Mutations That Improve the Binding of Yeast FLP Recombinase to Its Substrate

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ABSTRACT

When yeast FLP recombinase is expressed from the phage λ P_R promoter in a Salmonella host, it cannot efficiently repress an operon controlled by an operator/promoter region that includes a synthetic, target FLP site. On the basis of this phenotype, we have identified four mutant FLP proteins that function as more efficient repressors of such an operon. At least two of these mutant FLP proteins bind better to the FLP site *in vivo* and *in vitro*. One mutant changes the presumed active site tyrosine residue of FLP protein to phenylalanine, is blocked in recombination, and binds the FLP site about five-fold better than the wild-type protein. A second mutant protein that functions as a more efficient repressor retains catalytic activity. We conclude that the eukaryotic yeast FLP recombinase, when expressed in a heterologous prokaryotic host, can function as a repressor, and that mutant FLP proteins that bind DNA more tightly may be selected as more efficient repressors.

THE *Saccharomyces cerevisiae* FLP recombinase binds to specific sequences of DNA ("FLP sites"), and catalyses the cleavage and rejoining of these sequences to mediate a site-specific inversion within the 2μm circle (BROACH, GUARASCIO and JAYARAM 1982; ANDREWS *et al.* 1985; JAYARAM 1985; SENECOFF, BRUCKNER and COX 1985). FLP protein belongs to the integrase family of recombinases, which includes bacteriophage P1 Cre recombinase and a number of phage integrases (*e.g.*, λ Int protein) that carry out similar recombination events (ARGOS *et al.* 1986). The chemistry of the reactions that the eukaryotic FLP protein catalyzes is similar to that of the reactions catalyzed by prokaryotic members of this family (PRASAD, YOUNG and JAYARAM 1987).

The pathway of FLP-mediated recombination must involve at least five sequential steps (Figure 1). First, the nascent FLP polypeptide chain must fold. Then it may bind, perhaps as a dimer, to each of two identical sequences that consist of 13-bp inverted repeats separated by an 8-bp spacer region (JAYARAM 1985; GRONOSTAJSKI and SADOWSKI 1985a; SENECOFF, BRUCKNER and COX 1985). FLP cleaves its site twice, on opposite strands of the duplex, at the junctions of the spacer and the inverted repeats. As a consequence of the cleavage reaction, two FLP monomers become covalently attached to each site by 3′-phosphotyrosyl ester linkages (GRONOSTAJSKI and SADOWSKI 1985b). The cleaved intermediate undergoes a topological rearrangement requiring the synapsis of the two bound and cut sites (SENECOFF, BRUCKNER and COX 1985; PRASAD *et al.* 1986). FLP reseals the broken strands in a manner that inverts the DNA between the two bound and cut sites.

To dissect the pathway of FLP-mediated recombination, we would like to develop simple genetic selections for FLP mutations that affect specific steps in this pathway. These mutations should allow us to identify amino acid residues of FLP critical for each step. One of the more intriguing steps in the pathway is the binding of FLP to its side.

In this paper, we show that alteration of the presumed active site tyrosine residue of FLP to phenylalanine not only abolishes recombinase activity (PRASAD, YOUNG and JAYARAM 1987), but also enables FLP to function as a repressor in Salmonella, whereas the wild-type protein cannot. We have used this phenotype (repressor function) in Salmonella to select additional mutations in the FLP gene that increase the affinity of FLP recombinase for its site.

MATERIALS AND METHODS

Bacteria, phage, and plasmids: Salmonella strains are derivatives of *Salmonella typhimurium* LT2. DB7000 (*leuA-414* (*am*) *sup*^{0} (SUSSKIND, WRIGHT and BOTSTEIN 1971) and its *supE* derivative MS1363 (SUSSKIND 1980), used for the permissive growth of phages, have been described. MS1868 and MS1883 are otherwise isogenic with DB7000 and MS1363, respectively, but carry an additional mutation that inactivates the *hsdSB* restriction system (GRANA, YOUDERIAN and SUSSKIND 1985). MS1582 is an immune (*c2*$^+$ *mnt*$^+$) lysogen of prophage P22 *16-amH1455 sieA44 Ap68tpfr49* in MS1363, used for the selection of virulent mutants of P22 (GRANA, YOUDERIAN and SUSSKIND 1985). Challenge phages are derivatives of P22 *Kn9 arc-amH1605* phage and were constructed as described previously (BENSON *et al.* 1986);

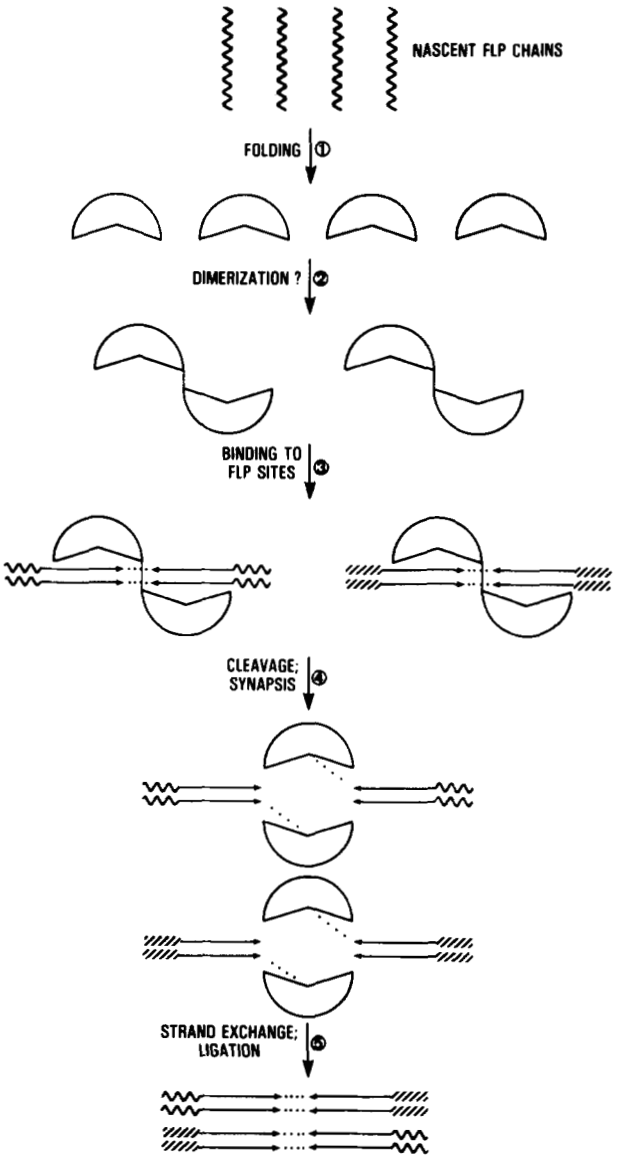

FIGURE 1.—The FLP recombination pathway. The steps that lead to a functional recombination complex must involve folding (1), dimerization (2), and binding of the FLP protein to its target site (3) within the 2-μm circle inverted repeats. After FLP binding, the subsequent steps require DNA cleavage and synapsis (4), followed by strand exchange and ligation (5) to complete the recombination event. The symmetry elements of the FLP target site are indicated by the arrows and the core sequence by the dotted lines. Sequences flanking the FLP site are represented by different symbols for the two repeats of 2-μm circle. The model is not intended to imply that cleavage precedes synapsis, or that cleavage and rejoining of strands occurs in a concerted (one-step) rather than in a sequential (two-step) fashion.

the synthetic site carried by the FLP challenge phage is D1 (5′ GAAGTTCCTATACTTTCTAGAGAATAGGAACTTC 3′) (PRASAD *et al.* 1986), which was cloned into the unique *Sma*I site of pPY190 (BASS *et al.* 1987) to construct the plasmid parent of the FLP challenge phage. Plasmid pSE156 is a derivative of plasmid pSC101 that carries the error-prone repair (*mucA* and *mucB*) genes from conjugative plasmid pKM101 (S. ELLEDGE, unpublished results). Compatible plasmid pMJ, a derivative of pBR322, was used as an inducible source of FLP protein (JAYARAM 1985). Plasmid pCS3 (C. SCHWARTZ and P. D. SADOWSKI, unpublished results) carries the FLP structural gene fused to the *tac* promoter on a pSC101-derived replicon, and was the kind gift of C. SCHWARTZ.

Media and chemicals: Media and general phage techniques have been described (SUSSKIND, WRIGHT and BOTSTEIN 1971; BOTSTEIN, CHAN and WADDELL 1972; YOUDERIAN and SUSSKIND 1980; YOUDERIAN *et al.* 1983). Green indicator plates were supplemented with ampicillin, and/or kanamycin sulfate (Sigma) to final concentrations of 50 and 20 μg/ml respectively. Restriction endonucleases, T4 DNA ligase, and *Escherichia coli* DNA polymerase I were from New England Biolabs. AMV reverse transcriptase was from Boehringer Mannheim; deoxy- and dideoxy-ribonucleoside triphosphates were from PL Biochemicals; and, α-^{32}P-ATP (700 Ci/mmol) was from ICN Biomedicals, Inc.

Mutagenesis and selection of mutant pMJ plasmids: To mutagenize pMJ, we prepared a high-frequency specialized transducing lysate of the plasmid packaged into P22 capsids. MS1868 cells carrying the plasmid were infected with phage P22 *sieA44 ant-amR222 Ap68tpfr138* (YOUDERIAN and SUSSKIND 1980), which carries a portion of the β-lactamase (*bla*) gene from transposon Tn*1* inserted in the P22 tail gene, at a multiplicity of 1 to 10 phages per cell. Ultraviolet (UV)-stimulated homologous recombination between a portion of the *bla* gene on plasmid pMJ and the replicating phage genome results in the formation of cointegrates that are packaged into P22 capsids by the headful packaging mechanism (ORBACH and JACKSON 1982; YOUDERIAN, BOUVIER and SUSSKIND 1982). Ampicillin-resistant transducing particles were found to represent about 0.1% of the plaque forming titer in the resulting lysates. Transducing lysates were irradiated heavily with UV (to a fluence of 1800–3600 ergs mm^{-2}) and adsorbed to recipient MS1868 cells carrying plasmid pSE156 at a multiplicity of infection of 10 phage/cell for 25 min at 25°. Plasmid pSE156 stimulates the error-prone repair of UV-induced lesions on the plasmid DNA; we find that the combination of UV-iradiation and pSE156-stimulated error-prone repair is an efficient way to induce a broad spectrum of all types of substitution mutations in Salmonella (YOUDERIAN, BOUVIER and SUSSKIND 1982). Infected cells were incubated overnight at 37°, in the absence of antibiotic election. Overnight cultures were diluted 50-fold into LB medium containing ampicillin (50 μg/ml) and grown to a density of 4 × 10^8/ml at 37°. Exponential cells were infected with FLP challenge phage at an input multiplicity of 25 phages/cell. After adsorption for 25 min at 25°, aliquots of the infections were spread on green indicator plates containing ampicillin and kanamycin, and incubated at 40° for 24 h. Single colonies from each of three independent transductions were purified on green ampicillin plates at 40°. Plasmid DNAs isolated from three lysogens were reintroduced into MS1868 by transformation, and challenge phage infections were repeated to confirm the plasmid-borne mutant phenotypes.

Challenge phage infections: MS1868 cells carrying plasmid pMJ or its derivatives were grown in LB medium containing ampicillin (50 μg/ml), or MS1868/F′*lacI*^{Q1} cells carrying plasmid pCS3 were grown in LB medium with chloramphenicol (20 μg/ml), at 37° to a density of 6 × 10^8 cells/ml. Cells were infected with a multiplicity of 25 phage/cell, and incubated at 25° for 20 min to allow for adsorption of the challenge phage. Infected MS1868(pMJ) cells were diluted and spread on green indicator plates with kanamycin (20 μg/ml) and ampicillin (50 μg/ml); infected MS1868/F′*lacI*^{Q1}(pCS3) cells were spread on green plates with kanamycin (20 μg/ml), chloramphenicol (20 μg/ml),

TABLE 1

Mutant FLP proteins function as more efficient repressors

Plasmid	Codon changes	Temperature 30°	37°	40°
Wild-type (pMJ)		$<10^{-6}$	3×10^{-3}	7×10^{-6}
YS343 (Tyr343→Ser)	TAT→TCT	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$
YF343 (Tyr343→Phe)	TAT→TTT	1×10^{-3}	10	8
AG51 (Ala51→Gly)	GCA→GGA	5×10^{-3}	2	0.5
IM298 (Ile298→Met)	ATA→ATG	1×10^{-5}	6×10^{-3}	8×10^{-4}
RQ258 (Arg258→Gln)	CGA→CAA	5×10^{-2}	0.3	0.6
Wild-type (pCS3)		1.3	8	1×10^{-5}

Cells producing wild-type and mutant FLP proteins were infected with challenge phage at high multiplicity (20 phage/cell) at 30°, 37° and 40°. Numbers represent the percent of surviving cells selected on green indicator plates with kanamycin.

and 10^{-5}M β-isopropylthiogalactoside. Plates were incubated at the indicated temperatures for 24 to 36 h.

Purification of wild-type and mutant FLP proteins: The steps for partial purification of FLP have been described (PRASAD, YOUNG and JAYARAM 1987). To determine the purity of FLP protein, preparations were resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, stained by Coomassie brilliant blue, and scanned by an LKB ultrascan XL laser densitometer. In this gel system, FLP migrates with an apparent mobility of 45 kD. The recombination and DNA binding assays *in vitro* were done using approximatively 20 to 25% pure FLP. Protein estimates were made by the method of LOWRY *et al.* (1951) as modified by PETERSON (1977).

Recombination assays: Recombination *in vivo* was assayed as described by GOVIND and JAYARAM (1987). Assays for recombination *in vitro* were carried out according to the method of PRASAD, YOUNG and JAYARAM (1987).

DNA-binding *in vitro*: The DNA substrate used in FLP binding (see Figure 3) and the details of the assay have been described (PRASAD, YOUNG and JAYARAM 1987). FLP protein was diluted to the required concentration in 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1 mM EDTA, 10% glycerol, and 20 mM β-mercaptoethanol. Glycerol and NaCl concentrations were constant in the binding reactions at 4% and 133 mM, respectively. The three FLP-DNA complexes with different mobilities were separated from the substrate by electrophoresis. Autoradiograms of the dried gels were scanned, and the amount of bound DNA was calculated as the sum of the areas under the peaks corresponding to all three complexes. Apparent disassociation constants were extrapolated from double reciprocal plots of bound DNA *vs.* FLP concentration, fit by linear least squares.

RESULTS

FLP recombinase can function as a temperature-sensitive repressor in Salmonella: We have described a general genetic selection for Salmonella cells that produce specific DNA-binding activities corresponding to synthetic binding sites. We have shown that this selection works for a variety of prokaryotic repressors, starting with their cognate operators (BENSON *et al.* 1986). To extend this selection to eukaryotic DNA-binding proteins, we tested whether we could select for the binding of yeast FLP recombinase to its site in a Salmonella host. We asked whether FLP protein, when produced from a plasmid construct in which the λP_R promoter is fused to the FLP coding sequence, could regulate a second operon fusion with a FLP site at the transcription startpoint.

To assay FLP binding, we constructed a derivative of the Salmonella phage P22, called a FLP challenge phage, in which the FLP-binding site is placed at the start of transcription of the P22 *ant* (antirepressor) operon. The site we used is the 34 bp sequence consisting of the two 13 bp inverted repeats and 8-bp spacer that define the minimal FLP site (JAYARAM 1985). For a challenge phage, the decision between lytic and lysogenic development is determined by the state of occupancy of the site contolling *ant* gene transcription. A Salmonella host will survive infection by a challenge phage (and establish the infecting phage as a prophage) only if it produces an activity that binds this site efficiently (BENSON *et al.* 1986). To express FLP activity in Salmonella, we transformed a Salmonella host with plasmid pMJ. pMJ carries a temperature-sensitive allele of the λ repressor gene that regulates a fusion of the λ P_R promoter to the FLP coding sequence (JAYARAM 1985). In Salmonella, active FLP protein is produced from the plasmid at 37° (data not shown).

As shown in Table 1, cells that produce wild-type FLP are lysogenized poorly by a challenge phage with the FLP site. Nonetheless, the low frequency of lysogeny seen depends on the expression of FLP, since it is observed only at temperatures (37° and 40°) that permit high-level expression of FLP from the λ P_R promoter. Cells producing wild-type FLP protein from plasmid pMJ are lysogenized less efficiently by a FLP challenge phage at 40° than at 37°. This result is suprising, since expression of FLP from the temperature-inducible λ P_R promoter should be higher at 40° than at 37°. FLP protein or mRNA production does not appear to be lethal to Salmonella at 40°, since cells producing FLP protein show no loss in viability at this higher temperature.

To determine whether FLP is temperature-sensitive when produced in Salmonella, we examined the ability of cells carrying a different FLP-producing

plasmid, pCS3, to be lysogenized by a FLP challenge phage. Plasmid pCS3 (C. SCHWARTZ and P. D. SADOWSKI, unpublished results) produces FLP protein from the inducible *tac* promoter. In the presence of 10^{-5} M IPTG (inducer), Salmonella host MS1868/F'*lacI*^{Q1} carrying plasmid pCS3 is lysogenized well by the FLP challenge phage at 30° and 37°, but not at 40° (Table 1). This result indicates both that FLP protein may be produced in sufficient levels at 37° to permit the efficient lysogeny of the challenge phage, and that FLP protein is temperature-sensitive in Salmonella at 40°.

A mutation that blocks FLP cleavage enables FLP to function as a more efficient repressor: A comparison of the amino acid sequences of the integrase family of recombinases reveals a modest degree of homology in the carboxyl-terminal regions of these proteins (ARGOS *et al.* 1986). Quite strikingly, three residues, corresponding to His305, Arg308, and Tyr343 of FLP, are invariant in all members of this family. These residues may represent conserved features of the active sites of these recombinases. In particular, the Tyr343 residue may be the active site tyrosine that forms a transient covalent attachment to DNA during recombination. To determine the functions of these three amino acids, we have changed them by site-directed mutagenesis. We found that the mutant YF343 (Tyr→Phe) protein has lost recombinase activity but not DNA-binding activity (PRASAD, YOUNG and JAYARAM 1987). This change removes the hydroxyl group of Tyr that may participate in the formation of the protein-DNA linkage. As shown in Table 1, the mutant YF343 protein can function as a repressor in Salmonella, permitting efficient lysogeny of a FLP challenge phage at 37°, and even at 40°. In contrast, a mutant FLP protein with a different change of the presumed active site tyrosine (to serine, YS343) can bind to the FLP substrate *in vitro* but is blocked in substrate cleavage (PRASAD, YOUNG and JAYARAM 1987). Yet, it does not function as a repressor in Salmonella (Table 1). We conclude that the loss of catalytic activity *per se* is not sufficient to turn FLP into a more efficient repressor.

New mutations that make FLP a more efficient repressor: We used the challenge phage selection to obtain several additional mutant FLP proteins that function as more efficient repressors in Salmonella. The plasmid donor of FLP protein, pMJ, was mutagenized with ultraviolet light and transduced into a Salmonella host proficient in error-prone repair. Transductants were then selected for the ability to be lysogenized by the challenge phage at 40°. Three independent mutants were isolated. DNA sequence analysis of both strands of the entire FLP gene of the mutant plasmids revealed that they have single base pair substitutions within the FLP coding sequence predicted to result in single amino acid changes in the protein: Ala51→Gly (AG51), Arg258→Gln (RQ258), and Ile298→Met (IM298) (Table 1).

TABLE 2

Three mutant FLP proteins are proficient in recombination *in vivo*

Plasmids	No. of transformants	
	Kan^S	Kan^R
Wild type	464	4
IM298	516	56
AG51	442	38
RQ258	368	2

A *recA*$^-$ *E. coli* strain with the substrate plasmid, pJK (Cam^R, Kan^R) (JAYARAM 1985), was transformed with pMJ or its variant FLP analogs (Amp^R) and selected on plates containing chloramphenicol and ampicillin. Transformants were replica-plated onto plates containing chloramphenicol, ampicillin, and kanamycin, to determine the frequency of loss of the Kan^R determinant. FLP-mediated recombination results in a deletion of the Kan^R determinant, so that the frequency of kanamycin-sensitive cells is a measure of FLP activity.

The three mutant FLP proteins we isolated using the challenge phage selection differ from wild-type FLP and each other in their abilities to permit the lysogeny of a FLP challenge phage. The mutant IM298 FLP is only slightly better than wild-type FLP at 37°, and is distinctly better than wild-type at 40°. Mutant AG51 and RQ258 FLP proteins bind a FLP challenge phage better than wild-type FLP protein at all temperatures.

Activity of mutant FLP proteins *in vivo* and *in vitro*: Three mutant plasmids, AG51, IM298, and RQ258, were extracted from their Salmonella hosts and transformed into *E. coli*. FLP proteins produced from these plasmids were tested for the ability to catalyse recombination *in vivo*, as described by GOVIND and JAYARAM (1987). Recombination assays were carried out in a *recA-E. coli* host with a pACYC184-derived substrate plasmid that has a kanamycin-resistance determinant flanked by direct repeats of the FLP site. In the presence of functional FLP, the kanamycin-resistance gene is lost upon recombination between the two FLP sites, rendering the host sensitive to kanamycin. As shown in Table 2, the mutant RQ258 FLP catalyses recombination to the same extent as wild-type FLP, whereas AG51 and IM298 show slightly lower levels of recombination *in vivo*.

We purified these mutant proteins using the protocol of PRASAD *et al.* (1986), and tested their abilities to catalyse recombination *in vitro* (PRASAD, YOUNG and JAYARAM 1987) (Figure 2). The substrates in this assay are two different linear molecules of identical length, each containing a FLP site. The relative placement of the FLP site with respect to the ends of the DNA is different for each substrate. Recombination between the two substrates (P_1 and P_2) gives

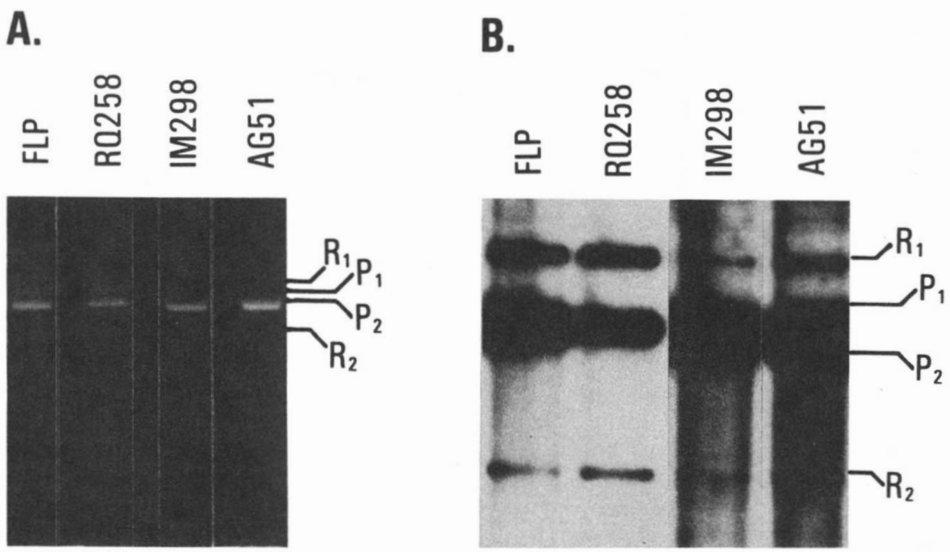

FIGURE 2.—FLP recombination *in vitro*. The plasmid substrate used for recombination assays *in vitro* contains the 900-bp *Hin*dIII fragment from the B form of the 2-μm circle (HARTLEY and DONELSON 1980) cloned into *Hin*dIII site of pBR322, and includes a single copy of the FLP site. This plasmid was cut separately with *Eco*RI and with *Pvu*II. An equimolar mixture of the two types of linear molecules was incubated with partially pure FLP preparations as described by PRASAD *et al.* (1986). Reaction mixtures were resolved on 1% agarose gels. A, Ethidium bromide staining of the fractionated DNA reveals the parental fragments (labeled P_1 and P_2) and the recombinant fragments (labeled R_1 and R_2). B, To detect low levels of recombination, the gel fractionated DNA was transferred to nitrocellulose and hybridized to a ^{32}P-labeled DNA fragment that contains the FLP site. The lanes corresponding to IM298 and AG51 are overexposed relative to those corresponding to FLP and RQ258.

rise to two products (R_1 and R_2), one longer and the other shorter than the substrates. When the products of a recombination reaction are fractionated by electrophoresis on an agarose gel, the extent of recombination is revealed by the appearance and abundance of R_1 and R_2. In this assay, the mutant RQ258 FLP protein is as active as wild-type FLP (Figure 2B). In contrast, the recombinant bands generated by AG51 and IM298 proteins are barely detectable after staining with ethidium bromide. To demonstrate that these two mutant proteins catalyse site-specific recombination *in vitro*, we transferred the gel-fractionated products of these reactions to nitrocellulose, and hybridized the transferred DNA's with a ^{32}P-labeled probe. As shown in Figure 2, a low level of recombination catalysed by these two proteins can be detected by this more sensitive assay.

It is not clear whether the decreased ability of these two proteins to catalyse recombination *in vitro* is due to a defect in the specific activity of these proteins, or merely reflects their relative concentrations in the preparations assayed. To distinguish these possibilities, "Western" blots of these preparations were probed with anti-FLP antisera and developed with a peroxidase-conjugated second antibody. No reactive bands with a mobility greater than or equal to that of the single band corresponding to wild-type FLP were seen for AG51 or IM298 proteins (data not shown), indicating that these proteins are produced in amounts less than one-tenth of the wild-type amount or are unstable in *E. coli*, perhaps by being rendered sensitive to proteolysis. In contrast, the YF343, YS343, and RQ258 proteins were detected as single bands present in wild-type amounts by this assay.

Two mutant FLP proteins bind the FLP target site with higher affinities than wild type: One explanation for the ability of the mutant FLP proteins to function as repressors in Salmonella is that they have increased affinities for the FLP site. To obtain a crude measure of the relative affinities of these proteins *in vitro*, we assayed their abilities to complex with a 100 bp restriction fragment of DNA from the 2-μm circle that includes the wild-type FLP site (PRASAD, YOUNG and JAYARAM 1987). Binding of FLP to this fragment results in the formation of three complexed species with different mobilities than the free DNA fragment, as determined by a gel retardation assay (ANDREWS, BEATTY and SADOWSKI 1987; PRASAD, YOUNG and JAYARAM 1987). This is partly because the wild-type FLP site is composed of three "half-sites" recognized by FLP monomers, two that are bound with slightly higher affinities than a third accessory binding site (ANDREWS *et al.* 1985).

The results of these assays are shown in Figure 3. If we plot the reciprocal of the fraction of bound DNA (the sum of the fractions of bound DNAs in the three complexes) *vs.* the reciprocal of the amount of FLP protein in each experiment, we find a reasonably good linear fit with our data. This result indicates that, to a first approximation, we may treat the assemblage of three "half-sites" that comprise the

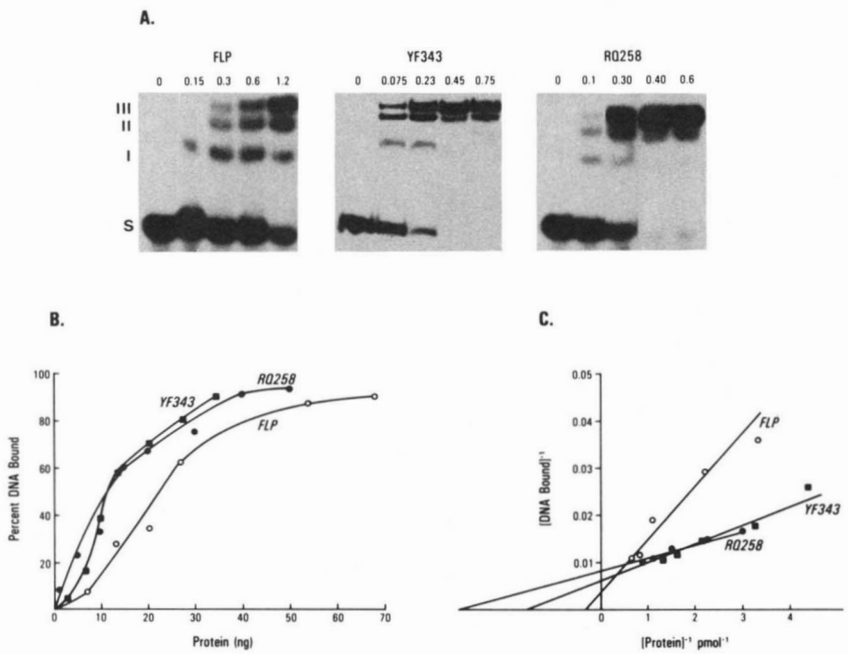

FIGURE 3.—Binding of FLP to its substrate *in vitro*. The partially purified protein preparations used in these binding experiments were the same as those used for recombination assays *in vitro*. The 100 bp *Hin*dIII-*Eco*RI fragment containing the FLP target site, used *in vitro* binding assays, has been described (PRASAD, YOUNG and JAYARAM 1987). The fragment (S) was labeled at the *Hin*dIII end on the bottom strand with α-^{32}P-dCTP by filling-in. Binding assays have been described by PRASAD, YOUNG and JAYARAM (1987). A, The three DNA-protein complexes resulting from the binding of FLP to its substrate (I, II and III) were visualized by polyacrylamide gel electrophoresis, followed by autoradiography. The FLP concentration in pmol is indicated above each lane. B, The binding patterns as a function of increasing protein concentration are shown for wild-type FLP, YF343 and RQ258. B, The amounts of free and bound substrates were estimated by densitometry. The bound substrate was calculated as the sum of complexes I, II and III. The percent of bound substrate with increasing FLP (wild-type or mutant) concentration is shown. C, The data in (B) are represented on double reciprocal plots.

wild-type FLP site as a single site, and assign overall apparent disassociation constants for each of these sets of reactions. The apparent disassociation constants for these mutant proteins with the wild-type FLP site calculated from Lineweaver-Burk plots are 8.2×10^{-9} M for RQ258 FLP and 1.3×10^{-8} M for YF343 FLP; both have significantly higher affinities than wild-type FLP (6.6×10^{-8} M). These values must be regarded with some reservation, due to uncertainty in the measurement of the active fractions of each of the wild-type and mutant FLP preparations; however, they are qualitatively consistent with the results of our binding assay *in vivo*.

DISCUSSION

Most prokaryotic repressors recognize specific, short DNA sequences (operators) that overlap RNA polymerase binding sites (promoters). Binding of a repressor to an operator sterically interferes with the binding of RNA polymerase to the promoter. Therefore, most repressors are competitive inhibitors of RNA polymerase binding.

We have shown that a number of different prokaryotic operators function to repress the Salmonella phage P22 *ant* operon, when placed at the startpoint of transcription of the *ant* promoter. Since the P22 *ant* gene can control the lysis/lysogeny decision of P22, we have been able to devise a general genetic selection for repressor activities, based on the properties of P22 phages that carry synthetic operators at the startpoint of *ant* transcription (the challenge phage selection) (BENSON *et al.* 1986). A P22 challenge phage carrying a synthetic operator selectively lysogenizes, and spares from death, a host that produces a specific DNA-binding activity corresponding to the synthetic operator, affording us a positive selection for DNA-binding activity.

The sequence-specific recognition of DNA by proteins in structural, catalytic, and regulatory cellular processes is as important in eukaryotes as it is in prokaryotes. To see if the challenge phage selection could be adapted to eukaryotic DNA binding activities, we examined whether we could detect the binding of yeast FLP protein to its target site by this selection. We chose FLP because active FLP can be expressed in *E. coli* from strong, regulated promoters, including the *tac* and λ P_R promoters (SADOWSKI *et al.* 1984; COX 1983; JAYARAM 1985), and the DNA sequence recognized by FLP has been characterized precisely (ANDREWS *et al.* 1985; JAYARAM 1985; SENECOFF, BRUCKNER and COX 1985).

Wild-type FLP recombinase, when expressed from the λ P_R promoter on plasmid pMJ in Salmonella, offers only partial protection to its host from an infecting challenge phage carrying the FLP target sequence. This is not particularly surprising, since the equilibrium binding constant of FLP to its substrate may be significantly higher in Salmonella than the binding constants of many prokaryotic repressors for a variety of reasons. At 37°, FLP is not expressed in sufficient levels from plasmid pMJ to function efficiently as a repressor, and at 40°, FLP appears to be temperature-sensitive. Nonetheless, we have shown that single codon changes in the FLP gene on plasmid pMJ (resulting in single amino acid changes in the FLP polypeptide) can result in mutant FLP proteins that function as more efficient repressors in Salmonella.

We have identified four mutations that turn wild-type FLP recombinase into more efficient prokaryotic repressors. One of these changes the presumed active site tyrosine to phenylalanine (YF343) and has a pleiotropic phenotype; it is affected in several different steps in the FLP recombination pathway. This mutation eliminates both strand cleavage and recombination, yet enables the mutant protein to bind the FLP substrate with a higher affinity than the wild-type protein (PRASAD, YOUNG and JAYARAM 1987; this paper). The ability of this mutant protein to function more efficiently as a repressor could be due either to loss of recombinase function or enhanced substrate binding.

A second mutant FLP, RQ258, also functions as a more efficient repressor in Salmonella. Unlike the mutant YF343 FLP, this variant protein retains activity in recombination assays. Assays *in vitro* show that RQ258 FLP is better than wild-type FLP in substrate binding. It appears that the ability of RQ258 FLP to function well as a repressor arises from its increased affinity for the FLP site. In *E. coli*, RQ258 FLP is produced in the same relative amounts as are wild-type and YF343 FLP proteins.

Two other mutant FLP proteins, AG51 and IM298, also are better repressors. Both of these altered proteins show slightly reduced recombinase activities *in vivo* (in *E. coli*). When partially purified, they are barely detectable by labeling with anti-FLP antibodies. If their induced levels in Salmonella are comparable to those in *E. coli*, their ability to function as repressors in the challenge phage selection implies that they must indeed have higher affinities for the FLP site. We note that two of the four mutations that permit FLP to function as a more efficient repressor in Salmonella are located in the carboxyl terminus of FLP, the region most highly conserved among the integrase family of recombinases.

These results represent two significant advances. First, they establish the power and utility of the P22 challenge phage selection in exploring DNA-protein interactions in eukaryotes. Second, they show that we can select for mutations that affect the DNA-binding step in the FLP recombination pathway (Figure 1), and provide us with several new genetic approaches for understanding the molecular mechanisms involved in the pathway of FLP-mediated recombination.

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