Substrate Recognition by the 2 μ m Circle Site-Specific Recombinase: Effect of Mutations within the Symmetry Elements of the Minimal Substrate

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Received 22 May 1986/Accepted 25 August 1986

The minimal substrate for the 2 μ m circle site-specific recombinase FLP consists of a nearly perfect 13-base-pair dyad symmetry with an 8-base-pair core. By using a series of chemically synthesized FLP substrates in *in vitro* FLP recombination and FLP-binding assays, we have identified four positions within each of the symmetry elements that are important contact points for the FLP protein. Furthermore, the binding and recombination data provide evidence for cooperativity between the two symmetry elements of a substrate and between the symmetry elements of two partner substrates during FLP recombination.

Sequence-specific recognition of DNA by proteins often plays regulatory, structural, and catalytic roles in key cellular processes. The *lac* repressor, the C1 and Cro repressors of bacteriophage λ , the Mnt repressor of phage P22, and the CAP protein (a regulator of catabolite sensitive operons of *Escherichia coli*) recognize consensus binding sequences with an approximate dyad symmetry (17, 18, 25). The three-dimensional structures of C1, Cro, and CAP strongly suggest that they have a common DNA recognition code in the form of an α -helix-turn- α -helix motif (for a review, see reference 18). Amino acid sequence analyses imply that this may be a general feature of almost all procaryotic and at least some eucaryotic DNA-binding proteins. A number of site-specific recombinases, the Int protein of phage λ (19), the resolvase of the related transposons Tn3 and $\gamma\delta$ (4), the Cre protein of phage P1 (10), the procaryotic invertases (8, 26; R. Kahmann and D. Kamp, personal communication, quoted in reference 7), and the FLP protein of the 2 μ m plasmid of *Saccharomyces cerevisiae* (5, 11, 22) recognize specific, relatively short DNA sequences which include elements of twofold symmetry. The substrates for Cre and FLP are remarkably similar, consisting of a 13-base-pair (bp) dyad symmetry with an 8-bp core; the similarity extends to the chemistry of the reactions catalyzed by them. Both FLP and Cre cleave their substrate DNA and become covalently attached to DNA through a 3'-phosphate linkage (1, 9, 22). The protein-DNA bridge formed during the FLP reaction is a phosphotyrosine (6). The active residue of Cre involved in covalent bonding to DNA has not been determined. It has been proven that the points of cleavage by Cre are also the points of strand exchange during the recombination reaction (9). It is assumed that the same rule holds for the FLP recombination system as well.

As already pointed out, the normal substrate for FLP consists of a nearly perfect 13-bp dyad symmetry plus an 8-bp core located within the 599-bp long repeated segment of the 2 μ m circle (Fig. 1). The 13-bp inverted repetition is flanked on one side by a second copy of the symmetry element. However, this is not essential for recombination *in vivo* or *in vitro* (5, 11, 22). *In vitro* assays have also demonstrated that at least three terminal base pairs of each

of the two essential symmetry elements can be deleted without abolishing recombination (22). The entire 13-bp inverted repeat together with the core region and the second copy of the symmetry element are protected by the FLP protein from DNase I digestion (1). Recent studies have shown that core homology is essential for efficient recombination and that the asymmetry within the core sequence determines the directionality of the reaction (23). However, a number of alterations, such as point mutations and single-base-pair insertions or deletions within the core, are tolerated by the recombination system as long as core homology between the recombining partners is maintained (22, 23). Thus, it would appear that the recognition of its substrate by FLP is mediated almost exclusively by the symmetry elements.

To analyze the interactions between the FLP protein and its target site, we have chemically synthesized a series of FLP substrates. These substrates have been used to examine the possible role of substrate symmetry in recombination and to identify those residues within the symmetry elements that are central to recognition by FLP. The results are summarized in this communication.

MATERIALS AND METHODS

Purification of FLP protein. FLP protein was purified by a modification of published procedures (2, 16; Prasad and Jayaram, unpublished data). The preparations used in binding and recombination assays were approximately 40% pure and were free of detectable nuclease activity.

Synthesis of oligonucleotides. The FLP substrates were synthesized in an Applied Biosystems 380-A DNA synthesizer with phosphoramidite chemistry (3). Each oligonucleotide was purified by polyacrylamide gel electrophoresis, and the accuracy of the sequence was verified before and after cloning into the plasmid vector pPY190.

Plasmids. Plasmid pJRI was constructed as follows. First, the 900-bp *Hind*III fragment from the A form of the 2 μ m circle, which includes one copy of the 599-bp repeat, was excised from pMJ20 (12) and gel purified. This fragment was then cloned into *Hind*III-digested pBR322.

Plasmid pPY190, a gift from Philip Youderian, is a pBR322 derivative that contains a 500-bp segment of phage P22 DNA which includes the *ant/arc* promoter and the 5'-proximal

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FIG. 1. The sequence of the FLP recombination region within the 2 μ m circle repeat is shown. The "minimal" FLP site is constituted by the dyad symmetry 1a–1'a. The singular imperfection at position 12 of the 1–1' dyad symmetry is indicated in lowercase letters. The second, nonessential copy of the symmetry element (1'b) follows 1'a with a single-base gap. The positions within the symmetry elements which are involved in FLP recognition (as defined by the studies described in this paper) are marked by ■.

portion of the *arc* gene (P. Youderian, personal communication). A single copy of each of the synthetic substrates was cloned into the *Sma*I site of pPY190 by the method of Seth (24) to create the pPYD series of plasmids. The unphosphorylated oligonucleotide substrate was ligated to *Sma*I-digested pPY190. The ligation mixture was heated to 90°C and quickly chilled on ice to dissociate the unligated complementary strands of the oligonucleotide. After removal of the unligated oligonucleotide by gel purification, the plasmid DNA (with the single-stranded oligonucleotide overhangs) was treated with kinase, ligated, and used to transform a suitable *E. coli* strain. The cloning strategy places the FLP substrate at the –3 position with respect to the start site of the *arc* mRNA. Although this aspect is not relevant to experiments described here, it is hoped that it will enable us to explore in vivo interactions between FLP and its substrate in a manner analogous to that described for the Mnt repressor and its operator (25).

Miscellaneous methods. Bacterial transformations, isolation of plasmid DNA, restriction enzyme digests, agarose gel electrophoresis, transfer of DNA to nitrocellulose, and hybridization to nick-translated DNA were done by published

procedures (13). DNA sequences were determined by the methods of Maxam and Gilbert (14) or of Sanger et al. (21).

RESULTS

Synthetic FLP substrates. The set of synthetic FLP substrates used in our experiments is listed in Fig. 2A. The complete sequence of the wild-type substrate D₁ is shown. The residues within the left-hand symmetry element are numbered 1 to 13, and those within the right-hand symmetry element are numbered 1' and 13'. The bases within the 8-bp core region (which is the same in all of the substrates) are not numbered. Although there is a single imperfection at position 12 of the dyad symmetry, D₁ is considered a symmetric substrate for the purpose of our discussion. Most of the mutant substrates (D₂ through D₉) contain a single mutation within the left symmetry element compensated for by a corresponding complementary mutation in the right symmetry element. These substrates are therefore symmetric. Substrates D₁₀ and D₁₁ contain a 1-bp substitution at position 9 and 9', respectively. These two substrates are therefore asymmetric. Since in vitro results have shown that several base pairs near the ends of the symmetry elements may not be critical to recombination (5), we have not tested the effects of mutations at positions 1 through 6 of the symmetry elements. Instead, the substrates (D₂ through D₁₁) were designed to harbor mutations at every position of the dyad symmetry from 7 through 12. The present studies do not include a mutation at position 13. In the mutant substrates shown in Fig. 2A, only residues that differ from the wild type are indicated. All of the substrates were cloned into the plasmid vector pPY190 as described in Materials and Methods to obtain the pPYD plasmids (Fig. 2B). Binding and recombination reactions involving the pPYD plasmids were compared with those involving pJR1 (Fig. 2B), which harbors an entire copy of the wild-type 2 μ m circle repeat.

FIG. 2. Synthetic FLP substrates. (A) Synthetic substrates used in this study. D₁ is the wild-type substrate, consisting of a nearly perfect dyad symmetry (1a and 1'a; see Fig. 1) with an 8-bp core. There is a single imperfection at position 12; however, D₁ is considered a symmetric substrate. In substrates D₂ through D₁₁, only those positions altered with respect to the wild type are indicated. In all of the substrates the core region is wild type. Chemical synthesis of the substrates was done in an Applied Biosystems 380-A DNA synthesizer with phosphoramidite chemistry (3). (B) Plasmid pJR1 contains the 900-bp *Hind*III fragment from the A form of the 2 μ m circle (hatched bar) (7), which includes a copy of the 2 μ m circle repeat (solid bar) cloned into the *Hind*III site of pBR322. The pPYD plasmids contain the synthetic FLP sites (solid bar; see panel A) introduced into the *Sma*I site located within a 500-bp *Eco*RI-*Hind*III fragment (stippled bar) derived from phage P-22 and cloned into pBR322. The cloning strategy places the FLP site at the –3 position with respect to the start site of the P22 *arc* mRNA (arrow). Abbreviations: P, *Pst*I; R, *Eco*RI; H, *Hind*III; AMP^R, ampicillin resistance gene.

In vitro binding of FLP to the synthetic substrates. The FLP-binding assay takes advantage of the retardation of the electrophoretic mobility of a DNA fragment when it is associated with FLP. After incubation of a mixture of DNA fragments with partially purified FLP at a protein-to-DNA ratio of 1:5 (wt/wt) or less, the fragment which includes the FLP site binds FLP specifically (for details, see the legend to Fig. 3). Assuming 40% purity of FLP, a protein-to-DNA mass ratio of 1:5 corresponds to approximately 4 to 5 monomers of FLP per recombination site. At higher protein concentrations, however, FLP behaves like a nonspecific DNA-binding protein. Since our FLP preparations were only partially pure, it is possible that the nonspecific binding was caused by a protein(s) other than FLP. Such nonspecific binding is dependent on the target size; the larger the DNA fragment, the more avid the binding. However, the nonspecific binding could be almost entirely eliminated by challenging the protein-DNA complexes with heparin, as described in the legend to Fig. 3.

To establish the specific binding of FLP to its substrate DNA, we first carried out the binding assay with plasmid pJR1. After digestion of pJR1 with *Pst*I and *Hind*III, three fragments were obtained: two pBR322-derived fragments (approximately 3,600 and 750 bp) and a 900-bp 2 μ m circle fragment containing the FLP site. When this mixture of fragments was incubated with FLP, challenged with heparin, and fractionated on an agarose gel, the 900-bp fragment alone was retarded in its mobility (Fig. 3A). For assaying the binding potential of the synthetic sites, the pPYD plasmids digested with *Eco*RI were used as substrates. The digestion gives a backbone fragment consisting exclusively of the vector sequences and a 340-bp fragment harboring the FLP site. The electrophoretic pattern of the latter fragment after incubation with FLP provided a clear distinction between a binding and a nonbinding substrate. Figure 3B shows the results for a good binder (D₂) and a nonbinder (D₈). Substrates D₁, D₄, D₅, D₁₀, and D₁₁ were similar to D₂ in FLP binding. On the other hand, with D₃, D₆, D₇, D₈, and D₉ it was hard to detect any shift in the migration of the 340-bp fragment in ethidium bromide-stained gels. Since the assay is not sensitive enough to detect weak binding, we also checked each of the substrates by Southern blot hybridization. The electrophoresed DNA fragments were transferred to nitrocellulose and probed with a ³²P-labeled DNA fragment that contained a copy of the 2 μ m circle repeat. The results of the assay for three representative substrates (D₁, D₃, and D₈) are shown in Fig. 3C. The method is capable of distinguishing between a weak binder and a nonbinder. For example, in this assay, D₃ showed a modest but finite level of binding, whereas D₈ showed no binding at all. Similarly, D₉ also tested as a nonbinder, whereas D₆ and D₇ showed a low level of binding, approximately 30 to 50% of that for D₃. It is interesting to contrast the binding properties of D₁₀ and D₁₁ with that of D₃. D₁₀ and D₁₁ may be looked on as the asymmetric counterparts of D₃. While D₃ contains a pair of symmetric base substitutions at position 9 or 9', D₁₀ and D₁₁ contain identical but asymmetric substitutions at positions 9 and 9', respectively. Both D₁₀ and D₁₁ bound FLP efficiently, whereas D₃ was a poor FLP binder.

Recombination potential of the synthetic FLP substrates. Each synthetic substrate was tested in two types of recombination assay: recombination with an intact copy of the wild-type 2 μ m circle repeat and recombination with a second copy of itself. The rationale of the assay is that recombination between the FLP sites on a pair of suitably designed parental DNA fragments gives rise to two new fragments.

FIG. 3. Binding of FLP to the recombination site. (A) Plasmid pJR1, digested with *Pst*I and *Hind*III, was incubated in 50 mM Tris hydrochloride (pH 7.5)–0.15 M KCl–10 mM MgCl₂–200 μ g of bovine serum albumin per ml–1 mM dithiothreitol with partially purified FLP protein (Prasad and Jayaram, unpublished) in a total volume of 25 μ l at 30°C for 30 min. After the addition of heparin (5 μ g/ml) and incubation for 5 min at room temperature, 5 μ l of 50% glycerol containing 0.1% bromophenol blue was added to the reaction mixture, and half of each sample was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Each assay mixture contained 4 μ g of the digested plasmid DNA. Lanes: 1, 0 μ g of FLP; 2, 0.25 μ g of FLP; 3, 0.5 μ g of FLP; 4, 0.75 μ g of FLP. At a protein-to-DNA ratio of less than or equal to 1.5 (wt/wt), FLP binds specifically to the fragment (indicated by the arrow) containing the 2 μ m circle repeat (which includes the FLP site). (B) Binding assays with the synthetic sites were carried out with *Eco*RI-digested plasmids of the pPYD series as described for panel A. The synthetic FLP sites are contained within the smaller of the two *Eco*RI plasmid fragments (340 bp long). Results for D₂, which is a symmetric substrate active in recombination, and for D₈, which is virtually inactive in recombination, are shown. D₄, D₅, D₁₀, and D₁₁ gave binding patterns similar to that of D₂. Hardly any binding could be detected for D₃, D₆, D₇, and D₉ in an ethidium bromide-stained gel. (C) True nonbinding substrate could be distinguished from a weak binder by transferring the DNA fragments (see panel B) to nitrocellulose and hybridizing them to a ³²P-labeled 900-bp *Hind*III fragment from the A form of the 2 μ m circle that includes a copy of the repeated segment (7). The autoradiograms for three representative substrates, D₁ (a good binder), D₃ (the best of the weak binders), and D₈ (a nonbinder), are shown. The pattern for D₆ and D₇ was similar to that of D₃, although the level of binding was only 30 to 50% of that for D₃; D₉ was indistinguishable from D₈. The two additional bands in the autoradiogram for D₁ must be the result of a trace contamination in the plasmid preparation used for the assay; since the hybridization probe is specific to the 2 μ m circle repeat, these DNA fragments most likely contain the FLP sites. The shift in their mobility with increasing FLP concentration was not unexpected.

The difference in size between the recombinant molecules and the parents permits their identification by electrophoresis in agarose gels. The parental DNA fragments are designated by F and the recombinant DNA fragments by R (Fig. 4 and 5).

(i) **Self-recombination.** In the self-recombination assays, the ability of a synthetic substrate or an intact 2 μ m circle repeat to undergo recombination with a second copy of itself

FIG. 4. Efficiency of synthetic substrates in self-recombination. Plasmid pJR1 (or pPYD) was cut separately with *EcoRI* and *PvuII*. After phenol extraction and purification, an equimolar mixture of the products of the two digestions were incubated with FLP in a volume of 50 μ l in 50 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$ –1 to 2% glycerol–50 mM NaCl at 30°C for 30 min. At the end of the incubation the reaction mixture was made 0.2% with respect to sodium dodecyl sulfate, 30 mM in EDTA, 10% in glycerol, and 0.02% in bromophenol blue and heated at 65°C for 5 min. Half of this mixture was electrophoresed on a 1% agarose gel. The DNA was visualized by ethidium bromide staining. Bands F_1 , F_2 , and F_3 correspond to parental fragments and R_1 and R_2 to recombinant products, respectively. Notice that the lack of perfect alignment of the DNA fragments for the various substrates results from the fact that not all of the samples were run on a single gel. Panel L: Results of self-recombination with increasing concentrations of FLP for pJR1 are shown. Lane 1, 0 μ g of FLP; lane 2, 0.5 μ g of FLP; lane 3, 1.0 μ g of FLP. DNA concentration per reaction was 3 to 4 μ g. Panel M: Reactivity of each of the synthetic substrates in self-recombination. Each lane represents the result of a 50- μ l incubation containing 1 μ g of FLP. Panel R: With some of the weak substrates the recombinant bands were not easily visible on ethidium bromide-stained gels. DNA was transferred from these gels to nitrocellulose and probed with ^{32}P -labeled pPY190 DNA.

was determined. The results for the 2- μ m circle repeat contained on plasmid pJR1 are shown in Fig. 4, panel L. Plasmid pJR1 was linearized with *EcoRI* or *PvuII*, and the two types of linear molecules were reacted with each other in the presence of FLP in equimolar amounts. Similarly, a pPYD plasmid was cut with *EcoRI* or *PvuII*, and an equimolar mixture of the products of the two digestions was incubated with FLP (Fig. 4, panel M). The assay detects only those recombination events that occur between two molecules cut with different restriction enzymes. Reaction between two molecules cut with the same restriction enzyme would be undetected as the products are indistinguishable from the reactants. As shown in Fig. 4 (panel M) and in Table 1, D_1 , D_2 , D_4 , D_5 , D_{10} , and D_{11} were all active in self-recombination. In contrast, D_3 , D_6 , and D_7 were considerably weaker substrates. However, they did undergo modest but significant levels of recombination, as demonstrated by Southern blot hybridization (Fig. 4, panel R). Among the three, D_3 was a slightly more reactive substrate than either D_6 or D_7 . The substrates D_8 and D_9 were almost completely inactive in self-recombination. For both of these substrates no recombinant bands could be detected even after Southern blotting and hybridization to a radiolabeled probe (Fig. 4, panel R).

(ii) Recombination of a synthetic substrate with a copy of the 2- μ m circle repeat. Recombination between a synthetic substrate and a copy of the intact 2- μ m circle repeat was carried out as follows. Plasmid pJR1 linearized with *PvuII* was mixed with an equimolar amount of the pPYD plasmid cut with *EcoRI* and reacted with FLP. The synthetic substrate is contained within the 340-bp *EcoRI* fragment of pPYD. Recombination between the FLP sites in linear pJR1 and the pPYD fragment would result in two recombinant fragments whose lengths can be accurately predicted. Each of the substrates active in self-recombination was also active in recombination with the 2- μ m circle repeat (Fig. 5, panel L). The interesting observation, however, is that D_3 , D_6 , and D_7 ,

which were poorly active in self-recombination, were normal or nearly normal in recombination with the 2- μ m circle repeat. Thus, the presence of the wild-type substrate appears to restore the reactivity of these mutant substrates. Even D_8 and D_9 , which were totally inactive in self-recombination, reacted with the 2- μ m circle repeat, albeit inefficiently. In an autoradiogram of the Southern blotted DNA hybridized to a ^{32}P -labeled probe, the recombinant fragments were seen as faint bands (Fig. 5, panel R).

DISCUSSION

Previous work permits the division of the FLP substrate into two structural parts—a core sequence and a dyad symmetry flanking it (5, 11, 22). Recent experiments strongly suggest that these two structural parts may play distinct functional roles as well (23). The core sequence is involved in the homologous alignment of two recombination sites via DNA pairing. The natural asymmetry of the core sequence allows the recombination system to orient the recombining sites and to impart directionality to the reaction. However, the fact that several types of alterations within the core are not deleterious to the recombination reaction strongly implies that little or no direct interaction takes place between the bases of the core region and the FLP protein. The DNA-protein interactions relevant to the recombination reaction appear to be confined entirely to the symmetry elements. The results presented here for synthetic substrates carrying point mutations within the symmetry elements clearly substantiate this inference.

The difference in the relative reactivity of the various substrates permits the identification of several positions within the symmetry elements that are specially pertinent to interactions with FLP. Substrates D_8 and D_9 , which carry symmetric mutations at position 7 and 7', a transversion from C · G to G · C in D_8 from C · G to A · T in D_9 , were virtually inactive in recombination and in FLP binding.

TABLE 1. Binding and recombination proficiency of synthetic FLP substrates

Substrate	Mutation position(s)	Nature of mutation	Symmetry ^a	Binding to FLP ^b	Recombination ^b	
					Self	With 2 μ m circle repeat
D ₁	None		S	+	+	+
D ₂	8 and 8'	C · G → T · A	S	+	+	+
D ₃	9 and 9'	T · A → C · G	S	±	±	+
D ₄	10 and 10'	A · T → G · C	S	+	+	+
D ₅	10 and 10'	A · T → C · G	S	+	+	+
D ₆	11 and 11'	T · A → C · G	S	±	±	+
D ₇	12 and 12'	A · T → G · C	S	±	±	+
D ₈	7 and 7'	C · G → G · C	S	—	—	±
D ₉	7 and 7'	C · G → A · T	S	—	—	±
D ₁₀	9	T · A → C · G	A	+	+	+
D ₁₁	9'	T · A → C · G	A	+	+	+

^a S and A, Symmetric and asymmetric substrates, respectively.

^b +, Good substrate; ±, poor substrate; ±±, extremely poor substrate; —, nonsubstrate.

Symmetric mutations close to the spacer region, T · A to C · G at position 11 (D₆) and A · T to G · C at position 12 (D₇), resulted in significant reduction in FLP binding and in the extent of self-recombination. However, both D₆ and D₇ showed almost normal reactivity in recombination with the 2 μ m circle repeat. Thus, the effects of these mutations are annulled when one of the reactants contains the wild-type sequence. A very similar situation was encountered with D₃, which harbors a symmetric pair of mutations at positions 9 and 9' (T · A to C · G). The mutation resulted in a large drop in FLP binding and in self-recombination. In contrast, the effect of this mutation in recombination with the 2 μ m circle repeat was minimal.

Together, our results imply that positions 8 and 10 of the symmetry element may be altered without sacrificing the binding and catalytic properties of the substrate; on the other hand, mutations at positions 7, 9, 11, and 12 reduce both binding and catalytic properties to various degrees. We interpret these results to mean that positions 7, 9, 11, and 12 represent important points of interaction between the FLP protein and its DNA substrate. These conclusions are substantiated by methylation protection and recombination interference experiments (R. C. Bruckner and M. M. Cox, personal communication). However, there is one notable discrepancy between our results and those of Bruckner and Cox. According to these authors, positions 8 and 8' of the symmetry elements are contact points for the FLP protein. Our studies show that a mutation at these positions (C · G to

T · A in D₂) has little effect on FLP binding or on recombination. In light of our results, we would like to consider position 8 as a point of incidental contact and positions 7, 9, 11, and 12 as points of critical contact. It must be pointed out that the most deleterious effect on FLP recombination was observed for mutations of position 7. Interestingly, a mutation of the FLP recombination site obtained by in vitro mutagenesis and identified by genetic selection in *S. cerevisiae* also maps to this position (15). As would be expected from the data presented here, this mutation also markedly reduces the ability of a substrate harboring the mutation to bind to FLP (P. D. Sadowski, personal communication).

The behavior of the synthetic substrates in FLP binding and in recombination clearly substantiates the role of the symmetry elements in mediating substrate recognition by FLP. This is brought out by the strong correlation between the ability of a mutant to bind to FLP and its efficiency in self-recombination. Among the substrates tested, there was not one that bound well to FLP but reacted poorly in recombination. Another feature of FLP recombination revealed by the synthetic substrates is the role of cooperativity in the reaction. First, there is intrasubstrate cooperativity, as illustrated by a comparison of D₃ with D₁₀ and D₁₁. D₃, which contains symmetric mutations at positions 9 and 9' of the two symmetry elements, was a weak substrate in binding to FLP and in self-recombination. In contrast, both D₁₀ and D₁₁, which contain the same point mutation as D₃ but in only one of the two symmetry elements, were almost indistin-

FIG. 5. Recombination of synthetic substrates with an intact 2 μ m circle repeat. Linear pJR1 (obtained by digestion with *Pvu*II) was reacted with *Eco*RI-digested pPYD plasmids as described in the legend to Fig. 4. F₁, F₂, and F₃ are parental plasmid bands; R₁ and R₂ are recombinant bands. Panel L: Reaction containing 1.0 μ g of FLP and substrates D₁ through D₁₁. Panel R: For a weakly reactive substrate, the sensitivity of the assay was increased by immobilization of gel-fractionated DNA on nitrocellulose and hybridization to ³²P-labeled pJR1.

guishable from the wild-type substrate in binding and in recombination. Thus, the defect in one symmetry element can be overcome by a good copy of the second symmetry element. There is also intersubstrate cooperativity. Each of the mutant substrates which was defective in FLP binding and in self-recombination was significantly improved in its reactivity when its partner in recombination was an intact 2 μ m circle repeat. Although the term cooperativity is not used here in its conventional enzymological connotation, it is not unlikely that the facilitation of reactivity within a substrate or between substrates observed here is mediated by interactions between multiple units of the FLP protomer.

Although symmetry is not an absolute requirement for sequence-specific DNA-protein binding (for example, RNA polymerase), several DNA-binding proteins, such as the *lac* repressor, Cro, CI, and CAP use symmetrically related subunits to interact with twofold-related sites in their operator sequences. Furthermore, a perfectly symmetric *lac* operator binds the *lac* repressor more tightly than the wild-type operator does (20). The experiments described here imply that such symmetric interactions may be germane to recognition by FLP of its substrate DNA. Several single point mutations of the FLP substrate within the symmetry element 1a, when compensated by complementary mutations in 1'a (thereby preserving symmetry), did not abolish FLP binding or catalysis by FLP. However, absolute structural symmetry within each of the participant sites is not essential for its recognition by FLP. D₁₀ and D₁₁, which contain asymmetric mutations at positions 9 and 9', respectively, behaved essentially like the wild-type substrate in FLP binding and in recombination. This is not particularly surprising, since most, if not all, DNA-binding proteins rely on approximate, not absolute, symmetry for recognizing their targets. It is interesting, therefore, to explore whether substrates containing stronger perturbations in the form of double or triple asymmetries would be inefficient or inactive in recombination and, if so, whether they could be revived by restoration of symmetry through compensatory mutations.

ACKNOWLEDGMENTS

We thank Philip Youderian for providing the pPY190 plasmid. We are grateful to P. D. Sadowski and M. M. Cox for sharing their results with us before publication.

This work was supported by Public Health Service grant GM 35654-01 from the National Institutes of Health.

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