Preferential Transposition of an IS630-Associated Composite Transposon to TA in the 5'-CTAG-3' Sequence

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A composite transposon, Tn4731, associated with IS630 has been shown to transpose preferentially to 5'-TA-3' sequences that are located at two sites in a rho-dependent transcription terminator in plasmid CoEl in Escherichia coli (T. Tenzen, S. Matsutani, and E. Ohtsubo, J. Bacteriol. 172:3830–3836, 1990). Here we demonstrated that Tn4731 preferentially transposes to TA sequences at four sites in plasmid pUC118 and its derivatives: the TA sequence (hot spot I) in the intergenic region of phage M13 within the pUC sequence, the TA sequence (hot spot II) in the XbaI site in multiple cloning sites of the lacZ coding region, the TA sequence (hot spot III) in a spacer region flanked by inverted repeat sequences of a transcription terminator located downstream of the bla gene, and the TA sequence (hot spot IV) in the middle of the lacZ promoter. Transposition of Tn4731 to hot spot III was found to require the inverted repeat sequences, which is located immediately downstream of the lacZ promoter, was not affected by mutations introduced into the promoter. There appear to be no particular sequences important for transposition of Tn4731 around each of the hot spots, except a palindromic sequence, 5'-CTAG-3', that contains the target sequence. Mutations introduced within the CTAG sequence at a hot spot inhibited Tn4731 from transposing to it, indicating that the CTAG sequence is responsible for the preferential transposition of Tn4731.

MATERIALS AND METHODS

Bacterial strains, plasmids, and a phage. Bacterial strains used were E. coli K-12 derivatives JE5519 (24, 25), MV1184 (30), and BW313 (13, 14).

Plasmid pTT4 was described previously (29). pUC118 and pUC119 (30) were purchased from Takara Shuzo. Helper phage M13K07 (Takara Shuzo) was used for site-directed mutagenesis as described by Kunkel et al. (14) to construct pUC118 derivatives.

Media. Culture media were used L broth, L-rich broth, medium (31), and 2× YT broth (21). L-agar plates contained 1.5% agar (Eiken) in L broth. Antibiotics were added to L broth or L-agar plates, if required, as follows: 150 µg of ampicillin (Wako) per ml, 5 to 8 µg of tetracycline (Sigma Chemical Co.) per ml, and 70 µg of kanamycin (Sigma) per ml. Dilution buffer (0.1% tryptone [Difco], 0.3% NaCl) was used for diluting the cell cultures.

Enzymes. Restriction endonucleases used were EcoRI, XbaI (Takara Shuzo), and Mael (Boehringer Mannheim). Phage T4 DNA ligase (Takara Shuzo) and RNase A (Sigma) were also used. The reaction conditions for these enzymes were as recommended by the suppliers.

DNA preparation. An E. coli strain harboring a plasmid was grown in L broth or L-rich broth. The alkaline lysis method (18) or the method of Ohtsubo et al. (23) was used to prepare plasmid DNA for DNA sequencing. The crude lysis method (17) was used to isolate a small amount of plasmid DNA.

Transposition assay. recA strain JE5519 harboring pTT4 and each of the recipient plasmids (pUC118, pUC119, pTT70, pTT71, pTT75, pTT80, pTT81, and pTT82) was prepared by transformation with L-agar plates containing tetracycline and ampicillin, as described previously (29). JE5519 harboring the two plasmids was inoculated in 5 ml of L broth and grown at 30°C overnight. The culture was diluted 106-fold, and 0.1 ml of the diluted culture was

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inoculated into each of 25 culture tubes containing 5 ml of L broth. After incubation at 30°C for 0.2 ml of the culture from each tube was spread onto a plate containing tetracycline (8 μg/ml), and the plates were incubated at 42°C for 2 days for selection of colonies of cells harboring the recipient plasmid having Tn4731.

Only one colony was picked up from each plate, and cells were grown in L-rich broth at 37°C overnight. Plasmids were extracted and analyzed for their sizes by 0.7% agarose gel electrophoresis. A few plasmids which had the same size as pTT4 were revertants of pTT4 (about 12.4 kb in size) for temperature sensitivity and could replicate at 42°C. The rest of the plasmids were larger than parental plasmids. Most of them were recipient plasmids carrying Tn4731 (about 14.7 kb) (Fig. 1); the others were cointegrates (about 16.5 kb), formed between pTT4 carrying IS element IS102 (26) and the recipient plasmid, in which IS102 was duplicated at the junctions of the two plasmid sequences. The cointegrates were readily distinguished from the plasmids carrying Tn4731 by digestion with EcoRI, as described previously (29). Only the plasmids with Tn4731 were further analyzed as described in Results. Frequency of transposition of Tn4731 was calculated from the number of colonies of cells harboring the plasmids with Tn4731 by a fluctuation test (16, 24).

Nucleotide sequencing. The dyeoxy chain termination method of DNA sequencing (21, 27) was employed. The plasmid DNA containing Tn4731 was used as template, and synthetic oligonucleotides which could hybridize near the site of transposition were used as primers (Table 1). To sequence the plasmid DNA, a 2'-deoxy-7-deazaguanosine triphosphate sequencing kit (Takara Shuzo) was used, and the ingenting DNA chains were labeled with [α-32P]dCTP (15 TBq/nmol) (Amersham). The labeled DNA fragments were electrophoresed in 6% acrylamide gels containing 8 M urea.

Mutagenesis of pUC118. The method of mutagenesis of pUC118 with strain BW313 was described previously (13, 14). Primers TER1, TER2, and PRO1 (Table 1) were hybridized to single-stranded pUC118 DNA to make pTT70, pTT77, and pTT75, respectively. Primers TER80, TER81, and TER82 were hybridized to single-stranded pTT70 DNA to make pTT80, pTT81, and pTT82, respectively. For the reaction, Muta-Gene (Bio-Rad) was used. Mutations in pUC118 or pTT70 were confirmed by nucleotide sequencing.

RESULTS

Transposition of IS630-associated transposon Tn4731 to pUC plasmids and locations of target sites. We have previously reported that the tetracycline resistance transposon Tn4731 in plasmid pTT4 (Fig. 1), a temperature-sensitive replication mutant originally derived from plasmid pSCI101, transposes to ColEl to give ColEl::Tn4731 plasmids, whose replication systems are temperature resistant and thus can

### Table 1. Primers used for sequencing and mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Position</th>
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</thead>
<tbody>
<tr>
<td>Blam1</td>
<td>ATGTAACGCGCCCTGATC</td>
<td>2496-2479</td>
</tr>
<tr>
<td>118 prime</td>
<td>GCTTTTCGTATGGCAAAGC</td>
<td>1882-1902</td>
</tr>
<tr>
<td>M4</td>
<td>GCTGCAGCTGCAACG</td>
<td>835-851</td>
</tr>
<tr>
<td>HISV1A</td>
<td>CTTCTCGAGGAAACCAT</td>
<td>187-203</td>
</tr>
<tr>
<td>TER1</td>
<td>TTCCACTAGGACGCTTAAAT</td>
<td>2025-2057</td>
</tr>
<tr>
<td>TER2</td>
<td>CACGTCGAGATTTACATGGTACCTA</td>
<td>2000-2032</td>
</tr>
<tr>
<td>TER80</td>
<td>ATCTTCAGGCTTTACGATGAAAA</td>
<td>2022-2044</td>
</tr>
<tr>
<td>TER81</td>
<td>GATCTCTAGGACGCTTAAAT</td>
<td>2023-2045</td>
</tr>
<tr>
<td>TER82</td>
<td>AGCTTCTCAGCTTAAAAGCATGATAA</td>
<td>2022-2047</td>
</tr>
<tr>
<td>PRO1</td>
<td>AGCGAGAATATAAGGTACCCTGGTGGTCC</td>
<td>998-1035</td>
</tr>
</tbody>
</table>

* Coordinate numbers to pUC118.

### Table 2. Transposition of Tn4731 to hot spots in pUC derivatives

<table>
<thead>
<tr>
<th>Recipient plasmid*</th>
<th>Transposition frequency/ division cycle</th>
<th>No. of plasmids with Tn4731 transposed to hot spotb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>pUC118</td>
<td>6.6 × 10^-8</td>
<td>2 (1)</td>
</tr>
<tr>
<td>pTT70</td>
<td>1.6 × 10^-7</td>
<td>3</td>
</tr>
<tr>
<td>pTT77</td>
<td>1.2 × 10^-7</td>
<td>0</td>
</tr>
<tr>
<td>pUC119</td>
<td>5.4 × 10^-8</td>
<td>0</td>
</tr>
<tr>
<td>pTT75</td>
<td>1.0 × 10^-8</td>
<td>1</td>
</tr>
<tr>
<td>pTT80</td>
<td>7.7 × 10^-8</td>
<td>2 (1)</td>
</tr>
<tr>
<td>pTT81</td>
<td>8.5 × 10^-8</td>
<td>1 (2)</td>
</tr>
<tr>
<td>pTT82</td>
<td>5.9 × 10^-8</td>
<td>1</td>
</tr>
<tr>
<td>ColEI</td>
<td>1.8 × 10^-9</td>
<td></td>
</tr>
</tbody>
</table>

* Plasmids pTT70, pTT71, and pTT75 were derived from pUC118. Plasmids pTT80, pTT81, and pTT82 were derived from pTT70.

* Transposition of Tn4731 to each hot spot was determined by nucleotide sequencing and by cleavage first with EcoRI and then with Mael and also with XbaI when Tn4731 transposed to hot spot II (Fig. 2). The numbers of plasmids examined by sequencing are in parentheses. pUC119 has a sequence containing the multiple cloning sites in the orientation opposite to the corresponding sequence in pUC118; thus, pUC119::Tn4731 plasmids show restriction patterns different from those of pUC118::Tn4731 derivatives, although the pUC119 derivatives with Tn4731 at each hot spot give unique restriction patterns, as observed in the pUC118 derivatives (Fig. 2).
To map the target sites for transposition, each of pUC118 derivatives carrying Tn4731 was digested with EcoRI, which cuts pUC118 at one site and cuts two sites within the Tn4731 sequence (Fig. 1), and electrophoresed in an agarose gel. Band patterns of the EcoRI fragments were identical to those from all pUC118::Tn4731 plasmids were of four kinds (Fig. 2), although each of the plasmids examined was isolated from an independent clone of cells. This result suggests that there exist hot spots (maybe four) for transposition of Tn4731 in the pUC sequence.

We then determined the exact positions of several target sites by sequencing junctions of Tn4731 with the pUC118 sequence and found that Tn4731 transposed to 5'-TA-3' sequences at four sites (here called hot spots I, II, III, and IV) to give rise to duplication of the TA sequence (Fig. 3 and Table 2). Hot spot I was located at the TA sequence within the intergenic region of phage M13 in pUC118; hot spot II was located at the TA sequence within the recognition sequence of restriction endonuclease MaeI in the multiple cloning sites; hot spot III was located at the TA sequence within a 6-bp spacer region flanked by 10-bp inverted repeat sequences, 60 bp downstream of the bla gene; and hot spot IV was located at the TA sequence within the bla gene.

None of the pUC118 derivatives carrying Tn4731, whose map position was assigned near the XbaI site by the EcoRI cleavage analysis, could be cleaved with XbaI. This indicates that Tn4731 in these plasmids transposed to hot spot II (Fig. 3 and Table 2). Note that the TA sequence in hot spot II as well as each in hot spots I, III, and IV is the core of a palindromic sequence 5'-CTAG-3' (Fig. 3B), which is the recognition site of restriction endonuclease MaeI. Cleavage analysis with MaeI showed that band patterns of the MaeI-digested fragments generated from all the pUC118::Tn4731 plasmids were essentially of four kinds (Fig. 2); a characteristic pair of fragments containing junctions of Tn4731 with pUC118 was generated, but a particular pair of the MaeI fragments of pUC118 which adjoined each MaeI site used as a transpositional target was not generated (see legend to Fig. 2). This indicates that Tn4731 has been transposed to TA in the four CTAG sequences almost exclusively in one orientation in pUC118 (see legend to Fig. 2).

Sequence requirement for the preferential transposition of Tn4731. We have previously observed that Tn4731 transposes preferentially to the TA sequence in each of the inverted repeat sequences of 13 bp in a rho-dependent transcription terminator in ColE1 (29). As described in the previous section, hot spot III in pUC118 was located in a 6-bp spacer sequence flanked by 10-bp inverted repeat sequences (Fig. 3B), which are entirely contained in the transcription terminator for bla (28). To test the possibility that the inverted repeats flanking a hot spot are responsible for preferential transposition of Tn4731, therefore, we introduced substitution mutations within each of the inverted repeat sequences flanking hot spot III and examined transposition of Tn4731 to the resulting mutant plasmids pTT70 and pTT71 (Fig. 4A). Tn4731 was found to transpose to hot spot III in these plasmids at even a slightly higher frequency than to the same site in pUC118 (Table 2). This result shows that the inverted repeats are not required for transposition of Tn4731 but rather inhibit it, suggesting that the transcription terminator in pUC118 is not responsible for the preferential transposition of Tn4731.

As for hot spot II, we examined transposition of Tn4731 to plasmid pUC119, which has the same sequence of 58 bp containing multiple cloning sites including the hot spot as that in pUC118 in the inverted orientation (30). Tn4731
found to transpose to hot spot II in pUC119 at a frequency similar to that in pUC118 (Table 2). This result indicates that the orientation of the sequence containing hot spot II is not important for transposition of Tn4731.

Bernardi and Bernardi (3) have reported that read-through transcription of the target sites enhances transposition of an insertion element. As described above, Tn4731 transposed to hot spot II, which is within the lacZ coding region in pUC118 or pUC119 and located immediately downstream of lac promoter. To test, therefore, whether transcription of hot spot II may have caused the preferential transposition of Tn4731 to the hot spot, we introduced substitution mutations in the −35 region of lac promoter in pUC118 to obtain a mutant plasmid, pTT75, with a completely different promoter consensus sequence (Fig. 4B). Tn4731 was found to transpose to hot spot II in pTT75 as well as to hot spots I, III, and IV at frequencies as high as those in pUC118 (Table 2). This indicates that transcription of hot spot II from lac promoter has little or no effect on transposition of Tn4731.

As described in the previous section, the four hot spots in pUC118 are the core of the CTAG sequence (Fig. 3). There seem to be no other common sequences in the region surrounding the palindromic sequences. It is, therefore, likely that the CTAG sequence is responsible for the preferential transposition of Tn4731. To prove this, we mutated the sequence in hot spot III in pTT70, in which Tn4731 could transpose most frequently (Table 2). A mutant plasmid, pTT80, has the CTAA sequence instead of CTAG in hot

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** FIG. 3.** Map locations of hot spots I, II, III, and IV and nucleotide sequences around the hot spots and a potential site for transposition of Tn4731 in pUC118. (A) Locations of hot spots. pUC118 contains multiple cloning sites within the lacZ coding frame. RNA primer for replication (RNA II) and the coding regions of lacZ and βl genes are shown by arrows. An intergenic region (IG), derived from M13 phage, is shown by a line. A dot represents the replication origin where DNA synthesis starts. An asterisk shows the potential integration site of Tn4731, where CTAG exists within the RNA II primer region. Abbreviations for cleavage sites of restriction endonucleases: E (EcoRI), X (XbaI), and M (MaeI). (B) Nucleotide sequences including hot spots. The dinucleotide TA boxed in each sequence of hot spots (I, II, III, and IV) is duplicated upon transposition of Tn4731. Thick solid lines near the target sites indicate palindromic sequences with the dyad axis in the middle of TA. A pair of inverted arrows in hot spot III shows 10-bp inverted repeat sequences of a transcription terminator (28). Positions of nucleotides are shown by the coordinate numbers to pUC118. Hot spots I to IV were sequenced by using primers HSIVA, M4 (purchased from Takara Shuzo), 118 prime, and Blal (Table 1), respectively.
spot which is within a transcription terminator downstream of the glmS gene in the *E. coli* chromosome (1, 6, 7, 15), but the termination function has also been shown not to be required for transposition of Tn7 (7, 20).

We have also shown here that the frequency of transposition of Tn4731 to hot spot II was not affected by mutations introduced into lac promoter which is located immediately upstream of the hot spot. This indicates that read-through transcription into hot spot II from the lac promoter did not change the frequency of transposition to the hot spot. However, we cannot rule out the possibility that read-through transcription of the target site from other promoters present in pUC plasmids still affects Tn4731 transposition.

Tn7 requires a sequence of about 50 bp located about 10 bp away from the hot spot (atTn7) for its transposition (7, 20). Some other insertion sites (pseudo-atTn7) share homology with the sequence required (12). Unlike for transposition of Tn7, however, we cannot find any homologous sequences in the neighborhood of the hot spots which appear to be important for transposition of Tn4731. We can find only a palindromic sequence, CTAG, which includes the target sequence TA, in the four hot spots in pUC118.

There are actually five CTAG sequences in pUC118 as well as in pUC119, one of which was not used as target for Tn4731 transposition. This exception is located within the region that is essential for the initiation of replication of the ColEl-type plasmids, including pUC plasmids (10) (Fig. 3). It is, therefore, quite likely that transposition of Tn4731 to this potential site could not be detected even if Tn4731 had transposed to it, since the resulting plasmid would not replicate. In ColEl, Tn4731 has been shown to transpose to the TA sequence which is the core of at least 4-bp palindromic sequences, such as TTAA and ATAT, in addition to CTAG (29). There are four CTAG sequences: three of them were used for transposition of Tn4731, and two of the three were hot spots located in a rho-dependent transcription terminator (29). The exception is located at the same essential region for replication of ColEl as that of pUC plasmids; thus, transposition of Tn4731 to this site might not be detected as well. There are 30 TTAA sequences and 31 ATAT sequences in ColEl, in which only three TTAA sequences and one ATAT sequence were used as targets for transposition of Tn4731. These and our present results indicate that TA in the CTAG sequence becomes a hot spot, although TA in the other 4-bp palindromic sequences is used as target very rarely. Our findings that mutations introduced within the CTAG sequence, including hot spot III in pUC, inhibited the transposition to it support this indication.

**DISCUSSION**

We have previously reported that Tn4731 always transposes to the dinucleotide 5'-TA-3' in ColEl. We have shown in this article that Tn4731 can transpose also to the TA sequence in pUC plasmids.

In our previous report, two hot spots for transposition of Tn4731 are present within each of the 13-bp stem sequences of a rho-dependent transcription terminator in ColEl (29). We have recently shown that the inverted repeats of the terminator in ColEl were not required for the preferential transposition of Tn4731 (our unpublished result). This and our present finding that transposition of Tn4731 to hot spot III in a transcription terminator in pUC118 does not require inverted repeats indicate that transcription termination is not a signal for the preferential transposition of Tn4731. It has been reported that transposon Tn7 has a transpositional hot

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**REFERENCES**