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Asymmetrical recognition and activity of the I-*Sce*I endonuclease on its site and on intron-exon junctions

Arnaud Perrin[2], Malcolm Buckle[1] and Bernard Dujon

Unité de Génétique Moléculaire des Levures, [1]Unité de Physicochimie des Macromolécules Biologiques (URA 1149/CNRS), Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France
[2]Corresponding author

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Group I intron-encoded endonucleases represent a new class of double strand cutting endonucleases whose function is to initiate the homing of introns by generating double strand breaks in site-specific sequences. We have studied the mechanism of interaction of the I-*Sce*I endonuclease with different DNA substrates derived from its natural site in the intron-less gene or from intron-exon junctions in the gene with an intron. We show that the enzyme recognizes its asymmetrical site with high affinity binding to the sequence corresponding to the downstream exon followed by binding to the upstream exon and catalysis of phosphodiester bond hydrolysis. Asymmetrical nicking activity is observed as an intermediate of the cleavage reaction. In the intron-containing gene, the enzyme recognizes the downstream intron-exon junction without any cleavage activity. This binding raises the possibility of a specific function of homing endonucleases in either gene expression or intron homing steps subsequent to DNA cleavage.
Key words: DNA-protein interaction/group I intron/intron homing site/I-*Sce*I/site-specific endonuclease

Introduction

Intron-encoded endonucleases were first discovered in yeast mitochondria with the demonstration of the endonucleolytic activity of I-*Sce*I, the protein encoded by the mobile group I intron of the large rRNA gene (Dujon *et al.*, 1985; Colleaux *et al.*, 1986). I-*Sce*I cleaves intron-less genes at the site of intron insertion and initiates intron homing, a DNA mediated process by which group I introns propagate. To date, several 'homing endonucleases' encoded by mobile group I introns of a variety of eukaryotes (yeasts, fungi, algae and protozoans) as well as bacteriophages have been characterized (reviewed in Dujon, 1989, 1990; Lambowitz, 1989; Scazzocchio, 1989; Belfort, 1991). There are at least three classes of intron homing endonucleases as judged from their structural motifs and their mode of cleavage. The most frequent one (called here the dodecapeptide family) is represented by a set of proteins with conserved dodecapeptide motifs, among which are I-*Sce*I, I-*Sce*II, I-*Sce*III, I-*Sce*IV, I-*Cre*I, I-*Ceu*I and I-*Csm*I (Dujon *et al.*, 1985; Colleaux *et al.*, 1986; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989; Durrenberger and Rochaix, 1991; Gauthier *et al.* 1991; Marshall and Lemieux, 1991; Sargueil *et al.*, 1991; Ma *et al.*, 1992; Seraphin *et al.*, 1992; for nomenclature conventions refer to Dujon *et al.*, 1989). They cleave DNA inside their recognition sequences, leaving 4 bp 3'-OH overhangs. The same applies to I-*Ppo*I (Muscarella and Vogt, 1989) except that no clear-cut dodecapeptide motif can be recognized. Proteins of the second class, such as I-*Tev*I and I-*Tev*II, cleave DNA outside their recognition sequences and leave 2 bp 3'-OH overhangs (Bell-Pedersen *et al.*, 1990; Chu *et al.*, 1990). Another protein, so far the only known one of its type, leaves 5' overhangs of 2 bp (Eddy and Gold, 1991).

The dodecapeptide family also contains a few site-specific endonucleases that are not encoded within group I introns. Some, like I-*Dmo*I in Archaebacteria, are encoded by another type of intron (Dalgaard *et al.*, 1993). Others, like Endo.*Sce*I (Shibata *et al.*, 1984; Nakagawa *et al.*, 1992) or HO (Kostriken *et al.*, 1983) are encoded by normal mitochondrial or nuclear genes in yeast, respectively. Finally, some are inserted in-frame within protein-coding genes in yeast (VDE1 in the *VMA1* gene), in *Mycobacterium tuberculosis* (RecA homologue) or in Archaebacteria (I-*Tli*I) and appear to undergo protein splicing (Shih *et al.*, 1988; Hirata *et al.*, 1990; Davis *et al.*, 1992; Gimble and Thorner, 1992; Hodges *et al.*, 1992; Perler *et al.*, 1992; Shub and Goodrich-Blair, 1992).

It has also been shown by extensive mutagenesis that the homing endonuclease I-*Sce*I recognizes an unusually long non-palindromic nucleotide sequence consisting of the junction between the two exons in the intron-less gene (Colleaux *et al.*, 1988). The same applies to other homing endonucleases subsequently studied (Muscarella *et al.*, 1990; Sargueil *et al.*, 1990; Marshall and Lemieux, 1991; Thompson *et al.*, 1992). This property distinguishes homing endonucleases from bacterial restriction endonucleases with short recognition sites and relates them to gene regulatory proteins that bind larger sites but do not cleave DNA (see Takeda *et al.*, 1983; Bennett and Halford, 1989; Harrison and Aggarwal, 1990). Thus, in-depth analysis of the mechanism of action of homing endonucleases is particularly interesting. But, apart from the demonstration of their endonucleolytic activity and the characterization of their recognition sequences, only a few homing endonucleases, or structurally related enzymes, have been purified and studied *in vitro* so far. Endo.*Sce*I has been shown to be a heterodimer with one subunit encoded by mitochondrial DNA in some *Saccharomyces cerevisiae* strains and the other subunit, related to hsp70, encoded by a nuclear gene (Morishima *et al.*, 1990; Nakagawa *et al.*, 1991). This enzyme cleaves DNA frequently, indicative of a low stringency in sequence recognition (Kawasaki *et al.*, 1991). On the other hand, the stringency of HO, the nuclear encoded protein responsible for mating type switching in yeast (Kostriken *et al.*, 1983), is extremely high, with only one site cleaved in the entire genome, yet partly degenerate. *In*

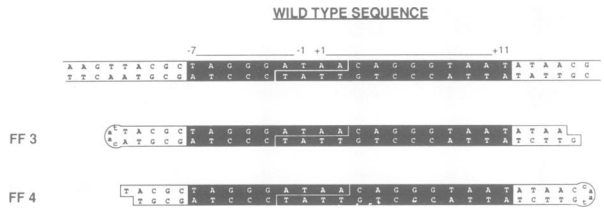

Fig. 1. Sequence and structure of oligonucleotides used as substrates. Oligonucleotides containing the wild-type recognition sequence were synthesized and purified as described in Materials and methods. **Top**: sequence of the wild-type 21S rRNA gene of yeast mitochondria containing the I-*Sce*I recognition site. The black box indicates the extent of the site (−7 to +11) as determined previously (Colleaux *et al.*, 1988). Coordinates are relative to the centre of cleavage. Cleavage is indicated by the broken line. The top strand is oriented 5′ to 3′, left to right. **Bottom**: FF3 and FF4 oligonucleotides have sequences identical to the wild-type site (capital letters) except for the four nucleotides in the loops (lower case). These two oligonucleotides possess a 5′ extension of one nucleotide to facilitate end-labelling.

vivo analysis of HO recognition revealed a core of eight non-contiguous bases near the Y−Z junction of MAT essential for binding and cleavage while other contacts are required, indicative of a complex phenomenon of recognition (Nickoloff *et al.*, 1990). Kinetics and substrate binding properties have also been partially characterized for I-*Sce*I (Monteilhet *et al.*, 1990) and for I-*Sce*II, the endonuclease encoded by intron Sc cox1·4 of yeast mitochondria (Wernette *et al.*, 1990, 1992). Sequence recognition stringency is very high for I-*Sce*I and much lower for I-*Sce*II.

Using a highly purified fraction of the I-*Sce*I endonuclease, prepared for large genome mapping, and a variety of artificial DNA substrates, we demonstrate that the very high sequence selectivity of I-*Sce*I for cleavage results from a two step recognition process involving strong binding to the downstream exon sequence and weaker binding to the upstream exon sequence. Consistent with the asymmetrical binding to exon sequences, we also demonstrate that I-*Sce*I recognizes the downstream intron−exon junction in the intron-containing gene, raising the possibility that homing endonucleases have a specific function in either gene expression or subsequent steps of intron homing.

Results

A two step kinetics of I-SceI activity suggests slow product release

Enzymatic properties of the endonuclease I-*Sce*I have been previously described using a partially purified fraction (Colleaux *et al.*, 1988; Monteilhet *et al.*, 1990). The enzyme shows two interesting properties. First, its activity is optimal at high pH (V_{max} at pH 9.5 is 7 times higher than that at pH 7.5). Second, partial reactions are often observed even after prolonged incubation. This last observation has been explained in part by the instability of the enzyme in the absence of substrate. But partial reactions have also been often observed for several other intron-encoded endonucleases. We have therefore re-examined the endonucleolytic activity of I-*Sce*I on double-stranded DNA, using a fully purified enzyme fraction and synthetic oligonucleotides (see Figure 1) as substrates of the reaction.

In a first series of experiments (Figure 2a), the amount of reaction products (cleaved oligonucleotides) was measured as a function of incubation time for two enzyme

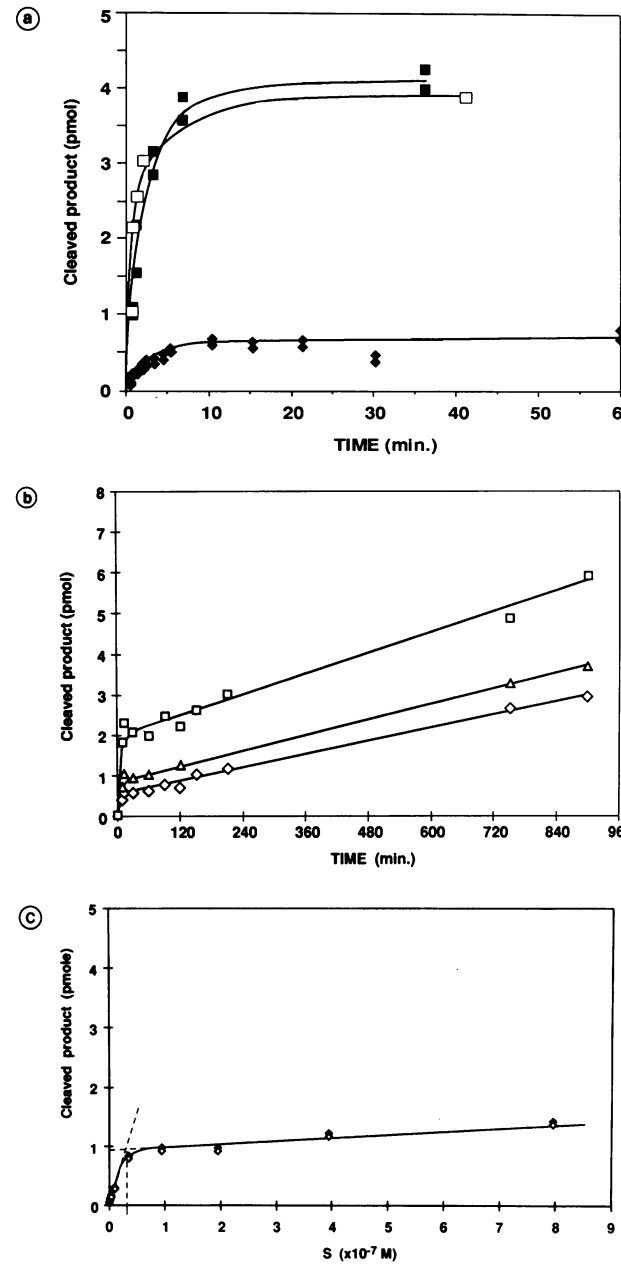

Fig. 2. Cleavage of wild-type site as a function of enzyme and substrate concentrations. (**a**) 5 pmol of 5′ end-labelled oligonucleotides FF3 or FF4 were incubated in the presence of 1 or 5 units of I-*Sce*I in a total volume of 10 μl. Samples were incubated at 37°C for various times with 0.008 M $MgCl_2$. Reactions were stopped and samples were analysed as described in Materials and methods. Measurements were done in duplicate (except for FF4). □: FF4, with 5 units of I-*Sce*I; ■: FF3, with 5 units of I-*Sce*I; ◆: FF3, with 1 unit of I-*Sce*I. (**b**) 50 pmol of 5′ end-labelled oligonucleotides FF4 were incubated in the presence of 1, 2 or 4 units of I-*Sce*I, in a total volume of 10 μl. Subsequent steps were as in panel a. ◇: 1 unit of I-*Sce*I; △: 2 units of I-*Sce*I; □: 4 units of I-*Sce*I. (**c**) Various amounts of 5′ end-labelled oligonucleotide FF4 (~0.5−8 pmol) were incubated in the presence of 1 unit of I-*Sce*I (◇) in standard incubation buffer in a total volume of 10 μl. Subsequent steps were as in panel a with a 1 h incubation.

concentrations (corresponding to the addition of 1 and 5 units, respectively). Experiments were done using a substrate concentration of 5×10^{-7} M (~14-fold higher than the apparent K_M, see Monteilhet *et al.*, 1990). It can be seen that the total amount of cleaved products increases very

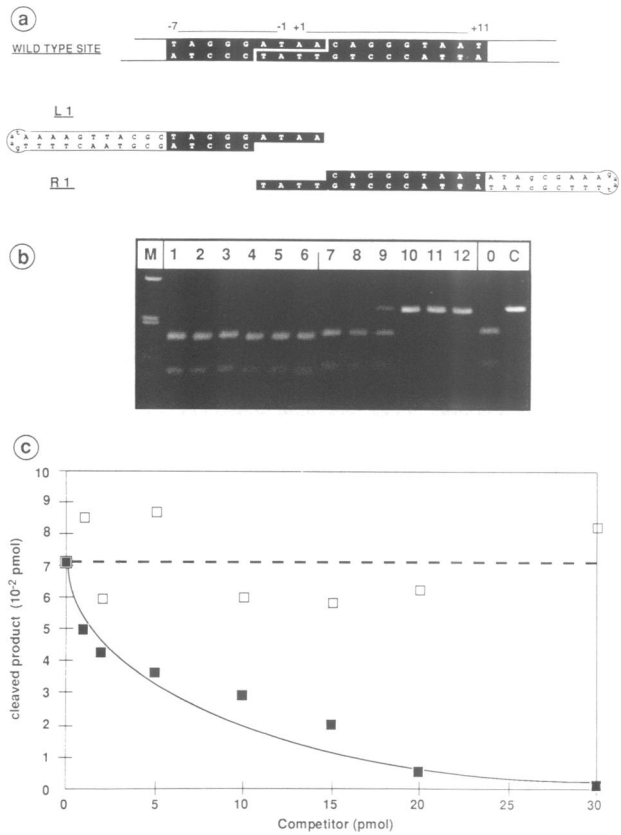

Fig. 3. Effects of the two reaction products on enzyme activity. (**a**) Top: sequence of the wild-type recognition site of I-*Sce*I in the intron-less gene. Bottom: oligonucleotides L1 and R1. Each oligonucleotide was synthesized as a hairpin with 3′-OH overhangs identical to the cleaved products. Capitals, lower case letters and black box are as in Figure 1. (**b**) 0.14 pmol of *Dra*I-digested plasmid pSCM522 (used as wild-type substrate) were mixed with various amounts of the oligonucleotides L1 (lanes 1−6) or R1 (lanes 7−12) at 0°C in standard incubation buffer and 1 unit of I-*Sce*I was added in a total reaction volume of 20 μl. Reactions were started by adding 0.005 M $MgCl_2$ and transfer to 37°C. Subsequent steps were as in Figure 2a with a 30 min incubation. Lanes 1−6: 0.4, 2, 4, 200, 400 and 800 pmol of L1, respectively. Lanes 7−12: 0.4, 2, 4, 200, 400 and 800 pmol of R1, respectively. Lane 0: control without oligonucleotide, lane C: control without enzyme, lane M: molecular weight marker (λ *Mlu*I digest). (**c**) 5 pmol of 5′ end-labelled FF3 were mixed with various amounts of the oligonucleotides L2 (□) or R2 (■) at 0°C in standard incubation buffer and 0.1 unit of I-*Sce*I was added in a total reaction volume of 10 μl. The reactions were started by addition of 0.008 M $MgCl_2$ and transfer to 37°C. Subsequent steps were as in Figure 2a with a 30 min incubation.

rapidly during the first 10 min of incubation and then reaches an apparent plateau, the value of which is ~5 times higher when 5 units of enzyme are used compared with when 1 unit is used. Thus, after the initial phase of the reaction, the total amount of product appears proportional to the amount of enzyme, indicating no turnover or very rapid enzyme decay. However, prolonged incubation (Figure 2b) reveals a slow but constant increase of products over several hours. Note that the slope of this latter activity increases with enzyme concentration, as expected for a typical enzyme. The kinetics of the reaction can, therefore, be separated into two phases: a first phase in which product formation is rapid, producing an amount of product which is roughly proportional to the amount of enzyme present, and a second phase in which product formation is very slow (~0.2 pmol/h/unit). From these results, one can calculate that 1 unit of enzyme must roughly correspond to 0.8 pmol of active protein. This is consistent with the concentration of I-*Sce*I in the purified fraction used as determined from gel electrophoresis staining. Note, however, that the amount of enzyme corresponding to 1 unit may vary slightly between different series of experiments due to the instability of the purified fraction. (Under the conditions used here, 1 unit corresponds to ~20 ng of protein or 0.8 pmol.)

In the second series of experiments (Figure 2c), we examined the effect of substrate concentration on the first phase of the kinetics. To do this, we measured the amount of product after 1 h of incubation (at this time, the total amount of product formed is very close to that resulting only from the first phase, see Figure 2b). It can be seen that, for substrate concentrations above the apparent K_M, the amount of product increases very moderately with increasing substrate concentration, whilst it decreases rapidly, as expected, for concentrations of substrate below the apparent K_M.

We conclude that during the first phase an excess of substrate saturates the enzyme. These results are consistent with the idea that the first phase of the kinetics represents binding of all available enzyme molecules to its substrate (which eventually results in cleavage), while the second phase represents the turnover of the enzyme. The low turnover can be explained by either inactivation of the enzyme after catalysis or slow product release.

Differential interaction of the two cleaved products with the enzymatic activity

To differentiate between these two possibilities, we have examined the effects of the reaction products on the enzyme activity. Cleavage of the wild-type site by I-*Sce*I liberates 4 bp extensions with free 5′ phosphate ends and 3′-OH ends as determined from the observation that it is possible to religate the two cleaved products (Colleaux *et al.*, 1988 and data not shown). Because in the intron-plus gene, the intron is inserted in the middle of the 4 bp cleavage extensions, one of the cleavage products corresponds to the upstream exon and the other to the downstream exon. Two oligonucleotides corresponding to the two products of the reaction were synthesized (Figure 3a). Interaction of the oligonucleotides with the enzyme was measured by competition experiments against the wild-type substrate (Figure 3b). Very different results were obtained for the two oligonucleotides. Increasing concentrations of L1 (up to 5700-fold more than the wild-type substrate concentration) do not interfere with the activity of I-*Sce*I. On the other hand, an excess of R1 of only 14-fold over the wild-type substrate concentration inhibits the activity of the enzyme (lane 8). The effect is more pronounced at 28-fold excess (lane 9). Quantification of the relative inhibition by the reaction products is difficult from the experiment described above because the concentration of the wild-type site is insufficient when using plasmid pSCM522 as substrate (a concentration of 7×10^{-9} M was used, which is below the apparent K_M). To quantify the degree of inhibition by each product of the reaction, experiments were repeated using a synthetic oligonucleotide as substrate. Figure 3c shows the results of a competition experiment using 5×10^{-7} M of end-labelled substrate (FF4) and increasing concentrations of unlabelled competitor oligonucleotides (1×10^{-7} to 3×10^{-6} M). In this experiment, oligonucleotides L2 and R2 (described in

Table I. Competition experiments with oligonucleotides L2 and R2

Competitor	Experiment 1	Experiment 2
None	0.036	0.42
L2	0.036	0.40
R2	0.011	0.14

3.3 pmol of labelled G13/G15 oligonucleotide (Experiment 1) or 5 pmol of labelled FF3 oligonucleotide (Experiment 2) were used with 10 pmol of unlabelled L2 or R2 competitor. Reactions were carried out at 37°C in the presence of 0.008 M of $MgCl_2$ for 3 min with 0.1 unit of enzyme (Experiment 1) or 1 h with 1 unit of enzyme (Experiment 2), in a total volume of 10 μl. Figures indicate the amount of cleaved product (in pmol).

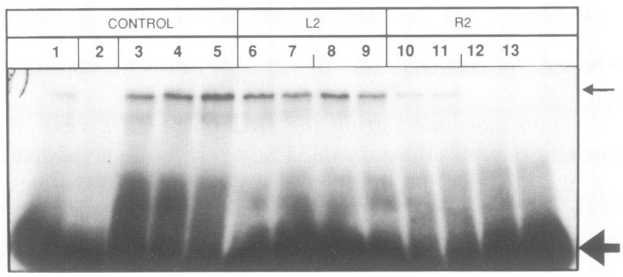

Fig. 4. UV laser cross-linking of I-*Sce*I–substrate complex in the presence of reaction products. 5 or 10 pmol of 5′ end-labelled FF4 oligonucleotide, with or without unlabelled competitor oligonucleotide, were mixed at 0°C with 5 units of I-*Sce*I in standard incubation buffer without magnesium ions, in a final volume of 10 μl and subjected to UV laser irradiation as described in Materials and methods. All experiments were done at least in duplicate. Samples were analysed as in Materials and methods. The thin arrow indicates the complex and the large arrow unbound FF4 substrate. Complexes were 3 cm away from the origin of migration. Lanes 1–5 are controls without competitor oligonucleotides; they contain 5 pmol (lanes 1 and 2), 10 pmol (lanes 3–5) of FF4; lane 2 is a control without UV irradiation. Lanes 6–13 show competition between 5 pmol of FF4 and unlabelled oligonucleotides: with 5 pmol of oligonucleotide L2 (lanes 6 and 7), 10 pmol of oligonucleotide L2 (lanes 8 and 9), 5 pmol of oligonucleotide R2 (lanes 10 and 11) or 10 pmol of oligonucleotide R2 (lanes 12 and 13).

Figure 5) were used in place of L1 and R1, respectively. The results obtained confirm the absence of inhibition by L2 as observed before for L1, and the competition effect of R2, as for R1. Examination of the inhibition curve by R2 indicates that 50% inhibition is obtained for concentrations of $\sim 5 \times 10^{-7}$ M. This value is equivalent to the concentration of the wild-type substrate, illustrating the high efficiency of R2 as competitor. These results have been confirmed by two independent experiments (Table I) using either a longer incubation time (1 h) or a different wild-type substrate (G13/G15, see Figure 5). Again, no inhibitory effect of L2 could be detected, whereas the presence of R2 inhibits the reaction.

To determine if the disymmetrical effect observed for the two reaction products reflects differential binding to the enzyme or possible modification of the enzyme by the right end product, I-*Sce*I–DNA complex formation was examined. Because standard gel shift assays have remained unsuccessful, UV laser cross-linking was used to study the complex (Hockensmith *et al.*, 1991). Complexes were allowed to form, in the absence of magnesium, between I-*Sce*I and a constant concentration (10^{-7} M) of labelled wild-type site in the absence or presence of unlabelled competitor oligonucleotides, L2 and R2, each at two different concentrations (5×10^{-7} and 1×10^{-6} M). Samples were irradiated by a single pulse at 266 nm using an Nd:YAG laser as described in Materials and methods. Separation on SDS–PAGE and quantitative densitometry using a PhosphorImager gave results shown in Figure 4. The cross-linked material gives rise to a labelled band migrating slightly more slowly than the free protein. Assuming that this band contains the protein cross-linked to the substrate, we calculated the extent of cross-linking with respect to free oligonucleotide. In accordance with previous results (Buckle *et al.*, 1991; Hockensmith *et al.*, 1991) we observe ~4% of the DNA in the cross-linked band, corresponding to the expected quantum yield for a protein making few contacts with the DNA. The simple fact that we observe a cross-link after UV laser irradiation is a powerful argument for the presence of intimate amino acid–nucleic acid interactions in our complex. The extent of cross-linking is invariant between different experiments carried out under identical conditions. However, whereas the presence of the L2 oligonucleotide does not reduce the amount of I-*Sce*I–substrate complex formed, identical concentrations of the R2 competitor significantly decrease complex formation.

From all these experiments, we conclude that the left hand product alone, which corresponds to the upstream exon, has low or no affinity for the enzyme whereas the right hand product, which corresponds to the downstream exon, retains a strong affinity for the enzyme and inhibits its activity. This is consistent with the idea that the right hand product of the reaction remains bound to the enzyme after cleavage. The two step kinetics experiments reported above and the affinity of the enzyme for one of its two products, can be symbolized as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} EP2 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P2$$
$$\qquad\qquad\qquad\qquad\qquad\quad P1$$

where P1 and P2 represent the left hand and the right hand products, respectively. The observed kinetics suggest that k_1 and k_{-3} are relatively rapid and roughly equivalent, and that k_3 is extremely low.

Specific interaction of the enzyme with downstream exon sequences

Because previous experiments indicate the importance of the downstream exon in the recognition of its site by I-*Sce*I, we synthesized different oligonucleotides reproducing the right hand part of the recognition site of I-*Sce*I with various mutations (Figure 5). In a first series of experiments, the competition of the oligonucleotides against the enzymatic activity was directly tested by mixing a constant amount of wild-type substrate (pSCM522) with various amounts of unlabelled oligonucleotide followed by addition of the enzyme (as described in Figure 3). Qualitative results are summarized in Figure 5. In agreement with the above results, we found that 10 pmol of oligonucleotide R2 inhibit the reaction by ~50%, whilst L2 has no effect. Oligonucleotides based on the right hand product but containing various mutations, affect the reaction to various extents: R5, R6 and R12 show no competition, R3 and R4 show very little competition, whereas R7, R8, R11, R13 and R14 are competitors of the reaction.

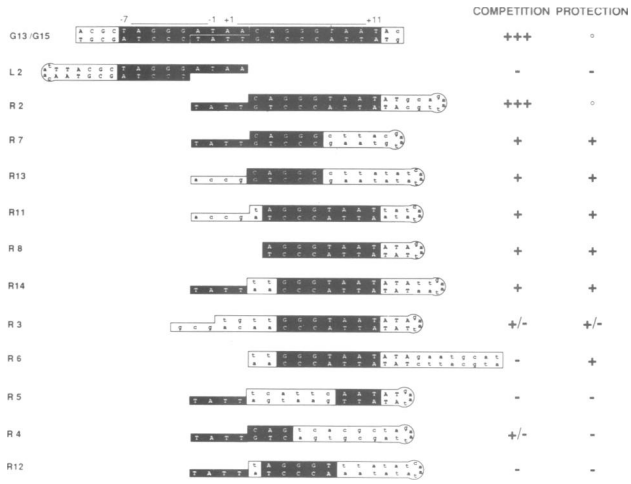

Fig. 5. Competition and protection of wild-type and various mutant cleaved products. Oligonucleotides were synthesized as described in Materials and methods. Capital letters, lower case letters and black box as in Figure 1. Results of several competition and protection experiments with various amounts of oligonucleotide are summarized qualitatively. Competition is estimated from the amounts of oligonucleotide giving rise to 50% inhibition of enzymatic activity for 0.14 pmol of wild-type substrate. The quantity of oligonucleotide is indicated as follows: +++, 1−10 pmol; +, 50−125 pmol; +/−, 150−500 pmol; −, >1000 pmol. Protection of the enzyme is estimated from the remaining enzymatic activity after 15 min of incubation prior to addition of wild-type substrate. Plus signs indicate a protection effect; minus signs indicate no protection and ° indicates that protection cannot be measured due to the very strong competitor effect.

In a second series of experiments, the binding of each oligonucleotide to I-*Sce*I was estimated from its ability to stabilize the enzyme in the reaction conditions prior to addition of the wild-type substrate. It has previously been shown that the presence of the recognition site or of some mutants thereof, increases the stability of the enzyme in the presence of magnesium ions (Monteilhet *et al.*, 1990). This protection is specific: random DNA sequences do not stabilize the enzyme. Therefore, protection of the enzyme can be used as an indirect measurement of binding of various substrates or derivatives. All oligonucleotides were added at the same concentration (1×10^{-6} M), mixed with I-*Sce*I and incubated at 37°C in the presence of magnesium ions for various times before addition of the wild-type substrate. The residual enzymatic activity was then measured. At time 0 (no preincubation) results represent a direct competition between the wild-type and mutant DNAs. We again find a strong competition for R2, a weak competition for R3, R7 and R8 and no competition for R5. After only 5 min of preincubation of the enzyme in the presence of magnesium ions, almost no activity remains for oligonucleotides L1, R2, R3 and R5, while addition of oligonucleotides R7 and R8 significantly protects the enzyme (data not shown). The results of different protection experiments are summarized qualitatively in Figure 5. In fact, only one oligonucleotide (R6) shows protection without competition.

Although the results shown in Figure 5 are not sufficient to draw more precise conclusions on the I-*Sce*I−DNA interaction, it appears that the downstream exon sequence can be roughly divided into two sectors: one composed of the right part of the recognition site (approximately positions +5 to +11) important for binding to I-*Sce*I, the other

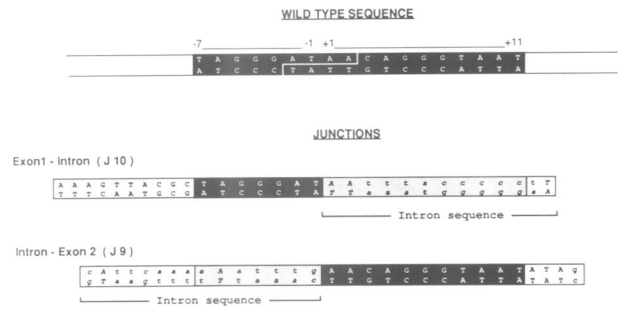

Fig. 6. Sequence of oligonucleotides representing intron−exon junction sequences. Two oligonucleotides (top and bottom) for each duplex were synthesized as described in Materials and methods. The black boxes indicate the extent of the recognition site in the wild-type sequence and its equivalent at the two intron−exon junctions. The grey box indicates the intron sequence extending from −7 to +11 with respect to the recognition site. Capital and lower case letters as in Figure 1. Italics are intron sequences.

(approximately positions −2 to +4) with a lesser effect on binding.

Interaction of the enzyme with the intron−exon junctions

The differential binding of I-*Sce*I to the two reaction products raises an interesting question since each half of the site is present at the intron−exon junctions in the intron-plus gene. We have therefore examined the possible interaction of the two junction sequences on I-*Sce*I using oligonucleotides representing the upstream and the downstream junctions (see Figure 6).

Competition experiments, performed as in Figure 3, show the same asymmetrical effect as for the corresponding reaction products: oligonucleotide J9 (intron−exon 2 junction) inhibits the enzymatic activity while oligonucleotide J10 (exon 1−intron junction) has no effect (see Table II). Protection experiments also reveal the same difference. Although a large excess of the upstream junction (J10) has no effect (no enzyme activity remains after 5 min of preincubation), the downstream junction (J9) protects the enzyme as well as R7 or R8 (data not shown).

This dissymmetry has again been confirmed by UV laser cross-linking experiments as shown in Figure 7. Complexes were allowed to form between I-*Sce*I and a constant concentration of labelled wild-type site (FF4) in the absence or presence of unlabelled competitor oligonucleotides. Following cross-linking, it can be seen that the addition of oligonucleotide J9 decreases complex formation with the wild-type site (~2-fold less for 5 pmol and 3-fold less for 10 pmol), whereas addition of identical concentrations of oligonucleotide J10 shows no significant decrease in complex formation.

In conclusion, I-*Sce*I binds to the downstream junction, but not to the upstream junction, of the intron-plus gene. This is consistent with the differential binding of the two cleaved products and also demonstrates that the presence of intron sequences attached to the downstream exon does not prevent binding to I-*Sce*I.

Differential cleavage of the two phosphodiester bonds

Because of the asymmetry of its binding, the possibility arises that catalysis of the two phosphodiester bond hydrolysis by I-*Sce*I may also be unequal. To determine if the cleavage

Table II. Results of competition and protection experiments with various amounts of oligonucleotides J9 and J10

Oligonucleotides	Competition (pmol for 50% inhibition)	Protection (% remaining activity at 15 min)
Junction I–E (J9)	10	68
Junction E–I (J10)	>1000	0.5

The table indicates (i) the amount of oligonucleotide competitor giving rise to 50% inhibition and (ii) the level of protection of the enzyme measured as the remaining activity after 15 min of incubation in the presence of oligonucleotide and magnesium ions, prior to addition of wild-type substrate. Figures are the average of several experiments.

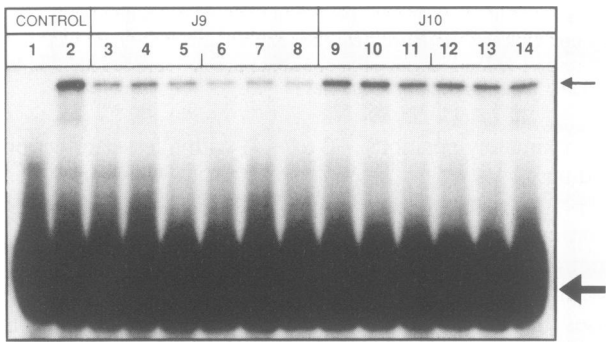

Fig. 7. UV laser cross-linking of protein–DNA complexes with the two junctions. Conditions were the same as in Figure 4, with 5 pmol of 5′ end-labelled FF4. Lane 1, control without enzyme; lane 2, control without competitor; lanes 3–14, competition between unlabelled oligonucleotides and labelled FF4 with 5 pmol of oligonucleotide J9 (lanes 3–5), 10 pmol of oligonucleotide J9 (lanes 6–8), 5 pmol of oligonucleotide J10 (lanes 9–11) or 10 pmol of oligonucleotide J10 (lanes 12–14).

of each phosphodiester bond occurs concomitantly or if molecules with single strand nicks are formed, FF3 and FF4 were labelled at either their 5′ end or their 3′ end and incubated with I-*Sce*I for various periods of time. The DNA was then denatured and electrophoresed on denaturing sequencing gels. Figure 8a shows that when FF4 is labelled at its 5′ end, a 50 nucleotide (nt) fragment is formed (visible after 2 min of incubation) in addition to the 14 nt fragment expected for the normal double strand break. The intensity of this fragment diminishes after 15 min of incubation. However, 3′ end labelling of FF4 does not reveal fragments other than the 10 nt fragment expected for the double strand break. Therefore, the 50 nt fragment must correspond to a nick in the bottom strand of the asymmetrical I-*Sce*I site (for definition of strands, refer to Figure 1). To confirm this interpretation, the FF3 oligonucleotide was labelled at its 3′ end and digested by I-*Sce*I as above (Figure 8b). As expected, a 41 nt fragment is transiently formed. This fragment is most intense after 15 s of incubation and gradually disappears. Labelling of FF3 at its 5′ end does not reveal any fragment other than the expected 18 nt fragment corresponding to the double strand break (data not shown). Therefore, I-*Sce*I generates a nick on the bottom strand of its asymmetrical site. Measurement of band intensity indicates a maximum of 4% of nicked molecules visible after a few seconds of reaction in the presence of magnesium ions. The fraction of nicked molecules is higher for the same incubation time when 0.004 M Mg^{2+} is used,

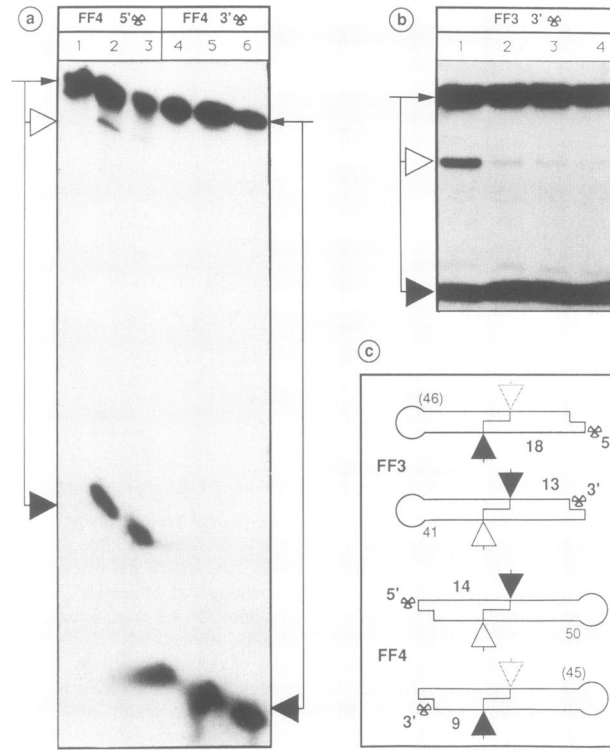

Fig. 8. Asymmetrical nicking activity of I-*Sce*I. (**a**) 4 pmol of 5′ end-labelled (lanes 1–3) or 3′ end-labelled (lanes 4–6) FF4 oligonucleotide, were mixed with 1 unit of I-*Sce*I at 0°C in standard incubation buffer. Reactions were started by adding 0.008 M $MgCl_2$ (except lane 4: 0.002 M $MgCl_2$). Subsequent steps as in Figure 2a. Lane 1: control without Mg^{2+}, 15 min incubation; lanes 2 and 5, 2 min incubation; lanes 3, 4 and 6, 15 min incubation. Thin arrows indicate uncleaved substrate, black arrows indicate cleaved product and white arrow, nicked fragment. (**b**) 5 pmol of 3′ end-labelled FF3 oligonucleotide were mixed with 5 units of I-*Sce*I at 0°C in standard incubation buffer. Subsequent steps as in Figure 2a except for incubation times (0.25, 0.5, 1 and 2 min, respectively for lanes 1–4). Arrows as in panel a. (**c**) Summary of 5′ and 3′ end-labelling of oligonucleotides FF3 and FF4. Black arrows indicate first cleavage from the label; cleavage corresponding to double strand break or to nicking cannot be distinguished. White arrows indicate the second cleavage from the label correspond to nicking (dashes: nicking not observed). The sizes of the labelled products corresponding to each possible cut are indicated.

than when 0.008 M Mg^{2+} is used (data not shown). Under the same conditions, no nicking activity is detectable on the top strand (see Figure 8a, lane 4). Figure 8c summarizes the results with the two labelling oligonucleotides.

Two possible models can be proposed to explain the asymmetrical nicking activity of I-*Sce*I. Either the nicked bottom strand is an obligatory intermediate prior to the formation of the double strand break or nicking of the two strands is independent, with the top strand being nicked less efficiently than the bottom strand which remains below detection level.

Discussion

Like all known intron-encoded endonucleases, I-*Sce*I recognizes a long and asymmetrical site made of the junction between two exons in the intron-less form of the gene. Our experiments show that the two reaction products corresponding, in the case of I-*Sce*I, to exon 1 and exon 2 respectively, exhibit drastically different binding properties

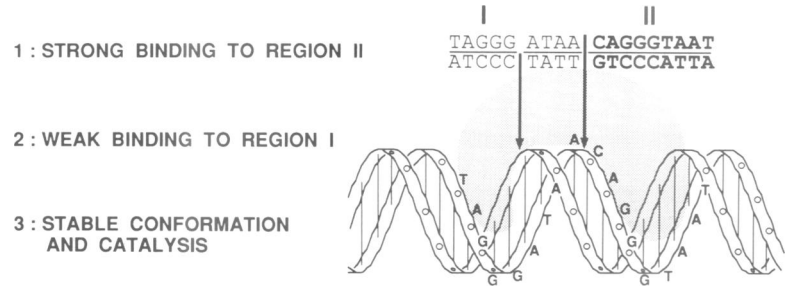

Fig. 9. A two step recognition model for I-*Sce*I. Two recognition regions (I and II) are identified. Binding of the enzyme is assumed to take place in region II, followed by binding to region I, if present. After tight binding has occurred, catalysis takes place as indicated by arrows. The catalytic domain of the enzyme is likely to face the minor groove while recognition domains could face the flanking major grooves in regions I and II.

with the enzyme. If these properties reflect binding prior to cleavage, then two different regions can be determined in the recognition sequence of I-*Sce*I: region I extends from −7 to −2, while region II extends from +2 to +11 (see Figure 9). The enzyme has a much higher affinity for region II than for region I. Consistent with the asymmetry of the site itself and of its recognition, the enzyme also shows preferential nicking of one of the two strands. Nicking activity on the other strand has not been detected, hence it is unclear whether the double strand break eventually formed results from sequential nicking of the two strands in an ordered fashion or from the sum of two independent nicking activities, one of which is more efficient than the other. Preferential nicking of the bottom strand is consistent with the higher reactivity of this bond to phenanthroline cleavage in free DNA (A.Spassky, personal communication). Asymmetrical nicking activity has also been described for another intron-encoded endonuclease of the dodecapeptide family: I-*Ceu*I (P.Marshall and C.Lemieux, personal communication). In this case, however, preferential nicking is found on the non-transcribed strand whereas in the case of I-*Sce*I, it is the transcribed strand that is nicked (see Figure 8). Hence, it is possible that the recognition of the two enzymes with respect to exon 1 and exon 2 sequences is inverted.

The differential binding on the two regions of its site, as well as the observed kinetics, suggest a model for the activity of I-*Sce*I (see Figure 9). It is assumed that the protein contains two recognition sites for DNA, one for region I and the other for region II, with a catalytic domain in between (Figure 9). Binding of the enzyme to region II is followed by binding to region I. Cleavage occurs only when both sites are tightly bound. We have verified the complete absence of both nicking and cleavage activity, when using the downstream intron−exon junction (oligonucleotide J9) as substrate (data not shown).

Because all known intron-encoded endonucleases of the dodecapeptide family have asymmetrical sites, it is possible that a similar mechanism could apply. Thus, the conserved dodecapeptides are unlikely to play a role in sequence recognition because all recognition sites differ. The specificity of recognition must lie with other parts of the endonucleases that remain to be characterized. It is possible that the two dodecapeptides of I-*Sce*I play a direct role in the catalysis of cleavage when the protein is tightly bound to its site.

Our experiments also indicate a very slow turnover of I-*Sce*I, which is likely to result from a slow release of the reaction product(s). Because the affinity for region II is higher than that for region I, it is likely that the downstream cleavage product (which corresponds to exon 2) rather than the upstream cleavage product, remains bound to the enzyme, after cleavage. Covalent binding is excluded as the two termini generated by I-*Sce*I cleavage can be religated *in vitro*. Another explanation of the low turnover observed is that an essential component of the reaction is missing in our *in vitro* assays. In the case of Endo.*Sce*I, another endonuclease of the 'dodecapeptide family' encoded by yeast mitochondria, it has been shown that a 75 kDa protein subunit, homologous to the hsp70 protein of *Escherichia coli*, significantly increases enzymatic activity (Morishima *et al.*, 1990). We have no indication so far that I-*Sce*I could be associated in mitochondria with a hsp protein or any other specific protein (or even RNA). Nevertheless, considering the total number of cleavage sites in yeast mitochondria *in vivo* (only one per mtDNA molecule), there may be no need for enzyme turnover.

The long recognition site of I-*Sce*I and of some other intron-encoded endonucleases (Colleaux *et al.*, 1988; Chu *et al.*, 1990; Muscarella *et al.*, 1990; Sargueil *et al.*, 1990; Gauthier *et al.*, 1991; Marshall and Lemieux, 1991; Moran *et al.*, 1992; Thompson *et al.*, 1992) makes it an attractive model for studying the mechanism of DNA sequence recognition by proteins. Bacterial restriction endonucleases recognize and cleave short sequences (4−8 bp) with no or limited degeneracy. Deleterious effects on the host genome are avoided by the existence of DNA methylases with the same recognition specificity (Bennett and Halford, 1989; Wilson and Murray, 1991). In contrast, gene regulatory proteins bind at larger sites (12−30 bp), which are often symmetrical, and some bind a series of related sites in graduated fashion with high internal degeneracy (Takeda *et al.*, 1983; Busch and Sassone-Corsi, 1990; Harrison and Aggarwal, 1990). Intron-encoded endonucleases have properties of both classes of proteins, with long recognition sequences and a catalytic activity that hydrolyses phosphodiester bonds. In the case of I-*Sce*I, there exists some sequence degeneracy but most mutant sequences are cleaved more poorly than the wild-type site (Colleaux *et al.*, 1988; Monteilhet *et al.*, 1990). Other intron-encoded endonucleases of the same family, e.g. I-*Sce*II, appear less stringent (Sargueil *et al.*, 1990; Wernette *et al.*, 1992). In the cases of *Eco*RI or *Eco*RV, sequence specificity is determined in part by contacts of the protein to the bases and in part by contacts to the DNA backbone (Lesser *et al.*, 1990; Thielking *et al.*, 1990; Heitman, 1992). Because I-*Sce*I as well as other dodecapeptide-containing endonucleases leaves 4 bp 3′ extensions, it seems likely that the enzyme contacts the DNA on the minor groove side at the point of cleavage. If so, recognition of the regions I and II defined

in this work is likely to involve the two flanking major grooves facing the same side of the DNA helix (see Figure 9). The UV cross-linking experiments described here, indicate the presence of direct contacts between amino acids of I-*Sce*I and nucleotides of its site.

The high sequence selectivity of I-*Sce*I over its 18 bp long recognition site makes it a potentially useful tool for genome mapping. No cleavage site exists in a complete eukaryotic nuclear genome like that of the yeast *Saccharomyces cerevisiae* (13.6×10^6 bp), while the enzyme cleaves artificially inserted sites (Thierry *et al.*, 1991; Thierry and Dujon, 1992). The same was recently reported for VDE1, a dodecapeptide-containing enzyme (Bremer *et al.*, 1992). The site-specific endonuclease activity of I-*Sce*I supports the idea that its role is to introduce a double strand break in the intron-less gene to initiate intron homing. But our finding that the protein also interacts with the downstream intron−exon junction (and not with the upstream one) suggests that I-*Sce*I may play other roles *in vivo*. Two possibilities exist. By binding the intron−exon junction of the intron-plus gene, I-*Sce*I might play a role in initiating or facilitating the strand transfer necessary for double strand break repair. Alternatively, binding of I-*Sce*I to the downstream intron−exon junction might interfere with the transcription of the intron-plus gene, for example, causing the polymerase to pause at the end of the intron sequence and resulting in a transiently shorter intron RNA sequence. This in turn may interfere with intron splicing if an incomplete group I intron could prepare for, or even perform, the first transesterification reaction which is normally rate limiting (Cech, 1990; Partono and Lewin, 1991). This might be one explanation for the fact that some group I intron encoded endonucleases of the dodecapeptide family also exhibit an RNA maturase property *in vivo* (Goguel *et al.*, 1992).

Materials and methods

*I-Sce*I enzyme

I-*Sce*I was overproduced in *E.coli* from a synthetic gene on plasmid pSCM525 (A.Thierry, personal communication) and purification was performed by Boehringer Mannheim (cat. no. 1 362 402). Aliquots of the enzyme at 10 units/μl were conserved at −20°C in phosphate buffer, 50% glycerol; 0.2 M Thesit (Boehringer Mannheim); 200 μg/ml bovine serum albumin.

Substrate

All oligonucleotides were synthesized in the laboratory using a Pharmacia LKB 'Gene assembler plus' and purified by electrophoresis on a (19:1) polyacrylamide−urea gel running in 0.5×TBE at 35 W for appropriate times. DNAs were revealed by UV shadowing. Acrylamide slices were cut and incubated in 0.5 M NH_4Ac, 0.01 M MgAc, 0.001 M EDTA, 0.1 % (w/v) SDS at 55°C, overnight. After brief centrifugation, samples were passed through siliconized wool−glass and water was extracted five times with butan-1-ol. DNA was precipitated in ethanol 100%, dried and recovered in 50−100 μl of water. Concentrations were measured by UV absorbance using a Perkin-Elmer lambda 5 spectrophotometer. For duplex oligonucleotides, equimolar amounts of two complementary single-stranded DNAs were dissolved in annealing buffer, heated at 96°C and slowly cooled to room temperature. The concentration of the duplex oligonucleotide was checked by measuring its optical density. Oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase from USB. 3′ end-labellings were performed with [α-^{32}P]ATP (Amersham) and Sequenase from USB.

Buffers and cleavage conditions

1×TBE (pH 8.3) is 0.1 M Tris−Cl, 0.1 M boric acid and 0.025 M EDTA, pH 8. Standard incubation buffer, without magnesium, for I-*Sce*I is 0.1 M diethanolamine−Cl, pH 9.5, 0.001 M dithiothreitol, 200 μg/ml BSA. Annealing buffer is 0.02 M Tris−Cl, pH 7.4, 0.05 M NaCl, 0.002 M $MgCl_2$. Optimum cleavage conditions were as follows. I-*Sce*I was added to DNA in standard incubation buffer at 0°C. The reaction was started by adding 0.005−0.008 M $MgCl_2$ and transferring at 37°C for various times. The reaction was stopped by adding 0.005 M EDTA and transfer to 0°C.

Quantitative measurements of activity

Cleavage experiments were performed either with plasmid substrate (*Hin*fI- or *Dra*I-digested pSCM522 DNA, see Monteilhet *et al.*, 1990) or with radioactively end-labelled oligonucleotides. Plasmid DNA substrates were analysed in 0.8% agarose gels (1×TBE) and revealed by ethidium bromide staining. Fluorescence at 254 nm was photographed on Polaroid 665 films. The relative amounts of DNA in the different bands of each sample were then determined by scanning negative films using a Bio-Rad video densitometer (Model 620) coupled to a Shimadzu Chromatopac C-R3A integrator. Labelled oligonucleotides were analysed by (19:1) polyacrylamide−urea gel electrophoresis (8−15%, 1×TBE) and revealed by autoradiography or quantified using a PhosphorImager (Molecular Dynamics) at 88 or 176 μm resolution (Image Quantifier) coupled to a Compaq computer.

Laser cross-linking of protein−nucleic acid complexes

The laser used was an Nd:YAG discontinuous laser (SpectraPhysics). The average power output generated by this machine is typically 46 mJ/pulse at 266 nm with 5 ns pulses, the beam width is 6 mm and the area is 0.28 cm^2. The incident dose was measured as $\sim2\times10^{17}$ photons/cm^2. All experiments were carried out in a final volume of 10 μl. 5 or 10 pmol of purified labelled oligonucleotides were mixed with 5 units of I-*Sce*I in standard incubation buffer without magnesium ions in the presence or absence of unlabelled competitor oligonucleotides. Components were incubated at 0°C in Eppendorf tubes for 10 min before irradiation. After UV laser irradiation, samples were kept on ice for a few minutes, denatured by heating at 95°C in 5 or 10 μl of SDS gel-loading buffer and analysed on a (37.5:1) polyacrylamide−SDS gel (10−15%). The gel was run at a constant temperature using a Pharmacia GE-2/4 gel electrophoresis apparatus. After electrophoresis, gels were covered with Saran wrap and directly scanned using a PhosphorImager.

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