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# Recognition of *Escherichia coli attTn7* by Transposon Tn7: Lack of Specific Sequence Requirements at the Point of Tn7 Insertion

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**Transposon Tn7 inserts at high frequency into a specific site in the *Escherichia coli* chromosome called *attTn7*. We show that the point of Tn7 insertion in *attTn7* lies within the transcriptional terminator of the bacterial *glmS* gene. We have exploited the *glmS* transcription terminator to isolate mutants with altered sequences at the point of Tn7 insertion and have used these mutants to show that the nucleotide sequence at the point of Tn7 insertion is irrelevant to *attTn7* target activity. Thus, the nucleotides which provide *attTn7* target activity are distinct from the point of Tn7 insertion. We have also examined the effect of transcription on the capacity of *attTn7* to act as a target for Tn7 transposition. Our results suggest that transcription of *attTn7* does not modulate its Tn7 target activity.**

Transposons are mobile DNA segments that insert at a variety of chromosomal and episomal target sites. Most elements display little target site specificity when they transpose to large DNA molecules, such as bacterial chromosomes (14, 20, 25). Transposon Tn7 (5), which encodes resistance to trimethoprim and to streptomycin and spectinomycin, is distinguished by its capacity to transpose at high frequency to a specific site in the *Escherichia coli* chromosome called *attTn7* (4, 5, 27), where it inserts in a single orientation (27). Site-specific insertion of Tn7 into the chromosomes of a number of other bacteria has also been observed (3, 7, 8, 11, 15, 23, 43, 44, 49).

*attTn7* is located at min 84 of the *E. coli* chromosome (4, 5, 27). The specific point of Tn7 insertion lies between two genes: *phoS*, which encodes a protein involved in phosphate transport, and *glmS*, which encodes a protein involved in cell wall biosynthesis (28, 46; also see Fig. 1). The specific point of Tn7 insertion is located downstream of the *glmS* coding sequence in a G+C-rich region of dyad symmetry, followed by a run of T residues (Fig. 1). This region is structurally reminiscient of simple transcription terminators (38, 48) and of stabilizers of the 3′ ends of mRNAs (35). Gay et al. (18) have shown that the 3′ ends of *glmS* mRNAs extend to the T residues. In this work, we directly show that the point of Tn7 insertion does lie within the *glmS* transcription terminator, as postulated by Gay et al. (18). We also show that the ability of *attTn7* to act as a target for Tn7 insertion is independent of transcription.

In our nomenclature for *attTn7*, the middle base pair of the 5-base-pair (bp) sequence usually duplicated upon Tn7 insertion (18, 28, 31) is designated 0, sequences towards *phoS* are designated as −, and sequences towards *glmS* are designated as + (Fig. 1). Other work from this laboratory (31) has shown that *attTn7*(−3 to +64) has *attTn7* target activity, i.e., can act as a target for high-frequency site- and orientation-specific Tn7 insertion, and that at least some nucleotides essential for *attTn7* target activity are located within the protein-coding sequence of the *glmS* gene. In this work, we further define the nucleotides required for *attTn7* target activity. We have isolated a number of mutants with altered sequences at the Tn7 point of insertion by exploiting the *glmS* transcription terminator. Analysis of these mutants has shown that the nucleotide sequence immediately surrounding the specific point of Tn7 insertion is irrelevant to *attTn7* target activity. Thus, the utilization of *attTn7* by Tn7 resembles the activity of certain restriction enzymes whose recognition sites and sites of cleavage are distinct (2, 21).

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## MATERIALS AND METHODS

**Media, chemicals, and enzymes.** LB broth and agar were as described by Miller (32), except that 1 mg of glucosamine per ml was added to agar. Iso-Sensitest agar (Oxoid Ltd.) was used with trimethoprim. Antibiotic supplements were as follows: carbenicillin, 100 μg/ml; kanamycin, 100 μg/ml; and trimethoprim, 100 μg/ml. MacConkey-galactose-carbenicillin indicator plates contained 40 g of MacConkey agar base (Difco Laboratories) per liter, 10 g of galactose per liter, and 100 μg of carbenicillin per ml. DNA-modifying enzymes were obtained from commercial sources and used as recommended by the manufacturer.

**Manipulation and characterization of DNA molecules.** Plasmid DNA isolation and restriction enzyme analysis were performed as described by Maniatis et al. (29). Cloning methods were as performed as described by Maniatis et al. (29), except that DNA fragments contained in slices excised from low-melting-point agarose (SeaPlaque) gels were used directly in the assembly of recombinant molecules, as described by Struhl (42). DNA sequence analysis was performed as described by Sanger et al. (40) with double-stranded DNA as a template (13). Because *attTn7* contains a G+C-rich region which complicates DNA sequencing, we included T4 gene 32 protein in the sequencing reactions (36), used Klenow fragment with deoxy-7-deazaguanosine triphosphate (American Bionetics) (34) or Sequenase (United States Biochemical Corp.) with dITP (33) in place of dGTP,

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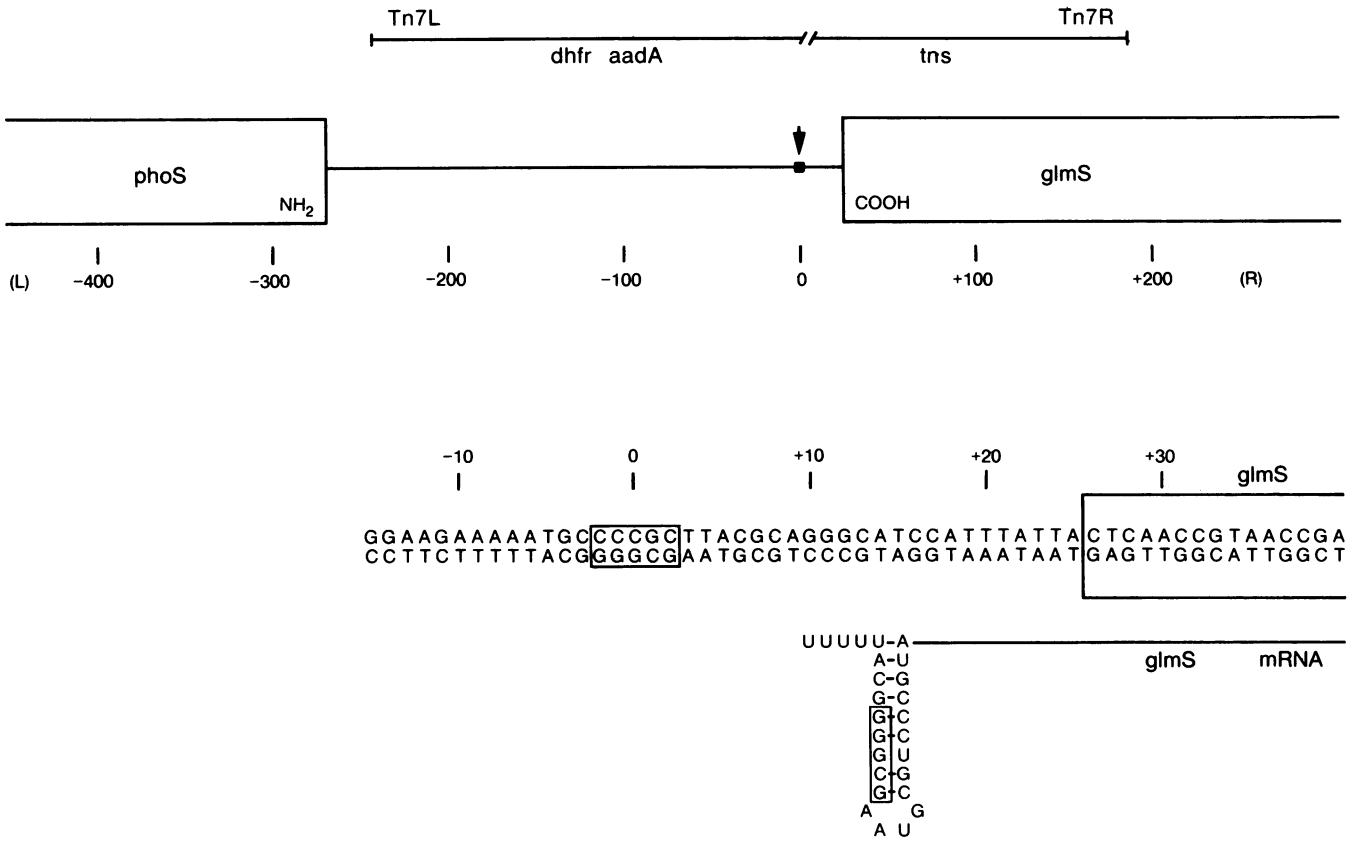



FIG. 1. Features of *att*Tn*7*. The upper line is Tn*7*, which encodes two antibiotic resistance genes, *dhfr* (17), which provides resistance to trimethoprim, and *aadA* (16), which provides resistance to streptomycin and spectinomycin, and five transposition (*tns*) genes (37, 45). The left (Tn*7*L) and right (Tn*7*R) ends of Tn*7* are indicated. The next line is the region of Tn*7* insertion in the *E. coli* chromosome (18, 28, 46). The middle base pair of the 5-bp sequence, usually duplicated upon Tn*7* insertion (■) (18, 28, 31), is designated 0, sequences rightwards (R), towards *glmS*, are designated +, and sequences leftwards (L), towards *phoS*, are designated − (31). The next line is the nucleotide sequence at the point of Tn*7* insertion, as determined by Gay et al. (18) and this laboratory (31). The 5-bp sequence duplicated upon Tn*7* insertion (narrow box) and the terminus of *glmS* (wide box) are indicated. The lower line shows the potential G+C-rich stem and loop, followed by U residues at the 3′ end of *glmS* mRNA (18). The nucleotides corresponding to the sequences duplicated upon Tn*7* insertion are boxed.

included 25% formamide in the sequencing gels, and ran the gels at high temperature.

**Bacterial strains.** HB101 is *E. coli galK* [$F^-$ *hsdS20* ($r_B^-$ $m_B^-$) *supE44 ara-14 galK-2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13*] (9). LA3 is *E. coli att*$Tn7_{84}$::Tn*7* [$F^-$*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* $Val^r$ *recA56 att*$Tn7_{84}$::Tn*7*] (31). The Tn*7* derivative used in this work (Tn*7*S) contains an IS*1* insertion near the drug resistance determinants of Tn*7*; however, the transposition properties of Tn*7*S are indistinguishable from those of canonical Tn*7* (22).

**Plasmids.** pKL600 (from K. McKenny, National Institutes of Health, Bethesda, Md.) is a carbenicillin-resistant terminator probe vector. It is the same as pKO1 (30), except that the *Eco*RI site was replaced by a *Sal*I linker, and a 300-bp *Pvu*II-*Pvu*II fragment containing the *lac* promoter (p*lac*) and polylinker from M13mp10 was inserted into the *Sma*I site to give *Sal*I-*Hin*dIII-p*lac*-*Eco*RI(polylinker)*Hin*dIII-*galK*. The *Eco*RI site was filled in to create a translation stop codon, preventing *lacZ* translation from crossing the polylinker. pKO500 (from K. McKenny) is a carbenicillin-resistant promoter probe vector. It is the same as pKO1, except that the *Eco*RI site was replaced by a *Sal*I linker, and a 300-bp *Pvu*II-*Pvu*II fragment containing p*lac* and the polylinker from m13mp11 was inserted into the *Sma*I site to give *Sal*I-*Hin*dIII-p*lac*-*Hin*dIII(polylinker)*Eco*RI-*galK*. The *Hin*dIII fragment was then deleted to give *Sal*I-*Hin*dIII(polylinker)*Eco*RI-*galK*.

pEG61 [p*lac*-*att*Tn*7*(+64 to −52)-*galK*] and pEG63 [p*lac*-*att*Tn*7*(−52 to +64)-*galK*] were constructed by introducing the *Hin*cII-*Hin*dIII *att*Tn*7* fragment from pKAO3 (31) after treatment with Klenow fragment into the *Sma*I site of pKL600. pEG10 [p*lac*-*att*Tn*7*(+64 to −4)-*galK*] and pEG11 [p*lac*-*att*Tn*7*(−4 to +64)-*galK*] (31) were constructed by introducing the *Sst*I-*Sst*I fragment of pKAO16 (31) into the *Sst*I site of pKL600.

pEG51 [*att*Tn*7*(+64 to −52)-*galK*] and pEG57 [*att*Tn*7*-(−52 to +64)-*galK*] were constructed by introducing the *Hin*cII-*Hin*dIII *att*Tn*7* fragment of pKAO3 after treatment with Klenow fragment into the *Sma*I site of pKO500.

pEG20 [p*lac*-terminator-*att*Tn*7*(+64 to −4)-*galK*] and pEG21 [p*lac*-terminator-*att*Tn*7*(−4 to +64)-*galK*] were constructed by introducing tandem direct copies of the *trpA* transcription terminator (Pharmacia) into the *Sma*I site of pKL600 and then introducing the *Bam*HI *att*Tn*7* fragment from pEG10 into the *Bam*HI site.

pEG31 [*att*Tn*7*(−52 to +64)] was constructed by introducing the *Bam*HI-*Sst*I *att*Tn*7* fragment from pEG61 into the

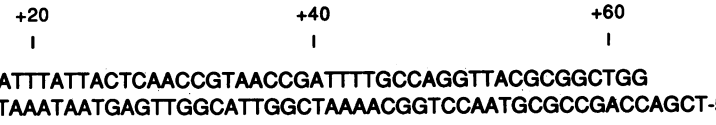



FIG. 2. *att*Tn*7* oligonucleotide. Sequence of oligonucleotide synthesized to create pEG30 [*att*Tn*7*(+7 to +64)], as described in Materials and Methods.

*Bam*HI-*Sst*I site of pSP64 (Promega Biotech). pEG30 [*att*Tn*7*(+7 to +64)] was constructed by synthesizing the *att*Tn*7* oligonucleotide shown in Fig. 2.

**Assay of Tn*7* transposition frequency.** *att*Tn*7* target activity was evaluated using a Tn*7* lambda hop assay (31) in which a Tn*7*-end derivative, containing the essential *cis*-acting transposition sequences of Tn*7*, transposes from an integration- and replication-defective lambda derivative upon infection into cells containing chromosomal Tn*7*, which supplies the Tn*7*-encoded transposition proteins. Assays were performed using lambda KK1 [lambda 780 *hisG9424*::Tn*10* *del*16 *del*17::*att*Tn*7*(−342 to +165)::Tn*7*L166-kanamycin resistance-Tn*7*R199) as the donor phage (31). *att*Tn*7*-containing plasmids to be assayed were introduced into the Tn*7*-containing strain LA3. A culture of cells to be assayed was grown to mid-log phase in LB broth plus 0.2% maltose, recovered by centrifugation, and suspended at about $1.6 \times 10^9$ cells per ml in 0.01 M $MgSO_4$. Phage was added at a multiplicity of 0.1 per cell, the resulting 0.2 ml of mixture was incubated without shaking at 37°C for 15 min, and 0.8 ml of LB broth plus 0.01 M sodium citrate was added and incubated with shaking for 1 h at 37°C. Samples from the mixture (or dilutions thereof in LB broth plus sodium citrate) were plated on kanamycin-trimethoprim plates and incubated for 1 day at 37°C before being counted. The transposition frequency is presented as transpositions (i.e., kanamycin-resistant colonies) per PFU.

**Mutagenesis of *att*Tn*7*.** Our strategy to isolate *att*Tn*7* mutants was to mutagenize pEG61 [p*lac*-*att*Tn*7*(+64 to −52)-*galK*], which has very low-level *galK* expression (white on MacConkey-galactose-carbenicillin indicator plates) because of the *glmS* transcription terminator within the *att*Tn*7* segment, and to isolate mutants with increased *galK* expression. (i) In vitro mutagenesis of pEG61 with hydroxylamine, which predominantly induces C-G to T-A transitions, was performed by the method of Freese as described by Busby et al. (10). Plasmid DNA isolated by a "mini-prep" procedure was incubated for 2 h at 75°C in 1 M hydroxylamine in a solution containing 50 mM sodium $PP_i$ (pH 7.0), 100 mM NaCl, and 2 mM EDTA. The DNA was then purified on an Elutip column (Schleicher & Schuell, Inc.), precipitated with ethanol, and resuspended. (ii) The mutagenized DNA was transformed into HB101(*galK*) cells, selecting for carbenicillin resistance. (iii) The transformants were screened by replica plating for those with increased *galK* expression (nonwhite on MacConkey-galactose-carbenicillin). (iv) Plasmid DNA was isolated from six nonwhite transformants and pooled; the *att*Tn*7* fragments were excised by digestion with *Sst*I and *Bam*HI, introduced into the *Sst*I-*Bam*HI site of unmutagenized pKL600, and transformed into HB101 cells, selecting for carbenicillin resistance. (v) The transformants were screened by replica plating to identify those with increased *galK* expression (nonwhite on MacConkey-galactose-carbenicillin indicator plates). The recloning step (step iv) was carried out so as to allow the identification of mutations only within the *att*Tn*7* segment.

A total of 16 independent transformations with mutagenized pEG61 DNA were performed at step ii to allow isolation of independent mutations (the pools at step iv included only the products of one step ii transformation). In some cases, step v transformants with very different levels of *galK* expression were recovered; in these cases, we further analyzed both mutant types. When unmutagenized pEG61 was transformed into HB101, about 2,000 transformants (all white) were recovered. With equivalent samples of mutagenized pEG61, 160 to 800 transformants were recovered at step ii and the number of nonwhite colonies ranged from 0 to 45. We also note that both nonwhite and white transformants were recovered at step v, indicating that some of the nonwhite transformants identified at step iii reflected vector mutations. We recovered nonwhite step v transformants from 13 of the 16 step ii transformations.

## RESULTS

**Point of Tn*7* insertion located within the *glmS* transcription terminator.** The specific point of Tn*7* insertion in *att*Tn*7* lies within a region structurally reminiscent of both transcription terminators and of stabilizers of the 3′ ends of mRNA, a G+C-rich region of dyad symmetry, followed by several T residues (35, 38, 48; also see Fig. 1). Gay et al. (18) have shown that the 3′ ends of *glmS* mRNA extend to these T residues. However, this observation does not reveal whether the region of Tn*7* insertion acts as a transcription terminator or as a stabilizer of a *glmS* mRNA that actually terminates at some distance downstream and is then exonucleolytically processed. We directly examined the termination capacities of various fragments containing this region by introducing the fragments into a terminator probe vector (Table 1). We found that the fragment *att*Tn*7*(−52 to +64) substantially reduced downstream gene expression when transcription enters this fragment from *att*Tn*7*(+64) (line 3) but has little detectable effect on downstream gene expression when transcription enters this fragment from *att*Tn*7*(−52) (line 4). As no promoters have been detected within the *att*Tn*7*(−52 to +64) segment (data not shown), such expression reflects readthrough of transcription from an upstream promoter. We also found that the fragment *att*Tn*7*(−4 to +64), which

TABLE 1. Properties of terminator probe plasmids containing *att*Tn*7* segments[a]

| Plasmid | Plasmid feature | Color on MacConkey-galactose-carbenicillin |
|---|---|---|
| pUC | No *galK* | White |
| pKL600 | p*lac*-*galK* | Red |
| pEG61 | p*lac*-*att*Tn*7*(+64 to −52)-*galK* | White |
| pEG63 | p*lac*-*att*Tn*7*(−52 to +64)-*galK* | Red |
| pEG10 | p*lac*-*att*Tn*7*(+64 to −4)-*galK* | Red |
| pEG11 | p*lac*-*att*Tn*7*(−4 to +64)-*galK* | Red |

[a] HB101 cells containing the indicated plasmid were plated on MacConkey-galactose-carbenicillin plates and incubated at 37°C for 18 h, and their color was observed.

TABLE 2. Effect of transcription on *att*Tn*7* target activity[a]

| Plasmid | *att*Tn*7* sequences | Transpositions/PFU (mean ± SEM [*n*]) |
|---|---|---|
| pKL600 | None | $<1 \times 10^{-6}$ (3) |
| pEG61 | p*lac*-*att*Tn*7*(+64 to −52) | $(2.5 \pm 0.2) \times 10^{-2}$ (3) |
| pEG63 | p*lac*-*att*Tn*7*(−52 to +64) | $(3.1 \pm 0.5) \times 10^{-2}$ (3) |
| pEG51 | *att*Tn*7*(+64 to −52) | $(3.1 \pm 0.1) \times 10^{-2}$ (3) |
| pEG57 | *att*Tn*7*(−52 to +64) | $(2.5 \pm 0.2) \times 10^{-2}$ (3) |
| pEG10 | p*lac*-*att*Tn*7*(+64 to −4) | $(3.0 \pm 0.1) \times 10^{-2}$ (3) |
| pEG11 | p*lac*-*att*Tn*7* (−4 to +64) | $(2.9 \pm 0.1) \times 10^{-2}$ (3) |
| pEG20[b] | p*lac*-terminator-*att*Tn*7* (+64 to −4) | $(2.5 \pm 0.1) \times 10^{-2}$ (3) |
| pEG21[b] | p*lac*-terminator-*att*Tn*7* (−4 to +64) | $(2.8 \pm 0.1) \times 10^{-2}$ (3) |

[a] The target activity of LA3 cells containing the indicated plasmids was determined using the Tn*7* lambda hop assay as described in Materials and Methods.

[b] Introduction of the *trpA* terminator between p*lac* and *galK* results in a white phenotype in HB101 cells on indicator plates; thus, transcription termination is highly efficient and few (if any) transcripts from p*lac* reach the *att*Tn*7* segments.

contains the G+C-rich dyad but lacks the downstream T residues, had little detectable effect on downstream gene expression in either orientation (lines 5 and 6). These results demonstrate that the *att*Tn*7*(−52 to +64) segment does encode a transcription terminator that is active when transcription enters the *att*Tn*7* region from *att*Tn*7*(+64), the same direction in which *glmS* transcripts enter this region (Fig. 1). They also suggest that the G+C-rich dyad, followed by T residues, provides the terminator, a view supported by further mutational analysis of *att*Tn*7* (see below). As also described below, we have exploited the *glmS* transcription terminator to examine the nucleotide sequence requirements of the capacity of this region to provide a highly reactive and specific target for Tn*7* insertion.

**Role of transcription in *att*Tn*7* target activity.** Since the point of Tn*7* insertion in *att*Tn*7* lies within the *glmS* transcription terminator, it is of interest to determine whether transcription of *att*Tn*7* influences its activity as a target for Tn*7* insertion. We have previously shown that *att*Tn*7* target activity is independent of the direction of transcription entering the *att*Tn*7* region (31). To determine whether such transcription is required for *att*Tn*7* target activity, we compared *att*Tn*7* target activity when transcription did and did not enter the *att*Tn*7* region. The target activity of several *att*Tn*7* fragments was equivalent in the presence and absence of transcription into the *att*Tn*7* region (compare lines 2 and 3 to 4 and 5 and compare lines 6 and 7 to 8 and 9; Table 2). Physical analysis of the transposition products obtained in the assays presented in Table 2 by restriction enzyme digestion showed that the Tn*7*-end derivative inserted in a site- and orientation-specific manner into *att*Tn*7* in all cases (data not shown). These experiments suggest that the target activity of *att*Tn*7* is not influenced by transcription of *att*Tn*7*. We also note that the target activity of an *att*Tn*7* segment containing an active transcription terminator at the point of Tn*7* insertion (line 2) is equivalent to the target activity of an *att*Tn*7* segment that lacks an active terminator (lines 6 and 7). Thus, *att*Tn*7* target activity is also independent of efficient transcription termination at the point of Tn*7* insertion.

The ends of many transposable elements encode outward-firing promoters that can activate the expression of genes adjacent to the transposon (14, 20, 25). By analysis of *galK* gene expression in the transposition products obtained in the experiments shown in lines 4 and 5 of Table 2, we have been unable to detect any highly active outward-firing promoters within the ends of Tn*7* that might provide transcriptional activation of adjacent genes (data not shown). We note that such a promoter may be positioned within intact Tn*7* so that it is not present in the Tn*7*-end derivative sequences.

**Mutagenesis of *att*Tn*7*.** We have previously established that the segment *att*Tn*7*(−3 to +64) has *att*Tn*7* target activity, i.e., promotes the high-frequency site- and orientation-specific insertion of Tn*7* (31). Which nucleotides within this region are essential to *att*Tn*7* target activity? Deletion analysis has shown that at least some essential nucleotides lie within *att*Tn*7*(+39 to +64) (31). To further define critical *att*Tn*7* nucleotides, we have taken advantage of the transcription termination capacity of the *att*Tn*7*(+64 to −52) segment. We isolated mutants with decreased transcription termination activity, hypothesizing that such mutations would likely lie in the *glmS* transcription terminator, i.e., the G+C-rich dyad region into which Tn*7* insertion occurs, and examined the *att*Tn*7* target activity of these mutants.

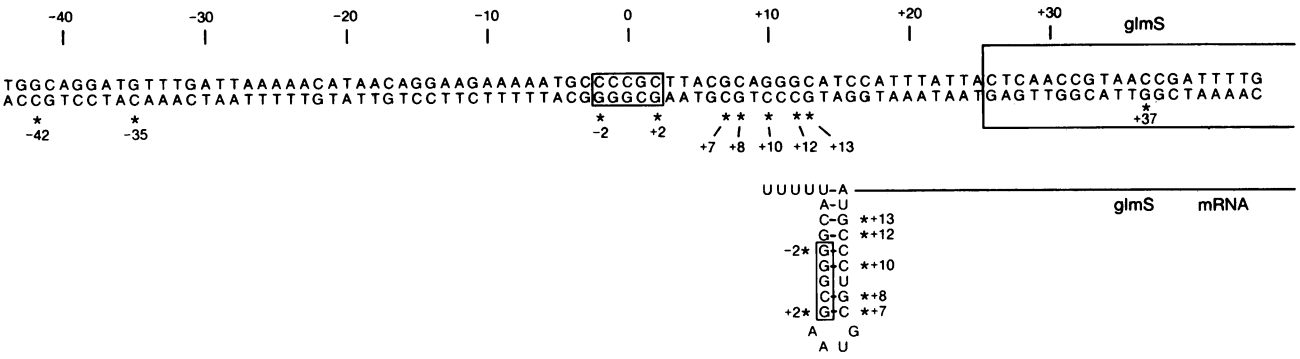
As described above, introduction of *att*Tn*7*(+64 to −52) into a terminator probe vector between a strong promoter and the downstream reporter gene *galK* efficiently blocks detectable *galK* expression. As described in detail in Materials and Methods, we isolated derivatives of *att*Tn*7*(+64 to −52) that allowed higher levels of *galK* expression. The 17 *att*Tn*7* mutants recovered and their *galK* expression phenotypes are shown in Table 3. The mutants show a wide range of *galK* expression. By comparing *galK* expression, we were able to identify five distinct mutant phenotypes (classes I through V). In some cases, only a small increase in *galK* expression was observed (class I), and in other cases, there was a marked increase in *galK* expression (class V). We determined the nucleotide sequence of the *att*Tn*7* segment in the mutants; each had a single-base-pair change (Fig. 3). This sequence analysis revealed 10 different mutations among the 17 mutants; some independently isolated mutants had the same base pair change. For example, mutants pEG61/7-1, pEG61/9-1, and pEG61/13-1 all contain a G-to-A change at *att*Tn*7*(+7). As expected, mutants with the same genotypic change had identical phenotypes. For example, the aforementioned *att*Tn*7*(+7) mutants displayed the same level of *galK* expression (Fig. 3).

TABLE 3. Properties of *att*Tn*7*(+64 to −52) mutant plasmids[a]

| Mutant class | Appearance | Plasmid[b] |
|---|---|---|
| | Red, intense halo | pKL600 |
| | White, no halo | pEG61 |
| I | Very light pink, no halo | pEG61/5-2 |
| II | Light pink, no halo | pEG61/4-1 |
| III | Light pink, faint halo | pEG61/2-1, pEG61/7-1, pEG61/9-1, pEG61/11-1, pEG61/13-1 |
| IV | Light pink, moderate halo | pEG61/1-1, pEG61/8-2 |
| V | Dark pink, intense halo | pEG61/3-1, pEG61/5-1, pEG61/6-1, pEG61/7-2, pEG61/8-1, pEG61/10-1, pEG61/12-1, pEG61/13-2 |

[a] HB101 cells containing the indicated plasmids were plated on MacConkey-galactose-carbenicillin plates and incubated for 18 h at 37°C. The color given refers to the colony color and the description of the halo refers to precipitate formed in the plates around the colonies.

[b] For the mutant plasmids, the first number indicates the step ii transformation (see Materials and Methods) from which the mutant was obtained and the second number indicates the isolate number.




| Downstream | | | Stem + Loop | | | Upstream | | |
|---|---|---|---|---|---|---|---|---|
| Change | Plasmid | Class | Change | Plasmid | Class | Change | Plasmid | Class |
| -42(G→A) | pEG61/5-2 | I | -2(C→T) | pEG61/3-1 | V | +37(C→T) | pEG61/1-1 | IV |
| -35(G→A) | pEG61/4-1 | II | +2(C→T) | pEG61/5-1 | V | | pEG61/8-2 | IV |
| | | | +7(G→A) | pEG61/7-1 | III | | | |
| | | | | pEG61/9-1 | III | | | |
| | | | | pEG61/13-1 | III | | | |
| | | | +8(C→T) | pEG61/6-1 | V | | | |
| | | | | pEG61/8-1 | V | | | |
| | | | +10(G→A) | pEG61/2-1 | III | | | |
| | | | | pEG61/11-1 | III | | | |
| | | | +12(G→A) | pEG61/10-1 | V | | | |
| | | | | pEG61/12-1 | V | | | |
| | | | | pEG61/13-2 | V | | | |
| | | | +13(C→T) | pEG61/7-2 | V | | | |

FIG. 3. *att*Tn*7* mutations. The upper line is the sequence of the region surrounding the point of Tn*7* insertion in *att*Tn*7* and is numbered as described in the legend to Fig. 1. The 5-bp sequence, usually duplicated upon Tn*7* insertion, and the protein-coding sequences at the terminus of *glmS* gene are indicated (narrow and wide boxes, respectively). The next line is the 3′ end of *glmS* mRNA with its proposed transcription terminator. The positions of the *att*Tn*7* mutations generated as described in the text are (*) indicated. The lower portion of the figure gives the positions of the *att*Tn*7* mutations with the base changed (top strand), the mutant plasmid containing each mutation, and the phenotype of mutant plasmids, using the designations of Table 3.

Most (7 of 10) of the mutations that allowed increased downstream *galK* expression occurred in the potential G+C-rich stem-and-loop structure (Fig. 3). This strongly suggests that this potential stem and loop and its following T residues are indeed the transcriptional terminator of the *glmS* gene. Mutational analyses of other transcriptional terminators have given similar results (12, 38, 48).

Several (3 of 10) mutations within the *att*Tn*7* segment that allowed increased downstream *galK* expression occurred at positions other than the G+C-rich dyad (Fig. 3). Mutants pEG61/5-2 and pEG61/4-1 contain changes considerably downstream [*att*Tn*7*(−42) and *att*Tn*7*(−35), respectively] of the G+C-rich dyad, and mutants pEG61/1-1 and pEG61/8-2 are both altered in an upstream base pair at *att*Tn*7*(+37). One possible explanation for the observed phenotypes is that these nucleotide changes create new promoters within the *att*Tn*7* segment. Alternatively, nucleotides other than those within the G+C-rich dyad may be involved in transcription termination (12, 48). We note that inspection of the sequence of the *att*Tn*7* region has not revealed any alternative stem-loop structures which might participate in an attenuationlike mechanism (26).

**Effects of *att*Tn*7* point mutations on *att*Tn*7* target activity.** A major purpose in this work was to use the *att*Tn*7* base pair changes obtained by the mutagenesis described above to identify nucleotides within the *att*Tn*7*(−52 to +64) segment critical to the capacity of this segment to act as a target for Tn*7* insertion. We found that base pair changes at *att*Tn*7*(−42) (line 2) and *att*Tn*7*(−35) (line 3) had no effect on the frequency of Tn*7* transposition to these *att*Tn*7* sites (Table 4); we also found, by restriction enzyme analysis of insertion products obtained in these assays, that the Tn*7*-end derivative inserted in a site- and orientation-specific manner into these *att*Tn*7* segments (data not shown). These results are consistent with other work from this laboratory (31), which has shown that the nucleotides critical for *att*Tn*7* target activity are contained within *att*Tn*7*(−3 to +64).

We have found that a mutation at *att*Tn*7*(+37) dramatically reduced the frequency of Tn*7* transposition (lines 17 and 18, Table 4). Although the frequency of transposition to such mutant *att*Tn*7* sites was reduced, restriction enzyme digestion revealed that insertion occurred in a site- and orientation-specific fashion (data not shown). Thus, *att*Tn*7*(+37) is a nucleotide critical to *att*Tn*7* target activity. We have previously shown that at least some nucleotides within *att*Tn*7*(+39 to +64) are also critical to *att*Tn*7* target activity (31).

The most striking finding from these experiments was that changes in base pairs immediately surrounding the point of insertion [from *att*Tn*7*(−2) to *att*Tn*7*(+13); lines 4 through 16, Table 4) did not alter the frequency of Tn*7* insertion to these *att*Tn*7* sites and that insertion into these sites occurred in a site- and orientation-specific fashion (data not shown). It is particularly notable that changes at *att*Tn*7*(−2) (line 4) and

TABLE 4. Target activity of mutant *att*Tn*7*(+64 to −52) segments[a]

| Plasmid | *att*Tn*7*(+64 to −52) segment | Transpositions/PFU (mean ± SEM [*n*]) |
|---|---|---|
| pEG61 | Wild type | $(2.0 \pm 0.4) \times 10^{-2}$ (6) |
| pEG61/5-2 | *att*Tn*7*(−42) (G → A) | $(3.0 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/4-1 | *att*Tn*7*(−35) (G → A) | $(2.1 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/3-1 | *att*Tn*7*(−2) (C → T) | $(2.8 \pm 0.1) \times 10^{-2}$ (3) |
| pEG61/5-1 | *att*Tn*7*(+2) (C → T) | $(3.3 \pm 0.4) \times 10^{-2}$ (3) |
| pEG61/7-1 | *att*Tn*7*(+7) (G → A) | $(1.3 \pm 0.2) \times 10^{-2}$ (3) |
| pEG61/9-1 | *att*Tn*7*(+7) (G → A) | $(3.8 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/13-1 | *att*Tn*7*(+7) (G → A) | $(2.9 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/6-1 | *att*Tn*7*(+8) (C → T) | $(1.5 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/8-1 | *att*Tn*7*(+8) (C → T) | $(1.5 \pm 0.5) \times 10^{-2}$ (3) |
| pEG61/2-1 | *att*Tn*7*(+10) (G → A) | $(3.0 \pm 0.5) \times 10^{-2}$ (3) |
| pEG61/11-1 | *att*Tn*7*(+10) (G → A) | $(3.4 \pm 0.3) \times 10^{-2}$ (3) |
| pEG61/10-1 | *att*Tn*7*(+12) (G → A) | $(3.6 \pm 0.6) \times 10^{-2}$ (3) |
| pEG61/12-1 | *att*Tn*7*(+12) (G → A) | $(3.8 \pm 0.4) \times 10^{-2}$ (3) |
| pEG61/13-2 | *att*Tn*7*(+12) (G → A) | $(1.2 \pm 0.2) \times 10^{-2}$ (3) |
| pEG61/7-2 | *att*Tn*7*(+13) (C → T) | $(3.2 \pm 0.6) \times 10^{-2}$ (3) |
| pEG61/1-1 | *att*Tn*7*(+37) (C → T) | $(1.4 \pm 0.5) \times 10^{-4}$ (5) |
| pEG61/8-2 | *att*Tn*7*(+37) (C → T) | $(1.0 \pm 0.1) \times 10^{-4}$ (4) |

[a] The target activity of LA3 cells containing the indicated plasmids was determined using the Tn*7* lambda hop assay as described in Materials and Methods.

at *att*Tn*7*(+2) (line 5), base pairs located within the 5-bp sequence usually duplicated upon Tn*7* insertion (18, 28, 31), had no detectable effect on *att*Tn*7* target activity. These results suggest that the nucleotides immediately surrounding the specific point of Tn*7* insertion are not essential to *att*Tn*7* target activity.

**Nucleotides essential for *att*Tn*7* target activity contained within *att*Tn*7*(+7 to +64).** The experiments presented above suggest that all the nucleotides required for *att*Tn*7* target activity lie rightwards of the specific insertion point, i.e., within *att*Tn*7*(+14 to +64). To directly test this hypothesis, we evaluated the target activity of an *att*Tn*7*(+7 to +64) segment contructed by oligonucleotide synthesis. As determined by the Tn*7* lambda hop assay (see Materials and Methods), we found that the frequency of transposition to *att*Tn*7*(+7 to +64) was equivalent to the frequency of Tn*7* transposition to *att*Tn*7*(−52 to +64) [$(4.6 \pm 0.3) \times 10^{-3}$ and $(4.6 \pm 1.1) \times 10^{-3}$ transposition per PFU for pEG30 and pEG31, respectively]. Previous work has shown that Tn*7* insertion into *att*Tn*7* is accompanied by a 5-bp duplication, usually of the sequences *att*Tn*7*(−2 to +2) (18, 28, 31). DNA sequence analysis of six independent insertions into *att*Tn*7*(+7 to +64) revealed that all the insertions occurred in an orientation-specific fashion and were accompanied by a 5-bp duplication of target sequence at positions nearly spatially identical to the positions of Tn*7* insertions into larger *att*Tn*7* segments (Fig. 4). With three insertions, the target sequences duplicated in *att*Tn*7*(+7 to +64) were equivalent in position to *att*Tn*7*(−2 to +2), i.e., of *att*Tn*7*"(−2 to +2)"; in two cases, the duplication was of *att*Tn*7*"(−1 to +3)"; and in one case, the duplication was of *att*Tn*7*"(+1 to +5)". We note that we have previously observed a small degree of wobble at the point of insertion into larger *att*Tn*7* fragments (31). We conclude that sequences essential for *att*Tn*7* target activity are contained within *att*Tn*7*(+7 to +64) and that the sequence of the nucleotides immediately surrounding the Tn*7* insertion point is irrelevant to *att*Tn*7* target activity.

## DISCUSSION

Tn*7*, unlike most other transposable elements (14, 20, 25), can insert at high frequency into a specific target site, *att*Tn*7* (4, 5, 27). Other mobile DNA segments, such as bacterio-

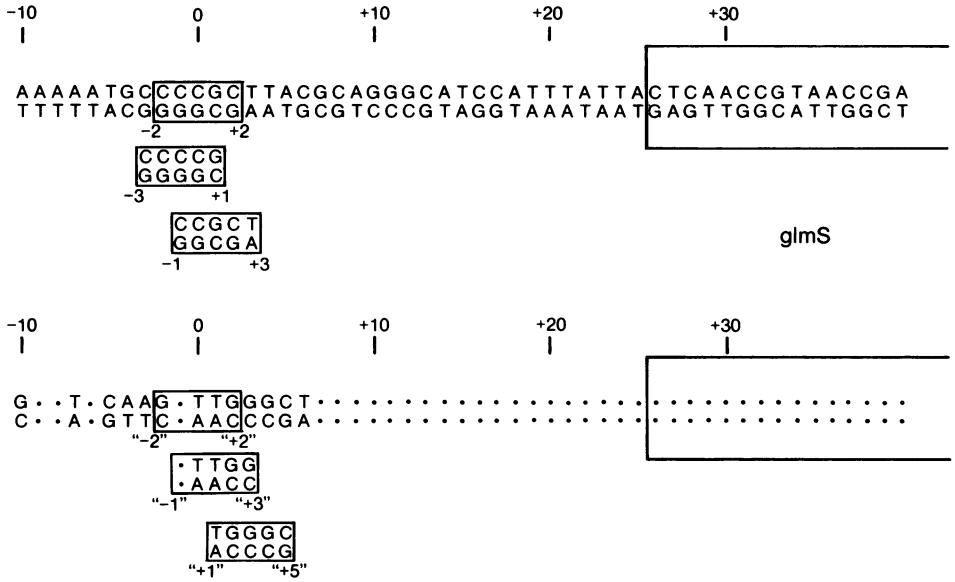



FIG. 4. Positions of Tn*7* insertions. The upper line is the sequence of intact *att*Tn*7*, and the lower line is the sequence of the *att*Tn*7*(+7 to +64) region in pEG30. Positions of identity with intact *att*Tn*7* are indicated (*); the sequence of nonidentical positions is shown directly. These sequences are numbered as described in the legend to Fig. 1. The protein-coding sequence at the terminus of *glmS* is indicated (wide box). The narrow boxed 5-bp sequences are those duplicated upon Tn*7* insertion. The duplications with intact *att*Tn*7* (below upper line) were determined in this laboratory (31); over 90% of the insertions occurred at *att*Tn*7*(−2 to +2). The boxed 5-bp sequences below the lower line are the duplications observed upon insertion into *att*Tn*7*(+7 to +64) (pEG30). Quotation marks indicate vector sequences in positions corresponding to *att*Tn*7* sequences. In three cases, duplication of *att*Tn*7*"(−2 to +2)" occurred; in two cases, duplication of *att*Tn*7*"(−1 to +3)" occurred; and in one case, duplication of *att*Tn*7*"(+1 to +5)" occurred.

phage lambda, also recognize specific insertion sites. However, such elements employ DNA-DNA homology between the recombining sites to direct insertion and insert via conservative site-specific recombination (39, 47). Tn*7* insertion into *att*Tn*7* is distinctive, because it results from a transposition reaction in which the ends of Tn*7* (19, 28), which participate directly in transposition, are structurally unrelated to *att*Tn*7* (18, 31).

Our results suggest that the nucleotide sequence immediately surrounding the specific point of Tn*7* insertion is irrelevant to *att*Tn*7* target activity. We have shown that *att*Tn*7*(+7 to +64), which lacks the sequences found at the specific insertion point of intact *att*Tn*7*, has the capacity to promote Tn*7* insertion at a frequency indistinguishable from those of much larger *att*Tn*7* fragments. Like insertion into larger *att*Tn*7* segments, Tn*7* insertion into *att*Tn*7*(+7 to +64) occurs in an orientation-specific fashion and is accompanied by duplication of a 5-bp pair chromosomal sequence. The positions of Tn*7* insertion in both cases are nearly spatially identical. We have also shown that a number of single-base-pair changes within the *att*Tn*7*(−2) to *att*Tn*7* (+13) region do not alter *att*Tn*7* target activity. These results suggest that the nucleotides critical to *att*Tn*7* activity lie at least 12 bp away from the point of insertion. We have also directly identified a particular nucleotide, *att*Tn*7*(+37), which is critical to *att*Tn*7* target activity. We have previously shown that at least some nucleotides within *att*Tn*7* (+39 to +64) are also essential to *att*Tn*7* target activity (31). The emergent picture of the Tn*7* transposition machinery which directs Tn*7* to *att*Tn*7* is that it operationally resembles certain restriction enzymes whose specific recognition sequences and sites of DNA cleavage are displaced from each other (2, 21). A notable difference between these two reactions is the number of proteins involved; while recognition and cleavage of DNA sequences within *att*Tn*7* are probably performed by multiple *tns* proteins, these functions are carried out by a single protein in the case of the restriction enzymes.

Interestingly, at least some of the nucleotides essential to *att*Tn*7* target activity are contained within the protein-coding sequence of the bacterial *glmS* gene (31 and this work). Specific sites of Tn*7* insertion have been observed in a number of bacteria (3, 7, 8, 11, 15, 23, 43, 44, 49). An attractive hypothesis (31) is that these sites reflect conservation of the *glmS* gene, which contains the essential *att*Tn*7* nucleotides within its coding sequence. Recent work by I. Qadri, C. Flores, and C. Lichtenstein (personal communication) has shown that specific Tn*7* insertion sites in other bacteria are located near sequences homologous to *E. coli glmS*. Why is a reactive and specific site for Tn*7* insertion provided by the *glmS* gene? Although it is not clear why this gene in particular is used, it is not unreasonable to suggest that a specific insertion site is advantageous to Tn*7*, as it provides a "safe" haven into which insertion can occur without lethal effects on the recipient bacterium.

Because the nucleotides required for *att*Tn*7* target activity are apparently distinct from the point of insertion, Tn*7* insertion into *att*Tn*7* does not physically disrupt nucleotides critical to *att*Tn*7* target activity. However, Tn*7* insertions into *att*Tn*7*::Tn*7* have not been observed (L. Arciszewska, K. Kubo, and N. L. Craig, unpublished observation). What prevents such multiple Tn*7* insertions into *att*Tn*7*? The presence of the ends of Tn*7* in a target replicon substantially reduces the frequency of subsequent insertion of Tn*7* anywhere within the target replicon (22; L. Arciszewska and N. L. Craig, unpublished observation). This phenomenon, called transposition immunity (14, 20, 25), has also been observed with some other transposons. It is tempting to speculate that transposition immunity provided by the ends of Tn*7* inactivates *att*Tn*7* upon Tn*7* insertion, thereby preventing multiple Tn*7* insertions into *att*Tn*7*.

In this work, we have also shown that the specific point of Tn*7* insertion lies within the transcriptional terminator of the *E. coli glmS* gene. This possibility was first suggested by the work of Gay et al. (18), who showed that the 3′ ends of *glmS* mRNA extend to near the Tn*7* insertion point. Their experiments, however, did not reveal whether these 3′ ends were generated by transcription termination or by RNA processing. The *glmS* transcription terminator is structurally reminiscent of simple transcription terminators which provide RNAs containing a G+C-rich region of dyad symmetry, followed by a string of U residues (12, 38, 48). Our analysis of the *att*Tn*7* region, like other analyses of transcription terminators with similar structures (12, 48), has identified nucleotides within this stem and loop and the downstream T residues as critical to transcription termination. Our mutational analysis has also identified nucleotides not in the stem and loop structure whose alteration changes downstream gene expression. These nucleotides may lie within recognition sites for cellular transcription factors (12, 48). Alternatively, these mutations may provide new promoters.

It has been suggested that transcription of recombination sites that direct other DNA rearrangements, such as mating-type switching in *Saccharomyces cerevisiae* (24) and immunoglobulin gene assembly (6, 41), influences recombination. The mechanism of this effect, direct or indirect, is unknown. We have observed no alterations in *att*Tn*7* target activity by variations in transcription or transcription termination. Although it is tempting to speculate that the location of a highly specific and reactive site for Tn*7* insertion in the transcription terminator of a gene intimately involved in cellular metabolism may provide a coupling of transposition to cell growth, our experiments provide no support for this view. Gay et al. (18) have suggested a different linkage between Tn*7* transposition and *glmS* transcription. They found that in *att*Tn*7*::Tn*7*, the chromosomal *glmS* terminator is destroyed and that *glmS* transcripts end about 100 bp into the end of Tn*7*, slightly beyond a promoter for a Tn*7*-encoded transposition gene. Gay et al. (18) suggest that the activity of this Tn*7* promoter may be affected through promoter occlusion (1) by the level of *glmS* transcription. Thus, alterations in *glmS* transcription might affect Tn*7* transposition through the expression of Tn*7*-encoded transposition genes, rather than by changing the reactivity of *att*Tn*7*.

In summary, this work has shown that sequences required for *att*Tn*7* target activity are distinct and separate from the actual point of Tn*7* insertion. It will be interesting to determine how sequences displaced from the specific site of insertion direct Tn*7* to that site.

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