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In Vivo Packaging of Cosmids in Transposon-Mediated Mutagenesis

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A technique was developed that permits the analysis of large regions of DNA by transposition mutagenesis. Large fragments of the pTiA6NC plasmid were cloned into the broad host range cosmid pHK17 and subjected to transposition mutagenesis by Tn*3*. Cosmids containing Tn*3* insertions were selected by in vivo packaging by λ *c*I857 and transduction to a new host. The insertions were localized by DNA restriction endonuclease analysis and transferred to the Ti-plasmid by marker exchange.

Advancements in recombinant DNA technology have provided powerful tools for the study of bacterial genomes. The construction of broad host range cloning vectors, the use of transposable antibiotic resistance markers as mutagenic agents, and the reintroduction of mutagenized DNA by the marker rescue technique have made many bacterial genomes available to genetic analysis (14). These methods have proved to be valuable in the genetic analysis of the large Ti (tumor-inducing) plasmids of *Agrobacterium tumefaciens*. Previous studies have provided functional and organizational maps of the plasmid pTiA6NC, an octopine-type Ti plasmid (3, 5, 6, 12). Two areas of the plasmid, excluding the region that is concerned with replication, are involved in tumorigenesis. One region, the T-DNA, is transferred to the plant genome upon infection of a plant wound by the bacterium (2, 16). This region was the subject of extensive genetic analysis by site-directed mutagenesis (6). Initial studies had found that the majority of Ti plasmid mutations affecting virulence are located in a second region of the plasmid (5, 12). This region, the virulence region, extends over approximately 35 kilobases (kb) of DNA and is separated from the T-DNA region by another 20 kb of DNA.

The method we describe here was developed to study more extensively the large virulence region of pTiA6NC. It is outlined in Fig. 1. It builds on the aforementioned technical advancements in the manipulation of bacterial genomes. A clone bank of large regions of pTiA6NC was constructed previously by using the vector pHK17 (8). The cosmid pHK17 contains the λ *cos* site, which permits the in vitro packaging of large DNA fragments into λ phage heads, and the replication functions of the broad host range plasmid pRK2, which permits plasmid replication in both *Escherichia coli* and *Agrobacterium* spp. Although cosmids were originally created for the selection of large recombinant DNA molecules by in vitro packaging, we exploited the ability of the temperature-sensitive phage λ *c*I857 to package cosmids in vivo during the lytic cycle under the derepressed conditions (17). The cosmid pHK210 is presented as an example. This cosmid contains the left end of the T-DNA and extends to just beyond the rightmost virulence gene, *virE* (Fig. 2). The cosmid also confers on the bacterium resistance to kanamycin and tetracycline. *E. coli* strain HB101(pHK210) was mated with *E. coli* strain HU679 containing F′::Tn*3*. Because pHK210 alone is nontransferable, this step effectively introduced Tn*3* into HB101(pHK210). The strains were mated on 0.45-μm nitrocellulose filters (Millipore Corp.) on L agar (11), and transconjugants were selected on L agar containing 50 μg of kanamycin and 50 μg of carbenicillin per ml at 30°C. One loopful of cells from the transconjugants (approximately 200 to 500 colonies per plate) was inoculated into 3 ml of L broth containing 50 μg of kanamycin and 50 μg of carbenicillin per ml, 0.10% maltose, and 0.01 M $MgCl_2$ and grown at 37°C to a density of 5×10^8 cells per ml. The cells were centrifuged and suspended in 0.5 ml of L broth plus 0.01 M $MgCl_2$. A 50-μl sample of a phage λ *c*I857 lysate (10^{10} PFU/ml) was added to the culture, which was allowed to stand for 15 min at 37°C. Five milliliters of L broth (37°C) was added to the culture. The culture was incubated for 20 min at 42°C and then incubated at 37°C with shaking. When lysis was evident (2 to 3 h), a transducing phage lysate was prepared by adding 0.1 volume of $CHCl_3$. The lysate was incubated another 15 min at 37°C. The debris was pelleted by centrifugation, and the lysate was decanted to a sterile tube. In addition to the

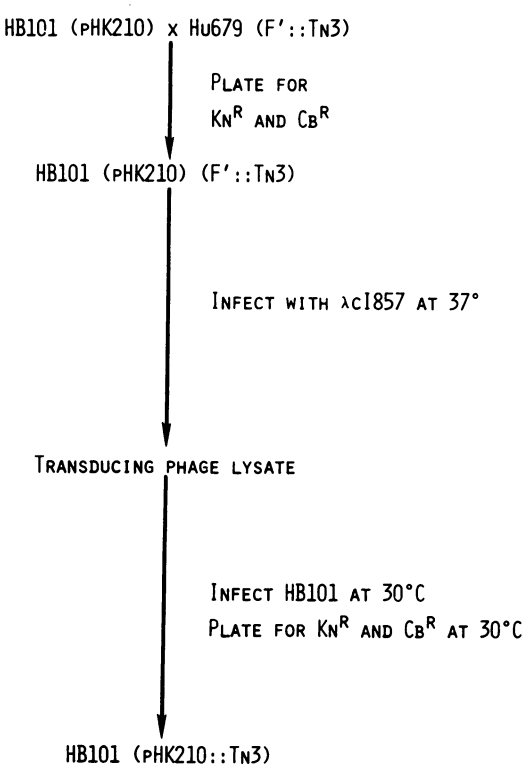

FIG. 1. In vivo packaging of cosmids in transposon-mediated mutagenesis. F'::Tn*3* and λ *c*I857 were kindly provided by Daniel Portnoy and Allison Weiss, respectively. Kn^R, kanamycin resistance; Cb^R, carbenicillin resistance.

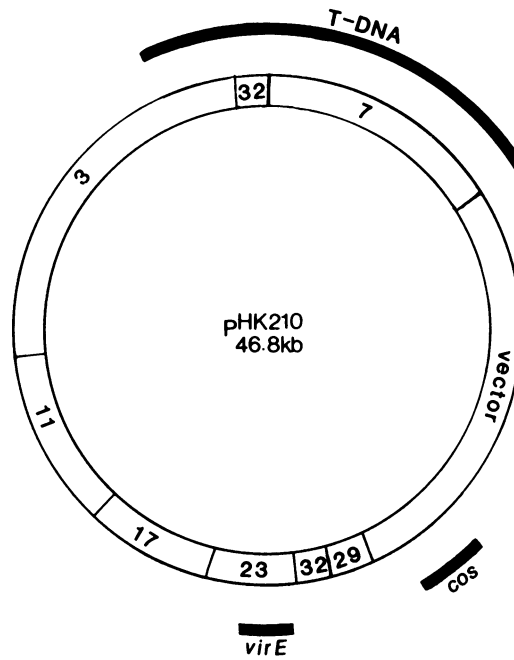

FIG. 2. *Eco*RI restriction map of pHK210.

packaging of λ *c*I857 during propagation on HB101(pHK210) (F'::Tn*3*), pHK210 with and without Tn*3* insertions was packaged into phage heads. The analysis of three independent transducing phage lysates indicated that the procedure yielded 1.7×10^9 PFU/ml of lysate. Approximately two phage per 1,000 PFU contained a cosmid, as indicated by the frequency of kanamycin resistance transduction (2.9×10^6 colonies per ml of lysate). One cosmid in 1,500 contained a Tn*3* insertion, as indicated by the cotransduction of kanamycin and carbenicillin resistance (1.9×10^3 colonies per ml of phage lysate).

The recovery of colonies containing pHK210::Tn*3* required the temperature-sensitive repressor of λ *c*I857. At 37°C, the phage lytic cycle is induced by inactivation of the repressor, whereas at 30°C the phage lysogenizes stably. Cosmid transductants are usually lysogenic for λ *c*I857 due to the excess of phage. Therefore, transductants were plated at 30°C, and all subsequent manipulations of the transductants were done at 30°C to prevent the induction of λ *c*I857. Killing due to λ *c*I857 was reduced by the use of a λ lysogen, and recovery of cosmid transductants improved.

A total of 94 pHK210 cosmid transductants were analyzed. In each case, the transductant appeared to contain an authentic Tn*3* insertion into the cosmid. These insertions were distributed throughout the cloned region. However, the insertions were not distributed randomly on the basis of fragment size since some fragments received a disproportionate number of insertions (Table 1). The locations of the transposons on

TABLE 1. Distribution of Tn*3* insertions of pHK210

Fragment[a]	% Cosmid (kb)	% Insertions (no.)
vector	27.4 (12.8)	13.8 (13)
3	24.4 (11.4)	16.0 (15)
7	15.6 (7.3)	8.5 (8)
11	11.4 (5.35)	27.6 (25)
17	8.2 (3.85)	13.8 (13)
23	5.6 (2.60)	9.6 (9)
29	2.9 (1.35)	0 (0)
32f	1.1 (1.05)	3.2 (3)
32g	1.1 (1.05)	3.2 (3)
—[b]	—	5.3 (5)

[a] Fragments are *Eco*RI restriction fragments of pHK210. The sizes were determined by De Vos et al. (4).

[b] Five cosmids contained deletions that presumably were generated during Tn*3* transposition.

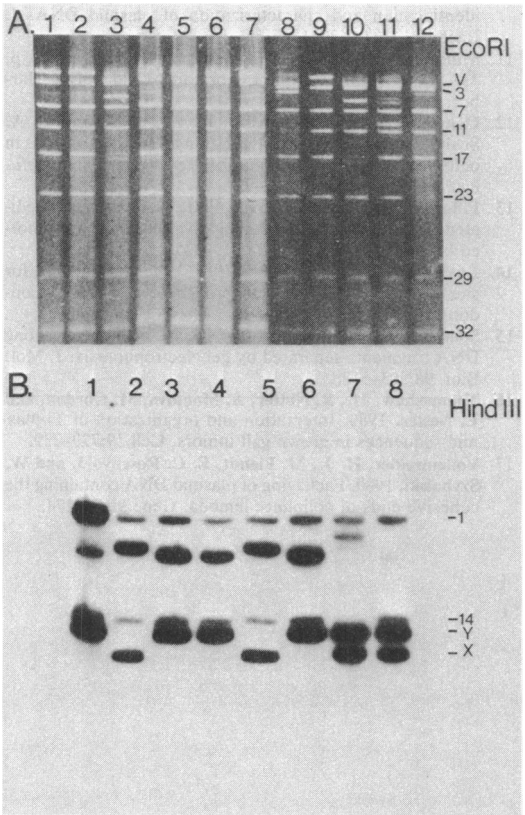

FIG. 3. (A) Gel of *Eco*RI-cut pHK210::Tn*3* that was stained with ethidium bromide. Plasmid DNA was isolated by the procedure of Birnboim and Doly (1). Electrophoresis was done according to Meyers et al. (10). Tn*3* insertions are located in the following *Eco*RI fragments: V (vector), lanes 1, 2, and 9; *Eco*RI-3, lanes 4 through 7; *Eco*RI-11, lane 8; *Eco*RI-17, lanes 3 and 10; *Eco*RI-32f, lane 11. (B) Hybridization analysis of site-directed Tn*3* mutagenesis of the Ti plasmid. Total bacterial DNA was isolated from *Agrobacterium* strains that contained Tn*3* introduced onto the Ti plasmid by the procedure of Ruvkun and Ausubel (13). The DNA was digested with *Hin*dIII and prepared for Southern hybridization analysis. The hybridization probe was pNW31-8,29-1, which contains the left end of the Ti plasmid T-DNA region (15). Tn*3* insertions are located in the following *Hin*dIII fragments: *Hin*dIII-X, lanes 1, 3, 4, and 6; *Hin*dIII-Y, lanes 2 and 5; *Hin*dIII-14, lane 7; wild type, lane 8.

the cosmid were determined by analysis with the proper combination of restriction endonucleases. *Eco*RI, for example, does not cut within Tn*3*, and the size of a fragment that contains Tn*3* is increased by 5 kb (Fig. 3A) (7). A second enzyme was used to locate the transposon within a particular *Eco*RI fragment (data not shown). The position was checked again once an insertion was transferred to the Ti plasmid by marker exchange. This was done by digesting total bacterial DNA and determining the presence of the appropriate restriction fragments by the method of Southern (Fig. 3B) (15).

The procedure described in this paper has several attractive features. The in vivo packaging procedure allows efficient selection of mutagenized cosmids and may circumvent difficulties associated with the cotransformation of plasmids that are used to introduce a transposon. The technique is not limited to Tn*3* nor is delivery limited to F′::Tn*3* but can be used with any transposon that confers a selectable trait. We chose F′::Tn*3* because the plasmid is too large for packaging and cotransduction by λ *c*I857. Cotransduction can occur presumably by a transposition-mediated cointegration event with small plasmids (13), and this might unnecessarily complicate the analysis of the cosmid insertions. Klee et al. used Tn*5* that was delivered by a defective λ::Tn*5* as the mutagenic agent (9). The method is relatively simple, and because cosmids contain inserts of 30 kb or greater, it is particularly well suited for site-directed mutagenesis. A large, yet limited, region of DNA is mutagenized; the long regions of DNA that flank an insertion provide extensive homology for marker exchange recombination; and insertions into small restriction fragments that are not adequately positioned within another larger fragment, and therefore not amenable to marker exchange, can be isolated. Klee et al. have used this method successfully to analyze the *vir* region of pTiA6NC and the region that extends between the *vir* region and the T-DNA (9). The insertions within the T-DNA portion of pHK210 extended the left boundary of the *tms* locus due to insertion of Tn*3* into regions missed by Tn*5* (6). It also has been used to generate mutations in large regions of the root-inducing plasmid of *A. rhizogenes*.

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