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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED...

Tn3 Transposition Immunity: Expression of Transposition Immunity in pACM Carrying a small Tn3 Derivative

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science

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Tn3 TRANSPosition immunity:
expression of transposition immunity in pACYC
CARRYING A SMALL Tn1 DERIVATIVE

by

Patrick J. Getty

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List of Abbreviations

Cm................................................. chloramphenical
Cb................................................ carbenicillin
Sm............................................... streptomycin
Su............................................... sulfonimide
Tc............................................... tetracycline
superscript, r............................... resistant
.............................................. sensitive

iel+............................................. colicin El immune
bp............................................... base pairs
kbp.............................................. kilobase pairs

Restriction endonucleases -

B............................................... Bam HI
C............................................... Cla I
G............................................... Bgl II
H............................................... Hinc II
K............................................... Kpn I
F............................................... Pst I
R............................................... Eco Rl
S............................................... Sac I
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INTRODUCTION

Transposable elements are discreet segments of DNA with the capacity to move, or transpose, from one genetic location to another (8). Transposons, bacterial transposable elements carrying at least one genetic marker, are generally grouped into three categories. The first is composite transposons which are composed of DNA sequences bracketed by direct or inverted repeats of IS elements. IS elements are relatively small segments of DNA (750-1500 base pairs long) that code for a protein necessary for their transposition but contain no genetic markers themselves. The termini of IS elements are inverted repeats of approximately 16-41 bp that are also required for transposition (3). Tn9 and Tn10 are examples of composite transposons. Tn9 carries DNA sequences that code for chloramphenical resistance (\text{Cm}^R) and are bounded by direct repeats of IS1 (3). Tn10 carries DNA sequences that code for tetracycline resistance (\text{Tc}^R) and are bounded by inverted repeats of IS10 (12). Transposition of composite transposons is mediated by the transposase protein coded in the IS element.

The second group of bacterial transposons is composed of bacteriophage such as Mu. Transposition is a normal step in the production of progeny replicons for these phages. Following infection, the phage DNA integrates itself into the host chromosome and replicates by transposing into other sites within the host chromosome. Thus one
copy of the phage DNA remains at the original site of integration and other copies are located at the sites of transposition. (22)

The third category of bacterial transposons is comprised of the Tn3 transposon family of which Tn3 and Tn1 are members. Tn3 type transposons do not have IS elements at their termini. Typically Tn3 family transposons carry one or more genetic markers, commonly drug resistance markers. They are bounded by small inverted repeat sequences (approximately 40 bp) and carry two genes that code for proteins involved in transposition (11). Tn1 and Tn3 are highly similar transposons. Their inverted repeat sequences are functionally identical and their gene products are interchangeable. They differ in a few known restriction endonuclease cleavage sites (see Figure 1). Both have 38 bp terminal inverted repeat sequences, an internal resolution site designated res involved in site specific recombination, and three genes: tnpA, tnpR, and bla. The tnpA gene product (TnpA protein) is a replicative recombinase that mediates the first step in transposition. The tnpR gene product (TnpR protein) is a repressor/resolvase that regulates transcription of tnpA and tnpR by binding to res and mediates site specific recombination at res, the second step in transposition. The bla gene product (beta-lactamase) determines resistance to carbenicillin (4,5,7).

Transposition of a Tn3 type transposon occurs via a two step process: 1) formation of a cointegrate structure, and 2) resolution of that cointegrate (see Figure 2). The formation of the cointegrate is mediated by the TnpA protein and requires both intact terminal inverted repeats. This step is characterized by the semi-conservative
Physical and restriction endonuclease cleavage maps of Tn1 and Tn3. Restriction endonuclease cleavage sites are given for Kpn I (K), Cla I (C), Pst I (P), Hinc II (H), and Bam H1 (B). IR marks the 38 base pair (bp) terminal inverted repeats, and res marks the internal resolution site (at least 130 bp) which overlaps the transcription initiation sites for both tnpA and tnpR. Labeled lines mark regions coding for the tnpA, tnpR, and bla gene products. Arrows indicate direction of transcription. Internal deletions of Tn1ΔAp, Tn1Δpp, and Tn3Δ596 are indicated. Numbers represent kilobase pair at center of restriction endonuclease cleavage site or end-point of internal deletion. (8)
Figure 2

Tn3-mediated transposition.
Donor and Recipient Replicons $\rightarrow$ Cointegrate Structure $\rightarrow$ Full Transposition Products

TnpA protein

TnpR protein

RecA protein
replication of the transposon DNA as well as a 5 bp sequence of the recipient DNA at the target site (18). There is no known sequence specificity for the target site. However, it has been observed that certain regions of a given recipient replicon are transposed into more frequently than other regions. It appears that A=T rich regions (i.e., regions rich in adenine and thymine) are more frequently targeted than other regions (23). The 5 bp sequence that is duplicated is present in the cointegrate as shown in Figure 3. Resolution of the cointegrate to simple transposition products is mediated by the TnpR protein and requires direct repeats of res in the cointegrate molecule. This is a site specific recombination system (19). This resolution step can also be mediated by the RecA protein or other homology-dependent recombination system, due to the approximately 5000 bp of homology provided by the two copies the transposon (16).

Tn3 is an active transposon. It will transpose at a rate of $10^{-1}$ to $10^{-2}$ transposition events per cell generation. Many bacterial transposons transpose at rates of $10^{-5}$ to $10^{-8}$ events per cell generation. This high rate of activity found for Tn3 could lead to nonviability of host cells due to transposon insertions into required regulatory or structural genes. There are, however, three basic mechanisms that act to curtail transposition and may promote maintenance of host cell viability. First, Tn3 does not transpose into the host chromosomal DNA at a high rate. However, high rates of transposition are observed into plasmid and bacteriophage replicons (1, 11). Second, the TnpA protein is temperature sensitive. It is active at temperatures up to 32°C and inactive at 37°C (12). The third mechanism that
Figure 3

Semi-conservative replication of Tn3 and five-base-pair target sequence. Small arrows indicate sites where single-stranded nicking of DNA occurs. Hatch markings indicate newly synthesized DNA. It should be noted that if A is connected to B and C is connected to D after DNA replication then the structure formed (prior to TnpR protein or Rec A protein mediated recombination) is a cointegrate.
Tarqot

Target Site

TnpA protein

RecA replication

TnpB protein

RecA protein
restricts transposition frequency is called transposition immunity (9). Tn3 sequences present in a replicon reduce the ability of Tn3 sequences in a second replicon to transpose into the first replicon. Transposition immunity has been observed to reduce transposition activity 20 to $10^6$ fold (9, 11). The level of transposition immunity seems to be related to the system in which it is studied as well as the Tnl/3 derivatives involved. Transposition immunity is a cis-acting phenomenon. Only the replicon in which the Tn3 resides is immune to further transposition. Any other replicons in the cell not carrying Tn3 sequences are not transposition immune (20). Furthermore, transposition immunity occurs in the absence of tnpR protein activity indicating that transposition immunity occurs in the first step of transposition, the step mediated by TnpA protein (14).

The actual mechanism of transposition immunity is unknown. Transposition immunity has been observed using systems in which only a 38 bp terminal inverted repeat sequence was present in the target replicon (14). Alternatively, there is evidence that in some systems internal deletions in Tn3 beginning in tnpA and ending in tnpR (thus removing res) result in no observable transposition immunity. In these cases terminal inverted repeat sequences are present. (14, Muster, C. personal communication)

Muster has determined frequency of cointegrate formation between pACM1 or pXJS972 and RSF1596 (see Figures 4 and 5). Cointegrate formation between pACM1 (no Tnl/Tn3 sequences) and RSF1596 (pMB8::Tn3Δ596), a non-transposition immune system, occurs at a frequency of about 0.38 while cointegrate formation between pXJS972 (pACM1::Tn1ΔAp) and RSF1596, a
Figure 4

Physical maps of pXCM and pXJS972. Maps are linearized at the cos site of λ. Letters indicate λ genes. pXCM is a derivative of λ with a Tn9 sequence inserted at 24.85 kilobase pairs on the standard λ map and adjacent deletions to the left and right of the Tn9 sequence. pXJS972 is a derivative of pXCM with TnIAP inserted at 9.4 kbp of the standard λ map and an adjacent deletion of λ of approximately 3 kbp. Numbers represent position in kbp on the standard λ map. Elongated box represents TnIAP. Squares represent IS1 elements of Tn9 that flank the chloramphenical resistance determinant of the Tn9 sequence. (10, 15)
Physical maps of RSF1596, RSF103, and RSF1050. pMB8 and RSF1010 are linearized at unique Eco RI (R) restriction endonuclease cleavage sites. RSF1596 was made by deletion of an internal sequence of RSF1050. Numbers indicate positions in kilobase pairs. CbR, SmR, and Iel+ indicate determinants for resistance to carbenicillin, streptomycin, and immunity to colicin E1, respectively.
potentially transposition immune system, occurs at a frequency of 0.15. Other pMB8::Tn3 deletion derivatives retaining res sequences yielded cointegrate formation frequencies with pXJS972 of 0.0014 and 0.0035 suggesting a 100 to 200-fold depression of transposition activity with respect to the system using pXCM and the same pMB8::Tn3 deletion derivatives. It was further shown that distribution of insertion sites for RSF1596 in pXCM and pXJS972 are equivalent. In the latter case the orientation of insertions is predominantly in the same direction as the resident Tnl derivative. In the former case the orientation of insertions appears to be approximately 41% in that direction (see Figure 5). (Muster, C. personal communication)

There are three disadvantages in using pXJS972 in these studies. First, the resident Tnl is transcriptionally active making it difficult to compare this system with those using only the 36 bp terminal inverted repeat in the target replicon. Second, pXJS972 contains an approximately 3000 bp deletion in the pXCM sequence so that pXJS972-RSF1596 cointegrates remain of acceptable size to be packaged into phage heads. Third, there is no version of pXCM containing a similar Tnl deletion in the same region of pXCM but in opposite orientation to the Tnl derivative in pXJS972. This situation excludes the possibility of determining directly whether orientation of the resident transposon affects orientation of additional transposition insertions. To resolve these difficulties, construction of a pXCM carrying a short Tnl derivative containing the 38 bp terminal inverted repeats but exhibiting no transcription was needed.
The purpose of this project was to construct derivatives of pACM carrying a short Tnl deletion sequence (pACM::TnlΔpp) and use them as follows: 1) to test for transposition immunity in transposition between pACM::TnlΔpp constructs and RSF1050 (pMB8::Tn3), 2) to test for transposition immunity in cointegrate formation between pACM::TnlΔpp constructs and RSF1596, and 3) to examine pACM::TnlΔpp-RSF1596 structures to determine donor and recipient replicons, distribution of insertions into recipient replicons, and orientation of insertions into the recipient replicon.
MATERIALS AND METHODS

Bacterial Strains

Bacterial strains used are described in Table 1. All those used are strains of *E. coli*. recA character was verified by testing for UV sensitivity. (24)

Plasmids

Characteristics of plasmids used are presented in Table 2. Physical maps of plasmids are presented in Figure 5. RSF103 is RSF1010::TnAp (Smr) tnpA+ res+ tnpR bla (6, 10). RSF1050 is pMB8::Tn3 (6, 8). Physical maps of Tn1 and Tn3 deletions are presented in Figure 1.

Bacteriophage Strains

AYS19 is λ b2 red3 cl857 and was obtained from M. Benedik and A. Campbell. The b2 deletion prevents lysogeny by the normal λ insertion mechanism. AYS19 was used to obtain lysates containing pλCM and derivatives of pλCM. These cosmids do not undergo independent lytic cycle. pλCM is a derivative of λ carrying an insertion of Tn9, a chloramphenical resistant bacterial transposon, and adjacent deletions to the left and to the right into the λ cistron. Deleted sequences include the att site, int and red functions, therefore, normal λ
Table 1. Bacterial Strains (E. Coli)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
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<tbody>
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<td>MV12</td>
<td>F- lac Y1 thi-1 thr 1 leu 6 dtrp E5 rpm L ton A21 sup E44 rec A</td>
<td>R. Gill (6)</td>
</tr>
<tr>
<td>M7124</td>
<td>F- thi dlac U169</td>
<td>J. Beckwith</td>
</tr>
<tr>
<td>XJS213</td>
<td>MV12 (RSF1596)</td>
<td>R. Gill (6)</td>
</tr>
<tr>
<td>XJS214</td>
<td>MV12 (RSF1596, RSF103)</td>
<td>R. Gill (6)</td>
</tr>
<tr>
<td>XJS797</td>
<td>MV12 (RSF1050)</td>
<td>M. Fennewald</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pMBB or RSF1010 Marker</td>
<td>Transposon Genotype</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>RSF1596 (pMBB::Tn34596)</td>
<td>Ie+</td>
<td>tnpA- res- tnpR- bla+</td>
</tr>
<tr>
<td>RSF103 (RSF1010::TnlΔAp)</td>
<td>Smr</td>
<td>tnpA+ res+ tnpR-bla-</td>
</tr>
<tr>
<td>RSF1050 (pMBB::Tn3)</td>
<td>Ie+</td>
<td>tnpA+ res+ tnpR+ bla+</td>
</tr>
</tbody>
</table>
integration into the host chromosome is not possible and λ recombination system is missing. pACM replicates as a plasmid and is dependent on λ replication functions (Q and P gene products). pACM can undergo lytic cycle when λ N gene function is provided by a helper phage (e.g. λYS19). pACM can be encapsidated in λ phage heads and is small enough (approximately 39 kbp) to accept inserts up to 13 kbp and remain packageable. A physical map of pACM is presented in Figure 4 (16).

Media

The primary medium used was complete tryptone-yeast extract (TYE). Antibiotic supplementation of complete media was performed as follows: 500μg/ml carbenicillin, 25μg/ml streptomycin, and 50μg/ml chloramphenical. Minimal PA salts medium supplemented with thiamine (final concentration, 0.001%) and glucose (final concentration, 0.2%) was also used for characterization of bacterial strains (17). When required, tryptophan, leucine, and threonine were added to final concentrations of 0.001%. TYE-deoxycholate medium was used for testing for colicin immunity.

Characterization of Bacterial Strains

Bacterial strains were characterized by streaking for isolation on TYE or antibiotic-TYE, and isolated colonies were picked with sterile tooth picks to a plate of the same medium which was then used as a master plate. The master plate was replica plated via sterile velvets to appropriate media, and colonies displaying the proper characteristics were selected for use in experiments. Some transduced cells which were
expected to be carrying a derivative of pMB8 were also tested for colicin immunity by streaking cells across a streak of colicin El. Growth of cells across the colicin El streak indicated colicin immunity. Colicin immune and non-colicin immune cells were also cross streaked as controls. Colicin immunity indicated the presence of pMB8.

**Preparation of Bacteriophage and Cosmid Lysates**

Lysates of AYS19 were prepared by confluent plate lysis. Cosmid lysates were prepared as follows: plates containing approximately 1000 colonies of cells carrying cosmids were scraped into 5ml of TYE broth and concentration was adjusted to $10^8$ cells/ml (Klett units = 30-34, red filter no. 64) using TYE broth. To 1.0ml of the $10^8$ cells/ml suspension was added AYS19 of a dilution to achieve a multiplicity of infection of 2.5. This mixture was incubated at room temperature ($25^\circ$C) for 15 minutes to allow absorption of AYS19. After the 15 minute incubation, 5ml of 42°C TYE broth was added to each infected culture and the cultures were placed in a 42°C water bath with aeration for 2 hours. After the two hour incubation, and clearing of the culture had been noted, CHCl$_3$ was added, and lysates were vortexed and centrifuged at 5000 rpm for 5-10 minutes to remove cell debris. The supernatant (lysate) was placed in a fresh tube and stored over CHCl$_3$ with 0.01M MgCl$_2$ final concentration added to protect the integrity of the phage head. "Mass lysate" indicates that AYS19 lysates were prepared on unpurified transductants from a single transduction. In this case, where cointegrate formation via transposition had occurred in the transductants, the results of many different events were present in the
lysates. Other lysates were prepared from a purified transductant clone where only a single structure resulting from a single TnpA protein-mediated event was expected.

Transduction

Method 1 - Recipient cells were cultured overnight at 32°C in 5ml TYE broth. The cultures were centrifuged at 5000 rpm for 5 minutes and the pelleted cells were resuspended in 0.01M MgSO₄ for a minimum of 4 hours with aeration. This process, known as starving the cells, was performed to allow for more efficient absorption of phage. 0.1ml of starved cells was added to 0.1 ml of appropriate dilution of a lysate containing cosmids and incubated at room temperature (25°C) for 60 minutes. After incubation, cells were spread on appropriate antibiotic medium, incubated at 32°C for 48 hours, and scored.

Method 2 - Recipient cells were prepared in the same manner as in method 1. 0.2ml starved cells were added to 0.2ml undiluted lysate. After incubation for 60 minutes at room temperature, appropriate 100-fold dilutions were made and spread on appropriate antibiotic medium, incubated for 48 hours at 32°C, and scored. This method resulted in more accurate numbers for serial dilutions of lysates.

Isolation of Plasmid DNA

Rapid isolation of plasmid DNA was performed using an alkaline extraction method modified from Birnboim-Doly and Maniatis (4, 15).
Restriction endonuclease analysis includes Bam HI (B), Cla I (C), Bgl II (G), Sac I (S), Kpn I (K), and Eco RI (R). Restriction endonuclease digestions were performed according to the recommendations of the supplier (International Biotechnologies, Inc. or Brisco). In general, 3-4 units of enzyme were used to digest 2-3μg of DNA. Gel electrophoresis of enzyme-digested DNA fragments was done in horizontal 0.7% agarose slabs with TEB buffer (0.09M boric acid, 0.09M Tris base, 2.55mM Na₂-EDTA) at a current of 75mAmps and 90 volts for 3 hours. DNA fragments were visualized by staining for 15 minutes with ethidium bromide and viewing under UV-light. Gels were photographed with a polaroid camera using a UV filter and type 55 polaroid film. Distances DNA bands migrated were measured in millimeters and compared to standard curves [log(kbp) vs. distance migrated] to determine sizes of DNA fragments. An example of how isolated putative cointegrates were analyzed is given in the appendix.
RESULTS

Construction of p\(\lambda\)CM-pCMX801 Cointegrates

Construction of p\(\lambda\)CM::Tnl\(\Delta\)pp derivatives with the transposon sequence in the left end of p\(\lambda\)CM was begun prior to the commencement of this project. The first step in this procedure, construction of p\(\lambda\)CM-pCMX801 cointegrates, was accomplished in the following manner.

RSF1010::Tnl (pXJS595) was partially digested with PstI restriction endonuclease and the DNA was religated with T\(_4\) DNA ligase. After ligation the DNA was used to transform MV12 to streptomycin resistance (Sm\(^R\)). Plasmid DNA from Sm\(^R\)Cb\(^8\) transformants was analyzed with restriction endonucleases to identify appropriate structures. pCMX801 was a product of this process (see Figure 6). The Tnl derivative in pCMX801 (Tnl\(\Delta\)pp, 1.45 kilobase pairs) was missing two internal PstI fragments which rendered the Tnl derivative tnpA\(^-\), res\(^-\), tnpR\(^-\) and bla\(^-\). The RSF1010 portion of pCMX801 was deleted for one small segment which resulted in loss of the sulfonamide resistance (Su\(^F\)) marker. E. coli MV12 containing pCMX801 and a TnpA protein-producing plasmid was then transduced to Cm\(^R\) with p\(\lambda\)CM to allow cointegrate formation between pCMX801 and p\(\lambda\)CM. This process depended on the TnpA protein produced in the MV12, which acted on the intact terminal inverted repeats of Tnl\(\Delta\)pp to form the cointegrate between pCMX801 and p\(\lambda\)CM. The cointegrate was relatively stable because no tnpR activity was present and because MV12
Construction of $\lambda$CM-pCMX801 cointegrates. $p$XJS595 is linearized at the *Eco* R1 (R) restriction endonuclease cleavage site. The sulphonamide resistance determinant ($Su^R$) is removed, and the *bla* gene deleted after *Pst* I partial digestion. The streptomycin resistance determinant ($Sm^R$) remained intact. pCMX801 was recombined into $\lambda$CM via TnpA protein-mediated recombination. Physical maps of 4 $\lambda$CM-pCMX801 cointegrate structures obtained are shown.
"Partial Pst I Digestion" followed by "T4 DNA ligase".

pCMX801

pCMX831

pCMX920

pCMX911

pCMX926
was not capable of homologous recombination. AYS19 was used to make cosmid lysates of \(C_{mr}Sm_{r}\) transductants. These lysates contained AYS19, pAXCM, and any pAXCM-pCMX801 cointegrates. The lysates were used to transduce MV12 to \(C_{mr}\), and \(C_{mr}Sm_{r}\) clones were identified. These clones contained putative pAXCM-pCMX801 cointegrates. Putative cointegrate DNA was isolated, and restriction endonuclease mapping identified seven structures in which pCMX801 was inserted in the left and of pAXCM. Two pairs of cointegrates, pCMX831/920 and pCMX911/926 (see Figure 6), represented structures in which pCMX801 sequences were present in both orientations in the same region of pAXCM. These structures were available at the commencement of this project.

**Isolation of pAXCM::TnI\Delta app Structures**

The first phase of this project was to isolate pAXCM::TnI\Delta app structures from the pAXCM-pCMX801 cointegrates. Homologous recombination between the direct repeats of TnI\Delta app in the pAXCM-pCMX801 cointegrates was expected to yield pAXCM::TnI\Delta app structures. Lysates made by growing AYS19 on CMX831, CMX920, CMX911 and CMX926 contained pCMX831, pCMX920, pCMX911 or pCMX926. Each of these four lysates was used to transduce M7124 to \(C_{mr}\) via Method 1 (see Material and Methods). Transductants were tested by replica plating for \(Sm_{r}\) which indicated the presence of a cointegrate. Homologous recombination in M7124 was expected to yield pCMX801 and pAXCM::TnI\Delta app. Recombinants were identified by disassociation of \(Sm_{r}\) and \(C_{mr}\) markers as follows. Lysates were made by growing AYS19 on the M7124 \(Sm_{r}C_{mr}\) transductants and were used to transduce MV12 to \(C_{mr}\) via Method 1. These MV12 \(C_{mr}\)
transductants were tested by replica plating for Sm sensitivity, and
Cm<sup>r</sup>Sm<sup>s</sup> clones were carrying putative pACM::TnⅠAp recombinants. Cosmid
DNA was isolated from Cm<sup>r</sup>Sm<sup>s</sup> clones and analyzed with Cla I-Kpn I
restriction endonuclease digests to identify true recombinant structures
(see Table 3, Figure 7). Recombinant structures were isolated from
pCMX831, pCMX920, and pCMX911. A recombinant structure from pCMX926 has
not yet been identified. Since pCMX948 (from pCMX831) and pCMX1075
(from pCMX920) represent structures where pACM carries TnⅠAp in either
orientation with respect to pACM, and in the same region of pACM (at
21.97 kbp of λ for pCMX948 and at 20.84 kbp of λ for pCMX1075),
transposition immunity studies were performed.

Transposition Immunity When pCMX948 Is Challenged with Tn3

pCMX948, a recombinant product of pCMX831, was challenged with
wild-type Tn3 in phB8 (RSF1050). To accomplish this, XJS797 was
transduced via Method 1 to Cm<sup>r</sup> using λYS19 helper lysates containing
encapsidated pCMX948. XJS797 was also transduced via Method 1 to Cm<sup>r</sup>
with pACM as a control. No transposition immunity was expected for the
system using pACM. λYS19 was grown on the Cm<sup>r</sup> XJS797 transductants to
make mass lysates which were expected to contain λYS19, pACM or pCMX948,
and any transposition products (pACM::Tn3 or pCMX948::Tn3). The mass
lysates were used to transduce MV12 to Cm<sup>r</sup> via Method 2 (see Materials
and Methods). Cm<sup>r</sup> MV12 transductants were screened for Cb<sup>r</sup> by replica
plating. Cm<sup>r</sup>Cb<sup>r</sup> transductants were expected to contain pCMX948J, or
pACM, into which Tn3 had transposed. Thus the ratio of Cm<sup>r</sup>Cb<sup>r</sup>/Cm<sup>r</sup>
transductants indicated the frequency at which transposition had
Tabl. 3. Isolation of pACM::TnlApp Structures

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<tr>
<th>Lysate from M7124 ( )</th>
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<sup>a</sup>Cosmid lysates made by growing XYS19 on M7124(pCMX831), M7124(pCMX920), M7124(pCMX911), or M7124(pCMX926) were used to transduce MV12 to chloramphenical resistance (Cm<sup>f</sup>).

<sup>b</sup>Cm<sup>f</sup> transductants were tested for streptomycin sensitivity (Sm<sup>f</sup>). Cm<sup>f</sup>Sm<sup>f</sup> transductants were carrying putative recombinant pACM::TnlApp structures.

<sup>c</sup>DNA was isolated from some Cm<sup>f</sup>Sm<sup>f</sup> transductants and tested by restriction endonuclease analysis (Cla I-Kpn I double digests) for expected recombinant structures.

<sup>d</sup>Recombinant pACM::TnlApp structures isolated from given pACM-pCMX801 cointegrates.
Figure 7

Restriction endonuclease cleavage maps of pCMX948 and pCMX1075. Cosmids are linearized at λ cos site. Restriction endonuclease cleavage sites are shown for Cla I (C) and Kpn I (K). Numbers indicate sizes of fragments (in kilobase pairs) obtained from a Cla I-Kpn I double digest. The site of Tn1Δpp insertion in the pXCM sequence is noted.
occurred. The results showed that Tn3 from RSF1050 transposed into p\text{\textalpha}CM at a frequency of 0.057. The frequency of Tn3 transposition into pCMX948 was 0.00011 (see Table 4, Part A). Thus transposition immunity was observed in this system since transposition into pCMX948 was approximately 518-fold lower than transposition into p\text{\textalpha}CM. In this experiment simple transposition was expected. Cointegrates should have been resolved because the Tn3 on RSF1050 was wildtype and therefore \textit{tnp}^{R+}, \textit{res}^{+}. Recombination between Tn3 and Tn\text{\textalpha}App was not expected because Tn\text{\textalpha}App was \textit{res}^{-} (excluding the possibility of Tn\text{\textalpha}R protein-mediated recombination) and the MV12 was \textit{recA}.

**Transposition Immunity When pCMX948 and pCMX1075 Are Challenged with Tn3\text{\textalpha}596**

In studies with pXJS972 it was observed that RSF1596 formed cointegrates with pXJS972 and p\text{\textalpha}CM at approximately the same frequency (Muster, C. personal communication). Therefore, it was decided to challenge pCMX948 and pCMX1075 with RSF1596 to determine if transposition immunity was observed in this system.

XJS214 (MV12 carrying RSF1596 and a source of TnpA protein) was transduced via Method 1 to Cm\textsuperscript{E} with p\text{\textalpha}CM (no transposition immunity expected), pCMX948, or pCMX1075. In XJS214, RSF103 was present to supply TnpA protein for cointegrate formation since both Tn3\text{\textalpha}596 and Tn\text{\textalpha}App were \textit{tnp}A by deletion. A parallel experiment was performed using XJS213 which was identical to XJS214 except that it did not carry RSF103. Therefore, that system had no source of TnpA protein and no TnpA protein-mediated cointegrate formation was expected. AYS19 was grown on Cm\textsuperscript{E} transductants of XJS214 and XJS213 to make lysates. These
<table>
<thead>
<tr>
<th>Lysates from YS19 Grown on:</th>
<th>Transductants/ ml Lysate</th>
<th>Transductants/ ml Lysate</th>
<th>Average Frequency</th>
<th>Fold Decrease</th>
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<td></td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Cb&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;Cb&lt;sup&gt;r&lt;/sup&gt;/d</td>
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<td>.057</td>
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<td>1.4x10&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>.00011</td>
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<tr>
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<td><strong>Part B</strong></td>
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<td><strong>Part C</strong></td>
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<td>XJS213 (pCM)</td>
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<td>&lt;.00006</td>
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</tbody>
</table>

*aTnpA protein-mediated recombination frequencies for RSF1050 and pCM or pCMX948.

*bTnpA protein-mediated recombination frequencies for RSF1596 and pCM, pCMX948, or pCMX1075.

*cRecombination frequencies for RSF1596 and pCM, pCMX948, or pCMX1075 in the absence of TnpA protein.

*dRatio of Cm<sup>r</sup>Cb<sup>r</sup> transductants/Cm<sup>r</sup> transductants is the frequency of TnpA protein-mediated recombination.

*eFold decrease of TnpA protein-mediated recombination with respect to pCM.
lysates were then used to transduce MV12 to Cm\(^r\) via Method 2. Cm\(^r\) MV12 transductants were screened for Cb\(^r\) by replica plating. Once again, determining the ratio of Cm\(^r\)Cb\(^r\)/Cm\(^r\) transductants indicated the frequency of cointegrate formation between RSF1596 and p\(\lambda\)CM, pCMX948, or pCMS1075. TnpA protein-mediated recombination of RSF1596 into p\(\lambda\)CM occurred at a frequency of 0.14, while recombination into pCMX948 occurred at a frequency of 0.00026 and into pCMX1075 at a frequency of 0.00023 (see Table 4, Part B). Thus using p\(\lambda\)CM carrying Tnl\(\lambda\)pp in either orientation and RSF1596, transposition immunity was observed. Using pCMX948 a 538-fold decrease in TnpA protein-mediated recombination was observed with respect to p\(\lambda\)CM, and using pCMX1075 a 609-fold decrease in tnpA protein-mediated recombination was observed with respect to p\(\lambda\)CM.

In this set of experiments, unlike the experiments using XJS797, cointegrates were expected because both Tn3\(\lambda\)596 and Tnl\(\lambda\)pp were \(\text{tnpR}\) and \(\text{res}\) by deletion. Also, XJS214 was \(\text{recA}\) excluding the possibility of homology-dependent recombination.

The results of the experiments using XJS213 indicated that Cm\(^r\)Cb\(^r\) transductants were not observed in the absence of TnpA protein (see Table 4, Part C). This result supported the expectation that Cm\(^r\)Cb\(^r\) transductants resulted from recombination structures mediated by TnpA protein.

**Determination of Insertion Site and Orientation for Putative pCMX948-RSF1596 and pCMX1075-RSF1596 Cointegrates**

Plasmid DNA was isolated from some of the Cm\(^r\)Cb\(^r\) MV12 transductants. These transductants were expected to contain either
pCMX948-RSF1596 or pCMX1075-RSF1596 cointegrates formed by the TnpA protein-mediated recombination of RSF1596 and pCMX948 or pCMX1075 with concurrent replication of donor transposon sequences. Isolated plasmid DNA was digested with restriction endonucleases and analyzed to determine donor and recipient replications, distribution of insertions into recipient replications, and orientation of insertions into recipient replications. The results obtained for individual putative cointegrate structures are presented in Table 5. Each of these structures represents an independent insertion event. Of the 10 putative pCMX948-RSF1596 cointegrates and 12 putative pCMX1075-RSF1596 cointegrates analyzed, all showed that insertion occurred via replicative recombination of Tn3Δ596 into pCMX948 or pCMX1075. Surprisingly, most of the structures analyzed were not cointegrates, but rather, simple transposition products (i.e. pCMX948::Tn3Δ596 or pCMX1075::Tn3Δ596). Orientation of insertions into pCMX948 (40% rightward, tnpA to bla, and 60% leftward, bla to tnpA) was very similar to that observed by Muster (personal communication) for insertions into pλCM (41% rightward, 59% leftward) (see Figure 8). Orientation of insertions into pCMX1075 was 50% in either orientation. There may be a hot spot for insertion into pCMX948 near the cos site since 3 of the 10 structures analyzed showed insertion within 250 bp of cos. For pCMX1075, there may be a hot spot in the Tn9 sequence since 3 of 12 structures analyzed showed insertion within a 1000 bp region of Tn9. These observations, however, may be due to the small number of structures studied. Analysis of more structures is needed before any apparent trends of insertion distribution and orientation can be verified.
Table 5. Physical Analysis of Putative RSF1596-pCMX948 and RSF1596-pCMX1075 Cointegrates

<table>
<thead>
<tr>
<th>Structure</th>
<th>Position on Cosmid</th>
<th>Orientation</th>
<th>Structure Observed</th>
</tr>
</thead>
<tbody>
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<td>Part A</td>
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</tr>
<tr>
<td>pCMX2006</td>
<td>48.4 kbp of λ</td>
<td>----</td>
<td>Simple</td>
</tr>
<tr>
<td>pCMX2007</td>
<td>46.7 kbp of λ</td>
<td>----</td>
<td>Simple</td>
</tr>
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<td>pCMX2008</td>
<td>0.45 kbp of tn9</td>
<td>----</td>
<td>Simple</td>
</tr>
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<td>pCMX2009</td>
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<td>----</td>
<td>Simple</td>
</tr>
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<td>Simple</td>
</tr>
<tr>
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<td>9.0 kbp of λ</td>
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<td>Simple</td>
</tr>
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<td>8.1 kbp of λ</td>
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<td>Simple</td>
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<td>pCMX2032</td>
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<td>Cointegrate</td>
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<tr>
<td>pCMX2056</td>
<td>6.1 kbp of λ</td>
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<td>Part B</td>
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<td>pCMX2014</td>
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<td>Cointegrate</td>
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</table>

^Distribution of insertion sites and orientation for TnpA protein-mediated recombination of RSF1596 and pCMX948.

^Distribution of insertion sites and orientation for TnpA protein-mediated recombination of RSF1596 and pCMX1075.

^Position of insertion sites is given in kilobase pairs (kbp) on lambda standard map (10), kbp in the Tn9 sequence, or kbp in the resident Tn1Δpp sequence of pCMX948 or pCMX1075 (see Figure 1).

^Orientation is indicated by arrows and refers to standard orientation of Tn3 sequences shown in Figure 1.

^"Structure Observed": "simple" indicates that the structure analyzed is the structure of a simple transposition product (i.e. Tn3Δ596 inserted into pCMX948 or pCMX1075), while "cointegrate" indicates a cointegrate structure of RSF1596 and pCMX948 or pCMX1075.
Figure 8

Insertion sites and orientations of RSF1596 sequences into p\(\lambda\)CM, pCMX948, or pCMX1075. Distribution of insertions is shown by vertical lines indicating the sites of independent insertions of RSF1596 sequences into the cosmids. Orientations of insertions are indicated by position of the vertical lines above or below the cosmid maps. Lines above the map indicate rightward (\texttt{tnpA} to \texttt{bla}) orientation, and lines below the map indicate leftward (\texttt{bla} to \texttt{tnpA}) orientation. The insertion map for RSF1596 into p\(\lambda\)CM is from Muster, C. unpublished data.
p\lambda CM

pCMX948

pCMX1075
DISCUSSION

Isolation of p\text{\textlambda}CM::Tnl\text{\Delta}pp Structures

The method used to isolate p\text{\textlambda}CM::Tnl\text{\Delta}pp structures was fairly simple and efficient. The reasons for this lie in the fact that TnpA protein recombined pCMX801 into p\text{\textlambda}CM efficiently, and RecA protein recombined the direct repeats of Tnl\text{\Delta}pp leaving a p\text{\textlambda}CM::Tnl\text{\Delta}pp structure. A large number of independent insertions of pCMX801 into p\text{\textlambda}CM were obtained, and restriction endonuclease cleavage analysis identified insertions into the left end of p\text{\textlambda}CM. RecA protein-mediated recombinants for six cointegrates were easily obtained. However, isolation of a pCMX926 recombinant has not yet been accomplished. Of 21 putative pCMX926 recombinants analyzed thus far, none have had the proper structure (p\text{\textlambda}CM::Tnl\text{\Delta}pp). These putative recombinants appear to have deletions, or other rearrangements. This observation is surprising since the Tnl\text{\Delta}pp sequence lacks any transposon-mediated recombination activity, and thus transposon-mediated rearrangements are not expected. It is possible that the position and orientation of the pCMX801 sequence in the p\text{\textlambda}CM cosmid somehow destabilizes the surrounding sequences. However, the pCMX801 of pCMX911 is inserted near the insertion site of pCMX926, although in opposite orientation, and no trouble was experienced in isolating correct recombinant structures from pCMX911. Furthermore, a general effect of orientation on ability to
isolate recombinants was not noted for the other pACM-pCMX801 cointegrates studied.

**Transposition Immunity with RSF1050**

In the experiments with XJS797 and pACM, or pCMX948, it was observed that the wild type Tn3 sequence of RSF1050 recognized pCMX948 as transposition immune. There was a 518-fold decrease in TnpA protein-mediated recombination between RSF1050 and pCMX948 when compared to RSF1050 and pACM. It is presumed that this transposition immunity was due to the presence of the TnlApp sequence of pCMX948 since that was the only difference between the pCMX948 and pACM replicons. Although recombinant structures from these experiments were not analyzed, the genetic data are consistent with the presumption that recombination occurred via transposon-mediated insertion of Tn3 into the cosmids. These results are consistent with the observation that only one copy of the terminal inverted repeat of Tn3 is sufficient for transposition immunity (14).

**Transposition Immunity with RSF1596**

In the experiments with XJS214 and pACM, pCMX948, or pCMX1075, it was observed that the Tn3A596 sequence of RSF1596 recognized pCMX948 and pCMX1075 as transposition immune. RSF1596 recombined with pCMX948 at a 538-fold lower frequency than it did with pACM, and it recombined with pCMX1075 at a 609-fold lower frequency than it did with pACM. This decrease in TnpA protein-mediated recombination frequency was due to the presence of the TnlApp sequence, the only feature that distinguishes
pCMX948 and pCMX1075 from pλCM. No effect on transposition immunity was observed for opposite orientation of the resident TnlΔAp sequence in pλCM.

These results are surprising since they are not consistent with Muster’s observations that Tn3Δ596 does not recognize pXJS972 (a pλCM carrying TnlΔAp) as transposition immune even though other pMB8::Tn3 deletion derivatives do recognize pXJS972 as transposition immune (Muster, C., personal communication). The reason for this inconsistency in results is unknown. The internal deletion of TnlΔAp (3.506 kbp) is much bigger than the internal deletion of TnlΔAp (0.89 kbp). Furthermore, the entire sequence deleted in TnlΔAp is also deleted in TnlΔpp. It is not clear why pλCM carrying TnlΔAp should not be transposition immune to RSF1596, while pλCM carrying TnlΔpp is. An additional difference between TnlΔpp and TnlΔAp is that TnlΔAp has transcription activity for the tnpA gene and a functional res site, while TnlΔpp has no transcription activity and is deleted for res. There is no reason to believe, however, that transcription of tnpA or the presence of a functional res site should repress transposition immunity character since wild type Tnl is tnpA+, res+ and transposition immune. It is possible, however unlikely, that the non-transposition immune character of pXJS972 with respect to RSF1596 is due to the region and/or orientation of insertion of TnlΔAp into pλCM. To test this possibility, a pλCM::TnlΔpp with TnlΔpp inserted in the same region and orientation as the insertion in pXJS972 can be challenged with RSF1596. A final possibility is that somehow the deletion of pλCM sequences in pXJS972 has affected its behavior.
The results of the experiments with XJS213 and pLCM, pCMX948, or pCMX1075 showed no recombination between RSF1596 and pLCM, pCMX948, or pCMX1075 to the level measured. This result supports the assumption that in the XJS214 experiments, recombination was mediated by TnpA protein.

Physical Analysis of Putative RSF1596-pCMX948 and RSF1596-pCMX1075 Cointegrates

All of the putative cointegrates analyzed showed that RSF1596 had inserted into the cosmid (pCMX948 or pCMX1075) via Tn\(^{\Delta} 596\). This result agrees with observations made by Muster that in all cases she examined, RSF1596 (or other pMB8::Tn3 deletion derivatives) recombined into pXJS972. Thus there is a clear directionality to the TnpA protein-mediated recombination event. In this system, the cosmids were in low copy number (1-2 copies per cell), and the plasmids were in high copy number (30-50 copies per cell). The cosmids are 40.5 kbp while RSF1596 is approximately 7 kbp. Thus, there is likely to be more plasmid DNA in the cell than cosmid DNA, and consistent insertion of RSF1596 into the cosmids should not be due to the presence of more cosmid DNA target. However, the 40.5 kbp of the cosmids do offer more different sequences than the 7 kbp of RSF1596, and this may affect the donor-recipient relationship. Neither the Tn3\(^{\Delta} 596\) of RSF1596 nor the TnL\(^{\Delta} \)pp of pCMX948 or pCMX1075 was transcription active for tnpA. Thus, recombination of both transposon deletion derivatives depends on TnpA protein provided in trans (from RSF103), and cis-activity of TnpA protein could not be a factor as it might have been in the pXJS972 system.
Due to the small number of structures analyzed, trends of distribution and orientation of insertions cannot be identified. However, since 3 of 10 pCMX948-RSF1596 recombinant structures analyzed showed insertions within 250 bp of the cos site, it appears that this may be a hot spot for TnpA protein-mediated insertion. Note however, that for pCMX1075-RSF1596 recombinant structures no indication of a hot spot near cos is observed. Instead, 3 of the 12 structures analyzed showed insertion within a 1 kbp region of the Tn9 sequence in pCMX1075. This preliminary data indicates approximately equal orientations of insertion in either direction. To show strong evidence for an effect of the Tnl^pp sequence on orientation of RSF1596 insertions, a larger number of insertions must be mapped.

Surprisingly, only 5 of the 22 structures analyzed were cointegrates. The other 17 structures were simple transposition products (i.e. pCMX948::Tn3Δ596 of pCMX1075::Tn3Δ596). Since the system was recA, tnpR, res no resolution of cointegrates by RecA protein or TnpR protein was expected. This instability of cointegrates, where both sequences between the transposon repeats contain an origin of replication, has been observed before (16). However, in this present study, a much higher rate was observed. The high rate of instability suggested by these results may be due to the fact that isolation of these putative cointegrates was performed at 37°C while in the previous study, isolation of cointegrates was performed at 32°C. To test this possibility, parallel experiments could be conducted at 32°C and 37°C for isolation of putative cointegrates of RSF1596 and pCMX948 or RSF1596 and pCMX1075.
These results suggest that conclusions concerning transposition immunity in a particular system cannot be easily related to other systems. Transposon sequences and the sequences of the replicons in which they reside appear to affect the frequency with which TnpA protein-mediated recombination occurs. Further, there is evidence for preferred donor and target replicons in a given system.
SUMMARY

The closely related transposable elements Tnl and Tn3 display a characteristic called transposition immunity. When transposition immunity is expressed, a Tn3 sequence residing in a replicon will inhibit Tn3 sequences in other replicons from transposing into the first replicon. Transposition immunity is a cis-acting phenomenon, and it occurs during the first step of transposition, the TnpA protein-mediated step. Although Tn3 is an active bacterial transposon, transposing at a rate of $10^{-1}$ to $10^{-2}$ transposition events per cell generation, its activity can be reduced 20 to $10^6$-fold by transposition immunity. Transposition immunity has been observed when only one copy of the 38 bp terminal inverted repeat of Tn3 was present in the target replicon. However, in some systems where one replicon carried an internal deletion derivative of Tn3 (leaving both inverted repeats intact), no transposition immunity was observed. The presence or absence of transposition immunity and the degree to which it is expressed appears to depend on the derivatives of Tn3 used and on the system in which transposition immunity is studied. In this project, derivatives of the cosmid pACM in which a Tnl deletion derivative (TnlΔpp) was inserted in either orientation and in the same region of pACM were made. Two of these structures, pCMX948 and pCMX1075, were used to study transposition immunity. pCMX948 expressed transposition immunity when challenged with
RSF1050 (pMB8::Tn3). pCMX948 and pCMX1075 both expressed transposition immunity when challenged with RSF 1595 (pMB8::Tn3Δ596). Putative pCMX948-RSF1596 and pCMX1075-RSF1596 cointegrate DNA molecules were isolated, and in all cases examined, RSF1596 acted as a donor replicon. Distribution of insertions into the recipient replicons and orientation of the insertions were determined for structures.
REFERENCES


APPENDIX

An example of how putative RSF1596-pCMX948 and RSF1596-pCMX1075 cointegrates were mapped using restriction endonuclease digestions. The structure mapped in this example is pCMX2007. Statements refer to the pictured gel, the restriction endonuclease cleavage maps, and standard curve. Fragments on restriction endonuclease cleavage maps are lettered from largest to smallest. Digestions of pCMX948 and RSF1596 were also performed to compare to pCMX2007 digestions. Standard abbreviations for restriction endonucleases are given at cleavage sites: Bgl II (G), Bam HI (B), and Cla I (C). A restriction endonuclease cleavage map for TnlA pp is given in Figure 1 of this paper.

1. Compare lanes 4 and 5. The insert is in Bgl II B fragment since that band is missing from lane 4.

2. Compare lane 3 with lanes 4 and 5. Sac I only cuts in Tn3Δ596. The Bgl II B fragment is missing. Three new fragments are present at 31.0mm, 32.5mm, and 34.25mm. From standard curve, junctions (new fragments) are approximately 8 kbp, 7 kbp, and 6.1 kbp. The 7 kbp fragment represents RSF1596 DNA from the Sac I site in one TnlΔ596 repeat to the Sac I site in the second TnlΔ596 repeat. This suggests four possible insertion sites/orientations in Bgl II fragment.
Figure for Appendix I. Mapping putative RSF1596-pCMX948 cointegrate structures.

**Top left:** 0.7% agarose gel stained with ethidium bromide and photographed to show restriction enzyme digests of DNAs to map pCMX2007. Lanes 1 and 14: no DNA. Lane 2: RSF1596 cleaved with Sac I. Lanes 3 and 4: pCMX2007 cleaved with Bgl II + Sac I and Bgl II respectively. Lane 5: pCMX948 cleaved with Bgl II. Lane 6: pCM cleaved with Bam HI and Eco RI. Lane 7: pCMX948 cleaved with Bam HI. Lanes 8 and 9: pCMX2007 cleaved with Bam HI and Bam HI + Sac I respectively. Lane 10: pCMX948 cleaved with Cla I. Lanes 11 and 12: pCMX2007 cleaved with Cla I and Cla I + Sac I respectively. Lane 13: RSF1596 cleaved with Cla I + Sac I.

**Top right:** Standard curve relating DNA fragment size in kilobase pairs to distance migrated on the gel. Points on the standard curve relate the measured distances of migration with the known fragment sizes for pCM cleaved with Bam HI + Eco RI, RSF1596 cleaved with Sac I or Cla I + Sac I, and pCMX948 cleaved with Bgl II, Bam HI or Cla I. New fragments generated when pCMX2007 was cleaved by various enzymes were measured for distance migrated, and the standard curve was used to estimate the size of each new fragment.

**Center:** Restriction enzyme cleavage maps for pCMX948 cleaved with Bgl II, Bam HI or Cla I. In each map, the cosmid has been linearized at cos. The sizes of the fragments generated are given in kilobase pairs. The heavy letters indicate the relative fragment sizes. The largest fragment (slowest moving) is labeled A in each case.

**Bottom:** Structure of the insertion expected in RSF1596-pCMX948 cointegrates mediated by RSF1596. This structure is expected to be inserted into cosmid sequences. Note the direct repeats of the Tn3 derivative join the pMB8 sequences to the cosmid sequences. Note that the insert carries one Sac I site, one Bam HI site and one Cla I site in each copy of the Tn3 sequences. The sizes of the fragments generated by cleavage with each enzyme are noted. In addition to these fragments, junction fragments between insert DNA and pCMX948 sequences are generated. The sizes of the junction fragments will depend on the site and orientation of the insertion.
Restriction maps for SphI, BamHI and SacI digests of pMB8 are shown above. Numbers below the lines designate fragment sizes in kbp. Numbers in parentheses indicate the location of the particular cleavage site on the lambda standard map, and are in kbp.

The insert found in Tn3596-mediated cointegrates is shown above. It yields the following fragments. Digestion with SacI, BamHI or SphI yields a 7.0 kbp fragment in each case. Digestion with BamHI and SacI yields two 2.5 kbp fragments, and a 4.5 kbp fragment. Digestion with SphI and SacI yields two 1.7 kbp fragments and a 5.0 kbp fragment (see figure 1 of this thesis).
3. Compare lanes 7 and 8. The **Bam HI** B fragment is missing indicating the presence of the insertion in that fragment. Three new fragments are present at 30.5mm, 31.5mm, and 32.5mm. From the standard curve, these correspond to the 7.0 kbp internal plasmid insert fragment and junctions of approximately 8.5 kbp and 7.6 kbp. These data suggest that the insert at 41.04 kbp (shown in 2.) is incorrect because that site is not in the **Bam HI** B fragment. Four possible insertion sites/orientations are suggested. Note that 0.67 kbp insertion site cannot be correct since that site is not in the **Bgi II** B fragment. Also note that the 46.56 kbp insertion site is approximately equal to the 46.75 kbp site given in **Bgi II** B fragment shown in 2. and that they are in the same orientation.
4. Compare lane 9 with lanes 7 and 8. Note that Sac I did not give a complete digestion. Three new fragments are present at 36.5mm, 38.5mm, and 47.5mm. These correspond to a 5.1 kbp junction fragment, a 4.5 kbp RSF1596 fragment, and a 2.5 kbp RSF1596 fragment. Note that the 8.5 kbp junction from Bam HI digestion (lane 8) is still present in Bam HI-Sac I digestion (lane 9). Calculate that the 5.1 kbp new junction fragment plus the 2.5 kbp new RSF1596 fragment equals the 7.6 Bam HI fragment.
This suggests two possible insertion sites/orientations. But the 
0.67 kbp insertion site has already been shown to be incorrect 
because it is not in the Bgl II B fragment. Expect insertion to be 
at 46.56 kbp site.

5. To prove that the expected site and orientation is correct, 
compare lanes 10, 11, 12, and 13. It is expected that the 
Cla I digest (lane 11) will be missing the Cla I C fragment 
and will have a plasmid insert fragment of 7.0 kbp, and 
junction fragments of 2.1 kbp and 8.3 kbp. Digestion with Cla 
I and Sac I is expected to affect the 2.1 kbp junction 
fragment, but not the 8.3 kbp junction fragment, to give a 
very small fragment of approximately .6 kbp and a 1.7 kbp 
fragment, as well as a 5.5 kbp plasmid fragment.
For the Cla I digestion three new fragments were observed: 8.2 kbp (junction fragment), 7.0 kbp (RSF1596 fragment), and 2.3 kbp (junction fragment). For the Cla I-Sac I digestion the 8.2 kbp junction fragment is observed as well as a 5.3 kbp RSF1596 fragment and a 1.7 kbp RSF1596 fragment. The very small fragment (approximately 0.6 kbp) is too small to see on the gel. Since smaller fragments most accurately indicate size (i.e. they fall on the most linear portion of the standard curve), use the 2.3 kbp fragment to map. Cla I cleaves Tn3Δ596 2.0 kbp from the left end. Therefore the insertion is 2.3 - 2.0 = .3 kbp from the 46.44 kbp Cla I cleavage site of pCMX948, and at approximately 46.7 kbp of the standard map (10).