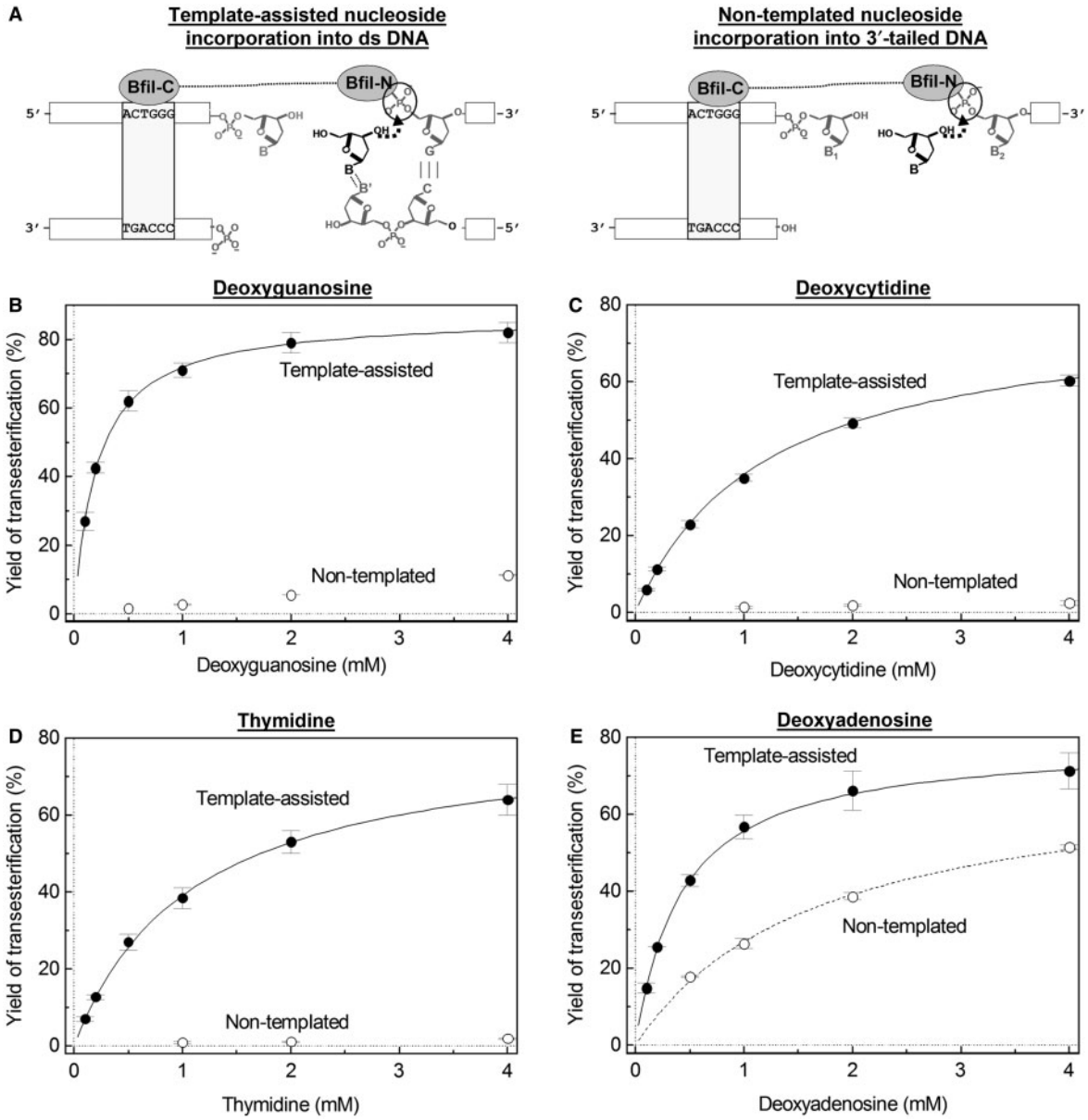

Figure 5. Role of DNA template in deoxynucleoside incorporation reactions. (**A**) Two schemes are shown for transesterification reactions. In both, the 3′-OH group of a nucleoside attacks the covalent intermediate between the BfiI protein and the 5′-phosphate of the cleaved DNA; in the left-hand scheme, template-assisted incorporation involving Watson–Crick pairing between the base on the incoming nucleoside and that on the 1-nt 3′-extension generated by BfiI cutting both strands (as in Figure 3); in the right-hand scheme, non-templated incorporation following BfiI cleavage of a 3′-tailed DNA substrate that is cut only in the top strand. (**B–E**) The reactions contained either a duplex DNA substrate for BfiI (one from Figure 1; for template-assisted incorporation) or a 3′-tailed DNA substrate (30/18, see text; for non-templated incorporation) and varied concentrations of a 2′-deoxynucleoside. After 2 h in reaction buffer with 20-nM BfiI enzyme, the reactions were stopped and the yield of transesterification product determined as a percent of the total level of DNA cleavage. In (B), the duplex was 30G/30C and the nucleoside dG. In (C), the duplex was 30C/30G and the nucleoside dC. In (D), the duplex was 30T/30A and the nucleoside dT. In (E), the duplex was 30A/30T and the nucleoside dA. In each plot, the yield of transesterification product from the duplex substrate is marked as template-assisted (filled circles) and that from the 3′-tailed substrate as non-templated (open circles). The lines are optimal fits to a rectangular hyperbola. The best fits were obtained with the maximal yields of transesterification product and with the K_D values for nucleoside binding shown in Table 1.

in the DNA helix and/or contacts within the binding pocket of BfiI.

The 3′-tailed substrate, 30/18, allows for only the non-templated incorporation of deoxynucleosides as it lacks the bottom strand that could otherwise act as a template (Figure 5A). The reactions of BfiI on 30/18 in the presence of either pyrimidine nucleoside yielded virtually none of their respective transesterification products. This shows that essentially all of the addition of dC to the cleaved product from 30C/30G, and likewise dT with 30T/30A, occurs by the DNA template-assisted pathway (Figure 5B and C). On the other hand, dG was added to the product from 30/18 at a low level (Figure 5A), while dA was attached at a significantly higher level (Figure 5D). The non-templated reaction on 30/18 yielded a K_D for dA (1.7 mM) that was only four times larger than that

Table 1. Comparison of BfiI transesterification reactions with various deoxynucleosides

Substrate	3′ nucleotide on bottom strand	Nucleoside added to top strand	P_{max} (%)	K_D (mM)
30G/30C	C	dG	87 ± 2	0.21 ± 0.02
30C/30G	G	dC	79 ± 2	1.2 ± 0.1
30T/30A	A	dT	82 ± 5	1.1 ± 0.2
30A/30T	T	dA	79 ± 3	0.42 ± 0.07
30/18	–*	dA	72 ± 5	1.7 ± 0.2

*Attachment of deoxyadenosine to the 30/18 substrate is a non-templated reaction.

from the template-assisted reaction on 30A/30T (K_D = 0.4 mM; Table 1).

Intriguingly, many DNA polymerases strongly favour dATP in non-template-directed reactions, e.g. addition of dNTPs to blunt ended double-stranded DNA (17,18) and incorporation of dNTP opposite an abasic site (19). A kinetic study to compare how polymerases responded to abasic sites confirmed the preference for dATP (20). It was suggested that dATP bound to the polymerase-DNA binary complex in the favoured *anti* conformation, whereas dGTP bound in the less-preferred *syn* form, accounting for the preference of dATP over dGTP. It was further postulated that incoming pyrimidines stacked less efficiently with neighbouring bases, resulting in non-optimal alignment with the active site and explaining why dATP is a better substrate than dCTP/dTTP. Such factors depend on the properties of free dNTPs and DNA, rather than the active site of the polymerase and may also account for the appreciable preference of BfiI for deoxyadenosine even in the absence of Watson–Crick base pairing with a template strand (Table 1). Alternatively, the active site of BfiI may have an appreciable affinity for purine residues, particularly dA. This may account for why both purine residues yielded lower K_D values than either pyrimidine even in the presence of a complementary base in the template.

At saturating nucleoside concentrations, the maximal yield (P_{max}) of nucleoside adducts from the template-directed reactions was only about 80% of the cleaved DNA (Table 1). This is most likely due to the fact that BfiI cuts the top DNA strand at several positions, with about 80% of its reactions occurring 5-nt downstream of the recognition site but with ~20% at alternate positions 6–7 nt away (7). Since the reaction mechanism depicted in Figure 3 is valid only for the fraction of the DNA cleaved 5-nt downstream of the BfiI site, the maximum yield of the corresponding transesterification products ought to be close to 80% rather than 100%.

Site-specific modification of 5′-DNA termini by BfiI

It is noteworthy that template-assisted transesterifications by BfiI can result in the addition of 5′-amino-5′-deoxythymidine to the 5′-phopshate of its cleaved DNA product with relatively high efficiency, even when the nucleoside is present at millimolar concentrations (Figure 4B). This is in marked contrast to the low efficiency of its transesterification reactions with alcohols such as ethanol or glycerol, which required molar concentrations of the alcohol (15). Specific labelling of DNA with an amino group provides a handle for the attachment of useful reporters such as fluorophores or biotin. The ability of BfiI to catalyse efficiently the addition of nucleoside derivatives to its DNA cleavage products raises the possibility that this enzyme could be a valuable tool for DNA manipulations, not only for cleaving DNA at specific sequences but also for modifying the resultant 5′-termini with a nucleoside analogue.

CONCLUSIONS

The type IIS restriction endonuclease BfiI, a member of the PLD superfamily (8) has a unique catalytic mechanism. BfiI does not require a divalent metal ion and uses an active site histidine to form an enzyme–DNA covalent intermediate (15). We previously showed that the phosphohistidine linkage between the protein and the 5′-terminus of the cleaved DNA is susceptible to attack by alcohols, yielding transesterification products (15). In this publication a novel transesterification is described, with the 3′-OH group of a deoxynucleoside acting as the alcohol and effectively replacing water as the nucleophile that attacks the covalent intermediate. This results in the attachment of the nucleoside to the 5′-phosphate of the DNA chain, giving a product extended by a single base and now possessing a 5′-OH terminus. The DNA products generated by the initial BfiI catalysed cleavage carry a 1 base 3′ single-strand extension, which acts as a template for the incoming deoxynucleoside in the subsequent transesterification reaction (Figure 3). Low concentrations of deoxynucleosides give strikingly high yields of transesterification products, governed by the formation of complementary Watson–Crick base pairs between the incoming deoxynucleoside and the unpaired 3′ single base extension. As anticipated, Watson–Crick base pairing also ensures considerable selectivity of BfiI for the inserted nucleoside. Thus, the exceptional mechanism of BfiI enables template-directed addition of a deoxynucleoside to a polynucleotide chain. To date, the template-directed polymerization of nucleotides has been the preserve of DNA and the RNA polymerases. However, the polymerases attach an activated nucleotide (dNTP or NTP) to the 3′-OH of the primer-terminus, extending the growing chain in the 5′-3′ direction. In contrast, BfiI uses an activated primer (the phosphohistidine covalent intermediate) for reaction with the 3′-OH of a deoxynucleoside, extending in the 3′-5′ direction. Polymerases are also capable of multiple base extensions, whereas BfiI is only able to add a single base. In common, though, is the use of Watson–Crick base pairs to govern selectivity.

A general technique in the manipulation of recombinant DNA employs DNA polymerases in conjunction with type II restriction endonucleases to generate blunt-ended DNA fragments (21): the restriction enzyme cleaves the DNA at a specific sequence to leave, in many instances, a staggered end with a single-strand extension; the polymerase is then used to fill in the staggered end to yield

the blunt end. With BfiI, both the endonuclease and the end-filling reactions can be catalysed by the same protein. The 'polymerase-like' activity of BfiI provides the potential for specific labelling of long DNA strands with useful reporters, illustrated here by the introduction of a 5′-amino group.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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