Genetic Mapping by Duplication Segregation in *Salmonella enterica*

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ABSTRACT

Mu*d*P and Mu*d*Q elements were used to induce duplications in *Salmonella enterica* by formation of a triple crossover between two transduced fragments and the host chromosome. The large size (36 kb) of Mu*d*P and Mu*d*Q is a favorable trait for duplication formation, probably because homology length is a limiting factor for the central crossover. Additional requirements are a multiplicity of infection of 2 or higher in the infecting phage suspensions (which reflects the need of two transduced fragments) and an exponentially growing recipient (which reflects the need of a chromosome replication fork). We describe a set of 11 strains of *S. enterica*, each carrying a chromosomal duplication with known endpoints. The collection covers all the Salmonella chromosome except the terminus. For mapping, a dominant marker (*e.g.*, a transposon insertion in or near the locus to be mapped) is transduced into the 11-strain set. Several transductants from each cross are grown nonselectively, and haploid segregants are scored for the presence of the marker. If all the segregants contain the transduced marker, it maps outside the duplication interval. If the marker is found only in a fraction of the segregants, it maps within the duplicated region.

A classical review of genetic duplications in bacteria (Anderson and Roth 1977) envisaged that a potential use of duplications might be genetic mapping. The logic of such a mapping system is simple: a marker introduced into a preexisting duplication will segregate together with the duplication, while a marker outside the duplication will not (Figure 1). In practice, the development of a procedure for mapping by duplication segregation requires the following tools: (i) a method to construct duplications with known endpoints; (ii) a procedure to introduce the marker to be mapped into one copy of the duplication; (iii) a screen to detect segregation of the duplication; and (iv) a screen to score cosegregation of the marker under study. All these tools are available for Salmonella, combining P22 HT transduction (Schmieger 1972), the use of transposable elements as genetic tools (Kleckner *et al.* 1977; Maloy *et al.* 1996), and a number of procedures developed by John Roth and co-workers for the construction and analysis of genetic rearrangements (reviewed by Roth *et al.* 1996).

This study describes the construction of duplications covering all regions of the *Salmonella enterica* chromosome except the terminus, where duplications were not obtained. Observations made by other authors suggest that duplications involving the terminus may be inviable (D. R. Hillyard and J. R. Roth, personal communication). All duplications have been constructed with the procedure of Hughes and Roth (1985), except that the Mu*d*-P22 prophages Mu*d*P and Mu*d*Q (Youderian *et al.* 1988) have been used instead of Mu*d*A elements. Mu*d*P and Mu*d*Q are hybrids between Salmonella phage P22 and coliphage Mu (Youderian *et al.* 1988). Each consists of about two-thirds of the phage P22 genome placed between the ends of Mu; the construction includes also a chloramphenicol resistance marker. The Mu*d*P and Mu*d*Q constructs contain the P22 immunity region but lack the P22 *sieA* (superinfection exclusion) gene; this permits DNA injection by phage P22 (Poteete 1988). Mu*d*P and Mu*d*Q elements also lack the P22 *mnt* gene, which encodes a repressor for the maintenance of lysogeny (Botstein *et al.* 1975). Absence of *mnt* permits the expression of lytic genes in superinfecting P22 phage (Botstein *et al.* 1975; Poteete 1988). Thus Mu*d*P and Mu*d*Q lysogens can be lysed by P22, and the resulting lysates are suitable for generalized transduction, since the 36-kb Mu*d*P and Mu*d*Q elements can be accommodated in a P22 head (Casjens and Hayden 1988).

We have constructed a collection of Mu*d*P/Q-held duplications that permits mapping of a dominant mutation to any of 11 chromosomal intervals. Mapping is carried out by P22 HT-mediated transduction, followed by segregation analysis. This technically simple method may be complementary to mapping with lysates of "locked-in" Mud-P22 prophages and can sort out intricacies derived from bidirectional packaging of markers flanking a Mu*d*-P22 hybrid (Benson and Goldman 1992; Flores and Casadesús 1995). The duplication collection can be also used for dominance/recessivity tests and other operations of genetic analysis. We also discuss experimental procedures and conditions that facilitate the construction of Mu*d*P/Q-induced duplica-

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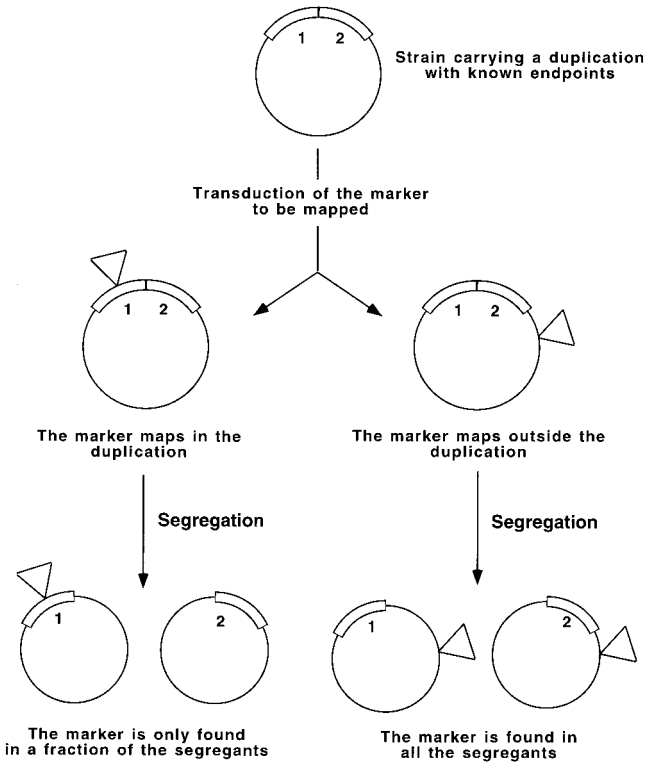

FIGURE 1.—Logic of genetic mapping by duplication segregation.

tions; use of such procedures may be helpful to add novel duplication-bearing strains to the current 11-strain set.

MATERIALS AND METHODS

Bacterial strains: Strains of *S. enterica*, serovar Typhimurium (usually abbreviated as *S. typhimurium*) listed in Table 1 are derived from the standard, wild-type strain LT2. Strain MST1202 was a gift from Stanley R. Maloy, University of Illinois, Urbana, Illinois. Strain TT16714 of *S. typhimurium* was obtained from Rafael Camacho, Universidad Nacional Autónoma de México, Mexico City, Mexico. The virulent strain *S. typhimurium* SL1344 (HOISETH and STOCKER 1981) was provided by Francisco García-del Portillo, Universidad Autónoma de Madrid, Cantoblanco, Spain. Strain 3246 of *S. dublin* was provided by Timothy S. Wallis, Institute for Animal Health, Compton, England. Strain SAO44 of *S. abortusovis* was obtained from Salvatore Rubino, Istituto di Microbiologia, Università degli Studi di Sassari, Sassari, Italy. Strain G9/4223 of *S. gallinarum* was a gift from John E. Olsen, Royal Veterinary and Agricultural University, Fredericksberg, Denmark. Mu*d*1-8[AmpLac] (HUGHES and ROTH 1984) and Mu*d*I1734[Kan-Lac] (CASTILHO *et al.* 1984) are transposition-deficient Mu derivatives. These elements have been renamed Mu*d*A and Mu*d*J, respectively (HUGHES and ROTH 1985). Mu*d*P and Mu*d*Q are P22-Mu hybrids that carry a chloramphenicol-resistance marker (YOUDERIAN *et al.* 1988). The nomenclature for duplications follows the rules of HUGHES and ROTH (1985).

Culture media: The E medium of VOGEL and BONNER (1956) was used as minimal medium. The carbon source was 0.2% glucose. Auxotrophic requirements and antibiotics were used at the concentrations described by MALOY *et al.* (1996). The rich medium was Difco nutrient broth (NB; 8 g/liter) with added NaCl (5 g/liter). Solid media contained Difco agar at 1.5% final concentration. Green plates were prepared according to CHAN *et al.* (1972), except that methyl blue (Sigma, St. Louis) substituted for aniline blue.

Bacteriophages and transduction: The transducing phage was P22 HT 105/1 *int201* (SCHMIEGER 1972; G. ROBERTS, unpublished data), henceforth referred to as "P22 HT." Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Phage NBP182 *erf (am1173)* is a derivative of P22 HT 105/1 provided by Nicholas R. Benson, Sidney Kimmel Cancer Center, San Diego, California. Erf$^-$ (essential recombination function) mutants of P22 are unable to grow on RecA$^-$ strains (BOTSTEIN and MATZ 1970). Transducing lysates were prepared according to DAVIS *et al.* (1980). The titers of phage suspensions were calculated from counts of plaque-forming units on strain LT2. The multiplicity of infection (MOI) is the number of input phage per colony-forming unit. For transduction, 0.2 ml of the recipient culture was mixed with 0.2 ml of phage suspension, diluted as needed. The mixtures were preincubated for 35 min at 37° with gentle shaking before spreading on nutrient agar (NA) supplemented with the appropriate antibiotic. To obtain phage-free isolates, transductants were purified by streaking on green plates.

Construction of "enlarged" Mu*d*J elements: The Mu*d*J elements carried by strains TT12313 and TT12316 were "enlarged" with a Tn*10*, introduced into their respective *lacZ* genes by homologous recombination. For this purpose, a P22 high-transducing (HT) lysate grown on strain MST1202 was used to transduce TT12313 and TT12316, selecting tetracycline resistance. Individual Tc^r transductants were made phage free and patched with toothpicks to appropriate media. Isolates with the appropriate phenotype (Km^r Tc^r Ap^s Lac$^-$) were propagated as strains SV4018 (derived from TT12313) and SV4019 (derived from TT12316). In both strains, the kanamycin resistance marker of Mu*d*J and the tetracycline resistance marker of Tn*10* proved to be 100% linked by cotransduction analysis.

Converting Mu*d*J elements to Mu*d*P or Mu*d*Q: The Mu*d*J element of strain TT12318 and the Mu*d*A element of strain TT16714 were both converted to Mu*d*Q elements to generate strains SV4130 and SV4131, respectively. SV4131 is actually the product of a second transductional cross using LT2 as the recipient; for this reason, it lacks the *argR9002*::Tn*10d*Tet mutation. Replacement of Mu*d*A or Mu*d*J with Mu*d*Q was performed according to BENSON and GOLDMAN (1992), using TT12916 as the donor strain. For tailing, the lysate obtained upon induction of TT12916 was mixed with an excess of P22 tails (YOUDERIAN *et al.* 1988).

Transductional test for the classification of duplication-carrying isolates: Individual transductants putatively carrying induced duplications were made phage free and lysed with P22 HT. The lysates were used to transduce LT2, selecting Cm^r transductants on NA-chloramphenicol plates. One hundred or more Cm^r transductants were then replica printed to minimal plates. Reconstruction of the duplication was detected by the formation of Cm^r prototrophic transductants.

RESULTS

Properties of P22 HT lysates grown on Mu*d*P/Q lysogens: Because Mu*d*P/Q elements lack the *sieA* and *mnt* genes of P22, Mu*d*P/Q lysogens can be lysed by infecting P22 HT phage. The resulting lysates have titers similar to those obtained on nonlysogenic hosts. If used for transduction, these lysates yield Cm^r transductants at

TABLE 1

Strain list

Strain	Genotype or phenotype	Reference or source[a]
MST1202	*putA1019*::Mu*d*1-8 (*lacZ*::Tn*10*)	S. R. Maloy
NB2	*leuA414 supE 20* Fels2$^-$	N. R. Benson
SV1235	*recA1*	Garzón *et al.* (1995)
SV1601	Dup [*cysG1573* *Mu*d*P**ilvA2642*]	
SV1603	Dup [*proA692* *Mu*d*Q**purE2164*]	
SV1604	Dup [*thr-469* *Mu*d*P**proA692*]	
SV1611	Dup [*purE2514* *Mu*d*P**purB1879*]	
SV2090	*hisC9968*::Mu*d*J	Flores and Casadesús (1995)
SV3038	*cysJIH3501*::Mu*d*J	Lab stock
SV3193	Dup [*hisH9962* *Mu*d*P**cysA1586*]	
SV4015	Dup [*cysA1586* *Mu*d*P**purG2149*]	
SV4018	*purE2154*::Mu*d*J (*lacZ*::Tn*10*)	
SV4019	*purB1879*::Mu*d*J (*lacZ*::Tn*10*)	
SV4130	*purA1881*::Mu*d*Q	
SV4131	*argA9001*::Mu*d*Q	
SV4142	Dup [*purA1881**Mu*d*P**thr-469*]	
SV4193	Dup [*purG2149**Mu*d*P**argA9001*]	
SV4194	Dup [*argA9000**Mu*d*P**cysG1573*]	
SV4195	Dup [*ilvA2648**Mu*d*P**purA1881*]	
SV4200	Dup [*trp-248* *Mu*d*P**uisD9953*]	
TR6934	*leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	
TT9636	*cysJ1567*::Mu*d*A	J. R. Roth
TT9686	*hsiC1264*::Mu*d*A	J. R. Roth
TT12313	*purE2154*::Mu*d*J	J. R. Roth
TT12316	*purB1879*::Mu*d*J	J. R. Roth
TT12318	*purA1881*::Mu*d*J	J. R. Roth
TT12916	*leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$ / F′114ts *lac*$^+$ *zzf-20*::Tn*10 zzf-3553*::Mu*d*Q	Benson and Goldman (1992)
TT15224	*thr-469*::Mu*d*Q *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15229	*proA692*::Mu*d*Q *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15231	*proA692*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15232	*purE2154*::Mu*d*Q *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15235	*purE2154*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15240	*putA1019*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15241	*purB1879*::Mu*d*Q *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	N. R. Benson
TT15242	*purB1879*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	N. R. Benson
TT15256	*purG2149*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15264	*cysG1573*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT15269	*ilvA2648*::Mu*d*P *leuA414 hsdL r*$^-$ *m*$^+$ Fels2$^-$	Benson and Goldman (1992)
TT16714	*argA9001*::Mu*d*A *argR9002*::Tn*10d*Tc	R. Camacho

[a] Omitted for strains described in this study.

frequencies around 10^{-5} per pfu. If the transduced Mu*d*P/Q insertion caused auxotrophy, all the transductants were auxotrophs. These observations suggest that Mu*d*P/Q elements can be transduced as ordinary transposon insertions. In addition, the following experiments indicated that lytic growth of P22 on a Mu*d*P/Q lysogen does not cause recombinational rearrangements at high frequency:

1. Twenty independent Cm^r transductants, generated upon transduction of the insertion *putA1019*::Mu*d*P to strain NB2, proved to be inducible by mitomycin C and able to package the nearby *serC* locus. Thus the Mu*d*P element appeared intact after transduction.
2. Formation of P22 HT derivatives able to perform specialized transduction of the Cm^r marker did not occur. In these experiments, six independent lysates of TT15240 were used to transduce a RecA$^-$ recipient (SV1235), selecting chloramphenicol resistance. Cm^r transductants were not obtained. Recombination is probably constrained by the position of the Mu*d*P chloramphenicol resistance determinant, which may lack homology for crossover at one side.
3. Evidence that recombination between Mu*d*P/Q elements and superinfecting phage occurred at low frequency was provided by the formation of Erf$^+$ recombinants during lytic growth of a P22 Erf$^-$ mutant on a Mu*d*P lysogen. Lysates of NBP182, an Erf$^-$

derivative of P22, were obtained on a pair of largely isogenic strains, TT15240 and TT6934. The resulting phage suspensions were then plaqued on RecA$^+$ and RecA$^-$ hosts (LT2 and SV1235). Only Erf$^+$ phage can form plaques on SV1235, because P22 Erf$^-$ mutants are unable to grow on RecA$^-$ strains (Botstein and Matz 1970). A higher frequency of Erf$^+$ phage was detected in the lysate of TT15240 (3×10^{-8} *vs.* 4×10^{-9} recombinants per pfu), providing evidence that the infecting Erf$^-$ mutant had recombined, albeit at low frequency, with the Mu*d*P (Erf$^+$) prophage.

Single- *vs.* double-lysate transduction as a screen for the formation of directed duplications: The method of Hughes and Roth (1985) for the construction of directed duplications employs two Mu*d*A elements that provide homology for a crossover between two fragments independently transduced into a single cell. If host sequences adjacent to each Mu*d*A element form two additional crossovers with a replication fork in the recipient chromosome, the triple recombination event results in the duplication of the chromosomal region between the original Mu*d*A insertions and the reconstruction of a single Mu*d*A element at the joint point of the duplicated segments. The resulting recombinants can be selected by their ability to form prototrophic, ampicillin-resistant colonies (Hughes and Roth 1985). We applied this procedure to duplication formation using Mu*d*P and Mu*d*Q prophages (Youderian *et al.* 1988). The advantages expected from the use of these elements will be discussed below.

As a screen to detect the occurrence of duplications induced by Mu*d*P/Q elements, we compared the frequencies of duplications obtained in ordinary, one-lysate transductions to the frequency obtained upon two-lysate transduction. The screen relies on the consideration that one-lysate transduction cannot induce duplication formation. However, prototrophic Cm^r transductants can be formed by one-lysate transduction if the incoming fragment carrying a Mu*d*P/Q element recombines with one copy of a duplicated gene already present in the infected culture (Anderson and Roth 1979, 1981). Thus an excess frequency of prototrophic transductants obtained upon two-lysate transduction (compared to one-lysate transduction) provides evidence of Mu*d*P/Q-directed duplications. In all cases, the frequency of duplications was measured as the ratio of the number of prototrophic Cm^r transductants over the total number of Cm^r transductants.

Because the incidence of spontaneous duplications varies among different chromosomal regions (Anderson and Roth 1981), comparisons between one-lysate and two-lysate transductions were performed in the following chromosomal intervals: *thr–proA* [centisomes (CS) 0-7], *proA–purE* (CS 7-12), *purE–purB* (CS 12-27), *hisH–cysA* (CS 44-53), and *cysG–ilvA* (CS 75-85). An experiment for each chromosomal interval is shown in Table 2. A significant increase in the frequency of Cm^r prototrophs by two-lysate transduction was observed in all intervals assayed except *cysG–ilvA*. In the latter interval, the absence of differences may be explained by the higher incidence of spontaneous duplications affecting the *ilvA* region. Chromosomal regions near the origin of replication undergo high frequencies of spontaneous duplication (Anderson and Roth 1979, 1981). The *ilvA* gene lies at CS 85, 3, relatively close to the origin of replication of the Salmonella chromosome, located at CS 84, 9 (Sanderson *et al.* 1995).

TABLE 2

Comparisons of the frequency of duplications upon one-lysate and two-lysate transductions

Donor strain	Transduced marker	Duplication frequency ($\times 10^{-3}$)[a]
TT15264	*cysG1573*::Mu*d*P	5
TT15269	*ilvA2642*::Mu*d*P	40
TT15264 + TT15269		38
TT15224	*thr-469*::Mu*d*Q	4
TT15231	*proA692*::Mu*d*P	1
TT15224 + TT15231		160
TT15251	*hisH9950*::Mu*d*P	0.8
TT15254	*cysA1586*::Mu*d*P	7
TT15251 + TT15254		30
TT15229	*proA692*::Mu*d*Q	2
TT15232	*purE1254*::Mu*d*Q	2
TT15229 + TT15232		180
TT15235	*purE2154*::Mu*d*P	3
TT15242	*purB1879*::Mu*d*P	0.8
TT15235 + TT15242		230

[a] Quotient between the number of prototrophic Cm^r transductants and the total number of Cm^r transductants.

Duplications across the terminus (between *purE* and *trp*) were not obtained (data not shown). This failure is in accordance with previous observations (D. R. Hillyard and J. R. Roth, personal communication).

In favorable cases, the two-lysate/one-lysate screen provides evidence that directed duplications have been formed. However, the screen does not identify the individual transductants that carry them: isolates carrying spontaneous duplications and those bearing duplications directed by Mu*d*P/Q are all Cm^r prototrophs. As described below, each type can be distinguished by its segregation pattern and by its behavior as a transductional donor.

Segregation patterns of spontaneous and directed duplications: Cm^r prototrophic transductants generated by recombination of a transduced fragment bearing the Mu*d* element with a preformed, spontaneous duplication will segregate Cm^s protrotrophs and Cm^r auxo-

trophs (Anderson and Roth 1981). In contrast, merodiploids carrying Mu*d*-held, directed duplications will segregate only chloramphenicol-sensitive protrotrophs (Hughes and Roth 1985). To analyze the segregation pattern of individual transductants, five or more Cm^r prototrophic transductants from each cross were inoculated in NB, grown to full density, diluted, and spread on nutrient agar. One hundred individual colonies derived from a single transductant were replica printed to minimal plates, minimal plates with chloramphenicol, NA plates, and NA plates with chloramphenicol. The data shown in Table 3 can be summarized as follows:

1. Isolates carrying duplications of either type (spontaneous or directed) were obtained in all crosses. In the examples shown in Table 3, all (5/5) Cm^r transductants from donors TT15224 and TT15231 carried directed duplications. The incidence of directed duplications was lower in other crosses: only one transductant (III) from donors TT15251 and TT15254 and two from donors TT15264 and TT15269 (IV and V) carried a directed duplication.
2. All isolates carrying duplications underwent segregation when grown in nonselective medium (NB), irrespective of the type of duplication carried. However, the frequency of segregants varied largely among different isolates. In the examples shown in Table 3, the slowest segregation rates (reflected in the higher number of Cm^r prototrophs) were found in crosses involving TT15269. One explanation might be that duplication of this region confers a selective growth advantage (Anderson and Roth 1981).

A cross-check for isolates putatively carrying induced duplications was their ability to yield Cm^r prototrophic transductants if used as donors. In these crosses, transduction of the duplication joint point reconstructs the duplication by a double crossover with a replicating chromosome (Hill *et al.* 1969; Anderson *et al.* 1976). In every cross, all transductants putatively carrying directed duplications (as judged from their segregation pattern) also passed the transductional test. Thus the congruence and reliability of both tests is high.

Effect of the multiplicity of infection on duplication formation: Formation of a triple crossover between two Mu*d*A elements and a replicating chromosome requires two independently transduced fragments (Hughes and Roth 1985). To investigate whether our Mu*d*P/Q-based procedure for duplication formation involved a similar mechanism, we studied the effect of MOI upon duplication formation. The underlying rationale was that, if duplication formation does require two transducing fragments, it should show a second-order dependence upon the MOI of the lysates (Miesel and Roth 1996). On these grounds, we performed transductional crosses in which a constant number of recipient cells was transduced with two lysates at various MOIs. To minimize the bias caused by spontaneous duplications, these experiments were carried out at the chromosomal region of CS 7-12, where the frequency of spontaneous duplications is low. The donor strains were TT15229 and TT15232; the recipient was LT2. A prediction is that the number of transductants (*T*) obtained will be a function of the phage multiplicity (*P*) raised to the

TABLE 3

Identification of isolates carrying duplications (spontaneous or directed) by their segregation patterns

Donors	Isolate	Cm^s prototrophs	Cm^r auxotrophs (I)[a]	Cm^r auxotrophs (II)[b]	Cm^r prototrophs
TT15251 + TT15254	I	31	32	0	35
	II	5	86	0	9
	III	97	0	0	3
	IV	0	100	0	0
	V	38	0	40	19
TT15224 + TT15231	I	47	0	0	53
	II	23	0	0	77
	III	27	0	0	73
	IV	90	0	0	10
	V	64	0	0	36
TT15264 + TT15269	I	11	0	4	85
	II	1	0	16	83
	III	4	0	9	87
	IV	1	0	0	99
	V	39	0	0	61

[a] Class I of Cm^r auxotrophs: TT15251 + TT15254: His$^-$; TT15224 + TT15231: Thr$^-$; TT15264 + TT15269: Cys$^-$.

[b] Class II of Cm^r auxotrophs: TT15251 + TT15254: Cys$^-$; TT15224 + TT15231: Pro$^-$; TT15264 + TT15269: Ilv$^-$.

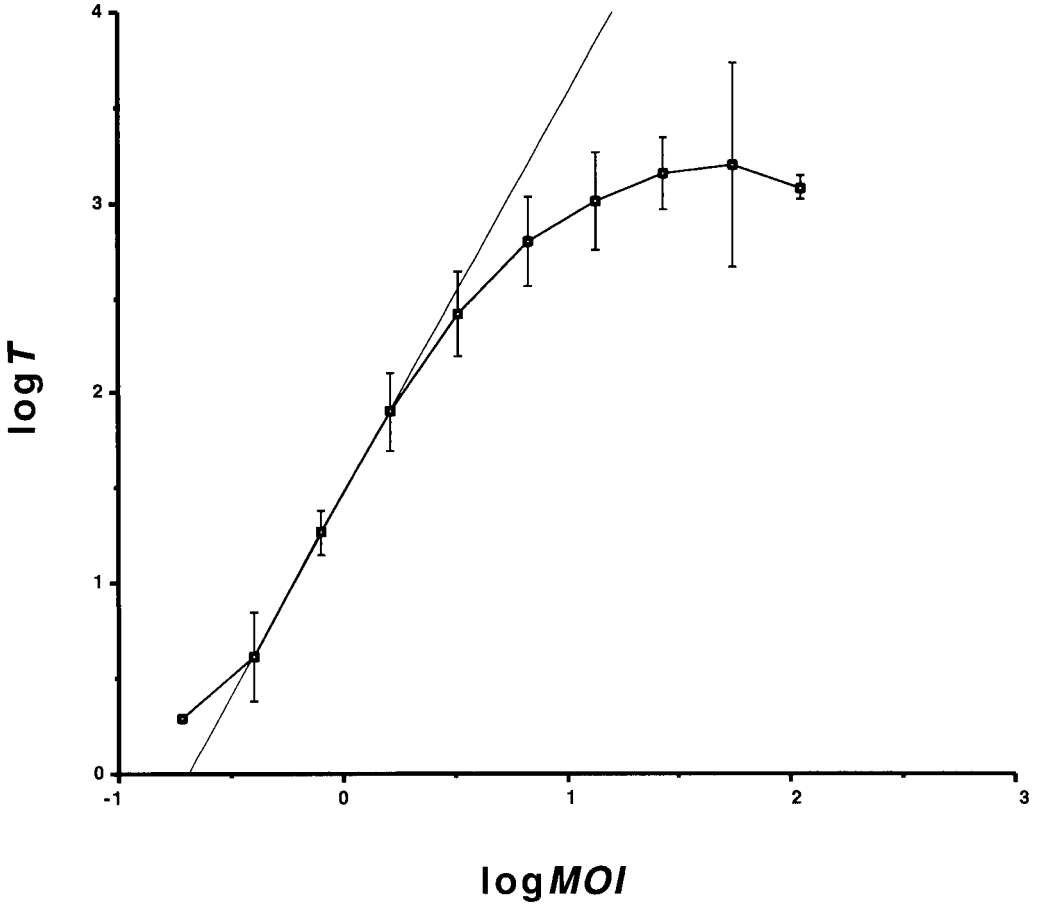

FIGURE 2.—Effect of the multiplicity of infection on the formation of directed duplications. MOI is the multiplicity of infection, calculated from counts of plaque-forming units. *T* is the number of prototrophic Cm^r transductants. Results are averages from four independent experiments; in each experiment, four assays (plates) per MOI were performed. Donors were TT15229 (*proA-692*::Mu*d*Q) and TT15232 (*purE2154*::Mu*d*Q); the recipient was LT2.

power of the number of transducing particles required (n),

$$T = kP^n,$$

where k is a proportionality constant. Plotting the logarithm of T *vs.* the logarithm of P will generate a straight line, whose slope will be the number of particles required to generate a transductant (HUGHES *et al.* 1987; MIESEL and ROTH 1996):

$$\log T = \log k + n \log P.$$

Data plotted in Figure 2 indicate that the slope is ~2. This result confirms that two transducing particles, each carrying a Mu*d*P/Q element, are required to generate a duplication. Because MOI values were calculated from plaque-forming units and P22 HT suspensions contain ~50% of transducing particles (MALOY *et al.* 1996), the actual MOI is likely higher than 2. Thus the possibility that additional infecting phage might be involved cannot be ruled out, as suggested by MIESEL and ROTH (1996) for ordinary, one-fragment transduction.

Effect of marker size on the formation of directed duplications: Because of the headful packaging mechanism of P22, all fragments transduced by P22 HT have a similar length, ~44 kb (CASJENS and HAYDEN 1988). Thus every fragment carrying a Mu*d* element will contain host sequences at one or both sides; the occurrence and length of these sequences will be constrained, among other factors, by the size of the Mu*d* element itself. If large Mu*d* elements are used (*e.g.*, Mu*d*A, 38 kb, or Mu*d*P/Mu*d*Q, 36 kb) transducing fragments containing the entire element can be expected to be rare. In contrast, small elements such as Mu*d*J (11 kb) will have more chances of being incorporated completely into the transducing particle. Transduced fragments carrying small elements will also contain flanking host DNA sequences at one of both sides, while fragments carrying large elements will likely contain flanking DNA at only one side. With these constraints in mind, one can envisage two possibilities:

1. That a limiting factor for the formation of a directed duplication is the length of the sequences required to form the flanking crossovers, as shown for ordinary, one-fragment transduction (MIESEL and ROTH 1994). If such were the case, directed duplications would be more efficiently formed by small elements.
2. That a limiting factor for the formation of a directed duplication is the length of the DNA sequence involved in the central crossover (*e.g.*, the Mu*d* element). If such were the case, directed duplications would be more efficiently formed by large elements. Furthermore, if a large size favors incomplete packag-

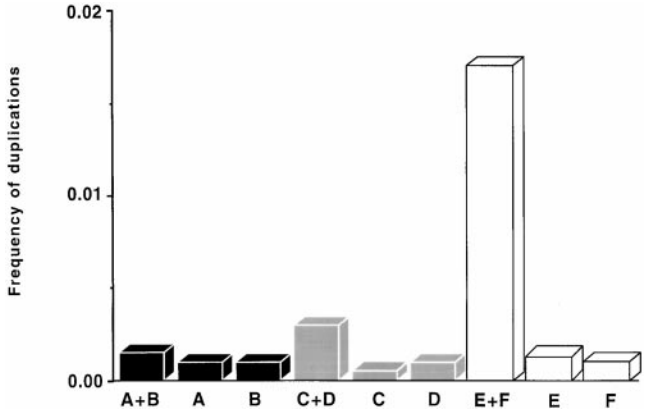

FIGURE 3.—Effect of marker size on the formation of directed duplications. The frequency of duplications is calculated as the quotient between the number of Cm^r prototrophic transductants and the number of Cm^r transductants. Because of the high numerical dispersion typical of these experiments, the median is shown instead of the average, and error bars are not included. Each median has been calculated from more than six independent experiments. P22 HT donor lysates were from the following strains: A, TT12313 (*purE2154*::Mu*d*J); B, TT12316 (*purB1879*::Mu*d*J); C, SV4018 (*purE2154*::Mu*d*J [*lacZ*::Tn*10*]); D, SV4019 (*purB1879*::Mu*d*J [*lacZ*::Tn*10*]); E, TT15235 (*purE2154*::Mu*d*Q); and F, TT15242 (*purB1879*::Mu*d*P). The recipient was LT2.

ing of Mu*d* elements, selection of Cm^r prototrophic transductants will enforce triple crossover (HUGHES *et al.* 1987).

To examine these possibilities, we compared the frequencies of duplication formation between *purE* to *purB* (centisomes 12 to 25) using markers of different sizes: Mu*d*J (11 kb), a Mu*d*J derivative enlarged with a Tn*10* insertion (11 + 10 kb = 21 kb) and Mu*d*P (36 kb). Formation of directed duplications was assessed by comparing double-lysate transductions to single-lysate transductions. The latter measures the background frequency of spontaneous duplications (see above and ANDERSON and ROTH 1981). The results, shown in Figure 3, were unambiguous: transduction with two lysates increased the frequency of Pur$^+$ Cm^r isolates in a size-dependent fashion, suggesting that triple crossover is facilitated by the use of large elements. Support for the conclusion that the element size is a limiting factor for triple crossover formation was also obtained in the *hisC–cysJ* interval: use of two Mu*d*A insertions (carried by strains TT9686 and TT9636) yielded duplications at a frequency ~17-fold higher than two Mu*d*J elements (carried by SV2090 and SV3038; data not shown). Size dependence admits two nonexclusive interpretations: (i) that a large size increases the homology length required for the central crossover and (ii) that a large size favors incomplete packaging, thus making the central crossover essential for the formation of Cm^r prototrophs.

Effect of the growth rate of the recipient culture on duplication formation: Besides the central crossover, directed duplication of a chromosomal region requires two flanking crossovers, each with a different daughter DNA duplex after passage of a replication fork (HUGHES and ROTH 1985). If this model applies to duplication formation induced by Mu*d*P/Q, the number of transductants should be a function of the number of replication forks present in the cell. In turn, the number of replication forks is a function of growth rate (SCHAECHTER *et al.* 1958). On these grounds, we compared the frequencies of duplication formation in exponential and stationary cultures. We chose a region in which the frequency of spontaneous duplications is low (CS 7-12) and another region in which spontaneous duplications are common (CS 72-83). To measure the background level of spontaneous duplications, transductions with single lysates were performed. The exponential cultures used had optical densities (OD_{560}) of 0.5–0.8 and were concentrated 10-fold before being mixed with the phage suspension. The stationary cultures had optical densities of 2. The conclusion from these experiments, summarized in Figure 4, is that both spontaneous and directed duplications occur more frequently in exponential cultures. Thus the formation of both duplication types may require a replicating chromosome.

Effect of marker orientation on the formation of directed duplications: A critical factor for duplication formation by Mu*d*A elements is their orientation; in fact, duplication formation by two Mu*d*A elements can be used to ascertain the direction of transcription of a gene or operon (HUGHES and ROTH 1985). An appropriate orientation is also required for duplication formation by Mu*d*P/Q elements. As shown in Table 4, double-lysate transductions with two Mu*d*P or two Mu*d*Q elements in opposite orientation failed to increase the frequency of Cm^r prototrophic transductants over those obtained with single lysates. However, the symmetrical nature of the Mu*d*P and Mu*d*Q constructs (YOUDERIAN *et al.* 1988; BENSON and GOLDMAN 1992) permits a more versatile use of these elements in duplication formation. A given Mu*d*P element can recombine either with a Mu*d*P element in the same orientation or with a Mu*d*Q element in the opposite orientation (Table 4). Recombination between a Mu*d*P and a Mu*d*Q element can be expected to generate hybrid elements of either type, whose ends will be both MuL or both MuR. These rearrangements are irrelevant for the formation of the duplication and for the stability and segregation capacity of the resulting merodiploid.

Construction of a collection of Mu*d*P/Q-held duplications covering the Salmonella chromosome: Using the procedures described above, 11 strains carrying duplications with known endpoints were constructed (Figure 5). Seven of these strains carry duplications held by a Mu*d*P element; 4 carry duplications held by Mu*d*Q. The collection covers all the Salmonella chromosome except

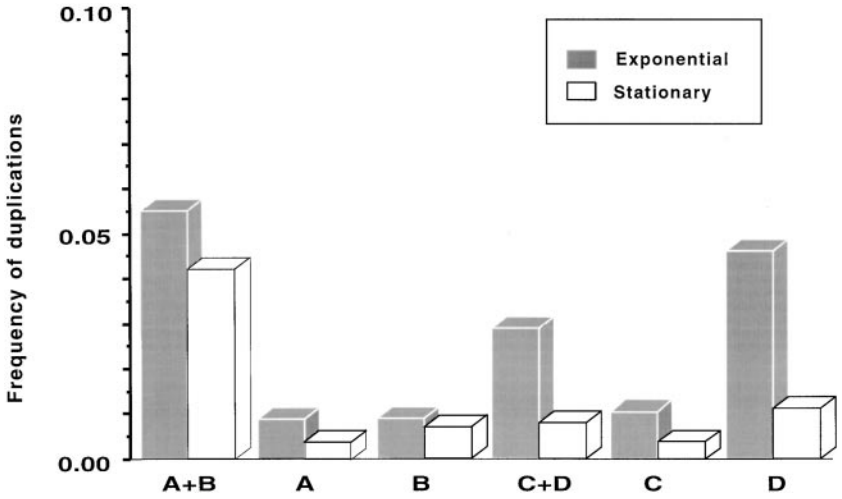

FIGURE 4.—Effect of growth rate on the formation of directed duplications. The median of more than six independent experiments is shown (see the legend of Figure 3). Donors were the following: A, TT15229 (*proA692*::Mu*d*Q); B, TT15232 (*purE2154*::Mu*d*Q); C, TT15264 (*cysG1573*::Mu*d*P); and D, TT15269 (*ilvA2648*::Mu*d*P). The recipient was LT2.

the *purB–trp* region (CS 27-38). The longest duplication (*purE–purB*) is 15 centisomes long; the shortest are *cysA–purG* (3 centisomes) and *proA–purE* (4 centisomes). The remaining duplications are all 7–10 centisomes long. All the duplication-carrying strains are stable if maintained in chloramphenicol-containing media. Long-term preservation can be carried out in frozen stocks, and massive segregation during recovery is not observed. An alternative preservation procedure is the storage of a P22 lysate grown on the merodiploid strain; high-titer lysates sterilized with chlorophorm are able to transduce the duplication joint point (and thus to reconstruct the duplication) after prolonged storage at 4°. For reconstruction, the original duplication-bearing strain is used as a donor in a transductional cross mediated by P22 HT, selecting Cm^r transductants in minimal medium (HILL *et al.* 1969; ANDERSON *et al.* 1976).

Duplications held by Mu*d*P/Q elements also could be reconstructed in genetic backgrounds other than LT2, such as the standard pathogenic strain *S. typhimurium* SL1344, and strains belonging to other Salmonella serovars of medical or veterinary importance, such as *S. dublin* 3246, *S. abortusovis* SAO44, and *S. gallinarum* G9/4223. Cm^r prototrophic transductants were obtained for all recipients tested, and the segregation patterns of individual transductants confirmed that all carried a directed duplication (data not shown). DNA fragments transduced by phage P22 undergo massive degradation at their ends before integration (EBEL-TSIPIS *et al.* 1972; MIESEL and ROTH 1994). Thus transduction of a duplication joint point to a genetically different host can be expected to introduce a small amount of heterologous DNA in the recipient chromosome aside from the Mu*d*P/Q element. A potential application of these constructions is the genetic analysis of chromosomal regions that are highly polymorphic among Salmonella serovars (CONNER *et al.* 1998).

Mapping by duplication segregation: Mapping is carried out in three steps. First, the marker to be mapped is transferred by P22 HT transduction to all members of the 11-strain collection. One or more isolates from each cross are then allowed to segregate. Finally, segreg-

TABLE 4

Effect of the orientation of Mu*d*P and Mu*d*Q elements on duplication formation

Donor	Transduced marker	Packaging direction[a]	Frequency of duplications[b]
TT15224	*thr-469*::Mu*d*Q	CCW	4×10^{-3}
TT15229	*proA692*::Mu*d*Q	CW	1×10^{-3}
TT15231	*proA692*::Mu*d*P	CCW	1×10^{-3}
TT15224 + TT15229			2×10^{-3}
TT15224 + TT15231			180×10^{-3}
TT15132	*purE2154*::Mu*d*Q	CW	1.7×10^{-3}
TT15235	*purE2154*::Mu*d*P	CCW	1.4×10^{-3}
TT15242	*purB1879*::Mu*d*P	CCW	1.2×10^{-3}
TT15232 + TT15242			1.2×10^{-3}
TT15235 + TT15242			215×10^{-3}

[a] CW, clockwise; CCW, counterclockwise.
[b] Number of prototrophic Cm^r transductants divided by the total number of Cm^r transductants.

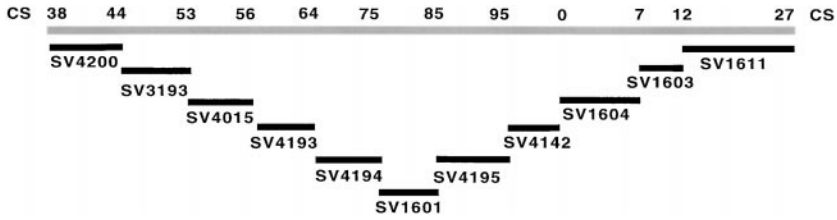

FIGURE 5.—A diagram of the 11-duplication set that covers most of the Salmonella chromosome (not drawn to scale). The endpoints of each duplication are indicated by the number of the corresponding centisomes (CS).

ants are scored for the presence of the marker to be mapped. The interpretation of the results is straighforward: if all the Cm^s segregants contain the transduced marker, it maps outside the duplication. If the marker is found only in a fraction of the segregants, it maps within the duplicated region (Figure 1).

In the example shown in Table 5, the insertion *ara-651*::Tn*10* was transduced into the 11 duplication-carrying strains, selecting Tc^r transductants on NA with tetracycline and chloramphenicol. Three individual Tc^r Cm^r transductants from each cross were grown to full density in NB to permit segregation. The cultures were then diluted and spread on NA plates to obtain single colonies. One hundred colonies from each culture were patched to the following media: (i) NA with chloramphenicol and tetracycline, to detect nonsegregating Cm^r Tc^r colonies; (ii) NA with tetracycline, to detect the presence of segregants (which are Cm^s); and (iii) NA, to detect Cm^s Tet^s segregants (in which the duplication and the Tn*10* element have cosegregated). Cm^s Tc^s segregants were found only in isolates derived from SV1604, indicating that the mutation *ara-651*::Tn*10* maps in the *thr–proA* interval between centisomes 0 and 7. This mapping location is correct, since the *araBAD* operon maps at CS 2.5 (SANDERSON *et al.* 1995). Strains SV1603 and SV4142, whose duplications lie at each side of the duplication of SV1604, have been included in Table 5 as representatives of the 11 strains that do not yield Cm^s Tc^s segregants.

In duplications affecting certain chromosomal regions, segregation is slow even in the absence of selection (see Table 3). In such cases, the number of Cm^r segregants found upon nonselective growth can be insufficient to score cosegregation of the transduced marker. To solve this problem, longer segregation periods can be allowed (*e.g.*, during 40–60 generations, which can be achieved by two or more serial cultures in NB). Slow segregation is the only potential complication of the method, which is otherwise unambiguous.

DISCUSSION

Genetic mapping in Salmonella can be performed by several procedures: cotransduction of nearby markers by high-transducing derivatives of phage P22 (SCHMIEGER 1972), conjugal transfer of DNA via Hfr formation (DEMEREC *et al.* 1955), and packaging of discrete chromosomal stretches upon induction of locked-in Mud-P22 prophages (BENSON and GOLDMAN 1992). Cotransduction with P22 HT is probably the easiest and most reliable mapping method ever described (MALOY *et al.* 1996). However, it can be applied only to closely linked markers. For large-scale mapping, mobilization of the Salmonella chromosome by the *Escherichia coli* F sex factor has been used (SANDERSON and MACLACHLAN 1987). F can be transferred to Salmonella, and Hfr strains can be obtained upon F integration into the chromosome (SANDERSON and MACLACHLAN 1987).

TABLE 5

Mapping of the mutation *ara-651*::Tn*10* by duplication segregation

Recipient	Isolate	Nonsegregants (Cm^r Tc^r)[a]	Cm^s Tc^r segregants[a]	Cm^s Tc^s segregants[a]
SV1603[b]	I	90	10	0
	II	75	25	0
	III	77	23	0
SV1604	I	83	3	14
	II	67	14	19
	III	59	27	14
SV4142[b]	I	63	37	0
	II	79	21	0
	III	71	29	0

[a] Among 100 patches.
[b] Included as representatives of strains in which the *ara-651*::Tn*10* mutation does not cosegregate with the duplication.

However, the efficiency of Hfr formation in Salmonella is reduced; one cause may be the absence of insertion elements IS*2* and IS*3* from the chromosome of *S. typhimurium* LT2 (Lam and Roth 1983; Mahillon and Chandler 1998). The development of transposon technology introduced a number of refinements for Hfr formation in Salmonella, such as the use of transposable elements as portable regions of homology to direct F integration (Kleckner *et al.* 1977; Chumley *et al.* 1979). However, Hfr mapping in Salmonella can still encounter problems derived from the instability of the Hfr donors.

A mapping procedure that can efficiently substitute for Hfr transfer in *S. typhimurium* was developed by Benson and Goldman (1992). The method employs Mu*d*P and Mu*d*Q, two hybrids between Salmonella phage P22 and coliphage Mu (Youderian *et al.* 1988). Mu*d*P and Mu*d*Q are inducible by DNA-damaging treatments. Upon induction, the entire prophage genome is replicated *in situ* but cannot excise. As a consequence, replication forks initiated at the P22 replication origin invade neighboring host DNA, causing a selective amplification of the chromosomal regions that flank the prophage (Youderian *et al.* 1988; Benson and Goldman 1992). Replicated regions are then packaged into P22 capsids by the "headful" mechanism characteristic of phage P22 (Casjens and Hayden 1988). The first headful will package a portion of the prophage and some adjacent host DNA; further headfuls will consist of chromosomal DNA only (Benson and Goldman 1992). The packaging direction depends on the orientation of the prophage itself. The Mu*d*P and Mu*d*Q constructs are identical except that each carries the central, P22-derived region in opposite orientation (Youderian *et al.* 1988). As a consequence, Mu*d*P will package in one direction and Mu*d*Q in the other when inserted at the same locus (Benson and Goldman 1992). Use of a set of Mu*d*P and Mu*d*Q lysogens scattered along the Salmonella chromosome permits mapping with a resolution of ~5 min, provided that a selection procedure is available (Benson and Goldman 1992). Bidirectional packaging of markers flanking a Mu*d*P/Q prophage is one of the few drawbacks of this technique (Benson and Goldman 1992; Flores and Casadesús 1995).

Mapping by duplication segregation, the procedure described in this study, was envisaged long ago (Anderson and Roth 1977) but has remained unexploited. The procedure is based on the rationale that a marker introduced into a preexisting duplication will segregate together with the duplication, while a marker outside the duplication will not (Figure 1). To assay this mapping procedure in Salmonella, we constructed a collection of duplications held by Mu*d*P/Q elements. Duplication construction was achieved by adapting the elegant method of Hughes and Roth (1985), originally devised for Mu*d*A elements, to Mu*d*P and Mu*d*Q. The choice of these elements over other transposons (*e.g.*, Mu*d*A) was based on the following criteria: (i) Mu*d*P and Mu*d*Q have a large size, which is a desirable trait to construct directed duplications (Figure 3); (ii) large collections of Mu*d*P and Mu*d*Q inserts exist (Benson and Goldman 1992; N. R. Benson, unpublished data); (iii) because Mu*d*P and Mu*d*Q carry a chloramphenicol resistance marker, duplications held by these elements can be used to map mutations tagged with tetracycline, ampicillin, and kanamycin resistance markers; and (iv) for the selection of rare recombinants, chloramphenicol resistance is a highly reliable marker, because it does not involve the production of extracellular enzymes.

The extensive homology between Mu*d*-P22 elements and P22 DNA permits recombination with superinfecting phage during lytic growth and/or transduction. Thus, isolates carrying transduced Mu*d*P/Q elements should not be used for induction of locked-in prophages. An additional reason to discourage induction of such lysogens is the presence of the mitomycin-inducible Fels prophage in the LT2 background. Last, it seems also advisable that the P22 HT lysates used for such transductions are not further propagated, because they might contain P22 HT recombinant derivatives. Aside from these caveats, transduction of Mu*d*P/Q elements by P22 HT appears to be a safe procedure, at least for operations like those described below, in which Mu*d*P/Q elements are used simply to provide homologies for recombination.

Duplication formation induced by Mu*d*P/Q elements followed relatively simple rules. One was the need to co-infect the recipient culture with two P22 HT lysates, each at a MOI of >2. This condition seems to reflect the need of two transduced fragments (Figure 2), as previously proposed for Mu*d*A elements (Hughes and Roth 1985; Hughes *et al.* 1987). Use of an exponentially growing culture increased the overall yield of duplications, both spontaneous and induced, suggesting that formation of both types requires a replication fork (Figure 4). The populations of Cm^r prototrophic transductants were always heterogeneous and contained both spontaneous and induced duplications (Table 3). However, each type could be easily distinguished by its segregation pattern (Anderson and Roth 1977, 1979; Hughes and Roth 1985; Roth *et al.* 1996). Directed duplications could be formed by two Mu*d*P or Mu*d*Q elements in the same orientation or by a Mu*d*P and a Mu*d*Q element in opposite orientation (Table 4). This orientation dependence for successful crossover confirmed that duplication formation was actually directed by the Mu*d*P/Q elements involved in the cross.

Each of the 11 strains shown in Figure 5 is a merodiploid carrying a duplication held by a Mu*d*P/Q element. If maintained under selection, these strains are stable; however, segregation can be easily achieved by growth under conditions that do not select the duplication. Segregation occurs fast in all strains but SV4195. For mapping, a dominant marker (*e.g.*, a transposon

insertion) is transferred by P22 HT transduction into the 11-merodiploid set. The resulting isolates contain the duplication and the marker to be mapped. These isolates are then grown nonselectively (*e.g.*, in NB) to permit segregation, and haploid segregants are scored for the presence of the insertion mutation. If all the segregants contain the insertion, it maps outside the duplication interval. If the insertion is found only in a fraction of the segregants, it maps within the duplicated region (Figure 1).

Aside from mapping, chromosomal duplications with known endpoints can be used for other operations of genetic analysis. For instance, the introduction of a mutation into one copy of a chromosomal duplication permits complementation analysis: the phenotype of a dominant mutation will still be observed in the merodiploid, while that of recessive mutation will require the formation of haploid segregants. An advantage of this complementation procedure (*e.g.*, over complementation with plasmids) is that it does not alter gene dosage. Duplications can also be useful to ascertain whether a mutation is lethal. For this purpose, a plasmid-borne allele can be recombined into one copy of a duplication; the segregation pattern of the resulting heterozygote will indicate whether haploid segregants carrying the mutation are viable. Another potential application of duplications is the analysis of gene fusions, especially for the study of genes that regulate their own transcription. In such cases, use of a duplication permits monitoring of gene expression in the presence of the normal dosage of the wild-type allele.

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