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# Selected Translocation of Plasmid Genes: Frequency and Regional Specificity of Translocation of the Tn3 Element

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A procedure is described that selects for the insertion of transposable antibiotic resistance elements in a variety of recipient replicons. The selected translocation procedure, which employs a plasmid having a temperature-sensitive defect in replication as a donor of transposable genetic elements, was used to investigate certain characteristics of the translocation process. Our results indicate that translocation of the Tn3 element from plasmid to plasmid occurs at a $10^3$- to $10^4$-times-higher frequency than from plasmid to chromosome. In both cases, continued accumulation of Tn3 on recipient genomes is prevented by development of an apparent equilibrium when only a small fraction of molecules in the recipient population contain Tn3. An alternative method for estimation of translocation frequency has shown that the translocation process is temperature sensitive and that its frequency is unaffected by the presence of host *recA* mutation. Insertions of Tn3 onto the $65 \times 10^6$-dalton R6-5 plasmid in *Escherichia coli* are clustered on *Eco*RI fragments 3 (8 of 23 insertions) and 9 (7 of 23 insertions), which contain 12 and 5%, respectively, of the R6-5 genome. The occurrence of multiple insertions of Tn3 within *Eco*RI fragment 9, which contains the IS1 element and a terminus of the Tn4 element, is consistent with earlier evidence indicating that terminal deoxyribonucleic acid sequences of already present transposable elements may provide recognition sequences for subsequent illegitimate recombinational events.

Recently it has been recognized that genetically and structurally defined segments of deoxyribonucleic acid (DNA) are capable of translocation among bacterial, phage, and plasmid genomes in the *Enterobacteriaceae* (for reviews, see references 6, 11, and 32). Two types of transposable elements have been identified: Tn and IS (insertion sequence). The Tn elements constitute a phenotypically and structurally diverse group of genetic units that carry genes specifying resistance to one or more antibiotics. They are usually at least several thousand nucleotide base pairs in length and frequently contain duplicated DNA segments at their termini in either direct or inverted nucleotide sequence orientation. The IS elements, which range from 700 to 1,400 base pairs in length, appear to be more limited in variety than the Tn elements, and no terminal DNA sequence duplications are detectable in IS elements by electron microscope analysis. Although IS units may carry signals for initiation or termination of ribonucleic acid synthesis, they are otherwise phenotypically cryptic.

Despite increasing evidence for the importance of Tn and IS elements in the evolution of procaryotic genomes, little is known about the mechanism of translocation of these elements or about control of the apparent site specificity of translocation events (6, 32). To study the frequency and specificity of translocation, and as an aid to investigating the mechanisms involved in the translocation process, we employed a simple method to accomplish the selective translocation of antibiotic resistance elements to a variety of recipient replicons. Although the procedure, which utilizes a donor plasmid having temperature-sensitive replication functions [Rep(Ts)], was developed for use with a transposable ampicillin-resistant element (Tn3) (4, 22), it should also be applicable for the study of translocation of other elements that contain selectable phenotypic properties. The present communication describes the selected translocation procedure and reports studies of the frequency and site specificity of translocation of Tn3.

## MATERIALS AND METHODS

**General procedures.** Bacterial strains and plasmids used in this study are described in Table 1. Nutrient agar (Difco) plates were supplemented with 0.5% tryptone (Difco). Antibiotic concentrations used were (per milliliter): ampicillin (Ap), 20

TABLE 1. *Bacterial strains and plasmids*

| Strain or plasmid | Relevant phenotype | Reference or source |
|---|---|---|
| Bacterium | | |
| C600 | | Bachmann (1) |
| JC1569 | RecA⁻ | Clark et al. (5) |
| CR34 Nal$^r$ | Thy⁻ Nal$^r$ | Cohen and Chang (7) |
| Plasmid | | |
| pSC50 | Ap Cm Sm Su, conjugative | Kopecko and Cohen (22) |
| pSC101 | Tc | Cohen and Chang (7); Cohen et al. (9) |
| pSC105 | Tc Km | Cohen et al. (9) |
| pSC175 | Ap Tc | pSC101 containing Tn3 insertion; Kopecko and Cohen (22) |
| pSC201 | Tc Rep(Ts) | Kretschmer et al. (23) |
| pSC204 | Tc Ap Rep(Ts) | This study |
| R6-5 | Km Nm Cm Sm Su | Silver and Cohen (29) |
| RSF1010 | Sm Su | Guerry et al. (15) |

$\mu$g; chloramphenicol (Cm), 20 $\mu$g; kanamycin (Km), 20 $\mu$g; streptomycin (Sm), 10 $\mu$g; tetracycline (Tc), 10 $\mu$g; and nalidixic acid (Nal), 100 $\mu$g. Conditions and procedures for bacterial growth in L broth (29), isolation of covalently closed circular (CCC) plasmid DNA by detergent lysis and dye-buoyant density centrifugation (33, 34), conjugal transfer (29) of plasmids, and transformation (10) by plasmid DNA have been described.

Isolation and use of *Eco*RI restriction endonuclease was described earlier (8, 14). The reaction mixture for *Bam*I restriction endonuclease (Miles Laboratories; isolated from *Bacillus amyloliquefaciens* RVB500) was 0.1 M tris(hydroxymethyl)aminomethane-hydroxychloride (pH 7.5) plus 0.01 M $MgCl_2$. *Bam*I endonuclease (10 U/200 $\mu$l of reaction mixture) was added to 3 to 5 $\mu$g of DNA and incubated at 37°C for 45 min. For *Bam*I/*Eco*RI double digestions, the *Bam*I reaction was stopped by heating the mixture at 65°C for 10 min; after the mixture cooled in ice for 5 min, *Eco*RI endonuclease was added, and the reaction mix was incubated for an additional 45 min at 37°C. This reaction was stopped by addition of 0.15 volume of BGS (0.25% bromophenol blue, 25% glycerol, 5% sodium dodecyl sulfate). The resulting DNA fragments were analyzed by electrophoresis in 0.7% agarose gels as previously described (8, 17, 28).

Electron microscopic procedures for contour length measurement (ammonium acetate spreading) and for heteroduplex analysis (50% formamide hyperphase and 17% formamide hypophase) of plasmid DNA have been described (13, 26, 27).

**Selected translocation.** The procedure used for selected translocation of Tn3 is illustrated in Fig. 1. A bacterial strain containing pSC204 in the presence or absence of an additional plasmid was grown at 32°C in L broth for 16 h (stationary-phase cells) or diluted 1:20 in L broth and growth to early log phase. Appropriate dilutions of stationary- or log-phase cells ($1 \times 10^9$ to $5 \times 10^9$ viable cells per ml) were plated on nutrient agar plates containing or lacking Ap and incubated for 24 to 48 h at 45 or 32°C. The equal numbers of cells observed on both Ap and plain agar plates at 32°C indicated the total cell count. In some experiments, the number of cells appearing on nutrient agar plates at 45°C was significantly less than the number seen at 32°C; this resulted from the effects of minimal variations in oven temperature in the 45°C range on cell growth (P. J. Kretschmer and S. N. Cohen, unpublished data), and such experiments were not continued. After 48 h at 45°C, colonies appearing on Ap plates were counted, and some were purified by streaking on Ap plates at 45°C. The translocation frequency was estimated as indicated in Table 2. CCC DNA was prepared from the Ap-resistant clones by inoculation of a single purified colony into 2 ml of L broth, incubation with shaking at 45°C for 6 h, a 1:20 dilution into 45°C L broth containing Ap, incubation for an additional 6 h, and finally a dilution of 1:500 into 100 ml of L broth containing Ap. After 16 h of incubation at 45°C, CCC plasmid DNA was isolated.

**Alternative method for estimation of translocation frequency.** The alternative method for estimating translocation frequency is illustrated in Fig. 2. C600 cells containing the pSC175 or pSC204 plasmid were transformed with RSF1010 plasmid DNA, and, immediately after the heat pulse step in transformation (10), 0.1-ml fractions (approximately $10^4$ trans-

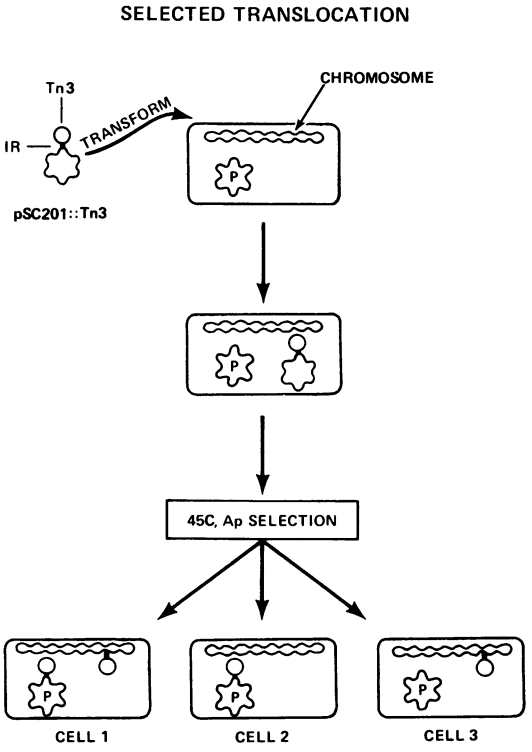


FIG. 1. *Procedure for selected translocation. See text for description. For simplicity, the double-stranded chromosome and plasmids are represented by single lines.*

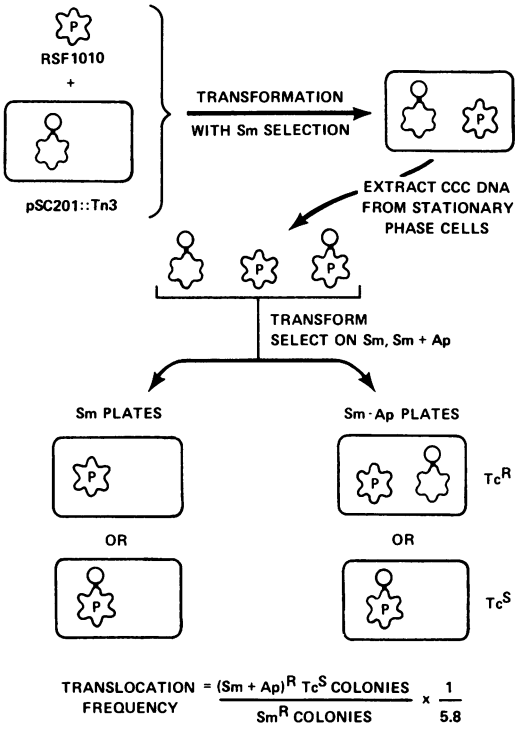


FIG. 2. *Alternative method for estimation of translocation frequency. See text for description. For simplicity, the chromosome has not been depicted, and double-stranded plasmid DNA is represented by a single line.*

formed cells) were transferred to a 100-ml prewarmed broth culture(s). After incubation for 120 min (to allow phenotypic expression), Sm (final concentration, 10 μg/ml) was added to select for transformed cells. After growth to stationary phase, CCC plasmid DNA (consisting of a mixture of the three molecules shown) was extracted and used to transform C600 cells. Transformants were selected on Sm and Sm-Ap plates. The translocation frequency was estimated by determining the fraction of $Sm^r$ colonies (Sm plates) that were $(Sm\ Ap)^r\ Tc^s$ (by "toothpicking" colonies on Sm-Ap plates to Tc plates) and dividing by 5.8 to express the frequency as translocation events per megadalton of recipient DNA (see also Table 4).

## RESULTS

**Isolation and characterization of the pSC204 plasmid.** Translocation of the Tn3 element to the mutant plasmid pSC201, which is temperature sensitive in replication (23), was accomplished as described earlier for translocation of Tn3 to pSC101 (22). A *recA*$^-$ *E. coli* strain (JC1569) containing the pSC50 plasmid was transformed with pSC201, and a Tc-resistant transformant clone was purified and mated with an Nal-resistant strain of *E. coli* (CR34$Nal^r$). A transconjugant colony resistant to Tc, Ap, and Nal was purified, and the plasmids present were separated by transformation of total CCC DNA into JC1569. Transformants that expressed resistance to both Ap and Tc were tested for the Cm, Sm, and Su resistance determinants of pSC50, and one clone lacking these three resistance determinants was selected. CCC DNA isolated from this transformant was shown by agarose gel electrophoresis to contain only a single species of plasmid having one *Eco*RI cleavage site (see Fig. 3). Electron microscope contour length measurements of this plasmid (which was designated pSC204) indicated a length of 13.84 ± 0.39 kilobases (kb) (i.e., molecular mass of 9.08 ± 0.29 megadaltons), with pSC101 plasmid DNA as a reference standard (contour length, 9.1 kb [30]). Heteroduplex studies between the parent pSC201 plasmid and its pSC204 derivative (21) indicated that pSC204 is homologous with pSC201 except for a loop characteristic of Tn3, 4.8 kb long and situated 3.34 kb from the *Eco*RI site. pSC204 plasmid DNA was transformed into *E. coli* C600, and a transformant clone, which was shown to have a Rep(Ts) phenotype indistinguishable from the Rep(Ts) phenotype of the pSC201 parent, was labeled SC350.

**Selected translocation of the Tn3 element to the *E. coli* chromosome.** Experiments involving selected translocation of Tn3 to the bacterial chromosome were carried out as indicated in Fig. 1, with the pSC204 plasmid of *E. coli* SC350 as the donor of the transposable element. As shown in Table 2, 0.01% of the total cells in the population were found to retain resistance to Ap after growth of cultures of SC350 overnight at 45°C, corresponding to a putative translocation frequency of $3 \times 10^{-8}$ translocation events per megadalton of *E. coli* chromosomal DNA. One in $10^7$ of the total cells (i.e., 0.1% of the $Ap^r$ colonies) was resistant to Tc in addition to Ap (as shown by plating total cells on Ap-Tc plates at 45°C). CCC DNA isolated from three such $Ap^r\ Tc^r$ colonies indicated that these clones resulted from reversion of the Rep(Ts) mutation of pSC204 to temperature insensitivity: this CCC DNA was shown to have the same molecular weight as pSC204 (gel electrophoresis after *Eco*RI digestion), and, furthermore, transformation of C600 cells with this DNA resulted in temperature-resistant $Ap^r\ Tc^r$ transformant cells (Kretschmer and Cohen, unpublished data). The remaining 99.9% of the $Ap^r$ cells (termed 45°C-$Ap^r$ clones) were Tc sensitive, consistent with loss of the pSC204 plasmid and with translocation of Tn3 to the chromosome.

Eight randomly selected 45°C-$Ap^r$ clones that

TABLE 2. *Frequencies of Tn3 translocation from pSC204*

| Strain | Recipient genome | Mol wt of recipient | Copy no. of recipient[a] | Ap$^r$ colonies at 45°C[b] (%) | Avg translocation frequency[c] |
|---|---|---|---|---|---|
| SC383—*E. coli* C600 (containing pSC201::Tn*3* + RSF1010) | RSF1010 | $5.8 \times 10^6$ | 25 | 1 | $10^{-4}$ |
| SC384—*E. coli* C600 (containing pSC201::Tn*3* + R6-5) | R6-5 | $65 \times 10^6$ | 1.0 | 1 | $10^{-4}$ |
| SC350—*E. coli* C600 (containing pSC201::Tn*3*) | Chromosome | $2.5 \times 10^9$ | 1.5 | 0.01 | $3 \times 10^{-8}$ |

[a] Copy numbers of plasmids in strains SC383 and SC384 were determined as described by Timmis et al. (33). The molecular weight values used to calculate copy number were $65 \times 10^6$ for R6-5 (18), $5.8 \times 10^6$ for RSF1010 (this study) and $9.2 \times 10^6$ for pSC204 (this study). The copy numbers so obtained were 3.9 and 25 for pSC204 and RSF1010, respectively, in SC383, and 4.8 and 1.0 for pSC204 and R6-5, respectively, in SC384 (P. Kretschmer, unpublished data). The molecular weight and copy number of the chromosome were obtained from Cooper and Helmstetter (12).

[b] The data presented are the average of at least five separate experiments. Variation of Ap$^r$ colonies at 45°C was 0.7 to 1.5% for SC383, 0.48 to 1.5% for SC384, and 0.006 to 0.018% for SC350.

[c] Translocation frequency (translocation events/megadalton of recipient DNA) was calculated as: frequency Ap$^r$ cells at 45°C/megadaltons of recipient DNA per cell. The effect of translocation to the chromosome on the translocation frequency in strains SC383 and SC384 is negligible in these calculations since only 1% of 45°C-Ap$^r$ colonies in these strains are due to translocation to the chromosome (see column 5).

were sensitive to Tc at both 32 and 45°C were purified further by streaking on Ap plates and growth at 45°C. DNA obtained from cultures of these cells by a procedure we employ routinely for the isolation of plasmid DNA (see Materials and Methods) showed no CCC DNA band in cesium chloride-ethidium bromide gradients. Moreover, attempts to transform *E. coli* C600 to Ap resistance by using total DNA isolated from 45°C-Ap$^r$ clones, or cesium chloride-ethidium bromide gradient fractions obtained from parts of the gradient that might be expected to contain CCC DNA, were unsuccessful.

To verify the absence of the pSC204 plasmid in Ap-resistant bacterial colonies that were grown at 45°C, the 45°C-Ap$^r$ clones were transformed with the Km-resistant pSC105 plasmid (9), which is incompatible with pSC204 (Kretschmer and Cohen, unpublished data), and a Km-resistant clone was selected. After growth of this clone for 20 generations in the absence of antibiotics, 100% (30/30) of colonies examined were resistant to both Km and Ap. In a control experiment, cells containing the Tn3 element on the pSC204 plasmid were transformed with pSC105 plasmid DNA, and a clone resistant to Ap, Tc, and Km was selected. After 20 generations of growth of this clone in the absence of antibiotics, only 2% (2/110) of colonies were resistant to Ap and Km. From these incompatibility experiments (for further details see Fig. 3 of reference 3), we conclude that the above-mentioned 45°C-Ap$^r$ clones both contain the Tn3 element on the chromosome and lack the pSC204 replicon.

The Tn3 element present on the chromosome of 45°C-Ap$^r$ clones was easily translocated onto the pSC201 or pSC101 plasmids. In these experiments, one of the 45°C-Ap$^r$ clones, labeled SC351, was transformed with either pSC201 or pSC101 plasmid DNA, and CCC plasmid DNA isolated after overnight growth (in the presence of Tc to select for transformants) was used to transform C600 cells; transformants were selected on nutrient plates containing either Tc or Tc together with Ap. The ratio of Ap-Tc-resistant transformants to Tc-resistant transformants was taken as an indication of translocation frequency of Tn3 from the chromosome to the plasmid ($5 \times 10^{-6}$ to $1 \times 10^{-5}$ translocation events per megadalton of recipient DNA). This frequency of translocation from chromosome to plasmid during overnight growth of cells was an order of magnitude less than the frequency of translocation between plasmids for the same 20 generations of growth (Table 2). Continued culture of SC351 cells carrying the pSC101 plasmid for 50 to 100 generations resulted in a progressive increase in the fraction of translocated plasmids, approaching the plasmid-plasmid translocation frequency (see below) and suggesting that a slower rate of translocation from the chromosome to the plasmid was responsible for the lower frequency observed during overnight growth. In contrast, the frequency of Tn3 translocation from pSC204 to the chromosome ($\sim 10^{-8}$, Table 2) as determined by the selected translocation method was the same whether cells were grown for 20 or 200 generations.

**Plasmid-plasmid translocation frequency.** The frequency of translocation from pSC204 to recipient plasmids was studied by using RSF1010 and R6-5 as recipient replicons (Tables 1 and 2). A C600 clone (SC383) containing both the pSC204 and RSF1010 plasmids and another clone (SC384) containing pSC204 and R6-5 were constructed by transformation. Translocation of Tn3 from pSC204 to the RSF1010 or R6-5 plasmids (Table 2) occurred in approximately 1% of cells, equivalent to a frequency of $10^{-4}$ translocation events per megadalton of recipient plasmid DNA.

To determine whether the frequency of translocation between plasmids was influenced by the length of time that the donor and recipient plasmids are present concurrently in the same bacterial cell, the frequency of translocation of Tn3 from pSC204 to RSF1010 using the selected translocation procedure was determined after growth of bacteria carrying the two plasmids at the permissive temperature (32°C) for 20 or 200 generations prior to plating on Ap plates at 45°C. A similar translocation frequency (about $10^{-4}$/megadalton of recipient DNA) was observed in both cases, suggesting that the upper limit of translocation frequency was reached soon after the recipient plasmid was introduced into a strain containing a Tn3 donor plasmid, and that some kind of equilibrium was established at this level. In the case of plasmid-to-plasmid translocation, the maximum translocation limit was reached prior to the time of the first assay of translocation frequency (i.e., after overnight growth). However, because approximately 100 generations of growth of cells carrying the donor and recipient genomes were required to reach an equilibrium in the translocation of Tn3 from chromosome to plasmid (see above), it would appear that the rate of translocation from chromosome to plasmid is slower than the rate of plasmid-to-plasmid translocation.

Our interpretation that the persistence of Ap resistance in SC383 cells grown at 45°C is a consequence of translocation of Tn3 from pSC204 to the RSF1010 plasmid was verified by isolation and examination of CCC DNA isolated from seven randomly selected Ap-resistant clones. All seven preparations contained a single plasmid that, after cleavage by the *Eco*RI endonuclease, migrated at a position corresponding to a molecular weight of $9.1 \times 10^6$ (Fig. 3, well b). Electron microscopy of this DNA indicated a contour length of $13.72 \pm 0.43$ kb, which is consistent with molecular weight values estimated from gels and with the plasmid size expected as a result of addition of Tn3 to RSF1010. Furthermore, a DNA segment having short inverted repeat segments characteristic of Tn3 (16, 21, 22) was observed in heteroduplexes between the recombinant and parent plasmids (Fig. 4).

**Specificity of Tn3 insertions.** Because the selected translocation procedure is a simple method for introducing Tn3 or other antibiotic resistance elements onto a variety of recipient plasmids, it provides a convenient tool for studying the site specificity of translocation events. Earlier investigations showed that multiple recipient sites for Tn3 are present on even small plasmids such as RSF1010 (16), ColE1 (31) and pSC101 (21, 22). In all of these plasmids, a clustering of recipient sites for the Ap resistance translocating element was observed. Inactivation of drug resistance determinants on recipient genomes as a consequence of insertion of IS and Tn elements (16, 19, 32) has enabled the mapping of gene sites on various plasmids (16, 26). In studying the inactivation of the Sm gene by Tn3, Heffron et al. (16) utilized sucrose gradients and sequential transformation to isolate plasmid molecules that contained insertions of Tn3 in the Sm resistance gene of RSF1010. In the present studies, we also observed inactivation of Sm resistance in about 10% (21 of 200) Ap-resistant clones isolated after growth of SC383 (which contains coexisting RSF1010 and pSC204 plasmids) at 45°C overnight. However, no inactivation of the Sm, Km, or Cm markers of the R6-5 plasmid was found in 250 Ap-resistant clones of SC384 (originally containing the coexisting R6-5 and pSC204 plasmids) at 45°C. The absence of detectable insertion into these genes is consistent with further studies described below, indicating that the majority of Tn3 insertions into R6-5 occur in one of two *Eco*RI fragments, 3 or 9, neither of which contains the genes for Km, Cm, or Sm (K. Timmis, F. Cabello, and S. N. Cohen, manuscript in preparation).

The sites of insertion of Tn3 into the R6-5 plasmid DNA molecule were studied further by using agarose gel electrophoresis to identify *Eco*RI endonuclease-generated DNA fragments that acquired Tn3 by selected translocation. CCC DNA was isolated from 10 45°C-Ap$^r$ clones derived from SC384 and from 12 additional 45°C-Ap$^r$ clones derived from independently transformed isolates of C600 carrying coexisting pSC204 and R6-5 plasmids. The R6-5::Tn3 plasmids present in each of the 23 DNA preparations were cleaved with the *Eco*RI endonuclease and examined by agarose gel electrophoresis (Fig. 3). In each instance, alteration of the size of one of the *Eco*RI-generated fragments of

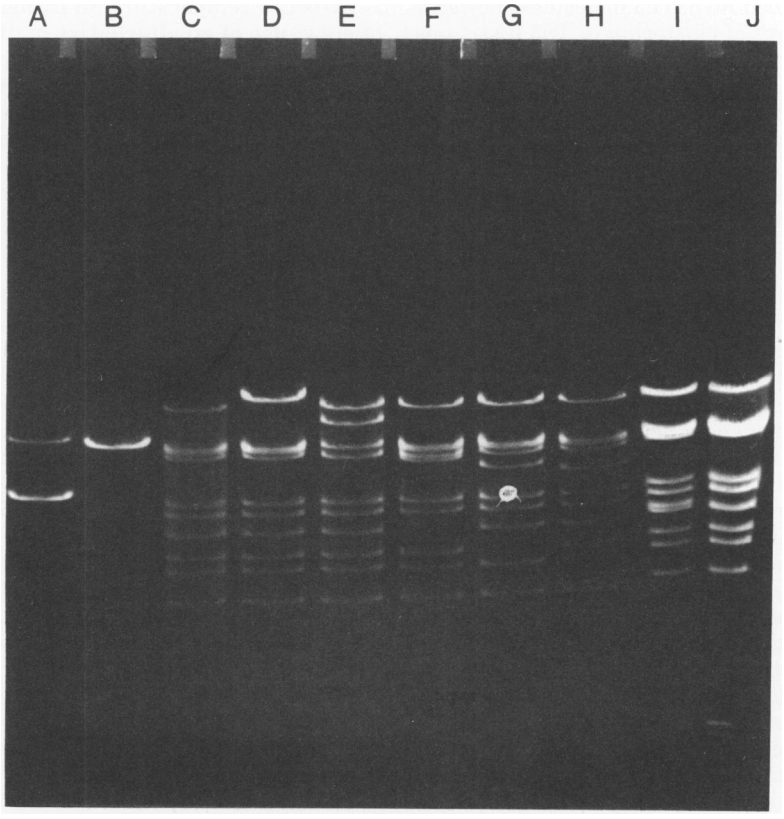


FIG. 3. *Agarose gel electrophoresis of EcoRI endonuclease digests of the donor plasmid pSC204 and recipient molecules containing Tn3. (A) CCC DNA isolated from C600 containing the pSC204 and RSF1010 plasmids (i.e., SC383, see text). RSF1010 DNA is contained in the lower band. The upper band represents the pSC204 plasmid. (B) Plasmid DNA isolated from a 45°C-Ap$^r$ clone of SC383 (i.e., containing RSF1010::Tn3 plasmid DNA). (C) Parental R6-5 DNA. (D through I) R6-5 containing Tn3 insertions in EcoRI fragments 1, 3 (or 2), 7, 8, 9, and 12, respectively. (J) Parental R6-5 DNA.*

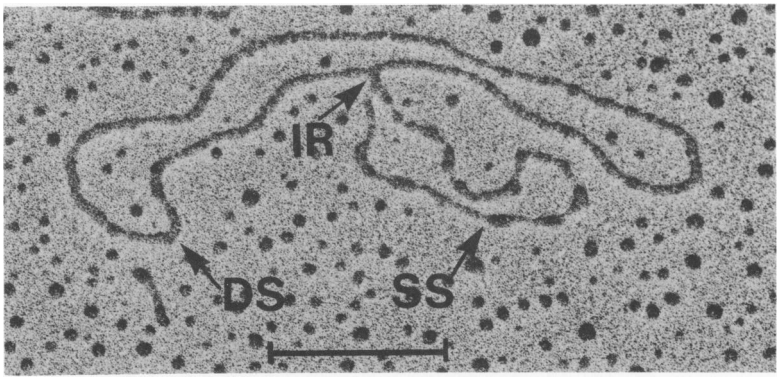


FIG. 4. *Heteroduplex analysis of RSF1010::Tn3 recombinant plasmid DNA (isolated from a 45°C-Ap$^r$ clone of SC383) with RSF1010 DNA. A representative heteroduplex is shown. IR, Inverted repeat; SS, single-stranded DNA; DS, double-stranded DNA. Bar represents 1 kb of double-stranded DNA.*

R6-5 as a consequence of Tn3 insertion was evident, and this event coincided with the appearance of a fragment migrating at the position expected from addition of a 3.2-megadalton segment of DNA to the altered fragment.

Gel electrophoresis of *Eco*RI digests of the

above-mentioned R6-5::Tn3 molecules showed a striking pattern of clustering of Tn3 insertions on certain *Eco*RI fragments of the R6-5 plasmid; furthermore, this pattern was the same for SC384-derived clones as for the independently transformed isolates. Of the 23 clones studied, 11 contained Tn3 insertions in fragment 2 or 3 (5 clones were from SC384), 7 in fragment 9 (3 clones were from SC384), 2 in fragment 8, and 1 in each of fragments 1, 7, and 12.

To distinguish between *Eco*RI fragments 2 and 3 of R6-5 and to determine whether independent insertions of Tn3 into a single *Eco*RI-generated fragment occurred at the same location within the fragment, double digestion of R6-5::Tn3 plasmid DNA with the *Eco*RI and *Bam*I restriction endonucleases was carried out (Fig. 5). Tn3 contains a single *Bam*I restriction endonuclease cleavage site located approximately 1.5 kb from the Tn3 terminus closest to the Ap resistance gene of the element (P. D. Nisen, D. J. Kopecko, and S. N. Cohen, submitted for publication). This cleavage site serves as a marker for the position and orientation of Tn3 insertions within the various *Eco*RI fragments of R6-5; insertions at the same site and in the same orientation would yield identical gel patterns after double digestion by the *Eco*RI and *Bam*I enzymes, whereas insertion of Tn3 at different locations within the same *Eco*RI fragment would yield different gel patterns and be distinguishable. Because of the asymmetrical position of the *Bam*I cleavage site within Tn3, the possibility of separate insertions occurring at the same site but in different orientations can also be examined.

*Bam*I/*Eco*RI double digestion has also enabled assignment of Tn3 insertions into either *Eco*RI fragment 2 or fragment 3. Other investigations (Kretschmer and Cohen, unpublished data) showed that *Eco*RI fragment 2 contains at least one *Bam*I site, whereas *Eco*RI fragment 3 lacks such a site. Using this information, we assigned 8 of the 11 fragment 2 or 3 Tn3 insertions to fragment 3 and 3 insertions to fragment 2. For example, in Fig. 5, DNA preparations 2a through 2g contain a Tn3 insertion in *Eco*RI fragment 3 (resulting in loss of the 9.1-megadalton *Bam*I/*Eco*RI fragment), whereas DNA preparation 2h contains a Tn3 insertion in *Eco*RI fragment 2 (thus not affecting the 9.1-megadalton *Bam*I/*Eco*RI fragment, which is in fact *Eco*RI fragment 3). Finally, the lengths of the fragments produced by *Bam*I/*Eco*RI double digestion indicate that the multiple insertions of Tn3 into fragment 3 occurred at different sites within *Eco*RI fragment 3 (Fig. 5 and Table 3, DNA preparations 2a through 2g).

**Effect of temperature on translocation frequency; use of an alternative method for estimating frequency.** Although Reif and Saedler recently showed that deletion formation promoted by the IS1 element is influenced by temperature (25), no information is currently available about the influence of temperature on the translocation of Tn elements. However, the frequency of translocation events we observed with the selected translocation procedure (Table 2) suggested the feasibility of a method that could be used to study the effect of temperature on the translocation of Tn3. In the alternative procedure for determining translocation frequency (see Materials and Methods and Fig. 2), bacterial cells containing the pSC175 plasmid as a donor for Tn3 were transformed with RSF1010. Immediately after the heat-pulse step in the transformation procedure, which accomplishes uptake of plasmid DNA (10), small volumes of cells were transferred to 100-ml prewarmed broth cultures. After 120 min was allowed for phenotypic expression of the Sm resistance carried by the RSF1010 plasmid, Sm was added and transformed cells were selected by growth of cultures overnight. The total CCC DNA was isolated from cell cultures, and the frequency of translocation of Tn3 to RSF1010 was determined by carrying out a subsequent transformation step as depicted in Fig. 2.

To determine the effect of temperature after the heat-pulse step in the first transformation, portions of cultures were immediately transferred to L broth and maintained at 32 or 45°C. Growth of cells carrying the two plasmids at 45°C resulted in at least a two-log reduction in translocation frequency compared with the frequency obtained when cells were grown at 32°C (Table 4). In three separate experiments in which cells were maintained at 45°C from the time the recipient plasmid was first introduced, the translocation frequency was determined to be less than $10^{-6}$, which was the limit of experimental detection.

Additional experiments excluded the possibility that the absence of detectable translocation of Tn3 at 45°C resulted from a reduced frequency of transformation of RSF1010 in cells grown at this temperature or from decreased stability of the RSF1010::Tn3 plasmid at 45°C. Equal numbers of $Sm^r$ RSF1010 transformants were observed routinely at 32 and 45°C (Table 4); moreover, no difference in stability of RSF1010::Tn3 in comparison with that of RSF1010 was observed at 45°C under these conditions. In contrast, during overnight growth of transformant cells at 45°C, the donor plasmid (pSC175) was eliminated from 30 to 60% of cells;

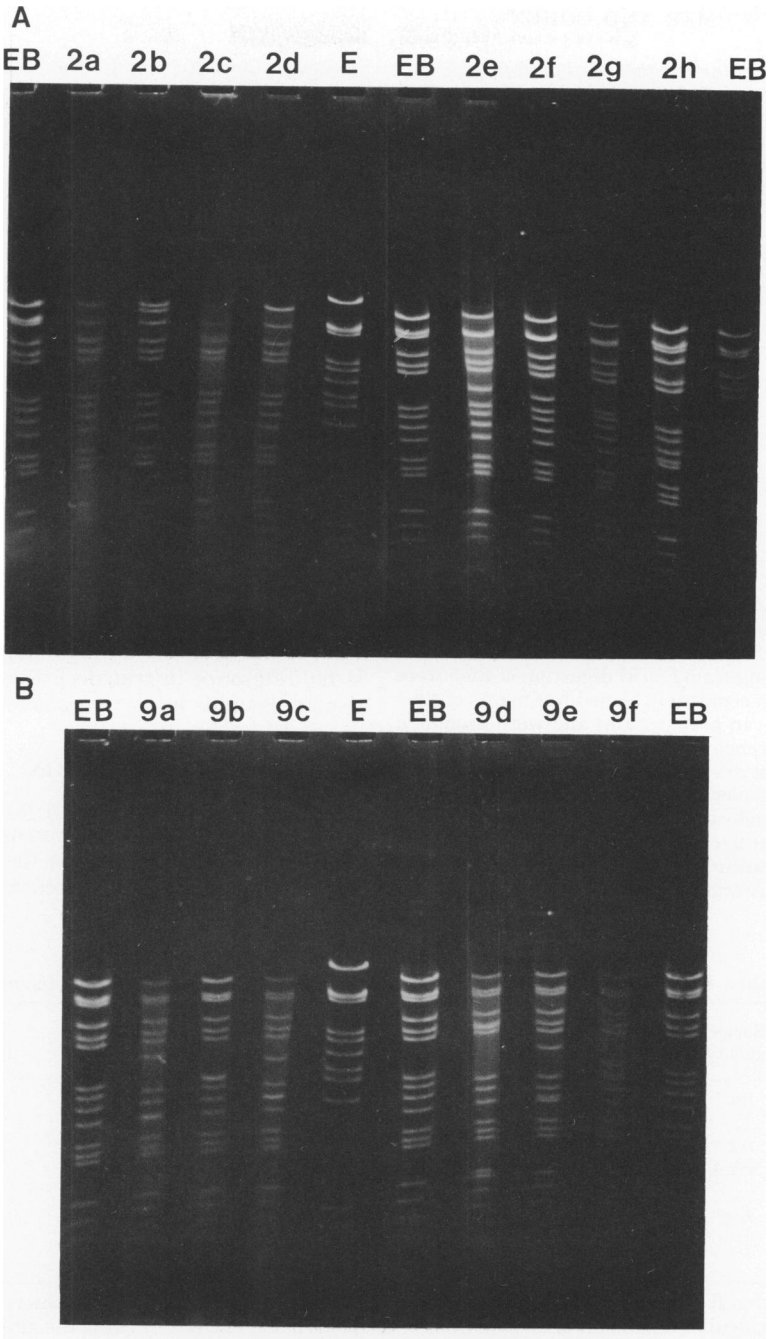


FIG. 5. *EcoRI/BamI double digests of R6-5 molecules containing a Tn3 transposable element in EcoRI fragment 2 or 3 (gel A) or EcoRI fragment 9 (gel B). The wells labeled E and EB contain the parental R6-5 DNA digested with EcoRI and doubly digested with EcoRI and BamI, respectively. The molecular weights of DNA fragments resulting from EcoRI/BamI double digestions were calculated from well E by using the revised molecular weight estimates of R6-5 EcoRI fragments (Timmis et al., in preparation). We identified 18 EcoRI/BamI fragments of R6-5, having calculated molecular weights ($\times 10^6$) of 12.3, 9.1, 8.4, 6.2, 5.3, 4.6, 3.2, 3.0, 2.7, 2.4, 2.1, 1.9, 1.8, 1.25, 1.2, 1.1, 0.88, and 0.65. Since there are 13 EcoRI-susceptible sites and 6 BamI-susceptible sites in independent digests of R6-5 (Kretschmer and Cohen, unpublished data), two additional fragments that were not resolved in these gels are likely to result from the double digest of R6-5 DNA. The calculated molecular weights of fragments altered by Tn3 insertion and of the resulting fragments (as calculated from these gels) are shown in Table 4 for DNA preparations 2a through h and 9a through f. In certain of these preparations (e.g., 2a, 2b, 2c, 2d, 2e, 2f, 9a, and 9b), the fragment resulting from Tn3 insertion migrates coincidentally with a preexisting fragment and is thus detected as a double-intensity band.*

TABLE 3. *Molecular weights of altered fragments resulting from BamI/EcoRI double digestion*[a]

| R6-5::Tn3[b] | Mol wt of altered fragment ($\times 10^6$) | Mol wt of new fragments ($\times 10^6$) | Total mol wt inserted ($\times 10^6$) |
|---|---|---|---|
| 2a | 9.1 | 6.2, 5.3 | 2.4 |
| 2b | 9.1 | 10.2, 1.8 | 2.9 |
| 2c | 9.1 | 6.8, 5.3 | 3.0 |
| 2d | 9.1 | 12.3, 0.9 | 4.1 |
| 2e | 9.1 | 8.4, 3.7 | 3.0 |
| 2f | 9.1 | 8.4, 3.6 | 2.9 |
| 2g | 9.1 | 8.9, 3.4 | 3.2 |
| 2h | 6.2 | 6.9, 2.5 | 3.2 |
| 9a | 3.2 | 4.1, 2.7 | 3.6 |
| 9b | 3.2 | 3.2, 3.2 | 3.2 |
| 9c | 3.2 | 3.9, 2.8 | 3.5 |
| 9d | 3.2 | 5.2, 1.4 | 3.4 |
| 9e | 3.2 | 4.0, 2.5 | 3.3 |
| 9f | 3.2 | 3.4, 3.1 | 3.3 |

[a] Fragment molecular weights were calculated from Fig. 5 by assuming that the migration distance was proportional to the log of the fragment molecular weight (17). These values of fragment molecular weights resulting from *Eco*RI digestion of R6-5 were calculated from separately cloned fragments of R6-5 (Timmis et al., in preparation) and were used as a standard reference for calculation of molecular weights. Values are the average of two experiments.

[b] R6-5::Tn3 molecules 2a through 2h are the R6-5::Tn3 DNA molecules containing an insertion in *Eco*RI fragment 2 (or 3); 9a through 9f are R6-5::Tn3 molecules containing an insertion in *Eco*RI fragment 9 (Fig. 5A and B, respectively).

however, a reduction in the number of donor plasmids of this magnitude could not account for a complete absence of detectable translocation at 45°C. Moreover, when cultures that had been reduced in Tn3 donors by growth overnight at 45°C were diluted and grown at 32°C, translocation of Tn3 to the RSF1010 plasmid was observed. Additional evidence supporting the interpretation that translocation per se is inhibited at 45°C is provided by preliminary experiments indicating that the translocation frequency for Tn3 at 37°C is only 10% of the frequency observed at 32°C. We conclude from these collective data that little or no translocation occurred at 45°C and that the bacteria identified by the selected translocation procedure contain plasmids that received Tn3 prior to the temperature shift.

The alternative procedure was also used to quantitate the previous qualitative finding that translocation of the Tn3 element occurs in the absence of the *E. coli recA* gene product (22). The results (Table 4) indicate that at 32°C there is no difference in translocation frequency between plasmids in *rec*$^+$ versus *recA*$^-$ cells.

## DISCUSSION

The ability to select conveniently for genomes that have received transposable genetic elements provides a means for investigating characteristics of the translocation process. The

TABLE 4. *Effect of temperature and the recA genotype on translocation frequency*[a]

| Temp[b] (°C) | Bacterial genotype | Donor plasmid | Transformants/ml | | Tc$^s$/Sm$^r$ Ap$^r$[c] | Translocation frequency |
|---|---|---|---|---|---|---|
| | | | Sm$^r$ | Sm$^r$ Ap$^r$ | | |
| 32 | *rec*$^+$ | pSC175 | $6.6 \times 10^4$ | $1.4 \times 10^3$ | 23/208 | $4.0 \times 10^{-4}$ |
| | | | $2.3 \times 10^4$ | $6.0 \times 10^2$ | 25/346 | $3.3 \times 10^{-4}$ |
| | *rec*$^+$ | pSC204 | $2.1 \times 10^4$ | $1.6 \times 10^2$ | 32/200 | $2.0 \times 10^{-4}$ |
| | *recA*$^-$ | pSC204 | $6.5 \times 10^4$ | $1.9 \times 10^3$ | 55/794 | $3.5 \times 10^{-4}$ |
| 45 | *rec*$^+$ | pSC175 | $4.0 \times 10^4$ | $2.0 \times 10^2$ | 0/250 | $<3.4 \times 10^{-6}$ |
| | | | $1.2 \times 10^4$ | $4.7 \times 10^2$ | 0/620 | $<1.1 \times 10^{-5}$ |
| | | | $2.3 \times 10^4$ | $2.4 \times 10^2$ | 0/510 | $<3.6 \times 10^{-6}$ |

[a] Translocation frequencies of Tn3 from the donor plasmid (pSC175 or pSC204) to the recipient plasmid RSF1010 were calculated in terms of megadaltons of recipient plasmid DNA by using the alternative method (Fig. 2). For example, in the top line above:

$$\text{translocation frequency} = \frac{23}{208} \times \frac{1.4 \times 10^3}{6.6 \times 10^4} \times \frac{1}{5.8} = 4.0 \times 10^{-4}$$

[b] Immediately after the heat pulse step for plasmid DNA uptake in the alternative method (Fig. 2), 0.1-ml fractions of the transformation mix were added to L broth medium prewarmed at 32 or 45°C. An equal number of Sm$^r$ transformants (ca. $10^4$/0.1-ml fraction) was observed at 45°C as at 32°C (assayed 120 min after the 42°C heat pulse but before the addition of Sm).

[c] Denominator is number of Sm$^r$ Ap$^r$ transformants examined by "toothpicking" to Tc plates. The numerator is the number that were Tc$^s$.

selected translocation procedure employs a plasmid having a temperature-sensitive mutation in replication as a donor of the Tn3 element. The procedure is potentially applicable to the study of translocation of other elements that carry assayable phenotypic properties. Recently, we isolated temperature-sensitive mutants of pSC101 and pSC105 that are defective in replication at 42°C (Kretschmer and Cohen, unpublished data). Replication of these plasmids, which have been designated pSC301 and pSC305, respectively, is inhibited several degrees below the maximum growth temperature for *E. coli* and thus appear to be easier to work with than the 45°C-Rep(Ts) plasmid used in the experiments reported here.

The selected translocation procedure has enabled comparison of translocation frequencies to different molecules from the same Rep(Ts) donor plasmid. The results of our investigations indicate that translocation of Tn3 occurs at a frequency similar to that of RSF1010, a small multicopy plasmid, or to R6-5, a large oligocopy plasmid. However, variability in its frequency of translocation of a related Ap resistance element (Tn1; 4) from the *E. coli* chromosome to different conjugative plasmids has been reported (2).

Translocation of Tn3 to the chromosome showed a frequency four orders of magnitude less than plasmid-to-plasmid transfer of the same element. That this difference was a true frequency difference, and not due simply to a lower rate of translocation to the chromosome, was shown by experiments in which cells carrying coexisting donor recipient genomes were grown for 20 or 200 generations and then assayed by the selected translocation procedure. Such experiments indicated that, by 20 generations of growth, plasmid-to-plasmid and plasmid-to-chromosome translocation of Tn3 had reached their respective maximum frequencies of $10^{-4}$ and $10^{-8}$ translocations per megadalton of DNA. Continued growth of cells carrying the donor and recipient genomes did not increase the translocation frequency further. The Tn3 element could be acquired from the chromosome by a plasmid at a maximum frequency of $10^{-4}$ translocations per megadalton of recipient plasmid DNA; however, in contrast to translocation of Tn3 from a plasmid, approximately 100 generations of growth were needed to attain this frequency. Thus, it appears that translocation of the Tn3 element from the chromosome occurs at a slower rate than translocation of the same element from a plasmid.

Our observation that only about 0.1% of RSF1010 plasmid molecules receive Tn3 despite prolonged growth of cells carrying coexisting donor and recipient plasmids indicates the attainment of an equilibrium between $Tn3^+$ and $Tn3^-$ recipients, such as an equilibrium between insertion and excision of the transposable element. However, we have no evidence that excision of Tn3 from the recipient plasmid molecules is the basis of the apparent equilibrium observed.

An alternative method for estimation of translocation frequency indicated that the frequency of translocation of Tn3 is unaffected by the *recA*$^-$ genotype, but that the translocation event is temperature sensitive. The temperature sensitivity of the Tn3 translocation event is useful experimentally in that it enables cessation of the translocation process at any point in time without affecting the number of translocations accumulated to that point (Kretschmer and Cohen, unpublished data).

The specificity and clustering of recipient sites for the translocation of Tn3 (i.e., regional specificity) that have been reported previously (16, 21, 22) was strikingly evident in the current experiments. Although 10% of insertions of this element into the 5.8-megadalton RSF1010 plasmid occurred in the Sm resistance gene, no insertions of Tn3 were found in the genes for Sm, Cm, or Km resistance on the large R6-5 plasmid. Instead, 15 of 23 separately occurring insertions of Tn3 into R6-5 were clustered within two *Eco*RI-generated fragments that together represent only about one-sixth of the R6-5 DNA molecule. It is of special interest that *Eco*RI fragment 9, which contains only 3 megadaltons of DNA, received fully a third of the translocations to the 65-megadalton R6-5 plasmid; this fragment contains the Tn4-IS1 junction (Timmis et al., in preparation), which has been shown previously (21) to represent a hotspot for illegitimate recombination of DNA sequences during evolution of plasmids. It is conceivable that fragment 3 similarly contains a hotspot for terminus-specific recombination.

The apparent ability of terminal DNA sequences of already present transposable elements to promote additional illegitimate recombinational events in their vicinity has been reported previously (21; Nisen et al., submitted for publication), and the present results are consistent with the earlier findings from our laboratory. Although the mechanism for such *recA*-independent recombination is not known, both the present and earlier findings indicate that terminus-specific illegitimate recombination may occur in the vicinity of, but at a short distance from, the putative recognition sequence. Potentially, this could be accomplished

by a mechanism analogous to that involved in the action of the *Hph* restriction endonuclease, which cleaves DNA eight or nine base pairs from the recognition sequence (20), or a mechanism analogous to the *E. coli* B restriction enzyme, which cleaves at variable nucleotide sites that are distinct from the recognition sequence (24).

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