Flp Ribonuclease Activities

MECHANISTIC SIMILARITIES AND CONTRASTS TO SITE-SPECIFIC DNA RECOMBINATION*

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Chong-Jun Xu, Yong-Tae Ahn, Shailja Pathania, and Makkuni Jayaram†‡

From the Department of Microbiology, and Institute of Cell and Molecular Biology, University of Texas, Austin, Texas 78712

The ribonuclease active site harbored by the Flp site-specific recombinase can act on two neighboring phosphodiester bonds to yield mechanistically distinct chain breakage reactions. One of the RNase reactions apparently proceeds via a covalent enzyme intermediate and targets the phosphodiester position involved in DNA recombination (Flp RNase I activity). The second activity (Flp RNase II) targets the phosphodiester immediately to the 3' side but appears not to involve an enzyme-linked intermediate. Flp RNase I is absolutely dependent upon Tyr-343 of Flp and is competitive with respect to the normal strand joining reaction. It can utilize the 2'-hydroxyl group from any one of the four ribonucleotides with comparable efficiencies in the cleavage reaction. On the other hand, the RNase II activity mediated by Flp(Y343F) is specific for U and cannot utilize the 2'-hydroxyl group from ribo-A, -G, or -C under standard reaction conditions. The RNase II activity is also sensitive to the 3'-neighboring base. Although dT is functional, the activity is stimulated by U or U-2'-OMe. The Flp RNase II reaction effectively competes with the normal strand cleavage reaction mediated by Tyr-343, even though their phosphodiester targets are not the same.

The Flp protein encoded by the 2-µm plasmid of Saccharomyces cerevisiae is a site-specific DNA recombinase that is thought to play a central role in the copy number control of this extrachromosomal element (reviewed in Ref. 1). Flp is a member of the integrase family of “conservative” site-specific recombinases. Members of this family utilize a common biochemical mechanism to carry out a wide range of biological functions (2–4).

The int(egrase) family recombinases harbor four invariant residues located within two domains of modest amino acid homology: an arginine-histidine-arginine triad (RHR) and a tyrosine residue, corresponding to Arg-191, His-305, Arg-308, and Tyr-343 of Flp (see sequence alignments in Refs. 5–7). The RHR triad and Tyr-343 of Flp appear to be directly involved in the catalytic steps of recombination (reviewed in Ref. 8). The tyrosine residue provides the nucleophile during the DNA strand cleavage reaction. The resulting products are a 3'-O-phosphotyrosyl bond between Flp and the broken DNA end, and a 5'-hydroxyl group. Strand joining is chemically equivalent to the reverse of the cleavage reaction. The 5'-hydroxyl acts as the nucleophile to displace the DNA-linked tyrosine, restoring the continuity of the DNA strand. During a normal recombination event, this 5'-hydroxyl group originates from the cleaved strand of the partner DNA molecule.

Mutations of the RHR triad residues or Tyr-343 of Flp result in the arrest of recombination at the cleavage step, or at the strand transfer step, or both (9–12). It has been generally believed that the likely catalytic role of the RHR triad is in orienting the target, the scissile phosphodiester in the DNA chain or the 3'-O-phosphotyrosyl bond formed as a result of cleavage, for nucleophilic attack. The recently solved crystal structure of the Cre recombinase from phage P1 (an integrase family member) complexed with its DNA substrate supports this notion (13). In the crystal structure, the two cleaved phosphates are hydrogen-bonded to the triad arginines and the histidine. The proximity of the RHR triad residues in the crystal structures of other integrase type recombinase proteins, lambda Int, the Int protein of the lambda-related HP1 phage, and the Xer D protein of Escherichia coli is consistent with a common functional role for the triad throughout the family (5, 14, 15). In the case of Flp, it has been demonstrated that, provided the scissile phosphate has been properly oriented, a mutant Flp lacking Tyr-343 can mediate strand cleavage when supplied with alternative nucleophiles such as the peroxy anion or a phenolate moiety (12, 16). Furthermore, in reactions that mimic the strand joining step, glycerol and other polyhydric alcohols can take the place of the 5'-hydroxyl group of DNA (17).

Consistent with the proposed mechanism for strand cleavage, it has recently been demonstrated that the Flp active site also harbors a cryptic RNase active site (18). Reactions using hybrid DNA-RNA substrates, containing specific ribonucleotide substitutions within a DNA chain, unveil two site-specific RNA cleavage activities by Flp. These activities are hereafter referred to as Flp RNase I and Flp RNase II (or type I and type II activities). The former is analogous to the site-specific RNA cleaving activity described by Sekiguchi and Shuman (19) for vaccinia topoisomerase I. It is believed that the type I reaction requires the formation of the 3'-O-phosphotyrosyl bond that is then attacked by the vicinal 2'-hydroxyl group. The reaction is therefore mechanistically related to the strand joining reaction. The shared structural homology between type IB topoisomerasers and the integrase type recombinases within their catalytic domains (20–22) legitimizes the closely related RNA cleavage activities exhibited by Flp and vaccinia topoisomerase I (23). However, the equivalent of the Flp RNase II has not been detected in the topoisomerase IB family. This activity does not require Tyr-343 of Flp and is thought to proceed via a direct attack by the adjacent 2'-hydroxyl group on the backbone phosphodiester (18).

In this report, we have characterized Flp RNase I and Flp RNase II with regard to their substrate requirements, target specificities, and their differential sensitivities to sequence con-
The half-sites, labeled at the 5′ end of a deoxyoligonucleotide were labeled using [γ-32P]ATP in a T4 polynucleotide kinase reaction. The unreacted ATP was removed by spin dialysis on a Sephadex G-25 column.

**Synthetic Half-site Substrates**—Synthetic hybrid oligonucleotides containing ribose substitutions at specific positions were purchased from Oligos Etc., Wilsonville, OR. Standard deoxyoligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA. Batches of the oligonucleotide preparations were gel-purified prior to individual sets of experiments.

The half-sites were assembled by hybridization between pairs of oligonucleotides under conditions standardized previously (24). The 5′ end of a deoxyoligonucleotide was labeled using γ-[32P]ATP in a T4 polynucleotide kinase reaction. The unreacted ATP was removed by spin dialysis on a Sephadex G-25 column.

**Half-site Cleavage Reactions**—The reactions were done according to the protocol described for Flp recombination assays by Chen et al. (25) with minor modifications. Each reaction contained 0.05–0.10 pmol of the labeled half-site and approximately 0.4 pmol of Flp or Flp(Y343F). Some of the reactions contained RNasin (80 units/ml; Promega, Madison, WI). Incubations were done at 30 °C for 30 min. Reactions were terminated by the addition of SDS (0.2% final concentration) and immersion in a boiling water bath for 30 s. After phenol/chloroform extraction, the DNA or the hybrid DNA-RNA was recovered by ethanol precipitation and subjected to electrophoresis in 12% denaturing polyacrylamide gels. The protein-DNA complexes were identified by autoradiography or by phosphorimaging.

The large majority of the remainder were variants of these, containing changes in the nucleotide positions of the spacer (the strand exchange region in recombination) or in the spacer-adjacent position of the Flp-binding element. Three of the substrates (called S1–S3 in Table I) were designed so as to eliminate Flp binding and served as controls for potential nonspecific RNase activity in Flp preparations. In each variant substrate, both strands were simultaneously altered to maintain base complementarity.

In order to simplify the description and interpretation of experimental results, the following conventions have been used. The bases written in bold capital letters denote deoxy to ribo substitutions at specific positions (in the Flp-binding element or in the spacer). The scissile phosphodiester target in recombination (also the target for the type I RNase of Flp) is denoted by p. The adjacent phosphodiester that is targeted by the type II RNase of Flp is denoted by p′. These general rules are followed in the figures and in the text. In addition, the 2′-hydroxyl groups are explicitly represented in the figures. Sequences unrelated to the Flp reaction are written in italics. The relevant and representative substrates is assembled in Table I.
Ribonuclease Activities of Flp Recombinase

The Role of Tyr-343 and of the Ribonucleotide Location in RNA Cleavage Reactions—The cleavage reactions for a series of hybrid DNA-RNA substrates in the presence of Flp or Flp(Y343F) are assembled in Fig. 2. They permit the distinction of Flp RNases I and II with respect to their target specificities, the requirement or dispensability of Tyr-343, and their utilization of separate 2'-hydroxyl groups from neighboring ribose units. These results, which expand upon those described in Fig. 1, provide strong evidence to reveal the essential substrate requirements for the two types of Flp RNase activities.

In Fig. 2A, the experimental outcomes corresponding to the schema in Fig. 1 are displayed. The recombinant product was formed in reactions of all three substrates, the DNA substrate (lanes 3, 6, and 9). As expected from the role of Tyr-343 in recombination, Flp(Y343F) did not yield R (lanes 2, 5, and 8). Similarly, the Flp RNase I activity was exhibited only by wild type Flp (CP2 in lane 6), but not in lane 5). By contrast, Flp RNase II activity was detected with Flp as well as with Flp(Y343F) (CP2 in lanes 8 and 9). Neither of the two activities yielding CP1 or CP2 was elicted with the DNA substrate (lanes 2 and 3). The yield of R was lower for the hybrid substrates containing three Us adjacent to p are depicted in Fig. 2B. Cleavage at p’ with the resultant formation of CP2 was promoted by Flp(Y343F) (lane 2). The yield of CP2 was insensitive to RNasin (lane 3), but CP2 production was abolished when boiled Flp(Y343F) was used in the assay (lane 4). The data in Fig. 2C demonstrate that three nonspecific RNA-DNA substrates (S1–S3, Table I) that could not be bound by Flp were not targets for cleavage by Flp (lanes 4, 7, and 10) or by Flp(Y343F) (lanes 5, 8, and 11). In a control reaction with the hybrid substrate (Cttt) and wild type Flp, CP1 was formed (lane 2).

The experiments in Fig. 2D reveal the essential substrate requirements for the two types of Flp RNase activities. Assays using the set of substrates depicted to the left of the marker lane M (all of which contained the indicated common ribo-C) demonstrate that the CP1 product could be made by Flp as long as the 2'-hydroxyl was vicinal to the 3',5’-phosphodiester p (presence of CP1 in lanes 2, 4, 6, 8, and 10). Similarly, reactions with the group of substrates (all of which contained a common deoxyribo-C, succeeded by a 3’ U in four cases) arranged to the right of the marker lane indicate that the formation of the CP2 product was predicated upon the 2'-hydroxyl group being adjacent to the p’ phosphodiester (presence of CP2 in lanes 18, 20 and 22). Note that, given this basic rule, CP2 formation was more efficient when the base to the 3’ side of p’ was U rather than t (compare the level of CP2 in lane 20 to that in lanes 18 and 22). With substrates that simultaneously satisfied the functional group requirements for the two cleavages, both CP1 and CP2 were produced (lanes 2 and 4). An apparent exception was the substrate (shown in lanes 5 and 6) in which the ribo-CU sequence was followed by t rather than U. In this case, CP2 was barely detectable, although CP1 formation was normal (compare lane 6 to lane 4). The combined effects of the
adjacent t inhibition (lane 20) and the intrinsic competition between the type I and type II cleavage reactions (see below) were likely responsible for almost completely eliminating CP2 formation. The relatively lower levels of R in lanes 10 and 16 were consistent with previous observations that half-sites containing a single spacer nucleotide in the cleavage strand were much less reactive in recombination than those containing 2 or 3 spacer nucleotides (29).

The sum of the data from Fig. 2 provides the framework for a more incisive comparison of the distinctive features Flp RNase I and Flp RNase II presented below.

Nucleotide Specificities for Flp RNase I and II—The scissile phosphodiester bond in the DNA target site for Flp recombination (p in the half-site D1, Table I) is flanked by a C on the 5' side and a T on the 3' side. The corresponding C-G and T-A base pair form, respectively, the last position of the Flp-binding element and the first position of the spacer sequence. Note that this separation of the Flp-binding element from the spacer (indicated in Table I) is convenient, even though it is somewhat arbitrary. Previous studies from the Cox laboratory (30) had shown that substitution of the C position by T is tolerated in recombination, whereas that by A affects recombination significantly (5-fold according to the Senecoff et al. (30) scale). Substitution by G virtually abolishes recombination (>100-fold).

This effect probably stems from unfavorable contacts induced by the latter base replacements, rather than by a direct effect on the chemical steps of recombination. Similarly, the optimal base pair at the first spacer position (which is also contacted by Flp; Ref. 31) is T-A; replacement by A-t results in a 3-5-fold decrease in recombination efficiency (30). We have therefore tested how different ribonucleotide substitutions at the normal C and t positions affect the type I and II RNase activities of Flp.

The Flp RNase I activity was assayed using wild type Flp and half-site substrates containing three spacer ts following the ribonucleotide position (Fig. 3A). Under the assay conditions, all four ribonucleotides, C, U, G or A, at the reactive position yielded comparable amounts of the CP1 cleavage product (lanes 2, 4, 6, and 8). To assay Flp RNase II, half-sites were assembled that contained ribo-substitutions at the first spacer position and deoxy-C at the immediate 5' position (Fig. 3B). Reactions were done with Flp(Y343F). Only the U-substituted substrate was active in the Flp RNase II reaction; the other three were inactive (presence of CP2 in lane 2 and its absence in lanes 4, 6, and 8).

Thus, the type I RNase of Flp is indifferent to the base at the reactive ribonucleotide position. In contrast, the type II RNase of Flp(Y343F) is highly base-specific and is expressed only when the reactive position is occupied by U.
O-methylation. Reactions were done under standard conditions, except that the indicated positions in the various substrates were blocked by 2'-O-methylation.

(18). Furthermore, the type II activity is stimulated considerably by the presence of a neighboring 3'-U relative to a 3'-t (for example, see lanes 20 and 22 of Fig. 2D).

In order to dissect further the role of the 2'-hydroxyl groups in Flp RNase activity and to explore the effects of sequence contexts on the type II activity, we have tested a number of half-sites containing 2'-O-Me substitutions at specific positions in the RNA cleavage reactions (Fig. 4, A and B). When the ribo-C position in the type I substrate (Fig. 4A) contained the O-Me block, the formation of CP1 was completely eliminated (compare lane 4 to lane 2). Nevertheless, the formation of the recombinant product was unaffected by the block (conversion of S into R in lanes 2 and 4). This result is consistent with the view that the phosphotyrosyl bond in the Flp-cleaved intermediate is a common target for the adjacent 2'-hydroxyl group or the 5'-hydroxyl group of an incoming DNA strand. The presence of the 2'-O-Me group apparently had little effect on the Tyr-343-mediated strand cleavage reaction, as inferred from the extent of R formation.

The unstimulated and adjacent U-stimulated type II Flp RNase cleavage reactions and their sensitivity to 2'-O-methylation are displayed in Fig. 4B. The presence of the 3'-U neighbor in the (CpUpUt) half-site resulted in approximately a 4–5-fold increase in the CP2 yield relative to that from the (CpUp'tt) half-site (lanes 2 and 10). However, there was also a corresponding decrease in the production of R from the former. The sum of R plus CP2 in the reactions shown in lanes 2 and 10 were approximately the same. This finding suggests that the recombination-specific strand cleavage activity (mediated by Tyr-343) and Flp RNase II activity (mediated by the 2'-hydroxyl group) are likely competitive. Since the two activities target distinct backbone phosphodiester bonds (p and p'), the probable cause for this competition is the sequestration of catalytic residues that are common to both reactions. When the 2'-hydroxyl at the reactive position was blocked (as in the [CU(OMe)Ut] half-site), CP2 formation was abolished (lane 4). Consistent with the competition hypothesis, there was an increase in the yield of R (compare lane 4 to lane 2). However, the extent of R formation was less than that obtained with the CUtt half-site (compare lane 4 to lane 10). The averaged results from a number of reactions indicate that the restoration of recombination was approximately 50% at best. Therefore the cleavage per se of the p' phosphodiester by Flp RNase II appears not to be a prerequisite for the inhibition of recombination (see also the results in Fig. 5). We cannot rule out some steric interference by the methyl group at the 2' position with the alignment of Tyr-343 during strand cleavage. It is unlikely that the 5'-hydroxyl attack during strand joining is affected, since the cleaved trinucleotide harboring the 2'-Ome group is not expected to remain stably hydrogen-bonded to the complementary strand under the assay conditions. When both of the adjacent U positions were 2'-O-methylated (as in the [CU(OMe)U(OMe)t] half-site), there was no CP2 formation as expected (lane 8). However, the recombination output was decreased with respect to the singly blocked substrate (compare the R bands in lanes 8 and 4), perhaps due to the additional steric impediment posed by the second OMe group. Finally, stimulation of the type II cleavage by the adjacent U was not dependent upon the 2'-hydroxyl group; an OMe-blocked U (in the [CtU(OMe)t] half-site) was as competent as U (compare CP2 in lanes 6 and 2). We suspect that the neighboring nucleotide effect on type II cleavage observed with U or U-2'-Ome (lanes 2 and 6), as contrasted with the t reaction (lane 10), is due to a distinct substrate conformation that facilitates the type II cleavage reaction.

**Fig. 4.** Type I and type II RNA cleavage activities of Flp in hybrid substrates containing specific OMe substitutions. Reactions were done under standard conditions, except that the indicated positions in the various substrates were blocked by 2'-O-methylation.

**Fig. 5.** Formation of Flp-cleaved covalent complexes in DNA half-sites and hybrid half-sites. Reactions were analyzed by electrophoresis in SDS-polyacrylamide gels to reveal the covalent Flp complexes (~Flp). In this assay, the recombinant (R) or the RNA cleavage products (CP1 and CP2) were not resolved from the substrate. They are collectively marked as S.
ence of an exogenously added DNA strand that could be utilized as a "ligator" during the strand joining reaction (Fig. 6).

In all of the previous assays, in order to assess the RNA cleavage activities of Flp without interference from the Tyr-343-mediated cleavage product, we had intentionally excluded the latter from the samples analyzed by electrophoresis (see "Materials and Methods"). By contrast, in the assays shown in Fig. 5, the reactions were directly fractionated in SDS-polyacrylamide gels so as to separate the covalent complex formed between Flp and the cleaved strand (*-Flp) from the substrate (S). Under these conditions, the products of the type I and type II RNase reactions (CP1 and CP2) as well as the recombinant product (R) were not resolved from the substrate. The extent of the covalent cleavage product obtained with the all-DNA substrate and Flp (lane 2) provides the reference for comparing the yields of this product from the hybrid substrates, two of which carried the indicated 2'-OMe modification.

The reactions with the type I hybrid substrates (for following the effects of Flp RNase I) are shown in lanes 3–6 of Fig. 5. When the half-site contained the ribo-C substitution, the cleaved covalent complex was not recovered (lane 4). (Prolonged exposure of the autoradiogram indicated a trace amount of *-Flp in this lane.) The absence of *-Flp was not due to lack of strand cleavage by Tyr-343 in this reaction. An identical sample analyzed in a denaturing gel (lane 2; Fig. 6A) showed the presence of both CP1 and R. We conclude that, under the reaction conditions used, the combined action of the adjacent 2'-hydroxyl group and the 5'-hydroxyl group from the non-cleaved strand drives all of the Tyr-343-mediated cleavage product into CP1 and R. Even when the 5'-hydroxyl was blocked by phosphorylation, there was little accumulation of *-Flp (data not shown). Thus, in these hybrid half-sites, the vicinal 2'-hydroxyl group is comparable to the 5'-hydroxyl group of the invading strand in its effective nucleophilicity plus its functional orientation with respect to the phosphotyrosyl bond. Support for this notion is provided by the half-site in which the ribo-C harbored a 2'-OMe block. In this case, there was an accumulation of the Flp-cleaved complex (compare lane 6 to lane 2). The extent of formation of the cleaved complex from the OMe-blocked substrate (COttt) was approximately 2-fold greater when compared with the DNA half-site (compare lane 6 to lane 2). It is conceivably that the methyl substituent poses some steric hindrance to the strand joining reaction.

From a physicochemical perspective, the apparent competition between the 2'- and 5'-hydroxyl groups for a common phosphodiester target is quite surprising (32). They are predicted to differ significantly in their chemical reactivities (one
being a primary, and the other a secondary alcohol; their spatial dispositions are also expected to be dissimilar. How then does the recombinase active site overcome the geometric constraints? And how does it establish equivalent reactive configurations using distinct functional groups? These questions remain open.

The results with the type II substrates (for assaysing the effects of the Tyr-343-independent Flp RNase II) are presented in lanes 7–10. There was a large drop in the level of the cleaved covalent complex (Tyr-343 linked to p) when the p' phosphodiester (the Flp RNase II target) was flanked on its 5' side by a U (lane 8). (As explained earlier, the presence of the U on the 3' side was not essential but was stimulatory for the Flp RNase II reaction.) Compared with the DNA half-site reaction (lane 2), the decrease in '*-Flp was approximately 4–5-fold. Two explanations can be offered for the above result. The half-site with the shortened spacer (resulting from the Flp RNase II reaction) is an intrinsically poorer substrate for cleavage by Tyr-343, as would be consistent with the previously known poor recombinase potential of half-sites containing a single spacer nucleotide on the cleavage strand (29). Alternatively, the presence of the ribonucleotide substituents directly inhibits the normal covalent cleavage reaction of Flp. The reaction shown in lane 10 tests these two possibilities. When the reactive 2'-hydroxyl was methylated (thus blocking the type II RNase), no marked increase in the covalent complex resulted. The averaged quantitative estimates from different experiments suggests that there was at best a 50–60% increase in '*-Flp production. Thus, we believe that the primary contribution to the suppression of cleavage by Tyr-343 is provided by the U substitutions present in the half-site (regardless of its susceptibility to Flp RNase II action), with some additional contribution from the type II cleavage reaction itself. As alluded to earlier, there might also be some effect of the OMe substitution per se on the Tyr-343 cleavage reaction, although its magnitude has not been determined.

To verify the relationship between the Flp RNase reaction and the strand joining reaction, we carried out a set of type I cleavage assays with the ribo-C-containing half-site in the presence of increasing concentrations of an exogenous ligator DNA strand. The 5'-terminal 8 nucleotides of this strand were perfectly complementary to the spacer sequence of the bottom strand (see Fig. 6). Therefore, it could use its 5'-hydroxyl end to attack the 3'-O-phosphotyrosyl bond formed by Flp cleavage, thus yielding the intermolecular recombinant product R' (as depicted schematically at the top of Fig. 6). The reactions were analyzed by denaturing polyacrylamide electrophoresis (Fig. 6A) and quantitated for the type I cleavage product (CP1) and the two recombinant products R and R' (Fig. 6B).

There was a clear-cut parallel relationship between the levels of CP1 and R produced, as would be expected for two mechanistically similar reactions acting on a common target (the 3'-O-phosphotyrosyl bond). Note that the absolute concentrations of the reactive 5'- and 2'-hydroxyl groups in the half-site were the same (1:1). However, as evidenced by the relative yields of R and CP1, the effective concentration was approximately 2:1 in favor of the 5'-hydroxyl. The effect of the externally added strand on the reaction was not felt until it was present at nearly a hundred-fold molar excess over the half-site substrate. The squelching of the competitor strand probably reflects the steric barrier posed by the half-site Flp complex within which the type I RNase cleavage or the recombinant formation occurs. At a thousand-fold molar excess, the competitor strand contributed significantly to recombinant production (increase in R accompanied by a drop in R). The net relative increase in the 5'-hydroxyl concentration over that of the 2'-hydroxyl was reflected in the net increase in recombination (R plus R') with an equivalent decrease in the type I RNase cleavage (CP1). This is precisely what is expected upon altering the relative concentrations of two competing nucleophiles without changing the concentration of their common target. Note that the formation of the presumed target, the phosphotyrosyl bond, was dependent only on the concentration of Tyr-343, which remained constant in the assay.

Overall, our results demonstrate that both the type I and type II RNase activities of Flp on the one hand, and the chemical steps of DNA recombination on the other, are mutually competitive. The type I activity competes directly with the strand joining reaction by virtue of the common phosphotyrosyl intermediate upon which each of them acts. On the other hand, the type II activity competes indirectly with the strand cleavage step mediated by Tyr-343. This competition results primarily from the requirement of the same active site (or at least common functional groups within overlapping active sites) to orient two separate phosphodiester bonds for nucleophilic attack by distinct nucleophiles, Tyr-343 of Flp or the vicinal 2'-hydroxyl of a ribonucleotide.

**DISCUSSION**

The recent finding that the Flp site-specific recombinase can function as a site-specific ribonuclease (18) is intriguing but not entirely unexpected. Several years ago, Parsons et al. (9, 10) had pointed out that the Flp catalytic triad of two arginines and a histidine (Arg-191, Arg-308, and His-305) is suggestive of possible mechanistic similarities between the recombinase and pancreatic ribonuclease or *Staphylococcus* DNase. However, the discovery of two distinct type of ribonuclease activities directed against two neighboring phosphodiester bonds within Flp (18) was completely unsuspected. The results presented in this study examine the features of each of these two activities as they relate to those of the chemical steps of the normal recombination pathway.

The Type I Flp RNase: Phosphodiester Hydrolysis via a Covalent Enzyme Intermediate—The data presented here provide strong evidence for the mechanism proposed by Xu et al. (18) for the type I RNase activity of Flp. This RNase activity is site-specific with respect to the ribonucleotide position and proceeds via the same covalent 3'-O-phosphotyrosyl intermediate that is formed during the recombination reaction. On the other hand, it is insensitive to the particular base present at this position. Mechanistically, the Flp RNase I reaction is analogous to the site-specific RNA cleavage that has been demonstrated for the vaccinia topoisomerase I enzyme (19). Thus, the recombinase and topoisomerase active sites can orient the vicinal 2'-hydroxyl group or the adjacent 5'-hydroxyl group (with respect to the target phosphotyrosyl bond) with comparable efficiencies. Although the recently revealed structural similarities between type IB topoisomerases and the integrase family recombinases can account for the shared mechanism, the structures do not offer an obvious explanation for the active site flexibility required to utilize two distinct nucleophiles (32). Whereas the end product of the vaccinia topoisomerase I reaction is the 2',3'-cyclic phosphate, the reaction with Flp yields the 3'-phosphate as the final product, presumably by hydrolysis of the cyclic intermediate. In this respect, the Flp RNase I resembles pancreatic ribonuclease more than it does topoisomerase IB. This finding is consistent with the Flp RNase II activity discussed below.

The Type II Flp RNase: Phosphodiester Hydrolysis without the Involvement of a Covalent Enzyme Intermediate—Since the type II RNA cleavage by Flp is independent of Tyr-343, we shall discuss this reaction based upon the properties of Flp(Y343F). Although this cleavage reaction (like the type I activity) is also...
site-specific, the scissile phosphodiester is the 3’ neighbor of the recombination target, and the cleavage product harbors a 3’-phosphate. In addition to being site-specific, the Flp(Y343F) reaction is also base-specific; only U is tolerated at the ribonucleotide position. Furthermore, the reaction is stimulated by the presence of U or by U-2'-OMe immediately 3’ to the scissile phosphodiester. Thus, Flp(Y343F) is an authentic “ribonuclease” whose activity is not contingent upon assistance by the first chemical step of recombination. Yet, when the type II reaction is carried out with wild type Flp (that is, in the presence of Tyr-343), the RNase activity competes with the DNA cleavage reaction even though they act on different phosphodiester bonds. The conformational flexibility in the substrate due to the ribonucleotide substitution appears to stabilize either one of two active site configurations, for attack of the recombination target (the phosphodiester p) by Tyr-343 or attack of the Flp RNase II target (the phosphodiester p’) by the 2’-hydroxyl group.

Evolution of Novel Reactivities from an Ancestral Catalytic Configuration—Cozzarelli (33) has suggested the possibility that a nuclease capable of cleaving the backbone phosphodiester in a nucleic acid chain via a covalent enzyme intermediate could, in principle, have served as the progenitor for the present day topoisomerases. The ability of vaccinia topoisomerase I and of Flp (a site-specific recombinase that follows a topoisomerase IB-like mechanism) to mediate a 2’-hydroxyl attack on a protein-nucleic acid adduct (18, 19) is consistent with this proposal. Once the essential functional groups for carrying out a basic chemical transaction have been placed in their proper context within an essential active site, it may only take relatively small evolutionary steps for an enzyme to manifest rather giant mechanistic leaps in its enzymology. Emergence of “novel activities in recombinases and topoisomerases” from “conserved chemical themes” has been reviewed recently (23).

Consistent with the above view, the active site of the Flp recombinase can orient a single phosphodiester within a DNA chain for attack by a number of nucleophiles as follows: Tyr-343 of Flp, hydrogen peroxide, and by tyrosine mimics such as tyramine and phenol (12, 16). Furthermore, a rather inefficient reaction mediated by Flp(Y343F) that leads to hairpin formation in half-sites is best accommodated by the direct attack of a precise positioning ribonucleotide, and glycerol as well as other polyhydric alcohols) on the cleavage intermediate of recombination, the 3'-O-phosphotyrosyl bond (17, 18). The type IB topoisomerases appear to share the catalytic versatility exhibited by the Flp recombinase. In addition to its principal activity of DNA relaxation, the reactions catalyzed by the vaccinia topoisomerase I include resolution of Holliday junctions and the production of a 2',3'-cyclic phosphate by a mechanism analogous to Flp RNase I (19, 35). The topoisomerase can mediate a strand joining reaction in which the 5'-hydroxyl end of a DNA chain attacks the cyclic phosphate (36) and can be made to act as an endonuclease by replacing its active site tyrosine with glutamic acid (37). The altered reactivity is reminiscent of the conversion of the endonuclease NseI into a topoiso merase or of E. coli topoiso merase IV into an endonuclease by single amino acid changes in each instance (38, 39).

What is remarkable about Flp is that it has retained an RNase activity (the type II reaction) that is uncoupled from recombination in its target specificity as well as its mechanism. This feature of the type II cleavage reaction suggests that the emergence of a tyrosine residue at position 343 might have played a key role in fundamentally changing the character of a nuclease and its evolution into a site-specific recombinase. Gain of a new catalytic function without the loss of an old one has also been observed during the in vitro evolution of a ribozyme into a DNA-cleaving enzyme without compromising its RNA-cleaving ability (40).

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