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Recombination by resolvase to analyse DNA communications by the *Sfi*I restriction endonuclease

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The *Sfi*I endonuclease differs from other type II restriction enzymes by cleaving DNA concertedly at two copies of its recognition site, its optimal activity being with two sites on the same DNA molecule. The nature of this communication event between distant DNA sites was analysed on plasmids with recognition sites for *Sfi*I interspersed with recombination sites for resolvase. These were converted by resolvase to catenanes carrying one *Sfi*I site on each ring. The catenanes were cleaved by *Sfi*I almost as readily as a single ring with two sites, in contrast to the slow reactions on DNA rings with one *Sfi*I site. Interactions between *Sfi*I sites on the same DNA therefore cannot follow the DNA contour and, instead, must stem from their physical proximity. In buffer lacking Mg^{2+}, where *Sfi*I is inactive while resolvase is active, the addition of *Sfi*I to a plasmid with target sites for both proteins blocked recombination by resolvase, due to the restriction enzyme bridging its sites and thus isolating the sites for resolvase into separate loops. The extent of DNA looping by *Sfi*I matched its extent of DNA cleavage in the presence of Mg^{2+}.

Keywords: action at a distance on DNA/DNA–protein interaction/DNA topology/restriction–modification/site-specific recombination

Introduction

Many genetic events in both prokaryotes and eukaryotes require proteins to mediate long-range communications between distant DNA sites (Wang and Giaever, 1988). The events include the repair and restriction of DNA (Au *et al.*, 1992; Bickle, 1993), gene expression and its control (Hochschild, 1990; Schleif, 1992) and both site-specific recombination (Landy, 1993; Grindley, 1994) and other genome rearrangements (Adzuma and Mizuuchi, 1989; Kanaar and Cozzarelli, 1992).

The communications often involve interactions between two or more proteins bound to separate sites on the DNA, looping out the intervening DNA (Hochschild, 1990). Alternatively, a DNA loop can be generated by a single protein with two DNA binding domains that recognize different sequences (Landy, 1993), or by an oligomeric protein that binds simultaneously to two target sequences (Lobell and Schleif, 1990; Friedman *et al.*, 1995). In principle, the type of interaction that leads to a DNA loop between two sites *in cis*, on the same molecule of DNA, could also occur *in trans*, bridging sites from separate molecules, the latter leading to a 'sandwich' complex of protein between the two DNA molecules (Krämer *et al.*, 1987). However, long-range interactions that connect two DNA sites through three-dimensional space operate much more efficiently *in cis* than *in trans*, simply because the local concentration of one site in the immediate vicinity of another site will be much higher when both sites are on the same chain (Adzuma and Mizuuchi, 1989). The effective concentration for two sites *in cis* will be enhanced further by supercoiling, since this compresses the volume occupied by the DNA (Vologodskii and Cozzarelli, 1994). Consequently, many of the proteins that mediate long-range interactions of this type only function with sites *in cis* on a supercoiled DNA (Schleif, 1992; Kanaar and Cozzarelli, 1992).

Another proposal for DNA communications is 'tracking' along the DNA from one site to the other (Yuan *et al.*, 1980; Krasnow and Cozzarelli, 1983; Au *et al.*, 1992). Both type I and type III restriction enzymes follow this scheme (Bickle, 1993). These ATP-dependent enzymes recognize specific DNA sequences but cleave the DNA elsewhere, as a result of translocating non-specific DNA past the protein bound to its recognition site (Yuan *et al.*, 1980; Meisel *et al.*, 1995). In contrast, type II restriction enzymes cleave DNA at fixed locations at or adjacent to their recognition sites (Roberts and Halford, 1993). The recognition sequences for type II enzymes are generally palindromes of 4–6 bp and the proteins are usually dimers of identical subunits, organized so that each active site is positioned to cleave one DNA strand (Aggarwal, 1995). Consequently, each recognition site for a typical type II enzyme, such as *Eco*RV, is cleaved in an independent reaction (Vipond and Halford, 1993). However, some type II enzymes, such as *Eco*RII and *Nae*I, are dimeric proteins that interact with two copies of their target sequences, looping out the intervening DNA (Topal *et al.*, 1991): one copy of the sequence appears to acts as an allosteric activator to allow the enzyme to cleave the other copy (Krüger *et al.*, 1988; Gabbara and Bhagwat, 1992; Jo and Topal, 1995). The *Sfi*I endonuclease, from *Streptomyces fimbriatus,* differs further from the conventional behaviour of a type II enzyme: first, it is a tetramer of identical protein subunits; secondly, it not only interacts with two copies of its recognition sequence but it can also cleave both copies in a concerted reaction (Nobbs and Halford, 1995; Wentzell *et al.*, 1995).

As with other type II endonucleases, *Sfi*I cleaves DNA at fixed locations and requires only Mg^{2+} as a cofactor, but its recognition sequence, GGCCnnnn↓nGGCC (where n is any base and ↓ the point of cleavage), is longer than those for most other restriction enzymes (Qiang and Schildkraut, 1984). However, no DNA cleavages seem to stem from the binding of *Sfi*I to this sequence alone

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(Nobbs and Halford, 1995). The inability of *Sfi*I to cleave DNA without interacting with two copies of its recognition site was first noted from the way in which this enzyme cleaved a plasmid with two sites separated by 1 kb: 80% of the DNA was converted in a concerted fashion directly to the products cut at both sites, while only 20% was liberated from the enzyme after cutting one site (Wentzell *et al.*, 1995). Moreover, the fraction that had been cut at one site was generated by a process that required both sites: the singly cut DNA may have come from the dissociation of a complex with two sites before both had been cleaved (Nobbs and Halford, 1995). In addition, the plasmid with two *Sfi*I sites was cut more rapidly than plasmids with one site, though the reactions on the latter were accelerated by the addition of duplex oligonucleotides carrying the *Sfi*I sequence (Wentzell *et al.*, 1995). The enzyme thus displays its optimal activity with two sites *in cis* on the same DNA, on which it presumably forms a 'looped' complex, but it can also act, albeit less efficiently, with sites *in trans* on separate molecules to possibly give a 'sandwich' complex. However, direct evidence showing that *Sfi*I can form complexes with two DNA sites has yet to be obtained.

We aim here to elucidate the nature of the interactions between distant DNA sites by the *Sfi*I endonuclease. A rigorous distinction between mechanisms that connect two sites through three-dimensional space and those involving a one-dimensional search along the DNA can be made by testing for the interaction on a DNA catenane, with one site on each ring (Craigie and Mizuuchi, 1986; Dunaway and Dröge, 1989; Wedel *et al.*, 1990). In a catenane, the physical proximity of two sites on the interlinked rings will be similar to that for two sites on one ring and the catenane should thus allow for a bridging interaction between the rings (Adzuma and Mizuuchi, 1989). In contrast, any process that follows the DNA contour from one site to the other is doomed to failure on the catenane. This strategy was applied to *Sfi*I by constructing plasmids with two *Sfi*I sites interspersed with two *res* sites for site-specific recombination by Tn*21* resolvase. Recombination by a resolvase from a Tn*3*-like transposon such as Tn*21* converts a circular DNA carrying two *res* sites to a singly interlinked catenane (Krasnow and Cozzarelli, 1983; Castell *et al.*, 1986) which, in this case, will carry one *Sfi*I site on each ring. Furthermore, the strict specificity of resolvase for two *res* sites *in cis*, in directly repeated orientation on a supercoiled DNA (Stark *et al.*, 1989), means that the plasmids constructed here can be used in another test for long-range interactions, one that has been used before both on the *lac* repressor and on Tn*3* resolvase. On a plasmid with two *lac* operators interspersed with two *res* sites, recombination by resolvase was blocked by the prior addition of *lac* repressor, due to the repressor spanning the operators and thus segregating the *res* sites into separate loops so that resolvase was no longer able to carry out its reaction (Saldanha *et al.*, 1987). Similarly, on a plasmid with two *res* sites for Tn*3* resolvase alternated with two for Tn*21* resolvase, the formation of the synaptic complex for Tn*3* resolvase prohibited recombination by Tn*21* resolvase (Parker and Halford, 1991). Hence, bridging interactions by *Sfi*I on the plasmid with two *Sfi*I sites and two *res* sites ought to result in the inhibition of recombination by resolvase.

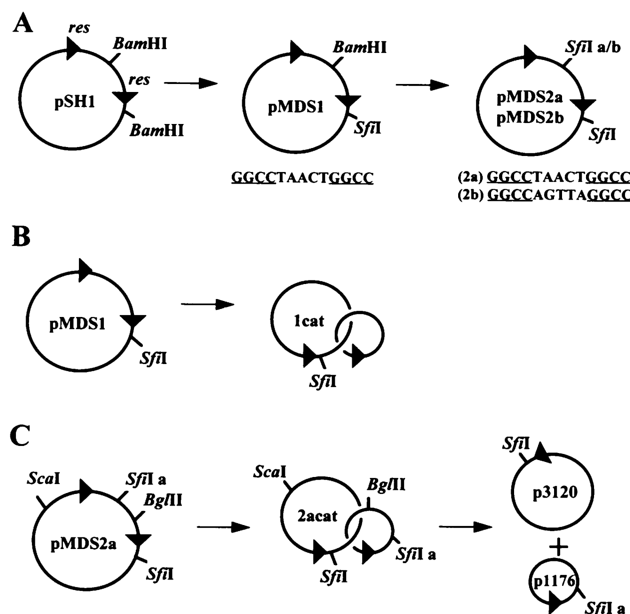

Fig. 1. Substrates for *Sfi*I. (**A**) The plasmid pSH1 (4234 bp; Hall and Halford, 1993) contains two *Bam*HI sites and two *res* sites from Tn*21*, the latter at the positions and in the orientations marked by the arrowheads. The plasmid pMDS1 (4265 bp) was constructed from pSH1 by cloning a DNA duplex containing a *Sfi*I site at one of its *Bam*HI sites. The sequence of the *Sfi*I site in the 'top' strand is indicated. The insertion of the same duplex at the remaining *Bam*HI site on pMDS1 produced two plasmids of 4296 bp, with the second insert either in the same orientation as the first (pMDS2a) or in the opposite orientation (pMDS2b). The 'top' strand sequences of the *Sfi*I sites in the second insertions are listed as (2a) and (2b) respectively. (**B**) Recombination by Tn*21* resolvase between the two Tn*21* *res* sites on pMDS1 yields a singly linked catenane (1cat) with rings of 3120 and 1145 bp. The *Sfi*I site is located on the larger ring. (**C**) Recombination by resolvase between the *res* sites on pMDS2a (or pMDS2b: not shown) yields a singly linked catenane (2acat or 2bcat) with rings of 3120 bp and 1176 bp, both of which carry a *Sfi*I site. The large and small rings from 2acat (p3120 and p1176) were purified by cleaving 2acat in one ring, with either *Bgl*II or *Sca*I, and then removing the linear DNA.

Results

Enhanced activity with two SfiI sites on the same ring

Substrates for the *Sfi*I endonuclease that could be converted to DNA catenanes, and which would permit the use of resolvase to analyse possible bridging interactions between *Sfi*I sites, were constructed from a plasmid that already had two *res* sites from Tn*21* but which lacked any natural *Sfi*I sites (pSH1: Figure 1A). A *Sfi*I site was introduced to this plasmid by cloning a synthetic DNA duplex in the long arc between the *res* sites (pMDS1: Figure 1A). To make substrates with two *Sfi*I sites, the same duplex was then cloned into pMDS1 in the short arc between the *res* sites. Two derivatives were obtained that were identical to each other apart from the orientation of the second insert (Figure 1A). In one case, pMDS2a, the second insert had the same orientation as the first, to give two *Sfi*I sites in direct repeat, i.e. with the same 5 bp spacer sequence in the 'top' strand at both sites (the 13 bp *Sfi*I site is symmetrical only in the two 4 bp segments at both ends). In the other case, pMDS2b, the two *Sfi*I sites were in inverted orientation, so that the 'top' strand sequence

for the 5 bp spacer at one site was complementary to the other site. The *Sfi*I sites on pMDS2a and pMDS2b are separated by 0.78 (or 3.5) kb, a distance which is long enough for the looping interaction not to be constrained by either the intrinsic bend or twist of the DNA but which is short enough to allow for loop formation without an excessive entropy penalty (Schleif, 1992).

The sequence for the synthetic duplex was chosen to be AT rich on either side of the *Sfi*I site, and also in the 5 bp spacer region in the middle of the site, in order to differ from previous studies on *Sfi*I where the substrates had contained both flanking and spacer sequences that were GC rich (Wentzell *et al.*, 1995). In the previous studies, supercoiled DNA with two *Sfi*I sites had been cleaved faster than supercoiled DNA with one site, the former being assigned to *cis* interactions between sites on the same DNA molecule and the latter to *trans* interactions across sites from separate molecules. However, the cleavage rates varied with ionic strength: the reactions assigned to *trans* interactions were only observed at low salt, but the same conditions also maximized the stability of the proposed *cis* interactions (Nobbs and Halford, 1995). To check whether *Sfi*I sites embedded in AT-rich sequences behaved in the same manner as sites in GC-rich regions, the supercoiled forms of the three new plasmids, pMDS1, pMDS2a and pMDS2b, were used as substrates for the *Sfi*I endonuclease (Figure 2A). The reactions were carried out at low salt to maximize any possible interactions between the *Sfi*I sites.

The supercoiled DNA with one *Sfi*I site, pMDS1, was cleaved at a slower rate than the plasmids with two sites, pMDS2a and pMDS2b (Figure 2A). On the latter pair, ~90% of the supercoiled DNA was cleaved within 15 min while the same extent of reaction on pMDS1 required 180 min. The reaction rates on both the one-site and the two-site substrates constructed here were similar to those observed previously on other plasmids under similar conditions (Nobbs and Halford, 1995; Wentzell *et al.*, 1995). Hence, the difference in DNA cleavage rates between substrates with one or two *Sfi*I sites cannot be a consequence of the particular sequences flanking and interrupting the *Sfi*I sites. Instead, it must be a general feature of the mechanism of this endonuclease. In addition, the identical rates on pMDS2a and pMDS2b (Figure 2A) show that the nature of the communication event that leads to the enhanced cleavage rate at two sites is independent of the orientation of the sequences.

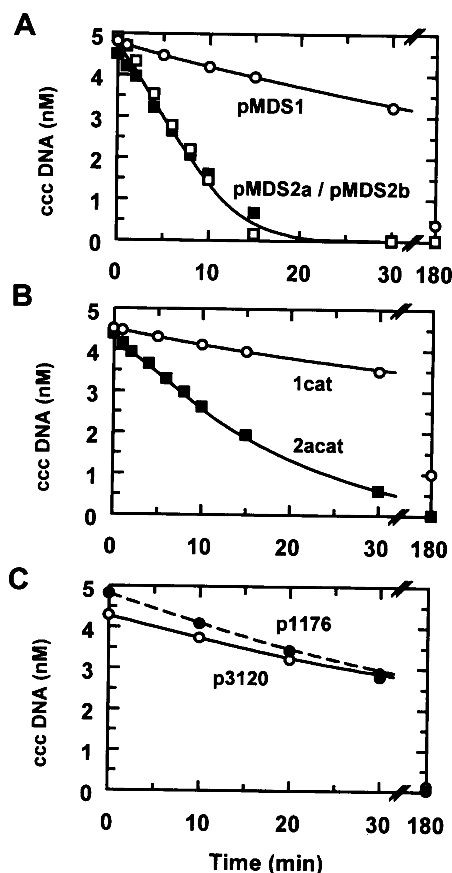

Fig. 2. *Sfi*I reactions on single or catenated rings of DNA. Reactions in buffer S at 50°C contained 0.125 nM *Sfi*I endonuclease and 5 nM DNA [in all cases, the DNA was ^{3}H-labelled and >85% of the preparation(s) was supercoiled]. For the three separate reactions shown in (**A**), the DNA was either pMDS1 (○), pMDS2a (■) or pMDS2b (□). For the two separate reactions shown in (**B**), the DNA was either 1cat (○) or 2acat (■). For the single reaction shown in (**C**), the DNA was a mixture of 5 nM p3120 (○) and 5 nM p1176 (●). Aliquots were removed from the reactions at the time points indicated on the *x*-axis and the concentration of the DNA substrate retaining its covalently closed circular structure (ccc DNA) is shown on the *y*-axis.

Enhanced activity with two SfiI sites on catenated rings

Long-range interactions between distant DNA sites in recombination and transcription have been analysed previously on catenanes carrying target sites for the proteins on interlinked rings (Craigie and Mizuuchi, 1986; Wedel *et al.*, 1990). This approach was applied to the restriction of DNA by *Sfi*I, by measuring its activity on the DNA catenanes generated by Tn*21* resolvase from either pMDS1, pMDS2a (Figure 2B) or pMDS2b (data not shown). The catenane from pMDS1, 1cat, contains one *Sfi*I site on the larger of the two rings. The catenanes from pMDS2a and pMDS2b, 2acat and 2bcat, again carry a *Sfi*I site on the larger ring but these also have a *Sfi*I site on the smaller ring. For some experiments, the individual rings from 2acat, p3120 and p1176 (Figure 1C), were isolated by using different restriction enzymes to cleave the catenane in either its large or small rings and then purifying the covalently closed form of the intact ring: an equimolar mixture of the two rings was also used as a substrate for *Sfi*I (Figure 2C).

The catenane with one *Sfi*I site on one of its two rings, 1cat, was cleaved by *Sfi*I exclusively in the ring that had the site (data not shown), but this reaction occurred at the same slow rate (Figure 2B) as that on the plasmid from which this catenane had been derived, pMDS1 (Figure 2A). The catenanes with one *Sfi*I site on each ring, 2acat and 2bcat, were cleaved in both rings to give primarily DNA that had been linearized in both rings as the initial product (see below, Figure 3). The rate of the reaction on 2acat (Figure 2B) was identical to that on 2bcat (data not shown) and, in both cases, these rates were about a factor of two slower than those on the original plasmids with two *Sfi*I sites (Figure 2A). However, the catenanes with one site on each ring were still cleaved faster than the catenane with a site on just one ring. In addition, when *Sfi*I reactions were carried out on the mixture of the two

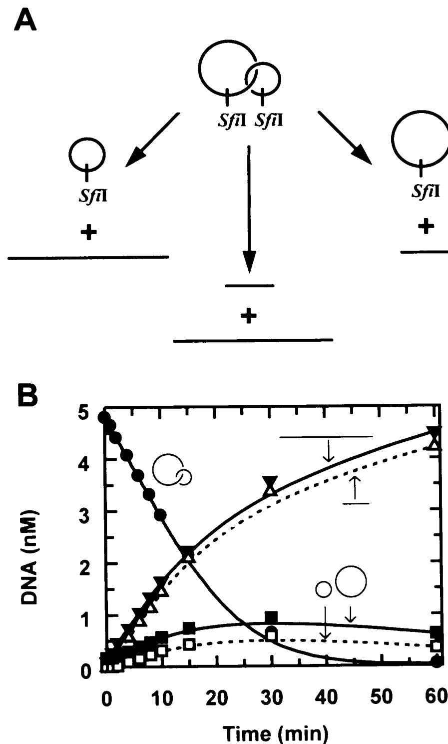

Fig. 3. Coupled reactions on catenaned *Sfi*I sites. (**A**) The cartoon shows the potential products from the initial reaction of the *Sfi*I endonuclease on a catenane containing a large ring of DNA linked to a small ring, with one *Sfi*I site on each ring. If *Sfi*I were to first cleave the site on the large ring, the initial products will be a small circular DNA and a large linear DNA (left hand pathway), while a reaction at the site on the small ring will produce a large circular DNA and a small linear DNA (right hand pathway). Conversely, a coupled reaction at both *Sfi*I sites produces the two linear forms as its initial product (central pathway). (**B**) The reaction in buffer S at 50°C contained 0.125 nM *Sfi*I endonuclease and 5 nM DNA from 2acat (^{3}H-labelled, with initially >90% of the catenanes supercoiled in both rings). Samples were withdrawn from the reaction at the timed intervals, quenched and analysed by gel electrophoresis to separate catenated DNA substrate (●), the large free circle (■), the small free circle (□), the large linear product (▼) and the small linear product (△). The concentrations of all of these forms of the DNA were measured at each time point sampled: each form is identified in (B) by the symbols from the cartoon in (A).

rings that had been isolated from 2acat, the separate rings were both cleaved at the slow rate characteristic of a *Sfi*I reaction on a circular DNA with one site (Figure 2C). The reaction mixture containing the two separate circles is identical in all respects to that containing 2acat, except for the fact that the two rings are not interlinked, yet *Sfi*I converted the catenane into the final product cleaved in both rings (see below, Figure 3) at a faster rate than the sum of the rates on the unlinked circles (2.6 mol *Sfi*I sites/mol enzyme/min in the former case, 0.8 in the latter).

The data in Figure 2 show first that the catenation of DNA has, by itself, no effect on *Sfi*I activity, since the catenated and the orthodox substrates with one *Sfi*I site were cleaved at the same rate. Secondly, the enhanced activity of *Sfi*I on a substrate with two sites cannot be due to any process that is restrained to the DNA contour between the two sites, since an enhanced activity was also observed on a catenane with sites on interlinked rings, where the sites are not connected to each other by a continuous sequence. However, the degree of enhancement with the catenane was not as high as that with a single molecule of supercoiled DNA carrying two sites: possible reasons for this are discussed below. Thirdly, the enhancement must stem from the physical proximity of the two sites, since two sites on separate circles of DNA were cleaved less efficiently than the same two sites on the same two circles held together topologically by catenation.

Concerted cleavages at two SfiI sites on catenated rings

Previous studies on the *Sfi*I endonuclease had indicated that a plasmid with two *Sfi*I sites was cleaved at both sites in a concerted reaction: the initial product was primarily the DNA cut at both sites, and the yields of the partial digest products, cleaved at either one of the sites, were lower than would have been expected for independent reactions at each site (Wentzell *et al.*, 1995). In addition, the kinetics for the formation and decay of the singly cut DNA were inconsistent with a sequential pathway in which the enzyme first cuts one site and then the other (Nobbs and Halford, 1995). A catenane such as 2acat (Figure 1C), with one site on each ring, permits the dissection of a reaction encompassing two recognition sites. If the enzyme were to cleave each site on the catenane in an independent reaction, it must give rise initially to both free circles and linear products in exactly equal yields; either the small circle from the catenane and a large linear DNA, should it first cut the site in the large ring, or vice versa (Figure 3A). [The residual circle with one *Sfi*I site will be linearized subsequently by *Sfi*I but this reaction is slow (Figure 2C) and thus makes no contribution to initial rates.] On the other hand, if both sites are cleaved concertedly by the same DNA–protein complex, the catenane will be converted directly to the two linear products without liberating the individual circles.

Samples were taken from a *Sfi*I reaction on 2acat at timed intervals and the concentrations of the 2acat substrate, the two individual circles (p3120 and p1176) and the two linear products were all measured (Figure 3B). [Essentially all of the starting catenane was covalently closed in both rings and the single circles that were liberated from the catenane remained as covalently closed DNA until cleaved to the linear forms (data not shown).] The initial rate for forming the two linear DNA products was considerably faster than that for forming free circles. Some of the catenanes must therefore have been cleaved in concerted reactions spanning both sites. The number of the *Sfi*I reactions that yield DNA cut at two sites, relative to the number of its reactions that yield DNA cut at just one site, can be calculated from the difference between the initial rates for forming either linear DNA products or free circles. This calculation revealed that ~66% of the catenated DNA molecules were cleaved in a concerted fashion, directly to the two linear products (i.e. the central pathway in Figure 3A), while 17% went through each of the two pathway that liberate singly cut DNA. Identical results were obtained with the other catenane carrying *Sfi*I sites in both rings, 2bcat (data not shown).

On a DNA with two *Sfi*I sites, the concerted cleavage of both sites could be due to a processive mechanism, of the type proposed for *Eco*RI (Terry *et al.*, 1987), in which the enzyme first cuts one site and then diffuses rapidly along the DNA to the next site. However, this scheme

was unable to account for why DNA molecules with two *Sfi*I sites were cleaved faster than molecules with one site, so it was suggested that the cleavage reaction required one molecule of the tetrameric protein to bind simultaneously to two copies of its recognition site (Wentzell *et al.*, 1995). The observation that coupled cleavages are also generated with *Sfi*I sites on separate rings within a catenane (Figure 3B) eliminates the possibility of a processive mechanism involving linear diffusion along the DNA from one site to the other. The data from the catenanes show that the coupling of the cleavage events at two *Sfi*I sites must stem from a direct physical interaction between the two sites that can operate through three-dimensional space. This provides strong support for the previous proposal of a bridging interaction with the protein bound to two segments of DNA. From the earlier data (Nobbs and Halford, 1995), it is likely that the reactions on the catenane that result in the cleavage of one of its two *Sfi*I sites are due to the dissociation of the complex with the protein bound to both sites before both are cleaved.

DNA looping by SfiI

If the *Sfi*I endonuclease is able to form a protein bridge between two recognition sites, then it ought to be possible to detect this interaction by measuring resolvase activity on the plasmids that carry both *Sfi*I sites and *res* sites. This assay is based on the rigid requirement of resolvase for a supercoiled DNA substrate with two *res* sites *in cis*, in directly repeated orientation. Resolvase has no activity with *res* sites in inverted orientation nor with sites *in trans* on different DNA molecules (Stark *et al.*, 1989). Hence, if a plasmid has two *res* sites for resolvase interspersed with two target sites for another protein that can form a bridge between its sites, the bridging interaction by the latter protein will segregate the *res* sites into separate loops: this effectively creates a DNA with two *res* sites *in trans* so the plasmid will no longer be a substrate for resolvase (Saldanha *et al.*, 1987; Parker and Halford, 1991). The plasmids constructed here (Figure 1A) permit the application of this strategy to *Sfi*I.

Reaction conditions were needed in which Tn*21* resolvase is active but where *Sfi*I has no DNA cleavage activity yet still binds specifically to its recognition sites. *Sfi*I has no activity without Mg^{2+} (Qiang and Schildkraut, 1984) but Tn*21* resolvase was reported to have its maximal activity in the absence of Mg^{2+} only in the presence of high concentrations of NaCl (Castell *et al.*, 1986; Parker and Halford, 1991), conditions that do not favour interactions between distant sites by *Sfi*I (Nobbs and Halford, 1995). However, buffers containing potassium glutamate in place of NaCl enable Tn*21* resolvase to achieve its full activity in the absence of Mg^{2+} at low ionic strengths (M.Oram and S.E.H, unpublished) and the same conditions allow for full *Sfi*I activity upon the addition of Mg^{2+} (Nobbs and Halford, 1995). The recombination reactions by Tn*21* resolvase in the presence or absence of *Sfi*I endonuclease were therefore carried out in buffer containing both potassium glutamate and EDTA.

In the absence of *Sfi*I, the rate of recombination by Tn*21* resolvase on pMDS2a, the plasmid with two *Sfi*I sites and two *res* sites in directly repeated orientations, was similar to that on other plasmids under comparable conditions (Castell *et al.*, 1986; Soultanas *et al.*, 1995): the reaction was ~80% complete in 2 min (Figure 4A). The addition of an equimolar amount of *Sfi*I endonuclease to this plasmid 5 s after the resolvase had no effect on the rate of recombination (Figure 4A). However, when this plasmid was incubated with an equimolar amount of *Sfi*I endonuclease for 10 min before the addition of resolvase, the rate of recombination was significantly reduced: only 30% of the DNA was now recombined in 2 min (Figure 4A). The plasmid with two *res* sites in direct repeat interspersed with two *Sfi*I sites in inverted orientation, pMDS2b, gave the same results as pMDS2a in all three situations (data not shown). The plasmid with two *res* sites and one *Sfi*I site, pMDS1, also gave the same rate for Tn*21* recombination in the absence of *Sfi*I but, this time, the pre-incubation of the plasmid with *Sfi*I failed to inhibit recombination (Figure 4B).

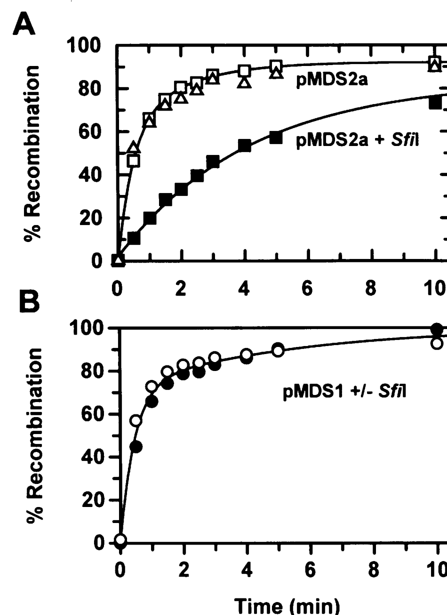

Fig. 4. Recombination by resolvase on plasmids with *res* sites and *Sfi*I sites. The reactions in buffer T at 25°C contained 9 nM (^{3}H-labelled) plasmid DNA as noted below, 180 nM Tn*21* resolvase and, where indicated, 9 nM *Sfi*I endonuclease. Aliquots were removed from the reactions at the time points indicated and analysed as described in Materials and methods in order to measure the fraction (%) of the supercoiled DNA that had undergone recombination by resolvase. For all three reactions in (**A**), the DNA was pMDS2a: one of the reactions (□) was carried out in the absence of *Sfi*I endonuclease; for another reaction (△), *Sfi*I was added to pMDS2a 5 s after the resolvase; for the third reaction (■), *Sfi*I was added to pMDS2a 10 min before the resolvase. For both reactions in (**B**), the DNA was pMDS1: one of the reactions (●) was carried out in the absence of *Sfi*I endonuclease; for the other reaction (○), *Sfi*I was added to pMDS1 10 min before the resolvase.

The fact that *Sfi*I inhibited recombination on the plasmids with two *Sfi*I sites, but had no effect on the plasmid with one *Sfi*I site, shows that the *Sfi*I endonuclease binds specifically to its recognition site(s) in the absence of Mg^{2+}. If the inhibition had been due to non-specific binding of *Sfi*I to the *res* sites or anywhere else on the DNA, it would also have been observed on the plasmid with one *Sfi*I site. Moreover, the data in Figure 4 demonstrate that this endonuclease can indeed bind to two copies of its recognition sequence at the same time. Since two *Sfi*I sites were required for the inhibition of resolvase, it

must have come from a bridging interaction between the *Sfi*I sites that segregated the *res* sites into separate loops of DNA, essentially as described in the previous applications of this approach (Saldanha *et al.*, 1987; Parker and Halford, 1991). This view is substantiated by the lack of inhibition when *Sfi*I was added to pMDS2a after the resolvase, since a bridging interaction by *Sfi*I would only inhibit recombination if it had been established before resolvase had formed its synaptic complex. The formation of the synaptic complex for resolvase is a rapid process, largely complete within a second, and it is also essentially irreversible on a DNA with *res* sites in direct repeat (Parker and Halford, 1991; M.Oram and S.E.Halford, unpublished). Consequently, in the reactions where *Sfi*I was added to the DNA before the resolvase, the dissociation of the complex between the two *Sfi*I sites will allow resolvase immediately to form its synaptic complex and carry out its recombination reaction, but the rate of recombination is then limited by the rate of dissociation of the looped complex for *Sfi*I. The half-time for the dissociation of the looped complex for *Sfi*I is thus ~4 min under these reaction conditions.

Correlation between DNA looping and DNA cleavage by SfiI

The identification of a looped complex spanning two recognition sites for the *Sfi*I endonuclease (Figure 4) leaves open the question of whether the looped complex is necessary for DNA cleavage by *Sfi*I. It could, alternatively, be just a consequence of its tetrameric structure, with the protein binding to either one or two copies of its site and then cleaving whatever number of sites it had bound to, though this latter possibility is difficult to reconcile to the kinetics of DNA cleavage by *Sfi*I (Nobbs and Halford, 1995). In addition, while the *Sfi*I enzyme is known to exist in solution as a tetramer (Wentzell *et al.*, 1995), it has yet to be determined whether a single tetramer is sufficient for loop formation.

To answer these questions, a plasmid with two *Sfi*I sites and two *res* sites was incubated with various concentrations of *Sfi*I endonuclease in buffer lacking Mg^{2+} ions. To one set of these incubations, Tn*21* resolvase was added and the recombination reactions were then stopped after 2 min, this being the optimal time to measure the inhibition of recombination caused by pre-incubation with *Sfi*I (Figure 4A). The amount of recombination by resolvase decreased linearly with increasing concentrations of *Sfi*I, reaching its minimum at a ratio of ~1.5 mol protein/mol DNA, but further increases in the *Sfi*I concentration restored the amount of recombination to the level seen in the absence of *Sfi*I (Figure 5A). To a parallel set of incubations, $MgCl_2$ was added to activate the *Sfi*I, and the DNA cleavage reactions were then stopped after 5 s, a time interval that allows for a significant fraction of the enzyme-bound DNA to be cleaved but which is too short for multiple turnovers of this enzyme [these reactions were at 25°C, well below the optimum of 50°C for DNA cleavage by *Sfi*I (Qiang and Schildkraut, 1984)]. Just as in the recombination reactions, the amount of uncleaved DNA left at the end of these 5 s *Sfi*I reactions decreased linearly with increasing concentrations of *Sfi*I, again giving a minimum at a ratio of ~1.5 mol protein/mol DNA, and the amount of uncleaved DNA was also restored to a higher level by further increases in the concentration of *Sfi*I (Figure 5B).

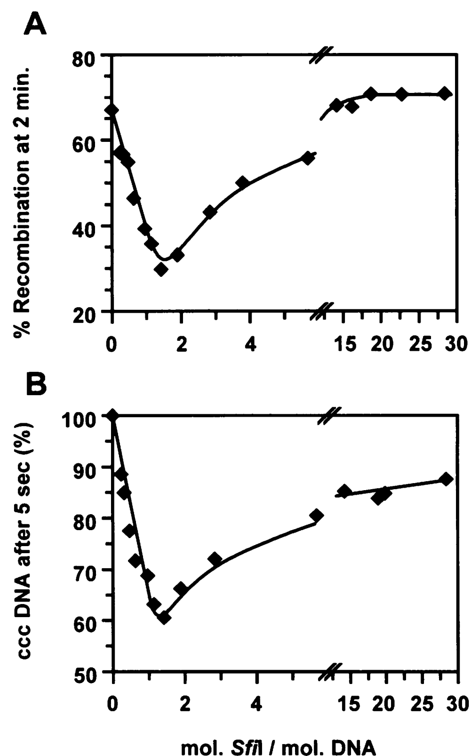

Fig. 5. Stoichiometry of the *Sfi*I synaptic complex. Varied concentrations of *Sfi*I endonuclease were added to solutions containing 9 nM pMDS2a in buffer T, to give the ratio of mol *Sfi*I endonuclease/mol DNA shown on the *x*-axes. The samples were then incubated for 10 min at 25°C. To one set of these samples (**A**), Tn*21* resolvase was added to a final concentration of 180 nM: the recombination reactions were allowed to proceed for 2 min before being stopped, and the fraction (%) of the supercoiled plasmid that had undergone recombination was measured as described in Materials and methods. To a parallel set of samples (**B**), $MgCl_2$ was added to a final concentration of 10 mM: the DNA cleavage reactions were allowed to proceed for 5 s before being stopped, and the fraction (%) of the substrate retaining its covalently closed circular structure (ccc DNA) was measured as described in Materials and methods.

The degree of DNA looping by *Sfi*I in the absence of Mg^{2+}, measured from the inhibition of recombination by resolvase (Figure 5A), thus varies with the concentration of *Sfi*I protein in the same manner as does the degree of DNA cleavage in the presence of Mg^{2+} (Figure 5B). The correlation between looping and cleavage indicates that the looped complex is a prerequisite for the reaction of *Sfi*I. This validates the conclusion deduced from its reaction kinetics, that no DNA cleavages stem from the binding of this restriction enzyme to one copy of its recognition site (Nobbs and Halford, 1995). At high concentrations of *Sfi*I, the extents of both DNA looping and DNA cleavage diminished progressively with further increases in protein concentration. The extent of looping declined to zero, since Tn*21* resolvase recovered its full activity at high *Sfi*I concentrations (Figure 5A). In contrast, the *Sfi*I reactions were partially diminished: some DNA cleavage was still observed at the highest enzyme concentrations tested (Figure 5B).

These results can be accounted for by the mechanism for DNA synapsis shown in Figure 6. The titration of a

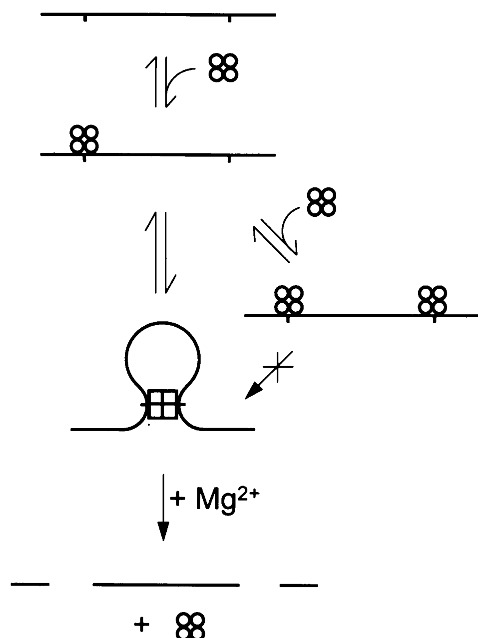

Fig. 6. Model for DNA synapsis by *Sfi*I. The DNA is represented as a solid line, with two *Sfi*I sites indicated by hatch marks, and the tetrameric *Sfi*I protein by four circles. The addition of a sub-stoichiometric amount of *Sfi*I to the DNA results in the initial binding of one tetramer to one of the two *Sfi*I sites. The unliganded subunits can then bind to the other *Sfi*I site *in cis* to give a looped complex, with a concomitant change in protein conformation in all four subunits (now indicated by squares) that allows each subunit to cleave one DNA strand. In the presence of Mg^{2+}, the looped complex can then proceed to cleave the DNA at both sites. Alternatively, at higher protein concentrations, the DNA carrying a *Sfi*I tetramer at one of its two sites can bind a second tetramer at the other *Sfi*I site, but this doubly bound form of the DNA cannot proceed directly to the looped complex. The only route from the doubly bound DNA to the looped complex is via the singly bound DNA.

DNA carrying two *Sfi*I sites with increasing concentrations of *Sfi*I will result initially, at sub-stoichiometric amounts of protein, in the binding of the endonuclease to just one of the two sites, in an interaction that perhaps involves only two subunits from the tetramer. The unliganded subunits in this initial complex may then bind to the second site and thus loop out the intervening DNA. In the presence of Mg^{2+}, the looped complex can proceed to cleave the DNA. However, at higher concentrations of the endonuclease, protein molecules will bind to both sites on the DNA but this doubly bound species is unable to form the loop. Hence, on this scheme (Figure 6), increasing protein concentrations must eventually disrupt the looped complex. However, excess protein will be more efficient at breaking the loop in the absence of Mg^{2+} (Figure 5A) than in its presence (Figure 5B) because, in the latter case, the irreversible DNA cleavage reaction will drag the equilibrium between the doubly bound DNA and the looped complex (via the singly bound species) towards the looped state. The transition between loop formation and loop disruption with increasing amounts of *Sfi*I was observed as its concentration was raised from 1 to 2 mol *Sfi*I/mol DNA (Figure 5). A single tetramer of the *Sfi*I endonuclease is thus sufficient for loop formation. If the loop came from a tetramer at both sites, then the transition would have needed $\geq$2 mol protein/mol DNA.

Discussion

Mechanisms of long-range interactions between distant DNA sites (Wang and Giaever, 1988) fall into two main categories: either one-dimensional processes that involve tracking along the DNA from one site to the other, or three-dimensional processes that connect the distant sites through space. The former are constrained to act exclusively *in cis*, between sites on the same DNA molecule, while the latter can, in principle, operate either *in cis*, by the protein(s) binding simultaneously to both sites and looping out the intervening DNA, or *in trans*, where the simultaneous binding will sandwich the protein between two DNA molecules. Both type I and type III restriction enzymes communicate between their specific recognition sites and their non-specific sites for DNA cleavage elsewhere on the molecule by one-dimensional mechanisms (Bickle, 1993; Meisel *et al.*, 1995). Type II restriction enzymes, on the other hand, generally act at individual sites in independent reactions (Roberts and Halford, 1993). However, this study has shown that the *Sfi*I endonuclease, an atypical type II enzyme whose DNA cleavage reaction spans two copies of its recognition sequence (Wentzell *et al.*, 1995), communicates between its sites by a three-dimensional mechanism.

It had been noted before that a particular plasmid carrying two *Sfi*I sites surrounded by GC-rich sequences had been cleaved by *Sfi*I at a faster rate than deletion mutants of the same plasmid with one *Sfi*I site (Nobbs and Halford, 1995; Wentzell *et al.*, 1995). However, it had not been established whether the unusual kinetics for DNA cleavage at two sites were due to the intrinsic mechanism of action of this endonuclease: the kinetics could, alternatively, have been due to some unique feature of the particular DNA molecule(s) used in the earlier studies. The initial observation reported here, that a different plasmid with two *Sfi*I sites in AT-rich environments is also cleaved faster than an isogenic plasmid with one site (Figure 2A), indicates that enhanced activity at two sites *in cis* is a general feature of the reaction mechanism for *Sfi*I.

The enhanced activity was observed with plasmids carrying two *Sfi*I sites in either directly repeated or inverted orientations. This aspect of *Sfi*I is common to several site-specific recombination enzymes, such as FLP or *cre,* that can catalyse DNA rearrangements between sites in either orientation. Both FLP and *cre* act at sites that have similar structures to *Sfi*I sites, two symmetrical target sequences separated by an undefined spacer, and they either delete or invert the intervening DNA depending on the orientation of the spacer sequence (Sadowski, 1993). In contrast, other recombinases, such as the resolvases from Tn*3*-like transposons and the DNA invertases, only act at sites in a particular orientation (Kanaar and Cozzarelli, 1992; Grindley, 1994). Resolvases and invertases form synaptic complexes in which the recombinational sites are wrapped around each other, and around the protein, in a stereospecific manner: this wrapping can only be achieved with sites in a specified orientation on account of the topological constraints that it imposes upon the rest of the DNA molecule (Stark *et al.*, 1989). Furthermore, resolvase can act on DNA catenanes only when the *res* sites on each ring are related by particular topologies (Benjamin and

Cozzarelli, 1990; Stark *et al.*, 1994). Yet *Sfi*I acted on catenanes containing, with respect to the *Sfi*I sequences, either negative nodes (2acat) or positive nodes (2bcat): only catenanes with negative nodes are substrates for resolvase (Stark *et al.*, 1994). The synaptic complex for *Sfi*I is therefore likely to involve a simple bridging interaction between the two DNA segments, perhaps similar to that proposed for the *lac* repressor (Friedman *et al.*, 1995), rather than a higher order structure with a defined topology.

When the long-range interactions between *Sfi*I sites were analysed on catenanes, following the strategy of Craigie and Mizuuchi (1986), the endonuclease was more active on a catenane with one *Sfi*I site in each ring than on the same two rings of DNA separated from each other (Figure 2B and C). The enhanced activity on a substrate with two sites therefore cannot come from any mechanism that follows the DNA contour from one site to the other. Instead, the local concentration of one *Sfi*I site in the vicinity of the other site must be higher when the two rings are held together topologically than when the same two rings are present at the same total concentrations but where the rings are not linked to each other. However, the activity of *Sfi*I on a catenane with a site in each ring was not as high as that on a single ring of supercoiled DNA with two *Sfi*I sites. Several reasons might account for this. First, the effective concentration of one DNA site in the vicinity of another is increased by supercoiling (Vologodskii and Cozzarelli, 1994) but catenanes generated by resolvase possess fewer negative supercoils than the initial plasmid, on account of its topoisomerase activity (Krasnow and Cozzarelli, 1983; Castell *et al.*, 1986). Secondly, resolvase produces singly interlinked catenanes while multiply interlinked catenanes would perhaps be better mimics of a supercoiled plasmid, due to their interwound structures (Benjamin and Cozzarelli, 1990). Thirdly, the mean distance between two loci on rings of a catenane is longer than that between loci on a supercoiled plasmid, particularly for a singly interlinked molecule (Levene *et al.*, 1995).

The fact that the majority of *Sfi*I reactions on a catenane with sites in each ring result in the concerted cleavage of both sites (Figure 3) can only be accounted for by the protein interacting simultaneously with both sites, physically creating a direct bridge between the sites. The experiments described here on the plasmid with two *res* sites and two *Sfi*I sites, showing that resolvase was inhibited when this DNA was pre-incubated with *Sfi*I (Figure 4), provide the first direct evidence for such a bridge. The latter experiments also showed that the *Sfi*I endonuclease binds to DNA, specifically to its recognition site(s), in the absence of Mg^{2+} ions. In this respect, *Sfi*I is similar to some other restriction enzymes, such as *Eco*RI, that also bind specifically to DNA in the absence of Mg^{2+} (Terry *et al.*, 1987), but it differs from *Eco*RV and many other restriction enzymes that show no specificity for their recognition sites in binding to DNA unless an appropriate metal ion is provided (Vipond and Halford, 1995).

The assay used here to detect long-range interactions between distant DNA sites had been used before to measure the formation of synaptic complexes by Tn*3* resolvase (Parker and Halford, 1991). However, the way in which synapsis by Tn*3* resolvase varies with the concentration of the protein differs radically from *Sfi*I. Tn*3* synapsis showed a sigmoidal dependence on the concentration of resolvase, with essentially all of the synaptic complexes being formed as soon as six dimers of the protein were added to the DNA: almost no complexes were detected at lower protein concentrations while higher concentrations had no further effect (Parker and Halford, 1991). This relationship can be explained by each *res* site having three binding sites for the resolvase dimer: all three binding sites at both *res* sites first need to be filled by resolvase, and the synaptic complex is then established by interactions between the protein molecules at each site (Grindley, 1994; Soultanas *et al.*, 1995). In contrast, *Sfi*I synapsis increased linearly with increasing concentrations of the endonuclease, up to a maximum at ~1.5 mol protein/mol DNA, but then decreased as the protein concentration was raised further (Figure 5A). The difference between resolvase and *Sfi*I in this respect illustrates the distinction between two major mechanisms for long-range interactions: either the binding of separate proteins to each site that are then held together by protein–protein interactions (Hochschild, 1990), as is the case with resolvase; or the simultaneous binding of an oligomeric protein to both sites (Schleif, 1992), as is the case with *Sfi*I. Only the latter scheme (Figure 6) can account for the disruption of the long-range interaction by excess protein.

The disruption of a looped complex by excess protein has been observed before with the *lac* repressor on a DNA containing two ideal *lac* operators (Krämer *et al.*, 1987), but many natural instances of looping interactions involve the protein binding simultaneously to non-identical DNA sequences, for example *O1*, *O2* and *O3* for the *lac* repressor or *araO* and *araI* for the *araC* protein (Schleif 1992). In these situations, the protein often has a higher affinity for one of its binding sites over the others, and the looping interaction will then be less sensitive to disruption by excess protein than was found for *Sfi*I (Figure 5). In addition, the looping interaction by *Sfi*I is comparatively unstable relative to those by repressor proteins in the absence of their inducers. The half-time for the dissociation of the DNA loop formed by *Sfi*I is ~4 min (Figure 4A), while that for *araC* is ~100 min (Lobell and Schleif, 1990). This difference is presumably a reflection of the need for catalytic turnover by *Sfi*I. However, the stability of the looped complex for *Sfi*I is similar to that for the non-productive complex formed by resolvase with two *res* sites in inverted orientation. On a plasmid with Tn*21 res* sites in direct repeat alternating with Tn*3 res* sites in inverted orientation, so that the DNA is a substrate for Tn*21* resolvase but not for Tn*3* resolvase, the addition of the Tn*3* enzyme before the Tn*21* enzyme reduced the rate of Tn*21* recombination over a 15 min period but it still eventually allowed for Tn*21* recombination (Parker and Halford, 1991), in a similar fashion to the inhibition of resolvase caused by the pre-incubation with *Sfi*I (Figure 4A).

A correlation between the enzymatic properties of the *Sfi*I endonuclease and its biological functions has yet to be established. The mechanism of action of *Sfi*I is clearly more elaborate than is necessary for the endonuclease from a type II restriction–modification system. Other endonucleases from type II systems employ much simpler

mechanisms (Roberts and Halford, 1993; Vipond and Halford, 1993). Moreover, several features of its mechanism suggest that *Sfi*I would be highly inefficient at defending *S.fimbriatus* against phage infections (Wentzell *et al.*, 1995). These include first the comparative infrequency of *Sfi*I sites on most DNA molecules relative to other restriction sites of 4–6 bp, since it has eight specified base pairs in its recognition sequence, yet *Sfi*I needs to find two copies of this sequence to cleave DNA. Secondly, in the ionic conditions likely to be found in the bacterial cytoplasm, *Sfi*I will have no activity across *Sfi*I sites from different DNA molecules and it will only be able to cleave DNA with two sites *in cis* (Nobbs and Halford, 1995). Thirdly, *Sfi*I is prevented from cleaving DNA by an excess of enzyme over DNA (Figure 5B). *Sfi*I could thus only combat infections at very high multiplicities by phage whose chromosomes carry two or more *Sfi*I sites. Even then, its temperature optimum *in vitro*, 50°C (Qiang and Schildkraut, 1984), is far above the maximal temperature for the growth of *S.fimbriatus*, 25°C (G.S.Baldwin, personal communication). Other type II restriction enzymes may have functions outside of restriction–modification (Jo and Topal, 1995; Naito *et al.*, 1995) and it seems unlikely that the biological function of the *Sfi*I endonuclease is solely the restriction of foreign DNA.

Materials and methods

Proteins

*Sfi*I endonuclease was purified to 98% homogeneity (by T.J.Nobbs and L.M.Wentzell, this laboratory) from an overproducing strain supplied by New England Biolabs (Vancott, 1991) as described previously (Wentzell *et al.*, 1995). Methods for the purification of Tn*21* resolvase and for measuring its concentration were from Ackroyd *et al.* (1990). The concentrations of *Sfi*I endonuclease (determined by absorbance at 280 nm) are given in terms of the tetrameric protein of M_r 124 176 and those for Tn*21* resolvase for the dimeric protein of M_r 42 610. All other enzymes were purchased from New England Biolabs and used as prescribed.

DNA

Two oligodeoxynucleotides were synthesized by L.Hall (this department) using reagents from Cruachem, purified on NAP-20 columns (Pharmacia) and then annealed to form the following duplex containing a *Sfi*I site (underlined base pairs in bold)

5′-GATCTCGATGCAT**GGCC**TAACT**GGCC**TATGT
AGCTACGTA**CCGG**ATTGA**CCGG**ATACACTAG-5′.

By using standard procedures (Sambrook *et al.*, 1989), the duplex was cloned at one of the two *Bam*HI sites on pSH1 (Hall and Halford, 1993), by ligating it to vector DNA that had been linearized previously by a partial *Bam*HI digest. A product from this ligation, pMDS1 (Figure 1A), was used as a vector to clone a second copy of the duplex at its residual *Bam*HI site to yield two derivatives, pMDS2a and pMDS2b (Figure 1A), that differed in the orientation of the second insert. The identity of the plasmids was confirmed by DNA sequencing (DuPont Genesis) from primers upstream of each *Sfi*I site. The plasmids were used to transform *Escherichia coli* HB101 (Sambrook *et al.*, 1989), the transformants grown in M9 minimal medium with 1 mCi/l [methyl-^{3}H]thymidine, and the covalently closed form of the DNA purified by density gradient centrifugations in CsCl–ethidium bromide (Castell *et al.*, 1986).

Catenanes were prepared from the plasmids (350 nM) by reactions with Tn*21* resolvase (8.3 μM) for 3 h at 37°C in buffer T supplemented with 10 mM $MgCl_2$. Buffer T is 10 mM Tris–HCl (pH 8.0), 150 mM potassium glutamate, 1 mM EDTA, 5 mM β-mercaptoethanol. Under these conditions, >98% of the supercoiled plasmid was converted to the catenane (data not shown), but the nicked DNA in the plasmid preparations (typically 5%) is not recombined by resolvase. To remove both the nicked (unrecombined) plasmids and any catenanes subsequently nicked in either one or both rings, the covalently closed forms of the catenanes (with respect to both rings) were purified from the recombination mixtures by centrifugation through CsCl–ethidium bromide. This procedure removed ~90% of the nicked DNA.

To isolate p1176, the covalently closed form of 2acat (50 μg) was first cleaved in its large ring by digestion with *Sca*I and the DNA then extracted with phenol/chloroform prior to precipitation with isopropanol. The linear DNA in this mixture was removed by an overnight digestion at 37°C with 1000 units of exonuclease III in 90 μl of the buffer supplied and the covalently closed DNA recovered by phenol/chloroform followed by isopropanol precipitation. The isolation of p3120 was by the same route, except that 2acat was cleaved initially in its small ring by *Bgl*II. The final preparations of p3120 and p1176 contained <1% linear DNA and <10% nicked molecules (data not shown).

Restriction reactions

DNA cleavage reactions by *Sfi*I were carried out at 50°C by adding the endonuclease (12.5 μl, diluted on ice to the requisite concentration in the dilution buffer from Wentzell *et al.*, 1995) to 5 nM ^{3}H-labelled DNA in buffer S (237.5 μl) already at 50°C. Buffer S is 10 mM Tris–HCl (pH 8.0), 5 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol. At timed intervals after the addition of *Sfi*I, aliquots (20 μl) were withdrawn from the reaction and mixed immediately with 10 μl of stop mix [0.1 M EDTA, 0.1 M Tris–HCl (pH 8.0), 40% (w/v) sucrose, 0.4 mg/ml bromophenol blue]. A zero time point aliquot was withdrawn before the addition of *Sfi*I. The DNA in each aliquot was analysed by agarose gel electrophoresis to separate the substrate(s) from the reaction product(s), and the concentration of each form of the DNA at each time point was measured by scintillation counting on slices excised from the gel (Halford *et al.*, 1994). Each data point in Figures 2 and 5B is the mean from three or four repeats of the same experiment.

Recombination reactions

Recombination by Tn*21* resolvase was measured in 250 μl reactions at 25°C containing Tn*21* resolvase (180 nM), on occasions *Sfi*I endonuclease (9 nM), and ^{3}H-labelled DNA (9 nM) in buffer T. The reactions were initiated by adding Tn*21* resolvase (25 μl, diluted on ice in buffer T supplemented with 500 μg/ml bovine serum albumin) to a solution containing the DNA (212.5 μl) that had been pre-mixed 10 min earlier with either *Sfi*I endonuclease or its dilution buffer (12.5 μl). (In some instances, *Sfi*I endonuclease was added to the reaction 5 s after the resolvase.) One aliquot (20 μl) was taken from the mixture before the addition of resolvase and further aliquots at timed intervals after the resolvase: each was mixed immediately with a solution (3 μl) containing 40 μg/ml ethidium bromide and 80 mM $MgCl_2$. [This amount of ethidium bromide stops resolvase (Castell *et al.*, 1986) while the $MgCl_2$ permits *Sfi*I activity. For reactions containing *Sfi*I dilution buffer, *Sfi*I endonuclease (1 μl) was added at this stage.] The aliquots were incubated for 2 h at 50°C and then analysed by electrophoresis through agarose, to separate the restriction fragments generated by *Sfi*I from either the plasmid substrates or the recombinant catenanes. (*Sfi*I cleaves pMDS1 to a linear DNA of 4265 bp and 1cat to a linear DNA of 3120 bp and a circular DNA of 1145 bp: pMDS2a yields two linear fragments of 3512 and 784 bp and 2acat linear fragments of 3120 and 1176 bp.) The amounts of the restriction fragments resulting from either substrate or catenane were measured by scintillation counting and recombination (%) evaluated as before (Castell *et al.*, 1986). Each data point in Figures 4 and 5A is the mean from three or four repeats of the same experiment.

Incubations of pMDS2a (9 nM) with various concentrations of *Sfi*I (0–120 nM) were carried out as above in 18 μl reactions in buffer T. Either 2 μl of Tn*21* resolvase (1.8 μM) was added to these followed, 2 min later, by 3 μl of 40 μg/ml ethidium bromide and 80 mM $MgCl_2$: recombination was then determined as above. Alternatively, 2 μl of $MgCl_2$ (100 mM) was added, followed 5 s later by 10 μl of the stop mix for the restriction reactions: DNA cleavage was then measured as in the restriction reactions.

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