

# Long-range Communications between DNA Sites by the Dimeric Restriction Endonuclease SgrAI

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The SgrAI endonuclease displays its maximal activity on DNA with two copies of its recognition sequence, cleaving both sites concertedly. While most restriction enzymes that act concurrently at two sites are tetramers, SgrAI is a dimer in solution. Its reaction at two cognate sites involves the association of two DNA-bound dimers. SgrAI can also bridge cognate and secondary sites, the latter being certain sequences that differ from the cognate by one base-pair. The mechanisms for cognate–cognate and cognate–secondary communications were examined for sites in the following topological relationships: *in cis*, on plasmids with two sites in a single DNA molecule; on catenanes containing two interlinked rings of DNA with one site in each ring; and *in trans*, on oligoduplexes carrying either a single site or the DNA termini generated by SgrAI. Both cognate–cognate and cognate–secondary interactions occur through 3-D space and not by 1-D tracking along the DNA. Both sorts of communication arise more readily when the sites are tethered to each other, either *in cis* on the same molecule of DNA or by the interlinking of catenane rings, than when released from the tether. However, the dimer bound to an oligoduplex carrying either a cognate or a secondary site could be activated to cleave that duplex by interacting with a second dimer bound to the recognition site, provided both duplexes are at least 30 base-pairs long; the second dimer could alternatively be bound to the two duplexes that correspond to the products of DNA cleavage by SgrAI.

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## Introduction

Type II restriction endonucleases recognise short DNA sequences, typically 4–8 bp long, and cut both strands at fixed positions within or adjacent to the sequence.<sup>1,2</sup> Most require only Mg<sup>2+</sup> as a cofactor. Many restriction enzymes have a unique recognition sequence with one particular base-pair at each position, but some recognise degenerate sequences where certain positions can be occupied

by an alternative base-pair.<sup>3</sup> Their target sequences are often symmetrical palindromes and the enzymes that act at palindromic sites are often dimers that interact symmetrically with the site: the active site in one subunit acts on one strand of the DNA, while the other active site cuts the complementary strand. These dimeric enzymes usually cut each site in an independent reaction, though they can function processively on DNA with two or more sites.<sup>4,5</sup> However, a large number of the Type II restriction enzymes interact with two copies of their target sites before cleaving DNA.<sup>6–9</sup> Some of these, the Type IIE enzymes such as EcoRII, NaeI, Sau3AI and FokI,<sup>10</sup> bind two sites but cleave only one; the second site functions as an allosteric activator.<sup>6,11–14</sup> Others, the Type IIF enzymes,<sup>10</sup> bind simultaneously to two target sites and then cut both in a concerted reaction; for example, SfiI, Cfr10I, NgoMIV, and BspMI.<sup>15–18</sup>

Like most proteins that bridge two DNA loci,<sup>7,19</sup> Type IIF restriction enzymes function best on sites

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Abbreviations used: SC, supercoiled; OC, open circle; FLL, full-length linear; L<sub>1</sub> and L<sub>2</sub>, linear DNA fragments from cutting a circular DNA at two sites; C<sub>L</sub> and C<sub>S</sub>, intact circular forms of the large and small rings, respectively, from a catenane with two rings of unequal size; OC<sub>L</sub>, open-circle form of the large ring; L<sub>L</sub> and L<sub>S</sub>, linearised forms of the large and small rings.

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*in cis*, in the same DNA molecule, though they can interact, albeit less readily, with sites *in trans*, in different molecules.<sup>15,20</sup> They usually cleave DNA with two sites more rapidly than DNA with one site, and convert the two-site DNA directly to the final product cut at both sites, cutting all four of the scissile phosphodiester bonds before dissociating from the DNA. On catenanes containing two interlinked rings of DNA, with one recognition site in each ring, the sites are tethered in physical proximity but without a direct path between them, yet Type IIF enzymes still act concertedly on such substrates.<sup>8,12,21</sup> They must therefore span the sites through 3-D space,<sup>22</sup> rather than following the 1-D path along the DNA.<sup>23</sup> The tetrameric Type IIF enzymes are organised as two “primary dimers” in back-to-back configuration, with identical DNA-binding sites facing in opposite directions.<sup>16,17,24,25</sup> They are fully active only when both clefts contain cognate DNA.<sup>26</sup>

The SgrAI endonuclease recognises an 8 bp degenerate sequence 5'-CR↓CCGGY-3' (where R indicates a purine, Y a pyrimidine and ↓ the point of cleavage).<sup>27</sup> It cleaves supercoiled plasmids with one copy of this sequence in the conventional manner of a dimeric restriction enzyme, albeit at an unusually low rate, but plasmids with two copies are cut at both sites in concert, like a tetrameric Type IIF enzyme.<sup>28</sup> However, unlike the other Type IIF enzymes, SgrAI is a dimer in solution.<sup>29</sup> Furthermore, the velocity of its steady-state reactions on a plasmid with one site increases linearly with enzyme concentration, but by proportionally more than the enzyme concentration on two-site plasmids.<sup>29</sup> At low concentrations, SgrAI cleaves one-site and two-site plasmids at equal rates, and shows the characteristic behaviour of Type IIF systems, faster cleavage of the two-site DNA, only at elevated concentrations. Hence, it seems that an SgrAI dimer bound to its recognition site cleaves that site at a slow rate but two dimers bound to separate sites in a two-site substrate associate with each other to form a tetramer with enhanced activity.<sup>29</sup> The termini from specific DNA cleavage by SgrAI can also activate the enzyme bound to its recognition site.<sup>30</sup> Though precluded by steric exclusion from *trans* interactions on supercoiled plasmids, as is often the case with DNA-looping proteins,<sup>7,15</sup> SgrAI can nevertheless mediate interactions *in trans* on 80 bp oligonucleotide substrates: these substrates give sigmoidal *v* versus [S] plots.<sup>31</sup>

Many restriction enzymes cleave their recognition sequence but also, at much lower rates, non-cognate sequences that differ from their cognate site by 1 bp, in a process called star activity.<sup>32,33</sup> With the archetypal Type II enzymes like EcoRV and EcoRI, the best non-cognate sites are cleaved a million times more slowly than the cognate site.<sup>34–36</sup> However, SgrAI cleaves certain non-cognate sequences relatively rapidly, at rates that are approximately 200-fold lower than the rate at the cognate site.<sup>30,31</sup> The sites that differ from the

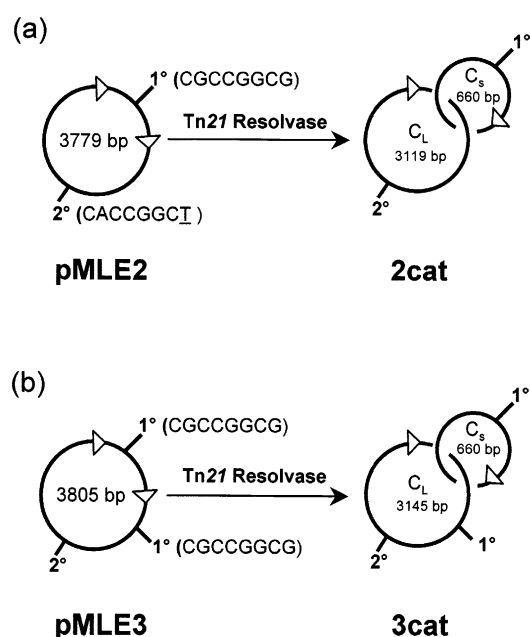
recognition site by 1 bp yet are still cleaved readily are CRCCGGY(A,T or C) and CRCCGGGG. These sites are called secondary sites, to distinguish them from other star sites.<sup>30</sup> As with the cognate sequence, 80 bp substrates with one of these secondary sites display sigmoidal *v* versus [S] plots.<sup>31</sup> Furthermore, the secondary sites are cleaved even more readily, at rates only tenfold lower than the canonical, if the DNA possesses *in cis* either the cognate sequence or the termini generated by SgrAI at a cognate site.<sup>30</sup> SgrAI thus appears to assemble into higher-order complexes during its reactions on all of the following combinations of sites: two cognate sites; two secondary sites; one cognate and one secondary site; and either a cognate or a secondary site with the termini from cleaving a cognate site.<sup>28–31</sup>

In this study, both cognate–cognate and cognate–secondary interactions were analysed by examining the reactions of SgrAI on a variety of DNA topologies: plasmids with two cognate sites, or one cognate and one secondary site, for interactions *in cis*; DNA catenanes comprised of two interlinked rings of DNA, with either a cognate site in each ring or a cognate site in one ring and a secondary site in the other; oligoduplexes containing either a cognate or a secondary site, or the termini corresponding to a cleaved cognate site, for interactions *in trans*. The activity of the enzyme on the catenane, relative to the substrates with, on one hand, sites *in cis* and, on the other, sites *in trans*, can reveal directly the mode of communication.<sup>22</sup> In a catenane, the mean distance between sites in each ring will be similar to that between sites in the equivalent molecule of supercoiled DNA.<sup>37</sup> Consequently, communications that rely on the random motion of the DNA chain to juxtapose the sites in 3-D space<sup>38</sup> ought to function almost as well on the catenane carrying one site in each ring as on the plasmid with two sites *in cis*, and both of these should be better than the reactions *in trans* across separate DNA molecules.<sup>12,21</sup> Conversely, processes that track along the 1-D contour of the DNA from one site to another are confined to a single ring in the catenane.<sup>39</sup>

## Results and Discussion

### Plasmids and catenanes

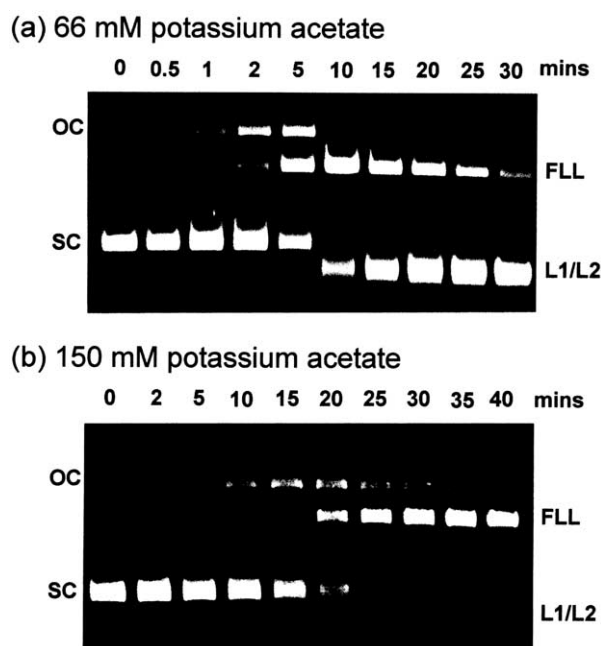
The plasmid pMLE2 (Figure 1) is a derivative of pUC19 that has one copy of a canonical sequence for SgrAI and several sites that differ from the canonical by 1 bp.<sup>12</sup> Only one of these, CACCGGCT, constitutes a secondary site for SgrAI (the underlined base marks the deviation).<sup>30</sup> It also has two *res* sites from the transposon Tn21 in directly repeated orientation. Tn21 resolvase can recombine the *res* sites *in vitro*<sup>40</sup> to convert the supercoiled form of this DNA into a catenane, named 2cat, that contains two interlinked rings of covalently closed DNA (Figure 1). The smaller of the two circles



**Figure 1.** Plasmids and catenanes. (a) Plasmid pMLE2 and (b) plasmid pMLE3 show, at the positions indicated: canonical recognition sites for SgrAI (marked 1°) with base sequence as shown; secondary site for SgrAI (marked 2°); *res* sites from the transposon Tn21 (arrowheads). The reaction of Tn21 resolvase converts these plasmids into catenanes, 2cat from pMLE2 and 3cat from pMLE3, that contain one large and one small circle of DNA ( $C_L$  and  $C_S$ , respectively). DNA sizes are noted in base-pairs.

incorporates the canonical site and the larger incorporates the secondary site. A further derivative, pMLE3, has the same cognate and secondary sites as pMLE2, and the same *res* sites, but also a further copy of the recognition site for SgrAI: the second copy is identical to that in pMLE2 with respect to both the degeneracies in the SgrAI site (both have the sequence CGCCGGCG) and the flanking sequences for 3 bp either side of the site. The catenane produced by the action of Tn21 resolvase on pMLE3, 3cat, has one cognate site in each ring (Figure 1).

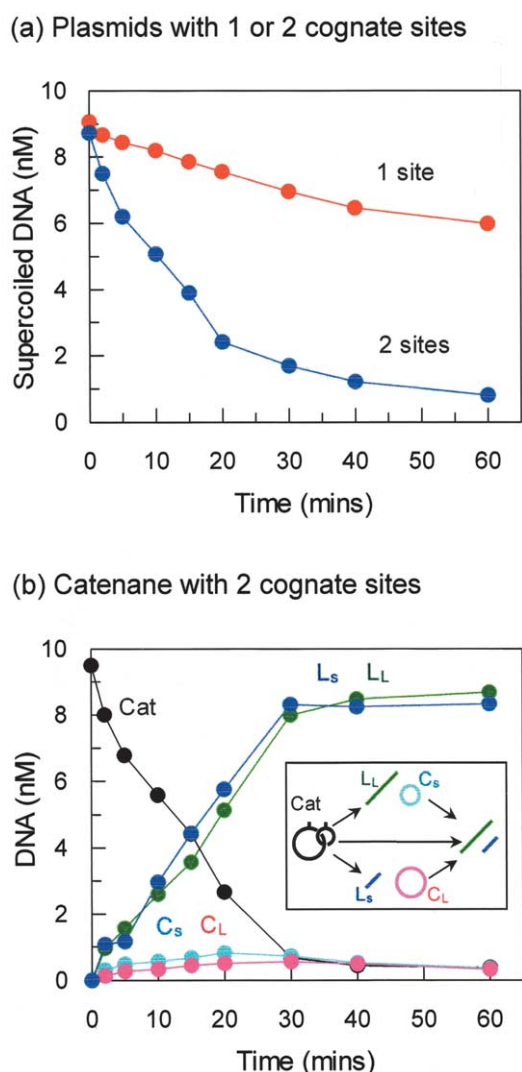
Due to the frequency of secondary sites for SgrAI in many plasmid vectors,<sup>30</sup> none of the currently available plasmids with Tn21 *res* sites had two cognate sites for SgrAI and no secondary sites. Consequently, before either pMLE3 or 3cat could be used to study cognate–cognate interactions, conditions had to be identified that minimised as far as possible SgrAI activity at secondary sites. Conversely, the interactions between cognate and secondary sites, on pMLE2 or 2cat, are best studied under conditions that give high activity at secondary sites. Star activity of restriction enzymes is generally optimal at low ionic strength and is detected most readily with elevated concentrations of enzyme.<sup>32,33</sup> SgrAI was therefore tested against pMLE2 at various ionic strengths in single-turnover reactions, with the enzyme in excess of the DNA



**Figure 2.** Control of secondary site activity. Reactions at 37 °C contained 80 nM SgrAI endonuclease and 10 nM supercoiled pMLE2 (one cognate and one secondary site) in reaction buffer supplemented with: for the reaction in (a), 66 mM potassium acetate; in (b), 150 mM potassium acetate. Aliquots were withdrawn at timed intervals, mixed immediately with EDTA stop-mix and analysed by electrophoresis through agarose. The agarose gels are shown, with the time for each withdrawal (in minutes) indicated above the lane. The electrophoretic mobilities of the open-circle (OC) and supercoiled (SC) forms of pMLE2 are shown on the left of the gels. On the right are marked the positions of: FLL, the full-length linear DNA of 3779 bp from cutting the cognate site; L<sub>1</sub>/L<sub>2</sub>, the two co-migrating fragments of 1932 and 1847 bp from cutting cognate and secondary sites.

(Figure 2). In the standard buffer for SgrAI, which contains 66 mM potassium acetate, the supercoiled (SC) form of pMLE2 was cleaved initially at one site to yield the full-length (3779 bp) linear form of the DNA (FLL) *via* a small amount of a nicked open-circle (OC) intermediate (Figure 2(a)). (The nomenclature for the various forms of the DNA analysed here is illustrated below (Figures 3 and 4)). Further studies (Figure 4(b)) revealed that this initial reaction occurs at the cognate site. The FLL DNA was then cleaved at an additional site to generate two linear fragments (marked L<sub>1</sub>/L<sub>2</sub>) of similar electrophoretic mobilities. The sizes of these products match those expected from cleavage at both the cognate and the secondary site, 1932 bp and 1847 bp.

With 150 mM potassium acetate in the reaction buffer (Figure 2(b)), SC pMLE2 was still cleaved to the FLL form, albeit at a slower rate and *via* more of the nicked OC form than had been the case at the lower ionic strength. At elevated ionic strength, the protein is more likely to dissociate from the DNA before cutting both strands.<sup>20,28</sup> However, in contrast to the reaction at low salt (Figure 2(a)),



**Figure 3.** Communications between cognate sites *in cis*. Reactions at 37 °C contained 5 nM SgrAI endonuclease and 10 nM [<sup>3</sup>H]DNA (~95% supercoiled) in reaction buffer with 150 mM potassium acetate. In (a), the DNA was either pMLE2 or pMLE3, with one or two SgrAI sites, respectively. In (b), the DNA was 3cat, a catenane with one SgrAI site in each ring. The inset in (b) shows the products from cutting this DNA: in the large ring, to give a large linear DNA (L<sub>L</sub>, in green) and a small circle (C<sub>S</sub>, in cyan); in the small ring, short linear DNA (L<sub>S</sub>, in blue) and large circle (C<sub>L</sub>, in mauve); in both rings, the two linear forms (L<sub>L</sub> and L<sub>S</sub>, as before). Samples were removed from the reactions at timed intervals and analysed as described in Materials and Methods to obtain concentrations of the following forms of DNA: (a) supercoiled pMLE2 in red and supercoiled pMLE3 in blue; (b) the 3cat substrate (marked Cat) in black; L<sub>L</sub> in green; C<sub>S</sub> in cyan, L<sub>S</sub> in blue and C<sub>L</sub> in mauve.

almost none of the FLL DNA was cleaved at the secondary site (Figure 2(b)). In high salt, SgrAI is impeded from cleaving secondary sites yet still cleaves cognate sites, so cognate–cognate interactions can be studied under these conditions without interference from cognate–secondary interactions.

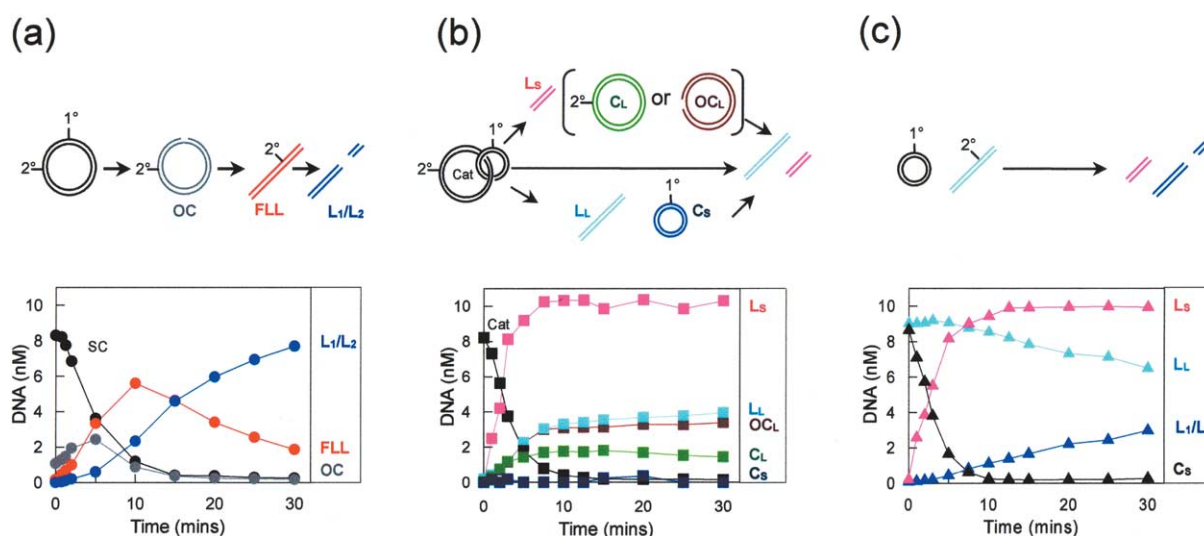
The reactions in Figure 2 also confirm the extraordinarily slow rate at which SgrAI cleaves plasmids with one copy of the recognition sequence. Under single-turnover conditions with enzyme in excess of the DNA, the conventional dimeric restriction enzymes such as EcoRV complete the cleavage of single-site plasmids in <10 s,<sup>41–44</sup> while SgrAI takes longer than ten minutes.

### Cognate–cognate communications *in cis*

To elucidate the mechanism of interactions between cognate sites, reactions were carried out in parallel on the plasmids pMLE2 and pMLE3, which contain one and two cognate sites for SgrAI, respectively, and on the catenane derived from pMLE3, 3cat, which has one canonical site in each ring (Figure 3). To ensure that the DNA cleavage events were due to cognate–cognate rather than cognate–secondary interactions, the reaction buffer contained 150 mM potassium acetate (Figure 2(b)). The enzyme was at a fourfold lower concentration than that of recognition sites on the DNA, which is sufficient to achieve steady-state conditions but which is still sufficiently high to result in enhanced activity on the two-site plasmid over the one-site plasmid, though not so high as to permit secondary-site reactions. Reaction velocities were measured from the initial linear decline in the concentration of substrate with time and normalised against the enzyme concentration to yield turnover numbers ( $k_{\text{cat}}$  values) in terms of mol DNA consumed per mol enzyme dimer per minute.

In its steady-state reactions on the plasmid with one cognate site, SgrAI first cleaved one strand of the DNA, to give the OC form, and then the second strand, to produce the FLL form (as in the single turnovers: Figure 2(b)). In contrast, SgrAI cleaved the two-site plasmid directly to the final products, the DNA cut in both strands at both sites: almost none of the OC form, cut in one strand at one or both sites, nor the full-length linear form cut in both strands at one site, was liberated during the reaction (data not shown).<sup>†</sup> Moreover, as in previous studies,<sup>28,29</sup> the  $k_{\text{cat}}$  of SgrAI on the two-site DNA,

<sup>†</sup> The highly concerted action of SgrAI on pMLE3 noted here differs from previous reports about its reactions on a different two-site plasmid.<sup>28</sup> At high ionic strength, as used here, that plasmid had been cleaved in a distributive manner, with the extensive liberation of intermediates cleaved at just one site during the course of the reaction, and it had been cleaved in a concerted manner only in reactions at low ionic strength. A possible reason for this discrepancy is that the sequences of the SgrAI recognition sites in the two plasmids differ with respect to their purine/pyrimidine degeneracies. Changes at the degenerate positions can alter markedly the kinetic parameters for DNA cleavage by SgrAI.<sup>31</sup> In addition, the two plasmids have different flanking sequences around their SgrAI sites, which can also affect restriction activity.<sup>46</sup>



**Figure 4.** Communications between cognate and secondary sites *in cis*. Reactions at 37 °C contained 80 nM SgrAI endonuclease and 10 nM [<sup>3</sup>H]DNA (~85% supercoiled) in reaction buffer with 66 mM potassium acetate. Samples were removed at timed intervals and analysed as described in Materials and Methods to obtain concentrations of the DNA forms illustrated in the reaction schemes above each panel: the forms are shown in the same colour as the data points for that DNA in the panel below. In (a), the DNA was pMLE2, with one cognate and one secondary site (marked 1° and 2°, respectively), and the following forms were measured: SC substrate (SC in black); DNA cut at the cognate site in one (OC in grey) or both strands (FLL in red); DNA cut at both cognate and secondary sites (L<sub>1</sub>/L<sub>2</sub> in blue). In (b), the DNA was 2cat, with a 1° site in the small ring (C<sub>S</sub>) and a 2° site in the large ring (C<sub>L</sub>), and the following were measured: SC substrate (Cat in black); the products from cutting the 1° site in the small ring, L<sub>S</sub> (in mauve), and either the intact (C<sub>L</sub> in green) or nicked (OC<sub>L</sub> in brown) forms of the large ring; the products from cutting the 2° site in the large ring, L<sub>L</sub> (in cyan), and the small ring (C<sub>S</sub> in blue); the products from cutting both sites, L<sub>S</sub> and L<sub>L</sub>. In (c), the DNA was 2cat that had been cut with SmaI in the large ring, and the following were measured: the intact small ring (C<sub>S</sub> in black) and the linear DNA from cutting the 1° site in this ring (L<sub>S</sub> in mauve); the linear DNA from the large ring (L<sub>L</sub> in cyan) and the products from cutting the 2° site in this DNA (L<sub>1</sub>/L<sub>2</sub> in blue).

0.06 mol/mol per minute, was larger than that on the one-site plasmid, 0.01 mol/mol per minute (Figure 3(a)).

The catenated substrate, 3cat, contains two cognate sites segregated onto its interlinked rings (Figure 1(b)). The initial rate for the utilisation of this substrate, 0.05 mol/mol per minute (Figure 3(b)), was similar to that on the plasmid with two cognate sites *in cis* and thus faster than that on the one-site plasmid. Moreover, if SgrAI were to act on the catenane like a conventional dimeric restriction enzyme, it will first cut a recognition site in one ring, though presumably without any major preference for any particular ring (the sites in each ring are identical), and then, in a separate reaction, the site in the other ring. Cutting the site in the large ring generates a large linear DNA and releases the small ring as a covalently closed circle, while cutting the site in the small ring gives the converse, a small linear DNA and a large circle (Figure 3(b), inset). However, SgrAI cleaved the catenane directly to the final product, the two linear species: it released none of the nicked forms cut in one strand in one or both rings, and almost none of the separate circles that would have arisen from a double-strand break in the other ring (Figure 3(b)).

Compared to its activity on a circular DNA with a single canonical site, SgrAI acts in a different

manner when that circle is tethered topologically to a separate circle that also carries an SgrAI site. The direct conversion of the two interlinked rings of the catenane to the two linear products means that the enzyme must act concurrently at both sites in the same molecule of catenane, presumably through dimers at the individual sites associating with each other through 3-D space. The protein complex spanning the two recognition sites must then cleave all four of the scissile phosphodiester bonds before it dissociates: otherwise, intermediates cleaved at one, two or three bonds would have been liberated. The behaviour of SgrAI on 3cat excludes the possibility that its cognate-cognate interactions occur by 1-D tracking along the DNA.

### Cognate–secondary communications *in cis*

For efficient cleavage of secondary sites, the SgrAI nuclease needs a cognate site *in cis*.<sup>30</sup> To examine the nature of the interactions between secondary and cognate sites, reactions were done on a plasmid with one canonical and one secondary site, pMLE2, and on a catenated form of this DNA with the cognate site on the smaller ring and the secondary on the larger, 2cat (Figure 4). For some experiments, the catenane was first cleaved with another restriction enzyme that cuts only one ring, so that

the secondary site was no longer linked to the cognate site. To boost SgrAI activity at the secondary site, the reaction buffer contained a low concentration of potassium acetate (Figure 2(a)). To facilitate further the detection of the products from cleaving the secondary site, single-turnover conditions were employed, with enzyme in excess of the DNA. Under these conditions, the concentration of each substrate followed an exponential decline during the reaction, and the rates cited here are the first-order constants from the decline.

The plasmid pMLE2 was cleaved initially at one site to yield FLL DNA *via* a nicked OC intermediate (Figure 4(a)). This initial reaction occurs exclusively at the cognate site (see below). After a lag phase, the FLL DNA was cleaved at an additional site, to give products consistent with cutting both the cognate and the secondary site (as in Figure 2(a)). Eventually (over a longer time base than that in Figure 4(a)), all of the FLL DNA was cleaved at the secondary site. On fitting these data to a consecutive reaction scheme,<sup>45</sup> the best fit was obtained with a 2.5-fold faster rate constant for cutting the cognate site over the secondary site, 0.20 and 0.08 min<sup>-1</sup>, respectively.

With the catenated form of this DNA, 2cat (Figure 4(b)), the rate of substrate utilisation, 0.27 min<sup>-1</sup>, was similar to that on pMLE2 (Figure 4(a)). The first reaction of SgrAI on this substrate could be at the cognate site in the small ring, or it could be at the secondary site in the large ring. But virtually none of the intact small circle was released ( $C_S$ , in Figure 4(b)). This shows that essentially all of the DNA is cleaved first at the cognate site to yield the linear DNA from this ring ( $L_S$ ): any reaction on the catenane that first cleaves the secondary site in the large ring must liberate a corresponding amount of the intact small circle. Yet in the first ten minutes of the reaction, during which >95% of DNA was cut in the small ring, ~80% of the DNA was also cleaved at the secondary site in the large ring. Over this length of time, the large ring was partitioned into three species (Figure 4(b)): ~20% remained as an intact covalently closed circle of DNA ( $C_L$ ); ~40% was cut in one strand to give its nicked open-circle form ( $OC_L$ ); and ~40% cut in both strands to give its linear form ( $L_L$ ). However, after this period of ten minutes, no further cleavage of the large ring occurred: neither the intact nor the open-circle forms of the large circle progressed through to the linear form (Figure 4(b)).

The catenane, with cognate and secondary sites on separate rings, thus differs from the plasmid that has cognate and secondary sites in the same ring: all of the plasmid was eventually cleaved by SgrAI at both cognate and secondary sites. One explanation for this difference is that, after cutting the cognate site on the plasmid, the termini generated by SgrAI remain connected to the secondary site by the linear structure of the DNA. By staying in close physical proximity to the secondary site, the termini *in cis* may continue to activate the enzyme at the secondary site. In contrast, after the enzyme has

cut the cognate site in one ring of the catenane and has dissociated from at least one terminus, the ring carrying the secondary site is free to diffuse away from the other DNA: the secondary site on one DNA is then no longer held near the termini on the other DNA. To test this hypothesis, the 2cat DNA was first cleaved with SmaI, which cuts only the large ring of this catenane, the ring with the secondary site. The SmaI-cleaved DNA was then used as a substrate for SgrAI under the same conditions as above (Figure 4(c)). On adding SgrAI to this equimolar mixture of two DNA molecules, the small ring with the cognate site was cleaved rapidly to its linear form, at a rate similar (0.29 min<sup>-1</sup>) to those on pMLE2 and on the intact 2cat (Figure 4). However, the linear DNA in this mixture, which has the secondary site, was cleaved at a much slower rate. On the plasmid, the secondary site was cleaved 2.5 times slower than the cognate site, but with the separate DNA molecules, this difference increased to 15-fold.

Similar experiments were performed after first using PstI to cut 2cat in the small ring, to leave the cognate site on a linear fragment and the secondary site on a circular DNA. As in Figure 4(c), the cognate site was cleaved rapidly but the secondary site was still cleaved slowly (data not shown). The different rates at cognate and secondary sites on separate DNA molecules do not depend on which site is on a circular (supercoiled) molecule and which is on a relaxed linear molecule.

The data in Figure 4 show that a DNA with one cognate and one secondary site is cleaved by SgrAI in a fixed sequential order: first the cognate site and then the secondary site. If any of the catenated DNA had been cleaved at the secondary site before the cognate site, the DNA carrying the cognate site ( $C_S$ ) would have been released as an intact circle, but none of this form was liberated (Figure 4(b)). Yet the secondary site is still cleaved more rapidly when tethered to the cognate sequence (Figure 4(a) and (b)) than when released from the tether (Figure 4(c)). Even so, the activation of cleavage of the secondary site cannot be due to an intact copy of the cognate site, since that site has already been cleaved. Instead, it must be due to the termini left after cutting the cognate site.

On the plasmid pMLE2, the product from cutting the cognate site is a linear DNA with SgrAI-specific termini at both ends. The maximal separation between the secondary site and termini is limited by the contour length of the intervening DNA and, in a worm-like coil, the mean distance in 3-D space will be considerably shorter than the contour length.<sup>5</sup> Therefore in 3-D space, the termini stay close to the secondary site. In contrast, on the catenane, the termini left after cutting the cognate site in one ring are held close to the secondary site in the other ring only whilst the enzyme remains bound to both termini. While bridging both termini, the enzyme maintains the topology of the catenane but as soon as it dissociates from either terminus, the two DNA chains can diffuse away from each

other and so will not remain in close proximity. Even so, the enhanced cleavage of the secondary site on one ring of the catenane, by the cleaved cognate site in the other DNA, which was observed prior to the dissociation of the enzyme, shows that this interaction must occur through 3-D space rather than along the 1-D contour.

### Oligonucleotide substrates

Any interaction that bridges two DNA sites *in cis* through 3-D space ought also to function *in trans*, by bridging two separate DNA molecules that each carry one site. However, the effective concentration of one DNA site in the vicinity of another is generally higher for sites *in cis* than *in trans*,<sup>19,22</sup> so interactions *in trans* are likely to require higher concentrations of DNA than those *in cis*. In addition, bridging interactions spanning two DNA molecules are impeded severely if both are supercoiled, and occur more readily when at least one of the DNA molecules is linear.<sup>7,15</sup> Consequently, our tests to examine cognate–cognate and cognate–secondary interactions *in trans* employed short duplexes of 20–40 bp. The duplexes were made from synthetic oligodeoxyribonucleotides with complementary sequences. They contained single copies of the requisite sequence: a cognate recognition site for SgrAI; a secondary site; a different non-cognate site with a 1 bp change from the canonical; and a non-specific sequence in which all 8 bp were reversed from the canonical (Table 1). Type II restriction enzymes typically contact 3–4 bp either side of their recognition site<sup>2,46</sup> so, to curtail effects from flanking sequences, all had the same sequences for 6 bp either side of the 8 bp site. The 20 bp, 30 bp and 40 bp duplexes with a given target sequence thus all had the same sequence over the innermost 20 bp and differed from each other only in the additional nucleotides needed to increase duplex length.

Previous studies had shown that SgrAI cleaves 80 bp duplexes with cognate or secondary sites by means of *trans* interactions with two molecules of the duplex.<sup>31</sup> But DNA just 20 bp long allows for the binding of SgrAI in the presence of Ca<sup>2+</sup> (in place of the catalytic cofactor Mg<sup>2+</sup>). The binding of SgrAI to 20 bp duplexes with the recognition sequence, such as COG<sub>20</sub> (Table 1), yields initially a complex with one dimer per duplex, though further increases in the protein concentration lead to high molecular mass aggregates with multiple dimers per duplex.<sup>29</sup> Cognate duplexes normally activate Type IIF enzymes to cleave plasmids with a single recognition site,<sup>15–18</sup> due to the enzyme mediating a *trans* interaction between its sites on the plasmid and on the duplex but, when added to a plasmid with one SgrAI site, COG<sub>20</sub> inhibited the cleavage of the plasmid.<sup>29</sup> Whether the duplex was itself cleaved was not determined. SgrAI also binds, with a ~25-fold lower affinity, to a 20 bp duplex with the secondary sequence, SEC<sub>20</sub> (Table 1), giving rise solely to the 1:1 complex, to the exclusion of multimeric complexes.<sup>47</sup>

**Table 1.** Oligoduplexes

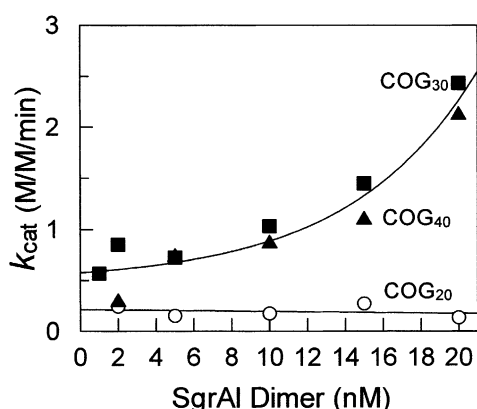
Name	Sequence
COG <sub>20</sub>	5'-GATCAT <u>CACCGCGC</u> ACCAG-3' 3'-CTCGTAGTGGCCGCGTGGTC-5'
SEC <sub>20</sub>	5'-GATCATCACCGGCTCACCAG-3' 3'-CTAGTAGTGGCCGAGTGGTC-5'
COG <sub>30</sub>	5'-GACAAGATCATCACCGCGCACCAGCACAC-3' 3'-CTGTTCTAGTAGTGGCCGCGTGGTCGAGAG-5'
SEC <sub>30</sub>	5'-GACAAGATCATCACCGGCTCACCAGCACAC-3' 3'-CTGTTCTAGTAGTGGCCGAGTGGTCGTTG-5'
NC <sub>30</sub>	5'-GACAAGATCATCACCGGTGCACCAGCACAC-3' 3'-CTGTTCTAGTAGTGGCCACGTTGGTCGAGAG-5'
NS <sub>30</sub>	5'-GACAAGATCATGTGGCCGCCACCAGCACAC-3' 3'-CTGTTCTAGTACACCGCGGTGGTCGAGAG-5'
COG <sub>30</sub> E	5'-GACAAGATCATCA-3' 3'-CTGTTCTAGTAGTGGCC-5'

Pairs of synthetic oligodeoxyribonucleotides with complementary sequences were annealed to give the duplexes shown. The duplexes are named as follows, with a suffix to denote its length in base-pairs: COG, duplexes with a cognate recognition sequence for SgrAI; SEC, duplexes with a 1 bp change from the recognition sequence that yields a secondary site;<sup>30</sup> NC, non-cognate sequences 1 bp different from the recognition sequence but at a position that does not create a secondary site; NS, non-specific duplexes with an inverted SgrAI site; COG<sub>30</sub>E, a duplex with the same terminal sequence as that from SgrAI cleavage of COG<sub>30</sub>, but lacking the 5'-phosphate group in the bottom strand. The recognition sequence is underlined and deviations from this are marked in bold. Unless stated otherwise, the top strand was <sup>32</sup>P-radiolabelled.

### Cognate–cognate communications *in trans*

To see if the cleavage of oligoduplex substrates for SgrAI requires the enzyme to interact simultaneously with two molecules of the duplex, the reaction rates of SgrAI on the cognate duplexes, COG<sub>20</sub>, COG<sub>30</sub> and COG<sub>40</sub>, were measured at various concentrations of enzyme (Figure 5). The reactions were carried out under steady-state conditions, with the DNA at a higher concentration than even the highest concentration of enzyme tested. The tests for cognate–cognate interactions *in trans* were carried out in a buffer containing a lower concentration of potassium acetate (cf. Figure 1(a)) than that used for the cognate–cognate interactions *in cis* (Figure 3), as the concurrent binding of a protein to two separate DNA molecules is in most cases inhibited strongly by increased salt.<sup>18,20</sup>

On the 20 bp duplex with the recognition sequence, COG<sub>20</sub>, the velocity of the DNA cleavage reaction, in terms of mol DNA cleaved per minute, increased linearly with the enzyme concentration, in the manner expected<sup>45</sup> for a steady-state reaction. The turnover number ( $k_{cat}$ , mol DNA/mol enzyme per minute) of SgrAI on the 20 bp substrate is thus independent of the concentration of enzyme (Figure 5). The reaction velocities on the 30 bp substrate were similar to those with the 20 bp DNA at the lowest concentrations of enzyme tested. But as the concentration of enzyme was raised, the velocities then increased by proportionally more than the rise in concentration. Consequently, instead of showing the normal behaviour of an



**Figure 5.** Communications between cognate sites *in trans*. The reactions at 37 °C, in reaction buffer with 66 mM potassium acetate, contained 200 nM cognate [<sup>32</sup>P]DNA duplex and the concentration of SgrAI endonuclease indicated on the *x*-axis. The DNA was: COG<sub>20</sub>, open circles; COG<sub>30</sub>, filled squares; COG<sub>40</sub>, filled triangles. Samples were removed from the reactions at timed intervals and analysed as described in Materials and Methods to determine the concentration of cleaved DNA product at each time-point sampled. Reaction velocities were evaluated from the initial linear increase in product concentration with time and were normalised against the enzyme concentration to obtain the turnover rate ( $k_{cat}$ ) for each reaction in terms of mol DNA cleaved per mol enzyme per minute.

enzyme under steady-state conditions, i.e. a  $k_{cat}$  that is independent of the enzyme concentration, the turnover number on COG<sub>30</sub> increased with the concentration of SgrAI (Figure 5). The 40 bp substrate, COG<sub>40</sub>, was cleaved in the same way, and at similar rates, to the 30 bp DNA, and thus again differed from the 20 bp DNA (Figure 5).

On the 20 bp substrate, the lack of variation in  $k_{cat}$  with enzyme concentration suggests that SgrAI cleaves this DNA as the 1:1 dimer:duplex complex, without associating to form a larger assembly. Hence, it seems unlikely that the enzyme interacts *in trans* with two molecules of this substrate. The SgrAI enzyme thus acts at its recognition site in the COG<sub>20</sub> oligoduplex in the same way as on a supercoiled plasmid with one site.<sup>29</sup> Moreover, the inability of the 20 bp DNA to support cognate–cognate interactions *in trans* accounts for why it failed to stimulate SgrAI activity against the one-site plasmid.<sup>29</sup>

On the 30 bp and 40 bp substrates, the increasing  $k_{cat}$  values with increasing concentrations of SgrAI show that these reactions must include a protein association step. Other restriction enzymes, FokI and Sau3AI, show increases in  $k_{cat}$  with enzyme concentration, due to their need to associate from monomer to dimer to cut DNA.<sup>13,14,48</sup> In the case of SgrAI, the association is most probably from dimer to tetramer, with the dimer bound to one duplex having low activity and the tetramer bound to two duplexes having a higher activity. The SgrAI

endonuclease thus seems to act on the COG<sub>30</sub> substrate by bridging two copies of its cognate sequence *in trans*, in a manner similar to that seen before on plasmids with two recognition sites, where it bridges sites *in cis*.<sup>28,29</sup> In addition, when added to SgrAI reactions on a plasmid with one canonical site, the COG<sub>30</sub> substrate stimulated the cleavage of the plasmid,<sup>47</sup> presumably as a result of its ability to support cognate–cognate interactions *in trans*.

### Cognate–secondary communications *in trans*

Secondary sites for SgrAI are cleaved more efficiently on DNA molecules that carry the recognition sequence for SgrAI than on DNA that lacks the canonical sequence,<sup>30</sup> though the stimulation of secondary-site cleavage is most likely due to the termini left *in cis* after cleaving the cognate site, rather than the intact site (Figure 4(b)). Experiments were carried out with oligoduplexes that have a secondary sequence for SgrAI, either in the presence or in the absence of cognate duplexes (Table 1), to see if cognate and secondary sites could also interact *in trans*. The secondary duplex was <sup>32</sup>P-labelled and the cognate duplex unlabelled, so that the cleavage of the former could still be measured in the presence of a large excess of the latter. On account of the slow rate of cleavage of secondary sites,<sup>31</sup> the reactions were carried out under single-turnover conditions with the enzyme in excess of the secondary site.

The addition of SgrAI to a 20 bp substrate containing a secondary site (SEC<sub>20</sub>), in the absence of any other DNA, led to very little cleavage of the secondary duplex; <6% in one hour, a level that is virtually indistinguishable from background (Table 2; Figure 6). The same minuscule levels of cleavage of SEC<sub>20</sub> were observed when these reactions were repeated in the presence of either 20 bp or 30 bp duplexes carrying the cognate site, COG<sub>20</sub> and COG<sub>30</sub>, respectively (Table 2). The rate of cleavage of the 30 bp secondary duplex, SEC<sub>30</sub>, was also very low in the absence of any other DNA and it remained very low on adding the 20 bp cognate duplex, COG<sub>20</sub> (Table 2). But in marked contrast, the rate on SEC<sub>30</sub> was enhanced massively by adding the 30 bp cognate duplex, COG<sub>30</sub> (Figure 6, Table 2). COG<sub>30</sub> failed, however, to stimulate the cleavage of 30 bp duplexes that had either a non-cognate sequence, with a 1 bp change from the canonical but at a position that differs from any secondary site, or a non-specific sequence that differs at all eight positions, NC<sub>30</sub> and NS<sub>30</sub>, respectively (Table 2). The activation thus seems to be specific for the secondary sequence, and it requires both the secondary substrate and the cognate activator to be embedded in 30 bp duplexes. Moreover, the cognate–cognate activation also required two 30 bp duplexes: COG<sub>30</sub> failed to stimulate the cleavage of COG<sub>20</sub> (data not shown).

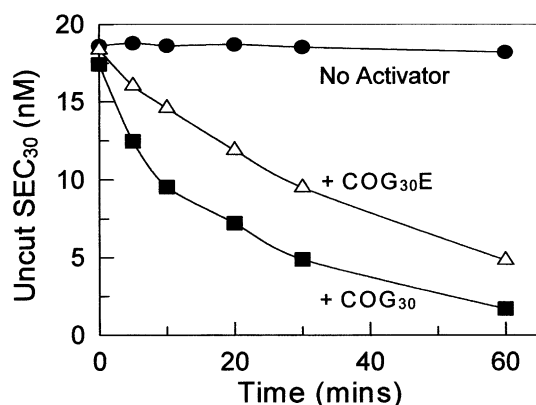
These experiments show that the SgrAI endonuclease can interact *in trans* with one duplex

**Table 2.** *Trans* activation of oligoduplex cleavage

Substrate	% Substrate cleaved, no activator	% Substrate cleaved with activator as below	
		COG <sub>20</sub>	COG <sub>30</sub>
SEC <sub>20</sub>	3	6	4
SEC <sub>30</sub>	6	3	91
NC <sub>30</sub>	3	nd	2
NS <sub>30</sub>	4	nd	5

Reactions at 37 °C, in reaction buffer with 66 mM potassium acetate, contained 0.2 μM SgrAI endonuclease, 20 nM <sup>32</sup>P-labelled substrate DNA and, in some cases, 0.2 μM activator DNA. The substrates were SEC<sub>20</sub>, SEC<sub>30</sub>, NC<sub>30</sub> or NS<sub>30</sub>. The activators were COG<sub>20</sub> or COG<sub>30</sub>. The combinations of substrate and activator not tested are indicated as nd. One hour after the addition of the enzyme, the samples were mixed with formamide stop-mix and subjected to electrophoresis through polyacrylamide as described in Materials and Methods. The gels were analysed by Phosphorimager to determine the fraction of the radiolabelled substrate that had been cleaved, as a percentage of the total for that substrate. Each datum point is the mean of three independent reactions.

carrying a secondary site and another with a cognate site, when both duplexes are 30 bp long, as it can also with 80 bp substrates.<sup>31</sup> However, if SgrAI is activated to cleave the secondary site by interacting with the termini from the cognate site, rather than the intact site, then its reaction on SEC<sub>30</sub> ought to be stimulated by adding duplexes that carry the terminal sequence. The duplex COG<sub>30</sub>E (Table 1) was therefore synthesised: it corresponds to one of the two products from SgrAI-cleavage of COG<sub>30</sub>, the “left-hand” end, except that it lacks the 5′-phosphate group that SgrAI would have left at



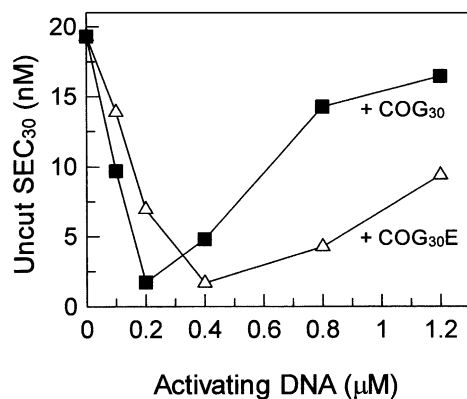
**Figure 6.** Communications between cognate and secondary sites *in trans*. The reactions at 37 °C, in reaction buffer with 66 mM potassium acetate, contained 0.2 μM SgrAI endonuclease and 20 nM [<sup>32</sup>P]SEC<sub>30</sub>, with either no other DNA present (filled circles) or one of the following unlabelled duplexes present at 0.2 μM: COG<sub>30</sub>, filled squares; COG<sub>30</sub>E, open triangles. Samples were removed from the reactions at timed intervals, quenched and subjected to electrophoresis through polyacrylamide as described in Materials and Methods. The gels were analysed by Phosphorimager to determine the amount of the intact [<sup>32</sup>P]SEC<sub>30</sub> left at each time-point.

the cut site†. When COG<sub>30</sub>E was added to SgrAI reactions on SEC<sub>30</sub>, at the concentration that had been used with the intact COG<sub>30</sub> duplex, it stimulated the rate of cleavage of the secondary duplex, though not to quite as large an extent as the intact DNA: by about tenfold rather than ~15-fold (Figure 6). This difference may, however, simply be a reflection of the fact that COG<sub>30</sub>E possesses only half of the recognition sequence for SgrAI, so it may need to be present at double the molarity of COG<sub>30</sub> in order to give an equivalent effect.

The abilities of COG<sub>30</sub> and COG<sub>30</sub>E to stimulate cleavage of SEC<sub>30</sub> were assessed by adding varied concentrations of these duplexes to reactions containing fixed concentrations of <sup>32</sup>P-labelled secondary DNA and SgrAI enzyme (Figure 7). The enzyme was in large excess of the secondary DNA, so that in the absence of any other DNA essentially all of the latter would be enzyme-bound yet this would still occupy only a small fraction of the DNA-binding sites in the enzyme. On adding progressively increasing amounts of the intact cognate duplex, COG<sub>30</sub>, the extent of cleavage of SEC<sub>30</sub> increased up to a maximum when the concentration of COG<sub>30</sub> equalled that of the enzyme: further increases then inhibited the cleavage of the secondary site (Figure 7). The complex of SgrAI protein bound to a cognate site thus seems to stimulate the catalytic activity of its complex with a secondary site in a 1:1 stoichiometry. The inhibition of SEC<sub>30</sub> cleavage by COG<sub>30</sub> concentrations in excess of the enzyme is then presumably due to direct competition between cognate and secondary duplexes for binding sites in the enzyme.

The duplex corresponding to a product from SgrAI-cleavage of COG<sub>30</sub>, COG<sub>30</sub>E, also gave progressively more cleavage of SEC<sub>30</sub> as its concentration was increased, up to a maximal value, prior to inhibiting the reaction as its concentration was increased further (Figure 7). However, the concentrations of termini needed to achieve any given level of activation were higher than those with the intact duplex. Hence, at low concentrations of the duplexes (as in Figure 6), the degree of activation with COG<sub>30</sub> was greater than that with COG<sub>30</sub>E. Moreover, the maximal activation was achieved with termini at double the concentration of the enzyme, instead of the equal concentration seen

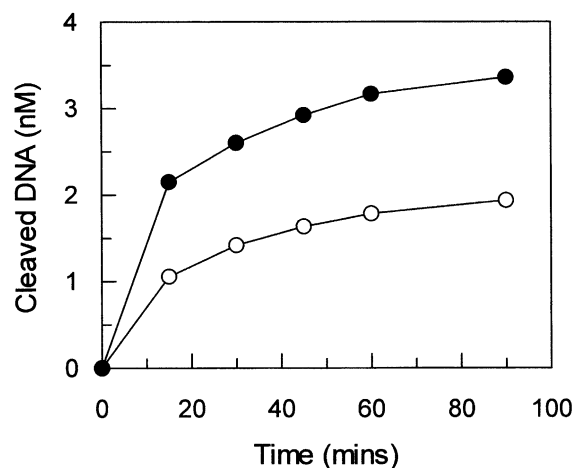
† The COG<sub>30</sub>E duplex was also constructed with an oligonucleotide for the “bottom” strand (Table 1) carrying a 5′-phosphate group, so as to give an exact replica of a terminus generated by SgrAI at its cognate site. Experiments with this duplex yielded results that were similar to those described here with the duplex lacking the 5′-phosphate group (data not shown), though it gave a lower degree of activation than the DNA without the phosphate group. Hence, SgrAI can interact with both phosphorylated and unphosphorylated termini, though perhaps more readily with the unphosphorylated DNA. This is similar to the Bse643I endonuclease, which also interacts better with termini lacking the 5′-phosphate group.<sup>25</sup>



**Figure 7.** Stoichiometry of cognate–secondary interactions. The reactions at 37 °C, in reaction buffer supplemented with 66 mM potassium acetate, contained 0.2 µM SgrAI endonuclease, 20 nM [<sup>32</sup>P]SEC<sub>30</sub> and one of the following activating DNAs at the concentration noted on the x-axis: COG<sub>30</sub>, filled squares; COG<sub>30</sub>E, open triangles. One hour after the addition of the enzyme, the reactions were stopped and the samples subjected to electrophoresis. The gels were analysed by Phosphor-imager to determine the amount of the intact [<sup>32</sup>P]SEC<sub>30</sub> left after the one hour reactions.

with the intact duplex (Figure 7). The simplest explanation of this observation is that the dimeric SgrAI enzyme bound to a secondary site can be activated to cleave that site by interacting with a second dimer that is bound to two SgrAI-specific termini from its cognate site. The dimer bound to one terminus would appear to be unable to activate the enzyme at the secondary site. Alternatively, SgrAI may have a lower affinity for the terminal duplex relative to the intact duplex, so that even the binding of a single terminus to the SgrAI dimer requires a relatively high concentration of the COG<sub>30</sub>E duplex.

To determine whether SgrAI needs to interact with one or with two cognate termini in order to cleave its secondary site, the activity of SgrAI at the secondary site on pMLE2 was measured on two linear forms of this DNA (Figure 8). One form was generated by using SgrAI to cleave the plasmid under conditions where it cleaves only the cognate site (Figure 2(b)), to leave a 3779 bp linear DNA carrying the secondary site at a central location, and with cognate termini at both ends. The other was generated by cleaving pMLE2 at both the cognate site for SgrAI (as above) and at its XhoI site, to leave one fragment of 3374 bp, with the secondary site and a cognate SgrAI terminus at one end, and a separate fragment of 405 bp carrying the other SgrAI terminus. Hence, if two termini are needed, SgrAI should cleave the secondary site more rapidly on the DNA with cognate termini at both ends than on the DNA with a cognate terminus at one end only. This is because the DNA with cognate termini at both ends allows for two termini to be recruited *in cis*, while the binding of two termini on



**Figure 8.** DNA with one or two SgrAI termini. The reactions at 37 °C, in reaction buffer supplemented with 66 mM potassium acetate, contained 80 µM SgrAI endonuclease and one of the following DNA at 5 nM: a 3779 bp linear DNA carrying a secondary site for SgrAI at a central location and cognate SgrAI termini at both ends, generated by using SgrAI to cleave pMLE2 at its canonical site; a 3374 bp DNA with the secondary site and a cognate SgrAI terminus at one end, and 405 bp DNA carrying the other SgrAI terminus, generated by using SgrAI and XhoI to cleave pMLE2 at their respective canonical sites. Samples were removed from the reactions at timed intervals and analysed as described in Materials and Methods to determine the extent of cleavage of the secondary site on: the 3779 bp DNA with cognate termini at both ends, filled circles; the 3374 bp DNA with a cognate terminus at one end, open circles. Each point represents the mean of two repeats.

the DNA with an SgrAI terminus at just one end requires an interaction *in trans*. The latter is inefficient relative to that *in cis* (Figure 4). But if only one end is needed, the two substrates should be cleaved at equal rates. The DNA with two cognate termini was cleaved to a greater extent than the DNA with a single cognate terminus (Figure 8).

## Conclusions

The Type II restriction endonucleases all cleave DNA at fixed positions in or adjacent to a specified DNA sequence,<sup>3,10</sup> but it is becoming increasingly apparent that they carry out this common reaction by a broad range of distinct mechanisms.<sup>1,6–9</sup> Even the use of Mg<sup>2+</sup> as a cofactor for phosphodiester hydrolysis is not unanimous amongst these enzymes.<sup>49</sup> The SgrAI endonuclease operates differently from most other restriction enzymes studied to date, so it serves to illustrate the variety of mechanisms used by these enzymes to cleave DNA.

SgrAI exists in free solution as a dimer of identical subunits<sup>29</sup> but it differs from the conventional dimeric restriction enzymes, such as EcoRV,

that act at individual sites. Instead, it can act concurrently at two copies of its cognate recognition site, to convert a DNA with two such sites directly to the products cut at both sites, without liberating intermediates cut at just one site (Figure 3(b)).<sup>28</sup> Yet SgrAI also differs from other restriction enzymes that act concurrently at two recognition sites, the Type IIF nucleases,<sup>10</sup> which are generally tetramers.<sup>15–18</sup> The Type IIF enzymes are usually composed of two “primary dimers”, each of which binds one copy of a symmetrical recognition site.<sup>17,25</sup> Another distinctive feature of SgrAI is that it can cleave certain sequences that differ from its canonical recognition sequence by 1 bp, its secondary sites, much more readily than is usual for a restriction enzyme, especially when the DNA also contains a copy of the cognate sequence.<sup>30,31</sup>

Though the Type II enzymes that act at individual sites, such as EcoRI and BamHI, are often considered as archetypal, it now seems that such enzymes are exceptions to the general behaviour of restriction nucleases, which usually need to interact with two sites before cutting DNA.<sup>7</sup> Two basic mechanisms exist for long-range interactions on DNA; either 1-D processes, diffusional “sliding” or NTP-dependent “tracking” along the DNA from one site to the other;<sup>23</sup> or 3-D processes, that rely on thermal fluctuations of the DNA to juxtapose distant sites.<sup>38</sup> The Type I and the Type III enzymes follow 1-D routes<sup>39</sup> while, among the Type II enzymes that need two sites, those that have been examined to date rely on 3-D juxtapositions.<sup>7</sup> In this study, the mechanisms for long-range communications by SgrAI were analysed for both cognate and secondary sites in a variety of spatial relationships: with sites *in cis*, on the same molecule of DNA; with sites *in trans* on separate oligoduplexes; and on catenanes in which the sites on the separate rings are connected topologically but not covalently. These experiments showed that SgrAI interacts with two cognate sites, and with cognate and secondary sites, through 3-D space and not by the 1-D route along the DNA. Furthermore, as the relative positioning of the two target sites in 3-D space differed amongst these substrates, the study yielded further insights into the mechanisms of long-range communications by SgrAI, in both cognate–cognate and cognate–secondary interactions.

### Cognate–cognate interactions

The SgrAI endonuclease was previously proposed to cleave a supercoiled plasmid with one copy of its cognate recognition sequence like a conventional restriction enzyme, binding to the site as a dimer and cleaving that solitary site very slowly, without interacting with another site *in trans*.<sup>29</sup> On the other hand, it was proposed to cleave a supercoiled plasmid with two cognate sites by means of dimers bound to each site interacting with each other *in cis*, to yield a tetramer spanning

the two sites: the tetramer then rapidly cleaves both sites before dissociating. The different kinetics of its reactions on the one-site and the two-site plasmids were thus taken to indicate that SgrAI can use two different mechanisms to cleave DNA. The studies reported here support this conclusion in several ways.

Firstly, SgrAI cleaved a catenane with two cognate sites, one in each ring, directly to the two linear products, whilst liberating virtually none of the individual circles that would have arisen from cutting just one ring (Figure 3(b)). This shows that SgrAI is indeed capable of concerted action at two spatially separate sites in DNA.

Secondly, the kinetic behaviour of SgrAI on a 20 bp duplex with the recognition site, COG<sub>20</sub> (Figure 5), matches that on a supercoiled plasmid with one cognate site:<sup>29</sup> both have  $k_{\text{cat}}$  values that do not vary with the enzyme concentration. Conversely, its behaviour on a 30 bp cognate duplex, COG<sub>30</sub> (Figure 5), is like that on a supercoiled plasmid with two recognition sites:<sup>29</sup> both yield  $k_{\text{cat}}$  values that increase with the enzyme concentration. This supports the view that SgrAI can indeed cleave DNA by two different mechanisms: either as a dimer at an individual site, without interacting with a second site; or, at a higher rate, as a tetramer after the association of two dimers bound to separate sites, either *in cis* or *in trans*. Though SgrAI can mediate *trans* interactions with cognate oligoduplexes of either 30 bp (Figure 5) or 80 bp,<sup>31</sup> it appears unable to mediate *trans* interactions to bridge two molecules of a ~4000 bp plasmid with one recognition site. This is most likely due to *trans* interactions being attenuated severely when both molecules are supercoiled: they occur more readily when at least one of the two DNA molecules is linear.<sup>15</sup> But why a 20 bp duplex with a recognition sequence for SgrAI fails to support *trans* interactions, while the same sequence embedded within a 30 bp duplex permits *trans* interactions, has yet to be determined. However, preliminary footprinting studies indicate that the binding of SgrAI to its recognition sequence protects a region of about 32 bp from DNaseI digestion, not only the 8 bp recognition site but also ~12 bp either side of the site (data not shown).<sup>47</sup> Thus, even though DNaseI footprinting generally overestimates the length of DNA in contact with the protein, the 20 bp substrate for SgrAI may be too short for all of the interactions with the protein.<sup>50</sup>

### Cognate–secondary interactions

SgrAI is capable of cleaving its canonical recognition site and its secondary sites, particularly if the secondary site is located on a DNA that has either a canonical site or the termini derived from cleaving a cognate site.<sup>30</sup> SgrAI cleaved a catenane with a cognate site in one ring and a secondary site in the other ring in a fixed order, first the cognate site and only then the secondary site: none of this DNA was cleaved at the secondary site before the cognate site

(Figure 4(b)). Consequently, on a DNA that has a cognate site and a secondary site *in cis*, the species that activates cleavage of the secondary site is not the intact cognate site but rather the termini left by SgrAI cutting the cognate site. Moreover, it seems that the molecule of this (dimeric) enzyme that activates the enzyme at the secondary site needs to bind to two molecules of the terminal sequence: one end is insufficient (Figures 7 and 8).

The activation of secondary site cleavage thus involves the synapsis of three loci in the DNA: the secondary site, most probably bound to one dimer of SgrAI; and two termini, both presumably bound to another dimer. Though this three-way synapse can be assembled from three oligoduplexes that each carry one of the requisite sites (Figures 6 and 7), it occurs most readily on a DNA with cognate and secondary sites *in cis* (Figure 4(a)): the linear DNA left after SgrAI has cleaved its cognate site carries SgrAI-termini at both ends, so they remain covalently attached to the secondary site (Figure 8). In contrast, in the catenane (Figure 4(b)), the SgrAI termini left after cleaving the ring with the cognate site remain tethered to the secondary site in the other ring for only as long as the enzyme stays bound to both termini.

Though the probability of juxtaposing three separate sites in a DNA molecule is lower than that for two sites,<sup>51</sup> several other genetic events require three-way synapses. For example, the transposition of phage Mu proceeds through a synaptic complex that incorporates sequences at both ends of the transposon and an internal enhancer sequence.<sup>52</sup> Similarly, site-specific recombination by Gin involves a synapse with two recombinational sites and an enhancer.<sup>53</sup>

## Materials and Methods

### Materials

The SgrAI endonuclease was purified as described,<sup>29</sup> as was the resolvase from the transposon Tn21.<sup>40</sup> Protein concentrations were evaluated from  $A_{280}$  measurements. Molar concentrations of SgrAI are given in terms of the dimeric protein of  $M_r$  75,806. Before reactions, it was diluted to the requisite concentration in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% (v/v) glycerol, 0.2% (v/v) Triton X-100.

The plasmids pMLE2 and pMLE3<sup>12</sup> were used to transform *Escherichia coli* HB101 and the transformants grown in M9 minimal medium containing 37 MBq/l of [*methyl*-<sup>3</sup>H]thymidine (Amersham Biosciences Ltd). Covalently closed DNA was purified by density gradient centrifugations.<sup>28,29</sup> The preparations contained mostly the SC form of the monomeric plasmid, with generally <10% as either dimer or OC DNA. Samples of the plasmids in which ~95% was SC monomer were subjected to reactions with Tn21 resolvase as described, under conditions where virtually all of the SC plasmid is converted to the catenane.<sup>12,21</sup> Only the SC form of the plasmid can undergo recombination with resolvase, so these preparations contained ~95% catenane and ~5%

unrecombined OC plasmid. DNA concentrations were determined from  $A_{260}$  measurements.

Oligodeoxyribonucleotides were purchased from MWG-Biotech AG or Cruachem Ltd. When required, the oligonucleotides were 5'-end labelled by using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP.<sup>26</sup> Duplexes were generated by heating complementary pairs of oligonucleotides to 95 °C, with a twofold excess of unlabelled bottom strand over labelled top strand, prior to overnight cooling to room temperature.

### Reactions on plasmids

Reactions on plasmids and catenated DNA were initiated by adding 5  $\mu$ l of SgrAI endonuclease (diluted as above) to 10 nM [<sup>3</sup>H]DNA in 200  $\mu$ l of reaction buffer at 37 °C. Reaction buffer was 33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate and 0.5 mM dithiothreitol; this was supplemented with either 66 mM or 150 mM potassium acetate. An aliquot (20  $\mu$ l) was removed before the addition of the enzyme, to act as a zero time-point. At timed intervals after adding the enzyme, further 20  $\mu$ l aliquots were removed and mixed immediately with 10  $\mu$ l of EDTA stop-mix.<sup>12</sup> The samples were analysed by electrophoresis through agarose (as in Figure 2), under conditions that separated, as far as possible, the substrate and all of the various products: for the plasmids, the SC substrate from the OC, the FLL and the L<sub>1</sub>/L<sub>2</sub> forms; for the catenane, the SC catenane from the OC forms nicked in either one or both rings, and the intact, OC and linear forms of each individual ring.<sup>8,21</sup> The identity of each of these products on the agarose gels was ascertained by using as markers the DNA from restriction digests of the catenane, with enzymes that cleave in only the large or only the small ring. The concentration of each form of the DNA at each time-point sampled was measured by scintillation counting.<sup>29</sup> For each series of substrates tested, parallel reactions were carried out in triplicate: Figures 2–4 show representative data from one set of parallel reactions. Steady-state and first-order reaction rates were evaluated from the changes in concentration with time by fitting to the appropriate function<sup>45</sup> in GRAFIT (Erithacus Software). For reaction schemes containing two or more sequential steps, the individual rate constants were determined by using SCIENTIST (MicroMath Software) to generate the optimal fit of the experimental data to the relevant rate equations,<sup>45</sup> as described.<sup>41,44</sup>

For some reactions, the 2cat catenane was first cleaved in either the large or the small ring with another restriction enzyme: SmaI in the large ring, PstI in the small. The restriction digests contained 10 nM catenane and 40 units of restriction enzyme in 240  $\mu$ l of reaction buffer with 66 mM potassium acetate. After one hour at the requisite temperature, the solution was heated to 65 °C for 20 minutes, and the cleaved DNA used directly as a substrate for SgrAI. For the experiments in Figure 8, the DNA was prepared by first digesting pMLE2 with SgrAI, or with SgrAI and XhoI, in reaction buffer containing 150 mM potassium acetate. It was then washed with phenol/chloroform and precipitated with propan-2-ol, prior to resuspension in reaction buffer containing 66 mM potassium acetate.

### Reactions on oligoduplexes

Reactions were initiated by adding 5  $\mu$ l of SgrAI (diluted as above) to 300  $\mu$ l of the oligoduplex(es) at the

requisite concentration(s), in reaction buffer with 66 mM potassium acetate at 37 °C. The oligoduplex being analysed was <sup>32</sup>P-radiolabelled: if another duplex was present, it was unlabelled. At timed intervals after the addition of the enzyme, aliquots (25 µl) were removed and quenched immediately with 15 µl of formamide stop-mix (80% (v/v) formamide, 2 mM NaOH, 10 mM EDTA and 100 µg/ml of bromophenol blue). Samples were heated to 95 °C, to melt the duplex into single strands, and loaded onto 20% (w/v) polyacrylamide gels with 4 M urea. The gels, in 45 mM Tris base, 45 mM boric acid, 2.5 mM EDTA, were run at 45 °C before loading the samples and were then run at a constant temperature of 50 °C for two hours, to separate the intact strands from the cleaved products.<sup>26</sup> The gels were fixed for ten minutes in 10% (v/v) acetic acid, 10% (v/v) methanol, 5% (v/v) glycerol and vacuum-dried. Gels were scanned in an 8100 Typhoon PhosphorImager (Amersham) and analysed using ImageQuant Software (Molecular Dynamics) to measure the concentrations of <sup>32</sup>P-labelled strand from the substrate and from the cleaved product. Each datum point in Figures 5–7 and in Table 2 is the mean from three separate experiments. Rates were evaluated by fitting the initial linear decline in the concentration of substrate, or increase in the concentration of the cleaved product, to a zero-order function in GRAFIT.

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