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Simple Assay for Quantitation of Tn*5* Inversion Events in *Escherichia coli* and Use of the Assay in Determination of Plasmid Copy Number

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In this report, we describe a simple method for measuring the frequency of sequence inversion in the transposable element Tn*5* as a result of recombination across its duplicated IS*50* elements. The structure of Tn*5* was manipulated so that the neomycin phosphotransferase gene of the transposon would be expressed only if a sequence inversion event occurred. This highly sensitive assay also served as the basis for a novel means of estimating plasmid copy number.

The bacterial transposon Tn*5* consists of a 2.8-kilobase (kb) central unique region which contains a neomycin-kanamycin phosphotransferase gene flanked by two inverted 1.5-kb IS*50* insertion elements. Many aspects of the molecular biology of this transposable element are well understood, and Tn*5* has been widely used for insertional mutagenesis in *Escherichia coli* and a number of other procaryotic species (for reviews, see references 3 and 7).

The original restriction site mapping of Tn*5* indicated that inversion of the central unique region of Tn*5* by recombination across its duplicated IS*50* elements does not readily occur in *E. coli* (11). The fixed orientation of the transposon in procaryotes is in marked contrast to the behavior of Tn*5* in several eucaryotic systems, such as the 2μm circle of yeast cells (10) and the herpes simplex virus type 1 genome (P. C. Weber, M. D. Challberg, N. J. Nelson, M. Levine, and J. C. Glorioso, Cell, in press), in which high levels of sequence inversion have been reported. However, the apparent inversion of Tn*5* sequences has been detected by using two specialized systems. In the first, inversion of a Tn*5* deletion derivative integrated in the *E. coli* chromosome resulted in the activation of an adjacent *lacZ* gene (2, 4), while in the second, inversion of a Chi^+ derivative of Tn*5* in the bacteriophage λ genome led to an altered plaque morphology (18). These observations indicate that Tn*5* does undergo sequence inversion in its bacterial host, albeit at a low frequency.

In this report, we describe a simple recombination assay to study the phenomenon of Tn*5* inversion in *E. coli*. This method employed a Tn*5* derivative which conferred normal levels of neomycin resistance on its host only if it underwent sequence inversion in vivo. The manipulations performed to generate this transposon are depicted in Fig. 1.

In the first step of the construction, the 5.4-kb *Hpa*I fragment of Tn*5* was inserted into the *Sma*I site of pCW522 to yield pTn*5*$\Delta1_{sv}$ (Fig. 1). This removed only 185 base pairs from either end of the transposon but also deleted the three transposase gene promoters as well as a cruciform DNA structure which terminates transcription originating outside of Tn*5* (12). The loss of these elements had no apparent effect on the frequency of Tn*5* inversion as determined by the relative intensities of inversion products detected by Southern blot analysis of plasmid DNA (data not shown). The neomycin phosphotransferase (Nm^r) gene of the transposon in pTn*5*$\Delta1_{sv}$ can be expressed by either the *lacZ* promoter of pUC18 (which operates constitutively in the *E. coli* DH1 or C600 strains used in this work) or by its natural promoter in IS*50*$_L$.

In the second step, the central 2.8-kb *Bgl*II fragment of pTn*5*$\Delta1_{sv}$ was inverted to yield pTn*5*$\Delta1_{sv}$(INV A) (Fig. 1). This caused the Nm^r gene to be under the control of the Nm^r promoter of IS*50*$_R$, which is considerably weaker than its IS*50*$_L$ counterpart due to a single-base nonhomology between IS*50* elements (13). As a result, the host cell resistance to neomycin conferred by pTn*5*$\Delta1_{sv}$(INV A) was significantly reduced (MIC, <25 μg/ml, compared with >150 μg/ml for pTn*5*$\Delta1_{sv}$; Rothstein and Reznikoff [13] have obtained a MIC of 10 μg/ml for an analogous construction in their studies).

If a sequence inversion event occurred in pTn*5*$\Delta1_{sv}$(INV A), then the Nm^r gene should be expressed by the *lacZ* promoter of pUC18, resulting in higher levels of neomycin resistance. To verify this prediction, an in vitro construction which mimicked an in vivo inversion event, pTn*5*$\Delta1_{sv}$(INV B), was generated by inverting the central *Hin*dIII fragment of pTn*5*$\Delta1_{sv}$(INV A) (Fig. 1). As expected, the host cell resistance to neomycin conferred by pTn*5*$\Delta1_{sv}$(INV B) was significantly elevated (MIC, 50 μg/ml, compared with <25 μg/ml before the inversion event).

Thus, the inversion of Tn*5* sequences in pTn*5*$\Delta1_{sv}$(INV A) results in activation of the Nm^r gene, so that selection for host cells carrying Tn*5* inversion events can be made on media containing neomycin. This formed the basis for a simple and highly sensitive recombination assay by which Tn*5* inversion events could be readily detected and quantitated. To illustrate its use, duplicate dilutions of an overnight culture of the recombination-proficient strain C600 (8) carrying pTn*5*$\Delta1_{sv}$(INV A) were plated on media containing either 100 μg of ampicillin or 35 μg of neomycin per ml. The frequency of neomycin-resistant colonies, and therefore the frequency of Tn*5* inversion events in C600, was calculated to be 3.4×10^{-2}.

Plasmid DNA was isolated from several neomycin-resistant colonies and analyzed by restriction endonuclease digestion to verify the occurrence of Tn*5* inversion events. As diagrammed in Fig. 1, the original and inverted orientations

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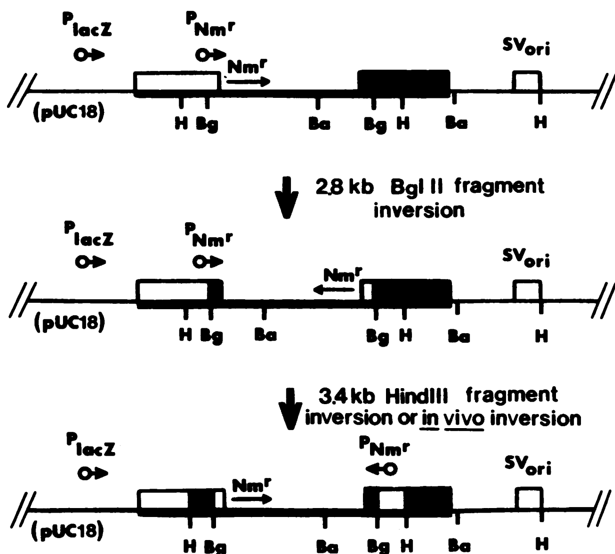

FIG. 1. Construction of plasmids pTn5Δ1_{sv} (top), pTn5Δ1_{sv}(INV A) (middle), and pTn5Δ1_{sv}(INV B) (bottom) used in the Tn5 inversion assay. All cloning steps, which are described in detail in the text, were done by using pCW522, a pUC18 derivative containing a simian virus 40 origin of replication (Weber et al., in press), so that this system could also be used to examine Tn5 inversion in a simian virus 40 minichromosome. The results of the simian virus 40 studies will be published elsewhere (Weber et al., in press). The resistance to neomycin conferred by each plasmid on a C600 or DH1 host was determined. MICs were as follows: pTn5Δ1_{sv}, >150 μg/ml; pTn5Δ1_{sv}(INV A), <25 μg/ml; and pTn5Δ1_{sv}(INV B), 50 μg/ml. Sizes of the *Bam*HI fragments were 6.0 and 2.5 kb in pTn5Δ1_{sv}, 5.4 and 2.9 kb in pTn5Δ1_{sv}(INV A), and 6.0 and 2.5 kb in pTn5Δ1_{sv}(INV B). Tn5 sequences are indicated by a heavy line in the diagrams, and the IS50_L and IS50_R repeats are shown as white and black boxes, respectively. Nm^r, Neomycin phosphotransferase gene; P_{lacZ}, *lacZ* promoter of pUC18; P_{Nm^r}, neomycin phosphotransferase gene promoter of IS50_L; SV_{ori}, simian virus 40 origin of DNA replication; Ba, *Bam*HI site; Bg, *Bgl*II site; H, *Hin*dIII site.

of Tn5 in pTn5Δ1_{sv}(INV A) should each yield two distinct *Bam*HI restriction fragments; these are shown in Fig. 2A, lanes 1 and 2. Plasmid DNA isolated from neomycin-resistant colonies typically consisted of an equal mixture of the native and inverted forms of pTn5Δ1_{sv}(INV A) (Fig. 2A, lane 3). This heterogeneity in the plasmid population remained after several rounds of colony purification on media containing neomycin and was also observed in neomycin-resistant colonies obtained after transformation of pTn5Δ1_{sv}(INV A) DNA (discussed below).

Interestingly, if these plasmids were electrophoresed undigested (Fig. 2B), the DNA isolated from neomycin-resistant colonies was found to exist exclusively as a dimer (Fig. 2B, lane 3), while the original and inverted orientations of pTn5Δ1_{sv}(INV A) existed predominantly as monomers (Fig. 2B, lanes 1 and 2). This indicates that the plasmids in the neomycin-resistant colonies are actually heterodimers which contain one Tn5 in the original orientation and the other in the inverted orientation. This finding also demonstrates that inversion of one of the two Tn5 constructions in the heterodimer is sufficient for conferring resistance to neomycin. We are currently investigating whether this dimerization is a precursor or a by-product of Tn5 inversion and whether it is required for the expression of the neomycin phosphotransferase gene of these constructions.

These results indicate that a single Tn5 inversion event in

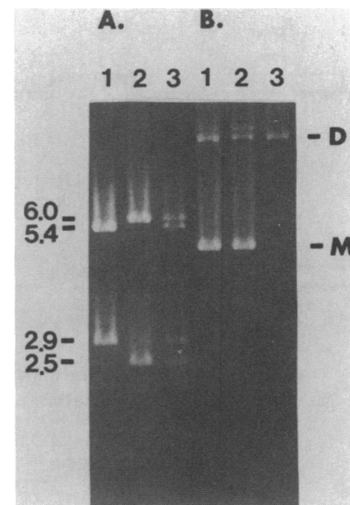

FIG. 2. Agarose gel electrophoresis of plasmid DNAs used in the Tn5 inversion assay. Plasmids were extracted from *E. coli* C600 by the procedure of Birnboim and Doly (5) and were either digested with *Bam*HI (A) or left undigested (B). Lanes 1, pTn5Δ1_{sv}(INV A); lanes 2, pTn5Δ1_{sv}(INV B); lanes 3, pTn5Δ1_{sv}(INV A) isolated from a neomycin-resistant colony generated in the Tn5 inversion assay. The sizes (in kilobases) of the *Bam*HI fragments in panel A are indicated at left, and the monomeric (M) and dimeric (D) forms of the plasmids in panel B are indicated at right.

the plasmid population of a host cell can give rise to the neomycin-resistant phenotype. However, the number of copies of the plasmid carrying the inversion event is subsequently amplified within the plasmid pool during neomycin selection. As a consequence, the frequency of neomycin-resistant colonies arising in C600 carrying pTn5Δ1_{sv}(INV A), 3.4×10^{-2}, is actually the number of host cells which carry a Tn5 inversion event rather than the true frequency of Tn5 inversion in the plasmid population. This difference, as well as the calculation of the latter index, will be discussed in greater detail below.

To control for false-positive neomycin-resistant colonies which may arise after overnight incubation, a noninverting analog of pTn5Δ1_{sv}(INV A) was constructed (Fig. 3). The 4.4-kb *Pst*I partial-digest fragment of Tn5 was cloned into the *Pst*I site of pCW522 to yield pTn5Δ3_{sv}. This plasmid was identical to pTn5Δ1_{sv} except that an additional 500 base pairs were deleted from either end of the transposon. The central 2.8-kb *Bgl*II fragment of pTn5Δ3_{sv} was then inverted to yield pTn5Δ3_{sv}(INV A) (Fig. 3). Like pTn5Δ1_{sv}(INV A), the Nm^r gene of this construction was under the control of the relatively weak Nm^r promoter of IS50_R, so that pTn5Δ3_{sv} (INV A) also conferred reduced levels of resistance to neomycin upon its host (MIC, <25 μg/ml).

The IS50_L element of pTn5Δ3_{sv}(INV A) was then removed by deletion of the 2.0-kb *Bam*HI fragment of the plasmid, yielding pTn5Δ3_{sv}(DEL A) (Fig. 3). This loss of one of the IS50 repeats prevented sequence inversion from occurring in this plasmid. The frequency of neomycin-resistant colonies observed in strains carrying pTn5Δ3_{sv}(DEL A) was never any greater than 3×10^{-4}. This background level of spontaneously arising neomycin resistance was insignificant in most determinations of Tn5 inversion frequency and was therefore ignored in calculations unless indicated otherwise.

The Tn5 inversion assay described above was used to search for hot spots of homologous recombination within the IS50 repeats of the transposon. Six sequential deletions in

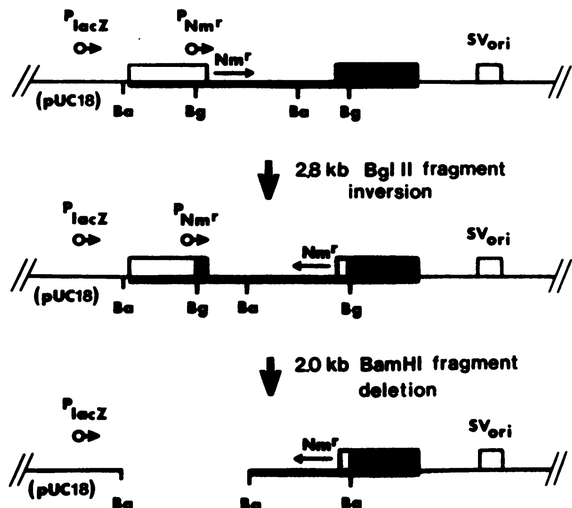

FIG. 3. Construction of pTn5Δ3$_{sv}$(DEL A), a plasmid used for quantitation of the background level of neomycin resistance in the Tn5 inversion assay. All cloning steps are discussed in detail in the text, and the abbreviations used are identical to those in the legend to Fig. 1. The MICs of neomycin for strains carrying pTn5Δ3$_{sv}$ (top), pTn5Δ3$_{sv}$(INV A) (middle), and pTn5Δ3$_{sv}$(DEL A) (bottom) were >150, <25, and <25 μg/ml, respectively.

the IS50 elements (pTn5Δ1 through pTn5Δ6; Fig. 4A) were constructed by cloning six restriction fragments of Tn5 generated by various restriction endonucleases (*Hpa*I, *Xho*I, *Pst*I, *Fsp*I, *Hin*dIII, and *Pvu*II, respectively) into pUC19 (Weber et al., in press). The 2.8-kb *Bgl*II fragment of each plasmid was inverted to yield pTn5Δ1(INV A) through pTn5Δ6(INV A), which were then used in neomycin selection assays to quantitate the frequency of Tn5 inversion for each IS50 deletion.

The six sequential deletions of 200 to 300 base pairs from the ends of the transposon resulted in a linear reduction in Tn5 inversion frequency (from 3.4×10^{-2} to 1.8×10^{-3}; Fig. 4B). This clearly indicated that the frequency of recombination events in Tn5 was dependent upon the extent of homology between IS50 repeats rather than specific sequences within these elements. These observations also suggested that Tn5 inversion was the result of *recA*-mediated homologous recombination.

The role of the *recA* function in Tn5 inversion was confirmed by examining the frequency of recombination events occurring in a *recA*-deficient host strain, DH1 (8). The frequency of neomycin-resistant colonies for DH1 carrying pTn5Δ1$_{sv}$(INV A) was calculated to be 1.3×10^{-4}, which was equivalent to the background level of neomycin resistance observed with pTn5Δ3$_{sv}$(DEL A), 3×10^{-4}. Comparable results were obtained by using a *recA* C600 mutant (16). Thus, the inversion of Tn5 sequences in *E. coli* appears to be mediated primarily by *recA*-dependent homologous recombination across the IS50 elements of the transposon. The observed absence of recombinational hot spots was in agreement with the studies of Hirschel and Berg (9),

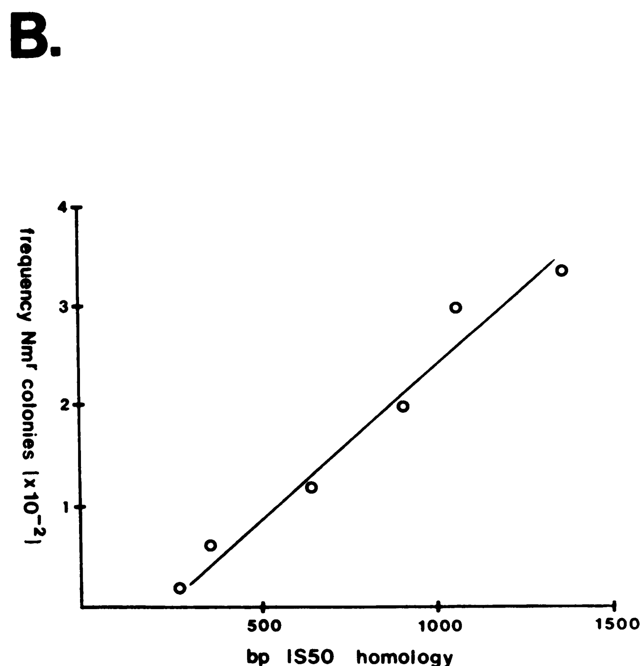

FIG. 4. (A) Construction of deletion derivatives of Tn5. The six complete and partial digest restriction fragments of Tn5 illustrated were cloned into pUC19 for use in neomycin selection assays (see text for details). The sizes of the deletions in the 1,534-base-pair IS50 elements are indicated below each plasmid name. (B) Relationship between inversion frequency and the extent of IS50 homology in the Tn5 deletion derivatives. The frequency of neomycin-resistant colonies resulting from inversion events in each of the six Tn5 deletions in panel A is shown as a function of the length (in base pairs) of undeleted IS50 sequences.

who suggested that the IS*50* elements of Tn*5* lacked the site-specific resolvase function found in other transposons (1, 14).

As described above, plating C600 cells containing pTn$5\Delta1_{sv}$ (INV A) onto selective media and then calculating the frequency of neomycin-resistant colonies yielded an accurate assessment of the number of host cells harboring a Tn*5* inversion event. However, this value (3.4×10^{-2}) was not the same as the actual number of Tn*5* inversion events within the plasmid population. This latter frequency was determined by transforming the *recA*-deficient host strain DH1 with pTn$5\Delta1_{sv}$(INV A) DNA isolated from the recombination-proficient strain C600. The frequency of neomycin-resistant transformants, and therefore the true frequency of Tn*5* inversion in *E. coli*, was calculated to be 1.6×10^{-3} (after correction for the background of spontaneous neomycin resistance, 3×10^{-4}). This value was in close agreement with the estimated frequency of Tn*5* inversion within the bacteriophage λ genome, 2×10^{-3} to 5×10^{-3} (18).

Thus, the frequency of neomycin-resistant colonies obtained by direct plating of cells was considerably higher than that observed after transformation of plasmid DNA. As discussed above, a host cell which gives rise to a neomycin-resistant colony contains a single Tn*5* inversion event within its plasmid pool. The noninverted plasmids within the pool are therefore counted as inversion events in a direct-plating assay, but not in a transformation assay, where they result in neomycin-sensitive colonies. By extension, the frequency of neomycin-resistant colonies calculated from the transformation assay is reduced by a factor which should correspond exactly to the copy number of the plasmid pool in the host cell. To illustrate, the copy number of pTn$5\Delta1_{sv}$(INV A) was equal to the frequency of host cells carrying Tn*5* inversion events (3.4×10^{-2}) divided by the actual frequency of Tn*5* inversion events within the plasmid population (1.6×10^{-3}), or 21 plasmids per cell.

This novel procedure for estimating plasmid copy number can be easily adapted for use on any plasmid. The 5.4-kb *Hpa*I fragment of Tn*5*, in which the 2.8-kb *Bgl*II fragment is inverted, can be inserted downstream of any active plasmid promoter for use in the measurement of inversion frequency. The ubiquitous nature of Tn*5* (3, 7) suggests that this construction would be functional in a wide variety of procaryotic species. This procedure for copy number determination is much simpler than dye-buoyant density centrifugation methods (17) and is much more accurate than approaches which utilize the measurement of antibiotic resistance levels (6, 15). In addition, the neomycin selection assay is sensitive enough to detect even slight changes in plasmid copy number, which would make this system invaluable in studies involving characterization of replication control mutants.

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