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Mutagenesis by Insertion of a Drug-resistance Element Carrying an Inverted Repetition

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A novel genetic element, which carries genes conferring tetracycline resistance (flanked by a 1400 base-pair inverted repetition), is capable of translocation as a unit from one DNA molecule to another. The tet^R element, which is found in nature on a variety of R-factors, was acquired by bacteriophage P22 (producing P22Tc-10 and P22Tc-106) and has now been observed to insert into a large number of different sites on the *Salmonella* chromosome. Insertion of the tet^R element is mutagenic when it occurs within a structural gene, and polar when it occurs within an operon. Insertion of the element is usually precise, occurring without loss of information on the recipient DNA molecule. Excision, on the other hand, is usually *not* precise, although excisions precise enough to restore a gene function can always be detected at low frequencies. Both insertion and excision processes are independent of the *recA* function.

1. Introduction

Bacteriophage P22 is a temperate phage whose normal host is *Salmonella typhimurium*. We described previously an unusual variant of P22 (called P22Tc-10) which transduces resistance to tetracycline at high frequency (Watanabe *et al.*, 1972; Chan *et al.*, 1972). Examination of P22Tc-10 DNA in the electron microscope showed that this specialized transducing variant contains a large (8·3 kilobase) insertion which has an unusual structure: in heteroduplex DNA molecules, the insertion forms a lariat-like structure with a double-stranded stem (about 1·4 kilobases long) and a single-stranded loop (Tye *et al.*, 1974). This is interpreted to mean that the insertion consists of an inverted duplication separated by non-repeated DNA sequences.

The tet^R insertion was acquired by P22 during a lytic cycle of growth in a *Salmonella* strain harboring a drug-resistance plasmid (R-factor) in whose DNA a similar non-tandem reverse duplication was found to be associated with the genetic determinant for tetracycline resistance (Watanabe *et al.*, 1972; Sharp *et al.*, 1973). Two independently-arising tetracycline-transducing P22 phages were examined in our previous studies and both had identical insertions (Watanabe *et al.*, 1972; Tye *et al.*,

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1974). In neither case was there any detectable (less than 100 bases) loss of P22 DNA accompanying the insertion.

More recently, we found that the site of the *tet*^{R} insertion in P22Tc-10 is not the phage attachment site: i.e. the insertion is not at the point at which the prophage is integrated by site-specific recombination into the *Salmonella* chromosome (Chan & Botstein, 1975). This made it unlikely that P22Tc-10 was formed by the mechanism normally associated with the production of specialized-transducing genomes of temperate phages (Campbell, 1962).

From these observations, and from other aspects of the genetic behavior of P22Tc-10, we formed the hypothesis that the *tet*^{R} element from the R-factor (with its inverted repeat) is capable of translocation, as a discrete unit, from one DNA molecule into any one of many different places on other DNA molecules. This paper describes both physical and genetic evidence in support of this idea.

Berg *et al.* (1975) have recently isolated coliphage λ-transducing variants carrying a kanamycin-resistant determinant which is associated with an inverted repetition. The structures of these phages is analogous to that of P22Tc-10. Heffron *et al.* (1975*a*,*b*) have recently shown that an ampicillin-resistance determinant, associated with a very small inverted repetition, is capable of translocation from one DNA molecule to another.

We suggest that genetic elements like the *tet*^{R}, *amp*^{R} and *kan*^{R} insertions play an important role in reassortment of drug-resistance determinants among resistance factors. The apparent ubiquity of inverted repetitions in both prokaryotic (Sharp *et al.*, 1972, 1973; Daniell *et al.*, 1973; Berg *et al.*, 1975; Heffron *et al.*, 1975*b*; Hsu & Davidson, 1975) and eukaryotic DNA (Garon *et al.*, 1972; Wolfson & Dressler, 1972; Locker *et al.*, 1974; Manning *et al.*, 1975; Wadsworth *et al.*, 1975) further suggests that such elements could play an important role in the mobilization and translocation of genetic information in many different biological systems.

2. Materials and Methods

(a) *Bacteriophage*

The P22Tc-10 and P22Tc-106 genomes are too long to fit into a single P22 phage head. The strains are therefore maintained as lysogens which, upon induction, give rise to particles which are defective on single infection (Chan *et al.*, 1972; Tye *et al.*, 1974). Tc-10 recombinants carrying the additional mutations c_2ts29 (heat-inducible repressor (Levine & Smith, 1964)); *erf-am*12B (recombination-deficient (Botstein & Matz, 1970)); or int_3 (integration-deficient (Smith & Levine, 1967)) were constructed by crosses with induced lysates (Chan *et al.*, 1972) or rescue of *tet*^{R} from prophage deletions (Chan, 1974).

P22int_3 HT 12/4 was constructed in a standard phage cross between P22c^+int_3 and P22c_2^- HT 12/4 (Schmieger, 1972), a high-frequency generalized transducing derivative of P22.

(b) *Bacteria*

Strains of *S. typhimurium* used:

(i) In isolation of *tet*^{R} auxotrophs: DB7000 = LT2 *leu*A414 (Susskind *et al.*, 1974); NK80 = LT2 *edd*$^-$ constructed for this work from two *his*$^-$*edd*$^-$ strains obtained from J. Roth); and DB143 = LT7 *pro*AB47 (deleted for P22 attachment site) *recA*$^-$ (Miyake & Demerec, 1960).

(ii) In complementation studies: the following strains were all the gift of John Roth: (1) derivatives of LT2 *trpA*8 *purE*801 *his*612 (BHAFIE deletion) carrying *Escherichia coli* F'*his*bG2377, F'*his*abD2381, and F'*his*abD2382; (2) derivatives of LT2 *ser*821 *arg*501

*his*712 (DCBHAFIE deletion) carrying *E. coli* F'*his*$^+$, F'*his*C2383, F'*his*C2385, F'*his* a(b)cdB2405, F'*his*abI2312 a(b)E, F'*his* bI2413, F'*his* a(b)E2414, and F'*his* a(b)cd B245. (The small letters denote deficiencies of the mutant with respect to intragenic complementation groups.)

(iii) In deletion mapping: for *his* deletion strains listed in Fig. 2, endpoints of deletions with respect to known *his* point mutations are shown in Hartman *et al.* (1971) and Scott & Roth (1975). Deletion strains were kindly supplied by J. Roth and P. Hartman.

(c) *Media*

Complete liquid medium: LB (Chan & Botstein, 1972); solid minimal medium: M9 + 1·5% agar (Smith & Levine, 1964); complete solid media: green plates (Chan & Botstein, 1972), trypticase plates (Stahl & Stahl, 1971). P22 phage stocks are stored and diluted in buffered saline (Chan & Botstein, 1972).

(d) *Visualization of DNA heteroduplexes in the electron microscope*

Procedures used for the isolation of phage DNA and for preparation and visualization of DNA in heteroduplex structures were as described by Tye *et al.* (1974).

(e) *Isolation of* tet^R *auxotrophs*

Stocks of P22Tc-10 derivatives were made by induction of corresponding lysogens (Chan *et al.*, 1972); lysates were purified once through discontinuous CsCl gradients and the concentration of particles determined from the A_{260} (Chan *et al.*, 1972). Exponentially growing recipient bacteria were mixed directly with phage at a multiplicity of 2 to 13 particles per cell, and after 10 to 30 min, were spread directly on green plates supplemented with 25 μg tetracycline hydrochloride (Calbiochem)/ml and 0·01 M-EGTA (Eastman). Plates were incubated at 37°C (experiment A) or 41°C (experiments B and C) and then replica plated onto M9 plates supplemented with glucose and any other nutrient required by the recipient strain (DB143 is *pro*$^-$ and DB7000 is *leu*$^-$). In accordance with the notation introduced by Bukhari & Metlay (1973) for mutations made by insertion of phage Mu, the symbol :: will be used here to indicate that the *tet*^{R} element is inserted at the locus preceding the symbol. For example, *his* : : *tet*^{R} means that the *tet*^{R} element is inserted into the histidine operon.

(f) *Phage P22-mediated generalized transduction*

P22 is capable of mediating generalized transduction of bacterial DNA from one host strain to another (Zinder & Lederberg, 1952). For all generalized transduction experiments described here, the P22 derivative int_3 HT 12/4 was used. The HT 12/4 mutation, isolated and characterized by Schmieger (1972) and Raj *et al.* (1974), greatly increases the proportion of generalized transducing particles in a phage lysate. The int_3 mutation (Smith & Levine, 1967) prevents integration of normal P22 genomes into the host chromosome in the course of the transduction experiment.

Transducing lysates were made by putting a seed stock through a single cycle of growth on appropriate donor strains. For transducing lysates used in deletion mapping, the seed stock used to make single-cycle stocks was grown on a strain deleted for the entire histidine operon in order to prevent carry-over of *his*$^+$ transducing particles from the seed stock to the transducing lysates.

For co-transduction experiments (Table 2), exponential cultures of the recipient strain were infected directly with the various transducing lysates at a multiplicity of 10. After 15 min adsorption at room temperature, the mixtures were diluted and spread on green plates +25 μg tetracycline/ml, and the plates were incubated at 37°C. Resulting *tet*^{R} colonies were then tested for growth in M9–glucose plates, and on either M9 glucose and leucine, M9–glucose and histidine, or M9–gluconate plates in order to detect the donor auxotrophy.

For deletion mapping (Fig. 2), recipient *his*-deletion strains were grown to 2×10^9 cells/ml in LB broth. 0·15 ml bacteria, 0·1 ml transducing lysate (at 1 to 3×10^{10} phage/ml), and 0·15 ml LB broth were then spread together directly on an M9–glucose plate (without

prior pre-adsorption of phage and bacteria). Plates were incubated at 37°C for 2 days before scoring for the appearance of *his*$^+$ recombinants. With this protocol, a positive result meant that *at least* 50 (and as many as 10^4) *his*$^+$ recombinants appeared on a single plate. A negative result meant that *no* (0) *his*$^+$ recombinants appeared. As expected for deletion mutants, no *his*$^+$ revertants of the recipient strains were ever observed.

(g) *Complementation of F'*his *strains by* his*::*tet^R *auxotrophs*

The histidine operons of *S. typhimurium* and *E. coli* can complement each other for all of the functions in the histidine biosynthesis pathway; however, recombinants between the two operons are almost never observed (Atkins & Loper, 1970). We asked which *his* functions can be provided by *his*::*tet*^{R} auxotrophs by replica plating patches of *Salmonella* strains carrying F'*his* episomes (which themselves carried *his*$^-$ mutations in various genes) onto minimal plates spread with cultures of the various *his*::*tet*^{R} auxotrophs. The donor strain was counter-selected by omission of required nutrients (serine and arginine or tryptophan and purines). The F'*his* episome should mate into the *his*::*tet*^{R} strain, but growth in the region of a patch will be seen only if the *his*::*tet*^{R} recipient can supply the *his* function(s) not made by the F'*his*.

Cultures of donor F'*his* strains were spotted onto trypticase plates, and grown for 16 h at 37°C prior to replica plating. *His*::*tet*^{R} auxotrophs were grown to late log phase and 0·15 ml was spread directly on M9 plates. After replica plating, plates were incubated for 48 h at 37°C prior to scoring.

Various F$^-$*his*-deletion strains were included as control recipients to verify that the internal promoters detected by Atkins & Loper (1970) in such experiments were also detected here. A strain carrying an F'*his*$^+$ episome was included among donors as a control for the ability of *his*::*tet*^{R} auxotrophs to function as recipients and for the absence of negative complementation.

3. Results

(a) *DNA heteroduplexes between two independent P22* tet^R *transducing phage*

When a mixture of DNA from the two independent P22 *tet*^{R}-transducing phages (P22Tc-10 and P22Tc-106) was denatured, slowly reannealed, and examined by electron microscopy, a number of unusual structures were seen. The structure shown in Plate I(a) is apparently a heteroduplex molecule containing one strand of Tc-10 and one strand of Tc-106, since the two complementary strands are not the same. From this structure, it is clear that Tc-10 and Tc-106 each contain a single insertion which is unaccompanied by any detectable loss of P22 DNA (less than 50 to 100 bases). The Tc-10 and Tc-106 insertions are indistinguishable; both are of the same total length and are bounded by inverted repetitions of similar size. The only difference between the two transducing phages appears to be the location of the insertion into the P22 genome. This indicates that there is more than one possible site for insertion of the *tet*^{R} element.

Another structure which is seen in Tc-10/Tc-106 DNA heteroduplex preparations is shown in Plate I(b). This structure differs from that in Plate I(a) in that there has been partial pairing between the two single-strand "loops" of the inserted material. The pattern of paired and unpaired regions is reproducible among the many such structures we have seen. We interpret these structures to mean that the insertions carried by Tc-10 and Tc-106 are actually totally homologous, and that such structures represent instances in which intra-molecular pairing between the self-complementary portions of each strand preceded inter-molecular pairing between the complementary strands of the loop. Complete pairing in the loop region is apparently obstructed by by steric constraints imposed by the prior pairing of the self-complementary regions.

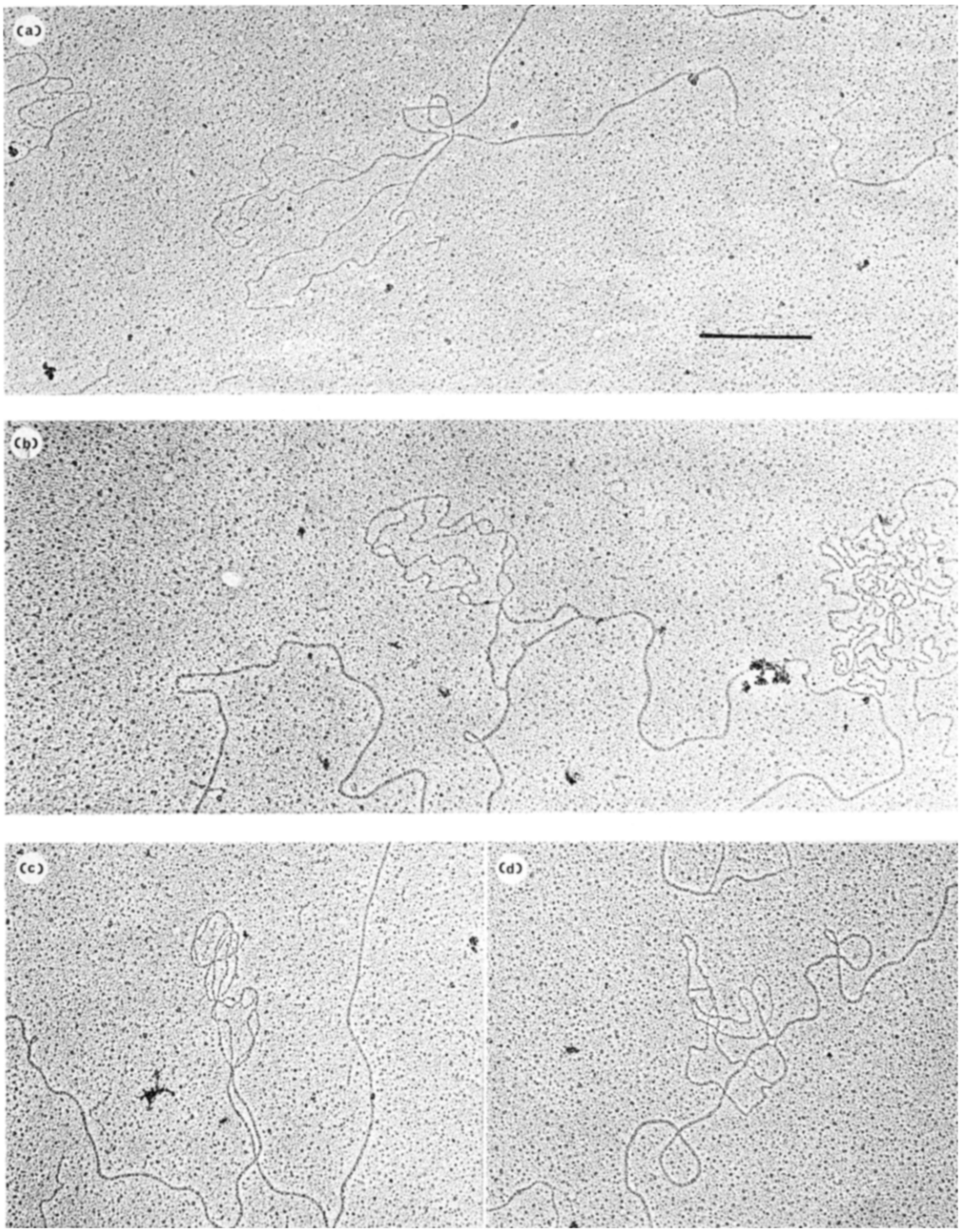

PLATE I. Heteroduplexes of Tc-10 and Tc-106.

(a) Tc-10/Tc-106 heteroduplex in which loops have not interacted.

(b) Tc-10/Tc-106 heteroduplex in which loops have interacted.

(c) Homoduplex (Tc-10/Tc-10 or Tc-106/Tc-106) in which loops have interacted.

(d) Homoduplex (Tc-10/Tc-10 or Tc-106/Tc-106) in which loops have not interacted but branch migration has occurred around the point of the insertions.

The bar represents 0·5 μm.

[*facing p. 564*

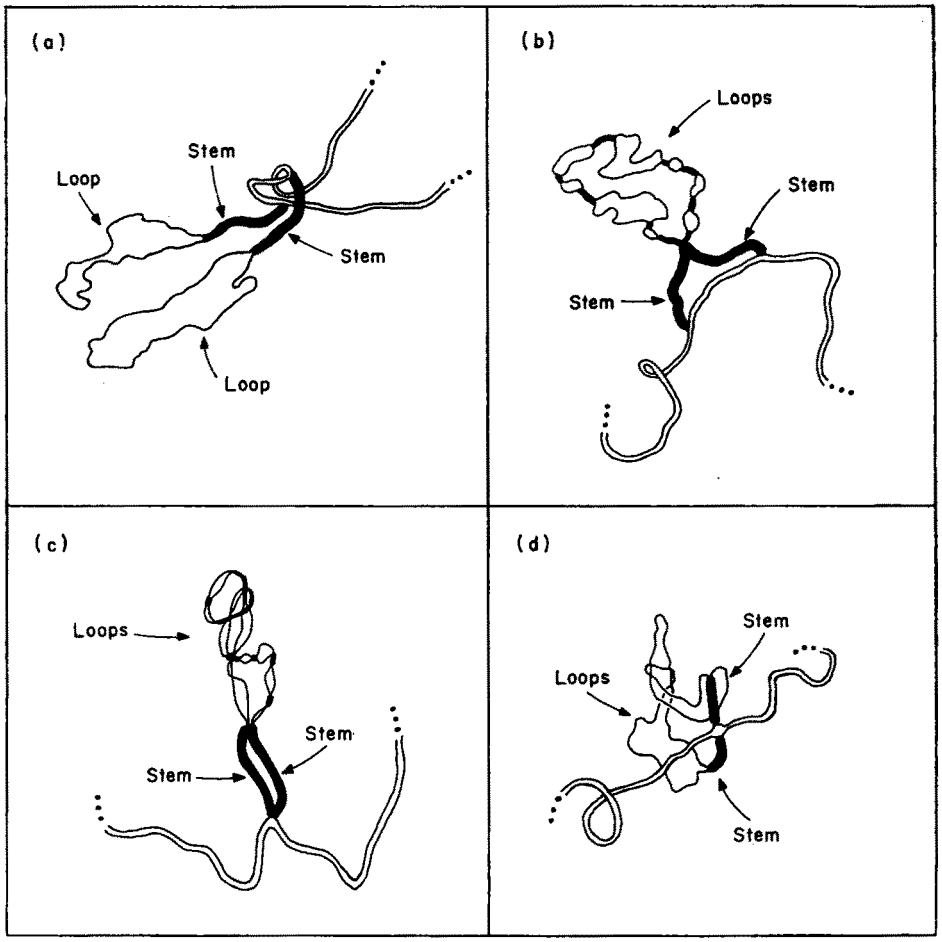

FIG. 1. Interpretation of heteroduplexes in Plate I.
(a) to (d) Correspond to micrographs (a) to (d) in Plate I. ▬▬, Paired stems (double-stranded); ▬▬, paired regions within the loop (double-stranded); ———, unpaired regions within the loop (single-stranded); ═══, P22 DNA (double-stranded).

Implicit in this interpretation is the idea that the insertions carried by P22Tc-10 and P22Tc-106 are not only homologous in base sequence but are also oriented in the same direction with respect to the P22 genome.

Alternative explanations for the structures in Plate I(b) involve partial homology within the loops. These alternative explanations are made less likely by the appearance of homoduplex molecules in the same experiment (Plate I(c)). In these structures, the stem and loop is seen emanating from the same point on the P22 genome; and here also the loops have interacted to give a partially paired structure whose pattern of paired and unpaired regions is very similar to that seen in heteroduplex molecules like the one shown in Plate I(b). Measurement of the lengths of the stems in these molecules indicates that "branch migration" (Lee *et al.*, 1970; Broker & Lehman, 1971) has occurred at the junction between the inserted DNA and the P22 DNA. For example, the lengths of both stems in Plate I(d) are about half the normal stem

length measured for other stems in the same field. Observation of branch migration (in about half of the twenty homoduplexes examined) verifies that the two insertions are actually located at the same point. The slightly separated structure (resembling a square), at the point of the insertion in Plate I(d), is typical of double-stranded branch migrations of this sort (Broker, personal communication).

In summary, P22Tc-10 and P22Tc-106 apparently contain identical insertions (unaccompanied by detectable deletion of P22 DNA) of foreign material at different locations on the P22 genome.

(b) *Mutagenesis of* Salmonella *by insertion of the* tet^R *element carried by Tc-10*

The above physical evidence suggests that the integrity of the *tet*^{R} insertion (bounded by its inverted repetition) has been maintained during translocation of the material from the R-factor onto the P22 genome. The genetic evidence which follows shows that this *tet*^{R} element is capable of further translocation *out* of the P22 genome and *into* many different locations on the chromosome of *S. typhimurium*.

When P22 Tc-10 infects *Salmonella* under conditions where the phage DNA is unable to maintain itself in the host cell, transductants carrying the *tet*^{R} determinant are still obtained. As shown in Table 1, *tet*^{R} transductants are obtained at low frequencies after infection even in the absence of phage (*erf*$^-$) and bacterial (*recA*$^-$) recombination functions, and even though the infecting phage is unable to integrate (*int*$^-$/*ataA*$^-$), or repress (*c2*^{ts}) (Watanabe *et al.*, 1972; Chan *et al.*, 1972; Tye *et al.*, 1974).

Since it seemed possible that these *tet*^{R} transductants had arisen simply by translocation of the *tet*^{R} element out of the P22 genome and into the *Salmonella* chromosome we sought to identify instances in which the insertion into the host chromosome had resulted in an identifiable mutation. We screened *tet*^{R} transductants (isolated on rich medium) for ones which had simultaneously acquired an auxotrophic mutation making them unable to grow on minimal medium. As shown in Table 1, roughly 1% of all *tet*^{R} transductants were auxotrophs. A wide variety of nutritional requirements is represented: out of 142 independent auxotrophs, 27 required proline, 18 methionine, 17 histidine, 12 purines, 8 isoleucine, 8 arginine, 7 tryptophan, 5 cysteine, 3 leucine,

TABLE 1

Isolation of tet^R *auxotrophs*

Expt	Phage	Bacterium	Frequency *tet*^{R} transductants per infecting phage particle	Percentage of auxotrophs among *tet*^{R} transductants
A	P22Tc-10*erf*$^-$	*recA*$^-$*ata*$^v_{P22}$	1×10^{-7}	~1 (14/~1500)
B	P22Tc-10*int*$^-$*c2ts*	*rec*$^+$*ata*$^+_{P22}$	not measured	2 (7/362)
C	P22Tc-10*int*$^-$*c2ts*	*rec*$^+$*ata*$^+_{P22}$	3×10^{-7}	1 (79/7892)

Bacteria were infected with derivatives of P22Tc-10 as indicated above and as described in Materials and Methods. *trt*^{R} transductants obtained from these infections were replica-plated directly onto minimal medium in order to identify auxotrophs. Candidate auxotrophs were then cloned and auxotrophy verified. In experiment C, 120 separate mixtures of phage and bacteria were made; transductants coming from different mixtures are assumed to be independent. In experiments B and C, different recipient strains were used (DB7000 and NK80, respectively).

3 thiamine, 2 alanine, 2 lysine, 1 phenylalanine, 1 tyrosine, 1 threonine, and 25 required nutrients as yet unidentified. This distribution, with its predominance of proline, methionine, purine, and histidine auxotrophs is similar to that obtained after standard chemical mutagenesis of *Salmonella*.

If these tet^R auxotrophs indeed represent simple insertion of the tet^R element alone into the *Salmonella* chromosome, these transductants should not carry an P22 genes. 18 of these tet^R auxotrophs have been tested by marker rescue for the presence of alleles in any of 15 different P22 genes spanning the known genetic map; no rescue was observed.

Two types of experiments provide direct evidence that in these tet^R auxotrophs the tet^R element is intimately associated, both physically and functionally, with the simultaneously acquired auxotrophic mutations:

(1) When the tet^R determinant from these auxotrophs is transferred to another *Salmonella* strain by P22-mediated generalized transduction, all of the recipient bacteria which have become tet^R have also acquired the corresponding auxotrophic requirement (Table 2). Absolute co-transduction of the auxotrophy with the tet^R suggests that the two determinants are physically very closely linked.

(2) A large number of independent tet^R auxotrophs carrying a wide variety of auxotrophic mutations have been reverted to prototrophy. Revertants were obtained at frequencies of 10^{-9} to 3×10^{-7}, depending upon the strain. For 33/37 auxotrophs tested, reversion to prototrophy was always accompanied by loss of the tet^R determinant (Table 3). Thus, not only have these tet^R transductants simultaneously acquired a new auxotrophic mutation, but revertants of the new mutations have simultaneously lost tet^R.

These data strongly suggest that the acquisition of tetracycline resistance and of auxotrophy are the consequence of the same event, namely, the insertion of the tet^R element into the affected gene. The wide distribution of auxotrophic requirements

TABLE 2

Co-transduction of auxotrophy with tetracycline resistance

Donor strain (tet^R auxotroph)	Donor auxotrophy	Proportion of tet^R transductants acquiring donor auxotrophy
NK144	*leu*	73/73
NK147	*leu*	82/82
NK120	*his*	64/64
NK127	*his*	82/82
NK219	*his*	71/71
NK231	*his*	78/78
NK114	*gnd*†	73/73
	Total	523/523

Seven independent tet^R auxotrophs were used as donors in P22-mediated generalized transduction experiments. Lysates of P22int_3HT12/4 were grown on each donor and used to transduce recipient strain NK80 ($edd^-leu^+his^+gnd^+$) to tetracycline resistance on complete medium. tet^R transductants were then tested for growth on appropriately supplemented minimal plates to determine how many had also acquired the donor auxotrophy.

† *gnd* = gluconate dehydrogenase; in the presence of an edd^- mutation, a gnd^- mutation renders *Salmonella* unable to use gluconate as a carbon source.

TABLE 3

Reversion of tet^R *auxotrophs to prototrophy*

	Expt A (*recA*$^-$)	Expt B (*rec*$^+$)	Expt C (*rec*$^+$)	Total
(a) Number of auxotrophs tested for reversion	10	6	34	50
Number yielding any revertants	6	5	34	45
(b) Tetracycline-resistance phenotypes of revertants				
Number of auxotrophs yielding:				
only *tet*^{S} revertants	6	5	22	33
only *tet*^{R} revertants	0	0	2	2
tet^R and *tet*^{S} revertants	0	0	2	2
Total	6	5	26	37

(a) Several colonies of each 50 *tet*^{R} auxotrophic strains were individually inoculated in LB broth, and grown to saturation; 0·1-ml samples were then spread directly on minimal plates. Revertants were obtained at frequencies of 3×10^{-9} to 5×10^{-7}, depending on the particular auxotrophic strain. No difference was seen in the frequencies or types of revertants obtained at temperatures from 28°C to 41°C.

(b) Many revertants were then directly tested for presence of the *tet*^{R} determinant (on minimal plates + 25 μg tetracycline/ml). A total of 397 revertants from 89 independent clones of 37 different auxotrophs were tested.

obtained means, therefore, that the *tet*^{R} element can insert into a large number of different sites on the *Salmonella* chromosome. The observation that nearly all of the *tet*^{R} auxotrophs can revert to prototrophy further suggests that, in most cases, insertion of the *tet*^{R} element is not accompanied by loss of genetic information on the bacterial chromosome. It seems most likely that the host nucleotide sequences on either side of the insertion are preserved exactly; although conceivably insertion could sometimes produce small alterations which do not preclude subsequent reversion to prototrophy.

(c) *Polarity of* tet^R *insertions in the* Salmonella *histidine operon*

In order to characterize some of the presumed *tet*^{R} insertions with respect to their precise locations and to ascertain their effects on gene expression, we chose to examine closely 16 independent *his*$^-$ auxotrophs isolated in our experiments. The *his* operon of *Salmonella* is convenient because it has been extensively characterized both genetically and biochemically (Hartman *et al.*, 1971; Brenner & Ames, 1971); the only genes known to be required for biosynthesis of histidine are the nine structural genes which comprise this operon.

The *his*$^-$ auxotrophs were first tested for their ability to complement a series of F′ factors (which carry heterologous *his* genes from *E. coli*), each of which was mutant in one of the *his* structural genes. This intergeneric complementation system, as described by Atkins & Loper (1970) allows the assessment of complementation in the virtual absence of recombination. Table 4 shows that the 16 histidine auxotrophs fall into only four categories on the basis of their complementation patterns. In three of these categories, the auxotrophs exhibit pleiotropic defects: they fail to complement with episomes carrying mutations in two or three contiguous genes in the operon.

TABLE 4

Complementation patterns of his$^-$ tet^R *auxotrophs*

		his$^-$ mutation on episome†									
Promoters:‡		G$^-$ →···	D$^-$	C$^-$	B$^-$ →···	H$^-$	A$^-$	F$^-$	I$^-$ →···	E$^-$	Inferred location of lesion
his$^-$*tet*^{R} auxotrophs											
Class	Number of strains										
1	8	−	−	−	+	N.T.	+	+	+	+	*G* (or promoter)
2	3	+	−	−	+	N.T.	+	+	+	+	*D*
3	1	+	+	+	+	N.T.	+	+	−	−	*I*
4	4	+	+	+	+	N.T.	−/+	−/+	+	+	*H* or *A*?

16 independent *his*$^-$*tet*^{R} auxotrophs fell into only 4 classes on the basis of their ability to complement various *his*$^-$ mutations carried on *E. coli* F′*his* episomes (see Materials and Methods). The locations of *his*$^-$ lesions are inferred from the observed complementation patterns, the position of the promoters in the *his* operon, and the assumption that the lesions are polar. N.T. = not tested.

†*His*$^-$ mutations are listed in the same order as the corresponding genes in the *his* operon. In this orientation, *his* operator/promoter region is just to the left of gene *G*.

‡ Positions of arrows indicate sites of primary promoter and two low-level internal promoters.

Auxotrophs in the fourth category (class 4) complement all *his*⁻ mutants tested, but complementation for genes *A* and *F* was quite poor. Complementation using episomes mutant in gene *H* was not done.

Since all of these *his*⁻ auxotrophs revert to prototrophy (data not shown), the pleiotropic effects of the *tet*^{R} insertions are not attributable to a deletion. Using the same intergeneric complementation system, Atkins & Loper (1970) identified two internal secondary promoters in the *his* operon at the positions shown in Table 4. One of these promoters is in gene *C* and promotes transcription of distal genes, while the other is farther "downstream." The observation that *tet*^{R} *his*⁻ auxotrophs of classes 1 and 2 are $G^-D^-C^-$ and $G^+D^-C^-$, respectively, but are still capable of expressing genes distal to gene *C*, suggested that the *tet*^{R} insertion mutation is polar on distal genes and that this polarity extends only as far as the next downstream promoter. The complementation pattern of the class 3 auxotroph is consistent with such an hypothesis, as is that of the class 4 auxotrophs if the partial complementation seen for mutants in *A* and *F* is attributed to weak polarity.

If the polarity hypothesis is correct, each of the *tet*^{R} insertions should map within the most operator–proximal of the genes it affects. Thus, class 1 mutants should map in the operator/promoter or in gene *G*; class 2 mutants in gene *D*; and the class 2 mutant in gene *I*. Deletion mapping (shown in Fig 2) places unambiguously all of these *tet*^{R} auxotrophs in the expected genes.

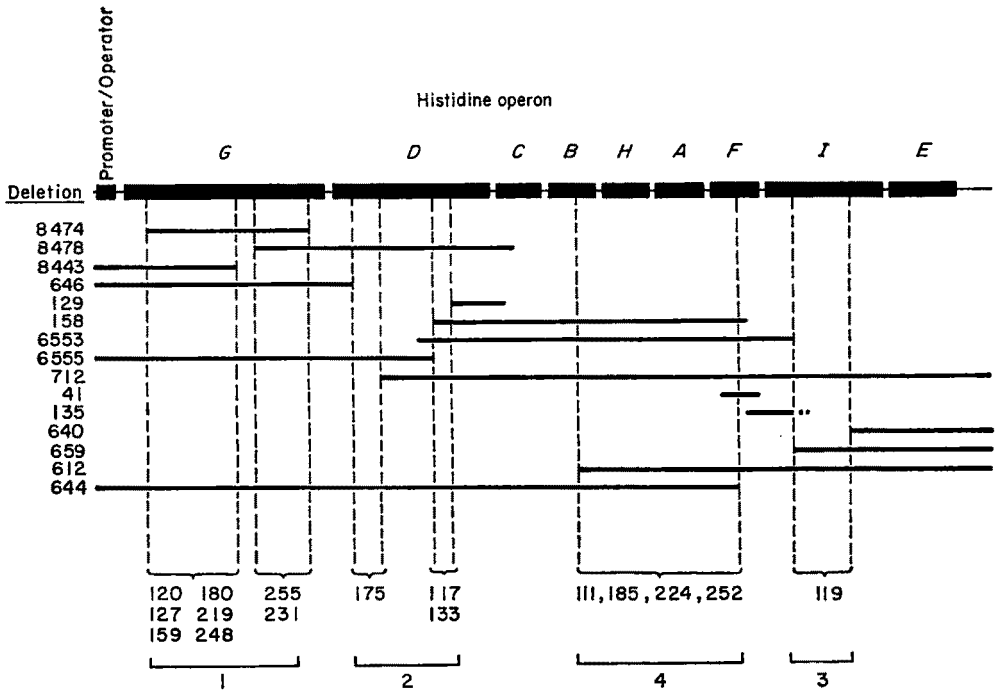

FIG. 2. Deletion mapping of *his*::*tet*^{R} auxotrophs.

Positions of 16 *his*::*tet*^{R} auxotrophs with respect to end points of known deletions in the histidine operon were determined by P22-mediated transductional crosses. Appearance of *his*$^+$ recombinants was scored after infection of appropriate deletion strains with transducing lysates grown on each of the *his*::*tet*^{R} auxotrophs. Horizontal bars indicate extent of *his* material deleted. Map is not drawn to scale. Bracketed numbers denote *his*::*tet*^{R} strains mapping in the indicated interval. Complementation classes 1 to 4 (from Table 4) also indicated.

The locations of *tet*^{R} auxotrophs in classes 1 and 2 have been confirmed by an independent method. The final step in the pathway for histidine biosynthesis, the conversion of histidinol to histidine, is catalyzed by the product of the *D* gene, histidinol dehydrogenase. Bacteria able to express *D* function can grow on minimal medium supplemented with histidinol even if they are defective in any other *his* function. For *tet*^{R} auxotrophs located in gene *D* itself, all revertants selected on histidinol should also be *his*$^+$. For *tet*^{R} auxotrophs located nearer to the promoter (i.e. "upstream"), it should be possible to obtain revertants which grow on histidinol in which the polar effect of the *tet*^{R} insertion on the expression of *D* function has been alleviated, but total operon function has not been restored and the "revertants" remain *his*$^-$.

Revertants of class 2 auxotrophs (mapping in *D*) which are able to grow on histidinol arise at frequencies of 10^{-7} to 10^{-9}. All such revertants tested (a total of 459 revertants picked from 7 to 10 clones of each of the three class 2 strains) had also become *his*$^+$, confirming that these *tet*^{R} insertions are in fact located in gene *D*. Revertants of class 1 auxotrophs (mapping in *G*) which grow on histidinol occur at frequencies of up to 10^{-3} and fewer than 1% of these revertants have become *his*$^+$, confirming that these insertions do *not* lie in gene *D*.

All of the above observations are consistent with the notion that the *tet*^{R} insertions in the *his* operon exert polar effects on the expression of distal genes.

All of the revertants of class 1 auxotrophs (in *G*) which were selected for ability to grow on histidinol had simultaneously lost the *tet*^{R} determinant. The vast majority of these revertants had not regained full *his*$^+$ function, and thus must represent some imperfect and/or partial excision of the *tet*^{R} element. Thus, *excision* of the *tet*^{R} element is usually not precise. This is in contrast to *insertion* of the element which is seen to be precise enough to allow restoration of gene function (as discussed above).

4. Discussion

The above experiments substantiate the idea that a genetic element which carries genes conferring tetracycline-resistance (flanked by an inverted repetition about 1400 base-pairs long) is capable of translocation as a unit from one DNA molecule to another. The *tet*^{R} element is found in nature on a variety of R-factors (Sharp *et al.*, 1973), was acquired by P22 (producing P22Tc-10 and P22Tc-106) and has now been observed to insert into a large number of different sites on the *Salmonella* chromosome, including six different sites in the *his* operon. Translocation of the *tet*^{R} element has the following genetic properties:

(1) When the *tet*^{R} element is inserted into a structural gene, gene function is abolished.

(2) When the *tet*^{R} element is inserted into a group of genes forming a single transcription unit (operon), it exerts a polar effect on the expression of promoter-distal genes.

(3) Insertion is relatively non-specific; the distribution on the *Salmonella* chromosome of *tet*^{R} insertion auxotrophs is similar to that obtained by chemical mutagenesis. On the other hand, there appears to be, within the *his* operon, some clustering of the sites of insertion.

(4) Insertion of the *tet*^{R} element virtually always occurs without loss of information from the molecule into which it inserts: most of the *tet*^{R} auxotrophs are capable of

reverting to prototrophy. Excision of the *tet*^{R} element, on the other hand, is usually not so precise: in the cases where we could test, there were hundreds of excisions which did *not* restore gene function for every excision that generated a prototrophic revertant.

(5) Insertion and excision of the *tet*^{R} element appears to be independent of the function of the *recA* gene of *Salmonella*.

Other laboratories have recently identified translocatable drug-resistance elements whose properties resemble those of the *tet*^{R} element. Heffron *et al.* (1975*a*,*b*) identified an ampicillin-resistance determinant which is flanked by a very short (150 base-pairs) inverted repetition. This element has been observed to translocate to at least a dozen sites within a 2800 base-pair segment of a small plasmid. Kopecko & Cohen (1975) report the *recA*-independent, apparently site-specific integration and translocation of a similar *amp*^{R} element. Most strikingly, Berg *et al.* (1975) describe two derivatives of coliphage λ which carry (as a simple insertion) a kanamycin-resistance element (derived from an R-factor) which is bounded by a 1400 base-pair inverted repetition. Thus, there appear to be many translocatable elements carrying drug-resistance determinants on R-factors which are associated with inverted repetitions.

However, Berg *et al.* also found derivatives of λ carrying a second type of kanamycin-resistance insertion (derived from a different R-factor) which do not have detectable inverted repetition; Gottesman & Rosner (1975) have also found a chloramphenicol-resistance determinant, which is translocatable into λ from coliphage P1, in which no reverse duplication was seen. These insertions may represent a different type of translocation element, or may be cases in which the inverted repetition is too short or too unstable to be detectable by standard visualization methods which depend on intra-molecular annealing.

In any case, it seems quite clear that translocatable genetic elements carrying drug-resistance determinants must make a substantial contribution to the reassortment of drug-resistant determinants which is seen among R-factors (Watanabe & Lyang, 1962; Clowes, 1972; Heffron *et al.*, 1975*a*).

In heteroduplex studies including the R-factor from which P22Tc-10 was made, Sharp *et al.* (1973) were able to correlate the *tet*^{R} determinant with a non-tandem inverted repetition having the same dimensions as the Tc-10 insertion. More recently, Ptashne & Cohen (1975) have shown that the inverted repetition associated with *tet*^{R} (on such an R-factor) is homologous with the IS3 insertion sequence of Malamy *et al.* (1972). The insertion sequences themselves were detected as individual insertion units which cause polar mutations (Shapiro, 1969; Jordan *et al.*, 1968; Malamy, 1970). In the cases of IS1 and IS2, polarity is attributable to the presence of transcription termination signals on the IS sequences themselves (Adhya *et al.*, 1974; Max Gottesman, personal communication). Thus, our *tet*^{R} element is bounded by two units which should be capable of functioning individually. This idea is supported by the preliminary observation that some of the *his*$^-$*tet*^{S} polarity relief revertants (obtained from class 1 *his*::*tet*^{R} insertions) can further revert to *his*$^+$ at high frequency. These could be instances in which excision of the *tet*^{R} determinant left behind a single IS sequence which can subsequently excise to restore *hisG*$^+$ function. (This explanation requires the additional assumption that the remaining IS sequence is non-polar or else can promote expression of *hisD* function; precedent exists for the idea that IS sequences might contain transcription termination signals when inserted in one

orientation, but serve as new promoters when inserted in the other (Saedler *et al.*, 1974).)

The IS sequences, singly and as non-inverted duplications, have recently been associated with a large number of unusual (often "illegitimate" and *recA*-independent) recombination phenomena—deletions, fusions, integration of F-factors, and amplification of R-factors (Sharp *et al.*, 1972; Ptashne & Cohen, 1975; Lee *et al.*, 1974; Davidson *et al.*, 1975; Hu *et al.*, 1975). The precise relationship of these phenomena to the properties of the inverted duplications of an IS3 sequence in the tet^R element remains to be worked out. We would like to suggest, based on our studies of the tet^R element, that a pair of inverted IS sequences might be essential for efficient and relatively stable translocation of the DNA between them to many new sites.

There are other bacterial systems for translocation of particular genetic elements which may or may not be mechanistically related to insertion of the tet^R element. The most striking example is bacteriophage Mu-1 which causes polar mutations by random insertion into the *E. coli* chromosome (for review, see Howe & Bade, 1975). Mutants of Mu which are capable of perfect excision much more frequently excise imperfectly (Bukhari, 1975), as is the case with the tet^R element. Coliphage λ is also capable of integration into a large number of sites (many of them structural genes) on the *E. coli* chromosome when the normal λ attachment site is missing (Shimada *et al.*, 1973). This integration is still dependent on the phage enzyme involved in site-specific integration at the normal site. Unlike Mu integration, however, integration of λ in the absence of the normal attachment site still shows considerable preference for particular sites, and excision of λ from these sites is usually precise (Shimada *et al.*, 1975). Both Mu and λ integration are, like that of the tet^R element, independent of *recA* function (Shimada *et al.*, 1975; Boram & Abelson, 1971).

Translocation of genetic material by such non-tandem inverted repetitions offers a powerful tool with which biological systems can perform substantial genetic rearrangements. Recombination between the inverted sequences themselves would generate an inversion of the material carried between them. Translocations by such elements could also result in duplications. Inverted repetitions (tandem and non-tandem) have been identified in the DNA of a large number of prokaryotic (Sharp *et al.*, 1972,1973; Berg *et al.*, 1975; Heffron *et al.*, 1975*a*,*b*; Daniell *et al.*, 1973) and eukaryotic (Wadsworth *et al.*, 1975; Wolfson & Dressler, 1972; Locker *et al.*, 1974; Manning *et al.*, 1975) organisms. In many of these cases, the inverted repetitions are associated with DNA rearrangements that can be explained in terms of translocatable segments. It seems possible therefore that a fundamental relationship may exist between translocatable segments of DNA and inverted repetitions.

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