Use of a Tn5 derivative that creates lacZ translational fusions to obtain a transposition mutant

(IS50; transposition screen; papillation; transposase inhibitor; ochre suppressor; fusion proteins; recombinant DNA)

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SUMMARY

We constructed a derivative of Tn5, Tn5ORFlac, that is capable of creating lacZ translational fusions upon transposition. Lac- strains carrying this construct formed red papillae when plated on MacConkey-lactose media. Lac+ cells isolated from independent papillae expressed distinct β-galactosidase fusion proteins, suggesting that the Lac+ phenotype resulted from transposition. In support of this, analysis of plasmids carrying Tn5ORFlac prepared from these cells indicated that the Lac+ phenotypes arose as a result of intermolecular rearrangements. Furthermore, a derivative of Tn5ORFlac that contains an ochre mutation in the transposase gene formed papillae only in a supB strain. Tn5ORFlac is useful for obtaining mutants that affect Tn5 transposition and for creating lacZ fusions. We used the papillation phenotype to isolate a spontaneous revertant of IS50L that promotes transposition at a 3.6-fold higher rate than IS50R. The mutation altered the amino acid sequence of both transposase and inhibitor.

INTRODUCTION

Tn5 is a transposable element that functions in numerous Gram-negative bacteria (Berg and Berg, 1983). It consists of two inverted repeats, IS50L and IS50R, that flank a region encoding several antibiotic resistance determinants (Auerswald et al., 1981; Beck et al., 1982; Mazodier et al., 1985). Each repeat expresses two proteins from the same ORF, but from independent transcripts (Rothstein et al., 1980; bp, base pair(s); CFU, colony-forming unit(s); EtBr, ethidium bromide; Gm, gentamycin; Ig, immunoglobulin; Km, kanamycin; Nal, nalidixic acid; NPT II, neomycin phosphotransferase II; ORF, open reading frame; PolIk, Klenow fragment of E. coli DNA polymerase I; R, resistance, resistant; SDs, sodium dodecyl sulfate; Sp, spectinomycin; Str, streptomycin; Tc, tetracycline; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactose; [ ] designates plasmid-carrier state.

Abbreviations: Ap, ampicillin; AMV, avian myeloblastosis virus;
Krebs and Reznikoff, 1986). Only the proteins expressed from IS50R are functional in transposition and its regulation. The larger protein, transposase, is required for transposition (Rothstein et al., 1980; Johnson and Reznikoff, 1984). The smaller protein has the same amino acid sequence but lacks 55 N-terminal amino acid residues and acts as an inhibitor of transposition (Johnson et al., 1982; Isberg et al., 1982; Johnson and Reznikoff, 1984).

The transposition rate of Tn5 is low, about $10^{-5}$ events per cell per generation as measured by a mating assay. The isolation of mutations that increase the transposition rate would contribute to the understanding of regulatory factors and would facilitate the development of an in vitro transposition assay. Few mutants that increase the Tn5 transposition rate have been isolated because a convenient genetic assay for transposition is not available, although several exist for other transposons. Host mutants that affect Tn10 transposition were identified by an assay that relies on the ability of Tn10 to delete flanking regions of the chromosome (Roberts et al., 1985). Colonies of cells bearing a Tn10 insertion neighboring the gal operon of a galE - strain were replica-plated on galactose media to determine the frequency of GalR revertants. Host mutants that affect transposition can also be obtained by a screen that relies on the ability of endogenous transposable elements (IS1 or IS5) to activate expression of the bgl operon, which allows metabolism of $\beta$-glucosides (Clements and Syvanen, 1981). Bgl + cells grow at faster rates than Bgl - cells on MacConkey plates containing salicin and form red papillae within Bgl- colonies. As this screen monitors the transposition of endogenous elements, mutant candidates must then be tested for their effect on other transposons. Mutants within Tn5 can be obtained by a mating assay that directly measures the transposition frequency, but this method requires efficient mutagenesis, and only mutants with decreased transposition rates have been obtained (Johnson and Reznikoff, 1984).

The transposition screen described here involves the formation of red papillae within otherwise Lac- colonies on MacConkey-lactose plates by a derivative of Tn5 that creates lacZ translational fusions upon transposition. This construct is analogous to bacteriophage Mu derivatives that have been used to obtain fusions of E. coli genes to lacZ and may have some applications in this regard. We describe the features of the screen and its use in isolating an IS50 mutant exhibiting a modest increase in transposition rate. A similar screen was developed independently for Tn10 (Huisman and Kleckner, 1987).

**MATERIALS AND METHODS**

(a) **Bacterial and phage strains**

*Escherichia coli* strain RZ211 (Δlac-pro ara recA56 thi srl- StrR) (Johnson et al., 1982) was used for cloning and papillation studies. RZ211 [FΔ15::Tn10], carrying a transposition of Tn10 onto an F deletion derivative (Yin et al., 1987), was used as a donor in mating assays. RZ224 (Δlac-pro ara polA thi SpR StrR NalR A') (R. Johnson) was used as the recipient. RZ708 (Δlac-pro ara thi StrR) and RZ710, an isogenic supB derivative obtained by P1 transduction (K. Kendrick), were also used. JM101 (Δlac pro supE thi [F' traD36 lacIqZAM15 proAB +]) was used as a host for bacteriophage M13mp19 (Messing, 1983).

(b) **Media**

Strains were grown in rich media at 37°C in L broth or on agar plates prepared as described (Miller, 1972) and supplemented with antibiotics when appropriate. Papillation assays were performed on thick MacConkey-lactose agar plates (approx. 40 ml/8-cm plate). Antibiotic concentrations were, per ml: Ap, 100 µg; Gm, 10 µg; Km, 40 µg; Tc, 15 µg. XGal was used at 40 µg/ml.

(c) **Enzymes**

Restriction enzymes were obtained from New England Biolabs. Calf intestinal alkaline phosphatase and PolIk were obtained from Boehringer-Mannheim. AMV reverse transcriptase was from Life Sciences and the bacteriophage T7 DNA polymerase sequencing kit was from United States Biochemicals. High-Mₖ standards and goat anti-rabbit IgG horseradish peroxidase conjugate were obtained from BioRad. Rabbit anti-$\beta$-galactosidase sera were obtained previously (Johnson et al., 1982).
Bacteriophage T4 DNA ligase was a gift of Dr. M. Cox (University of Wisconsin).

(d) Plasmid constructions

DNA manipulations were performed essentially as described (Maniatis et al., 1982). To construct pRZ620, a BamHI digest of pRZ465 (Johnson and Reznikoff, 1983) was treated with PolIk in the presence of the four dNTPs. After digesting with SalI, this plasmid was ligated with a SmaI + SalI digest of pMC1403 (Casadaban et al., 1980). Transformants that formed papillae (Pap+) on MacConkey-lactose Tc plates were analyzed by restriction digestion, and one was retained as pRZ620. The outer end-lacZ junction was sequenced with reverse transcriptase as described by Inoue and Cech (1985), except that plasmid DNA prepared as described (Chen and Seeburg, 1985) was used as a template. A transposition-defective version of pRZ620, pRZ621, was constructed by replacing the HindIII-SalI fragment from pRZ309 that includes IS50L and the KmR determinant from Tn5. pRZ622 is a spontaneous Pap+ revertant of pRZ621. The location of the pRZ622 mutation was mapped as follows. pRZ992 (Yin et al., 1987), which contains IS50R lacking terminal repeats and a complementable transposon marked with a GmR determinant, was digested with BglII in the presence of EtdBr (Parker et al., 1977). After treatment with HindIII and calf intestinal alkaline phosphatase, the correct fragment was combined with the small HindIII-BglII fragment from either pRZ621 or pRZ622. The fragments were obtained from low-gelling-temperature agarose and ligated within the gel. ApR transformants of RZ211[FΔ15::Tn10] were screened by a mating assay for their transposition phenotype relative to pRZ992. Candidates that showed very low transposition from the ligation with pRZ621 were retained as pRZ623, and those that showed high transposition from the ligation with pRZ622 were retained as pRZ624. The HindIII-BglII fragment used in these constructs was also cloned into M13mp9 for sequencing with T7 DNA polymerase according to the manufacturer’s instructions.

(e) Immunoblotting and transposition assays

Immunoblotting of β-galactosidase fusion proteins was performed as described (Krebs and Reznikoff, 1986). Transposition was quantitated by a mating assay essentially as described (Yin et al., 1987). A qualitative measure of relative transposition rates could be obtained by monitoring the accumulation of papillae over several days. Overnight cultures of cells carrying various constructs were plated on MacConkey-lactose media to give 10 to 30 colonies per plate. Colonies were examined under a dissecting light microscope for the appearance of distinct red papillae. The number of papillae on each colony was recorded at various times until individual papillae could no longer be distinguished. Data from three or more plates were combined to calculate the average number of papillae per colony.

RESULTS AND DISCUSSION

(a) Construction of TnSORFluc

The strategy used to construct Tn5SORFluc is similar to that used in the construction of other transposable fusion vectors, such as MudII301 (Casadaban and Chou, 1984) and TnphoA (Manoil and Beckwith, 1985). Essentially, a region from the left half of Tn5 is replaced by a version of the IucZ gene lacking transcriptional and translational initiation signals. The outer end of ISSOR is fused to IucZ so that an uninterrupted reading frame results and the functional integrity of the end is preserved. The transposon that is created should yield translational fusions to the lacZ gene when transposition occurs in the proper orientation relative to an ORF. To construct Tn5SORFluc, a fragment from the lac operon beginning at the eighth codon of lacZ and ending in lacA was obtained from pMC1403 (Casadaban et al., 1980). This was inserted into pRZ465, a deletion derivative of Tn5 that retains only 25 bp of the left end (Johnson and Reznikoff, 1983), to yield pRZ620 as shown in Fig. 1. The sequence of the junction between the left end of Tn5 and lacZ in pRZ620 demonstrates that an uninterrupted reading frame results from this fusion (Fig. 1). However, we found two unexpected sequence dif-
Fig. 1. Structure of TnS0RFlac. Thick lines and solid boxes show regions from Tn5. The lacZYA" and TcR (tet') open boxes represent DNA derived from pMC1403 (Casadaban et al., 1980) and Tn10, respectively. pRZ620 contains this construct on ColEl. The sequence of the junction between the outer end of TnS and lacZ is shown with amino acids that would be encoded in a β-galactosidase fusion protein. Bent arrows refer to sequence derived from the outer end of IS50 or from the lacZ gene. Outlined nucleotides indicate unexpected sequence changes, which include the presence of an extra C and the loss of a G.

ferences, the gain of a C in the region from pRZ465 and the loss of a G in the region from pMC1403. These changes do not perturb the overall reading frame and do not introduce any in-frame initiation or termination codons.

(b) Papillation of pRZ620

On MacConkey-lactose indicator medium, Lac+ cells that arise within a Lac− colony grow at an accelerated rate, forming projections on the colony surface (papillae) that become red as lactose fermentation continues. Colonies of RZ102 carrying pRZ620 form new papillae continuously over a period of several days, as shown in Fig. 2. The distribution of papillae among colonies at a given point in time is approximated by the Poisson distribution. The two distributions were typically well-correlated; for example, a statistical analysis of papillae on 67 colonies after 57 h gave \( \chi^2 = 2.25 \), df (degrees of freedom) = 3, \( 0.5 < P < 0.9 \). This fit is consistent with the expectation that papillae form as a result of transposition, although other mutational processes that occur independently at a fixed rate could yield the same result (Luria and Delbrück, 1943).

The formation of translational fusions by TnS0RFlac was tested by monitoring cells grown from papillae for the presence of fusion proteins to β-galactosidase (Fig. 3). Fusion proteins to β-galactosidase were detected in all cases, although the size and amount of the proteins varied widely. Most of the isolates yielded fusion proteins that were only slightly larger than β-galactosidase. Several isolates contained larger fusion proteins. In some cases these were accompanied by a smaller protein that was close to β-galactosidase in size, probably the product of proteolytic cleavage at the fusion junction. Efficient cleavage in the majority of cases could explain the prevalence of isolates that produce a fusion protein the size of β-galactosidase. Alternatively, the pool of Lac+ colonies may be biased if there exists a preferred transposition target or if papillae formation favors events that place lacZ close to the N-terminal coding region of a target gene.

To address whether or not papillae form as a result
Fig. 2. Papillation time course. Colonies of RZ211[pRZ620] were photographed at (a) 25 h, (b) 50 h, (c) 75 h, and (d) 100 h after plating on MacConkey-lactose media at 37°C. Papillae appear as dark spots on the surface of the colonies in these photographs.

of transposition, we prepared plasmid DNA from 54 Lac + colonies isolated from independent papillae and transformed RZ211. In 44 cases, all of the transformed colonies were Lac -, indicating that rearrangement of the plasmid was not responsible for the Lac + phenotype. In the remaining ten cases, all transformants were Lac +. Restriction analysis indicated that nine of these were larger by an amount equal to the size of Tn5ORFlac and one was even larger (data not shown). These results suggest that papillae form as a result of intermolecular transposition events, either onto the chromosome or onto a plasmid.

The effect of a mutation in the transposase gene of Tn5ORFlac on papillation was also determined. Colonies of a nonsuppressing strain (RZ708) bearing pRZ621, which carries an ochre mutation in the transposase gene, form papillae at a much lower rate than colonies of the same strain bearing pRZ620 (Fig. 4). In a supB strain (RZ710) papillation in
Fig. 3. Immunoblot of β-galactosidase fusion proteins expressed in papillae. Cultures were grown from 15 independent papillae (lanes a to o) that formed on RZ211[pRZ620] to an A₅₅₀ of 0.5 to 0.8 in L broth. Equivalent A₅₅₀ units of each sample (approx. 0.75 A₅₅₀ units) were pelleted and resuspended in 75 µl of sample buffer (Laemmli, 1970). After heating at 100°C for 5 min, the samples were electrophoresed in 0.1% SDS-5% polyacrylamide, transferred to nitrocellulose and probed with rabbit anti-β-galactosidase IgG. Goat anti-rabbit IgG horseradish peroxidase conjugate was used for detection. Arrowheads show faint bands observed in some lanes. The Mᵣ (in kDa) and location of protein standards are given on the right; the 116-kDa standard corresponds to β-galactosidase.

Fig. 4. Papillation time course of TnSORFlac and an ISSOL derivative in supB and supB strains. The average number of papillae per colony was determined at various times after plating. Black circles, RZ708[pRZ620]; open circles, RZ710[pRZ620]; black squares, RZ710[pRZ621]; open squares, RZ710-[pRZ621]. Strain RZ708 is su⁺, RZ710 is supB.

(c) Isolation of a transposition mutant

We demonstrated the use of papillation in isolating transposition mutants by obtaining a transposition-proficient pseudorevertant of pRZ621. Colonies of cells bearing pRZ621 exhibit papillae only after extensive incubation (1–2 weeks) on MacConkey lactose medium. Some of these papillae may result from mutagenic events that allow transposition, such as reversion of the ochre codon. Lac⁺ colonies were isolated from 32 independent papillae that formed in cells bearing pRZ621. Plasmid DNA prepared from them was used to transform RZ211 and the papillation phenotype was examined; in each case, the transformant phenotype was homogeneous. Of the 29 candidates which were Lac⁺, one formed papillae at high frequency and was retained as pRZ622. Papillation occurred more frequently in pRZ622 than in pRZ620, suggesting that the transposition rate of this mutant is higher than wild type (Fig. 5).

The region responsible for the Pap⁺ phenotype of pRZ622 was mapped by replacing a HindIII-BglII fragment of pRZ621 that includes the ochre codon with the same fragment from the revertant. This subcloning yielded Pap⁺ colonies, indicating the fragment was sufficient to confer the phenotype. The only sequence difference between pRZ621 and pRZ622 on this fragment is a T → C transition at bp 1443 of IS50L, which changes the ochre codon to a glutamine codon (Table I). The sequence of IS50R in this region is shown for comparison; a glutamate codon is present at this position (Table I). Since the ochre codon and the -10 region of the NPT II promoter overlap (Rothstein and Reznikoff, 1981), the mutation might be expected to affect KmR. Both RZ211[pRZ621] and RZ211[pRZ622] grew well on plates containing 40 µg Km/ml, but only the former grew well on plates containing 80 µg Km/ml. This suggests that the mutation in pRZ622 results in decreased transcription of NPT II.
Fig. 5. Papillation phenotype of Tn5orfFlac derivatives. Colonies of RZ211 carrying pRZ620, pRZ621, pRZ622, or pBR322 were photographed 75 h after plating on MacConkey-lactose agar as in Fig. 2. The key indicates colony genotype as determined from antibiotic resistance, which is different for all of the plasmids used except pRZ621 and pRZ622 (colonies carrying these were identified on the basis of their papillation phenotype). Open shapes, pBR322; light gray shapes (hatched), pRZ621; dark gray shapes (densely striped), pRZ620; solid black shapes, pRZ622.

### TABLE I

Transposition activity encoded by IS50 derivatives

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>IS50</th>
<th>Sequence</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRZ992</td>
<td></td>
<td>GAA GGT</td>
<td>1</td>
</tr>
<tr>
<td>pRZ623</td>
<td>IS50L</td>
<td>TAA GGT</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>pRZ624</td>
<td>IS50L-r</td>
<td>CAA GGT</td>
<td>3.6</td>
</tr>
</tbody>
</table>

| a | The plasmids are described in MATERIALS AND METHODS, section d. |
| b | The version of IS50 that provides transposase or a derivative thereof is described. IS50L-r was obtained from pRZ622, a spontaneous Pap⁺ pseudorevertant of IS50L in pRZ621. |
| c | The nucleotide sequence starting at position 1443 of IS50 and the amino acids encoded are given for the three derivatives. In IS50L, the sequence shown corresponds to the −10 hexamer of the NPT II promoter. |
| d | Transposition frequencies were determined by a mating assay. Independent transformants of RZ211[F::Tn10] were grown overnight in the presence of Gm and diluted 1:100 with a 1:100 dilution of RZ224 in 1 ml of L broth. After 6 h mating with slow shaking, the cultures were vortexed, diluted, and plated on L agar plates containing Sp (200 μg/ml) and Tc (15 μg/ml) or containing Sp (200 μg/ml), Nal (50 μg/ml), Tc (5 μg/ml) and Gm (5 μg/ml). The transposition frequency is given by \(f = \frac{\text{number of Sp}^R \text{Nal}^R \text{Tc}^R \text{Gm}^R \text{CFU}}{\text{number of Sp}^R \text{Tc}^R \text{CFU}}\). The transposition rate (a) is related to the frequency by \(a = f / (\ln N_1 - \ln N_0)\), where \(N\) is the number of cells at a time, \(t\). The relative transposition rate is given by \(f\) (mutant) / \(f\) (wild-type) assuming that mutant and wild-type cultures grow at the same rate. Values were averaged from six cultures, and the relative error was ~40%. The transposition frequency measured for pRZ992 was \(1.2 \times 10^{-3}\) Gm⁺ exconjugants / total exconjugants. |

(d) Transposition of the IS50L pseudorevertant

To measure the transposition frequency of this mutant and to compare it with IS50L and IS50R, the HindIII-BglII fragment from pRZ621 and pRZ622 was used to replace the same fragment in pRZ992 to yield pRZ623 and pRZ624, respectively. In these
vectors, IS50-encoded proteins complement a defective Tn5 derivative located on the same plasmid. As a result, the effect of mutations in IS50 may be attributed to changes in the activity of the transposase or inhibitor, and not to alteration of a site involved in the transposition reaction. The relative transposition rates in cells carrying pRZ992, pRZ623 and pRZ624 are given in Table I. pRZ623 exhibits the expected low rate of transposition relative to pRZ992, but pRZ624 shows a 3.6-fold increase in the transposition rate. These measurements correlate with the papillation phenotypes of pRZ620, pRZ621, and pRZ622 (Fig. 5). The increase in transposition rate could arise as a result of an increase in the stability or specific activity of transposase, or a decrease in the stability or specific activity of the inhibitor. We favor an effect on specific activity, as the substitution of gln for glu in the mutant is unlikely to have a major effect on protein conformation and thereby proteolytic degradation.

The increase in transposition rate resulting from the glu → gln substitution changes the interpretation of previous results concerning the control of Tn5 transposition. In a study of the effect of changes in the expression of IS50-encoded proteins (Johnson and Reznikoff, 1984), ochre suppressor strains of varying efficiency were used to achieve different levels of IS50L expression. The transposition frequency of IS50L in these strains was close to that of IS50R, suggesting that coordinate decreases in the level of IS50 proteins do not affect the transposition rate. This conclusion was contingent upon the demonstration that the activity or stability of the proteins was not altered. Our results indicate that in at least one suppressor strain, supB, the protein activity is altered. Ochre codons are misread in a supB background as gln, so the transposase and inhibitor proteins would contain the same substitution expected in proteins encoded by pRZ622 and pRZ624. This substitution clearly affects the overall transposition activity of IS50 proteins, and argues against the previous conclusion. The effect of supB on IS50L transposition can be explained if a coordinate decrease in expression of IS50 proteins does decrease the transposition rate. Then the effect of inefficient protein expression on transposition would be compensated by an increase in overall protein activity. These compensating effects, low expression (18.5% IS50R, due to inefficient suppression) and increased activity (360% IS50R), yield an expected transposition frequency relative to IS50R of 67%, which agrees well with the observed value of 70% (Johnson and Reznikoff, 1984). It is likely that the effect of other suppressor strains is similar, and that coordinate decreases in IS50 protein levels decrease the transposition rate.

(e) Conclusions

The primary objective in constructing Tn5 ORF/lac was to develop a convenient genetic screen for transposition. The results indicate that papillae formation in colonies of Lac - cells bearing Tn5 ORF/lac is a reliable predictor of the transposition rate. The qualitative comparison of papillation rates correlates well with relative transposition rates measured by a mating assay. The lactose-papillation screen is likely to be useful in obtaining mutants in both the host and the transposon, and has some advantages over existing assays. It does not require mating or preparation of phage from mutant candidates to assay transposition. The lactose-papillation screen monitors transposition of the element directly, unlike the salcin-papillation screen (Clements and Syvanen, 1981). Mutant candidates can be screened in a single plating, unlike the screen devised for obtaining host mutants that affect Tn10 transposition (Roberts et al., 1985). Finally, because Tn5 ORF/lac is plasmid-borne, mapping and sequencing of mutations in the transposon is facilitated. A similar approach has been adopted successfully for Tn10 (Huisman and Kleckner, 1987).

A secondary objective in constructing Tn5 ORF/lac was to develop a Tn5 derivative capable of creating translational fusions to the lacZ gene. The use of gene fusions in molecular biology is extensive (Bassford et al., 1978), and a number of excellent vectors have been constructed for this purpose. Tn5 is attractive because it transposes in a broad range of Gram-negative hosts whose genetic characterization has only recently been initiated. Use of Tn5 derivatives that create translational and transcriptional fusions (Kroos and Kaiser, 1984) should facilitate the study of these organisms. Tn5 ORF/lac yields stable fusion proteins in some cases and could be used both to monitor translational control of expression and to isolate β-galactosidase fusion proteins.
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