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Gene Replacement and Retrieval with Recombinant M13mp Bacteriophages

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We have developed an allele exchange system for shuttling sequences of DNA to and from their original chromosomal loci. Cloned segments of the histidine operon of *Salmonella typhimurium* and the lactose operon of *Escherichia coli* served as target sequences and were used to develop the system. Replacement and retrieval of target sequences used the phage M13mp vectors and proceeded through an M13 lysogen intermediate. The intermediates and products of allele exchange were characterized by genetic and hybridization analyses. Several unique properties of M13 lysogens were exploited to devise positive selections to detect integration and excision. These positive selections were used to manipulate phenotypically silent alleles.

Recent innovations in recombinant DNA technology have greatly facilitated the ability to modify DNA sequences in vitro (7). The biological consequences of these mutations can be studied in vitro in cell-free systems or in vivo in strains carrying plasmid or viral vectors containing the modified DNA. Both of these approaches have potential problems. The experimental conditions in a cell-free system may not accurately reflect the situation in vivo. Analysis of function of cloned genes in vivo can be complicated by gene dosage effects (multicopy plasmids) and alterations of the natural chromosomal environment (flanking DNA sequence context, DNA supercoiling and higher-order DNA conformations, etc.). To circumvent these problems, it has been important to develop allele replacement and retrieval methods for analysis of DNA sequence alterations in genes at their normal chromosomal locations.

Methods for allele replacement in *Escherichia coli* have been reported. Plasmids carrying the ColE1 origin of replication fail to replicate in *polA* mutant strains (16), and antibiotic resistance conferred by a plasmid gene can be used to select and maintain the integrated plasmid following transformation (8, 11, 37). Double-stranded linear DNA fragments containing an antibiotic resistance gene can be stably transformed into *recB recC sbcB* mutants, and chromosomal recombinants can be selected (36). In this paper, we describe allele replacement and retrieval methods using the phage M13mp vector system.

The phage M13mp vector system (22, 38) has found wide application in recombinant DNA technology. Large quantities of single- and double-stranded DNA are easily produced and can be used for dideoxy DNA sequencing, site-directed mutagenesis in vitro, hybridization analysis, and shotgun cloning. Several of the M13mp phages carry nonsense mutations in phage genes I and II. These phages replicate only in tRNA suppressor host cells. Infection of cells lacking a tRNA suppressor prevents autonomous replication of the phage and provides the basis of our allele replacement and retrieval methods (2). In the course of studies on strains carrying integrated M13mp prophages, we observed several phage-conferred phenotypes. Manipulation of these phenotypes has allowed us to extend the use of the phage M13mp vector system to facilitate replacement and retrieval of any target sequence, including phenotypically silent and unselectable alleles.

MATERIALS AND METHODS

Bacterial strains, phages, media, and chemicals. The genotypes and sources of bacterial strains and phages are described in Table 1. Phage R17 lysates were prepared (23) by using strain KLF23. M13 phage lysates were prepared and sterilized as described elsewhere (22). Complex media were either LB medium, YT medium (23), or nutrient broth (NB; Difco Laboratories) as indicated, and minimal liquid medium was E medium (34) containing 0.4% (wt/vol) glucose and 50 μg of amino acids per ml as needed. All solid media contained 1.5% (wt/vol) agar. Solid media containing 2% (wt/vol) glucose were used for the identification of smooth or wrinkled cell colonies. Phage indicator plates (32), modified as described previously (9), were used as an aid to score the M13 lysogen phenotype in *Salmonella typhimurium*, and MacConkey base plates (Difco) were used to select segregants of *S. typhimurium* and *E. coli* M13 lysogens. As an alternative to MacConkey base plates, segregants were selected on nutrient agar plates (NA; Difco) containing sodium deoxycholate (0.15 to 0.3%, wt/vol). These plates were prepared by the addition of autoclaved solutions of sodium deoxycholate in 150 ml of water to autoclaved and cooled solutions of NA (23 g/liter) plus NaCl (5 g/liter) in 850 ml of water. For purifying M13 lysogens away from R17 phage, NA plus EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (20 mM) was used; the EGTA was added after the medium was autoclaved. Top agar contained 0.6% (wt/vol) agar and 0.5% NaCl. Colicin E1 (Sigma Chemical Co.) was hydrated in 100 mM phosphate buffer (pH 7.0) and kept at 4°C for up to 1 month prior to use.

Construction of F′123 *trp*$^+$ *finP301*. Efficient M13 plaquing in *S. typhimurium* required use of the F factor *finP* mutation isolated in F′42 *lac* (10). The *finP* mutation derepresses F factor functions and overcomes F factor repression by the coresident cryptic plasmid of *S. typhimurium* (28). To avoid

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TABLE 1. Bacterial strains and phages

Strain or phage	Genotype	Comments and source
S. typhimurium		
AZ193	Δ*hisGa1242 hisG2101 zee-1256*::Tn*10 hsdSA12 hsdLT30 proC90 rpsL* (F'42 *finP301 lac*$^+$)	(2)
AZ1457	Δ*hisGa1242 hisD3337 zee-1256*::Tn*10 hsdSA12 hsdLT30 proC90 supE rpsL* (F'42 *finP301 lac*$^+$)	(2)
AZ1516	*trp*::Tn*5* (F'123 *finP301 trp*$^+$)	See Materials and Methods
AZ1524	*trp*::Tn*5 hsdSA12 hsdLT30 proC90 rpsL* (F'123 *finP301 trp*$^+$)	This work
AZ1549	Δ*hisGD trp*::Tn*5 hsdSA12 hsdLT30 proC90 rpsL* (F'123 *finP301 trp*$^+$)	Isogenic with strain AZ1524 but lacking a 1.5-kbp *Hin*cII fragment spanning the C-terminal end of *hisG* and the N-terminal end of *hisD*; constructed by allele replacement with M13mp9:*his*4 donor (see Results)
E. coli K12		
TA4282	(Δ*lac pro*) *thi rpsL hisG213*::Tn*10* (F' Δ*lacZM15 proAB*$^+$)	
TA4283	*trpC22*::Tn*10* Δ*lacZM15 thi rpsL* (F'123 *finP*$^+$ *trp*$^+$)	
KLF23	*trp-1 pyrD34 thi-1 his-1 recA1 mtl-1 xyl-1 malA1 gal-6 rpsL118* (F'123 *finP*$^+$ *trp*$^+$)	
Phages		
M13mp9		Carries UAG mutations in genes I and II (22)
M13mp9::*his*1	*hisGa*$^+$ *hisG*$^+$ *hisD*$^+$	(2)
M13mp9::*his*2	Δ*hisGa1242 hisG*$^+$ *hisD*$^+$	(2)
M13mp9::*his*3		Isogenic with M13mp9::*his*2 but lacking a *Sal*I site in the insert (2)
M13mp9::*his*4		Isogenic with M13mp9::*his*3 but lacking a 1.5-kbp *Hin*cII fragment spanning the C-terminal end of *hisG* and the N-terminal end of *hisD* (2)
M13*am* 4-H38		Carries a UAG mutation in phage gene IV but is otherwise wild type (26)

undesirable and potentially misleading homologous recombination between the F'42 *lac*$^+$ genes and the *lac* fragment in the M13mp phages, the *finP301* mutation was recombined onto F'123 *trp*$^+$, which does not carry the *lac* operon. This was accomplished as follows. Strain AZ1510 (F'42 *lac*$^+$ *zzf-20*::Tn*10 finP301*) was mated with strain AZ1514 (*trp*::Tn*5* [F'123 *trp*$^+$]). Both strains were grown in 2 ml of NB at 37°C to stationary phase. AZ1514 was then washed, suspended, and incubated under starvation conditions for 4 h at 37°C with aeration to enrich for F$^-$ phenocopies (23), while AZ1510 was subcultured in NB to obtain log-phase donor cells. Samples (0.5 ml) of each culture were mixed and gently agitated for 40 min at 37°C. Dilutions were spread on E glucose plates containing 5 μg of tetracycline per ml and 75 μg of kanamycin per ml and were incubated at 37°C for 24 h; a 100-fold dilution gave approximately 200 colonies per plate. (These medium conditions select for maintenance of the two F' factors in the strain AZ1514 background and select against growth of strain AZ1510. Incompatibility between the two F' factors necessitates their recombination for retention of the Trp$^+$ and tetracycline resistance [Tet^r] phenotypes). These plates were then replica plated to MacConkey lactose plates (to score the Lac phenotype) and to phage indicator plates seeded with 10^9 PFU of phage R17 (to score the FinP phenotype); incubation was at 37°C for 24 h. Both types of replica plates contained tetracycline and kanamycin at the concentrations used in the initial selection. Fifty percent of the colonies (100 of 200) were Lac$^-$ FinP$^+$ (white on MacConkey lactose plates and resistant to phage R17). Forty-nine percent (98 of 200) were Lac$^+$ FinP$^-$ (red on MacConkey lactose plates and sensitive to phage R17). One percent (2 of 200) had the desired Lac$^-$ FinP$^-$ phenotype (white on MacConkey lactose plates and sensitive to phage R17); these were purified once on NA plates, and their phenotypes were confirmed. Both purified isolates were Trp$^+$ Tet^r Kan^r FinP$^-$ Lac$^-$. Upon subsequent transfers of the F'123 *trp*$^+$ *finP301 zzf-20*::Tn*10* episomes, derivatives were isolated that had lost tetracycline resistance, presumably by spontaneous deletion of the Tn*10*. One of these isolates, strain AZ1516 (Table 1), was used as the source of F'123 *trp*$^+$ *finP301* for experiments involving allele replacement.

Chromosomal DNA preparation. *S. typhimurium* chromosomal DNA was prepared as follows. Cells were grown in 50 ml of LB medium to an optical density at 650 nm of approximately 0.5, harvested by centrifugation, washed in 50 ml of J buffer (0.1 M Tris hydrochloride [pH 8.0]–0.1 M EDTA–0.15 M NaCl), pelleted, and suspended in 0.8 ml of J buffer. A fresh solution of lysozyme (5 mg/ml in 0.05 M Tris hydrochloride [pH 8.0]–0.01 M EDTA) was added to a final concentration of 1 mg/ml. After incubation at 37°C for 10 min, RNase A (10 mg/ml in 10 μM sodium acetate [pH 5.1]; boiled for 2 min) was added to a final concentration of 0.1 mg/ml, and incubation was continued at 37°C for 10 min followed by heating to 70°C for 3 min. The lysis solution was adjusted to 2% Sarkosyl and was incubated at 70°C for 20 min and then at 37°C for 1 h. Pronase was added to 2 mg/ml, and the samples were held at 37°C for 2 h. The samples were then centrifuged to equilibrium in a CsCl gradient (1.7 g/ml) with a VTi50 vertical rotor at 45,000 rpm for 18 h. Chromosomal DNA was extracted with butanol, was dialyzed against low-Tris buffer (0.02 M Tris hydrochloride [pH 7.5]–0.02 M NaCl–0.001 M EDTA) for 2 days, was extracted with

TABLE 2. Comparison of M13mp phage lysogen and nonlysogen phenotypes

M13mp phage	Phenotype						
	Generation time[a] (min)	Phage R17 sensitivity[b]	Phage M13IV*am* propagation[b]	Bile salt sensitivity[c]	Unstable duplicated sequence[d]	*supE*-dependent production of M13mp phage[e]	Colicin E1 sensitivity[b,f]
Lysogen	48	Resistant	Yes	Sensitive	Yes	Yes	Resistant
Nonlysogen	37	Sensitive	No	Resistant	No	No	Sensitive

[a] Cultures of strain AZ1524 and an M13mp9::*his*1 lysogen of strain AZ1524 were grown in NB at 37°C and were monitored spectrophotometrically (optical density at 650 nm).

[b] See Materials and Methods.

[c] A culture of an M13mp9::*his*1 lysogen of strain AZ1524 growing in NB at 37°C was subcultured 1:6 at mid-log phase into prewarmed MacConkey broth (Difco) which contained 0.5% oxgall bile or into NB and was allowed to reach late log phase. Samples were diluted in 0.9% NaCl (wt/vol), spread onto NA plates for single colonies, and incubated for 48 h at 37°C. The NA plates were replicated to phage indicator plates containing 5 mM $CaCl_2$ and spread with 10^9 phage R17. After 48 h of incubation at 37°C, the number of lysogens (R17-resistant colonies) was counted and expressed as percentage of total viable cells (determined as colonies on NA plates). Of the cells in the NB culture, 99% (134 of 136) were M13 lysogens, whereas only 2% (2 of 112) of the cells in the MacConkey broth culture were lysogens.

[d] See Fig. 1 and 2.

[e] An M13mp9::*his*4 lysogen was transduced to tetracycline resistance by using a phage P22 lysate of strain TT2342 (*supE zbf-98*::Tn*10*). The *zbf-98*::Tn*10* is 74% linked to *supE*, and a comparable percentage of the tetracycline-resistant colonies produced M13mp9::*his* phage. This was assayed by ability of the purified transductants to produce PFU and by the ability of the phage produced to transduce strain AZ193 to His^+ at high frequency.

[f] Applies to *E. coli* but not *S. typhimurium* strains, which are naturally resistant to colicin E1.

phenol, was precipitated with ethanol, and was dissolved in low-Tris buffer at a final concentration of 1 mg/ml.

For hybridization analyses (33), DNA samples were digested with the appropriate restriction enzymes, were electrophoresed in a 0.8% agarose gel, and were transferred to Zeta-probe paper with a Bio-Rad Trans-blot system by using the protocol suggested by the manufacturer. Radioactive probes were prepared as described elsewhere (14) by using approximately 0.5 μg of M13mp9::*his*2 single-stranded DNA as a template and a hybridization primer purchased from New England BioLabs, Inc. Prior to hybridization, the reaction mixtures were chromatographed on a P60 column (Bio-Rad) to separate the probe from [α-^{32}P]dATP. The hybridization reactions were done as described previously (14), except that prehybridization was done for 6 to 8 h. The hybridized DNA was visualized by autoradiography with Kodak XAR-5 film.

Selection of *S. typhimurium* M13mp phage lysogens. Prior to infection, host strains were grown in a medium selective for retention of the F factor or were inoculated from selective medium and grown under nonselective conditions (NB medium) to mid-log phase (optical density at 650 nm ≈ 0.5 ≈ 5 × 10^8 cells per ml) at 37°C. Samples (100 μl) of cells diluted 10^2, 10^3, or 10^4 in 0.9% (wt/vol) NaCl were mixed with 10 μl of recombinant M13mp phage lysates at multiplicities of infection of 500 or greater and were incubated for 60 min at 37°C to allow phage adsorption. The infected cells were spread onto phage indicator plates containing 5 mM $CaCl_2$ and approximately 10^9 PFU of phage R17 (by spreading about 30 μl of an undiluted phage R17 lysate). Controls included plates spread with cells uninfected with M13mp phage and plates lacking phage R17. Plates were incubated at 37°C for 48 h, and well-isolated colonies were examined for the presence of M13mp phage lysogens. Selection for phage R17 resistance on phage indicator plates produced colonies of two distinct types: M13mp phage lysogenic colonies were dark green, and colonies of F^- segregants were pale green. Lysogens were picked and were purified twice on NA plates containing 20 mM EGTA to chelate Ca^{2+} and prevent subsequent infection by phage R17.

Selection of *E. coli* M13mp9 phage lysogens. The procedure for growth and infection of *E. coli* strains was identical to that for *S. typhimurium*, but the selection for M13mp phage lysogens differed. Dilutions of infected cells (100 μl) were spread on LB plates spread previously with 80 U of colicin E1, and the plates were incubated at 37°C for 48 h. Alternatively, infected cells and colicin E1 (10 U) were mixed in 2.5 ml of molten top agar and were poured onto LB plates. (It is recommended that a range of colicin E1 concentrations be tested to optimize the selection with different strains.) Well-isolated colicin-resistant colonies were picked and purified once on LB plates spread with colicin E1 to eliminate false-positive isolates. Spontaneous colicin E1-resistant mutants (*tolB* and *tolC*) occurred at low frequencies. Mutants of *tolC* are particularly sensitive to methylene blue (24) and were discarded on the basis of this phenotype. Unlike *tol* mutants, M13mp phage lysogens were phage R17 resistant (Table 2; see below).

Confirmation of the phage M13mp lysogen phenotype in *S. typhimurium* and *E. coli*. The lysogen phenotype was confirmed by spot testing for resistance to phage R17 killing. Putative lysogens were patched (50 per plate) on NA plates containing 5 mM $CaCl_2$. The patches were spotted with 5-μl volumes of phage R17 (10^{11} PFU/ml) and were incubated at 37°C for 4 to 6 h or until clearing due to lysis became evident. Nonlysogens were included as phage R17-sensitive controls. Alternatively, M13 lysogens were identified by their ability to propagate a superinfecting M13 gene IV_{UAG} mutant (M13 *am* 4-H38). The putative lysogen was inoculated into YT medium (1 ml) and grown at 37°C until slightly turbid. Phage M13 *am* 4-H38 (100 PFU) in 10 μl was added to the culture, and growth was continued to stationary phase. Cells were removed by centrifugation at 10,000 × *g* for 5 min, the supernatant was sterilized by heating at 70°C for 20 min, and the phage titer was determined on top-agar overlays of strain AZ1457 on YT plates. After 12 h of incubation at 37°C, M13mp phage lysogens were identified as having produced M13 *am* 4-H38 lysates with at least a 10-fold increase over the input titer.

Segregation of M13mp phage lysogens in *S. typhimurium* and *E. coli*. M13mp phage lysogens have enhanced sensitivity to bile salts, and media containing these salts were used to select for segregants that had lost the prophage (Table 2). Lysogens were streaked twice successively for single colonies on bile salt plates and were incubated at 37°C for 48 h. Lysogens of *S. typhimurium* LT2 and LT7 were differentially sensitive to bile salts; MacConkey base plates containing 0.15% bile salts were used for LT7 (and *E. coli*), and NA plates containing 0.3% sodium deoxycholate were used for LT2. Concentrations of sodium deoxycholate greater than

0.5% strongly inhibited growth of *S. typhimurium* strains. Nonlysogens were included as bile salt-resistant controls. Loss of the prophage was confirmed by the phage R17 spot test.

Retrieval of chromosomal mutations. Bacterial strains carrying the chromosomal *his* mutations of interest were lysogenized with an M13mp phage carrying a wild-type allele of the target sequence by using the procedure for construction of M13mp lysogens. Cultures (50 ml) of the M13mp phage lysogens were grown in LB medium at 37°C with shaking and were harvested during mid-log phase (optical density at 650 nm, 0.5). Chromosomal DNA was isolated as described above through the pronase treatment. The sample was treated with pronase a second time and was dialyzed against 1 liter of low-Tris buffer overnight at 37°C. The dialyzed sample was extracted four times with equal volumes of phenol-chloroform (1:1) and was then dialyzed against low-Tris buffer for 6 h at 4°C. DNA was purified by cesium chloride gradient centrifugation as described above and was then dialyzed against low-Tris buffer overnight at 4°C. After dialysis, DNA was ethanol precipitated, washed once with 70% ethanol, and dried. The DNA was dissolved in sterile water to a final concentration of 150 μg/ml, with a final yield of 15 μg/50 ml of culture.

Portions of the chromosomal DNA were digested to completion with *Sal*I or *Eco*RI and were then treated with phage T4 DNA ligase at 8°C overnight (19). The ligated DNAs were used to transfect competent cells (12) of the *supE* mutant strain JM107 (22). Transfection frequencies ranged between 14 and 56 plaques per 100 ng of DNA.

RESULTS

A general scheme for recombination between homologous plasmid vector and host chromosomal sequences is shown in Fig. 1. The vector carries a chromosomal fragment demarcated by restriction sites A and B. Aligned beneath the vector is the homologous chromosomal region which differs by the presence of a mutation at "X." A single reciprocal crossover between the chromosomal DNA and vector DNA results in a nontandem duplication of the target DNA separated by vector DNA. Integration can occur by crossover 1 or 2 with respect to the mutation in the chromosomal target sequence. Excision of the vector DNA and one copy of the target DNA occurs by the analogous single reciprocal crossovers that resulted in integration. If excision occurs by the same crossover, then the original chromosomal allele of the target sequence is restored (recombinant a); if the alternative crossover occurs, then allele replacement is accomplished (recombinant b).

Retrieval of chromosomal alleles can be accomplished by a related scheme. The vector carrying the wild-type cloned sequence is used to construct an integrated vector intermediate, again resulting in a nontandem duplication. Chromosomal DNA is isolated from this intermediate and separately digested with each of the restriction endonucleases (indicated by A and B in Fig. 1) used originally to clone the insert. Intramolecular ligation of these excised DNAs followed by transformation of the two samples separately into a suitable recipient strain generates two populations of plasmids, one containing the chromosomal copy of the target sequence and the other containing the original cloned copy.

Efficient detection of the integrated vector intermediate requires a method of preventing autonomous replication of the vector. With the M13mp series of vectors, autonomous replication is prevented by the presence of an amber mutation in gene II (2); phages M13mp7, 8, 9, 10, and 11 are available with the gene II amber mutation (22, 38). The gene II product is a site-specific DNA nickase essential for replication of the M13 viral genome (17). After infection of a sup^+ host (lacking an amber suppressor), the M13 plus-stranded DNA of the gene II mutant is converted to double-stranded replicative form (RF) DNA which cannot be replicated and which will be diluted out by cell division unless it is recombined into the bacterial chromosome. Only gene II nonsense mutations are suitable for this purpose, as nonsense mutations in other phage M13 genes result in host cell killing (26). The selections used for integration and excision and examples of allele replacement and retrieval using these selections are described in the following sections.

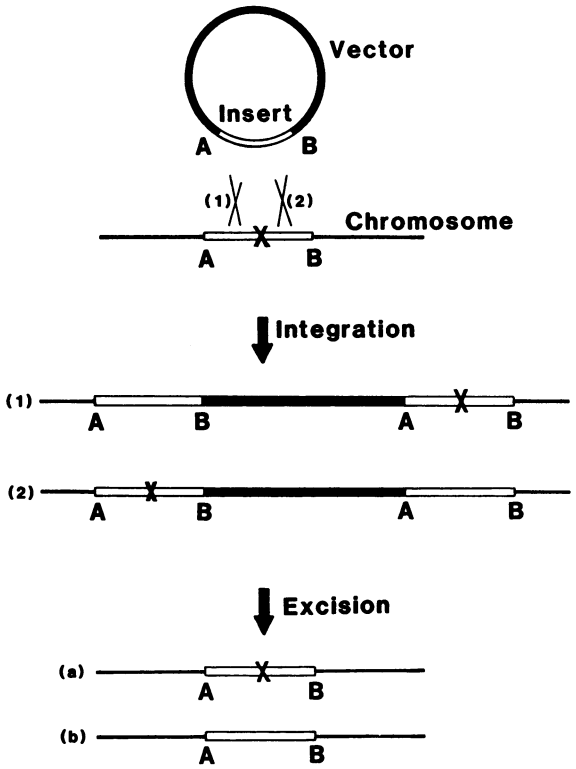

FIG. 1. A generalized scheme for allele replacement showing crossovers (1 and 2) between chromosomal and vector DNA and possible recombinants (a and b). A and B, restriction sites; X, mutation. For details, see Results.

Genetic and physical evidence for integration (lysogenization) of an M13mp::*his* phage. To determine the feasibility of using M13mp recombinant phages in allele replacement, we initially made use of well-characterized histidine operon phenotypes in *S. typhimurium*. Phage M13mp9::*his*2 was used as a donor in crosses with the *his* auxotrophic strain AZ193 (Table 1; Fig. 2). Selection on minimal medium plates gave rise to His^+ prototrophs (at a frequency depending on the conditions of infection; see below). The prototrophic colonies could not occur by extrachromosomal complementation since strain AZ193 lacks an amber suppressor and cannot support replication of phage M13mp9::*his*2. Histidine prototrophy therefore requires recombination between the phage and bacterial genomes.

Analysis of the appearance of the His^+ recombinant colonies and their segregants (Fig. 2, legend) indicated that recombination occurs according to the scheme shown in Fig.

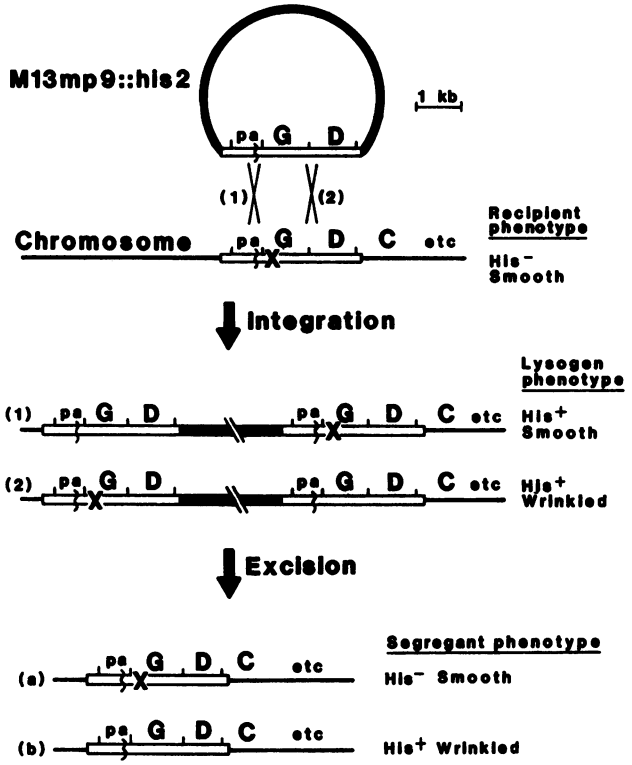

FIG. 2. Genetic evidence for integration of an M13mp::*his* phage. The donor phage M13mp9::*his*2 carries a 3.1-kbp insert containing the *his* operon regulatory region (with the Δ*hisGal242* attenuator deletion) and the intact *hisG* and *hisD* genes. The recipient strain AZ193 carries the Δ*hisGal242* mutation which results in constitutive derepression of the *his* operon. Strains that are constitutively derepressed for the *his* operon produce wrinkled colonies on high-glucose (2%) plates because of overproduction of the downstream *hisH* and *hisF* gene products (25). Strain AZ193 produced smooth colonies because of polarity caused by the *hisG2101* mutation. His$^+$ lysogens of strain AZ193 produced either smooth or wrinkled colonies, depending on the position of *hisG2101* with respect to the intact operon. p, Promoter; a, attenuator; x, *hisG2101*.

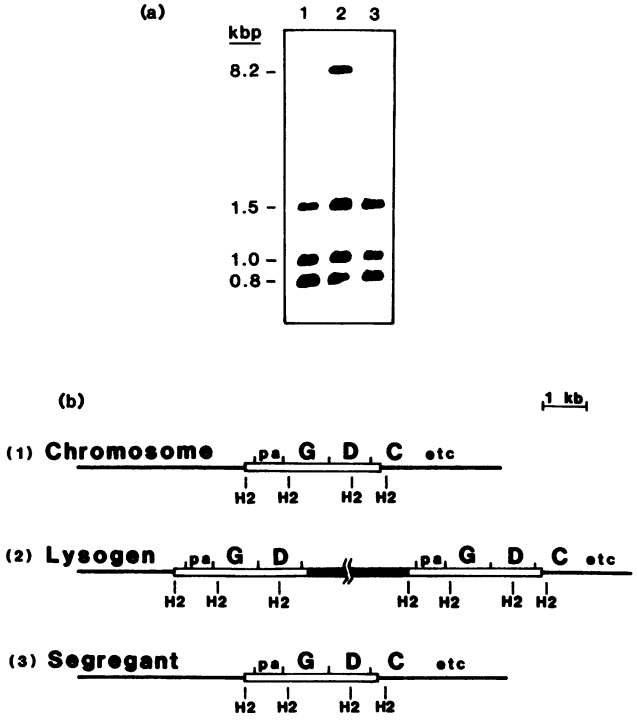

FIG. 3. Physical evidence for integration of an M13mp9::*his* phage. (a) Hybridization analysis of chromosomal DNA from strain AZ193, a lysogenized derivative of the strain, and a segregant of the lysogen, as described in the text. (b) Restriction maps of the three strains. Fragment sizes were estimated by comparison with phage λ *Hin*dIII fragments.

2. Wrinkled and smooth His$^+$ colonies appeared at a ratio of about nine to one (several thousand were analyzed). Smooth His$^+$ colonies must result from a single reciprocal crossover to the left of *hisG2101* (crossover 1); wrinkled His$^+$ colonies could result from a single reciprocal crossover to the right of *hisG2101* (crossover 2) or by a recombination event involving both crossovers 1 and 2 with a concomitant loss of the M13 vector sequence. The observed nine-to-one ratio of wrinkled to smooth recombinant colonies is consistent with the extent of DNA homology available for single reciprocal crossovers on each side of the *hisG2101* mutation. (There is about 0.6 kilobase pair [kbp] of homologous DNA to the left of *hisG2101* and 2.5 kbp to the right).

The instability of the recombinants provided further support for the scheme in Fig. 2. The vast majority (greater than 95%) of the His$^+$ recombinants segregated His$^-$ colonies spontaneously at frequencies of 1 to 10%. In addition, most wrinkled and smooth recombinants segregated colonies having the alternative appearance. These results strongly support the presence of a lysogen intermediate.

Additional evidence of M13mp9::*his*2 phage integration and excision was obtained by hybridization analysis (Fig. 3). Chromosomal DNA was isolated from the parental His$^-$ smooth strain AZ193, a lysogenized derivative of strain AZ193 (a His$^+$ smooth colony), and a segregant of this lysogen (a His$^+$ wrinkled colony). The DNA was digested with *Hin*cII, electrophoresed, transferred to nitrocellulose paper, and hybridized with radiolabeled M13mp9::*his*2 phage DNA (Fig. 3a). Restriction maps of the *his* chromosomal regions of the three strains are shown in Fig. 3b. The *hisOGDC* region contains four *Hin*cII restriction sites (2), and when parental-strain DNA was probed, the three expected fragments were detected (lane 1). Hybridizing *Hin*cII-digested DNA from the lysogen (lane 2) detected a fourth fragment of 8.2 kbp which is composed of M13 DNA (7.6 kbp) and the *hisDC* end of the insert (0.6 kbp). The segregant (lane 3) lost this 8.2-kbp fragment, regenerating the parental pattern. Hybridizing undigested DNA from the lysogen failed to detect a molecule comparable in size to intact M13mp9::*his*2 RF (not shown), indicating that maintenance of the free phage RF DNA does not occur. These results confirm the existence of the integrated prophage intermediate in the allele replacement process.

Optimization of lysogen formation. Optimal conditions for lysogen formation were determined. Two variables were examined, the phage-to-cell ratio (multiplicity of infection [MOI]) and the duration of phage adsorption. The frequency of lysogenization under selective and nonselective conditions increased rapidly with the log MOI (Fig. 4). The frequency of lysogenization under selective conditions was measured as the number of His$^+$ recombinant colonies on minimal medium plates and under nonselective conditions was measured as the number of wrinkled colonies on NA

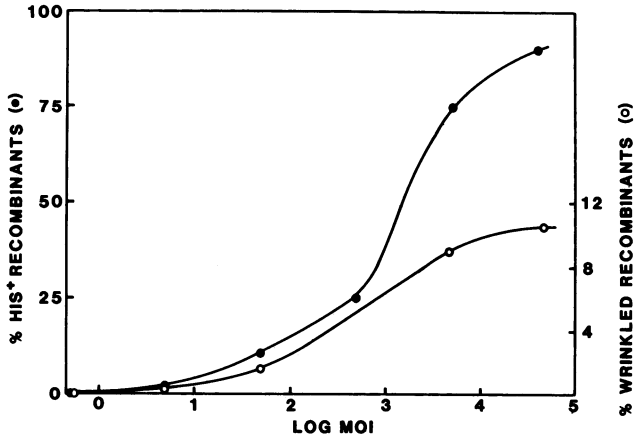

FIG. 4. Phage M13mp::*his* lysogenization frequency as a function of MOI. The donor phage was M13mp9::*his*2, and the recipient strain was AZ193 grown to stationary phase. Adsorption was for 60 min at the indicated MOIs and a cell density of 10^6/ml. The infected cells were diluted and spread either on minimal E medium plates containing 2% (wt/vol) glucose (●) (selective conditions) or on NA plates containing 2% (wt/vol) glucose (○) (nonselective conditions).

plates containing 2% glucose. Recombination frequencies approaching 100 and 10%, respectively, were obtained with an MOI of about 10^5, while an MOI of less than 10 resulted in few recombinants. Phage adsorption time was optimized by using a constant MOI (Fig. 5). Maximal recombination required a minimum of 20 min with exponential-phase cells and 60 min with stationary-phase cells. Further increases in adsorption time did not enhance recombination frequency, and 60 min was used in subsequent experiments. Similar kinetics and a necessity for a high MOI in efficient phage M13 binding to *E. coli* cells have been reported (20).

Phenotypes of phage M13mp lysogens. Since allele replacement proceeded through a lysogenic intermediate, we examined lysogens for distinguishing properties, including those that might be used as selections for phage integration and excision (Table 2). The lysogen grew 30% more slowly than its nonlysogenic parent. Lysogens were resistant to killing by superinfection with male-specific phage R17 and also gained the ability to rescue by complementation a superinfecting M13IV*am* mutant. In addition, lysogens were sensitive to bile salts; after one cycle of growth in medium containing bile salts, cells having lost the prophage constituted 98% of the population, compared with 1% in medium without bile salts. Segregation of target sequence duplications can be readily observed. Introduction of a tRNA suppressor by generalized transduction resulted in translational suppression of the M13 amber mutations, permitting autonomous phage replication and production of infectious M13 phage. Finally, M13mp lysogens of *E. coli* were resistant to colicin E1.

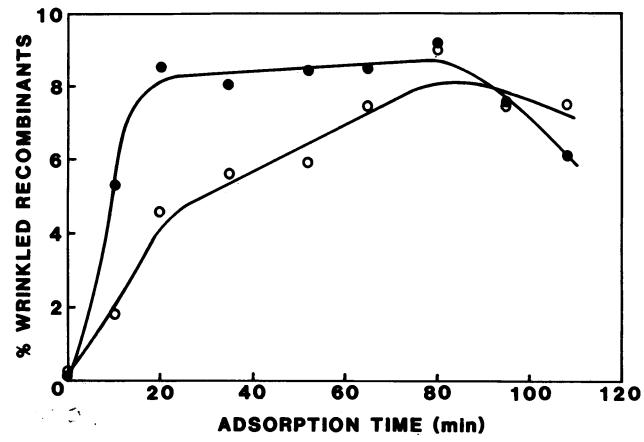

FIG. 5. Phage M13mp::*his* lysogenization frequency as a function of adsorption time. The transduction was the same as that described in the legend to Fig. 4. The recipient was grown to exponential phase (●) (MOI, 450) or stationary phase (○) (MOI, 166); adsorption was done at a cell density of 10^6/ml. Wrinkled and smooth colonies were scored on NA plates containing 2% (wt/vol) glucose.

The resistance of *S. typhimurium* phage M13mp lysogens to killing by R17 phage and of *E. coli* lysogens to killing by colicin E1 provides selections, independent of phenotype conferred by the cloned fragment, for the integrated prophage intermediate. The sensitivity of lysogens of both organisms to bile salts provides a selection for segregants of the prophage. These positive selections were used in subsequent experiments to facilitate the allele exchange procedures.

Allele replacement in *S. typhimurium* by using lysogen-specific positive selections. To demonstrate the use of selections for M13mp lysogens, we replaced a wild-type chromosomal *his* sequence with a *his* null mutation (Δ*hisGD*) carried on phage M13mp9::*his*4 (Table 1). Details of the manipulation are described in Materials and Methods; the basic procedure was as follows. M13mp9::*his*4 phage was mixed at a high MOI with the prototrophic strain AZ1524 and was allowed to adsorb for 60 min. Infected cells were spread on complex medium plates containing a lawn of R17 phage and were incubated until phage R17-resistant colonies appeared. The majority of the colonies were shown to be lysogens carrying the integrated M13mp9::*his*4 prophage by their ability to complement superinfecting M13 phage containing a gene IV amber mutation, M13*am* 4-H38; a minority of the colonies were F′ *trp* segregants (tryptophan auxotrophs) which were also resistant to killing by male-specific phage R17. Segregants of the M13mp9::*his*4 prophage were selected by streaking for single colonies on bile salt plates, and survivors were tested for their His phenotypes. His^- segregants were characterized further.

Hybridization analysis of *Hin*dIII-digested chromosomal DNA from the parent strain AZ1524, an M13mp9::*his*4 phage lysogen of strain AZ1524, and a His^- segregant of the lysogen is shown in Fig. 6a. Restriction maps of the three strains are shown in Fig. 6b. Hybridization with radiolabeled M13mp9::*his*2 phage DNA detected a single fragment of 7.0 kbp in blots of the parent AZ1524 (lane 1), two fragments of 13.8 and 2.3 kbp in the lysogen (lane 2), and a single fragment of 5.4 kbp in the His^- segregant (lane 3). These results provide physical evidence for the integrated prophage intermediate and for replacement of the wild-type sequence by the appropriate-sized deletion in the segregant. In addition, the His^- segregant failed to revert to His^+ and failed to recombine with several *hisG* and *hisD* point mutations in generalized transduction crosses using phage P22. The Δ*hisGD* mutant was named AZ1549 (Table 1).

Allele replacement in *E. coli* by using colicin E1 resistance. The M13mp phages carry an 800-bp fragment of *E. coli lac* DNA that includes the 5′ end of the *lacZ* gene (22). This fragment provides α-complementing activity in host strains lacking N-terminal β-galactosidase sequences such as the *lacZ* M15 deletion, which is used to follow insertional

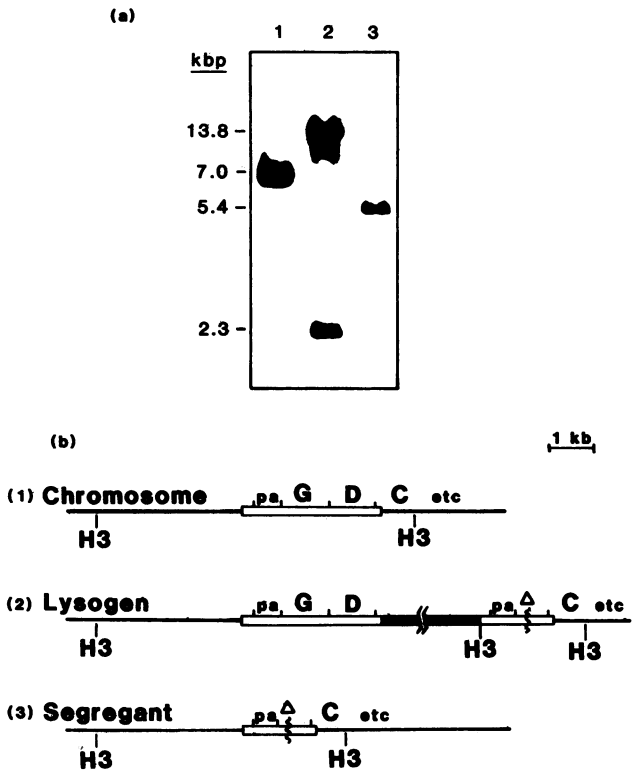

FIG. 6. Physical evidence for replacement of a wild-type allele with a *his* null mutation (Δ*hisGD*). (a) Hybridization analysis of *Hin*dIII-digested chromosomal DNA from strain AZ1254, a lysogen of the strain, and a His$^-$ segregant of the lysogen, as described in the text. (b) Restriction maps of the three strains. Fragment sizes were estimated by comparison with phage λ *Hin*dIII fragments.

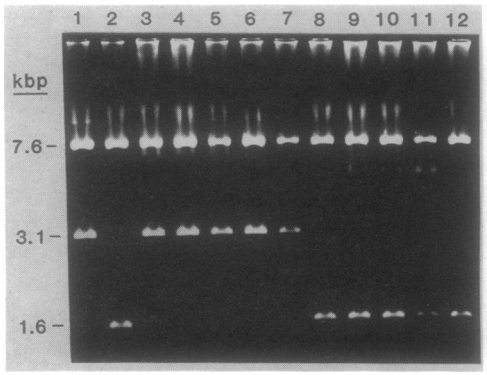

FIG. 7. Physical evidence for retrieval of a *his* null mutation (Δ*hisGD*). The RF DNAs were cleaved with *Eco*RI and *Hin*dIII to release the *his* insert from the vector (the unique *Hin*dIII site lies 14 bp from the *Sal*I site in the polylinker of the vector). RF DNAs from phage M13mp9::*his*1 containing the wild-type *his* fragment and from phage M13mp9::*his*4 containing the same Δ*hisGD* as strain AZ1549 (Table 1) were cleaved with *Eco*RI and *Hin*dIII and were included as size standards. Cleavage of M13mp9::*his*1 RF DNA released the 3.1-kbp wild-type *his* fragment plus the 7.6-kbp vector DNA (lane 1); cleavage of M13mp9::*his*4 RF DNA released the 1.6-kbp Δ*hisGD* fragment plus the 7.6-kbp vector DNA (lane 2). RF DNA samples derived from *Sal*I (lanes 3 to 7) and *Eco*RI (lanes 8 to 12) chromosomal DNA digests were cleaved with *Eco*RI and *Hin*dIII. The 1.6-kbp fragment from the M13mp9::*his*4 RF DNA (lane 2) ran slightly faster than the analogous fragments obtained following retrieval from the chromosome (lanes 8 to 12). The difference is due to the presence of the Δ*hisGal242* 35-bp deletion in phage M13mp9::*his*4 (2); the donor phage (M13mp9::*his*1) and recipient strain (AZ1549) used in the retrieval experiment have the wild-type *his* attenuator sequence.

inactivation in the use of the M13mp phages for cloning. Phage M13mp9 was used to repair by allele replacement a chromosomal *lacZ* M15 deletion and an F′ *lacZ* M15 deletion in *sup*$^+$ *E. coli* TA4283 and TA4282, respectively. M13mp lysogens of *E. coli* were selected by their resistance to colicin E1 (Table 2; see Materials and Methods). Lac$^-$ recipient cells were infected with phage M13mp9 and were spread on plates containing colicin E1. Recombinants were scored as Lac$^+$ colonies on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates. Of several hundred colicin-resistant colonies analyzed, 90% were Lac$^+$ and 10% were Lac$^-$ (the Lac$^-$ colonies included colicin-sensitive cells that had escaped the selection and occasional *tolB* and *tolC* mutants which are resistant to several colicins, including colicin E1). The use of colicin E1 resistance circumvents selection of F$^-$ segregants which occurs in the R17 selection. Segregation of *E. coli* M13 lysogens was performed as for *S. typhimurium* on sodium deoxycholate plates; segregants were confirmed by their sensitivity to R17 phage (see Materials and Methods). These results demonstrate the application of the phage M13mp allele replacement system in *E. coli*.

Chromosomal allele retrieval. The M13mp phage system can be used to retrieve chromosomal sequences independent of phenotypes conferred by the target sequence. The method depends on the ability to construct an integrated prophage intermediate in a *sup*$^+$ host strain (lacking a tRNA suppressor) by using phage M13-specified positive selections. Chromosomal DNA is isolated (see Materials and Methods) from a single lysogenic colony, and two samples of this DNA are digested to completion with each of the two restriction endonucleases originally used to construct the M13mp recombinant phage (e.g., enzymes A and B in Fig. 1). These two samples are treated with DNA ligase and are transfected separately into a *sup* mutant host strain (carrying a tRNA suppressor) where translational suppression of the phage nonsense mutations permits phage replication and production of phage plaques. Both alleles of the target sequence are recovered in this process; each transformation mixture generates a population of phages carrying one of the two alleles. A single plaque is picked from each population of phages and is used to determine the identity of the retrieved allele. The method is demonstrated here by the retrieval of a *his* null (Δ*hisGD*) mutation.

Chromosomal DNA was isolated from an M13mp9::*his*1 lysogen of strain AZ1549 (Δ*hisGD*; Table 1) and was cleaved at the unique *Sal*I or *Eco*RI sites that flank the *his* insert in phage M13mp9::*his*1 (Fig. 1, sites A and B). The two digests were then ligated and used to transfect the suppressing host strain JM107. Phage RF DNA was prepared from plaques derived from each digest, and restriction analysis of five examples from each digest is shown in Fig. 7. The RF DNAs were cleaved to release the *his* insert from the vector. All five RF DNA samples derived from *Sal*I chromosomal DNA digests released the 3.1-kbp wild-type *his* fragment (lanes 3 to 7), while all five samples derived from *Eco*RI chromosomal DNA digests released the 1.6-kbp Δ*hisGD* fragment (lanes 8 to 12). These results demonstrate directed retrieval of the unselectable chromosomal Δ*hisGD* allele.

DISCUSSION

E. coli cells infected with phage M13 display numerous altered properties, including changes in detergent sensitivity, colicin tolerance, and resistance to superinfection by male-specific phages (5, 21, 39). The presence of phage M13 gene III cloned on a multicopy plasmid is sufficient to confer all of these altered properties (5). (The gene III protein is a minor virion component located at one end of the virus particle which is required for infectivity of the virus; during phage multiplication, most of the protein is found in the inner and outer cell membranes [5]). Our results indicate that an integrated copy of M13mp phage provides sufficient gene III protein to confer the altered properties on the lysogen. The resistance of M13-infected cells to superinfection by other male-specific phages has been suggested to result from pilus retraction and loss of phage binding sites (21). Resistance to colicin E1, which kills by depolarizing the cytoplasmic membrane, may result from interference by the gene III protein with the action of the host *tol* gene products that mediate sensitivity to colicin E1.

Using the properties of M13-infected cells, we have extended the use of the phage M13mp vector system to facilitate allele replacement and retrieval of any target DNA sequence. The results show that allele exchange most commonly proceeds via an integrated M13mp prophage intermediate. The observation of phenotypes conferred on the host by expression of M13 genes allowed the development of selections to efficiently detect phage integration and segregation and provided the basis for the generalized allele replacement and retrieval methods. A distinguishing feature of the phage M13mp system is the high efficiency of introducing the DNA into the cell by infection, compared with that of methods that rely on transformation; recombination frequencies approaching unity can be obtained with a sufficiently high MOI (Fig. 4). It should be possible, however, to introduce recombinant M13mp phage DNA (either single-stranded or duplex forms) by transfection into organisms that cannot produce the F pili required for infection to expand the applicable host range of the methods.

The use of recombinant M13 phages in allele retrieval has previously been demonstrated (2, 4, 6, 15, 18, 29). We demonstrated the use of the phage M13mp gene II mutant to obtain integration of the M13mp::*his* genome into the homologous *his* region of the bacterial chromosome, followed by segregation of the prophage to replace a chromosomal mutation with the wild-type sequence (2). In all of these earlier experiments, selections depended on phenotypes conferred by the bacterial DNA inserted in the M13 vector and the applications were, therefore, limited.

The more generally applicable methods described in this paper have already been exploited to study a number of *S. typhimurium* and *E. coli* genes having different chromosomal locations. Insert DNA sequences, including phenotypically silent sequences, ranging in size from 0.4 to 3.1 kbp have been manipulated successfully with this system. The methods have been applied to retrieve 40 independently isolated *his* operon promoter mutations (1, 2) and to recombine into the chromosome several *his* operon promoter mutations generated by site-directed mutagenesis in vitro (27; unpublished results). Mutations generated in vitro in the regulatory region of the adenylate cyclase gene (*cya*) were recombined into the chromosomal *cya* locus and used for characterization of *cya* regulation in vivo (J. Fandl, L. Blaha, and S. Artz, unpublished results). Allele replacement of leucine operon leader peptide codons has been used to study tRNA base modification (*miaA*)-dependent leucine operon repression (P. Blum and B. Ames, unpublished results). Our colicin E1 selection has been used to recombine into the *E. coli* chromosome mutations in the gene (*birA*) for the biotin operon repressor-biotin holoenzyme synthetase protein (13). In addition, wild-type and mutant alleles of the *S. typhimurium* chemotactic aspartate receptor gene (*tar*) have been integrated into the *E. coli lac* operon by recombination between *lac* sequences present in the M13mp vector and homologous chromosomal sequences. (R. Weis and D. Koshland, personal communication).

The frequency of *recA*-dependent recombination in *E. coli* has been shown to vary directly with DNA length down to a lower limit of about 20 to 27 bp (3, 30, 35). We have observed excision of an M13mp::*cya* prophage by crossover within a 40-bp region of homology. We anticipate, therefore, that the M13mp phage system for allele replacement and retrieval should be applicable for manipulation of very small cloned inserts such as those that can be obtained with current DNA-synthesizing machines.

Homologous recombination is known to be the predominant recombination mechanism only in bacteria and a few other organisms (e.g., the yeast *Saccharomyces cerevisiae*). In *Aspergillus*, *Neurospora*, and higher eucaryotic cells, much recombination occurs by nonhomologous pathways (7, 31). Reconstruction of intact-eucaryotic genes in situ with short DNA fragments mutagenized by in vitro methods, therefore, is problematic. Such manipulations can, however, be accomplished in bacteria carrying the cloned eucaryotic gene. Because of the advantages of single-stranded DNA for in vitro mutagenesis and the high efficiency of introducing the mutagenized DNA by phage M13mp infection of cells that perform homologous recombination as the primary pathway, the M13mp phage vector system should be useful for manipulation of eucaryotic as well as procaryotic genes.

Finally, we have very recently constructed derivatives of phages M13mp10 and M13mp11 that carry a *cat* gene (encoding chloramphenicol transacetylase) (J. K. Moore and S. Artz, unpublished results). The *cat* gene was inserted into the *lacI* region of the phages and was modified by removal of a cleavage site for the *Eco*RI restriction enzyme. The modified *cat* gene does not contain restriction sites that interfere with use of the phages as cloning vectors. *S. typhimurium* lysogens have been obtained by selecting for chloramphenicol resistance following infection with M13mp10*cat* or M13mp11*cat* phage carrying cloned *S. typhimurium his* DNA. The chloramphenicol resistance phenotype, therefore, should provide an alternative to the phage R17 and colicin E1 resistance phenotypes for selecting and maintaining *S. typhimurium* and *E. coli* strains with integrated M13mp recombinant prophages. The *cat* phage derivatives may be of general use for performing gene replacements with other host strains able to express the *cat* gene.

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