ARTICULOS SOBRE TECNICAS

GENETIC APPROACH FOR BINDING ACTIVITY DETECTION OF THE FLP RECOMBINASE

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SUMMARY

We have devised a novel assay to determine *in vivo* FLP binding activity to the FRT site using a genetic selection. This genetic approach was previously described *(Elledge et al.*, 1989), as a general selection procedure to facilitate the cloning of genes encoding sequence-specific DNA-binding proteins.

The specific DNA-binding site (FRT) have been placed near the start of transcription of the strong synthetic ConII promoter at a position for repression mediated by the FLP recombinase. The binding activity is detected when cells expressing the FLP protein, repress transcription from the ConII promoter, so the adjacent drug-resistance gene, *aadA* could be transcribed, resulting in drug resistance in cells bearing the FLP gene.

RESUMEN

En este trabajo hemos desarrollado un experimento novedoso de selección genética para la determinación de la actividad de enlace de la proteína FLP al sitio de unión FRT *in vivo*. Esta metodología genética ha sido descrita previamente (Elledge *et al.*, 1989) como un procedimiento general de selección para facilitar el clonaje de genes que codifican proteínas que se unen al ADN por un sitio específico. El sitio de unión específico (FRT), fue subclonado cerca del inicio de la transcripción del promotor sintético ConII, en una posición que permite la represión mediada por la recombinasa FLP. La actividad de enlace se detecta cuando en las células que expresan la proteína FLP, se reprime la transcripción del promotor ConII, y el gen de resistencia adyacente *aadA* se puede transcribir, resultando en una resistencia de las células a la droga.

INTRODUCTION

The FLP protein of the 2 μ plasmid of the yeast *Saccharomyces cerevisiae* is a site-specific recombinase that is involved in the amplification of the plasmid *in vivo* (Broach *et al.*, 1982). The protein performs efficient site-specific recombination *in vitro* (Vetter *et al.*, 1983).

FLP has been extensively studied *in vitro* and it has been possible to divide the recombination reaction into a number of steps. The first step is binding to the FRT site (Beatty and Sadowski, 1988; Pan *et al.*, 1991). The FRT consists of three 13 bp symmetry elements flanking an 8 bp core (figure 1).

Formation of FLP-DNA complexes is most commonly assayed by mobility shift of bound fragments in an acrylamide gel (Andrews *et al.*, 1987). Several methods of foot printing have been used to analyze the interaction of FLP with the FRT site (Andrews *et al.*, 1985).

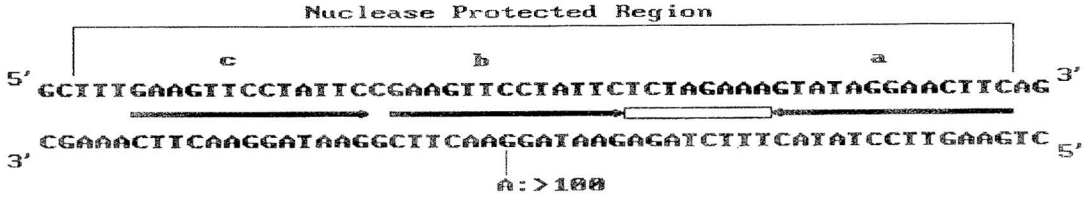

Fig. 1 Nucleotide sequence of the FLP recombinase target (FRT site). Horizontal bracket indicates region protected by FLP from DNase digestion. Horizontal arrows are the three 13 bp symmetry elements (labeled a, b, and c). The open box is the 8 bp core region. The vertical line shows the residue which when changed as indicated cause a dramatic fall in FLP activity. The number after the changed residue indicates the fold reduction of activity.

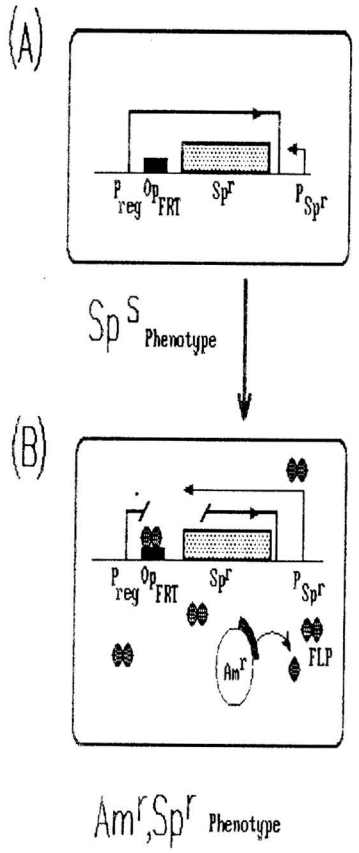

Fig. 2 Genetic selection for FLP binding activity. **a)** No FLP present in the cell. The rightward transcript (heavy arrow) from the Preg promoter stops transcription of the Sp^r gene (stippled box) from the Sp^r promoter (P_{Sp^r}; thin arrow). Since there is no FLP present, the operator (Op_{FRT}) is unoccupied. The cell is Spectinomycin-sensitive. **b)** FLP present in the cell. FLP is produced from a plasmid-encoded gene, (Am^r, filled box) and diffuses to occupy the FRT operator (filled circles, here shown as dimers or monomers in solution). This represses transcription from Preg and allows transcription of the Sp^r gene (thin leftward arrow). The cell is now Am^r and Sp^r. When the wild type FRT sequence is replaced by a mutated site that is unable to bind FLP, these cells will be Sp^s. Arrows indicate the sense of transcription from each promoter.

The recombination DNA target (FRT site) have been well-characterized, but the localization of the DNA binding domain of the FLP protein has proved difficult, because these appear to be no discrete domains. There are none of the well-recognized DNA-binding motif such as zinc-fingers, helix-turn-helix motifs or leucine zippers (Amin and Sadowski, 1989), so FLP seems to present a novel mode of site-specific DNA recognition.

In this study, we have devised a direct assay for FLP binding *in vivo* to the target DNA. We developed the genetic selection for the FLP system based on a general strategy for genetic selection of genes encoding sequence-specific DNA-binding proteins (Elledge and Davis, 1989). This system could be used for an *in vivo* selection of novel mutant proteins able to bind more or less tightly than wild type FLP, for the identification of regions of FLP involved in DNA binding.

Basically, the genetic selection works as follows, (figure 2): A strong constitutive promoter (Preg) drives transcription in an antisense direction through a gene encoding resistance to an antibiotic (spectinomycin resistance, Sp^r) which is driven from its own promoter. When Preg is active, normal transcription through the Sp gene is occluded and the cells are Sp-sensitive. When the FRT site is placed near the Preg promoter (Op), FLP is expected to bind to this operator site and to abrogate the antisense transcript, so that the Sp^r gene can be transcribed. The cell is thus Sp-resistant.

MATERIALS AND METHODS

Bacteria, plasmids, and genetic techniques

E. coli JM107 (F'*tra*D36*lac*I^q (*lac*Z)M15*pro*AB/e14 (mcrA$^-$)*lac-pro*AB)**thigyr**A96(Nal^r)*end*A 1 *hsd*R17($r^-_k m^+_k$)*rel*A1 *sup*E44 *mcr*A) was used as the host for the genetic selection.

Plasmids PNN388, PNN396, (Elledge and Davis, 1989) and PNN402 were a gift from S. Elledge.

PDV64 (Babineau *et al.*, 1985), was used for the expression of FLP protein in the cells, under the control of Tac promoter.

PGP25 (Proteau *et al.*, 1986), was used as a source for the FRT symmetry b element.

For drug-sensitivity measurements. Luria Bertani (LB) agar plates were supplemented with either chloramphenicol (Cm, 40 μg/ml), spectinomycin (Sp, 60 μg/ml), Kanamycin (Km, 40 μg/ml), or ampicillin (Ap, 50 μg/ml). All plates contained an additional 100 μg of L-tryptophan per ml. When necessary, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

Construction of regulated promoters and plasmids

Construction of ConII promoter was described previously (Elledge and Davis, 1989).

Construction of ConII-b symmetric element of FRT site was made in two steps. First, blunt-ended-BamHI insert, (b element), from PGP25, 5'-CTAGAGAATAGGAACTTCGCCG-3', was cloned into KpnI (flushed)/ BamhI-cut PNN396. This placed the b element following the ConII promoter. Then the NotI-HindIII ConII promoter-b element fragment was cloned into NotI/HindIII-cleaved PNN388. This plasmid, PNN18 (figure 3) confers the Cm^r, Sp^s phenotype.

Construction to place the assay cassette onto the host chromosome

To place the assay cassette on lambda phage and creating a lysogen, (because it provides an stable and low copy number system), we used a method proposed by S. Elledge (personal communication). First the NotI filled in PvuII fragment from PNN18 containing the assay cassette was ligated to EcoRI- filled in cleaved PNN402 (figure 3). The assay

Table 1
Selection of the optimal Sp concentration

LYSOGEN[a]	Sp (μg/ml)						
	0	10	20	30	40	50	60
JM107 (host strain)	+	+	-	-	-	-	-
PNN402 (no assay cassette)	+	+/-	-	-	-	-	-
PλNN18 (wild type FRT)	+	+	+	+/-	-	-	-
PλCR4 (altered FRT)	+	+	+	+/-	-	-	-

(a) This result is after 3 days of incubation at 30°C; plating around 500 cells
+ : growth, +/-: partial growth, - : no growth

Table 2
Detection of binding activity by Sp^r phenotype

LYSOGEN+flp PLASMID(pdv64)	1 mM IPTG,colony No.	No. IPTG,colony No.
PλNN18[(a)]	580	6
PλCR4[(b)]	1	1

(a): Were plated 10^3 cells per plate.(b): Were plated 10^4 cells per plate

cassette is now linked to the neo gene (Km^r), and is flanked by homology to a non-essential region of lambda. The entire region will recombine by a double crossover event. The recombinant phage can be selected by infection at low MOI into the host strain (JM107) by the Km^r phenotype of the PλNN18 lysogen (figure 4).

The PλCR4 lysogen was used as the negative control for binding and was done by site directed mutagenesis by PCR (Erlich, 1989).

In vivo binding assay

The lysogen (PλNN18) bearing the assay cassette was transformed with the PDV64 plasmid containing the FLP gene and the cells were plated after washing with 0.9% NaCl solution in LB plates Am (50 μg/ml), trp (100 μg/ml), Sp (60 μg/ml), with 1 mM IPT and without IPTG.

The negative control (lysogen PλDCR4) contains a single point mutation into the b element, which is known to decrease FLP binding activity in more than 100 fold (Senecoff *et al.*, 1987).

Wild Type FRT 5'-CTTCAAGGATAAG-3'

Altered FRT 5'-CTTCAAAGATAAG-3'

RESULTS AND DISCUSSION

General selection scheme for FLP binding activity *in vivo*

Elledge and Davis (1989) described a transcriptional interference assay in which transcription from the strong ConII promoter can be interfered with the phenotypic expression of a convergently transcribed drug-resistance gene, *aadA* (Hollingshead and Vapnek, 1985). This gene encoded aminoglycoside 3'-adenylyltransferase, an enzyme that confered resistance to the aminoglycosides Sp and streptomycin. Transcriptional interference can be detected by measuring Sp resistance; the level of drug resistance is inversely correlated with the level of transcription from ConII. Therefore, repression of ConII activated expression of *aadA* and confered resistance to Sp. In this case the target FRT (b element), worked as an operator for the ConII promoter, so when FLP was induced inside the cells (by adding IPTG), acted as a repressor when bounds at the FRT element; and becomed the cell Sp^r. When FLP did not bind to the b element then the cells remained Sp^s.

To make this technique work, we first titrated the Sp^r phenotype of the lysogens to determine the optimal Sp concentration (table 1).

The cells of an overnight culture of each lysogen were collected and washed with 0.9% NaCl , plated in LB trp (100 μg/ml) at different Sp concentrations.

This table shows the sensitivity to spectinomycin; in case of the host strain and the lysogen (PNN402), with no Sp gene, the sensibility is greater (partial resistance at 10 μg/ml) than for the lysogens bearing the wild type and the altered cassette. After 40 μg/ml these two last are no longer resistance. So we select for the *in vivo* binding assay 60 μg/ml.

In vivo binding assay

This experiment consisted in a transformation with the FLP plasmid (PDV64), the lysogens bearing the wild type FRT (PλNN18) and the altered FRT (PλCR4, as negative control). The cells after the transformation protocol were washed with 0.9% NaCl and then plated in LB Am (50 μg/ml), trp (100 μg/ml), Sp (60 μg/ml) in induced (1 mM IPTG) and uninduced conditions.

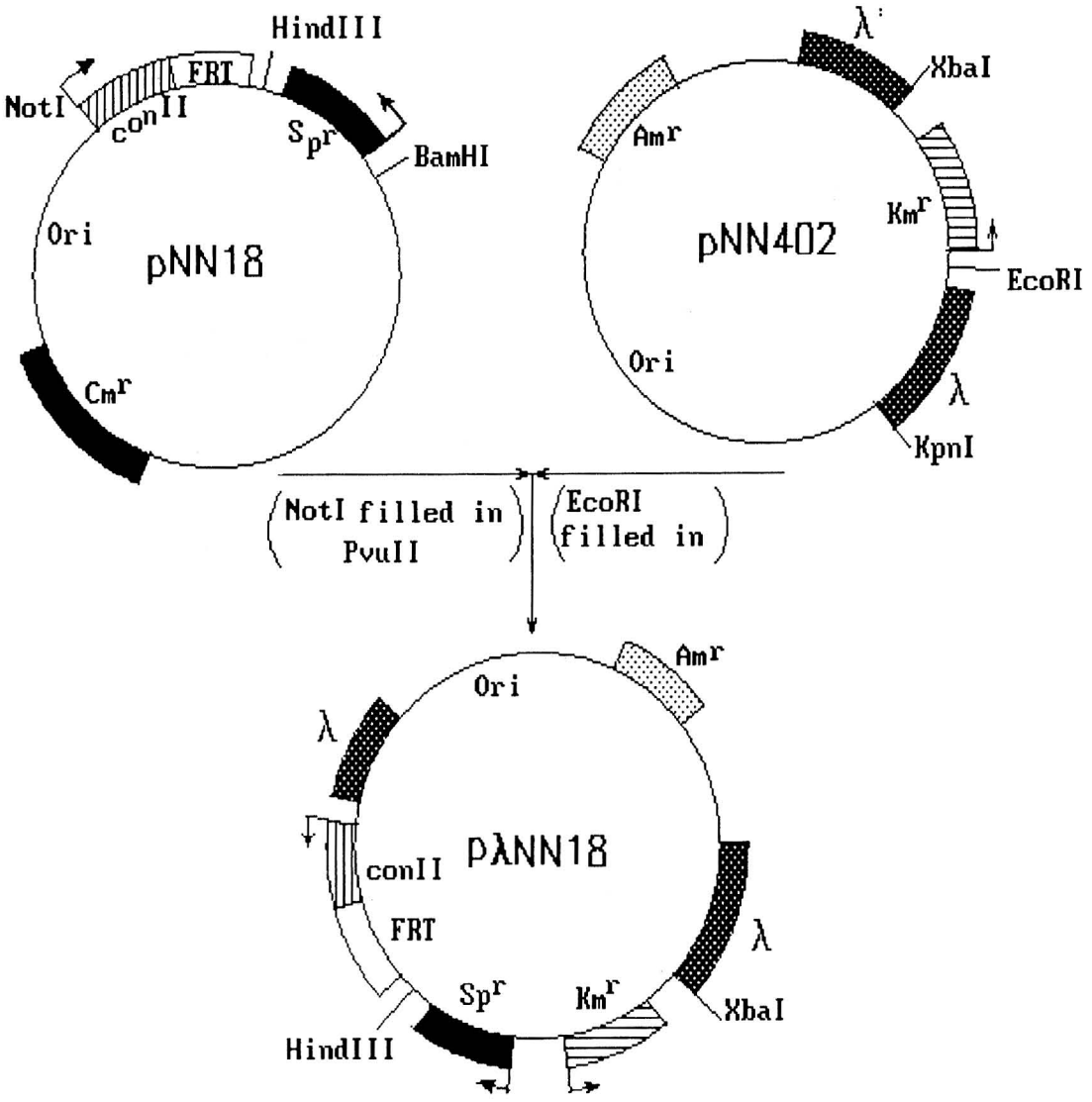

Fig. 3 Construction to place the assay cassette on lambda phage. The NotI filled in-PvuII fragment from PNN18 containing the assay cassette was cloned into the EcoR1-filled in cleaved PNN402. The assay cassette is now linked to the neo gene (Km^r) and is flanked by homology to a non-essential region of lambda. This plasmid PλNN18 was used to integrate the assay cassette onto the *E. coli* chromosome. Arrows indicate the sense of transcription from the promoters.

The results after 3 days of incubation at 30°C are shown in table 2. The experiment most be done at 30°C because at 37°C FLP is not active *in vivo* (data not shown).

This experiment demonstrated that when FLP is present into the cells is able to bind strong enough to one b symmetric element to permit enough Sp^r expression for thegrowth of Sp-resistant cells. When FLP is not present (no IPTG) or the target recognition element has been changed, the cells remain Sp^s.

We have found a position of the FRT-site 'operator' good enough for repression of the ConII (at the -5 position of the start of transcription), and we also can conclude that a single symmetric element surrounding a partial core is enough for binding *in vivo*.

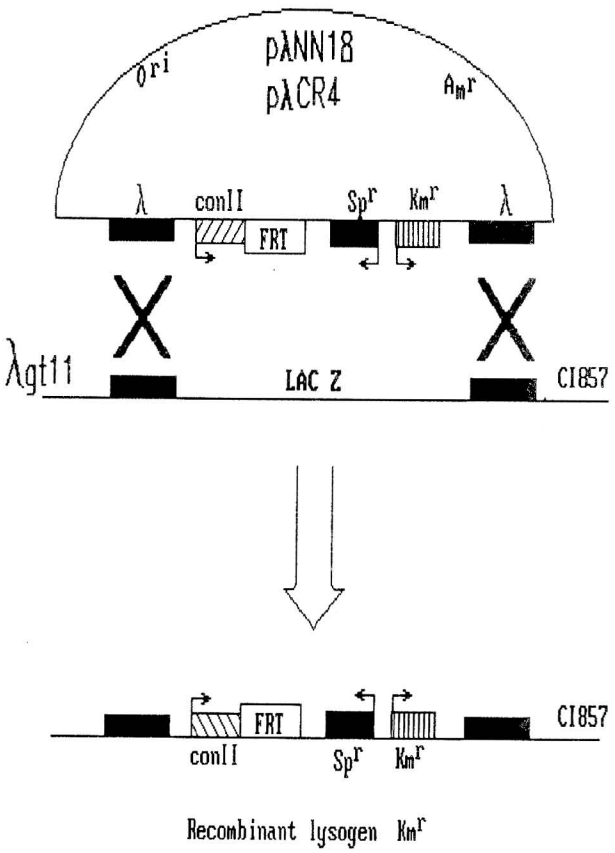

Fig. 4 Recombination event to integrate into the host chromosome. Here is shown the recombination by a double crossover event of the entire region flanked by the non-essential region of λgt11 phage (dark bigger boxes). The recombinant phage (PλNN18) can be selected by infection into the JM107 strain, by the Km^r phenotype of the lysogens. Arrows indicate the sense of transcription from the promoters.

This binding assay could be used for the *in vivo* detection of FLP mutants with altered binding activity and then could help in the understanding of the DNA-protein interaction of the site specific recombinase FLP as a model of the integrase family.

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REFERENCES

AMIN, A.A. and P.D SADOWSKI (1989). Synthesis of an Enzymatically Active FLP Recombinase *In vitro*: Search for a DNA-Binding Domain. *Mol. Cell. Biol.* 9:1987-1995.

ANDREWS, B.J.; L.G. BEATTY and P.D. SADOWSKI (1987). Isolation of Intermediates in the Binding of the FLP Recombinase of the Yeast Plasmid 2-micron Circle to its Target Sequence. *J. Mol. Biol.* 193:345-358.

ANDREWS, B. J.; G.A. PROTEAU; L.G. BEATTY and P.D. SADOWSKI (1985). The FLP Recombinase of the 2-micron Circle DNA of Yeast: Interaction with Its Target Sequences. *Cell* **40**:795-803.

BABINEAU, D.; D. VETTER; B.J. ANDREWS; R.M. GRONOSTAJSKI; G.A. PROTEAU; L.G. BEATTY and P.D. SADOWSKI (1985). The FLP Protein of the 2-micron Plasmid of Yeast. *J. Biol. Chemistry* **260**:12313- 12319.

BEATTY, L.G. and P.D. SADOWSKI (1988). The Mechanism of Loading of the FLP Recombinase onto its DNA Target Sequence. *J. Mol. Biol.* **204**:283-294.

BROACH, J.R.; V.R. GUARASCIO; M. JAYARAM (1982). Recombination within the Yeast Plasmid 2 Circle is site-specific. *Cell* **21**:501-508.

ELLEDGE, S. J. and R.W. DAVIS (1989). Position and density effects on repression by stationary and mobile DNA-binding proteins. *Genes Dev.* **3**:185-197.

ELLEDGE, J.S.; P. SUGIONO; L. GUARENTE and R.W. DAVIS (1989). Genetic selection for genes encoding sequence DNA-binding proteins. *Proc. Natl. Acad. Sci. USA* **56**:3689-3693.

ERLICH, H.A. (1989). PCR Technology. *Principles and Applications for DNA Amplification.* Stockton Press.

HOLLINGSHEAD, S. and D. VAPNEK (1985). Nucleotide Sequence Analysis of a Gene Encoding a Streptomycin/Spectinomycin Adenyltransferase. *Plasmid* **13**:17-30.

PAN, H.; D. CLARY and P.D. SADOWSKI (1991). Identification of the DNA-binding Domain of the FLP Recombinase. *J. Biol. Chemistry* **266**:11347-11354.

PROTEAU, G.; SINDENBERG and P.D. SADOWSKI (1986). The minimal duplex DNA sequence required for site-specific recombination promoted by the FLP protein of yeast *in vitro*. *Nucleic Acids Res.* **14**:4787-4802.

SENECOFF, J.F.; P.J. ROSSMEISSL and M.M. COX (1987). DNA Recognition by the FLP Recombinase of theYeast 2μ Plasmid. A Mutational Analysis of the FLP Binding Site. *J. Mol. Biol.* **201**:405-421.

VETTER, D.; B.J. ANDREWS; L.G. BEATTY and P.D. SADOWSKI (1983). Site-specific recombination of yeast 2μ DNA *in vitro*. *Pro. Natl. Acad. Sci., USA* **80**:7284-7288.