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Transcription and Translation of the Kanamycin Resistance Transposon Tn903

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Ribonucleic acid and protein syntheses directed by the kanamycin resistance transposon Tn903 were examined in both in vitro and in vivo systems. The structural gene conferring kanamycin resistance (kan) was actively transcribed and translated, and its expression was enhanced by the powerful promoter lacUV5. On the other hand, the structural gene for transposase (tnp) determining transposition function was rarely translated even when it was fused in-frame to the amino-terminal portion of lacZ gene under the control of lacUV5. By using various chimeric tnp genes, it was demonstrated that the amino-terminal 70 base-pair portion and central 520 base-pair portion of tnp as well as its regulatory regions negatively affected on expression.

KEY WORDS: Transposon/ Tn903/ Transposase/ Aminoglycoside 3'-phosphotransferase/ Maxicell/ Gene fusion/ Plasmid vector

INTRODUCTION

Plasmid-mediated antibiotic resistance in bacteria has often been shown to reside on a discrete DNA segment capable of transposing from one replicon to another in the bacterial cell (for reviews see refs 1, 2). One such transposable element Tn903 confers Km by coding for APH on its host bacterium. Tn903 can be transposed to many different sites even in small replicons, and it appears that no specific sequences are required for target sites. We have previously determined the entire nucleotide sequence of Tn903 and found that Tn903 is 3094 bp in length and at both extremities, possesses two identical inverted 1057 bp sequences named IS903. Combining these sequence data with genetic analyses of Tn903 together, a reading frame (kan) present at the central part (980 bp) flanked by two IS903s has been assumed to correspond to APH, and one (tnp) involved in each IS903 to putative TNP (Fig. 1). In order to identify these gene products, we have now examined syntheses of RNA and protein directed by Tn903 in both in vitro and in vivo systems. In addition, effects of a powerful promoter, lacUV5 (a mutant promoter of Escherichia coli lac operon) were studied on expression of kan and tnp. The results obtained indicate that kan is actively transcribed and translated and its expression is enhanced by lacUV5 to a reasonable

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† Abbreviations used: Km, kanamycin resistance; APH, aminoglycoside 3'-phosphotransferase; APH', modified APH; bp, base-pairs; kb, kilobases or kilobase-pairs; Km, kanamycin sensitivity; TNP, transposase; TNP', modified TNP.
Fig. 1. The structure of pAO65 including representative restriction sites is shown at the top, and those of various pAO65-derivatives are at the lower part. The structures are oriented with respect to the integration site of Tn903. Thick and thin bars indicate Tn903 and other moieties, respectively, and black bars show IS903. “UV5” with an arrow indicates insertion and transcriptional direction of the EcoRI-fragment that carries lacUV5 and the first 25 bp of lacZ derived from pKB252. Thin and broken arrows, respectively, under the pAO65 bar are coding proteins predicted from the nucleotide sequence and previously identified transcriptional products derived from pAO3. “A” to “E” over the pICR871 bar represent restriction fragments used for in vitro experiments. A DNA segment transferred from pAO142 to pAO195 is shown at the bottom.

degree. On the other hand, tnp was translated only at a very low efficiency even when it was placed under the control of the lacUV5 promoter or was fused in-frame to the amino-terminal portion of lacZ, which is downstream from lacUV5. The aminoterminal residue of APH was deduced from characterization of various fused APH derivatives.

MATERIALS AND METHODS

(a) General methods

Methods for bacterial transformation, preparation of plasmid DNA, restriction endonuclease digestion, repair synthesis, ligation, repair ligation, gel-electrophoresis with agarose and polyacrylamide, extraction of DNA fragments from gels, clone analysis, and DNA sequencing have been described previously.

(b) Culture media

The culture medium used was L broth and L agar unless otherwise noted. L broth contained 10 g of polypeptone (Daigo Eiyo), 5 g of yeast extract (Difco), 5 g of NaCl, 1 g of glucose and 20 mg of thymine per liter (pH 7.2). E broth used for preparations of plasmid DNA was composed of 10 g of polypeptone, 1 g of yeast extract, 10 g of glucose, 1 g of NH₄Cl, 3 g of NaCl, 0.1 g of Na₂SO₄, 0.1 g of MgCl₂ • 6H₂O, 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 5 mg of thiamine and 20 mg of thymine per liter.
M9-CAA medium used in labeling experiments contained 6 g of Na$_2$HPO$_4$, 3 g of KH$_2$PO$_4$, 5 g of NaCl, 1 g of NH$_4$Cl, 0.5 g of MgSO$_4$·7H$_2$O, 2 g of glucose, 0.01 g of CaCl$_2$, 5 mg of thiamine, 20 mg of thymine, 1 g of casamino acids (Difco) and 50 mg of L-tryptophan per liter.

(c) Bacteria, phages and plasmids

Escherichia coli K-12 strains used were C600 (F$^-$ thr leu thi lacY tonA supE44),$^8$ GM31 (F$^-$ thr leu dcm his thi galK galT xyl ml tronA tsx supE),$^9$ N1790 (F$^-{recA99}$ uvrA54 gal trp str),$^{10}$ JM109 (dlac$^-$ pro recA1 endA1 thi1 gyrA96 hsdRI7 relA1 supE44/F$^'$ carrying traD36 proA$^+$B$^+$ lacI$^q$ lacZAM15),$^{11}$ and HfrH5 (ColEI)/E1. Phages used were ${\phi}b$5,$^5$ M13mp7,$^{12}$ M13mp10 and M13mp11.$^{13}$ Plasmids used were pAO65,$^5$ pBR322$^{14}$ and pKB252.$^{15}$

(d) Synthetic linker-mediated ligation

About 700 pmoles of synthetic linker in a buffer (30 µl) containing 70 mM Tris.HCl (pH 7.6), 10 mM MgCl$_2$ and 5 mM dithiothreitol was heated for 2 min at 70°C followed by quick cooling. After addition of ATP (0.7 mM) and T4 polynucleotide kinase (5 units; Takara Shuzo Co., Ltd), the mixture was incubated for 1 h at 37°C, and then heated for 2 min at 70°C followed by slow cooling. The solution was mixed with a restriction fragment (about 20 pmoles) and T4 DNA ligase (5 units; Takara Shuzo Co., Ltd) in a reaction mixture (0.1 ml) containing 70 mM Tris.HCl (pH 7.6), 10 mM MgCl$_2$, 0.1 mM ATP and 10 mM dithiothreitol, and incubation was performed for 15 h at 12°C. The products were digested with the restriction endonuclease corresponding to the linker, and DNA fragments joined to the linker were separated by 5% polyacrylamide gel electrophoresis.

(e) RNA synthesis in vitro

RNA synthesis was carried out in a reaction mixture (0.1 ml) containing 40 mM Tris.HCl (pH 7.9), 8 mM MgCl$_2$, 150 mM KCl, 1 mM dithiothreitol, 0.05 mM [$\alpha$-32P]UTP (50 µCi; New England Nuclear), 0.1 mM each of three other NTPs, about 3 pmoles of template DNA and 8 units of E. coli RNA polymerase (New England Biolabs, Inc.). After incubation for 1 h at 37°C, the reaction was terminated by shaking with phenol. The aqueous layer was mixed with 25 µg of E. coli tRNA and passed through a Sephadex G100 column (0.6 cm by 20 cm), and fractions containing RNA was isolated.

(f) Labeling of plasmid-derived proteins in maxicells$^{16,17}$

N1790 cells harbouring a plasmid were grown in 2.5 ml of M9-CAA medium to a density of 2 × 10$^8$ cells/ml, and were irradiated with ultraviolet light (0.1 J/m$^2$/sec) for 20 sec. D-cycloserine (200 µg/ml) was added 1 h after irradiation and the cells were incubated for an additional 15 h. The cells were then collected by centrifugation and washed twice with M9 buffer. They were resuspended in half the original volume of M9 medium lacking sulfate, and incubated for 30 min. One µl of [35S]methionine (1100 Ci/mmol; 10 mCi/ml; New England Nuclear) was added, and incubation was continued for 30 min followed by chase with 30 min-incubation in the presence of supplementary amino acids. The labeled maxicells were harvested, suspended in 50 µl of a sample buffer composed of 63 mM Tris.HCl (pH 6.8), 3% sodium dodecylsulfate, 5% β-mercaptoethanol and 10% glycerol, and lysed by boiling for 3 min.
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at 95°C followed by quick cooling. Maxicell proteins were analyzed by electrophoresis on 15% sodium dodecylsulfate-polyacrylamide gels.

(g) Coupled transcription-translation in vitro

The system used here is a modification18 of the DNA-dependent, protein synthesizing system described by Zubay et al.19 Protein synthesis was carried out for 1 h at 37°C in 0.1 ml of a reaction mixture containing 40 mM Tris-acetate (pH 8.2), 60 mM potassium acetate, 30 mM NH₄Cl, 15 mM magnesium acetate, 1.5 mM dithiothreitol, 3% polyethylene glycol (No. 6000), 0.5 mM each of CTP, GTP, UTP and cyclic AMP, 2 mM ATP, 25 mM phosphoenol pyruvate, 0.1 mM [³⁵S]methionine (50 µCi), 0.2 mM each of 19 other amino acids, 6 µg of folinic acid, 5 µl of S-30 extract (27.5 mg protein/ml), 5 µl of crude ribosome fractions (0.57 A₅₉₀/µl), 0.7 µg of pyruvate kinase (Boehringer), and 1.5 pmole of template DNA. The reaction mixture was mixed with 20 µl of 1 N NaOH and incubated for 15 min at 37°C. Proteins were precipitated by the addition of 1 ml of 5% trichloroacetic acid, and precipitates produced were collected by centrifugation, washed with 0.5% trichloroacetic acid and then with ethyl ether, and dissolved in 0.1 ml of cracking buffer containing 50 mM Tris.HCl (pH 6.8), 1% sodium dodecylsulfate, 1% β-mercaptoethanol and 5% glycerol. The sample was heated for 3 min at 95°C prior to gel electrophoresis. The S-30 extract and crude ribosome fractions were prepared as described previously.18

RESULTS AND DISCUSSION

(a) RNA synthesis in vitro

pAO65 is composed of a quarter ColE1 (pAO3) and Tn903, and carries phenotypic markers Imm⁺ (immunity to colicin E1), Inc⁺ (incompatibility) and Km.5 RNA synthesis in vitro was carried out on this plasmid DNA as a template and the resulting transcripts were analyzed by urea-polyacrylamide gel electrophoresis. In essence, three classes of RNA were produced, of which two small RNA species (bands I and II) corresponded to RNA—I (0.11 kb; incompatibility RNA) and RNA—II (0.37 kb; mRNA for immunity protein) previously identified on ColE1 and pAO320 (Fig. 2a; RNA—I was run off in this photogram). Therefore, RNA transcribed from Tn903, if any, should be involved in the non-discrete band III with sizes larger than 1 kb. These results suggest that both tnp- and kan-mRNAs read-through into downstream regions, being consistent with the previous observation that no sequence characteristic for transcription termination is present.6 In order to find the initiation sites of tnp- and kan-mRNAs, truncated transcripts were synthesized on restriction fragments derived from pAO65 (Fig. 2b–d). If there are mRNAs for tnp and kan which start near the 5’ end of respective genes, truncated transcripts with various lengths should be observed. For instance, when the HindIII.EcoRI-fragment of 3304 bp (A-fragment; see Fig. 1) is used as a template, RNA—I, RNA—II, kan-mRNA truncated at the HindIII site (about 0.55 kb), and read-through tnp-mRNA (about 2.0 kb) should appear, while when B-fragment of 1482 bp and C-fragment of 682 bp are used as a template, read-through tnp-mRNA (about 1.1 kb) and truncated tnp-mRNA (about 0.3 kb) are respectively expected to be seen. The results show that RNA—I, RNA—II

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and truncated kan-mRNA (band IV) are actually synthesized on appropriate fragments. However, neither truncated nor read-through tnp-mRNA was detected under the conditions used. The longest RNA synthesized on each template is unlikely to be initiated at promoters, because their sizes roughly correspond to that of their respective template DNA fragments. This was interpreted as that in the in vitro transcription system, RNA synthesis frequently starts at the termini of linear DNA templates and ends around the other extremity of DNA fragments if no appropriate termination signal is present. Thus we concluded that tnp-mRNA synthesis rarely occurred on Tn903, whereas kan-mRNA synthesis was frequently initiated at about 550 bp upstream from the HindIII site.

(b) Protein synthesis in vivo and in vitro

Plasmid-derived proteins synthesized in vivo were analyzed by the maxicell procedure. Proteins directed by pAO65 (Km\(^+\)Tn\(^+\)), pICR871 (Km\(^+\)Tn\(^-\)) and pICR10 (Km\(^+\)Tn\(^+\)) were specifically labeled with \(^{35}\)S]methionine, and after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein bands resolved were visualized by fluorography (Fig. 3a–c). The patterns with pAO65 and pICR871 were almost identical, showing two characteristic bands i and ii that corresponded to molecular weights of 31000 and 29000, respectively. However, pICR10, in which the kan gene was inactivated by insertion of a foreign DNA segment carrying lacZ\(_\alpha\) at the HindIII site, did not give these two bands, but instead two other bands iii and iv were found. The difference of bands iii and iv from bands i and ii were confirmed by co-electrophoresis of the mixture (data not shown). Another Km\(^+\) plasmid, pAO164 (see below) failed to create bands corresponding to bands i and ii. It was therefore
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Fig. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of maxicell proteins directed by pAO65 (a), pICR871 (b), pICR10 (c), pAO142 (d), pAO146 (e), pAO170 (f), pAO164 (g), pAO195 (h), pAO176 (i), pKB252 (j) and pBR322 (k). TET, AMP and cl indicate the gene product of tet, bia and cl, respectively. Labeling of maxicells and electrophoresis were separately performed with each group of photograms.

concluded that these two bands correspond to the *kan* gene product, APH. Since no genomic spaces separately determining these two proteins are present on Tn903,6) the smaller one seems to be either degraded from the larger one or prematurely terminated. In any cases, we never found a band corresponding to TNP, consistent with the results of RNA synthesis described in the preceding section. Similar results (Fig. 4) were obtained by coupled transcription-translation *in vitro*19) When A-fragment

Fig. 4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of proteins synthesized *in vitro* on A-fragment of pAO65 (a), B-fragment of pAO65 (b), D-fragment of pICR871 (c), and E-fragment of pICR871. “i” and “ii” indicate positions of reference APH, and “i”” and “ii” correspond to APH'.

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(3304 bp) derived from pAO65 and the corresponding D-fragment (2784 bp) from pICR871 (see Fig. 1) were used as a template, two APH bands truncated at the HindIII site were identified (bands i' and ii'). On the other hand, no TNP band was observed with any of A-, B-, D- and E-fragments.

(c) Effects of lacUV5 on expression of tnp and kan

As the next step, we tried to express tnp more efficiently by placing the lacUV5 promoter in front of tnp or by replacing the amino-terminal 11 residues of tnp by the amino-terminal portion of lacZ, which is downstream from lacUV5 (Fig. 5a). The kan gene was similarly modified for control (Fig. 5b). The structure of four plasmids thus constructed from pAO65 are shown in Fig. 1 (see APPENDIX). In pAO164 and pAO146, tnp and kan were respectively under the control of lacUV5 about 210 bp apart. In pAO142 and pAO170, tnp and kan were respectively fused to 10 and 13 amino acid residues of lacZ so as to yield the same reading frame. Proteins directed by these four plasmids were analyzed by the maxicell procedure. The results (Fig. 3d—g) indicate that APH could be synthesized by all these plasmids except for Km" plasmid pAO164. As expected, the amount of APH was enhanced by pAO146 and pAO170, several times that by pAO65 (band vi corresponds to APH', the product of lacZ':: kan'). However the amount by pAO142 was significantly reduced. This reduction seems to be attributable to competition between promoters for RNA polymerase because the tet gene expression was also reduced when it was combined with lacUV5 in an opposite orientation on a single replicon (Fig. 3j—k). On the contrary, TNP was detected as a very faint band only in the case of pAO142 (band v corresponds to TNP', the product of lacZ':: tnp'), indicating that not only the replacement of promoter by lacUV5 but also the additional fusion of lacZ to tnp is required for visualization of TNP. This expression is however unexpectedly low in comparison to that of the similar fusion of kan as seen in Fig. 3 (bands v and vi). The low level expression of TNP appears not to be ascribed to the instability of TNP in maxicells, for the yield of TNP' synthesized by pAO142 was not affected by chasing for various periods after labeling.
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with [35S]methionine (data not shown). It is known that lacUV5 generally enhances the expression of other genes like as kan (e.g. the λcI gene in pKB252 (Fig. 3j)). Therefore, the expression of tnp is an exceptional case, and it is likely that both the structural gene and its regulatory regions give a strong inhibitory effect on expression by some unknown mechanisms. To examine this possibility further, we constructed two chimeric plasmids. One (pAO176) was made by inserting in-frame, the 520 bp-fragment generated from the inner part of tnp at the ClaI site within kan of pAO170, and the other (pAO195) was by linking the lacZ'-tnp' of pAO142, in-frame, to the carboxyl-terminal portion of tet on pBR322 (Fig. 6). These two plasmids were examined by the maxicell procedure as above. Neither chimeric tnp genes were expressed as efficiently as lacZ'-kan' of pAO170 (Fig. 3h–i). Only traces of TNP' (band vii) was detected with pAO176, and none with pAO195. The results demonstrate that at least the amino-terminal 70 bp portion and central 520 bp portion of tnp have inhibitory effect on expression.

![Diagram of plasmid structures](image)

**Fig. 6.** Structure of the chimeric tnp genes in pAO176 and pAO195. Arrows with numerals represent possible reading frames and the number of constituent amino acid residues. Restriction cleavage sites before ligation are shown in parentheses. For the others, see the legend to Fig. 1.

(d) Determination of amino-terminal residue of APH

Nucleotide sequence of Tn903 presented in our previous paper could not pinpoint the amino-terminal residue of APH since four initiation codons at positions 1162, 1216, 1366 and 1396 appeared in the same reading frame. The longest frame was the most likely candidate because of the presence of Shine-Dalgarno-like sequence. During the course of cloning experiments with either XhoI site at position 1191 or HindIII site at position 1711 on Tn903, we noticed that though insertion of any DNA segments at the HindIII site abolished Km', insertion of short DNA segments at the XhoI site sometimes retained Km'. This observation allows us to imagine that the XhoI site lies in the upstream region from the structural gene for APH and abolishment of Km' by insertions at this site results from transcription attenuation. To check this possibility, we replaced the upstream region from the XhoI site in kan by the lacUV5-lacZ' region. If the second or downstream initiation codons can be used for translation initiation for APH, the resulting chimeras should retain Km regardless of fusion in-frame (pAO170) or out-frame (pAO168), due to the presence of the strong promoter lacUV5 (Fig. 5b). However, only pAO170 and not pAO168 made cells Km'. Thus it is obvious that the real initiation codon for APH is the first one at position 1162. This result also implies that the upstream region from the XhoI site...
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does not contain active sites of the APH enzyme. Moreover, pAO170 producing APH’
made cells Km’ more than 1500 µg/ml, while pAO65 carrying the normal kan promoter
mediated Km’ up to 150 µg/ml. Absence of active sites around the XhoI site was
also confirmed by the observation that pAO184 (Fig. 5b), which carried 12 bp insertion
at the XhoI site without disruption of the kan reading frame, conferred Km’ as well as
pAO65. Absence of active sites upstream from the XhoI site is consistent with the fact
that two kan genes of Tn903 and another kanamycin resistance transposon Tn5(22)
were highly conservative along the sequences except for their amino-terminal portions.
(e) Transposition of Tn903 with the fused tnp gene

Tn903 of pAO142 carried the fused tnp gene in one IS903, while the complete tnp
gene was located in the other IS903. Transposition frequency of this mutant Tn903
from pAO142 to the λb5 genome was measured in Rec+ cells. As control, pAO65,
pAO146 and pICR871 were used as the donor of Tn903. Relative frequency of
transposition was pAO65 = 100; pAO142 < 5; pAO146 = 10; pICR871 = 70. The
structure of transposition products differed with donor plasmids. With transposition
from pAO65, only Tn903 moiety was integrated in λb5, whereas the products with
pAO146 and pICR871 were cointegrates between donor and recipient replicons.
These cointegrates were presumably formed by the host Rec function because no such
molecules were detected with Rec− cells. Thus the presence of lacUV5 inhibited not
only transposition of Tn903 mediated by TNP but also formation of cointegrate
by Rec. These results suggest that the powerful promoter generally prohibits
recombination reactions probably through changes of tertiary structure of DNA due
to active transcription. A similar phenomenon was reported with transposition of
IS1(28).

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APPENDIX

Construction of various plasmids appeared in the article (see Fig. 1)

(a) pICR871
pAO65 DNA carries four PvuII-susceptible sites, all of which are within IS903s. pAO65 DNA was digested with PvuII and then joined with ligase. After transformation of C600 cells to Km', plasmid DNA carried by respective transformants was analyzed by restriction endonuclease digestion. Plasmids in which the 520 bp-PvuII-fragment had been ejected from both IS903s and the polarity of kan against the pAO3 moiety was the same as pAO65, was selected and its representative was named pICR871.

(b) pICR10
A DNA segment (AvaII plus BglII digest) carrying lacOP and lacZu regions was purified from replicative form DNA of M13mp7, and inserted in the HindIII site of pAO65 by HindIII-linker-mediated-ligation. Upon transformation, Imm+ Lac+ clones were selected and one of them was named pICR10. The polarity of lacZ was opposite to that of kan.

(c) pAO142 and pAO146
Three out of four AvaII sites in pAO65 were destroyed by three successive partial AvaII digestion/filling in cohesive ends by repair synthesis/self-cyclization with ligase. The resulting pICR152 retaining only single AvaII site at position 945 was linearized by AvaII digestion and joined by repair ligation to an EcoRI-fragment carrying lacUV5 and the first 25 bp (lacZ') of the structural gene for β-galactosidase derived from pKB252. Upon transformation, Km'Lac+ colonies were selected, and plasmids carrying lacUV5 with two kinds of polarities were isolated (pAO142P and pAO146P). Each plasmid DNA was separated into two portions by AccI plus XhoI digestion and the smaller one containing lacUV5 was joined to the longer one similarly prepared from pAO65. The recombinants thus constructed (pAO142 and pAO146, respectively)
had a structure identical to that of pAO65 except for the presence of lacUV5-segment at the previous AvaI site at position 945. The nucleotide sequence around the junction in pAO142 was determined and found that the amino-terminal portion of lacZ was fused in-frame to tnp as expected (Fig. 5a).

(d) pAO170, pAO168, pAO164

The EcoRI-fragment carrying lacUV5 and lacZ' was purified from pKB252 and joined to the XhoI site of pAO65 by XhoI-linker-mediated-ligation. After transformation, several Km\textsuperscript{r} colonies were picked and their plasmid DNA was examined by restriction analysis and by sequencing. All had an identical structure that tandem contained XhoI-linker at the downstream junction of lacUV5 as shown in Fig. 5b. Note that another junction upstream from lacUV5 contained a single copy of XhoI-linker. A representative of such plasmids was named pAO170.

pAO170 DNA was digested with XhoI and re-cyclized followed by transformation with Imm\textsuperscript{+}Lac\textsuperscript{c} selection. All transformants thus obtained were Km\textsuperscript{r}. The polarity of lacUV5-segment in plasmid carried by respective transformants were determined by restriction analysis, and a clone with the same polarity as pAO170 and one with the opposite polarity were named pAO168 and pAO164, respectively. The presence of only one copy of XhoI-linker at each junction in these two plasmids was confirmed by sequencing (Fig. 5b).

(e) pAO176 and pAO195

The 520 bp PvuII-fragment carrying an inner part of tnp of pAO65 was inserted at the Clal site of pAO170 by repair ligation. Upon transformation with Imm\textsuperscript{+}Km\textsuperscript{r} selection, pAO176 was obtained. In-frame fusion between kan and tnp was confirmed by sequencing.

pBR322 carries two HinclI-susceptible sites, one in bla and the other in tet. pBR322 DNA was linearized by partial digestion with HinclI and joined to the StuI-fragment carrying kan and lacUV5 of pAO142 (marked at the bottom in Fig. 1). Note that pAO142 DNA used was prepared from GM31 cells because the StuI site overlaps with the target site of DNA cytosine methylase (dcm), at which methylation disturbs digestion by StuI. After transformation with Ap\textsuperscript{r}Km\textsuperscript{r}Tc\textsuperscript{r}Lac\textsuperscript{c} selection, pAO181 was obtained. Whether the fusion between tnp and tet is in-frame was checked by sequencing. To remove the amino-terminal portions of both bla and tet, pAO181 DNA was digested with PstI plus BamHI, and self-cyclized by repair ligation. Upon transformation with Km\textsuperscript{r}Lac\textsuperscript{c}Ap\textsuperscript{r} selection, pAO195 was isolated.

(f) pAO184

The lacUV5 segment in pAO168 was punctuated with EcoRI sites as well as with XhoI sites. Therefore most part of the lacUV5 segment was ejected by EcoRI digestion followed by self-cyclization. After transformation with Imm\textsuperscript{+} selection, a recombinant plasmid pAO184 was obtained which carried 12 bp insertion at the XhoI site in kan. Two XhoI sites and one EcoRI site were created during the process of construction as shown in Fig. 5b. This plasmid became suitable for cloning EcoRI fragments in addition to HindIII- and XhoI-fragments by insertional inactivation selection of Km\textsuperscript{r}. 

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