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The FLP Recombinase of the *Saccharomyces cerevisiae* 2μm Plasmid Attaches Covalently to DNA via a Phosphotyrosyl Linkage

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The FLP recombinase, encoded by the 2μm plasmid of *Saccharomyces cerevisiae*, promotes efficient recombination in vivo and in vitro between its specific target sites (FLP sites). It was previously determined that FLP interacts with DNA sequences within its target site (B. J. Andrews, G. A. Proteau, L. G. Beatty, and P. D. Sadowski. Cell 40:795–803, 1985), generates a single-stranded break on both DNA strands within the FLP site, and remains covalently attached to the 3′ end of each break. We now show that the FLP protein is bound to the 3′ side of each break by an *O*-phosphotyrosyl residue and that it appears that the same tyrosyl residue(s) is used to attach to either DNA strand within the FLP site.

Site-specific recombination plays an important role in a variety of cell-developmental and -regulatory processes. In procaryotes, site-specific inversion events control such diverse functions as flagellar-phase variation (28) and bacteriophage host range specificity (20). In eucaryotes, site-specific recombination events generate deletions of DNA that are required for the production of immunoglobulins (4, 10), the antigen receptors of T-lymphocytes (8, 13), and other products of the immune system (15). The biochemical mechanism of such recombination events is poorly understood, and we have been studying the site-specific recombinase FLP, encoded by the 2μm plasmid of *Saccharomyces cerevisiae* to gain insight into these processes (1, 23, 26).

The FLP protein promotes efficient recombination between two identical FLP recombination sites (FLP sites) present on the 2μm plasmid in vivo (6) and in vitro (23, 26). In the 2μm plasmid, recombination in vivo between the two sites produces an inversion event so that two forms of the plasmid (A and B) are present within the cell. In vitro, the FLP protein promotes an inversion event when the two FLP sites are in an inverted orientation and a deletion event when the two sites are in a direct orientation (18, 23, 26). At high concentrations of FLP protein, intermolecular as well as intramolecular recombination occurs (18, R. M. Gronostajski and P. D. Sadowski, J. Biol. Chem., in press). In addition, we have characterized the interaction of the FLP protein with its FLP site DNA target and demonstrated that the protein produces two single-stranded nicks at specific nucleotides in the FLP site to produce an 8-base-pair (bp) staggered break about which recombination presumably occurs (1). Under defined conditions, the protein remains covalently bound to the DNA at the 3′ termini of these nicks. Since the 5′ termini of such FLP-induced nicks bear free hydroxyl groups, it appears likely that the FLP protein is covalently bound to the DNA via a 3′-phosphoryl linkage. In this report, we present evidence that the FLP recombinase is attached to DNA via a nucleotide 3′-phosphotyrosyl linkage and that the same tyrosyl residue(s) is used for attachment of the protein to either DNA strand.

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MATERIALS AND METHODS

Preparation of FLP. FLP protein was purified from *Escherichia coli* JM103 harboring the plasmid pDV64 as described previously (23, D. Babineau, D. Vetter, B. J. Andrews, R. M. Gronostajski, G. Proteau, L. G. Beatty, and P. D. Sadowski, J. Biol. Chem., in press). The protein fraction was taken through the second Bio-Rex column step and was >70% pure as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzymes. Exonuclease III was from New England Biolabs, and restriction endonucleases *Eco*RI and *Bam*HI and the large fragment of DNA polymerase I (Klenow fragment) were from International Biotechnologies, Inc. Nuclease P1 was from P-L Biochemicals.

Preparation of labeled DNA. The plasmid pBA112 (1) contains a 74-bp FLP recombination site cloned into the *Sma*I site of plasmid pUC9 (27). Because the fragment is between an *Eco*RI site and a *Bam*HI site, digestion of the plasmid with *Eco*RI or *Bam*HI produces a linear molecule with one terminus adjacent to the right and left side, respectively, of the FLP site (Fig. 1). To label the upper strand of the FLP site, 5 μg of *Eco*RI-digested pBA112 DNA was incubated at 23°C for 15 min in 60-μl reactions containing 66 mM Tris hydrochloride (pH 8.0), 3.3 mM $MgCl_2$, 90 mM NaCl, 1 mM dithiothreitol, and 41 U of exonuclease III (14). Reactions were terminated by the addition of 40 μl of stop solution (1 M sodium acetate, 10 mM EDTA) extracted with 100 μl of phenol-chloroform (1:1), the phenol phase was back extracted with 33 μl of H_2O, and the two aqueous phases were combined and extracted with 500 μl of H_2O-saturated ether. The DNA was precipitated at −70°C by the addition of 400 μl of ethanol, collected by centrifugation at 12,000 × *g* for 10 min, and dissolved in 20 μl of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). The 3′-recessed termini generated by exonuclease digestion were labeled by incubating 1 μg of digested DNA at 23°C for 30 min in 50-μl reactions containing 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 4 mM dithiothreitol, bovine serum albumin (50 μg/ml), 400 μCi of [α-^{32}P]TTP (>3,000 Ci/mmol), 25 μM each of dATP, dGTP, and dCTP, and 1 U of Klenow polymerase. The reactions were terminated by extraction with phenol-chloroform, and the DNA was precipitated from the aqueous phase by the addition of 1.5 μl of 0.5 M EDTA, 15 μl of 5 M ammonium acetate, 15 μl of a 1-mg/ml solution of calf thymus DNA, and 240 μl of ethanol. The samples

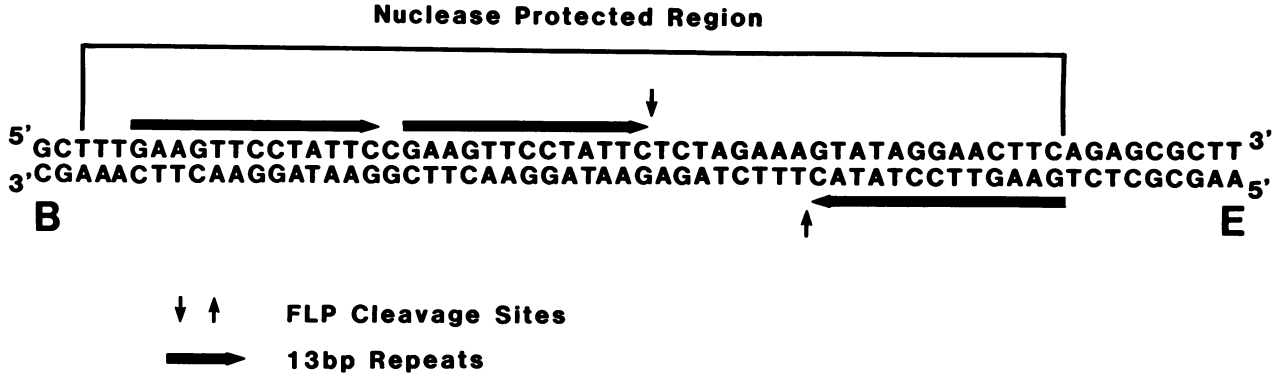

FIG. 1. DNA sequence of a FLP recombination site. The diagram shows the DNA sequence active in FLP-mediated recombination along with some important structural features of the site. The sequence represents nucleotides 671 to 732 from the 2μm plasmid and constitutes part of the 74-bp FLP site (nucleotide no. 657 to 731) cloned into the *Sma*I site of pUC9 to generate pBA112. The FLP site is composed of three 13-bp symmetry elements (heavy arrows) arranged about an 8-bp core region. We have previously shown that FLP makes single-stranded breaks at the junctions of the core and the 13-bp symmetry elements and remains attached to the 3' end of each break (thin vertical arrows) (1). The region of the DNA sequence protected from DNase I digestion by the presence of FLP is shown by the large bracket above the site. All references in the text to top and bottom strands of the FLP site refer to those strands of this diagram. *Eco*RI site (E) is to the right of fragment pictured here; *Bam*HI site (B) is to the left.

were cooled for 1 h in a dry ice-ethanol bath, the DNA was collected by centrifugation at 12,000 × *g* for 10 min, and the pellet was washed once with 70% ethanol and dissolved in 30 μl of TE. To label the lower strand of the FLP site, an identical procedure was performed with *Bam*HI-digested pBA112 in place of *Eco*RI-digested DNA. When the DNA was labeled with [α-^{32}P]dATP rather than [α-^{32}P]TTP (see Fig. 6), the labeling reaction was identical to that described above, except that 400 μCi of [α-^{32}P]dATP (>3,000 Ci/mmol) and 25 μM unlabeled TTP were used in place of [α-^{32}P]TTP and unlabeled dATP.

FLP attachment reaction. The formation of a covalent protein-DNA complex was performed under conditions previously shown to promote cleavage of the FLP site by FLP protein (1). Reactions (50-μl final volume) contained 50 mM Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 0 or 1 μg of FLP protein. Formation of the protein-DNA complex was initiated by the addition of 10 μl of a solution containing 0.33 μg of labeled pBA112 DNA (~8 × 10^7 cpm/reaction) and 5 μg of unlabeled calf thymus DNA in TE. Reactions were incubated for 30 min at 30°C and terminated by the addition of 25 μl of stop solution (50 mM EDTA [pH 8.0], 0.2% SDS, tRNA [50 μg/ml]), 8 μl of 2 M sodium acetate, and 250 μl of ethanol. The samples were cooled immediately in a dry ice-ethanol bath for 1 h, and the protein-DNA complex was isolated by centrifugation at 12,000 × *g* for 10 min, washed once with 70% ethanol, and dissolved in 40 μl of 0.1 M ammonium acetate (pH 5.3). The FLP-DNA complex was then digested with nuclease P1 at 37°C for 2 to 4 h in reactions (50 μl) containing 0.1 M ammonium acetate (pH 5.3) and 4 μg of P1 nuclease. Digestions were stopped by the addition of 50 μl of a solution containing 50 mM EDTA (pH 8.0), calf thymus DNA (1 mg/ml), and 150 μl of 20% trichloroacetic acid. The samples were cooled on ice for 10 to 30 min, and the FLP-nucleotide complexes were isolated by centrifugation at 12,000 × *g* for 10 min at 4°C. The resulting pellet was washed once with 500 μl of H_2O-saturated ether and then dissolved in 30 to 50 μl of SDS electrophoresis sample buffer (17) and analyzed by SDS-PAGE on a 12.5% polyacrylamide gel.

The gel was subjected to autoradiography either before or after being stained with Coomassie blue, with or without drying. The migration of labeled material was compared with that of unlabeled standard proteins analyzed on the same gel. Protein standards were detected by staining with Coomassie blue. Labeled material migrating at the position of FLP protein was eluted from the gel by rehydration and homogenization of the gel slice in 0.1 M NH_4HCO_3 (pH 8.0)–0.1% SDS, boiling the homogenized gel slice in 0.1 M NH_4HCO_3 (pH 8.0)–0.1% SDS–5% β-mercaptoethanol for 5 min, and subsequent incubation at 37°C with continuous mixing for 12 to 24 h (2). Residual polyacrylamide was pelleted by centrifugation at 12,000 × *g* for 5 min, and the supernatant containing the radioactive material was decanted. The efficiency of recovery of label from gel slices was 80 to 90%. A single gel slice (~3 by 10 mm) yielded ~800 μl of supernatant, and the labeled material was precipitated by the addition of 50 μg of bovine serum albumin and trichloroacetic acid to a final concentration of 20%. The samples were chilled for 1 h on ice and spun at 12,000 × *g* for 10 min at 4°C. The pellet was suspended in 50 μl of 0.1 M NH_4HCO_3 (pH 8.0)–0.1% SDS either directly after precipitation or after washing the pellet with 500 μl of H_2O-saturated ether. Washing the pellet with ether did not affect any of the subsequent analyses.

Acid hydrolysis and high-voltage paper electrophoresis. Samples of material eluted from a polyacrylamide gel were dried in vacuo and dissolved in 200 μl of 6 N HCl. The glass sample tubes were sealed under vacuum and incubated at 110°C for the various intervals. After hydrolysis, the tubes were opened, and samples were dried in vacuo and redissolved in 20 to 40 μl of a solution containing 0.33 mg each of *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine per ml. For analysis, 10 to 20 μl of sample was spotted on a sheet of Whatman 3MM chromatography paper and subjected to electrophoresis at 2 to 3 kV for 60 to 120 min in pyridine-acetic acid-H_2O (5:50:945) (pH 3.5). The paper was dried, developed with ninhydrin to stain the phosphoamino acid standards, and subjected to autoradiography with an intensifying screen for 1 to 5 days at −70°C. In all high-voltage analyses shown, the anode is at the top of the figure.

RESULTS

Isolation of FLP covalently linked to ^{32}P-labeled nucleotide. The DNA sequence shown previously (1) to be required for FLP-mediated recombination in vitro is depicted in Fig. 1. Andrews et al. (1) demonstrated that when this DNA sequence (FLP site) is incubated with FLP, the protein generates single-stranded breaks (vertical arrows) at the junctions between the two 13-bp inverted symmetry elements and the 8-bp core. This cleavage event produces a free 5′-OH group on one side of the break and leaves the FLP protein covalently attached to the 3′ terminus. It seemed probable that this attachment occurred via a 3′-phosphate linkage to the cytidine residue present on this side of the break. To determine the nature of this 3′-phosphate linkage, we first isolated FLP protein covalently linked to radiolabeled DNA by the procedure shown in Fig. 2. The plasmid pBA112 (1) contains a 74-bp FLP site isolated from 2μm plasmid DNA and inserted into the *Sma*I site of pUC9. Digestion of pBA112 with *Eco*RI generates a 2.8-kilobase linear molecule with the FLP site adjacent to one terminus. Figure 2A shows a schematic diagram of the end of the pBA112 molecule containing the FLP site. To specifically label the upper strand of the FLP site, *Eco*RI-digested pBA112 was digested briefly with exonuclease III to generate a single-stranded region through the site (Fig. 2B). The recessed 3′-OH termini were then repaired with the large fragment (Klenow fragment) of DNA polymerase I, [α-^{32}P]TTP, and unlabeled dATP, dCTP, and dGTP (Fig. 2C). This labeled DNA was then incubated with FLP under conditions previously shown to promote the covalent attachment of FLP to DNA (Fig. 2D). To generate FLP protein covalently linked to a radiolabeled mono- or oligonucleotide, the FLP-DNA complex was exhaustively digested with nuclease P1 (Fig. 2E). The resulting FLP-nucleotide complex was then analyzed by SDS-PAGE (Fig. 3). We had previously shown that unlabeled FLP protein migrates with a molecular size of about 45 kilodaltons (kDa) during SDS-PAGE (23, Babineau et al., in press). When radiolabeled DNA was incubated with FLP, a major band migrating at a position corresponding to a molecular size of about 45 kDa was generated (Fig. 3, lane 2). No band of this size was generated when the labeled DNA was subjected to an identical procedure but was incubated in the absence of FLP (lane 1).

We had also shown that FLP produces single-stranded breaks on both strands of the FLP site at the junctions of the core and the two 13-bp inverted symmetry elements (1). To ensure that the attachment of FLP to labeled DNA showed this same site specificity, we labeled the upper strand of the

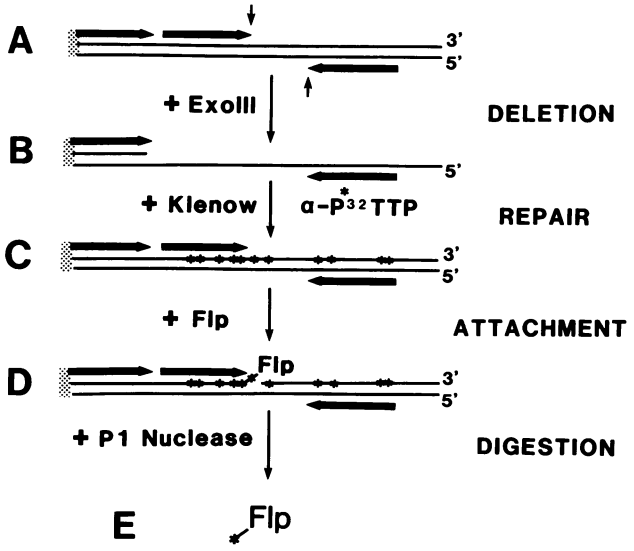

FIG. 2. Protocol for labeling of a unique strand of the FLP site. Plasmid pBA112 contains a 74-bp FLP site cloned into the *Sma*I site of pUC9 (1). (A) A schematic diagram of *Eco*RI-digested pBA112. The *Eco*RI site is at the terminus on the right, and the dotted box on the left represents the remaining 2.7 kilobases of plasmid DNA. The thick horizontal arrows and thin vertical arrows represent the 13-bp symmetry elements and FLP cleavage sites, respectively, of the FLP site on pBA112 (see Fig. 1). (B) Incubation of *Eco*RI-digested pBA112 with exonuclease III generates a single-stranded deletion through the FLP site (and also at the other end of the plasmid [data not shown]). (C) Repair of the DNA with Klenow polymerase [α-^{32}P]TTP and three unlabeled dinucleoside triphosphates incorporates a labeled phosphate 5′ to each thymidine. (D) Incubation of the labeled DNA with FLP causes cleavage between the C and T residues shown by the vertical arrows in Fig. 1 and attachment of FLP to the DNA via a 3′-phosphate linkage. (E) Digestion of the FLP-DNA complex with nuclease P1 generates FLP covalently linked to a mono- or oligonucleotide. The protein attached to the labeled DNA strand is detected by SDS-PAGE.

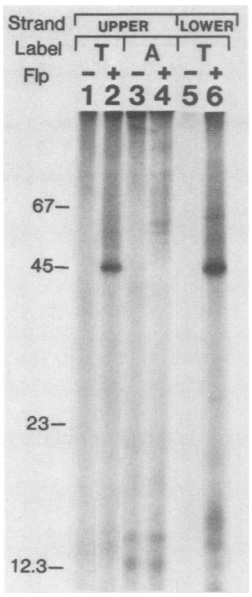

FIG. 3. Specificity of FLP-DNA complex formation. Three forms of pBA112 DNA labeled with ^{32}P in three different ways were incubated in the presence and absence of 1 μg of FLP, and the formation of FLP-DNA complex was measured as described in Materials and Methods and in the legend to Fig. 2. Reactions analyzed in lanes 1 and 2 contained 0.33 μg (~8×10^7 cpm) of pBA112 DNA labeled in the upper strand (Fig. 1) with [α-^{32}P]TTP (T) minus (−) and plus (+) FLP, respectively. Reactions analyzed in lanes 3 and 4 contained 0.33 μg (~8×10^7 cpm) of pBA112 DNA labeled in the upper strand (Fig. 1) with [α-^{32}P]dATP (A) minus and plus FLP, respectively. Reactions analyzed in lanes 5 and 6 contained 0.33 μg (~8×10^7 cpm) of pBA112 DNA labeled in the lower strand (Fig. 1) with [α-^{32}P] TTP minus and plus FLP, respectively. The numbers to the left of lane 1 indicate the positions of migration of various marker proteins in kilodaltons: 67, bovine serum albumin; 45, ovalbumin; 23, chymotrypsinogen; and 12.3, cytochrome *c*. The somewhat reduced background labeling in lane 5 is apparently due to less material being loaded. Overexposure of this lane does not reveal a labeled band at 45 kDa.

FLP site with [α-^{32}P]dATP rather than [α-^{32}P]TTP and measured the attachment of FLP to these DNA substrates. Since the nearest dAMP residue is 3 bases 5′ to the dCMP at the known FLP cleavage site (Fig. 1), digestion of the FLP-DNA complex with P1 nuclease should remove this labeled dAMP, and a 45-kDa band should not be detected. In accordance with this prediction, when the attachment of FLP to an [α-^{32}P]dATP-labeled FLP site was measured, no labeled 45-kDa band was present (Fig. 3, lane 4). These data demonstrate both the specificity of the attachment reaction and that the digestion of the FLP-DNA complex with nuclease P1 leaves an oligonucleotide with fewer than four residues attached to FLP.

FLP attaches to both DNA strands of the FLP site. The above analyses were performed with DNA labeled uniquely in the strand corresponding to the upper strand in Fig. 1. Since the FLP site is asymmetric, we compared the products obtained from attachment of FLP with the upper and lower strands of the FLP site shown in Fig. 1. To measure the attachment of FLP to the lower strand of the site, an analysis identical to that shown in Fig. 2 was performed, with the exception that the plasmid was linearized with *Bam*HI rather than *Eco*RI. The *Bam*HI cleavage site is on the opposite side of the FLP site from the *Eco*RI site. Thus, cleavage with *Bam*HI followed by digestion with exonuclease III and subsequent repair with [α-^{32}P]TTP labels the lower strand of the FLP site depicted in Fig. 1 and 2. A 45-kDa band was detected when DNA labeled with TTP in either the upper or lower strand of the FLP site was incubated with FLP (Fig. 3, lanes 2 and 6). No such bands were present when the same DNAs were incubated in the absence of FLP (lanes 1 and 5). Thus, FLP attaches to each strand of the FLP site in a similar manner.

Stability of the FLP-nucleotide complex. The resistance of the 45-kDa band to exhaustive digestion with nuclease P1 strongly suggested that the label was covalently linked to FLP protein. Different amino acid-phosphate linkages possess characteristic labilities to chemical treatment (5, 19). Ser-P and most Thr-P residues are very labile in alkali (19) while phosphoramidates such as Arg-P, His-P, and Lys-P are acid labile (5, 12). Acyl phosphates (acetyl-P, citryl-P, etc.) are unstable at either pH extreme and are also cleaved by hydroxylamine (12). Tyr-P is stable to the conditions listed above. To assess the stability of the FLP-phosphate linkage, we determined what fraction of the label remained in an acid-precipitable form after incubation under a variety of such conditions (Table 1). The linkage was completely stable to incubation for 1 h at 37°C in 0.25 M Tris hydrochloride (pH 7.4)–1 N NaOH–1 M HCl–4 M hydroxylamine (pH 4.5) or 0.25 M hydroxylamine–0.25 M Tris hydrochloride (pH 7.4). This high degree of stability of the FLP-DNA linkage thus suggested that the label was covalently attached to FLP by a phosphodiester or monoester bond to a tyrosine residue on the protein (19).

TABLE 1. Stability of FLP-phosphate linkage[a]

Condition	pH	% Acid precipitable
0.25 M Tris hydrochloride	7.4	99
1.0 N NaOH	>12	99
1.0 N HCl	<1	97
4.0 M NH_2OH	4.5	99
0.25 M Tris hydrochloride–0.25 M NH_2OH	7.4	100

[a] All incubations were at 37°C for 1 h. ^{32}P-labeled FLP-DNA complex eluted from a polyacrylamide gel was incubated in reactions (200 μl) under the conditions given above. The incubations were terminated by the addition of 20 μl of bovine serum albumin (5 mg/ml) and 55 μl of 100% trichloroacetic acid, cooled on ice for 1 h, and spun at 12,000 × *g* for 10 min; the label present in the supernatant and pellet were detected by Cerenkov counting. The reaction containing 1.0 N NaOH was neutralized by the addition of 16.5 μl of 11.6 M HCl before acid precipitation. Approximately 670 cpm of labeled material was used in each reaction.

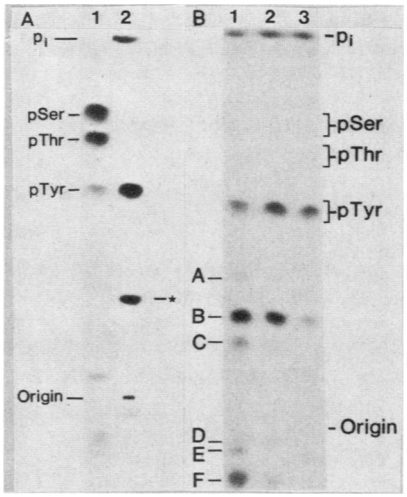

FIG. 4. Acid hydrolysis of FLP-DNA complex. (A) A gel slice containing material migrating with a molecular size of about 45 kDA was excised from an SDS-polyacrylamide gel similar to that shown in Fig. 3 (lane 2). The labeled material was eluted from the gel slice, and a portion (~4,000 cpm) was subjected to hydrolysis in 6 N HCl for 2 h at 110°C, dried, and analyzed by high-voltage paper electrophoresis as described in Materials and Methods. The paper was dried, stained with ninhydrin, and subjected to autoradiography at −70°C for 18 h. Lane 1, Ninhydrin staining of *O*-phosphoserine (pSer), *O*-phosphothreonine (pThr), and *O*-phosphotyrosine (pTyr) markers added to the sample before electrophoresis. Lane 2, Autoradiograph of lane 1 showing the radioactive material present in the sample. To the left of lane 1, the spotting origin and the areas of migration of P_i, pSer, pThr, and pTyr are denoted. The asterisk on the right of lane 2 denotes the presence of a putative intermediate in hydrolysis. The faint ninhydrin staining spots below pTyr are from bovine serum albumin present as carrier in the sample. (B) FLP-DNA complex eluted from a polyacrylamide gel was subjected to hydrolysis in 6 N HCl at 110°C for 1 h (lane 1), 2 h (lane 2), or 4 h (lane 3). The samples were then analyzed by high-voltage paper electrophoresis as described above and subjected to autoradiography at −70°C for 5 days. Each sample contained about 700 cpm of ^{32}P-labeled material. To the right of lane 3, the spotting origin and the regions of migration of P_i, pSer, pThr, and pTyr are shown. The letters A to F to the left of lane 1 indicate a number of intermediates formed during acid hydrolysis of the FLP-DNA complex.

DNA attaches to FLP through a phosphotyrosine bond. To measure directly the protein-phosphate linkage, the 45-kDa material was subjected to limited acid hydrolysis (6 N HCl, 2 h at 110°C), and the products were analyzed by high-voltage paper electrophoresis (Fig. 4). The labeled sample was mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine before electrophoresis. The migration of these unlabeled standards was determined by staining the paper with ninhydrin (Fig. 4A, lane 1). Autoradiography of the same lane of paper revealed a strong spot of labeled material comigrating with the phosphotyrosine standard (Fig. 4A, lane 2). No label was detected at the points of migration of phosphoserine and phosphothreonine. In addition to the phosphotyrosine, label was detected migrating in the position of P_i and at least one

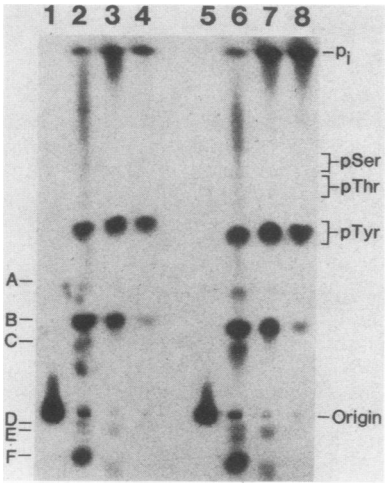

FIG. 5. Time course of hydrolysis of upper- and lower-strand FLP-DNA complexes. FLP-DNA complexes prepared from DNA labeled in the upper or lower strand of the FLP site (Fig. 1) were hydrolyzed in 6 N HCl at 110°C for 0 h (lanes 1 and 5), 1 h (lanes 2 and 6), 2 h (lanes 3 and 7), and 4 h (lanes 4 and 8) and then analyzed by high-voltage paper electrophoresis. Reactions analyzed in lanes 1 to 4 contained DNA labeled in the upper strand; those in lanes 5 to 8 contained DNA labeled in the lower strand. The discrete spots in and between lanes 1 and 2 below spots A and C are stray contaminant spots and are not seen in other analyses of the same material (see Fig. 4B, lane 1). Abbreviations to the right of lane 8 and letters to the left of lane 1 are defined in the legend to Fig. 4. Approximately 1,200 cpm of labeled material was applied in each lane, and autoradiography was for 5 days at −70°C.

putative phosphopeptide. The presence of label in material other than phosphotyrosine was expected, since the limited hydrolysis conditions used are known to generate about 25% of the product as phosphopeptides and 25 to 50% of the product as P_i (19).

To determine whether the putative phosphopeptide was an intermediate in the reaction, a time course of hydrolysis was performed (Fig. 4B). After 1 h of acid hydrolysis, a number of labeled products, including phosphotyrosine, were detected (Fig. 4B, lane 1). When hydrolysis was continued for 2 h, a pattern similar to that seen in Fig. 4A was obtained, with phosphotyrosine and a single major intermediate (labeled B) being detected (Fig. 4B, lane 2). After 4 h of hydrolysis, phosphotyrosine and P_i were the major products, and the amount of putative phosphopeptide B was substantially decreased (lane 3). The intensity of the labeled phosphate spot was relatively constant during the time course, because most of the phosphate migrated off the end of the paper during electrophoresis (producing the sharpness of the P_i spot). Thus, the complex series of products seen early in hydrolysis was apparently converted to phosphotyrosine and then later to P_i. If no acid hydrolysis was performed, all of the label remained at the origin (see Fig. 5, lane 1).

FLP-DNA attachment to both strands of a FLP site is via a phosphotyrosine. FLP-DNA complexes prepared from DNA labeled in either the upper or lower strand of the FLP site were subjected to limited acid hydrolysis and high-voltage paper electrophoresis (Fig. 5). For both strands, the major hydrolysis product comigrated with phosphotyrosine (Fig. 5, lanes 4 and 8). With less extensive hydrolysis, labeled material that corresponded in mobility to the putative hydrolysis intermediates seen in Fig. 4B was also detected in both samples (Fig. 5, lanes 2, 3, 6, and 7). The migration pattern of the putative intermediates was identical in material prepared from DNA labeled in the upper and lower strands. The similarity of the partial hydrolysis products of FLP-DNA complexes prepared from either labeled strand strongly suggested that the same tyrosine residue(s) was labeled in each case.

DISCUSSION

The findings presented here provide a basis for comparison for other site-specific recombination systems. The discovery of a 3′-phosphotyrosine linkage between FLP protein and DNA places FLP in the category of topoisomerases that apparently use reversible DNA-tyrosine bonds as reaction intermediates. Proteins that covalently attach to DNA via a phosphodiester bond to tyrosine can be subdivided into two classes: those that use 5′-phosphotyrosyl linkages and those that use 3′-phosphotyrosyl bonds. Among the former are the type I topoisomerases of *E. coli* and *Micrococcus luteus* (22), DNA gyrase of *M. luteus* (25), and the φX174 gene A protein (22, 24). The only protein previously shown to make a 3′-phosphotyrosine linkage with DNA is the type I topoisomerase found in rat liver (7) and other eucaryotes (3). Thus, FLP represents an important addition to this second class of proteins.

Clearly, the mechanisms of a site-specific recombinase such as FLP and a general type I topoisomerase differ considerably. The FLP protein cleaves and attaches to DNA only at two specific nucleotides within the FLP recombination site (Fig. 1). For this reason, a FLP-oligonucleotide complex can be detected with [α-^{32}P]TTP-labeled DNA but not [α-^{32}P]dATP-labeled DNA (Fig. 3). The rat liver and other eucaryotic type I topoisomerases show a nonrandom pattern of cleavage (3), but cleavage and attachment apparently occur with all four types of nucleotide (3, 11). Thus, it will be of interest to determine whether single base changes at the cytidine residues presumed to be the sites of FLP protein attachment to DNA (Fig. 1) affect the cleavage and attachment reactions.

A comparison of the cleavage mechanism of the FLP protein with several procaryotic recombinases reveals both similarities and differences. The λ Int protein (9) and phage P1 Cre protein (16) each cleave their respective recognition sites and remain covalently bound to the 3′ end of the break. However, the amino acid involved in these DNA-protein bonds is not known. Surprisingly, the resolvase protein of transposon γδ remains attached to the 5′ end of its cleavage site, and this linkage is via an *O*-phosphoserine residue (21). Thus, the two recombinases, FLP and γδ resolvase, can each promote the excision of DNA from between directly oriented recombination sites but utilize both different amino acid residues and a different polarity of linkage to the DNA. Clearly, it will be important to determine the amino acids used by other site-specific recombinases before a general model will be possible.

Our results suggest that the same tyrosine residue(s) is used for the attachment of FLP to the upper and lower strand of the FLP site (Fig. 1 and 5). In addition, we have preliminary evidence that the tyrosyl residue(s) used is present on a single small cyanogen bromide fragment located near the carboxy terminus of FLP (data not shown). Since a double-stranded break and exchange is required for recombination, these findings suggest that at least two molecules of FLP protein are needed for this event. In combination with our previous studies on the interaction of FLP with DNA sequences at a recombination site (1, 22; Gronostajski and

Sadowski, in press), we believe it is likely that two or more molecules of FLP bind symmetrically about the 8-bp core region of the FLP site. Symmetric binding then leads to either independent or concerted cleavage and attachment events on each strand, followed by strand exchange and religation to acceptor sites at an adjacent FLP recombination site. Further studies are required to test this model and to determine precisely which tyrosyl residue(s) is used in the FLP-DNA linkage. Site-directed mutagenesis of this residue may then yield further insight into the mechanism of recombination.

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