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A Tn*10* Derivative (T-POP) for Isolation of Insertions with Conditional (Tetracycline-Dependent) Phenotypes

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A new Tn*10*-based transposon has been constructed and used to isolate insertion mutations with tetracycline-conditional phenotypes. Classes of mutants include conditional lethal mutations, conditional auxotrophs, and conditional mutants of the *eut* (ethanolamine utilization) operon. The described mutations were made with a new derivative of Tn*10*dTet that we have called Tn*10*d(T-POP). Others have noted that transposon Tn*10*dTet directs weak tetracycline-inducible transcripts out of both ends of the element into adjacent sequences. We have increased this level of outward transcription from Tn*10*dTet by selecting deletion mutations within the element that presumably remove transcription-termination signals. Insertion of the Tn*10*d(T-POP) element within an operon disrupts the target gene and makes expression of distal genes dependent on induction of outward transcription by tetracycline. Insertion mutations made with Tn*10*d(T-POP) can cause tetracycline-correctable conditional phenotypes based on expression of distal genes.

Transposable elements have been widely used in bacterial genetics. The most commonly used elements are derived from transposon Tn*10* or from bacteriophage Mu (for a review, see reference 19). Insertion of these elements within an operon has an extremely strong polar effect on the expression of genes downstream of the insertion site. This absolute polarity limits the usefulness of transposon insertion mutations in complementation tests because multiple genes can be inactivated. Such polarity effects likewise prevent recovery of insertions in operons that include essential downstream genes. These limitations would be removed by a transposon that provided for the expression of distal genes.

The Tn*10*dTet element is a derivative of transposon Tn*10* made by introducing two deletions which remove the inside ends of both IS*10* elements and eliminate both transposase genes (31). The deletions leave intact the outside IS*10* end sequences (transposase substrates) and the central region which encodes resistance to tetracycline. This central region includes two genes, *tetA* and *tetR*, which are transcribed from divergent tetracycline-inducible promoters. The *tetA* gene encodes a tetracycline efflux protein (for a review on Tn*10*-based tetracycline resistance, see reference 16). The *tetR* gene encodes a regulatory protein which prevents transcription from the *tetA* and *tetR* promoters in the absence of tetracycline. Tetracycline interacts with the TetR protein to relieve repression and induce transcription of both the *tetR* gene and the *tetA* gene.

Studies of the *rnc* operon using the Tn*10*dTet element indicated that transcripts initiated at the *tet* promoters extend beyond the boundaries of this element and provide low-level regulated transcription of adjacent genes (30). This conclusion was based on the fact that an insertion of Tn*10*dTet within the *rnc* operon allows tetracycline-dependent expression of the distal, essential *era* gene. However, the level of outward transcription from Tn*10*dTet, as measured by *lacZ* operon fusion plasmids, was low.

In this paper, we describe Tn*10*dTet derivatives (called T-POP) that provide higher regulated expression of adjacent genes due to deletion mutations that allow more tetracycline-induced transcription to proceed out of the transposon. Isolation of Tn*10*d(T-POP) insertions between any gene and its promoter renders expression of that gene dependent on tetracycline. These new Tn*10*d(T-POP) elements should prove useful in genetic analysis since they avoid the problems of polarity and allow isolation of single-gene null insertion mutations in multigene operons.

MATERIALS AND METHODS

Bacterial strains. All strains are derivatives of *Salmonella typhimurium* LT2 (Table 1). The Tn*10*dTet element is a transposition-defective derivative of transposon Tn*10* from which the transposase gene and internal ends of IS*10* have been deleted (31). The MudA element is a transposition-defective derivative of phage Mu that provides ampicillin resistance and carries a *lacZYA* operon for creating transcriptional fusions (6, 18). The MudJ element is a transposition-defective derivative of phage Mu described by Castilho et al. (7) encoding resistance to kanamycin. Plasmids encoding Tn*10* transposase were kindly provided by Nancy Kleckner.

Media. Complex medium was nutrient broth (NB; 0.8%; Difco Laboratories) supplemented with NaCl (0.5%). Minimal medium was Vogel and Bonner E medium (12) with added glucose (0.2%) as a carbon and energy source. When analyzing the metabolism of carbon sources other than glucose, minimal E medium lacking citrate (NCE) (3) supplemented with the appropriate carbon source (0.2%) was used. When required, nutritional supplements were added to E and NCE media at previously recommended final concentrations (12). The chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to solid minimal lactose medium (final concentration, 20 μg/ml) to help visualize Lac^+ colonies. Antibiotics were added at the following concentrations: tetracycline, 20 μg/ml (in complex medium) or 10 μg/ml (in minimal medium); kanamycin, 50 μg/ml; ampicillin, 30 μg/ml for single-copy resistance determinants (MudA elements) or 100 μg/ml when maintaining multicopy plasmids with resistance determinants. When selecting Lac^{TD} mutants (tetracycline-dependent Lac^+; see below), tetracycline was used at a final concentration of 2 μg/ml. Solid media were prepared by the addition of agar (1.5%; Difco) to NB or minimal medium.

Genetic techniques. Transductional crosses were mediated by the high-frequency generalized transducing phage mutant P22 HT105/1 *int-201* (27). Transductants were single colony purified and made phage-free by streaking on nonselective green indicator plates (8). Cross-streaking to check phage sensitivity was done with a P22 clear-plaque mutant, H5.

Selection of mutants that relieve termination of transcription from Tn*10*dTet. A Mud-*lac* insertion was placed downstream of a Tn*10*dTet insertion within either the *eut* or the *his* operon. The orientation of the Tn*10*dTet element was determined by PCR as described below. Selection for mutants that expressed the *lac* genes of the Mud-*lac* reporter was carried out by plating 0.1 ml (approximately 10^8 cells) from independent, saturated NB cultures onto minimal NCE–

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TABLE 1. Bacterial strains used

Strain	Genotype (strain LT2)[a]
TR1810	*proAB47*/F′128 (*pro lac*)
TR5656	*proA36 strA1*
TR8353	Wild-type LT2/pLP103-6-3 (Amp^r MuA$^+$ MuB$^+$)
TT14544	Wild-type LT2/pNK972 (Amp^r *tnpA*$^+$)
TT14545	*recA1*/pNK972 (Amp^r *tnpA*$^+$)
TT17428	*recA1*/pZT380 (Amp^r *lacI* ptac-*tnpA*)
TT17437	Wild-type LT2/pNK2881 (Amp^r *tnpA**)
TT18778	*eutA208*::Tn*10*dTet (R) *eutB8*::MudA
TT18779	*eutA208*::Tn*10*dTet[del20] (R) *eutB8*::MudA
TT18780	*eutA208*::Tn*10*dTet[del21] (R) *eutB8*::MudA
TT18781	*eutA208*::Tn*10*dTet[del22] (R) *eutB8*::MudA
TT18782	*eutA208*::Tn*10*dTet[del23] (R) *eutB8*::MudA
TT18784	*hisG10175*::Tn*10*dTet (A) *hisC9955*::MudJ
TT18785	*hisG10175*::Tn*10*dTet[del24] (A) *hisC9955*::MudJ
TT18786	*hisG10175*::Tn*10*dTet[del25] (A) *hisC9955*::MudJ
TT18787	*hisG10175*::Tn*10*dTet[del26] (A) *hisC9955*::MudJ
TT18788	*hisG10175*::Tn*10*dTet[del27] (A) *hisC9955*::MudJ
TT18789	*hisG10175*::Tn*10*dTet[del28] (A) *hisC9955*::MudJ
TT18792	*hisG10176*::Tn*10*dTet[del25] (R) *hisC9955*::MudJ *eut-240*
TT18793	*hisG10175*::Tn*10*dTet[del20,del25] (R)*{*eutAB*}* *hisC9955*::MudJ *eut-240*(*eut-cysA* deletion)
TT18794	*recA1 zzz-3831*::Tn*10*Tet[del20,del25]
TT18795	*proAB47*/F′128 (*pro lac*) *zzf-3832*::Tn*10*Tet[del20]
TT18796	*proAB47*/F′128 (*pro lac*) *zzf-3833*::Tn*10*Tet[del25]
TT18797	*proAB47*/F′128 (*pro lac*) *zzf-3834*::Tn*10*Tet[del20,del25] (T-POP)
TT19179	*hisG10180*::Tn*10*dTet[del20,del25] (A) *hisC9955*::MudJ
TT19180	*hisG10181*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19181	*hisG10182*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19182	*hisD10183*::Tn*10*dTet[del20,del25] (A) *hisC9955*::MudJ
TT19183	*hisD10184*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19184	*hisD10185*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19185	*hisD10186*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19186	*hisC10187*::Tn*10*dTet[del20,del25] (R) *hisC9955*::MudJ
TT19194	*recA1 zzz-3839*::Tn*10*dTet[del20,del26]
TT19195	*proAB47*/F′128 (*pro lac*) *zzf-3840*::Tn*10*dTet[del26]
TT19196	proAB47/F′128 (*pro lac*) *zzf-3841*::Tn*10*dTet[del20,del26] (T-POP2)
TT19197	*hlpA1*::Tn*10*dTet[del20,del25]
TT19764	*purB2372*::Tn*10*d(T-POP) Pur^{TD}
TT19765	*pyrE2822*::Tn*10*d(T-POP) Pyr^{TD}
TT19766	*serA1477*::Tn*10*d(T-POP) Ser^{TD}
TT18767	*purB2371*::Tn*10*d(T-POP) Pur^{TD}
TT19768	*serA1478*::Tn*10*d(T-POP) Ser^{TD}

[a] Tn*10*dTet insertion orientation relative to downstream mud fusion: (R), *tetR* gene transcribed toward Mud-*lac* fusion; (A), *tetA* gene transcribed toward Mud-*lac* fusion. TD (superscript), tetracycline dependent.

lactose–X-Gal medium containing tetracycline (2 μg/ml). Tetracycline was included to induce the *tet* promoters, allowing isolation of mutants in which transcription of the *lac* genes was dependent on tetracycline. Mutant Lac$^+$ colonies arose on the plates after 2 days at 37°C, with additional mutants appearing upon additional incubation for up to 4 days. These Lac$^+$ colonies were tested to determine whether their Lac$^+$ phenotype depended on the presence of tetracycline.

Genetic characterization of Lac^{TD} mutants. Transductional linkage between the Tet^r phenotype of the Tn*10*dTet element and the drug resistance of the downstream Mud element (Kan^r for MudJ; Amp^r for MudA) was tested. Linkages substantially greater than those observed for the parent strain indicated that the Lac$^+$ mutant strain contained a deletion of the intervening region. For mutant strains obtained from the *hisG*::Tn*10*dTet insertion, a loss of *hisD* gene expression (required for growth on minimal histidinol medium) also indicated deletion of intervening material.

Any mutation (deletion or point mutation) that altered or removed the transposase binding sites at the ends of a Tn*10*dTet element or MudJ element would be expected to eliminate the ability to transpose. To test for Tn*10*dTet transposition, P22 lysates prepared on the Lac$^+$ mutant strains were used as donors in a cross with a RecA$^-$ recipient strain (TT14545) that expresses the Tn*10* transposase from plasmid pNK972. The recipient *recA* mutation prevents inheritance of the donor Tn*10*dTet insertion by homologous recombination; all Tet^r transductants are due to transposition of the Tn*10*dTet element from the transduced particle. To test the ability of the MudJ element to transpose, lysates from the Lac$^+$ mutant strains were used in crosses with recipient strain TT8353, which expresses the MuA and MuB genes from plasmid pLP103-6-3 (obtained from P. Van de Putte). Kanamycin-resistant transductants with a His$^+$ phenotype indicated Mud transposition to a new chromosomal site.

Reconstruction experiments were done to determine if the Lac^{TD} phenotype resulted from modification of the Tn*10*dTet element or from unlinked mutations that relieved transcription termination. By use of the mutant strains as donors, transductional crosses were done to recombine the Tn*10*dTet insertions into a clean MudJ background and to determine the linkage of the Lac^{TD} phenotype to the Tet^r phenotype of the Tn*10*dTet element. Final evidence that the mutations were confined to the Tn*10*dTet element was obtained by transposing the Tn*10*dTet element out of its original insertion site and then transposing it back into the original site in the *his* operon, a hot spot for Tn*10* insertion (15). The first transposition event was obtained by a transductional cross into the *recA* recipient strain TT14545 (as described above). The resultant strain (carrying an insertion of the element at an unknown site) was then used to create a random pool of Tn*10*dTet derivative insertion mutants in a *his*$^+$ background by a second cross into strain TT14545. This pool (containing at least 20,000 independent transposition events) was screened for His$^-$ insertions as outlined below.

The Tet^r transductants were pooled, and the free phage titer was reduced by washing the cells twice with E salts. A P22 lysate was prepared on this pool and used to transduce wild-type LT2. The resultant Tet^r transductants were replica-printed to identify His$^-$ auxotrophs. Because the *hisG* gene includes a major hot spot for Tn*10* insertion (15), roughly half of the His$^-$ insertions are at this single site within the *hisG* gene. This made it possible to return a mutant element (in either orientation) to the same site at which it originated.

Determining the orientation and position of Tn*10*dTet insertions. To determine which *tet* promoter was directed towards the Mud-*lac* reporter fusion, Tn*10*dTet insertions were oriented by the PCR. These reactions were run by use of an internal Tn*10*dTet primer specific for one side of the element and a second primer specific for a neighboring *hisG* or *eutB* sequence. This allowed determination of both the orientation of the insertions and their physical location within the *his* or *eut* operons.

Molecular characterization of Tn*10*dTet derivatives. A PCR was performed with primers internal to the Tn*10*dTet element to investigate changes in the regions of Tn*10*dTet distal to the *tetR* and *tetA* coding regions. The PCR products obtained from these reactions were purified with the Wizard PCR purification kit (Promega) and sequenced with the Mn^{2+} Reagent Kit for DNA Sequencing (Sequenase version 2.0, kit no. 70130; U.S. Biochemicals).

Placement of Tn*10*dTet derivatives on F′ episomes. To construct large pools of random insertion events, the Tn*10*dTet derivatives were moved onto F′ plasmids by transposition. The F′ episome carries the *pro* and *lac* genes and was maintained by selection for prototrophy in strains carrying chromosomal mutations in the *proA* (TR5656) or the *proAB* (TR1810) genes. Chromosomal Tn*10*dTet elements were transduced into a strain (TR1810) carrying the F′(*pro lac*) plasmid. We then introduced into this strain plasmid pZT380, which carries an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible Tn*10* transposase. The addition of IPTG (1.0 mM) to an exponentially growing culture of the resultant strain induces transposition of the chromosomal Tn*10*dTet insertion. Following the addition of IPTG, the culture was grown to saturation and the F′ element was moved to a new strain (TR5656) by conjugation, with selection for Pro$^+$ and counterselection of the donor with streptomycin. Exconjugants (Pro$^+$ Str^r) were screened for tetracycline resistance to identify those that carry a Tn*10*dTet insertion on the transferred F′ episome. Strains carrying this F′ plasmid with the Tn*10*dTet element were used as donors in subsequent transposition experiments; because the F′ plasmid is of *Escherichia coli* origin, it does not recombine with the *Salmonella* chromosome and the Tn*10*dTet element can be transductionally inherited only by transposition.

Determination of Tn*10*d(T-POP) insertion sites. Sequences adjacent to the Tn*10*d(T-POP) insertion in the *hlpA* gene were amplified by single-primer PCR done under low-stringency conditions (45 cycles of 1 min at 94°C, 1 min at 45°C, and 45 min at 72°C; Idaho Technologies Thermocycler). Internal, outward-directed primers specific to each side of the Tn*10*d(T-POP) element were used individually for this initial PCR. PCR products having one endpoint within the Tn*10*d(T-POP) element were identified by comparison with the bands produced with template DNA from an isogenic strain lacking the Tn*10*d(T-POP) element. The unique PCR products were excised and purified with the QIAquick gel extraction kit. The sequences were reamplified by high-stringency PCR with a nested primer within Tn*10*d(T-POP) used in 100-fold excess with the original primer used for single-primer PCR. The final, somewhat-smaller PCR product was purified and sequenced as described above. This was done for both sides of the Tn*10* insertion.

Isolation of Tn*10*d(T-POP) insertions in the *eut* operon. New insertions of the Tn*10*d(T-POP) element in the *eut* operon were isolated by transducing the Tn*10*d(T-POP) element (from strain TT18797) into a recipient strain expressing the Tn*10* transposase from plasmid pNK2881 (19). The recipient also carried a Mud-*lac* fusion element in the last gene of the *eut* operon (*eutR*); the Mud-*lac* fusion is not expressed since *eut* operon transcription depends on the EutR protein (26). Insertions of the Tn*10*d(T-POP) element were isolated as Lac$^+$ transductants on minimal NCE-lactose-tetracycline medium and shown to be

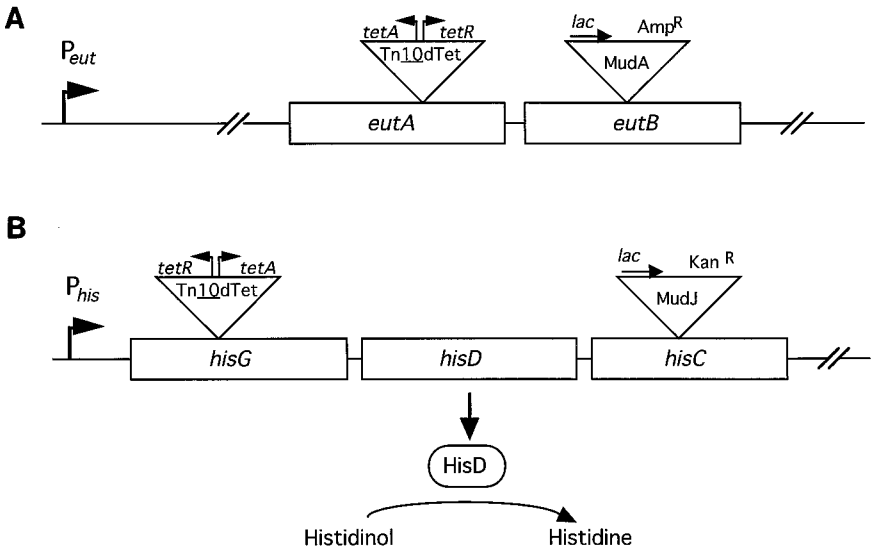

FIG. 1. Selection systems for modification of Tn*10*dTet. These abbreviated maps show the positions of Tn*10*dTet and MudJ insertions within the ethanolamine (*eut*) (A) and histidine (*his*) (B) operons of the parent strains. The *eutA* and *eutB* genes are the 11th and 12th genes in the ethanolamine operon. The *hisG*, *-D*, and *-C* genes are the first three genes in the histidine operon. The *hisG*::Tn*10*dTet insertion is at a hot spot for Tn*10* insertion.

Lac$^-$ on medium lacking tetracycline. These candidate insertions were then transduced into *eutR*$^+$ backgrounds to test their Eut phenotype with and without tetracycline.

RESULTS

Modification of left and right ends of Tn*10*dTet. Insertions of Tn*10*dTet were placed upstream of Mud-*lac* insertions, and the double mutant strains were used to select Lac$^+$ derivatives with increased transcription extending from the ends of the Tn*10*dTet element into the Mud-*lac* element. Modification of the left (*tetR*) end of Tn*10*dTet was selected by use of insertions in the *eut* operon (Fig. 1A). The Tn*10*dTet insertion was in the *eutA* gene and oriented so that *tetR* transcription proceeds in the same direction as that of the *eut* operon. The MudA element was located in the *eutB* gene immediately downstream. The Tn*10*dTet element blocks transcription of the MudA *lac* operon from the main *eut* promoter; induced transcription from Tn*10*dTet is insufficient to provide a Lac$^+$ phenotype. The Mud-*lac* fusion is not expressed unless a new mutation provides for increased transcription.

For mutations affecting the right (*tetA*) end of Tn*10*dTet, a similar system was used (Fig. 1B) but in the *his* operon; the Tn*10*dTet element was located in the promoter-proximal *hisG* gene and was oriented such that the *tetA* gene is transcribed in the same direction as that of the *his* operon. The MudJ insertion was located in the downstream *hisC* gene. Transcription from the *hisG*::Tn*10*dTet element could be monitored by expression of the *hisC*::MudJ *lac* genes or by expression of the intervening *hisD* gene. Since the HisD enzyme converts histidinol to histidine, its expression allows a His$^-$ strain to grow on medium containing histidinol.

Assay of β-galactosidase activity allowed quantification of the tetracycline-induced transcription emerging from the Tn*10*dTet element. For the original Tn*10*dTet element, the addition of tetracycline to the growth medium results in a 4.5- to 5-fold increased transcription of the Mud *lac* reporter adjacent to the *tetR* or *tetA* ends of (see Table 3, rows 1 and 6, respectively); this was originally demonstrated by Takiff et al. (30). However, the induced levels of β-galactosidase activity are insufficient to allow the parental strains to grow on minimal lactose-tetracycline medium. In the presence of tetracycline, the parental strain TT18784 containing the *hisG*::Tn*10*dTet insertion became HisD$^+$, indicating that the low level of tetracycline-induced transcription of *hisD* from the parental transposon is sufficient to produce histidine from histidinol.

Mutants that expressed the *lac* genes to a much higher level were selected on minimal lactose medium with tetracycline. Revertants (Lac$^+$) appeared at a frequency of 10^{-6} to 10^{-7}/cell plated. The majority of the revertants were shown to be Lac$^+$ with or without tetracycline. The Lac^{TD} strains were tested for linkage between the Tn*10* and MudJ elements and for the ability of the Tn*10* element to transpose. Eight independent Lac^{TD} mutants from the *eutA*::Tn*10*dTet insertion and 16 from the *hisG*::Tn*10*dTet insertion were analyzed and 13 of them behaved as expected for having mutations within the Tn*10* element. Classification of the Lac^{TD} mutants is de-

TABLE 2. Mutant classes obtained in Lac^{TD} selection

Parent strain	Classification	No. of Lac$^+$ mutants
TT18778 (*eut* operon)	Lac$^+$ constitutives	>500
	Lac^{TD} mutants (total)[a]	28
	Extended deletion[b]	0
	Alterations of IS*10* ends[c]	2
	Mutations internal to Tn*10*dTet[d]	6
TT18784 (*his* operon)	Lac$^+$ constitutives	43
	Lac^{TD} mutants (total)[a]	16
	Extended deletion[b]	3
	Alterations of IS*10* ends[c]	6
	Mutations internal to Tn*10*dTet[d]	7

[a] Total number of Lac^{TD} mutant classes from 10 independent saturated cultures of the parent strain TT18778. Mutations characterized in footnotes *b* to *d* are a subset of these Lac^{TD} mutants.

[b] Tn*10*dTet and MudJ elements were 100% linked (inseparable in transductional crosses).

[c] The Tn*10*dTet element was unable to transpose when provided with transposase.

[d] Lac^{TD} mutation showed 100% linkage to the Tn*10*dTet element. The Tn*10*dTet element was able to transpose when provided with transposase.

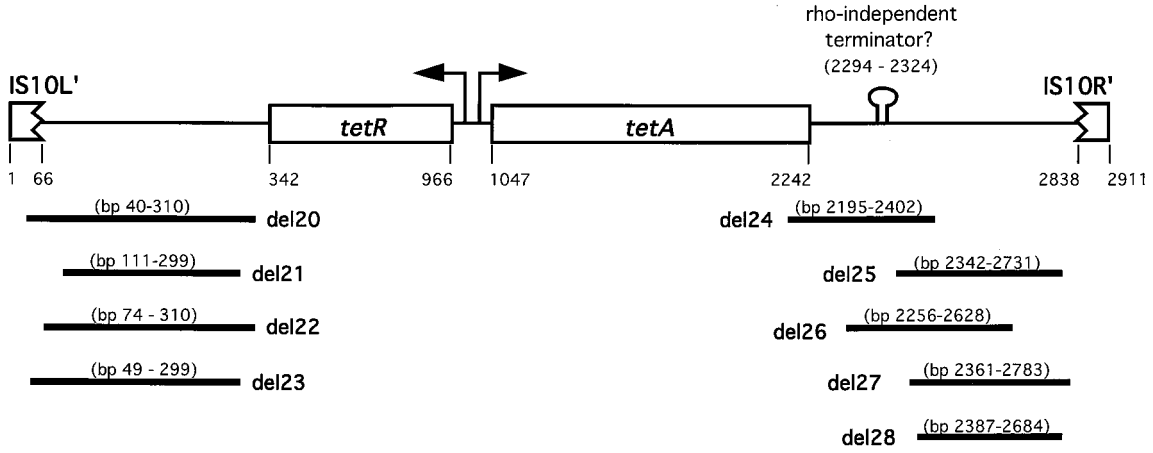

FIG. 2. Position of deletion mutations within Tn*10*dTet. Deletions at the *tetR* end (left) were selected by use of the insertions in the ethanolamine operon; those at the *tetA* end (right) were selected by use of the insertions in the histidine operon. Numbers indicate the positions of each feature in the DNA base sequence of the parent transposon, Tn*10*dTet.

scribed in Materials and Methods, and the results are summarized in Table 2. Six *eutA*::Tn*10*dTet mutants and five *hisG*::Tn*10*dTet mutants were sequenced; the chosen strains were those showing the greatest induced expression of *lacZ*.

Based on the inducibility of the Lac^+ phenotype and the tetracycline resistance, the Lac^{TD} mutations were expected to lie outside of the *tetR* and *tetA* coding regions. Oligonucleotides were designed to prime PCR amplification of the regions between the *tetR* or *tetA* gene and the IS*10* ends. All PCR products obtained from the mutant strains were smaller than those from the respective parent strains, indicating that the Lac^{TD} phenotype resulted from deletions within the Tn*10*dTet element. Sequencing of the PCR products and comparison of the sequence to the parental sequence localized the endpoints.

Five of the six *tetR* end deletions were characterized. Two of the deletions appeared to be identical, leaving four different deletions (Fig. 2, del20 to del23). As expected, none of the deletions disrupts the *tetR* coding sequence. Two deletions (del20 and del23) extend into IS*10* material, but neither removes the end sequences necessary for transposition. The collection of deletions at the *tetA* side was more heterogeneous (Fig. 2, del24 to del28). The published sequence (17) shows a stem-loop structure, characteristic of rho-independent terminators, just downstream of the *tetA* gene. Although this structure seemed a likely candidate site for mutations that allow increased transcription out of the right end of Tn*10*dTet, only two of the five deletions (del24 and del26) removed this putative transcription terminator.

Quantification of transcription levels from Tn*10*dTet derivatives. Transcription extending from the derivatives of Tn*10*dTet was determined by β-galactosidase assays. Without tetracycline, transcription from both *tet* promoters is essentially the same as that from the Tn*10*dTet element of the unmodified parent strains (Table 3, column 5), which is consistent with the tight repression previously described for the *tet* promoters (5, 10). The addition of tetracycline dramatically increased transcription of downstream *lacZ* reporter genes to levels well over 300 β-galactosidase units (Table 3, column 6). For many of the strains containing deletions within the Tn*10*dTet elements, expression was induced at least 30-fold, reaching a level 5- to 10-fold higher than the induced level of the parent strains

TABLE 3. Expression of *eut*::MudA and *his*::MudJ *lac* fusions from Tn*10* promoters

Strain no.	Tn*10*dTet location	Tn*10*dTet derivative	Tn*10*dTet orientation[a]	Level of transcription (β-galactosidase activity)[b]		Induction (fold)
				−Tetracycline	+Tetracycline[c]	
TT18778	*eutA208*	Original Tn*10*dTet	(R)	15 ± 2	75 ± 17	5.0
TT18779	*eutA208*	del20	(R)	13 ± 0.3	409 ± 33	31.5
TT18780	*eutA208*	del21	(R)	31 ± 2	471 ± 30	15.2
TT18781	*eutA208*	del22	(R)	23 ± 6	387 ± 12	16.8
TT18782	*eutA208*	del23	(R)	16 ± 1	356 ± 23	22.3
TT18784	*hisG10175*	Original Tn*10*dTet	(A)	9 ± 2	41 ± 12	4.5
TT18785	*hisG10175*	del24	(A)	13 ± 2	502 ± 73	38.6
TT18786	*hisG10175*	del25	(A)	14 ± 5	484 ± 20	34.6
TT18787	*hisG10175*	del26	(A)	17 ± 2	766 ± 8	45.1
TT18788	*hisG10175*	del27	(A)	14 ± 6	390 ± 99	27.9
TT18789	*hisG10175*	del28	(A)	9 ± 1	366 ± 51	40.7

[a] Tn*10*dTet insertion orientation relative to downstream Mud fusions: (R), *tetR* gene transcribed towards Mud-*lac* fusion; (A), *tetA* gene transcribed towards Mud-*lac* fusion.

[b] Transcription of downstream *lac* fusion (in Miller units). For *eut*::Tn*10*dTet insertions, a *eutB*::MudA element was used, for *his*::Tn*10*dTet insertions, a *hisC*::MudJ element was used.

[c] Inducing tetracycline was provided in growth medium at 2.0 μg/ml.

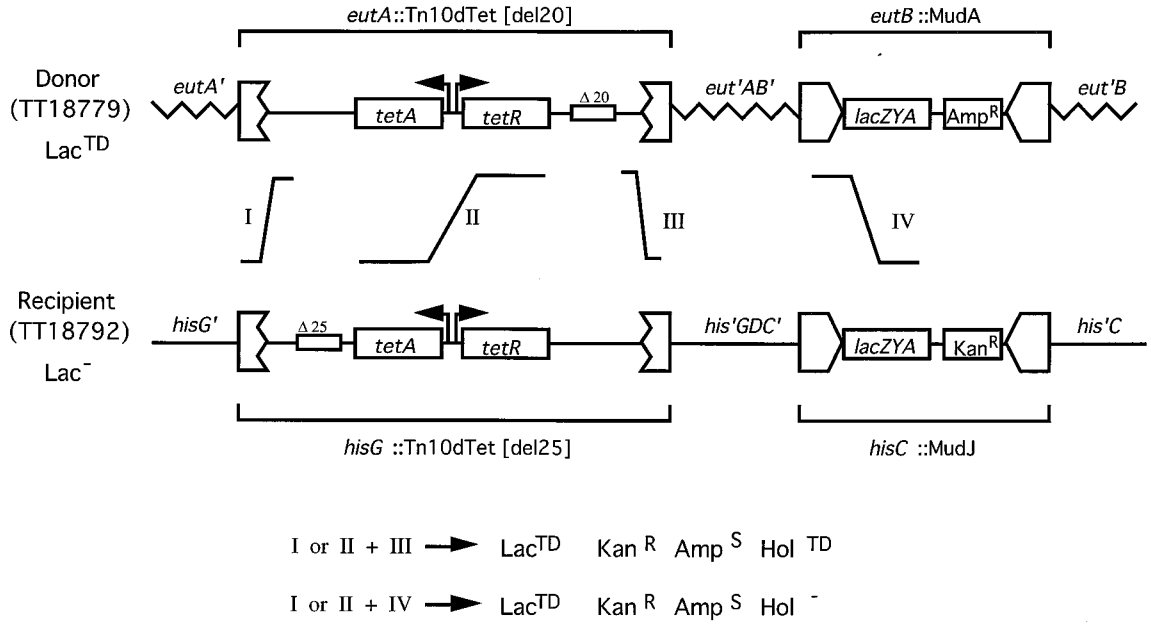

FIG. 3. Construction of a transposon with two deletion mutations. The diagrammed cross was used to construct T-POP, the double deletion mutant Tn*10*dTet (del25, del20). The recipient carries mutation del25 but is oriented such that the end needed for expression of the MudJ *lac* genes has no deletion; this strain is phenotypically Lac$^-$. The donor strain has mutation del20 at the end nearest the MudA-*lac* operon. Therefore, this strain is phenotypically Lac^{TD}. In the cross, selection is made for Lac^{TD} recombinants; this demands inheritance of the donor mutation del20 (events allowing this are indicated by roman numerals). The donor elements cannot be inherited at the recipient *eut* locus because it carries a large *eut* deletion mutation. All recombination events must occur within the two transposons sequences shared by donor and recipient.

(Table 3, compare rows 1 and 6 to rows 2 to 5 and rows 7 to 11).

Construction of recombinant Tn*10*dTet derivatives with a deletion at both ends of the element. A transductional cross was done to form the desired double-mutant element by recombination between two single-mutant Tn*10*dTet elements, one in the *eutA* gene and one in the *hisG* gene. This cross is diagrammed in Fig. 3. The donor strain (TT18779) carries a *eutA*::Tn*10*dTet[del20] insertion and a *eutB*::MudA(*lac* Amp^r) insertion; this strain is phenotypically Lac$^+$ in the presence of tetracycline. The recipient strain (TT18792) contains an insertion of the Tn*10*dTet[del25] element in the *hisG* gene with its unmodified (*tetR*) end oriented towards the *hisC*::MudJ element; this strain is phenotypically Lac$^-$. The transduction was done by selecting for Lac$^+$ recombinants on minimal lactose-tetracycline medium supplemented with histidine and cystine. The Lac$^+$ donor and the Lac$^-$ recipient strains have different sequences flanking their Tn*10*dTet and Mud elements (either *eut* or *his*), and recombination in the *eut* region is prevented by the *eut-cysA* deletion in the recipient. Therefore, all Lac$^+$ recombinants must arise by exchange between donor and recipient Tn*10*dTet and Mud transposons. The desired recombinant type with deletions at each end is expected to have both the Tn*10*dTet and Mud elements within the *his* operon, separated by an intervening *eut* sequence.

Lac$^+$ transductants were isolated and tested for the Lac^{TD} phenotype. All recombinants were Hol$^-$ (even with tetracycline) due to loss of the *hisD* gene, and all showed 100% linkage of the Tet^r and Kan^r phenotypes since nonhomology of the intervening *eut* sequence prevents recombination between the two elements. The Tn*10* elements of several Lac$^+$ transductants were screened by PCR for possession of the deletions at either side of the Tn*10*dTet. A recombinant (TT18793) which showed the bands predicted for each of the two deletions was saved for later use (data not shown). This composite Tn*10*dTet element was moved out of the *his* operon and subsequently onto an F′ plasmid by transposition as described in Materials and Methods. The element carrying both deletions (Tn*10*dTet[del20,del25]) has been named Tn*10*d(T-POP) or T-POP for the fact it confers (t)etracycline resistance and utilizes the divergent (p)romoter-(o)perator-(p)romoter system for providing expression of adjacent genes. By similar manipulations, a second double-mutant element that carries the same *tetR* end deletion (del20) in combination with a different *tetA* end deletion (del26) has been constructed; the second element has been designated T-POP2.

Characterization of *his* mutants generated by the Tn*10*d(T-POP) insertion. Insertions of the new Tn*10*d(T-POP) element at new sites in the *his* operon were isolated by screening approximately 35,000 independent insertion mutants. These His$^-$ insertions were mapped by a set of *his* deletions to identify insertions in the *hisGDC* region.

Each of these new insertions of Tn*10*d(T-POP) was moved into the *hisC*::MudJ background by transduction. The location and orientation of the new insertions were determined more precisely by PCR. Based on the strong insertion site specificity of the Tn*10* transposase (15), strains producing similarly sized

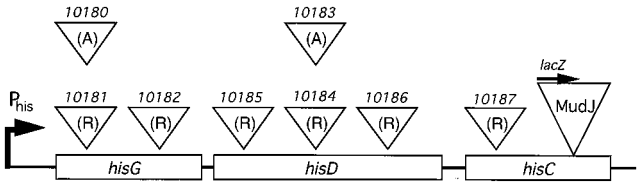

FIG. 4. Positions of T-POP insertions in the histidine operon. Random insertions in the operon were isolated to test the behavior of T-POP at a variety of sites. Positions were estimated by PCR. The behavior of these insertions is described in Table 4.

TABLE 4. Transcription levels from *his*::Tn*10*d(T-POP) insertions

Strain no.	Tn*10*dTet location	Tn*10*dTet orientation[a]	Level of transcription (β-galactosidase activity)[b]		Induction (fold)
			−Tetracycline	+Tetracycline[c]	
TT19179	*hisG10180*	(A)	15 ± 1.9	482 ± 39	32.1
TT19182	*hisD10183*	(A)	10 ± 0.2	378 ± 7.3	37.8
TT19180	*hisG10181*	(R)	10 ± 0.9	64 ± 0.4	6.4
TT19181	*hisG10182*	(R)	9 ± 1.0	11 ± 0.5	1.1
TT19183	*hisD10184*	(R)	12 ± 0.5	150 ± 3.5	12.5
TT19184	*hisD10185*	(R)	9 ± 0.6	35 ± 0.3	3.9
TT19185	*hisD10186*	(R)	9 ± 0.8	81 ± 5.0	9.0
TT19186	*hisC10187*	(R)	0.6 ± 0.2	6 ± 0.9	10.0

[a] Tn*10*d(T-POP) insertions orientation relative to downstream Med fusion: (A) *tetA* promoter directed towards MudJ insertion (R), *tetR* promoter directed towards MudJ insertion.

[b] Transcription of downstream *lac* fusion (in Miller units); a *hisC*::MudJ element was used.

[c] Tetracycline was provided in growth medium at 2.0 μg/ml.

PCR products are likely to be insertions at the same site. A set of Tn*10*d(T-POP) insertions is diagrammed in Fig. 4. These strains were chosen based on their having either a distinct insertion site or a distinct orientation. Tetracycline-induced transcription levels from these Tn*10*d(T-POP) elements are presented in Table 4. Strains in which the *lacZ* gene is expressed from the *tetR* promoter showed variability in the levels of transcription depending on the insertion site. Insertion of Tn*10*d (T-POP) at the *hisD10184* site exhibited high levels of induced transcription (Table 4, row 5), yet insertion at the *hisG10182* site generated virtually no tetracycline-induced expression of β-galactosidase (Table 4, row 4). In contrast, transcription from the *tetA* promoter consistently showed a high level of induction for *his* operon insertions (Table 4, rows 1 and 2) as well as for insertions obtained in other operons (data not shown).

Investigation of the effects of antisense transcripts from Tn*10*d (T-POP) elements. With an element that produces transcripts extending out of both ends, the possibility exists that the message directed upstream (opposite that of the target operon) reduces expression of genes upstream of the insertion site. To investigate this, we examined the HisD phenotype following induction of transcription from a Tn*10*d(T-POP) element inserted in the downstream *hisC* gene (TT19186). Strain TT19186 showed no difference in growth on minimal E medium-glucose-histidinol plates than on identical plates with added tetracycline (2.0 μg/ml). This result and those obtained from Tn*10*d (T-POP) insertions in the *eut* operon (see below) show that antisense messages from the Tn*10*d(T-POP) element do not cause major interference with the expression of genes upstream of the insertion site. It should be kept in mind that only a few *his* sites were tested and that the method used for those sites is not very sensitive, since low levels of HisD enzyme are sufficient for growth. However, in many more tests of the *eut* operon, we have seen no evidence of antisense effects on upstream gene expression (data not shown).

Isolation and characterization of conditional mutations generated by Tn*10*d(T-POP) insertion. To test the general usefulness of the Tn*10*d(T-POP) element, other chromosomal insertions were isolated from a pool of random Tn*10*d(T-POP) insertions. A lysate prepared on this pool of insertions was used to transduce LT2 on NB-tetracycline medium. Tetracycline-resistant transductants were replica-printed to minimal E-glucose medium lacking tetracycline to identify insertion mutants that caused auxotrophy or lethality in the absence of tetracycline. From this initial screen, 21 Tn*10*d(T-POP) insertions were isolated. The classification of these mutants is given in Table 5. This same general distribution of mutant types has been observed for a larger set of Tn*10*d(T-POP) insertions isolated by a genetics laboratory course. We mapped the mutants whose growth phenotype was corrected by tetracycline; the mapping method was that of Benson and Goldman (2).

One Tn*10*d(T-POP) insertion (TT19197) exhibited a tetracycline-correctable, lethal phenotype and mapped to the 4- to 7-min region of the *Salmonella* chromosome. To identify the exact location of this conditional lethal Tn*10*d(T-POP) insertion, a low-stringency single-primer PCR (as described in Materials and Methods) was used to amplify DNA adjacent to the insertion site for sequencing. A FASTA analysis of the sequences flanking the Tn*10*d(T-POP) insertion with sequences from the *E. coli* genome database indicated that the Tn*10*d(T-POP) insertion was located in the *Salmonella* homolog of the *E. coli hlpA* gene. Alignments of the *Salmonella* sequence on either side of the Tn*10*d(T-POP) element and the *E. coli hlpA* sequence showed 84 and 85% nucleotide sequence identity. Furthermore, the *hlpA* gene is located at min 4.4 of the *E. coli* genetic map (4). The lethal phenotype of the *hlpA* insertion is likely due to a polar effect on expression of essential downstream genes; this expression could be restored by the addition of tetracycline. The *hlpA* gene is part of an operon that includes essential functions required for the synthesis of lipid A, the hydrophobic anchor for lipopolysaccharides in the outer membrane (for reviews, see references 22 and 28). Thus, the use of the T-POP element provides in vivo evidence that the *hlpA* gene is dispensable but is located within an operon that includes distal genes for essential proteins.

Identification of phenotypically silent genes in the *eut* operon. Genetic analysis of the ethanolamine utilization operon (*eut*) of *Salmonella* indicated six genes whose mutation caused an aerobic Eut$^-$ phenotype (25). Subsequent sequence determination (14, 21, 29) has revealed 16 genes in the operon,

TABLE 5. Characterization of Tn*10*d(T-POP) insertions

General mutant class[b]	No. of mutants	Specific characterization[a]
Standard auxotrophs	11	2 histidine auxotrophs, 2 tryptophan auxotrophs, 2 leucine auxotrophs, 2 proline auxotrophs, 1 methionine auxotroph, 1 arginine auxotroph, 1 with undetermined zusotrophy
Tetracycline-corrected auxotrophs	5	2 serine auxotrophs (*serA*), 2 early purine auxotrophs (*purB*), 1 uracil auxotroph (*pyrE*)
Tetracycline-corrected lethal insertion	1	
Tetracycline-enhanced growth rate	4	

[a] Mutants classified by auxanography as described previously (13).

[b] Standard auxotroph, able to grow on NB medium but not on minimal medium with or without tetracycline; tetracycline-correctable auxotroph, able to grow on NB medium with or without tetracycline but grows on minimal E-glucose medium only if tetracycline is provided (specific gene classification based on auxotrophic requirement and map position; includes strains TT19764 to TT19768); tetracycline-correctable lethal insertion, able to grow on either minimal or NB medium in the presence of tetracycline [strain TT19197, *hlpA1*::Tn*10*d(T-POP)]; tetracycline-enhanced growth rate, growth on NB medium improved by but not requiring the presence of tetracycline.

suggesting that many genes lack mutant phenotypes and therefore were missed by the genetic analysis. The Tn*10*d(T-POP) element was used to test this hypothesis and generate single gene knockouts in this operon.

Insertions of the Tn*10*d(T-POP) element in the *eut* operon were isolated and tested for correctability of their Eut^- phenotype by the addition of tetracycline. Two classes of mutants were obtained. Insertions in the previously identified *eutD*, *-E*, *-A*, *-B*, *-C*, and *-R* genes were phenotypically Eut^- with or without tetracycline. Insertions of Tn*10*d(T-POP) in the *eutS*, *-Q*, *-M*, *-J*, *-G*, *-K*, and *-L* genes showed tetracycline-correctable aerobic Eut phenotypes as determined with MacConkey ethanolamine B_{12} indicator medium; previous mutant hunts had failed to identify these genes. The new Tn*10*d(T-POP) insertions demonstrate that these genes were missed because they do not encode functions necessary for aerobic metabolism of ethanolamine.

A secondary use of Tn*10*d(T-POP) insertions is to select in-frame deletions of the identified target region. This has been applied to insertions in the *eut* operon to reinforce the conclusion that some genes are nonessential. Tetracycline-correctable *eut*::Tn*10*d(T-POP) insertion mutants were plated, without tetracycline, on medium that selects for a Eut^+ phenotype. Among the revertant clones were deletions which removed the T-POP element and target gene sequences; the remaining genes were sufficient for a Eut^+ phenotype. Sequencing of these revertants showed that the deletions did not disturb the reading frame or else had endpoints near the distal end of genes; these deletions must be essentially nonpolar to provide the Eut^+ phenotype. Using this deletion procedure, we have confirmed that the *eutQ*, *eutT*, *eutM*, *eutN*, *eutJ*, *eutG*, *eutH*, and *eutK* genes are nonessential for aerobic utilization of ethanolamine. The implications of *eut* gene insertions and deletions will be detailed elsewhere (20).

DISCUSSION

We describe derivatives of transposon Tn*10*dTet that allow regulated, high levels of transcription of chromosomal sequences adjacent to the insertion site. The increased transcription results from deletion mutations within the Tn*10* element that presumably remove transcription termination signals. These elements (T-POP) should be useful in genetic analysis of bacteria and in the study of transcription termination.

The use of Tn*10*d(T-POP) elements permits isolation of insertion mutations with conditional (tetracycline-correctable) phenotypes due to transcriptional fusions between the regulated *tet* promoters of the element and a functional chromosomal gene nearby. In principle, these could be formed for any gene by an insertion between that gene and its promoter. However, since most genes are very close to their promoter, such fusions are more common for operons in which an extensive target region of dispensable genes is located between the gene providing the phenotype and its normal promoter. We describe several auxotrophs, mutants defective for ethanolamine utilization, and a lethal mutant, whose growth phenotypes are corrected by tetracycline. Tn*10*d(T-POP)-generated conditional mutations differ from traditional temperature-sensitive (i.e., missense) mutations in that nonpermissive conditions prevent gene expression instead of altering the native protein structure; such conditional expression avoids some potential complications inherent in the use of temperature-sensitive mutations. The elements described here improve a general utility of Tn*10*dTet elements first pointed out earlier (30).

Understanding how the described deletions increase transcription out of Tn*10*dTet may provide some insight into the general mechanisms of transcription termination. Currently, some termination is thought to occur at particular stem-loop structures in RNA by mechanisms that are independent of dedicated protein factors; alternatively, termination can be caused by rho factor acting at sites that have proved difficult to identify (11, 23, 24). Inspection of the sequences removed by the Tn*10* deletions reveals differences between the ends of the element. The region downstream of the *tetA* gene includes a stem-loop structure characteristic of rho-independent terminators. Our deletion mutations indicate that this structure is not a major contributor to transcription termination since only two of the five deletions (del24 and del26) remove it. Deletions distal to this stem-loop may remove a region necessary for rho-dependent transcription termination which is of major importance here. In support of this idea, we have isolated unlinked suppressor mutations that allow *tetA*-promoted transcripts to extend out of the original Tn*10*dTet element. Of 20 mutants isolated, 19 carried a *rho* mutation (data not shown). The sequence removed by the Tn*10*dTet-internal deletions does not fit well with the features of rho termination sites proposed by Alifano et al. (1); they provided evidence that rho-dependent terminator regions encode mRNA rich in cytosine and poor in guanine. The two deletions removing the stem-loop show a slightly higher transcription read-through, suggesting that the stem-loop structure may play a minor role in addition to a more important rho-mediated termination. The sequences between the *tetR* gene and the end of the element agree with general features of rho-dependent terminators in that they show a significant excess of cytosine over guanine. It is interesting to note that only deletions (no point mutations) were found to reduce termination of messages. This is reminiscent of the several mutations (all deletions) found to eliminate a rho-dependent termination site within the *hisG* gene (9).

Transcription from the *tetA* end of Tn*10*d(T-POP) provides strong, regulated expression of adjacent genes in a variety of contexts. This is surprising since many adjacent regions would be expected to include rho termination sites. It is possible that transcripts emerging from the *tetA* end of Tn*10*d(T-POP) may be intrinsically resistant to rho termination; alternatively, the *tetA* promoter may be of sufficient strength that commonly encountered rho sites do not reduce transcription significantly. Transcription from the *tetR* side of Tn*10*d(T-POP) shows lower expression and more variability in its ability to provide tetracycline-induced expression of downstream Mud-*lac* fusions than that from the *tetA* end. Studies on the *tet* promoters have shown that mRNA transcripts from the *tetR* promoter have a shorter half-life than transcripts from the *tetA* promoter (21 compared to 41 s) and that the *tetR* promoter initiates transcription 7 to 11 times less frequently than the *tetA* promoter (10). Insertions at the same *his* target site but in opposite orientation allowed comparison of transcription levels from the *tetR* and *tetA* promoters. These results indicate that the *tetR* promoter is 2.5 to 7.5 times less effective than the *tetA* promoter (Table 4, rows 1, 2, 3, and 5) at expressing the nearby *lacZ* gene. The difference between the earlier promoter studies and the ability of T-POP promoters to express nearby genes probably reflects factors other than promoter strength; that is, some termination signals may remain within the element or the two transcripts may have different sensitivities to degradation or termination at sites within sequences adjacent to the insertion site.

Both of the T-POP elements described here have been moved onto F′ plasmids (TT18797 and TT19196) for use in construction of pools of random Tn*10*d(T-POP) insertions in *Salmonella*. These plasmids make it possible to introduce T-

POP elements into *E. coli* by conjugation. Transposition of T-POP in *E. coli* could be selected by transducing the T-POP element from an F′ plasmid-bearing strain into an F^- $RecA^-$ recipient strain carrying a transposase plasmid (pNK972, pNK2881, or pZT380). This is essentially the method used here to move elements in and out of particular sites in the Salmonella *his* operon.

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