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Genetics

Tn*5*supF, a 264-base-pair transposon derived from Tn*5* for insertion mutagenesis and sequencing DNAs cloned in phage λ

(dideoxynucleotide sequencing/recombinant DNA cloning/suppressor tRNA)

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ABSTRACT **We constructed a derivative of transposon Tn*5* called Tn*5*supF for insertion mutagenesis and sequencing DNAs cloned in phage λ. This element carries a *supF* amber-suppressor tRNA gene. Its insertion into λ can be selected by plaque formation by using nonsuppressing (*sup*°) *Escherichia coli* for amber mutant λ phage and *sup*° *dnaB*-amber *E. coli* for nonamber λ phage. Tn*5*supF is just 264 base pairs long. It transposes efficiently and inserts quasi-randomly into DNA targets. The unique sequences near its termini can be used as primer binding sites for dideoxynucleotide DNA sequencing, thus permitting the direct sequencing of DNAs cloned in phage λ without subcloning.**

Much of the power of modern molecular genetics stems from efficient means of generating easily characterized mutations and from the ability to determine DNA sequences with efficiency and precision. In the frequently used chain-termination method, short oligonucleotides are used to prime *in vitro* DNA synthesis from specific sites in template DNA in the presence of chain-terminating dideoxynucleotides (1). Up to 1 kilobase (kb) of DNA can be read from a single primer (2), but additional manipulations are needed to sequence longer stretches of DNA—e.g., random subcloning, the isolation of nested deletions, or the repeated synthesis of oligonucleotide primers (3–5). In alternative strategies transposons have been inserted into many sites in target DNAs in plasmids, and unique DNAs close to their ends have been used as binding sites for DNA sequencing primers (6–10). Transposons have not, in contrast, been used to sequence DNAs cloned in phage λ, because most λ cloning vectors grow only lytically and kill their infected host cells, whereas the resistance markers in most transposons are generally used by selecting for cells that form colonies in the presence of antibiotic. In addition, insertion of a several-kilobase-long transposon into very large recombinant phage DNAs would make the phage inviable.

We describe here an efficient transposon-based method of sequencing DNAs cloned in λ phage. A derivative of transposon Tn*5* called Tn*5*supF† was made that is only 264 base pairs (bp) long and that contains unique subterminal sites and the *supF* nonsense suppressor tRNA gene as a selectable marker (Fig. 1). Our results show that transposition of Tn*5*supF to λ is easily selected, that the inserts are widely distributed in target DNAs, and that Tn*5*supF inserts facilitate the sequencing of DNAs in λ phage.

MATERIALS AND METHODS

Strains and General Techniques. The bacterial strains, plasmids, and λ phage derivatives used in this study are listed in Table 1. The media and conditions for bacterial and phage growth, DNA extractions, and recombinant DNA cloning were as described (11, 18, 19). The following oligonucleotides were used: O primer, 5′-TAGGATCCCCTACTTGTGTA for sequencing from the "outside" (O) end of Tn*5*supF into adjacent target DNA; I primer, 5′-TAGGATCCCGAGATC-TGATC, for sequencing from the inside (I) end of Tn*5*supF into adjacent target DNA (see Fig. 1); and 5′-CTGGCA-CGCGCTGGACGCG, specific for vector sequences 30 bp upstream of the Tn*5*supF I end of plasmids pBRG1308 and pBRG1310, for verifying the sequence of Tn*5*supF. Plasmid p3 was transferred between strains by conjugation as described (14).

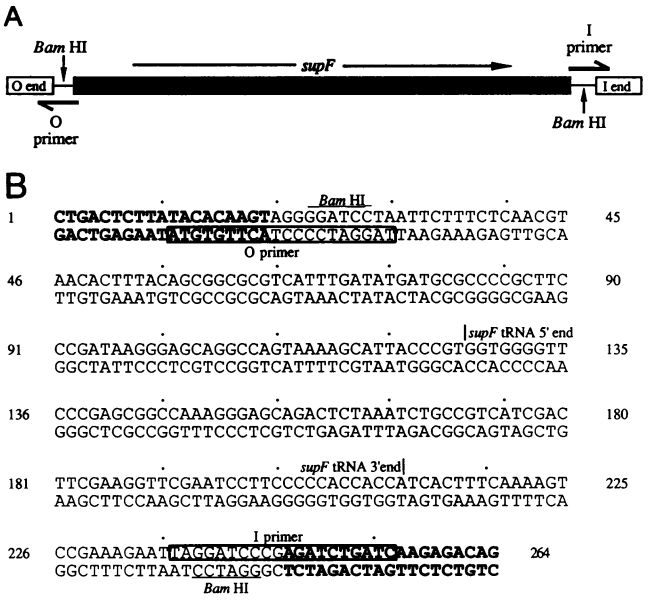

FIG. 1. Transposon Tn*5*supF. (*A*) Schematic diagram of Tn*5*supF. (*B*) Sequence of Tn*5*supF. The 19 bp at each end correspond to the O- and I-end segments of IS*50* that are needed for transposition. Boxed sequences indicate the primers used for sequencing outward from the O and I ends. The 5′ and 3′ ends of the mature (processed) *supF* tRNA product are indicated, but sequences needed for tRNA synthesis and processing extend from position 52 to position 231. Tn*5*supF was referred to as Tn*5*seq2 in ref. 10.

Construction of Tn*5*supF. A small Tn*5*-related transposon containing the *supF* suppressor tRNA gene as its selectable marker (Fig. 1*A*) was constructed starting with the pBR322-derived multicopy plasmid pBRG1306 (11). This plasmid contains the 19-bp O- and I-end segments of IS*50* (Tn*5*) that are needed as sites for transposition and the transposase (*tnp*) gene (for review, see ref. 20). pBRG1306 DNA was digested with *Bam*HI, which cleaves between the O and I ends of IS*50*,

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25496).

Table 1. Bacterial and plasmid strains

Name	Description/derivation	Ref./source
Bacterial strain		
DB973	*supF*	11
DB4494	MC1061 [p3] [pBRG1308]	This study
DB4495	MC1061 [p3] [pBRG1310]	This study
DB4496	MC1061 *dam*::Tn*9* [p3] [pBRG1310]	12 and this study
MC1061	F^- *sup*^{o}	13
DK21	*sup*^{o} *dnaB*am266 ($\lambda imm21$–ban_{P1}) cold sensitive	D. Kurnit
Plasmid		
p3	*kan*^{R} *amp*am *tet*am, conjugative	14
pDF41	miniF (single copy) *trpE*	15*
pBRG1306	pBR322 derivative *amp*^{R}, contains O and I ends of IS*50*	11
pBRG1308	*supF* gene from Proteus 13 in *Bam*HI site of pBRG1306	This study
pBRG1310	pDF41–Tn*5*supF, *tnp* nearby	This study
Proteus 13	pBR322 derivative, *supF* in a 211-bp *Alu* I fragment	H.V.H.
Phage		
Charon 4A	*A*am32 *B*am1 *lac5 bio256* KH54 *nin5*	16
$\lambda b221$ *c*I857		17

kan^R, Kanamycin resistance; amp, ampicillin; tet, tetracycline. *pDF41 was isolated as a 12.7-kb *Eco*RI fragment from pDF42, a chimaera that had been made by joining pDF41 to a derivative of the multicopy colE1 plasmid (see ref. 15).

the recessed 3′ DNA ends were filled using the Klenow fragment of DNA polymerase I, and the resultant linear DNA was ligated with a 211-bp *Alu* I fragment containing a synthetic *E. coli supF* gene from plasmid Proteus 13 (H.V.H., unpublished data) to generate plasmid pBRG1308. *Bam*HI sites flanking *supF* are regenerated by this ligation. Plasmid pBRG1308 was selected by transformation of the nonsuppressing strain MC1061 carrying the compatible p3 plasmid (which contains amber mutations in *amp* and *tet* genes; ref. 14) to tetracycline resistance (suppression of the *tet* amber allele), and the sequence of Tn*5*supF (Fig. 1*B*) was verified by chain-termination DNA sequencing (1) using reverse transcriptase on denatured double-stranded DNA (21). Preliminary tests showed that this element could transpose to phage λ.

Most cells in young cultures of strain DB4494, which carries Tn*5*supF on a multicopy plasmid, made small colonies. These cultures were usually overgrown by healthier cells during overnight incubation with or without tetracycline to select for retention of *supF*. λ infection of these faster growing strains did not result in λ–Tn*5*supF transposition derivatives, and further tests showed that the multicopy plasmids in these strains lacked the *Bam*HI sites that flank *supF* in the parental plasmid. The large colony phenotype may thus be due to selection for cells with lower levels of suppressor tRNA: this may be achieved by Tn*5*supF transposition to the chromosome, deletion of *supF* from the multicopy plasmid, and segregation of cells with the *supF*-deleted plasmid.

A low-copy-number plasmid containing Tn*5*supF and its nearby transposase gene was made by ligating a 3.1-kb *Eco*RI–*Tth*111I segment from pBRG1308 containing Tn*5*supF and the *tnp* gene to a 9-kb *Eco*RI–*Hin*dIII fragment containing the F factor-derived replication origin of the low-copy-number plasmid pDF41 (15) (the 5′ extensions left by *Tth*111I and *Hin*dIII digestion are not complementary and were filled-in with the Klenow fragment of DNA polymerase I before ligation). The resultant plasmid, pBRG1310 (Fig. 2),

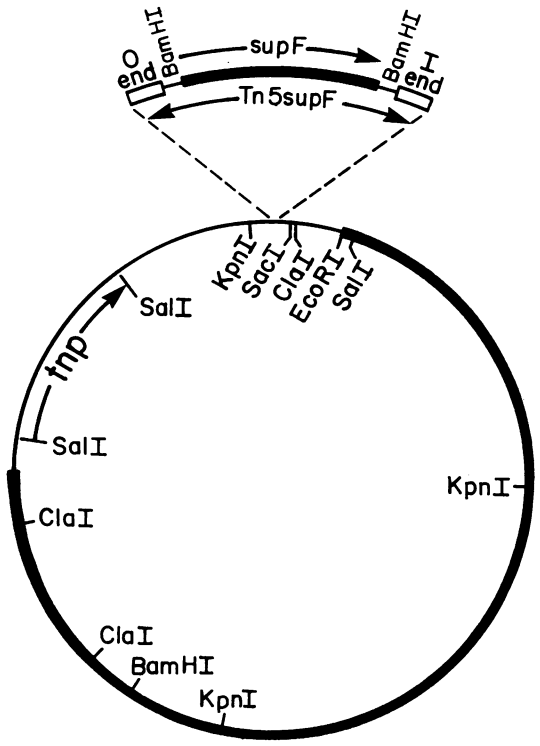

FIG. 2. Restriction map of the pBRG1310 plasmid containing Tn*5*supF. Thick line in plasmid, an 8.7-kb *Eco*RI–*Hin*dIII fragment (*Hin*dIII site is filled-in) from pDF41; thin line including Tn*5*supF on expanded scale, a 3.4-kb *Eco*RI–*Tth*111I (*Tth*111I is filled-in) fragment from pBRG1308; solid and open boxes in the expanded scale diagram of Tn*5*supF are as in Fig. 1*A*. The estimated position of restriction sites (clockwise from the *Eco*RI site, in bp) are: *Sal* I, 136; *Kpn* I, 2591; *Kpn* I, 5940; *Bam*HI, 6708; *Cla* I, 7082; *Cla* I, 8242; *Sal* I, 8878; *Sal* I, 10,413; *Kpn* I, 11,513; *Sac* I, 11,803; *Eco*RI, 12,133. The restriction fragment lengths were deduced from published nucleotide sequences of pDF41 (15), pBRG1306 (11), and the *supF* gene (14), and verified by restriction digests. In these coordinates Tn*5*supF extends between positions 11,518 and 11,783. The F origin of replication is between two *Kpn* I sites (positions 2591 and 5940).

was stable and was used as the donor plasmid in subsequent transpositions of Tn*5*supF to λ (*Results*).

Transposition of Tn*5*supF to Amber Mutant Phage λ. Insertions of Tn*5*supF into the amber mutant phage Charon 4A were obtained by infecting exponentially growing cells containing pBRG1310 at multiplicities of about one phage per cell and growing the infected cells for 90 min at 37°C. Aliquots of the lysate were plated on a lawn of *sup*^{o} strain MC1061 to select for *sup*-independent phage. Since Charon 4A contains amber mutations in phage genes A and B, only phage with inserts of Tn*5*supF or amber$^+$ revertants form plaques on MC1061.

Transposition of Tn*5*supF to Nonamber Phage. Because many λ phage vectors carry no amber mutations, an alternative selection was developed using the nonamber phage $\lambda b221$ *c*I857. This phage was grown on pBRG1310-containing cells, as above, and derivatives containing Tn*5*supF were selected by plating on the *dnaB*-amber *ban* strain DK21, a gift of D. M. Kurnit (Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI). This selection exploits the dependence of λ phage replication on the host *dnaB* function, and hence the inability of wild-type λ to form plaques on a *dnaB*-amber strain (22). Infection with *supF*-containing λ phage results in enough suppression of the *dnaB* amber mutation to permit phage replication and plaque formation (D. M. Kurnit, personal communication). DK21, like other strains carrying *dnaB* alleles suppressed by the P1 *ban* gene (22), is cold-sensitive and makes relatively small colonies at

37°C. Mutants that formed faster growing colonies were present at 10^{-5} to 10^{-3} in young cultures, caused a severe reduction in λ::Tn*5*supF plaque size, and thus were not suitable for the selection of Tn*5*supF transposition derivatives.

DNA Sequencing. Phage into which Tn*5*supF had transposed were plaque-purified once on the selective host strain (MC1061 or DK21, as appropriate); liquid lysates of the purified phage (5 ml) were grown on MC1061, and DNA was extracted in a rapid "miniprep" procedure (23). Phage DNA was denatured by heating 1 μg in 10 μl of distilled water to 100°C for 3 min with a 100-fold molar excess of primer (about 15 pmol; 0.1 μg), quick-chilled on ice, and used for chain-termination DNA sequencing, either with reverse transcriptase (21) or Sequenase (24), with comparable results. The sequencing results obtained with heat denaturation were generally better than those obtained with alkali denaturation of the phage DNA.

RESULTS

Rationale. To be used for insertion mutagenesis and the sequencing of DNAs in phage λ a transposon should have the following characteristics: (*i*) transpose efficiently and relatively randomly; (*ii*) specify a trait that is easily selected during lytic growth; (*iii*) contain unique sequences near each end to serve as primer binding sites; and (*iv*) be very small, so that it will not make the λ DNA larger than the capacity of the phage head. Tn*5*supF (Fig. 1) was designed to meet these criteria. As a derivative of Tn*5*, it should insert into many sites within a gene and exhibit less target specificity than most other transposons (10, 20). It contains a *supF* amber-suppressor tRNA gene for selection, the 19-bp segments from the O and I ends of IS*50* (Tn*5*) that are needed for transposition (20, 25, 26), and a pair of *Bam*HI sites, for easy restriction mapping of inserts; it is only 264 bp long, in contrast to the nearly 6 kb of wild-type Tn*5*. The 1.5-kb cis-acting transposase (*tnp*) gene (27, 28) is in the donor plasmid outside Tn*5*supF. Transposition of Tn*5*supF to λ phage was selected by plaque formation on appropriate host strains, as detailed below.

Characterization. Because the multicopy plasmid pBRG-1308 that carried Tn*5*supF was unstable, we cloned a segment containing Tn*5*supF and the nearby *tnp* gene into pDF41, a derivative of the *E. coli* F factor (which is maintained at about one copy per chromosome), to generate pBRG1310 (Fig. 2). Cells carrying pBRG1310 formed large colonies, equivalent to those formed by plasmid-free cells (in antibiotic-free medium), and no loss of pBRG1310 was detected when overnight cultures were grown without selection for *supF*.

Transpositions of Tn*5*supF to the amber mutant phage Charon 4A were selected by infecting cells carrying pBRG1310 and plating the resulting lysate on the nonsuppressing (*sup*°) strain MC1061. The frequency of Tn*5*supF transposition to Charon 4A was 2×10^{-7} in strain DB4595 (Dam^+) and 10^{-6} in strain DB4496 (Dam^-). This is in accord with other studies showing that Tn*5* transposition is decreased by Dam methylation (12, 29). Burst-size measurements indicated that an average of three Tn*5*supF-containing phage were produced per cell from cells that produced any transposition products (total burst size, 60 phage per cell).

Many λ cloning vectors do not contain amber mutations in essential genes, and thus form plaques on *sup*° MC1061. They do not form plaques on the *dnaB*-amber mutant strain DK21 because λ DNA replication depends on the host *dnaB* function (30). λ with inserts of *supF*, in contrast, does form plaques on DK21, due to suppression of the host *dnaB*-amber allele after phage infection (D. M. Kurnit, personal communication). Inserts of Tn*5*supF into the nonamber phage λ *b*221 *c*I857 were selected on DK21. Phage able to form plaques on DK21 were obtained at a frequency of 3×10^{-7} after a single cycle of phage growth in strain DB4495.

Analyses of Tn*5*supF-Containing Phage. The specificity of Tn*5*supF insertion was monitored genetically and physically. In plaque morphology tests using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) medium (16, 18), 9 of 148 (6%) Tn*5*supF transposition derivatives of Charon 4A formed white ($LacZ^-$) plaques, indicating insertion into *lacZ*; 30 $LacZ^-$ plaques would have been expected if insertion had been completely random, since the 3-kb *lacZ* segment constitutes about one-fifth of the nonessential part of Charon 4A (16). Digestions of the DNAs of Tn*5*supF-containing phage with *Bam*HI (which cleaves Tn*5*supF and Charon 4A) plus *Eco*RI (which cleaves Charon 4A but not Tn*5*supF) confirmed that Tn*5*supF could insert into numerous target sites. Five separate sites of insertion are evident in the six phage shown in Fig. 3, with the isolates in lanes 3 and 5 possibly being siblings from a single insertion event.

In another experiment the positions of 16 independent Tn*5*supF transpositions to Charon 4A were determined by restriction mapping and DNA sequencing (isolates 1–12) and are shown in Fig. 4. The insertion events were widely distributed in the dispensable segments of Charon 4A. DNA sequencing from each end of Tn*5*supF in 12 Charon 4A::Tn*5*supF phage and also in seven Tn*5*supF transposition derivatives of λ*b*221 *c*I857 (Table 2) showed that (*i*) no two insertions were at the same site (although inserts 7 and 8 in Charon 4A were separated by just a single base pair); (*ii*) 9 bp of the phage target sequence was duplicated by insertion at each site, and (*iii*) G+C pairs were present at each end of the duplicated segment in 9 cases, and at one end of the duplicated segment in the other 10 cases. None of the duplications contained A+T pairs at both ends, and no other features common to these insertion sites were found. An equivalent wide distribution of insertion sites and preference for G+C pairs at the ends of target duplications is also seen with other Tn*5*-based elements (20, 32, 33).

One of the seven λ*b*221::Tn*5*supF phage (λ*b*221 *c*I857 isolate 1, Table 2) is exceptional in containing Tn*5*supF in λ gene *T*, which is essential for phage tail formation. The insert

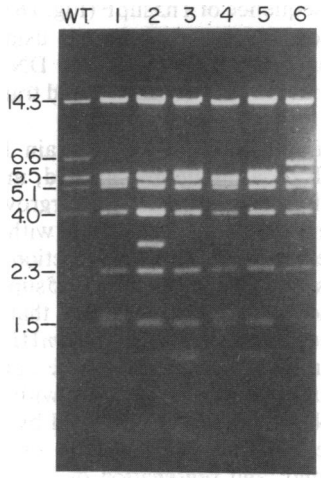

FIG. 3. Restriction mapping of sites of Tn*5*supF insertion. The DNAs of six Charon 4A::Tn*5*supF phage obtained after a single cycle of growth on strain DB4495 and plating for single plaques on the *sup*° strain MC1061 were digested with *Eco*RI and *Bam*HI and electrophoresed in an agarose gel. Lanes: WT, parental Charon 4A; 1–6, Charon 4A::Tn*5*supF phage. The DNAs in lanes 3 and 5 may be from a single transposition event. The absence of the 6.6-kb *Eco*RI–*Bam*HI fragment of Charon 4A from each of the six Tn*5*supF-containing phage indicates that each insert is in the same 6.6-kb region. This type of restriction analysis can be used to choose particular insertions for subsequent sequencing.

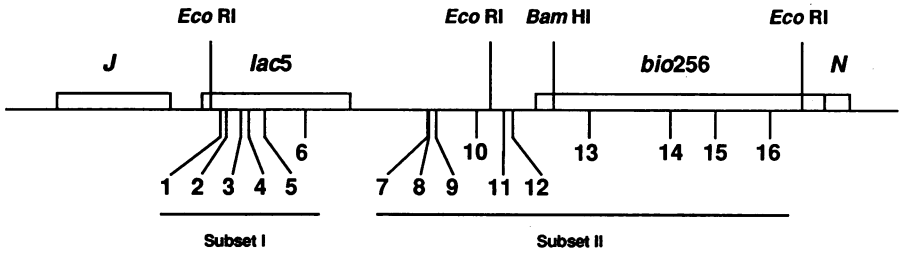

FIG. 4. Positions of independent Tn*5*supF insertions in Charon 4A. The 14.3-kb stuffer region of Charon 4A, between the essential λ genes *J* and *N*, contains most of the sequences in this phage that are not needed for plaque formation and was taken as representative of cloned DNA sequences. The Tn*5*supF insertions were obtained after single cycles of infection of strain DB4595 and selection of suppressor-containing phage on *sup*° strain MC1061. The phage with inserts designated as subset I (inserts 1–6) made LacZ⁻ plaques on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) indicator agar, whereas phage designated as subset II made Lac⁺ plaques on X-gal agar. All 16 insertions tested were in the 14.3-kb stuffer region; insertions 1–12 were sequenced (see Table 2), and the positions of the remaining four were estimated by digestion with *Eco*RI, *Bam*HI, and *Hin*dIII.

created an in-frame TGA termination codon 11 codons before the end of gene *T*. Since this phage formed plaques the last 11 amino acids of T protein must not be essential for activity.

DISCUSSION

Transposable elements serve as potent single-site mutagens and as mobile sources of primer binding sites for efficient sequencing of large DNA segments (10). The Tn*5*supF transposon described here should be extremely useful for the insertion mutagenesis and sequencing of DNAs cloned in phage λ for the following reasons. (*i*) Tn*5*supF specifies a nonsense suppressor tRNA, and its transposition to both amber mutant and nonamber phage targets can be selected efficiently. (*ii*) Insertions of Tn*5*supF are widely distributed in cloned DNA but are not generally found in λ vector sequences, which contain essential phage genes. (*iii*) Unique sequences near the ends of Tn*5*supF provide primer binding sites for dideoxynucleotide sequencing in both directions from each insertion. (*iv*) Tn*5*supF is just 264 bp long, so that its insertion will only rarely make even a very large λ genome exceed the packaging capacity of the phage head. (*v*) Tn*5*supF insertions subdivide long stretches of DNA into smaller easily sequenced segments but without the loss of linkage information that is inherent in random subcloning strategies. For the occasional DNAs that are not amenable to chain-termination sequencing, Tn*5*supF possesses restriction sites for end-labeling DNA fragments for the chemical cleavage sequencing method (34).

Table 2. Tn*5*supF insertions in Charon 4A and λ *b*221 *c*I857

Insertion*	Position†	Duplicated sequence‡	Orientation§
	Charon 4A		
Subset I			
(*lacZ* portion)			
1	1,663–1,671	CATTTAATG	B
2	1,803–1,811	CTGAGCGCA	B
3	2,026–2,034	GCGGCGAGT	A
4	2,328–2,336	CCGTTGCTG	B
5	2,588–2,596	GGCGATGAG	B
6	3,410–3,418	GTCAGAAGC	A
Subset II			
(non-*lacZ* portion)			
7	25,199–25,217	GGTTGTAGG	A
8	25,226–25,234	GTCGTAGGT	B
9	25,445–25,453	GGTTCGACC	A
10	26,330–26,338	GGGTTGGTG	A
11	26,847–26,855	CAAAGACAG	A
12	26,928–26,936	CTGCAGGAA	A
	λ *b*221 *c*I857		
1	10,504–10,512	GATGACAGT	A
2	33,340–33,348	CTCCTGGCT	B
3	34,002–34,010	GACATTGCT	B
4	34,003–34,011	ACATTGCTC	B
5	34,636–34,644	TTCCTCATG	A
6	34,894–34,902	AGCTTGGTG	B
7	36,772–36,780	GATCGGATG	B

*Positions of insertions 1–12 (in Charon 4A) are diagramed in Fig. 4.
†For insertions in *lacZ* (subset I), position refers to nucleotide sequence in the *lac* operon (31); for other insertions, position refers to nucleotide sequence in wild-type λ (17).
‡The 9 bp of target sequence duplicated by Tn*5*supF insertion. The sequences are presented 5′ to 3′ and correspond to the coding strand of *lacZ* (subset I), and the strand which is oriented left to right in λ.
§Orientation A, λ–left O–*supF*–I λ–right; orientation B, λ–left I–*supF*–O λ–right.

The best current DNA sequencing methodologies permit up to 1 kb to be read from a given primer (2). Hence 60 independent inserts should generally be sufficient for about 95% sequence coverage of both strands in a typical 20-kb segment cloned in λ. The remaining sequences could be obtained by using mapped transposon insertions, by bridging the gap with new oligonucleotides, or by subcloning that exploits the *supF* marker or the *Bam*HI sites in Tn*5*supF.

We anticipate additional valuable applications for Tn*5*supF and related elements. (*i*) Insertions of Tn*5*supF into plasmid or cosmid clones could be selected by suppression of a nonsense mutation in the *tet* and *amp* genes of the p3 plasmid used here or in a chromosomal gene. (*ii*) Tn*5*supF derivatives containing diverse unique subterminal sequences could permit the "multiplex" sequencing of λ clones, equivalent to the highly efficient procedure developed for plasmid subclones (35). (*iii*) UGA stop codons are present in Tn*5*supF in each reading frame and in each orientation; inserts of Tn*5*supF should, therefore, interrupt translation and result in nested sets of amino-terminal peptides that could be useful in epitope mapping of complex proteins. (*iv*) Systematic functional analyses of the *E. coli* genome should be feasible using derivatives of Tn*5*supF containing a reporter gene and a regulated promoter (20, 39), a λ phage library of *E. coli* DNAs (36), and homologous recombination to put insertion mutant alleles into the bacterial chromosome (37, 38).

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