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iiak, P. J. Barr, R. Fletterick, and W. J.

J. McClary, *in* Book, in press. IRL Press,

Res. **17,** 5408 (1989).
ison, *J. Biol. Chem.* **258,** 11165 (1983).
J. Biol. Chem. **262,** 16212 (1987).
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5′ end of the oligonucleotide primer. Consistent with a lack of strand displacement synthesis by T4 DNA polymerase or native T7 DNA polymerase, we have obtained mutant frequencies in excess of 50% even when DNA ligase is intentionally omitted from the reaction. Nevertheless, we routinely do include ligase, because at least under some circumstances it does improve the efficiency of mutagenesis.

Conclusion

Uracil-containing DNA can be prepared for any vector that can be passaged through an *E. coli dut*$^-$ *ung*$^-$ strain. We have presented here a simple oligonucleotide-directed mutagenesis protocol to demonstrate the utility of the uracil selection technique for efficiently generating mutants. This DNA can be used in conjunction with a variety of established methodologies for site directed mutagenesis (e.g., gapped duplexes, double priming, degenerate oligonucleotides). If more details are sought on any aspect of site-directed mutagenesis, or on alternatives to the use of uracil-containing DNA for improving efficiency, the comprehensive reviews by Smith are recommended.[1,2]

[7] Uses of Transposons with Emphasis on Tn*10*

By NANCY KLECKNER, JUDITH BENDER, and SUSAN GOTTESMAN

I. General Considerations

A. Introduction

As transposable elements have become indispensable tools for bacterial genetics, many different types of specialized transposon derivatives have been constructed. The most widely used constructs are derived from insertion sequence (IS)-based elements (Tn*10* and Tn*5*) or from bacteriophage Mu; constructs based on cointegrate-forming elements (Tn*3* and gamma-delta) are also available. Details regarding the transposition mechanisms of these elements can be found in a recent collection of review articles.[1]

One goal of this chapter is to summarize the major types of transposon

[1] D. E. Berg and M. M. Howe (eds.), "Mobile DNA." American Society for Microbiology, Washington, D.C., 1989.

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constructs available and to provide general guidance as to how best to choose the construct which is most appropriate to the desired application. Classic applications of transposable elements to bacterial genetics were originally outlined by Kleckner *et al.*[2] General considerations for transposon mutagenesis have also been reviewed by Berg and Berg.[3] A second goal is to describe in some detail both the methods used for Tn*10*-derived transposon vehicles and the most recent set of useful Tn*10* vehicles themselves. With respect to the latter goal, this chapter supplements and updates a previous article.[4] Vehicles derived from Tn*5*, Mu, and Tn*3*/gamma-delta are described in detail elsewhere.[3,5–10] Also, a Tn*3* derivative specially adapted for making short in-frame insertions has recently been described.[11] The reader is also referred to the chapter on construction and analysis of fusions by Slauch and Silhavy[12] in this volume.

The use of transposon insertions or transposon-promoted deletions to provide mobile priming sites for DNA sequence analysis of cloned genes is not considered here. Specific vector systems for this purpose are described by Liu *et al.*,[13] Nag *et al.*,[8] Phadnis *et al.*,[6] and Ahmed.[14] In general, the most important parameter limiting the use of transposons for this purpose will probably be the extent to which insertions or deletions occur preferentially at particular sites or into particular regions (see below).

B. Types of Insertions

1. General. Transpositions of an element from one DNA molecule to another are usually isolated by selecting for stable maintenance of a genetic marker present on the transposon under conditions where the DNA mole-

[2] N. Kleckner, J. Roth, and D. Botstein, *J. Mol. Biol.* **116,** 125 (1977).
[3] C. M. Berg and D. E. Berg, *in* "*Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology" (F. C. Neidhardt, J. L. Ingraham, B. Magasanik, K. B. Low, M. Schaechter, and H. E. Umbarger, eds.), Vol. 2, p. 1071. American Society for Microbiology, Washington, D.C., 1987.
[4] J. C. Way, M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner, *Gene* **32,** 369 (1984).
[5] C. Sasakawa and M. Yoshikawa, *Gene* **56,** 283 (1987).
[6] S. H. Phadnis, H. V. Huang, and D. E. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 5908 (1989).
[7] W. Y. Chow and D. E. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6468 (1988).
[8] D. K. Nag, H. V. Huang, and D. E. Berg, *Gene* **64,** 135 (1988).
[9] H. S. Seifert, E. Y. Chen, M. So, and F. Heffron, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 735 (1986).
[10] E. A. Groisman, [8], this volume.
[11] M. Hoekstra, D. G. Burbee, J. D. Singer, E. E. Mull, E. Chiao, and F. Heffron, *Proc. Natl. Acad. Sci. U.S.A.* in press (1991).
[12] J. M. Slauch and T. J. Silhavy, this volume [9].
[13] L. Liu, W. Whalen, A. Das, and C. M. Berg, *Nucleic Acids Res.* **15,** 9461 (1987).
[14] A. Ahmed, *Gene* **75,** 315 (1989).

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[16] N. Kleckner, D. F. Barker, D.
[17] C. Manoil and J. Beckwith, *Pr*

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Nucleic Acids Res. **15,** 9461 (1987).

cule which donates the transposable element (the delivery vehicle) is lost. Transposition of an element from a nonreplicating phage or plasmid genome into a stable replicon are the two most popular approaches.

2. Fusion Transpositions. A number of specialized transposon derivatives are available in which transposition of the element to an appropriate target site results in activation of an otherwise silent gene. Fusion vehicles can be used in three different ways. (1) They are most commonly used as genetic tools for the isolation of particular desired fusion constructs. (2) Such vehicles are also very useful for obtaining insertions without the necessity of a specialized delivery vehicle. If the transposon originates from a context in which the marker gene is not expressed, transpositions into new sites can be specifically selected without destruction or elimination of the donor molecule (see below). This approach is particularly useful for isolating transposition events in organisms or situations where no general delivery system exists, although it does yield only a subset of all possible insertions. (3) Such vehicles also facilitate analysis of the transposition process per se. They make it possible to compare endogenous transposition rates in different strains or under different conditions without having to resort to a "mating-out"[15] or "λ-hop"[16] assay.

Four different types of fusions can be isolated with existing tools (see also [9], this volume): promoter fusions in which transposition has placed the target gene under control of a transposon-borne *lac* operon promoter, transcriptional fusions between the target gene and a *lacZ* gene on the transposon, translational fusions between the target gene and either the *lacZ* gene or a *kan* gene on the transposon, and translational fusions to the *phoA* gene on the transposon. Expression of the *phoA* gene requires that the target gene product cross the inner membrane to the periplasm, and the Tn*5*–*phoA* construct can thus be used to identify specifically genes whose products are secreted or localized to the membrane.[17]

C. Choosing Delivery Vehicle: Choice of Target Molecule Determines Choice of Donor Molecule

1. Insertions into Bacterial Chromosome. For isolation of transposition events into the bacterial chromosome, bacteriophages are the most convenient type of delivery vehicle. A phage carrying the transposon can be introduced into the host cell under conditions where the phage genome neither replicates, kills, nor (in many cases) stably integrates into the host cell. λ vehicles are used for isolation of Tn*10*, Tn*5*, and Mu insertions; Mu

[15] T. Foster, V. Lundblad, S. Hanley-Way, S. Halling, and N. Klecker, *Cell* (*Cambridge, Mass.*) **23,** 215 (1981).
[16] N. Kleckner, D. F. Barker, D. G. Ross, and D. Botstein, *Genetics* **90,** 427 (1978).
[17] C. Manoil and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8129 (1985).

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is also used directly. For λ vehicles, the donor is crippled by nonsense mutations in phage replication genes (which necessitate the use of a non-suppressing host for isolation of transposition events), by a mutation in the λ repressor gene, and often by deletion of the phage integration system. These λ derivatives are referred to below as hop phages. For Mu, every lysogen is a transposition event, so lysogenization of phage carrying Mu ends is sufficient to produce insertions. Mu vectors generally are defective for phage growth and therefore must be grown with a helper phage. Tn*3*/gamma-delta vectors are not useful for this purpose because the bacterial chromosome is specifically immune to insertion of these elements.

An alternative to phage delivery vehicles are so-called suicide plasmids which are thermosensitive for replication or which replicate in a donor strain but fail to replicate in the recipient strain where transposon insertions are to be isolated. Such plasmids, some of which have a broad host range, are widely used in strains other than *Escherichia coli* that are not sensitive to bacteriophage λ; they are available but used less frequently in *E. coli* or *Salmonella*.[5]

2. Insertions into Multicopy Nonconjugative Plasmids. For isolation of insertions into nonconjugative multicopy plasmids, bacteriophages are also the delivery vehicles of choice. In the most general approach, a strain harboring the target plasmid of interest is infected with the phage vehicle, and a large number of colonies resulting from transposition are selected, exactly as for chromosomal insertions. About 1% of such colonies contain a transposition event into the plasmid unless a specific enrichment for transposon insertions is used (see below); also, it should be remembered that cells in a single colony may contain a mixture of plasmids with and without the transposon insertion. Plasmid insertions are specifically identified in a subsequent step. Many independent pools of about 1000 such transposition colonies are made, and plasmid DNA isolated from each pool is used to transform a new host. Transformants selected for expression of a marker on the transposon contain insertion-bearing plasmids.

Specific plasmid vehicles have also been constructed for the isolation of Tn*3*-based transpositions into target genes on multicopy plasmids.[9] In this case, isolation of insertions into a plasmid involves multiple steps. First cointegrate insertion products are identified, and then cointegrates are resolved to simple insertion products. Standard pBR-based Amp^R cloning vectors cannot be used as target plasmids in this system; they are all immune to Tn*3* transposition by virtue of the presence of a single Tn*3* terminal inverted repeat sequence.

3. Transpositions into Bacteriophage λ or Conjugative Plasmid. When the target molecule is a phage or conjugative plasmid, the transposon

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delivery vehicle can be any type of molecule or replicon other than the target itself. The most efficient transposon donor molecule is a multicopy nonconjugative plasmid because such vehicles usually provide the highest levels of transposition. Also, when there are limitations on the size of the phage genome, as for λ, use of a small minitransposon is almost always essential. Transpositions into a bacteriophage are obtained by growing a stock of the phage on a cell harboring the transposon, and phage derivatives carrying the transposon marker are identified in a subsequent step using any of several approaches (see below). Transpositions into a conjugative plasmid are obtained by constructing a strain carrying both the transposon and the target plasmid, mating this strain with a suitable recipient, and selecting for transfer of the transposon marker into a new host, with appropriate counterselection against the donor strain.

D. Choosing Transposon: General Properties of Transposon Derivatives

The specific choice of transposon element will depend on the ultimate goal of insertion mutagenesis and on the specific features of the transposon derivatives available. In this section we discuss the general features of transposon derivatives which are important if the primary goal is the isolation of stable insertions into a target gene or region of interest. Specific elements for fusion analysis and for isolation of transposon-promoted rearrangements are considered subsequently.

1. Stability of Inserts: Use Minitransposons Whenever Possible. Stable transposon insertions that are unable to undergo additional rounds of transposition can be obtained by using a delivery system in which the transposase gene is located outside of the transposon itself. In the ideal case, the transposase gene is located on the transposon donor molecule and is thus lost along with that molecule following transposition. In a less ideal case, the transposase gene is located on a separate replicon, usually a multicopy plasmid, from which the transposon insertion can eventually be separated (by plasmid segregation or by transfer of the insertion to a new strain). Transposons which do not contain a transposase gene within their boundaries are generically referred to as minitransposons; most of these elements are in fact also smaller than the corresponding wild-type transposon.

It is always preferable to use a minitransposon construct whenever other considerations permit. Since the transposase gene can be separated from the insertion, problems resulting from secondary transposition events are eliminated. Four types of problems are particularly troublesome. (1) Attempts to move an insertion to a new strain or to map an insertion will

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be confounded if the transposon marker is present at more than a single location at any stage. (2) Intact transposons have the capacity to promote the rearrangement (deletion or inversion) of adjacent material; such rearrangements occur at significant frequencies, often as high or higher than the frequency of transposition of the element itself. (3) For composite transposons such as Tn*10* and Tn*5*, transposition of the individual component insertion sequences (IS) also occurs at a much higher frequency than transposition of the entire transposon, about 10^{-3} and 10^{-2} per element per cell per generation for IS10 and IS50, respectively (e.g., Shen *et al.*[18]). Thus, a strain will tend to accumulate multiple copies of the (unmarked) insertion sequence which have the potential to cause complications during subsequent genetic or physical analysis. (4) P1 transduction of transposition-proficient insertions does not always lead to cotransduction of the transposon marker and the donor site mutation, probably because the transducing lysate contains significant numbers of P1 phages carrying the transposon which yield nonfaithful transductants carrying P1::Tn lysogens (N. Kleckner and S. Gottesman, unpublished observations, 1977; Berg *et al.*[19]). The proportion of "unfaithful" transductants can be very large, especially when the desired insertion is transduced at a relatively low frequency. This problem is eliminated by use of a minitransposon; for transposition-proficient insertions, the problem can be reduced by use of P1*vir* instead of P1*kc* or P1*clr*100 to minimize formation of P1 lysogens or by use of P1-HFT[20] to specifically increase the frequency of true transductants.

2. Insertion Specificity. The hallmark of a transposable element is its ability to insert in many different locations. However, no transposon really chooses its target sites completely at random, and some transposons exhibit a significant degree of target site preference. In all cases, the degree of specificity is low enough that insertions in a several kilobase (kb) region of interest or a single insertion in a specific gene of interest can always, or almost always, be identified. For Tn*5*, Mu, Tn*3*, Tn*9*, and the newly isolated Tn*10*-ATS derivatives described below, the specificity of insertion is sufficiently low that isolation of insertions at many different sites within a single gene is straightforward. However, these elements do still exhibit some preference for particular sites. Wild-type Tn*10* exhibits the highest degree of specificity; an occasional gene may be "cold" for insertion altogether or may be dominated by insertions into a single favored hot spot. If an available Tn*10*-ATS derivative cannot be used, these problems

[18] M. Shen, E. A. Raleigh, and N. Kleckner, *Genetics* **116**, 359 (1987).
[19] C. M. Berg, C. A. Grullon, A. Wang, W. A. Whalen, and D. E. Berg, *Genetics* **105**, 259 (1983).
[20] N. L. Sternberg and R. Maurer, this volume [2].

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[2].

can usually be overcome by screening a larger than usual number of insertions, since the element does insert into lower affinity sites at a lower frequency.[21] Tn*10* insertion specificity is discussed in detail below.

For Mu and Tn*3*/gamma-delta, insertion within any particular region can be considered essentially random. However, these elements do exhibit preferences for certain regions over others, for reasons that are not clear (reviewed by Kleckner[22]).

3. Selectable Markers. Most transposons contain as an expressed selectable marker either an antibiotic resistance determinant, a *supF* gene, or *lacZ*. In choosing a selectable marker, it is important to consider the types of transposon delivery systems available, the need to save particular markers for other aspects of the analysis, and other factors. Transposons used to generate fusions usually contain an expressed selectable marker in addition to the determinant involved in generating the fusion; thus, random insertions can be isolated in the usual way and then screened in a subsequent step for presence of an active fusion.

Transposon insertions carrying drug resistance markers are especially useful for analysis of mutations in the bacterial chromosome that produce phenotypes which are difficult to screen or that involve hosts with multiple modifications (see Kleckner *et al.*[2] for general discussion). Once an appropriate linked insertion is available, the drug resistance marker can be used to map the location of the mutation and to move the mutation from strain to strain. Sets of mutations from a given selection can be shown to be linked to each other by demonstrating their linkage to a common transposon insertion, even if the transposon itself has not been mapped.

4. Other Issues. Several other considerations may be important in selecting a transposition strategy or in analyzing the insertions obtained.

(a) Size. Smaller transposons are generally more tractable than larger ones. Size may be an overriding factor if insertions are isolated in, or eventually will be transferred to, bacteriophage λ, whose genome size is limited to the amount that can be packaged efficiently. Large transposons will also be harder to handle on multicopy plasmids, since larger plasmids will have lower transformation efficiencies and may give rise to deletion variants more frequently than smaller plasmids. Also, for Tn*10*- and Tn*5*-based transposons, the frequency of transposition decreases about 40% for every kilobase of transposon length. Thus, for example, a 9-kb element will transpose at approximately 1% the frequency of a 2-kb element. The smallest available transposons contain only a 265–390 base pair (bp) *supF* gene between the ends of the transposon (see Phadnis *et al.*[6] and below).

[21] A. Wang and J. R. Roth, *Genetics* **120,** 875 (1988).
[22] N. Kleckner, *Annu. Rev. Genet.* **15,** 341 (1981).

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(b) Inverted repeats. All of the transposable elements except for Mu have inverted repeats at their ends. The lengths of these repeats vary from 38 bp (Tn*3*/gamma-delta) to 70 bp (mini-Tn*10* elements) to 400–1400 bp for other Tn*10* and Tn*5* elements. The existence of such repeats is limiting in two ways. First, long inverted repeats are very unstable in single-stranded phage vectors and are somewhat unstable even in multicopy plasmid vectors; short inverted repeats (<100 bp) are not a significant problem. Second, even short inverted repeats interfere with DNA sequence analysis of transposon/target insertion junctions if the dideoxy method is used. On single-stranded templates used for dideoxy sequencing, intramolecular pairing between inverted repeats precludes DNA sequencing across the transposon/target DNA junction. The same may be true of double-stranded templates as well. Furthermore, on a double-stranded template, where the same sequence is present at both termini of the element, synthesis must be primed from inside the element and thus must extend across the inverted repeat before the target junction is reached. However, all of these problems are eliminated either by subcloning each transposon/target junction fragment into a separate vector prior to sequence analysis or by using Maxam–Gilbert sequencing of isolated restriction fragments containing the junction of interest.

(c) Rearrangements that occur on insertion. A small but significant fraction of bacteriophage Mu insertions (<10%) are accompanied by deletion or duplication of sequences adjacent to the insertion site. For other transposons, the initial insertion event is virtually always correct. All elements can give rise at low frequencies to rearrangements in secondary events subsequent to transposition (discussed in detail below), but this is not usually a major practical concern.

(d) Polarity and gene turn-on. Large transposons (the wild-type versions of Tn*10*, Tn*5*, Tn*3*, and Mu) are usually polar on expression of distal genes when inserted into an operon. Minitransposons may also be polar. The degree of polarity of an element depends on the nature and strength of transcription termination signals within the transposon, the presence or absence of promoters within the element which direct transcription outward beyond its ends, the precise location of the insertion with respect to internal Rho-dependent termination sites in the target gene, and the possibility that new promoters might be created or revealed by juxtaposition of transposon and target sequences at the new insertion junction.[21,23–26]

[23] M. S. Ciampi, M. B. Schmid, and J. R. Roth, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 5016 (1982).
[24] D. E. Berg, A. Weiss, and L. Crossland, *J. Bacteriol.* **142,** 439 (1980).
[25] P. Prentki, B. Teter, M. Chandler, and D. J. Galas, *J. Mol. Biol.* **191,** 383 (1986).
[26] R. W. Simons, B. Hoopes, W. McClure, and N. Kleckner, *Cell (Cambridge, Mass.)* **34,** 673 (1983).

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acteriol. **142,** 439 (1980).
Galas, *J. Mol. Biol.* **191,** 383 (1986).
N. Kleckner, *Cell (Cambridge, Mass.)* **34,**

Transposon insertions have been used to get rough information about operon structure and have successfully identified internal promoters in several cases. However, the general usefulness of this approach is limited by the many possible complications.

(e) Specific features of Tet^R, Kan^R, and supF selections. Each of the three selectable markers that are most commonly used to select insertions have certain peculiarities which influence the selection procedures. Selection of insertions into the *E. coli* genome is discussed here; selection of insertions into phage λ is discussed in Section III below.

Tet^R. The tetracycline resistance determinant of Tn*10* has the peculiar property that the level of tetracycline resistance conferred is dramatically reduced when the determinant is present on a multicopy plasmid.[27–29] Thus, use of this determinant as the sole selectable marker on such a plasmid should be avoided whenever possible. In particular, tetracycline resistance should not be used as a marker for isolation of transposon insertions into a multicopy plasmid. However, Tn*10–tet* insertions present in single copy in the bacterial chromosome can be routinely cloned into a multicopy cloning plasmid by selection for tetracycline resistance if certain precautions are followed. (1) The level of tetracycline should be reduced to the lowest possible level (2–5 μg/ml). (2) A favorable strain background should be used, as the magnitude of the effect varies considerably from strain to strain; *E. coli* MM294 is particularly good. (3) Transformants should be selected first using another marker on the cloning vector. Tetracycline-resistant transformants can be identified among these by replica plating onto (low) tetracycline plates; inclusion of (low) tetracycline as a second drug in the transformation plates is also a possible strategy but does not work very well and often yields junk.

Kan^R. (1) A multicopy plasmid carrying a kanamycin resistance gene confers resistance to a higher level of antibiotic than that conferred by a single copy of the resistance gene in the chromosome. Thus, when insertions of a *kan* element are made into a strain carrying a multicopy plasmid, the proportion of selected colonies containing an insertion of the transposon into the plasmid can be increased by including a high concentration of antibiotic in the selective plates (300 versus 50 μg/ml). (2) Most *E. coli* strains cannot become kanamycin resistant by spontaneous mutation. However, many or all streptomycin-resistant strains can mutate to kanamycin resistance. If transposon insertions are relatively rare, the frequency of spontaneous Kan^R derivatives may be high enough to cause significant background. The frequency of such derivatives is lower if the

[27] L. D. Smith and K. P. Bertrand, *J. Mol. Biol.* **203,** 949 (1988).
[28] H. S. Moyed, T. T. Nguyen, and K. P. Bertrand, *J. Bacteriol.* **155,** 549 (1983).
[29] D. C. Coleman and T. J. Foster, *Mol. Gen. Genet.* **182,** 171 (1981).

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selective medium is rich [e.g., Luria Bertani broth (LB)] than if it is minimal. Increasing the kanamycin concentration is not effective in eliminating spontaneous mutants.

supF. Insertions of the *supF* gene into the *E. coli* chromosome can be selected using a host strain which carries two *supF*-suppressible nonsense mutations (see, e.g., Seed[30]).

(f) Nonsense mutations and su⁺ hosts. All λ phage delivery vehicles are disabled by one or more nonsense mutations in the phage replication genes *O* and *P*. Most vehicles contain amber mutations and thus cannot be used in hosts containing an amber suppressor. However, if use of such a host is unavoidable, one λ::Tn*10* vehicle is available which is disabled with a UGA mutation (λNK370, see below); since UGA mutations are not suppressed by amber or ochre nonsense suppressors, this phage can be used to isolate insertions of wild-type Tn*10* in a host containing an amber or ochre suppressor.[4]

E. Using Tn10 to Create Deletions

1. Tn10-Promoted Adjacent Deletions. Most transposons are capable of generating deletions of chromosomal sequences adjacent to their site of insertion. Tn*10* is the transposon of choice for generating such deletions. Formation of Tn*10*-promoted deletions requires that the transposon contain two intact IS10 sequences; this type of rearrangement occurs by an interaction between the two "inside" IS10 ends of the element and a target site located in adjacent sequences outside of the element.[31] Wild-type Tn*10* is almost always used for this purpose. However, the IS10 elements may be present either in inverted orientation or in direct orientation, so other types of Tn*10* derivatives could also be used.[32]

Tn*10*-promoted deletions result in removal of a continuous segment extending from one of the inside ends of the transposon, across the internal region of the transposon including the tetracycline resistant determinant, across the distal IS10 sequence, and into adjacent chromosomal sequences.[31] Such deletions occur at a frequency of about 10^{-4} in an overnight culture grown from a single colony.[33] Since the Tet^R determinant is lost, a population highly enriched in such deletions can be obtained either by penicillin selection in the presence of bacteriostatic levels of tetracy-

[30] B. Seed, *Nucleic Acids Res.* **11**, 2427 (1983).
[31] N. Kleckner, *in* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), p. 225. American Society for Microbiology, Washington, D.C., 1988.
[32] E. A. Raleigh and N. Kleckner, *J. Mol. Biol.* **173**, 437 (1984).
[33] N. Kleckner, K. Reichardt, and D. Botstein, *J. Mol. Biol.* **127**, 89 (1979).

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[34] B. R. Bochner, H.-C. Hua
[35] S. R. Maloy and W. D. N
[36] V. François, J. Louarn, J.

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d M. M. Howe, eds.), p. 225. American
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73, 437 (1984).
Mol. Biol. **127,** 89 (1979).

cline[2] or by a selection for fusaric acid resistance under conditions where the *tetA* gene is induced.[34,35]

Tetracycline-sensitive derivatives obtained by either of these methods include not only Tn*10*-promoted deletions, but also related rearrangements which occur at roughly the same frequency, Tn*10*-promoted deletion/inversions (see Kleckner *et al.*[33]; reviewed in Kleckner[31]). In the case of the deletion/inversions, one IS10 element and a segment of adjacent chromosomal material has been inverted relative to its original orientation, rather than deleted as is the case in Tn*10*-promoted adjacent deletions. Deletions must be specifically distinguished from deletion/inversions by secondary tests. All such tests rely on the fact that deletions will have eliminated a contiguous segment of adjacent DNA, whereas inversions will have only a single new break point and will still retain all of the material between the transposon and that breakpoint. Any derivative which inactivates two adjacent independently expressed genes can be assumed to be a deletion. More generally, the presence or absence of sequences originally adjacent to the transposon can be determined either physically, by Southern blotting with a probe specific to those sequences, or genetically, by asking whether the Tet^S derivative can donate markers from the adjacent region in a phage-mediated transductional cross.[33]

Tetracycline-sensitive derivatives will also include a number of other types of variants. Nearly precise excisions,[15] which are specific deletions within the transposon, occur at about 10% the frequency of deletions and deletion/inversions. Spontaneous deletions and precise excisions of the transposon (see below) are much rarer.

2. Selecting Deletions with Predetermined End Points by Recombination between Modified Tn10 Insertions. Transposons can be used as portable regions of homology.[2] Recombination between two Tn*10* elements located at different sites in the genome as either direct or inverted repeats can generate either a deletion or an inversion of the intervening material. Special tools which facilitate the isolation of such recombinants have been developed.[36] These tools permit the replacement of the original *tet* region in a chromosomal insertion with either a *tet*::*str* or *tet*::*kan* disruption. The *str* and *kan* disruptions are at different locations in the *tet* segment. Thus, in a strain carrying a *tet*::*str* insertion at one site and a *tet*::*kan* insertion at another site, the desired deletion or inversion between the two sites can be obtained by selecting for Tet^R recombinants and then analyzing the recombinants to identify those having the desired structure. These

[34] B. R. Bochner, H.-C. Huang, G. L. Schieven, and B. Ames, *J. Bacteriol.* **143,** 926 (1980).
[35] S. R. Maloy and W. D. Nunn, *J. Bacteriol.* **145,** 1110 (1981).
[36] V. François, J. Louarn, J. Patte and J.-M. Louarn, *Gene* **56,** 99 (1987).

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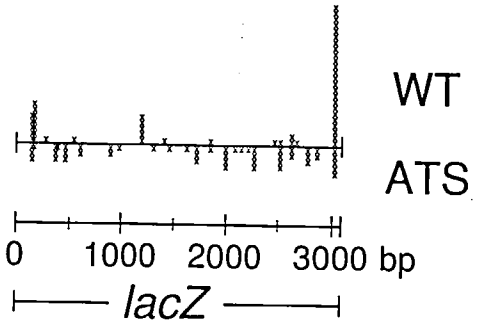

FIG. 1. Target specificity of wild-type and altered target specificity (ATS) mutant transposases in the *lacZ* gene. Fifty independent mini-Tn*10* Kan^R insertions into the 3.1-kb *lacZ* gene carried on a pGEM-3 plasmid vector were isolated from either a "hop phage" carrying wild-type transposase [λNK1105; J. C. Way, M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner, *Gene* **32**, 369 (1984)] or from a hop phage carrying ATS transposase (λNK1316, derivative 103 in Fig. 2). Insertion sites were determined by sequencing. Approximate positions of insertion are shown; base pair 1 of the scale is at the A of the ATG start codon for *lacZ*. The strongest insertion site for both transposases is at bp 3026–3034.

tools are as applicable to insertions of mini-Tn*10–tet* elements as to the full Tn*10* elements on which they were tested. Use of mini-Tn*10* elements should eliminate a residual background of Tet^R recombinants that appear to have undergone Tn*10*-promoted rearrangements.

II. Tn*10* Transposition Vehicles

A. Tn10 Transposase Mutation Which Decreases Target Site Specificity

Wild-type Tn*10* inserts preferentially into so-called hotspots.[37–40] This phenomenon is illustrated by the spectrum of Tn*10* insertions into the 3.1-kb *lacZ* gene which contains a single very strong hotspot plus several less preferred sites (Fig. 1, top). Two factors contribute to Tn*10*'s selection of particular sites. (1) Tn*10* insertion involves recognition, cleavage, and duplication of a specific 9-bp target site sequence. Comparison among many different 9-bp target sites reveals a consensus sequence, 5′ NGCTNAGCN 3′. Particular target sites can differ significantly from this sequence, but hotter sites match more closely while colder sites match

[37] N. Kleckner, D. A. Steele, K. Reichardt, and D. Botstein, *Genetics* **92**, 1023 (1979).
[38] S. M. Halling and N. Kleckner, *Cell (Cambridge, Mass.)* **28**, 155 (1982).
[39] O. Huisman, W. Raymond, K. Froehlich, P. Errada, N. Kleckner, D. Botstein, and M. A. Hoyt, *Genetics* **116**, 191 (1987).
[40] S. Y. Lee, D. Butler, and N. Kleckner, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7876 (1987).

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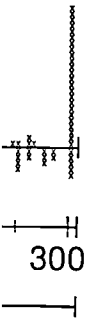

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Errada, N. Kleckner, D. Botstein, and

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less well. (2) The efficiency of insertion into a particular consensus sequence varies over several orders of magnitude according to the sequence of the 8–10 bp located immediately adjacent to the consensus sequence on either side. These base pairs may influence DNA structure, since no simple correlation with DNA sequence is obvious by inspection.

The nature and combination of these two factors make it difficult or impossible to predict potential sites of Tn*10* insertion from inspection of the target DNA sequence. However, one general rule follows from the fact that four of the six base pairs in the consensus sequence are GC pairs: very AT-rich DNA will have relatively few hot spots, so Tn*10* insertions will generally be more randomly distributed except in the unfortunate case where a single GC-containing hotspot occurs.[39]

A significant improvement in Tn*10* transposon mutagenesis is provided by the recent isolation of mutant IS10 transposases that exhibit a much lower degree of insertion specificity than wild type. These altered target specificity (ATS) transposases, *ats1* and *ats2*, result from G to A transition mutations at base pairs 508 and 853 of IS10 (TRN10IS1R.BACTERIA, GenBank J01829). The mutations change cysteines to tyrosines at amino acids 134 and 249 of the protein (bp 400 and 745 of the transposase gene[41]). The double-mutant ATS transposase exhibits only a slight decrease in transposition activity (3-fold), so its usefulness for insertion mutagenesis is not affected. The spectrum of insertion sites selected by the double-mutant ATS transposase in the *lacZ* gene is compared with that of wild type in Fig. 1. The mutant transposase utilizes a much larger number of sites than wild type; 50 insertions are relatively evenly distributed among 23 different sites in the 3.1-kb region. Tn*10* derivatives for transposon mutagenesis that contain the *ats1 ats2* double mutation in transposase are described in the following section and in Fig. 2 (derivatives 102–108).

B. Current Tn10 Transposon Derivatives

1. Obtaining Tn10 Derivatives. The Tn*10* derivatives described below and *E. coli* strain NK5336 can be obtained by communicating *in writing* with Dr. Nancy Kleckner, Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138 (FAX: 617-495-8308). (Requests made by telephone will not be accepted. If you are not sure which elements are appropriate for your needs or if you have general questions, write a letter or a FAX explaining your problem and giving your telephone and/or FAX number.) Bacterial strains N6377 and SG12021 and further information about λD69 procedures can

[41] S. M. Halling, R. W. Simons, J. C. Way, R. B. Walsh, and N. Kleckner, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2608 (1982).

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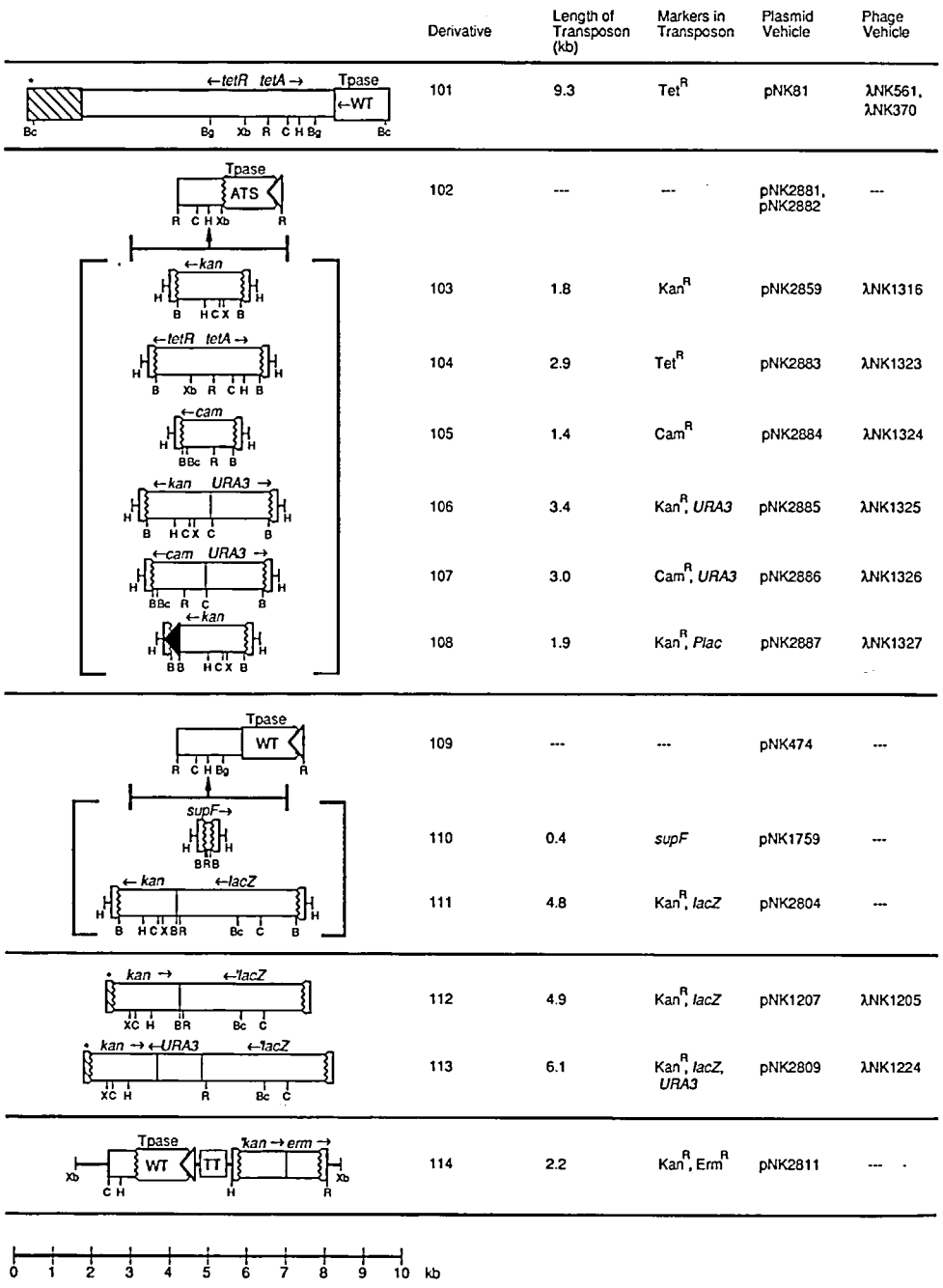

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FIG. 2. Useful Tn*10* deriv
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Morisato, D. E. Roberts, a
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IS10 Right with *Bcl*I (bp 66,
Ptac promoter [E. Amann,
in site so that the transposa
*Eco*RI site at bp 3140 of Tn*1*
110 and 111. Derivative 102
exceptions. First, the transp
a G to A transition at bp 508
Right). Second, the sequence
Tn*10* has been deleted fro
junction. Derivative 102 prov
103–108, 110, and 111 are ea
of IS10 Right (generated by
*Bam*HI site). The 70-bp tra
gene sequence terminating i
*Hin*dIII fragment which is in
(*Ptac*–ATS transposase) or
Tn*10* in cis to a transposase
carries a *Bam*HI Kan^R frag
J01839, bp 697 to 2392, *Pvu*II
in the backbone so that the
Ptac promoter. Derivative 1
fragment from Tn*10* (Fig. 4a
bp 3402 to 627, *Bgl*II fragme
backbone so that the *tetR* ge
gene promoter is transcribing
(mini-Tn*10* *cam*/*Ptac*–ATS t
pACYC 184 (P18XCYC18.SY
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element also carries a *Saccha*
YEP24 (YEP24.VEC, GenB
*Bam*HI site at bp 3784). The
107) or the *cam* gene (for d
opposite direction from the *k*
transposase) is identical to
fragment downstream of the
same direction as the *kan* gen
transposon end through the

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Length of Transposon (kb)	Markers in Transposon	Plasmid Vehicle	Phage Vehicle
9.3	Tet^R	pNK81	λNK561, λNK370
---	---	pNK2881, pNK2882	---
1.8	Kan^R	pNK2859	λNK1316
2.9	Tet^R	pNK2883	λNK1323
1.4	Cam^R	pNK2884	λNK1324
3.4	Kan^R, *URA3*	pNK2885	λNK1325
3.0	Cam^R, *URA3*	pNK2886	λNK1326
1.9	Kan^R, *Ptac*	pNK2887	λNK1327
---	---	pNK474	---
0.4	*supF*	pNK1759	---
4.8	Kan^R, *lacZ*	pNK2804	—
4.9	Kan^R, *lacZ*	pNK1207	λNK1205
6.1	Kan^R, *lacZ*, *URA3*	pNK2809	λNK1224
2.2	Kan^R, Erm^R	pNK2811	---

FIG. 2. Useful Tn*10* derivatives. The structure of each transposon-containing restriction fragment (or for derivatives 101, 112, and 113, the transposon itself) is drawn to scale. The backbones into which these restriction fragments (or transposons) are inserted to create each transposon vehicle are described in Fig. 3. In *lacI*$^+$ or *lacI*^{Q} strains, *Ptac* or *Plac–UV5* promoters on these constructions can be fully induced by the addition to the medium of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 m*M*. Derivative 101 (wild-type Tn*10*) constructions have been described previously [J. C. Way, M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner, *Gene* **32**, 369 (1984)]. The open reading frame for transposase protein is bp 108–1313 of IS10 Right (TRN10IS1R.BACTERIA, GenBank J01829). Derivative 109 (*Ptac*–wild-type transposase) was constructed by cleaving Tn*10* in IS10 Right with *Bcl*I (bp 66, Fig. 4a) and ligating a *Pvu*II to *Eco*RI fragment containing the *Ptac* promoter [E. Amann, J. Brosius, and M. Ptashne, *Gene* **25**, 167 (1983)] to this filled-in site so that the transposase gene is under *Ptac* control. The derivative extends to the *Eco*RI site at bp 3140 of Tn*10* (Fig. 4a). Derivative 109 provides a backbone for derivatives 110 and 111. Derivative 102 (*Ptac*–ATS transposase) is identical to derivative 109 with two exceptions. First, the transposase gene in derivative 102 carries two ATS mutations (*ats1*, a G to A transition at bp 508 of IS10 Right, and *ats2*, a G to A transition at bp 853 of IS10 Right). Second, the sequence between a *Xho*II site at bp 1319 and a *Bgl*II site at bp 1942 of Tn*10* has been deleted from derivative 109 and an *Xba*I linker inserted at this deletion junction. Derivative 102 provides a backbone for derivatives 103–108. Mini-Tn*10* derivatives 103–108, 110, and 111 are each bounded by identical inverted repeats of the outermost 70 bp of IS10 Right (generated by cleaving IS10 Right with *Bcl*I and converting the *Bcl*I site to a *Bam*HI site). The 70-bp transposon end in these derivatives is embedded in 40 bp of λ *c*I gene sequence terminating in a *Hin*dIII site. Thus each complete transposon is carried on a *Hin*dIII fragment which is inserted into the *Hin*dIII site (bp 2272, Fig. 4a) of derivative 102 (*Ptac*–ATS transposase) or of derivative 109 (*Ptac*–wild-type transposase) to put the mini-Tn*10* in cis to a transposase source. Derivative 103 (mini-Tn*10 kan*/*Ptac*–ATS transposase) carries a *Bam*HI Kan^R fragment from Tn*903* (Fig. 4b, TRN903.BACTERIA, GenBank J01839, bp 697 to 2392, *Pvu*II fragment converted to a *Bam*HI fragment with linkers), oriented in the backbone so that the *kan* gene promoter is transcribing in the same direction as the *Ptac* promoter. Derivative 104 (mini-Tn*10 tet*/*Ptac*–ATS transposase) carries a *Bam*HI Tet^R fragment from Tn*10* (Fig. 4a, bp 1942–4717, TRN10TETR.BACTERIA, GenBank J01830, bp 3402 to 627, *Bgl*II fragment converted to a *Bam*HI fragment with linkers), oriented in the backbone so that the *tetR* gene promoter is transcribing in the same direction and the *tetA* gene promoter is transcribing in the opposite direction as the *Ptac* promoter. Derivative 105 (mini-Tn*10 cam*/*Ptac*–ATS transposase) carries a *Bam*HI Tn*9*-derived Cam^R fragment from pACYC 184 (P18XCYC18.SYN, GenBank X06403, bp 3500 to 580, *Hae*II fragment converted to a *Bam*HI fragment with linkers), oriented in the backbone so that the *cam* gene promoter is transcribing in the same direction as the *Ptac* promoter. Derivative 106 (mini-Tn*10 kan URA3*/*Ptac*–ATS transposase) is identical to derivative 103, and derivative 107 (mini-Tn*10 can URA3*/*Ptac*–ATS transposase) is identical to derivative 105 except that in each case the element also carries a *Saccharomyces cerevisiae Bgl*II to *Bam*HI *URA3* gene fragment from YEP24 (YEP24.VEC, GenBank VB0067, *Eco*RI site at bp 2241 converted to *Bgl*II to the *Bam*HI site at bp 3784). The *URA3* gene is inserted upstream of the *kan* gene (for derivative 107) or the *cam* gene (for derivative 108) and is oriented so that it is transcribed in the opposite direction from the *kan* or *cam* gene. Derivative 108 (mini-Tn*10 kan Plac*/*Ptac*–ATS transposase) is identical to derivative 103 except that it also carries a *Plac–UV5 Bam*HI fragment downstream of the *kan* gene oriented so that the promoter is transcribing in the same direction as the *kan* gene promoter out across the transposon end. The sequence of the transposon end through the promoter fragment is shown in Fig. 5e. Derivative 110 (mini

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be obtained by communicating in writing with Dr. Susan Gottesman, Bldg. 37 Rm 4B03, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 (FAX: 301-496-0260).

2. Descriptions of Tn10 Derivatives. The structures of a number of Tn*10* derivatives useful for various types of transposon mutagenesis are described briefly below and in detail in Fig. 2 and its legend. These derivatives are carried on plasmid and/or phage vehicles, the structures of which are shown in Fig. 3.

Wild-type Tn*10*: Derivative 101. Wild-type Tn*10* has inverted repeats of insertion sequences IS10 Left and IS10 Right at its ends. The intervening material includes genes for tetracycline resistance (Tet^R) and other unknown determinants. This transposon can be used to generate insertions, deletions, or deletion/inversions. A partial restriction map of wild-type Tn*10* is shown in Fig. 4a; further details about the intervening material are summarized by Kleckner.[31] Wild-type Tn*10* is available on a pBR322-derived ampicillin-resistant (Amp^R) plasmid (pNK81), on a λ $O_{am}29$ $P_{am}80$ "hop phage" vehicle (λNK561), and on a λ O_{UGA} "hop phage" vehicle

Tn*10* *supF*/*Ptac*–wild-type transposase) carries a 248-bp *Xho*II *supF* fragment from bp 845 to 208 of PiAN7 (PIAN7.VEC, GenBank VB0066). Ligation of this *Xho*II fragment between the *Bam*HI sites of the transposon ends restores the *Bam*HI sites. The *supF* gene is oriented in the backbone so that its promoter transcribes in the opposite direction from the *Ptac* promoter. Derivative 111 (mini-*Tn10 lacZ kan*/*Ptac*–wild-type transposase) carries a promoterless *lacZ* *Bam*HI fragment and a Kan^R *Bam*HI fragment. The promoterless *lacZ* fragment consists of leader sequences from the *trpA* gene followed by the ribosome binding site and coding sequence of the *lacZ* gene [R. W. Simons, F. Houman, and N. Kleckner, *Gene* **53**, 85 (1987)]. The sequence from the transposon end through the leader region into the *lacZ* gene is shown in Fig. 5d. The fragment extends to the end of the *lacZ* gene (converted to a *Bam*HI site) at bp 4373 of ECOLAC.BACTERIA, GenBank J01636. The *lacZ* fragment is oriented in the backbone so that it would be transcribed in the same direction as read by the *Ptac* promoter. Downstream of this promoterless *lacZ* *Bam*HI fragment is a 1.5-kb Tn*903*-derived Kan^R *Bam*HI fragment from pUC4K (Pharmacia, Piscataway, NJ) oriented so that the *kan* gene promoter is transcribing in the same direction as the *Ptac* promoter. (A version of derivative 111 that is marked only with the promoterless *lacZ* fragment is also available as pNK2803.) Derivative 112 (Tn*10*–LK) carries the *'lacZ* fragment (ECOLAC.BACTERIA, GenBank J01636, bp 1309 to 4373, missing the first eight codons of the *lacZ* gene) oriented so that the gene is fused to the IS10 Right-derived end of the transposon. The sequence across the transposon end into the *'lacZ* gene is shown in Fig. 5b. The same Kan^R *Bam*HI fragment that marks derivative 103 is carried within the element downstream of the *'lacZ* gene but oriented so that it is transcribed in the opposite direction from the *'lacZ* gene. The second end of this element consists of the outermost 70 bp of IS10 Left. Derivative 113 (Tn*10*–LUK) is identical to derivative 112 except that an *S. cerevisiae URA3* *Bgl*II fragment (YSCODCD.PL, GenBank K02206, bp 1 to 1170) has been inserted at the *Bam*HI site

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ing with Dr. Susan Gottesman, ecular Biology, National Cancer Bethesda, MD 20892 (FAX: 301-

. The structures of a number of s of transposon mutagenesis are ig. 2 and its legend. These deriva- vehicles, the structures of which

d-type Tn*10* has inverted repeats Right at its ends. The intervening resistance (Tet^R) and other un- in be used to generate insertions, tial restriction map of wild-type bout the intervening material are Tn*10* is available on a pBR322- nid (pNK81), on a λ $O_{am}29$ $P_{am}80$ a λ O_{UGA} "hop phage" vehicle

248-bp *Xho*II *supF* fragment from bp 845 . Ligation of this *Xho*II fragment between e *Bam*HI sites. The *supF* gene is oriented in the opposite direction from the *Ptac* –wild-type transposase) carries a promot- agment. The promoterless *lacZ* fragment llowed by the ribosome binding site and F. Houman, and N. Kleckner, *Gene* **53**, l through the leader region into the *lacZ* the end of the *lacZ* gene (converted to a , GenBank J01636. The *lacZ* fragment is ribed in the same direction as read by the *lacZ Bam*HI fragment is a 1.5-kb Tn*903*- rmacia, Piscataway, NJ) oriented so that irection as the *Ptac* promoter. (A version noterless *lacZ* fragment is also available *'lacZ* fragment (ECOLAC.BACTERIA, t eight codons of the *lacZ* gene) oriented d end of the transposon. The sequence own in Fig. 5b. The same Kan^R *Bam*HI in the element downstream of the *'lacZ* posite direction from the *'lacZ* gene. The nost 70 bp of IS10 Left. Derivative 113 at an *S. cerevisiae URA3 Bgl*II fragment) has been inserted at the *Bam*HI site

(λNK370). In pNK81 the transposon is inserted in the *hisG* gene of *Salmonella typhimurium*; in both λ phages, the transposon is inserted in the *c*I gene.

Unlike the other λ vehicles described in Fig. 2, λNK561 and λNK370 are both suitable for transposon mutagenesis at any reasonable temperature. The transposon insertion in these phages confers an absolute defect in the *c*I gene, and the phage attachment site is deleted; other vehicles carry the *c*I857 mutation, so they can form abortive lysogens at temperatures below 37°.

ATS transposase fused to *Ptac*: Derivative 102. Derivative 102 contains the *ats1 ats2* transposase gene fused to the strong isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Ptac* promoter (*Ptac*–ATS transposase). This derivative should be used to complement in trans mini-Tn*10* constructs carried on vehicles that lack transposase (derivatives 112 and 113 below). It is available on a pBR322-derived Amp^R plasmid (pNK2881) and on a pACYC184-derived Tet^R plasmid (pNK2882). Derivative 102 also

between *'lacZ* and the Kan^R markers oriented to be transcribed in the same direction as the *'lacZ* gene. Derivative 114 carries a $'Kan^R$ fragment and an Erm^R fragment between the outermost 70 bp of IS10 Right. The $'Kan^R$ fragment consists of a leader sequence and the Tn*5* neomycin resistance gene starting at the second codon (TRN5NEO.BACTERIA, GenBank J01834, bp154) and extending through a *Sal*I site 1130 bp downstream [S. J. Rothstein, R. A. Jorgensen, K. Postle, and W. S. Reznikoff, *Cell* (*Cambridge, Mass.*) **19**, 795 (1980)]. The sequence of the transposon end through the start of the *'kan* gene is shown in Fig. 5c. A 1-kb selectable erythromycin resistance (Erm^R) fragment [B. Martin, G. Alloing, V. Mejean, and J. Claverys, *Plasmid* **18**, 250 (1987); K. Josson, T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon, *Plasmid* **21**, 9 (1989)] has been inserted into the *Sma*I site 970 bp away from the start of the *'kan* gene oriented so that it is transcribed in the same direction as the *'kan* gene. The entire transposon has been cloned on a *Hin*dIII to *Eco*RI fragment into *Salmonella hisG* and *hisD* sequences (indicated by a heavy line). Cloned upstream of the *'kan* end of the transposon are four tandem repeats of a 180-bp transcriptional termination sequence from the *rrnB* operon [J. Brosius, T. J. Dull, D. D. Sleeter, and H. F. Noller, *J. Mol. Biol.* **148**, 107 (1981)] that prevent expression of the *'kan* gene by nonspecific transcription from the vector (represented by a box containing TT). Transposase is provided to the transposon from a *Ptac*–wild-type transposase fusion (analogous to derivative 109) missing the innermost end of IS10 Right from a *Xho*II site at bp 1319 to a *Bgl*II site at bp 1942 of Tn*10* and extending to the *Cla*I site at bp 2591 of Tn*10* (Fig. 4a). This *Ptac*–transposase fusion is inserted immediately upstream of the transcriptional terminators and is oriented so that it is transcribed in the opposite direction from the *'kan* gene. Beyond the transposase-proximal end of the construction is another 740 bp of *Salmonella his* DNA terminating in an *Xba*I site. Beyond the mini-Tn*10*-proximal end of the construction is another 250 bp of *his* DNA terminating in an *Xba*I site. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; R, *Eco*RI; X, *Xho*I; Xb, *Xba*I. Open triangle, *Ptac*; filled triangle, *Plac–UV5*.

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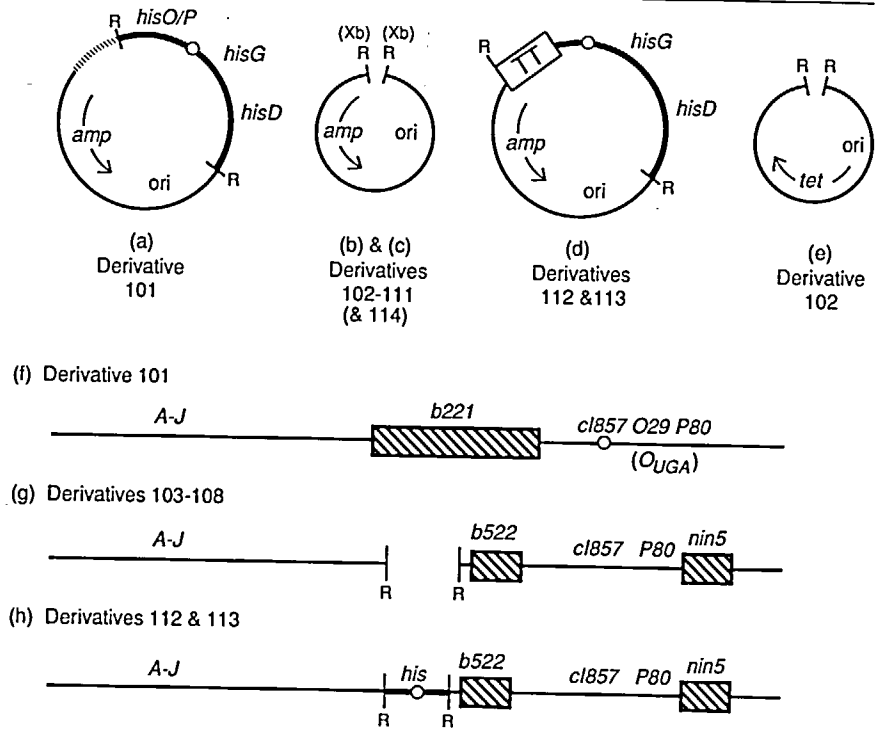

FIG. 3. Plasmid and λ vehicles for Tn*10* derivatives. (a) The pBR322-derived Amp^R plasmid vehicle for derivative 101 (pNK81) has been previously described [T. J. Foster, M. A. Davis, D. E. Roberts, K. Takeshita, and N. Kleckner, *Cell* (*Cambridge, Mass.*) **23**, 201 (1981)]. It carries 3.1 kb of the *Salmonella his* operon (indicated by a heavy line) containing the promoter/operator (*hisO/P*), *hisG*, and *hisD* genes on an *Eco*RI fragment with the transposon inserted by transposition into a hotspot in the *hisG* gene (STHISOP.EMBL, X13464, bp 379 to 387) oriented so that IS10 Right is closest to the *his* operon promoter. The site of transposon insertion is marked by an open circle. The *his* fragment is ligated at the *Eco*RI site of a deletion derivative of pBR322 described in (b) below which carries *amp* and the plasmid origin oriented so that the *amp* promoter is transcribing in the opposite direction from the *his* promoter. Between the promoter end of the *amp* gene and the promoter end of the *his* DNA on this plasmid are 1050 bp of uncharacterized "mystery DNA" (indicated as a shaded line). (b) The Amp^R plasmid vehicle for derivatives 102–111 is a deletion of bp 75 to 2353 of pBR322 (PBR322.VEC, GenBank VB0001). The *Hin*dIII site in this plasmid has been destroyed by filling in. The *Eco*RI fragments shown in Fig. 2 are inserted into the *Eco*RI site of this plasmid (at bp 4360 of the pBR332 sequence) oriented so that the *Ptac* promoter in the insert is transcribing in the opposite direction from the *amp* promoter. (c) Derivative 114 is inserted as an *Xba*I fragment into a derivative of the plasmid described in (b) where a 100-bp deletion has been made between the *Cla*I site (at bp 22 of pBR322) and the *Aat*II site (at bp 4285 of pBR322) and an *Xba*I linker has been inserted at the deletion junction. The insert is oriented so that the *Ptac* promoter is transcribing in the same direction as the *amp* promoter. (d) The Amp^R plasmid vehicle for derivatives 112 and 113 is derived from pNK81 [see (a) above] as follows. The transposon elements are inserted in the same *hisG*

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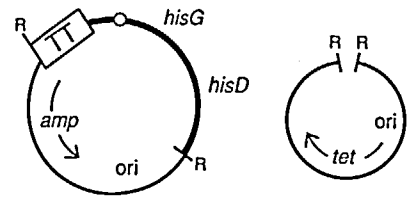
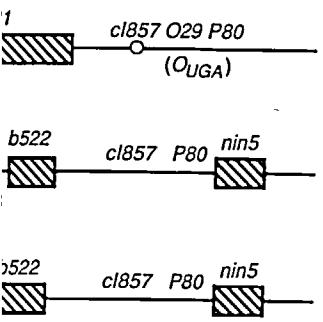


(d) Derivatives 112 &113

(e) Derivative 102



rivatives. (a) The pBR322-derived Amp^R been previously described [T. J. Foster, N. Kleckner, *Cell* (*Cambridge, Mass.*) **23**, ı *his* operon (indicated by a heavy line) and *hisD* genes on an *Eco*RI fragment with ıtspot in the *hisG* gene (STHISOP.EMBL, is closest to the *his* operon promoter. The ı circle. The *his* fragment is ligated at the cribed in (b) below which carries *amp* and ter is transcribing in the opposite direction l of the *amp* gene and the promoter end of ıracterized "mystery DNA" (indicated as derivatives 102–111 is a deletion of bp 75 001). The *Hin*dIII site in this plasmid has shown in Fig. 2 are inserted into the *Eco*RI uence) oriented so that the *Ptac* promoter on from the *amp* promoter. (c) Derivative ive of the plasmid described in (b) where I site (at bp 22 of pBR322) and the *Aat*II as been inserted at the deletion junction. ; transcribing in the same direction as the derivatives 112 and 113 is derived from n elements are inserted in the same *hisG*

forms the starting point for derivatives 103–108 below. The *Ptac*–transposase fusion is carried on a *Eco*RI fragment which extends from a site just upstream of the promoter to the *Eco*RI site at bp 3140 of Tn*10* (Fig. 4a) except that bp 1329–1942 at the inside end of IS10 Right have been deleted; this deletion removes the transposase binding site but does not affect the transposase structural gene.

Mini-Tn*10* with ATS transposase provided in cis: Derivatives 103–108. Mini-Tn*10* elements are generally short (400–3000 bp), can be engineered to carry a wide assortment of markers, and give rise to stable insertions because they do not carry a transposase gene. Mini-Tn*10* elements have at each terminus short segments carrying Tn*10* ends in inverted orientation; these Tn*10* ends flank one or more selectable markers. For mini-Tn*10* derivatives 103–108, the two ends are perfect inverted repeats of a 70-bp segment carrying the outside end of IS10 Right. These six elements are constructed on delivery vehicles that also carry a *Ptac–ats1 ats2* transposase gene in cis. Each element is carried on a *Hin*dIII fragment which is cloned in appropriate orientation into the *Hin*dIII site of derivative 102

site as wild-type Tn*10* in pNK81 and are oriented so that the *'lacZ* gene in each transposon would be transcribed in the same direction as the *his* operon. The site of transposon insertion is marked with an open circle. All of the "mystery DNA" and the *his* promoter sequences upstream of the transposon have been replaced with four tandem repeats of the transcriptional terminator sequences (represented by a box containing TT) from the *rrnB* operon which prevent nonspecific transcription from expressing the *'lacZ* gene on this plasmid. (e) The 4.2-kb Tet^R pACYC 184 plasmid vehicle for derivative 102 contains the *Eco*RI insert cloned in the *Eco*RI site at bp 1 of pACYC 184 (P18XCYC18.SYN, GenBank X06403) oriented so that *Ptac* is transcribing in the same direction as the *tet* gene. (f) The $O_{am}29\ P_{am}80$ λ hop phage vehicle for derivative 101 (λNK561) consists of the transposon inserted by transposition into the *c*I gene of a λ phage that is *b*221 *c*I857 $O_{am}29\ P_{am}80$; λNK370 is the same phage except that it carries an O_{UGA} mutation rather than the $O_{am}29\ P_{am}80$ mutations and can be used for making insertions into strains carrying amber or ochre nonsense suppressors. The site of transposon insertion in each phage is marked with an open circle. (g) The $P_{am}80$ λ hop phage vehicle for derivatives 103–108 is λ *b*522 *c*I857 $P_{am}80$ *nin5* with the appropriate *Eco*RI fragment from Fig. 2 substituted for the *Eco*RI fragment of λ DNA between bp 21226 and 26104 [R. W. Hendrix, J. W. Roberts, R. W. Stahl, and R. A. Weisberg (eds.), "Lambda II." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1983]. All inserts have been oriented in this phage vehicle so that the mini-transposon-proximal end of the insert is closest to the λ *J* gene. The *b*522 deletion removes the phage attachment site. (h) The $P_{am}80$ hop phage vehicle for derivatives 112 and 113 is λgt7–*his b*522 *c*I857 $P_{am}80$ *nin5* [λNK780; T. J. Foster, M. A. Davis, D. E. Roberts, K. Takeshita, and N. Kleckner, *Cell* (*Cambridge, Mass.*) **23**, 201 (1981)]. The λgt7–*his* backbone carries the same *Eco*RI fragment of the *Salmonella his* operon DNA as described for pNK81 [see (a) above] substituted in an unknown orientation for the λ DNA between *Eco*RI sites at bp 21226 and 26104. The transposons are inserted in the same *hisG* site (marked with an open circle) as in the corresponding plasmid constructions. The *b*522 deletion removes the phage attachment site.

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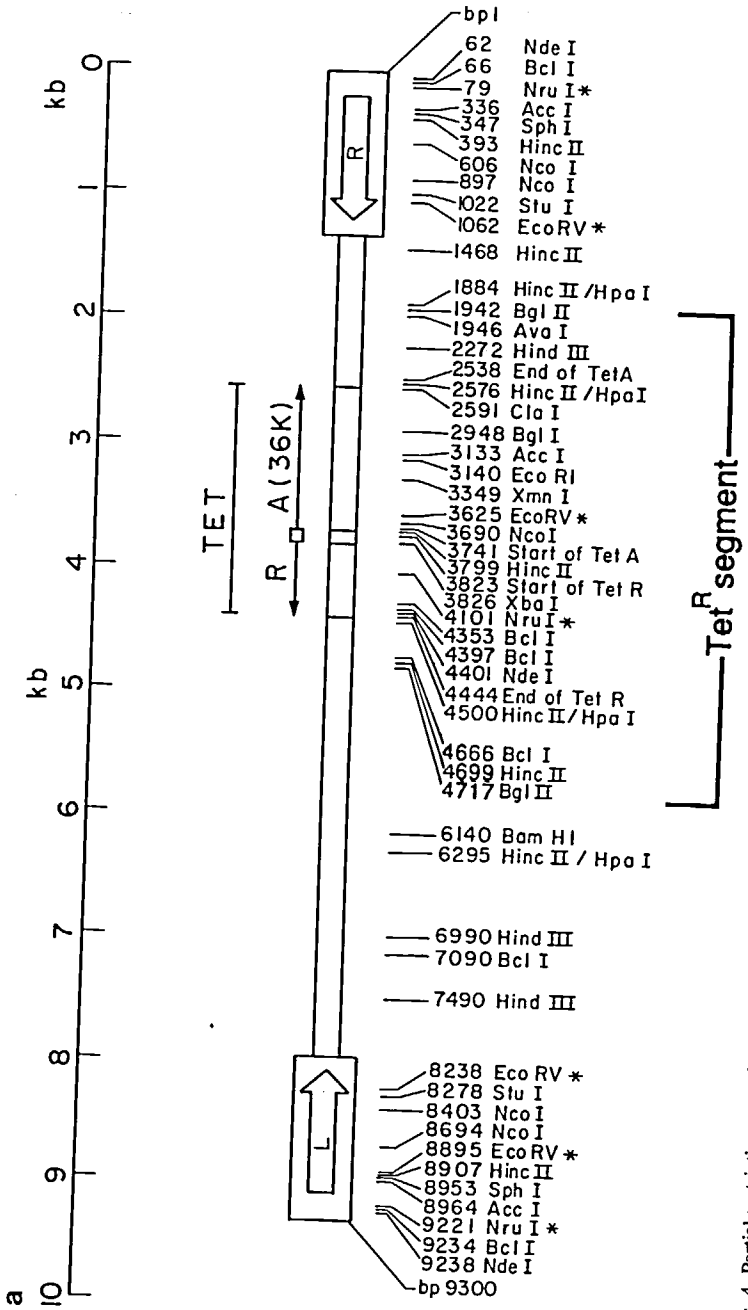

FIG. 4. Partial restriction maps for Tn*10* and the Tn*903* Kan^R segment. (a) Tn*10* includes IS10 Right, IS10 Left, and the tetracycline resistance determinant, which is composed of two divergently transcribed genes: *tetR*, the repressor, and *tetA*, the structural gene for a 36-kDa protein. The positions and orientations of the two genes are indicated by the two correspondingly marked divergent arrows. This map was compiled as described by Way *et al.* (1984), except that sites beyond the *HincII*/*HpaI* site at bp 4500 have been repositioned based on recent sequence data. The sequence of IS10 Right from bp 1 of this map to bp 1329 is given in TRN10IS1R.BACTERIA, GenBank J01829. Sequences of the *tet* genes corresponding to bp 5435 to 1406 on this map are given in TRN10TETR.BACTERIA, GenBank J01830. Asterisks indicate that Tn*10* contains *Eco*RV and/or *Nru*I sites in addition to those shown. The *Bgl*II Tet^R segment excised from Tn*10* to mark derivative 104 is indicated. (b) The Tn*903* Kan^R segment that marks derivatives 103, 106, 112, and 113 is shown. The first base pair of this map corresponds to TRN903.BACTERIA, GenBank J01839, bp 697. At the ends of the segment are 360-bp inverted repeats of the inner termini of two IS903 elements. The *kan* gene in this segment starts at bp 465 and extends to bp 1277 (GenBank J01839, bp 1162 to bp 1974). The *Stu*I* sites in the IS903 inverted repeats overlap with sites of *dcm* methylation and therefore can only be cleaved if DNA is prepared from a *dcm*$^-$ strain.

b

IS90

1675 Bam HI (formerly Pvu II)

(bp 2272 of Tn*10*, Fig. 4a).
analogous to each other in

Derivatives 103–105 a
transposon mutagenesis in
mycin resistance (Kan^R) fr
marked with a Tet^R fragme
marked with a chloramphe

Special care must be t
because a portion of the Tet
site at bp 1942 of Tn*10* (cor
Tet^R marker as drawn in F
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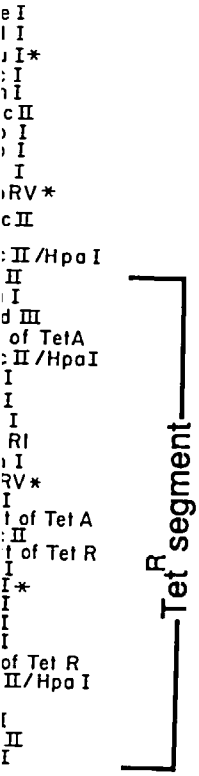

FIG. 4. Partial restriction maps for Tn*10* and the Tn*903* Kan^R segment. (a) Tn*10* includes IS10 Right, IS10 Left, and the tetracycline resistance determinant, which is composed of two divergently transcribed genes: *tetR*, the repressor, and *tetA*, the structural gene for a 36-kDa protein. The positions and orientations of the two genes are indicated by the two correspondingly marked divergent arrows. This map was compiled as described by Way *et al.* (1984), except that sites beyond the *Hin*cII/*Hpa*I site at bp 4500 have been repositioned based on recent sequence data. The sequence of IS10 Right from bp 1 of this map to bp 1329 is given in TRN10IS1R.BACTERIA, GenBank J01829. Sequences of the *tet* genes corresponding to bp 5435 to 1406 on this map are given in TRN10TETR.BACTERIA, GenBank J01830. Asterisks indicate that Tn*10* contains *Eco*RV and/or *Nru*I sites in addition to those shown. The *Bgl*II Tet^R segment excised from Tn*10* to mark derivative 104 is indicated. (b) The Tn*903* Kan^R segment that marks derivatives 103, 106, 112, and 113 is shown. The first base pair of this map corresponds to TRN903.BACTERIA, GenBank J01839, bp 697. At the ends of the segment are 360-bp inverted repeats of the inner termini of two IS903 elements. The *kan* gene in this segment starts at bp 465 and extends to bp 1277 (GenBank J01839, bp 1162 to bp 1974). The *Stu*I* sites in the IS903 inverted repeats overlap with sites of *dcm* methylation and therefore can only be cleaved if DNA is prepared from a *dcm*$^-$ strain.

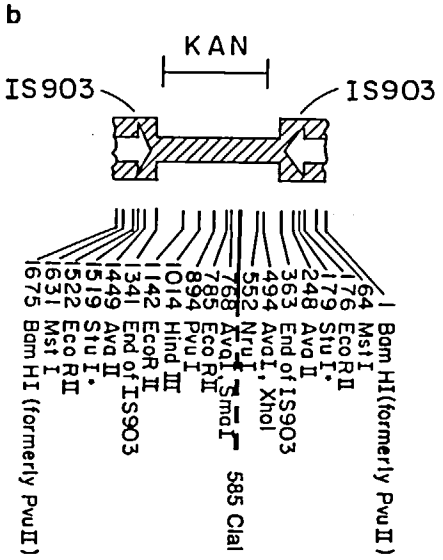

FIG. 4b

(bp 2272 of Tn*10*, Fig. 4a). The six mini-Tn*10* derivatives are thus exactly analogous to each other in structure.

Derivatives 103–105 are mini-Tn*10* constructs useful for general transposon mutagenesis in *E. coli*. Derivative 103 is marked with a kanamycin resistance (Kan^R) fragment from Tn*903* (Fig. 4b), derivative 104 is marked with a Tet^R fragment from Tn*10* (Fig. 4a), and derivative 105 is marked with a chloramphenicol resistance (Cam^R) fragment from Tn*9*.

Special care must be taken to verify the structure of derivative 104 because a portion of the Tet^R marker inside the transposon [from the *Bgl*II site at bp 1942 of Tn*10* (converted to a *Bam*HI site at the right end of the Tet^R marker as drawn in Fig. 2) to the *Eco*RI site at bp 3140 of Tn*10*] is directly repeated in the adjacent sequences outside the transposon. The sequence to the right of the transposon as drawn in Fig. 2 extends from the *Bgl*II site at bp 1942 to the *Hin*dIII site at bp 2272 of Tn*10*. Homologous recombination between this flanking sequence and the Tet^R marker will result in loss of the intervening transposon end. The sequence to the left of the transposon as drawn in Fig. 2 extends from the *Hin*dIII site at bp

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2272 to the *Eco*RI site at bp 3140 of Tn*10*. Homologous recombination between this sequence and the Tet^R marker will result in loss of *tet* genes and the other transposon end. The λNK1323 version of derivative 104 is particularly unstable since λ recombination functions are extremely active. Thus, stocks of this phage must each be grown from a single plaque and carefully checked for transposition activity.

Derivatives 106 and 107 are mini-Tn*10* constructs designed specifically for generating insertion mutations into cloned yeast genes. They carry both a marker that is selectable in *E. coli*, for isolation of insertions, and a marker that is selectable in yeast, for subsequent integration of the disrupted gene back into the yeast genome. Derivative 106 is marked with a Kan^R fragment from Tn*903* and the *Saccharomyces cerevisiae URA3* gene. Derivative 107 is marked with a Cam^R fragment from Tn*9* and the *S. cerevisiae URA3* gene.

Derivative 108 is a mini-Tn*10* marked with a Kan^R fragment from Tn*903* and contains the strong IPTG-inducible *Plac–UV5* promoter oriented to read out across one end of the transposon. This derivative can be used to generate promoter fusions of target genes to *Plac–UV5*, which will arise when the transposon inserts in the proper orientation upstream of the gene. The DNA sequence from the end of the transposon through the *Plac–UV5* promoter is shown in Fig. 5e.

Derivatives 103–108 are each available on a pBR322-derived Amp^R plasmid (pNK2859, pNK2883, pNK2884, pNK2885, pNK2886, and pNK2887) and on a $P_{am}80$ λ hop phage vehicle (λNK1316, λNK1323, λNK1324, λNK1325, λNK1326, and λNK1327). The λ vehicle for these elements is deleted for the phage attachment site and carries the temperature-sensitive *c*I857 repressor gene; insertions should be isolated at 37° or above, at which temperature the *c*I857 mutation will prevent formation of abortive lysogens. Plasmid and phage versions of mini-Tn*10* constructions analogous to derivatives 103–105 (mini-Tn*10 kan*, mini-Tn*10 tet*, and mini-Tn*10 cam* complemented to transposase by *Ptac*–ATS transposase) but each carrying a polylinker containing rare-cutting restriction enzyme sites are available to facilitate physical mapping of insertions by pulsed-field gel electrophoresis (J. Mahillon and N. Kleckner, unpublished observations).[41a]

Wild-type transposase fused to *Ptac*: Derivative 109. Derivative 109 consists of a wild-type transposase gene from IS10 Right fused to the strong IPTG-inducible *Ptac* promoter (*Ptac*–wild-type transposase). It is analogous to derivative 102 except that it lacks the *ats1* and *ats2* mutations and the small deletion at the end of IS10. It is carried on a pBR322-derived Amp^R backbone (pNK474). pNK474 is used as the starting point for construction of derivatives 110 and 111. The *Eco*RI fragment carrying

(a) Outside End IS10 Right

bp 1
CTG ATG AAT CCC CTA ATG
ATC TTG TCA TAT GAT C . .

(b) *'lacZ* Translational Fusion

... *CC GTC GTT* ...
codon 11

(c) *'kan* Translational Fusion

... CG GCC AAG CTA GCT TGG
leader sequence

(d) *lacZ* Transcriptional Fusion

... (*) GGATC CGGAC CGATG A
GCTGG CGGCA TTTTA ACTTT C
GAT TCA CTG GCC GTC GTT ...
codon 1

(e) *Plac-UV5* Promoter Fusion

... (*) GGATC CTGTT TCCTG TG
AGCCG GAAGC ATAAA GTGTA A
-35'

FIG. 5. Sequences through th
70 bp of IS10 Right is shown in
in the derivatives indicated. (b)
bases in the *lacZ* coding region
amino acids of the wild-type pr
bases in the *kan* coding region
acid of the wild-type protein. (d)
The bases indicated by (*) are t
ligated at the *Bcl*I site of the o
sequences is present in the tra
Shine–Dalgarno sequences are u
promoter in derivative 108. The
or CGC of the *Bam*HI linker lig
known which of the three sequen
to the first base of the transcript
−35′ region are indicated.

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Tn*10*. Homologous recombination
irker will result in loss of *tet* genes
K1323 version of derivative 104 is
ion functions are extremely active.
ıe grown from a single plaque and
ivity.
10 constructs designed specifically
ı cloned yeast genes. They carry
ɔli, for isolation of insertions, and
or subsequent integration of the
me. Derivative 106 is marked with
Saccharomyces cerevisiae URA3
Cam^R fragment from Tn*9* and the

l with a Kan^R fragment from Tn*903*
: *Plac–UV5* promoter oriented to
ɔn. This derivative can be used to
es to *Plac–UV5*, which will arise
ɔper orientation upstream of the
ıd of the transposon through the
ɜ.
ıble on a pBR322-derived Amp^R
884, pNK2885, pNK2886, and
ɡe vehicle (λNK1316, λNK1323,
NK1327). The λ vehicle for these
nent site and carries the tempera-
rtions should be isolated at 37° or
ıutation will prevent formation of
rsions of mini-Tn*10* constructions
n*10 kan*, mini-Tn*10 tet*, and mini-
ɜ by *Ptac*–ATS transposase) but
e-cutting restriction enzyme sites
ɔing of insertions by pulsed-field
Kleckner, unpublished observa-

Derivative 109. Derivative 109
e from IS10 Right fused to the
tac–wild-type transposase). It is
lacks the *ats1* and *ats2* mutations
It is carried on a pBR322-derived
sed as the starting point for con-
The *Eco*RI fragment carrying

(a) Outside End IS10 Right

bp 1
CTG ATG AAT CCC CTA ATG ATT TTG GTA AAA ATC ATT AAG TTA AGG TGG ATA CAC ATC TTG TCA TAT GAT C . . .

(b) *'lacZ* Translational Fusion

. . . *CC GTC GTT* . . .
codon 11

(c) *'kan* Translational Fusion

. . . CG GCC AAG CTA GCT TGG *ATT GAA CAA GAT GGA TTG CAC GCA GGT TCT* . . .
leader sequence codon 2

(d) *lacZ* Transcriptional Fusion

. . . (*) GGATC CGGAC CGATG AAAGC GGCGA CGCGC AGTTA ATCCC ACAGC CGCCA GTTCC
GCTGG CGGCA TTTTA ACTTT CTTTA ATGTT CACAC AGGAA ACAGC T *ATG ACC ATG ATT ACG*
GAT TCA CTG GCC GTC GTT . . .
codon 11

(e) *Plac-UV5* Promoter Fusion

+1
. . . (*) GGATC CTGTT TCCTG TGTGA AATTG TTATC CGCTC ACAAT TCCAC ACATC ATACG
operator -10'
AGCCG GAAGC ATAAA GTGTA AAGCC TGGGG TGCCT AATGA GTGAG AATTA ATTCC GGATC C . . .
-35'

FIG. 5. Sequences through the transposon end into fusion constructions. (a) The outermost 70 bp of IS10 Right is shown in bold type. This sequence is fused to the following sequences in the derivatives indicated. (b) Sequence into the *'lacZ* gene of derivatives 112 and 113. The bases in the *lacZ* coding region are italicized. The *'lacZ* fragment is missing the first eight amino acids of the wild-type protein. (c) Sequence into the *'kan* gene of derivative 114. The bases in the *kan* coding region are italicized. The *'kan* fragment is missing the first amino acid of the wild-type protein. (d) Sequence into the promoterless *lacZ* gene of derivative 111. The bases indicated by (*) are three possible sequences C, CG, or CGC of the *Bam*HI linker ligated at the *Bcl*I site of the outer end of IS10 Right. It is not known which of the three sequences is present in the transposon. The bases in the *lacZ* coding region are italicized. Shine–Dalgarno sequences are underlined. (e) Template/antisense sequence of the *Plac–UV5* promoter in derivative 108. The bases indicated by (*) are three possible sequences C, CG, or CGC of the *Bam*HI linker ligated at the *Bcl*I site of the outer end of IS10 Right. It is not known which of the three sequences is present in the transposon. The positions corresponding to the first base of the transcript from this promoter, the operator, the −10′ region, and the −35′ region are indicated.

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Ptac–wild-type transposase extends from a site just upstream of *Ptac* to an *Eco*RI site at bp 3140 of Tn*10* (Fig. 4a). The mini-Tn*10* elements of derivatives 110 and 111 are cloned on *Hin*dIII fragments into the *Hin*dIII site of derivative 109 (bp 2272 of Tn*10*, Fig. 4a).

New mini-Tn*10* constructs with wild-type transposase provided in cis: Derivatives 110 and 111. Derivative 110 is a mini-Tn*10* marked with the *supF* gene from PiAN7 (C. Jain and N. Kleckner). This derivative can be used to mutagenize a cloned gene in a λ phage carrying one or more amber mutations; phages that have acquired the *supF* transposon can be selected by virtue of their ability to make plaques on a nonsuppressing host strain. This derivative is also useful when a very short transposon is desired. Derivative 110 is only available on a pBR322-derived Amp^R plasmid (pNK1759).

Derivative 111 is a mini-Tn*10* marked with a promoterless *lacZ* gene and a *kan* gene from Tn*903* (J. Oberto and R. Weisberg). This derivative can be used to generate "operon fusions" in which the *lacZ* gene in the transposon is transcribed from the promoter of the gene into which it inserts. The sequence from the transposon end up through the start of the *lacZ* gene in this derivative is shown in Fig. 5d. Derivative 111 is only available on a pBR322-derived Amp^R plasmid (pNK2804). A version of this element that lacks the *kan* gene is also available on a pBR322-derived Amp^R plasmid (pNK2803). In derivatives 110 and 111 the markers are located between inverted repeats of the outermost 70 bp of IS10 Right, as in derivatives 103–108.

Other mini-Tn*10* elements complemented by wild-type transposase have been described previously. Some of these elements are complemented by wild-type transposase in cis.[4] Others require transposase provided in trans and contain various *E. coli* and yeast markers.[4,42] Most of these constructions are superseded by the derivatives above.

Mini-Tn*10* constructs which generate translational fusions of *lacZ* to target genes: Derivatives 112 and 113. Derivatives 112 and 113 are used to make *lacZ* translational fusions to a target gene. These two derivatives have been described previously as Tn*10*–LK[43] and Tn*10*–LUK,[39] respectively. Each derivative contains a *lacZ* gene lacking the appropriate transcription and translation start signals (*'lacZ*). The *'lacZ* gene is not expressed when the transposon is in its original (donor) site on the plasmid or phage vehicle, but $LacZ^+$ fusions can result from transpositions into an expressed target gene in appropriate orientation and reading frame. Such fusions can be isolated either as pure red colonies on MacConkey

[42] M. Snyder, S. Elledge, and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 730 (1986).
[43] O. Huisman and N. Kleckner, *Genetics* **116**, 185 (1987).

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ıds from a site just upstream of *Ptac* to) (Fig. 4a). The mini-Tn*10* elements of l on *Hin*dIII fragments into the *Hin*dIII Tn*10*, Fig. 4a).

ı wild-type transposase provided in cis: ve 110 is a mini-Tn*10* marked with the d N. Kleckner). This derivative can be n a λ phage carrying one or more amber ed the *supF* transposon can be selected aques on a nonsuppressing host strain. n a very short transposon is desired. on a pBR322-derived Amp^R plasmid

narked with a promoterless *lacZ* gene rto and R. Weisberg). This derivative usions" in which the *lacZ* gene in the promoter of the gene into which it sposon end up through the start of the wn in Fig. 5d. Derivative 111 is only p^R plasmid (pNK2804). A version of is also available on a pBR322-derived vatives 110 and 111 the markers are the outermost 70 bp of IS10 Right, as

plemented by wild-type transposase ome of these elements are comple- cis.[4] Others require transposase pro- *coli* and yeast markers.[4,42] Most of by the derivatives above.

rate translational fusions of *lacZ* to . Derivatives 112 and 113 are used to target gene. These two derivatives n*10*–LK[43] and Tn*10*–LUK,[39] respec- Z gene lacking the appropriate tran- s (*'lacZ*). The *'lacZ* gene is not ex- original (donor) site on the plasmid can result from transpositions into iate orientation and reading frame. s pure red colonies on MacConkey

oc. Natl. Acad. Sci. U.S.A. **83**, 730 (1986). 185 (1987).

lactose medium or, if they arise during growth of a clone, as red (Lac$^+$) papillae within white (Lac$^-$) single colonies.

Derivative 112 (Tn*10*–LK) carries an unexpressed *'lacZ* fragment and a Kan^R fragment from Tn*903*; derivative 113 (Tn*10*–LUK) is identical to derivative 112 except that it contains a *URA3* fragment from *S. cerevisiae* between the *'lacZ* and Kan^R markers. For these two mini-Tn*10* constructs, the ends of the transposon are 70-bp segments from the left and right ends of Tn*10*. The *'lacZ* gene is fused to the 70-bp end derived from IS10 Right. The sequence from the end of the transposon through the *'lacZ* gene for both derivatives is shown in Fig. 5b. Derivatives 112 and 113 are available on pBR322-derived Amp^R plasmids (pNK1207 and pNK2809) and on λ P_{am}80 hop phage vehicles (λNK1205 and λNK1224). Neither of these mini-Tn*10* constructions carries its own transposase. Transposase must be supplied in trans to these constructions from a plasmid carrying derivative 102.

Mini-Tn*10* which generates translational fusions to *kan* gene: Derivative 114. Derivative 114 carries a *kan* gene lacking the appropriate transcription and translation start signals (*'kan*).[44] The *'kan* gene is not expressed when the transposon is in its original (donor) site on the plasmid or phage vehicle, but Kan^R fusions can result from transpositions into an expressed target gene in appropriate orientation and reading frame. This derivative thus allows selection of transpositions without requiring destruction or elimination of the transposon donor molecule.

Derivative 114 is a mini-Tn*10* (L. Signon and N. Kleckner) which is marked with both an unexpressed *'kan* gene from Tn*5* and an erythromycin resistance (*erm*) gene from pAMβ1; these markers are located between inverted repeats of the outermost 70 bp of IS10 Right as in derivatives 103–108, 110, and 111. A *Ptac*–wild-type transposase fusion is present in cis on the transposon vehicle. The sequence through the transposon end into the *'kan* gene is shown in Fig. 5c. Derivative 114 is available on a pBR322-derived Amp^R plasmid vehicle (pNK2811).

C. Construction of New Mini-Tn10 Derivatives with Ptac–ATS Transposase

Plasmid-borne mini-Tn*10* derivatives analogous to derivatives 103–108 can be constructed in a single step from the plasmid version of derivative 103 (pNK2859) by substitution of any desired fragment of interest for the *Bam*HI fragment carrying the *kan* gene. Substitutions are particularly easy because the *Bam*HI backbone fragment does not give a viable plasmid if it is religated without an insert at the *Bam*HI site; in this case, the pair of inverted IS10 ends forms an inverted repeat which is lethal to the replicon.

[44] J. K. Sussman, C. Masada-Pepe, E. L. Simons, and R. W. Simons, *Gene* **90**, 135 (1990).

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A λ P_{am}80 hop phage version of such new plasmids can also be constructed by crossing the appropriate segment of the plasmid onto λNK1316 (or onto λNK1324 if the new plasmid is marked by Kan^R) using homology to each side of the mini-Tn*10*. (Note that mini-Tn*10* elements longer than about 5 kb are too big to fit into this λ genome.) Such crosses involve several steps. First, a stock of λNK1316 must be grown on a *supE* strain transformed with the new plasmid to allow recombination between the phage and plasmid. Second, the resulting λ stock should be titered for single plaques on a *supE* strain lacking the new marker at 30°. Under these conditions the *att*⁻ *c*I857 phage particles can form abortive lysogens, which are present as cells in the center of each (slightly turbid) plaque. Third, these plaques should be replica plated at 30° onto medium that selects for the new transposon marker. Replica plating of a phage lawn is exactly analogous to replica plating of bacterial colonies except that care should be taken that the layer of top agar remains on the original plate. The desired recombinants should occur at a frequency of about 1% of all plaques; if the frequency is much lower, something is wrong. Phages carrying the new marker can be recovered either by picking the corresponding plaque on the original plate or by purifying lysogenic colonies and recovering phage after heat induction. For heat induction, cultures should be grown in LB medium to early log phase at 30°, heated to 42° for 15 min, grown with aeration at 37° 1 h, and treated with chloroform and centrifuged to pellet debris; phage are recovered in the supernatant. The recombinant phage thus recovered should be tested for transposition by the usual procedure (below). Recombinant phage can also be selected in abortive lysogens by mixing phages with *supE* host cells and plating directly on selective medium at 30°. Phage can then be recovered by heat induction.

III. Procedures for Isolation and Processing of Transposon Insertions

A. Generating Transpositions from λ into Escherichia coli chromosome

1. Preparing Transposon Delivery Phage Lysate. A stock of the λ transposition vehicle should be grown on a suitable host in either a liquid or plate lysate. One procedure is given below; others are provided by Way *et al.*,[4] Silhavy *et al.*,[45] Arber *et al.*,[46] and Maniatis *et al.*[47] For vehicles

[45] T. J. Silhavy, M. L. Berman, and L. W. Enquist, "Experiments with Gene Fusions." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984.
[46] W. Arber, L. Enquist, B. Hohn, N. E. Murray, and K. Murray, *in* "Lambda II" (R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg, eds.), p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1983.
[47] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning, a Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1982.

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f such new plasmids can also be con-
segment of the plasmid onto λNK1316
d is marked by Kan^R) using homology
e that mini-Tn*10* elements longer than
his λ genome.) Such crosses involve
1316 must be grown on a *supE* strain
to allow recombination between the
sulting λ stock should be titered for
ıg the new marker at 30°. Under these
ırticles can form abortive lysogens,
nter of each (slightly turbid) plaque.
lica plated at 30° onto medium that
er. Replica plating of a phage lawn is
ɔf bacterial colonies except that care
ɔ agar remains on the original plate.
ɔur at a frequency of about 1% of all
lower, something is wrong. Phages
overed either by picking the corre-
e or by purifying lysogenic colonies
ıction. For heat induction, cultures
ly log phase at 30°, heated to 42° for
h, and treated with chloroform and
e recovered in the supernatant. The
ıould be tested for transposition by
ɔinant phage can also be selected in
with *supE* host cells and plating di-
ıage can then be recovered by heat

ɛssing of Transposon Insertions

into

Phage Lysate. A stock of the λ
on a suitable host in either a liquid
below; others are provided by Way
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ıuist, "Experiments with Gene Fusions."
Harbor, New York, 1984.
y, and K. Murray, *in* "Lambda II" (R. W.
A. Weisberg, eds.), p. 433. Cold Spring
York, 1983.
Molecular Cloning, a Laboratory Manual."
pring Harbor, New York, 1982.

that contain amber mutations $P_{am}80$ and/or $O_{am}29$, an *sulI* (*supE*) strain is best; *E. coli* C600 and LE392 are both usable. Strain NK5336, containing a UGA suppressor, is available on request for growth of λNK370. For λ stocks to be useful in generating transpositions, the titer of the stock should be at least 5×10^9 per ml, but titers of $1–2 \times 10^{10}$ should be obtainable routinely. Stocks should always be grown from a single plaque to reduce the frequency of O^+ or P^+ revertants and to reduce the possibility of losing the transposon construct, which occurs at a significant frequency for various vehicles for a variety of different reasons. The presence of the transposon construct in the lysate should also be verified either by measuring the ability of the lysate to give transpositions at a reasonable frequency or by scoring genetically for the presence of an associated transposon marker.

Stocks should be stored in the presence of 10 m*M* $MgSO_4$ at 5° and should be stable for many months. Protocols for freezing phage lysates are also available.[45] However, because λ phages of abnormal genome size are sometimes unstable, losses in titer of a phage stock can mean enrichment for aberrant types of phages (deletions or duplications). One of the most important reasons that phage lysates should always be made from single plaques is to avoid accumulation of such aberrant derivatives.

Procedure 1: Growing Phage λ Lysates

Solutions

TBMM: Tryptone Bl broth with maltose and magnesium. Composition per liter distilled water, 10 g tryptone, 5 g NaCl; autoclave, add filtered maltose to final concentration of 0.2% (w/v) and $MgSO_4$ from sterile stock to final concentration of 10 m*M*. Add filter-sterilized thiamin to final concentration of 1 μg/ml.

LB (Luria broth): Per liter distilled water, 10 g tryptone, 5 g yeast extract, 5 g NaCl.

TB1 agar plates: Make up tryptone broth without maltose or $MgSO_4$; add 11 g/liter Difco (Detroit, MI) agar before autoclaving; pour into petri plates when agar is partially cooled. Use the plates while relatively fresh (within 1 week). For growing phage that make very small plaques, substitute BBL trypticase (Fisher Scientific, Pittsburgh, PA) for tryptone.

Top agar: Tryptone broth with 7 g/liter agar.

TMG buffer: Tris, magnesium, gelatin. Composition per liter of distilled water, 1.2 g Tris base, 2.46 g $MgSO_4 \cdot 7H_2O$, 0.1 g gelatin. Adjust to pH 7.4; heat to dissolve gelatin and autoclave.

Preparing the Lysate. Grow permissive host to saturation in TBMM. Make serial dilutions of phage lysate in TMG and plate appropriate quantities in 2.5 ml top agar with 0.1 ml of an overnight culture of a permissive

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bacterial host (C600) on a TB1 agar plate. Incubate overnight at 37°. Pick a single plaque with a micropipette, transfer it to a 50-ml flask containing 10 ml LB plus 10 m*M* $MgSO_4$ and 0.1 ml of a fresh overnight culture of the permissive host, and shake the flask at 37°–39° for 4–5 hr. The culture should gradually become somewhat cloudy, then clear. Add a few drops of chloroform, shake, let sit 10 min, and then centrifuge at 5000 rpm for 10 min; save the supernatant.

Checking the Lysate. The phage lysate should be checked in the following ways: (1) It should have the appropriate titer on the permissive host. (2) It should have a titer below 10^{-4} of the permissive host titer on a nonsuppressing host. This confirms that the phage still carries amber mutations in essential genes. (3) The simplest way to check for the presence of the transposon is to test the lysate for transposition. Colonies resulting from stable incorporation of the transposon marker should arise at a reasonable frequency; furthermore, approximately 1% of such colonies should have acquired a new auxotrophic marker. Separate lysates made from individual plaques can be made in parallel and checked with a small-scale experiment; once the best lysate and appropriate multiplicity of infection (moi) are defined, a larger scale experiment can be done. A test for the antibiotic resistance marker itself can be done by mixing a sample of the lysate with a host which is lysogenic for λ and incubating for 1 hr to allow time for expression; double lysogens will form by homologous recombination between the infecting phage and the prophage at a frequency of 10^{-3} to 10^{-5} per infecting phage, and they can be detected on the appropriate antibiotic plates.

2. *Isolating Insertions.* In addition to considerations specific to the experiment of interest, the bacterial host should have the following characteristics. (1) It must be able to adsorb and inject λ. (2) It must not be permissive for lytic phage growth, that is, must not contain an inappropriate nonsense suppressor (see above). (3) Also, it should not contain a λ prophage. If having a prophage is unavoidable, then the strain should be *recA*$^-$ to prevent transfer of transposon markers to the prophage by homologous recombination. (4) The strain should be able to grow at 39°, since transpositions should be carried out at this temperature whenever possible. At lower temperatures, many of the phage vehicles are able to make abortive (unintegrated) lysogens because most of them carry the *c*I857 mutation which renders the repressor protein temperature-sensitive. λNK561, which carries a full Tn*10* element inserted into the λ *c*I gene, can be used at all temperatures. (5) The host must be sensitive to the transposon marker to be selected.

Transpositions from λ delivery vehicles are isolated by infecting sensitive cells with the phage under conditions which preclude lysogen formation and selecting for cells which have acquired the marker from the

transposon element. Usu
so the selection can be or

One general problem
a significant number of re
also be present. One ca
phage stock by titering o
of 10^{-7} or less is desirabl
be a problem; from the
the reversion frequency
revertants per plate. If t
grow on the plated cells a
tions. A second problem
sorbed phage on the sel
cells containing the desir
addressed by including s
destabilize phage particle
can also be removed by
procedures are described

Procedure 2: Transpositio

Materials

LB–antibiotic plates
Just before pourin
solution to a final
ml tetracycline (filt
mycin (filter-sterili
col (filter-sterilized
(filter-sterilized 5%

If sodium pyrophosph
final concentration of 2.5
solution. Plates which do
weeks after pouring if sto
be used within about 24
divalent cations eliminate
contain transpositions.

Protocol

(See Way *et al.*[4] for a

1. Grow cells overnigh
resuspend in 1/10 volume

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r plate. Incubate overnight at 37°. Pick
, transfer it to a 50-ml flask containing
0.1 ml of a fresh overnight culture of
flask at 37°–39° for 4–5 hr. The culture
t cloudy, then clear. Add a few drops
ı, and then centrifuge at 5000 rpm for

lysate should be checked in the follow-
propriate titer on the permissive host.
0^{-4} of the permissive host titer on a
ıs that the phage still carries amber
ıe simplest way to check for the pres-
he lysate for transposition. Colonies
of the transposon marker should arise
ıore, approximately 1% of such colo-
ıuxotrophic marker. Separate lysates
e made in parallel and checked with a
st lysate and appropriate multiplicity
ger scale experiment can be done. A
ırker itself can be done by mixing a
h is lysogenic for λ and incubating for
ble lysogens will form by homologous
ıg phage and the prophage at a fre-
phage, and they can be detected on

on to considerations specific to the
ıost should have the following charac-
orb and inject λ. (2) It must not be
at is, must not contain an inappropri-
). (3) Also, it should not contain a λ
ınavoidable, then the strain should
sposon markers to the prophage by
strain should be able to grow at 39°,
d out at this temperature whenever
ny of the phage vehicles are able to
ns because most of them carry the
essor protein temperature-sensitive.
ment inserted into the λ *c*I gene, can
ost must be sensitive to the transpo-

ıicles are isolated by infecting sensi-
ions which preclude lysogen forma-
ave acquired the marker from the

transposon element. Usually, the marker will confer antibiotic resistance, so the selection can be on rich plates containing the appropriate antibiotic.

One general problem in isolating transpositions from λ vehicles is that a significant number of replication-proficient O^+/P^+ revertant phage may also be present. One can determine the frequency of revertants in the phage stock by titering on a nonsuppressing host; a reversion frequency of 10^{-7} or less is desirable. Even with this low frequency, revertants may be a problem; from the total number of phage present on the plate and the reversion frequency one should be able to calculate the number of revertants per plate. If there are more than a few, these revertants will grow on the plated cells and lyse the cells containing the desired transpositions. A second problem is that the presence of a large number of unadsorbed phage on the selective plates can result in nonspecific killing of cells containing the desired transpositions. Both of these problems can be addressed by including sodium pyrophosphate in the selective plates to destabilize phage particles by chelation of Mg^{2+}; excess unadsorbed phage can also be removed by washing infected cells prior to plating; both procedures are described below.

Procedure 2: Transpositions from λ into Chromosome of *Escherichia coli*

Materials

LB–antibiotic plates: Add to LB broth 15 mg/ml agar and autoclave. Just before pouring, add the appropriate antibiotic from a sterile solution to a final concentration as indicated and mix well: 15 μg/ml tetracycline (filter-sterilized 1% stock in ethanol); 30 μg/ml kanamycin (filter-sterilized 3% stock in water); 25 μg/ml chloramphenicol (filter-sterilized 2.5% stock in ethanol); 100 μg/ml ampicillin (filter-sterilized 5% stock in water).

If sodium pyrophosphate is to be included, add after autoclaving to a final concentration of 2.5 m*M* by dilution from a 125 m*M* sterile stock solution. Plates which do not contain pyrophosphate can be used for 1–3 weeks after pouring if stored at 5°. Plates containing pyrophosphate must be used within about 24 hr of being poured, as continued chelation of divalent cations eliminates growth of all cells regardless of whether they contain transpositions.

Protocol

(See Way *et al.*[4] for a slightly different procedure.)

1. Grow cells overnight in TBMM, concentrate by centrifugation, and resuspend in 1/10 volume LB.

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2. Adsorb 0.1 ml of concentrated cells and various quantities of phage for 15 min at room temperature and 15 min at 37°. Assume a concentration of cells of about 10^9–10^{10}/ml in the concentrated culture, and add phage to give an moi of between 0.1 and 1 phage per cell.

It usually pays to do a small-scale experiment first to determine the multiplicity of infection which maximizes transpositions and minimizes killing by the phage. The experiment can then be repeated with multiple tubes at the best multiplicities of infection.

3. As for removing unadsorbed phage (which is optional), it is usually not necessary to wash away free phage or allow time for expression of transposon markers. However, both steps can be accomplished by the following simple procedure. Add 5 ml LB with sodium citrate (50 m*M*), centrifuge the cells, and resuspend in 5 ml fresh LB plus citrate. Citrate will inactivate free phage that have not adsorbed; washing also removes free phage. Grow the infected culture for about 1 hr at 37°; this allows expression of antibiotic resistance.

4. Plate 0.1 ml of the infected cell mixture on antibiotic selection plates at 39° overnight. If transposition is low (i.e., as with wild-type Tn*10*) the cells can be concentrated before plating. The frequency of insertions obtained per infecting phage should be approximately 10^{-3} to 10^{-4} for most of the minitransposon constructs, somewhat lower for constructs in which transposase is provided from a plasmid, and about 10^{-7} for wild-type Tn*10*.

3. Obtaining Insertions of Interest by Direct Screening or Selection. In some cases, colonies that arise on selective plates as the result of transposition will be screened for a phenotype introduced by the insertion mutation, either by visual inspection of the original selective plates or by replica plating. On the original selective plates, colony morphology markers can be scored. Expression of *lacZ* or other sugar fermentation genes can also be scored on pH indicator plates such as MacConkey lactose as long as the total number of cells plated is not too high. However, screening for β-galactosidase or alkaline phosphatase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) or 5-bromo-4-chloro-3-indolyl phosphate (XP) is not practical at this stage because of background expression from transiently infected cells which do not give rise to stable transpositions. If colonies are screened by replica plating, the condition (i.e., antibiotic selection) used to select the primary transpositions should be included in the screening plates as well. Cells which have not undergone a transposition event are always present on the original selective plate along with the colonies of interest.

Identification of a transposon insertion in a particular typical gene in

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cells and various quantities of phage
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ertion in a particular typical gene in

E. coli usually requires screening of about 10,000 transposition events. There are approximately 1000 such nonessential target genes. For Tn*5*, Tn*10*–ATS, Mu, and Tn*3*, a modest level of redundancy is required to ensure that the gene of interest is hit. For insertions of Tn*10* elements with wild-type transposase insertion specificity, it may be necessary to screen a larger number. An example of the screening of initial transposon insertions for colony morphology is found in Trisler and Gottesman[48]; an example of screening fusions for expression under particular circumstances can be found in Kenyon and Walker.[49]

4. Screening and Selecting for Insertions in Secondary Hosts. For many applications, identification of insertions of interest requires transfer of the insertions from the host in which they were selected to a secondary host. One general approach, described below, is to pool a large number of cells carrying independent insertions, grow P1 on the pool, and then transduce the mixture of insertions into a secondary host by selecting for the transposon marker. The resulting transductants are screened or selected for the phenotype of interest. A second approach, described by Silhavy *et al.*,[45] involves direct isolation of transposon insertions in a strain lysogenic for a temperature-inducible P1 followed by pooling and generation of a transducing lysate by induction. In either case, a good P1 lysate grown on a large number of pooled transposition events generated in a wild-type host should be a standard reagent for any laboratory carrying out genetic analysis in *E. coli*.

A frequent application in which transduction of pooled insertions is necessary is for isolation of an insertion which is linked to, rather than within, a gene of interest. In this case, the secondary recipient carries a marker in the region of interest and transductants are screened for loss of that marker. Since P1 carries about 1 min of an *E. coli* chromosome, even a pool of a few hundred independent transpositions is likely to contain at least one insertion that is within P1 transducing distance of any point of interest. Insertions into a particular gene may also be identified in a secondary host if the phenotype of interest requires a property incompatible with isolation of insertions, for example, *su*$^+$ or the presence of a λ prophage. In this case, the number of pooled transposition events must be as great as for direct isolation of insertions (see above).

For identification of insertions in very specific locations, either within or tightly linked to a gene of interest, a two-step approach is often useful. Insertions into the general region of interest are selected by looking for

[48] P. Trisler and S. Gottesman, *J. Bacteriol.* **160**, 184 (1984).
[49] C. J. Kenyon and G. C. Walker, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2819 (1980).

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transduction of a general marker such as an auxotrophy, and the resulting transductants are then screened in a second step to identify the specific subset desired.

Procedure 3: Making P1 Lysates on Pools of Chromosomal Insertions

Select at least a few thousand independent chromosomal insertions; more is better. Flood each selective plate with 2–3 ml of LB plus 0.1 *M* citrate which has been chilled to 4°. Use a glass spreader to resuspend the colonies in the broth. Pipette off the broth into a centrifuge tube and wash the plate once again. Pooled cells will usually give a dense cell suspension. Cells should be washed with LB at 4°, at least twice and preferably 3 or 4 times; 0.1 *M* citrate should be included in the first washes but omitted from the last one. Cells can then be resuspended at about 10^9 cells/ml and frozen with dimethyl sulfoxide (DMSO) or in 50% glycerol for future use.

For a P1 lysate of the pool, resuspend cells at about 10^{10}/ml in 0.1 ml LB plus 10 m*M* $MgSO_4$ and 5 m*M* $CaCl_2$ with P1*vir* (or P1 HFT[20]) at an moi of about 1; let adsorb 10 min, dilute into 10 ml LB with 10 m*M* $MgSO_4$ and 5 m*M* $CaCl_2$, and shake at 37°–39° for 2 hr, or until lysis. Treat with chloroform, centrifuge at 5000 rpm, and save the supernatant. This lysate, which should be stored at 5°, can be kept for many months.

5. Identifying Linked Transposon Insertions. To identify an insertion near a gene of interest, one should select a strain which carries a mutation in that gene and in which loss of the mutation can be selected or screened. This strain is used as a recipient for P1 transduction with the lysate grown on the transposon insertion library from a wild-type host (see above). Transductants receiving the marker on the transposon are selected and screened or selected for loss of the mutation in question. Approximately 1/100 transductants should bring the wild-type allele of the selected marker in along with the transposon insertion. Linkage of the transposon to the marker of interest should be verified by purifying candidate transductants and using them as donors in P1 transductional crosses with the original mutant strain as recipient. Insertions which show a linkage of at least 30% are easiest to use. Insertions that exhibit less than 5% linkage can be used if necessary, but further confirmation that the linkage is real is wise. From crosses that exhibit significant linkage, it is useful to save transductants which both have and have not lost the marker allele; the latter can be used to transfer the mutation to new hosts. Examples of the identification of insertions in or near a gene of interest by transposition followed by P1

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ı as an auxotrophy, and the resulting
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Pools of Chromosomal Insertions

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plate with 2–3 ml of LB plus 0.1 *M*
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:st by transposition followed by P1

transduction from pooled insertions can be found in Trun and Silhavy[50] and Lemotte and Walker.[51]

B. Isolating Transpositions from λ to Multicopy Plasmid

The general procedure for isolating transpositions into a multicopy plasmid is the same as for isolating transpositions into the *E. coli* chromosome (Procedure 2) except that the recipient strain contains the target plasmid of interest. It is best if the plasmid is present only as a monomer in the population of cells used to isolate transpositions. When dimers or higher multimers are present, isolation of insertion mutations by loss of function is impossible, many insertions go into essential plasmid backbone sequences rather than into the region of interest, and restriction mapping of insert plasmids is more difficult. Plasmids growing in *recA*$^+$ hosts frequently exist as head-to-tail dimers, so special procedures are required to ensure that the host cells used for transposition contain primarily monomer plasmids. Essentially pure monomer plasmid populations can be obtained by transforming a *recA*$^-$ or *recJ*$^-$ host and examining the DNA from a handful of transformants; monomers are easily distinguished from higher forms by gel electrophoresis of undigested plasmid DNA. These purified monomers can then be used to transform a second strain in which transpositions occur. If possible, a *recA*$^-$ or *recJ*$^-$ strain should be used. However, it is acceptable to transform a *recA*$^+$ strain with pure plasmid monomers and carry out transpositions in the resulting transformants as long as they are not subcultured any more than necessary before the transposition experiment. In a small culture grown directly from a single transformant, the majority of plasmids are still monomers. The general strategy for identification of plasmid inserts involves extraction of DNA from pools of insertion-containing colonies and retransformation into a new host.

Procedure 4: Generating Insertions into Plasmids

Carry out Procedure 2 using a suitable strain carrying the plasmid of interest. It is not necessary to select for a marker on the plasmid, but it is desirable in order to eliminate the possibility of transposon insertions into that marker. Make many independent pools of cells and isolate plasmid DNA; minipreps are sufficient. Approximately 0.1% of the plasmid DNA molecules in these preparations will contain an insert, since 1% of insertions occur into plasmid DNA and since only a subset of the plasmids in a given colony will contain the insert. Use these

[50] N. J. Trun and T. J. Silhavy, *Genetics* **116**, 513 (1987).
[51] P. Lemotte and G. C. Walker, *J. Bacteriol.* **161**, 888 (1985).

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DNAs to transform a strain in which the transposon marker can be selected. Also, it is best if this recipient strain is unable to adsorb λ, as there may be a surprisingly significant amount of phage DNA and/or phage particles in the DNA preparation, and infection of the strain plasmid and bacterial collections with phage is not desirable. Ideally, insertions into the target gene of interest are scored genetically; less ideally, physical analysis is used.

C. Isolating Insertions into Phage λ

To isolate insertions into bacteriophage λ, a lysate of the target phage is made on a host strain carrying the transposition vehicle. Phage which have acquired the plasmid transposon marker are then selected in one of several possible ways, some of which are described below and none of which is foolproof.

1. Integration of Transposon-Carrying Phage into Chromosome by Phage-Mediated Integration. If the phage in question is integration-proficient (*att*$^+$ *int*$^+$), the phage lysate can be used to infect an appropriate λ-sensitive host and lysogens carrying the transposon marker selected. If the phage is defective for making repressor, Int protein, and/or in the phage attachment site, these determinants can be provided in trans by a helper phage. Recovery of the desired phage from resulting lysogens may be complicated. The target phage may recombine with the helper phage prior to integration. Furthermore, the helper phage may still be present in the final lysogen; in fact, if it is providing *att* function, it must be present. [Technical note: Efficient lysogenization of λ requires that each infected cell receives at least 5 phages.] In some cases, use of a defective prophage to provide some functions can minimize the problems of helper phage (see below for such a procedure using the λ D69 vector).

2. Integration of Transposon-Carrying Phage into Chromosome of λ Lysogen by Homologous Recombination with Resident Prophage. In this case, the phage lysate is used to infect a previously established λ lysogen, and cells that acquire the transposon marker are selected. This method has the advantage that it will work for a phage of any genotype, regardless of whether it has the ability to replicate, lysogenize, or establish repression on its own. It has the disadvantage that the efficiency of recovery of marker-containing phage is low (10^{-3} to 10^{-5} per transposon-containing genome), which means that a large volume of phage lysate may have to be processed. Furthermore, the complications inherent in the helper coinfection approach above apply here as well.

3. Selection of Transductants Arising from nin$^+$ *Phage Vectors.* If the phage vector has an intact *nin* region (*nin*$^+$), the phage will grow as a

stable plasmid in a host w
transductants can be sele
vector carries nonsense
selected in a standard *su*
phage carry a deletion in
phage genome.

4. Direct Detection o
scribed by R. Maurer t
plaques.[52] A tetracycline
maltose (0.2%) and MgS
trated 10-fold in 10 m*M* M
containing 7 μg/ml tetracy
to 0.5 ml of the concentra
Tet^R phage forming plaq
should be checked with e

5. Direct Selection of
quired an insertion of a
phage carries *supF*-suppr
insertion can be isolated a
phage carries no nonsen's
using conditions in which
nonsense mutation in the h
selection is described by
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on selective minimal plat
defective gene; strains bea
used for selection. The an
host strains carrying a tetr
mutation (Maurer *et al.*[52];

Procedure 5: Generating I

Transform the plasmid
the minitransposon donors
a *lacI*^{Q} host such as *E. c*
which might occur with lo
transformed cells in TBM
the plasmid itself and infec

[52] R. Maurer, B. C. Osmond, E.
(1984).
[53] M. Snyder, S. Elledge, and R.
[54] J. Messing, this series, Vol. 10

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stable plasmid in a host which interferes with N function (*nusA*), and stable transductants can be selected in such a host. Alternatively, if a *nin*$^+$ phage vector carries nonsense mutations in the *N* gene, transductants can be selected in a standard *su*$^-$ host.[16] Unfortunately, most standard cloning phage carry a deletion in the *nin* region in order to reduce the size of the phage genome.

4. Direct Detection of tet Transductants. A procedure has been described by R. Maurer to detect phage carrying *tet* genes directly in plaques.[52] A tetracycline-sensitive bacterial lawn is grown in LB with maltose (0.2%) and $MgSO_4$ (10 m*M*) to 2–3 × 10^8 cells/ml, then concentrated 10-fold in 10 m*M* $MgSO_4$. The phage lysate is titered on LB plates containing 7 μg/ml tetracycline in top agar without drugs, after adsorption to 0.5 ml of the concentrated cells. A very faint lawn forms, with only the Tet^R phage forming plaques. The precise concentration of tetracycline should be checked with each batch of drug.

5. Direct Selection of supF-Carrying Phage. λ phage which have acquired an insertion of a *supF* element can be selected directly. If the phage carries *supF*-suppressible nonsense mutations, phage carrying the insertion can be isolated as plaque-forming phage on a *su*$^-$ host.[6,53] If the phage carries no nonsense mutation, the desired phage can be isolated using conditions in which plaques form only if the phage suppresses a nonsense mutation in the host strain used as the plating bacteria. One such selection is described by Phadnis *et al.*[6] Alternatively, if the host strain carries an auxotrophic mutation in a gene such as *his* or *trp*, plaques arise on selective minimal plates only if the phage permits expression of the defective gene; strains bearing nonsense mutations in such genes might be used for selection. The analogous approach should also be applicable for host strains carrying a tetracycline resistance determinant with a nonsense mutation (Maurer *et al.*[52]; D. Botstein, personal communication, 1990).

Procedure 5: Generating Insertions into λ

Transform the plasmid donor into a λ-sensitive host. Because many of the minitransposon donors use a *Ptac*–transposase fusion, it is best to use a *lacI*^{Q} host such as *E. coli* JM101,[54] to avoid plasmid rearrangements which might occur with long-term expression of transposase. Grow the transformed cells in TBMM with the appropriate antibiotic to select for the plasmid itself and infect with the target phage by the method of Proce-

[52] R. Maurer, B. C. Osmond, E. Shecktman, A. Wong, and D. Botstein, *Genetics* **108,** 1 (1984).
[53] M. Snyder, S. Elledge, and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 730 (1986).
[54] J. Messing, this series, Vol. 101, p. 20.

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dure 1. IPTG can be included during preparation of the phage lysate to increase transposase expression. The resulting lysate should be considered a low-frequency transducing lysate for the transposon insertion in question; about $1/10^6$ phage will be likely to contain the desired marker.

Procedure 6: Isolating Transpositions into λ D69 Carrying Cloned Insert

λ D69 is an *att*$^+$ *imm21* phage which has been widely used for cloning purposes.[45,55,56] An *imm* λ *c*I857 derivative of λ D69 is also available.[56] In these phage, foreign DNA is cloned into the phage *int* gene, so the resulting transducing phage are *int*$^-$. Integration of such transducing phage can be specifically targeted to *attB*, the normal site of phage integration, by providing Int function in trans; this approach is used for the initial identification of phage derivatives carrying transposon insertions as described here. Alternatively, integration can be targeted into the bacterial region homologous to the cloned *E. coli* insert by forcing integration in the absence of Int function. In this case, integration usually occurs by homologous recombination at the desired site, although occasional integrations into other sites also occur. Integration via bacterial homology is useful for obtaining transposon insertions in the relevant region of the bacterial genome as described in the following section.

In Procedure 6, a pool of transposon insertions into the phage is generated by growing a lysate of λ D69 on a strain carrying the desired transposon construct as in Procedure 5, using this pool to infect an appropriate host strain, and selecting for a marker on the transposon. Phage can then be recovered from such lysogens, individually or in pools, by an appropriate induction procedure. Phage carrying transposon insertions in the cloned segment can be distinguished from those carrying insertions in nonessential regions of the phage genome by subsequent tests.

The host strain used for isolation of λ D69::Tn insertions is N6377, which carries a defective prophage that provides both Int function and the bacterial attachment site (*attB*). λ D69 phage integrate very efficiently into this host, which makes it easy to collect large numbers of λ D69::Tn derivatives. Subsequent recovery of integrated phages by prophage induction is also easy because the prophage carries the temperature-sensitive *c*I857 repressor allele.

It may be desirable to select for λ D69::Tn phages in a host other than N6377 where, for example, the phenotypic effects of the transposon insertion can be assayed. The defective prophage carried in N6377 can be transferred to another strain by P1 transduction using as a donor strain

[55] S. Mizusawa and D. Ward, *Gene* **20**, 317 (1982).
[56] J. A. Brill, C. Quinlan-Walshe, and S. Gottesman, *J. Bacteriol.* **170**, 2599 (1988).

SG12021, which is identi
insertion linked to the pr
selected in the new host
and can be detected by
prophage (see genotype
the new strain by selecti

Bacterial Strains

N6377: *att B.B'*
(SG12021 is N637

Protocol

1. To generate and r
transducing phage on the
N6377 or another host ca
supplemented with biotin
heat to 42° for 15 min to
ml cells and 0.1 ml of the
lysogens on LB–antibioti

2. Recovery of λ D69:
gens will yield lysates pr
*c*I857 derivative of λ D69
10 m*M* $MgSO_4$ to a densi
shake at this temperature
centrifuge at 5000 *g* for 1
*c*I$^+$ D69 derivative was us
will require both raising t
prophage and synthesize I
induce the *imm21* *c*I$^+$ pha

About 5–50% of the λ
(λ D69) lysogen will have
prophage in such a way a
will necessarily have beco
from the Int$^-$ phage by a
test of Enquist and Weisbe
ing the cloned insert provi
transposon insert is within
was inserted within the b
should also have lost the
red plaque test should carr

[57] L. W. Enquist and R. A. Weis

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preparation of the phage lysate to esulting lysate should be considered r the transposon insertion in ques- to contain the desired marker.

into λ D69 Carrying Cloned Insert

ch has been widely used for cloning ative of λ D69 is also available.[56] In o the phage *int* gene, so the resulting n of such transducing phage can be rmal site of phage integration, by proach is used for the initial identifi- transposon insertions as described e targeted into the bacterial region sert by forcing integration in the tegration usually occurs by homolo- e, although occasional integrations via bacterial homology is useful for ie relevant region of the bacterial section.

n insertions into the phage is gener- strain carrying the desired transpo- g this pool to infect an appropriate ker on the transposon. Phage can is, individually or in pools, by an ge carrying transposon insertions in ed from those carrying insertions in ome by subsequent tests.

of λ D69::Tn insertions is N6377, t provides both Int function and the phage integrate very efficiently into llect large numbers of λ D69::Tn tegrated phages by prophage induc- e carries the temperature-sensitive

λ D69::Tn phages in a host other henotypic effects of the transposon e prophage carried in N6377 can be ansduction using as a donor strain

82).
sman, *J. Bacteriol.* **170,** 2599 (1988).

SG12021, which is identical to N6377 except that it carries a *nadA*::Tn*10* insertion linked to the prophage. If tetracycline-resistant transductions are selected in the new host, 20% will have acquired the defective prophage and can be detected by virtue of a *bio*$^-$ marker which accompanies the prophage (see genotype below). The Tn*10* element can be eliminated from the new strain by selecting for a Nad$^+$ (Tet^S) revertant.

Bacterial Strains

N6377: *att B.B' bio936 int*$^+$ *xis*$^+$Δ(*Sal–Xho*) *c*I857Δ*bio r*$^-$*m*$^+$ (SG12021 is N6377 *nadA*::Tn*10*)

Protocol

1. To generate and recover λ D69::Tn phage, grow a lysate of the transducing phage on the transposon-donating host (Procedure 5). Grow N6377 or another host carrying the defective prophage at 32° in TBMM supplemented with biotin (1 μg/ml) to approximately 2 × 10^8 cells/ml, heat to 42° for 15 min to induce Int synthesis, and return to 32°. Mix 0.1 ml cells and 0.1 ml of the phage lysate at 32° for up to 1 hr, then select lysogens on LB–antibiotic plates.

2. Recovery of λ D69::Tn phage from lysogens. Induction of the lysogens will yield lysates predominantly containing the desired phage. If a *c*I857 derivative of λ D69 was used, grow the lysogenic cells in LB plus 10 m*M* $MgSO_4$ to a density of about 2 × 10^8 cells/ml, heat to 40°, and shake at this temperature for 90 min. Add a few drops of chloroform, centrifuge at 5000 *g* for 10 min, and save the supernatant. If a λ *imm21* *c*I$^+$ D69 derivative was used to isolate the lysogens, induction of lysogens will require both raising the temperature to 40° (to induce the defective prophage and synthesize Int) and treating the cells with ultraviolet light to induce the *imm21* *c*I$^+$ phage.

About 5–50% of the λ D69 phage produced on induction of an N6377 (λ D69) lysogen will have undergone recombination with the defective prophage in such a way as to lose the cloned insert; such recombinants will necessarily have become Int$^+$. Such Int$^+$ phage can be distinguished from the Int$^-$ phage by analysis of individual plaques in the red plaque test of Enquist and Weisberg.[57] Comparison of phage containing and lacking the cloned insert provides a simple way of determining whether the transposon insert is within the cloned segment or outside. If the transposon was inserted within the bacterial DNA insert, *int*$^+$ recombinant phage should also have lost the transposon. All phage which are white on the red plaque test should carry the insertion.

[57] L. W. Enquist and R. A. Weisberg, *Virology* **72,** 147 (1976).

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D. Moving Insertions After They Are Isolated

1. From Plasmid to Chromosome. An insertion isolated in a cloned bacterial fragment on a plasmid can be transferred directly to the chromosome in several ways. (1) The insert region can be transferred by direct DNA transformation after cleavage of the plasmid on one or both sides of the transposon; a *recBC sbcB, recD* host must be used.[58–60] (2) The insert region can be integrated by recombination between the intact plasmid and the chromosome, with appropriate selection for both integration and excision events[61]; a *polAts* host must be used. (3) The insert can also be moved into the chromosome by first moving it into λ and then following the procedure in the next section. In all of these cases, use of a minitransposon is advantageous because it prevents stable transposon integration into the host chromosome by transposition and thus eliminates an unwanted source of background. In the case of linear transformation, the frequency of desired events is so low that use of a minitransposon is essential.

2. From Phage λ to Chromosome

(a) Moving insertions one at a time. A transposon insertion into a bacterial segment carried on λ can be moved into the chromosome in a two-step process. First the phage is integrated into the bacterial chromosome by recombination between the cloned insert and the homologous region of the chromosome, creating a nontandem duplication of the cloned segment, one copy of which is inactivated by the transposon insertion. Then, in a second step, the prophage is eliminated by recombination between the repeated segments. The resulting recombinants may have retained or lost the transposon insert depending on where recombination occurred; recombinants carrying the transposon can be identified by the presence of the transposon marker and/or their mutant phenotype. Occurrence of recombinants of the latter type at high frequency is taken as reasonable evidence that the disrupted gene is not essential.

This procedure is most easily carried out using a *c*I857 (temperature-inducible) derivative of *int*⁻ phage such as λ D69 (see Maurizi *et al.*[62] for an example). For the first step, lysogens are selected at low temperature in a wild-type, nonlysogenic host, and a handful of such lysogens is isolated

[58] S. Kanaya and R. J. Crouch, *Proc. Natl. Acad. Sci U.S.A.* **81**, 3447 (1984).
[59] S. C. Winans, S. J. Elledge, J. H. Krueger, and G. C. Walker, *J. Bacteriol.* **161**, 1219 (1985).
[60] C. B. Russell, D. S. Thaler, and F. W. Dahlquist, *J. Bacteriol.* **171**, 2609 (1989).
[61] N. I. Gutterson and D. E. Koshland, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4894 (1983).
[62] M. R. Maurizi, P. Trisler, and S. Gottesman, *J. Bacteriol.* **164**, 1124 (1985).

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3. Cloning Insertions ou

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n, *J. Bacteriol.* **164,** 1124 (1985).

and purified. Lysogens arise via the desired integration events if normal phage integration is blocked by inactivation of the phage *int* gene. If the phage is *int*$^+$, the majority of integrations by the Int pathway must be eliminated by deletion of the bacterial attachment site in the host chromosome; however, since insertions occur at a significant frequency into secondary attachment sites, lysogens isolated in an *att*Δ*int*$^+$ situation must be characterized to be sure that the prophage is located in the appropriate place.

For the second step, temperature-resistant derivatives of appropriate lysogens can be isolated by subjecting a culture of cells to a heat treatment and looking for survivors. It is best to identify cured cells first by virtue of their loss of phage immunity and then to score for the transposon marker in a second step. If one selects directly for temperature resistance and antibiotic resistance, abnormal events or secondary suppressing mutations may be obtained, particularly if the disrupted gene is essential.

(b) Isolating multiple transposon insertions in a cloned bacterial segment in a single step. If a bacterial gene has been cloned in λ *c*I857 D69 (or an equivalent *c*I857 integration-deficient phage vector), large numbers of transposon insertions into the cloned bacterial gene can easily be obtained from an unpurified pool of λ D69::Tn phage. In brief, a large number of λ D69::Tn lysogens are made (Procedure 6, Step 1), pooled, and induced (Procedure 6, Step 2). The resulting mixed lysate, which will contain a number of different transposon insertions into the cloned segment as well as insertions into the phage genome, is used to infect a nonlysogenic host; cells which have stably acquired the transposon marker are selected at low temperature. Most of the resulting colonies will contain phage integrated by bacterial homology within the cloned segment. However, some will contain no prophage but will have arisen because a transposon insertion present in the cloned segment of a λ D69::Tn phage has been substituted for the homologous region in the host chromosome by virtue of recombination on both sides of the insert. Cells of the latter type will be viable at temperatures which induce the *c*I857 prophage; they can be identified by pooling colonies carrying the transposon marker, replating on agar plates at 40°–42°, and screening survivors for retention of the transposon marker. (In fact, additional cells of the desired type may also be generated during the temperature induction procedure itself.) Some of the clones that survive heat selection will have become resistant to λ owing to the presence of phage released by induction of nonsurviving cells. It should be noted that insertions into an essential gene cannot be isolated in this way. Applications of this method are described by Maurizi *et al.*[62] and Brill *et al.*[56]

3. Cloning Insertions out of Bacterial Chromosome. In general, cloning

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insertions out of the bacterial chromosome is straightforward. Special precautions that must be taken when cloning out *tet* insertions are discussed in Section I above. Recently, new tools which facilitate rapid cloning and subsequent sequencing of Tn*10* and mini-Tn*10* insertions have been developed.[63] A recombinant M13mp vector carrying the central portion of the appropriate *tet* or *kan* segment is integrated into the chromosome at the site of the insertion by recombination; recombinants are selected using a chloramphenical resistance determinant present on the vector. Appropriate digestion, ligation, and transformation of chromosomal DNA from such integrants yields M13 phage carrying the segment of interest in a form suitable for sequencing and probing of other libraries for the wild-type gene.

A PCR (polymerase chain reaction) strategy for cloning genes disrupted by Tn*10* or mini-Tn*10* insertions has also recently been described.[64]

E. Eliminating Transposon Insertions

If a linked transposon insertion has been used to bring in a mutation of interest, it is often desirable to then eliminate the transposon (and its associated selectable marker). This can be done in several ways. The easiest and most general method is to plan strain constructions in such a way that markers can be removed by subsequent P1 transduction. For example, one can introduce a *recA* mutation by a single transduction using a linked Tn*10* insertion; however, a better strategy is to first introduce a *srl*::Tn*10* or *srl*::Tn*5* insertion, which is closely linked to *recA*, and then use a non-transposon-containing *recA*$^-$ donor strain to bring in that mutation linked to Srl$^+$.

However, transposon insertions which are located in appropriate genes can be eliminated by direct selection for restoration of gene function. These precise excision events occur at frequencies of 10^{-6}–10^{-10} depending on the particular insertion site. Revertants can be isolated by growing cells overnight in rich broth, concentrating each culture 25-fold, and plating 0.2 ml onto a selective plate. For very low reversion frequencies, larger volumes and multiple cultures (to take advantage of possible jackpots) may be necessary. For transposon insertions that carry tetracycline resistance, the drug marker (but rarely the transposon) can be eliminated by direct selection for tetracycline sensitivity as described in Section I,E,1 on Tn*10*-promoted adjacent deletions.

[63] M. L. Michaels, *Gene* **93**, 1 (1990).
[64] C. S. J. Hutton, A. Seirafi, J. C. D. Hinton, J. M. Sidebotham, L. Waddell, G. D. Pavitt, T. Owen-Hughes, A. Spassky, H. Buc, and C. F. Higgins, *Cell* **63**, 631 (1990).

F. Mapping of Insertion Approaches

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[66] D. Roberts, "Genetic Analysis of
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F. Mapping of Insertions in Bacterial Chromosomes: Special Approaches

It is easy to use transposon insertions as mapping markers because of the presence of the associated easily selectable marker. In addition to the classic approaches, some additional approaches have been developed which take special advantage of transposon insertions as genetic tools.

1. Mapping Transposon Insertions Located Near or Within Gene of Interest. The general location of the insertion can be determined by transducing it into a number of different Hfr strains having different points of origin. The resulting derivatives can be analyzed in either of two ways. They can be tested for their ability to transfer the transposon marker in a short interrupted mating (30 min); the marker will be transferred efficiently only in those Hfr strains in which it is proximally located and transferred early. Alternatively, the derivatives can be used to transfer a series of prototrophic markers and the selected exconjugants screened for the transposon marker; this is essentially the classic approach.

2. Mapping Nontransposon Mutations Using a Standard Set of Hfr Strains Bearing Transposons. Singer *et al.* have constructed a set of Hfr strains, each of which contains an antibiotic resistance transposon less than 20 min from its origin of transfer.[65] Crosses are performed using these strains as donors and an appropriate mutant strain as the recipient; exconjugants that have received the transposon insertion are selected and then screened for loss of the mutation of interest. Hfr strains which yield wild-type recombinants at a frequency of 50% indicate that the mutation is between the point of origin of the Hfr and the transposon. Both Tet^R and Kan^R Hfr derivatives are available.

3. Mapping Nontransposon Mutations with Hfr Strains and Minitransposon Plasmids. The same approach can be used even without employing a set of stably isolated transposon-containing Hfr derivatives. In this case, an active transposon is introduced on a multicopy plasmid into a series of standard Hfr derivatives. Cultures of such strains contain random transpositions into the Hfr genome. Thus, when they are mated with an appropriate mutant strain for a short period of time, the resulting exconjugants all carry proximally located transposon insertions, and Hfr strains with points of origin near the mutation of interest will frequently transfer the marker of interest along with these transposon insertions as well.[66]

[65] M. Singer, T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross, *Microbiol. Rev.* **53**, 1 (1989).

[66] D. Roberts, "Genetic Analysis of Mutants of *Escherichia coli* Affected for Tn*10* Transposition." Ph.D. Thesis, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA (1986).

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4. Physical Mapping of Insertions by Pulsed-Field Gel Electrophoresis. If a chromosomal transposon insertion contains a site for a restriction enzyme that cuts only rarely in the bacterial chromosome, it is possible to map the position of the insertion physically by cleaving chromosomal DNA with the enzyme and analyzing the restriction fragments by pulsed-field gel electrophoresis. For example, the *E. coli* chromosome is cleaved into 22 fragments by the enzyme *Not*I, and these fragments have been ordered around the chromosome; a Tn*5* insertion, which contains *Not*I sites in each of its IS50 inverted repeats (or any other transposon insertion containing a *Not*I site), can be mapped to the appropriate fragment of the chromosome by determining which fragment in the insertion-bearing strain is cleaved internally by *Not*I.[67] The same strategy can be used for a number of other rare-cutting enzymes with sites of cleavage that have been positioned on a chromosomal map.[68] Tn*10* derivatives that carry a polylinker containing rare-cutting restriction enzyme sites are described in Section II,B,2.

Acknowledgments

We are grateful to Nancy Trun, James Kirby, and Douglas Bishop for comments on the manuscript and to Howard Benjamin for helping with computer graphics. Research on transposons by J. Bender and the laboratory of N. Kleckner are supported by grants from the National Institutes of Health (5 RO1 GM25326-12) and the National Science Foundation (DMB-8820303).

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[68] Y. Kohara, K. Akiyama, and K. Isono, *Cell* (*Cambridge, Mass.*) **50**, 495 (1987).

[8] *In Vivo* Genetic Engineering with Bacteriophage Mu

By EDUARDO A. GROISMAN

Introduction

Bacteriophage Mu was discovered as a temperate phage which upon lysogenization generated mutations in the host with a high frequency (Taylor, 1963); its name stands for mutator phage. Mu has become the subject of study of several groups and the starting material for a series of derivatives and strategies for *in vivo* genetic engineering which are discussed in this chapter. A brief review on Mu biology is presented first to facilitate understanding of the particular features of phage Mu which have been crucial for the development of the different tools and techniques.

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Overview of Mu Biolog

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