

## Organization of Complex Transposon Tn2610 Carrying Two Copies of *tnpA* and *tnpR*

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**Transposon Tn2610 has two elements of 3.5 kilobase pairs as inverted repeats, one set at each end. This unique terminal element contained the transposition genes *tnpA* and *tnpR*. Only the *tnpA* gene in the left element was functional for transposition, whereas both *tnpR* genes were active. Possible evolutionary relationships among class II transposable elements are proposed on the basis of the genetic and structural organization of Tn2610.**

Tn2610 was discovered as a transposable element involved in spread of the carbenicillin-hydrolyzing PSE-1  $\beta$ -lactamase-encoding gene (14, 23). Tn2610 is 24 kilobase pairs (kbp) long and is flanked by long inverted repeat (IR) sequences. The intervening 17.0-kbp nonrepeated region includes, in addition, genes for resistance to streptomycin and sulfonamide.

Transposable elements have been found to have repetitive sequences arranged in inverted or direct orientation at the ends (for reviews, see references 7, 11, and 12). Most prokaryotic transposable elements can be conveniently grouped into two classes on the basis of mechanistic properties and DNA homology. Class I elements, comprising Tn5, Tn9, and Tn10 (10, 11), are flanked by structurally intact insertion sequences (IS) which encode information required for transposition. Thus, the essential functions for transposition are encoded by the IS element. Class II elements, e.g., Tn3, Tn501, Tn21, and Tn2603 (9, 11), are flanked by a pair of short IRs at both ends which lack genes for transposition, although the intact IR sequences are required for normal transposition. The genes essential for transposition are encoded in the central, nonrepeated region.

Since Tn2610 appeared to be distinguishable from known transposable elements because of unique IR elements at both ends (23), I examined the functions of both IR elements.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains used were *Escherichia coli* K-12 strains AB2463 (*recA thr leu thi lac gal ara xyl mtl pro his arg str tsx sup*); P3478 Rif<sup>r</sup>, a rifampin-resistant mutant of P3478 (*thy polA*); and C600 RecA<sup>-</sup> Nal<sup>r</sup>, a RecA<sup>-</sup> and nalidixic acid-resistant mutant of C600 (*thr leu thi lac tonA supE*) which was kindly provided by H. Danbara. Plasmid pTKY141 is a pBR322 (3) derivative carrying Tn2610. Plasmid R388 (5), which encodes resistance to trimethoprim and sulfonamide, was used in the mating assay described below.

**Growth of culture.** Routine growth of bacteria was performed in L broth (13) and on L agar containing appropriate antibiotics for selection at 37°C. For selection with trimethoprim, Mueller-Hinton agar (Difco Laboratories) was used with 0.5% (vol/vol) lysed horse blood.

**Antibiotics.** For selection of resistant colonies, antibiotics

were used at the following concentrations: ampicillin, 10  $\mu$ g/ml; nalidixic acid, 25  $\mu$ g/ml; rifampin, 25  $\mu$ g/ml; streptomycin, 25  $\mu$ g/ml; spectinomycin, 25  $\mu$ g/ml; sulfonamide, 100  $\mu$ g/ml; tetracycline, 12.5  $\mu$ g/ml; trimethoprim, 50  $\mu$ g/ml.

**Preparation of plasmid DNA.** Plasmid DNA was isolated by alkaline extraction by the method by Birnboim and Doly (2).

**Restriction endonuclease digestion of DNA.** The restriction endonucleases used were purchased from Takara Shuzo Co., Ltd, Kyoto, Japan. DNA was digested in buffer containing 10 mM Tris hydrochloride (pH 7.5), 10 mM MgCl<sub>2</sub>, 50 mM NaCl, and 6 mM 2-mercaptoethanol at 37°C for 2 h for complete digestion, heated at 65°C for 10 min to stop the reaction, and chilled. For partial digestion of plasmid DNA, the reaction was performed for 5 or 10 min.

**Analytical gel electrophoresis.** Gel electrophoresis was performed on a 1% agarose gel with a running buffer containing 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA (pH 8.3).

**Heteroduplex analysis by electron microscopy.** The heteroduplex molecule was prepared by a method previously described (23).

**Determination of transposition frequency.** A mating assay was used to determine transposition frequency. The donor strain was AB2463, harboring pTKY141 or a derivative and R388. Strain P3478 Rif<sup>r</sup> was used as the recipient. Overnight cultures of each strain were diluted 100-fold with fresh broth and incubated to an optical density of 0.5 at 610 nm. Portions of donor cells (0.2 ml) and recipient cells (2 ml) were mixed and filtered with a membrane filter (Millipore type HA; 0.45- $\mu$ m pore size). The filter was placed on an agar plate prewarmed at 37°C and incubated for 6 h at 37°C. The filter was transferred to a tube containing 2.0 ml of physiological saline, and the cells were suspended. After appropriate dilution, the cells were plated on selective agar plates. The transposition frequency was expressed as the number of Ap<sup>r</sup> colonies among the transconjugants that acquired R388. The transposition frequency of Tn21 as a control in this assay system was expressed as the number of Sm<sup>r</sup> colonies among the transconjugants carrying R388.

### RESULTS

**Determination of genes for transposition of Tn2610.** In the course of a structural analysis of IR elements of Tn2610, I found by heteroduplex analysis that the right IR (IR-R) region was highly homologous to the region encoding genes

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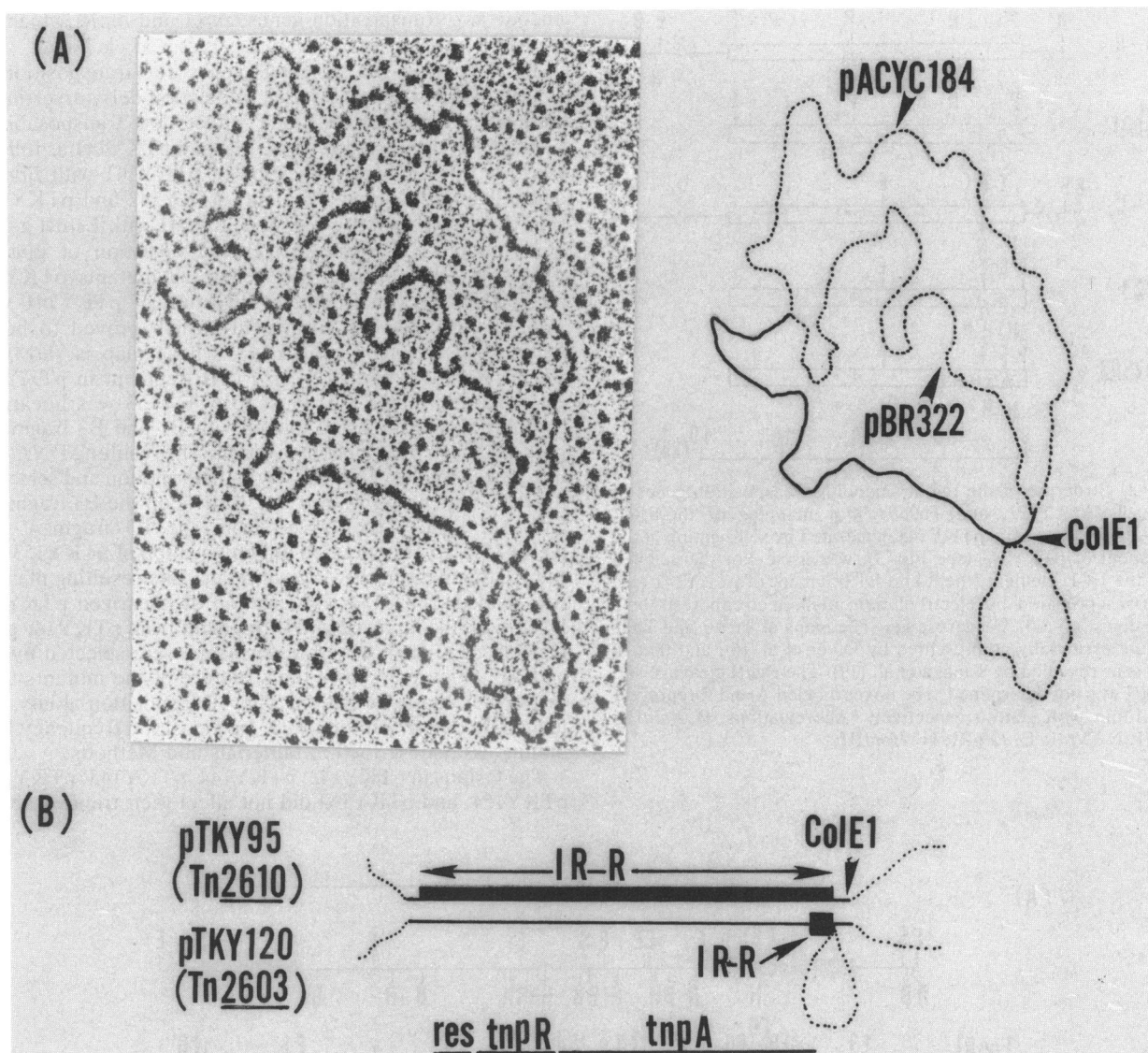


FIG. 1. Heteroduplex analysis of IR-R of Tn2610 and Tn2603. (A) A heteroduplex molecule was formed with pTKY95 and pTKY70 (previously termed pTY70; 19) DNAs. Plasmid pTKY95 consists of plasmid pBR322 with a P1 fragment from pTKY140 (previously termed pTY140; 23). In this fragment, Tn2610 is within the ColE1 structural gene (23). Plasmid pTKY70 is a pACYC184 derivative with the fragment encoding the *tnpA*, *tnpR*, and *res* genes of Tn2603 (19). In this fragment, these genes are 1.8 kbp apart from the ColE1 gene (21). Each part of the ColE1 gene in both fragments formed the short, double-stranded structure shown in the electron micrograph. In the tracing to the right, the solid and dotted lines represent double- and single-stranded DNAs, respectively. (B) Schematic representation of the homologous region in the heteroduplex molecule. The *tnpA*, *tnpR*, and *res* genes of Tn2603 were determined previously (16, 19).

*tnpA*, *tnpR*, and *res* on transposon Tn2603. The IR-R element and Tn2603 share a highly homologous region spanning 3.5 kbp (Fig. 1).

Tn2603 is a multiple resistance transposon encoding resistance to ampicillin, streptomycin, sulfonamide, and mercury (21, 22). We have previously demonstrated that Tn2603 requires at least two genes, *tnpA* and *tnpR*, for transposition (16). The product of gene *tnpA* is involved in the formation of a cointegrate structure of donor and recipient replicons joined by directly repeated copies of the transposon. This cointegrate is then resolved by the *tnpR* gene product, which catalyzes *res* site-specific recombination, giving a final transposition product (19). We also reported that the *tnpA* and *tnpR* mutations of Tn2603 could be complemented by transposons Tn501 and Tn21, suggesting that they are closely related in their transposition functions (17-19). The hetero-

duplex analysis suggested that IR elements of Tn2610 encode transposition genes analogous to genes *tnpA*, *tnpR*, and *res* on these transposons. To confirm this possibility, the structures of both IR elements were analyzed in detail by restriction endonuclease digestion.

Figure 2 shows the maps of Tn501, Tn21, and Tn2603 and the IRs of Tn2610. They are aligned with respect to the regions encoding the genes necessary for transposition of these elements. The results indicate that the IR-R element of Tn2610 is structurally related to the transposition genes of Tn501. In contrast, the left IR (IR-L) element could be related to those of Tn21 and Tn2603. The demonstration by heteroduplex analysis of homology between the IR-R element and Tn2603 is not surprising, since Tn21, Tn501, and Tn2603 are closely related in the functions of their transposition genes (6, 8, 19). Therefore, Tn2610 is assumed to

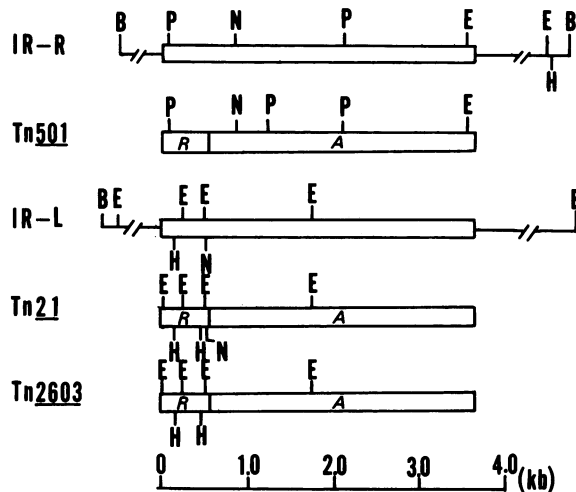


FIG. 2. Structure of the region encoding transposition genes of Tn2610, Tn501, Tn21, and Tn2603. For mapping of the IR-R element, 6.4-kbp plasmid pTKY143, generated by self-ligation of the B2 fragment of pTKY141 (see Fig. 3), was used. For the detailed map of the IR-L element, the 8-kbp B1 fragment of pTKY141 (see Fig. 3) was prepared by electroelution after electrophoresis of a *Bam*HI digest on a 0.7% agarose gel. The maps of Tn501 and Tn21 shown are essentially as presented by Diver et al. (6), and that of Tn2603 is as revealed by Tanaka et al. (19). The *Pvu*II cleavage site in Tn2603 was not determined. The boxes labeled A and R represent the *tnpA* and *tnpR* genes, respectively. Abbreviations: B, *Bam*HI; P, *Pvu*II; N, *Nru*I; E, *Eco*RI; H, *Hind*III.

encode two transposition genes, *tnpA* and *tnpR*, which are arranged in inverted orientation, one set at each end.

To determine the essential genes for transposition of Tn2610, I constructed a series of deletion derivatives in the elements and examined their effects on transposability. Deletions were introduced into plasmid pTKY141 as follows (see Fig. 3). (i) Partial digestion of pTKY141 with *Bam*HI and religation generated mutants pTKY142 and pTKY145. (ii) Partial digestion of plasmid pTKY141 with *Eco*RI generated mutant pTKY144. (iii) Partial digestion of plasmid pTKY145 DNA with *Eco*RI generated mutants pTKY131 and pTKY149. (iv) Complete digestion of pTKY141 with *Bam*HI yielded the B2 fragment, which proved to be an autonomous replicon, pTKY143, whose map is shown in Fig. 2. Removal of the 2-kbp *Pvu*II fragment in pTKY143 gave plasmid pTKY153. Plasmid pTKY153 was linearized by *Bam*HI digestion and joined with B1 and B3 fragments prepared from pTKY141 by ligation. The resulting DNA was introduced into C600 RecA<sup>-</sup> by transformation and selection for Ap<sup>r</sup>, Sm<sup>r</sup>, and Tc<sup>r</sup>. The Sm<sup>r</sup> gene is on the B3 fragment, and Ap<sup>r</sup> genes are derived from the B3 fragment and pTKY153. Tc<sup>r</sup> is expressed only by ligation of pTKY153 and the B1 fragment in the original order. The resulting plasmid was named pTKY154. (v) Ligation of linearized pTKY153 DNA with the largest *Bam*HI fragment from pTKY149 gave plasmid pTKY164. The transformants were selected by Tc<sup>r</sup> and Ap<sup>r</sup>. Figure 3 shows the structure of the mutants used for further characterization. Their transposition ability was judged by determination of the transposition frequency by a mating assay described in Materials and Methods.

The lesions in pTKY142, pTKY144, pTKY145, pTKY149, pTKY154, and pTKY164 did not affect their transposability

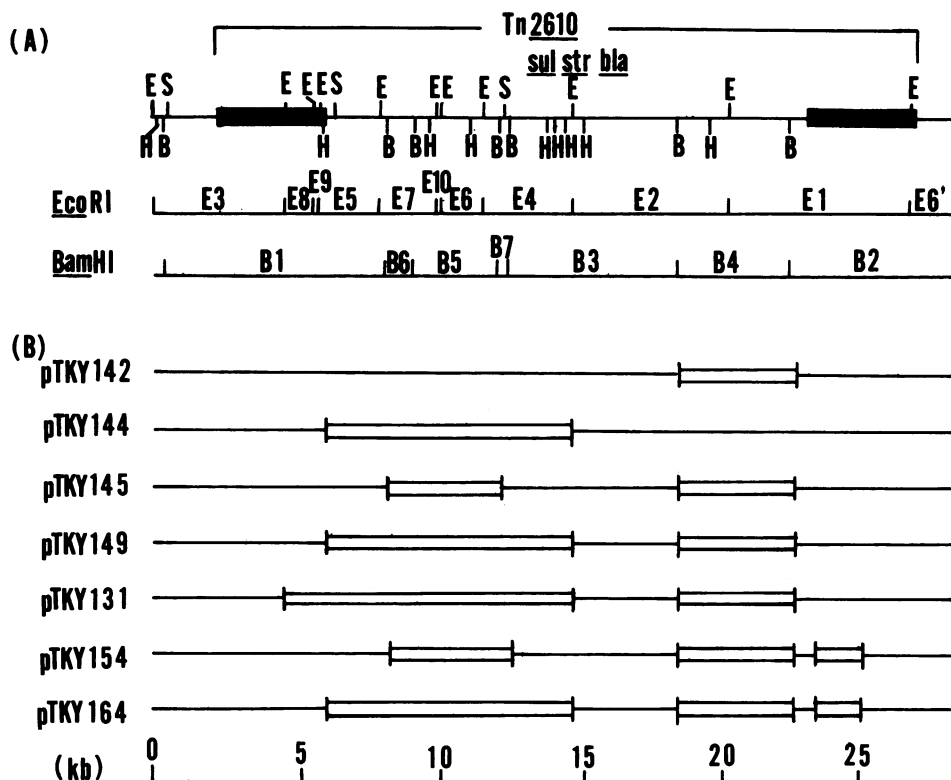


FIG. 3. Functional map of pTKY141 and structure of deletions in Tn2610. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hind*III; S, *Sall*; bla,  $\beta$ -lactamase gene; str, Sm<sup>r</sup> gene; sul, Su<sup>r</sup> gene. ■, IR-L and IR-R elements; □, deleted region in Tn2610.

TABLE 1. Transposition proficiency of deletion derivatives of Tn2610

Plasmid <sup>a</sup>	Transposition frequency (relative value) <sup>b</sup>	No. of Tc <sup>r</sup> colonies <sup>c</sup>
pTKY141	$6.79 \times 10^{-6}$ (1.00)	5
pTKY142	$1.18 \times 10^{-5}$ (1.73)	4
pTKY144	$1.40 \times 10^{-4}$ (20.6)	0
pTKY145	$1.13 \times 10^{-5}$ (1.16)	6
pTKY149	$1.14 \times 10^{-3}$ (212)	0
pTKY131	$<10^{-9}$	
pTKY154	$2.00 \times 10^{-5}$ (2.94)	3
pTKY164	$5.50 \times 10^{-4}$ (81.0)	100
pTKY166	$1.69 \times 10^{-5}$ (2.50)	

<sup>a</sup> Plasmid pTKY141 is a pBR322 derivative with an intact Tn2610. Plasmid pTKY166 is pBR322::Tn21 and was used as a control in the transposition assay. The other plasmids are deletion mutants derived from pTKY141 whose structures are shown in Fig. 3.

<sup>b</sup> Transposition frequency was determined as described in Materials and Methods.

<sup>c</sup> Strain C600 RecA<sup>-</sup> was used as a recipient for the conjugal mating assay of transposition. One hundred Ap<sup>r</sup>, Sm<sup>r</sup>, and Tp<sup>r</sup> transconjugants were tested for Tc<sup>r</sup>.

(Table 1). On the contrary, the transposition frequency increased in proportion to the increase in deletions. This agrees with the previous finding that the transposition frequency for transposons in the Tn2603 family depends systematically on the size of the element (19). Since deletion tends to flank the insert with promoters from the vector moiety, the increase in transposition frequency might be due not only to the decreased size but also to augmented transcription.

In contrast, pTKY131 could not transpose at all. The longer deletion in this mutant removed Tn2610 material from the central region to the left side EcoRI site. The region including this EcoRI site has already been assumed to encode the *tnpA* gene through mapping by restriction endonuclease digestion (Fig. 2). The deletion introduced into IR-R did not impair transposition. Therefore, the *tnpA* gene in IR-L is functionally involved in its transposition but that in IR-R must be defective. As mentioned previously, the first stage of transposition of a class II transposon is formation of a cointegrate structure of two replicons joined by two copies, which is mediated by transposase. The second stage is resolution of the cointegrate structure mediated by the resolvase. If a lesion occurs in the *tnpR* gene, the transposition reaction stops at the first stage and accumulates cointegrate structures. Such intermediates can be detected only in a RecA<sup>-</sup> host strain. To analyze the structure of transposition products, C600 RecA<sup>-</sup> was used as the recipient for the conjugal mating assay. The structure of the product can be monitored genetically by examining the linkage of markers; thus, the donor plasmid with Tn2610 confers Tc<sup>r</sup> (the marker on pBR322), Ap<sup>r</sup>, and Sm<sup>r</sup> (the marker on Tn2610), and R388 as an acceptor plasmid confers Tp<sup>r</sup>. Normal resolution of the intermediate in transposition results in loss of linkage between Tc<sup>r</sup> and other determinants on the transposon, Ap<sup>r</sup> and Sm<sup>r</sup>. Formation of a cointegrate intermediate results in linkage of all resistance determinants on the donor plasmid and Tp<sup>r</sup> on R388.

pTKY141 predominantly gave products resistant to ampicillin, streptomycin, and trimethoprim, suggesting authentic transposition. Structural analysis of plasmid DNAs prepared from 10 of these transconjugants by EcoRI digestion showed that Tn2610 transposed onto one of several sites in R388 (data not shown). Five percent of the transconjugants simultaneously acquired Tc<sup>r</sup>, suggesting cointegrate formation

TABLE 2. Integrity of pTKY99 in the presence of various derivatives of Tn2610

Complementing plasmid <sup>a</sup>	No. of Sp <sup>r</sup> colonies/no. of Ap <sup>r</sup> colonies
pTKY141 .....	100/100
pTKY143 .....	100/100
pTKY153 .....	0/100
pTKY155 .....	50/50
pTKY164 .....	0/50

<sup>a</sup> pTKY141, pBR322::Tn2610; pTKY143, self-ligation of the B2 fragment from pTKY141 (Fig. 3); pTKY153, deletion of 2-kbp PvuII fragment in pTKY143; pTKY155, pBR322 derivative with B1 fragment of pTKY141 (Fig. 3).

between pTKY141 and R388. Structural analysis of plasmid DNAs from five such transformants showing linkage of Tc<sup>r</sup>, Ap<sup>r</sup>, Sm<sup>r</sup>, and Tp<sup>r</sup> by EcoRI digestion indicated that the original structure of the transposon was not preserved in the cointegrate. Therefore, cointegrate formation occurred in a way other than authentic transposition of Tn2610. An analysis in greater detail will be published elsewhere.

Despite deletion of the E5 and E9 fragments, which probably cover the *tnpR* gene in IR-L, mutants pTKY144 and pTKY149 could generate products carrying Ap<sup>r</sup>, Sm<sup>r</sup>, and Tp<sup>r</sup>, suggesting normal transposition. Similarly, pTKY154, with deletion mutations in the *tnpA* and *tnpR* genes in IR-R could produce plasmids expressing Ap<sup>r</sup>, Sm<sup>r</sup>, and Tp<sup>r</sup> as a product of normal transposition. These results suggest that the *tnpR* genes on both ends of Tn2610 are functional. When mutant pTKY164, in which both *tnpR* genes are assumed to be impaired, was subjected to the mating assay, all of the transconjugants showed linkage of Tc<sup>r</sup>, Ap<sup>r</sup>, Sm<sup>r</sup>, and Tp<sup>r</sup>. Restriction cleavage analysis of plasmid DNAs prepared from 10 of the resulting transconjugants with EcoRI indicated that these structures contain two transposons in direct orientation at the junctions of two plasmids, suggesting that the cointegrate is an intermediate in transposition (data not shown).

The possibility that the *tnpR* genes in both IR elements encode a resolvase functional for transposition was further confirmed by using a previously established complementation system (19). Plasmid pTKY99 is a plasmid pACYC184 derivative carrying Ap<sup>r</sup> and Sp<sup>r</sup>. It has the two *res* sites of Tn2603 in direct orientation and can be stably maintained in the RecA<sup>-</sup> strain AB2463 in the absence of *tnpR*. If the *tnpR* gene encoded by Tn2610 is functionally analogous to that of Tn2603, it is expected to give an autonomous plasmid resistant only to ampicillin because of the recombination between two *res* sites promoted by the resolvase from Tn2610. The results are shown by loss of the linkage of Ap<sup>r</sup> and Sp<sup>r</sup> in Table 2. The results indicated that both *tnpR* genes in Tn2610 encode a functional resolvase and are also freely interchangeable with *tnpR* of Tn2603.

## DISCUSSION

In this study, complex transposon Tn2610 was found to be a class II transposon, like Tn3 and Tn21, despite its unique structure flanked by long IR sequences of 3.5 kbp at each end. One element, IR-R, was closely related to transposition genes *tnpA* and *tnpR* of Tn501 (4, 6), whereas IR-L was very close to those encoded by Tn21 (6, 20). Only the *tnpA* gene of IR-L was involved in transposition, since the *tnpA* gene in IR-R was defective. Alternatively, each *tnpA* gene might recognize the end of its own element to promote transposi-

tion, but the gene in IR-R would not be able to recognize the end of the IR-L element. This hypothesis is supported by the study of Grinsted et al. (8), in which Tn21 could complement a *tnpA* mutation of Tn501 in *trans* but Tn501 did not complement a *tnpA* mutation of Tn21 in *trans* at a detectable frequency. Alternatively, it has been reported that a single IR sequence of Tn21 or Tn1721 can fuse efficiently with other replicons (one-ended transposition) in the presence of the cognate transposase (1, 15). The IR-L element of Tn2610 could also cause one-ended transposition in the presence of the *tnpA* gene (data not shown).

Diver et al. reported that the DNA sequences of *tnpR* genes of Tn21 and Tn501 are similar and that the two genes are interchangeable for resolvase activity (6). Actually, both *tnpR* genes of Tn2610 were involved in the resolution of intermediates giving the normal products, even though only a *tnpA* gene in IR-L is involved in its transposition. Moreover, both *tnpR* genes were functional for the *res* site of Tn2603.

On the genesis of Tn2610, it is speculated that the particular sequences encoding Ap<sup>r</sup>, Sm<sup>r</sup>, and Su<sup>r</sup> could be flanked by two copies of the element consisting of the *tnpA* and *tnpR* genes in inverted orientation, thus generating a new transposable element. Therefore, its structure would be similar to that of composite transposons like Tn5, Tn9, and Tn10 (11). This hypothesis leads to the possibility that both IR elements are flanked by short IRs, like a 38-base-pair IR of Tn3 or Tn21, so that each element is independently transposable. This possibility was checked by examining inverse transposition, that is, transposition of the Tc<sup>r</sup> determinant on the pBR322 moiety of pTKY141, but not successfully, suggesting that both inside ends were not intact. In evolution, minor sequential alterations would give the present element. The present results support the notion that class II transposons have evolved by a mechanism analogous to that involved in the generation of a composite class I transposon and then have been compactly organized like Tn3 (8, 9).

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