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Resolution of cointegrates between transposons γδ and Tn*3* defines the recombination site

(internal resolution site/repressor function/site-specific recombination/λ integration)

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ABSTRACT **Transposition of the genetically related insertion elements γδ and Tn3 is thought to involve two steps. In the case of transposition from one replicon to another, the first step is fusion of the parent and target replicons with the element appearing in direct orientation at the two junctions. In a subsequent reaction, the cointegrate structure is resolved via a site-specific recombination event. I have constructed two plasmids, each carrying segments of γδ and Tn3, that contain the internal resolution site. The *tnpR* gene product encoded by either Tn3 or γδ mediates intramolecular recombination between these two sites. The product of this recombination is a hybrid region that contains γδ and Tn3 sequences fused at the point of crossover. DNA sequence analysis of such recombinants indicates that the recombination occurs within a 19-base-pair (bp) region of exact homology between γδ and Tn3. The site lies in the 160-bp center intercistronic region, 50 bp before the beginning of the *tnpA* gene. My results therefore suggest a model for the coupled regulation of the repressor (*tnpR*) and the transposase (*tnpA*) genes and site-specific recombination of transposition intermediates. The Tn3/γδ recombination system and bacteriophage λ integration are compared.**

Transposable elements are specific segments of DNA that can move from one chromosomal site to another. Transposition and other element-associated events are independent of *recA* mediated homologous recombination (1).

The ampicillin (Ap) resistance transposon, Tn*3*, and γδ, a 5.8-kilobase (kb) element originally identified on the F factor of *Escherichia coli* (2), are closely related in genetic organization and nucleotide sequence (3). Both encode two functions intimately involved in the transposition process: *tnpA* protein, a transposase, and the *tnpR* gene product, a repressor of both its own expression and that of the *tnpA* gene (4, 5). Experimental evidence suggests that both γδ and Tn*3* transpose from one replicon to another in a two-step process (Fig. 1A) (6). In the first step, element-mediated fusion of the two replicons produces a cointegrate structure containing two directly repeated copies of the transposon, one at each junction between the replicons (4). This fusion, which requires the *tnpA* protein (7), is necessarily a replicative process; cointegrates contain an additional copy of the element (3) and a duplication of a 5-base-pair (bp) target sequence. The second step in transposition is the resolution of the cointegrate by site-specific recombination between two copies of the duplicated transposon; the result is a copy of the element at its original locus and a new copy at the target site (4, 8). Recent studies have suggested that cointegrate resolution is not *tnpA* dependent but instead requires the *tnpR* gene product (8, 9). This site-specific, *recA*-independent event appears to be reciprocal and conservative; unlike the *tnpA*-mediated replicon fusion, DNA replication would not be formally required. In this respect, resolution is analogous to recombination between the bacteriophage λ genome and its bacterial attachment sites (10).

Resolution of transposition-generated cointegrate intermediates is normally rapid, and therefore these structures are not easily detected (4). Such cointegrates are stable if functional *tnpR* gene product is not present in the cell or if the site of recombination, the internal resolution site (IRS), is deleted (3, 4, 8). The IRS of Tn*3* has been localized to a central region of about 500 bp within which the *tnpA* and *tnpR* genes are divergently transcribed (3).

Early DNA sequence studies demonstrated that the termini of γδ and Tn*3* were highly homologous (11). My more recent work (unpublished data) has shown that the γδ *tnpR* gene has a DNA sequence predicting a product with 80% amino acid sequence homology to the analogous Tn*3* protein. Moreover, these proteins are interchangeable for known functions. First, functional Tn*3* *tnpR* protein can act as a repressor to decrease the frequency with which $tnpR^-$ γδ mutants transpose. Second, the site-specific recombination activity deficient in a $tnpR^-$ Tn*3* element can be complemented by $tnpR^+$ γδ (P. Kitts, personal communication).

In order to determine the site of *tnpR*-mediated recombination, I used the fact that the DNA sequences of the γδ and Tn*3* IRS regions are highly homologous but not identical. I constructed plasmids containing the IRS regions from Tn*3* and γδ in direct orientation. DNA sequence analysis of the hybrid structures formed upon *tnpR* protein-mediated resolution of these cointegrate constructs provided information on the precise location and nature of the crossover site.

METHODS

Bacterial Strains and Plasmids. The strains RR1012 ($\gamma\delta^+$) and RR1031 ($\gamma\delta^-$) (12) are acridine orange-cured *recA1* derivatives of W1485E (CGSC no. 4276) and W1485 (CGSC no. 5024), respectively (13). NG135 is a *gal*, F^-, recA56, Sm^R, $\gamma\delta^-$ strain obtained from Nigel Grindley (Yale University).

The plasmid pBR322 was purified from strain RR1 (14). RSF1010 is a ColE1-compatible plasmid encoding resistance to sulfonamide and streptomycin (Sm). RSF1010::Tn*1* (15) and ΔAp (=RSF103) (15), an Ap-sensitive mutant of RSF1010::Tn*1* (15) shown to be $tnpR^-$, were obtained from N. Grindley. The Ap^R transposons Tn*1* and Tn*3* appear to encode nearly identical proteins (4).

Mutations in the *tnpR* and *tnpA* genes of γδ were generated by *Eco*RI linker insertions into *Hae* III sites [a modification of the procedure used by Heffron *et al.* (16)] and identified by genetic analysis. The F deletion pOX38 consists of the largest *Hin*dIII fragment of F religated and transformed into a *recA* $\gamma\delta^+$

Abbreviations: Tc, tetracycline; Sm, streptomycin; Ap, ampicillin; bp, base pair; kb, kilobase; IRS, internal resolution site.

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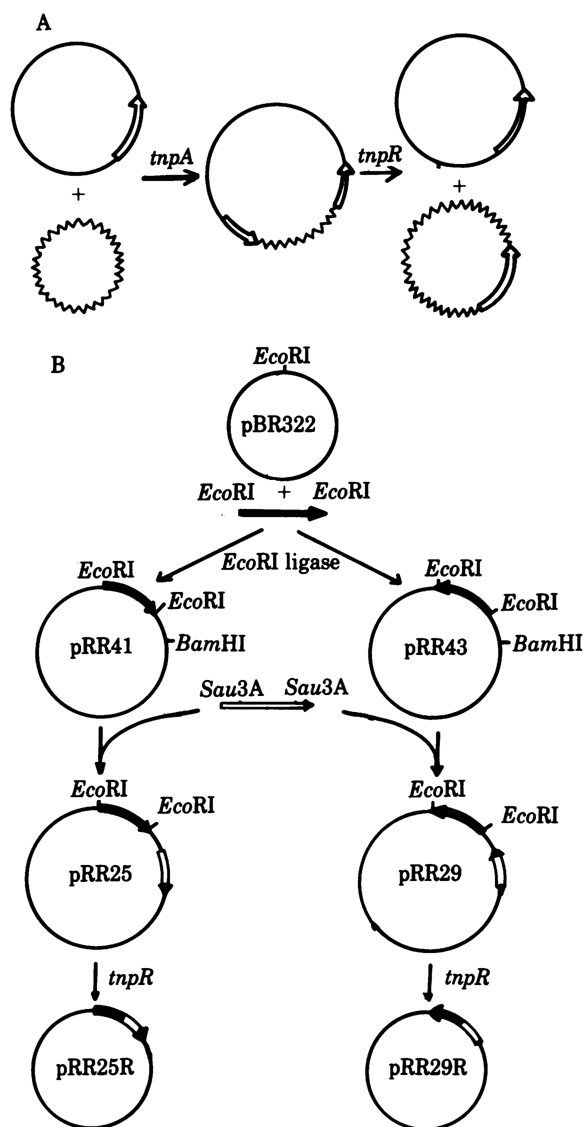

FIG. 1. (A) Diagram of the two-step mechanism proposed for transposition of $\gamma\delta$/Tn3 type elements. The intermediate structure is the cointegrate described in the text. Open arrow, transposable element. (B) Construction and structure of plasmids containing internal resolution sites as described in the text. Arrows, internal resolution site-containing $\gamma\delta$ (solid) or Tn3 (open) fragments; head of each arrow, *tnpR*-coding end of the fragment.

strain. The resulting strain, obtained from Mark Guyer (Genex), is called MG1345 (12). This conjugative plasmid (FΔ) no longer contains any identified insertion elements. The pOX38 derivatives were generated by placing pBR322 carrying *tnpR*$^-$, *tnpA*$^-$, or wild-type $\gamma\delta$ into MG1345 and selecting for transfer of tetracycline (Tc) resistance in a mating using a *recA*$^+$ recipient. These F derivatives were retransferred into NG135. Gal$^-$ colonies that were Tc sensitive and sensitive to male-specific phage yielded pOX38 derivatives carrying $\gamma\delta$ or its mutants.

Cloning of the IRS Fragments. The $\gamma\delta$ IRS site was cloned by using an 890-bp *Eco*RI/*Sma* I (2) fragment (see Fig. 4A) purified by gel electrophoresis from a $\gamma\delta$ mutant (pRR12) that had been created by insertion of an *Eco*RI linker into the *Hae* III site in the *tnpR* gene. An *Eco*RI linker was ligated onto this fragment at the *Sma* I end and subsequently cleaved with *Eco*RI, and the fragment was purified (16). This *Eco*RI/*Eco*RI (*Sma* I) fragment was ligated into the *Eco*RI site of pBR322 and used for transformation of RR1031 to Ap^R (17). Plasmids corresponding to the two orientations of the inserted fragment were designated pRR41 and pRR43 (Fig. 1B).

The 355-bp *Sau*3A fragment (3) containing the Tn3 IRS site was purified from the plasmid RSF1050 (15) by acrylamide gel electrophoresis and then ligated into *Bam*HI-digested pRR41 and pRR43 (Fig. 1B). After subsequent transformation of the $\gamma\delta^-$ strain RR1031, Tc-sensitive colonies were screened by restriction analysis for the presence of plasmids corresponding to the two directly repeated orientations of the $\gamma\delta$ and Tn3 IRS sites, which were designated pRR25 and pRR29.

Restriction Analysis and DNA Sequence Determination. Plasmids were purified after chloramphenicol amplification as described (11). *Eco*RI, *Hin*dIII, *Sal* I, *Bam*HI, *Pvu* II, and *Sma* I were obtained from New England BioLabs, *Sau*3A was from Bethesda Research Laboratories (Rockville, MD), and *Cla* I was from Boehringer Mannheim; each was used according to manufacturers' recommendations. T4 DNA ligase was purchased from P-L Biochemicals. *Hinf* was kindly supplied by Margaret Rosa (Yale University). Gel electrophoresis was performed in 1% agarose or 5% acrylamide, and restriction fragments were isolated as described (11). DNA sequence analysis was performed according to Maxam and Gilbert (18).

RESULTS

Resolution of Hybrid Plasmids of $\gamma\delta$ and Tn3. In order to determine the sequence at which site-specific recombination occurred, plasmids containing IRS regions from Tn3 and $\gamma\delta$ were resolved by $\gamma\delta$ *tnpR* protein to generate hybrid recombinants. Two plasmids carrying the directly repeated IRSs from $\gamma\delta$ and Tn3 in two different orientations were constructed (pRR25 and pRR29). These plasmids were introduced into an *E. coli* strain carrying a single chromosomal copy of $\gamma\delta$ (RR1012) (12), and the resolved plasmids (pRR25R and pRR29R) were purified. Note that, of the two products expected from resolution, only one contains the origin of replication of pBR322 and is therefore recovered. Restriction analysis of pRR25R and pRR29R indicated that they were smaller by approximately 950 and 700 bp, respectively, than their parent plasmids (Fig. 1B).

The $\gamma\delta$ *tnpR* gene product was demonstrated to be essential for resolution of the cointegrate plasmids as follows. Copies of $\gamma\delta$ or of *tnpA*$^-$ or *tnpR*$^-$ $\gamma\delta$ mutants were transposed onto an F factor in which the region containing the resident $\gamma\delta$ had been deleted. Each of these conjugative plasmids (FΔ, FΔ::$\gamma\delta$, FΔ::$\gamma\delta$ *tnpA*$^-$, and FΔ::$\gamma\delta$ *tnpR*$^-$) was introduced into a $\gamma\delta^-$ cell which was subsequently transformed with pRR25. Approximately 25 generations later, the fate of pRR25 was examined by restriction analysis. Fig. 2 shows that only those strains containing a functional $\gamma\delta$ repressor (FΔ::$\gamma\delta$ and FΔ::$\gamma\delta$ *tnpA*$^-$) gave the products expected from resolution of the *in vitro* constructed plasmid—i.e., loss of the *Eco*RI restriction site in the region between the two inserts (see Fig. 1B) and hence the disappearance of fragment C. In contrast, strains containing FΔ and FΔ::$\gamma\delta$ *tnpR*$^-$ retained the *Eco*RI restriction site and yielded fragment C. Moreover, use of an enzyme that cleaves pRR25 only once demonstrated that the samples analyzed in lanes 1 and 3 contained no detectable (<10%) resolved plasmid (not shown).

Finally, the Tn3/1 *tnpR* protein was shown to be competent for resolution of the hybrid construct in a similar experiment. Introduction of the compatible plasmids RSF1010, RSF1010::Tn1, or ΔAp RSF1010::Tn1 *tnpR*$^-$ (15) into cells containing pRR25 resulted in resolution of the cointegrate plasmids only when a functional Tn1 repressor was present (Fig. 2, lanes 5 and 6).

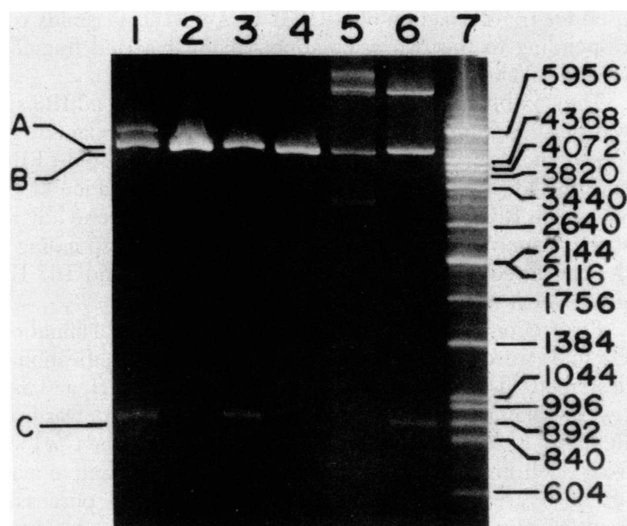

FIG. 2. Gel electrophoresis of *Eco*RI-digested plasmids after introduction of pRR25 into various *tnpR*$^+$ or deficient backgrounds. The strain in each case is NG135 containing: lane 1, FΔ; lane 2, FΔ::γδ; lane 3, FΔ::γδ*tnpR*$^-$; lane 4, FΔ::γδ*tnpA*$^-$; lane 5, RSF1010::Tn*1*; lane 6, RSF1010::Tn*1 tnpR*$^-$. Absence of band C, the *Eco*RI fragment containing the γδ IRS, is diagnostic of resolution. Band A, the longer *Eco*RI fragment, is only slightly longer than the linear resolved plasmid (band B). Markers are phage T7 LG3/H3 DNA cleaved with *Hpa* I (sizes from M. Rosa, personal communication).

Sequences of the γδ Intercistronic Region and Fused Recombinants. The DNA sequence of the γδ intercistronic region was determined as described in the legends to Figs. 3 and 4. Comparison with the Tn3 sequence (3) reveals 77% homology in the 160-bp region between the initiation codons for *tnpA* and *tnpR* gene products (Fig. 4*B*).

The DNA sequence of resolved plasmids recovered from strain RR1012 was determined in the region that restriction analysis indicated contained the crossover site. Two independent examples of pRR25R plasmid DNA were obtained from separate transformations of RR1012 with pRR25. Each of these isolates was subjected to sequence analysis from the *Sal* I site (Fig. 4*A*). Additionally, the sequence of this region in the plasmid pRR29R, derived from pRR29 in which the two IRS regions were cloned in the opposite orientation, was determined from the *Pvu* II site.

The site at which the DNA sequence switches from γδ to Tn3 in all three cases was within a 19-bp region of exact homology between the two elements (Figs. 3 and 4*B*). Within this site are 16 contiguous A·T base pairs and the longest stretch of homology between the γδ and Tn3 intercistronic regions (Fig. 4*B*).

DISCUSSION

The experiments described here indicate that, during resolution of γδ/Tn3 cointegrates, the site-specific crossover occurs within a 19-bp sequence conserved between the two related transposable elements. In fact, this sequence represents the longest stretch of perfect homology in the entire intercistronic region (160 bp) between the *tnpA* and *tnpR* genes. Therefore, one could argue that the hybrid plasmid was forced to resolve at this site whereas in "normal" resolution (e.g., between two copies of γδ) another recombination locus is used. However, three points suggest that the recombination event I have analyzed reflects the natural situation. First, the repressor gene

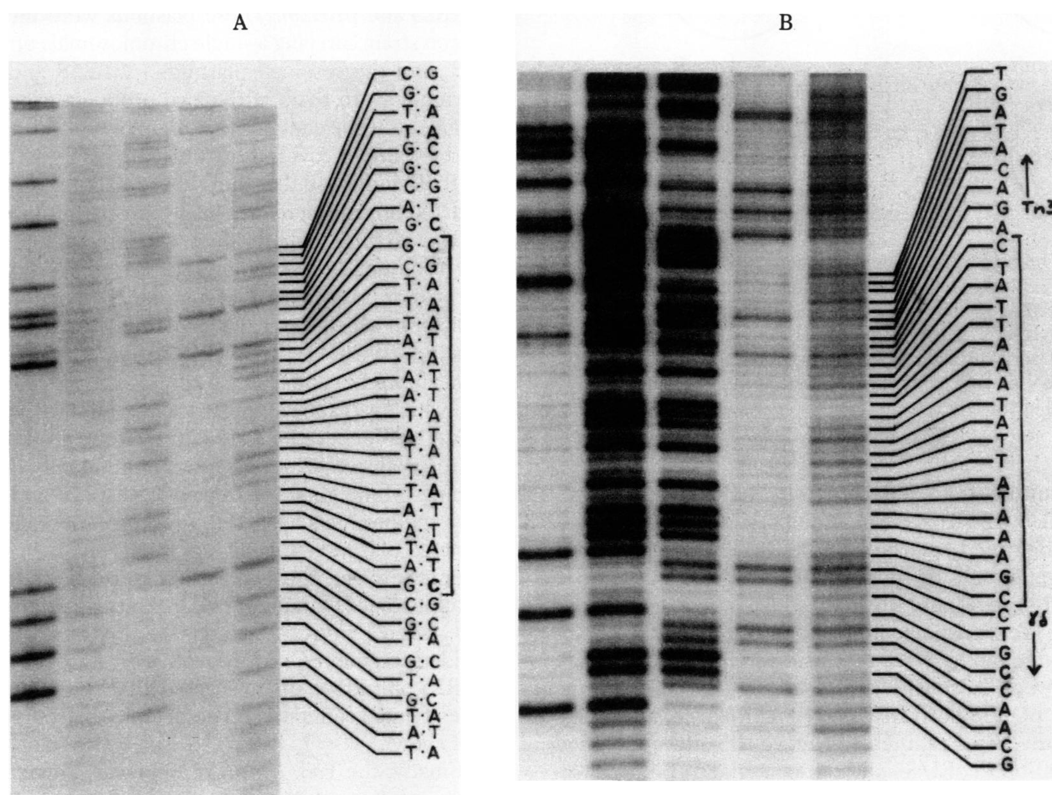

FIG. 3. (*A*) DNA sequence analysis of a portion of the γδ intercistronic region. The lanes from left to right are: G, G+A, A>C, C, C+T. The fragment was labeled at the *Cla* I site and extended toward the *Sal* I site (Fig. 4*A*). The bracketed region denotes the crossover site with Tn*3*. (*B*) DNA sequence analysis of the pRR25R plasmid in the crossover region. The reactions in each lane were as in *A*. The fragment was labeled at the *Sal* I site and contained γδ sequences (near the bottom of the gel), the crossover region (indicated by a bracket), and Tn3 sequences (near the top of the gel).

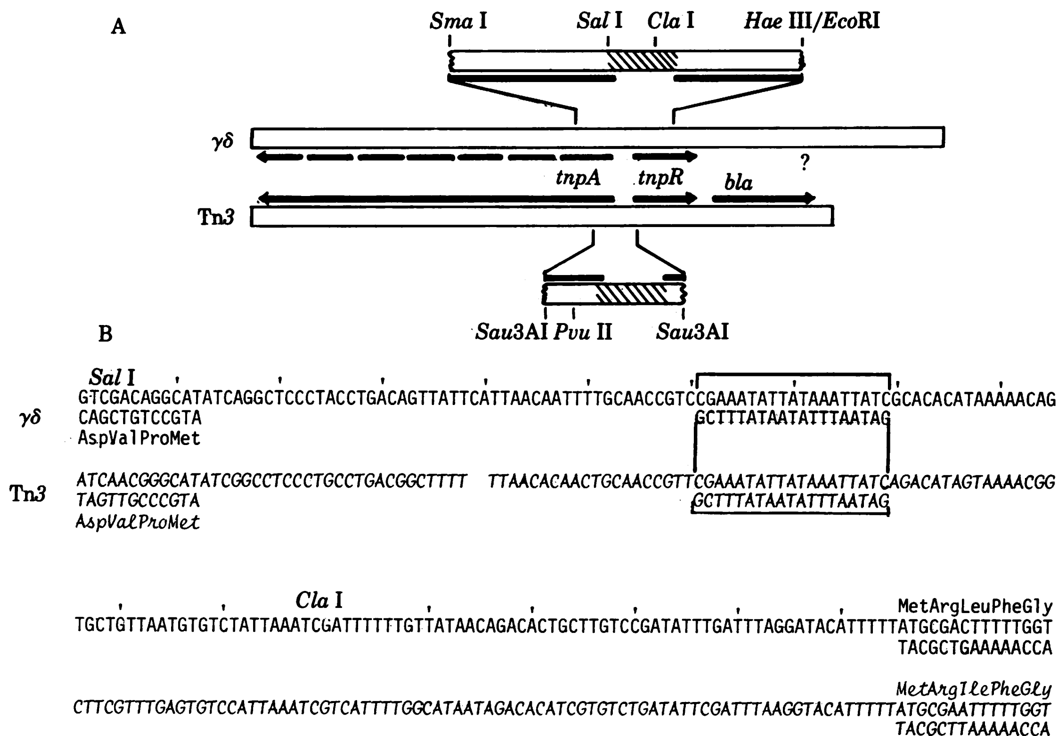

FIG. 4. (A) Genetic and restriction endonuclease maps of γδ and Tn3. DNA sequence data for the γδ *tnpA* gene is not complete. No functions have been assigned to the right-hand end of γδ. The expanded segments show that portion of each element present in the cointegrate constructs of Fig. 1B. Bold lines indicate protein encoding regions. The DNA sequence of the hatched area (including the entire intercistronic region) is presented in B. (B) DNA sequence of intercistronic regions of γδ and Tn3. The γδ sequence was determined from the *Sal* I site for the entire region from the *Cla* I site in both directions and from a *Pvu* I site located early in the repressor gene. The Tn3 sequence is from ref. 3. The region in which the crossover occurs is enclosed by a box. The NH_2 termini of *tnpA* and *tnpR* are at the left and right ends, respectively.

product of either γδ or Tn3 is able to resolve the hybrid plasmid as well as cointegrates formed solely from the other element; i.e., Tn3 *tnpR* protein resolves γδ::γδ cointegrate structures and vice versa. Second, the 19-bp sequence resembles other sites where *recA*-independent recombination occurs (19, 20). Third, the conservation of the intercistronic region in general, and of the 19-bp sequence in particular, suggests an important function; in contrast, there is almost no homology between γδ and Tn3 beyond the 3′ terminus of the repressor gene. However, one cannot rule out the possibility that normal resolution can occur anywhere within a given stretch of DNA and that the 19 bp of exact homology only strongly favors the generation of recombinants at that sequence.

Mechanism of Resolution. The finding that repressor is required for site-specific resolution of cointegrate structures (9), taken together with my demonstration that the crossover site is located in the intercistronic region, suggests an attractive model for the action of this protein. Genetic studies with γδ and Tn3 originally suggested that the *tnpR* gene product acts as a classical repressor (5, 7): namely, mutations of various types in the *tnpR* coding region lead to overproduction of the transposase and of the repressor itself. These mutations can be complemented in *trans* by a functional copy of the repressor gene. Another class of *tnpR* mutation (Tn3 cis_{10}) is located upstream from the *tnpR* cistron and appears to affect an operator: *tnpR* protein provided in *trans* does not fully repress expression of the mutant gene (5). These results suggest that the *tnpR* product interacts with an operator or operators located in the intercistronic region to regulate expression of the two divergently transcribed genes. It would not be at all surprising if the 19-bp sequence were to function dually as the resolution crossover site and as an operator. It is strategically located 50 bp before the *tnpA* coding region and contains a tantalizing Pribnow box sequence (21) (T-A-T-A-A-T-A) (Fig. 4B). Preliminary experiments suggest that *tnpA* transcription *in vitro* utilizes this promotor.

Many experiments have suggested that the transposon-mediated recombination event is reciprocal—i.e., the resolution of a single fused replicon yields two covalently closed circles. The studies presented here essentially examine the structure of both resulting replicons at the nucleotide level and confirm this notion. The resolved plasmid pRR25R contains a fusion of the *tnpR* region from Tn3 and *tnpA* from γδ; likewise, pRR29R contains the *tnpR* region from γδ and *tnpA* from Tn3. Such recombinant fusions are formally identical to the two product replicons expected after resolution of a single cointegrate structure carrying γδ and Tn3. The finding that both plasmids resolve in the same 19-bp sequence provides further evidence that the recombination event is reciprocal. Preliminary experiments performed in such a way that both products of the resolution could be recovered indicated that each was a covalently closed circle.

Analogies to Other Site-Specific Recombination Processes. Site-specific recombination in prokaryotes has been clearly demonstrated for the interaction of bacteriophage λ with its bacterial attachment site. The resolution of transposition-generated cointegrates appears, at least formally, to be analogous to phage λ integration into the host chromosome. Both of these small site reactions are independent of the general homologous (*recA*) recombination pathway and do not *a priori* require DNA synthesis. Similar site-specific recombination processes appear to be utilized in switching of the flagellar antigen in *Salmonella* (20) and in inversion of the G loop in phage Mu (22). There is little doubt that such mechanisms will be found to be wide-

spread throughout prokaryotes and probably eukaryotes as well.

The recombination site in γδ/Tn3 and the λ attachment site share more than just a formal analogy. The 15-bp λ attachment site and the 19-bp cointegrate resolution region are both striking in their A+T richness. There is 10/15 bp sequence homology between the recombination site in γδ/Tn3 and the λ attachment site (Fig. 5), comparable to the homology between λ*att* and the most efficient λ secondary attachment sites (23). The underlying reason for the apparent conservation among these sequences in two different site-specific recombination systems is unclear but may simply reflect a requirement for a region that could easily melt and permit protein interaction. The phage encoded *int* product, essential for λ integration (24), does not exhibit amino acid homology to *tnpR*. However, it is possible that a host protein is essential for both λ integration and cointegrate resolution. The host encoded *himA* gene product, required in the λ system (25), is an obvious candidate, but results of an experiment in which a plasmid containing two copies of the γδ IRS was introduced into a strain carrying a deletion of the *himA* gene indicate that resolution of cointegrate structures by the repressor-mediated pathway still occurs (N. Grindley, personal communication).

The flagellar antigen switch in *Salmonella* utilizes a recombination system similar to that of λ and γδ/Tn3 except that the crossover sites are present as inverted repeats (20). As a result, the recombination event results in the inversion of the intervening DNA. Zieg and Simon (20) noted homologies among the 14-bp inverted repeats in that system, the λ attachment site, and a region near one end of Tn3 distinct from the Tn3 internal resolution site. The actual crossover site in γδ/Tn3 exhibits an 8/14 bp match (Fig. 5) with the inverted repeat of the *Salmonella* system. Perhaps more intriguing is that the *hin* gene product of *Salmonella*, encoded between the inverted repeats and essential for inversion of the region, exhibits 30% amino acid homology with both the γδ and Tn3 protein (26).

The nonconjugative plasmid pSC101 contains a 200-bp transposable sequence (IS101) able to mediate replicon fusions between the plasmid and phage f1 (27). The termini of this element are homologous to those of Tn3 and γδ. A sequence present within the interior of IS101 (-A-T-T-A-T-G-T-T-T-C-G-) shows homology with the Tn3/γδ resolution site and may be responsible for *recA*-independent resolution of f1/pSC101 cointegrates (28).

λ core	GCTTTTTTATACTAA
γδ/Tn3	GATAATTTATAATATTTCG
Flagellar switch	AAGGTTTTTGATAA

FIG. 5. Sequence at which site-specific recombination occurs in three systems. In the λ attachment site core and *Salmonella* flagellar antigen switch, nucleotides in bold type indicate homology with the γδ/Tn3 crossover site. In each case, the sequences were aligned to provide greatest homology without deleting or inserting bases.

The results presented here, together with data on λ integration, the flagellar antigen switching phenomenon in *Salmonella*, and the recent data on G-loop inversion in phage Mu, suggest some conservation among *recA*-independent site-specific recombination events. Although these processes probably do not occur via identical pathways, further analysis may well identify additional common elements in the molecular mechanisms.

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