Transcription and Translation of the Kanamycin Resistance Transposon Tn903

Minami MATSUI and Atsuhiro OKA*

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[r]† by coding for APH on its host bacterium.[3] Tn903 can be transposed to many different sites even in small replicons, and it appears that no specific sequences are required for target sites.[4,5] 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.[6] 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).[6,7] 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

* 松井 南, 岡 穆宏: Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, Uji-shi 611, Japan
† Abbreviations used: Km[r], kanamycin resistance; APH, aminoglycoside 3′-phosphotransferase; APH′, modified APH; bp, base-pairs; kb, kilobases or kilobase-pairs; Km[s], kanamycin sensitiveness; TNP, transposase; TNP′, modified TNP.
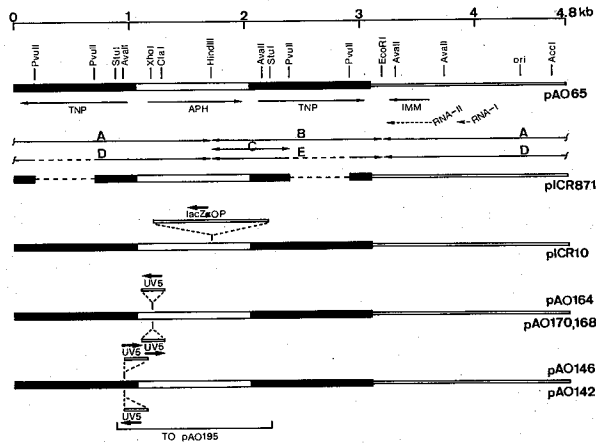
Fig. 1. The structure of pAO65 including representative restriction sites is shown at the top, and those of various pAO65-derivatives are at the lower part. The structures are oriented with respect to the integration site of Tn*903*. Thick and thin bars indicate Tn*903* and other moieties, respectively, and black bars show IS*903*. "UV5" with an arrow indicates insertion and transcriptional direction of the *Eco*RI-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[6] and previously identified transcriptional products derived from pAO3[20]. "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.[8]

(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 NH_4Cl, 3 g of NaCl, 0.1 g of Na_2SO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 mg of thiamine and 20 mg of thymine per liter.

M9-CAA medium used in labeling experiments contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 1 g of NH_4Cl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 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 ara lac galK galT xyl mtl str tonA tsx supE*),[9] N1790 (F⁻ *recA99 uvrA54 gal trp str*),[10] JM109 (*Δlac∼pro recA1 endA1 thi1 gyrA96 hsdR17 relA1 supE44*/F' carrying *traD36 proA⁺B⁺ lacIᵠ lacZΔM15*),[11] and HfrH5 (ColE1)/E1. Phages used were *λb5*,[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 [α³²P]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/m²/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 dode-cylsulfate, 5% β-mercaptoethanol and 10% glycerol, and lysed by boiling for 3 min

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 modification[18] of the DNA-dependent, protein syn-thesizing 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 NH_4Cl, 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 [^{35}S]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 A_{260}/μl), 0.7 μg of pyruvate kinase (Boehringer), and 1.5 pmoles 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-mercaptothanol 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 Tn*903*, and carries phenotypic markers Imm$^+$ (immunity to colicin E1), Inc$^+$ (incompatibility) and Km.^{r}[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 pAO3[20] (Fig. 2a; RNA-I was run off in this photogram).   Therefore, RNA transcribed from Tn*903*, 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 *Hind*III.*Eco*RI-fragment of 3304 bp (A-fragment; see Fig. 1) is used as a template, RNA-I, RNA-II, *kan*-mRNA truncated at the *Hind*III 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
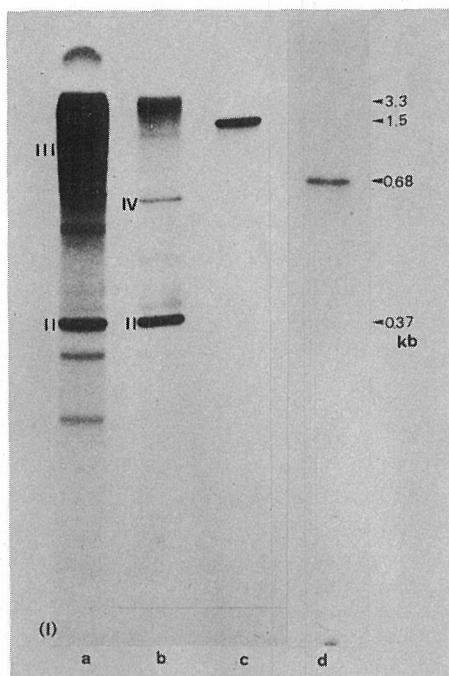
Fig. 2. Urea-polyacrylamide gel electrophoresis of RNA synthesized *in vitro* on template of covalently closed circular pAO65 DNA (a) and its restriction fragments A (b), B (c), and C (d).

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 Tn*903*, whereas *kan*-mRNA synthesis was frequently initiated at about 550 bp upstream from the *Hind*III site.

(*b*) *Protein synthesis* in vivo *and* in vitro

Plasmid-derived proteins synthesized *in vivo* were analyzed by the maxicell procedure.[16,17] Proteins directed by pAO65 (Km^{r}TnP$^+$), pICR871 (Km^{r}Tnp$^-$) and pICR10 (Km^{s}TnP$^+$) were specifically labeled with [^{35}S]methionine, and after sodium dodecylsulfate-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 *Hind*III 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^s plasmid, pAO164 (see below) failed to create bands corresponding to bands i and ii. It was therefore
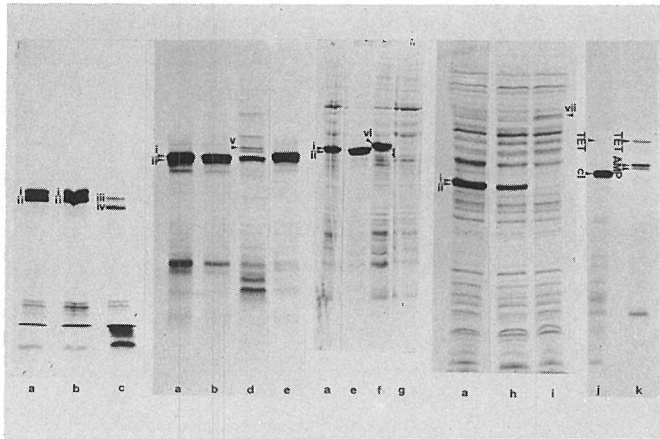
Fig. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of maxicell proteins directed by pAO65 (a), pICR871 (b), pICR10 (c), pAO142 (d), pAO146 (e), pAO170 (f), pAO164 (g), pAO195 (h), pAO176 (i), pKB252 (j) and pBR322 (k). TET, AMP and cI indicate the gene product of *tet*, *bla* and *cI*, 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 Tn*903*,[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
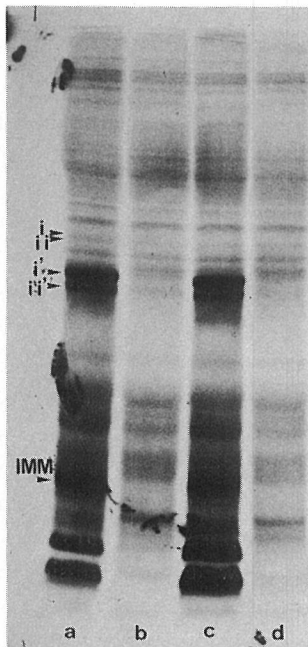

Fig. 4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of proteins synthesized *in vitro* on A-fragment of pAO 65 (a), B-fragment of pAO65 (b), D-fragment of pICR871 (c), and E-fragment of pICR871. "i" and "ii" indicate positions of reference APH, and "i′" and "ii′" correspond to APH′.

(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 *Hind*III 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*

```
a                                           AvaII
tnp            GTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACC-
(pAO65)        MetAlaLysGlnLysPheLysIleThrAsnTrpSerThr-

                                 (EcoRI)(AvaII)
lacZ'::tnp'    ATGACCATGATTACGGATTCACTGGAATTGTCCACC-
(pAO142)       MetThrMetIleThrAspSerLeuGluLeuSerThr-

b                                                XhoI
kan            ATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGA-
(pAO65)        MetSerHisIleGlnArgGluThrSerCysSerSerArgProArg-

                                      EcoRI XhoI       XhoI
lacZ'::kan'  ATGACCATGATTACGGATTCACTGGAATTCCTCGAGGCCTCGAGGCCGCGA-
(pAO170)     MetThrMetIleThrAspSerLeuGluPheLeuGluAlaSerArgProArg-

                                      EcoRI XhoI
lacZ'::kan'   ATGACCATGATTACGGATTCACTGGAATTCCTCGAGGCCGCGA-
(pAO168)      MetThrMetIleThrAspSerLeuGluPheLeuGluAlaAla-

                                   XhoI  EcoRI  XhoI
kan Ω12bp  ATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGAATTCCTCGAGGCCGCGA-
(pAO184)   MetSerHisIleGlnArgGluThrSerCysSerArgAsnSerSerArgProArg-
```

Fig. 5. Nucleotide and amino acid sequences of the amino-terminal portion of *tnp* in pAO65 and pAO142 (a), and those of the amino-terminal portion of *kan* in pAO65 and its derivatives (b).

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^s 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

with [^{35}S]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 *Cla*I 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.
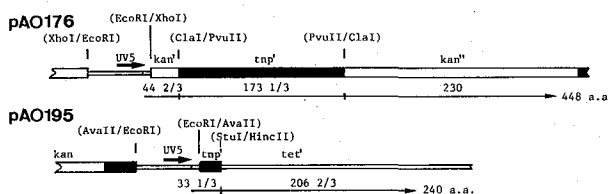

Fig. 6. Structure of the chimeric *tnp* genes in pAO176 and pAO195. Arrows with numerals represent possible reading frames and the number of constituent amino acid residues. Restriction cleavage sites before ligation are shown in parentheses. For the others, see the legend to Fig. 1.

(d) Determination of amino-terminal residue of APH

Nucleotide sequence of Tn*903* presented in our previous paper[6] 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.[21] During the course of cloning experiments with either *Xho*I site at position 1191 or *Hind*III site at position 1711 on Tn*903*, we noticed that though insertion of any DNA segments at the *Hind*III site abolished Km^r, insertion of short DNA segments at the *Xho*I site sometimes retained Km^r. This observation allows us to imagine that the *Xho*I site lies in the upstream region from the structural gene for APH and abolishment of Km^r by insertions at this site results from transcription attenuation. To check this possibility, we replaced the upstream region from the *Xho*I 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^r 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^r. 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 *Xho*I site

does not contain active sites of the APH enzyme. Moreover, pAO170 producing APH′ made cells Km^r more than 1500 μg/ml, while pAO65 carrying the normal *kan* promoter mediated Km^r up to 150 μg/ml. Absence of active sites around the *Xho*I site was also confirmed by the observation that pAO184 (Fig. 5b), which carried 12 bp insertion at the *Xho*I site without disruption of the *kan* reading frame, conferred Km^r as well as pAO65. Absence of active sites upstream from the *Xho*I site is consistent with the fact that two *kan* genes of Tn*903* and another kanamycin resistance transposon Tn*5*[22] were highly conservative along the sequences except for their amino-terminal portions.

(e) *Transposition of Tn*903 *with the fused* tnp *gene*

Tn*903* of pAO142 carried the fused *tnp* gene in one IS*903*, while the complete *tnp* gene was located in the other IS*903*. Transposition frequency of this mutant Tn*903* from pAO142 to the λ*b5* genome was measured in Rec$^+$ cells. As control, pAO65, pAO146 and pICR871 were used as the donor of Tn*903*. 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 Tn*903* moiety was integrated in λ*b5*, 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 Tn*903* 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 IS*1*.[23]

ACKNOWLEDGEMENTS

REFERENCES

(1) M. P. Calos and J. H. Miller, *Cell*, **20**, 579 (1980).

(2)′ P. Starlinger, *Plasmid*, **3**, 241 (1980).

(3) N. Nomura, A. Oka, M. Takanami and H. Yamagishi, "The single-stranded DNA phages", Cold Spring Harbor Laboratory, New York, 1978, pp. 467–472.

(4) N. Nomura, H. Yamagishi and A. Oka, *Gene*, **3**, 39 (1978).

(5) A. Oka, N. Nomura, K. Sugimoto, H. Sugisaki and M. Takanami, *Nature (London)*, **276**, 845 (1978).

(6) A. Oka, H. Sugisaki and M. Takanami, *J. Mol. Biol.*, **147**, 217 (1981).

(7) N. D. F. Grindley and C. M. Joyce, *Cold Spring Harbor Symp. Quant. Biol.*, **45**, 125 (1980).

(8) A. Oka, H. Sasaki, K. Sugimoto and M. Takanami, *J. Mol. Biol.*, **176**, 443 (1984).

(9) M. G. Marinus, *Mol. Gen. Genet.*, **127**, 47 (1973).

(10) T. Horii, T. Ogawa and H. Ogawa, *Cell*, **23**, 689 (1981).
(11) C.Y. Perron, J. Vieira and J. Messing, *Gene*, **33**, 103 (1985).
(12) J. Messing, R. Crea and P. H. Seeburg, *Nucl. Acids Res.*, **9**, 309 (1981).
(13) J. Norrander, T. Kempe and J. Messing, *Gene*, **26**, 101 (1983).
(14) F. Bolivar, R. L. Rodriguez, P. J. Greene, M. C. Betlach., H. L. Heyneker and H. W. Boyer, *Gene*, **2**, 95 (1977).
(15) K. Backman, M. Ptashne and W. Gilbert, *Proc. Natl. Acad. Sci. USA*, **73**, 4174 (1976).
(16) A. Sancer, A. M. Hack and W. D. Rupp, *J. Bacteriol.*, **137**, 692 (1979).
(17) A. Sancer, R. P. Wharton, S. Seltzer, B. M. Kacinski, N. D. Clarke and W. D. Rupp, *J. Mol. Biol.*, **148**, 45 (1981).
(18) M. Shibuya and Y. Kaziro, J. *Biochem.*, **86**, 403 (1979).
(19) G. Zubay, D. A. Chambers and L. C. Cheong, "The lactose operon", Cold Spring Harbor Laboratory, New York, 1970, pp. 375–391.
(20) M. Morita and A. Oka, *Eur. J. Biochem.*, **97**, 435 (1979).
(21) J. Shine and L. Dalgarno, *Proc. Natl. Acad. Sci. USA.*, **71**, 1342 (1974).
(22) E. Beck, G. Ludwig, E. A. Auerswald, B. Reiss and H. Schaller, *Gene*, **19**, 327 (1982).
(23) C. Machida, Y. Machida, H.-C. Wang, K. Ishizaki and E. Ohtsubo, *Cell*, **34**, 135 (1983).

APPENDIX

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

(a) *pICR871*

pAO65 DNA carries four *Pvu*II-susceptible sites, all of which are within IS*903*s. pAO65 DNA was digested with *Pvu*II and then joined with ligase. After transformation of C600 cells to Km^r, plasmid DNA carried by respective transformants was analyzed by restriction endonuclease digestion. Plasmids in which the 520 bp-*Pvu*II-fragment had been ejected from both IS*903*s 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 (*Ava*II plus *Bgl*II digest) carrying *lacOP* and *lacZ$_\alpha$* regions was purified from replicative form DNA of M13mp7, and inserted in the *Hind*III site of pAO65 by *Hind*III-linker-mediated-ligation. Upon transformation, Imm$^+$ Lac^c 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 *Ava*II sites in pAO65 were destroyed by three successive partial *Ava*II digestion/filling in cohesive ends by repair synthesis/self-cyclization with ligase. The resulting pICR152 retaining only single *Ava*II site at position 945 was linearized by *Ava*II digestion and joined by repair ligation to an *Eco*RI-fragment carrying *lacUV5* and the first 25 bp (*lacZ′*) of the structural gene for β-galactosidase derived from pKB252. Upon transformation, Km^{r}Lac^c 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 *Acc*I plus *Xho*I 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 *Ava*II 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 *Eco*RI-fragment carrying *lacUV5* and *lacZ'* was purified from pKB252 and joined to the *Xho*I site of pAO65 by *Xho*I-linker-mediated-ligation. After transformation, several Km^r colonies were picked and their plasmid DNA was examined by restriction analysis and by sequencing. All had an identical structure that tandem contained *Xho*I-linker at the downstream junction of *lacUV5* as shown in Fig. 5b. Note that another junction upstream from *lacUV5* contained a single copy of *Xho*I-linker. A representative of such plasmids was named pAO170.

pAO170 DNA was digested with *Xho*I and re-cyclized followed by transformation with Imm^+Lac^c selection. All transformants thus obtained were Km^s. 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 *Xho*I-linker at each junction in these two plasmids was confirmed by sequencing (Fig. 5b).

(*e*) *pAO176 and pAO195*

The 520 bp-*Pvu*II-fragment carrying an inner part of *tnp* of pAO65 was inserted at the *Cla*I site of pAO170 by repair ligation. Upon transformation with Imm^+Km^s selection, pAO176 was obtained. In-frame fusion between *kan* and *tnp* was confirmed by sequencing.

pBR322 carries two *Hinc*II-susceptible sites, one in *bla* and the other in *tet*. pBR322 DNA was linearized by partial digestion with *Hinc*II and joined to the *Stu*I-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 *Stu*I site overlaps with the target site of DNA cytosine methylase (*dcm*), at which methylation disturbs digestion by *Stu*I. After transformation with Ap^rKm^rTc^sLac^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 *Pst*I plus *Bam*HI, and self-cyclized by repair ligation. Upon transformation with Km^rLac^cAp^s selection, pAO195 was isolated.

(*f*) *pAO184*

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