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Targeting individual subunits of the FokI restriction endonuclease to specific DNA strands

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ABSTRACT

Many restriction endonucleases are dimers that act symmetrically at palindromic DNA sequences, with each active site cutting one strand. In contrast, FokI acts asymmetrically at a non-palindromic sequence, cutting 'top' and 'bottom' strands 9 and 13 nucleotides downstream of the site. FokI is a monomeric protein with one active site and a single monomer covers the entire recognition sequence. To cut both strands, the monomer at the site recruits a second monomer from solution, but it is not yet known which DNA strand is cut by the monomer bound to the site and which by the recruited monomer. In this work, mutants of FokI were used to show that the monomer bound to the site made the distal cut in the bottom strand, whilst the recruited monomer made in parallel the proximal cut in the top strand. Procedures were also established to direct FokI activity, either preferentially to the bottom strand or exclusively to the top strand. The latter extends the range of enzymes for nicking specified strands at specific sequences, and may facilitate further applications of FokI in gene targeting.

INTRODUCTION

Endonucleases commonly possess the same number of active sites as the number of phosphodiester bonds they cleave per turnover. The enzymes that cut one strand of nucleic acid are usually monomeric proteins with a single active site (1,2). Those that cut two phosphodiester bonds are often dimers with one active site in each subunit (3–5), though some carry two active sites in a single polypeptide (6–8), while the enzymes that make double-strand breaks at two separate loci, to excise the intervening segment, are usually tetramers with one active site per subunit (9,10). These organizations can all be exemplified with the Type II restriction enzymes (11–14). The Type II endonucleases (15) recognize specific DNA sequences, generally 4- to 8-bp long, and cut both strands of the duplex at fixed positions, commonly within but sometimes displaced from the recognition site (16).

Many restriction enzymes are dimers of identical subunits that bind palindromic sequences to form complexes in which the DNA and the protein share an axis of 2-fold symmetry (11,13). Such an arrangement can place the active site from one subunit against the scissile phosphodiester bond in one strand of the duplex and the other against the complementary strand (3). Similar arrangements apply to the tetrameric restriction enzymes that interact with two copies of a palindromic sequence; in these cases, two subunits bind one site and the other two the second site (14). Normally, the two catalytic centres in the dimer, or four in the tetramer, all display the same rate constants for phosphodiester hydrolysis (17–19). Only a small number of the enzymes that act at palindromic sequences are monomers: these seem to cut just one strand at a time and need two independent reactions to make a double-strand break (20,21). However, some restriction enzymes such as BbvCI are heterodimeric proteins that cleave DNA at asymmetric sequences (16), with each subunit attacking a specified strand (22): the two subunits hydrolyse their target phosphodiester bonds at different rates (23).

A further group of restriction enzymes that act at asymmetric sites are the Type IIS enzymes (15), which cut the DNA at fixed locations downstream of their sites (16). These are exemplified by the FokI restriction endonuclease, which cuts 'top' and 'bottom' strands 9 and 13 nt (nucleotides) away from its recognition site, as follows:

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5′-G-G-A-T-G-N-N-N-N-N-N-N-N-N↓N-N-N-N-N
3′-C-C-T-A-C-N-N-N-N-N-N-N-N-N-N-N-N-N↑N
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where N indicates any nucleotide and the arrows the scissile bonds. The FokI nuclease is a monomer in solution and binds DNA as a monomer (24). The monomer contains separate domains for DNA recognition and for catalysis (25) but the latter possesses only one active site (26), so it can cut only one strand at a time. Moreover, in the crystal structure of the FokI–DNA complex, the catalytic domain is held against the recognition domain

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a long way from the scissile bonds in either strand so it has to be repositioned before it can cut the DNA (26).

The Type IIS enzyme BfiI cuts both strands by switching a single active site between strands (27). An alternative to switching is to assemble a structure with two active sites, one for each strand. Many Type IIS enzymes do the latter. For example, FokI cuts a DNA with one cognate site by forming a dimer at that site: the protein bound to the site, the primary (1°) monomer, has no activity by itself, not even a nicking activity, until it associates with another subunit from free solution, the secondary (2°) monomer (28–30). Dimerization occurs via the catalytic domains but involves a relatively small surface area (29), so that at any one time only a small fraction of the enzyme bound to a solitary site is dimeric (31). Conversely, on a DNA with two FokI sites, the dimer is created *in cis* between monomers at each site and, as these are tethered together by the intervening DNA (9), they form a stable dimer (32). Consequently, FokI cleaves DNA with two sites more rapidly than DNA with one site (33).

Within the dimer of catalytic units, the two active sites in FokI have the same relative positions as those in a dimeric restriction enzyme like BamHI that, like FokI, cuts both strands to leave a 4-nt 5′ extension (29). But unlike BamHI or other dimeric restriction enzymes on palindromic sites (3,11), the FokI dimer at a single site is asymmetric: only one of the two DNA recognition domains, that of the 1° monomer, is bound to the recognition site while the other hangs free. One active site presumably attacks one strand of the DNA and the other the opposite strand, assuming neither switches between strands. But it is not yet known which subunit cuts which strand, nor even whether each active site can be assigned to an individual strand. These questions could be resolved unambiguously if a structure of a FokI–DNA complex had been obtained in which the catalytic domain was located at a scissile phosphodiester bond, but no such structure is available at present. Nevertheless, extrapolation from the solved structure of FokI bound to its recognition site (26) suggests that it may be easier to move the catalytic domain to the scissile bond 13 nt distant in the bottom strand than to the bond 9 nt away in the top strand (26,30). By using derivatives of FokI defective in either DNA binding or catalysis, or both, we show here that the 1° monomer cuts one particular strand and the 2° monomer the other strand, and we identify the strands in question.

Only a small number of endonucleases are currently available that cut DNA in a sequence-specific and strand-specific manner (16). Of these, several were constructed from heterodimeric enzymes in which each subunit attacks an individual strand in a non-palindromic sequence: heterodimers were assembled with one catalytically inactive but dimerization-proficient subunit and one native subunit, so that the hybrid cleaved only the strand targeted by the native subunit (22,34). This strategy is inapplicable to homodimers as the hybrid cannot select a particular strand and will eventually cut both strands by means of two successive nicks (35), like a monomer at a palindromic site (20,21). Nevertheless, some of the homodimeric Type IIS enzymes have been converted into strand-specific nicking enzymes by genetically altering the protein to prevent dimerization: the resultant monomers cleave just one strand, usually the top strand (36,37). However, this approach requires the monomer to be catalytically active, which is not the case with FokI: mutations at its dimerization interface inactivate the enzyme (28). In spite of this, it has been hypothesized, albeit without as yet any experimental support, that a combination of two mutants of FokI may specifically nick DNA at its recognition site: one of the mutants required for this scenario is defective for DNA recognition but retains both dimerization and catalytic functions, while the other is catalytically inactive yet still able to dimerize and bind DNA (38). A further objective of this study is to test this scheme and, if found to be valid, to determine which strand of the DNA is cut by the mixture of mutant proteins.

MATERIALS AND METHODS

Proteins

Escherichia coli over-producing strains for wt (wild-type) FokI, for the D450A variant (39) and for a N13Y-intein fusion protein (28) were gifts from W. Jack and J. Bitinaite (NEB: New England Biolabs). The N13Y mutation was also introduced into the plasmid encoding the D450A protein by the QuikChange method (Stratagene), to create the N13Y-D450A double mutant. The N13Y protein was purified by the IMPACT-CN system (28). All other forms of FokI were purified by column chromatography (31): phosphocellulose P11 (Whatman), then Mono S (GE Healthcare). Protein concentrations were assessed from A_{280} readings using an extinction coefficient of $72\,520\,M^{-1}\,cm^{-1}$ for the FokI monomer. Prior to reactions, enzymes were diluted to the requisite concentration in FokI Dilution Buffer (31).

Immobilized oligonucleotides

The oligodeoxyribonucleotides used to create BIO-42 (see Results section) were obtained as HPLC-purified samples from Sigma Genosys. Standard methods (40) were employed to phosphorylate 5′ termini, with T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP, and to fill in recessed 3′ termini, with Klenow polymerase (NEB) and the relevant dNTPs (with [α-^{32}P]dATP when required): radiolabelled nucleotides were from NEN Radiochemicals. The BIO-42 duplex (0.08 pmol), ^{32}P-labelled in either top or bottom strand, was added to 10 pmol streptavidin on streptavidin-coated magnetic beads (Promega) in 80 µl SSC [7.5 mM sodium citrate (pH 7.2), 25 mM NaCl] (41). After 5 min at 25°C, the beads were washed three times in 300 µl SSC and then resuspended in Buffer 4 (NEB).

Reactions on BIO-42 contained 1 nM immobilized DNA and FokI protein as required in 200 µl Buffer 4 at 20°C. After initiating the reactions by adding the FokI protein(s), samples (15 µl) were removed at timed intervals (0, 0.5, 1, 2, 3, 4, 5, 10, 15 and 20 min) and quenched by mixing with 10 µl Loading Mix (10 mM NaOH, 100 mM EDTA, 95% formamide, 0.05% bromophenol blue and

0.05% xylene cyanol): the zero time point was taken before adding the enzyme. The samples were incubated at 95°C for 10 min and on ice for 15 min before being analysed by denaturing gel electrophoresis at ~40 V/cm through 12% polyacrylamide in 45 mM Tris–borate (pH 8.3), 1 mM EDTA, at 55°C. The gels were fixed in 20% (v/v) acetic acid and 20% (v/v) methanol, dried and exposed overnight on an activated storage phosphor screen. The screens were scanned in a Typhoon 9400 imager (Molecular Dynamics) and the amounts of intact and cleaved ^{32}P-labelled strands quantified by using ImageQuant software (Molecular Dynamics). Exponential decays were fitted in GRAFIT (Erithacus Software).

Plasmids

Transformants of *E. coli* HB101 carrying either pSKFokI (28) or pIF190 (31) were grown in minimal media containing [methyl-^{3}H] thymidine and the monomeric supercoiled (SC) form of the plasmids purified by CsCl density gradient centrifugations (18,33).

Reactions to measure rates of plasmid cleavage contained 5 nM SC plasmid (^{3}H-labelled) and FokI protein as required in 200 µl Buffer 4 at 37°C. The protein was either wt FokI or a mixture of equal concentrations of the N13Y and the D450A mutants. A zero time point was taken before adding the protein and further samples (15 µl) removed at intervals thereafter. The samples were mixed immediately with 10 µl EDTA Stop-Mix (32) and subsequently analysed by electrophoresis through agarose under conditions that separated the SC substrate from the reaction products. The concentrations of the SC, open-circle (OC) and linear (LIN) species of the DNA were evaluated by scintillation counting (23,41).

To assess the abilities of the mutant FokI proteins to nick DNA, 2 µl aliquots containing varied concentrations of either the N13Y or the D450A proteins, or mixtures of the two, were added to 18 µl samples containing 5 nM SC pSKFokI in Buffer 4. After 1 h at 37°C 10 µl EDTA Stop-Mix was added and the samples analysed by electrophoresis through agarose.

RESULTS

Experimental strategy

To meet the objectives of this study, two experimental strategies were employed that, between them, required three derivatives of the FokI endonuclease (Figure 1): one with a mutation in the DNA recognition domain that blocked sequence-specific binding but which retained a functional cleavage/dimerization domain, N13Y (28); a second with a mutation at the active site that abolishes catalytic activity without affecting dimerization or DNA recognition, D450A (39); a third with both mutations, N13Y-D450A (this study).

Asn13 interacts with the adenine in the GGATG recognition sequence (26) and the replacement of this residue with a bulky Tyr not only eradicates this interaction but may also dislodge the entire recognition surface of the protein (28). On account of its inability to bind specific DNA, the N13Y protein cannot by itself cut both DNA strands at a single site, though it can at high concentrations nick DNA (31). However, the N13Y protein can stimulate the wt enzyme (28). As noted above, a single monomer of wt FokI bound to its recognition site has no activity until it associates with a second monomer but the latter can be, with equal facility, either the native or the N13Y protein (31). Hence, the cleavage/dimerization domain of N13Y must be fully functional. Variants of FokI with the N13Y mutation therefore cannot act as the 1° monomer but can function as the 2°.

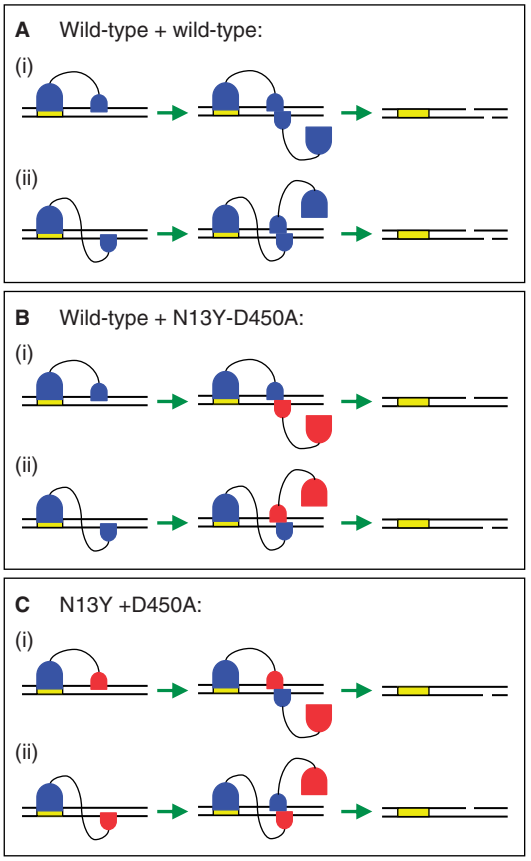

Figure 1. Experimental strategy. In all three panels, both strands of the DNA duplex are shown, with gaps to indicate cleaved products: the yellow box marks the recognition site for FokI. The FokI monomer is shown as two domains connected by a flexible linker: a (large) DNA recognition domain for specific binding and a (small) catalytic domain for dimerization and DNA cleavage. Functional domains are in blue. Domains inactivated by mutation for either DNA binding (in the large domain) or for catalysis (in the small domain) are in red. The 1° monomer bound to the recognition site could in principle use its catalytic domain to engage the scissile bond in the top strand, leaving the 2° monomer to attack the bottom strand (pathway i in all three panels). Alternatively (pathway ii), the 1° monomer attacks the scissile bond in the bottom strand, in which case the 2° monomer cuts the top strand. (**A**) With two monomers of wt FokI, both pathways (i) and (ii) lead to the cutting of both strands. (**B**) With a mixture of wt FokI and the N13Y-D450A double mutant, only the wt enzyme can act as the 1° monomer, while either the wt or the double mutant might act as the 2° monomer. Hence, the double mutant ought to inhibit the reaction on the strand cut by the 2° monomer: the bottom strand in pathway (i); the top in pathway (ii). (**C**) With the D450A and the N13Y mutants, D450A can bind to the specific site but N13Y cannot. As only N13Y is active, D450A and N13Y have to function as 1° and 2° monomers, respectively so this mixture ought to cut only one strand: the bottom strand in pathway (i); the top in (ii).

Asp450 forms part of the PD....(D/E)XK motif commonly found at the active sites of restriction and other endonucleases (5,13). Its substitution by Ala obliterates activity whilst still allowing the FokI protein to bind DNA specifically (39). Hence, the D450A protein can occupy the position of the 1° monomer at the recognition site but cannot cut either strand. Yet, the D450A protein at the specific site might still be able to recruit a catalytically active form of FokI and the latter might then be able to function as the 2° monomer (Figure 1).

The double mutant, N13Y-D450A, cannot act as the 1° monomer as the N13Y mutation blocks specific DNA binding. Nevertheless, if the recognition site is occupied by another form of FokI, the double mutant may be able to associate with the DNA-bound protein even though it cannot cut the DNA, due to its D450A mutation. Hence, in a reaction containing both wt FokI and N13Y-D450A, only the wt enzyme can bind to the recognition site and operate as the 1° monomer but the double mutant might compete with the wt enzyme for the position of 2° monomer and so inhibit cleavage of the strand attacked by the 2° monomer (Figure 1B).

In contrast, with a mixture of the two proteins that each carry one of these defects, N13Y and D450A, respectively, only D450A can take up the 1° position but only N13Y has an intact catalytic domain, so whatever DNA cleavage is observed with this mixture can only come from N13Y as the 2° monomer. If the 2° monomer is limited to cutting one particular strand of the DNA, then this combination of mutants will cut only the strand attacked by the 2° monomer (Figure 1C). These procedures thus have the potential to direct FokI activity to a specific strand: either preferentially to the strand cut by the 1° monomer (Figure 1B) or exclusively to the strand cut by the 2° monomer (Figure 1C).

Substrate for strand selection

To monitor separately the reactions of the FokI restriction endonuclease at its scissile phosphodiester bonds in the top and in the bottom strands, a DNA duplex was constructed by annealing two oligodeoxynucleotides: one 39 nt long, with a biotin moiety at its 5′-end, and another of 42 nt. The annealed species,

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5′-BIO-CTGGCGAAAGGGGGATGTGCTGCAAG↓GCGA TTAAGTTGG   -3′
   3′ -GACCGCTTTCCCCCTACACGACGTTC CGCT↑AATTCAACCTTG-5′
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carries the recognition sequence for FokI (in bold, underlined) and its downstream sites of cleavage (arrows). The complete footprint of FokI bound to DNA (39,42) also falls within this sequence. The 3 nt recession at the 3′-end of the top strand was filled in by using Klenow polymerase with dATP and dCTP to give a blunt-ended duplex 42 nt long, BIO-42. The duplex was radiolabelled in either top or bottom strand. For the top strand, the dATP in the Klenow reaction was replaced with [α^{32}P]dATP: cleavage of this strand liberates a 16 nt product (P_{16}) from the 42 nt substrate (S_t). For the bottom strand, the 42 nt oligonucleotide was phosphorylated at its 5′-end prior to the annealing step by using polynucleotide kinase and [γ^{32}P]ATP: cutting this strand (S_b) releases a 12 nt product (P_{12}). These products were identified by their electrophoretic mobilities relative to synthetic oligonucleotides of known lengths (data not shown).

The cleavage of BIO-42 by wt FokI requires one molecule of the protein to bind to the recognition site and a second to associate with the DNA-bound protein, but the 2° monomer has an unfilled recognition domain which could also bind a molecule of BIO-42. In this situation, no distinction can be made between the two monomers because both are 1° monomers bound to specific DNA and both are also 2° monomers associating with DNA-bound protein. To avoid this situation, and to record only reactions by dimers at solitary sites, the BIO-42 was first immobilized via its biotin tag to streptavidin-coated magnetic beads (43): BIO-42 was added to the beads at about a 100-fold lower concentration than that of streptavidin on the bead (41), so that the individual molecules of the immobilized DNA are held too far apart from each other for a dimer of FokI to be able to bridge two molecules (43). It is highly improbable, on entropic grounds, that a single dimer of FokI could tether two beads.

Strand selection by wt FokI

Immobilized samples of BIO-42, ^{32}P-labelled in either top or bottom strand, were tested in comparable reactions with wt FokI. Single-turnover conditions were employed with the enzyme in 5-fold excess over the DNA, to permit dimer formation at all of the recognition sites. Aliquots were taken from the reactions at varied times, subjected to denaturing PAGE and the gels then analysed by phosphorimager to expose the labelled strand (Figure 2A). With both reactions, the only species observed on the gels were the intact strand, 42 nt long in both cases (S_t in one instance, S_b in the other), and a product commensurate with cleavage at the appropriate phosphodiester bond (in the top strand, 9 nt downstream to yield P_{16}; in the bottom, 13 nt away to generate P_{12}). Though some Type IIS restriction enzymes can cut DNA at multiple positions (16,27), no products due to non-canonical cleavages were detected in these reactions. In both cases, the relative amounts of the intact strand and the corresponding cleaved product were evaluated from the phosphorimager records and the amount of the intact material recorded as a fraction of the total (Figure 2B). The decline in this fraction was fitted to an exponential decay to yield a rate constant for cutting that strand.

For wt FokI, the rate constant for cutting the scissile bond in the top strand of BIO-42 equalled, within experimental error, that for the bottom strand. The two active sites in the dimeric protein therefore operate at the same speed even though only one of the two subunits is bound directly to the upstream recognition sequence. Moreover, since the reaction on each strand of BIO-42 followed an exponential progress curve starting from zero time, the two active sites must operate simultaneously, in parallel with each other. If FokI had worked sequentially and had to cut one particular strand before the other, as is the case with BfiI (27), then the product from cleaving the second strand would have been formed only after an initial lag

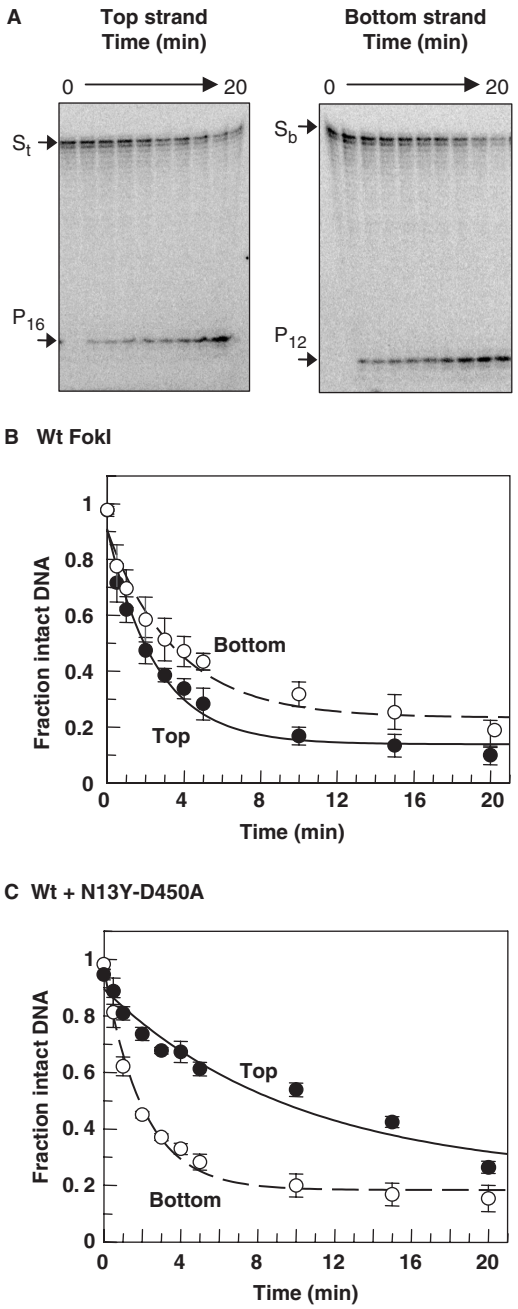

Figure 2. Strand selection by wt FokI. (**A**) The reactions, in Buffer 4 at 20°C, contained 5 nM wt FokI and 1 nM immobilized BIO-42, ^{32}P-labelled in either top or bottom strand. At various times after adding the enzyme, samples were removed from the reactions, quenched and subjected to denaturing PAGE. Phosphorimager records of the gels are shown: left, top-strand label; right, bottom-strand label. Time ranges are indicated above each gel and the electrophoretic mobilities of the intact (S_t or S_b) and cleaved strands (P_{16} or P_{12}) marked on the left. (**B**) The amounts of the intact and the cleaved forms of the labelled strands were measured and the amounts of intact DNA are shown as a fraction of the total; top strand, black circles; bottom strand, white circles. (**C**) The reaction was identical to that in (B) except that it also contained 100 nM N13Y-D450A. In both (B) and (C), error bars denote standard deviations from ≥3 independent repeats and the lines drawn through each data set are best fits to single exponentials: top strand, solid line; bottom strand, dashed line. The best fits were obtained with: in (B), wt FokI, 0.4 min^{-1} and 0.3 min^{-1} for top and bottom strands, respectively; in (C), wt FokI and N13Y-D450A, 0.1 min^{-1} and 0.5 min^{-1} for top and bottom strands, respectively.

phase, in the characteristic manner of a two-step sequential pathway (44). Instead, the first cut occurs with equal probability in either top or bottom strand.

Cleavage of immobilized BIO-42 by wt FokI was studied further in the presence of the N13Y-D450A protein (Figure 2C), which carries mutations that incapacitate it for both specific DNA binding (N13Y) and catalysis (D450A). The concentration of wt FokI was held constant at the same level as above (5 nM), while the concentration of the mutant was increased systematically to much higher levels than the wt enzyme. As the concentration of the double mutant was raised, the rate for cutting the top strand of BIO-42 decreased progressively while that for the bottom strand rose, albeit by a small margin. While the native enzyme had by itself given approximately equal rates for cutting each strand, the same reaction in the presence of a 20-fold excess of mutant over native enzyme gave a 5-fold faster rate for the bottom strand compared to the top (Figure 2C). However, it proved impossible with this procedure to block top-strand cleavage completely: even at the highest accessible concentrations of the N13Y-D450A protein, the top strand was still cleaved at a detectable rate.

The N13Y-D450A protein has no catalytic activity and cannot bind to the recognition sequence. The only way that it can modulate the activity of the wt enzyme is by competing with free wt protein for binding to the 1° monomer already on the DNA: if so, it would inhibit the reaction on the strand cleaved by the 2° monomer (Figure 1B). Figure 2C thus shows that the 2° monomer cuts the top strand. Moreover, as the rate of cutting the bottom strand is not inhibited by N13Y-D450A, the bottom strand must be cleaved by the 1° subunit. The rate of bottom-strand cleavage by wt FokI is in fact slightly enhanced by the double mutant, probably for the same reason as the N13Y protein itself (28,31): high concentrations of the double mutant presumably drive dimer formation with the wt protein at the recognition site, in the same way that the single mutant can also drive dimerization.

Strand selection by FokI mutants

It has been suggested (38) that a combination of two separate variants of FokI, one defective in DNA binding and the other in catalysis, might cut just one strand of the DNA (Figure 1C). To test this idea, mixtures containing equal concentrations of the N13Y and the D450A proteins were added to immobilized BIO-42 labelled in either top or bottom strand (Figure 3). The top strand of BIO-42 is cleaved by wt FokI to yield a 16 nt product, P_{16}, and a product of this size was also generated by the mixture of the two mutant proteins (Figure 3A). The bottom strand is cut by wt FokI at the same rate as the top strand: cleavage of the scissile phosphodiester bond in this strand, 13 nt downstream of the recognition site, releases a 12 nt product, P_{12}. However, the addition of the mixture of N13Y and D450A to BIO-42 labelled in the bottom strand failed to generate any of the 12 nt product, nor any other product (Figure 3A). Instead, the bottom

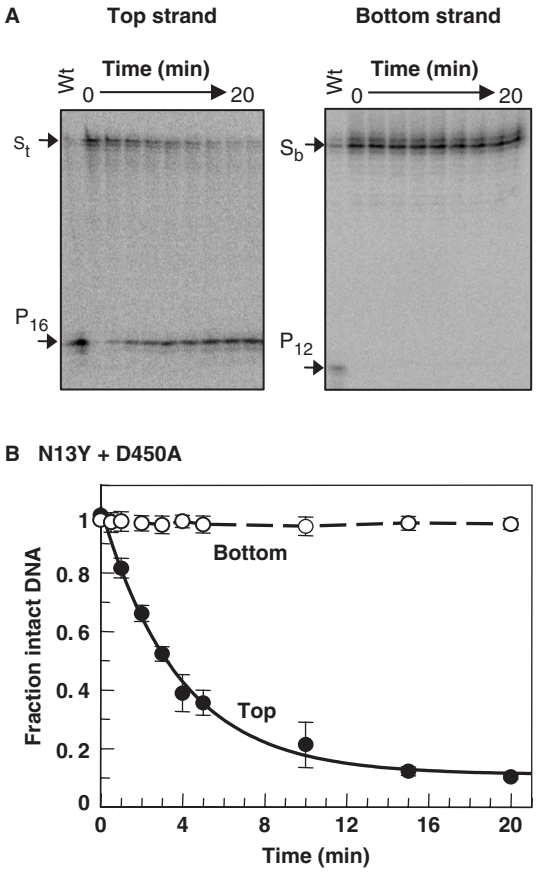

Figure 3. Strand-specific nicking. (**A**) A mixture of the N13Y and D450A mutants of the FokI endonuclease was added to immobilized BIO-42 to give a reaction with 10 nM N13Y, 10 nM D450A and 1 nM DNA in Buffer 4 at 20°C. The BIO-42 was ^{32}P-labelled in either the top (left-hand gel) or the bottom strand (right-hand). At various times after adding the mixture, samples were removed from the reactions, quenched and subjected to denaturing PAGE. Phosphorimager records of the gels are shown. The electrophoretic mobilities of the intact strands (S_t and S_b, from top- and bottom-strand labelled BIO-42, respectively) are noted on the left of the gels and the lanes marked Wt show the products from reactions of wt FokI on the same DNA species (P_{16} from the top strand, P_{12} from the bottom). (**B**) The fraction of the total amount of radiolabel in each lane still present as the intact DNA were measured and these values plotted as a function of reaction time: top strand, black circles; bottom strand, white circles. Error bars denote standard deviations from ≥3 independent repeats. The line drawn through the data from the top strand (solid line) is the best fit to a single exponential, which gave a rate constant of 0.3 min^{-1}. Data points for the bottom strand are connected by a dashed line.

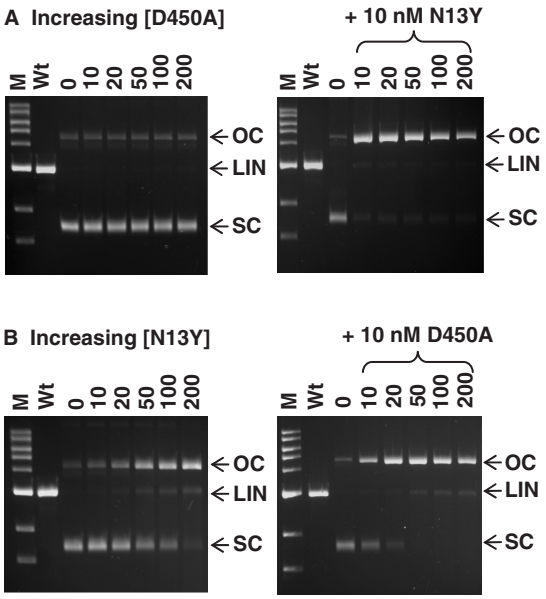

Figure 4. Specificity of nicking. The reactions, in Buffer 4, contained 5 nM SC pSKFokI (a plasmid with one recognition site for FokI) and FokI protein as indicated below. Reactions were stopped after 1 h at 37°C and the samples analysed by electrophoresis through agarose. The symbols SC, OC and LIN on the right of each gel mark the electrophoretic mobilities of the intact SC DNA, the nicked OC form cut in one strand and the LIN form cut in both strands at one site. The lanes marked M contain 1 kb electrophoresis markers (NEB), and the lanes marked Wt are from equivalent 1 h reactions of 10 nM wt FokI on pSKFokI. (**A**) Left-hand gel: the reactions contained D450A at the concentrations indicated above each lane (0 → 200 nM). In the right-hand gel, the reactions with 10 → 200 nM D450A also contained 10 nM N13Y. (**B**) As (A) except that the protein whose concentration was varied was N13Y and that, in the right-hand gel, the samples with varied N13Y also contained 10 nM D450A.

strand remained intact throughout the time needed to cleave virtually all of the top strand (Figure 3B).

These experiments validate the proposal that a mixture of a binding-defective mutant and a catalytically defective mutant might cleave only one strand of the DNA. In this combination, only the N13Y protein possesses a functional active site, so only this protein can be responsible for cutting the top strand, but N13Y cannot act as the 1° monomer because it cannot bind to the recognition site. On the other hand, D450A can bind to the specific site, so presumably recruits N13Y to that site where N13Y then acts as the 2° monomer. Hence, the FokI protein recruited to the recognition site by associating with the DNA-bound protein makes the proximal cut 9 nt away in the top strand, which leaves the 1° monomer bound to the recognition site to make the distal cut 13 nt away in the bottom strand (Figure 1C, pathway ii).

Regardless of mechanism, the combination of the N13Y and the D450A variants of FokI seems to cut DNA exclusively at one particular phosphodiester bond, that in the top strand 9 nt downstream of a FokI recognition site. It might therefore be possible to use this combination as a sequence-specific strand-specific nicking reagent.

Specificity of nicking

To be useful as a specific nicking reagent, the nuclease needs to display minimal activity against the non-cognate strand and against non-cognate DNA sequences. The D450A and the N13Y variants of FokI were therefore tested against a 3 kb plasmid that carries a single recognition site for FokI, pSKFokI (28). Varied concentrations of each protein were incubated with this DNA for 1 h by themselves and in the presence of a fixed concentration of the other protein (Figure 4). By itself, the D450A protein had no detectable activity on this plasmid. Even at the highest concentration tested, it failed to convert any

of the SC DNA to either the nicked OC form cut in one strand or to the LIN form cut in both strands at a single site. When increasing concentrations of D450A were incubated with the plasmid in the presence of a fixed—relatively low concentration—of N13Y, essentially all of the SC plasmid was converted to the nicked form: across the range of D450A concentrations tested, no other products were observed (Figure 4A).

In contrast, while the lowest concentration of the N13Y protein tested (10 nM) cut virtually none of the DNA, increasing concentrations cleaved progressively larger fractions of the SC plasmid: primarily to the nicked OC form but also, at the highest concentrations, to the LIN form (Figure 4B). To test whether these cleavage events occur at the FokI site or at random locations, the plasmid was first cleaved with BsaHI and the linear DNA then digested with various concentrations of N13Y: at concentrations that cause double-strand breaks, the linear DNA was converted into a heterogeneous smear of products (data not shown). Hence, N13Y cuts DNA at random rather than specifically and the LIN form most probably arises from two co-localized nicks, one in each strand. The extent of nicking at relatively low concentrations of N13Y ($\leq$100 nM) was, however, increased substantially by adding a fixed amount of the D450A protein (Figure 4B). Hence, the nicking of the SC plasmid seen with N13Y alone is probably due to the low level of non-specific nuclease activity seen before with this mutant (31) and with the isolated catalytic domain of FokI (28). The enhanced nicking seen in the presence of D450A can then be assigned to the combination of proteins acting at the FokI recognition site in one strand only.

The combination of the N13Y and the D450A proteins therefore can be used as a strand-specific sequence-specific nicking reagent provided that the concentration of the N13Y protein is kept to levels low enough for its non-specific nicking activity to be unobtrusive. Conversely, there seems to be no upper limit for the concentration of the D450A protein in this mixture.

Rates of nicking

The rates at which the combination of N13Y and D450A proteins cleaved SC plasmids with one or two FokI sites, pSKFokI and pIF190 (31), respectively, were compared to wt FokI (Figure 5). The combination cuts just one strand of the DNA at each FokI site, to generate as the final product the nicked OC species. On the other hand, the wt enzyme cuts both strands at each site to generate from the plasmid with one FokI site a single linear species, and from the plasmid with two FokI sites two linear fragments. Since the final products of these reactions differ in each case, rates were assessed from the decline in the concentration of SC DNA with time. The reactions were studied first under steady-state conditions, with enzyme at a lower concentration than the DNA, so that substrate utilization requires multiple turnovers of the enzyme.

Wt FokI cleaved the SC substrate with one FokI site at a relatively slow rate though over 3 h it eventually cleaved all of it, while the plasmid with two target sites was cleaved at a faster rate, in ~15 min (Figure 5A).

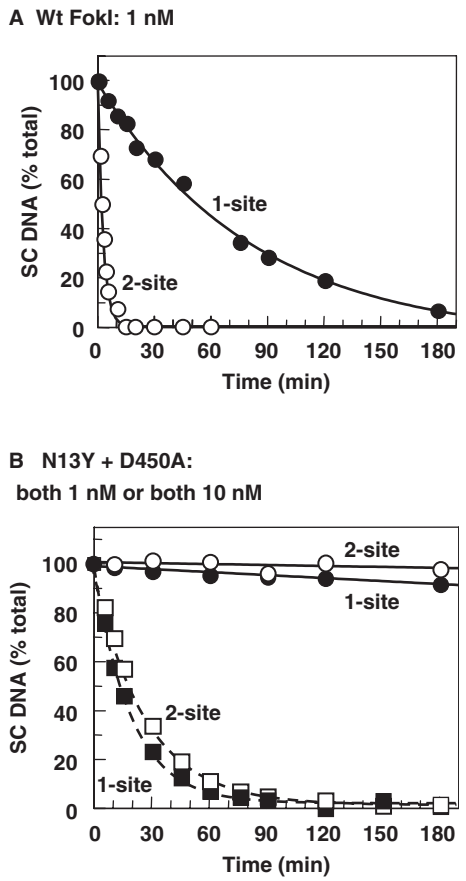

Figure 5. Reaction rates. The reactions, in Buffer 4 at 37°C, contained 5 nM ^{3}H-labelled DNA and FokI protein at either 1 nM or 10 nM, as indicated below. At timed intervals after adding the protein(s) to the DNA, samples were removed, quenched and analysed as described in Materials and methods section to obtain the concentrations of the SC, OC and LIN DNA. The residual concentration of SC DNA at each time point is given as a percentage of the total DNA in that sample. (**A**) Reactions of 1 nM wt FokI on: pSKFokI (one recognition site), black circles; pIF190 (two FokI sites), white circles. (**B**) Reactions with a mixture of N13Y and D450A, both at 1 nM, on pSKFokI (black circles) and on pIF190 (white circles). Also shown in (B) are the reactions with the mix of N13Y and D450A, both at 10 nM, on pSKFokI (black squares) and on pIF190 (white squares), both with dashed lines.

To compare the mixture of N13Y and D450A with the wt enzyme, both mutants were kept at the same concentration (1 nM) as used for the wt protein, to leave unaltered the concentrations of functional active sites and of functional recognition domains. Yet, the combination of the two mutants cleaved the one-site plasmid much more slowly than wt FokI, and a slow rate was also observed on the two-site substrate (Figure 5B). At these concentrations of mutant proteins, the steady-state velocities were too low to measure accurately: only a small fraction of either DNA was cleaved in 3 h. However, with 10-fold higher concentrations of mutant proteins, to give single-turnover conditions with enzyme in excess over the DNA, both plasmids were cleaved to completion in <3 h (Figure 5B). The latter allowed for a more accurate assessment of the relative rates on the one-site and two-site plasmids: unlike wt FokI, no significant difference was observed.

When the active dimeric form of wt FokI assembles at a solitary recognition site, the DNA recognition domain of the 2° monomer is not bound to specific DNA (Figure 1A) but on a DNA with two cognate sites a single dimer can span the two sites (30) and loop out the intervening DNA (31). As the monomers at each site on the two-site DNA are tethered to each other, they dimerize much more readily than is the case with one DNA-bound and one free monomer (32). Wt FokI thus cleaves substrates with two sites more rapidly than DNA with one site (33: Figure 5A). In contrast, an active dimer formed from the D450A and N13Y proteins cannot span two recognition sites as its activity has to come from the N13Y protein as the 2° monomer but N13Y cannot bind the specific sequence. Hence, unlike wt FokI, the combination does not show enhanced activity on the two-site substrate. The slow rates with the two mutants may be due to the D450A protein not only binding to the recognition site like a 1° monomer but also competing with N13Y for the 2° role, to give a dimer with two inactive D450A subunits.

DISCUSSION

Reaction pathway

The pathway for the reaction of wt FokI at its recognition sequence involves first one monomer of the protein, the 1° monomer, binding to the target site to give an initial complex that cannot cleave either strand of the DNA, possibly because its catalytic domain is held against the recognition domain distant from the scissile phosphodiester bonds (26). Even if it was active at this stage, the enzyme could cleave only one bond at a time as it possesses only one active site. To remedy this situation, the 1° monomer at the recognition site associates with another subunit, the 2° monomer, to give a dimer with two active sites (28–30). The resultant assembly is an asymmetric homodimer, in which only one of its two recognition domains contacts the cognate sequence. For both recognition domains to bind cognate sites, the DNA needs two or more such sites (31,32). However, this study relates primarily to reactions at solitary FokI sites, on DNA molecules that were physically separated from each other by attachment to a solid surface (43), so as to preclude the possibility of the dimer bridging two molecules.

With a homodimeric restriction enzyme at a palindromic sequence, the two subunits are positioned identically on the DNA and cut identical phosphodiester bonds (11,13) so they would be expected to cut their target bonds at identical rates (17,19). However, the heterodimeric enzyme BbvCI uses two different subunits to cleave two different phosphodiester bonds, one in each strand of its non-palindromic site (22), and cuts the strands at different rates (23). The FokI nuclease forms a homodimer at its cognate site but the two subunits are positioned differently and the nucleotide sequences at the sites of top- and bottom-strand cleavage generally differ from each other. Hence, one might expect the two active sites in this asymmetric dimer to act at different rates, like BbvCI. Contrary to this view, the two active sites in the FokI dimer show the same rates for phosphodiester hydrolysis (Figure 2B).

The rate at which FokI nicks a one-site plasmid was shown previously to equal the rate at which it converts the nicked species to the final product cut in both strands (31), but these experiments had not revealed whether the first cut was in a unique strand or distributed between the strands: i.e., whether FokI follows a sequential pathway cutting one particular strand before the other, like BfiI (27), or a parallel pathway with concurrent reactions on the two strands, like BamHI or BbvCI (19,23). This question was resolved here by using an oligoduplex substrate radiolabelled in either top or bottom strand. Moreover, the reaction on a one-site plasmid in free solution might have involved two molecules of the plasmid binding to the protein at the same time, as happens with enzymes like SfiI and Bse643I (9,14,41,43), but this cannot occur with the immobilized duplex. On the immobilized duplex labelled in individual strands, the reactions on both strands followed exponential progress curves that started directly after adding the enzyme (Figure 2B). Neither reaction began with a detectable lag phase, so wt FokI must cut the two strands in parallel rather than sequential reactions. As the parallel reactions proceed at equal rates, the first cut can be—equally—in either top or bottom strand.

The parallel reactions on the two strands can be assigned to individual subunits in the asymmetric dimer. The 1° monomer at the recognition site cuts the bottom strand 13 nt downstream of the sequence and the 2° monomer the top strand 9 nt away (Figures 2C and 3B). This result concurs with the crystal structure of a single subunit of FokI bound to its recognition sequence, in effect a 1° monomer. In that structure (26), the catalytic domain is located far away from the target phosphodiester bonds in either strand, but the structural adjustments required to relocate it to the scissile bond in the bottom strand are less demanding than those needed to move it to the top strand (26,30). The possibility that either 1° or 2° monomers could switch their active sites between the strands can be excluded. If the 1° monomer could switch strands, then the addition of the N13Y-D450A mutant to wt FokI would have inhibited the cutting of both top and bottom strands, rather than just the top (Figure 2C). Likewise, if the 2° subunit had been able to switch, the N13Y protein would have cleaved both strands in the presence of D450A rather than just the top (Figure 3B). Moreover, the 1° and 2° monomers carry out their respective reactions in parallel with each other, rather than sequentially: the 1° monomer can act before the 2° or vice versa.

Among the Type IIS enzymes in which a monomeric form that contains both DNA recognition and catalytic domains can be persuaded to cleave DNA as a monomer, that monomer generally cuts the top strand (36,37,45,46). In contrast, the DNA-bound monomer of FokI cuts the bottom strand. The switch is probably due to a different orientation of the recognition domain on the target sequence. The recognition domain of BpuJI, another Type IIS enzyme, has a similar structure to FokI but binds its target sequence in the opposite orientation to

FokI, as if it had been rotated through 180° around an axis perpendicular to the helical axis of the DNA (47). The same change in orientation may also apply to other Type IIS enzymes (46).

A specific nicking reagent

Endonucleases that cleave one particular strand of DNA at a specific sequence have acquired numerous applications: in strand displacement amplification (48); in the detection and labelling of specific DNA sequences (49,50); and in the manipulation of individual strands (51). In the latter, the endonuclease(s) is commonly used to make two closely spaced nicks in the same strand, and the resultant gap is then either left unfilled, to study the effect of the gap (52), or filled with an oligonucleotide carrying a modified base(s) (53).

Two procedures were developed here to direct the nuclease activity of FokI to a specific DNA strand. In one, the addition of the N13Y-D450A protein to a reaction of wt FokI inhibited cleavage of the top strand while slightly stimulating the reaction on the bottom strand. While the wt enzyme alone cleaves the two strands at equal rates (Figure 2B), the double mutant causes wt FokI to cut the bottom strand more rapidly than the top (Figure 2C). This result concurs with pathway (ii) in Figure 1B. However, to use this procedure to block completely the cleavage of the top strand, it would have been necessary to add an infinite concentration of the N13Y-D450A protein, which is clearly impracticable. Even with a 20-fold higher concentration of N13Y-D450A over wt FokI, the top strand was still cleaved only five times more slowly than the bottom.

The second procedure employed the same two mutations but in separate polypeptides, the N13Y and the D450A proteins. These two proteins contain only one functional catalytic centre, that in N13Y and, in the mixture of proteins, that centre cleaves only one strand of the DNA. The strand in question is the top strand, which indicates that the combination follows pathway (ii) in Figure 1C. The top strand was cleaved exclusively at the appropriate location, 9 nt downstream of the site (Figure 3). No products were detected due to reactions at inappropriate positions in the top strand of the 42 bp duplex tested here, nor any from cutting the bottom strand. The mixture of the N13Y and the D450A proteins thus meets the requirement of a sequence-specific strand-specific nicking reagent, and so can be added to the relatively short list of such activities that have been identified to date (16). However, the use of this system as a specific nicking reagent is limited to a relatively narrow range of concentrations of the N13Y protein: too high a concentration leads to random non-specific nicking and even double-strand breaks due to multiple nicks, though no such limitation applies to the D450A protein (Figure 4); too low a concentration leads to reactions that are impracticably slow (Figure 5B).

Potential applications in gene targeting

Derivatives of the FokI endonuclease are widely used to target specific genes in large genomes *in vivo* (54). For such applications, the DNA recognition domain of the FokI nuclease is replaced by a zinc-finger unit (55) tailored to the selected DNA sequence (56). Since the nuclease domain has to dimerize before cutting DNA, the zinc-finger nuclease (ZFN) acts optimally on DNA with two copies of the cognate sequence for the zinc-finger unit: ideally, with two closely spaced sites in head-to-head orientation to allow the monomers to interact directly without looping out the intervening DNA (57). The ZFN then cuts both strands of the DNA between the two sequences. *In vivo*, the double-strand break can be repaired by non-homologous end-joining, a highly mutagenic process that often inactivates the targeted gene (58). Alternatively, it can be repaired accurately by homologous recombination with the corresponding sequence from a second DNA supplied *in trans*, thus replacing the original gene with the second copy (59,60).

Single strand breaks can initiate homologous recombination (61,62) but cannot lead to the introduction of mutations by non-homologous end-joining. Consequently, a nuclease engineered to cleave the requisite sequence in just one strand might be a better reagent for gene replacements, as it would exclude the potentially toxic effects of double-strand breaks. This could be achieved by using a ZFN with an inactive catalytic domain that could bring in an active domain from a second protein that by itself was unable to bind to DNA, in the way that D450A recruits N13Y. The second catalytic domain could alternatively be attached covalently: ZFNs have been constructed with a zinc-finger unit followed by two catalytic domains from wt FokI in tandem repeat and these introduced double-strand breaks at the cognate site for the zinc fingers (63). However, if one of the catalytic domains had been derived from the D450A mutant, the resultant ZFN should cut just one specific strand of the DNA at the targeted sequence, while a tandem ZFN with a mutation in the other domain should cut only the opposite strand.

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