Biochemical and Kinetic Analysis of the RNase Active Sites of the Integrase/Tyrosine Family Site-specific DNA Recombinases

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In this study, we have used multiple strategies to characterize the mechanisms of the type I and type II RNA cleavage activities harbored by the Flp (pronounced here as “flip”) site-specific DNA recombinase (Flp-RNase I and II, respectively). Reactions using half-sites pre-bound by step-arrest mutants of Flp agree with a “shared active site” being responsible for the type I reaction (as is the case with normal DNA recombination). In a “pre-cleaved” type I substrate containing a 3’-phosphothiophosphoryl bond, the Flp-RNase I activity can be elicited by either wild type Flp or by Flp(Y343F). Kinetic analyses of the type I reaction are consistent with the above observations and support the notion that the DNA recombinase and type I RNase active sites are identical. The type II RNase activity is expressed by Flp(Y343F) in a half-site substrate and is unaffected by the catalytic constitution of a Flp monomer present on a partner half-site. Reaction conditions that proscribe the assembly of a DNA bound Flp dimer have no effect on Flp-RNase II. These biochemical results, together with kinetic data, are consistent with the reaction being performed from a “non-shared active site” contained within a single Flp monomer. The Flp-related recombinase Cre, which utilizes a non-shared recombination active site, exhibits the type I RNA cleavage reaction. So far, we have failed to detect the type II RNase activity in Cre. Despite their differences in active site assembly, Cre functionally mimics Flp in being able to provide two functional active sites from a trimer of Cre bound to a three-armed (Y-shaped) substrate.

The yeast site-specific recombinase Flp is encoded by the 2-μm circle, a highly persistent, multicopy extrachromosomal DNA element found in most strains of Saccharomyces cerevisiae (reviewed in Ref. 1). The Flp recombination system is believed to play a critical role in safeguarding the plasmid against downward fluctuations in its copy number (2-4). Based on the chemistry of recombination and the active site residues involved in the reaction (5, 6), Flp has been assigned to the integrase/tyrosine family of recombinases. Members of this family carry out DNA exchange with the help of four recombinase monomers acting in unison. One round of recombination is completed in two temporally distinct chemical steps, each step exchanging one pair of strands between the DNA partners. A Holliday junction is therefore an obligatory intermediate in the reaction. The recently solved structure of the Flp-Holliday junction complex (7) reveals close similarities to the structures of related complexes formed by the phage P1 recombinaseCre (8, 9). It is reasonable to suppose that the functional DNA-protein assemblies formed at the core recombination sites by all members of the integrase/tyrosine family have nearly identical geometry and topology.

Despite the differences in their primary amino acid sequences, the carboxyl-terminal catalytic domains of Flp and Cre have roughly the same three-dimensional folds. This similarity also extends to the catalytic domain of human and vaccinia topoisomerase I proteins (10, 11). This is not surprising because Flp and Cre utilize the basic type IB topoisomerase mechanism for strand cutting and joining. The active site nucleophiles in Flp and Cre (Tyr-343 and Tyr-324, respectively) form 3’-phosphothiophosphoryl bonds during the DNA cleavage reaction. The 5’-hydroxyl groups generated by cleavage provide the active nucleophiles for the strand exchange step. They attack the phosphothiophosphoryl bonds across DNA partners to bring about strand joining in the recombinant mode. In addition to the invariant tyrosine nucleophile, the active sites of Flp and Cre include a conserved catalytic pentad consisting of two arginines, a lysine, a histidine, and a tryptophan (7, 12, 13). In Flp, the pentad residues correspond to Arg-191, Lys-223, His-305, Arg-308, and Trp-330 (RKHWR). There is one important difference, however, between Flp and Cre in the manner in which the pentad cluster and the tyrosine nucleophile are utilized during active site assembly. In Cre, all active site residues are provided by a single monomer so that the catalytic tyrosine attacks the scissile phosphodiester bond immediately adjacent to the DNA-bound Cre monomer. This mode of DNA cleavage has been termed cleavage in cis. In Flp, the tyrosine is delivered across the 8-bp strand exchange region (or spacer) to effect cleavage in trans at the distal scissile phosphodiester bond. Thus, the Flp active site assembled on the FRT (Flp Recombination Target) DNA is a “shared” one. The monomer bound to one side of the spacer shares its pentad moiety with the Tyr-343 residue of the monomer bound on the opposite side. The catalytically active entity of Flp is a recombinase dimer positioned on the left and right binding elements flanking the spacer DNA (14, 15). Even in Cre, the reactivity of the cis-acting monomer is dependent on its allosteric activation via

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1 The abbreviations used are: bp, base pair; TEMED, N,N′N′,N′′-tetramethylethylenediamine; 2’-ACE, bis(acetoxyethoxy)-methyl ether substituent; CP, cleavage product.
contacts made by the second monomer on the far side of the spacer (8).

Although Flp is a DNA recombinase, it harbors two types of cryptic RNA cleavage activities, type I and type II, that can be exposed by providing it substrates containing appropriate ribonucleotide substitutions (16, 17). The type I activity (also called Flp-RNase I) attacks the same phosphodiester bond that normally takes part in DNA recombination and is absolutely dependent on Tyr-343. However, whether the reaction proceeds via a covalent 3'-phosphotyrosyl intermediate has not been established. A similar activity, with a minor distinction, has been observed with vaccinia topoisomerase I as well (18). The final product of the type I cleavage reaction contains a 3’-phosphate in the case of Flp, whereas it is a 2’,3’-cyclic phosphate in the case of the vaccinia enzyme. The type II reaction of Flp is peculiar in that the phosphodiester bond that it uniquely exposes by providing it substrates containing appropriate ribose substitutions at specific positions was identified by autoradiography, excised from the gel and extracted overnight in Flp reaction buffer. The bound intermediate was analyzed by using 32P-labeled DNA half-site (0.01–0.02 pmol) incubated at each temperature, with the incubation time was 10 min at each temperature.

Preparation of Hairpin Substrates—The hairpin substrates were obtained as pre-made oligonucleotides or by carrying out Flp recombination in desired half-site substrates. In the latter procedure, a half-site was incubated with wild type Flp under standard recombination conditions for 30 min at 30 °C to generate the recombinant hairpin product. It was separated by electrophoresis in a denaturing 10% polyacrylamide gel (19:1 acrylamide to bisacrylamide, 50% urea), visualized by autoradiography, excised from the gel and ethanol precipitated prior to electrophoresis in 12% denaturing polyacrylamide gels. It was then hybridized to its partner deoxyoligonucleotide using standard procedures.

Preparation of Pre-cleaved RNase I Substrate—A “pre-cleaved” RNase I half-site substrate was generated as follows. A Flp-RNase I half-site was first assembled with its reactive 2’-hydroxyl group blocked by a bis(acetoxyethyl)methyl ether substituent (2’-ACE protection). Furthermore the substrate was inactivated in strand transfer by phosphorylation of the 5’-OH group of the strand that is not subject to cleavage by Flp. This substrate was cleaved by tyramine in the presence of Flp at 50 °C to generate the 3’-O-phosphotyrosyl intermediate (23).

Following cleavage, the 2’-protecting group was removed by incubation in 100 mM TEMED acetic acid (pH 3.8) at 50 °C for 30 min. The deprotected, tyramine-linked strand was isolated by denaturing polyacrylamide gel electrophoresis. It was then hybridized to its partner deoxyoligonucleotide using standard procedures.

Half-site and Full-site Flp Reactions—All reactions were performed as described by Chen et al. (24) with minor modifications. The DNA substrates (0.05–0.10 pmol) were incubated with ~0.4–0.8 pmol of Flp or Flp(Y343F) for 30 min at 30 °C in standard Flp recombinase buffer. The reactions were terminated by the addition of SDS (0.2% final concentration). The samples were chloroform/phenol-extracted and EtOH-precipitated prior to electrophoresis in 12% denaturing polyacrylamide gels (19:1 acrylamide to bisacrylamide; 50% urea). The radioactive bands were identified by autoradiography or by PhosphorImaging.

It should be pointed out that, during assays of Flp-RNase activities, samples were not treated with proteinase K prior to chloroform/phenol extraction. The covalent cleavage complex formed between the substrate and wild type Flp protein was thus eliminated from the aqueous phase and did not interfere with the Flp-RNase cleavage bands during subsequent analysis. In certain experiments that monitored covalent DNA cleavage by Flp, the proteinase K treatment was included (24).

Complementation Reactions on RNase I and II Substrates—The reactions were performed essentially as described by Chen et al. (24). The unlabeled DNA half-site (0.01–0.02 pmol) and the radioactively labeled Flp(Y343F) half-site (0.01–0.02 pmol) were incubated separately with 0.05 pmol of the appropriate Flp variant for 10 min on ice. Approximately 0.5–0.1 pmol each of the respective unlabeled half-sites was added to each incubation mixture, and the two were quickly combined into a reaction tube maintained at 30 °C. After incubation at this temperature for 15 min, reactions were stopped by addition of SDS (0.2% final), and DNA was recovered by chloroform/phenol extraction and ethanol precipitation. Further analysis was performed by denaturing gel electrophoresis and autoradiography (or PhosphorImaging) as described earlier.

Steady State Kinetic Experiments—The steady state kinetic experiments were carried out at a fixed concentration of the substrate (0.0087 μm) and varying concentrations of wild type Flp or Flp(Y343F). In the assays for obtaining the Lineweaver-Burk plots, the protein concentration was maintained higher relative to substrate concentration. Reactions were carried out at 30 °C. Product yields were linear up to 20 min under these conditions. In the temperature dependence experiments, the incubation time was 10 min at each temperature.

DNA Binding, Resolution, and RNA Cleavage Assays with Cre or Cre(Y324F)—The binding reactions were done using Cre(Y324F) under conditions that had been previously used for Flp (19, 25). The DNA bound complexes were resolved by electrophoresis in an 8% native polyacrylamide gel (acrylamide to bisacrylamide, 29:1). The resolution assays with the Y junction substrates and Cre and RNA cleavage assays with half-site substrates and Cre or Cre(Y324F) were also done under conditions previously described for similar assays with Flp (17, 19).
**RESULTS**

Conventions Used in Substrate Representations—For clarity and uniformity, the following conventions are followed in schematically depicting the various half-site substrates used for type I or type II Flp-RNase assays. The “top strand” (arbitrarily designated as the one containing the scissile phosphodiester bond) is represented by a bold line. The complementary “bottom strand” is drawn as a thin line. The relevant nucleotide bases present in the Flp-binding element and in the spacer are shown by upper and lowercase letters, respectively. A ribonucleotide in the binding element or the spacer is denoted by a bold letter with an attached OH. The 5’ and 3’ ends of each strand are indicated, and a radioactive end label (when present) is denoted by an asterisk. The unlabeled DNA half-site used in assays containing a pair of Flp variants is not a source of cleavage products relevant to this study. Representation of the hairpin substrates follows the same rules as the half-site substrates.

Two phosphodiester positions in the half-site substrates are of significance to the experiments described below. The first one is located immediately 3’ to the terminal C of the Flp-binding element that abuts the spacer segment (indicated by p in Fig. 1, A and B). The second one is the immediate 3’ neighbor of the first and is 3’-linked to the first spacer nucleotide (p’ in Fig. 5A).

For the bulge containing Flp full-sites, as they are drawn here, the top strand (shown in bold) is defined as the one containing the scissile phosphodiester bond at the left end of the spacer. The bottom strand, containing the scissile phosphodiester bond at the right end of the spacer, is drawn as a thin line.

In the lox half-sites (substrates for Cre), the target phosphate for the type I Cre-RNase is indicated by p and is immediately to the 5’ side of the strand exchange segment. The neighboring base on the 5’ side is an A. The bases that form part of the strand exchange region during Cre recombination (equivalent to the Flp spacer segment) are written in lowercase letters. The target phosphate for the putative Cre-RNase, based on the corresponding Flp activity, is indicated as p’.

**Flp Type I RNase, Reaction in Half-sites Pre-bound by “Step Arrest” Mutants of Flp**—The currently favored mechanism for the type I Flp-RNase activity proposes a 3’-phosphotyrosyl intermediate for the reaction (Fig. 1, A and B). This covalent linkage between Flp and the nucleic acid backbone results from nucleophilic attack by the catalytic tyrosine (Tyr-343) of Flp on the scissile phosphodiester bond that is normally involved in DNA recombination (indicated as p in Fig. 1, A and B). Upon cleavage of a half-site substrate by Tyr-343, the short spacer segment of the cleaved strand will dissociate and diffuse away. The 5’-hydroxyl group of the other strand can then attack the phosphotyrosyl bond to give a hairpin recombinant (Fig. 1A). The strand joining step of the reaction can be mediated efficiently by Flp(Y343F), even though it is inactive in cleavage.

During recombination, a functional Flp cleavage pocket is assembled from a “pro-active site” containing the KKHWR catalytic pentad (Arg-191, Lys-223, His-305, Arg-308, and Trp-330) provided by one Flp monomer and Tyr-343 delivered in trans by a partner Flp monomer. As a result, a Flp point mutant in the pentad cluster can complement Flp(Y343F) in a half-site recombination reaction (24, 26). The Chen et al. (24) experiments tested Flp substituents at Arg-191, His-305, Arg-308, and Tyr-343 individually and in all possible pairwise combinations. The outcomes showed that the single mutants as well as the binary combinations among mutants of the pentad residues are inactive in half-site cleavage (and hairpin formation). However, Flp(Y343F) can be rescued in half-site recombination by pairing it with each mutant of the pentad class. According to the model in Fig. 1B, the type I RNase activity of Flp may be expressed when the half-site contains a vicinal 2’-hydroxyl group that can attack the 3’-phosphotyrosyl bond formed by the action of Tyr-343 in trans.

If the type I Flp-RNase is a variation of the basic DNA recombination mechanism, two predictions follow. First, pairwise combinations of the Flp pentad mutants with Flp(Y343F) should elicit this activity. Second, the RNase cleavage should occur only on the half-site bound by Flp(Y343F), which is the recipient of Tyr-343 from the pentad mutant. Results of an assay that test these predictions are shown in Fig. 1C. One of the substrates used in these assays was an unlabeled DNA half-site (HS-D) that is not subject to Flp-RNase I cleavage. The second substrate (HS-R1) contained a ribo-C as the terminal nucleotide of the Flp-binding element abutting the spacer and is a substrate for Flp-RNase I. The radioactive label placed at the 5’ end of the cleavage strand in HS-R1 permitted the detection of Flp-RNase cleavage and Flp recombination products. Reactions in lanes 2–4 of Fig. 1C were done by pre-binding
Figure 2. Flp-RNase I activity on a tyramine-cleaved type I half-site. A, cleavage of the type I half-site (HS-D, indicated at the bottom) was treated with tyramine and Flp(Y343F) as in A. The tyramine-cleaved, 2'-protected product is denoted by (rCP-Tyr). The CP1 product in lane 2 is due to the contaminating deprotected half-site present in the original substrate or produced during the cleavage reaction. C, the type I half-site assembled from the tyramine-cleaved intermediate (after deprotection of the 2' position) is represented at the top. Reactions were done with Flp, Flp(Y343F), Flp(R191S), or Flp(H305L), as indicated below the respective lanes. The level of CP1 containing the substrate preparation can be judged from lane 1, which represents a control incubation without Flp or a Flp variant. In all reactions (A–C), the 5' end of the bottom strand was phosphorylated. Product analyses in A–C were done in 10% denaturing polyacrylamide. The bands marked as HS-D and HS-R1* refer to the labeled strands of the all DNA half-site and the 2'-protected type I half-site, respectively.

Flp(Y343F) to HS-R1 and Flp(R191S), Flp(R308G), or Flp(H305L) to HS-D and then mixing the two complexes in recombination buffer (26). In each reaction with this binary protein combination, the half-site recombination product (R) and the Flp-RNase I cleavage product (CP1) were formed. When the protein-half-site partners were switched (Flp(Y343F) now being bound to HS-D), half-site recombination and Flp-RNase I activity were reduced to barely detectable levels (Fig. 2C, lanes 5–7). The very faint bands of R and CP1 in lanes 5–7 were likely because of some recycling of the Flp variants on the half-site substrates.

The results of the complementation assay are consistent with the scheme proposed in Fig. 1B. In the composite Flp-RNase I active site, the orientation of the scissile phosphodiester is achieved by one Flp monomer, and Tyr-343 is provided by the second Flp monomer. The phosphotyrosyl intermediate formed by strand cleavage may be attacked in the second step of the reaction either by the vicinal 2'-hydroxyl group (Flp-RNase I; Fig. 1B) or the 5'-hydroxyl group from the opposite strand (half-site recombination; Fig. 1A).

Flp as Well as Flp(Y343F) Can Yield the Type I RNase Cleavage Product from a Half-site That Is Pre-cleaved by Tyramine—One important criterion for the validity of the reaction scheme in Fig. 1B is the demonstration that Flp-RNase I is able to hydrolyze the predicted 3'-O-phosphorylated intermediate to the 3'-phosphate product. Although isolation of the cleaved DNA intermediate of recombination is straightforward (23, 27), attempts to obtain the corresponding cleavage product with the Flp-RNase I substrate have not been successful. Hence a structural or non-covalent catalytic role for Tyr-343 during the Flp-RNase I reaction cannot be ruled out. Alternatively, the presumed intermediate has a very short lifetime because of the reactivity of the neighboring 2'-hydroxyl group and does not accumulate during the reaction. We have circumvented this problem by protecting the 2'-hydroxyl group during the initial tyrosine cleavage step. The protecting agent was a bis(ace-toxyethoxy)-methyl ether substituent (2'-ACE protection) (28).

In reality, we used tyramine as the cleavage nucleophile assisted by Flp(Y343F) according to the reaction protocol described by Lee and Jayaram (23) (see Fig. 2, A and B). The strand containing the covalently linked tyramine was gel-purified and, after removal of the 2'-protecting group at low pH, it was hybridized to the bottom strand to obtain the pre-cleaved half-site used for the assays shown in Fig. 2C. Further details of the methodology are given under “Materials and Methods.”

Cleavage of the DNA half-site by tyramine in the presence of Flp(Y343F) gave the tyramine-linked product (CP-Tyr; lane 2 of Fig. 2A), which provides a convenient marker band during electrophoresis of reaction products. A similar reaction with the “protected type I half-site substrate” gave two prominent cleavage bands (lane 2 of Fig. 2B). One of these corresponded to the Flp-RNase I product CP1 (lane 2 of Fig. 2B) and indicates the level of the deprotected substrate contaminant in the reaction. The second band (rCP-Tyr), lane 2 of Fig. 2B) migrated slightly above CP-Tyr (compare lanes 2 of Fig. 2A and B), as would be consistent with the presence of the protecting group at the 2'-position. Following removal of the 2'-ACE moiety, its mobility shifted to that of CP-Tyr (compare rCP-Tyr, lane 1 of Fig. 2C to CP-Tyr in lane 2 of Fig. 2A). Note that the isolated rCP-Tyr intermediate was contaminated with a significant amount of the CP1 product. At this time, we suspect that the contamination is due to spontaneous breakdown of rCP-Tyr during the processing steps prior to substrate assembly for the assays in Fig. 2C. Reactions of the tyramine-cleaved and deprotected half-site with wild type Flp, Flp(Y343F), Flp(R191S), and Flp(H305L) are shown in Fig. 2C. Whereas Flp and Flp(Y343F) converted the “presumed” reaction intermediate into the final product of the Flp-RNase I reaction (disappearance of rCP-Tyr and increasing conversion in CP1 lanes 2 and 3, Fig. 2C), neither of the two pentad mutants tested here, Flp(R191S) or Flp(H305L), was able to bring about this conversion (lanes 4 and 5, Fig. 2C).

The above results support the reaction pathway outlined in Fig. 1A. They demonstrate that the 3'-phosphotyrosyl bond of...
the cleaved strand is a target for nucleophilic attack by either the adjacent 2'-hydroxyl group (if one is present) or the incoming 5'-hydroxyl group of the complementary strand. Neither of these two reactions requires Tyr-343, as demonstrated by the reactivity of Flp(Y343F) in processing a pre-cleaved substrate to the CP1 product (this study) or to a recombinant DNA strand (23, 29). It is known that glycerol, ethylene glycol, and a set of polyhydric alcohols can also substitute the 5'-hydroxyl group in polyacrylamide gels under denaturing conditions. The radioactively labeled strands from the substrates are indicated by S. The slower migration of the Cl band in lane 4 is because of the additional three nucleotides contributed by the A3 bulge.

Flp-RNase I Activity in FRT Sites Containing Directed DNA Bends—The normal Flp recombination reaction proceeds via two steps of single strand exchanges. A double strand cleavage and joining mechanism is not possible because, at any given time, Flp can cleave only one of the two labile phosphodiester bonds of an FRT site. However, either one of them has a roughly equal probability of being targeted for cleavage. In a Flp dimer, which is the cleavage-competent entity (14), the activation of one of the two potential active sites excludes the other from activation. Solution studies and recent crystallographic data indicate that a directed bend in DNA, and the consequent relative orientations of the Flp monomers, is responsible for this “half-of-the-sites” activity (7, 22, 31).

Lee et al. (22) placed strand-specific nucleotide bulges within the FRT spacer to force the DNA to bend in one orientation or the opposite and demonstrated the operation of active site exclusion in these substrates. When bound by a Flp dimer, a bulged substrate is cleaved at one scissile phosphodiester position with a large bias over the other. Furthermore, it is the bulge-containing strand that is cleavage-susceptible, thus defining the direction of the active DNA bend. Lee et al. (22) also noted that either of the two phosphodiester bonds can be cleaved in a bulged substrate bound by Flp(Y343F) when supplied with a small diffusible nucleophile such as hydrogen peroxide (the peroxide anion being the active agent). They concluded that the binding of a Flp monomer is sufficient to prepare the adjacent phosphodiester for nucleophilic attack. It is the misplacement of Tyr-343 that is the basis of active site exclusion.

From the crystal structures of Cre and Flp tetramers complexed with DNA (7, 8), it is apparent that the two inactive tyrosine nucleophiles are oriented incorrectly with respect to their phosphate targets. The misalignment is corrected during the isomerization step of recombination, which likely involves some flexing of the DNA arms and limited relative rotation of the protein subunits. Can the altered structural context of the scissile phosphate in a type I RNase substrate relax the restriction on tyrosine positioning? We have now assayed Flp-RNase I in synthetic FRT sites containing spacer bulges to test whether strand cleavage is biased and, if so, what the direction of the bias is.

The bulge-containing substrates for Flp-RNase I (Fig. 3) were very similar to the ones previously described by Lee et al. (22), except for the indicated single substitution of a deoxy-C by a ribo-C at each of the two cleavage positions, on the top and bottom strands. The reactions in lanes 1–4 of Fig. 3 confirm the previous observation that an A3 top strand spacer bulge restricted cleavage to the left end of the spacer in a DNA substrate (Cl in lane 4). In the corresponding bulge-free DNA substrate, cleavage was observed at the left or the right end (Cr and C0 in lane 2). The control reaction with an unbulged type I RNase substrate revealed cleavage at either end of the spacer in roughly equal amounts (Cl and Cr in lane 6, Fig. 3). In contrast to the DNA substrates, selective cleavage at a single
scissile phosphodiester position was not observed for the type I substrates containing the A3 spacer bulge on the top strand or the bottom strand (C_L and C_R in lanes 8 and 10, Fig. 3).

Let us assume that, as expected from the DNA reactions, initial cleavage by Flp occurs exclusively on the bulge-containing strand even in the Flp-RNase I reaction. The product of nicking has protein-free ends, a 5′-hydroxyl, and a 3′-phosphate. The lack of protein linkage and the consequent structural flexibility of the nicked substrate may then allow the assembly of the second active site to induce cleavage on the bulge-free strand. To verify this possibility, we tested the action of Flp on substrates where the bulge-containing strand was all DNA, and the ribonucleotide substitution was present only in the opposite strand (Fig. 4A). Because cleavage of DNA results in a Flp-linked 3′ end, these substrates will not have the conformational freedom of their nicked counterparts unlinked to protein. As shown in Fig. 4A, cleavage by Flp of the ribonucleotide-containing strand (without the bulge) was not observed even in these substrates (C_L and C_R in lanes 2 and 4, Fig. 4A).

In Fig. 4B, we have schematically diagrammed the substrate conformations that accommodate the contrasting results obtained with the “all DNA” and the “mixed DNA-RNA” substrates. Two oppositely directed bends, positioned asymmetrically within the spacer, can determine which of the two scissile phosphates of the DNA substrate will be correctly aligned with its target phosphodiester bond. The asymmetric bending of the spacer and the placement of the bend fulcrum are arbitrary designations. L and R refer to the Flp-binding arms at the left and right sides of the spacer, respectively. Bottom, a possible scheme that accounts for the Flp-RNase I activity on the bulge-containing strand or the bulge-free strand is outlined. Two identically bent substrates, each associated with a Flp dimer, are assembled into an antiparallel synaptic complex (7, 32). The ribonucleotide may sufficiently relax the structural restrictions posed by an all DNA substrate to permit Tyr-343 to assume its active orientation with respect to the scissile phosphate of the bulge-free strand. The trans donation of Tyr-343 in this case is across substrate partners.

FIG. 4. Flp active site exclusion operates in bulge-containing DNA half-site but not in type I half-sites. A, the substrates are depicted as in Fig. 3. Note that the bulge-containing strand is all DNA. Only the bulge-free strands contain the potential target phosphate for the type I Flp-RNase activity. Reactions were done with wild type Flp as in Fig. 3. The DNA strand was labeled at the 3′ end, and the DNA-RNA hybrid strand was labeled at the 5′ end. B, top, directed DNA bends located within the spacer are responsible for excluding one of the two possible active sites of a Flp dimer (22). An A3 bulge, depending on its location, selects for a given bend orientation by enforcing the flanking DNA arms away from itself. In the non-functional active site, the catalytic tyrosine is mispositioned with respect to its target phosphodiester bond. The curved arrows indicate the direction of donation of the reactive Tyr-343 in trans. The asymmetric bending of the spacer and the placement of the bend fulcrum are arbitrary designations. L and R refer to the Flp-binding arms at the left and right sides of the spacer, respectively. Bottom, a possible scheme that accounts for the Flp-RNase I activity on the bulge-containing strand or the bulge-free strand is outlined. Two identically bent substrates, each associated with a Flp dimer, are assembled into an antiparallel synaptic complex (7, 32). The ribonucleotide may sufficiently relax the structural restrictions posed by an all DNA substrate to permit Tyr-343 to assume its active orientation with respect to the scissile phosphate of the bulge-free strand. The trans donation of Tyr-343 in this case is across substrate partners.
As shown in Fig. 5B, the product of the type II RNase cleavage (CP2) was formed when Flp(Y343F) was bound to the type II half-site substrate HS-R2, and the presence of a partner protein on the DNA half-site (HS-D) had no effect on the reaction (lanes 2–5, Fig. 5B). No type II cleavage was detected when HS-D was bound by Flp(Y343F) and HSR2 by one of the Flp pentad variants (lanes 6–8, Fig. 5B).

To test further whether a monomeric Flp/half-site complex is the active entity for the type II RNase activity, reactions were carried out with the hairpin substrates shown in Fig. 5C. The DNA hairpin mimics the product of a half-site recombination reaction (see Fig. 1A) and can bind a Flp monomer, but the Flp-hairpin complex cannot dimerize.2 When treated with Flp, the DNA hairpin did not yield the Flp cleavage product (lane 2, Fig. 5C). This result was expected because the absence of a functional Flp dimer would preclude the trans donation of the Tyr-343 nuclease. The hairpin substrate for type II Flp-RNase was readily cleaved by both Flp and Flp(Y343F) (lanes 2 and 3, Fig. 5D).

We interpret the results from Fig. 5 to mean that a single Flp(Y343F) monomer is competent to carry out the type II RNase reaction. The inactivity of the pentad Flp mutants (lanes 6–8, Fig. 5B) in type II cleavage suggests that, except for the tyrosine nuclease, the active sites for the strand cutting step of DNA recombination as well as the type I and type II Flp-RNase reactions are either the same or utilize multiple overlapping catalytic residues. Tyr-343 serves as the nucleophile for DNA recombination and the initial step of type I RNA cleavage; the respective vicinal 2'-hydroxyl groups act as the nucleophiles for type II RNA cleavage and the second step of type I RNA cleavage. Thus, Flp-RNase I and Flp-RNase II represent instances of a common cluster of active site amino acids being able to target two distinct but adjacent phosphodiester bonds under different substrate contexts.

Steady State Kinetic Analyses of Flp-RNase I and Flp-RNase II, Tests for the Proposed Mechanisms—The Lineweaver-Burk plots for the type I RNase cleavage and the hairpin recombination assay were carried out simultaneously from a single half-site substrate. Typical values for $K_m$ and $V_{max}$ at 28 °C were $22 \text{ nM}$ and $0.23 \text{ nM min}^{-1}$ for Flp-RNase I. The corresponding values for the hairpinning reaction (recombination) were $46 \text{ nM}$ and $0.40 \text{ nM min}^{-1}$, respectively. The similar magnitudes of $V_{max}$ for RNA cleavage and recombination are consistent with both reactions proceeding via a common intermediate or intermediates.

Steady state kinetic experiments in the 16–34 °C range were carried out for the RNA cleavage reaction alone by phosphorylating the 5'-hydroxyl group of the bottom strand and thus blocking hairpin formation (Fig. 7). The $V_{max}$ at 28 °C under these conditions was $1.6 \text{ nM min}^{-1}$. The roughly 7-fold increase in $V_{max}$ for RNase I cleavage over that observed under recombination-permissive conditions agrees with the two reactions competing for a common intermediate. The logarithmic plot of $V_{max}$ versus $1/T$ (Fig. 7A) was non-linear, indicating that $V_{max}$ is controlled by more than one rate constant. To obtain a reasonable estimate of the number of possible intermediates during type I cleavage, $1/k_1$ was plotted against $1/T$ (Fig. 7B). Assuming at least one intermediate in the conversion of the [Flp-S]$_2$ complex into free enzyme and CP1 (the cleavage product), the rate expression may be related to the Arrhenius Equation 1 as follows:

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\frac{1}{V_{max}} = \frac{1}{k_1} + \frac{1}{k_1} \exp(-E'/RT) + \frac{1}{A_1} \exp(E'/RT) + \frac{1}{A_1} \exp(E'/RT) \quad \text{(Eq. 1)}
$$

Non-linear least squares fit to the experimental data yields $E' = 17.9 \text{ kcal mol}^{-1}$; $E' = 35.7 \text{ kcal mol}^{-1}$; $A_1 = 4.1 \times 10^{38} \text{ min}^{-1}$; and $A' = 1.04 \times 10^{38} \text{ min}^{-1}$. In principle, the data in Fig. 7B may be accommodated by the two activation energies being either positive or of opposite signs. Imposing the latter condition always resulted in poor fit to the data. The kinetic results therefore argue in favor of at least one intermediate in the conversion of the Flp-half-site complex into the RNase I cleavage product. Based on previous results, we argue that this intermediate is the covalent complex formed between Tyr-343 and the 3'-phosphate at the cleavage point, which is common to the recombination reaction as well. The proposed 2',3',5'-cyclic phosphate intermediate, the immediate precursor to CP1 (see Fig. 1A), is likely to be too transient to contribute significantly to the kinetics of the reaction.

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2. J. Lee and M. Jayaram, unpublished data.
The Lineweaver-Burk plot for the type II cleavage reaction by Flp(Y343F) (Fig. 8A) gives 49 nM for \( K_a \) and 0.37 nM min\(^{-1} \) for \( V_{max} \) at 28 °C. The temperature dependence of \( V_{max} \) was followed for the 16–34 °C interval and graphed as a logarithmic plot versus 1/\( T \) (Fig. 8B). The linear fit of the experimental points indicates that \( V_{max} \) is controlled by a single rate constant. The kinetic data thus support the mechanism proposed in Fig. 5A in which the vicinal 2'-hydroxyl group directly attacks the scissile phosphodiester bond to form the cleavage product CP2 via the short-lived 2',3'-cyclic phosphate intermediate. Application of the Arrhenius equation to the experimental data yields the activation energy \( E_a \) related to \( V_{max} \) as 13.5 kcal mol\(^{-1} \).

**Effect of Substrate Context on the Type II RNase Activity of Flp**—Our earlier work (16, 17) had indicated that the Flp-RNase II activity strongly prefers the base U at the ribonucleotide position, whereas the Flp-RNase I activity is relatively unaffected by the presence of any of the four bases (A, G, C, or U) at the cleavage point. Furthermore, placement of a second “ribo-U” rather than a “deoxyribo-T” immediately 3’ to the type II scissile phosphate was found to cause a significant stimulation of cleavage by Flp-RNase II. Because of the structural resemblance between U and the phenolic ring of tyrosine (the cleavage nucleophile in DNA recombination), we suspected possible interactions between this base at the cleavage position and the RNase I active site of Flp. Such interactions may be critically sensitive not only to the specific base at the cleavage site but also to the neighboring sequence context, local base pairing effects, and potential backbone distortions.

In the assays shown in Fig. 9, the series of type II substrates tested contain U, G, A, or C at the cleavage position. Each class is composed of three substrate subtypes as follows: (a) the base at the cleavage position has a normal complementary partner on the other strand; (b) it has an opposing non-complementary partner; and (c) this base is present as a bulge, with no partner (matched or mismatched) on the other strand. Each of these substrate subtypes, a–c, is arranged left to right within an assay set, and their reactions with Flp(Y343F) are displayed. The relevant comparisons apply to the panels captioned U, G, A, and C. In all substrates of these four panels, the 2 bp 3’ to the cleavage point are kept constant as 5’TT3/3’AA5’. The only difference between the U’ and U panels is that substrates of the former contain a “U-A” pair, rather than a “T-A” pair as the immediate 3’ neighbor of the scissile phosphate.

Whereas all three of the U’ panel substrates yielded type II cleavage (CP2) with Flp(Y343F), the activity was greatly enhanced in the corresponding U panel substrates. This result is consistent with the previous observations of Xu et al. (16) regarding the nearest 3’ neighbor effects of “U” versus “T” on Flp-RNase II. For substrates of the G, A, and C panels, no reaction was observed when the ribonucleotide base and the one opposite to it were complementary (G-C, A-T, and C-G in the G, A, and C panels, respectively). However, when the ribonucleotide base was presented as a bulge or as a mismatch (G-A, A-A, or C-A), the type II cleavage was readily detected. The most impressive stimulation of cleavage occurred with the “A” bulge and the “A-A” mismatch. The levels of CP2 formed in these cases were comparable with those seen with the U’ panel substrates.

The assays shown in Fig. 10 test the effects of the paired versus bulged ribonucleotide in type II RNase cleavage by Flp.
in the context of a 3' G-C neighbor. Reactions were done with Flp(Y343F). None of the substrates with the paired ribonucleotide were targets for Flp-RNase II. It is interesting to note that the presence of the 3' G-C bp antagonized U (paired with A), normally the highly preferred base for the type II reaction (16). In the bulged state of the ribonucleotide, all four bases tested positive for Flp-RNase II activity. The cleavage efficiency followed the order U > A > C > G.

The results of the analyses in Figs. 9 and 10 highlight how structural features at or adjacent to the ribonucleotide position modulate the efficiency of the Flp-RNase II reaction. For U, which is the most reactive in substrates that are fully base paired at the first three spacer positions (16), the cleavage efficiency changes significantly as the adjacent 3'-bp changes. Among the substrates tested, U-A is the best 3' neighbor, T-A is tolerated, and G-C is unsuitable. Furthermore, when the ribonucleotide base is unpaired or unopposed by a base on the opposite strand, all four bases are accepted by Flp-RNase II. Strikingly, the activity of A now closely parallels that of U. Consistent with the relatively high reactivity of unpaired or bulged A, we have occasionally seen very faint bands of the type II cleavage product (CP2) when wild type Flp is reacted with substrates in which the A is paired with T (results not shown). Because U can partially mimic tyrosine (which normally accesses the Flp active site), there is a higher likelihood of it being forced out of its paired state and correctly positioned for the type II RNase reaction. For the other three bases, relieving the base pairing constraints may predispose them toward acquiring the functional cleavage configuration, which may be further stabilized by hydrogen bonding, hydrophobic forces, or via stacking interactions.

**The Cre Recombinase Exhibits the Type I RNA Cleavage Activity**—If the RNA cleavage activities of Flp represent the evolutionary vestiges of the elaboration of a recombinase active site from an elementary nuclease active site, it is likely that similar activities are harbored by other members of the integrase/tyrosine family as well. As noted earlier, vaccinia topoisomerase I (a type IB topoisomerase) has been shown to possess an RNA cleavage activity that closely resembles Flp-
RNase I (18). We have now probed the Flp-related Cre recombinase for cryptic RNase active sites using half-sites containing ribonucleotides incorporated at specific positions.

Synthetic 5′ half-sites (substrates for Cre) containing appropriate ribonucleotide substitutions for assaying type I and type II RNA cleavage were treated with Cre or Cre(Y324F) (Fig. 11). Wild type Cre gave the CP1 product, expected from type II RNA cleavage were treated with Cre or Cre(Y324F) appropriate ribonucleotide substitutions for assaying type I and containing ribonucleotides incorporated at specific positions.

Inter-protomer Interactions between Recombinase Monomers during the Assembly and Orientation of Flp and Cre Active Sites—As already indicated, one likely explanation for the bi-specificity of Flp and the mono-specificity of Cre in their RNase activities is the difference in the manner in which the two recombinases assemble their active sites. In the case of Cre, all catalytic residues including the cleavage nucleophile (Tyr-324) are supplied by a single monomer, which cleaves in cis the scissile phosphodiester bond adjacent to it. In the case of Flp, the cleavage occurs in trans, Tyr-343 being delivered by the Flp cleavage pocket might be interfered with by a cis-acting tyrosine. This interference would be absent in the case of a trans-acting tyrosine.

The contrast between Flp and Cre in their active site architectures and their RNA cleavage activities has implications in the functional interactions between recombinase monomers bound to distinct, multiple DNA arms, as would be the case in the pre-catalytic synaptosome complex or the Holliday junction intermediate of recombination. Whereas only a single active site can be derived from a dimer of Flp at a time, two active sites can be arranged by either a Flp trimer bound to three DNA arms or a Flp tetramer (or a dimer of dimers) bound to four DNA arms (19, 20, 22).

In Fig. 12, we have summarized the action of Cre on a synthetic Y junction DNA substrate with three Cre-binding arms and three potentially labile phosphodiester bonds. The resolution of this artificial substrate, in a reaction analogous to that performed by Flp (19), would require the action of two active sites. The resulting two cleavage and joining events would give a linear product and a hairpin product (Fig. 12B). In a binding assay, the Y substrate yielded three complexes with Cre(Y324F) (CI, CII, and CIII) corresponding to the filling of one, two, and all three DNA arms, respectively (Fig. 12B). In a resolution assay with wild type Cre (at a Cre to Y molar ratio as in lane 2 of Fig. 12B), the linear (LR) and hairpin (HP) resolution products were formed (Fig. 12C). Because only a single strand of the Y substrate was labeled at the 3′ end (asterisk), the radioactive linear product must arise by cleavage at positions 1 and 3 and the radioactive hairpin product by cleavage at positions 1 and 2 (see Fig. 12A).

Thus, despite their cis versus trans cleavage modes, both Flp and Cre can yield two functional active sites from three recombinase monomers. The representation of the plausible trimer form of Cre active in Y junction resolution (Fig. 12D) is based on the crystal structure of the cleaved Cre-DNA complex (8).

**DISCUSSION**

We briefly highlight here the implications of the present findings for the organization and mode of action of the RNA cleavage active sites resident within the integrase/tyrosine

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**FIG. 10. Further effects of substrate context on Flp-RNase II.** The substrates labeled at the 5′ end on the strand containing the scissile phosphate are shown. The substrate and cleavage product are designated as in Fig. 9 (HS-R2 and CP2, respectively).

**FIG. 11. Probing cryptic RNA cleavage activities in the Cre recombinase.** A, the half-site substrate designed to probe the type I RNase activity of Cre is schematically shown. The top strand containing the scissile phosphate was labeled at the 5′ end. The 5′ end of the bottom strand was phosphorylated. The ribonucleotide base A is immediately to the 5′ side of the phosphodiester bond (p) that takes part in Cre-mediated DNA recombination. Consistent with the Flp nomenclature, the type I half-site substrate and the corresponding cleavage product are called HS-R1 and CP1, respectively. B, in the type II RNase substrate for Cre (HS-R2; represented at the top), the ribonucleotide U was positioned immediately 5′ to the presumed target phosphate (p′).
The two cleavage-active Cre monomers are shown in the Y junction and a Cre trimer shown here is based on the crystal structure. The position of the 3' hydroxyl is labeled as an asterisk. D, a plausible representation of the active complex between Cre and DNA. The type I RNA cleavage reaction could be readily detected in Cre, but so far the type II reaction has proven to be elusive. As already noted, the type I RNase has also been characterized in vaccinia topoisomerase I (18). This activity appears to be present in most, if not all, of the integrase/tyrosine family recombinases and other enzymes that utilize a type IB topoisomerase mechanism. The generality of this reaction suggests that the evolution of the recombinases is likely to have started with the emergence of an elementary nuclease active site and proceeded through the intermediary formation of a topoisomerase active site (17, 34, 35). Based on the Flp results, we suspect that the Cre-RNase I active site is also the same as the DNA recombinase active site, its chemical competence being dependent on inter-partner activation within a Cre dimer (8).

The "Y" junction resolution assay reveals the assembly of two functional active sites by a trimer of Cre. The situation is analogous to that of a Flp trimer bound to a Y substrate (19). Thus, regardless of the cis and trans cleavage modes, the number of active sites that can be assembled from Flp or Cre in a given oligomeric state is restricted by the structural constraints of the DNA-protein complexes. The assays in lanes 2 and 3 contained Cre(Y324F) at a molar ratio of 4 and 8 per DNA arm, respectively. The control assay in lane 1 did not contain Cre(Y324F). Fractionation of DNA-protein complexes was done in an 8% native polyacrylamide gel. S refers to the unbound substrate. The hierarchical Cre-DNA complexes are labeled as CI, CII, and CIII. C, the resolution reaction was carried out with wild type Cre and subjected to electrophoresis in a 10% polyacrylamide gel. The two equal included angles between the Y arms permit them to contact their Cre neighbor by extending their carboxyl-terminal portions of Cre, including the catalytic Tyr-324 (Y), are shown. The two cleavage-active Cre monomers are shown in gray and their target phosphates as white circles. The two equal included angles between the Y arms permit them to contact their Cre neighbor by extending their carboxyl-terminal N helix in the counterclockwise direction. This inter-protomer interaction is responsible for conferring cleavage competence on one Cre monomer within an adjacent pair. The Cre monomer in white is inactive because the third included angle (unequal to the first two) prevents its N helix from contacting the adjacent Cre. The inert phosphate is shown in gray. There are three possible isomers of this structure, each containing two active Cre monomers and an inactive one. Thus, resolution is possible by cleavage of any two of the three scissile phosphodiester bonds.

family of DNA recombinases and their functional relationship to the corresponding DNA recombination active sites.

The Type I RNase Active Site of Flp, Congruence with the DNA Recombination Active Site—Several lines of evidence support the active site congruence and mechanistic identity between DNA recombination and type I RNA cleavage mediated by Flp. The Flp-RNase I cleavage is performed by a shared active site in which the target phosphate is oriented by a Flp monomer bound adjacent to it (in cis) but is cleaved by Tyr-343 provided by a Flp partner bound distal to it (in trans). These features of the reaction are identical to those observed for DNA recombination in half-sites (24, 33). Consistent with Tyr-343 being involved in covalent catalysis during the first cleavage step of the Flp-RNase I reaction, Flp or Flp(Y343F) can convert a type I half-site substrate containing a 3'-phosphotyramine bond to the 3'-phosphate product (CP1). The non-linear temperature dependence of log Vmax for Flp-RNase I is accommodated by two rate constants determining the conversion of the Flp-half-site complex into CP1; the first controls the formation of the covalent tyrosyl intermediate, and the second determines its conversion to the 3'-phosphate product.

The DNA cleavage and the type I RNase active sites of Flp assemble differently in geometrically restricted substrates containing spacer bulges. Whereas the DNA cleavage pocket is excluded from the target phosphodiester on the bulge-free strand, the same exclusion does not apply to the Flp-RNase I pocket. Solution studies and the Flp crystal structure indicate that it is the mispositioning of Tyr-343 that is responsible for half-of-the-sites activity of Flp (7, 22). The ribonucleotide substitution present in the type I RNase substrate appears to sufficiently relax structural constraints to avoid Tyr-343 misalignment.

The Type II RNase Active Site of Flp Is Housed within a Single Monomer but Is Modulated by the Substrate Context—The type II RNase cleavage by Flp does not require Tyr-343 and is not blocked when the assembly of a dimeric half-site complex is prevented by using hairpin substrates. The linear dependence of log Vmax on temperature suggests that the breakdown of the Flp-half-site complex into CP2 is determined by a single rate constant. Taken together, these results argue for a cis-acting Flp-RNase II active site, in which a single Flp monomer executes the reaction, with the vicinal 2'-hydroxyl providing the cleavage nucleophile.

Unlike Flp-RNase I, Flp-RNase II is quite sensitive to the nature of the base present at the cleavage position and whether it is in a paired, unpaired, or bulged state. The observed preference for U at the cleavage position would be consistent with its closer structural relatedness to tyrosine than the other three bases. In the crystal structure of the Flp-Holliday junction complex, the Tyr-343 residue, delivered in trans, is stabilized by its stacking interaction with His-309 of the monomer that receives this cleavage nucleophile (7). Perhaps the same mechanism is also utilized to position the aromatic ring of U within the Flp-RNase II active site. The relatively high activity of an unpaired or bulged A and modest activities of unpaired or bulged C and G, when present at the cleavage position, further suggest the need for these bases to be distorted from their normal configuration before they can be functional in type II cleavage.

RNA Cleavage Activity and Active Site Configuration of Cre—The type I RNA cleavage reaction could be readily detected in Cre, but so far the type II reaction has proven to be elusive. As already noted, the type I RNase has also been characterized in vaccinia topoisomerase I (18). This activity appears to be present in most, if not all, of the integrase/tyrosine family recombinases and other enzymes that utilize a type IB topoisomerase mechanism. The generality of this reaction suggests that the evolution of the recombinases is likely to have started with the emergence of an elementary nuclease active site and proceeded through the intermediary formation of a topoisomerase active site (17, 34, 35). Based on the Flp results, we suspect that the Cre-RNase I active site is also the same as the DNA recombinase active site, its chemical competence being dependent on inter-partner activation within a Cre dimer (8).
straints imposed by their DNA substrates. Although the Flp trimer can provide two functional active sites (as demonstrated by resolution of a Y substrate), the same is not true when the trimer is part of a tetramer bound to the four DNA arms of a Holliday junction (36). The active form in the tetrameric state of Flp is a dimer of dimers. This feature of the recombinase is consistent with the structural data of Cre and Flp in association with DNA (7, 8).

Given that the chemical mechanisms of the Flp and Cre reactions are virtually identical, the absence of the type II RNase activity in Cre (which has retained the type I activity) is rather difficult to explain. It is possible that the presence of the cis-acting Tyr-324 blocks the cleavage pocket and precludes the functional interactions required to position the ribonucleotide in the proper cleavage orientation. We are currently testing whether Cre can be induced to perform the type II cleavage when Tyr-324 is replaced by glycine or alanine rather than by phenylalanine, as was done in this study. The local context effects observed for Flp-RNase II suggest that Cre-RNase II may be more difficult to unveil, even if it is present in a cryptic state. In the lox half-sites that we have tested, the base pair neighboring the labile type II phosphate on its 3′ side is G-C, which has a large negative effect on the Flp-RNase II activity (as shown in this study). Mispairing or bulging out the ribonucleotide at the type II cleavage position or changing the bp to its 3′ side are under consideration. A combination of the above strategies that tweak both the recombinase and its DNA substrate should reveal whether the type II RNase reaction is more pervasive in the integrase/tyrosine family than is suggested by the present results.

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Note Added in Proof—A recent paper by Woods et al. (37) describes the crystal structure of a trimer form of the catalytically inactive Cre(Y324F) complexed with a Y-shaped DNA substrate. Our interpretation of the resolution of Y junctions by wild type Cre is consistent with the structural data. Woods et al. have also reported the ability of wild type Cre to resolve Y-shaped substrates.

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Page 46618: Equation 1 was incorrect. The correct equation is shown following.

\[
\frac{1}{k_1} = \frac{1}{k_1^1} + \frac{1}{k_1^1} \exp \left( \frac{E_1}{RT} \right) + \frac{1}{A_1^1} \exp \left( \frac{E_1^2}{RT} \right) \quad (Eq. 1)
\]

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