Selection of novel, specific single-stranded DNA sequences by Flp, a duplex-specific DNA binding protein

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ABSTRACT

Flp is a member of the integrase family of site-specific recombinases. Flp is known to be a double-stranded (ds)DNA binding protein that binds sequence specifically to the 13 bp binding elements in the FRT site (Flp recognition target). We subjected a random pool of oligonucleotides to the in vitro binding site selection method and have unexpectedly recovered a series of single-stranded oligonucleotides to which Flp binds with high affinity. These single-stranded oligonucleotides differ in sequence from the duplex FRT site. The minimal length of the oligonucleotides which is active is 29 nt. This single strand-specific DNA binding activity is located in the same C-terminal 32 kDa domain of Flp in which the site-specific dsDNA binding activity resides. Competition studies suggest that the apparent affinity of Flp for single-stranded oligonucleotide is somewhat less than for a complete duplex FRT site but greater than for a single duplex 13 bp binding element. We have also shown that Cre, another member of the integrase family of site-specific recombinases, also exhibits single-stranded DNA binding activity similar to that of Flp.

INTRODUCTION

The selfish 2μm plasmid DNA of Saccharomyces cerevisiae encodes the Flp protein, which is a member of the integrase family of conservative site-specific recombinases (1–3). Flp mediates a site-specific recombination event between two 599 bp inverted repeats on the 2μm plasmid (4) and functions to amplify the plasmid in the cell (5–7).

The Flp recognition target site (FRT) is confined to 48 bp within the 599 bp inverted repeats of the 2μm plasmid (8,9). The FRT site includes an 8 bp core region flanked by three Flp binding sites that are 13 bp in length (symmetry elements a, b and c; Fig. 1). Biochemical studies with purified Flp and DNA substrates show that recombination initiates by Flp binding specifically to the FRT site (10–13) and inducing a bend in the DNA (14,15). Protein–protein interactions between Flp molecules bring two FRT sites together to form a synapatic complex (16). Site-specific cleavage results from a nucleophilic attack of Tyr343 on two phosphodiester bonds at the margins of the core region (17). A phosphotyrosine bond is formed between the 3′-phosphoryl end of the nicked DNA and the Flp protein (18,19). Strand exchange and ligation occur when the free 5′-OH of the nick from the partner DNA acts as a nucleophile to attack the phosphotyrosine bond and regenerate a new phosphodiester bond. The Holliday junction resulting from the first strand exchange (20–23) is resolved through a second round of strand cleavage and ligation (24,25).

DNase I and chemical footprinting studies showed that all three symmetry elements and the core region of the duplex FRT site are protected by Flp (10,12,26). Flp binds first to symmetry element b; then to element a and finally to element c. The C-terminal domain of Flp (P32) is responsible for the sequence-specific ssDNA binding property of Flp, whereas the N-terminal peptide P13 has non-specific DNA binding activity and stimulates binding to >90% purity. Wild-type Flp protein was either a Sephacryl S300 fraction or a Mono S fraction, purified as described previously (28). Histidine-tagged fusion peptides of P13 and P32 were purified on Ni–NTA columns as described by A.Shaikh (30) and were generous gifts of A.Shaikh. Expression and purification

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of the Cre protein was essentially as described by Shaikh and Sadowski (31) with some modifications. The Cre protein was purified by heparin–agarose and Mono S chromatography and was a generous gift of J. Walker. The concentration of protein was estimated using the method of Bradford (32).

**Synthetic substrates**

Oligonucleotides were synthesized by the Biotechnology Service Centre, University of Toronto. Duplex FRT sites were prepared by annealing the appropriate oligonucleotides in 5 mM MgCl₂, 100 mM NaCl as described previously (33, 34). A 10-fold excess of unlabelled over labeled oligonucleotides was used in the annealing reactions. Where appropriate, the 5′-termini of oligonucleotides were labeled with [γ-³²P]ATP by T4 polynucleotide kinase.

DNA binding assays

A radiolabeled DNA probe (0.02 pmol) was incubated with protein in 30 μl binding buffer (50 mM Tris–HCl, pH 7.5, 33 mM NaCl, 1 mM EDTA) containing 2 μg sonicated and denatured calf thymus DNA (CT DNA). When competition experiments were performed an additional excess of specific DNA competitor was also added to the reactions. After 25 min incubation at room temperature the reaction mixture was loaded onto an 8% non-denaturing polyacrylamide gel. The amount of Flp protein in each reaction was ~5.8 pmol, whereas the amount of Flp peptides (P13 and P32) and the Cre protein used was varied as stated in the figure legends. The results of the competition experiments were quantitated using a Molecular Dynamics PhosphorImager.

When DNA binding assays were performed using the in vitro transcription–translation mixtures 20 or 30 μl translation mixture were combined with 0.02 pmol radiolabeled DNA probe in 40 μl binding buffer (50 mM Tris–HCl, pH 7.5, 33 mM NaCl, 1 mM EDTA) containing 2 μg CT DNA and 240 pmol non-specific oligonucleotide ssDNA [SAD3499 (43mer), TAGGGCCCATGGAATTTTGTAAACACCACC].

**In vitro binding site selection**

**In vitro transcription and translation assays**

In vitro expression of Flp was achieved using the Promega TNT® T7 coupled reticulocyte lysate system. Approximately 2 μg supercoiled plasmid pLD3 DNA (28), which contains the Flp gene under control of the T7 promoter, was used as template with 40 μCi [³⁵S]methionine (1000 Ci/mmol) in 50 μl reticulocyte lysate. As a control the in vitro transcription and translation assays were also carried out using plasmid pAR3038, which has the same transcriptional promoter and terminator as pLD3 but lacks the Flp gene (35). The translation mixtures were used immediately for DNA binding and DNA recombination assays.

**Figure 1.** The sequence of the Flp recognition target site (FRT). The FRT site is 48 bp in length and includes 13 bp symmetry elements (a, b and c, indicated by horizontal arrows) and an 8 bp core region (rectangle). Each symmetry element is a Flp binding element. Elements a and b are inverted repeats which differ in 1 bp. Element c is a direct duplication of element b in both sequence and orientation. Flp cleaves DNA at the junctions of the symmetry elements and the core sequence as indicated by the two vertical arrows.

![Diagram of Flp recognition target site (FRT)](image)

**Table 1.** Synthetic oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLP-10-T(56mer)</td>
<td>TCGACTGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>FLP-10-B(56mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>SS-1 (18mer)</td>
<td>CAATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<tr>
<td>SS-2 (18mer)</td>
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<tr>
<td>SS-3 (18mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<tr>
<td>SS-4 (18mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>SS-5 (9mer)</td>
<td>CAATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>SS-6 (10mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>SS-7 (9mer)</td>
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<tr>
<td>FRTB-T(54mer)</td>
<td>TCGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<td>FRTB-B(54mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<tr>
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<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<td>FRTB-B(55mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
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<tr>
<td>FRTA-T(5mer)</td>
<td>TCGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
<tr>
<td>FRTA-B(5mer)</td>
<td>CGACATCGAGTGGAGATGAGGTGAGGTAATGCTAGTGAAGCC</td>
</tr>
</tbody>
</table>

The left-hand flanking sequence is indicated in italics and the right-hand flanking sequence is shown in lower case. The underlined nucleotides represent sequences that were randomized during site selection. The shaded nucleotides indicate the sequence of the symmetry elements; the core sequence of the FRT site is double underlined. Oligonucleotides FRTB-T and FRTB-B were hybridized to give duplex FRTs. Oligonucleotides FRTB-T and FRTB-B were annealed to form duplex FRTab. The concentrations of the oligonucleotides were measured spectrophotometrically using the relation 1 OD₆₀₀ = 30 μg oligonucleotide as specified by Sambrook et al. (37).
was removed from the gel slice, extracted twice with phenol/chloroform and then precipitated with ethanol using 20 µg glycogen (Boehringer) as carrier. PCR amplification of the eluted DNA was performed using the Perkin-Elmer Cetus amplification kit in the presence of primers P1 (see above) and P2. Primer P2 (5'-CGATGTCGACTCGAGTCGA-3') is complementary to the 5' non-random sequence of the template. The amplification used a 30 s denaturation step at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C for 25 cycles. The amplified oligonucleotides were extracted twice with phenol/chloroform, precipitated with ethanol and purified on a non-denaturing polyacrylamide gel. The dsDNA fragment was then radiolabeled by T4 polynucleotide kinase in the presence of [γ-32P]ATP and used as a substrate for binding by Flp in the next round of selection. As a negative control a blank slice of the gel was excised after each round and treated in the same manner as the band containing bound protein and DNA. No PCR product was amplified from the control, indicating that there was no template contamination. The selection procedure was repeated for a total of four rounds. After the final round of selection the enriched DNA pool was digested with SalI and EcoRI and cloned into SalI/EcoRI-digested pUC19. DNA from individual clones was isolated using a Wizard Minipreps DNA purification system from Promega and then sequenced using a sequencing kit obtained from US Biochemicals.

RESULTS

Recovery of Flp binding sites by in vitro binding site selection

We originally designed experiments to select DNA sequences to which certain mutant Flp proteins might bind and we used wild-type Flp as a control. Our selection strategy used an electrophoretic mobility shift assay to enrich for DNA sequences that were bound by Flp (36).

A pool of DNA sequences which contained a randomized 18 bp internal region flanked by two constant regions was incubated with Flp protein and the protein–DNA complexes were resolved from the free DNA on a non-denaturing polyacrylamide gel. The bound DNA was eluted, amplified by PCR and used in subsequent rounds of selection. After four rounds of selection a large amount of complex was formed from the selected pool of DNA that was subjected to the gel mobility shift assays in the presence of Flp (Fig. 2A, lanes 2 and 3).

Figure 2. (A) Binding of Flp to the mixture of oligonucleotides before and after four rounds of binding site selection. The binding reactions were done as described in Materials and Methods. Lane 1 was a control, where the DNA substrate was double-stranded FRT site (Flp-FRTab) containing two symmetry elements flanking the core sequence. Binding of Flp to Flp-FRTab generates two complexes (CI and CII), representing Flp molecules binding to one element and two symmetry elements respectively. Lanes 2 and 3 contained Flp and oligonucleotides with random sequences. Lane 2 shows the first binding reaction in which random oligonucleotides had undergone no rounds of selection (none), whereas lane 3 was the binding reaction in which the mixture of oligonucleotides had gone through four rounds of selection and PCR enrichment (four). The DNA oligonucleotides from the complex in lane 3 were eluted, amplified by PCR and cloned into the pUC19 vector. S refers to free labeled oligonucleotides. (B) Analysis of binding of Flp to selected sequences. Synthetic oligonucleotides corresponding to Flp-10-T, Flp-10-B, FRTb-T and FRTb-B (Table 1) were used to assemble selected double-stranded sequences. DNA sequences were 5'-end-labeled with [32P] (asterisks). The binding assays were done in the presence of Flp and DNA substrates as described in Materials and Methods. DNA substrates and the presence or absence of Flp are indicated above each lane. In this and subsequent figures CP represents protein–DNA complexes, DS refers to labeled dsDNA substrates, SS represents labeled ssDNA oligonucleotides.
Figure 3. Summary of DNA sequences selected by Flp. Thirty one site-selected oligonucleotides were sequenced after cloning into pUC19, revealing the existence of three families of binding sequences. Bases that were randomized during the binding site selection are highlighted in bold. Sequences that are not in bold represent the left flanking sequences that were invariant during site selection. The invariant right flanking sequences are not shown. The first 7–9 conserved nucleotides of each family are underlined. Studies in this paper were done using the oligonucleotide Flp-10-T, which corresponds to the sequence of F-10 (bold, left column). Oligonucleotides corresponding to F-14, F-16 and F-19 (bold, left column) also showed top strand-specific, single strand-specific DNA binding by Flp.

The enriched DNA sequences were cloned into pUC19 and inserts from 31 independent clones were sequenced. As shown in Figure 3, sequences selected by wild-type Flp fell into three families. To our surprise none of the sequences resembled the Flp binding symmetry elements of the FRT site (Fig. 1). Although each family had its own consensus sequence, all three families showed a striking common feature, i.e. the first 7 nt of the randomized internal region within each family were absolutely conserved. This seemed to imply that the left-hand flanking sequence may have played a role in binding site selection.

We also did mobility shift assays on several of the selected sequences from the first two families. These assays showed that Flp failed to bind strongly to any of the selected sequences when the selected sequences were dsDNA, whether excised from a plasmid or formed from annealed synthetic oligonucleotides (see Fig. 2B and data not shown).

Flp binds to ssDNA sequence specifically

While wild-type Flp bound efficiently to the duplex FRTb site (Fig. 2B, lane 2), it was not able to form a large amount of protein–DNA complex when assayed with one of the duplex selected sequences (Fig. 2B, lane 4). However, the small amount of binding complex seen in lane 4 of Figure 2B seemed to have arisen from Flp binding to a DNA species which migrated faster than the dsDNA substrates, since the intensity of these bands diminished upon incubation with Flp (triangles, lane 4). These DNA bands likely correspond to ssDNA resulting from some denaturation of dsDNA substrates. Therefore, we decided to investigate whether Flp would bind to ssDNA of the selected sequence. We selected Flp-10 sequence and made DNA oligonucleotides corresponding to its top and bottom strands. Each of the oligonucleotides was 32P 5' -end-labeled and incubated with Flp. As shown in Figure 2B, Flp bound only to the top strand of the Flp-10 sequence but not to the complementary bottom strand (lane 4 and lanes 6, 8). As a control the first 13 bp in the random core region of the selected Flp-10 sequence were replaced by the 13 bp Flp binding symmetry element b and this new sequence was named Flp-FRTb. As expected, Flp bound to dsDNA Flp-FRTb readily (Fig. 2B, lane 2). However, when the oligonucleotide corresponding to either the top or the bottom strand of the Flp-FRTb sequence was incubated with Flp protein Flp failed to bind to either of these single-stranded substrates (Fig. 2B, lanes 6 and 8). These results suggest that Flp has a sequence-specific ssDNA binding activity. We obtained identical results with three other selected sequences, Flp-14, 16 and 29 (data not shown).

Figure 4. Binding of Flp peptides to ssDNA. ssDNA (SS, Flp-10-T and Flp-10B), as well as duplex DNA Flp-FRTb (DS), was 5'-end-labeled with 32P. Lanes 1, 5 and 10 contained only the labeled DNA substrates. Flp and Flp peptides were added as indicated above the lanes. Triangles indicate increasing amounts of Flp peptides. The amounts of Flp peptides were as follows: lanes 6 and 11, 77 pmol P13; lanes 3, 7 and 12, 231 pmol P13; lanes 8 and 13 and 13, 31.25 pmol P32; lanes 4, 9 and 14, 62.5 pmol P32.
(SAD3499) had no effect on binding of Flp to the single-stranded Flp-10-T sequence (data not shown). The fact that addition of Flp-10-B abolished binding of Flp to Flp-10-T was likely due to annealing of the complementary strand to the labeled single-stranded probe to form dsDNA. Since this duplex sequence is different from the duplex Flp binding symmetry elements, Flp could not bind to it. These results further support the idea that Flp binds to ssDNA in a sequence-specific manner.

**The single strand-specific DNA binding activity is attributable to Flp**

Although binding site selection was done using highly purified Flp protein (>90% pure), it was formally possible that the activity was due to a contaminant in the Flp preparation. However, several preparations of purified wild-type Flp protein and mutant Flp proteins, all of which have normal binding activity to the duplex FRT site, bound to the Flp-10-T sequence but not to Flp-10-B (data not shown), suggesting that this single strand-specific DNA binding activity is not peculiar to one protein preparation.

We next tested the ability of Flp peptides to bind to ssDNA. The Flp protein can be partially proteolyzed into an N-terminal domain of 13 kDa (P13) and a C-terminal domain of 32 kDa (P32) (28,29). The N-terminal P13 peptide does not display site-specific DNA binding activity but has been demonstrated to stimulate binding of P32 to the Flp binding symmetry element. On the other hand, the C-terminal P32 peptide retains the site-specific DNA binding property of Flp. Highly purified, recombinant His-tagged peptides P13 and P32 were each incubated separately with Flp-10-T and Flp-10-B. Only the P32 peptide showed sequence-specific ssDNA binding activity (Fig. 4, lanes 8 and 9). Weak binding was also detected when P32 was incubated with Flp-10-B (Fig. 4, lanes 13 and 14). This was likely due to the non-specific ssDNA binding activity of P32, since binding of P32 to Flp-10-B was abolished when excess non-specific ssDNA competitor (SAD3499) was added to the binding reactions (data not shown), whereas this non-specific ssDNA competitor had no effect on P32 binding to Flp-10-T (data not shown). These results strongly support our claim that Flp has an intrinsic sequence-specific ssDNA binding activity. Furthermore, the single-strand-specific DNA binding activity resides in the 32 kDa C-terminal portion of the Flp protein (P32).

To further substantiate the evidence that the ssDNA binding activity is specifically associated with Flp, the wild-type Flp protein was synthesized in vitro using an in vitro transcription and translation system in the presence of [35S]methionine. SDS–PAGE revealed that production of Flp was dependent on addition of a plasmid containing a Flp gene (data not shown). No Flp was made in the absence of plasmid DNA or with a plasmid (pAR3038) that lacked the Flp gene (data not shown). Like Flp generated in vivo, the Flp protein made in vitro exhibited site-specific dsDNA binding activity to the FRT sequence (Fig. 5A, B).

**Figure 5.** Analysis of DNA binding activity of the wild-type Flp protein generated in vitro in a reticulocyte lysate. (A) Gel mobility shift assays using duplex DNA as substrates. dsDNA Flp-FRTab containing two symmetry elements flanking the core sequence was 5′-end-labeled with 32P. Lane 1 contained only the labeled DNA substrate. The contents in each reaction were indicated above the lanes. Triangles indicate increasing amounts of translation products or purified Flp. Lanes 2, 4 and 6, 10 µl translation products; lanes 3, 5 and 7, 20 µl translation products; lanes 8 and 9, 2.9 and 5.8 pmol purified Flp. CI and CII were as defined in Figure 2. (B) Analysis of binding of ssDNA by translation products and purified Flp. ssDNA Flp-10-T was 5′-end-labeled with 32P. The contents of each reaction are shown above the lanes. The amounts of translation products and purified Flp in lanes 1–9 were identical to those in (A).
Flp protein also exhibits single strand-specific DNA binding activity. To confirm this it will be necessary to produce highly purified Cre protein of phage P1. As shown in Figure 6, Cre, like Flp, also bound only to Flp-10-T and not Flp-10-B (lanes 4 and 7 versus lanes 6 and 8). Furthermore, binding of Cre to the Flp-10-T sequence was not affected by addition of a large excess of non-specific DNA SAD3499 (data not shown). This suggests that Cre also exhibits a sequence-specific ssDNA binding activity. To confirm this it will be necessary to carry out random site selection with Cre recombinase.

The length of sequence required for binding of Flp to ssDNA

We have shown that Flp binds single-stranded oligonucleotides Flp-10-T in a sequence-specific manner. To define the minimal length of sequence necessary for binding of Flp we carried out gel mobility shift assays using synthetic oligonucleotides derived from Flp-10-T and the results are summarized in Figure 7. The sequence of Flp-10-T ssDNA can be divided into three parts (Fig. 7): (i) the internal region of 18 nt which was randomized during site selection; (ii) the left flanking sequence (Lf, 19 nt); (iii) the right flanking sequence (Rf, 19 nt). Both the left and right flanking sequences were invariant. Flp failed to bind to single-stranded oligonucleotides containing either the internal region alone (SS-1) or the internal region and the Rf region (SS-3, Fig. 7). However, Flp bound to an oligonucleotide containing the internal region and the Lf region (SS-2) as efficiently as to Flp-10-T. This suggested that a portion of the Flp recognition site lay within the sequence of SS-2 (Fig. 7). To define the left-hand boundary of the Flp binding site we made a series of oligonucleotides with deletions of the 5'-region in the left flanking sequence of SS-2. As shown in Figure 7, SS-5, SS-9, SS-10, SS-11 and SS-8 contained deletions of 7, 6, 5, 4, and 3 nt respectively from the 5'-end of SS-2. Mobility shift assays showed that only SS-8 (3 nt deletion) was able to bind Flp as efficiently as SS-2 (data not shown and Fig. 7). Although SS-5 was bound weakly by Flp, SS-9, SS-10 and SS-11, which contained smaller deletions in the Lf region than did SS-5, were not bound by Flp at all. Therefore, we conclude that SS-8 contained the left-hand boundary of the ssDNA binding site of Flp.

We also examined the right-hand boundary of the binding site of Flp. As shown in Figure 7, oligonucleotides SS-12, SS-13 and SS-14 contained deletions of 7, 5, and 3 nt respectively from the 3'-end of SS-8. Mobility shift assays showed that Flp failed to bind to SS-12 but it bound to both SS-13 and SS-14 as efficiently as it bound to SS-8 (data not shown and Fig. 7). Although SS-5 was bound weakly by Flp, SS-9, SS-10 and SS-11, which contained smaller deletions in the Rf region than did SS-5, were not bound by Flp at all. Therefore, we conclude that SS-8 contained the right-hand boundary of the ssDNA binding site of Flp.

Relative binding of Flp to ssDNA versus to dsDNA

It is known that Flp binds specifically to the duplex 13 bp symmetry elements of the FRT site. Therefore, it was of interest to compare the binding of Flp to ssDNA with that to dsDNA. To perform this comparison a series of competition experiments was carried out.

First, duplex DNA containing either one Flp binding symmetry element (Flp-FRTb) or two Flp binding symmetry elements in inverted orientation (Flp-FRTab) were used as competitors added directly to a binding reaction containing labeled ssDNA (Flp-10-T). For comparison, the unlabeled ssDNA (Flp-10-T) was also used as competitor. The specific complex formed by binding of Flp to ssDNA (Flp-FRTb) was competed efficiently with Flp-FRTab (Table 2, line 2). Less than 1 pmol Flp-FRTab was needed to give 50% inhibition of complex formation. However, ~50 pmol Flp-FRTb were required to inhibit formation of the complex by 50% (Table 2, line 2). When Flp-10-T itself served as competitor DNA ~5 pmol were required to inhibit complex formation by 50% (Table 2, line 2). These results indicate that Flp binds ~10-fold more efficiently to the ssDNA (Flp-10-T) than to dsDNA containing one Flp binding symmetry element. The dsDNA (Flp-FRTab) was the most effective...
**Figure 7.** Analysis of the minimal sequence of the ssDNA (Flp-10-T) for binding of Flp. The sequence of Flp-10-T is diagramed above. The core sequence randomized prior to site selection is underlined, separating the invariant left and right flanking sequences (Lf in italics and Rf in lower case respectively). Stippled bars represent the regions of the Flp-10-T sequence that are present in the oligonucleotides. The relative ability of Flp to bind to these single-stranded oligonucleotides is summarized on the right: +, binding approximately equal to that shown by Flp-10-T; –, no binding; +/-, reduced binding. Vertical arrows above the sequence of Flp-10-T denote the minimal sequence required for binding of Flp to the ssDNA.

A competitor, probably due to cooperative interactions between two Flp molecules bound to the two 13 bp symmetry elements (11). Similar relative affinities were obtained by examining the ability of single-stranded Flp-10-T and duplex Flp-FRTb to inhibit formation of complexes formed by Flp and Flp-FRTb and the ability of excess Flp-10-T, Flp-FRTb and Flp-FRTab to inhibit formation of complex II by Flp and Flp-FRTab (Table 2, lines 1 and 3). These results confirm that Flp binds to Flp-10-T more efficiently than to Flp-FRTb.

**Table 2.** Approximate amounts of competitor required to give 50% inhibition of complex formation by Flp and the DNA probe

<table>
<thead>
<tr>
<th>DNA probea</th>
<th>DNA competitor (pmol)</th>
<th>Flp-FRTab</th>
<th>Flp-10-T</th>
<th>Flp-FRTb</th>
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<tr>
<td>Flp-FRTab</td>
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<td>50c</td>
<td></td>
</tr>
<tr>
<td>Flp-FRTb</td>
<td>ND</td>
<td>1c</td>
<td>10c</td>
<td></td>
</tr>
</tbody>
</table>

- a0.02 pmol 32P-labeled DNA was used in competition experiments.
- bThe amount of competitor needed to reduce the amount of complex II (CII) by 50%.
- cThe amount of competitor needed to reduce the amount of complex I by 50%.
- ND, not determined.

**DISCUSSION**

In this paper we report the use of the in vitro binding site selection method to discover novel ssDNA sequences to which Flp binds in a sequence-specific manner. Flp is known to bind specifically to the duplex FRT site. We have shown that the ability of Flp to bind the ssDNA sequence of Flp-10-T was superior to its ability to bind duplex DNA containing only Flp binding element b. This may be one of the reasons for selection of the ssDNA sequences when the experiment was designed originally to select dsDNA sequences containing one Flp binding element. Another may have been that the 18 bp random region was insufficiently long to accommodate two 13 bp binding sites that might mediate cooperative binding between two Flp molecules. Competition studies also indicated that Flp possesses a single DNA binding site for both duplex and ssDNA (data not shown).

The minimum length of the sequence (SS-13) of ssDNA that is required for binding of Flp is 29 nt. This sequence includes 16 nt from the invariant left flanking sequence and 13 nt from the internal sequence that was randomized prior to site selection. The fact that the left flanking sequence serves as part of the ssDNA binding site for Flp may be the reason that all the sequences selected by Flp share an exact junction with the left flanking sequence. Missing nucleotide interference experiments revealed that the Flp protein makes extensive interactions with all the nucleotides in the sequence of SS-13 (X.-D.Zhu, unpublished data). Although these interactions are essential for DNA binding, they may not be base specific. It is possible that the sequence of bases specifies a novel secondary structure of the oligonucleotide that is in turn recognized by Flp.

It is known that Flp binds to the duplex FRT site in a sequence-specific manner. However, neither the invariant left flanking sequence of Flp-10-T nor the selected internal region resembles the sequence of the FRT site. Indeed, Flp does not bind to the single strands of the FRT site.
What does the ssDNA binding activity of Flp mean? There are at least three possibilities. First, the ssDNA binding activity of Flp may play a role in the recombination reaction itself. However, our attempts to perturb recombination of duplex FRT sites in vitro by incubation with the single-stranded oligonucleotide (Flp-10-T) resulted simply in inhibition of the reaction. We presume that this was due to interference with binding of Flp to the FRT site, since no specific interference with a subsequent step (e.g. cleavage, ligation or strand exchange) could be detected (data not shown).

Second, the activity may regulate some other biological process (e.g. ligation or strand exchange) could be detected (data not shown). No specific interference with a subsequent step (e.g. cleavage, ligation or strand exchange) could be detected (data not shown). We presume that this resulted simply in inhibition of the reaction. We presume that this resulted simply in inhibition of the reaction. We presume that this resulted simply in inhibition of the reaction. We presume that this resulted simply in inhibition of the reaction.

Further experiments will be necessary to dissect the relative roles of base specificity versus structure specificity in contributing to binding affinity. However, it is possible that some motifs in Flp that recognize the duplex FRT site also recognize some structures in the single-stranded oligonucleotide that mimic the duplex FRT site. Such a structure may be dictated by both sequence-specific and structure-specific components. There are a few examples in the literature of proteins that recognize ssDNA sequence specifically, including single-stranded telomere end binding proteins and mammalian PurC proteins (38–40). It will be interesting to see if the repertoire of such proteins can be expanded by deliberately searching for such activities among proteins that bind duplex DNA site specifically. It is also possible that single-stranded oligonucleotides to which proteins bind sequence specifically could have novel therapeutic applications.

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