

Chemical probe and missing nucleoside analysis of Flp recombinase bound to the recombination target sequence

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ABSTRACT

The Flp protein catalyzes a site-specific recombination reaction between two 47 bp DNA sites without the assistance of any other protein or cofactor. The Flp recognition target (FRT) site consists of three nearly identical sequences, two of which are separated by an 8 bp spacer sequence. In order to gain insight into this remarkable protein–DNA interaction we used a variety of chemical probe methods and the missing nucleoside experiment to examine Flp binding. Hydroxyl radical footprints of Flp bound to a recombinationally-competent site fall on opposite faces of canonical B-DNA. The 8 bp spacer region between the two Flp binding sites becomes reactive towards 5-phenyl-1,10-phenanthroline-copper upon Flp binding, indicating that once bound by Flp, this segment of DNA is not in the B-form. Missing nucleoside analysis reveals that within each binding site the presence of two nucleosides on the top strand and four on the bottom, are required for formation of a fully-occupied FRT site. In contrast, loss of any nucleoside in the three binding sites in the FRT interferes with formation of lower-occupancy complexes. DNA molecules with gaps in the 8 bp spacer region are over-represented in complexes with either two or three binding sites occupied by Flp, evidence that DNA flexibility facilitates the cooperative interaction of Flp protomers bound to a recombinationally-active site.

INTRODUCTION

The enzyme Flp recombinase is encoded on the yeast 2 μ m circle. Flp catalyzes a recombination reaction that serves to amplify this extrachromosomal element (reviewed in 1,2). Each of the two segments of DNA which undergoes recombination contains three nearly identical 13 bp Flp binding sites. This set of binding elements together with the included strand exchange region is called an Flp recognition target (FRT) site (Fig. 1). The recombination reaction is precise. Flp first cleaves and religates

two of the four strands of the DNA in the synaptic complex to produce a Holliday intermediate (3,4). The enzyme then cleaves and exchanges the remaining two strands, at positions exactly 8 bp away from the first exchange points, yielding recombinant product.

The amino acid sequence of Flp bears no apparent resemblance to any known DNA binding motif. Chemical protection and interference studies and a complete mutational analysis have provided information on where Flp sits on DNA (5–8), but a detailed model for the structure of the Flp–DNA complex that is consistent with the existing data has yet to be proposed.

Two different experimental points of view, focusing either on function or structure, have been adopted in previous studies of the effect on the Flp system of changes in DNA (made either via mutagenesis or chemical modification). It is clear that there is not always a strict correspondence between the two assays. For example, the 8 bp DNA spacer region that is surrounded by recombinationally-active Flp binding sites (Fig. 1) can be changed in length or sequence without destroying Flp binding capacity (9). Yet changes in the sequence of the 8 bp spacer, namely alterations in the polypyrimidine tracts or diminution of the A/T content, can impose restrictions on Flp recombination activity (9). Thus, sequence-dependent properties of DNA contribute to Flp activity.

Association of Flp with its target site induces two types of DNA bending, called type I and type II (10). The type I bend is caused by the interaction of a single Flp monomer with DNA while the type II bend (likely >140°) is mediated by interaction of two Flp monomers bound across the spacer from each other (10,11). This large distortion of DNA apparently is not utilized to physically strain the phosphodiester involved in the breakage/exchange reaction; rather the DNA distortion establishes the correct Flp interface at which a functional active site is assembled (11,12). It is therefore important to consider both how Flp recognizes its binding sites and what structural consequences follow once it binds DNA.

We report here chemical probe and missing nucleoside experiments on the Flp–FRT complex that are aimed at addressing these questions. By comparing our results with the results of previous work, we show that, like the spacer DNA, DNA in the

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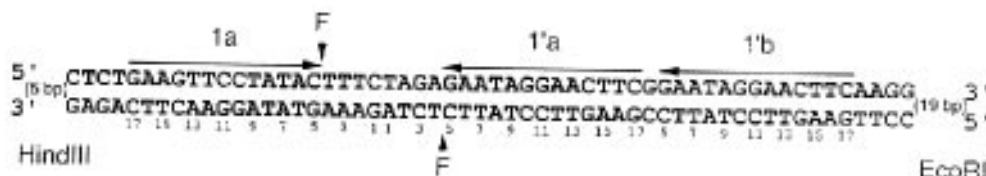


Figure 1. The FRT site. Horizontal arrows above the sequence delineate the three 13 bp symmetry elements, 1a, 1'a and 1'b, to which Flp binds. The points where Flp catalyzes strand exchange are indicated by F. The spacer DNA is the 8 bp that lie between the Flp strand-exchange points. Nucleotide positions in element 1a are numbered as defined by Bruckner and Cox (5). Positions in elements 1'a and 1'b are given the same numbers as the corresponding positions in 1a. In all the experiments presented in this paper DNA was labeled at the *Hind*III site shown at the left.

Flp binding sites participates in the Flp reaction according to its capacity to undergo specific structural alterations.

MATERIALS AND METHODS

Purification of Flp

Flp and mutant Flp proteins were expressed in *Escherichia coli* from the pMJ plasmids (13). Site-specific mutagenesis of Flp is described by Parsons *et al.* (14). Flp proteins were purified from cell extracts by affinity chromatography (15). The final preparations were judged to be >90% pure by staining of sodium dodecyl sulfate polyacrylamide gels with Coomassie Brilliant Blue.

Hydroxyl radical footprinting

An 85 bp *Hind*III–*Eco*RI restriction fragment from pJ3 (13) (Fig. 1) containing the wild-type FRT site was radiolabeled with ^{32}P at either the 5' or 3' end of the *Hind*III site following standard procedures (16).

Flp protein (45 ng) and 30 fmol of radiolabeled DNA were incubated together at 30°C for 30 min in 45 μl of a buffer consisting of 52.5 mM Tris–HCl (pH 7.5), 62.5 mM NaCl, 200 $\mu\text{g}/\text{ml}$ BSA, 100 $\mu\text{g}/\text{ml}$ calf thymus DNA, 1 mM DTT, 0.05 mM EDTA, 1 mM 2-mercaptoethanol and 1% glycerol. In the reaction mixture the concentration of Flp monomer was 20 nM and the concentration of DNA binding sites was 2 nM. Mobility shift gel electrophoresis of a typical binding reaction mixture showed that under these conditions >80% of the DNA is present as the fully-occupied (cIII) Flp–DNA complex.

The $[\text{Fe}(\text{EDTA})]^{2-}$ complex was prepared just before the footprinting experiment by mixing equal amounts of stock solutions of ferrous ammonium sulfate (Aldrich) (2 mM) and EDTA (4 mM), which had been stored frozen. The Flp–DNA sample was brought to room temperature and 6.5 μl of 20 mM sodium ascorbate, 6.5 μl of 3% H_2O_2 and 6.5 μl of 1 mM Fe(II): 2 mM EDTA were mixed on the inside wall of the Eppendorf tube containing the sample. The cleavage reagent was then mixed with the sample. After 2 min, 6.5 μl of 0.1 M thiourea were added to stop the reaction. TE buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA] was added to bring the sample volume to 200 μl . The mixture was extracted with phenol. The phenol layer was back-extracted with TE and the combined aqueous layers were extracted 3-fold with ether. The DNA was precipitated twice with ethanol. The DNA pellet was rinsed with ethanol and lyophilized.

The DNA was dissolved in 4 μl of 90% formamide/dye mixture and electrophoresed on a 12% polyacrylamide (1:19) denaturing gel. The gel was made with 1 \times TBE (100 mM Tris base, 100 mM

borate, 2.2 mM EDTA) and 8.3 M urea. The gel was dried and exposed to Kodak XAR-5 film. The autoradiograph was scanned on a Joyce Loebel Chromoscan 3 densitometer or a Molecular Dynamics Model 300E Computing Densitometer.

Methidiumpropyl EDTA•Fe(II) footprinting

Methidiumpropyl EDTA (MPE) was the generous gift of Professor Peter Dervan (California Institute of Technology). Flp and DNA were incubated together as described above. The MPE•Fe(II) reagent was prepared by mixing equal volumes of stock solutions of ferrous ammonium sulfate (1 mM) and MPE (1.03 mM) and then diluting with water to the desired final concentration. Footprinting was performed at 30°C by adding 6.5 μl of 20 mM ascorbate, 6.5 μl of 0.3% H_2O_2 and 6.5 μl of 20.6 μM MPE:20 μM Fe(II). The reaction was stopped after 2 min by adding 35 μl of 0.1 M thiourea. DNA was recovered and electrophoresed on a denaturing gel as described above.

5-Phenyl-1,10-phenanthroline•copper reactions

Flp and DNA were incubated together as described above. The sample was brought to room temperature and 5 μl of a solution containing 5-phenyl-1,10-phenanthroline (Sigma) and CuSO_4 were added, resulting in final concentrations of 100 μM 5-phenyl-1,10-phenanthroline and 24 μM copper ion. Since the Flp binding reaction mixture already contained 1 mM DTT, no additional reducing agent was added. After 30 min, 5 μl of 28 mM 2,9-dimethyl-1,10-phenanthroline (Sigma) were added to quench the reaction. DNA was prepared for denaturing gel electrophoresis as described above.

Missing nucleoside analysis

A batch of gapped DNA was generated by adding 10 μl of 20 mM ascorbate, 10 μl of 3% H_2O_2 and 10 μl of 250 μM Fe(II):500 μM EDTA to 120 fmol of end-labeled DNA in 70 μl of TE. After 2 min, 10 μl of 0.1 M thiourea were added. The DNA was precipitated with ethanol twice, rinsed, lyophilized and brought up in TE buffer. Flp binding reactions were performed as for hydroxyl radical footprinting except that 3 ng Flp was used and radiolabeled, gapped DNA was used in place of radiolabeled, intact DNA. The various Flp–DNA complexes were separated on a 5% polyacrylamide (1:29 bisacrylamide:acrylamide, 1 \times TBE) mobility shift gel that was run at 140 V at 4°C. The gel was autoradiographed to reveal the positions of the free DNA and Flp–DNA complexes. Densitometry of the autoradiograph gave the following percentages for the various species: free DNA, 22%; cI, 19%; cII, 31%; cIII, 28%. Bands containing unbound

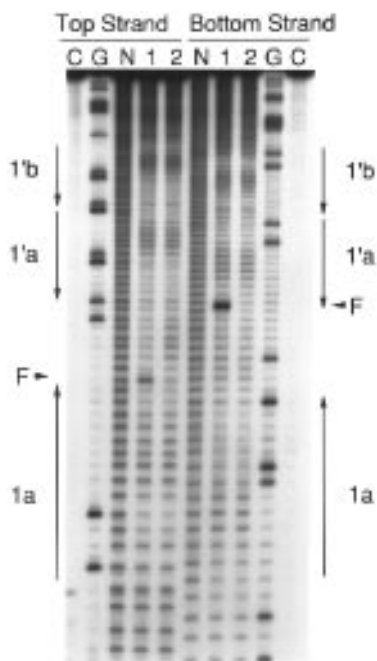


Figure 2. Hydroxyl radical footprints of Flp bound to the FRT site. To produce these footprints, either the top strand (left) was radiolabeled at the 5' end at the *Hind*III site (Fig. 1) or the bottom strand (right) was labeled at the 3' end, also at the *Hind*III site. Control lanes (labeled C) contain untreated DNA. Lanes labeled G contain products of the Maxam–Gilbert guanine-specific sequencing reaction. Lanes labeled N, 1 and 2 contain DNA treated with the hydroxyl radical. DNA in lanes N was treated in the absence of Flp, in lanes 1 with wild-type Flp bound and in lanes 2 with Flp(Y343F) bound. The three Flp binding sites are indicated by vertical arrows at the sides of the autoradiograph. With wild-type Flp (lanes 1) cleavage is seen at the strand-exchange points on each strand (indicated by F). No Flp-induced cleavage is seen in lanes 2, which contain footprints of the cleavage-deficient Y343F mutant protein.

DNA and the cI, cII and cIII Flp–DNA complexes (17) were excised from the gel. DNA was extracted from the gel slices, denatured and run on a sequencing gel.

RESULTS

The hydroxyl radical footprints of wild-type Flp and the mutant protein Flp(Y343F) are shown in Figure 2. Flp(Y343F) (18), in which phenylalanine replaces the active site tyrosine that nucleophilically attacks the DNA backbone, is inactive in recombination but still capable of binding to DNA. Densitometer scans of the footprints are shown in Figure 3. Within each 13 bp Flp binding element there are two short footprints on each strand, separated by ~10 bp. The footprints on one strand are offset from corresponding footprints on the other strand by 2–3 bp in the 3' direction. This set of footprints shows that within each binding element one face of the DNA helix is protected by bound Flp.

The footprints of wild-type Flp and the Y343F mutant protein are very similar, but they differ in one place. While the 8 bp spacer is strongly protected on the top strand by bound Flp, Flp(Y343F) leaves the spacer region on the top strand quite exposed to hydroxyl radical cleavage.

We looked for evidence of an altered DNA structure in Flp–DNA complexes by treating bound DNA with the structure-sensitive cleavage reagents MPE•Fe(II) (19) and 5-phenyl-

1,10-phenanthroline•copper (20). The copper reagent has been used successfully to detect changes in DNA structure induced by RNA polymerase (20) and the mercury-responsive gene regulatory protein MerR (21). Binding of $\gamma\delta$ resolvase to the active site, but not to two inactive sites, causes the central non-contacted core DNA to become hyperreactive towards MPE (22). This hyperreactivity is correlated with the DNA bending that accompanies binding of $\gamma\delta$ resolvase.

Although Flp also bends DNA (10), we find no enhancement of MPE cleavage in the Flp–DNA complex (Fig. 4). In fact, both wild-type Flp and Flp(Y343F), each of which bends DNA to the same extent, give a large MPE footprint that extends from site 1'b to 1a (Fig. 4). In particular, very little MPE cleavage is seen in the 8 bp spacer that separates sites 1'a and 1a. We also studied a Flp mutant, Flp(Y343S) (18), that bends DNA to a lesser extent than wild-type Flp (10). In contrast to wild-type Flp and Flp(Y343F), we find that MPE cleaves readily throughout the spacer region when Flp(Y343S) is bound (Fig. 4), while still giving footprints in sites 1a, 1'a and 1b.

All three forms of Flp mentioned above, as well as the step-arrest mutant Flp(R308K), induce hyperreactivity of 5-phenyl-1,10-phenanthroline•copper in the spacer region (Fig. 5). Four positions on the top strand and three on the bottom are strongly cut. Weaker cuts also are seen in the spacer, especially on the top strand. The most reactive positions on each strand are offset 1–2 bp in the 3' direction. This remarkable reactivity suggests that the structure of the spacer DNA is indeed perturbed by Flp.

As a final analysis of the nature of Flp binding we sought to identify which nucleosides are important for formation of the various Flp–DNA complexes. To do this we employed the missing nucleoside experiment (23). In this experiment a DNA molecule containing the FRT site is treated with the hydroxyl radical in order to remove nucleosides (base plus deoxyribose) randomly from the DNA molecule. The gap in the DNA backbone that is introduced by this treatment is flanked by the two phosphates that were originally connected to the missing nucleoside. The conditions of the hydroxyl radical reaction are adjusted so that a DNA molecule has at most one gap in its backbone. Mobility shift gel electrophoresis of the Flp–DNA complexes formed with gapped DNA, followed by denaturing gel electrophoresis of DNA isolated from the bands of the mobility shift gel, produces a pattern that reveals which nucleosides are essential for formation of Flp–FRT complexes.

We concentrated our analysis on sites 1'a and 1'b. Gaps in site 1a are near the labeled end of the DNA (Fig. 1) and others have shown that short DNA fragments can dissociate during precipitation and thus be lost (24). Since gapped DNA was precipitated four times during the experiment, the recovery of labeled DNA fragments resulting from gapping in site 1a was unlikely to be quantitative. We therefore did not consider further the details of signals within site 1a.

Native polyacrylamide gel electrophoresis of a mixture of Flp(Y343F) and DNA containing the FRT site gives rise to three retarded bands, representing Flp bound to one, two or all three Flp binding sites (Fig. 1). Because one step of the missing nucleoside experiment involves isolation of protein–DNA complexes from a mobility-shift gel (23), we had the opportunity to study separately the populations of gapped DNA that were associated with each of the three possible Flp–DNA complexes.

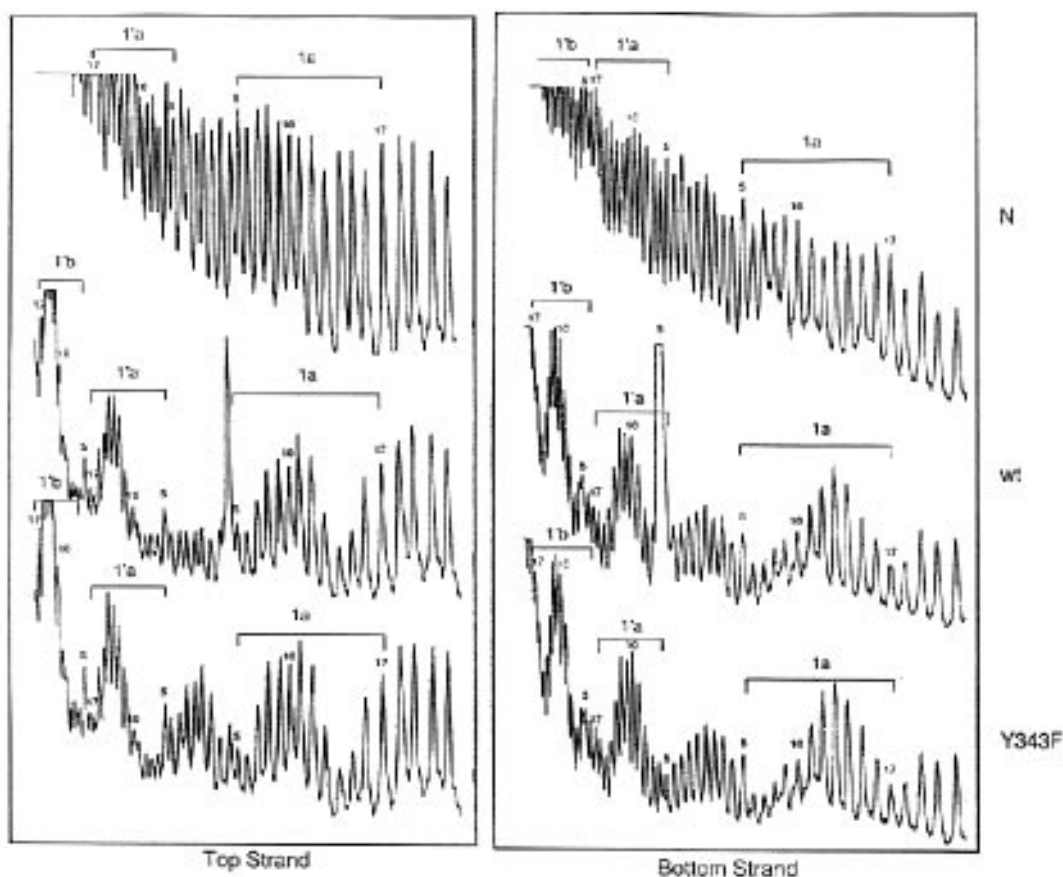


Figure 3. Densitometer scans of hydroxyl radical footprints of Flp. Left, top strand. Right, bottom strand. Top, hydroxyl radical cleavage pattern of free DNA; middle, hydroxyl radical footprint of wild-type Flp; bottom, footprint of the Y343F mutant. The three 13 bp Flp binding sites in the FRT are labeled and marked by brackets. The intense peaks in the middle of the scans of the footprints of the wild-type protein are due to cleavage by Flp at the strand exchange sites.

Densitometer tracings of the missing nucleoside data for the bottom strand are shown in Figure 6. The population of DNA that supports full Flp binding (complex cIII) shows modulated peak intensities within binding element 1'a that are exactly duplicated in 1'b. In this experiment, peaks of lower intensity represent positions where the loss of a nucleoside causes a reduction of protein binding, in turn causing these gapped DNAs to be under-represented in the bound fraction. The nucleosides that are identified by this method as being essential for full occupancy of the FRT by Flp(Y343F) are A9 and G10 on the top strand (data not shown) and T7, A8, G14 and A15 on the bottom strand (Fig. 6). This result means that for Flp to form a complex with all three sites, each of these nucleosides must be present in the DNA. Conversely, gaps at other positions (for example, nucleosides 5, 17 and the nucleoside between these two) can be present in the cIII complex.

The missing nucleoside patterns we see for the intermediate (cI and cII) complexes are somewhat more complicated to explain and we therefore leave this to the Discussion.

DISCUSSION

The hydroxyl radical footprints presented here complement the existing methylation interference and protection data (5,6,8) and confirm the assignment of the DNA binding surface proposed

previously (5). Our results show that Flp protects backbone positions on one face of the DNA within each 13 bp binding element. The positions protected from hydroxyl radical attack surround the two minor and central major grooves identified by Bruckner and Cox (5). Protection by a bound protein of one face of DNA from hydroxyl radical attack is a commonly-observed pattern. Repressor proteins containing the helix–turn–helix DNA binding motif also protect one face of DNA, covering backbone positions that surround the part of the major groove that is recognized by the protein (25,26).

The Flp recognition sequence is shorter, at 13 bp, than a typical recognition sequence for a helix–turn–helix protein. For example, the bacteriophage lambda repressor binds (as a dimer) to a 17 bp operator sequence and as a consequence protects two segments of the major groove and the minor groove in between. The backbone positions that Flp protects surround a single major groove surface and two minor groove surfaces (Fig. 7). The Flp footprint more resembles the hydroxyl radical footprint of the yeast Mat α 2 protein (27), which recognizes adjacent parts of the major and minor groove through a single homeodomain (28).

Two of the three Flp binding sites in the FRT, labeled 1a and 1'a in Figure 1, are necessary and sufficient for recombination (29). The hydroxyl radical footprint suggests that the surfaces of DNA that are protected by Flp bound to sites 1a and 1'a fall on opposite

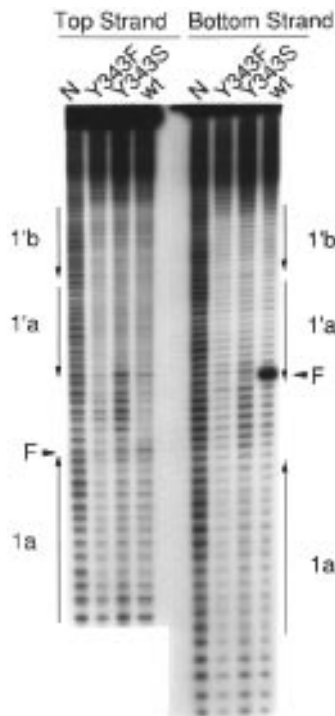


Figure 4. Cleavage patterns of DNA treated with MPE•Fe(II). Lanes are marked according to the species of Flp protein present during the cleavage reaction. N, no protein.

faces of the DNA, if the DNA is not distorted from the B-form. To demonstrate this point, we map our hydroxyl radical footprinting data on a B-form DNA helix in Figure 7. Flp is known to bind to DNA cooperatively (7,15,17,30). By analogy to other DNA–protein complexes that exhibit cooperativity, such as the lambda repressor-operator system (31), one would have expected the protein-binding surfaces of elements 1a and 1'a to lay on the same DNA face (32). In order for Flp protomers bound to sites 1a and 1'a to interact, either the protein must be able to reach around the DNA or the DNA must deform.

We also note that in our experiments using wild-type Flp, the enzyme appears to cleave the bottom strand (at site 1'a) to a greater extent than the top strand (at site 1a) (e.g. Fig. 2). Since Flp is known to covalently attach to the 3'-phosphate end of a cleaved strand, this might lead to underrepresentation of the 5'-radiolabeled top strand cleavage product as observed in a denaturing gel. However, we find that even with proteinase K treatment of top-strand cleavage products there still is more bottom-strand cleavage product apparent. This discrepancy in cleavage extent of the two strands has also been noted by others (33).

To determine which nucleosides are necessary for Flp binding we performed a missing nucleoside experiment (23). The power of this approach has been demonstrated for the bacteriophage lambda repressor and cro proteins, which give different missing nucleoside signals with the same DNA sequence, reflecting their different specificities. The nucleosides identified by these experiments as contacts agreed with those that would be expected from the co-crystal structures of the repressor and cro proteins and from complete mutational analyses.

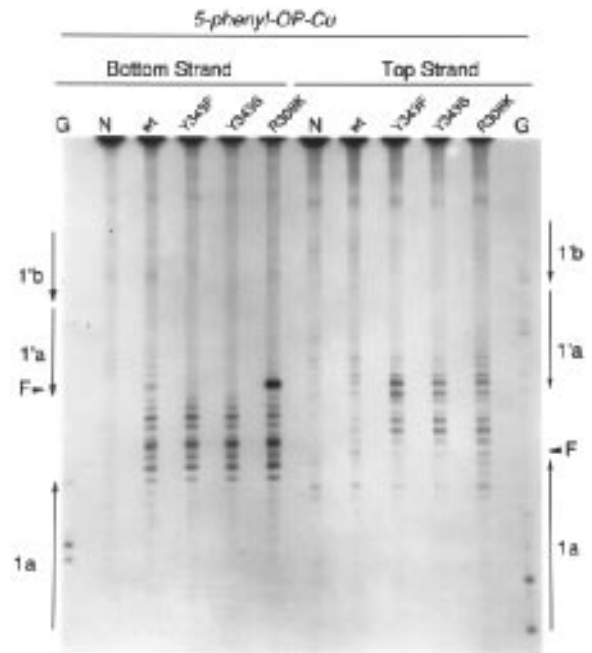


Figure 5. Cleavage patterns of DNA treated with 5-phenyl-1,10-phenanthroline•copper. Lanes are marked according to the species of Flp protein present during the cleavage reaction. N, no protein. G, products of the Maxam–Gilbert guanine-specific sequencing reaction.

The missing nucleoside signals in sub-saturated Flp–DNA complexes (cI or cII) show roughly similar effects for the removal of each nucleoside within a binding site (Fig. 6). For example, in complex cI each of the 13 peaks in site 1'b is reduced in intensity relative to the input gapped DNA. This observation agrees with the results of a missing contact experiment (34) on Flp binding to a single site, in which every base was found to contribute to binding (8).

Upon forming the fully-occupied Flp–DNA complex, complex cIII, a different missing nucleoside pattern appears. A strong modulation of peak intensity is seen within each of the binding sites. This experiment shows that Flp is unable to form complex cIII if any of the nucleosides T7, A8, G14 or A15 on the bottom strand, or A9 or G10 on the top strand, is missing (Fig. 7). Other nucleosides can be removed from a binding site and a fully-bound Flp–DNA complex can still form (Fig. 6). The essential nucleosides do not coincide with those base pairs identified as critical for Flp recombination activity, as determined from a complete mutational analysis (7). In those experiments the identities of base pairs 5, 6, 7 and 11 were found to be essential for activity. The basis for the discrepancy between our missing nucleoside results and the mutational analysis of recombination is not obvious.

The base pair substitutions that interfere with Flp recombination activity (7) do not follow the pattern that would be predicted if these substitutions simply interfere with base recognition by Flp. The rules for base recognition by hydrogen bonding in the major and minor grooves were predicted by Seeman *et al.* (35) and have been borne out in subsequent work (36). Rather, the recognition of a single binding site by Flp seems to involve all 13 base pairs in a binding site, as reflected by diminished Flp binding

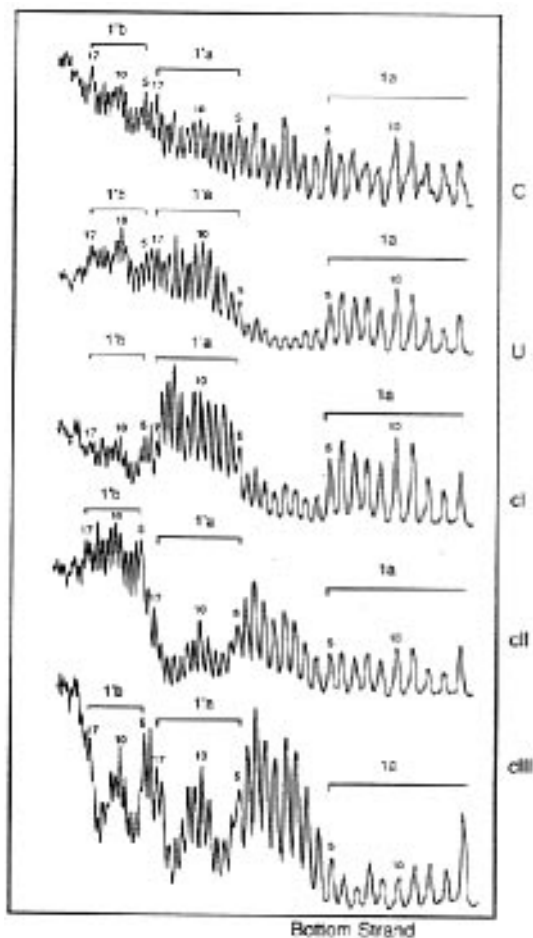


Figure 6. Missing nucleoside analysis of Flp(Y343F). Shown are densitometer tracings of lanes of a denaturing gel on which was separated the various DNA fractions obtained in a missing nucleoside experiment. The DNA used in this experiment was radiolabeled on the bottom strand. C, cleavage pattern of the gapped DNA that was used in the Flp binding reactions. U, cleavage pattern of gapped DNA that was incubated with Flp, run on a mobility shift gel and isolated from a band that ran as unbound DNA. cI, cII and cIII: cleavage patterns of gapped DNA that was incubated with Flp, run on a mobility-shift gel and isolated from bands corresponding to complexes cI, cII and cIII respectively. (Complex cI corresponds to the least-retarded band on the mobility-shift gel, cIII to the most retarded.)

to a site lacking any single base (8) or nucleoside (Fig. 6, complexes cI or cII). On the other hand the mutagenesis experiment, which demonstrated that the identities of only some of the base pairs within a binding site are essential for recombination activity (7) and the missing nucleoside experiment, which shows that the presence of only a few nucleosides are required for full Flp occupancy (Fig. 6), both seem to reflect a process that occurs after initial site recognition.

Recent experiments on *E.coli* integration host factor (37), which also strongly distorts DNA as a necessary aspect of its function, support a two-step model for DNA recognition and binding by this protein. In the case of Flp, our data suggest the possibility that a two-step process is operative in the formation of a stable and functional Flp-FRT complex. The rate of the initial association reaction of Flp with a binding site is determined by

one set of contacts; the stability of the fully-occupied complex (which is related to the off-rate) is governed by another set of contacts.

Analysis of the unbound DNA (U) and the intermediate occupancy (cI and cII) complexes in the missing nucleoside experiment provides additional information concerning the requirements for formation of Flp-FRT complexes. We first consider the average peak height within each of the sites. In contrast to the even intensities of peaks in the input (C) DNA, in the unbound DNA fraction (U) the peaks within each Flp binding site are more intense than those in the flanking and spacer DNA. This observation suggests that the loss of a single nucleoside from any of the three binding sites lowers the overall potential for Flp binding, and therefore increases the probability that such a molecule will remain unbound. This is a somewhat surprising result, even though in simpler cases [e.g. the lambda repressor/O_R1 complex (23)] an apparently similar result is seen. That is, for the lambda complex the unbound DNA fraction contains several intense bands, which correspond to missing nucleosides that are important for repressor binding. The interpretation is that these gaps are incompatible with complex formation, so they are over-represented in the unbound fraction. The lambda system that was studied consisted of a single DNA binding site, though, in contrast to the Flp FRT site, which supports binding at three separate sites. One might have expected that a gap in any one site in the FRT would not preclude Flp binding to the other two sites. Thus no single gap would be over-represented in unbound DNA. This reasoning leads to the prediction that the bands in the unbound fraction would be uniform in intensity, contrary to the observation (Fig. 6, U). Thus our tentative conclusion is that there must be interaction between the Flp protomers bound to the FRT, since a single gap in any one of the three binding sites interferes with the formation of bound complexes, while gaps outside the binding sites (e.g. the 8 bp spacer) do not interfere with Flp binding.

However, damaged DNA can be bound by Flp. The position of the gap directs the occupancy of the DNA. For example, consider a DNA molecule which has a missing nucleoside in site 1'b. In such a damaged DNA molecule Flp can still bind to site 1a or to site 1'a. Flp binding to sites 1a and 1'a exhibits positive cooperativity (7,15,17,30). Therefore once one of these sites is occupied, binding of a second Flp to the other site will be especially favorable and will produce a doubly-occupied DNA species that is gapped in site 1'b. When DNA from the doubly-bound population is examined, damage in site 1'b indeed predominates (Fig. 6, cII). Molecules that are damaged in sites 1a or 1'a, on the other hand, are compromised in binding to one of these two sites and thus are unable to form as stable a doubly-occupied complex as DNA molecules with sites 1a and 1'a intact. DNA molecules damaged in site 1a or 1'a are less able to support formation of complex cII than are molecules damaged in site 1'b and thus are under-represented in cII. DNA molecules damaged either in site 1a or 1'a are correspondingly overrepresented in the tracing of DNA isolated from complex cI (Fig. 6). In this way, positive cooperativity in binding to two of the three sites explains the peak heights within binding sites that we observe (Fig. 6).

We noticed that the extent of gapping in the 8 bp spacer region is higher in complexes cII and cIII than it is in unbound or cI DNA. To better illustrate this point we compare the densitometer scan of the gapped DNA that partitioned into complex cII with the

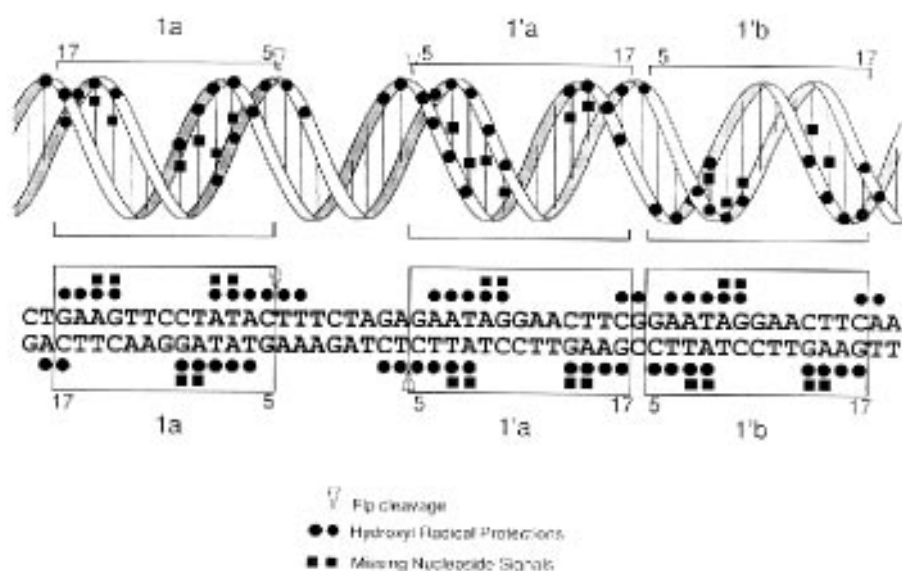


Figure 7. Representation of hydroxyl radical footprinting and missing nucleoside data for Flp. ●, Mark deoxyriboses that Flp(Y343F) protects from hydroxyl radical cleavage. ■, Indicate positions giving missing nucleoside signals. Top, the data are mapped on a B-DNA helix (10.5 bp/turn). Bottom, the sequence of the FRT is aligned with the helix above. The three Flp binding sites are boxed. Open arrowheads above the helix and above and below the sequence mark the strand-exchange sites where Flp cleaves.

scan of the input gapped DNA (C) (Fig. 8). These two DNA samples show nearly identical peak heights throughout binding sites 1a and 1'a. This is most easily seen in the overlay of the two scans at the bottom of Figure 8. In contrast, peaks corresponding to the eight nucleosides in the spacer region and to the 13 nucleosides in site 1'b, clearly are enhanced in intensity in the cII sample. Outside the binding sites the peak intensities in the two samples again are similar. This comparison reveals preferential partitioning into complex cII of DNA molecules gapped either in the spacer or in site 1'b.

The effect seen at site 1'b reflects the positive cooperativity of Flp binding to sites 1a and 1'a, as discussed above. That is, it is less likely that doubly-bound DNA molecules will have Flp bound at site 1'b than at site 1a or 1'a, so DNA molecules gapped at site 1'b are disproportionately represented in complex cII.

The preferential formation of multiply-bound Flp-DNA complexes with DNA gapped in the spacer is most simply interpreted as being the result of the increased flexibility of this region when it is gapped (38). Since hydroxyl radical footprinting shows that Flp molecules bound to sites 1a and 1'a would be on opposite faces of B-form DNA (Fig. 7), it is logical that stress is induced in the spacer DNA separating them when they interact cooperatively. Presumably Flp protomers bound at sites 1a and 1'a can interact with less energetic penalty when the DNA between them is more flexible. Increased DNA flexibility resulting from a discontinuous backbone has been shown to raise the association constant for the bacteriophage 434 repressor with its operator (39). The higher degree of Flp binding to DNA molecules gapped in the spacer region is not as dramatic as is seen for *E. coli* RNA polymerase when the -10 region is gapped in a missing nucleoside experiment (40). However, the enhancement of Flp binding to DNA gapped in the spacer region does parallel the observation of significant DNA bending that occurs upon formation of the multiply-bound complexes cII and cIII (10).

In summary, the missing nucleoside experiment gives patterns that are consistent with positive cooperativity in the binding of Flp to the inverted repeat sequences 1a and 1'a. The experiment also indicates that Flp bound to the third element, 1'b, does not participate in these strong cooperative interactions (7,15,17,30). However, gaps in site 1a or 1'a must have some effect on Flp binding to site 1'b, since the cleavage pattern of the unbound DNA fraction (Fig. 6, U) is not uniform. In other work from our laboratory it has been shown that a protein which fills adjacent DNA binding sites without cooperativity gives no missing nucleoside signal in subsaturated DNA samples (Dixon, W. PhD. Dissertation, The Johns Hopkins University). Thus the missing nucleoside experiment provides a simple way to identify DNA sites that are filled in a cooperative manner.

The intense reactivity of 5-phenyl-1,10-phenanthroline-copper towards the 8 bp spacer DNA in a Flp-DNA complex (Fig. 5) indicates that this segment of DNA exists in an altered conformation. Such a conformation may serve to allow Flp protomers, bound on opposite faces of DNA, to interact across the spacer. While Flp bends DNA, the reactivity of 5-phenyl-1,10-phenanthroline-copper is not strictly related to DNA bending. The Flp(Y343S) mutant, which produces a less-severe type II DNA bend (10), still supports high reactivity of 5-phenyl-1,10-phenanthroline-copper. Also, unlike the cases of *E. coli* RNA polymerase (20) and MerR (21), the induction of DNA reactivity towards 5-phenyl-1,10-phenanthroline-copper is not correlated with increased sensitivity towards another cleavage reagent, MPE-Fe(II) (Fig. 4), that has been used previously to detect DNA distortion (22).

It is unlikely that the distorted spacer DNA revealed by 5-phenyl-1,10-phenanthroline-copper is single-stranded in nature. In experiments with two other chemical probes, Sadowski *et al.* found no evidence for single-stranded DNA in the Flp-DNA

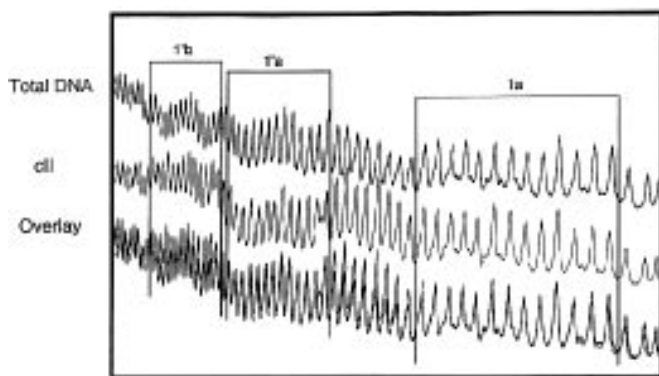


Figure 8. DNA with gaps in the 8 bp spacer is over-represented in multiply-occupied Flp–FRT complexes. Densitometer scans of the cleavage patterns of total gapped DNA (top scan, heavy line) and gapped DNA that was isolated from complex cII (middle scan, light line) are shown. The data were taken from a missing nucleoside experiment on Flp(Y343F), similar to that depicted in Figure 6. The bottom scan is simply an overlay of the two tracings shown above, so that differences in band intensities can more easily be seen. The two scans overlay nearly perfectly, except in site 1'b and in the 8 bp spacer region between sites 1'a and 1'a, where the lighter line (complex cII) is noticeably more intense.

complex (8). A more likely possibility is that spacer DNA is unwound when Flp occupies sites 1'a and 1'a. The MerR protein, which upon mercury binding induces remarkable reactivity of 5-phenyl-1,10-phenanthroline•copper, has been definitively demonstrated to unwind DNA (41). Furthermore, the induction of DNA cleavage by 5-phenyl-1,10-phenanthroline•copper was shown to be directly associated with unwinding; a mutant of MerR that was capable of unwinding DNA in the absence of mercury also supported DNA cleavage by 5-phenyl-1,10-phenanthroline•copper (42). In the case of Flp, unwinding of the intervening DNA would facilitate the cooperative interaction of two Flp molecules bound to opposite faces of the DNA duplex.

A most interesting aspect of this study is the lack of correspondence between the critical missing nucleosides we find and those nucleotides believed to have key roles in Flp binding on the basis of other kinds of studies. Missing nucleoside signals observed for the bacteriophage lambda repressor and cro proteins (23) and for the *Drosophila* homeodomain protein engrailed (Draganescu, A. and Tullius, T.D. manuscript in preparation), correspond nearly one-to-one with the contacts observed in the co-crystal structures of these proteins and to essential base pairs identified in mutagenesis experiments. The difference might lie in the fact that Flp is a protein that not only binds to DNA, but also catalyzes a specific covalent rearrangement of DNA, without the benefit of an energy-providing cofactor. Flp changes the structure of DNA and the nature of the Flp–DNA interaction is dynamic. Not only must the binding of Flp to DNA yield the potential to overcome the activation barrier for DNA cleavage, this complex must also be structurally amenable to the subsequent formation of a Holliday intermediate. The different response of the Flp protein to base substitutions, base methylation and loss of nucleosides is evidence for DNA structural perturbation that accompanies even the first step of the complex series of reactions that results in recombination, the formation of the fully bound Flp–DNA complex.

CONCLUSIONS

We have used the results of chemical probe and missing nucleoside experiments to characterize the complex of the Flp recombinase with the FRT site. Hydroxyl radical footprinting shows that Flp covers one face of the DNA helix in each of the three binding sites in the FRT. The footprints also show that Flp would be bound to opposite faces of the DNA in sites that participate in recombination, if the DNA is not distorted from the B-form structure. Flp binding induces DNA cleavage by 5-phenyl-1,10-phenanthroline•copper in the 8 bp spacer region between Flp binding sites, providing evidence that the DNA in the recombinationally-active complex indeed is distorted, most likely by unwinding. Missing nucleoside analysis of FRT DNA with one, two or three sites occupied by Flp provides evidence for cooperative interaction between Flp molecules bound on opposite sides of the 8 bp spacer sequence. The unique missing nucleoside pattern of the fully-occupied Flp–FRT complex, compared to the singly- or doubly-occupied complexes, suggests that there are special structural requirements for forming a recombinationally-active Flp–DNA complex.

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